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THE INFLUENCE OF FATTY ACIDS *IN VITRO* ON MAMMALIAN CELLS FROM SPECIES DIFFERING IN THEIR FATTY ACYL DESATURASE CAPABILITIES.

VOLUME I

ALFREDO GIANGREGORIO

THE INFLUENCE OF FATTY ACIDS *IN VITRO* ON MAMMALIAN CELLS FROM SPECIES DIFFERING IN THEIR FATTY ACYL DESATURASE CAPABILITIES.

ALFREDO GIANGREGORIO

A Thesis Submitted to the Faculty of Medicine, in Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

in the Department of Chemical Pathology, School of Pathology and the South African Institute for Medical Research, University of the Witwatersrand Medical School, Johannesburg, South Africa.

December 1991.

ABSTRACT.

Numerous studies have assessed the effects of single fatty acids on various aspects of lipid metabolism, particularly cancer. Established cell lines have largely been used for this purpose. The choice of control cells, however, has often been inappropriate. There is also a surprising lack of knowledge of the effects of fatty acids in the "real world", in which normal cells *in vivo* are presented with mixtures of dietary fatty acids. Before transformed cells can be used as models of disease states, it is essential to fully understand fatty acid metabolism in normal (control) cells. Only then can experimental findings be extrapolated to the clinical situation with some certainty. This thesis has therefore, assessed the effects of exogenous fatty acid mixtures on the growth/viability of normal mammalian tissues all cultured under standard conditions, and attempted to elucidate the mechanisms underlying such effects.

As different mammalian species exhibit different fatty acyl desaturase capabilities, cells from three species were chosen, viz. rat, Man and cat, with desaturase capability decreasing with species, respectively. A wide range of different cell types from each species were studied due to the known differences in their lipid

profiles and metabolism. The cells studied in the rat and cat included those derived from the cerebral cortex, white adipose tissue, skin, lung, skeletal muscle and aortic endothelium, while the human cell types obtainable included those derived from skin, skeletal muscle and lung. Erythrocytes and lymphocytes were studied in all 3 species.

Each of the cultured normal mammalian tissues studied were shown to exhibit different proliferation rates. Cultured cells were dosed with fatty acid mixtures mimicking the fatty acid composition of dietary oils; these mixtures were termed 'pesudo-oils', were bound to bovine serum albumin as carrier, dosed at concentrations ranging from 0 to 100mg/l, and cultures incubated for 48 hours. Viable cell numbers were assessed and related to control cell numbers at the time of dosing. This was termed the 'cytostatic number', and its implications were discussed.

pseudo-Oils modulated cell viability, but the extent thereof varied considerably in magnitude with pseudo-oil and concentration dosed. Furthermore, differences in cell viability were shown both between tissues from a particular species, as well as between identical cultured tissues derived from different species. In general, pseudo-oil supplementation of cultures induced an overall concentration dependant growth limiting

and/or cytotoxic effect. Proliferation of certain cell types were, however, stimulated with some pseudo-oils, particularly at low to intermediate concentrations in the range dosed. Differences in cell viability were also related to the degree of pseudo-oil unsaturation; the two most saturated oils, meat and coconut, were in general, least effective in limiting and/or promoting cell viability, while the effects induced with pseudo-oils rich in polyenoic fatty acids were more marked. The possibility that the cell viability changes induced were related to albumin as fatty acid carrier, or fetal calf serum as a supplement in the incubation medium, were investigated, and the possibility discounted. The use of pseudo-oils rather than single fatty acids, however, warrants consideration of fatty acid synergism and antagonism.

To establish the possible mechanism(s) whereby the dosed pseudo-oils influenced cell viability, various cell parameters were subsequently examined. These included total protein, the incorporation, desaturation and elongation of both pseudo-oil, and single C18, fatty acids, as well as the production of lipoperoxides and prostanoids.

Total cellular protein concentrations varied both between tissues and species, and reflected changes in cell number in dosed cells. However, pseudo-oils were

also shown to modulate absolute protein synthesis in nucleated mammalian cells, primarily by enhancing such. It was postulated that this related to the induction of lipid metabolising enzymes.

Variations in cellular fatty acid composition were found both between the mammalian tissues and species studied. Evidence to support the capability of cultured mammalian cells to incorporate exogenous fatty acids was shown. The extent of incorporation, however, varied with cell type, species and fatty acid structure. Desaturase cascade enzyme capability was also assessed by comparison of dosed cell FA profiles with that of controls. Once again, desaturation and elongation capability varied with different cell types within a particular species, independent of cell proliferation rates, but was generally greatest in dividing rat, lower in human, and least in cat, tissues. In all three species, however, erythrocytes and lymphocytes failed to efficiently perform these reactions. This correlated with the lower threshold for cytotoxicity in lymphocytes and erythroctyes than in dividing cells, although desaturation capability did not correlate directly with proliferation rates in growing cells. It was nevertheless clearly shown that desaturase cascade enzyme expression with pseudo-oil dosage was more limited than, and could not be fully predicted by, the results

obtained utilising single FA's. This phenomenon was related to synergism and antagonism between pseudo-oil FA's.

Cultured mammalian tissues were found to vary in their capability to form both lipoperoxides and eicosanoids. Pico-molar amounts of total eicosanoids were quantitated compared to nano-molar amounts of lipid peroxides. Overall, lipid peroxide production was not directly related to control cell proliferation rates or total unsaturated FA levels. However, total molar eicosanoid concentrations in control cells and the eicosanoid:MDA ratio suggested a correlation with the desaturation capabilities of the three species, decreasing in the order rat > human > cat. Each cell type studied exhibited a unique prostanoid profile, which was modulated (enhanced, suppressed or inhibited) to a greater or lesser extent with pseudo-oil incubation. However, no correlation between cell viability, the degree of pseudo-oil unsaturation, or any other biochemical parameter studied, except pseudo-oil concentration dosed, could be proven to relate to the changes in prostanoid levels induced with pseudo-oil supplementation. This implied that endogenously bio-synthesised prostanoids were not directly responsible for effects induced. On the other hand, lipoperoxide production generally increased with pseudo-oil

concentration dosed and was greater with pseudo-oils rich in polyenoic than monoenoic or saturated FA's. Furthermore, lipoperoxide involvement in the modulation mammalian cell proliferation was proposed. This, however, was shown not to be the sole mechanism, and the involvement of membrane structural changes, eg. fluidity was additionally suggested and discussed. The findings indicated that the modulation of cell proliferation by FA's was multifactorial.

The variations found between rat, cat and human tissues with regard to the parameters investigated indicated that extrapolation of experimental results between mammalian tissues and species, as well as the use of cells from different tissues and particularly from different species as controls for other cells, is potentially misleading, and should be avoided if reliable interpretation of results is desired. Furthermore, we recommend fatty acid analysis of all culture media prior to use, and appropriate supplementation with polyenoic, or at least essential, fatty acids if required.

Since p-oil FA composition reflected that of dietary oils, the data from this thesis serves as an *in vitro* model and guide to how normal genetically entire mammalian cells *in vivo* may respond when similar oils form part of the dietary intake.

I hereby declare that this thesis is my own work and that the results contained herein were obtained by me while a student at the University of the Witwatersrand, and has not been submitted to any other University.



14-12-91

A. GIANGREGORIO. DATE.

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LITERATURE CITED.

APPENDIX.

Refereed publications to date arising from this thesis.

LIST OF ABBREVIATIONS.

Sat.	=	Saturate.
Monos.	=	Monoenoic/Monounsaturate.
Polys.	=	Polyenic/Polyunsaturate.
TAG	=	Triacylglycerol.
PGL	=	Phosphoglyceride.
FA	=	Fatty acid.
FFA	=	Free fatty acid.
EFA	=	Essential fatty acid.
PUFA	=	Polyunsaturated fatty acid.
PDFA	=	Post-delta-6-desaturation fatty acid.
FAME	=	Fatty acid methyl ester.
p-	=	pseudo.
p-oil	=	pseudo-oil.
CO	=	Coconut oil.
MO	=	Meat oil.
OO	=	Olive oil.
SSO	=	Sunflower seed oil.
LO	=	Linseed oil.
EPO	=	Evening primrose seed oil.
FO	=	Fish oil.
PA	=	Palmitic acid.
SA	=	Stearic acid.
OA	=	Oleic acid.
LA	=	Linoleic acid.
ALA	=	Alpha (α) linolenic acid.

GLA	=	Gamma (γ) linolenic acid.
DGLA	=	Dihomogamma (γ) linolenic acid.
AA	=	Arachidonic acid.
ETA	=	Eicosatrienoic acid.
EPA	=	Eicosapentaenoic acid.
DHA	=	Docosahexaenoic acid.
Δ9D	=	Delta-9-desaturase.
Δ8D	=	Delta-8-desaturase.
Δ6D	=	Delta-6-desaturase.
Δ5D	=	Delta-5-desaturase.
Δ4D	=	Delta-4-desaturase.
MDA	=	Malondialdehyde-equivalent.
TBA	=	Thiobarbituric acid.
TBARM	=	Thiobarbituric acid reactive material.
PG	=	Prostaglandin.
HETE	=	Hydroxy eicosatetraenoic acid.
HPETE	=	Hydroperoxy eicosatetraenoic acid.
GLC	=	Gas liquid chromatography.
HPLC	=	High pressure liquid chromatography.
Conc.	=	Concentration.
[p-Oil]	=	pseudo-Oil concentration.
[MDA]	=	MDA-equivalent concentration.
mg/l	=	mg of a compound per litre growth medium.
n	=	Number of experiments.
s.e.m.	=	Standard error of the mean.
" - "	=	No value obtained for the variable at

this point.

DMEM = Dulbecco's modification of Eagle's medium.

HBBS = Hank's balanced salt solution.

PBS = Phosphate buffered saline.

EDTA = Ethylene-diamine-tetra-acetic acid.

DMSO = Dimethylsulphoxide.

BSA = Bovine serum albumin.

FCS = Fetal calf serum.

FBS = Fetal bovine serum.

RS = Rat serum.

CS = Cat serum.

HS = Human serum.

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CHAPTER 1: INTRODUCTION.

1.1 Lipids.

1.1.1 General.

Lipids are a heterogeneous group of compounds consisting of fats, oils, waxes and related compounds. They are relatively insoluble in water, but are soluble in organic solvents. They are important not only because of their high energy value, but also because of the essential fatty acids they contain and the fat soluble vitamins they transport. Lipids act as structural components of membranes, as storage metabolic fuels, as insulating materials, and as cell-surface components conferring, for example, tissue and species specificity (Op den Kamp 1979, Spector et al 1981a and Traill et al 1984).

Almost 95% of dietary lipids are triacylglycerols (TAG's), while the remaining 5% is composed primarily of phosphoglycerides (PGL's) and sterols. TAG's consist of a glycerol backbone to which 3 fatty acids (FA's) of variable length and degree of unsaturation are attached. TAG's are the main storage form of lipids primarily stored *in vivo* in adipocytes for subsequent use as fuels, or for membrane synthesis (Spector et al 1981a and Crawford 1983). PGL's are primarily found in structural lipids, eg. membranes (Spector et al 1967, 1981a, 1985 and Crawford 1983). Like the TAG's, they

contain a glycerol backbone, but with a phosphate/base group substituted for FA three. Typically, PGL molecules contain a saturated or monounsaturated FA in the 1-position, and a monounsaturated or polyunsaturated FA in the 2-position (Holman 1971). PGL's are not only the main components of cell membrane lipids, but also serve as a source of eicosanoid precursors (Spector et al 1981a). Body PGL FA composition (the relative proportions of saturates, monounsaturates and polyunsaturates) varies in response to limiting dietary changes (eg. Yavin 1977, Yavin et al 1979 and McGee 1981). Holman (1971) proposed that this is a homeostatic response, tending to minimise the changes in unsaturation of the PGL's caused by the diet. Sterols, of which cholesterol is by far the most common, have a rigid structure, and tend to stabilise cell membranes by counteracting the membrane fluidising effect of polyunsaturated FA's (Arbogast et al 1976, Stubbs 1983 and Shinitzky 1984). The living organism/cell can satisfy its need for these lipids by synthesising its own de novo from other 'raw materials', by obtaining them intact from the diet, or by modifying dietary lipids to suit requirements.

1.1.2 Lipids in Foods.

Dietary lipid is derived from both plants and animals. Generally, foods of animal origin contain more lipid

than do those derived from plants (eg. Giangregorio et al 1988a). Some lipid is "visible", eg. butter, while other is not, eg. lipid in plant or animal membranes. While the amount of lipid present in a food is important, its quality is of greater significance.

1.1.3 Lipids in Serum.

Serum lipid levels in Man can range from 5.0 to 9.0g/l (Documenta Geigy 1962 and Geigy 1984), while in other mammalian species, between 3.0 and 20.0g/l have been reported (Spector et al 1981a). In serum, hydrophobic lipids are transported in an aqueous environment as part of lipoproteins. Lipoproteins contain TAG's, PGL's, cholesterol and cholesteryl esters, and cultured cells utilise these lipids when they are supplemented (Spector et al 1981a).

1.1.4 Fatty Acids.

1.1.4.1 General.

The IUPAC-IUB Commission on Biochemical Nomenclature of Lipids (1977) designates fatty acids (FA's) as consisting primarily of a hydrocarbon chain with a terminal carbonyl group. Naturally occuring FA's usually contain an even number of carbon atoms (12 to 24), are straight chained, and may be saturated or unsaturated (from 0 to 6 methylene-interrupted double bonds).

There are 2 main series of polyunsaturated fatty acids (PUFA's) produced by mammals from the plant-derived essential FA's (linoleic acid and α-linolenic acid). All other PUFA's are made from these essential fatty acids (EFA's) by reaction sequences of desaturation and elongation (see section 1.3). Further, all naturally occurring PUFA's of benefit to mammals contain double bonds in the 'cis-' configuration (Crawford et al 1972 and Mahfouz et al 1980a, 1980b).

In general, the terrestrial food chain is rich in C18 FA's, viz. LA and ALA; the marine food chain, however, provides an abundence of C20 and C22 FA's, containing up to 6 double bonds, eg. EPA and DHA. The marine environment, therefore, provides more ω3 series FA's, compared to more ω6 series on the land (Williams et al 1977a, 1986).

Of the total serum lipid (5.0-9.0g/l), FA's comprise 3.5-4.0g/l (12.0-14.0mmol/l) (Geigy 1984). Normally the plasma free fatty acid (FFA) concentration is about 500μmol/l, or 150mg/l, in the range 180-1650μmol/l (Geigy 1984). Most FFA is carried bound to albumin, the concentration of which is normally about 800μmol/l. Albumin possesses 8 binding sites for FFA's, thus 800μmol/l albumin is equivalent to 6400μmol/l FFA. Thus, plasma albumin is almost never saturated with FFA's. While the concentration of FFA's in the circulation is

low, this unesterified fraction is metabolically important. Plasma FFA's contain a mixture of long-chain FA's, about 95% of which is 16 and 18 carbon saturated, monounsaturated and dienoic FA's (Spector 1971 and McDaniel et al 1981).

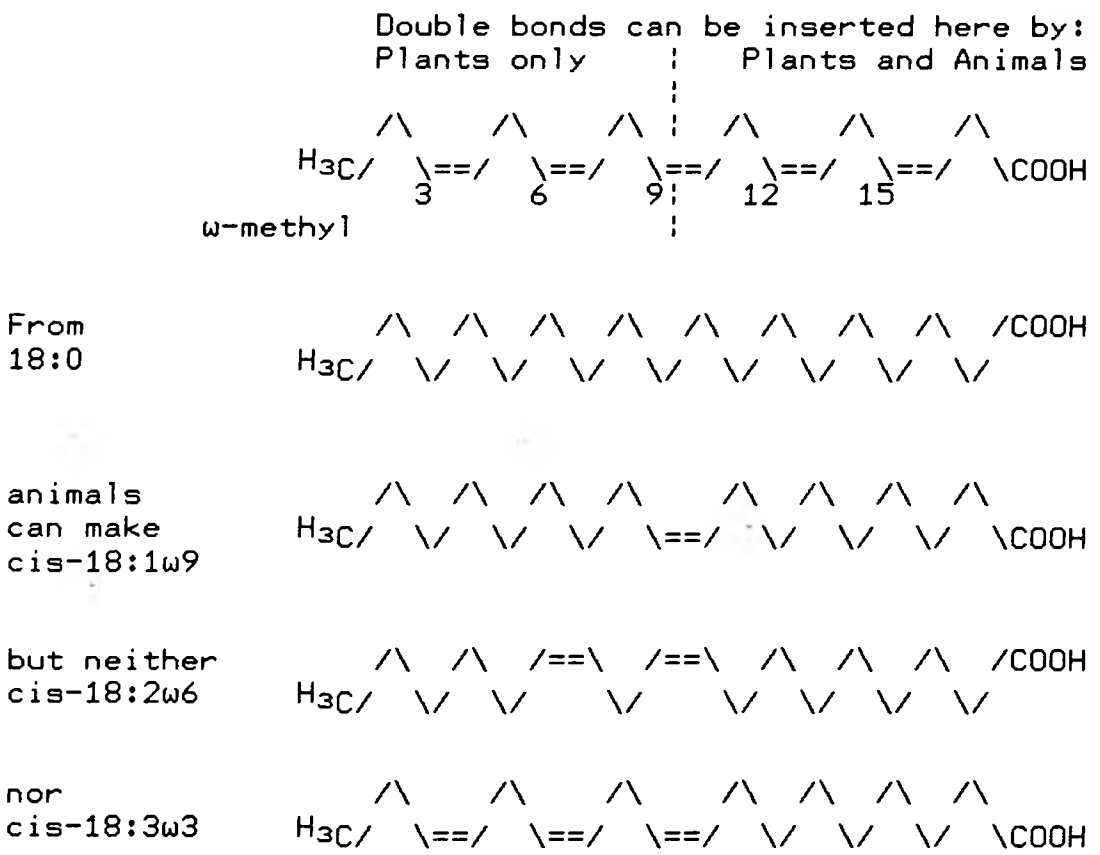
1.1.4.2 Essential Fatty Acids.

In 1930, Burr et al established that particular PUFA's are required in the diet of growing rats. They described the development of a number of symptoms which appeared when fat was excluded from the diet for several weeks, and which disappeared when linoleic acid was supplemented. This FA, and alpha-linolenic acid, have become known as the essential fatty acids (EFA's) since they either cannot be biosynthesised, or are synthesised in inadequate amounts, by organisms requiring them, and therefore, need to be supplied through the diet (eg. Mead et al 1976).

Plants and some invertebrate animals possess the intracellular enzymes capable of inserting double bonds between the ω-terminal and the ω9-carbon atom (Fig. 1.1.4.2.1.). Mammals cannot perform this desaturation step, but require the reaction products for normal metabolism (Holman 1968, Crawford et al 1971, Crawford 1983). Double bonds between the ω8 and carbonyl carbons can be introduced by both plants and animals, and these double bonds are thus, non-essential. As both plants and

Fig. 1.1.4.2.1.

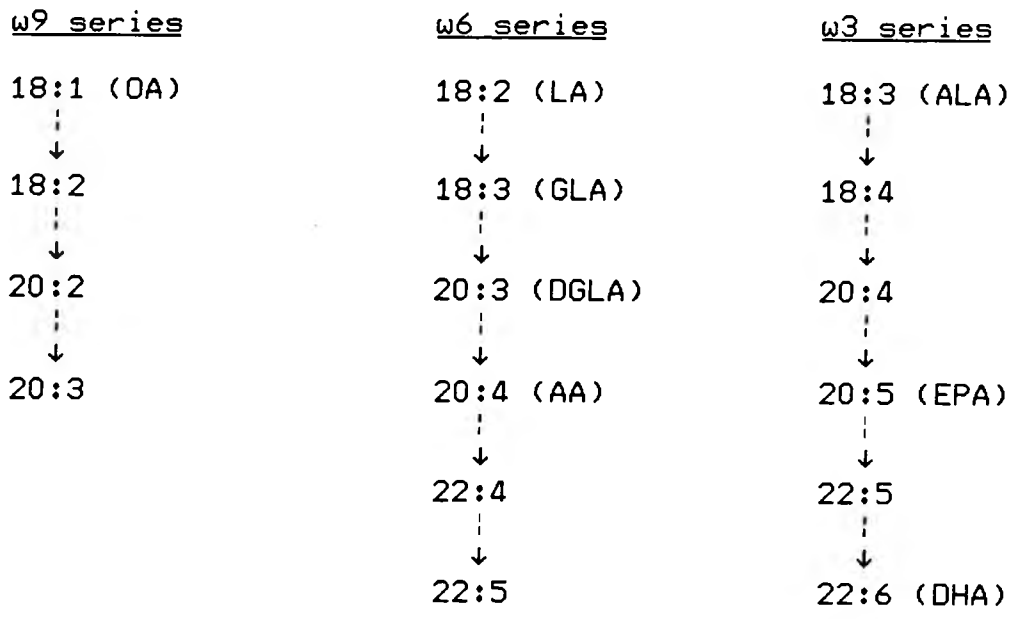
The formation of unsaturated fatty acids from saturated precursors, showing the ability of animals only to desaturate fatty acids in positions higher than ω8 (Crawford et al 1971).



animals can make both saturated and monounsaturated FA's, these FA's are also non-essential. The inability to introduce ω3 double bonds implies inability to convert ω6 to ω3 PUFA's, thus there are 2 EFA's, linoleic and alpha-linolenic acids (Holman 1968, 1971 and Crawford et al 1971, 1972). Mammals are, therefore, either directly, or indirectly, dependant on plant sources for their dietary EFA requirements. Although animals cannot make EFA's de novo, they can perform limited metabolic conversions on the EFA's which are presented to them (eg. Brenner 1971, Rivers et al 1975a and Horrobin 1983). The metabolism of these C18 FA's consists of a series of alternating desaturation and elongation reactions (see section 1.3), in which 2 hydrogen atoms are removed to create a new double bond, followed by the addition of 2 carbon atoms to lengthen the chain to form C20 and C22 FA's with 4, 5, and 6 double bonds (Mead 1968). This sequential process leads to the formation of 2 distinct families/series of PUFA's which cannot be interconverted, the ω6 series and the ω3 series (Fig. 1.1.4.2.2.) (Mead et al 1976 and Kanau et al 1977). There is another family of PUFA's, the ω9 series, derived from oleic acid, which is not an EFA; this series appears only to be significant during EFA deficiency (Holman 1960).

Fig. 1.1.4.2.2.

The metabolically important fatty acid families.



1.1.4.2.a Dietary Sources of Essential Fatty Acids and their Derivatives.

The ω3 series FA's are found mainly in marine fish oils and terrestrial leaf lipids, while the ω6 series are found mainly in terrestrial seed oils (eg. Garcia et al 1965, Willis et al 1976, McCance et al 1978, Gibson et al 1981a, Giangregorio et al 1988a and Horrobin 1990). These investigations showed that 18:2ω6 is abundant in meat, offal, seed oils and dairy products, with lower amounts found in olive and linseed oils. 18:3ω6 is rare, but is nevertheless metabolically important. Human milk contains 0.3-1.0% 18:3ω6, compared to 8.0-9.0% 18:3ω6 in evening primrose seed oil and 18.7% 18:3ω6 both in borage and blackcurrant oils. Moderate 20:3ω6 amounts are found in organs and human milk, while red meat, egg yolk and certain seaweeds contain high 20:4ω6 levels. Seeds, grains, green leafy plants, linseed and some fish oils are 18:3ω3-rich, while long chain ω3 PUFA's, especially 20:5ω3 and 22:6ω3, are abundant in marine oils.

It is often overlooked that when a terrestrial mammal eats the tissues of another organism, the cell membranes of those tissues supply EFA's and post-Δ6-desaturation fatty acids (PDFA's). Herbivores get a preponderance of 18:3ω3 from the green leaf material in their diet, a lot of 18:2ω6 from seeds on a seasonal basis, but none of the PDFA products of the EFA's. The combination of

direct incorporation, chain elongation and desaturation results in a profile of FA's in the liver PGL's which includes both parent (plant) and long-chain FA derivatives, but which is weighted towards the parent acids. Carnivores get a preponderence of 18:2ω6, as well as its PDFA derivatives, with minimal amounts of ω3 FA's. When carnivore eats herbivore, it consumes lipids that already contain long-chain FA's, and its liver FA profile shifts in favour of the long-chain PUFA's. Thus, there is a progression of the FA's through the food chain (Fig. 1.1.4.2.3.) (Crawford et al 1971).

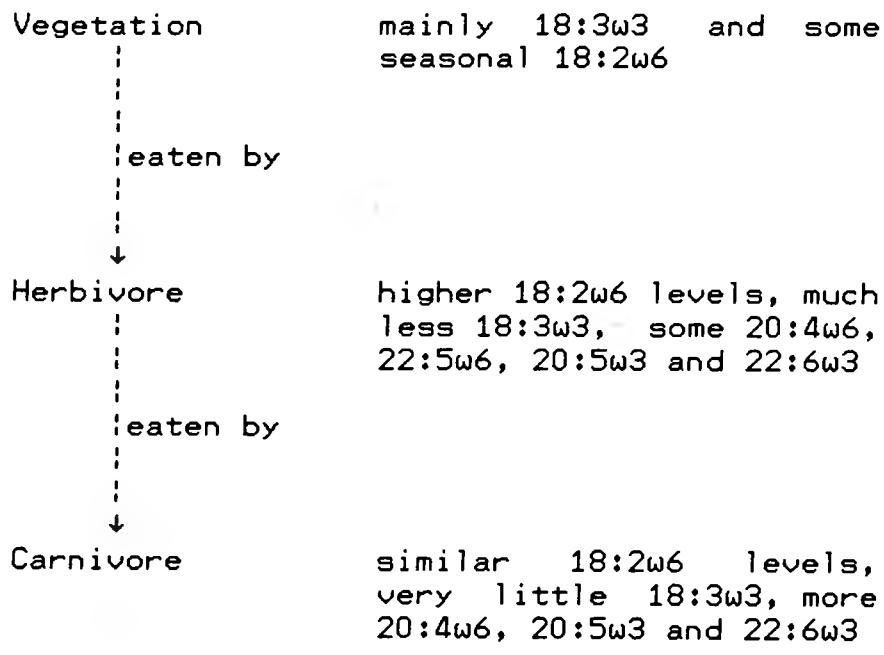
1.1.4.2.b The Importance of Essential Fatty Acids.

The current theories of cellular function depend heavily on membrane function, and thus indirectly on the FA composition of those membranes. Williams et al (1974) showed that the physical state of membrane lipids are altered when the FA composition of the cell membrane was changed. By modulating membrane PGL structure, EFA's confer and dictate the membrane properties of fluidity and flexibility, which are key determinants of membrane permeability and of the behaviour of membrane-bound enzymes and receptors (eg. Burns et al 1979 and Spector et al 1985).

EFA's also serve as substrates for the desaturase enzymes, which produce longer-chain, more unsaturated FA's. These FA's are important, themselves, but three of

Fig. 1.1.4.2.3.

The progression of fatty acids through the food chain (Crawford et al 1971).



them also are the precursors of the eicosanoids (Crawford 1983).

Many authorities suggest that EFA intake should be a minimum of 1.0-1.5% of total calories (eg. Holman 1971), although the FAO/WHO (1977) suggested a minimum intake of 3.0% in adults, and 5.0% in children, pregnant and lactating women.

1.1.4.2.c Abnormalities in Essential Fatty Acid Metabolism.

Abnormalities of EFA metabolism can result from deficient dietary intake, or an intracellular block of the desaturase enzymes primarily responsible for the conversion of the precursor FA to its biologically active product. In the latter case, even a substantially increased dietary intake of EFA's will not alleviate the resultant metabolic abnormalities.

Experimentally induced EFA deficiency induces characteristic symptoms in mammals, which usually include some, or all, of the following (eg. Crawford et al 1972, Rivers et al 1974a, 1974b, Sinclair 1974 and Davidson et al 1987a):

a) Increased metabolic rate
b) Impaired growth
c) Dry scaly skin
d) Poor coat condition
e) Dull encrusted eyes
f) Lassitude
g) 'Wire' hair
h) Hair loss
i) Reproductive abnormalities
j) Open skin lesions
k) Irritability
l) Haemorhage
m) Maldigestion/malabsorbtion
n) Death

Chronic impairment of EFA intake, or blockade of derivative formation, leads to general systemic deterioration, especially the central nervous and cardiovascular systems, ultimately causing death (eg. Davidson et al 1987a). However, all symptoms prior to death are reversible by the administration of EFA's/PDFA's, thus highlighting the critical importance of these substances for the maintenance of normal cellular function.

Three techniques have been commonly used to assess EFA status: measurement of cis-LA intake, measurement of tissue and/or blood LA levels, and measurement of the triene/tetraene (20:3ω9/20:4ω6) ratio. In most species, a ratio of 0.4, or less, is considered normal (Holman 1960, 1971). The use of 20:3ω9 as a marker of dietary EFA deficiency, however, is only valid for species exhibiting an intact desaturase cascade (Holman 1977 and Sprecher 1977).

1.1.4.2.d Essential Fatty Acid Deficiency in the Etiology of Disease.

Many human diseases involve EFA deficiency or distortion of EFA/PDFA equilibrium. In an in depth review, Holman (1986a) reported that EFA/PDFA equilibrium may be distorted by many factors, including the level of dietary FA's, non-optimal intake/uptake of essential nutrients, hormonal effects, and drug therapy. As many of these factors can be assessed, it is possible to re-establish the equibibrium by therapeutic manipulation.

High levels of EFA intake have not been shown to induce any harmful side effects (Anon 1973), and have been shown to reduce both plasma cholesterol and platelet aggregation, and to have anti-thrombotic properties (eg. Dyerberg et al 1978, 1982 and Dyerberg 1986). Considerable evidence exists that suggests EFA involvement in a wide variety of other abnormalities and diseases states (eg. Mead et al 1976, FAO/WHO 1977, Crawford 1980, Horrobin 1982, 1983, Sinclair 1982 and Horrobin et al 1983), including the following:

Poor skin condition	(eg. Hansen 1933)
Cardiovascular disorders	(eg. Sinclair 1956)
Diabetes	(eg. Mercuri et al 1966)
Acrodermatitis enteropathica	(eg. Cash et al 1969)
Abetalipoproteinemia	(eg. Barnard et al 1970)
Cystic fibrosis	(eg. Rivers et al 1975b)

Multiple sclerosis	(eg. Field et al 1978)
Cancer	(eg. Carroll et al 1979)
Schizophrenia	(eg. Obi et al 1979)
Artherosclerosis	(eg. Sinclair 1980a)
Hyperactivity	(eg. Colquhoun et al 1981)
Sjogren-Larsson syndrome	(eg. Holman et al 1982)

Wene et al (1975) observed that EFA deficiency is characterised by a series of changes in the FA pattern of the PGL's. The levels of EFA's fall, but are counterbalanced by an increase in monounsaturates, and by the production of 20:3ω9 (Collins et al 1971 and Holman 1960, 1968, 1971). The ratio's of 18:1ω9/18:0 and 20:3ω9/20:4ω6 thus increase. Peifer et al (1959) showed that EFA deficient rats fed a coconut oil diet (rich in lauric acid) exhibited poor growth, suggesting impairment of EFA metabolism. Similarly, cholesterol addition to EFA deficient diets induces early onset of EFA deficiency symptoms (Takasugi et al 1966), while animal studies indicate that dietary trans-octadecanoic acids exaggerate the signs and symptoms of EFA deficiency (Privett et al 1977 and Hill et al 1979).

Tissue culture can be used to model EFA deficiency in mammalian tissues at a cellular level. Bailey et al (1973a) showed that cells lose most of their LA and AA when grown in FA-free medium, and dependant on the cell type, 20:3ω9 accumulation may occur (Bailey et al 1973a

and Hyman et al 1981a). While studies have shown that normal cell growth and function *in vivo* requires EFA's (eg. Gerschenson et al 1967), some reports indicate that some cell lines can grow normally in lipid-free medium, and with no EFA's detectable in cell lipids (eg. Evans et al 1965). Takaoka et al (1971) showed that in 10 cell lines cultured in lipid free medium EFA's were not detected, that monounsaturated FA levels increased, and no imparement of growth or mitochondrial function was detectable. Bailey et al (1973a) suggested that EFA's were not essential for normal cell division, and that EFA-deficient cultured cells show normal growth rate and morphological appearance. Similarly, respiration, mitochondrial function and other physical and metabolic characteristics were not significantly different from controls. However, the cell membranes were less stable than controls, and thus the long term stability of EFA deficient cells *in vitro* is debatable.

1.1.5 Lipid and Fatty Acid Uptake.

Only monoacylglycerols (MAG's) can be absorbed unmodified through the intestinal wall, while remaining dietary lipid must first be digested to form FFA's and MAG's. This means that of the FA's absorbed from dietary intake, only one definitely remains in its original position in the TAG molecule, while all the others have the potential for re-arrangement during absorbtion

(Clement 1980). Subsequent to absorbtion into the brush border of the jejunum, FA's are resynthesised into TAG's, and chylomicrons formed by addition of apoproteins, cholesterol and PGL's. These chylomicrons then enter the lymphatic system, and subsequently the general circulation.

In the capillaries of adipose tissue or muscle, lipoprotein lipase hydrolyses the TAG's of the chylomicrons, and the FFA's are absorbed into the cells, where TAG's are resynthesised using glycerol-3-phosphate derived from glycolysis. Subsequent mobilisation of TAG's produces FFA's again, which are transported in plasma bound to albumin (Spector 1975), which complex is freely dissociable. The liver posseses albumin receptors which bind the FFA-albumin complex prior to FFA uptake by hepatocytes (Weisiger et al 1981); all other tissues take up FFA's readily without the need for specific receptors (Spector 1968, 1971).

Spector et al (1981a) report that cultured cells also hydrolyse TAG's contained in lipoproteins by the enzyme lipoprotein lipase. Cells take up both FFA's and TAG's, and <u>in vitro</u> studies indicate that FA uptake into cells occurs in a two stage process involving two FA 'pools' (Goodman 1958, Spector et al 1965a, Switzer et al 1965, Bailey et al 1973b, Brenneman et al 1974, Howard et al 1974, 1976, Shohet 1976, Weyman et al 1977, de la Llera 1979, Dise et al 1980 and Morand et al 1982a, 1982b,

1985). Initially, albumin-bound FFA is in equilibrium with a membrane surface pool (F1) of freely exchangeable FFA's. In an energy requiring process, FA's from the F1 pool can then be transposed to a 'deeper', non-exchangable, inner membrane pool of FFA (F2). Studies with erythrocyte ghosts showed that the uptake of radiolabelled palmitic acid was significantly greater in the presence of CoA and ATP, than with ATP alone (Morand et al 1985). The synthesis of acetyl CoA occurred inside these ghosts, suggesting that acyl-CoA synthetase was located in the inner layer of the plasma membrane. The presence of long chain acyl CoA synthetase in plasma membranes of a range of cell types has since been shown by Davidson (1986). Once the FA is across the plasma membrane, an intracellular FA binding protein facilitates its movement into the cytoplasm and its subsequent metabolism (Ockner et al 1974, Mishkin et al 1977 and Brandes et al 1983).

1.2 Lipids in Cells.

Intracellular FA levels in cultured cells reflect a combination of both exogenous FA incorporation and de novo synthesis (Bailey et al 1972 and Howard et al 1974). Thus, lipid composition is regulated by interaction between intracellular metabolism and trans-membrane permeability. When extracellular FA's are depleted, TAG's, and possibly PGL's, act as sources of intracellular lipid; however, de novo synthesis often occurs simultaneously (Bailey et al 1972 and Howard et al 1974).

Although most cultured cells synthesise lipids from amino acids and glucose in the incubation medium, cells derive most of their requirements by uptake if a source of lipid is available (Spector et al 1981a). Numerous studies have shown that cultured cells derive most of their lipids from the serum present in the growth medium (Mackenzie et al 1970, Bailey et al 1972, Howard et al 1974 and Spector et al 1981a). Under conditions in which adequate amounts of lipid are available to cultured cells from the incubation medium, endogenous (de novo) FA synthesis is minimal (eg. Bailey et al 1972). When cells are cultured in a lipid-free, chemically defined medium, however, increased rates of FA synthesis are seen (eg. Bailey et al 1972). These studies thus indicate that cultured cells have the capability to

selectively regulate de novo FA biosynthesis.

Generally, the FA compositions of a variety of cells grown in medium containing serum reflected the FA composition of the medium (eg. Gerschenson et al 1967). Rothblat (1969) reports that under standard culture conditions, serum FA's contributed 80-95% of the FA carbon incorporated into cellular glycerides in WI-38 human fibroblasts. FA supplementation has also been shown to modify the FA composition of many different cultured cell types, and both TAG's and PGL's may become enriched with the FA's supplemented (Glaser et al 1974, Horwitz et al 1974, Spector et al 1979, 1980a, 1980b, Hyman et al 1981b, Kaduce et al 1982, Needleman et al 1982, Bourre et al 1983).

The degree of fluidity of cellular plasma membranes has been shown to be an important factor in many cellular processes, including mitogenesis (Ferber et al 1975), differentiation (de Laat et al 1977), proliferation (Cheng et al 1979) and transformation (Hilgers et al 1978), while interactions between esterified and non-esterified FA's modulate membrane structure (Jeffcoat 1979 and Stubbs et al 1984). Spector et al (1985) describe how altering the physical properties of the membrane with supplementation of different FA's affects carrier mediated transport, receptors, membrane bound enzyme activity, phagocytosis, endocytosis, exocytosis, cytotoxicity, eicosanoid production and cell growth.

Since biological membranes perform a wide variety of functions, including transport, signal transduction, endocytosis, secretion, and also act as permeability barriers, the functional integrity of membranes has been recognised as critical to cell survival (Lodish et al 1979, Op den Kamp 1979 and Storch et al 1985). Complex membrane structures have evolved to perform such functions, and the importance of lipids in the physicochemical properties of biological membranes has been extensively studied (eg. Shinitzky et al 1976, Op den Kamp 1979, Storch et al 1985).

1.2.1. Cellular Triacylglycerols.

Excess lipid/FA in the culture medium inhibits de novo lipid synthesis (eg. Bailey et al 1972). In parallel, others have shown an accumulation of lipid/FA in cells under such conditions, which appears as cytoplasmic droplets of TAG and cholesterol esters (Mackenzie et al 1967, Rothblat 1969, Schneeberger et al 1971, Spector et al 1979, 1981a and Miller et al 1980). The studies of McGee (1981), examining the neutral lipid FA composition of neuroblastoma x glioma hybrids (NG 108-15), showed that in the absence of added FA, little neutral lipid could be detected in the cells. However, upon addition of unsaturated FA's, a large accumulation of neutral lipids occurred, and the FA composition of this lipid reflected the exogenously supplied FA to a greater exent

than did the PGL's. Gavino et al (1981a, 1981b) showed that treatment of human skin fibroblasts with PUFA's increased the numbers of cytoplasmic lipid droplets and lysosomes, while TAG and cholesterol doubled when cells were grown in medium containing 20%, compared to 10%, FBS. Although FA's exchanged with fatty acyl groups of the PGL fraction, the total FA content of the PGL's remained unchanged. This supported the hypothesis that microsomal fatty acyl intermediates can be shunted into neutral lipid droplets when cells are stimulated to accumulate lipid.

The accumulation of TAG has been suggested to occur because the FA's present in the culture medium are in excess of that required for PGL synthesis (Rothblat 1969), or may reflect overloading of the cells ability to degrade lipids (Morand et al 1985). The extent of FA accumulation and TAG droplet formation appears to vary with cell type. When human foreskin fibroblasts or guinea pig aorta smooth muscle cultures were incubated with PUFA's, fibroblasts accumulated much less FA's than did smooth muscle cells (Gavino et al 1981b). This accumulation reverts rapidly after the removal of the supplemented FA from the culture medium. Spector et al (1980a) showed that 45% of TAG's in human skin fibroblasts were degraded within 4 hours after transfer of cells to a medium containing either 10% FBS, or lipid depleted serum. In parallel, Denning et al (1983) showed

that within 6 hours after removal of supplemented FA's, TAG levels fell by 40% in bovine aortic endothelial cells. Nevertheless, both TAG and cholesterol accumulation have been shown to cause cell damage (Howard et al 1974).

1.2.2 Cellular Phosphoglycerides.

PGL's, as major components of cell membranes, are rarely utilised as metabolic fuels, except in pathological circumstances (Rothblat 1969). This, however, does not imply metabolic inertia. Spector et al (1967), using Ehrlich's ascites cells, showed rapid turn over of membrane PGL FA's, mainly in the phosphatidylcholine fraction. The work of Peterson et al (1969, 1970) not only implied cycling of cellular PGL, but also showed an exchange of cellular PGL between membrane structures of adjacent cells. There are reports that exogenous FA dosage can lead to extensive alterations in cell PGL FA composition (eg. Spector et al 1979, 1985), with changes as large as 40 fold reported in the monounsaturated:polyunsaturated FA ratio in membrane PGL's when FA's were added directly to serum-containing culture media (McGee 1981). On the other hand, human umbilical vein endothelial cells exhibited reductions in PGL LA, from 27% to 12%, during the first 48 hours after transfer to a medium containing 20% FBS (Spector et al 1980b). Spector et al (1981a) reported that cells

regulate the PUFA content of their PGL's to between 15% and 50%, and that an increase in PGL FA polyunsaturation may be responsible for controlling cell growth. This supports previous studies which also indicated a possible role for PGL's in the regulation of cell division, based on the fact that membranes contain nearly all of the PGL's that occur in cells and that membrane structures are intimately involved in DNA replication in mammalian cells (Spector et al 1967, Hanaoka et al 1971, Mizuno et al 1971, Cunningham 1972 and Manzoli et al 1974).

1.3 Fatty Acid Desaturation and Elongation.

Since the first description of the oxidative desaturation of FA's in animals (Bernhard et al 1959) and in bacteria (Bloomfield et al 1960), several reviews have been devoted to the subject (eg. Brenner 1971, 1974, 1982, James 1977, Jeffcoat 1979a and Jeffcoat et al 1984). In mammals, cis-FA's can be converted to longer chain, more unsaturated derivatives by a process of sequential desaturation and chain elongation. The subsequent FA products are important as themselves, as well as being the precursors for many other essential biochemicals involved in metabolism. This cascade is under strict metabolic control, both directly and indirectly, and serves as a mechanism for regulating the lipid composition of cells.

1.3.1 The Location and Mechanism of Fatty Acid Desaturation and Elongation.

Mammalian long chain FA's are desaturated and elongated in membrane bound vesicles formed from the endoplasmic reticulum (microsomes) (Ayala et al 1973 and Brenner 1982). The enzymes responsible, the fatty acyl desaturases and elongases, are integral proteins of the microsomes. They are tightly bound to the inner microsomal membrane and require an intact membrane structure for their activity (Bloomfield et al 1960,

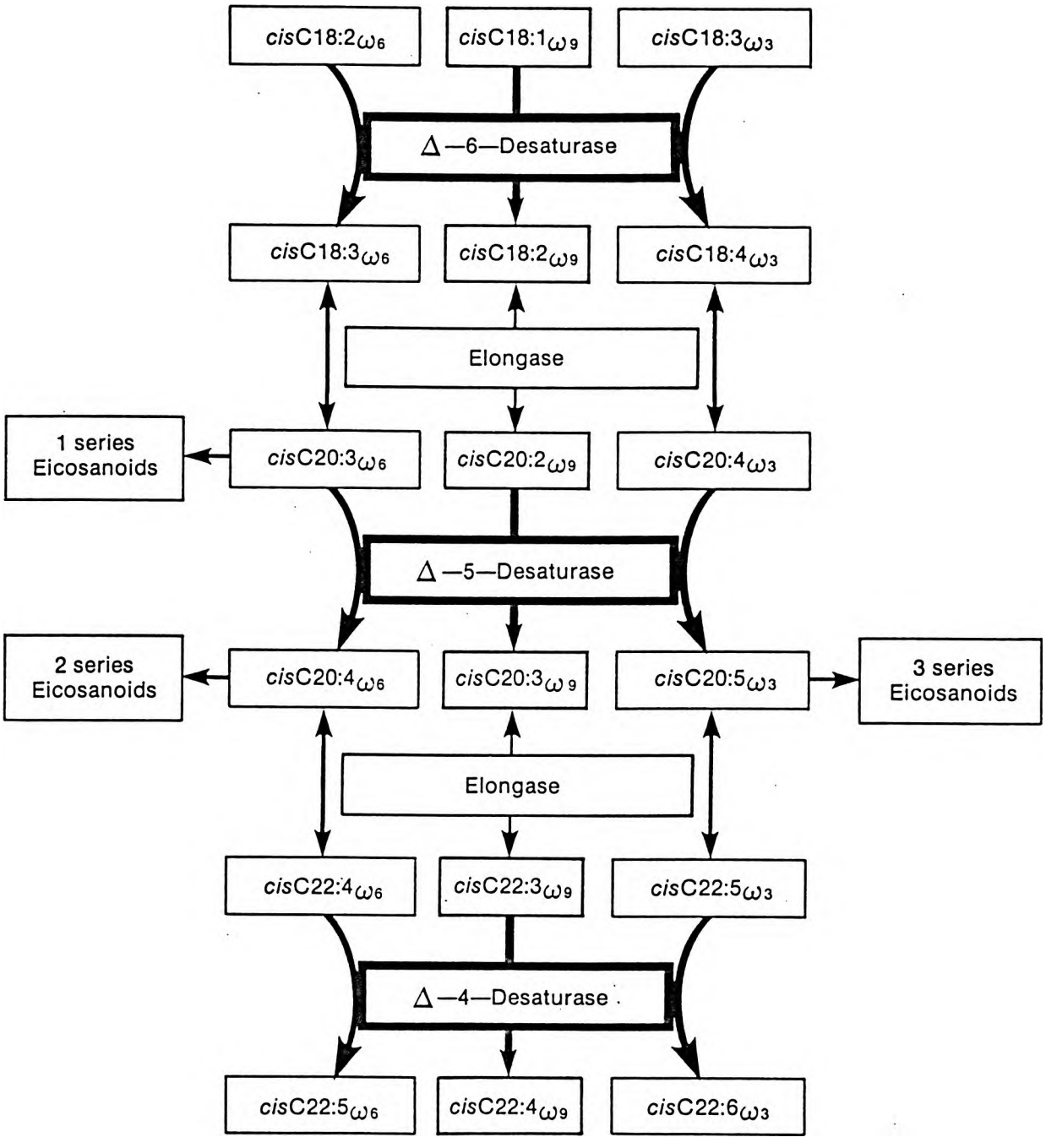
Nugteren 1960, 1962, 1965, Stoffel 1961, Oshino et al 1971, 1972, Ayala et al 1973, Brenner 1974, 1982 and Fulco 1974).

Unsaturated FA's are synthesised aerobically from saturated FA's by a microsomal, 3 component enzyme complex, consisting of NADH-cytochrome b_5 reductase, cytochrome b_5 and the terminal desaturase enzyme, often referred to as the cyanide sensitive factor (Holloway 1963, Gellhorn et al 1964, Holman 1964, Oshino et al 1966, 1971, 1972, 1973, Brenner et al 1969, Holloway 1971, Shimokata et al 1972, Strittmatter et al 1974, Lee et al 1977, Okayasu et al 1976 and Brenner 1982). This process requires both ATP and Mg^{2+} (Nugteren 1962, Brenner 1974 and Cook 1978a).

Desaturation requires prior activation of FA's to acyl-CoA, via plasma membrane acyl:CoA synthetase (Brenner 1971). This enzyme has been demonstrated in several rat and human tissue plasma membranes by Davidson et al (1986a, 1986b), is non-rate-limiting, and shows a substrate preference of ω3 PUFA's > ω6 PUFA's > ω9 monoenoics > saturated FA's. This activity profile mirrors that of the desaturase enzymes (Kanau et al 1977). Not only is acyl-CoA formation many times more rapid than desaturation (eg. Pande et al 1968, Marcel et al 1972 and Sprecher 1981), but the NADH-cytochrome b_5 electron transferring system is common to all the desaturases (Brenner 1982). In addition, the electron

Fig. 1.3.2.1.

The desaturation pathway for unsaturated fatty acids.



flux generated is many times greater than the requirements of the desaturases (Sprecher 1981 and Brenner 1982), thus the enzyme itself is the rate limiting factor (Brenner 1971 and Raju et al 1972).

The enzymes which catalyse these desaturations appear to have a Coenzyme A binding site, which recognises the fatty acyl-CoA substrate. It seems likely that the methylene chain of the FA fits into a restrictive cleft of finite length (Brett et al 1971 and Do et al 1975). For the full activity of these enzymes to be expressed, cytoplasmic protein factors are required (Catala et al 1975, 1977, Jeffcoat et al 1976, 1977a, 1978 and Leikin et al 1979, 1986, 1989). Crude microsomal preparations showed delta-9-, delta-6- and delta-5-desaturase activity, which activities were lost with repeated washing, but were then restored by the addition of cytoplasmic proteins.

Chain elongation of unsaturated FA's occurs at the carboxyl terminal, while desaturation occurs between the carboxyl group and the first double bond from the carboxyl group. Thus, the number of carbon atoms between the methyl terminal and the double bond nearest this remains constant (Davidson et al 1985a).

Since microsomal membranes contain lipids, the composition of which may be modulated (eg. Nervi et al 1968 and Garg et al 1988a, 1988b, 1988c), it has been

suggested that the microsomal lipid composition may be involved in regulation of the desaturase enzymes (Garda et al 1984, 1985 and Kurata et al 1980). Membrane PGL's may also serve as sources of enzyme substrates (Pugh et al 1984).

1.3.2 The Biosynthetic Pathway of Unsaturated Fatty Acid Synthesis.

The desaturase cascade primarily consists of three desaturase enzymes, delta-6-desaturase (Δ6D), delta-5-desaturase (Δ5D), and delta-4-desaturase (Δ4D) (Fig. 1.3.2.1.). These enzymes, and especially Δ6D, are the rate limiting steps in the desaturase cascade (Marcel et al 1968, Brenner 1974, Hassam et al 1975 and de Gomez Dumm et al 1976). The first enzyme is specific for C18 FA's, the second for C20 FA's and the third for C22 FA's, and the enzymes exhibit greater activity with ω3 than with ω6, and least activity with ω9 substrates (Brenner et al 1966, Mead et al 1976 and Kanau et al 1977). It is also apparent, since mammals cannot interconvert ω3, ω6, and ω9 FA's (eg. Crawford et al 1972), that the desaturase/elongase pathway will produce 3 distinct families of related compounds (Crawford et al 1971, Sprecher 1977 and Sinclair 1984). Different products of the pathway do, however, compete with each other (Holman et al 1963, Holman 1964 and Mohrhauer et al 1963a). This competition can be displaced in either

direction, and the FA favoured in this competition is dependant on relative substrate levels. In general, ω3 inhibit the metabolism of ω6 and ω9 FA's, and ω6 inhibit that of ω9 (eg. Bernert et al 1975). Consequently, the metabolites of oleic acid are only found in trivial amounts under normal physiological conditions. The ability not only to distinguish clearly between FA's, but at the same time to appreciate the relationships between members of a homologous series, is therefore, vitally important.

Within any unsaturated FA metabolic pathway, rates of desaturation are generally slower than those for chain elongation (Bernert et al 1975, Brenner 1971, Sprecher 1977 and Naughton 1981), and there is evidence that the desaturation rate for membrane-bound substrate may be greater than that for exogenously added FA's (Aeberhard et al 1981). Thus, misleading conclusions may arise about enzyme activity when isolated enzymes are assayed (Aeberhard et al 1981 and Hill et al 1982). Furthermore, the elongation steps are generally easily reversible, thus retroconversion probably depends on substrate availability, while the desaturation steps are usually not reversable <u>in vivo</u> (Mead et al 1960, Stearns et al 1967, Ayala et al 1973 and Gavino et al 1981b).

Although a number of different pathways for desaturation and elongation have been reported (eg. Brenner 1971 and

Gurr et al 1971), the preferred route appears to be the one described (Fig. 1.3.2.1.), with initial desaturation followed by chain elongation (Marcel et al 1968, Bernert et al 1975 and Sinclair 1984). Other pathways appear to be of minor importance in mammals (Brenner 1971, Ullman et al 1972, Lands et al 1977 and Sprecher 1981).

1.3.3 The Desaturase Enzymes.

1.3.3.1 Δ-6-Desaturase.

Δ6D is the first, and rate-limiting, enzyme of the desaturase enzyme cascade (Stoffel 1961, Holloway 1963, Marcel et al 1968, Oshino et al 1972, Brenner 1974, Hassam et al 1975, de Gomez Dumm et al 1976, Mead et al 1976, Kanau et al 1977 and Sprecher 1981). This enzyme catalyses the formation of 18:3ω6 and 18:4ω3 (Fig. 1.3.2.1.), is essential for the full expression of EFA biological activity (Frankel et al 1975), and requires zinc (Huang et al 1982).

Okayasu et al (1981) isolated and purified the enzyme from rat liver microsomes and showed it to be a single polypeptide chain of 66000 Daltons. The liver enzyme exhibits the greatest activity, especially during prenatal brain growth, when PUFA requirement is high, and maternal supply may be limiting (Brenner 1971, Sinclair 1974, 1975a, 1975b, Naughton 1981 and Novelot et al 1986).

The regulation of Δ6D has been reviewed extensively by Jeffcoat (1979) and Brenner (1982), and evidence to date indicates that Δ6D exhibits a particular substrate specificity pattern (Brenner et al 1969). As with the other desaturates, Δ6D is more active with ω3, than with ω6 or ω9, substrates (eg. Kanau et al 1977). This may reflect a selective mechanism whereby the much lower dietary levels of ω3 FA's are preferentially utilised. Δ6D activity is extremely sensitive to slight changes in the metabolic and physical environments (Cook 1979), and has a circadian rhythm (Actis Dato et al 1972). In vivo and in vitro studies indicate that Δ6D is inhibited very easily by a wide range of different factors. These are summarised in Figure 1.3.3.1.1., and include dietary, hormonal and pathological factors. While Δ6D is the rate limiting step and is easily inhibited (eg. Brenner 1982), few factors inhibit/suppress elongation. In fact, while conditions such as diabetes and malnutrition suppress Δ6D activity, elongation is either conserved or enhanced (Gellhorn et al 1964, Peifer et al 1965, Brenner et al 1968a, Ayala et al 1973 and Holman et al 1981).

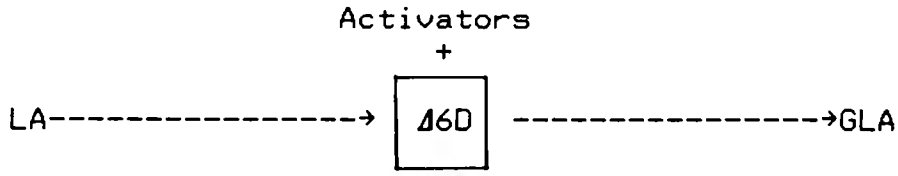
Fig. 1.3.3.1.1.

Factors that modify Δ6D activity.

Dietary
Moderate food restriction (1)
EFA deficiency (1)
Fat-free diet (1)
Carbohydrate deficient diet (1)
Protein rich diet (2)

Hormonal
Low insulin levels (1)
ATP (1)

Other
Temperature decrease (1)



Dietary
Fasting (1)
Protein deficiency (3)
High glucose levels (4)
High carbohydrate levels (2)
High cholesterol levels (5)
High saturated FA levels (6)
High trans-FA levels (7)
FA reaction products (1)

Pathological
Diabetes (8)
Transformation (9)
Atopic eczema (10)

Hormonal
cAMP mediated mechanisms (11)
Glucagon (11)
Thyroxine (12)
Epinephrine (13)

Other
Temperature increase (1)
Zinc deficiency (14)
Mg^{2+} deficiency (15)
Alcohol (16)
Aging (17)

(1) eg. Brenner (1982)
(2) eg. de Gomez Dumm et al (1970)
(3) eg. de Tomas et al (1980)
(4) eg. Brenner et al (1968a)
(5) eg. Garg et al (1986)
(6) eg. Brenner et al (1966)
(7) eg. Cook (1981)
(8) eg. Gellhorn et al (1964)
(9) eg. Dunbar et al (1975)
(10) eg. Manku et al (1982a)
(11) eg. de Gomez Dumm et al (1975a)
(12) eg. Hoch (1981)
(13) eg. de Gomez Dumm et al (1986)
(14) eg. Huang et al (1982)
(15) eg. Mahfouz et al (1989)
(16) eg. Nervi et al (1980)
(17) eg. Bordoni et al (1988)

1.3.3.2 Δ5-Desaturase.

Δ5D catalyses the conversion of 20:3ω6 and 20:4ω3 to 20:4ω6 and 20:5ω3, respectively (Fig. 1.3.2.1.). As with Δ6D, Δ5D activity varies depending on animal species (Marcel et al 1968, Danon et al 1975, Rivers et al 1975a, 1976, Stone et al 1979 and Chapkin et al 1984). The enzyme is more active with ω3, than with ω6, substrates (eg. Kanau et al 1977), and thus exhibits a much higher affinity for 20:4ω3 than for 20:3ω6. Thus, 20:4ω3 is rapidly converted to 20:5ω3, while only some 20:3ω6 is converted to 20:4ω6. 20:3ω6 and 20:4ω6 are the precursors for the 1- and 2-series eicosanoids, respectively, while 20:5ω3 is the precursor for 3-series eicosanoids (eg. Crawford 1983 and Lands 1986). Thus, the activity of Δ5D may regulate the balance between the different series of eicosanoids. Indeed, Δ5D has been suggested to act as a secondary regulatory point in the desaturase cascade (Castuma et al 1972).

The regulation of Δ5D has been reviewed by Jeffcoat (1979) and Brenner (1982). Many of the inhibitors of Δ6D are also effective inhibitors of Δ5D. Large intakes of cholesterol, oleic acid and ethanol inhibit Δ5D function (Lowry et al 1966, Alfin Slater et al 1968 and Nervi et al 1980), and there is also evidence that excess vitamin A suppresses this reaction (Huang et al 1985). As in the case of Δ6D, a low protein intake inhibits, and a high

protein intake stimulates, Δ5D (Castuma et al 1972, de Gomez Dumm et al 1972, Mercuri et al 1979 and de Tomas et al 1980). Contrary to Δ6D, however, EFA deficiency induces decreased Δ5D activity (Peluffo et al 1976 and Brenner et al 1982). Rats maintained on a fat-free diet exhibit a significant reduction of Δ5D activity compared to animals on a balanced diet (de Gomez Dumm et al 1983). In contrast, 18:2ω6 supplementation of an EFA deficient diet increases Δ5D activity to almost control levels, while Δ5D activity is not affected by dietary carbohydrate (Jeffcoat et al 1977b and Castuma et al 1982). Jeffcoat et al (1977c) have, however, found that triolein is a very good activator of Δ5D, but a depressor of Δ6D.

Unlike Δ6D, Δ5D does not appear to be suppressed by cell transformation (Dunbar et al 1975). In diabetes, the activity of Δ5D is only slightly reduced (Gellhorn et al 1964, Brenner et al 1968, Peluffo et al 1971 and Castuma et al 1972). Insulin increases Δ5D, but most other hormones decrease its activity. Like Δ6D, Δ5D activity is reduced by glucocorticoids, epinephrine and dibutyryl cAMP (de Gomez Dumm et al 1979, 1980). It has been reported that Δ5D activity is lost in Man after puberty (eg. Dyerberg et al 1980a and Horrobin 1982, 1983), although Mest et al (1983) report the opposite.

1.3.3.3 Δ4-Desaturase.

The formation of 22:5ω6 from 20:4ω6 via Δ4D (Fig. 1.3.2.1.) has been studied considerably less than the desaturation of 20:5ω3 to 22:6ω3, and Δ4D has been studied much less than Δ6D and Δ5D. FA's of 22 carbons are, however, important as they contribute considerably to the lipids of cell membranes in general, and brain cells in particular (eg. Svennerholm 1968). Desaturation and chain elongation of 20:4ω6 to 22:5ω6 has been demonstrated in liver microsomes of rats fed a fat-free diet (Hagve et al 1984). A separate study feeding radiolabelled [^{14}C]-20:5ω3 showed the formation of 13% 22:6ω3 in rat hepatocyte microsomes, but no Δ4D activity with [^{14}C]-22:4ω6 as substrate (Hagve et al 1986). Testis Δ4D activity appears to be greater than in the liver (Clejan et al 1982), but activity has been shown to be absent in many cell lines, including the C1300 neuroblastoma (Yavin et al 1975). This may be surprising since neural tissue is reported to contain large amounts of PGL 22:6ω3, but may reflect a requirement for dietary supply. Traces of Δ4D activity have, however, been reported by others in human skin fibroblasts (eg. Aeberhard et al 1978). While the varied Δ4D activities found in these studies may reflect experimental differences, it is more likely that Δ4D activity varies with cell type.

It appears that many factors influencing Δ6D alter Δ4D activity in a similar manner, eg. zinc deficiency (eg. Clejan et al 1982). While a diet rich in LA has been found to cause depletion of long chain ω3 FA's (Lepran et al 1981), a number of workers have also found that EFA deficiency depletes cellular C22 FA's (Dunham et al 1978 and de Schriver et al 1982), while low levels of C22 FA's have been demonstrated in many pathological states, including diabetes (eg. Brenner et al 1968a).

1.3.3.4 Δ9-Desaturase.

Δ9D catalyses the conversion of either de novo synthesised or dietarily derived 18:0 to 18:1ω9 (Fig. 1.3.2.1.) (Bernert et al 1975, Brenner 1982, Sinclair 1984). The enzyme consists of a three-component protein and the rat liver enzyme has a molecular weight of 53000 Daltons (Strittmatter et al 1974).

Control of Δ9D activity shows a different pattern from those of Δ6D or Δ5D (Brenner 1974, Jeffcoat 1979a and Nervi et al 1980). For example, Δ9D is not inhibited by ethanol, as are both Δ6D and Δ5D, and is only modulated significantly by dietary composition. A high protein diet decreases Δ9D activity (Peluffo et al 1984), while a high cholesterol diet enhances its activity (Garg et al 1986). The activity of Δ9D is depressed by fasting (Inkpen et al 1969 and Oshino et al 1972) and also by

diets containing cis-linoleic acid (Jeffcoat et al 1977, 1978, 1979, Brenner 1982 and Sinclair 1984). Conversely, Δ9D activity is increased dramatically by feeding diets high in carbohydrate (de Gomez Dumm et al 1970, Peluffo et al 1971, Oshino et al 1972, de Tomas et al 1973, Brenner 1982 and Sinclair 1984). A fat-free diet also enhances Δ9D activity (Paulsrad et al 1970, Brenner 1974 and Jeffcoat et al 1977), but addition of EFA's to a fat-free diet inhibits the enzyme (Jeffcoat et al 1977, 1978 and de Gomez Dumm et al 1982). Saturated FA effects on Δ9D activity are less clear, with both increased (Inkpen et al 1969 and Mercuri et al 1974) and decreased (Jeffcoat et al 1977a) activities reported, possibly reflecting differences in experimental design. While the rate of delta-9-desaturation is markedly depressed in diabetics, this reflects low levels of the desaturase rather than impairment of activity (eg. Gellhorn et al 1964, Brenner et al 1968b, Peluffo et al 1971, Castuma et al 1972 and Prasad et al 1979).

1.3.4 Fatty Acid Regulation of the Desaturase Cascade.

Brenner (1974) showed that 18:2ω6 and 18:3ω3 compete for Δ6D, with 18:3ω3 being the preferred substrate. 18:3ω3 inhibits the metabolism of ω6 FA's by competitively inhibiting Δ6D (Mohrhauer et al 1963b, Brenner et al 1966, 1977 and Garg et al 1988a), while 18:2ω6 only weakly inhibits conversion of 18:3ω3 (Holman et al 1964 and Actis Dato et al 1970). Thus, dietary 18:3ω3 may decrease the amount of 20:4ω6 in tissues primarily by limiting Δ6-desaturation of 18:2ω6 (Holman et al 1963 and Mohrhauer et al 1963a). Budowski (1981) reported that a diet rich in linseed oil depressed 20:4ω6, and raised 18:2ω6, concentrations in liver PGL's of chicks and in plasma lipids of both rats and chicks. In parallel, constant dietary 18:2ω6 levels, with increasing 18:3ω3, decreased the proportion of 20:4ω6 in rat liver and plasma FA's (Hwang et al 1980). However, competitive inhibition between ω3 and ω6 FA's depends largely on the dietary ratio's of these FA's (Holman et al 1964 and Cook et al 1987). Kramer (1980) suggested that dietary 18:3ω3 only inhibits Δ6-desaturation of 18:2ω6 if the 18:3ω3 content of the diet is greater than 15%, further implicating the importance of dietary EFA levels in the control of desaturation. Nevertheless, both 18:2ω6 and 18:3ω3 inhibit 18:1ω9 desaturation, thus preventing 20:3ω9 synthesis (Brenner 1966, 1969, 1982

and Holman 1986b). However, when 18:2ω6 and/or 18:3ω3 levels are low, the inhibition is removed, and 18:1ω9 is metabolised to 20:3ω9 (Brenner et al 1965a, 1965b and Sprecher 1981). While it has been suggested that 20:3ω9 could substitute for long chain ω3 and ω6 FA's in maintenance of cell membrane flexibility during EFA deficiency (eg. Horrobin 1983), it is known to inhibit prostaglandin synthesis (Okazaki et al 1974, 1978), and also may block Δ5D activity (Lowry et al 1966).

Chang et al (1973) used rat liver microsomes to show the effect of double and triple bonds in FA inhibition of stearic acid desaturation. The position of one cis-double bond in an 18 carbon FA chain affected the ability of that acid to inhibit stearic acid desaturation. The isomers found to be most inhibitory were those of cis-octadecenoic acid; in those cases in which both cis- and trans-isomers were tested, the trans-acids were either equally, or less, inhibitory than the corresponding cis-isomers (Chang et al 1973). Kinsella et al (1981) reported that the trans-isomers of 18:1, other than elaidic acid, can be desaturated by Δ9D to yield trans,cis dienes. Dietary trans-18:1ω9 also inhibits conversion of 18:1ω9 to 20:3ω9 and of 18:2ω6 to 20:4ω6 by acting as a competitive inhibitor of the desaturase (Kinsella et al 1981).

The decrease in monounsaturated FA's in rat tissues

(16:1ω7 and/or 18:1ω9) induced by diets containing high levels of 18:2ω6 and/or 18:3ω3, is consistent with PUFA inhibition of Δ9D activity (Mahfouz et al 1984 and Pugh et al 1984). Ullman et al (1971a) prepared microsomes from male weanling Sprague-Dawley rats and investigated the role ω6 FA's play in inhibiting the ω9-desaturation step. 18:2ω6, 20:3ω6 and 20:4ω6 inhibited Δ9-desaturation of 18:0, while 20:3ω6, 18:3ω3 and 20:4ω6 inhibited Δ5-desaturation of 20:2ω9. When Garg et al (1988b) studied Δ9D activity in liver microsomes of rats fed diets enriched with either saturated FA's, 18:3ω3 (linseed oil), or 20:5ω3 and 22:6ω3 (fish oil), both linseed and fish oils inhibited Δ9D activity, with fish oil being more effective.

Both 18:2ω6 and 20:3ω6 suppress 18:2ω9 formation, but 20:4ω6 does not (Brenner et al 1966, 1969 and Mohrhauer et al 1967). 20:4ω6 does, however, inhibit desaturation of EFA's (eg. Irvine 1982), thus high dietary levels of this FA may inhibit Δ6D. 20:4ω6-rich foods, such as red meat, may well inhibit desaturases by negative feedback, and this can generate an imbalance of both PDFA's and eicosanoids (Crawford et al 1970). In contrast, while 18:3ω6 only weakly inhibits 18:3ω3 desaturation, it effectively inhibits desaturation of both 18:2ω6 and 18:1ω9 (Brenner 1971, 1974 and Cook et al 1987). There is also monounsaturated FA involvement in the control of polyenoic FA desaturation. Dhopeshwarkar et al (1963)

demonstrated that dietary monoenoic FA's suppressed LA desaturation, while Mahfouz et al (1980a, 1981) showed that liver microsomal desaturation of both LA and DGLA were inhibited by positional isomers of 18:1ω9.

Long chain ω3 FA's also inhibit/suppress desaturation; both EPA and DHA act as analogues of AA to inhibit Δ6D by a feedback mechanism, and DHA is a highly effective inhibitor of Δ6D (eg. Garcia et al 1965, Brenner et al 1966, 1967, Brenner 1974 and de Schrijver et al 1982). Studies suggest that EPA is less effective than DHA in inhibiting Δ6D (Brenner et al 1966, Alfin-Slater et al 1968 and Bernert et al 1975), but more effective than DHA in inhibiting conversion of DGLA to AA via Δ5D (Nassar et al 1986). Still other studies showed that liver microsomes from rats fed EPA showed depressed Δ6D activity (Kurata et al 1980), while DHA decreased OA, LA and ALA desaturation (Brenner et al 1966). While EPA effectively inhibits desaturation of 20:4ω3 by feedback mechanisms, DGLA desaturation is also inhibited (Garcia et al 1965 and Alfin-Slater et al 1968). Others showed competitive inhibition of Δ6D in liver microsomes of rats fed linseed or fish oils (Garg et al 1988a), apparently by competition with 18:2ω6 for Δ6D. Fish oil was significantly more inhibitory than linseed oil. In a subsequent study, Garg et al (1988c) showed that Δ5D desaturation of DGLA to AA was greater than control levels in rats fed linseed oil and lower in those fed

fish oil. These studies demonstrate that while both linseed and fish oils limit Δ6D, only fish oil limits Δ5D; the lower levels of AA when linseed oil was fed thus reflects reduced Δ5D substrate formation due to suppression of Δ6D.

While desaturase cascade FA's have the capability to inhibit/suppress desaturase activity, there is also evidence to suggest that FA's enhance enzyme expression. In a series of studies on rat heart, liver, adipose tissue, erythrocytes and brain, Mohrhauer et al (1963a, 1963b, 1963c, 1963d, 1963e) showed that as the amount of dietary 18:2ω6 or 20:4ω6 increased, their ω6 PUFA derivatives increased in tissue lipids, while ω3 PUFA's decreased. Furthermore, Holman (1971) reported that 18:2ω6 promotes desaturation of 20:3ω6 to 20:4ω6. On the other hand, Marshall et al (1982) showed that increasing the dietary level of 18:2ω6 eventually led to a decreased level of 20:4ω6, implying suppression of the cascade. Thus, the exact roles of substrate and products of the desaturases in the regulation of the enzyme activity is unclear.

While the desaturation steps are rate limiting and under direct control by FA substrate/product levels, the elongation steps may also be modulated by FA's. Christiansen et al (1968) studied the effects of unsaturated FA's on chain elongation of 18:3ω6 in microsomes, and showed that 18:2ω6, 20:2ω6 and 20:4ω6

enhanced the reaction, while 18:3ω3 and 22:6ω3 were inhibitory. Although 18:1ω9, 20:3ω3 or 20:5ω3 were not effective, two unusual PUFA's, 18:3ω7 and 17:3ω6, were both inhibitory. The latter was the most effective, suggesting that the ω1-ω6 structure was the important factor. In addition, Banerjee et al (1986) showed that while 20:5ω3 is an effective inhibitor of 20:4ω6 elongation, both 20:4ω6 and 20:5ω3 had the capability to modulate their own elongation.

The influence of trans-C18 FA's on *in vivo* and *in vitro* desaturation and elongation in rat brain was studied by Cook (1981). Linoelaidate was highly inhibitory of chain elongation *in vitro*. *In vivo*. both desaturation and elongation of linoleate were inhibited by both oleate and linoelaidate, while elaidate and stearate were stimulatory. Mahfouz et al (1980a, 1981) investigated the effects of trans-C18:1 FA's on Δ9D, Δ6D and Δ5D using rat liver microsomes, and showed that not only were these FA's inhibitory, but the position of the double bond in the trans-C18:1 FA appeared to be important in determining the degree of inhibition. Although chain elongation of some positional isomers of trans-C18:1 were shown, other studies demonstrated considerably slower rates of elongation than Δ9 desaturation (Kameda et al 1980).

These studies illustrate that the balance between ω3, ω6

and ω9 FA's are an important regulatory factor for the modulation of desaturase cascade enzyme activity, and that high levels of a desaturase reaction product may block desaturation of other FA's of both the same and different families by earlier enzymes in the cascade. At the same time, high levels of any FA substrate may stimulate desaturation of other FA's in the cascade. To date, almost every FA substrate/product in this cascade has been shown to be involved in the control of overall desaturation/elongation. A blockade of any step, but primarily Δ6D, will lead to functional PDFA deficiency, even if EFA levels, or the triene:tetraene ratio, are normal. Consequently, impairment of enzyme activity and subsequent changes in PDFA levels have been reported to lead to altered membrane structure, interference with membrane associated processes, EFA deficiency symptoms, impaired eicosanoid metabolism and the perturbation of eicosanoid mediated responses (Blank et al 1976, Horrobin 1983, Cantrill et al 1984 and Jones et al 1986), involving the cardiovascular and immune systems, brain function and cell proliferation, among others.

1.3.5 Fatty Acid Desaturation in Disease States.

A substantial volume of evidence implicates derangement of the desaturase enzyme cascade in diverse clinical conditions. Diabetes mellitus is one of the most common conditions in which Δ6D activity is deranged. Mercuri et al (1966) and de Alaniz et al (1969) demonstrated that the conversion of 18:2ω6 to 18:3ω6 was blocked, that 20:4ω6 and longer chain 22-carbon FA's were depleted, and that desaturation could be restored by low doses of insulin injected into alloxan diabetic rats. While elongation appears to be conserved in diabetes (Ayala et al 1973), Poisson et al (1978) reported 70% inhibition of Δ6D in alloxan diabetic rats, and Faas et al (1980) demonstrated that the conversion of 18:2ω6 to 18:3ω6 was only 50% of controls in streptoyotocin diabetic rats. Elevated 18:2ω6 and lowered 20:4ω6 levels were found by Jones et al (1983) in platelets from diabetic patients, implicating a Δ6D blockade, subsequently confirmed by others (eg. Horrobin 1983 and El-Boustani et al 1986). EFA requirements are considerably enhanced in diabetes, since excess 18:2ω6 is required to overcome the partial Δ6D blockade (Peifer et al 1965).

Defective desaturase, particularly Δ6D, function has also been implicated in pathological conditions such as:

Cancer	eg. Yavin et al (1975)
Alcoholism	eg. Nervi et al (1980)
Liver cirrhosis	eg. Blond et al (1981)
Scaly dermatoses	eg. Ziboh (1981)
Pre-menstrual syndrome	eg. Brush (1982)
Crohn's Disease	eg. Holman et al (1982)
Cystic fibrosis	eg. Holman et al (1982)
Atopic eczema	eg. Manku et al (1982b, 1984)
Hypercholesterolemia	eg. Garg et al (1986, 1988a)
Atopic asthma	eg. Rocklin et al (1986)

In most of the above, plasma linoleate levels are raised, while Δ6D products are low, indicating enzyme blockade. In an extensive report, Horrobin (1982a) indicated that in some of these conditions, oral treatment with GLA produced significant improvements in both the biochemical and clinical state, further supporting the suggestion of Δ6D impairment.

1.3.6 Fatty Acid Desaturation in Mammalia.

Some species of the Rodentia show marked ability to desaturate PUFA's, and EFA's in particular (Brenner 1971, 1974 and Sinclair 1974). Desaturation capability, however, varies considerably between different rat tissues. Brenner (1971) showed that the percentage desaturation of LA to GLA in isolated microsomes from young rats was 20% in adrenals, 14% in liver, 6% in testes and less than 2% each in heart, kidney and brain. No desaturation could be detected in lung or epididymal fat (Brenner 1971), although Gellhorn et al (1964) found that rat adipose tissue was able to desaturate SA to OA. Δ5D is active in the rat, but appears to be more active in the mouse (Anon 1979, Stone et al 1979 and Blond et al 1981). Both species, however, exhibit greater Δ5D activity than other mammalia, eg. rabbit, guinea pig or Man (Stone et al 1979). Δ5D catalyses a slow reaction (eg. Bernert et al 1975), and rat tissues such as testes (Ayala et al 1973) and adrenals (Brenner et al 1967 and Takayasu et al 1970) often accumulate DGLA, particularly in the fed state. Danon et al (1975) showed that while DGLA levels are generally low in the rat, an increased ratio of DGLA to AA, with only a small increase in AA, was found when DGLA was fed. Thus, it appears that Δ5D activity is not stimulated by DGLA-rich diets. Little attention has being focussed on Δ4D, although the enzyme has been found in rat testes by Bridges et al (1970)

and Ayala et al (1973), who also showed retroconversion of 22:4ω6 to 20:4ω6.

Rivers et al (1975a) published biochemical evidence indicating that the cat lacked Δ6D; this confirmed the obligate carnivore nature of the domestic cat. Numerous subsequent studies showed that GLA is essential for the cat (MacDonald et al 1983a, 1984). Other species of the Felinae, such as the lion and cheetah, have been shown to exhibit a similar enzyme deficiency (Rivers et al 1976 and Davidson et al 1986c, 1986d). The lack of Δ6D is thus probably a feature common to the Felinae as a Family. At least one wild species of the Canidae (the wolf) appears to possess an active form of Δ6D, while some breeds of domestic dog, however, appear to have impaired Δ6D activity (Horrobin et al 1990 and Lloyd 1990). The exact extent of the deficiency within the Carnivora is thus, not certain. Frankel et al (1978) showed that while chain elongation was unimpaired, the cat also lacked Δ5D. Stone et al (1979) similarly provided evidence to suggested the absence of Δ5D, but this has been largely contradicted by other <u>in vivo</u> studies, which showed signs of Δ5D and Δ4D activity in the cat (Rivers et al 1978, Sinclair 1979, Kane et al 1981, MacDonald et al 1983a, 1984 and Davidson et al 1989, 1990). Thus, it appears that PDFA's can probably be metabolised by the cat to form eicosanoids and their precursors.

The presence of long chain PDFA derivatives of LA and ALA in vegans suggests that Man can metabolise parent EFA's (Sanders et al 1978). While human liver has been shown capable of desaturating [^{14}C]-LA and [^{14}C]-ALA, the rate is slow (de Gomez Dumm et al 1975b). Others also report limited Δ6D activity in Man (Dyerberg et al 1980 and Horrobin 1983). Humans also exhibit limited Δ5D activity, even in liver (Willis et al 1976, Anon 1979, Stone et al 1979, Blond et al 1981 and El-Boustani et al 1986). This may reflect limited Δ6D activity, although numerous studies suggest that Δ5D itself is not very active. Despite administering large doses of DGLA to humans, both Kernoff et al (1977) and Willis (1981) found only a small rise in AA levels, while the ratio's of DGLA and PGE_1 to AA and its products increased significantly. Horrobin (1983) reported that while administration of GLA to humans significantly raised DGLA, AA levels were only marginally raised. Even administration of large amounts of ALA resulted in only a small change in EPA and DHA (de Gomez Dumm et al 1975b, Blond et al 1981, Mest et al 1983 and Neuringer et al 1984), implying suppressed Δ4D function. This limited desaturation capability makes the extreme "Western" human very close to being an obligate carnivore. In addition, since Eskimo's appear to either lack or have suppressed Δ6D and Δ5D, and exhibit elevated LA and DGLA, and decreased AA, levels (Gibson

et al 1981b and Sinclair 1981), it has been suggested that they may be obligate carnivores, the human equivalent of the cat (Sinclair 1981).

The lack of Δ6D makes the cat an ideal experimental model for assessing the relative importance of different PDFA's in metabolism. Other animal models possessing an active form of this enzyme are, unlike the cat, difficult to make deficient in specific FA's since they are capable of endogenous PDFA production from their plant derived precursors. The cat, however, can be made PDFA-deficient by dietary modification, even when fed the classic EFA's, without this being redressed by in vivo production. This species therefore serves as a model for the extreme "Western" human. Further, the cat also shows a very close degree of genetic homology with Man between all gene loci mapped to date, and exhibits a very high incidence of hereditary diseases paralleling similar conditions in humans (O'Brien 1986).

While different mammalian species exhibit different desaturase capabilities, no quantitative data is available to date on the relative contribution of individual organs to their own, or total, body PDFA requirements, or whether the tissue distribution of desaturases varies in different species of the mammalia. The available evidence suggests that the desaturases are not distributed evenly between tissues (Gellhorn et al 1964 and Brenner 1971), implying dependence of some

tissues on others *in vivo* for a supply of PDFA's (eg. Nervi et al 1965, Cook et al 1978a, Chapkin et al 1984, 1986a, 1986b and Ziboh et al 1987); thus, the assessment of desaturation potential *in vivo* may not necessarily reflect the desaturation capability of the particular tissue studied, but may reflect PDFA's transported from tissues exhibiting desaturation capability to tissues exhibiting limited or no desaturation potential.

1.3.7 Fatty Acid Desaturation in Cultured Cells.

Menkes (1972) showed that rat brain cells cultured in a lipid-free medium take up exogenous [^{14}C]-palmitic, [^{14}C]-stearic and [^{14}C]-lignoceric acids, and such could be desaturated and/or elongated. Desaturation in brain *in vivo* has not been conclusively proven, although the Δ9D desaturation capability of brain cells *in vitro* under specific culture conditions implies that this enzyme may be inactive in the brain *in vivo*. and activated, at least partly, when brain PUFA levels are depleted.

Dunbar et al (1975) showed that normal human lung fibroblasts (WI-38) readily desaturate and elongate PUFA's via Δ6D and Δ5D. There also is evidence that human skin fibroblasts exhibit Δ6D, Δ5D and even some Δ4D activity in culture (Dunbar et al 1975 and Aeberhard et al 1978). Rosenthal et al (1983a, 1984) showed that

Δ9-, Δ6- and Δ5-desaturation of [^{14}C]-stearate, [^{14}C]-linoleate and [^{14}C]-γ-eicosatrienoate, respectively were enhanced by prior growth of human skin fibroblasts in lipid-free medium. These workers also showed that addition of exogenous FA's to the culture medium modulated desaturase activity, depending on the FA and the particular desaturase. Δ9D activity was enhanced with elaidic acid, but was not modulated upon dosing the incubation medium with either myristic, pentadecanoic, palmitic, stearic, trans-vaccenic (trans-18:1ω7) or linoelaidic acids. Addition of cis-vaccenic, oleic or linoleic acids, however, inhibited Δ9D activity in the order 18:2ω6 > 18:1ω9 > 18:1ω7. On the other hand, both elaidic and linoelaidic acids were effective inhibitors of Δ6-desaturation, while trans-vaccenic acid was only half as effective in inhibiting Δ6D. Oleic, cis-vaccenic and γ-linolenic acids only marginally inhibited [^{14}C]-linoleate desaturation. Elaidate was a potent inhibitor of Δ5D, as were all ω9-trans FA's, while ω7 trans-FA's were much less inhibitory. This supported previous evidence concerning the involvement of the position of the double bond in the inhibition/suppression of desaturation (Mahfouz et al 1981).

Contrary to the above, Spector et al (1980a) showed that when the culture medium of human skin fibroblasts was enriched with 18:2ω6, 20:4ω6 decreased by 45%, while 20:2ω6 accumulated, implying suppression of desaturase

activity. They suggested that human skin fibroblasts possibly could not efficiently convert 18:2ω6 to 20:4ω6, although such may also reflect the ability of these cells to selectively control 20:4ω6 levels. Other reports similarly indicate that both cultured human skin fibroblasts and guinea pig aorta smooth muscle cells were unable to desaturate 18:2ω6, although elongation was retained (Gavino et al 1981b and Morisaki et al 1982a). Human skin fibroblasts have also been shown to elongate numerous other FA's, including 16:0, 18:0, 20:0, 22:0, 20:3ω6, 20:4ω6 and 20:5ω3 (Gavino et el 1981a, Tsuji et al 1984 and Banerjee et al 1986).

Rosenthal et al (1983b, 1986) demonstrated that [^{14}C]-18:2ω6 was desaturated and chain elongated to 20:3ω6, 20:4ω6 and 22:4ω6 in cultured human umbilical vein endothelial cells, indicating Δ6D, Δ5D, and elongase expression. Desaturation and elongation of [^{14}C]-18:3ω3, however, was more extensive than for [^{14}C]-18:2ω6. This supported the similar desaturation pattern found in human skin fibroblasts (Aeberhard et al 1978 and Rosenthal et al 1982). Endothelial cells exhibited greater fatty acyl CoA Δ6D activity when grown in medium containing 2.5%, instead of 10% or 20%, FBS, while medium supplementation with 20-100μM oleate inhibited arachidonate synthesis by up to 67% (Rosenthal et al 1983b). The concentration of serum and serum lipids also influenced desaturase activity in human skin fibroblasts

(Rosenthal et al 1982, 1983a, 1984). However, the mechanism by which serum inhibits desaturase expression awaits further clarification, although Rosenthal et al (1983b) suggested that serum lipids provide sufficient arachidonate to inhibit endogenous synthesis, at least partially.

The evidence for desaturase capability in human umbilical vein endothelial cells was directly contradicted by Spector et al (1980b, 1981b, 1981c, 1983). They showed that these cells either exhibit very weak, or lack, significant Δ6D activity, and implied that vascular endothelium requires preformed PDFA's, particularly arachidonate, from the serum for eicosanoid synthesis. The lack of detection of long chain more unsaturated FA's did not appear to relate to ineffective FA uptake as their studies showed that dosed FA's were taken up by these cells and incorporated into the PGL's selectively from a mixture of FFA's. Furthermore, structurally unrelated FA's did not appear to interfere with FFA incorporation, even when present in large amounts (Spector et al 1981b, 1981c). Thus the presence or absence of desaturation capability in this cell type is unclear, and may indicate that the conditions of culture significantly influence the results obtained.

Maeda et al (1978) studied the metabolic conversions of 18:2ω6 to 20:4ω6 and 18:3ω3 to 22:6ω3 in six different cultured mammalian cell lines. These included Chang

liver cells (human adult liver cells), LM cells (a strain of mouse fibroblast), WI-38 cells (human embryonic lung fibroblasts), L5178Y cells (mouse leukemia cells), FM3A cells (mouse mammary tumor cells) and CHO cells (Chinese hamster ovary cells). Only WI-38, L5178Y and FM3A cells converted 18:2ω6 to 20:4ω6, while all cells converted 18:3ω6 to 20:4ω6. Thus, Δ6D was absent in LM, Chang liver and CHO cells, unlike Δ5D. L5178Y and WI-38 cells metabolised 18:3ω3 to 22:5ω3, whereas LM, Chang liver, FM3A and CHO cells only converted 18:3ω3 to 20:4ω3. However, 22:5ω3 was produced by all cells lines upon addition of 20:5ω3, but only FM3A cells formed 22:6ω3. Thus, it appeared that Δ5D activity dissapeared in LM, Chang liver, FM3A and CHO cells, while Δ4D was found only in FM3A cells.

Dunbar et al (1975) and Bailey (1977) showed that normal diploid human fibroblasts exhibited significant fatty acyl Δ6D and Δ5D capability, whereas Δ6D activity was absent in several transformed mouse cell lines. Others showed that transformed cell lines have a greatly reduced ability to convert LA to GLA compared to normal diploid cells (Collins et al 1965, Howard et al 1974, Yavin et al 1975, Burns et al 1978, Maeda et al 1978, Morton et al 1979, Daniel et al 1980, Montaudon et al 1981 and Horrobin 1983). Chiappe et al (1974) reported that in addition to Δ6D, Δ9D was also depressed in two rat hepatoma cell lines, compared to normal liver. Such

transformed cells are, therefore incapable of producing eicosanoid precursors from EFA's, and Spector et al (1981a) proposed that the loss or reduction of Δ6D activity may relate to malignant transformation. Other studies which demonstrated Δ6D activity in transformed cells (Yavin et al 1975, Burns et al 1978 and Maeda et al 1978), however, suggested that the deletion of Δ6D may not be a requirement for, or a characteristic of, transformation, rather a side effect in some cases.

The evidence reviewed showed that while many normal and transformed cell types in culture desaturate FA's, others have been desaturase-deficient. Furthermore, culture conditions and the presence of exogenous FA's are all factors which may modulate fatty acyl desaturase capability.

1.4 Lipid Peroxides and Lipoperoxidation.

Lipid peroxidation can be defined as the oxidative deterioration of unsaturated lipids, particularly those containing more than 2 covalent carbon-carbon double bonds (Halliwell et al 1985). PUFA's are susceptible to oxidation, and their metabolism to eicosanoids involves the formation of free radicals, lipid hydroperoxides, and malondialdehyde (MDA) as a breakdown product (Mead 1976, Pryor 1976, Tappel 1980, Witting 1980, Carpenter 1981 and Lands et al 1981). The enzymes responsible for lipid peroxidation are microsomal, and incorporated exogenous FFA's, or those released from cell membranes during PGL cycling, may serve as substrates for cellular peroxidation (eg. Mounie et al 1986). Studies on the mechanism of lipid peroxidation suggest that MDA and other peroxidation products are formed in two sequential steps, involving the formation of peroxy radicals or lipid hydroperoxides in the first instance, followed by formation and breakdown of cyclic endoperoxides (Pryor et al 1976, Dumelin et al 1977 and Svingen et al 1979).

Enzymic lipid peroxidation is common in most cell types, but auto-oxidation may also occur (Pryor et al 1976, Tappel 1980 and Frankel 1984). When Begin et al (1986a) incubated culture medium without cells, but with GLA, AA, EPA or DHA for a period of 7 days, significant MDA was detected compared to control medium, which increased

in direct correlation with the number of double bonds in the PUFA's dosed. Spontaneous oxidation should therefore always be considered as a possible mechanism whereby lipid peroxides are generated.

When unsaturated FA's are components of complex lipids, eg. membrane PGL's, they become relatively unaccessible to oxidation compared to FFA's. Another factor leading to a reduced ability for cellular lipoperoxidation includes a slow rate of PGL turnover, which limits both FFA incorporation and release from membrane lipids into the cellular pool. Beta-carotene, alpha-tocopherol, superoxide dismutase, glutathione peroxidase and catalase form the anti-oxidant and enzymic lines of protection against free radical attack. It has been sugested that cellular peroxidases act to keep hydroperoxidase levels low, as such may limit eicosanoid formation (Lands et al 1971 and Smith et al 1972). PGL's allow glutathione peroxidase to reduce and detoxify hydroperoxides in membranes, thereby protecting cell membranes from oxidative injury. Peroxide formation readily occurs in the body if PUFA's are fed in large amounts without appropriate vitamin E supplementation. It is not certain exactly how much vitamin E is required to prevent oxidation, but animal studies suggest 2.4-3.2mg/100 calories of food per day, although this should be increased to 3.7-6.4mg for high PUFA diets (Jager 1972).

Lipid oxidation products are toxic, and their reactions with biological cell components can affect vital cell functions (Mead 1976). Secondary products, such as MDA, are powerful cross linking agents, and react with amino groups of enzymes, proteins and DNA (Chio et al 1969, Tappel 1980, Frankel 1984 and Draper et al 1986). Reactions between peroxidised lipids or free radicals with proteins leads to changes in membrane protein cross linking (Beppu et al 1987), polypeptide chain scission (Zirlin et al 1969), destruction of labile amino acids (Roubal 1966a), protein polymerisation and denaturation (Desai et al 1963, Andrews et al 1965, Roubal et al 1966a, 1966b and Roubal 1971), inactivation of enzyme activities (Wills 1961, Chio et al 1969, Garda et al 1985 and de Groot et al 1985), disruption of biological membranes and release of destructive cellular enzymes, and the formation of age pigments in damaged membranes, amongst others (Chio et al 1969, Mead 1976, Tappel 1980, Gavino et al 1981c, Morisaki et al 1982b and Frankel 1984). It has been suggested that the hydroperoxide free radical may be responsible for tissue damage (Tappel 1975), and reviews have appeared on the biological effects of lipid oxides and their relevance to aging, strokes, atherosclerosis, inflammation and cancer (Hopkins et al 1976 and Frankel 1984), although the nature of many interactions <u>in vivo</u> are not, as yet, fully understood.

Rapidly dividing cells, with a high mitotic index, such as regenerating rat liver, generate low amounts of lipoperoxides compared to non-regenerating tissues; however, these concentrations increase when growth slows down, or stops (Wolfson et al 1956). Cheeseman et al (1984) found lower lipid peroxide levels induced by tumor, than by normal, cells in vitro. even when dosed with potent oxidants. Their findings were consistent with a higher alpha-tocopherol content in tumor cells. Others showed that the mean lipid peroxide amounts generated by control human breast tumor (ZR-75-1) or normal simian kidney (CV-1) cultures over a 7 day period were similar, although lipoperoxides amounts generated by tumor cultures dosed with PUFA's (20mg/l GLA, AA, EPA or DHA) approximated to, or were significantly greater than, amounts induced by dosed normal cultures (Begin et al 1986a). Work in our laboratory on a normal (HSF), two benign (3T6, 3T3), and a transformed (3T6D), cell lines showed that peroxidation of C18 FA's related to the number and position of double bonds in the FA chain (Girao et al 1988, 1989). No more lipid peroxides were induced by cells dosed with either saturated, cis- or trans-monoenoic FA's than was produced by controls, but the highest MDA levels were produced with 18:3w6. However, as the growth rate of the cell lines increased, so increased incorporation and peroxidation of dosed polyenoic FA's in the order HSF, 3T3, 3T6 and 3T6D cells (Girao et al 1988, 1989). We suggested that this may

relate to impairment of desaturation capability in transformed cells, resulting in a build-up of peroxidation substances. Alternatively, HSF and 3T3 cells may possess efficient mechanisms to remove excess lipid peroxides, or possess enzymes such as peroxidase, catalase and/or superoxide dismutase which remove different species of activated oxygen that promote lipid peroxidation. It was also possible that 3T6D and 3T6 cells lost, due to enzyme loss or anti-oxidant activity, the relevant mechanisms for controlled lipoperoxide formation.

The biological significance of lipid peroxidation reactions in vivo are still unclear, but a number of breakdown products, including MDA, fatty aldehydes and alkanes, appear to be involved in the aetiology of cell proliferation (eg. Mead 1976 and Tappel 1980). Miller et al (1980) and Gavino et al (1981c) showed that addition of PUFA's (20:3ω6, 20:4ω6 or 22:4ω6) to normal guinea pig aorta smooth muscle or human skin cultures induced significant and increasing amounts of MDA with time. The finding that lipoperoxide concentrations could be increased, and cell division inhibited with PUFA dosage, led to the proposal that the extent of cell proliferation was controlled, in part, by lipid peroxidation. Since 90% of the MDA, or its precursor, remained within the cells, and only 10% was found in the media, such implied that dosed FA's were

incorporated and cell growth inhibited primarily by intracellularly generated lipid peroxides (Gavino et al 1981c). When Morisaki et al (1982b) incubated primary cultures of guinea pig aortic smooth muscle with different ω3, ω6, or ω9 FA's, all FA's which inhibited cell proliferation generated lipid peroxides. However, lipid peroxidation correlated with the degree of growth inhibition induced only with ω9 series FA's. Other studies indicated that FA preparations containing traces of hydroperoxy FA's inhibited cell proliferation to a greater extent than did pure FA's (Cornwell et al 1979 and Morisaki et al 1984). Nevertheless, challenging guinea pig aortic smooth muscle cultures with 20:4ω6, in the presence of various anti-oxidants and inhibitors of lipid peroxidation pathways, suggested that cell proliferation was partially controlled by general peroxidation reactions; peroxides generated via cyclo-oxygenase, lipoxygenase or cytochrome P_{450} related reactions were not involved (Morisaki et al 1984). This supported the proposal of Cornwell et al (1979) and Gavino et al (1981c) that general peroxidation reactions, rather than those involved in eicosanoid synthesis, may be involved in the control of normal cell proliferation. Inhibition of cancer cell proliferation by PUFA's has similarly been shown to involve lipid peroxides, and possibly even HPETES, while PG's and LT's have been ruled out as important in this mechanism (Begin et al 1985a, 1986a, 1988).

1.5 Eicosanoids.

Eicosanoids are unsaturated derivatives of C20 FA's, comprising prostaglandins (PG's), thromboxanes (TX's), prostacyclins (PGI's), leukotrienes (LT's) and various hydroxy acids, formed via microsomal enzymes (Bergstrom et al 1964, van Dorp et al 1964, Samuelsson et al 1975, Lands et al 1977, Horrobin 1980a, Moore 1985 and Higgs et al 1986). Being lipid soluble, eicosanoids act both as intra-cellular and extra-cellular hormone-like local messengers, which regulate numerous metabolic pathways and the second by second functioning of cells and tissues (eg. Ferreira et al 1976 and Crawford 1980). Eicosanoid synthesis occurs in most mammalian organs, but not necessarily in all cells comprising that organ (Christ et al 1972, Sun et al 1978 and Smith 1987). Any eicosanoid-forming cell usually produces more than 1 of these compounds, and this may be characteristic of that particular cell type (Hammarstrom 1981). Eicosanoids are involved with all body systems, have myriad effects, including the control of inflammation and thrombosis, and are often species-, sex- and tissue-dependant (Abdel-Halim et al 1979 and Johnson et al 1983). Thus far, no tissue or bodily process has been shown to be completely independant of eicosanoid involvement at some point.

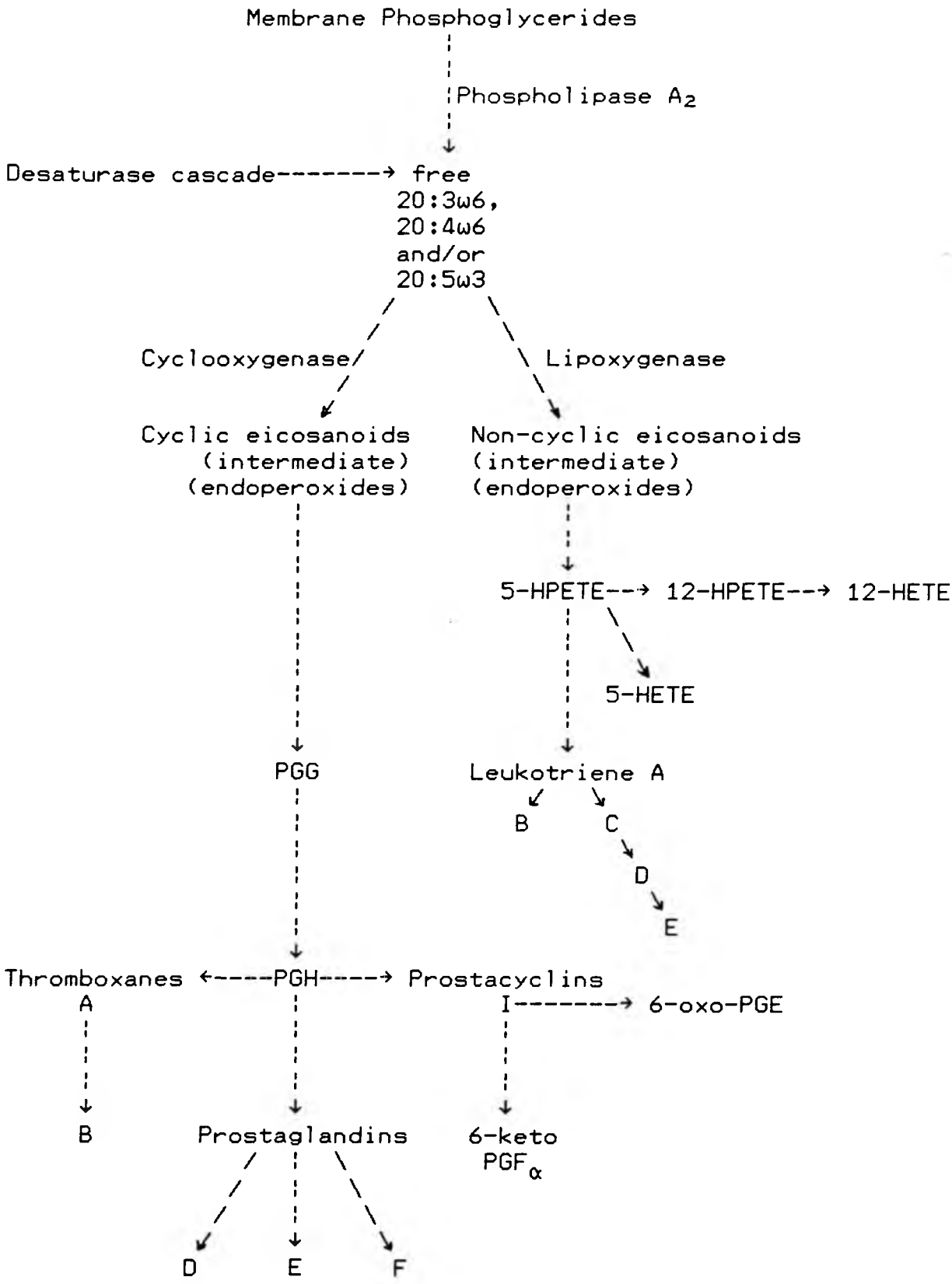
1.5.1 Essential Fatty Acid Precursors and Eicosanoids.

There are 3 distinct eicosanoid series, each derived from a different C20 PUFA, containing double bonds in at least the ω6, ω9 and ω12 positions. DGLA, AA and EPA are the 1-, 2- and 3-series eicosanoid precursors, respectively, usually provided as substrates for eicosanoid biosynthesis after chemical/hormonal, neurological, mechanical or pathological stimuli induce phospholipase A_2 release from cellular lysosomes; this cleaves the PUFA's from membrane PGL's in a Ca^{2+} dependent process (Bergstrom et al 1964, van Dorp et al 1964, Lands et al 1968, Rahman et al 1970, Needleman et al 1979, Wolfe et al 1979, Schwartzman et al 1981, Irvine 1982, Crawford 1983, Fischer et al 1983, Spector et al 1983, Moore 1985, Lands 1986, Rosenthal et al 1986 and Willis 1987).

Unesterified C20 PUFA metabolism to eicosanoids is catalysed either by microsomal cyclooxygenase or lipoxygenase (Fig. 1.5.1.), and produce either cyclic or non-cyclic eicosanoid families, respectively (Lands et al 1971, 1977, Horrobin 1980a, Kuehl et al 1981, Johnson et al 1983, Moore 1985, Higgs et al 1986, Lands 1986). Within each eicosanoid series, there are 4 classes, viz. PG's, PGI's, TX's and LT's, as well as many different individual compounds of each class.

Fig. 1.5.1.

The biosynthesis of primary eicosanoids.



1.5.2 Cyclooxygenase Products (Prostanoids).

Cyclooxygenase (hydroperoxidase) requires lipoperoxides as activators (Lands et al 1971, Smith et al 1972), tryptophan and heme as co-factors (Kuehl et al 1981), and catalyses the oxygenation and cyclisation of free DGLA, AA and EPA to PGG (a 15-hydroperoxy endoperoxide), followed by peroxidation and reduction by PGH-synthetase to PGH (a 15-hydroxy endoperoxide) (Samuelsson et al 1975 and Lands et al 1977). The hydroxy group on carbon 15 allows prostanoids to be active. Both PGG and PGH (cyclic endoperoxides) are unstable in aqueous solution, and may undergo chemical decomposition to products such as MDA and 12L-hydroxy-5,8,10-heptadecatrienoic acid (Moore 1985). PGH is also the precursor for all prostanoids.

During prostanoid (PG, PGI or TX) synthesis, two double bonds are lost from the precursor FA, one double bond is converted from cis- to trans-, and two oxygen molecules are incorporated, with concommitant cyclisation of the carbon chain. Hence, prostanoids derived from PUFA precursors with 3 double bonds (DGLA) have only 1 double bond and form part of the 1-series, while those derived from AA (4 double bonds) or EPA (5 double bonds) give rise to 2- and 3-series eicosanoids, respectively. There are at least 30 prostanoids and related substances, differing in the number of double bonds and/or chemical

structure of their rings or side chains (Moore 1985). Prostanoids have been implicated in numerous physiological and pathophysiologic processes in every mammalian organ system, including regulation of cell function, enzyme activity, intracellular calcium and cyclic nucleotide levels (eg. Crawford 1980 and Horrobin 1980b).

1.5.2.1 Prostaglandins.

Microsomal PGH-PGD isomerase, PGH-PGE isomerase and PGH-PGF_{α} reductase convert PGH to PGD, PGE, PGI and PGF_{α}, respectively (Fig. 1.5.1.). These are the classical and most common PG's. Prostaglandins A, B and C are dehydration products of PGE, and have been reported not to occur naturally (Moore 1985), but this has not been conclusively proven. As with all prostanoids, PG's are not stored, but are synthesised, released, act and are then rapidly inactivated.

Most mammalian tissues synthesise PG's (eg. Christ et al 1972), and total daily PG production has been quoted to be about 1mg/d in an adult (Horrobin 1983). PGD_2 formation has been shown in many tissues, including lung (Christ-Hazelhof et al 1976), brain (Abdel-Halim et al 1977 and Moore 1985) and skin (Puzicka et al 1982), and has been claimed to be the main PG formed from rat lung, stomach, intestine and skin DGLA (Nugteren et al 1973). Moore (1985) reported PGE_2, $PGF1_{\alpha}$ and $PGF2_{\alpha}$ in lung,

PGE_2 and $PGF_{2\alpha}$ in kidney, PGD2, PGE2 and $PGF_{2\alpha}$ in platelets, and PGE_2 and $PGF_{2\alpha}$ in adipocytes. Thus it is clear that characteristic PG's are produced in particular tissues, and that different tissues vary in the types of PG's they produce. There is also evidence that PG production varies with species. PGD_2, PGE2 and $PGF_{2\alpha}$ have been detected in rat and cat brain, with PGD2 predominant in rat and PGE_2 in cat (Abdel-Halim et al 1977, 1979). On the other hand, $PGF_{2\alpha}$ and PGE2 are major cyclooxygenase products in guinea pig brain (Nicosia et al 1975 and Wolfe et al 1976).

1.5.2.2 Prostacyclins.

These bicyclic products of the cyclooxygenase reaction exhibit 2 five membered rings (Moncada et al 1976a, 1984), and are formed when prostacyclin synthetase catalyses the conversion of PGH to PGI (Fig. 1.5.1.). Most tissues produce PGI, and generally only PGI_2 and PGI_3 occur (Sun et al 1978). PGI2, the main product of PG endoperoxide metabolism in vascular endothelium, is a potent vasodilator and inhibitor of platelet aggregation (Moncada et al 1976a, 1976b, 1984 and Kuehl et al 1981). However, PGI_2 is unstable in aqueous solution, and has a half life of about 3 minutes (Herold et al 1986), but less than 1 minute in the vascular system (Moncada et al 1976a, 1976b, 1984), and either undergoes non-enzymic hydrolysis to yield a stable inactive metabolite, 6-oxo-

(keto-)$PGF_{1\alpha}$, or may be converted to a stable product, 6-oxo-(keto-)PGE_1, by 9-hydroxy PG dehydrogenase.

1.5.2.3 Thromboxanes.

These cyclooxygenase products contain a 6 membered oxane ring (Hamberg et al 1975 and Moore 1985), formed when TX synthetase converts PGH to TXA (Fig. 1.5.1.). TXA_2 has a half life of only 30 seconds, and is rapidly transformed by spontaneous non-enzymic hydrolysis into TXB_2, a stable, but inactive metabolite (Hamberg et al 1975 and Herold et al 1986). TX's are primarily involved in thrombocyte aggregation and vasoconstriction (Hamberg et al 1975 and Kuehl et al 1981), but have been reported in a number of other tissues, including leukocytes, lung, spleen, brain and kidney (Nicosia et al 1975, Wolfe et al 1976, Abdel-Halim et al 1977, 1979 and Moore 1985). Sun et al (1978) report that most tissues synthesise TX, but others indicate that the TX metabolic pathway has limited distribution, being found mainly in platelets (Hamberg et al 1975) and lung (Svensson et al 1975).

1.5.3 Lipoxygenase Products (Leukotrienes).

LT's are non-cyclic biologically active molecules, containing a conjugated triene, and are derived from precursor PUFA's (DGLA, AA and EPA) containing a penta-1,4-cis-diene system by microsomal 5'-lipoxygenase (Fig.

1.5.1.); this catalyses the hydroperoxidation of PUFA's to hydroperoxy derivatives, viz. hydroperoxy eicosatetraenoic acid (HPETE) (Nugteren 1975, Borgeat et al 1976 and Taylor et al 1983). These hydroperoxides may be metabolised to a series of less toxic metabolites, viz. hydroxy eicosatetraenoic acid (HETE), or converted into biologically active LT's (Borgeat et al 1979, Goetzl 1980, Samuelsson 1981, 1986 and Taylor et al 1983). As no ring structure is introduced, no double bonds are lost, thus LT's contain the same number of double bonds as their precursor PUFA's. 2-series LT's thus have 4 double bonds, and are named appropriately (eg. LTA_4). LT's are of interest in relation to the slow reacting substance of anaphylaxis (SRS-A), which is considered an important intermediate in acute hypersensitivity reactions (Samuelsson 1981).

1.5.4 The Control of Eicosanoid Biosynthesis.

Eicosanoid biosynthesis can be influenced by various factors, including physiological, nutritional and pathological (Hwang et al 1975, 1979, 1980, Brenner 1982 and Jeffcoat et al 1984).

Lipid hydroperoxides are modulators of the conversion of FA's to eicosanoids; low concentrations (<1μM) seem essential for prostanoid formation, whereas greater amounts inhibit activity of enzymes including cyclo-oxygenase and PGH-synthetase (Lands et al 1971, Cook et

al 1976, Moncada et al 1976b, Hemler et al 1978, 1979, Siegel et al 1979 and Kulmacz et al 1983). Inactivating enzymes also play a role in the control of eicosanoid production. Most PG's are physiologically inactivated by the same enzyme, 15-hydroxy-prostaglandin dehydrogenase; this cytoplasmic enzyme generally found in most tissues irreversibly converts the hydroxyl group on carbon number 15 to a keto group (eg. Moncada et al 1976a).

Numerous drugs have been reported to influence enzymatic steps in the eicosanoid pathway (Vane 1971, Kuehl et al 1981, Horrobin 1983, Taylor et al 1983, 1986 and Smith 1989). Phospholipase A_2 is inhibited by cortisol, glucocorticoids, corticosterone, dexamethasone and the anti-malarial drug, Mepacrine, all of which either inhibit FFA release from membrane PGL's or bind directly to the enzyme. Lipoxygenase activity is inhibited by retinoids for example, while cyclooxygenase is inhibited by aspirin, paracetamol, most other non-steroidal anti-inflammatory agents, and reducing agents as well, usually by blocking enzyme oxygen uptake (Vane 1971, Smith et al 1972, Samuelsson et al 1975, Huttner et al 1977, Lands et al 1977 and Kuehl et al 1981). Imidazole inhibits TX-synthetase, while 15-hydroxy-prostaglandin dehydrogenase is inhibited by carbenoxolone and indomethacin (Smith et al 1972). Animal studies indicate that the inhibitory effects reported are exacerbated in EFA deficiency (Hansen 1981).

Eicosanoid biosynthesis is largely dependent on the size of the PUFA precursor pools, and it is generally believed that the relative availability of the precursor FFA's determines, to a greater or lesser extent, which eicosanoids are synthesised and the rate of product formation (Hong et al 1979, Irvine 1982 and Laposata 1988). There is also evidence that the size of the precursor pool can be modulated by dietary manipulation (Galli 1980, Goodnight et al 1982, Herold et al 1986). GLA, for example, enhances 1-series eicosanoid formation (Willis 1981), while EFA deficiency depresses prostanoid synthesis due to unavailability of precursor FA's (Weston et al 1978) and/or inhibition of cyclooxygenase by 20:3ω9 (Ziboh et al 1974 and Dunham et al 1978). On the other hand, PG-synthetase activity is increased in EFA deficiency (Kaa 1976).

Animal studies indicate that eicosanoid synthesis is inhibited when dietary LA intake is increased to about 1% (Lepran et al 1981). Others report that dietary LA supplementation inhibits PGI_2 release from cultured human endothelial cells (Spector et al 1980b) and decreases human platelet TBX_2 (Needleman et al 1982), while enhancing PGI_2 and TXB_2 production in the cardiovascular system (Agradi et al 1981). These studies suggest that eicosanoid production is modulated in different ways in the presence of dietary LA, and varies with cell type.

The Western diet does not provide an adequate balance of PUFA precursors for the 1-, 2- and 3-series eicosanoids, but rather a preponderance of AA, the 2-series precursor found in large amounts in red meat (McCance et al 1978 and Giangregorio et al 1988a). Doyle et al (1982) report that the average AA intake approximates to 200mg/day, whereas the requirement for eicosanoid synthesis is only about 1mg/d. Thus, dietary AA can more than supply all eicosanoid production. High AA levels, however, exert negative feedback control on Δ6D and limit both 1- and 3-series eicosanoid precursor production, predisposing to eicosanoid imbalance. As body AA stores are high compared to DGLA, partial EFA deficiency leads to DGLA and PGE_1 deficiency before a deficit of AA and 2-series eicosanoids develops. PGE_1 partly regulates cAMP formation, and this normally regulates 2-series eicosanoid formation by inhibiting AA mobilisation from PGL's (Feinstein et al 1977, Minkes et al 1977 and Horrobin 1980b). If PGE_1 and thus cAMP levels fall, then AA is mobilised and converted to 2-series eicosanoids (Horrobin 1980b). EFA administration lowers 2-series eicosanoid production by restoring PGE_1 and cAMP control of AA (Horrobin 1980b), while manipulation of the DGLA/AA balance also selectively regulates eicosanoid biosynthesis (Horrobin et al 1984a).

18:3ω3 can suppress conversion of 18:2ω6 to 20:3ω6 and 20:4ω6, and thus impair 2-series eicosanoid production

(Hwang et al 1975, 1980 and Marshall et al 1982). Furthermore, eicosanoid synthesis from ω6 series PUFA's appears to be reduced by competitive inhibition of cyclooxygenase by 18:3ω3 (Adam et al 1986). However, 18:3ω6, 20:3ω6, 18:1ω9, 20:3ω9, trans-isomers of eicosanoid precursors, and to a lesser extent 18:2ω6, may also irreversibly occupy the active site of cyclo-oxygenase and inhibit prostanoid synthesis (Pace-Asciak et al 1968, Nugteren 1970, Lands et al 1973, 1977, Ziboh 1973, Mathias et al 1979, Wicha et al 1979, Lands 1982, Morita et al 1983).

Long chain ω3 series PUFA's are potent inhibitors of 2-series eicosanoid synthesis by competing with AA both at the desaturase level and for binding sites on cyclo-oxygenase (Lands et al 1973, 1977, 1981, Hamazaki et al 1982, Lands 1982, 1986, Corey et al 1983, Fischer et al 1984 and Croft et al 1986), as well as by stimulating 2-series LT formation (Hwang et al 1980, Lands 1982 and Lee et al 1985). EPA in particular, is a poor cyclo-oxygenase, but a relatively good lipoxygenase, substrate under normal conditions (Club et al 1979, Needleman et al 1979, Lands et al 1981, Murphy et al 1981, Spector et al 1983, Lokesh et al 1984 and Lee et al 1985). Evidence suggests that EPA, therefore, acts as a physiological control of eicosanoid biosynthesis (Marshall et al 1982, Lands 1986 and Nassar et al 1986). The effects of EPA on cyclooxygenase product formation,

however, are still unclear, as EPA reduces the synthesis of cyclooxygenase products in tissues such as human endothelial cells (Spector et al 1983), has no effect on the conversion of AA to PGI_2 in umbilical blood vessels (Dyerberg et al 1980), and increases PGI_2 production in rat aorta (Hamazaki et al 1982). Such findings are a further indication that different tissues respond differently to particular eicosanoids.

1.5.5 Eicosanoids and Disease Processes.

Eicosanoid levels are largely dependant on the dietary supply and metabolism of EFA's, thus any EFA/PDFA imbalance could alter the eicosanoid profile. Many of the clinical conditions previously ascribed either to EFA and/or desaturase imbalance may therefore also relate to alterations in eicosanoid production. Impaired Δ6D expression in diabetes, for example, affects the negative regulation of 2-series PG production by PGE_1, thus diabetes is associated with reduced PGE_1, and increased 2-series PG, formation (Chase et al 1979 and Lagarde et al 1981).

Cancer is a pathological condition in which eicosanoid production may be altered (eg. Panje 1981 and Bennett 1984, 1986). Raised blood prostanoid levels have been shown in the development of prostatic carcinoma, with an encrement in 6-keto-$PGF_{1\alpha}$ levels with progression of the

disease (Khan et al 1982). Many malignant cells do not express Δ6D, and thus produce little PGE_1, but extensive amounts of 2-series PG's (Collins et al 1965, Dunbar et al 1975, Maeda et al 1978, Horrobin 1980a, 1988 and McLemore et al 1988). This phenomenon could explain the enhanced proliferation found in malignancy since PGE_1 has an anti-proliferative effect (Horrobin 1980a and Huttner et al 1977). Normal regulation of intracellular Ca^{2+} levels and movements by PGE_1 are also altered in malignancy (Horrobin 1980a).

Medical opinion recognises an imbalance of 1- and 2-series eicosanoids as contributary to numerous other clinical conditions including schrizophrenia, asthma, cystic fibrosis, disorders of inflammation and immunity (Horrobin et al 1978, Hyman et al 1978, Horrobin 1979a, 1980b, Chase et al 1980 and Hubbard 1983). However, manipulating dietary EFA/PDFA supplies has shown significant improvements in restoring the eicosanoid imbalance without using drugs (Horrobin 1983).

1.5.6 Eicosanoids in Cultured Cells.

Many cultured cells produce eicosanoids (eg. Pong et al 1977, Marcus et al 1978, Ali et al 1980, Spector et al 1980b, 1981c, 1983, Morisaki et al 1984, van Rollins et al 1984, Campbell et al 1985, Milks et al 1985 and Durant et al 1988), especially when stimulated by agents such as serum, epidermal growth factor, thrombin,

bradykinin or mechanical manipulation (Dunn et al 1976, Hong et al 1976, Hammarstrom 1977, Hirata et al 1985 and Wickremasinghe 1988). These agents appear to act by increasing precursor PUFA release from cellular lipids via cAMP (Alexander et al 1976). Serum may additionally stimulate eicosanoid production by providing free AA (Dunn et al 1976, Hong et al 1976 and Hammerstrom 1977). Not only are there differences in eicosanoid production between normal cells, but also between normal and transformed cells. Many normal cells produce low amounts of eicosanoids in the absence of stimulatory agents, while certain virus-transformed cells have a much higher basal rate of eicosanoid synthesis, despite poor desaturation capability (Dunbar et al 1975, Ritzi et al 1976 and Hammarstrom 1977). In the case of SV40 or polyoma virus-transformed baby hamster kidney and 3T3 mouse embryo fibroblasts, PGE_2 and $PGF2_\alpha$ concentrations were higher than in non-transformed parent cells (Ritzi et al 1976 and Hammarstrom 1977). In addition, elevated PGE_1, $PGF1_\alpha$, PGA, PGB and 13,14-dihydro-15-keto-$PGF1_\alpha$ levels were found in polyoma and SV40-transformed 3T3 cells (Ritzi et al 1975, 1976). However, PGE_2 or $PGF2_\alpha$ levels were not elevated in a different clone of virus-transformed 3T3 cells (Hammarstrom 1977), implying that eicosanoid production may vary not only with cell type, but also with different clones derived from the same parent cell.

There is evidence that PGE_1 and PGE2 stimulate prepubertal guinea pig aortic smooth muscle cell proliferation at low or physiological concentrations, and inhibit proliferation at high or pharmacological concentrations (Cornwell et al 1979). Other PG's such as $PGF_{1\alpha}$ and $PGF2_{\alpha}$ have no effect on cell proliferation at low concentrations, but stimulate proliferation at high concentrations (Cornwell et al 1979 and Bettger et al 1981). Some studies implicate that PG's exert a balanced effect on fibroblast growth <u>in vitro</u>, with a stimulatory action of $PGF_{2\alpha}$ (de Asua et al 1975) and a negative action of PGE_2 (Ko et al 1977 and Korn et al 1980). On the other hand, Durant et al (1989) were unable to demonstrate this effect, since both exogenous PGE_2 or $PGF_{2\alpha}$ increased the rate of cell division in mouse embryo fibroblasts, as well as the maximum density reached at confluence. However, these PG's had no effect on rat hepatocyte viability, despite modulating DNA synthesis (Skouteris et al 1988). These studies showed that exogenous PG's may induce potent effects on the multiplication of a variety of cell types, and some workers have proposed that eicosanoid secretion may represent an autocrine mode of growth regulation for some fibroblasts (Taylor et al 1977 and Smith et al 1984). However, Thomas et al (1974) and Cornwell et al (1979) suggested that endogenous PG biosynthesis was not required for multiplication of human diploid cells

in vitro. Later studies, using guinea pig aorta smooth muscle, confirmed that inhibition of cell proliferation with exogenous FA dosage did not correlate with enhanced or inhibited PG synthesis (Morisaki et al 1982b). Recent studies also suggested that endogenous PG secretion does not constitute an important mode of control of normal cell proliferation (Durant et al 1989).

Botha et al (1985) suggested that elevated TXA_2 and/or PGE_1 levels may be involved in the growth inhibitory effects induced with GLA supplementation in malignant cells, but subsequent studies indicated that PUFA-induced destruction of tumor cells also does not involve eicosanoids, and have rather implicated involvement of other oxidation products (Begin et al 1985b, 1986a, 1988 and Botha et al 1989).

Some studies have implicated lipoxygenase metabolites as mediators of normal cell proliferation (Huttner et al 1977, Cornwell et al 1979 and Mayer et al 1984). Smith et al (1984) reported that 12-HETE was more potent at inhibiting cultured rabbit aortic smooth muscle cell proliferation than a range of PG's or TXB_2, which either did not exhibit growth inhibitory potential, or were only weakly active. Others found 15-HPETE, but not PGE_2, PGD_2 or LTB_4, to be toxic to rat hepatocyte cultures, and increasing its concentration from 0.1 to 50μM decreased cell viability from 70% to 30% after only 90 minutes incubation (Sakagami et al 1989).

Others believe that neither cyclooxygenase nor lipoxygenase products, nor the peroxides generated from these pathways, or cytochrome P_{450} related reactions, are involved in the inhibition of cultured cell growth mediated by exogenous FA's, but rather that peroxisomal peroxidation of unsaturated FA's may be involved (Horrobin 1980a, Morisaki et al 1984 and Fujiwara et al 1986). Still others have proposed that FA metabolism, anti-oxidant status and eicosanoid production all contribute to the regulation of cell proliferation (Bettger et al 1981).

The work reviewed illustrates that different cultured cell types exhibit a particular pattern of eicosanoid production, and that each cell type responds differently to exogenous and/or endogenous eicosanoids. Although some studies proposed that the regulation of cell proliferation may involve eicosanoids, the mechanism, if true, is complicated and as yet unclear.

1.6 Influence of Polyunsaturated Fatty Acids in Mammalian Cells In Vitro.

1.6.1 Normal Cells in Culture.

There are numerous reports that exogenous FA's modulate the proliferation of a variety of cultured normal cells. Dubin et al (1965) found that the growth of cultured chicken embryo macrophages could be stimulated with albumin-bound LA, and that the greatest proliferative effects were induced with a concentration of 0.07mg/l. They suggested that the beneficial effects of LA stemmed from the provision of essential elements deficient in the serum, such as FA's, and that inhibitory substances present in the serum could be removed by binding with the albumin present.

Later studies found that 16μM 20:3ω6 inhibited the proliferation of human foreskin fibroblasts cultured in medium with 10% fetal bovine serum (Huttner et al 1978). Supplementation with up to 50μM 18:1ω9 or 20:4ω6, however, progressively enhanced cell proliferation to about 150% and 120%, respectively, but higher amounts markedly inhibited cell viability in a concentration dependent manner to <30% with 100μM FA (Huttner et al 1978). Using human skin fibroblasts grown in medium with 10% dialysed fetal calf serum, Spector et al (1979) found that 18:1ω9 or 18:2ω6 did not affect cell growth

when supplemented in the range 10 to 100μM, while cell viability was inhibited by about 25% with 18:3ω3 and 50% with 16:0 or 20:4ω6 dosage. Others reported that human neonatal foreskin fibroblast growth was only inhibited with ≥40μM 20:4ω6, while 20:3ω6 and 22:4ω6 were equally cytotoxic in the range 1.6-160μM dosed (Gavino et al 1981a). The same study showed that 20:3ω6, 20:4ω6 and 22:4ω6 were toxic and almost equally effective in inhibiting pre-pubertal guinea pig aorta smooth muscle proliferation. However, the effects induced varied quantitatively between skin and muscle, implying that FA modulation of cell growth varies with cell type.

The susceptibility of guinea pig aorta smooth muscle cell proliferation to 20:3ω6, 20:4ω6 and 22:4ω6 toxicity was previously demonstrated by Huttner et al (1977). This group subsequently showed that while these triene and tetraene PUFA's inhibited cell growth, monoene (18:1ω9) and diene (18:2ω6) FA's stimulated cell growth in the range 20-120μM (Huttner et al 1978 and Cornwell et al 1979). However, saturated FA's (14:0, 16:0, 18:0) progressively limited cell proliferation with increased chain length, and the concentrations required to significantly inhibit growth were 220μM for 14:0, 95μM for 16:0 and 18μM for 18:0.

Morisaki et al (1982b) confirmed increased cell growth when guinea pig aorta smooth muscle cells were incubated with 30, 60 or 90μM 18:1ω9, but other ω9 FA's inhibited

cell proliferation in a concentration dependant manner, increasing in the order 18:2ω9 < 20:2ω9 < 20:3ω9. Both 18:2ω6 and 18:3ω3 were least growth inhibitory for their particular series and only induced inhibition at high concentrations, but the inhibition increased when desaturation or elongation products of these FA's were dosed. The most potent inhibitors of cell proliferation in the ω9, ω6 and ω3 series were eicosatrienoic acids, although 20:3ω9 was more potent than 20:3ω6, which in turn was more inhibitory than 20:3ω3. This implied the involvement of FA carbon chain length, number and position of double bonds in the modulation of cell viability. PG synthesis was inhibited by 22:4ω6 and 22:6ω3, but enhanced by 18:3ω6, 20:3ω6 and 20:4ω6, indicating FA modulation of PG production. Inhibition of cell proliferation, however, did not correlate with enhanced or diminished PG synthesis, and peroxides were implicated as possibly being responsible since all FA's which killed cells generated lipoperoxides (Morisaki et al 1982b).

Fetal mouse brain cell growth was modulated when Bourre et al (1983) dosed 18:2ω6, 18:3ω3, 20:4ω6 or 22:6ω3 at the time of seeding. A FA concentration of 1mg/l was optimal for cell proliferation, while greater concentrations were toxic. However, the extent of growth enhancement varied with FA, and increased in the order 22:6ω3 < 18:2ω6 < 18:3ω3 < 20:4ω6 = 20:4ω6 + 22:6ω3. Our

laboratory found that C18 FA supplementation to cultured rat brain cells had little effect on proliferation at low concentrations (1mg/l), but amounts up to 100mg/l inhibited growth in a concentration dependent manner in the order 18:0, 18:4ω3, 18:1ω9, 18:2ω6, 18:3ω3 and 18:3ω6 (Davidson et al 1988a and Girao 1988).

Blood cells appear particularly susceptible to the effects of exogenous FA's. Ruck et al (1986) found that 18:0 had no effect on resting human monocyte viability in culture, whereas 18:3ω6 and 18:2ω6 induced toxicity at much lower concentrations than 18:1ω9 or 18:3ω3 (ID_{50}=4.4, 5, 12 and 13mg/l, respectively). Earlier work by Buttke (1984) indicated that low concentrations (20-60μM) of saturated FA's suppressed mitogen-induced DNA synthesis in mouse lymphocytes, while both saturated and unsaturated FA's were inhibitory at high concentrations (100-120μM). However, T and B cells responded differently, with mitogen-activated B cells being less inhibited by FFA's. Weyman et al (1975, 1977) found that unsaturated (18:1ω9, 18:2ω6 and 20:4ω6) and saturated (16:0, 17:0 and 18:0) FA's inhibited PHA-stimulated uptake of [^{14}C]-uridine in human lymphocytes in the order 18:1 < 16:0 < 20:4 < 18:2 < 18:0 < 17:0, while uridine uptake was not affected by any FA in unstimulated cells. When saturated and unsaturated FA's were present together in the medium, the inhibition was much less pronounced, or even abolished, while when 2 or

more saturated, or unsaturated, FA's were added, the inhibition was the same, or greater, than for single FA's. This implied that synergistic or competitive effects may be induced by the presence of other FA's. Furthermore, the finding of Kitagawa et al (1984) that 18:1ω9, 18:2ω6 or 18:3ω3 cause platelet aggregation at concentrations just below that inducing cell lysis (>100μM) suggests that certain FA's could modulate particular cell functions at specific concentrations.

The studies reviewed illustrate that exogenous FA's modulate normal cell growth and function in vitro, although the extent varies with the FA dosed, its concentration, the particular cell type and conditions of culture. Certain studies also suggest that factors other than a pure individual FA effect may be involved in the control of cell growth or function.

1.6.2 Transformed Cells in Culture.

Horrobin (1980a) reported that malignant cells exhibit a common group of metabolic abnormalities which include the control of calcium movements, formation, destruction and action of cAMP, cholesterol synthesis, control of glycolysis, EFA desaturation and eicosanoid synthesis. His group later proposed that the inability to make GLA, and therefore PGE_1 (a naturally occuring endogenous reverse transforming agent), may contribute to the

etiology of cell transformation (Horrobin et al 1984b).

Early studies using 3 mouse ascites cell lines showed in vitro anti-tumour activity with FA's of chain length ranging from C6 to C20 (Tolnai et al 1962). Considerable variations were found in the relative effectiveness of the FA's against the cell lines, but LA and ALA consistently exhibited the greatest anti-tumor activity. The data suggested that FA chain length, number and position of double bonds were important factors determining in vitro anti-tumor activity, while the lack of organic peroxide detection implied that the effects reflected the action of the FA's themselves.

McGee (1981) reported that PA and SA were toxic to neuroblastoma x glioma hybrid (NG108-15) cells, whereas addition of OA, LA, ALA or AA stimulated growth. There is evidence that these unsaturated FA's also stimulate the proliferation of HeLa and rodent cancer cell lines (Gerschenson et al 1967 and Spector et al 1981a). Others however, report marked inhibition of tumor cell growth in culture with PUFA dosage. Booyens et al (1984a) found that OA enhanced, while LA, GLA, ALA, AA, EPA and DHA suppressed, the rate of human osteogenic sarcoma growth. Supplementation with up to 20mg GLA/ml culture medium also suppressed benign kidney tumor, mouse melanoma, human hepatoma, human oesophageal and laryngeal carcinoma cell proliferation (Dippenaar et al 1982a, 1982b, Leary et al 1982, Booyens et al 1984b, 1984c and

Cantrill et al 1986). Studies examining the influence of C18 FA's (1-100mg/l) on the growth of human hepatoma, NG105-15 neuroblastoma, SP210 mouse myeloma, mouse 3T6-derived (3T6D), 3T6 and 3T3 benign, cells showed that ω9, ω6 and ω3 series unsaturated FA's were growth inhibitory, with the greatest toxicity induced with GLA or ALA dosage (Girao et al 1986, 1987a and Girao 1988).

Neither SA nor OA killed cultured breast carcinoma cells, but LA, GLA, ALA, AA, EPA and DHA each induced concentration dependent cytotoxicity, although FA's containing 3, 4 or 5 double bonds were most effective (Begin et al 1985a, 1987). When Botha et al (1989) studied the effect of 50mg/l GLA or DGLA on a human breast cell line (NUB 1), only GLA inhibited cell growth, although both FA's, especially DGLA, enhanced PGE and PGF production. This implied that the effects of GLA were unrelated to eicosanoid formation, which was consistent with findings that indomethacin enhanced the growth inhibitory effects of GLA (Botha et al 1985).

The ability of certain exogenous PUFA's to enhance tumor cell growth in culture with particular FA concentrations may relate to evidence suggesting that dietary fat is important in the promotion, but not initiation, of tumorogenesis, and that unsaturated fat is a more potent promotor than saturated fat (Hopkins et al 1976, Carroll 1984, Karmali 1986 and Hill 1987). Despite differences

in the sensitivity of tumor cells to exogenous FA's, studies suggest that GLA, DGLA, AA and EPA are generally the most cytotoxic FA's to transformed cells (Dippenaar et al 1982a, 1982b, Booyens et al 1984a, 1984b, Cantrill et al 1986 and Begin et al 1985a, 1985b, 1986b, 1986c). The work presented suggested that the effect of a FA on transformed cells in vitro related to the number, position and geometric configuration of double bonds in that FA. The susceptibility of a transformed cell line to growth inhibition by exogenous FA's also appears to relate to the particular cell line and degree of transformation of that cell line (Fujiwara et al 1986, Girao 1988 and Begin et al 1989).

1.6.3 Normal vs Transformed Cells in Culture.

Numerous studies have shown that normal and transformed cells take up exogenous FA's from the surrounding medium; once incorporated, the FA's can either be found in the TAG or PGL fractions, or in the free form, but the effects they induce vary (eg. Mackenzie et al 1964, Yavin et al 1974, Weyman et al 1977, Rosenthal et al 1978, Spector et al 1979, 1980a, 1980b and Rosenthal 1981).

Wicha et al (1979) found that both normal and neoplastic rat mammary epithelial cell proliferation was stimulated when the culture medium was supplemented with 0.1-10mg/l unsaturated FFA's (OA, LA, ALA or AA), but inhibited by

all concentrations of saturated FFA's (PA or SA). LA and ALA were most stimulatory to normal cells, enhancing proliferation by more than 100% at concentrations of 1.0 and 0.1mg/l, respectively, whereas tumor cell growth was enhanced most effectively with 0.5mg/l OA.

Other workers found that supplementation of culture medium with 20mg/l LA, GLA, DGLA, AA, ALA, EPA or DHA inhibited the proliferation of cancer cells derived from human lung, prostate or breast, but did not kill normal human fibroblasts, monkey or canine kidney cells, although the rate of normal cell division was lowered dependent on cell type (Begin et al 1985a, 1985b and 1986b). EPA and AA inhibited normal cell growth with concentrations greater than 20mg/l, although no marked cytotoxicity was induced with other PUFA's, even with concentrations 3 fold greater than required to kill cancer cells (20mg/l). Co-cultures of human cancer cells and normal human fibroblasts resulted in overgrowth of normal fibroblasts by the carcinoma cells (Begin et al 1985a, 1985b, 1986b and 1986c). However, when these co-cultures were supplemented with 20mg/l GLA, AA or EPA, malignant cell growth was selectively inhibited, and normal fibroblasts outgrew the malignant cells, even when tumor cells had being growing for up to 72 hours longer than normal cells. Subsequent studies using genetically related embryonic or transformed rat brain cells dosed with GLA or EPA (0-60mg/l) confirmed that

tumor cells were considerably more sensitive to PUFA toxicity (Begin et al 1989).

A series of studies in our laboratory showed that C18 FA's, dosed up to 100mg/l, were generally least growth limiting with normal fibroblasts (HSF), more so with benign lines (3T3 and 3T6), and most effective in limiting transformed (3T6D) cell growth (Davidson et al 1988b). At the same time, the growth inhibitory effects increased with increasing degree of FA unsaturation. C20 FA's, however, were most growth limiting with normal fibroblasts, less so with 3T6D, and least effective with 3T3 and 3T6 (Giangregorio et al 1988b). Once again, the inhibitory effects increased with increasing degree of unsaturation of the FA's, although ω3 PUFA's were more effective than ω6 PUFA's, even when of equal degree of unsaturation, implying that the ω3 configuration may be involved in determining the extent of cytotoxicity. The 3 eicosanoid precursors were not markedly more effective than other FA's, suggesting that there was no direct eicosanoid involvement (Giangregorio et al 1988b).

Overall, the evidence to date suggests that normal and transformed cells vary in sensitivity to exogenous FA's. It it also clear that the extent to which FA's modulate cell proliferation relates to factors such as FA structure, the concentration dosed, culture conditions and the particular cell type.

1.7 Summary of Introduction and Objectives.

EFA's are the precursors for a variety of compounds, each performing essential functions. However, the nature of the compounds produced and efficiency of the metabolic sequences involved in lipid metabolism appear to be species and tissue dependent, while physiological, nutritional and pathological conditions can influence the relative concentrations of these compounds. Since cell culture systems provide a less variable, more defined, more homogeneous and easily manipulated system for experimentation than *in vivo* models, tissue culture systems have been used extensively to model various aspects of lipid metabolism.

In recent years, numerous studies have focused on the functional interactions between lipids and cells (eg. Rosenthal 1978, 1981 and Spector et al 1981a). Some effects have been dependant on whether the FA's were saturated or unsaturated (eg. Gill et al 1980); other effects were brought about by both saturated and unsaturated FA's and with no apparent correlation between the effect and the chain length or degree of unsaturation (eg. Weyman et al 1977). There is a large body of evidence which indicates that normal, benign and transformed cells respond to exogenous polyenoic FA's (eg. Horrobin 1983, Booyens et al 1984a, Begin et al 1985a, Girao et al 1986, Davidson et al 1987b and

Giangregorio et al 1988b). However, two schools of thought have arisen concerning the effect of exogenous FA's on cellular proliferation, viz. those which found that particular FA's stimulated the proliferation of cultured cells (eg. Gerschenson et al 1967, Wicha et al 1979, McGee 1981 and Spector et al 1981a), and those which found that FA's selectively inhibited cell growth (eg. Booyens et al 1984b, Begin et al 1986b, Davidson et al 1988b and Giangregorio et al 1988b).

To date, no satisfactory mechanism or site of action for such FA's has been conclusively demonstrated. Contrary to the original postulation that the effects were produced via cell membrane or membrane-related processes, on investigation of a large number of plasma membrane parameters, only changes in lipid peroxides correlated with FA concentrations (Girao 1988). Lipid peroxide quantitation showed a direct correlation between the degree of FA unsaturation and concentration of lipoperoxide formed (Girao et al 1988). The effects produced, therefore, do not appear to be directly associated with the plasma membrane; the initial site of action of these FA's is probably intracellulary mediated and probably involves the desaturase/elongase pathway. Considerable evidence suggests that unsaturated FA's are more effective in limiting cell proliferation than saturated FA's (eg. Cornwell et al 1979, Morisaki et al 1982b, Ruck et al 1986, Begin et al 1987 and Girao

1988); thus, the ability to further desaturate dietary FA's may be an important factor in this process.

Fatty acyl desaturase enzymes are located in microsomes, as are the enzymes involved in lipid peroxide and eicosanoid formation. Assuming desaturase, eicosanoid and/or lipoperoxide involvement, then cells from a species known to exhibit high desaturase activity, eg. Rattus norvegicus (Brenner 1971, 1974), would be expected to react differently to those from a species lacking such activity, eg. Felis domesticus (Rivers et al 1975a, 1976, Frankel et al 1978, Sinclair 1979). Homo sapiens, however, is considered to be in an intermediate position (Horrobin 1983), but with the enzyme activity deteriorating and susceptible to inhibition related to age and lifestyle patterns. The inherent ability of tissues to respond to FA's may thus, vary dependent on the species concerned.

Many workers have assessed the effects of individual exogenous FA's as a means of limiting neoplastic cell growth (eg. Tolnai et al 1962, Dippenaar et al 1982a, Leary et al 1982, Booyens et al 1984a, Cantrill et al 1986, Girao et al 1986, Begin et al 1987 and Botha et al 1989). Considerably less data, however, is available on the effects of FA's on normal cell growth (eg. Huttner et al 1978, Cornwell et al 1979 and Ruck et al 1986). It is thus difficult to relate many of the neoplastic cell studies to the 'real world' if similar studies have not

been performed with normal cells, particularly since the ability to respond to FA's may be impaired by transformation. Further, examination of the literature illustrates that many studies have, unfortunately, compared the effects induced by individual FA's in a transformed cell line with that of a "control cell line" which was either benign in origin, or normal but unrelated to the transformed one studied; a more valid comparison would be to use the normal cell type from which the transformed one was derived since evidence suggests that cells even of the same source and degree of transformation respond differently to exogenous FA's. This is clearly evident from previous studies in our laboratory which compared the effects of exogenous C18 FA supplementation on the proliferation of 3T3 and 3T6 cells, both benign and originally derived from the same source (Swiss mouse embryo) (Davidson et al 1987c). Although the cells were morphologically similar, the effects induced by the added FA's were not alike, implying differences in FA metabolism. Studies have shown that the FA composition of different tissues, both within and between mammalian species, vary considerably (eg. de Gier et al 1961, Crawford et al 1970, 1971 and Horrobin et al 1984c). Evidence additionally suggests that cells derived from different mammalian species and their tissues, as well as cells of different degrees of transformation, possess varying degrees of desaturation

capability (Brenner 1971, Dunbar et al 1975, Rivers et al 1975a, Horrobin 1983), and thus would be expected to respond differently to exogenous FA's.

Most studies to date have ignored this fact. Booyens et al (1984b), for example, used benign bovine kidney cells (MDBK) as a control for osteogenic sarcoma (MG63) and eosophageal carcinoma cell lines in studying the effects of GLA toxicity on cultured cells, while a human hepatoma cell line was compared with a benign monkey kidney cell line (LLCMK), used as control, in a similar study (Booyens et al 1984c). Other studies have only investigated lipid metabolism in a single cancer cell line without a suitable normal control. For example, the importance of the stearoyl-CoA desaturase system in octadecanoate metabolism was studied in a hepatoma cell line (7288C) by Zoeller et al (1985), while Gaspar et al (1975) used the same cell line to study uptake and metabolism of eicosa-8,11,14-trienoic acid; no effort was made to compare with normal hepatocyte cultures. Fujiwara et al (1986) compared the anti-tumor effects of GLA on 4 neuroblastoma cell lines, without an attempt to relate their data to normal brain cells, while Ikai et al (1987) only studied inhibition of cell proliferation by PG's in transformed mouse epidermal cells (PAM 212) without examining whether similar effects occurred in normal epidermal cells.

Indeed, these studies make a significant contribution to

understanding particular aspects of lipid metabolism, but it is unfortunate that they have generally ignored the importance of relating data to the original normal cell type and thus, to the 'real world'. Such literature findings support Howard et al (1974), who reported that there have been very few direct comparisons of lipid metabolism between various related cell lines. Only recently, Begin et al (1989) commented in one of their articles that for valid comparisons to be made between normal and tumorigenic cell lines, the corresponding normal cell type should be used. Yet, many studies continue to fall short in this respect; the significance of such data is, therefore, uncertain in relation to the situation *in vivo*.

In the 'real world', however, individual FA's are not consumed *per se*. but rather as a combination of FA's, derived from a variety of food sources, usually in the form of plant and/or animal derived lipid contained in the product consumed, eg. oils. Thus, cells *in vivo* are rarely exposed to individual FA's in isolation, but rather to combinations, with different chain lengths and degrees of unsaturation. While knowledge of the behaviour of individual FA's is essential to quantitate individual effects, the effects of combinations are just as important. Few workers have used more than one FA at a time, and the effects of more complex combinations have been ignored *in vitro*: animal studies, however,

indicate that different dietary oils influence cell metabolism to a greater or lesser extent (eg. Barzanti et al 1986). Studies to date using cultured cells have not examined the effects of FA mixtures which mimick the FA composition of dietary oils. It is thus difficult to relate many of the studies to *in vivo* where normal cells of all tissues are continuously exposed to FA's derived from a variety of food sources.

The aims of this study were, therefore, to investigate the effect of exogenously added FA mixtures which mimick the FA composition of natural oils (termed pseudo-oils) on the viability of a range of normal cell types derived from mammalian species differing in desaturation capability. Furthermore, since evidence suggests that exogenous FA supplementation does not modulate plasma membrane associated parameters (Girao 1988), and the indications are that the microsomal desaturase/elongase, cyclooxygenase/lipoxygenase and/or lipid peroxidation pathways may be involved, it was hypothesised that the intracellular processes of polyenoic FA metabolism may be the route whereby growth limitation effects are induced; thus, it was also decided to assess these aspects of lipid metabolism.

The direct assay of the desaturase enzymes, *in vitro*. by means of incubation of subcellular fractions with radiolabelled substrates (eg. Actis Dato et al 1972) is

the most absolute means of assessing such enzyme activity, and can be applied to subcellular fractions from any cell type. Numerous studies have been conducted on isolated microsomes *in vitro*. and the direct assay procedure is well documented, at least for Δ6D (eg. Brenner et al 1966, Brenner 1971, Castuma et al 1972, Peluffo et al 1976, de Gomez Dumm et al 1975b, 1976, 1980, 1983, 1986). This assay, however, merely reflects total enzyme activity, not the activity actually expressed in the cell. An indirect assessment of desaturase activity is more realistic and is more relevant to normal and disease conditions since it more closely parallels the intact organism. This view is shared by Mahfouz et al (1981) who described difficulties in interpreting microsomal studies, and suggested cell culture systems to overcome this as such show significant parallels to the *in vivo* situation. The following year, Hill et al (1982) advocated measurement of relative substrate and product levels in cells as more accurate and reliable indications of true desaturase activity, rather than *in vitro* microsomal assays.

Three species of mammalia, viz. Rattus norvegicus (rat), Felis cattus (cat) and Homo sapiens (Man), were selected for this study. The selection of species was based on their known differing desaturation capabilities. The rat is reported to exhibit high activity (eg. Brenner 1971,

1974), Man has limited activity (eg. Dyerberg et al 1980 and Horrobin 1983), while the cat is reported to be Δ6D deficient (eg. Rivers et al 1975a, Frankel et al 1978 and Sinclair 1979). A wide range of tissues from each species were selected because of the known differences both in lipid profile and metabolism between tissues. Erythrocytes, lymphocytes, aortic endothelium, skeletal muscle, brain, lung, skin and white adipose tissues were studied in culture. By using available cells from all 3 species, it was intended to generate comparative data on the role of the desaturase enzymes, and the products derived from such reactions, in the modulation of cell proliferation both within and between species. Such data would direct more light on the involvement of these enzymes in the growth promoting or limiting effects of FA's.

Each cell type was to be incubated with a wide range of concentrations of each pseudo-oil. Proliferative, growth limiting, cytostatic and/or cytotoxic effects were to be assessed. Subsequent quantitative measurements of total protein, FA's, lipid peroxides and eicosanoids were to be performed to establish the possible mechanism(s) by which the pseudo-oils influenced cell viability. Protein was to be used as an indication of cell viability and as to the influence of pseudo-oils on total protein synthesis. FA analyses would permit assessment of exogenous FA incorporation into cellular lipids and

desaturase cascade enzyme capability. Lipid peroxide quantitation would assess the production of those moieties, as well as the cells ability to produce such. Finally, eicosanoids were to be quantitated to assess conversion of FA's to eicosanoids, giving an indication of cyclooxygenase/lipoxygenase enzyme function.

Interpretation of the data would be performed in the light of the known processes of lipid metabolism, in an attempt to determine the possible site(s) and mechanism(s) of the action of the pseudo-oil FA's. By employing a multi-parameter approach it was expected to obtain a greater insight into these mechanisms; furthermore, by using FA mixtures, it was expected to get closer to the situation <u>in vivo</u>.

CHAPTER 2: MATERIALS AND METHODS.

2.1 MATERIALS.

2.1.1 Animal and Human Ethics Assurance.

The protocol and all procedures carried out in this study were approved by the University of the Witwatersrand Animal and Human Ethics Controlling Committees. Clearance certificates obtained to use rat and cat tissues were assigned protocol numbers 87/21 and 88/7, and that for human blood collection was 21/2/89.

2.1.2 Source of Animals.

Animals were obtained from the Central Animal Service of the University of the Witwatersrand Medical School, Johannesburg, South Africa. They included inbred strains of Wistar specified pathogen-free (SPF) albino male rats, either 2 day old, 2 week old, or adult. Cats were approximately 2 day old kittens, or adults.

The animals were housed in cages at constant temperature and humidity, and were exposed to continuous 12 hour light/dark cycles with free access to food and water.

2.1.3 Source of Human Tissues.

Blood specimens were obtained with informed consent from human volunteers at the University of the Witwatersrand Medical School and adjoining Johannesburg Hospital.

2.1.4 Source of Cells for Tissue Culture.

With the exception of those acknowledged, all cells used in this thesis were isolated and cultured by the author. In most cases, young animals were used as sources of tissue to initiate primary cultures since cells survive the isolation procedure and adapt to *in vitro* culture conditions to a greater extent when obtained from young, than old, animals. It was generally possible to use 2 day old rat pups or kittens to provide sufficient tissue from which large numbers of viable cells could be isolated. However, 2 week old rat pups had to be used as a source of white adipose cells since younger animals had insufficient fat to yield the cell numbers required. Similarly, adult rats (>300g) or cats were utilised to obtain maximum numbers of aortic endothelial cells, erythrocytes and lymphocytes.

All cells were cultured under standard conditions with the same batch of serum, and without the addition of hormones or growth factors. Liver cells were excluded from the study since they require various growth factors for optimal proliferation, and financial constraints prevented the inclusion of such factors in the growth media.

Following is a description of the cells used and their origin:

Rat erythrocytes	Isolated from adult rats.
Rat lymphocytes	
Rat aortic endothelial cells*	
Rat skeletal muscle cells*	All isolated from 2-day old rat pups.
Rat brain cortical astrocytes	
Rat lung cells*	
Rat skin cells*	
Rat adipose cells*	Isolated from 2-week old pups.
Cat erythrocytes	Isolated from adult cats.
Cat lymphocytes	
Cat aortic endothelial cells*	
Cat skeletal muscle cells*	All isolated from 2-day old kittens.
Cat brain cortical astrocytes	
Cat lung cells*	
Cat skin cells*	
Cat adipose cells*	
Human erythrocytes	Isolated from adult volunteers.
Human lymphocytes	
Human (fetal) skeletal muscle cells*	Purchased from Highveld Biologicals (Pty) Ltd., South Africa.
Human (fetal) lung cells*	
Human (fetal) skin cells*	
3T3 benign Swiss mouse embryo fibroblasts	

*Largely take a fibroblastic morphology in culture.

While every attempt was made to obtain further human tissue types, ethical and logistical constraints limited the range of tissue types available.

Normal cells derived from primary cultures survive only for a finite number of passages, afterwhich they die. All experimental procedures employing normal dividing cells were therefore executed using cells subconfluent in the log growth phase at the lowest passage number possible, but never exceeding 50 population doublings. Practically, this was about 10-12 passages, usually obtained from split ratios of 1:2.

As cell culture is a dynamic system in which cells are infinitely capable of change, prior to their use in experimentation, dividing cells were cultured for a few passages in which any changes in growth rate and morphology were monitored. Samples of logarithmically growing cells were subsequently frozen so that, if necessary, any changes in characteristics could be related to passage number and the cells re-examined.

2.1.5 Tissue Culture Media, Reagents and Culture Vessels.

All sterile tissue culture plasticware, viz. 10ml and 50ml centrifuge tubes, 25, 75 and 175cm^2 vented culture flasks, 24-multi well culture plates and 1.8ml cryotubes were obtained from Sterilin Ltd., Feltham, England.

Hank's balanced salt solution (with 0.35g/l $NaHCO_3$, Ca^{2+}/Mg^{2+} free) was purchased from Flow Laboratories Ltd., Scotland.

Fetal calf serum, EDTA (0.1%+0.1% glucose in Dulbecco buffer, Ca^{2+}/Mg^{2+} free) and trypsin (0.25% in Dulbecco modification, Ca^{2+}/Mg^{2+} free) were obtained from the National Institute for Virology, South Africa.

Collagenase (0.3-0.4U/mg lyophilised from Clostridium histolyticum) was obtained from Boehringer Mannheim Biochemica, Mannheim, West Germany.

Nylon mesh (250 and 150µm) was obtained from Nybolt, Switzerland, while 0.45 and 0.22µm filters were bought from Millipore Corporation, Massachusetts, U.S.A.

Streptomycin sulfate (750U/mg), penicillin-G (sodium salt 1662U/mg), bovine serum albumin (BSA) fraction V (essentially fatty acid free), and trypan blue were bought from Sigma Chemical Company, St. Louis, Missouri, U.S.A., while gentamycin sulphate (Garamycin 10mg/ml) was obtained from Scherag, Isando, South Africa.

Sterile venoject evacuated blood collecting tubes (uncoated or with 0.5ml 3.8% Na-citrate solution as anti-coagulant), needles and syringes were obtained from the Johannesburg General Hospital.

Ficoll-Paque was purchased from Pharmacia Laboratory Separation Division, Uppsala, Sweden.

Dimethylsulphoxide (DMSO) was obtained from Merck, Darmstadt, West Germany.

2.1.6 Other Research Products, Chemicals and Reagents.

100% ethanol, 14% boron trifluoride in methanol, 2,6-di-tert-butyl-p-cresol (BHT), 1,1,3,3-tetramethoxypropane, malondialdehyde (MDA), thiobarbituric acid (TBA), trichloroacetic acid and Folin and Ciocalteau's phenol reagent were purchased from Merck, Darmstadt, West Germany.

Whatman (No. 1) filter paper circles (18.5cm) were purchased from Whatman Ltd., England.

The scintillation cocktail used was Aquagel I, obtained from Chemlab (Pty) Ltd., Johannesburg, South Africa.

Gasses (air, helium, hydrogen, propane and medical carbon dioxide) were obtained from Afrox, South Africa.

The 6 foot x 3mm ID GLC column, on 100/120 chromabsorb, was purchased from Anatech Instruments (Pty) Ltd., South Africa.

Sephadex LH-20 (particle size 25-100µm) was obtained from Pharmacia Laboratory Separation Division, Uppsala, Sweden.

HPLC grade methanol and acetonitrile, 0.22 and 0.45µm filters, and the C_{18} HPLC column (100mm x 8mm ID) were obtained from Waters Millipore Corporation, U.S.A.

Eicosanoids standards, viz. PGI_2, TXB_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGE_1 and PGD_2 were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Fatty acids, as ≥95% pure free acids, were also obtained from this source. The fatty acids (all in the cis-configuration) are listed below:

10:0	decanoic (capric) acid
12:0	dodecanoic (lauric) acid
14:0	tetradecanoic (myristic) acid
16:0	hexadecanoic (palmitic) acid
16:1ω9	9-hexadecenoic (palmitoleic) acid
17:0	heptadecanoic (margaric) acid
18:0	octadecanoic (stearic) acid
18:1ω9	9-octadecenoic (oleic) acid
18:2ω6	9,12-octadecadienoic (linoleic) acid
18:3ω6	6,9,12-octadecatrienoic (γ-linolenic) acid
18:3ω3	9,12,15-octadecatrienoic (α-linolenic) acid
18:4ω3	6,9,12,15-octadecatetraenoic (morotic) acid
20:2ω6	11,14-eicosadienoic acid
20:4ω6	5,8,11,14-eicosatetraenoic (arachidonic) acid
20:5ω3	5,8,11,14,17-eicosapentaenoic acid
22:6ω3	4,7,10,13,16,19-docosahexaenoic acid

The following cis-radiolabelled fatty acids (≥95% pure) were obtained from Amersham International Ltd., England:

9-[1-^{14}C]-18:1ω9, 53.8mCi/mmol.

9,12-[1-^{14}C]-18:2ω6, 53.6mCi/mmol.

9,12,15-[1-^{14}C]-18:3ω3, 53.9mCi/mmol.

Methylated fatty acid standard mixtures for GLC, viz. GLC-3A, GLC-08A and GLC-68A, were obtained from Nu Chek Prep, Inc., Minnesota, U.S.A.

Unless otherwise stated, all other chemicals used were purchased from Merck, South Africa, or the University Central Stores, and were of analytical grade.

All weighing of chemicals was carried out on a Sartorius 4-digit electronic digital balance.

2.1.7 Fatty Acid Nomenclature in Relation to the Study.

There are 3 commonly used systems of nomenclature for FA's (Davidson et al 1985a):

a) 'trivial' nomenclature, which names the FA on the basis of some factor other than structure. For example, adrenic and nervonic acids, so called as they were first isolated from adrenal glands and nerve tissue, respectively.

b) 'systematic' nomenclature, which names the FA according to the number of carbon atoms and degree of unsaturation. However, this creates complications in recognising members of homologous series as the double bonds are numbered from the carboxyl terminal end of the chain; thus when a FA is chain elongated and further desaturated the double bonds appear to change position when in fact they do not.

c) 'ω', or 'n-' nomenclature, which numbers the double bonds from the carbon atom at the methyl ('ω') terminal. As FA's are always elongated at the carboxyl end of the chain, this system circumvents any desaturation/elongation problems and thus allows members of a homologous series to be easily recognised. By convention, FA nomenclature therefore relates the position of a double bond within a FA to its distance from the methyl, or ω, terminal carbon atom.

In this thesis, the 3 systems will be used interchangeably. The list in 2.1.6 includes the names of each FA used, and this may be used as a reference to cross-correlate between the three systems of nomenclature. Unless otherwise stated, all FA's mentioned in the text are in the cis-configuration.

2.2 METHODS.

2.2.1 Tissue Culture.

The standard tissue culture techniques employed in this study were those in general use in this and other laboratories around the world, and were based on the general methods described by Flow (1983), Freshney (1983, 1986) and Gibco (1984).

2.2.1.1 Sterility Procedures.

Glassware, filtration units and all instruments were thoroughly washed in deionised water prior to sterilisation by autoclaving at 15 pounds/square inch for 15 minutes, followed by a 10 minute drying cycle. Liquids were sterilised by filtration through 0.22µm Millipore membrane filtration units.

The tissue culture fascility was routinely exposed to an ultra-violet germicidal lamp at least 30 minutes prior to use, and continuously when not in use, to maintain a sterile enviroment.

2.2.1.2 Preparation of Tissue Culture Medium.

All culture media was prepared in our own laboratory from high grade chemicals according to reported specifications (Flow 1983 and Gibco 1984). The standard

medium routinely employed for culturing all cells was Dulbecco's Modification of Eagle's Medium (DMEM) (with glutamine, but without $NaHCO_3$). Powdered medium was appropriately reconstituted with deionised, double distilled water, buffered to a pH of 7.4 with sodium bicarbonate, and then immediately sterilised by membrane filtration under positive air pressure, to prevent loss of carbon dioxide. The media was incubated for 72 hours at 37°C to ensure sterility, and subsequently stored at 2°C to 8°C for a maximum period of one month. Medium was free of antibiotics, and only a supplementation of 2mM glutamine and 10% (v/v) heat inactivated fetal calf serum (FCS) was routinely added for fortification. It appears that the presence of serum fascilitates the adherence of cells to tissue culture vessels, especially from newly established primary cultures (Bonney et al 1974 and McGowan et al 1981). Following addition of 10% FCS to DMEM, this completed 'growth/incubation medium' was again filtered through a sterile 0.22µm Millipore hand filter prior to use. Total protein in growth medium (90% DMEM+10% FCS) was assayed using the method of Lowry et al (1951).

2.2.1.3 Collection and Deactivation of Rat, Cat and Human Serum.

Whole blood was collected aseptically from suitable adult rat, cat and human donors in sterile blood

collecting tubes and allowed to coaggulate. The plasma clot was removed and the serum subsequently centrifuged at 200xg for 30 minutes in a clinical benchtop centrifuge. Supernatant serum was carefully collected without disturbing the cell debris pellet, was inactivated by heating to 56°C for 30 minutes and subsequently cooled to room temperature. This was centrifuged at 200xg for 10 minutes to remove any particulate material, and the supernatant serum filtered first through a 0.45µm, then a 0.22µm sterile hand filter, and subsequently stored at -20°C until used.

2.2.1.4 Tissue Dissociating Solution.

This was prepared fresh every time by dissolving 0.05% collagenase in Hank's balanced salt solution (HBSS). An antibiotic 'cocktail' consisting either of 100U/ml penicillin and 100µg/ml streptomycin, or alternatively 200µg/ml gentamycin sulphate, was added. Gentamycin was the antibiotic of choice as Schafer et al (1972) showed that it exhibits broad spectrum bactericidal activity, is heat and pH stable, and does not induce any notable influence on cell metabolism.

Prior to use, the dissociating solution was gassed with 95% O_2/5% CO_2 for at least 30 minutes, the pH was adjusted to 7.4, the solution subsequently sterilised by filtration twice through a 0.22µm filter, and then warmed to 37°C.

2.2.1.5 Initiation of Primary Cell Cultures.

The general tissue isolation procedure employed a combination of mechanical and enzymatic tissue dis-aggregation techniques as described by Sly et al (1979), Flow (1983) and Freshney (1983, 1986). The general procedure described below was used for the isolation of cells derived from aortic endothelium, skeletal muscle, brain, lung, skin and white adipose tissue. Slight modifications of the procedure, if necessary, were used for certain tissue types to ensure maximal viable cell yield (see 2.2.1.5.a-g for modifications).

All cell isolations were conducted between 10am and 12 noon, as suggested by Actis Dato et al (1972), to minimise differences created by diurnal variations. During the entire tissue isolation procedure, a bunsen flame was kept burning close to the work area, and strict sterile procedures were maintained throughout.

Rats were transferred to the aseptic tissue culture environment and placed in a sterile plastic container lined on its base with adsorbent paper. This container, together with all the instruments required in the isolation procedure, were previously exposed to an ultra-violet germicidal lamp for at least 30 minutes to destroy any surface pathogens.

Rat pups were killed by decapitation without anesthesia

using a pair of sharp sterile dissecting scissors, while older rats were stunned and killed by cervical dislocation. Animals were placed onto a clean absorbant cotton pad layered over a simple cork board (cooled to 4°C prior to use), and secured. The appropriate animal surface was thouroughly swabbed with 70% ethanol.

Prior to use, disecting instruments were briefly emersed in 70% ethanol, flamed over a bunsen (to evaporate all ethanol and fix any loose particulate matter), and cooled in ice. A second set of sterile operating instruments was always available as backup in the event of accidental contamination of the first. The required organ/tissue was removed surgically by making a midline incision at the appropriate site, using a separate set of sterile dissecting instruments for making incisions through the skin, muscle layer, into the body cavity and to free and lift the organ/tissue out of the animal.

The isolated organ/tissue was placed into a 25ml glass beaker and rinsed with preheated (37°C) sterile saline containing 100U/ml penicillin and 100µg/ml streptomycin (to decontaminate the organ/tissue in the event of any contamination either <u>in situ</u> in the animal, or as a result of the dissection and isolation procedure). The organ/tissue was stirred at half speed with the aid of a magnetic stirring bar in an Agrimatic Stirring apparatus at 37°C for 5 minutes, to remove as much blood as possible.

Following decanting of the saline, the organ/tissue was chopped into 1-5mm cubes with a sterile scalpel. Any blood was washed off by several changes of cold saline. Dissociation of the tissue pieces was initiated by incubation with 20ml preheated dissociating solution (section 2.2.1.4) in a 25ml glass Ehrlenmeyer flask with gentle magnetic stirring (100±20rpm) at 37°C, as recommended by Herring et al (1983). The temperature was carefully monitored during the entire procedure.

Liberation of cells was manifested by an increased turbidity of the dissociating solution. Digestion of the tissue was allowed to continue for about 10-15 minutes, after which time the flask was briefly placed on ice to allow the larger undissociated tissue fragments to settle. The supernatant was gently decanted into a sterile 50ml plastic centrifuge tube, an equal volume of DMEM+10% FCS was added to terminate enzyme action, and this placed on ice.

The above procedure was repeated about 5 times with fresh dissociating solution. Generally, 90 minutes after initiation of the tissue dissociation procedure, most of the tissue was digested.

The dissociated cells obtained after each 10-15 minute incubation were immediately filtered through a sterile 250µm nylon gauze mesh, followed by passage through a 150µm mesh into sterile 50ml plastic centrifuge tubes.

After gentle inversion, the suspension was centrifuged at 100-200xg for 5 minutes in a clinical benchtop centrifuge with swing-out buckets, as recommended by Williams et al (1977b). This low-speed centrifugation removed most contaminating blood, while pelletting the larger isolated cells.

The supernatant was decanted, packed cells were resuspended in warm DMEM+10% FCS, washed again at 100-200xg for 5 minutes, followed by a third centrifugation at 200xg for 10 minutes to completely pellet the cells. The final pellet was resuspended in 10ml DMEM+10% FCS containing 200µg/ml gentamycin (Williams et al 1977b), and plated into 25cm^2 sterile tissue culture flasks with vented caps. Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C in a water-jacketed incubator (Labotec, South Africa). Time from surgery to plating of isolated cells was approximately 2 hours.

After allowing 3-4 hours for cell attachment to the culture vessel, cells were examined microscopically for for viability, adhesion and any microbial contamination. Subsequently, the contents of the flask were gently swirled and decanted, thus fascilitating the removal of any cellular debris, non-adherent, loosely adherent or dead cells. Viable cells were subsequently fed with warm antibiotic-free 90% DMEM+10% FCS, and checked every day. Growth medium was replaced with fresh medium on the

second day, and subsequently every 48-72 hours (to remove any loosely attached/dead cells, cellular debris and potentially toxic metabolic products) until a near confluent monolayer was established.

The above technique maximises the viable cell yield, and thus shortens the period to which cells are exposed to disaggregating enzymes. Mechanical procedures were kept to a minimum as enzymatically isolated cells retain more characteristic features of the tissue in vivo, in contrast to mechanically prepared cells (Jezyk et al 1969 and Ontko 1972).

Cells were allowed to divide several times, resulting in the formation of islands of cells which eventually formed a monolayer. The cell populations obtained from a particular primary culture were heterogeneous and thus representative of the whole organ/tissue in the 'real world'.

Further growth and maintenance of cells were carried out employing the principles described by Sly et al (1979), Flow (1983) and Freshney (1983, 1986). The different cell types were cultured chronologically separate to avoid the possibility of cross contamination. Proliferating cells were regularly monitored for doubling time between passages.

2.2.1.5.a Modifications for the Isolation of Cat Tissues for Primary Culture.

Cats were transferred from their cages to the autopsy facility of the Animal Unit at about 9am, and killed with an intravenous overdose (1.0ml/kg IV) of Euthanase solution (sodium pentobarbital). The appropriate surface was immediately swabbed with 70% ethanol and an incision made using strict sterile procedures, as before. The removed organ/tissue was placed in pre-cooled saline solution containing 100U/ml penicillin and 100µg/ml streptomycin, and rapidly transported on ice to the tissue culture facility, where cells were isolated as described previously in 2.2.1.5.

2.2.1.5.b Modifications for the Isolation of Cells from Aortic Endothelium.

These modifications included those described by Wechezak et al (1973), Bar et al (1984) and Hoshi et al (1986).

The aorta from adult animals was removed, rinsed with saline to remove blood, and cut into 5-10mm pieces. The vessels were everted so that the endothelium was exposed to the dissociating solution. After each incubation period, the dissociating solution was pipetted repeatedly over the endothelial surface to dislodge the cells, which were collected and washed with growth medium as before.

2.2.1.5.c Modifications for the Isolation of Cells from Skeletal Muscle.

The modifications of Bullaro et al (1976) and Koningberg (1979) were employed.

Skeletal muscle from the thigh was used. Following collagenase digestion, incubation of remaining diced muscle fragments with 0.1% EDTA:0.25% trypsin (1:1 v/v) for 30 minutes increased cell yields.

2.2.1.5.d Modifications for the Isolation of Cells from Brain.

The modifications employed were those described by Poduslo et al (1975), Walker et al (1984) and Bjerkvig et al (1986).

Whole brain was freed from its meninges and removed from the animal. The cerebellum was discarded and cells were isolated from the cortex only. Increased cell yields were obtained when brain fragments were incubated with dissociating solution saturated with oxygen for up to 60 minutes, followed by incubation with 0.1% EDTA:0.25% trypsin (1:1 v/v) for a further 30 minutes. When necessary, remaining tissue fragments were mechanically disaggregated through a 250µm and then a 150µm mesh. The dissociating solutions and initial growth media were all supplemented with additional glucose (final

concentration 1g%). This method yielded mainly glial cell progenators, which could be identified as largely astrocytes with some oligodendrocytes.

2.2.1.5.e Modifications for the Isolation of Cells from Lung.

The modifications of Tompa et al (1979) and Kumar et al (1987) were employed.

Only the distal 2/3 of lungs were used to eliminate the bronchii, etc. Lungs were washed three times in growth medium to flush out as much blood from the organ as possible. After collagenase incubation for 60 minutes, remaining cells were dissociated from the diced organ with 0.1% EDTA:0.25% trypsin (1:1 v/v) for 30 minutes to increase the cell yield.

2.2.1.5.f Modifications for the Isolation of Cells from Skin.

The modifications of Fujimoto et al (1977), Sly et al (1979) and Yen-Chow et al (1984) were employed.

Skin biopsies were obtained from the anterior abdominal wall and from the forearms. Any hair was shaved off the skin at the appropriate site prior to sample removal. Cell isolation was carried out as described in 2.2.1.5.

2.2.1.5.g Modifications for the Isolation of Cells Cells from Adipose Tissue.

These modifications were based on the techniques utilised by Rodbell (1964), Adebonojo (1975) and Fain (1975).

Adipose tissue was removed from non-obese young animals from the supra-testicular, forearm and scapular regions, which contain white adipose tissue only. All procedures were identical to those outlined previously, but the dissociating solution and initial culture medium contained 5.0% albumin.

Centrifugation pelleted blood, endothelial and some stromal-vascular cells, while the supernatant contained whole adipocytes, lipid droplets from damaged adipocytes and some stromal-vascular cells. There is evidence that the stromal-vascular fraction contains fibroblasts which exhibit potential to become adipocyte precursors, and acquire morphological and biochemical characteristics similar to mature adipocytes (Van et al 1976). Further, when mature adipocytes lost their intracellular lipid, they aquired characteristics of stromal-vascular cells (Van et al 1976).

2.2.1.6 Determination of Cell Viability.

Cell viability was determined using the trypan blue exclusion method, as described by Paul (1975). A suspension of 1 part cells (10µl) to 1 part 0.1% trypan blue in 0.9% saline was made. After 2 minutes at room temperature, cell counts were performed by counting the number of viable cells (which exclude the dye) in an improved Neubauer Hemocytometer (1mm x 1mm x 0.1mm).

2.2.1.7 Trypsinisation of Cell Monolayers.

When nearly confluent monolayers of logarithmically growing cells were obtained, subcultures/trypsinisations were performed (Sly et al 1979, Flow 1983 and Freshney 1983, 1986).

Existing medium was decanted from the tissue culture vessel. Sufficient sterile 0.1% EDTA at 37°C was added to just cover the cells, and the culture vessel returned to the incubator for 5 minutes; thereafter, an equal volume of sterile 0.25% trypsin solution at 37°C was added and the cells once again returned to the incubator. Trypsinisation was allowed to continue for a period not exceeding 15 minutes, during which time the cells were regularly monitored for detachment.

As soon as the cells became detached, an equal volume of warm DMEM+10% FCS was added to the culture vessel to

inhibit the action of the trypsin. The cell suspension was decanted into a 50ml sterile plastic centrifuge tube and centrifuged at 200xg for 10 minutes. Subsequently, the supernatant was decanted, cells were resuspended in warm growth medium and re-centrifuged. After decanting the supernatant, the cell pellet was resuspended in a small volume of growth medium and cell viability determined as described in 2.2.1.6. Cells were either diluted with warm growth medium to $1x10^5$/ml, plated into sterile tissue culture flasks, incubated and allowed to grow until a new monolayer was established, or frozen.

2.2.1.8 Cell Freezing for Long Term Storage.

The general principles described by Sly et al (1979), Flow (1983) and Freshney (1983, 1986) were used. Cells at the lowest possible passage number from each primary culture were frozen for long term storage.

Following the initial trypsinisation and subsequent washing of cultures (2.2.1.7), the final cell pellet was reconstituted with a small volume of growth medium and a cell count was performed. Cells were frozen in sterile 1.8ml cryotubes at a concentration between 2 and $5x10^6$ viable cells/1ml growth medium, in an equal volume of a 70% DMEM:10% FCS:20% dimethylsulphoxide (DMSO) solution (v/v/v), to give a final DMSO concentration of 10%. Cryotubes were placed in a polystyrene container and put into a -70°C deep freeze overnight to allow for a

cooling rate of approximately 1°C/minute. Subsequently, cells were stored in the vapour phase of a liquid nitrogen container (cryobank).

2.2.1.9 Resuscitation of Frozen Cells.

Cryotubes were removed from the cryobank and thawed as quickly as possible in a 37°C waterbath as described by Sly et al (1979). The contents of the cryotube were added to sufficient growth medium at 37°C to dilute the DMSO at least tenfold. The cell suspension was centrifuged at 200xg for 10 minutes, the supernatant decanted, and the cells re-washed in warm growth medium. The resulting cell pellet was re-suspended in sufficient warm growth medium to perform a cell count (2.2.1.6), diluted to $1x10^5$/ml, plated into sterile tissue culture flasks and incubated. Cells were fed 24 hours later and subsequently every 2-3 days as necessary. When cultures reached near confluence, they were subcultured or frozen as described in 2.2.1.7 and 2.2.1.8, respectively.

2.2.1.10 Isolation of Peripheral Erythrocytes and Lymphocytes.

2.2.1.10.a Collection of Blood from Rats.

Adult rats were killed with an intraperitoneal overdose (1.0ml/kg) of Euthanase solution (sodium pentobarbital) rather than cervical dislocation so that blood vessels in the neck remained intact, thus maximising the volume of blood collected (normally 3-10ml). The thorax was immediately opened once the appropriate reflexes dissappeared, and the animal bled from the inferior vena cava into a sterile citrated syringe and needle.

2.2.1.10.b Collection of Blood from Cats.

Cats were anaethetised with Saffan intramuscularly. As soon as the required reflexes were diminished/lost, blood was drawn from the jugular vein into a sterile citrated needle and syringe. Cats were injected with an antagonist, monitored for the return of reflexes and subsequently returned to their cage. Cat donors used for this purpose were bled no more than once a week, with the blood collection volume not exceeding 5.0ml per collection per animal.

2.2.1.10.c Collection of Blood from Humans.

Venous blood from normal human volunteers was collected

into sterile citrated blood collecting tubes at the adjoining Johannesburg General Hospital according to standard clinical procedures.

2.2.1.10.d Blood Separation.

Whole blood was immediately centrifuged at 1000rpm (200xg) in a Beckman TJ-6 clinical benchtop centrifuge for 15 minutes at room temperature. Platelet rich plasma was subsequently carefully removed and discarded, while the buffy coat and erythrocytes were carefully separated using sterile pipettes, and placed into separate tubes.

2.2.1.10.e Erythrocyte Preparation.

Erythrocytes were washed 3 times with growth medium at 37°C by centrifugation at 100xg for 10 minutes. Cells were subsequently diluted with warm growth medium to $1x10^5$/ml, plated into 24-multiwell plates, incubated, and dosed an hour later (see 2.2.3).

2.2.1.10.f Lymphocyte Isolation.

Mononuclear cell isolation was based upon the original principle put forward by Boyum (1968). The buffy coat was suspended in growth medium:citrate (9:1 v/v). This suspension was carefully layered onto Ficoll-Paque in a ratio of 4:3 (v/v), and centrifuged at 400xg for 30-40 minutes at 18-20°C in a clinical benchtop centrifuge

with swing-out buckets.

The upper phase was removed leaving the mononuclear layer at the Ficoll-Paque interface undisturbed. The mononuclear layer was collected and transferred to a new tube. It is essential to remove as much interface as possible but a minimum of Ficoll-Paque and supernatant to reduce unnecessary granulocyte, platelet and plasma protein contamination in the mononuclear fraction. Cells were washed three times with at least 3 volumes of warm growth medium, at not more than 200xg for 10 minutes (to minimise platelet contamination) at room temperature.

The cells were resuspended in warm growth medium, plated into 25cm^2 culture flasks, and incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Approximately 3 hours later, unattached cells (lymphocytes) were pooled, and washed with warm growth medium at 200xg for 10 minutes. Cells were resuspended in warm growth medium, diluted to $1x10^5$/ml, plated into 24-well multidishes, incubated, and dosed 60 minutes later (see 2.2.3).

2.2.1.11 Light Microscopy of Cells.

All cultures were examined daily with a Nikon Diaphot phase contrast microscope (with camera attachment). Parameters monitored included any sign of contamination, pH of medium, cell morphology, proliferation and degree of confluency.

2.2.2 Experimental 'pseudo-Oil' Preparation.

The fatty acid compositions of a wide range of natural oils, and of potential oil sources, have been reported by McCance et al (1978) and Giangregorio et al (1988a). Seven natural oils were chosen for this study as rich supplies either of ω9, ω6 or ω3 series unsaturated, or saturated, FA's.

Individual FFA's (from the list in 2.1.6) were initially made up as stock solutions (100mg/ml) in 100% ethanol, and maintained at -20°C under an atmosphere of nitrogen. These individual FFA stock solutions were mixed in appropriate proportions to mimick the FA spectra of different natural oils, viz. coconut oil (CO), meat oil (MO), olive oil (OO), sunflower seed oil (SSO), linseed oil (LO), evening primrose seed oil (EPO) and fish oil (FO). These FA mixtures were termed 'pseudo-oils' (p-oils), and their composition paralleled that of oils commonly ingested by humans. The p-oils were abbreviated to p-CO, p-MO, p-OO, p-SSO, p-LO, p-EPO and p-FO, with each having different degrees of unsaturation, corresponding to the natural oil. The FA spectra of each p-oil was checked by GLC (see 2.2.7.2) to ensure it mimicked the FA spectra of the natural oils.

Working solutions of these p-oils were made up fresh prior to use. From the stock p-oil mixtures in 100% ethanol, 40µl was aliquoted into a glass vial and the

ethanol evaporated under a stream of nitrogen gas in a 37°C waterbath. To mirror the in vivo situation as closely as possible, p-oil FA's were complexed with bovine serum albumin to facilitate their delivery to the cells (McGee et al 1982). This was achieved by adding 200µl of a sterile 5.0% essentially FA free BSA (fraction V) solution in distilled water to act as FA carrier, and this incubated for 5 minutes at room temperature. To this, 1.8ml growth medium at 37°C was added to bring the mixture to a total volume of 2.0ml. The p-oil mixture was gently heated prior to use as recommended by Weyman et al (1977) so that the FA's remained in solution during dosing, especially in the case of p-oils rich in saturated FA's.

2.2.3 Experimental 'pseudo-Oil' Dosing.

Adherent cells in the log phase of growth were trypsinised as described in 2.2.1.7, and a cell count was performed (2.2.1.6). Each cell type was seeded at 1.0×10^5 viable cells/ml in 24-well tissue culture plates in a total volume of 1.0ml growth medium. Adherent cells were incubated with growth medium for a period of 24 hours prior to dosing with p-oils to allow for cell recovery from trypsinisation. Cells in suspension, viz. erythrocytes and lymphocytes, did not require this recovery period, since trypsinisation was not necessary.

Appropriate volumes of the albumin-bound p-oil solutions were added directly to the culture medium to achieve the indicated p-oil concentration required. Cultures were dosed at 10mg/l intervals with each p-oil working solution, to yield final p-oil concentrations ranging from 0 to 100mg/l. The maximum albumin concentration utilised as FA carrier was 0.025% (250mg/l). After dosing, the plates were gently swirled to evenly disperse the p-oil throughout the wells, and cultures were incubated for 48 hours at 37°C in an atmosphere of 90% humidity, 5% carbon dioxide:95% air.

Following the incubation period, the existing medium was removed, cells were trypsinised if adherent (2.2.1.7), and cell viability determined (2.2.1.6). The percent cell viability at each p-oil concentration was calculated by assuming the mean number of viable control cells to correspond to 100%. The ID_{50} was calculated for each p-oil with every cell type studied, and was taken as the p-oil concentration required to reduce cell viability to 50% of controls.

To ascertain the approximate concentration of p-oil required to prevent nett cell proliferation, the number of viable control cells at the end of the 24 hours allowed for recovery from trypsinisation was compared to the number of viable control cells at the end of the 48 hour incubation period with p-oils. This was expressed

as a percentage, and represented the 'cytostatic number'. Only p-oil concentrations reducing cell viability significantly below the cytostatic number would be cytotoxic.

2.2.3.1 Albumin Effects on Cell Viability.

The effects of albumin were investigated on cell viability to eliminate albumin involvement in the effects induced with p-oil dosage, and thus to justify under the chosen culture conditions the use of albumin as FA carrier with each of the cell types used.

Cells were set up as previously described in 2.2.3, and dosed with varying concentrations of albumin without p-oil to yield final concentrations ranging from 0 to 250mg albumin/l growth medium. After 48 hours incubation, cell counts were performed (2.2.1.6), and this compared to controls.

2.2.3.2 Varying the Albumin Concentration with a Fixed pseudo-Oil Concentration.

The effect of altering the albumin concentration at a fixed p-oil concentration was investigated on cell viability to eliminate the possibility that any of the effects induced with p-oil dosage were modulated by different albumin concentrations.

Cells selected from each species were seeded as

described in 2.2.3, and dosed at the concentration corresponding to the ID_{50} for that p-oil which induced a significant effect on cell viability. At least 5 points were chosen around which to vary the albumin concentration, keeping the p-oil concentration fixed, viz. 2 points below, 2 points above, and the ID_{50} of the chosen p-oil. The appropriate amount of albumin was added to the cultures to correspond to that level used at the points chosen, and cultures were incubated for 48 hours at 37°C in an atmosphere of 5% carbon dioxide:95% air. Cell viability was subsequently determined, and this compared to controls.

2.2.3.3 Effect of Serum Type on Cell Growth.

To ensure that the routine use of 10% FCS in culture medium did not adversely influence cell viability, cells derived from rat, cat and human skin were plated at $1x10^5$ cells/ml in DMEM+10% rat serum (RS), DMEM+10% cat serum (CS) and DMEM+10% human serum (HS), respectively. The growth of these cells in DMEM containing serum from their own species was monitored by determination of cell viability after 24, 48 and 72 hours incubation, and compared with that of the same cells grown in DMEM+10% FCS over a 72 hour period.

Total protein (2.2.5), lipids (2.2.6) and FA's (2.2.7) were quantified in the above growth media to determine their possible contribution to the results obtained.

2.2.4 Bulk Dosing of Cultures for Quantitative and Qualitative Analyses.

2.2.4.1 Selection of Cells Types.

Cells from each species previously studied viz. erythrocytes, lymphocytes, aortic endothelium, skeletal muscle, brain, lung, skin and adipose tissue, were cultured to provide sufficient material for further analytical procedures.

For each available adherent cell type, frozen cell stocks at low passage numbers were resuscitated as previously described in 2.2.1.9. and each grown in bulk. Erythrocytes and lymphocytes were freshly isolated.

2.2.4.2 Selection of the Dosing pseudo-Oil Concentrations.

For each of the cell types in 2.2.4.1, between $5\text{-}10\times10^6$ cells/175cm^2 tissue culture flask were seeded. Each cell type was subsequently incubated with the appropriate amount of p-oil, as described before, corresponding to four different concentrations, viz. 0, 20, 40 or 60mg/l, which were found to best reflect the relationship between cell viability and p-oil concentration across the spectrum of cells studied previously.

2.2.4.3 Harvesting of Bulk Cultures.

Following the 48 hour p-oil incubation period, the growth medium was removed from each flask, and this centrifuged at 1000xg for 10 minutes in a Beckman TJ-6 centrifuge. Supernatants (spent incubation medium) from the same concentration of each dosed p-oil were pooled together and stored at -20°C until required for further experimentation. Prior to use, each aliquot of spent medium was centrifuged at 5000xg for 10 minutes.

Adherent cells were trypsinised and the resulting cell pellet obtained after centrifugation was combined with any obtained from centrifugation of the spent incubation medium. For each cell type, cell viability was assessed as described before, and compared to that previously obtained. The cell suspension was subsequently centrifuged at 1000xg for 10 minutes. The supernatant was discarded and the cells washed three times by resuspension in 20.0ml physiological saline (0.85%) and centrifugation at 1000xg for 10 minutes each; supernatants were discarded following each wash.

Following the final centrifugation, the cell pellet was resuspended and swollen in 2.0ml of a solution at pH 7.4 containing 1mM $NaHCO_3$, 2mM $CaCl_2$ and 5mM $MgCl_2$, and frozen to -20°C to lyse the cells. Lysed cells were subsequently stored at -20°C until required for further analysis.

Prior to further experimentation, the frozen cells were thawed and homogenised with 25 strokes of a Dounce homogeniser.

2.2.5 Total Protein Determination.

100µl aliquots of lysed cells obtained in 2.2.4.3 were assayed for total protein by the method of Lowry et al (1951). Absorbances were read at 600nm in 1cm quartz cells in an LKB Biochrom Ultrospec 4050. Total protein was calculated with reference to bovine serum albumin (fraction V), in the range 0-100µg, assayed in parallel with the samples. The results were compared with those of controls.

2.2.6 Quantitative Lipid Analysis.

2.2.6.1 Total Lipid Extraction.

Total lipids were extracted from aliquots of p-oils, DMEM+10% FCS, DMEM+10% RS, DMEM+10% CS, DMEM+10% HS and spent incubation media. Once small aliquots of the lysed cells at each of the p-oil concentrations from 2.2.4 were taken for total protein (2.2.5) and lipid peroxide (2.2.9) assays, the remaining cell suspensions were also extracted for lipids.

Total lipids were extracted from samples with 20 volumes chloroform:methanol (2:1 v/v) containing 0.01% (w/v) 2,6-di-tert-butyl-p-cresol (BHT) as antioxidant, and the lipids left to extract overnight at 4°C, as per the method of Folch et al (1957).

Lipid extracts were filtered into glass separating funnels using Whatman (No.1) filter paper circles, and washed by adding 20% (v/v) physiological (0.85%) saline. The solutions were thoroughly mixed in stoppered separating funnels, and the phases allowed to separate overnight at 4°C. The lower chloroform (lipid) phase was subsequently removed and taken to dryness under vacuum at 37°C in an all glass Heidolph rotary evaporator at 190rpm. Resulting concentrated lipid extracts were redissolved in a small volume of chloroform, and transferred into clean 20ml screw-cap glass vials. All

extracts were diluted to 20.0ml with chloroform, stored as stock solutions at -20°C, and used for all further qualitative and quantitative lipid analyses.

2.2.6.2 Determination of Total Lipid Dry Weights.

Total lipid dry weights were determined in triplicate as per the method of Kates (1972). Clean 7.5ml glass vials were kept overnight in a Salvis oven at 100°C to evaporate any moisture present. Empty vials were removed from the oven, allowed to cool to room temperature, and weighed in a 4-decimal digit analytical electronic balance to 0.5mg. Empty vials were returned to the oven for 1 hour and subsequently reweighed. This procedure was repeated until vial weight was constant to within ±1.0mg.

1.0ml aliquots of stock lipid extract from 2.2.6.1 were pipetted into the empty glass vials and taken to dryness in a 37°C waterbath under a slow stream of nitrogen gas. The vials were returned to the oven for 1 hour at 100°C to ensure the chloroform was taken to complete dryness, then weighed and dried repeatedly as before until the weight was constant to within ±1.0mg.

Total lipid dry weight was calculated by subtracting the weight of the empty vial from that with lipid, and multiplying the resultant weight by the total volume of stock lipid extract in 2.2.6.1.

2.2.7 Qualitative Lipid Analysis.

2.2.7.1 Fatty Acid Methylation.

During methylation, FA's are removed from the glycerol backbone, and derivatised to methyl esters. This process was described by Moscatelli (1972) and is essential as it increases FA volatility and thus enables these moieties to be separated chromatographically.

10.0ml of stock lipid extract from 2.2.6.1 were pipetted into clean glass vials and the chloroform taken to dryness in a 37°C waterbath under nitrogen gas. To each vial was added 0.2ml petroleum ether (bp 40-60°C), followed by 1.0ml 14% boron trifluoride in methanol. Vials were briefly gassed with nitrogen to remove any air, immediately sealed and placed in a 100°C oven for 30 minutes to facilitate FA transmethylation. The vials were subsequently cooled to room temperature, their contents washed with 1.0ml saturated NaCl solution, followed by extraction of the fatty acid methyl esters (FAME's) with 5.0ml petroleum ether. The vials were sealed, shaken and the phases allowed to separate for at least 15 minutes at room temperature. The upper petroleum ether phase was removed using a pasteur pipette (making sure not to take up any interface), placed into a new 7.5ml glass vial, sealed under nitrogen gas and stored at -20°C until analysed by GLC.

2.2.7.2 Gas-Liquid Chromatographic Analysis of the Fatty Acid Methyl Esters.

In gas-liquid chromatography (GLC), volatile compounds are passed in a stream of inert gas (mobile phase) through a column in which a liquid (stationary phase) is immobilised on a solid support. Separation is based on the partition of compounds between the gas and liquid phases. The compounds emerge from the column as distinct peaks that can be quantitated.

All GLC analyses were performed as one group to eliminate between-batch variations. Prior to GLC analysis, the FAME's from 2.2.7.1 were concentrated by reducing their volume to dryness at 37°C under nitrogen gas. The FAME's were redissolved in 500µl petroleum ether added down the sides of each vial, this again taken to dryness, and subsequently redissolved with 100µl petroleum ether.

FAME's were analysed by means of a Varian 3400 gas chromatograph equipped with a flame ionisation detector (FID) and a SP-2330 column (2m x 3mm internal diameter, with Chromosorb 100/120 WHP-SP as support) using helium as carrier gas. 1µl of the concentrated FAME sample was injected manually onto the GLC column. The column temperature was set isothermally at 200°C with both the injector and detector set at 220°C. Run time was set at 31 minutes. Peaks were quantitated using a Varian 4270

integrator, and the relative percentage total area of each FAME recorded. Identification of the FAME's was made by comparison with the retention times of known saturated and unsaturated methylated FA standards. GLC analyses were often repeated at random to check the repeatability of the system.

2.2.8 Studies with Radiolabelled Fatty Acids.

2.2.8.1 Cellular Incorporation of [^{14}C]-18:2ω6 with Time.

Prior to radiolabelled FA studies with normal cells (see 2.2.8.2), a pilot investigation was conducted to assess the incorporation of dosed radiolabelled FA into cells over a 48 hour incubation period. This would ascertain whether sufficient radiolabelled FA was detectable in dosed cells for further analysis with the 48 hour incubation period routinely employed.

A rapidly growing 3T3 benign cell line was chosen for the pilot study since its growth rate was significantly faster than every normal cell type used, thus its metabolism was presumed to be higher. Detection of sufficient radiolabel in 3T3 cells 48 hours post-incubation with radiolabelled FA would indicate that this incubation time could also be satisfactorily used with normal cells.

3T3 cells were seeded at $1.0x10^5$ cells/ml growth medium in $75cm^2$ flasks and incubated for 24 hours at 37°C in an atmosphere of 5% carbon dioxide:95% air. The cultures were subsequently dosed with 2µCi [^{14}C]-18:2ω6 bound to sufficient albumin to act as carrier, and incubated.

Cultures were harvested at 6, 24 and 48 hours after dosing. At the end of each incubation period, the spent medium was carefully removed and stored at -20°C. The cells were trypsinised as described in 2.2.1.7, and the resulting pellet washed 3 times with physiological saline. Supernatants from each washing were pooled together and stored at -20°C. The final cell pellet was resuspended in a small volume of phosphate buffered saline (PBS, pH 7.4) and stored at -20°C. Frozen cell suspensions were subsequently thawed and briefly homogenised prior to further analysis.

100µl aliquots of homogenised cells, spent incubation media and pooled washings from each harvest period were placed into separate 20ml glass scintillation vials with 10.0ml Aquagel I scintillation cocktail and capped. Subsequently, the radioactivity of each fraction was measured in an LKB RackBeta scintillation counter. Standards, blanks and controls were run in parallel to give a measure of efficiency and for comparison to dosed cultures.

2.2.8.2 Studies with Radiolabelled C18 Fatty Acids and Normal Cells.

The cell types selected for dosage with radiolabelled C18 FA's were adherent fibroblasts derived from lung, skin and adipose tissue from each species, cultured from resuscitated frozen stocks (2.2.1.9). These cells were subsequently trypsinised, seeded at 1.0×10^5 cells/ml growth medium in $75cm^2$ flasks and incubated for 24 hours at 37°C in an atmosphere of 5% carbon dioxide:95% air. The cultures were subsequently dosed either with 2µCi [^{14}C]-18:1ω9, [^{14}C]-18:2ω6, or [^{14}C]-18:3ω3 together with unlabelled 18:1ω9, 18:2ω6 or 18:3ω3, respectively, all bound to sufficient albumin to act as carrier. The final FA concentration was 20mg/l. Cultures were then incubated for a further 48 hours, afterwhich the spent medium was carefully removed, centrifuged at 1000xg for 10 minutes in a Beckman TJ-6 centrifuge, and stored at -20°C. The cells were trypsinised, and the resulting pellet combined, as before, with any obtained from centrifugation of the spent incubation medium. Cells were washed 3 times with physiological saline, and the supernatants from each washing pooled together and stored at -20°C. The final cell pellet was resuspended and swollen in 2.0ml of a solution at pH 7.4 containing 1mM $NaHCO_3$, 2mM $CaCl_2$ and 5mM $MgCl_2$, and frozen to -20°C to lyse the cells. Lysed cells were stored at -20°C until further analysed.

Aliquots (100µl) of homogenised cell suspension, spent media and pooled washings from each normal cell type dosed with each radiolabelled FA were placed into separate 20ml glass scintillation vials with 10.0ml Aquagel I scintillation cocktail, and the radioactivity of each fraction measured as described in 2.2.8.1.

The remaining cell suspensions were extracted for total lipid (2.2.6.1) and methylated (2.2.7.1), with aliquots of sample taken at each step of the methodology for analysis of radioactivity to account for any loss of total radioactivity which may have occured. The resulting FAME's were subsequently concentrated to 50µl in petroleum ether, and analysed by injecting 2µl of this solution into a SP-2330 GLC column, connected to a GRC Radioactive Flow Detector (Flo-One\Beta) having a split ratio of 1:1 and a detector cell size of 5.0ml. Radioactivity was detected utilising the ^{14}C channel over a run period of 35 minutes. Quantitation of the peaks was made using an Acer 500^{+} personal computer and Epson LX-800 printer, and peaks identified by comparison with retention times of known FA standards.

Control and dosed cell samples were analysed, with random samples re-analysed to obtain a measure of reproducibility between runs. Standards and blanks were run in parallel under identical conditions to obtain a measure of efficiency of the instrument.

2.2.9 Lipid Peroxide Quantitation.

The thiobarbituric acid (TBA) reaction, described by Gavino et al (1981c), is a commonly employed method for the detection of peroxidation of unsaturated lipids. It is dependant on the development of a red pigment resulting from the reaction of TBA with oxidised lipids and products such as malondialdehyde (MDA). Cells and their corresponding spent growth media at each of the p-oil concentrations supplemented in 2.2.4 were assayed seperately for MDA-equivalent material by adding 2.0ml of 20% trichloroacetic acid (w/v) to 2.0ml spent medium or to 200µl homogenised cell suspension (from 2.2.4.3) in 1.8ml PBS (pH 7.4). To this was added 4.0ml of 0.67% TBA (w/v), followed by incubation of this mixture for 20 minutes at 97°C and subsequent centrifugation at 12000xg for 10 minutes at 4°C in a Beckman J2-21 centrifuge. The absorbance of the supernatant was measured in a LKB Biochrom Ultrospec 4050 at 532nm using 1cm quartz cells, with new incubation medium and PBS (pH 7.4) used as reference for spent growth media and cell samples, respectively. Absorbances were translated to nmoles MDA from a standard graph produced using 1,1,3,3-tetra-methoxypropane assayed in parallel with the samples, and results expressed as nmoles MDA/$1.0x10^6$ cells for both the spent incubation medium from which these cells were obtained and the cell samples themselves. The efficiency of the TBA assay was also determined.

2.2.10 Eicosanoid Quantitation.

The procedure used for the quantitation of eicosanoids was based on the method described by Rydzik et al (1984).

2.2.10.1 Eicosanoid Extraction.

10.0ml aliquots of the cell lipid stock extracts from 2.2.6.1 were placed into clean glass vials and dried at 37°C under a stream of nitrogen gas. Samples were reconstituted with 0.5ml chloroform:ethyl acetate (85:15 v/v) and applied onto a 1.0g chloroform pre-equilibrated LH20 column. The column was washed sequentially with three 1.0ml volumes of chloroform:ethyl acetate (85:15 v/v), followed by 10.0ml chloroform (to elute FA's). Eicosanoids were eluted by washing the column with 10.0ml HPLC grade methanol and collected in new glass vials. Samples were gassed with nitrogen and stored at -20°C until HPLC analysis.

2.2.10.2 Eicosanoid Analysis.

All HPLC analyses were performed as one group to eliminate between-batch variations. Prior to analysis, eicosanoid samples from 2.2.10.1 were concentrated by reducing their volume to dryness at 37°C under a stream of nitrogen gas and subsequently redissolved in 1.0ml HPLC grade methanol added down the sides of the vial.

Each sample was filtered through a 0.45µm filter into a new 7.5ml glass vial, this again taken to dryness, and the eicosanoids finally redissolved in 100µl HPLC grade methanol. Eicosanoids were separated by injecting 30µl aliquots of this solution into a 100mm x 8mm ID C_{18} column, and separation continued for 40 minutes. The HPLC running buffer (mobile phase) was 2mM $NH_4H_2PO_4$ buffer (pH to 4.2 with 0.0174M H_3PO_4):acetonitrile (69:31 v/v). New HPLC running buffer was made each day, filtered through a 0.22µm Millipore filter and degassed for approximately 10 minutes before use. HPLC running buffer was delivered from a reservoir, at a flow rate of 1.0ml/minute, via a LKB 2150 HPLC pump to the injector equipped with a 20µl sample loop.

Absorbance detection of eicosanoids at 192nm was accomplished using a Walters Millipore Lambda-Max 481 LC Spectrophotometer with variable wavelength adjustment capability. Sensitivity on the detector was adjusted to 2.0 absorbance units full scale (a.u.f.s.). Absorbance changes were quantitated using a Hewlett Packard 3390A integrator. Eicosanoid identification and quantitation was accomplished by comparison with the retention times and peak areas of standard eicosanoids (PGI_2, TXB_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGE_2 and PGD_2) run under identical conditions in known concentrations.

2.2.11 Statistical Analyses.

Results were analysed by standard statistical techniques, and included calculation of means, standard deviations (S.D.), standard error of the mean (s.e.m.), percentages and analysis of variance. These calculations were performed on an Apple 11e microcomputer utilising custom written software.

2.3 Observations, Justifications and the Efficiencies of Methodologies Employed.

2.3.1 Normal Cell Growth in Culture.

The cells isolated from the tissues/organs in 2.2.1.5 required less than 12 hours to attach to the culture vessel after initial plating and incubation. Plating efficiency of primary isolated cells was generally >50% after 3 hours initial incubation, and the proportion increased up until about the twelfth hour, as was also found by Tompa et al (1979). Thereafter, any unattached cells would not adhere to the culture vessel, even with further incubation. Light microscopy showed that most fibroblast-like cells adhered to the culture vessel within the first 6 hours after plating, followed by attachment of more epithelial-like cells.

Erythrocytes and lymphocytes isolated from each species were maintained in culture over a 48 hour period, while all cells derived from primary cultures proliferated *in vitro* under the conditions described in 2.2.1. Table 2.3.1.1. lists the mean population doubling times calculated for each adherent cell type cultured, seeded at 1.0×10^5 cells/ml growth medium in $2cm^2$ multiwell plates and incubated for 72 hours at 37°C in an atmosphere of 5% carbon dioxide:95% air. Cells derived from rat lung exhibited the fastest (66±5hrs), and human

Table 2.3.1.1.

The population doubling times of normal rat, cat and human cells, seeded at $10x10^4$ cells/ml growth medium and cultured in 1.0ml DMEM+10% FCS for 72 hours at 37°C in $2cm^2$ multiwell plates, expressed as mean±s.e.m.

Tissue	Population doubling time (hrs)		
	Rat (n=10)	Cat (n=10)	Human (n=10)
Adipose	88±5	131±11	
Brain	70±6	124±12	
Endothelium	125±7	110±6	
Lung	66±5	77±5	69±7
Muscle	103±11	84±9	141±12
Skin	96±6	77±8	75±8

muscle the slowest (141±12hrs), growth rate. The data in Table 2.3.1.1. compared favourably with literature reports for similar cell types (eg. Tompa et al 1979, Wicha et al 1979, Dugail et al 1986 and Vignikin et al 1989), and indicated that population doubling times varied with different cell types within a species and between similar cell types from different species.

The rate of cell proliferation in vitro was dependant, to a certain extent, on cell size for a particular cell type. Given the same surface area and cell number seeded, larger cells grew slower than cells of a smaller size, eg. human muscle and rat lung, respectively, because their growth became contact-inhibited far more quickly than smaller cells. Hence, cell size and the surface area available for growth (ie. cell density) are important factors contributing to the rate of cell proliferation.

2.3.2 The Total Protein Content of Complete Incubation Media.

The results obtained from the quantitation of total protein in DMEM+10% FCS, DMEM+10% RS, DMEM+10% CS and DMEM+10% HS are shown in Table 2.3.2.1.

Medium total protein concentrations ranged from 7.2±0.9g/l for DMEM+10% FCS to 7.7±0.2g/l for DMEM+10% CS. Analysis of variance showed no statistically significant difference between the protein concentrations of the 4 types of complete incubation media ($F=4.667$, $p=0.1$). Thus any differences in cell proliferation when rat, cat and human skin fibroblasts were grown in 2.2.3.3 in DMEM supplemented with 10% RS, CS or HS, respectively, compared to FCS, would not be relatable to medium protein concentrations.

Table 2.3.2.1.

The total protein content of complete incubation media, expressed as g/l growth medium (mean±s.e.m.).

Complete Incubation Medium	Protein content (g/l) (mean±s.e.m.) (n=3)
DMEM + 10% FCS	7.2±0.9
DMEM + 10% RS	7.5±0.7
DMEM + 10% CS	7.7±0.2
DMEM + 10% HS	7.6±0.3

2.3.3 Fatty Acid Analyses.

With the GLC column and running conditions employed in 2.2.7.2, 20:1ω9 co-chromatographed with 18:3ω3. Peaks coinciding with the retention time for both FA's were, however, reported as 18:3ω3 since 20:1ω9 occurs rarely.

2.3.3.1 The Fatty Acid Spectra of Natural Oils and of Potential Oil Sources.

The FA compositions of commercially available olive oil (OO), sunflower seed oil (SSO), linseed oil (LO), evening primrose seed oil (EPO), fish oil (FO), and lipids (oils) extracted from coconut (CO) and meat (MO), were determined as described in 2.2.6. The FA spectra of these 7 plant or animal derived oils are listed in Table 2.3.3.1., expressed as a percentage of the total area recorded.

Each oil exhibited a unique FA composition, and supplied large amounts either of ω3, ω6 or ω9 series unsaturated, or saturated, FA's. Noteworthy amounts of 10:0 and 12:0 were found only in CO (5.6% and 53.1%, respectively), with significant levels of 14:0 in MO, CO and FO (61.2%, 27.3% and 12.9%, respectively). 16:0 proportions were fairly consistent between oils, and ranged from 5.7% to 11.1%. A small amount of 17:0 was detected only in CO and MO (1.4% and 6.9%, respectively), and all oils contained <3.0% 18:0. 18:1ω9 levels ranged from 5.3% in

Legend to Table 2.3.3.1.

The fatty acid spectrum of 7 natural oils are shown. All values are tabulated as relative percent total area. Values are reported as mean±s.e.m., where 'n' is the number of experiments.

Table 2.3.3.1.

The fatty acid spectrum of 7 natural oils, expressed as mean±s.e.m.

FATTY ACID SPECTRUM (%)		CO (n=3)	MO (n=3)	OO (n=3)	SSO (n=3)	LO (n=3)	EPO (n=3)	FO (n=3)
SATURATES.	10:0	5.6±0.31	-	-	-	-	-	-
	12:0	53.1±2.00	-	-	-	-	0.1±0.03	0.6±0.03
	14:0	27.3±0.60	61.2±1.29	0.2±0.03	0.8±0.05	0.3±0.05	0.2±0.03	12.9±0.75
	16:0	6.8±0.40	5.7±0.37	11.1±0.15	7.2±0.30	7.0±0.60	6.8±0.13	10.9±0.90
	17:0	1.4±0.03	6.9±0.37	-	-	-	-	-
	18:0	0.6±0.05	2.7±0.15	0.2±0.03	1.6±0.10	1.0±0.10	1.7±0.09	0.6±0.05
	20:0	-	-	-	-	-	-	-
	22:0	-	-	-	-	-	-	-
	24:0	-	-	-	-	-	-	-
MONO.	16:1	-	-	-	-	-	-	30.5±1.90
	18:1	5.3±0.15	11.1±0.30	70.4±3.65	20.0±0.20	11.5±1.15	8.7±0.12	13.0±1.05
ω6 POLYS.	18:2	0.1±0.00	7.9±0.25	15.8±1.80	70.5±1.10	17.9±1.40	72.5±0.22	10.7±0.45
	18:3	-	-	-	-	-	9.1±0.29	-
	20:2	0.1±0.00	-	1.9±0.20	-	-	0.1±0.03	0.5±0.01
	20:3	-	0.1±0.05	0.2±0.05	0.1±0.05	-	0.3±0.05	-
	20:4	-	2.0±0.14	-	-	-	-	0.2±0.01
	22:4	-	0.1±0.03	-	-	-	0.4±0.05	0.1±0.00
	22:5	-	0.1±0.00	-	-	-	-	0.2±0.01
ω3 POLYS.	18:3	-	-	-	-	62.3±0.45	-	0.7±0.05
	18:4	-	-	-	-	-	-	0.9±0.03
	20:4	-	-	-	-	-	-	0.1±0.03
	20:5	-	0.1±0.00	-	-	-	-	17.7±1.00
	22:5	-	0.3±0.02	-	-	-	-	0.1±0.03
	22:6	-	0.1±0.00	-	-	-	-	0.3±0.01

CO to 70.4% in OO, while the highest 18:2ω6 levels occurred in EPO (72.5%) and SSO (70.5%), and the lowest in CO (0.1%). Only EPO, LO and MO provided significant amounts of 18:3ω6, 18:3ω3 or 20:4ω6, respectively (9.1%, 62.3% and 2.0%, respectively), while significant 16:1ω9 and 20:5ω3 proportions occured only in FO (30.5% and 17.7%, respectively). FO was significantly low in 22:6ω3, whereas higher levels have been reported abroad (eg. McCance et al 1978). This reflected the lower content of 22:6ω3 in local compared to foreign FO's, which the literature confirms (McCance et al 1978 and Giangregorio et al 1988a).

2.3.3.2 Pseudo-Oil Fatty Acid Composition.

The data in Table 2.3.3.1. was used to prepare p-oil stock solutions from individual FFA's in ethanol. FA's were only included in a p-oil if such contributed >0.5% to the total spectrum. p-Oil FA composition was checked by GLC, and the results listed in Table 2.3.3.2. p-Oil FA composition mimicked that of the natural oils, and was within ±1S.D. of the data in Table 2.3.3.1.

Legend to Table 2.3.3.2.

Pseudo-oil fatty acid spectra are tabulated as relative percent total area detected.

Table 2.3.3.2.

The fatty acid composition of the pseudo-oils.

FATTY ACID SPECTRUM (%)	PSEUDO-OILS						
	CO	MO	OO	SSO	LO	EPO	FO
10:0	5.6	-	-	-	-	-	-
12:0	53.1	-	-	-	-	-	0.6
14:0	27.3	62.7	-	0.8	-	-	13.0
16:0	6.8	5.8	11.2	7.2	7.0	6.9	11.0
17:0	1.4	7.1	-	-	-	-	-
18:0	0.6	2.8	-	1.6	1.0	1.7	0.6
Total sats.	94.8	78.4	11.2	9.6	8.0	8.6	25.2
16:1ω9	-	-	-	-	-	-	30.8
18:1ω9	5.3	11.4	71.0	20.0	11.5	8.8	13.1
Total mono.	5.3	11.4	71.0	20.0	11.5	8.8	43.9
18:2ω6	-	8.1	15.9	70.4	18.0	73.4	10.8
18:3ω6	-	-	-	-	-	9.2	-
20:2ω6	-	-	1.9	-	-	-	0.5
20:4ω6	-	2.1	-	-	-	-	-
Total poly.	-	10.2	17.8	70.4	18.0	82.6	11.3
18:3ω3	-	-	-	-	62.5	-	0.7
18:4ω3	-	-	-	-	-	-	0.9
20:5ω3	-	-	-	-	-	-	17.9
Total poly.	-	-	-	-	62.5	-	19.5

2.3.3.3 The Fatty Acid Spectrum and Total Lipid Dry Weight of Complete Incubation Media.

The results obtained from the analysis of FA's and quantitation of total lipids in DMEM+10% FCS, DMEM+10% RS, DMEM+10% HS and DMEM+10% CS are shown in Table 2.3.3.3., and were carried out to determine their possible contribution to the data obtained with cells.

Large proportions of 14:0 were present in all incubation media, ranging from 97.9% to 98.7%. Of the remaining FA's detected, 17:0 contributed 1.4%, 0.9%, 0.8% and 0.6% in DMEM containing 10% FCS, RS, CS or HS, respectively, while 16:0, 18:0, 18:1ω9, 18:2ω6, 20:4ω6 and 22:4ω6 contributed ≤0.5% each. Hence, the FA compositions of the 4 types of complete incubation media were similar, irrespective of the source of serum concerned.

Total medium lipid concentrations ranged from 0.56g/l for DMEM+10% FCS to 0.76g/l for both DMEM+10% RS and DMEM+10% CS. Variance analysis showed no statistically significant difference between the lipid contents of the 4 types of complete incubation media ($F=121$, $p=0.001$). Thus any differences in cell proliferation when rat, cat and human skin fibroblasts were cultured in DMEM supplemented with 10% RS, CS or HS, respectively, compared to FCS (2.2.3.3), would not be relatable to medium lipid concentrations.

Legend to Table 2.3.3.3.

Fatty acid values are tabulated as relative percent total area. Medium lipid amounts are expressed as grams total lipid per litre of culture medium. All values are reported as mean±s.e.m., where 'n' is the number of experiments.

Table 2.3.3.3.

The fatty acid spectrum and total lipid content of complete incubation medium, expressed as mean±s.e.m.

FATTY ACID SPECTRUM (%)		90% DMEM + 10% FCS (n=3)	90% DMEM + 10% RS (n=3)	90% DMEM + 10% HS (n=3)	90% DMEM + 10% CS (n=3)
S	10:0	-	-	-	-
A	12:0	-	-	-	-
T	14:0	98.4±0.05	98.5±0.15	97.9±0.19	98.7±0.21
U	16:0	-	0.1±0.02	0.3±0.03	-
R	17:0	1.4±0.03	0.9±0.11	0.6±0.03	0.8±0.07
A	18:0	-	-	0.2±0.03	0.2±0.09
T	20:0	-	-	-	-
E	22:0	-	-	-	-
S	24:0	-	-	-	-
ω9	14:1	-	-	-	-
M	16:1	-	-	-	-
O	18:1	0.1±0.03	0.1±0.02	0.3±0.03	0.1±0.00
N	20:1	-	-	-	-
O	22:1	-	-	-	-
S.	24:1	-	-	-	-
	16:2	-	-	-	-
ω6	18:2	-	0.1±0.02	0.5±0.07	0.2±0.03
P	18:3	-	-	-	-
O	20:2	-	-	-	-
L	20:3	-	-	-	-
Y	20:4	-	0.1±0.00	0.1±0.00	-
S.	22:4	-	0.1±0.02	0.1±0.03	0.1±0.00
	22:5	-	-	-	-
ω3	18:3	-	-	-	-
P	18:4	-	-	-	-
O	20:4	-	-	-	-
L	20:5	-	-	-	-
Y	22:5	-	-	-	-
S.	22:6	-	-	-	-
LIPID WEIGHTS (g/l)		0.56±0.02	0.76±0.04	0.58±0.02	0.76±0.06

2.3.4 Radiolabelled Fatty Acid Studies.

In all work conducted using radiolabelled FA's, the total counts detected (as cpm) for control samples approximated to that of the background.

2.3.4.1 Incorporation of [^{14}C]-18:2ω6 with Time.

Benign 3T3 cells were incubated with 2μCi [^{14}C]-18:2ω6 for 6, 24 and 48 hours to determine whether sufficient radiolabel would be present in the cells for further analysis. The radioactivity recovered from the cells and corresponding spent incubation media are shown in Table 2.3.4.1. Over the time period used, there was a consistent increase in detection of radiolabel in the cells, with 5.4% of total counts detected incorporated at 6hrs, 23.3% at 24hrs and 49.6% at 48hrs, representing an uptake of approximately 1% per hour. In contrast, the amount of radioactivity detected in the spent incubation medium decreased consistently with increased incubation time. At 6 hours, 80.3% of total counts dosed were recovered, and this dropped to 59.7% and 37.3% at 24 and 48 hours, respectively. Significantly more radioactivity was detected in the spent incubation medium than in the cells at 6 and 24 hours, whereas the amount of radiolabel detected in the cells and spent incubation medium at 48 hours were approximately equal (1719951cpm and 1750940cpm, respectively). Total counts

Table 2.3.4.1.

The radioactivity (cpm) recovered from 3T3 cells and the spent incubation medium dosed with 2μCi [^{14}C]-18:2ω6. expressed as mean (±s.e.m.), n=3.

FRACTION COUNTED	INCUBATION TIME		
	6hr	24hr	48hr
CELLS	214335 (±1212)	850914 (±819)	1719951 (±5574)
INCUBATION MEDIUM	3774320 (±6400)	2805060 (±16520)	1750940 (±15780)
TOTAL	3988655	3655974	3470891

Total 18:2 counts dosed to medium = 4.7 x 10^6

recovered also decreased with incubation time, although a fair proportion (about $0.5x10^6$cpm) of this loss was accounted for in the washing solutions, possibly due to non-specific binding. The data presented nevertheless indicates that incubation of cultures with 2µCi radiolabelled FA for 48 hours will yield sufficient radiolabel in the cells for reliable detection and quantitation.

2.3.4.2 Efficiencies of Radiolabelled Studies.

2.3.4.2.a Efficiency of the Lipid Extraction and Fatty Acid Methylation Procedures.

The procedure for lipid extraction from cell samples dosed with radiolabelled FA's in 2.2.8.2 was monitored to determine whether the technique, at any step, was responsible for a loss of radioactivity. Upon washing the lipid extracts in chloroform:methanol with saline, insignificant amounts of radioactivity (0.03%) were detected in the upper phase compared to the lower lipid phase. Monitoring methylation steps similarly showed no significant loss of radioactivity.

2.3.4.2.b Efficiency of Scintillation Counter.

The efficiency of the scintillation counter in detecting [^{14}C]-label was determined. Repeated analysis using known [^{14}C]-standards showed counter efficiency to be

97%. The manufacturers state that [^{14}C] counter efficiency is at least 90%. Since every step in biological sample preparation always involves up to about 5% loss or error, the recovery obtained was acceptable.

2.3.4.2.c Efficiency of Radiomatic Flow Counter.

The manufacturers specifications state a 10% counting efficiency, which was confirmed *in situ*.

2.3.5 The Thiobarbituric Acid (TBA) Assay.

2.3.5.1. Sensitivity and Specificity.

The TBA reaction measures the amount of MDA formed as a breakdown product of FA peroxidation and eicosanoid biosynthesis (Flower et al 1973, Mead 1976, Pryor 1976, Tappel 1980 and Witting 1980). Studies have shown that MDA is the aldehyde that is most reactive with TBA (eg. Esterbauer et al 1982). Although MDA detection has been widely used both *in vivo* (Tappel 1975) and *in vitro* (Gray 1978) for the detection of lipid peroxidation, the reaction is, unfortunately, not specific for MDA, and several compounds other than MDA (eg. metal ions) give positive results (Tappel 1980 and Nair et al 1984). The quantitative evaluation of MDA is further complicated by its rapid binding to proteins (Draper et al 1986). Thus, despite the sensitivity of the assay, the results should merely be interpreted as a crude indication of the overall level of lipid peroxidation. Measurement of TBA-reactive material (TBARM) nevertheless continues to be the most widely used assay in studies involving lipid peroxidation (Gavino et al 1981c, Liepkalns et al 1982, Morisaki et al 1982b, Begin et al 1986a and Girao 1988).

2.3.5.2 Determination of the Efficiency of the TBA Assay.

A random medium sample from those assayed in 2.2.9, containing a known amount of MDA, was selected. The sample contained 83nmoles MDA, or 3.32nmoles MDA/2ml assayed. To 2.0ml of this sample was added a volume of 1,1,3,3-tetramethoxypropane solution (at 5000nmoles/2ml) corresponding to the equivalent MDA concentration of the sample (ie. 3.32nmoles/2ml). Samples were assayed by the method described before in parallel with standards and controls. The efficiency of the TBA assay was determined as follows:

Theoretical [MDA] = known [MDA] in sample + [MDA] added

= 3.32nmoles/2ml + 3.32nmoles/2ml

= 6.64nmoles.

Recovered [MDA] (mean ± s.e.m., n=6) from standard curve

= 6.634 ± 0.368nmoles MDA/2ml.

$$\text{Efficiency} = \frac{\text{assayed amount}}{\text{known amount}}$$

$$= \frac{6.634}{6.640}$$

= 99.9%

Thus, the assay showed a 99% efficiency in this laboratory.

2.3.6 Eicosanoid Analyses.

With the HPLC column and running conditions employed in 2.2.10.2, $PGF_{1\alpha}$ co-chromatographed with $PGF2_{\alpha}$. Peaks coinciding with the retention time for both PG's were, therefore, reported either as $PGF_{1\alpha}$ + $PGF2_{\alpha}$, or merely as PGF_{α}.

CHAPTER 3: THE RAT: RESULTS AND DISCUSSION.

3.1 THE EFFECTS OF PSEUDO-OILS ON RAT ERYTHROCYTES.

3.1.1 Effects of pseudo-Oils on Cell Viability.

Microscopic examination showed the rat erythrocyte cultures to be free of platelet or white blood cell contamination. Although most erythrocytes retained their biconcave disc shape, some lost this characteristic after 48 hours in culture. About 50% of rat erythrocytes also lost their distinct 'red' colour after 2 days in culture, and thus became difficult to visualise. Only those erythrocytes which retained their pigmentation were hence regarded as viable, normal and representative of the situation *in vivo*.

The effect of albumin, with final medium concentrations ranging from 0 to 250mg/l, were investigated on rat erythrocyte viability. The results showed no significant changes in cell viability with dosage of up to 250mg albumin/l culture medium, nor were any synergistic effects observed (Fig. 3.1.1.1.). The effects induced with exogenous p-oil dosage were therefore a result of the FA's in the p-oil mixture and not the albumin used as FA carrier.

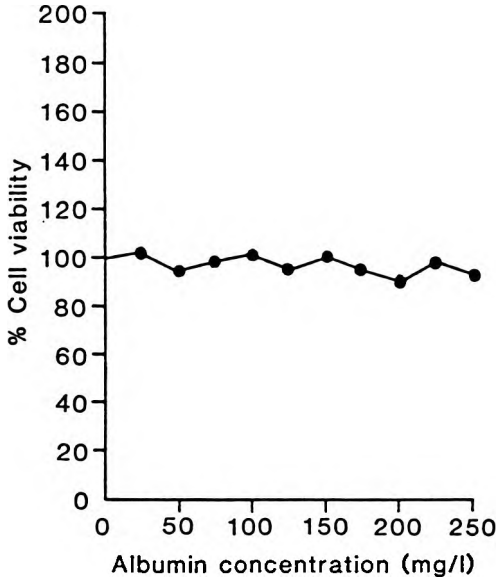
Since erythrocytes do not divide and did not require trypsinisation, no 24 hour post-trypsinisation recovery period was needed, and erythrocytes were dosed

Legend to Figs. 3.1.1.1-3.1.1.8.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.1.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.1.1.2-3.1.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l).

Fig. 3.1.1.1.

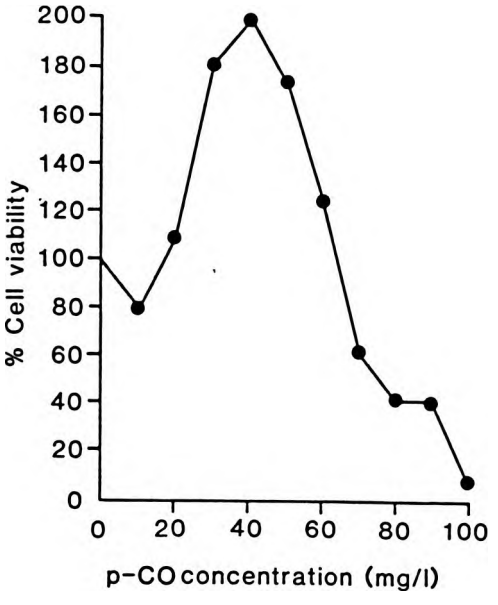
The percentage viability of rat erythrocytes incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.0	12
25	101.6	5.1	12
50	95.1	4.7	12
75	98.9	6.6	12
100	101.6	5.9	12
125	95.9	4.0	12
150	100.6	4.8	12
175	96.1	5.2	12
200	91.2	7.0	12
225	98.6	6.3	12
250	94.1	5.6	12

Fig. 3.1.1.2.

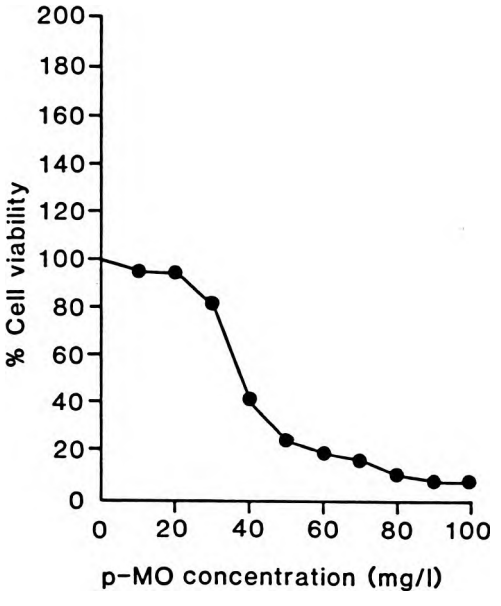
The percentage viability of rat erythrocytes incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.9	6
10	78.7	6.0	6
20	108.6	6.4	6
30	180.4	7.0	6
40	199.8	7.1	6
50	174.1	6.5	6
60	124.1	3.8	6
70	62.2	6.2	6
80	42.3	5.3	6
90	40.4	7.2	6
100	7.7	1.8	6

Fig. 3.1.1.3.

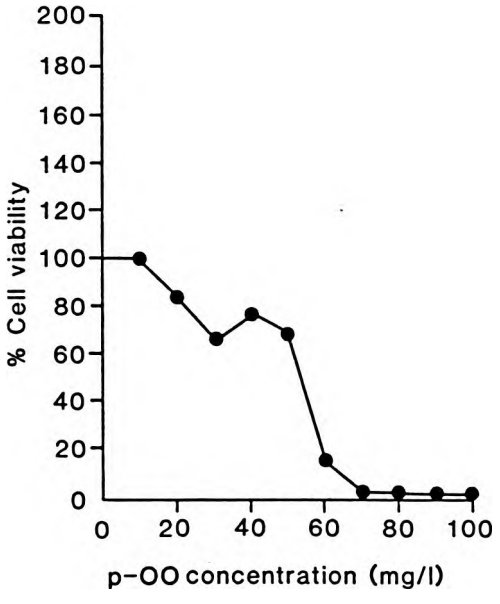
The percentage viability of rat erythrocytes incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.7	6
10	95.0	5.5	6
20	94.4	5.4	6
30	81.1	2.8	6
40	41.2	3.4	6
50	25.0	1.5	6
60	18.6	0.8	6
70	16.8	1.0	6
80	10.3	0.9	6
90	7.6	0.6	6
100	7.6	0.6	6

Fig. 3.1.1.4.

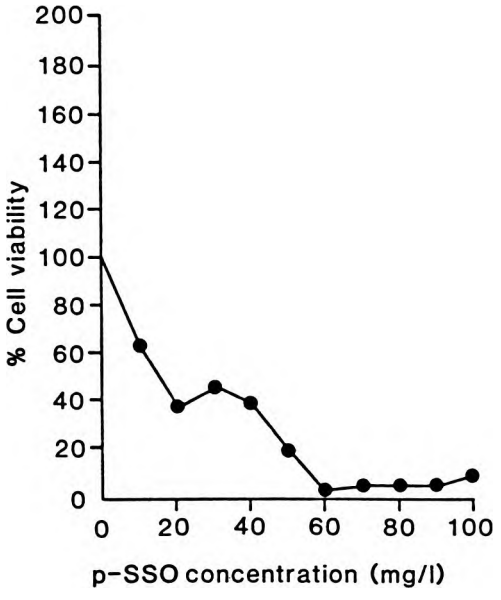
The percentage viability of rat erythrocytes incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.8	6
10	99.0	7.0	6
20	83.1	5.6	6
30	66.2	3.1	6
40	75.9	4.5	6
50	68.4	4.5	6
60	15.8	2.5	6
70	1.9	0.4	6
80	0.1	0.0	6
90	0.0	0.0	6
100	0.0	0.0	6

Fig. 3.1.1.5.

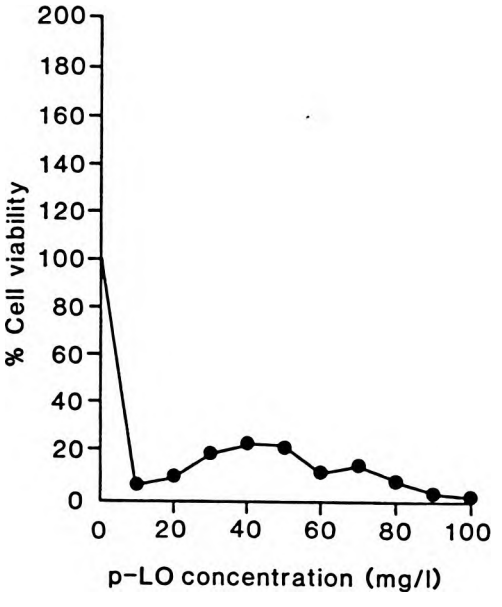
The percentage viability of rat erythrocytes incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	6.0	6
10	63.2	2.3	6
20	37.2	1.9	6
30	45.5	2.5	6
40	38.8	1.0	6
50	18.7	4.2	6
60	1.5	0.2	6
70	4.1	0.7	6
80	4.9	0.9	6
90	4.4	1.0	6
100	8.5	1.1	6

Fig. 3.1.1.6.

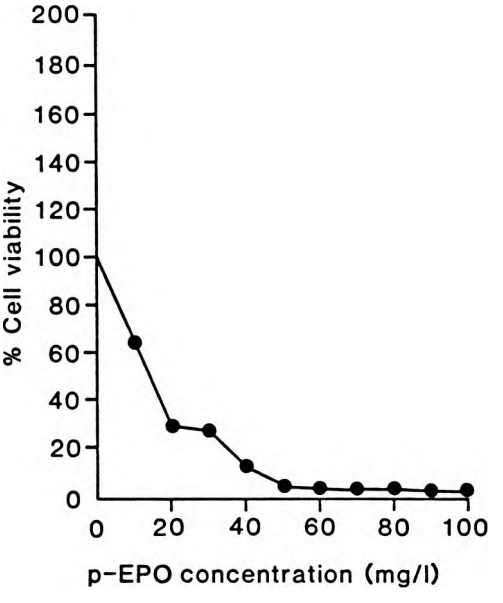
The percentage viability of rat erythrocytes incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.6	6
10	6.2	0.4	6
20	9.8	0.7	6
30	19.3	1.7	6
40	22.8	2.5	6
50	22.3	2.9	6
60	11.5	1.2	6
70	13.9	2.2	6
80	7.6	2.0	6
90	1.8	0.7	6
100	0.0	0.0	6

Fig. 3.1.1.7.

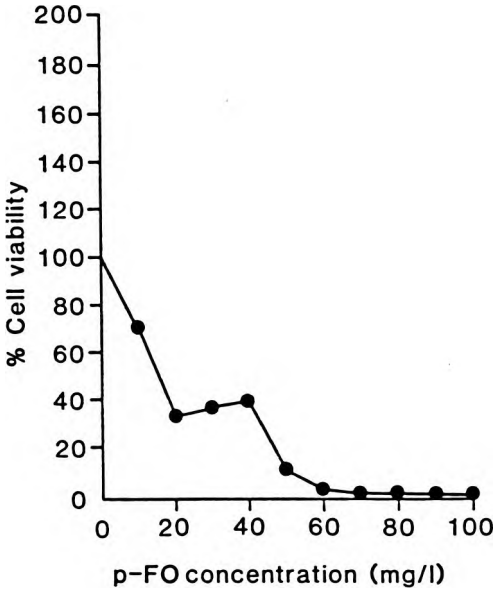
The percentage viability of rat erythrocytes incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.4	6
10	64.7	3.5	6
20	29.2	1.5	6
30	27.5	1.0	6
40	12.4	2.0	6
50	4.3	0.3	6
60	3.0	0.3	6
70	3.5	0.4	6
80	3.6	0.3	6
90	2.3	0.2	6
100	2.3	0.3	6

Fig. 3.1.1.8.

The percentage viability of rat erythrocytes incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	6.1	6
10	70.3	5.6	6
20	34.3	4.6	6
30	38.2	4.4	6
40	40.0	4.1	6
50	10.6	1.6	6
60	2.7	0.4	6
70	1.3	0.3	6
80	0.5	0.2	6
90	0.3	0.1	6
100	0.0	0.0	6

immediately subsequent to plating. Thus the seeding concentration was the cytostatic number.

All p-oils exhibited cytotoxic potential when incubated with rat erythrocytes (Figs. 3.1.1.2-3.1.1.8.). However, p-oils containing significant amounts of PUFA's, viz. p-FO, p-EPO, p-LO and p-SSO, induced greater cytotoxicity than p-oils rich in monounsaturated (p-OO), or saturated (p-MO and p-CO), FA's.

p-CO increasingly induced cell stabilisation in the range 20 to 40mg/l (Fig. 3.1.1.2.). With 40mg/l p-CO, erythrocyte morphology was maintained, all cells retained their biconcave disc shape and characteristic 'red' colour, thus cell viability was about 100% of that initially plated. Concentrations between 40 and 70mg/l p-CO progressively lowered the number of morphologically normal cells to control levels, while greater amounts induced considerable cytotoxocity, and relative cell viability was 7.7% with 100mg/l p-CO. The ID_{50} for p-CO was about 75mg/l, the highest value calculated for any p-oil dosed to rat erythrocytes.

Supplementation with p-MO or p-OO induced intermediate effects compared to other p-oils. Relative cell viability paralleled controls with low concentrations, followed by a dramatic reduction in viability with concentrations between 20 and 70mg/l (Figs. 3.1.1.3. and 3.1.1.4., respectively). About 38mg/l p-MO and 53mg/l

p-OO killed 50% of cells (ID_{50}), while 100mg/l p-oil reduced viability to 7.6% and 0%, respectively.

p-Oils containing significant amounts of PUFA's induced the greatest concentration dependent cytotoxic effects (Figs. 3.1.1.5-3.1.1.8.). p-SSO, p-EPO and p-FO induced similar effects, having ID_{50} values in the range 14 to 16mg/l. Concentration dependent cytotoxicity with p-LO dosage was most dramatic, evident by the very low ID_{50} (approximately 5mg/l). p-SSO, p-LO, p-EPO and p-FO concentrations greater than 20mg/l caused further reductions in viability, but with a more gradual effect. Cell viability with 100mg/l p-oil was 8.5% and 2.3% for p-SSO and p-EPO, respectively, while no viable cells were seen with this concentration of p-LO or p-FO.

Subsequent to these studies, rat erythrocytes were plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient quantities for all quantitative and qualitative analyses to be performed. Upon harvesting, cell numbers relative to controls were compared with those in Figs. 3.1.1.1-3.1.1.8., and were found not to be statistically different. All further biochemical assays were performed on these samples.

3.1.2 Effects of pseudo-Oils on Total Protein.

Total erythrocyte protein was determined for each of the p-oil concentrations dosed and compared with controls. Results are shown in Table 3.1.2.1. as µg total protein/10^6 cells seeded.

Control erythrocytes contained 13.0µg protein/10^6 cells seeded. Except for cells dosed with 40mg/l p-CO, all dosed erythrocytes exhibited less total protein than controls. Cells dosed with 20mg/l p-OO, p-MO or p-CO exhibited similar protein levels to controls, compared to very low concentrations with 20mg/l p-SSO, p-LO, p-EPO or p-FO dosage (<5.0µg/10^6 cells seeded). In general, erythrocyte protein concentrations decreased with increasing p-oil concentration dosed, so that cells supplemented with 60mg/l p-oil exhibited the lowest protein levels. The only significant increase in erythrocyte protein occurred with dosage of 40mg/l p-CO (23.8µg/10^6 cells seeded); this was the highest protein level measured, while the lowest was for erythrocytes dosed with 60mg/l p-EPO (0.5µg/10^6 cells seeded).

Table 3.1.2.1.

The protein content of rat erythrocytes, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	13.0			
CO		12.3	23.8	12.3
MO		11.8	4.8	2.0
OO		10.1	10.4	1.7
SSO		4.5	4.1	0.6
LO		1.3	1.8	1.7
EPO		3.3	1.9	0.5
FO		3.6	4.3	0.9

3.1.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Rat Erythrocytes.

Table 3.1.3.1. shows the FA spectra of rat erythrocytes dosed with 0, 20, 40 or 60mg/l of each of the p-oils. FA's contributing ≥5.0% to the FA spectrum in control erythrocytes were 16:0 (16.7%), 18:0 (8.6%), 16:1ω9 (5.9%), 18:1ω9 (15.3%), 22:4ω6 (35.3%) and 20:4ω3 (6.0%). Varying amounts of these FA's were found in dosed erythrocytes.

16:0 and 18:1ω9 proportions in dosed erythrocytes were generally parallel to, or greater than, control levels, while the opposite was true for 16:1ω9. 18:0 percentages were more variable, but generally greater in dosed cells than controls. Variable amounts of 18:2ω6 were also present in dosed cultures, but the levels of all other ω6 and ω3 PUFA's detected were generally parallel to, or below, control levels.

Erythrocytes dosed with p-CO showed an increase in the major FA components of that p-oil being incorporated with concentration. The amount of 16:0 detected increased from 36.8% when erythrocytes were dosed with 20mg/l p-CO, to 52.8% and 52.2% with 40 and 60mg/l p-CO, respectively (16.7% in controls). Changes in 18:0 and 18:1ω9 levels were small compared to controls (8.6% and 15.3%, respectively), and PUFA levels decreased with p-CO dosage.

Legend to Table 3.1.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.1.3.1.

The fatty acid spectrum of rat erythrocytes.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	16.7±0.45	36.8	52.8	52.2	34.9	33.8	29.1	15.2	15.4	20.3	19.3	17.3	25.0	21.7	16.9	31.6	20.3	33.4	28.2	33.3	26.0	18.7
	18:0	8.6±0.60	7.7	8.8	9.0	10.5	12.1	13.3	3.4	6.5	5.4	9.4	12.2	8.7	14.1	10.0	9.3	10.9	13.5	10.4	4.9	2.6	1.3
	20:0	0.2±0.10	-	0.1	0.1	-	-	-	0.2	0.4	-	-	0.4	-	-	0.4	-	-	0.1	-	-	-	-
	22:0	0.3±0.05	0.1	-	0.1	0.3	0.1	0.1	0.2	-	-	0.1	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.1	-	-	-
	24:0	-	-	0.7	0.5	1.0	1.0	0.7	1.0	2.6	0.5	0.8	1.5	0.8	1.0	2.2	0.4	0.4	-	1.3	-	-	-
ω9 MONOS.	16:1	5.9±0.05	-	-	0.9	0.5	0.7	1.5	1.1	1.1	5.7	0.9	2.0	4.9	1.4	0.9	4.6	1.2	1.6	1.8	18.8	32.2	32.0
	18:1	15.3±0.05	15.0	19.4	18.5	9.4	13.2	13.7	33.7	35.2	38.8	22.5	20.0	20.4	16.1	17.5	19.0	13.9	22.3	22.0	18.9	21.8	21.9
	24:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	3.9±0.00	1.4	0.8	0.8	3.2	3.8	3.1	2.5	2.9	2.0	10.5	5.9	6.2	2.0	2.7	3.0	9.4	18.8	27.7	5.1	8.2	10.5
	18:3	-	-	-	-	0.2	0.1	0.1	-	-	-	-	-	-	-	-	-	-	0.4	1.2	-	-	-
	20:2	1.0±0.00	-	-	-	-	0.1	-	-	0.7	0.1	-	-	-	-	-	-	-	0.1	-	0.3	0.8	1.1
	20:3	1.2±0.05	0.6	0.1	0.4	1.3	0.1	0.6	0.8	-	0.1	0.6	-	-	1.1	-	0.5	-	-	0.2	-	0.1	0.1
	20:4	0.8±0.05	0.4	0.4	0.4	-	0.4	1.0	-	0.1	-	-	0.1	-	-	0.1	-	-	0.3	0.1	0.3	0.2	0.3
	22:4	35.3±0.05	34.3	14.4	15.1	35.3	30.6	33.5	35.1	28.8	23.6	31.7	34.1	30.7	34.6	38.2	26.3	39.5	6.9	5.4	15.5	2.9	4.9
	22:5	3.9±0.55	-	0.2	-	-	1.5	0.2	-	3.5	0.2	0.2	2.8	0.2	0.4	4.5	0.1	-	0.6	-	0.1	0.2	0.1
ω3 POLYS.	18:3	0.3±0.00	-	0.1	0.1	0.2	0.2	0.3	-	0.2	0.1	0.1	0.2	-	0.6	1.1	2.6	-	0.1	-	0.3	0.5	0.8
	18:4	0.5±0.00	0.3	0.6	0.2	0.3	0.6	-	0.5	0.7	0.3	0.4	0.4	0.4	0.5	0.8	0.3	0.3	0.4	0.2	0.1	0.1	0.2
	20:4	6.0±0.05	3.6	1.7	1.4	2.9	1.7	2.8	6.2	2.1	2.9	3.6	3.0	2.7	6.3	4.7	2.2	3.9	1.5	0.6	1.7	0.7	0.6
	20:5	0.3±0.00	0.1	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	3.3	7.0
	22:5	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1
	22:6	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.0	0.1	0.1	0.4

34.9%, 33.8% and 29.1% 16:0 was detected when erythrocyes were dosed with 20, 40 or 60mg/l p-MO, respectively, compared to 16.7% in controls. 18:0 percentages also increased (10.5%, 12.1% and 13.3%, respectively vs 8.6% in controls), but 18:1ω9 and PUFA levels were parallel to controls, or decreased.

The major component of p-OO was 18:1ω9. With all p-OO concentrations dosed, 18:1ω9 levels were at least double control levels (15.3%), and percentages increased slightly with increasing concentration dosed (33.7%, 35.2% and 38.8%, respectively). A marginal decrease, followed by an increase in 16:0 was found in the same range (15.2%, 15.4% and 20.3%, respectively), compared to controls (16.7%). 18:0 levels ranged from 3.4% to 6.5% (8.6% in controls), while between 0.5% and 2.6% 24:0 was found (none in controls). 16:1ω9 was decreased with 20 and 40mg/l (1.1% each), but parallel to controls (5.9%) with 60mg/l (5.7%). However, all PUFA levels dropped as the p-OO concentrations dosed increased, especially that of 22:4ω6 (35.1%, 28.8% and 23.6%, respectively vs 35.3% in controls).

16:0 incorporation increased from 19.3% to 17.3% and 25.0% when erythrocytes were dosed with 20, 40 or 60mg/l p-SSO, respectively (16.7% in controls). 18:0 levels also increased with 20 and 40mg/l p-SSO (9.4% to 12.2%, respectively), but levels paralleled controls (8.6%)

with 60mg/l (8.7%). 24:0 was absent in controls, while 0.8% to 1.5% was detected in dosed cells. 18:1ω9 levels dropped slightly from 22.5% to 20.0% and 20.4%, respectively with increasing p-SSO concentrations dosed, and only 15.3% was detected in controls. On the other hand, 16:1ω9 levels decreased to below control levels (5.9%) with 20, 40 and 60mg/l p-SSO (0.9%, 2.0% and 4.9%, respectively). 18:2ω6 incorporation amounted to 10.5% with 20mg/l p-SSO, but dropped slightly to 5.9% and 6.2% with 40 and 60mg/l p-SSO, respectively, although these percentages were higher than in controls (3.9%). Other PUFA levels, however, were not increased.

16:0 levels increased significantly with 20 or 60mg/l p-LO incubation (21.7% and 31.6%, respectively), but paralleled control levels (16.7%) with 40mg/l (16.9%). 18:0 percentages were greater than control levels (8.6%) with all concentrations dosed, but decreased as greater amounts of p-LO were dosed (14.1%, 10.0% and 9.3%, respectively). 24:0 was absent in controls, but up to 2.2% was detected in dosed cultures. With 20 or 40mg/l p-LO supplementation, 0.6% and 1.1% 18:3ω3 was detected, respectively, while 18:3ω3 levels increased to 2.6% with 60mg/l, compared to 0.3% for controls. Relative 18:2ω6 levels increased with p-LO concentration dosed (2.0%, 2.7% and 3.0%, respectively), but were nevertheless below control levels (3.9%). Both 22:4ω6 and 20:4ω3 percentages approximated to controls (35.3% and 6.0%,

respectively) with 20 and 40mg/l p-LO, but were decreased with 60mg/l (26.3% and 2.2%, respectively). 22:5ω6 levels were 4.5% with 40mg/l, but <0.5% with 20 or 60mg/l p-LO, compared to 3.9% in controls.

A marked increase in 18:2ω6 incorporation was observed when erythrocytes were dosed with 20, 40 or 60mg/l p-EPO (9.4%, 18.8% and 27.7%, respectively, 3.9% in controls). 18:3ω6 was absent in control erythrocytes, but small amounts were incorporated when p-EPO dosage increased (0%, 0.4% and 1.2%, respectively). There was a marked fall in 22:4ω6 levels with increased concentration dosed (39.5%, 6.9% and 5.4%, respectively, 35.3% in controls), with increased 16:0, 18:0 and 18:1ω9 levels, compared to controls (16.7%, 8.6% and 15.3%, respectively).

The amount of 16:0 incorporated into erythrocytes with 20 and 40mg/l p-FO was 33.3% and 26.0%, respectively, but only 18.7% with 60mg/l, paralleling control amounts (16.7%). 18:0 levels were reduced from 8.6% in controls to 4.9%, 2.6% and 1.3% with 20, 40 and 60mg/l p-FO, respectively. There was, however, a marked increase in 16:1ω9 incorporation (18.8%, 32.2%, 32.0%, respectively vs 5.9% in controls), and 18:1ω9 levels increased similarly (18.9%, 21.8% and 21.9%, respectively vs 15.3% in controls). The increase in 18:2ω6 was more linear (5.1%, 8.2% and 10.5%, respectively, 3.9% in controls). 22:4ω6 proportions were reduced by at least 2 fold (15.5%) with 20mg/l p-FO, compared to controls (35.3%),

while considerably lower amounts were found with 40 and 60mg/l (2.9% and 4.9%, respectively). 0.3% 20:5ω3 was detected in control cells, but 0.7%, 3.3% and 7.0% when 20, 40 or 60mg/l p-FO were dosed, respectively. 22:5ω3 and 22:6ω3 were absent from controls, and only trace amounts were detected in dosed cells (<0.5%).

3.1.4 Effects of pseudo-Oils on Lipid Peroxide Formation.

Lipoperoxide production was measured in rat erythrocytes dosed with p-oils and their respective spent media, and this compared to controls. Results are shown in Table 3.1.4.1. as nmoles MDA/10^6 cells, but in the case of the spent media, this represents the nmoles of MDA in the volume of medium from which $1x10^6$ cells were obtained.

No lipoperoxides were detected in control erythrocytes. Those p-oils which predominantly contained FA's of one double bond or less, viz. p-CO, p-MO and p-OO, did not exhibit a significant change in lipid peroxide formation relative to controls. With incubation of 60mg/l p-OO, however, 1.4 nmoles MDA/10^6 cells was detected. p-Oils containing significant amounts of PUFA's induced greater amounts of cellular lipid peroxides, which increased markedly with the p-oil concentration dosed, and was greatest for erythrocytes incubated with 60mg/l p-FO or p-EPO (41.2 and 33.8nmoles MDA/10^6 cells, respectively).

Lipoperoxides were absent from spent medium in which control erythrocytes had been maintained, and in spent medium dosed with 20, 40 or 60mg/l p-CO, p-MO or p-OO. However, spent media from cells dosed with 40 or 60mg/l PUFA-rich p-oils generally contained lipoperoxides, and the highest amounts measured were with 60mg/l p-EPO and p-SSO (41.4 and 43.5nmoles MDA/10^6 cells, respectively).

Table 3.1.4.1.

Lipid peroxide formation by rat erythrocyte cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	-			
CO		-	-	0.1
MO		0.1	0.1	0.2
OO		0.1	0.1	1.4
SSO		2.8	1.9	10.8
LO		0.1	0.6	3.1
EPO		6.9	12.2	33.8
FO		1.7	7.8	41.2
pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	-
MO		-	-	-
OO		-	-	-
SSO		-	0.9	43.5
LO		-	2.9	-
EPO		-	25.5	41.4
FO		0.6	-	16.1

3.1.5 Discussion.

Supplementation of rat erythrocytes with p-oils induced hemolysis in every instance (Figs. 3.1.1.1-3.1.1.8.). However, the extent of cytotoxicity induced was relatable to p-oil PUFA composition (Table 2.3.3.2.), as hemolysis was greatest with p-oils rich in PUFA's (p-SSO, p-LO, p-EPO and p-FO), and less extensive with p-OO and p-MO which contained smaller amounts of PUFA's. Furthermore, the stabilisation of erythrocyte membranes with dosage of low and intermediate p-CO concentrations correlated with the lack of PUFA's in this p-oil. When large amounts of p-CO were dosed, the incorporation of saturated FA's into erythrocyte membranes may have caused rigidification, and this possibly explains the hemolysis induced. The similar effects induced overall with p-SSO or p-EPO dosage probably relates to the large amount of 18:2ω6 (about 70%) in these p-oils, while the small amount of 18:3ω6 (about 9%) in p-EPO only may account for the slightly greater cytotoxicity of p-EPO. As the concentration of 18:3ω3 in p-LO was similar to that of 18:2ω6 in p-SSO and p-EPO, the significantly lower amount of p-LO than p-SSO or p-EPO required to reduce cell viabililty to 50% of controls suggests that 18:3ω3 is more cytotoxic than 18:2ω6, and that such relates to differences in the number and position of double bonds in these C18 FA's. p-FO induced similar effects to p-EPO and p-SSO, despite a different FA

composition, suggesting that synergism and/or antagonism between p-oil FA's probably also played a role in the modulation of erythrocyte viability.

Supplementation of cultures with p-oils induced changes in erythrocyte total protein concentrations (Table 3.1.2.1.) which largely mirrored the changes reported in cell viability (Figs. 3.1.1.1-3.1.1.8.). Hemoglobin comprises at least 95% of total erythrocyte protein (Geigy 1984), thus cell lysis and subsequent loss of hemoglobin would be consistent with the marked reduction in erythrocyte protein reported with hemolytic p-oil concentrations.

The effects induced with p-oil supplemenation were consistent with reports indicating that FA's modify erythrocyte morphology, their ability to change shape and to resist hemolysis (Rao et al 1979, Csordas et al 1984, Cartwright et al 1985 and German et al 1985). Csordas et al (1984) investigated the effect of C18 unsaturated FA's on erythrocyte osmotic resistance, and showed that 18:1ω9, 18:2ω6 and 18:3ω3 induced hemolysis with concentrations from 0.03 to 80mg/l. With 3-8mg/l FA, however, 18:1ω9 protected erythrocytes from rupture, 18:2ω6 was much less effective, but 18:3ω3 was hardly protective at all. These findings correlated with those from our study. Firstly, p-oils rich in 18:1ω9 (p-OO), 18:2ω6 (p-SSO and p-EPO), or 18:3ω3 (p-LO)

induced extensive hemolysis overall. Secondly, with 10mg/l p-oil, p-OO induced no hemolysis, p-SSO and p-EPO induced approximately 40% hemolysis, while p-LO induced about 95% hemolysis. We additionally showed that 10 and 20mg/l p-MO were protective against hemolysis, although 20 to 60mg/l p-CO was most effective. Protection against hemolysis thus seems to relate directly to the degree of p-oil saturation, while cytotoxicity relates to the presence of PUFA's.

Numerous workers have investigated the manipulation of erythrocyte FA profiles. Monsen et al (1962) showed that varing the nature of dietary fat caused changes in the 18:1ω9 and 18:2ω6 content of rat erythrocyte PGL's. Rats fed safflower oil had twice as much 18:2ω6 in erythrocyte PGL's than did animals fed coconut oil, whereas the PGL's of the latter group had twice as much 18:1ω9. 20:4ω6 levels were unchanged, however, although others reported an accumulation of 20:4ω6 relative to 18:2ω6 in a ratio of at least 5:1 in erythrocytes of rats fed a source of 18:2ω6 (Mohrhauer et al 1963e). On the other hand, Watson (1963) found that feeding rats an EFA-deficient diet resulted in erythrocytes deficient both in 18:2ω6 and 20:4ω6, with increased membrane permeability, although cell morphology was unaltered. Latter work, however, proved that PUFA-deficiency in rats caused a concommitant decrease in erythrocyte PUFA content, and that EFA-deficiency had pronounced effects

on erythrocyte morphology (Rao et al 1979). Ehrstrom et al (1981) showed that erythrocyte membranes exhibited increased osmotic sensitivity when rats were fed a low EFA content diet. These dietary changes, however, did not alter membrane fluidity, whereas cod liver oil or oils rich in 20:5ω3 and 22:6ω3 increased both the degree of unsaturation of erythrocyte PGL's and cell deformability (Popp-Snijers et al 1984, 1986a, 1986b, Cartwright et al 1985 and German et al 1985).

We found about 4% total EFA's in control erythrocytes (Table 3.1.3.1.), compared to approximately 12% reported by Horrobin et al (1984c) in rat erythrocyte PGL's. In the light of the work reviewed above, the morphological changes reported in 3.1.1 for some erythrocytes after the 48 hour incubation period may therefore relate to EFA-deficiency and resulting changes in membrane permeability. Rat erythrocytes incorporated dosed p-oil FA's (Table 3.1.3.1.), probably into the PGL fraction. The overall erythrocyte FA profile was significantly altered compared to controls by both the amount and nature of FA's supplied (Table 3.1.3.1.), thus it seems likely that membrane fluidity, permeability and/or osmotic sensitivity were altered, and contributed to the effects induced in Figs. 3.1.1.2-3.1.1.8.

The data in Table 3.1.3.1. confirmed the lack of desaturase and elongase activity in rat erythrocytes (Lehninger 1982). These cells <u>in vivo</u> are therefore

dependent on dietary intake and/or on other tissues capable of desaturation as a source of PUFA's to maintain membrane fluidity.

The absence of lipoperoxides in control erythrocytes (Table 3.1.4.1.), despite the detection of approximately 75% unsaturated FA's in control cells (Table 3.1.3.1.) and the capability for non-enzymic lipid peroxidation in erythrocytes (Lehninger 1982), suggested that cellular FA's were not susceptible to peroxidation. Such may have occurred if erythrocyte unsaturated FA's were components of complex lipids such as membrane PGL's, if the rate of PGL turnover and therefore also the rate of FA uptake and release were slow, and/or if the erythrocytes contained sufficient vitamin E to prevent oxidation of FFA's. Evidence of increased lipid peroxidation has indeed been reported in erythrocytes membranes derived from vitamin E-deficient rats (Barker et al 1975). On the other hand, the lack of lipoperoxides in spent control medium reflected the large amounts of saturated FA's in growth medium (Table 2.3.3.3.).

The absence of significant lipoperoxides in erythrocytes and the corresponding spent medium of cultures dosed with p-CO or p-MO was consistent with the presence of only small amounts of unsaturated FA's in these p-oils (Table 2.3.3.2.). On the other hand, detection of small amounts of cellular lipoperoxides with p-OO or p-LO

dosage, despite the large amounts of 18:1ω9 and 18:3ω3 present, respectively, suggested that incorporated p-oil FA's were possibly components of complex membrane lipids such as PGL's, and thus not so readily accessible to oxidation. The lack of significant lipoperoxides in the corresponding spent culture medium, despite significant hemolysis, supports this. The large amounts of cellular lipoperoxides reported with p-SSO, p-EPO or p-FO dosage were consistent with the susceptibility and availability of free PUFA's to oxidation, and this has been shown to increase with deficiencies in erythrocyte vitamin E (Lubin et al 1982). The lipoperoxides formed with p-SSO dosage probably reflect oxidation of the large amount of 18:2ω6 present in this p-oil (approximately 70%). p-SSO and p-EPO exhibited similar FA compositions and induced similar changes in cell viability, thus the significantly greater lipid peroxide amounts generated overall with p-EPO supplementation probably relates to the presence of about 9% 18:3ω6 in p-EPO only. Rat erythrocytes were shown to incorporate exogenous FA's (Table 3.1.3.1.), thus the lipid peroxides in the corresponding spent medium of cultures dosed with PUFA-rich p-oils probably related to the release of cellular lipoperoxides as a direct result of hemolysis.

PUFA-rich p-oils induced large amounts of lipoperoxides and were responsible for the most extensive destruction of erythrocytes, while both lipoperoxide production and

hemolysis were less pronounced with more saturated p-oils (Figs. 3.1.1.1-3.1.1.8. and Table 3.1.4.1.). Reports that lipoperoxides have been implicated in hemoglobin and membrane damage, and that lipoperoxide-induced membrane protein cross-linking affects rat erythrocyte deformability (Chio et al 1969, Mead 1976, Tappel 1980, Trotta et al 1982, MacDonald et al 1983b, Frankel 1984 and Sakai et al 1990) support lipid peroxide involvement in the modulation of p-oil-induced hemolysis.

3.2 THE EFFECTS OF PSEUDO-OILS ON RAT LYMPHOCYTES.

3.2.1 Effects of pseudo-Oils on Cell Viability.

Rat lymphocyte cultures were free of erythrocyte or platelet contamination. Microscopic examination of control lymphocytes before, during and after each experiment showed no morphological changes at any time, and p-oil dosed lymphocytes were morphologically similar to controls.

The effect of albumin on rat lymphocyte viability was investigated with final medium concentrations ranging from 0 to 250mg/l. The results indicated that albumin alone had no significant effect on cell viability with any concentration (Fig. 3.2.1.1.). Hence, the effects observed with p-oil supplementation were induced by the exogenous FA's and not the albumin used as FA carrier.

Lymphocytes do not normally divide in culture without appropriate mitogen stimulation, and did not require trypsinisation, thus no 24 hour post-trypsinisation recovery period was needed. Lymphocytes were dosed immediately subsequent to plating, thus the seeding concentration was the cytostatic number.

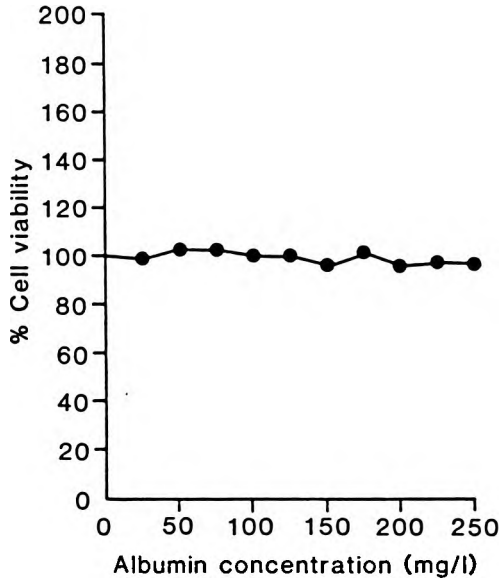
The results obtained from the incubation of lymphocytes with p-oils are shown in Figs. 3.2.1.2-3.2.1.8. All

Legend to Figs. 3.2.1.1-3.2.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.2.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.2.1.2-3.2.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.2.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.2.1.1.

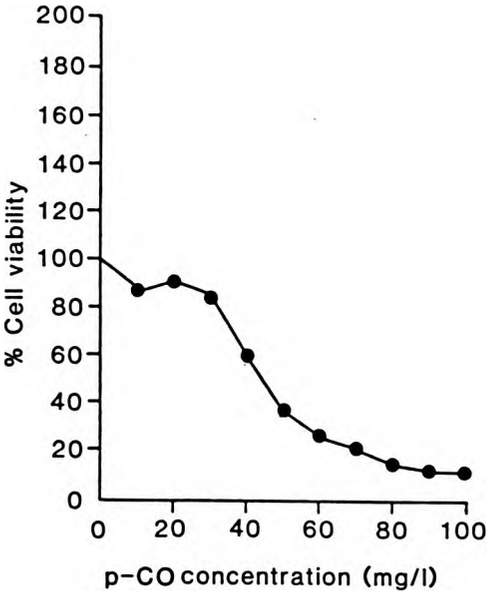
The percentage viability of rat lymphocytes incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.2	12
25	99.2	6.3	12
50	103.2	4.2	12
75	102.8	5.1	12
100	100.1	5.2	12
125	99.7	5.4	12
150	96.6	5.3	12
175	100.9	5.8	12
200	95.5	5.6	12
225	97.6	3.8	12
250	95.5	4.6	12

Fig. 3.2.1.2.

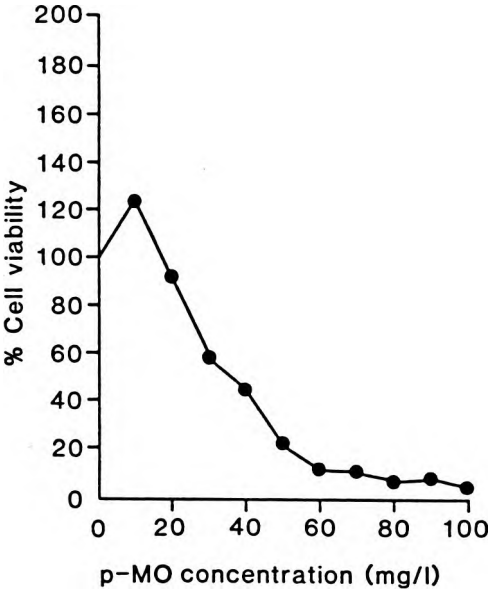
The percentage viability of rat lymphocytes incubated incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.0	6
10	86.4	4.1	6
20	90.2	2.7	6
30	83.8	4.4	6
40	60.7	6.3	6
50	38.1	4.7	6
60	27.1	3.6	6
70	22.5	2.4	6
80	17.6	3.8	6
90	12.9	2.4	6
100	10.6	1.8	6

Fig. 3.2.1.3.

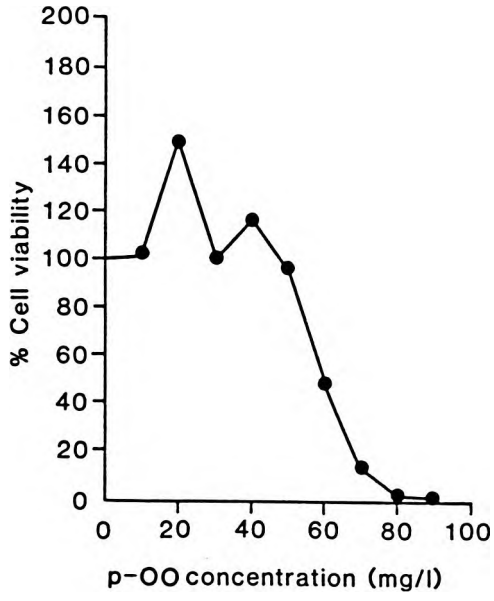
The percentage viability of rat lymphocytes incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.8	6
10	124.0	1.6	6
20	92.7	4.5	6
30	59.0	4.1	6
40	45.9	3.1	6
50	23.4	2.9	6
60	12.9	1.2	6
70	11.4	2.0	6
80	7.7	1.1	6
90	8.8	1.5	6
100	4.9	2.0	6

Fig. 3.2.1.4.

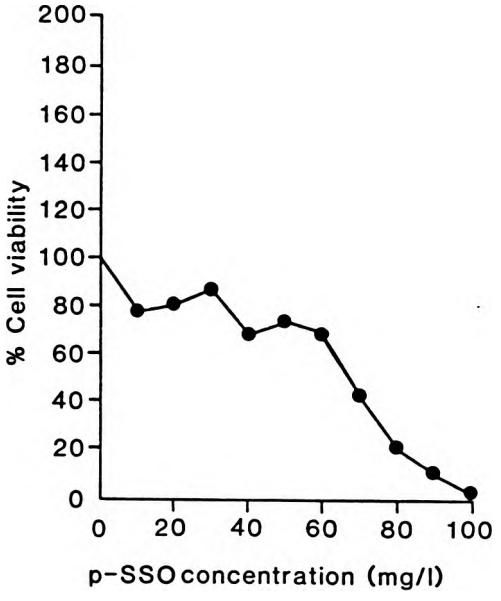
The percentage viability of rat lymphocytes incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.5	6
10	102.2	3.6	6
20	148.8	5.1	6
30	99.6	4.9	6
40	116.2	3.4	6
50	96.4	4.9	6
60	48.5	4.2	6
70	14.0	3.2	6
80	2.6	1.2	6
90	0.0	0.0	6
100	0.0	0.0	6

Fig. 3.2.1.5.

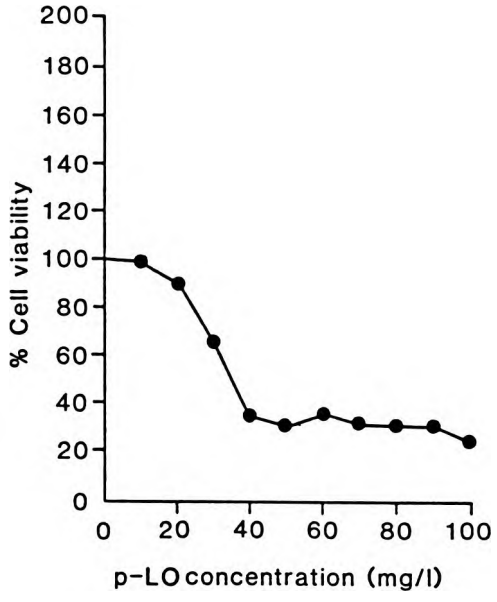
The percentage viability of rat lymphocytes incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.1	6
10	77.5	4.2	6
20	80.1	6.0	6
30	86.6	6.0	6
40	68.6	5.7	6
50	73.5	5.2	6
60	67.8	5.0	6
70	43.3	4.1	6
80	21.2	3.7	6
90	11.4	2.1	6
100	1.6	1.0	6

Fig. 3.2.1.6.

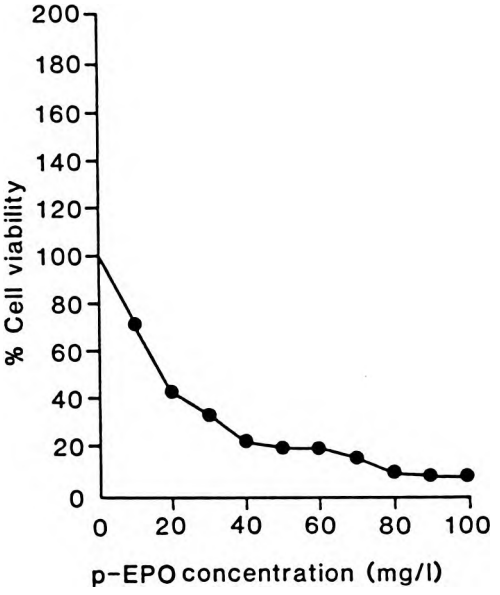
The percentage viability of rat lymphocytes incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.6	6
10	98.8	5.8	6
20	90.0	4.5	6
30	65.9	5.1	6
40	34.5	3.3	6
50	30.5	2.2	6
60	36.8	2.6	6
70	32.9	1.9	6
80	31.4	2.1	6
90	31.2	1.5	6
100	24.9	2.4	6

Fig. 3.2.1.7.

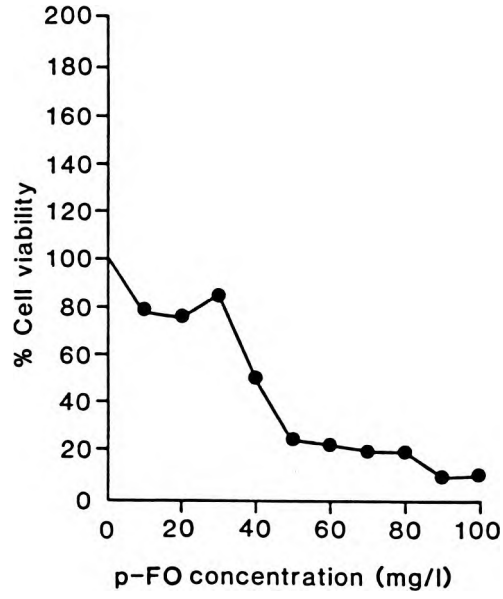
The percentage viability of rat lymphocytes incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.4	6
10	71.7	5.5	6
20	45.0	5.0	6
30	34.5	2.6	6
40	22.7	3.2	6
50	20.9	2.3	6
60	21.0	3.2	6
70	16.5	5.6	6
80	10.4	3.0	6
90	8.7	1.9	6
100	8.7	3.5	6

Fig. 3.2.1.8.

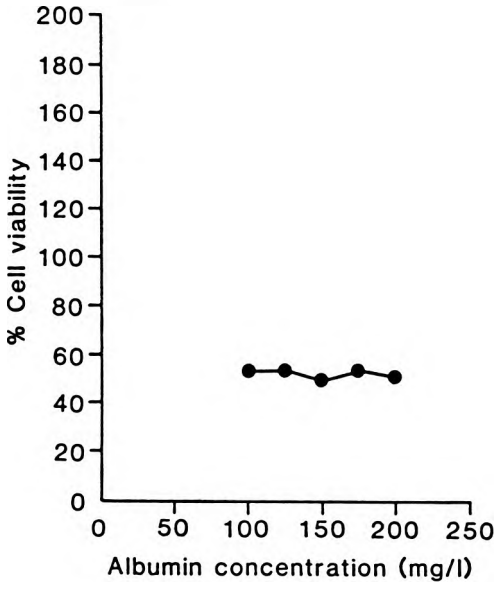
The percentage viability of rat lymphocytes incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.0	6
10	78.7	3.9	6
20	76.8	4.9	6
30	85.7	5.3	6
40	51.4	2.8	6
50	26.2	2.8	6
60	23.5	2.5	6
70	21.1	1.8	6
80	20.0	5.3	6
90	10.5	2.4	6
100	11.4	1.1	6

Fig. 3.2.1.9.

The percentage viability of rat lymphocytes incubated with albumin at the ID_{50} of p-SSO.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	7.1	6
100	53.6	4.3	6
125	53.8	5.4	6
150	50.2	5.6	6
175	53.0	6.3	6
200	50.7	4.4	6

p-oils induced concentration dependent cytotoxicity, but it was unclear whether PUFA-rich p-oils exhibited greater cytotoxic potential than monounsaturated, or saturated, FA-rich p-oils.

Only p-CO concentrations greater than 30mg/l caused marked concentration dependent inhibition of lymphocyte viability (Fig. 3.2.1.2.). On the other hand, 10mg/l p-MO increased cell viability to 124.0%, followed by rapid concentration dependent cytotoxicity in the range 20 to 60mg/l (Fig. 3.2.1.3.). ID_{50} values for cells dosed with p-CO or p-MO were approximately 45 and 38mg/l, respectively, with 10.6% and 4.9% viability found with 100mg/l p-CO or p-MO, respectively.

p-OO supplementation induced the most dramatic changes in lymphocyte viability (Fig. 3.2.1.4.). Relative cell viability was greater than, or equal to, controls with concentrations up to 50mg/l, and stimulated to a maximum of 148.8% with 20mg/l p-OO, and to 116.2% with 40mg/l p-OO. Cytostasis was induced with 50mg/l p-OO, and concentration dependent cytotoxicity with greater amounts dosed. Approximately 59mg/l p-OO reduced cell viability to 50% of controls (ID_{50}), and no viable lymphocytes were present with 90 or 100mg/l p-OO.

A gradual inhibitory response was induced with p-SSO dosage up to 60mg/l, with lymphocyte viability reduced to 67.8% (Fig. 3.2.1.5.). Cytotoxicity became more

pronounced with greater concentrations dosed, and only 11.4% and 1.6% of lymphocytes were viable with 90 and 100mg/l p-SSO, respectively. p-SSO dosed lymphocytes nevertheless exhibited the highest ID_{50} (about 67mg/l). On the other hand, 10mg/l p-LO induced cytostasis, and concentrations from 20 to 40mg/l were considerably more cytotoxic than was p-SSO in the same range (Fig. 3.2.1.6.). The ID_{50} was approximately 36mg/l p-LO, and cell viability was reduced to 34.5% with 40mg/l p-LO. Thereafter, a plateau effect was noted, with viability only marginally influenced with further concentration increases (30.5% to 36.8% viability over the range 40 to 90mg/l). About 25% of lymphocytes were viable with 100mg/l p-LO, and this was at least double that found with any other p-oil dosed at this concentration.

Low concentrations of p-EPO were very cytotoxic, and only 18mg/l p-EPO was required to reduce the viable cell count to 50% of controls (Fig. 3.2.1.7.). This was the lowest ID_{50} obtained with rat lymphocytes. The toxicity induced with p-FO was less pronounced than with p-EPO dosage, and lymphocyte viability was reduced to 50% with approximately 40mg/l p-FO (Fig. 3.2.1.8). The cytotoxic potential of both p-EPO and p-FO increased slowly with concentrations greater than 50mg/l, and viability was about 10% with 100mg/l p-oil.

To exclude the possibility that any of the effects induced with p-oil dosage were influenced by the amount

of albumin bound to the FA's, lymphocytes were incubated with p-SSO to correspond with its ID_{50} (about 67mg/l). Five points were chosen around which to vary the albumin concentration keeping the p-SSO concentration fixed, viz. two points above, two points below, and the ID_{50} of p-SSO. The appropriate amount of albumin was added to these cultures to give the desired concentrations. The results are shown in Fig. 3.2.1.9., and indicated that the amount of p-SSO dosed reduced lymphocyte viability to approximately 50% of controls, and that such was not influenced by the amount of albumin present as FA carrier. Hence, synergistic effects of albumin could be excluded, and the effects induced with the p-oils were solely due to the exogenous FA's.

Subsequent to these studies, rat lymphocytes were plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient amounts for all qualitative and quantitative analyses to be carried out. Upon harvesting, cell viabilities relative to controls were compared to those in Figs. 3.2.1.1-3.2.1.8., and were found not to be statistically different. All further biochemical assays were performed on these samples.

3.2.2 Effects of pseudo-Oils on Total Protein.

Total lymphocyte protein was determined for each of the p-oil concentrations dosed, and the data shown in Table 3.2.2.1. as µg total protein/10^6 cells seeded.

Control rat lymphocytes contained 6.5µg protein/10^6 cells seeded, while levels in dosed lymphocytes ranged from 5.2 to 97.3µg protein/10^6 cells seeded. All dosed cells, except those supplemented with 20mg/l p-EPO or p-SSO, exhibited total protein concentrations greater than controls.

Protein concentrations increased progressively with the amount of p-oil dosed. The saturated p-oils, p-CO and especially p-MO, induced the greatest overall increases in protein, particularly with a concentration of 60mg/l. Dosage with any PUFA-rich p-oil generally induced the smallest overall increment in protein, while that with p-OO supplementation was intermediary between the two extremes. With dosage of 60mg/l p-oil, the overall capability to enhance lymphocyte protein was in the order p-MO > p-CO > p-OO > p-SSO > p-EPO > p-FO > p-LO.

Table 3.2.2.1.

The protein content of rat lymphocytes, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	6.5			
CO		11.3	15.8	36.7
MO		11.9	50.8	97.3
OO		15.3	16.7	29.5
SSO		5.7	10.5	20.2
LO		8.8	8.4	11.2
EPO		5.2	7.8	19.3
FO		13.8	14.2	18.2

3.2.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Rat Lymphocytes.

The FA spectra of rat lymphocytes dosed with 0, 20, 40 or 60mg/l of each p-oil are shown in Table 3.2.3.1. Control lymphocytes exhibited a FA spectrum in which one FA, 22:4ω6, was present at 52.8%. FA's which contributed >5.0% to the spectrum included 16:0 (11.7%), 18:0 (7.2%), 18:1ω9 (5.5%), 22:5ω6 (5.5%) and 20:4ω3 (6.0%). Dosed cells contained varying amounts of these FA's.

The FA spectra showed that the major p-oil FA components dosed were incorporated. In general, 16:0, 18:1ω9 and 18:2ω6 levels were greater in dosed lymphocytes than in controls, more 16:1ω9 was found in control lymphocytes, while 18:0 levels were more variable. 18:2ω6 desaturation and elongation products were generally decreased in dosed cells, as were the long chain more unsaturated products of 18:3ω3.

16:0 proportions increased from 11.7% in controls, to 24.3%, 45.1% and 48.6% with supplementation of 20, 40 or 60mg/l p-CO, respectively. 18:1ω9 levels increased similarly (15.2%, 15.3% and 16.4%, respectively vs 5.5% in controls), but the increment in 18:0 was only slight (8.3% to 8.7% vs 7.2% in controls). In contrast, 22:4ω6, 22:5ω6, 18:4ω3 and 20:4ω3 percentages decreased significantly compared to controls (52.8%, 5.5%, 3.8% and 6.0%, respectively).

Legend to Table 3.2.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.2.3.1.

The fatty acid spectrum of rat lymphocytes.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	11.7±0.55	24.3	45.1	48.6	21.3	32.8	33.9	14.6	16.7	15.6	10.7	23.4	16.7	16.4	25.6	23.7	9.4	26.0	21.2	24.6	30.7	23.6
	18:0	7.2±0.00	8.3	8.5	8.7	7.8	18.1	10.1	5.0	1.5	1.3	9.3	9.0	6.0	5.5	7.8	5.6	12.9	10.1	7.5	6.0	3.9	2.4
	20:0	0.1±0.00	-	0.2	-	-	-	-	2.9	-	-	-	-	-	-	-	-	-	-	-	2.7	-	-
	22:0	0.7±0.00	0.7	0.1	0.1	0.3	0.1	0.1	-	-	-	0.1	-	-	0.4	-	-	-	0.1	0.1	-	-	-
	24:0	-	0.8	-	-	0.4	-	-	-	-	-	0.4	0.7	0.6	-	0.5	-	-	1.6	0.5	-	-	-
ω9 MONOS.	16:1	1.5±0.00	-	0.3	1.1	-	-	3.0	2.7	-	0.5	1.0	0.2	0.1	0.6	1.5	0.1	0.2	0.2	0.9	13.6	25.9	33.6
	18:1	5.5±0.10	15.2	15.3	16.4	23.0	26.6	24.7	37.9	71.6	73.4	10.5	34.0	33.1	23.1	34.0	27.9	12.2	21.4	18.9	12.7	23.3	23.7
	24:1	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	2.1±0.00	1.7	1.3	1.0	6.7	6.7	4.8	2.2	4.1	4.6	7.7	20.0	37.0	3.2	7.6	11.5	14.2	31.3	42.4	1.5	7.2	8.1
	18:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.9	1.6	-	-	-	-
	20:2	-	-	-	-	-	-	-	1.2	1.7	1.4	-	-	-	-	0.2	-	-	-	-	-	0.9	0.8
	20:3	0.4±0.05	-	-	0.1	-	-	0.1	2.7	-	-	-	-	-	-	-	0.1	-	-	-	2.0	0.2	0.1
	20:4	1.1±0.00	-	0.2	0.2	0.4	0.7	0.4	-	-	-	-	0.2	0.1	-	0.2	0.1	-	0.3	0.1	-	0.2	0.1
	22:4	52.8±0.65	40.2	23.1	21.0	34.7	11.3	20.6	23.2	2.9	2.4	53.4	8.4	5.2	41.1	10.6	8.7	41.5	4.4	5.7	27.0	4.2	4.3
	22:5	5.5±0.65	0.3	1.0	-	-	0.4	-	-	0.2	-	-	0.5	-	0.3	0.6	-	0.4	1.8	-	0.7	0.1	-
ω3 POLYS.	18:3	0.3±0.06	0.3	0.2	0.2	1.0	0.5	0.3	-	0.2	0.2	-	0.3	0.1	1.0	8.2	20.9	-	0.2	-	0.3	0.4	0.5
	18:4	3.8±0.15	0.9	1.2	0.3	0.4	1.0	0.3	4.4	0.5	0.2	0.5	1.3	0.2	0.7	1.2	0.3	4.2	0.9	0.1	4.0	0.5	0.1
	20:4	6.0±0.55	7.4	3.8	2.3	4.1	1.8	1.1	3.1	0.7	0.2	6.3	1.4	0.6	7.5	1.5	0.7	5.3	1.0	0.5	2.8	0.5	0.5
	20:5	1.0±0.00	-	-	0.2	-	-	0.7	-	0.1	0.1	-	0.6	-	0.4	0.4	0.2	-	-	-	1.6	1.7	2.1
	22:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-
	22:6	-	-	-	-	-	0.1	-	-	-	-	-	-	0.2	-	0.1	0.2	-	-	0.5	0.5	0.2	0.1

When lymphocytes were dosed with 0, 20, 40 or 60mg/l p-MO, 16:0 incorporation increased from 11.7% to 21.3%, 32.8% and 33.9%, and 18:0 increased from 7.2% to 7.8%, 18.1% and 10.1%, respectively. A considerable increase in 18:1ω9 was also found (from 5.5% to 23.0%, 26.6% and 24.7%, respectively), but 16:1ω9 increased only with 60mg/l (3.0% vs 1.5% in controls). 18:2ω6 incorporation increased from 2.1% in controls to between 4.8% and 6.7% over the range dosed, but 20:4ω6 and particularly 22:4ω6 levels were decreased compared to controls (1.1% and 52.8%, respectively).

37.9% 18:1ω9 was found with dosage of 20mg/l p-OO, and this amount almost doubled to 71.6% and 73.4% with 40 and 60mg/l p-OO, respectively, compared to only 5.5% in controls. 16:0 levels increased only marginally over the range dosed (14.6%, 16.7% and 15.6%, respectively vs 11.7% in controls), 18:0 proportions were reduced from 5.0% to 1.3% (7.2% in controls), while 2.9% 20:0 and 2.7% 16:1ω9 were detected with 20mg/l p-OO (0.1% and 1.5% in controls, respectively). 18:2ω6 percentages with 20mg/l p-OO were similar to controls (2.2% and 2.1%, respectively), but levels increased about two fold with 40 and 60mg/l. 20:2ω6 levels ranged from 1.2% to 1.7% in dosed cells, but this FA was absent from controls, while 2.7% 20:3ω6 was detected with 20mg/l p-OO (0.4% in controls). Less than half the control level (52.8%) of 22:4ω6 was found with supplementation of 20mg/l p-OO

(23.2%), and this decreased further to 2.9% and 2.4% with 40 and 60mg/l p-OO, respectively.

16:0 levels were parallel to controls with 20mg/l p-SSO, but increased with dosage of 40 or 60mg/l p-SSO (11.7%, 10.7%, 23.4% and 16.7%, respectively). 18:0 percentages were not significantly different with 20 or 40mg/l (9.3% and 9.0%, respectively), while only 6.0% was shown with 60mg/l (7.2% in controls). 18:1ω9 levels increased about 2 fold with 20mg/l p-SSO compared to controls (10.5% and 5.5%, respectively), and this increased to at least six fold with 40 and 60mg/l (34.0% and 33.1%, respectively). 18:2ω6 incorporation into lymphocytes dosed with 20, 40 or 60mg/l p-SSO increased from 7.7% to 20.0% and 37.0%, respectively (2.1% in controls), but other ω6, and all ω3, PUFA levels were parallel to, or significantly lower than, controls.

16:0 and 18:1ω9 percentages ranged from 16.4% to 25.6% and 23.1% to 34.0%, respectively with p-LO dosage (11.7% and 5.5% in controls, respectively), while 18:0 levels were parallel to controls (1.5%) or decreased. 18:2ω6 incorporation increased from 3.2% to 7.6% and 11.5% with 20, 40 and 60mg/l p-LO, respectively (2.1% in controls), but other ω6 PUFA levels were decreased. The amount of 18:3ω3 incorporated into lymphocytes dosed with 20mg/l p-LO was 1.0%, but increased significantly to 8.2% with with 40mg/l and 20.9% with 60mg/l (0.3% in controls).

18:4ω3 percentages ranged from 0.3% to 1.2% (3.8% in controls), and 7.5% 20:4ω3 was detected with 20mg/l p-LO (6.0% in controls), but no significant increases in other ω3 PUFA's were found.

16:0 proportions changed from 11.7% to 9.4%, 26.0% and 21.2% with 0, 20, 40 or 60mg/l p-EPO dosage, compared to 7.2%, 12.9%, 10.1% and 7.5% 18:0, respectively. 1.6% 24:0 was found with 40mg/l p-EPO (0% in controls), while 18:1ω9 levels ranged from 12.2% to 21.4% (5.5% in controls). 18:2ω6 incorporation increased significantly to 14.2%, 31.3% and 42.4% with 20, 40 or 60mg/l p-EPO, respectively (2.1% in controls). No 18:3ω6 was found in control lymphocytes, but 0.9% and 1.6% were detected with 40 and 60mg/l, respectively. However, other ω6 PUFA levels were significantly decreased, and no significant increase in ω3 PUFA's were found.

When lymphocytes were dosed with 20, 40 or 60mg/l p-FO, the amount of 16:0 incorporated ranged between 2 to 3 fold that of controls (11.7%), 18:0 levels decreased from 6.0% to 2.4%, respectively (7.2% in controls), and 2.7% 20:0 was detected with 20mg/l (0.1% in controls). 16:1ω9 incorporation was concentration dependent (1.5%, 13.6%, 25.9% and 33.6% with 0, 20, 40 and 60mg/l p-FO, respectively), as was 18:1ω9 uptake (5.5%, 12.7%, 23.3% and 23.7%, respectively). 18:2ω6 was increased only with 40 and 60mg/l p-FO (7.2% and 8.1%, respectively vs 2.1% in controls), a significant amount of 20:3ω6 was

detected with 20mg/l p-FO (2.0% vs 0.4% in controls), but no increases in 20:4ω6 or 22:4ω6 were found. Dosed lymphocytes incorporated 20:5ω3 in p-FO (1.6%, 1.7% and 2.1%, respectively vs 1.0% in controls), but only trace amounts (≤0.5%) of 22:5ω3 and 22:6ω3 were detected.

3.2.4 Effects of pseudo-Oils on Lipid Peroxide Formation.

Results obtained from the measurement of lipid peroxides both in rat lymphocytes and their respective spent media are shown in Table 3.2.4.1. The values are reported as nmoles MDA/10^6 cells, but in the case of spent growth media, this represents the nmoles of MDA in the volume of medium from which 1x10^6 cells were obtained.

Lipoperoxide concentrations for dosed lymphocytes ranged from a low of 1.2nmoles MDA/10^6 cells (with 20mg/l p-MO or p-EPO) to a high of 102.6nmoles MDA/10^6 cells (with 60mg/l p-LO), compared to 0.4nmoles MDA/10^6 control lymphocytes. Lipid peroxide amounts increased in every instance with increments in p-oil concentration dosed, and the greatest increase was detected with ω3 PUFA rich-p-oils (5.0, 26.1 and 102.6nmoles MDA/10^6 cells with p-LO, and 7.3, 12.7 and 65.2nmoles MDA/10^6 cells with p-FO, respectively). On the other hand, lymphocytes supplemented with 20, 40 or 60mg/l p-MO or p-EPO induced the smallest concentration dependent increase in lipid peroxides, although the amounts generated were similar (1.2, 3.0 and 8.3nmoles MDA/10^6 cells, and 1.2, 5.1 and 8.4nmoles MDA/10^6 cells, respectively). Lipid peroxide production with p-CO, p-SSO or p-OO dosage was intermediate between the two extremes, ranging from 2.9 to 18.2nmoles MDA/10^6 cells.

Table 3.2.4.1.

Lipid peroxide formation by rat lymphocyte cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	0.4			
CO		2.9	11.1	15.3
MO		1.2	3.0	8.3
OO		2.8	11.2	18.2
SSO		4.4	9.9	12.4
LO		5.0	26.1	102.6
EPO		1.2	5.1	8.4
FO		7.3	12.7	65.2
pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	-
MO		-	-	-
OO		-	-	-
SSO		-	-	-
LO		-	31.5	102.7
EPO		-	-	-
FO		-	-	10.4

Only spent medium derived from cultures incubated with ω3 PUFA-rich p-oils contained lipid peroxides. Medium derived from cultures incubated with p-FO contained fewer lipoperoxides than the lymphocytes themselves, with significant amounts measured in spent medium only with 60mg/l p-FO (10.4nmoles MDA/10^6 cells). As with the cells, the greatest amounts of medium lipoperoxides were found with 40 or 60mg/l p-LO dosage (31.5 and 102.7 nmoles MDA/10^6 cells, respectively), which correlated with cellular levels.

3.2.5 Discussion.

No reference has been found in the literature with regard to the influence of exogenous FA's on normal rat lymphocyte viability in culture. However, investigation of the effects of exogenous FA's on mitogen-induced DNA synthesis and cell activation in the modulation of mouse T and B lymphocyte proliferation showed that saturated FA's alone were more potent inhibitors of these processes than unsaturated FA's, and that the simultaneous addition of equimolar amounts of a saturated and an unsaturated FA abrogated the inhibitory effect of the former (Tsang et al 1977 and Buttke 1984). The finding that mitogen-activated B cells were much less susceptible to inhibition by FFA's than T cells (Buttke 1984) implied differential sensitivity of lymphocytes to the effects of FFA's. This was clear from comparison with our data. p-Oil supplementation affected rat lymphocyte viability (Figs. 3.2.1.2-3.2.1.8.), but it was unclear whether PUFA-rich p-oils exhibited greater cytotoxic potential than monoenoic or saturated FA-rich p-oils, since the effects induced were p-oil-specific rather than dependent on the degree of p-oil unsaturation. This implied that the modulation of rat lymphocyte viability related both to the nature (type) and proportion of saturated, monoenoic and/or polyenoic FA's dosed (Table 2.3.3.2.). p-EPO was most, and p-SSO least, cytotoxic, despite similar FA compositions,

implying involvement of both major and minor p-oil FA components, and possibly also p-oil FA synergism and/or antagonism, in the effects induced. Comparison of cell viability with p-EPO, p-SSO or p-LO dosage with the respective p-oil FA profiles indicated that GLA was a more effective cytotoxic agent than ALA, and both GLA and ALA exhibited greater cytotoxic potential than LA. This illustrated that both FA chain length and the number and position of double bonds present were involved in the modulation of rat lymphocyte viability.

Lymphocytes do not normally divide in culture without appropriate mitogenic stimulation, thus increased cell viability only with supplementation of 10mg/l p-MO, or 20 or 40mg/l p-OO, indicated FA involvement in the stimulation of rat lymphocyte proliferation at low concentrations. This, together with the finding of Dubin et al (1965) that an optimum concentration of only 0.07mg/l LA effectively promoted macrophage growth in culture, suggests that p-oil concentrations below 10mg/l may also effectively stimulate rat lymphocyte proliferation.

The data in Table 3.2.3.1. confirmed the ability of rat lymphocytes to incorporate exogenous FA's (Tsang et al 1977, Stubbs et al 1980 and Conroy et al 1986). A mechanism by which p-oils affected lymphocyte viability may therefore relate to differential uptake of p-oil

FA's and alterations in membrane fluidity. Studies have indeed shown that modification of rat diets either with olive oil or fish oil altered lymphocyte membrane fluidity, with the fish oil diet more effective in increasing the unsaturation index of the membrane than olive oil (Conroy et al 1986). p-Oil dosed lymphocyte FA compositions were modified compared to controls, thus it was likely that overall membrane fluidity was altered and this involved in the viability changes reported in Figs. 3.2.1.2-3.2.1.8. Supplementation with optimal proportions of saturated and unsaturated p-oil FA's at low concentrations may have allowed the cells to maintain a degree of membrane fluidity compatable with viability, whereas addition of large amounts of highly saturated or unsaturated p-oils may have led to pronounced changes in membrane fluidity such that lymphocyte viability could not continue. However, it was unlikely that eicosanoids were directly involved in the modulation of lymphocyte viability with p-oil dosage since this mechanism could not explain the effects of p-oils rich in saturated or monoenoic FA's, which can not be metabolised by this route. Even EFA-rich p-oils could not act as sources of eicosanoid precursors since the data in Table 3.2.3.1. indicated that rat lymphocytes exhibited no significant capability for PUFA desaturation (Δ6D, Δ5D and Δ4D) or elongation. Others have similarly shown that neither [^{14}C]-18:2ω6 nor [^{3}H]-20:4ω6 were further metabolised by these enzymic

pathways in rat leukocytes, despite considerable and rapid uptake into lipid pools, and suggested that the desaturase cascade was a minor route for FA metabolism in these cells (Brenner et al 1966, Mead et al 1976, Kanau et al 1977 and Cunnane et al 1984).

Detection of small, but significant, increases in 20:3ω6 levels with p-OO or p-FO dosage, which contained small amounts of 20:2ω6 (Table 2.3.3.2.), implied possible Δ8D capability. This may reflect an attempt by the cells to maintain membrane PUFA levels by activating Δ8D when Δ6D expression is suppressed/inhibited. However, it appeared that this mechanism was unable to sufficiently maintain lymphocyte PUFA levels since Δ8D substrate availability was limited by the inability to elongate 18:2ω6 incorporated with p-oil supplementation.

Zevallos et al (1989) showed that when rat lymphocytes were incubated with either [^{14}C]-18:0 or [^{14}C]-18:2ω6, in the presence of either diheptadecanoylphosphatidyl-choline or dioleylphosphatidylcholine, the fluidity of all membranes, including those of the microsomes, increased. Such membrane fluidisation was associated with depressed conversion of 18:0 to 18:1ω9, and 18:2ω6 to 18:3ω6 via Δ9D and Δ6D, respectively. The data in Table 3.2.3.1. showed that p-oil incorporation altered rat lymphocyte FA profiles. This may have altered the physical state (fluidity) of microsomal membranes and

depressed desaturase activity. Such may have contributed to absence of significant desaturase/elongase activity reported, and in particular, may explain the lack of PDFA formation from 18:2ω6 or 18:3ω3 incorporated with p-SSO, p-EPO or p-LO incubation (Table 3.2.3.1.). 18:1ω9 levels were significantly increased with p-oil dosage, but such probably reflects decreased PDFA levels and the fact that all p-oils contained 18:1ω9 in amounts ranging from about 5% to 70% (Table 2.3.3.2.), rather than 18:1ω9 formation from 16:0, 16:1ω9 and/or 18:0. It was unlikely, however, that the desaturase and elongase activity reported related to p-oil induced cytotoxicity since no significant evidence for such enzyme capability was found even when p-oils induced little or no toxicity.

Rode et al (1982) found between 4.5 and 6.0μg total protein/10^6 control lymphocytes, and this was in close agreement with the data reported in Table 3.2.2.1. The results presented also indicated that rat lymphocyte protein synthesis was significantly stimulated in a concentration dependent manner with increased p-oil concentration dosed (Table 3.2.2.1.), despite the inverse correlation with cell viability (Figs. 3.2.1.2-3.2.1.8.). p-Oils were thus potent agents for enhancing rat lymphocyte protein synthesis, and the extent to which such was stimulated was relatable to p-oil FA composition (Table 2.3.3.2.). The finding that saturated

FA-rich p-oils were generally most, and PUFA-rich p-oils least, effective in enhancing protein synthesis implied that the ability to modulate rat lymphocyte protein production related partly to p-oil unsaturation. This, did not explain why p-MO, for example, induced more protein than p-CO, although it was possible that such differences related to the mediation of synergistic or antagonistic effects between p-oil FA's.

Increased lymphocyte protein synthesis may reflect promotion of lymphocyte activation, enhanced protein synthesis in different cell components, as was shown by Rode et al (1982) in concanavalin A stimulated lymphocytes, increased enzyme synthesis to metabolise incorporated p-oil FA's, and/or enhanced biosynthesis of proteins required to maintain the integrity of cell membranes damaged directly with p-oil dosage, or indirectly by lipoperoxide formation (Table 3.2.4.1.). However, it was unlikely that increased protein synthesis related to enhanced desaturase or elongase expression in light of the data reported in Table 3.2.3.1.

The data presented in Table 3.2.4.1. was consistent with intact cellular enzymic and/or non-enzymic mechanisms for the production of lipoperoxides. Large amounts of PUFA's were detected in control lymphocytes (Table 3.2.3.1.), thus the small amounts of lipoperoxides measured may reflect cellular anti-oxidant and enzymic

mechanisms of protection against free radical attack. It was also possible that cellular PUFA's were present as components of complex lipids, eg. membrane PGL's, which would therefore not be so readily accessible to oxidation. On the other hand, saturation of the cellular anti-oxidant mechanisms and/or increased cellular FA availability may have occurred with incorporation of supplemented p-oils, and the lipoperoxides measured thus reflected the p-oil concentration dosed, p-oil FA composition (Table 2.3.3.2.) and FA susceptibility to oxidation. Measurement of the largest amounts of cellular and medium lipoperoxides with p-LO or p-FO dosage correlated with the significant ω3 PUFA levels in these p-oils. The significantly greater amount of ALA in p-LO relative to EPA in p-FO (approximately 63% and 18%, respectively) was consistent with the greatest lipid peroxide levels measured, both in the cells and spent medium, despite the greater susceptibility of EPA to oxidation. Rat lymphocytes were shown to incorporate p-oil FA's (Table 3.2.3.1.), thus the medium lipid peroxides measured with p-LO or p-FO dosage probably reflect cellular lipoperoxides released upon cell lysis, and/or released cellular FA's oxidised extracellularly by released cellular peroxidising enzymes.

Mosconi et al (1988) reported that superoxide production by white blood cells increased significantly when rat diets were supplemented with fish oil compared to olive

oil, and this correlated with the findings in Table 3.2.4.1. The significantly lower amounts of cellular lipoperoxides measured with p-MO, p-OO, p-SSO or p-EPO, compared to p-LO or p-FO, suggested that more substrates for peroxidation were lipid bound and stable with incubation of the former group, despite all these p-oils containing significant amounts of unsaturated FA's. This finding was consistent with the absence of lipoperoxides in the corresponding spent medium with p-MO, p-OO, p-SSO or p-EPO dosage, despite the mediation of cell lysis. The cellular lipid peroxides formed with p-SSO or p-EPO incubation possibly related to oxidation of incorporated LA or other cellular FA's. Both p-oils exhibited similar FA compositions, thus the greater lipoperoxide amounts induced with p-SSO suggested enhanced PGL cycling, which would have increased the rate of FA incorporation, release and pool of FFA's available for peroxidation. Alterations in PGL turnover could also have been consistent with the greater amounts of lipid peroxides measured with dosage of p-CO or p-OO than p-MO or p-EPO.

Current evidence suggests that lipid peroxides affect normal cell metabolism and cause membrane damage (Chio et al 1969, Mead 1976, Tappel 1980, Gavino et al 1981c, Morisaki et al 1982b and Frankel 1984), thus it was possible that such compounds were involved in the modulation of cytotoxicity induced with p-oil dosage. This may explain the inverse relationship between

lipid peroxide levels (Table 3.2.4.1.) and lymphocyte viability (Figs. 3.2.1.2-3.2.1.8.) with increased p-oil dosage. However, the finding that the greatest lipid peroxide levels did not correlate with the greatest cytotoxicity implied that these compounds were only partly involved in the modulation of rat lymphocyte viability. The data presented rather suggested that both lipoperoxides and alterations in membrane fluidity were involved in the effects induced.

3.3 THE EFFECTS OF PSEUDO-OILS ON CELLS DERIVED FROM RAT AORTIC ENDOTHELIUM.

3.3.1 Effects of pseudo-Oils on Cell Viability.

No morphological changes were observed between control cells before, during or after each experiment, and p-oil dosed cells were morphologically similar to controls, although cytoplasmic droplets were observed with p-oil incubation at high concentrations in some instances.

Investigation of the effects of 0 to 250mg albumin/l growth medium showed no significant changes in rat aortic endothelial cell viability (Fig. 3.3.1.1.), nor were any synergistic effects evident. The effects induced with p-oil dosage were thus a result of the mixture of exogenous FA's and not the albumin used as FA carrier.

The number of control cells, seeded at 10×10^4/ml, at the end of the 24 hour post-trypsinisation recovery period was 10.2×10^4/ml. This equated to approximately 89% of the final control cell number at the end of the 48 hour incubation period and represented the cytostatic number. Only p-oil concentrations limiting cell viability to significantly below 89% were considered cytotoxic.

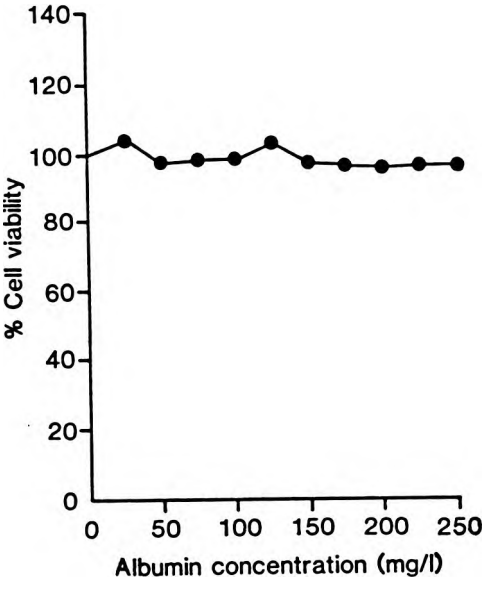
Figs. 3.3.1.2-3.3.1.8. show the results obtained from the incubation of rat endothelial cells with p-oils.

Legend to Figs. 3.3.1.1-3.3.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.3.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.3.1.2-3.3.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.3.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.3.1.1.

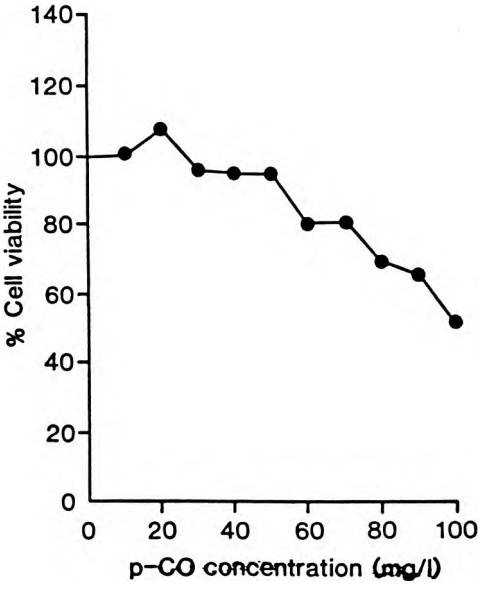
The percentage viability of cells derived from rat aortic endothelium incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.3	12
25	103.4	5.5	12
50	96.5	3.0	12
75	98.0	3.4	12
100	97.7	2.3	12
125	102.8	5.1	12
150	97.1	3.7	12
175	95.9	3.3	12
200	94.9	2.6	12
225	95.5	2.8	12
250	96.0	4.4	12

Fig. 3.3.1.2.

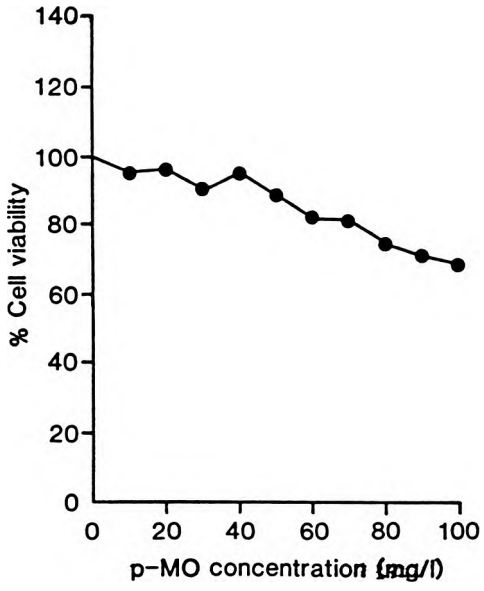
The percentage viability of cells derived from rat aortic endothelium incubated with p-CO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.4	6
10	100.5	2.1	6
20	108.4	3.6	6
30	95.6	2.7	6
40	95.3	4.1	6
50	95.0	3.5	6
60	80.3	4.4	6
70	81.2	4.6	6
80	69.0	1.8	6
90	65.6	2.7	6
100	51.2	4.3	6

Fig. 3.3.1.3.

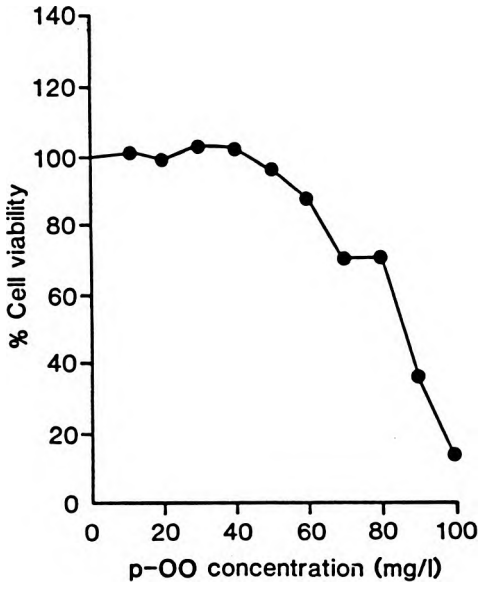
The percentage viability of cells derived from rat aortic endothelium incubated with p-MO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.3	6
10	96.0	2.0	6
20	97.0	2.3	6
30	90.9	2.6	6
40	96.3	1.0	6
50	89.9	2.4	6
60	82.8	2.3	6
70	82.5	2.8	6
80	75.4	2.3	6
90	72.1	2.1	6
100	68.7	1.5	6

Fig. 3.3.1.4.

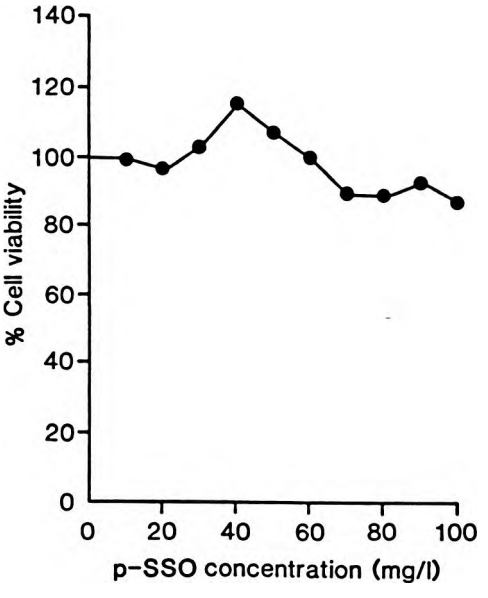
The percentage viability of cells derived from rat aortic endothelium incubated with p-OO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.3	6
10	101.2	3.5	6
20	99.6	2.9	6
30	103.2	3.2	6
40	102.0	2.7	6
50	97.2	2.9	6
60	88.5	2.4	6
70	70.8	2.2	6
80	72.3	2.8	6
90	36.8	3.2	6
100	13.4	2.0	6

Fig. 3.3.1.5.

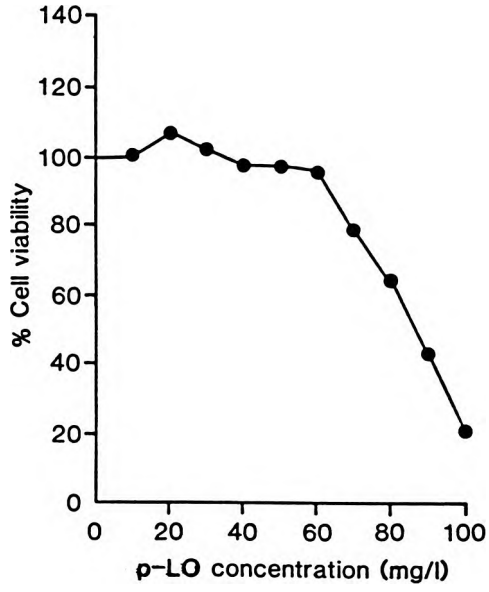
The percentage viability of cells derived from rat aortic endothelium incubated with p-SSO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.6	6
10	99.2	3.7	6
20	96.1	6.8	6
30	102.0	5.7	6
40	115.4	2.3	6
50	106.7	4.5	6
60	99.8	4.4	6
70	89.5	3.6	6
80	88.3	3.3	6
90	92.6	6.3	6
100	86.4	4.0	6

Fig. 3.3.1.6.

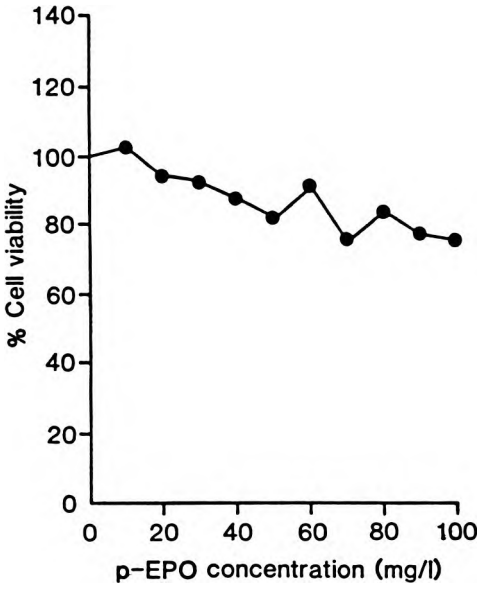
The percentage viability of cells derived from rat aortic endothelium incubated with p-LO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.8	6
10	101.0	3.5	6
20	107.5	3.8	6
30	102.0	3.3	6
40	98.0	3.5	6
50	97.5	4.2	6
60	96.0	4.0	6
70	79.1	3.5	6
80	64.7	2.6	6
90	42.8	3.2	6
100	20.9	3.2	6

Fig. 3.3.1.7.

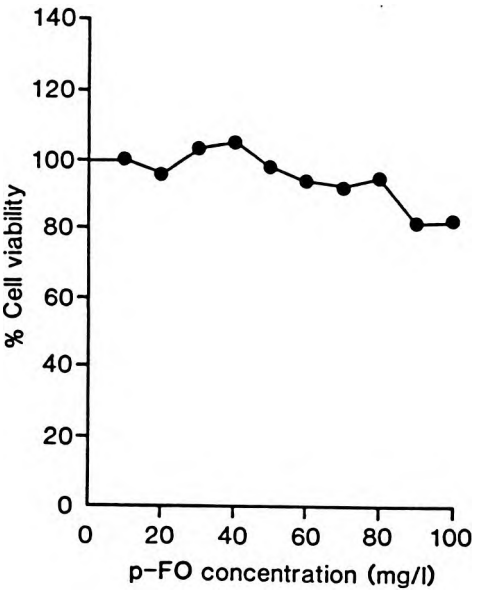
The percentage viability of cells derived from rat aortic endothelium incubated with p-EPO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.5	6
10	102.8	3.5	6
20	93.9	4.0	6
30	92.1	3.7	6
40	87.7	4.2	6
50	82.1	1.8	6
60	91.5	4.6	6
70	75.9	3.3	6
80	83.5	5.1	6
90	77.5	4.0	6
100	75.6	5.1	6

Fig. 3.3.1.8.

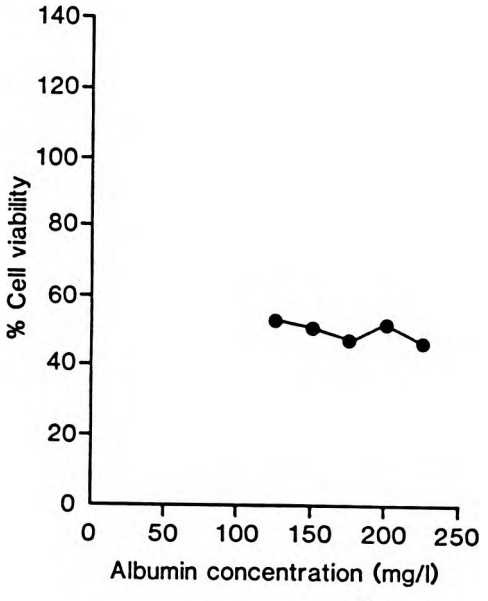
The percentage viability of cells derived from rat aortic endothelium incubated with p-FO.



pseuso-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.1	6
10	100.9	4.2	6
20	96.4	3.0	6
30	103.3	3.6	6
40	105.4	3.1	6
50	97.9	2.5	6
60	94.3	5.1	6
70	92.5	4.2	6
80	94.9	4.0	6
90	82.2	3.4	6
100	83.4	3.3	6

Fig. 3.3.1.9.

The percentage viability of cells derived from rat aortic endothelium incubated with albumin at the ID_{50} of p-QQ.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.1	6
125	53.4	2.5	12
150	51.7	5.1	12
175	47.7	4.5	12
200	52.2	3.1	12
225	46.4	2.2	12

Certain concentrations of some p-oils stimulated cell proliferation, and all p-oils limited cell viability to varying extents, but only p-CO, p-OO and p-LO induced significant cytotoxicity.

Dosage with 20mg/l p-CO stimulated cell proliferation to 108.4%, whereas p-MO did not cause any significant rise in cell viability with any concentration supplemented (Figs. 3.3.1.2. and 3.3.1.3., respectively). Both p-oils induced cytostasis in the range 50 to 60mg/l and cytotoxicity with greater concentrations, although p-CO was slightly more effective than p-MO. Cell viability was reduced to 51.2% and 68.7% with 100mg/l p-CO and p-MO, respectively, thus ID_{50} values could not be calculated.

No significant change in cell viability occurred with dosage of up to 50mg/l p-OO or 60mg/l p-LO, although 20mg/l p-LO stimulated cell proliferation to a maximum of 107.5% (Figs. 3.3.1.4. and 3.3.1.6., respectively). These p-oils induced the greatest cytotoxicity with concentrations greater than 60mg/l, and reduced cell viability almost linearly to 13.4% and 20.9% with 100mg/l p-oil, respectively. ID_{50} values for p-OO and p-LO were alike (about 86mg/l and 87mg/l, respectively), and this supported the similar effects these p-oils induced.

p-SSO dosage induced the greatest growth stimulatory and least limiting effects (Fig. 3.3.1.5.). Incubation with

10 or 20mg/l p-SSO had little effect on cell viability, but such was stimulated in the range 20 to 60mg/l p-SSO to a maximum of 115.4% with 40mg/l, and limited to approximate the cytostatic number (about 89%) with concentrations greater than 60mg/l p-SSO.

10mg/l p-EPO had little effect on cell viability, but concentrations of 20 to 60mg/l p-EPO were slightly growth limiting or cytostatic (Fig. 3.3.1.7.). Cell viability was not significantly altered with p-FO dosage up to 80mg/l, but was marginally stimulated to 105.4% with 40mg/l p-FO (Fig. 3.3.1.8.). Both p-oils, however, induced little cytotoxicity as relative cell viability was only slightly lower than the cytostatic number with 100mg/l p-oil (75.6% and 83.4%, respectively).

Cells were incubated with p-OO to correspond with its ID_{50} (about 86mg/l) and dosed with varying amounts of albumin to exclude the possibility that the effects induced with p-oil dosage were influenced by the amount of albumin bound to the FA's. Five points were chosen around which to vary the albumin concentration, keeping the p-oil concentration fixed, viz. two points above, two points below, and the ID_{50} of p-OO. The appropriate amount of albumin was added to these cultures to give the desired concentrations. The results are shown in Fig. 3.3.1.9., and indicated that the concentration of p-OO supplemented reduced cell viability to about 50% of controls, and that such was not significantly influenced

by different albumin concentrations. Exclusion of the synergistic involvement of albumin therefore implied that the effects induced with the p-oils were attributed solely to the exogenous FA's.

Rat aortic endothelial cells were subsequently plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient amounts for all qualitative and quantitative analyses to be performed. Cell viabilities were assessed upon harvesting, and found not to be statistically different from those in Figs. 3.3.1.1-3.3.1.8. All further biochemical assays were performed on these samples.

3.3.2 Effects of pseudo-Oils on Total Protein.

The results obtained from the determination of total protein in control and dosed cells are shown in Table 3.3.2.1. as µg total protein/10^6 cells seeded.

181.1µg protein was measured per 10^6 control cells seeded, while cells incubated with 60mg/l p-CO exhibited the lowest protein levels (174.2µg/10^6 cells seeded). All other dosed cells contained more protein than controls, and the highest amount measured was 254.7µg protein/10^6 cells seeded (with 40mg/l p-SSO).

p-Oil dosage induced concentration dependent changes in cellular protein. Total protein concentrations increased in direct correlation with the amount of p-OO dosed, but were generally inversely related to the p-CO, p-MO, p-LO or p-EPO concentration supplemented. With p-SSO or p-FO incubation, however, the highest protein concentrations measured were with a concentration of 40mg/l.

Table 3.3.2.1.

The protein content of cells derived from rat aortic endothelium, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	181.1			
CO		212.3	188.1	174.2
MO		195.0	195.5	188.5
OO		198.4	215.7	226.1
SSO		201.5	254.7	201.9
LO		227.9	195.0	187.5
EPO		201.9	195.4	194.2
FO		184.6	227.9	219.3

3.3.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Cells Derived From Rat Aortic Endothelium.

Table 3.3.3.1. shows the FA spectra of rat aortic endothelial cells incubated with 0, 20, 40 or 60mg/l of each p-oil.

Control cells exhibited a FA spectrum in which no one FA was present in amounts greater than 20.5% (for 16:0). Other FA's contributing ≥5.0% to the total control spectrum included 18:0 (19.4%), 16:1ω9 (6.1%), 18:1ω9 (15.0%) and 22:4ω6 (13.5%). The remaining 25.5% was made up of small amounts (<5.0%) of 18:2ω6, 18:3ω6, 18:3ω3, 18:4ω3, 20:3ω6, 20:4ω6, 20:4ω3, 20:5ω3, 22:5ω6, 22:5ω3 and 22:6ω3. The above FA's were detected in varying amounts in the dosed cells.

In general, 16:0 levels were variable, and 18:1ω9 levels similar, between control and dosed cells, while 16:1ω9 and 18:0 percentages were higher in control cells. Dosed cells contained at least twice as much 18:2ω6 than controls, ω6 PDFA levels were variable depending on the p-oil and concentration supplemented, while small but significant increases in 22:5ω3 and 22:6ω3 were detected in dosed cells.

16:0 levels were increased with dosage of 20mg/l p-CO (27.5%), but parallel to controls (20.5%) with 40mg/l

Legend to Table 3.3.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.3.3.1.

The fatty acid spectrum of cells derived from rat aortic endothelium.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	20.5±0.20	27.5	19.9	16.7	24.6	20.2	26.1	23.4	17.8	21.6	12.9	17.8	24.3	27.3	18.3	6.5	27.7	16.3	24.1	29.4	20.4	23.7
	18:0	19.4±0.70	14.3	14.2	26.1	13.7	15.1	16.3	11.5	10.7	10.3	14.3	12.8	13.9	13.9	15.8	28.0	16.6	15.9	13.5	14.4	14.3	11.9
	20:0	-	-	0.2	2.5	-	-	-	-	-	-	-	0.2	-	-	0.1	1.2	-	-	-	-	-	-
	22:0	-	0.2	0.2	-	0.1	0.2	0.1	-	-	-	-	-	-	0.1	0.2	-	-	-	-	-	-	-
	24:0	-	-	0.5	-	-	0.3	-	-	0.3	-	-	0.5	-	-	0.2	-	-	0.2	-	-	-	-
ω9 MONOS.	16:1	6.1±0.20	6.1	1.5	1.4	4.7	4.6	4.1	6.0	5.6	3.8	0.3	1.5	5.0	6.9	1.0	0.7	6.2	1.4	5.2	8.9	7.8	9.8
	18:1	15.0±0.15	15.9	15.6	24.2	14.7	15.9	18.9	21.5	29.5	35.1	15.9	14.8	16.0	15.6	15.8	22.5	14.9	13.3	13.7	15.1	16.1	16.3
	24:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	2.8±0.05	6.9	4.7	11.6	8.2	9.2	10.8	8.2	8.6	10.2	19.4	21.6	19.0	10.5	10.9	15.6	13.4	20.1	19.4	7.6	5.6	8.2
	18:3	0.8±0.05	0.9	-	-	0.9	0.2	0.7	0.6	0.1	0.6	1.7	-	0.7	1.2	-	-	1.1	0.3	0.9	1.2	0.2	0.8
	20:2	-	-	-	-	-	0.2	-	0.4	0.6	1.2	0.5	1.5	0.8	-	-	-	0.3	1.0	0.4	-	0.3	0.2
	20:3	4.7±0.35	1.3	2.2	0.8	1.1	1.7	1.2	1.0	1.5	1.0	1.2	1.6	1.1	0.9	1.5	2.1	2.1	5.0	3.6	0.7	1.5	1.0
	20:4	4.1±0.05	3.0	5.0	2.4	3.7	6.0	3.5	2.3	3.7	2.2	2.4	2.7	2.0	2.8	5.1	2.3	1.8	2.8	1.7	2.0	3.7	2.2
	22:4	13.5±0.30	15.1	19.7	6.4	18.9	16.3	11.1	18.0	11.0	7.2	20.4	15.0	10.7	10.7	12.1	0.7	9.4	11.6	11.3	10.4	12.8	10.2
	22:5	0.9±0.00	-	2.3	0.3	0.1	0.9	-	0.2	1.3	0.1	0.4	1.5	0.2	0.2	1.4	0.2	0.2	2.7	0.2	0.3	1.6	-
ω3 POLYS.	18:3	0.2±0.01	0.8	0.6	2.2	1.1	0.7	0.7	1.0	1.2	1.0	1.5	0.5	0.6	3.8	8.4	11.7	0.9	0.3	0.5	1.1	0.5	0.9
	18:4	3.9±0.30	0.4	1.2	0.5	0.4	1.0	0.2	0.3	1.3	0.2	0.7	0.6	0.3	0.3	2.0	7.3	0.4	1.7	0.3	0.5	0.1	0.3
	20:4	2.0±0.10	1.5	0.9	1.3	2.4	0.8	0.9	1.5	0.2	0.7	3.5	0.4	1.2	1.8	0.5	0.1	1.7	0.7	1.6	2.4	0.7	2.1
	20:5	3.3±0.10	0.9	2.5	0.5	0.5	0.7	0.5	0.3	1.2	0.7	0.2	1.6	0.2	0.5	0.9	0.1	0.2	1.7	0.1	1.6	3.8	3.7
	22:5	1.2±0.10	2.1	3.3	1.4	2.0	2.6	2.0	1.6	2.4	1.6	2.0	2.3	1.8	1.6	2.5	0.4	1.4	2.2	1.5	3.1	8.0	6.6
	22:6	1.7±0.30	3.1	5.3	1.9	2.9	3.4	3.0	2.4	3.4	2.4	2.8	3.3	2.5	2.3	3.5	0.5	1.9	2.9	2.1	1.4	2.8	2.3

p-CO (19.9%), and decreased with 60mg/l p-CO (16.7%). 16:1ω9 levels decreased considerably with 40 and 60mg/l (1.5% and 1.4%, respectively vs 6.1% in controls), while notable increases in 18:0, 20:0 and 18:1ω9 occurred with 60mg/l (26.1%, 2.5% and 24.2% vs 19.4%, 0% and 15.0% in controls, respectively). Dosed cells contained 4.7% to 11.6% 18:2ω6 and 0.6% to 2.2% 18:3ω3 (2.8% and 0.2% in controls, respectively), and the greatest increase in 22:4ω6, 22:5ω6, 22:5ω3 and 22:6ω3 occurred with 40mg/l p-CO (19.7%, 2.3%, 3.3% and 5.3% vs 13.5%, 0.9%, 1.2% and 1.7% in controls, respectively).

p-MO dosage induced an overall increase in 16:0 levels (20.2% to 26.1% vs 20.5% in controls), a decrease in 16:1ω9 and 18:0 (4.1% to 4.7% and 13.7% to 16.3% vs 6.1% and 19.4% in controls, respectively), but little change in 18:1ω9 (14.7% to 18.9% vs 15.0% in controls). 18:2ω6 incorporation increased significantly from 8.2% to 9.2% and 10.8% with 20, 40 and 60mg/l p-MO, respectively (2.8% in controls). 20:4ω6 was only increased with 40mg/l (6.0% vs 4.1% in controls), although 18.9% and 16.3% 22:4ω6 was found with 20 and 40mg/l, respectively (13.5% in controls). Small but significant increases in 22:5ω3 (2.0% to 2.6%) and 22:6ω3 (2.9% to 3.4%) were also found (1.2% and 1.7% in controls, respectively).

Supplementation with 20, 40 or 60mg/l p-OO resulted in concentration dependent 18:1ω9 incorporation (21.5%, 29.5% and 35.1%, respectively vs 15.0% in controls),

followed by a concommitant decrease in both 16:1ω9 (6.0%, 5.6% and 3.8%, respectively vs 6.1% in controls) and 18:0 (11.5%, 10.7% and 10.3%, respectively vs 19.4% in controls), but little overall change in 16:0 levels (23.4%, 17.8% and 21.6%, respectively vs 20.5% in controls). Neither 18:3ω6, 20:3ω6 nor 20:4ω6 levels were increased, despite 18:2ω6 and 20:2ω6 incorporation, although 18.0% 22:4ω6 and 1.3% 22:5ω6 were detected with 20 and 40mg/l p-OO (13.5% and 0.9% in controls, respectively). Dosed cells also contained slightly elevated proportions of 22:5ω3 and 22:6ω3, especially with 40mg/l (2.4% and 3.4% vs 1.2% and 1.7% in controls, respectively).

p-SSO supplementation had little effect on 18:1ω9, but decreased overall 16:0, 16:1ω9 and 18:0, levels (15.0%, 20.5%, 6.1% and 19.4% in controls, respectively). 2.8%, 19.4%, 21.6% and 19.0% 18:2ω6 were found with dosage of 0, 20, 40 or 60mg/l p-SSO, with small amounts of 20:2ω6 detected over the entire range dosed (0.5% to 1.5% vs 0% in controls). 18:3ω6 was increased only with dosage of 20mg/l p-SSO (1.7% vs 0.8% in controls), and 20:3ω6 and 20:4ω6 were decreased over the entire range dosed. However, 20.4% and 15.0% 22:4ω6 were found with 20 and 40mg/l, respectively, and 1.5% 22:5ω6 with 40mg/l (13.5% and 0.9% in controls, respectively). Increases in 22:5ω3 and 22:6ω3 were also detected, especially with 40mg/l (2.3% and 3.3% vs 1.2% and 1.7% in controls,

respectively).

The results obtained for cells dosed with 20, 40 or 60mg/l p-LO indicated concentration dependent 18:3ω3 incorporation (3.8%, 8.4% and 11.4%, respectively vs 0.2% in controls). 18:4ω3 increased significantly only with 60mg/l (7.3% vs 3.9% in controls), although 22:5ω3 and 22:6ω3 were enhanced slightly with 20mg/l, and approximately two fold with 40mg/l, compared to controls (1.2% and 1.7%, respectively). 16:0 levels dropped from 27.3% to 18.3% and 6.5%, and 18:0 increased from 13.9% to 15.8% and 28.0% with 20, 40 and 60mg/l p-LO (20.5% and 19.4% in controls, respectively). 6.9% 16:1ω9 was found with 20mg/l, but this decreased to 1.0% and 0.7% with 40 and 60mg/l, respectively (6.1% in controls), and 18:1ω9 levels increased significantly only when 60mg/l p-LO was dosed (22.5% vs 15.0% in controls). 18:2ω6 percentages ranged from 10.5% to 15.6% (2.8% in controls), with 1.2% 18:3ω6 detected with 20mg/l (0.8% in controls), and 5.1% 20:4ω6 with 40mg/l (controls 4.1%). There was, however, a concentration dependent decrease in 22:4ω6 from 13.5% in controls to 0.7% with 60mg/l, although 1.4% 22:5ω6 was detected with 40mg/l (0.9% in controls).

16:0 amounts ranged from 16.3% to 27.7% with p-EPO incubation (20.5% in controls), while overall 16:1ω9, 18:0 and 18:1ω9 levels were slightly decreased (6.1%,

19.4% and 15.0% in controls, respectively). 18:2ω6 incorporation was marked (13.4%, 20.1% and 19.4% with 20, 40 and 60mg/l p-EPO, respectively vs 2.8% in controls), with up to 1.0% 20:2ω6 detected (0% in controls). With the exception of 2.7% 22:5ω6 with 40mg/l p-EPO (0.9% in controls), ω6 PDFA levels were parallel to controls, or decreased, although up to 2.2% 22:5ω3 and 2.9% 22:6ω3 were detected (1.2% and 1.7% in controls, respectively).

Incubation with 20mg/l p-FO resulted in significant 16:0 incorporation (29.4%), while 18:0 percentages were slightly decreased over the entire range dosed, in relation to controls (20.5% and 19.4%, respectively). 18:1ω9 levels were parallel to controls (15.0%), with 7.8% to 9.8% 16:1ω9 and 7.6% to 8.2% 18:2ω6 detected (6.1% and 2.8% in controls, respectively). With the exception of 1.6% 22:5ω6 with 40mg/l p-EPO (0.9% in controls), ω6 series PDFA levels were parallel to controls, or decreased. 1.6%, 3.8% and 3.7% 20:5ω3 were detected with 20, 40 and 60mg/l p-FO, respectively (3.3% in controls), although 22:5ω3 levels were significantly, and 22:6ω3 slightly, increased compared to controls (1.2% and 1.7%, respectively).

3.3.4 Effects of pseudo-Oils on Lipid Peroxide Formation.

Table 3.3.4.1. shows the lipoperoxides measured in the cultured cells and their respective growth media. The values are shown as nmoles MDA/10^6 cells, but in the case of the spent incubation media, this reflects the nmoles of MDA in the volume of medium from which $1x10^6$ cells were obtained.

Lipid peroxide formation in dosed cells ranged from 0.7 to 2.7nmoles MDA/10^6 cells (with 20mg/l p-FO and 60mg/l p-LO, respectively) compared to 0.7nmoles MDA/10^6 controls cells. Lipid peroxide formation increased in an overall concentration dependent manner with increased amount of p-oil dosed. Cells incubated with 20mg/l p-oil generally induced <1.0nmole MDA/10^6 cells, while amounts ranged from 1.1 to 2.3 and 1.1 to 2.7nmoles MDA/10^6 cells with supplementation of 40 or 60mg/l p-oil, respectively. No clear relationship between lipid peroxidation and degree of p-oil unsaturation was evident, although cells incubated with 40 or 60mg/l p-LO generated the largest lipoperoxide amounts at these concentrations (2.3 and 2.7nmoles MDA/10^6 cells, respectively). Lipid peroxides were, however, absent from all spent media.

Table 3.3.4.1.

Lipoperoxide formation by rat aortic endothelial cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	0.7			
CO		1.1	1.8	1.7
MO		0.9	1.1	1.4
OO		1.3	1.3	1.9
SSO		0.9	1.3	1.1
LO		0.9	2.3	2.7
EPO		0.9	1.1	1.7
FO		0.7	1.1	1.8
pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	-
MO		-	-	-
OO		-	-	-
SSO		-	-	-
LO		-	-	-
EPO		-	-	-
FO		-	-	-

3.3.5 Discussion.

No reference has been found in the literature in which the effects of FA's on the growth of rat aortic endothelial cells has been found. This study showed that p-oil supplementation affected rat aortic endothelial cell viability (Figs. 3.3.1.2-3.3.1.8.). However, it was unclear whether PUFA-rich p-oils generally exhibited greater growth stimulatory or cytotoxic potential than monoenoic or saturated FA-rich p-oils as the effects induced were not consistent with the degree of p-oil unsaturation. This may relate to differences in p-oil FA composition (Table 2.3.3.2.) and to FA's in the p-oils acting synergistically or antagonistically with each other to modulate the effects induced. Such may explain why p-oils of similar FA composition (p-EPO and p-SSO) induced different effects. The large amount of LA in p-EPO and p-SSO (about 70%) probably accounted for the effects these p-oils induced, although the greater capability for growth limitation and inability to stimulate cell proliferation significantly with p-EPO dosage may have related to the presence of about 9% GLA in p-EPO only. These findings implied LA involvement in the stimulation of cell proliferation and that GLA was a more potent growth inhibitory agent than LA. The large proportion of ALA in p-LO and OA in p-OO (approximately 63% and 71%, respectively) probably accounted largely for the extensive toxicity these p-oils induced with

high concentrations dosed, unlike both p-EPO and p-SSO. These moieties therefore appeared considerably more cytotoxic than LA, and such could reflect differences in the number and position of double bonds in these C18 FA's.

Rat aortic endothelial cells incorporated the exogenous FA's dosed (Table 3.3.3.1.), thus the p-oils may have induced their effects by alterations in membrane fluidity as there is substancial evidence to suggest that dietary supplementation with FA's induces changes both in membrane physical properties and membrane-associated cell functions and (King et al 1971, Ginsberg et al 1981, Simon et al 1982 and Stubbs et al 1984). Incubation with large amounts of p-OO or p-LO may have increased the membrane unsaturation index to such an extent that cell viability could not continue. In contrast, the large complement of saturated FA's (approximately 95%) and absence of PUFA's in p-CO could have inhibited cell viability with high concentrations by shifting membrane fluidity to a less stable state by increasing its rigidification. Such may explain the greater inhibition of cell viability with high concentrations of p-CO than p-MO, p-SSO, p-EPO or p-FO. On the other hand, the finding that p-FO had little overall effect on cell viability may relate to its more even balance of saturated, monoenoic and polyenoic FA's.

The cellular protein concentrations reported with p-oil dosage (Table 3.3.2.1.) generally paralleled the changes observed in cell viability (Figs. 3.3.1.2-3.3.1.8.). The highest protein concentration was for cells dosed with 40mg/l p-SSO, which corresponded to the greatest cell viability increase. This may have represented increased cell number, but with constant or increased protein per cell. The latter possibility seemed more likely as enhanced protein levels were measured even when cell proliferation was parallel to controls, or limited, with p-oil supplementation. The finding that dosed cells contained more protein than controls when cell viability was not significantly impaired indicated that p-oil FA's stimulated protein synthesis, possibly when the requirement for lipid metabolising enzymes increased. On the other hand, decreased protein only with 60mg/l p-CO dosage could have represented decreased cell numbers either with constant or decreased protein per cell.

Endothelial cells play a central role in eicosanoid, and especially prostacyclin, production, and therefore in vascular homeostasis (Moncada et al 1976a). Thus, the ability to desaturate FA's to generate eicosanoid precursors is of prime importance. The data in Table 3.3.3.1. indicated that cultured rat endothelium has the capability to desaturate and elongate PUFA's. Evidence for Δ6D, Δ5D, Δ4D and elongase expression were found with both ω3 and ω6 series PUFA's with p-oil dosage. The

finding that Δ6D and Δ5D product levels were generally lower, and that Δ4D substrates and products were higher, in dosed cells than in controls implied that the initial reaction steps in the desaturase cascade occurred rapidly. Δ4D product formation was more limited with ω6 than ω3 PUFA substrates, and this confirmed the preference for ω3 desaturase substrates (eg. Brenner 1974 and Kanau et al 1977). The formation of 22:5ω3 and 22:6ω3 even with p-oils which did not contain ω3 PUFA's implied that such p-oils enhanced desaturation and elongation of cellular ω3 PUFA's, and such may reflect a mechanism to restore membrane fluidity altered with exogenous FA incorporation.

Hornstra et al (1981) showed the endothelium of the rat to be very specific in the way in which it metabolises long chain polyenoic FA's. These workers found that rat aorta endothelium PGL 20:4ω6 levels were low when fed diets rich in 18:2ω6, implying inability of these cells to express the rate limiting enzyme, Δ6D. Low 20:4ω6 levels were also found with dosage of p-oils rich in 18:2ω6 (p-EPO or p-SSO) in our study (Table 3.3.3.1.). However, increased 22:4ω6 detected with 20mg/l p-SSO, and enhanced 22:5ω6 levels with 40mg/l p-SSO and p-EPO were indeed consistent with at least some expression of desaturase cascade enzymes. Hornstra et al (1981) did not comment on the levels of these moieties, and decreased 20:4ω6 may merely have reflected further

metabolism to 22:4ω6 and/or 22:5ω6. Hornstra et al (1981) also found low 20:4ω6 levels when fish oil was fed, but 20:5ω3 and 22:6ω3 increased up to 4.0% in the vessel walls. Their findings supported the data in Table 3.3.3.1. with p-FO dosage, although the increments we showed in 20:5ω3 and 22:6ω3 were not as great. On the other hand, 22:5ω3 levels increased by up to 6.8% with p-FO dosage, and 22:5ω6 increased almost 2 fold with 40mg/l p-FO. The data from both studies were nonetheless consistent with elongase and Δ4D expression.

Decreased 16:0 and increased 18:0 levels when rat aortic endothelial cells were incubated with 60mg/l p-CO or p-LO suggested that 16:0 was elongated, and the concommitant increase in 18:1ω9 indicated the potential for Δ9D expression (Table 3.3.3.1.). 16:1ω9 elongation could possibly be excluded as contributing to the enhancement of 18:1ω9 since the incorporation of 16:1ω9 with p-FO supplementation did not produce a concommitant increase in 18:1ω9. Both 18:3ω3 and 18:2ω6 competitively inhibit desaturation of 18:1ω9 (Brenner et al 1966, 1969, Brenner 1977, 1982 and Holman 1986b). Relative EFA percentages were enhanced with p-oil incubation (Table 3.3.3.1.), and such probably explains the lack of 20:3ω9 formation despite significant 18:1ω9 incorporation with p-OO supplementation, significant 18:1ω9 formation with 60mg/l p-CO or p-LO dosage, and the capability of these cells to express Δ6D and Δ5D.

Supplementation with 20, 40 or 60mg/l p-oil induced no significant growth limitation or cytotoxicity (Figs. 3.3.1.2-3.3.1.8.), thus it was unlikely that such limited the extent of desaturase cascade enzyme activity reported (Table 3.3.3.1.). It was more likely that the relative amounts of cellular FA's, both of the same and different series, modulated desaturase expression by competitive interactions which have been well documented (eg. Garcia et al 1956, Brenner et al 1966, Brenner 1974, Ullman et al 1971, de Schriver et al 1982 and Nassar 1986). ω3 PUFA's were desaturated and elongated to a greater extent than ω6 PUFA's (Table 3.3.3.1.), thus it was possible that the ω3 PDFA's formed suppressed, or inhibited, ω6 PUFA metabolism, depending on the overall ω3/ω6 PUFA balance in the cell. On the other hand, incubation of cells with saturated FA-rich p-oils (p-CO or p-MO) could have induced a relative EFA/PDFA deficiency, and such may have enhanced desaturase cascade enzyme expression in an attempt to restore membrane PUFA levels.

The results presented in Table 3.3.4.1. indicated that rat aortic endothelial cells exhibit intact enzymic and/or non-enzymic mechanisms for the production of lipoperoxides. Control cell lipid peroxides reflected oxidation of endogenous unsaturated FA's, whereas increased lipid peroxide production in dosed cells reflected oxidation of incorporated exogenous (p-oil)

unsaturated FA's. The finding that lipid peroxide generation increased overall as greater amounts of p-oil were supplemented was consistent with saturation of the protective mechanisms against free radical production, eg. glutathione peroxidase, and therefore increased FA availability for oxidation. However, the similar lipid peroxide amounts formed with incubation of p-CO or p-MO compared to p-OO, p-SSO, p-EPO or p-FO, despite differences in p-oil FA composition and unsaturation (Table 2.3.3.2.), suggested that the substrates for lipoperoxidation in the latter group were not so readily accessible to oxidation, possibly because they were present as components of complex membrane lipids. The absence of lipoperoxides in the spent medium of all dosed cultures indeed supported p-oil FA incorporation. It was also possible that incubation with p-CO or p-MO increased, or that p-OO, p-SSO, p-EPO or p-FO decreased, the rate of PGL turnover and therefore the size of the FFA pool. The above mechanisms could also explain the greatest cellular lipoperoxide amounts measured with 40 or 60mg/l p-LO supplementation, which probably related to the large amount of ALA in p-LO (about 63%) and its susceptibility to oxidation.

Measurement of relatively low lipoperoxide levels in dosed cells and their absence in all spent media (Table 3.3.4.1.) correlated with the lack of significant cytotoxicity induced with dosage of up to 60mg/l p-oil

(Figs. 3.3.1.2-3.3.1.8.). This suggested lipoperoxide involvement in the modulation of cell proliferation, although no correlation with cytotoxicity could be established. Lipoperoxide amounts greater than those reported with dosage of 60mg/l p-oil should, however, not be overlooked as a possible mechanism involved in the modulation of cytotoxicity. Overall, it seems likely that the modulation of rat aortic endothelial cell viability relates both to lipoperoxides and alterations in membrane fluidity.

3.4 THE EFFECTS OF PSEUDO-OILS ON CELLS DERIVED FROM RAT SKELETAL MUSCLE.

3.4.1 Effects of pseudo-Oils on Cell Viability.

Cells were examined microscopically before, during and after each experiment, and at no time were changes seen in the morphology of control or p-oil dosed cells, except for cytoplasmic droplets observed with p-oil incubation at high concentrations in some instances.

Final medium albumin concentrations ranging from 0 to 250mg/l induced no significant effect on cell viability (Fig. 3.4.1.1.). The effects induced with the p-oils dosed were therefore a result of the exogenous FA's and not the albumin used as FA carrier.

The number of control cells, seeded at $10x10^4$/ml, at the end of the 24 hour post-trypsinisation recovery period was $10.6x10^4$/ml. This equated to about 76% of the final control cell number at the end of the 48 hour incubation period, and represented the cytostatic number. Only p-oil concentrations limiting cell viability to significantly below 76% were considered cytotoxic.

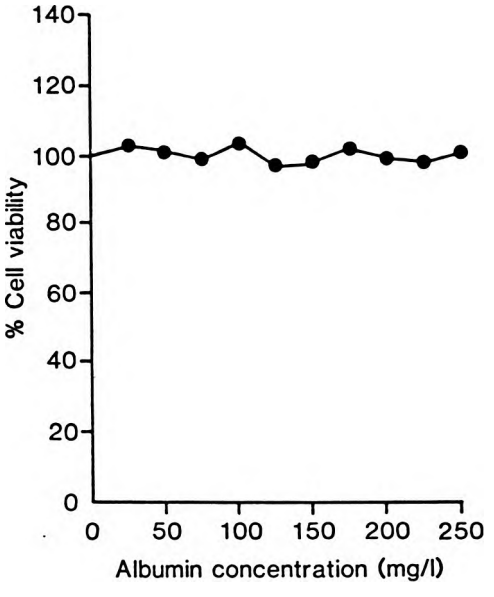
The results of the incubation of cells with p-oils are depicted in Figs. 3.4.1.2-3.4.1.8. Cell proliferation was enhanced only with certain concentrations of p-SSO, p-EPO or p-LO, all p-oils exhibited growth limiting

Legend to Figs. 3.4.1.1-3.4.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.4.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.4.1.2-3.4.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.4.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.4.1.1.

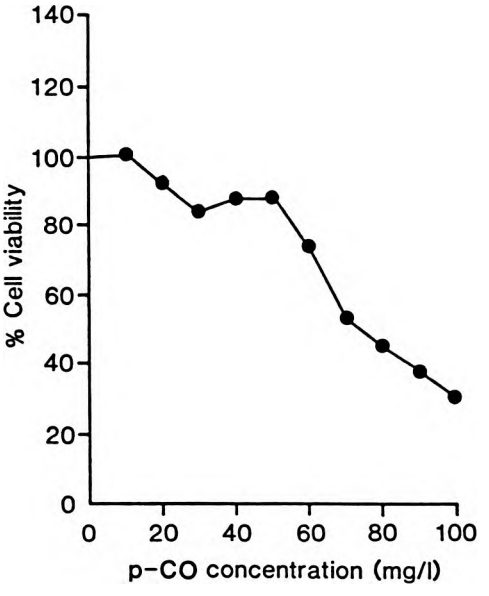
The percentage viability of cells derived from rat skeletal muscle incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.4	12
25	102.3	4.2	12
50	100.7	4.2	12
75	98.9	4.2	12
100	103.6	4.6	12
125	96.7	5.1	12
150	97.9	6.2	12
175	101.4	4.4	12
200	99.1	2.3	12
225	98.1	4.5	12
250	100.8	3.7	12

Fig. 3.4.1.2.

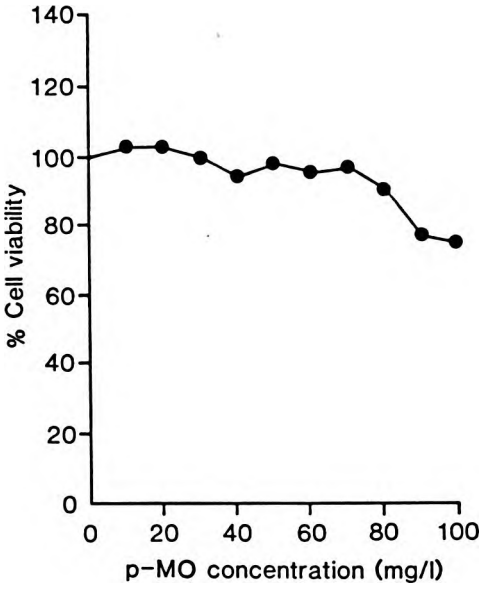
The percentage viability of cells derived from rat skeletal muscle incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.8	6
10	100.2	5.7	6
20	92.2	2.4	6
30	83.6	3.0	6
40	87.6	4.7	6
50	88.4	4.4	6
60	73.7	4.0	6
70	53.5	3.3	6
80	45.4	2.3	6
90	38.0	3.2	6
100	30.4	1.8	6

Fig. 3.4.1.3.

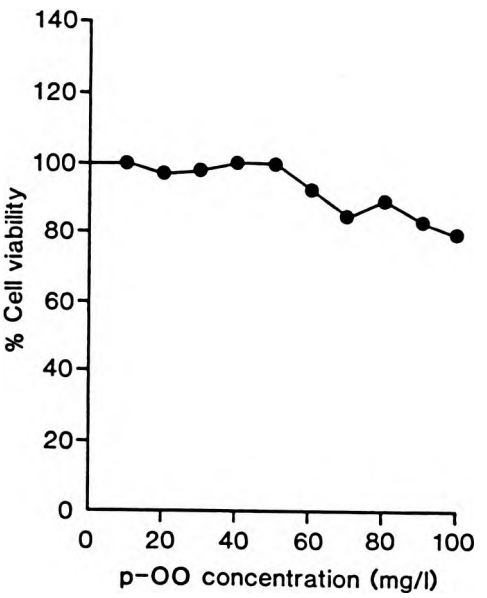
The percentage viability of cells derived from rat skeletal muscle incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.9	6
10	102.7	2.2	6
20	102.1	2.1	6
30	100.0	2.8	6
40	94.2	2.5	6
50	97.6	2.4	6
60	95.4	2.7	6
70	97.3	2.9	6
80	90.6	2.7	6
90	77.2	1.5	6
100	74.8	2.7	6

Fig. 3.4.1.4.

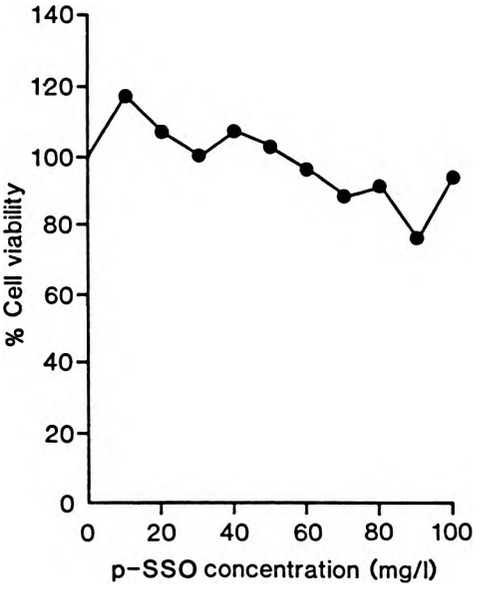
The percentage viability of cells derived from rat skeletal muscle incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.1	6
10	99.8	2.7	6
20	96.8	1.4	6
30	98.0	1.8	6
40	99.8	1.7	6
50	99.8	1.2	6
60	92.6	1.5	6
70	84.7	2.0	6
80	89.6	3.1	6
90	83.5	2.3	6
100	79.0	3.0	6

Fig. 3.4.1.5.

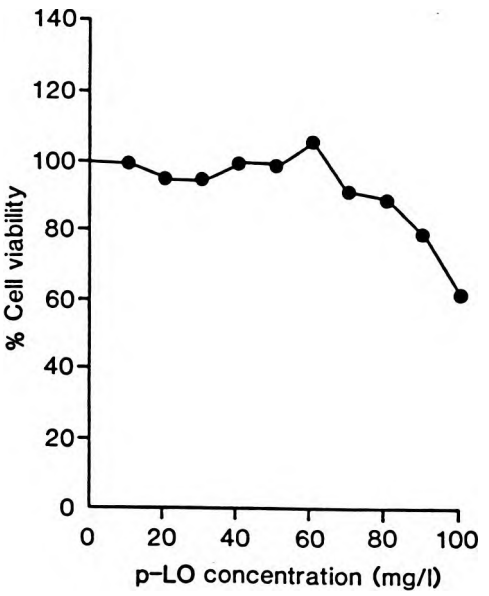
The percentage viability of cells derived from rat skeletal muscle incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.1	6
10	116.9	3.9	6
20	106.7	3.7	6
30	99.8	2.6	6
40	107.2	4.3	6
50	102.8	5.3	6
60	96.4	5.2	6
70	88.3	4.2	6
80	91.0	6.2	6
90	75.1	2.8	6
100	94.3	4.8	6

Fig. 3.4.1.6.

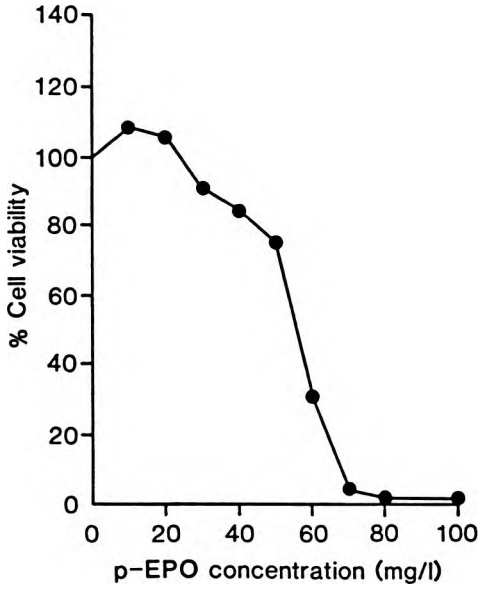
The percentage viability of cells derived from rat skeletal muscle incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.8	6
10	99.6	2.7	6
20	95.0	3.4	6
30	94.6	5.2	6
40	99.3	4.2	6
50	98.4	4.0	6
60	106.1	1.6	6
70	90.9	1.2	6
80	89.6	1.7	6
90	79.7	2.6	6
100	61.4	3.0	6

Fig. 3.4.1.7.

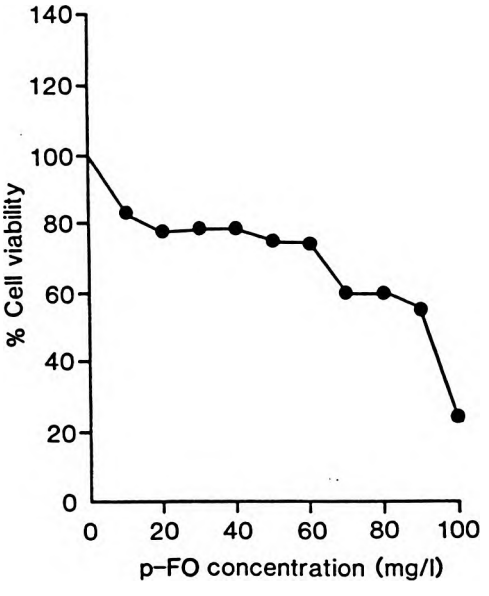
The percentage viability of cells derived from rat skeletal muscle incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.7	6
10	107.9	2.3	6
20	105.1	3.8	6
30	90.7	3.2	6
40	83.6	3.3	6
50	74.5	3.4	6
60	29.9	2.5	6
70	3.0	1.1	6
80	0.2	0.2	6
90	0.0	0.0	6
100	0.0	0.0	6

Fig. 3.4.1.8.

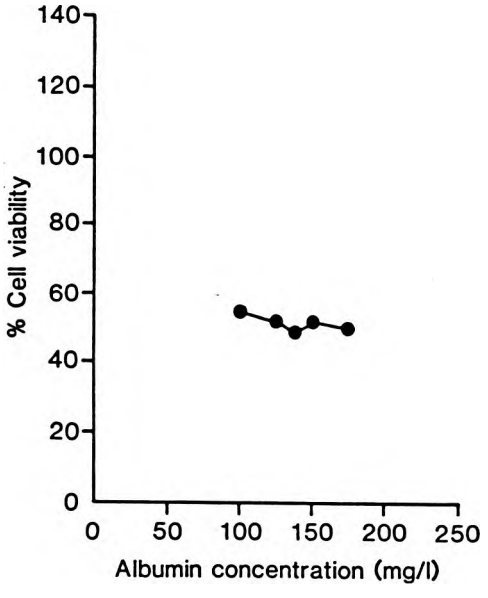
The percentage viability of cells derived from rat skeletal muscle incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.2	6
10	84.0	3.1	6
20	77.9	3.3	6
30	79.3	4.3	6
40	79.5	3.5	6
50	75.9	2.6	6
60	74.2	3.2	6
70	60.8	3.1	6
80	60.8	3.4	6
90	56.2	2.5	6
100	25.0	2.3	6

Fig. 3.4.1.9.

The percentage viability of cells derived from rat skeletal muscle incubated with albumin at the ID_{50} of p-EPO.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.9	6
100	55.5	5.6	6
125	52.1	4.4	6
137.5	50.0	3.7	6
150	52.3	3.6	6
175	50.5	4.4	6

potential dependent on the concentration dosed, while significant cytotoxicity was induced only with p-CO, p-FO or p-EPO incubation.

10mg/l p-CO had little effect on cell proliferation, but greater amounts dosed induced an overall concentration dependent reduction in cell viability (Fig. 3.4.1.2.). Cytostasis was induced with 60mg/l p-CO, while 50% of cells remained viable with about 75mg/l p-CO (ID_{50}), and 30.4% with 100mg/l p-CO.

Cell proliferation approximated to controls with dosage of up to 80mg/l p-MO or 60mg/l p-OO (Figs. 3.4.1.3. and 3.4.1.4., respectively). Greater amounts incubated were growth limiting, and 74.8% and 79.0% of cells remained viable with 100mg/l p-MO or p-OO, respectively, which approximated to the cytostatic number (about 76%). Cell viability was stimulated to 116.9% with 10mg/l p-SSO, ranged from 99.8% to 106.7% with concentrations between 10 and 60mg/l p-SSO, and was limited with greater amounts dosed to a minimum of 75.1% with 90mg/l p-SSO (Fig. 3.4.1.5.). No cytotoxicity was induced with p-MO, p-OO or p-SSO dosage, thus ID_{50} values could not be calculated.

Cell viability ranged from 94.6% to 106.1% with dosage of up to 60mg/l p-LO, but was reduced in a concentration dependent manner to 79.7% with 90mg/l p-LO (Fig. 3.4.1.6.). Despite cytotoxicity with 100mg/l p-LO, no

ID_{50} value could be calculated as cell viability was 61.4%.

The cell viability changes induced with p-EPO incubation followed a sigmoidal relationship (Fig. 3.4.1.7.). Cell proliferation increased to 107.9% and 105.1% with 10 and 20mg/l p-EPO, respectively, was progressively reduced to equate the cytostatic number (about 76%) with 50mg/l p-EPO, and was markedly inhibited with greater amounts dosed. p-EPO was the most cytotoxic p-oil dosed since a concentration of about 55mg/l reduced cell viability to 50% of controls (ID_{50}), and practically all cells took up Trypan blue with concentrations of 70mg/l or greater.

Cell viability was effectively limited to 84.0% with 10mg/l p-FO (Fig. 3.4.1.8.), and ranged from 74.2% to 79.5% when amounts of 20 to 60mg/l p-FO were incubated (cytostasis). Supplementation with higher concentrations induced cytotoxicity. Cell viability was reduced by half with about 92mg/l p-FO (ID_{50}), and by three quarters with 100mg/l p-FO.

To exclude the possibility that any of the effects observed with p-oil dosage were influenced by the amount of albumin bound to the FA's, cells were incubated with a fixed amount of p-oil (55mg/l p-EPO, ID_{50}) and dosed with varying amounts of albumin. Five points were chosen around which to vary the albumin concentration, keeping the p-oil concentration fixed, viz. two points above,

two points below, and the ID_{50} of p-EPO. The appropriate amount of albumin was added to these cultures to give the desired concentrations. The results shown in Fig. 3.4.1.9. indicated that the concentration of p-EPO dosed reduced cell viability to approximately 50% of controls, and that such was not influenced by different albumin concentrations. Exclusion of the synergistic involvement of albumin indicated that the effects induced with the p-oils could be attributed solely to the exogenous FA's.

Rat skeletal muscle cells were subsequently plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient quantities for all the quantitative and qualitative analyses to be carried out. Upon harvesting, cell viabilities relative to controls were compared and found not to be statistically different from those obtained in Figs. 3.4.1.1-3.4.1.8., thus all further biochemical assays were performed on these samples.

3.4.2 Effects of pseudo-Oils on Total Protein.

The protein concentrations for cells supplemented with 0, 20, 40 or 60mg/l p-oil are shown in Table 3.4.2.1. as µg total protein/10^6 cells seeded.

Cells dosed with 60mg/l p-EPO contained only 362.4µg protein/10^6 cells seeded compared to 401.7µg protein/10^6 control cells seeded. All other dosed cells, however, contained significantly more protein than controls, with amounts ranging from 451.4 to a maximum of 607.4µg protein/10^6 cells seeded (with 60mg/l p-FO and 20mg/l p-EPO, respectively). The changes reported in cellular protein were p-oil concentration dependent. Total protein levels increased in direct correlation with the amount of p-LO incubated, but were generally inversely related to the p-MO, p-SSO, p-EPO or p-FO concentration supplemented. With p-CO or p-OO dosage, however, the highest protein amounts measured were with a concentration of 40mg/l.

Table 3.4.2.1.

The protein content of cells derived from rat skeletal muscle, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	401.7			
CO		524.2	537.7	498.0
MO		551.1	537.9	537.9
OO		499.7	597.7	504.4
SSO		602.5	569.8	481.4
LO		516.8	548.7	600.1
EPO		607.4	501.5	362.4
FO		563.5	499.7	451.4

3.4.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Cells Derived From Rat Skeletal Muscle.

Table 3.4.3.1. shows the FA spectra of rat skeletal muscle cells incubated with 0, 20, 40 or 60mg/l of each of the p-oils. The greatest amount of any single FA in control cells was 22.7% 22:4ω6, but significant levels of 16:0 (17.8%), 18:0 (13.7%), 18:1ω9 (13.7%) and 20:4ω6 (9.5%) were also found. Varying amounts of these FA's were found in the dosed cells.

16:0 and 16:1ω9 percentages in dosed cells generally approximated to controls, or were decreased, while the converse was true for 18:0, 18:1ω9 and 18:2ω6. ω6 PDFA levels were raised only with certain concentrations of some p-oils, although no increments in 22:5ω6 were found. Dosed cells generally contained 18:4ω3 amounts parallel to, or greater than, controls, but 20:4ω3, 20:5ω3, 22:5ω3 and 22:6ω3 were again increased only with certain concentrations of some p-oils.

15.1%, 16.8% and 24.0% 16:0 was found when cells were dosed with 20, 40 or 60mg/l p-CO, respectively (17.8% in controls), although 16:1ω9 was decreased (0.6% to 2.3% vs 3.9% in controls). 18:0 and 18:1ω9 levels, in contrast, were significantly increased with 20mg/l p-CO (32.4% and 22.4%, respectively vs 13.7% in each control), but approximated to controls with 40 or

Legend to Table 3.4.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.4.3.1.

The fatty acid spectrum of cells derived from rat skeletal muscle.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	17.8±0.05	15.1	16.8	24.0	13.3	15.6	25.4	16.0	19.4	12.3	10.4	18.7	7.9	16.6	20.5	22.4	3.6	18.6	4.1	10.6	17.7	4.8
	18:0	13.7±0.10	32.4	13.9	16.8	12.0	16.1	16.2	12.8	11.9	24.1	13.6	12.2	25.8	20.7	13.6	10.6	13.3	13.1	12.8	13.0	13.8	29.9
	20:0	0.3±0.00	-	0.3	-	-	0.4	0.3	-	0.4	-	-	0.1	-	-	0.2	-	-	0.2	-	-	0.1	-
	22:0	0.2±0.05	-	0.2	0.1	0.1	0.1	0.1	-	0.1	-	-	0.1	-	-	0.1	-	-	-	-	-	0.2	-
	24:0	0.3±0.00	-	0.6	-	-	0.2	-	-	0.1	-	-	-	-	-	-	-	-	0.1	-	-	0.1	-
ω9 MONOS.	16:1	3.9±0.25	0.6	1.6	2.3	1.2	0.8	5.9	1.0	1.4	1.1	0.4	1.1	0.8	0.7	1.9	2.8	0.3	2.4	0.2	2.1	4.1	0.9
	18:1	13.7±0.20	22.4	12.3	14.8	13.0	13.3	15.5	23.1	27.5	28.3	30.4	15.5	24.7	18.3	15.4	18.5	31.7	14.1	30.0	26.1	12.6	23.8
	24:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	3.8±0.10	3.6	3.2	3.6	5.3	4.9	5.1	5.5	6.0	18.2	24.7	9.0	26.8	5.4	4.0	7.2	32.1	6.9	32.7	17.9	3.6	24.2
	18:3	-	-	-	-	-	-	-	0.2	-	-	-	-	-	0.1	-	-	-	-	-	-	0.1	-
	20:2	-	-	-	-	-	-	-	0.4	1.0	2.0	-	0.2	-	-	-	-	-	0.1	-	0.2	0.1	-
	20:3	1.6±0.10	0.9	1.7	2.1	1.2	1.7	1.4	1.4	1.4	1.9	2.5	1.7	0.2	1.5	1.4	-	2.2	1.5	0.2	3.3	2.4	1.9
	20:4	9.5±1.00	7.1	11.7	12.2	10.4	11.7	9.5	9.3	8.9	2.8	3.2	7.6	1.7	8.2	7.1	7.8	2.9	5.6	2.3	4.3	10.7	2.4
	22:4	22.7±1.40	10.9	26.4	10.3	32.7	21.9	13.8	18.8	9.6	2.3	4.9	22.0	3.1	16.1	24.5	17.2	3.5	26.0	2.2	3.2	15.9	1.9
	22:5	2.8±0.00	0.4	1.8	0.3	0.5	2.5	-	0.8	2.5	0.4	0.4	2.7	0.2	1.9	1.4	1.4	0.3	2.2	0.3	0.4	2.3	0.1
ω3 POLYS.	18:3	0.5±0.05	-	0.4	0.5	0.4	0.4	0.4	0.6	0.8	3.0	-	0.2	-	1.4	1.5	6.0	-	0.5	-	0.7	0.4	-
	18:4	1.1±0.30	1.1	1.9	1.0	0.7	3.2	0.5	0.7	1.6	2.2	6.3	1.6	7.3	0.8	2.4	2.5	7.7	2.3	12.7	12.5	2.3	7.5
	20:4	3.3±0.00	2.1	1.5	2.5	4.9	3.2	1.8	3.3	2.3	0.2	1.9	3.5	0.8	3.0	3.0	1.6	1.8	3.6	2.3	1.4	2.3	0.6
	20:5	0.4±0.10	0.2	1.7	3.0	0.2	0.2	0.3	0.2	0.2	0.1	-	0.2	-	0.2	0.1	-	-	0.4	-	1.0	2.3	0.7
	22:5	2.1±0.10	1.7	3.2	3.2	2.2	2.8	2.3	3.2	2.6	0.5	0.7	2.0	0.3	3.3	1.6	1.2	0.4	1.4	0.1	2.8	6.9	1.1
	22:6	2.1±0.10	1.6	0.9	3.3	1.9	0.9	1.9	2.7	2.3	0.6	0.6	1.7	0.2	1.9	1.2	0.8	0.3	1.0	0.1	0.6	2.1	0.3

60mg/l p-CO. No significant changes in 18:2ω6 or 18:3ω3 percentages were observed, but small increases in some PDFA's were found with incubation of 40 or 60mg/l p-CO. The most significant increases were for 22:4ω6 (26.4% with 40mg/l vs 22.7% in controls) and 20:5ω3 (1.7% and 3.0% with 40 and 60mg/l, respectively vs 0.4% in controls), with smaller increments shown for 20:3ω6, 20:4ω6, 18:4ω3, 22:5ω3 and 22:6ω3.

Both 16:0 and 16:1ω9 percentages were decreased with dosage of 20 or 40mg/l p-MO, but increased with 60mg/l p-MO (13.3%, 15.6% and 25.4%, and 1.2%, 0.8% and 5.9% vs 17.8% and 3.9% in controls, respectively). 18:0 and 18:1ω9 levels ranged from lows of 12.0% and 13.0% with 20mg/l p-MO, to highs of 16.2% and 15.5% with 60mg/l p-MO, respectively (13.7% in each control), while 4.9% to 5.3% 18:2ω6 was found (3.8% in controls). Only 10.4%, 11.7% and 9.5% 20:4ω6 were detected with 20, 40 and 60mg/l p-MO, respectively (9.5% in controls), although the elongation product, 22:4ω6, was significantly raised with 20mg/l (32.7% vs 22.7% in controls). However, no increase in 22:5ω6 was found in dosed cells (2.8% in controls). ω3 PUFA's were absent in p-MO, yet 18:4ω3 and 22:5ω3 were increased in cells dosed with 40mg/l p-MO (3.2% and 2.8% vs 1.1% and 2.1% in controls, respectively), while 20:4ω3 was increased with 20mg/l p-MO (4.9% vs 3.3% in controls).

Concentration dependent 18:1ω9 incorporation occurred with 20, 40 or 60mg/l p-OO incubation (23.1%, 27.5% and 28.3%, respectively vs 13.7% in controls). 16:0, 16:1ω9 and 18:0 levels were generally lower than controls (17.8%, 3.9% and 13.7%, respectively), although 18:0 was significantly increased with 60mg/l p-OO (24.1%). Concentration dependent increases in 18:2ω6 and 20:2ω6 were observed (5.5% to 18.2% and 0.4% to 2.0% vs 3.8% and 0% in controls, respectively), but ω6 PDFA levels were parallel to controls, or significantly decreased. On the other hand, small increases in 18:3ω3, 18:4ω3, 22:5ω3 and 22:6ω3 were found with p-OO incubation.

p-SSO dosage decreased overall 16:0 and 16:1ω9 levels, significantly increased 18:0 only with 60mg/l (25.8% vs 13.7% in controls), and raised 18:1ω9 levels with all concentrations dosed (15.5% to 30.4% vs 13.7% controls). 24.7% and 26.8% 18:2ω6 were detected with 20 and 60mg/l p-SSO, respectively, but only 9.0% with 40mg/l (3.8% in controls). 18:3ω6 was absent both in control and p-SSO dosed cells, 2.5% 20:3ω6 was found with 20mg/l p-SSO (1.6% in controls), but no increases in 20:4ω6, 22:4ω6 or 22:5ω6 were detected. Surprisingly, 18:4ω3 amounts were increased with p-SSO incubation (1.6% to 7.3% vs 1.1% in controls), but this did not induce significant increments in 20:4ω3, 22:5ω3 or 22:6ω6.

16:0 levels increased to 16.6%, 20.5% and 22.4% with

20, 40 or 60mg/l p-LO incubation, respectively (17.8% in controls), while 18:0 decreased from 20.7% to 13.6% and 10.6%, respectively (13.7% in controls). 16:1ω9 amounts were decreased (0.7% to 2.8% vs 3.9% in controls), while 18:1ω9 percentages increased slightly (15.4% to 18.5% vs 13.7% in controls). 4.0% to 7.2% 18:2ω6 was found (3.8% in controls), but ω6 PDFA levels were not significantly increased. Cellular 18:3ω3 amounts increased to 1.4%, 1.5% and 6.0% with addition of 20, 40 or 60mg/l p-LO, respectively (0.5% in controls). Furthermore, increased levels of 18:4ω3 were detected with 40 and 60mg/l p-LO (2.4% and 2.5%, respectively vs 1.1% in controls), and 22:5ω3 with 20mg/l p-LO (3.3% vs 2.1% in controls), but no increment in 22:6ω3 was found.

Supplementation with 20 or 60mg/l p-EPO caused a marked decrease in 16:0 (3.6% and 4.1%, respectively vs 17.8% in controls), but a significant increase in 18:1ω9 (31.7% and 30.0%, respectively vs 13.7% in controls). p-EPO had little effect on 18:0 levels, induced a reduction in 16:1ω9, and increased 18:2ω6 levels nearly 2 fold with 40mg/l and 10 fold with 20 and 60mg/l (6.9%, 32.1% and 32.7%, respectively vs 3.8% in controls). 18:3ω6 was absent in control and dosed cells, but 2.2% 20:3ω6 was found with 20mg/l p-EPO (1.6% in controls) and 26.0% 22:4ω6 with 40mg/l p-EPO (22.7% in controls). 18:4ω3 amounts were significantly increased over the range dosed (2.3% to 12.7% vs 1.1% in controls), yet the

levels of its products were not significantly raised.

16:0 and 16:1ω9 levels were parallel to controls (17.8% and 3.9%, respectively), or significantly decreased, with p-FO incubation, whereas marked increases in 18:0 were found with 60mg/l p-FO (29.9% vs 13.7% in controls) and 18:1ω9 with 20 and 60mg/l p-FO (26.1% and 23.8% vs 13.7% in controls). Significant increments in 18:2ω6 and 20:3ω6 were detected with p-FO dosage, yet overall 20:4ω6, 22:4ω6 and 22:5ω6 percentages were decreased. 22:6ω3 levels were not increased, despite raised amounts of 22:5ω3 found with 20 and 40mg/l p-FO (2.8% and 6.9%, respectively vs 2.1% in controls), and 18:4ω3 and 20:5ω3 detected with all p-FO concentrations supplemented (2.3% to 12.5% and 0.7% to 2.3% vs 1.1% and 0.4% in controls, respectively).

3.4.4 Effects of pseudo-Oils on Lipid Peroxide Formation.

The results obtained from the quantitation of lipid peroxides in rat skeletal muscle cultures are shown in Table 3.4.4.1., expressed as nmoles MDA/10^6 cells. In the case of spent incubation media, however, this represents the nmoles of MDA in the volume of medium from which $1x10^6$ cells were derived.

Lipid peroxide concentrations in dosed cells ranged from 0.1 to 8.0nmoles MDA/10^6 cells (with 20mg/l p-CO and 60mg/l p-EPO, respectively), compared to 0.1nmoles MDA/10^6 control cells. The lipoperoxide amounts measured both in the cells and spent medium varied with the p-oil supplemented, and generally increased in a concentration dependent manner. Cultures dosed with p-CO, p-MO or p-OO induced 0.1 to 0.8nmoles MDA/10^6 cells, and a maximum of 0.6nmoles MDA/10^6 cells was measured in the spent medium with p-MO (none in control medium). Surprisingly, cells supplemented with 20, 40 or 60mg/l p-LO contained only 0.2nmoles MDA/10^6 cells, although corresponding spent media lipid peroxides were the largest amounts measured (0.7, 2.1 and 3.9nmoles MDA/10^6 cells, respectively). Similarly, p-FO induced only 0.2 to 0.6nmoles MDA/10^6 cells, but 0.1, 0.5 and 1.4nmoles MDA/10^6 cells in the corresponding spent media, respectively. Only 0.3 and 0.4nmoles MDA/10^6 cells were measured with incubation of

Table 3.4.4.1.

Lipoperoxide formation by rat skeletal muscle cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	0.1			
CO		0.1	0.2	0.8
MO		0.2	0.3	0.4
OO		0.5	0.7	0.6
SSO		0.3	0.4	7.7
LO		0.2	0.2	0.2
EPO		1.8	1.9	8.0
FO		0.2	0.2	0.6
pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	-
MO		0.6	0.4	-
OO		-	-	-
SSO		-	-	-
LO		0.7	2.1	3.9
EPO		-	-	-
FO		0.1	0.5	1.4

20 or 40mg/l p-SSO, respectively, compared to 7.7nmoles MDA/10^6 cells with 60mg/l p-SSO, whereas p-EPO induced the largest cellular lipid peroxide amounts measured at any concentration (1.8, 1.9 and 8.0nmoles MDA/10^6 cells with 20, 40 or 60mg/l, respectively). However, neither p-SSO nor p-EPO induced any lipid peroxides in the corresponding spent media.

3.4.5 Discussion.

The effects of FA's on the growth of rat skeletal muscle have not been found in the literature, and this is the first report describing the modulation of such cell growth in culture with supplementation of exogenous FA mixtures mimicking the composition of dietary oils. The data reported in Figs. 3.4.1.2-3.4.1.8. showed that rat skeletal muscle cell viability was affected with p-oil supplementation. The effects induced, however, were not consistent with the degree of p-oil unsaturation, and it was unclear whether the potential to modulate cell proliferation was greater with p-oils rich in polyunsaturated, monounsaturated or saturated FA's. The p-oil-specific effects induced rather appeared to relate to the unique FA composition of each p-oil (Table 2.3.3.2.), although the possibility also exists that certain effects could have been enhanced or deminished by the mediation of synergistic or antagonistic effects between FA's within any p-oil. The FA spectra of p-OO, p-SSO and p-LO suggested that the effects induced with dosage of each p-oil related to the large amount of OA, LA or ALA present, respectively, and that these C18 FA's exhibited little, or no, growth inhibitory capability at the concentrations dosed. The FA composition of p-EPO was similar to that of p-SSO, although the mediation of significantly more growth limitation and cytotoxicity

with p-EPO dosage was consistent with the presence of approximately 9% GLA in p-EPO only. It was apparent from the above, therefore, that GLA exhibited greater growth limiting and cytotoxic ability than LA, ALA and OA. This may relate to the presence of a double bond at the ω12-position in GLA, and the fact that this moiety is a PDFA. Such may also explain the marked cytotoxicity induced with high concentrations of p-FO, which contained 18% EPA. Indeed, the finding that pronounced growth inhibition was induced with supplementation of p-EPO or p-FO, but not with p-MO, p-OO, p-SSO or p-LO which contained insignificant PDFA amounts, implicated PDFA involvement in the modulation of cytotoxicity. On the other hand, the finding that cell viability was stimulated with certain concentrations of p-SSO, p-EPO or p-LO only, rich either in LA or ALA, implied EFA involvement in the enhancement of cell proliferation.

Evidence to support exogenous FA incorporation was found with p-oil supplementation (Table 3.4.3.1.), hence the changes reported in cell viability could relate to differential uptake of p-oil FA's, alterations in membrane unsaturation and fluidity. It was possible that the abundance of saturated FA's (about 95%) in p-CO inhibited cell viability at high concentrations by increasing membrane rigidification, whereas addition of large amounts of p-EPO or p-FO elevated the unsaturation index of the membrane sufficiently to induce cell death.

Modifications of the membrane PGL FA composition may desatabilise the membrane and induce changes in the activity of membrane-bound enzymes (Stubbs et al 1984 and Spector et al 1985). A possible mechanism whereby these p-oils induced cell death may therefore relate to uncoupling of calcium ATPase activity and altered calcium transport. At this stage, however, the effects of FA's on sarcoplasmic reticulum calcium ATPase are unclear (Hidalgo et al 1982, East et al 1984, Almeida et al 1986, Hidalgo 1987, Infante 1987 and Stubbs et al 1990). On the other hand, maintenance of basal membrane fluidity may explain why p-MO, p-OO, p-SSO and p-LO were not significantly cytotoxic. During periods demanding extra energy, skeletal muscle TAG stores are mobilised, releasing FFA's which this tissue can utilise as an energy source (Lehninger 1982). Skeletal muscle may therefore be well equipped to tolerate high FFA concentrations, and this could also partly explain the findings that many p-oils had little effect on cell viability, stimulated cell proliferation, or were merely growth limiting.

The changes in cellular protein concentrations reported with p-oil supplementation (Table 3.4.2.1.) reflected the changes induced in cell viability (Figs. 3.4.1.2-3.4.1.8.). Enhanced cellular protein amounts with 20 and 40mg/l p-SSO, 20mg/l p-EPO and 60mg/l p-LO correlated with increased cell viabilities at these concentrations,

but it was apparent that increments in cell number alone could not justify the marked protein increases. This implied FA involvement in the stimulation of rat skeletal muscle protein synthesis, particularly since protein concentrations were significantly increased even when p-oils induced growth limitation and cytostasis. The extent to which protein synthesis was stimulated varied with dosage of identical amounts of different p-oils, even when such yielded similar cell numbers. This suggested that the capability to enhance protein synthesis varied with FA, and was probably relatable to FA structure and concentration in any p-oil. The enhancement of protein synthesis with p-oil dosage may, nevertheless, reflect the extremely high metabolic activity of skeletal muscle in vivo (Lehninger 1982) and a mechanism whereby the cells enhanced the production of lipid metabolising enzymes in an attempt to metabolise the exogenous FA's incorporated with p-oil incubation. On the other hand, the reduction of cellular protein concentations only with 60mg/l p-EPO correlated with the marked cytotoxicity this p-oil induced, and reflected decreased cell numbers either with constant or decreased protein per cell.

Comparison of control cell FA spectra with those derived from p-oil-dosed cells indicated the potential of cultured cells derived from rat skeletal muscle to express desaturase and elongase enzymes (Table 3.4.3.1.). The

capability for 16:0 desaturation and elongation, and 18:0 desaturation to 18:1ω9 via Δ9D, were suggested. However, it was apparent that delta-6-desaturation of 18:1ω9 was competitively inhibited by 18:2ω6 and/or 18:3ω3 since evidence for Δ6D and subsequent elongase activities were found with both ω3 and ω6 series PUFA's as substrates. PDFA product formation was clearly more limited with ω9 than ω6, and ω6 than ω3, series FA's, and this confirmed the established substrate preference (Brenner 1974, Mead et al 1976 and Kanau et al 1977). Increased 18:4ω3 levels in cells supplemented with p-oils deficient in 18:3ω3 was consistent with delta-6-desaturation of endogenous 18:3ω3 and/or extramicrosomal retroconversion of 20:4ω3. The significant increase in 20:5ω3 amounts with p-CO dosage implied Δ5D capability, and the finding that Δ5D expression was more restricted with supplementation of PUFA-rich p-oils supported the formation of 18:4ω3 by retroconversion of 20:4ω3. Gavino et al (1981b) similarly showed limited conversion of 20:3ω6 to 20:4ω6 via Δ5D in rodent smooth muscle cell cultures, which elongated 20:4ω6, but did not desaturate 22:4ω6. The present study demonstrated elongation both of 20:4ω6 and 20:5ω3, but the formation of small amounts of 22:6ω3 only with p-CO or p-OO incubation reflected limited/suppressed Δ4D activity. This explained the accumulation of the Δ4D substrates, 22:4ω6 and 22:5ω3, particularly with dosage of p-MO or p-FO, respectively.

Desaturation is regulated by competitive interactions between FA's of the same and different families (eg. Garcia et al 1965, Brenner et al 1966, Ullman et al 1971, de Schriver et al 1982 and Nassar et al 1986), and this partly determined the pattern of desaturation and elongation reported in Table 3.4.3.1. with p-oil dosage. Studies have also shown that dietary FA's alter the lipid composition of microsomal membranes and that such is involved in the modulation of desaturase enzyme activity (Nervi et al 1968, Kurata et al 1980, Garda et al 1984, 1985 and Garg et al 1988a, 1988b, 1988c). Detection of increased PDFA levels with p-CO incubation, which contained about 95% saturated FA's and no PUFA's, implied enhanced desaturase cascade enzyme expression and subsequent desaturation and elongation of endogenous PUFA's. This reflected an attempt to re-establish membrane fluidity altered with uptake of exogenous saturated FA's. On the other hand, PDFA formation with dosage of PUFA-rich p-oils was consistent with the availability of desaturase cascade enzyme substrates. However, once optimal PUFA levels were established to maintain normal membrane fluidity and cellular functions, it was likely that desaturase cascade enzyme expression was suppressed by negative feedback reactions which have been well documented (Garcia et al 1965, Brenner et al 1966, Brenner 1974, Ullman et al 1971, de Schriver et al 1982 and Nassar 1986). Such probably

explains why long chain polyenoic FA levels were not markedly raised with dosage of PUFA-rich p-oils, despite incorporation of such moieties. The potential of these cells to desaturate and elongate FA's nevertheless reflects a mechanism to maintain its own FA levels.

Detection of large proportions of unsaturated FA's in control cells (Table 3.4.3.1.), but only small amounts of lipid peroxides (Table 3.4.4.1.) implied chemical and enzymic mechanisms of protection against free radical attack, eg. vitamin E and/or superoxide dismutase. It was also possible that most cellular unsaturated FA's in control cells were present as components of complex lipids, eg. membrane PGL's, which would therefore not be so readily accessible to oxidation. Increased lipid peroxide measurement in dosed cells, however, could have reflected saturation of the cellular anti-oxidant mechanisms induced with exogenous unsaturated FA uptake and/or increased cellular unsaturated FA availability. The lipid peroxides detected in dosed cells were nevertheless consistent with intact cellular enzymic and/or non-enzymic mechanisms for their generation, and reflected the p-oil concentration incubated, p-oil FA composition (Table 2.3.3.2.), and FA availability and susceptibility to oxidation.

The small increment in cellular lipoperoxides detected with p-CO or p-MO dosage (Table 3.4.4.1.) correlated with the large concentration of saturated FA's these

p-oils contained (Table 2.3.3.2.). However, quantitation of small cellular lipoperoxide amounts also with p-OO, p-LO or p-FO incubation, despite these p-oils containing significant levels of unsaturated FA's, implied that the substrates for peroxidation were lipid bound and stable. Furthermore, the finding that overall lipid peroxide levels were lowest in the cells and greatest in the spent medium with p-LO supplementation in the absence of cytotoxicity (Fig. 3.4.1.6.) suggested that the cellular lipid peroxides formed with incorporation of this p-oil were transfered into the incubation medium through the intact plasma membrane. This correlated with studies which have implicated lipid peroxides in increasing membrane permeability (Chio et al 1969, Mead 1976, Tappel 1980, Gavino et al 1981c, Morisaki et al 1982b and Frankel 1984). This may also explain why cellular lipid peroxide levels were greater in the spent medium than in the cells with p-FO supplementation. It was also possible that these medium lipoperoxides reflected released cellular FFA's which were oxidised extra-cellularly by released cellular peroxidising enzymes. Nevertheless, measurement of the largest medium lipid peroxide amounts with p-LO or p-FO dosage correlated with the significant ω3 PUFA levels in these p-oils. The significantly greater concentration of ALA in p-LO than EPA in p-FO (approximately 63% and 18%, respectively) was consistent with the greatest medium lipoperoxide

levels measured, despite the greater susceptibility of EPA to oxidation.

The cellular lipid peroxides formed with p-SSO or p-EPO supplementation (Table 3.4.4.1.) probably related mainly to oxidation of incorporated free LA. Both p-oils exhibited similar FA compositions (Table 2.3.3.2.), but the presence of about 9% GLA in p-EPO only probably explains the greater lipid peroxides amounts induced overall with this p-oil. Morisaki et al (1984) have indeed shown that GLA generates large quantities of lipoperoxides in rodent smooth muscle cell cultures, and that such could induce cytotoxicity. The greatest cellular lipoperoxide levels quantitated with dosage of 60mg/l p-EPO correlated with the most extensive growth inhibition induced at this p-oil concentration (Fig. 3.4.1.7.), and suggested lipid peroxide involvement in the mediation of cytotoxicity. This supported current evidence which implicates that lipoperoxides affect normal cell metabolism and cause membrane damage (Chio et al 1969, Mead 1976, Tappel 1980, Gavino et al 1981c, Morisaki et al 1982b and Frankel 1984). Demonstration of cell lysis and the absence of lipoperoxides in the spent medium with p-EPO supplementation implied that the cytotoxicity induced arose partly as a result of oxidation of membrane FA's. Lipoperoxides were also absent in the spent medium with p-SSO incubation, and similar amounts of cellular lipid peroxides were

quantitated with 60mg/l p-EPO or p-SSO supplementation, yet cell viability was not significantly limited with this amount of p-SSO (Figs. 3.4.1.5. and 3.4.1.7.). These findings suggested that a critical lipid peroxide concentration may have to be attained before lipid peroxides become membrane damaging and detrimental to cell survival, dependant on the overall state of cellular equilibrium. On the other hand, it may indicate that lipoperoxides are less involved in FA mediated cytotoxicity than postulated, and that changes in membrane fluidity are equally or more important. The absence of significant amounts of unsaturated FA's in p-CO would indeed imply that the reduction of cell viability this p-oil induced related to alterations in membrane fluidity rather than lipoperoxide production. However, the finding that the low concentrations of cellular lipoperoxides measured with p-MO, p-OO, p-LO or p-FO dosage (Table 3.4.4.1.) correlated with the absence of cytotoxicity (Figs. 3.4.1.3., 3.4.1.4., 3.4.1.6. and 3.4.1.7., respectively), indicated the possiblity that lipoperoxides were partly involved in the modulation of cell viability.

3.5 THE EFFECTS OF PSEUDO-OILS ON CELLS DERIVED FROM RAT BRAIN.

3.5.1 Effects of pseudo-Oils on Cell Viability.

Cells isolated from rat cerebral cortex were largely astrocytic, and microscopic examination of control and p-oil dosed cells before, during and after each experiment showed no morphological changes at any time, except for cytoplasmic droplets observed with p-oil incubation at high concentrations in some instances.

Final medium albumin concentrations ranging from 0 to 250mg/l had no significant effect on cell viability (Fig. 3.5.1.1.), nor were any synergistic effects evident. Thus, the cell viability changes induced with the p-oils were a result of the exogenous FA's, and not the albumin used as FA carrier.

The number of control cells, seeded at $10x10^4$/ml, at the end of the 24 hour post-trypsinisation recovery period was $12.8x10^4$/ml. This equated to approximately 62% of the final control cell number at the end of the 48 hour incubation period and represented the cytostatic number. Only p-oil concentrations limiting cell viability to significantly below 62% were thus considered cytotoxic.

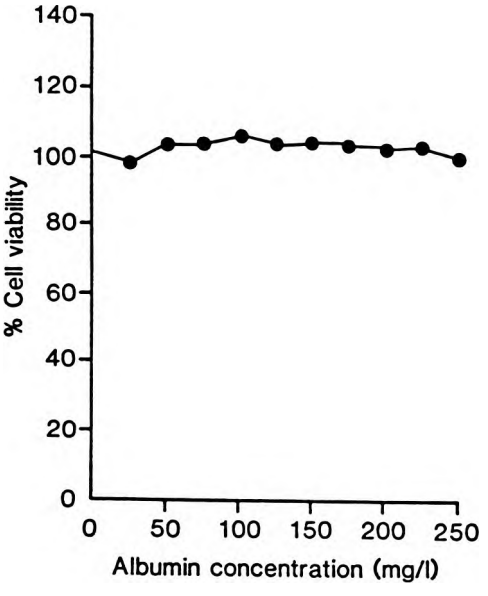
Figs. 3.5.1.2-3.5.1.8. depict the data derived following incubation of rat brain cells with p-oils. Certain

Legend to Figs. 3.5.1.1-3.5.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.5.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.5.1.2-3.5.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.5.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.5.1.1.

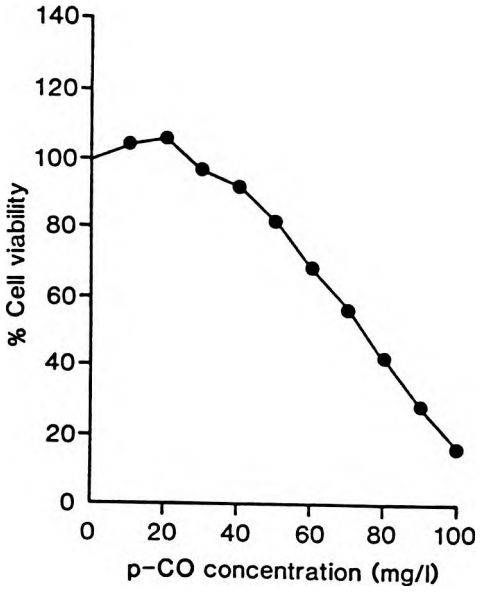
The percentage viability of cells derived from rat brain incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.8	12
25	96.8	5.4	12
50	102.5	3.3	12
75	102.9	4.3	12
100	104.9	2.7	12
125	102.9	3.2	12
150	103.5	5.2	12
175	102.8	4.4	12
200	101.5	5.0	12
225	102.9	3.1	12
250	99.0	2.9	12

Fig. 3.5.1.2.

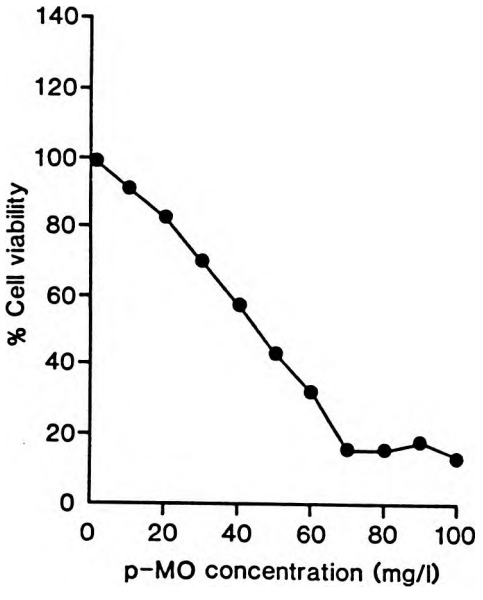
The percentage viability of cells derived from rat brain incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.2	6
10	104.4	2.6	6
20	105.8	1.6	6
30	97.1	1.9	6
40	91.8	3.0	6
50	82.0	1.8	6
60	67.9	1.3	6
70	55.8	1.6	6
80	42.4	1.9	6
90	28.4	1.5	6
100	15.8	1.0	6

Fig. 3.5.1.3.

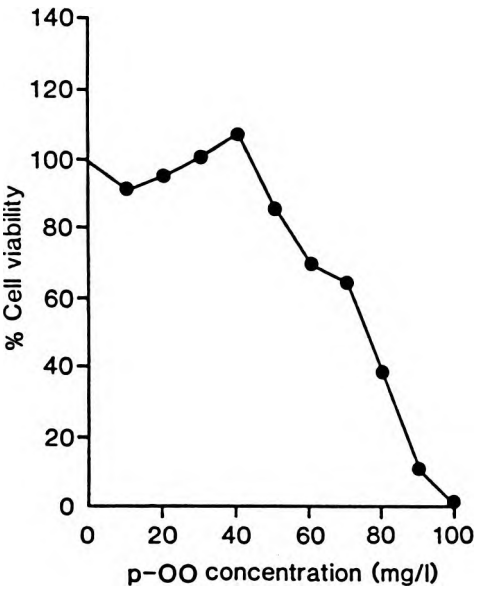
The percentage viability of cells derived from rat brain incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.7	6
10	91.3	3.5	6
20	83.1	2.6	6
30	69.6	6.3	6
40	56.8	5.1	6
50	43.5	2.5	6
60	32.3	2.4	6
70	15.3	2.4	6
80	15.4	4.6	6
90	18.0	2.8	6
100	12.5	1.8	6

Fig. 3.5.1.4.

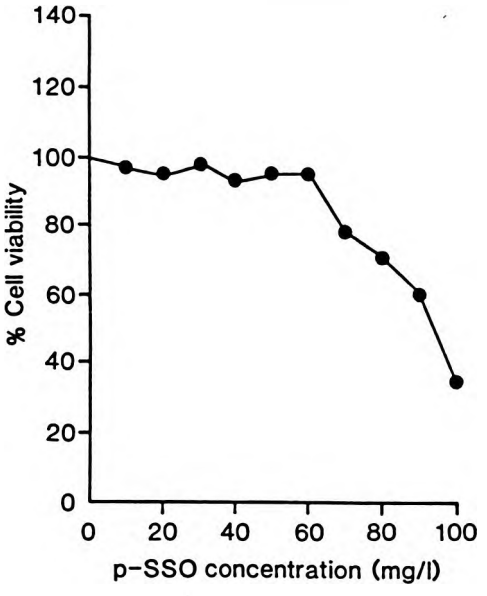
The percentage viability of cells derived from rat brain incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.9	6
10	90.9	4.8	6
20	94.8	4.9	6
30	100.4	5.8	6
40	107.3	4.5	6
50	86.2	5.7	6
60	70.4	3.7	6
70	64.9	3.0	6
80	38.2	4.1	6
90	10.8	2.4	6
100	0.0	0.0	6

Fig. 3.5.1.5.

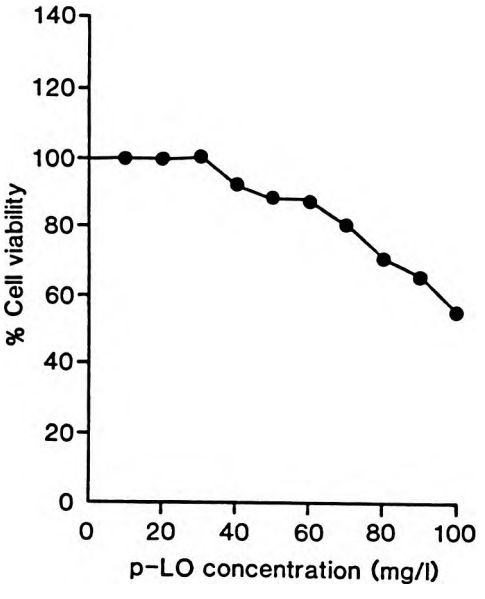
The percentage viability of cells derived from rat brain incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.4	6
10	96.7	2.3	6
20	95.0	2.0	6
30	98.0	1.9	6
40	93.5	3.1	6
50	95.2	2.8	6
60	95.2	2.4	6
70	78.3	5.3	6
80	70.8	3.0	6
90	60.1	2.4	6
100	34.5	1.3	6

Fig. 3.5.1.6.

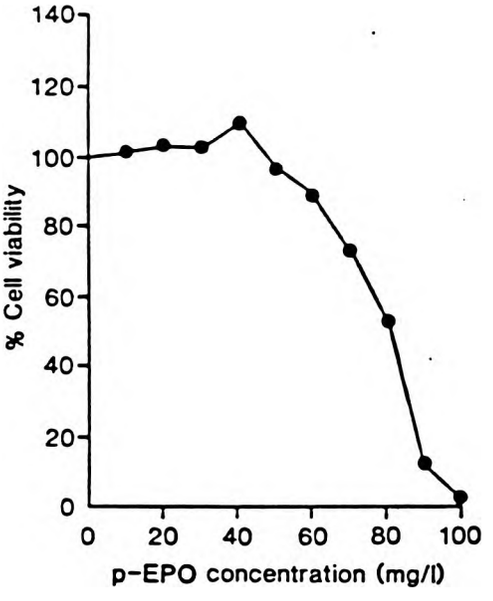
The percentage viability of cells derived from rat brain incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.8	6
10	99.2	2.8	6
20	99.1	2.6	6
30	99.5	2.3	6
40	90.9	2.4	6
50	87.5	2.7	6
60	86.8	2.3	6
70	80.6	3.4	6
80	70.3	1.4	6
90	64.5	2.2	6
100	54.3	1.4	6

Fig. 3.5.1.7.

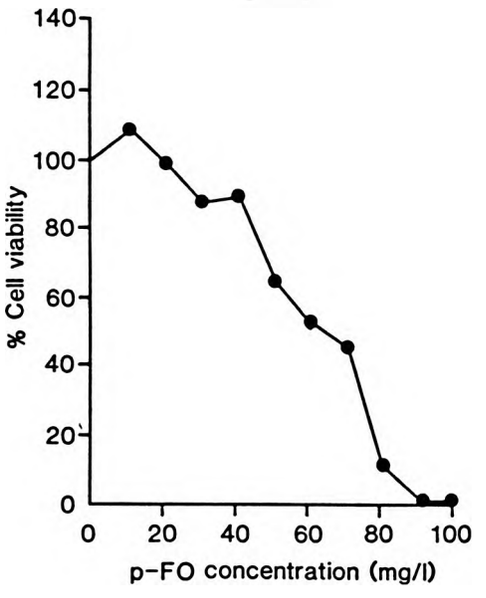
The percentage viability of cells derived from rat brain incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.8	6
10	100.2	2.9	6
20	102.1	2.7	6
30	101.3	3.0	6
40	109.2	2.5	6
50	96.3	2.3	6
60	89.0	2.1	6
70	73.1	2.7	6
80	52.1	2.9	6
90	11.9	0.9	6
100	1.0	0.3	6

Fig. 3.5.1.8.

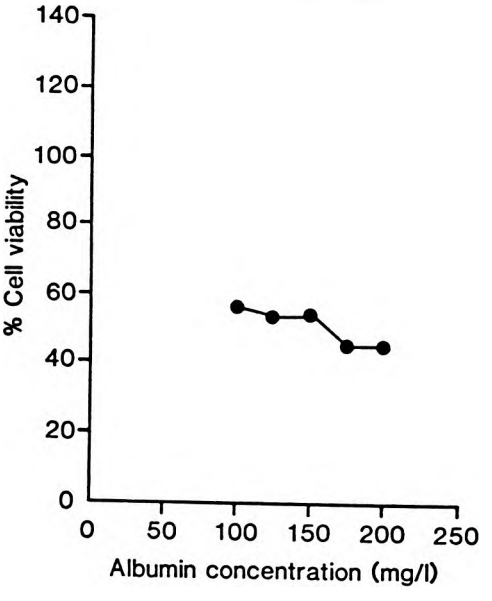
The percentage viability of cells derived from rat brain incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.8	6
10	109.1	3.4	6
20	99.0	3.1	6
30	88.0	2.5	6
40	89.8	2.7	6
50	64.8	2.3	6
60	52.6	5.5	6
70	45.1	2.1	6
80	10.5	3.3	6
90	0.0	0.0	6
100	0.0	0.0	6

Fig. 3.5.1.9.

The percentage viability of cells derived from rat brain incubated with albumin at the ID_{50} of p-00.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.5	6
100	56.6	3.1	6
125	54.6	5.7	6
150	55.3	3.6	6
175	46.1	4.8	6
200	45.8	4.0	6

concentrations of p-CO, p-OO, p-EPO and p-FO stimulated cell proliferation slightly, whereas all p-oils limited and inhibited cell growth. The magnitude of the effects induced, however, varied with the nature of the p-oil and concentration dosed.

Cell proliferation was stimulated to a maximum of 105.8% with 20mg/l p-CO and to 107.3% with 40mg/l p-OO (Figs. 3.5.1.2. and 3.5.1.4., respectively). Incubation with greater amounts of p-CO or p-OO limited cell viability in a concentration dependent manner, while amounts greater than 70mg/l p-CO or p-OO induced cytotoxicity and reduced the viable cell yield, almost linearly, to 15.8% and 0% with 100mg/l p-CO and p-OO, respectively. The ID_{50} for both p-oils was approximately 75mg/l, which reflected the similar effects these p-oils induced.

Dosage with up to 70mg/l p-MO reduced cell viability in an approximately linear concentration dependent manner (Fig. 3.5.1.3.). p-MO was the most effective p-oil to impair cell proliferation as cytotoxicity was induced with amounts greater than 30mg/l p-MO, and 50% of cells were killed with only 45mg/l p-MO (ID_{50}). The cytotoxic effect of p-MO plateaued with concentrations of 70mg/l to 100mg/l as cell viability ranged from 12.5% to 18.0%.

Cell proliferation was not significantly influenced with supplementation of up to 60mg/l p-SSO or 30mg/l p-LO (Figs. 3.5.1.5. and 3.5.1.6., respectively). Higher

concentrations limited cell growth almost linearly, but cytotoxicity was induced only with amounts greater than 80mg/l p-SSO and 90mg/l p-LO. Half the cells were viable with about 94mg/l p-SSO (ID_{50}), compared to 34.5% with 100mg/l p-SSO. More than half the cells, however, were still viable with 100mg/l p-LO (54.3%), thus no ID_{50} could be calculated. This was the least cytotoxic p-oil.

Supplementation with up to 30mg/l p-EPO had little effect on cell viability and increased cell numbers were found with 40mg/l p-EPO (109.2%), whereas proliferative effects were induced only with 10mg/l p-FO (109.1%) (Figs. 3.5.1.7. and 3.5.1.8., respectively). These were the greatest increments in cell proliferation found. Concentrations greater than 50mg/l p-EPO and 20mg/l p-FO induced growth limitation, but amounts greater than 70mg/l p-EPO and 50mg/l p-FO were cytotoxic. Calculation of ID_{50} values showed that p-EPO was less cytotoxic than p-FO (approximately 81mg/l and 65mg/l, respectively), and practically all cells took up the Typan blue with 100mg/l p-EPO and 90mg/l p-FO.

To exclude the possibility that any of the effects induced with p-oil dosage were influenced by the amount of albumin bound to the FA's, cells were incubated with a fixed amount of p-OO corresponding to its ID_{50} (about 75mg/l). Five points were selected around which to vary the albumin concentration, viz. two points above, two points below, and the ID_{50} of p-OO, and the appropriate

amount of albumin added to these cultures to give the desired concentrations. The results are depicted in Fig. 3.5.1.9., and indicated that the amount of p-OO dosed reduced cell viability to approximately 50% of controls, and that such was not influenced by the concentration of albumin present as FA carrier. Hence, synergistic effects of albumin could be excluded, and the effects induced with the p-oils could be attributed solely to the exogenous FA's.

Subsequent to these studies, cells derived from rat brain were plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient amounts to permit all the quantitative and qualitative analyses to be performed. Upon harvesting, cell viabilities relative to controls were compared and found to be statistically similar to those in Figs. 3.5.1.1-3.5.1.8. All further biochemical assays were thus performed on these samples.

3.5.2 Effects of pseudo-Oils on Total Protein.

Table 3.5.2.1. shows the total protein concentrations of cells incubated with 0, 20, 40 or 60mg/l p-oil, expressed as µg total protein/10^6 cells seeded.

455.5µg protein/10^6 control cells seeded was detected, whereas dosed cells contained protein amounts ranging from 198.7 to 500.5µg/10^6 cells seeded (with 60mg/l p-MO and 20mg/l p-EPO, respectively). Cells supplemented with 20mg/l p-oil (except p-OO) exhibited the highest protein concentrations quantitated over the range dosed (422.1 to 500.5µg/10^6 cells seeded), which approximated to control amounts. Cellular protein levels were related inversely to the p-oil concentration dosed, although slightly more protein was found in cells incubated with 40mg/l than with 20mg/l p-OO (478.2 vs 461.5µg/10^6 cells seeded). Cells dosed with 40mg/l p-OO or p-EPO exhibited enhanced protein levels, whereas significantly reduced protein concentrations were found in all cells incubated with 60mg/l p-oil, compared to controls.

Table 3.5.2.1.

The protein content of cells derived from rat brain, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	455.5			
CO		471.9	351.2	297.9
MO		451.5	254.9	198.7
OO		461.5	478.2	309.7
SSO		450.5	428.1	397.7
LO		474.9	409.9	387.1
EPO		500.5	496.5	409.9
FO		422.1	400.8	223.2

3.5.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Cells Derived From Rat Brain.

Table 3.5.3.1. lists the FA profiles of cells incubated with 0, 20, 40 or 60mg/l p-oil. The greatest percentage of any single FA detected in control cells was 18:1ω9 (25.9%). FA's comprising >5.0% of the total spectrum in control cells included 16:0 (20.5%), 18:0 (18.6%), 18:2ω6 (5.4%) and 22:4ω6 (6.4%).

p-Oil dosed cells contained 16:0 and 18:1ω9 percentages parallel to, or greater than, those in control cells, whereas 16:1ω9 proportions were generally, and 18:0 levels were always, lower in dosed cells. 18:2ω6, 18:3ω6, 20:4ω6, 18:3ω3 and 20:5ω3 percentages were generally only comparable to, or higher than, control amounts if the p-oils dosed contained these moieties, and the levels of most other polyenoic FA's were parallel to, or lower than, control levels.

Incubation with 20, 40 or 60mg/l p-CO increased 16:0 levels to 27.9%, 29.0% and 41.8%, respectively (20.5% in controls), while 16:1ω9 and 18:1ω9 amounts ranged from 1.0 to 5.5% and 23.8% to 29.6%, relative to 3.8% and 25.9% in controls, respectively. 18:0 and 18:2ω6 levels were both decreased (12.9% to 13.2% and 2.8% to 4.6% vs 18.6% and 5.4% in controls, respectively), although up to 4.7% 20:4ω6 and 8.6% 22:4ω6 were found (3.5% and 6.4% in controls, respectively).

Legend to Table 3.5.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.5.3.1.

The fatty acid spectrum of cells derived from rat brain.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	20.5±0.50	27.9	29.0	41.8	22.8	23.1	30.7	17.7	19.3	17.9	23.1	25.8	17.8	19.0	32.3	22.9	16.8	26.9	22.1	19.5	41.1	33.5
	18:0	18.6±0.30	13.2	12.9	13.0	11.9	12.1	18.0	7.3	6.2	3.4	11.3	13.5	16.8	10.6	15.3	7.9	9.5	14.6	11.8	9.1	10.2	6.1
	20:0	1.2±0.05	-	0.2	0.1	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1
	22:0	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24:0	0.3±0.05	-	0.3	-	-	-	0.1	-	-	-	-	0.2	-	-	0.1	-	-	0.2	0.2	-	0.1	-
ω9 MONOS.	16:1	3.8±0.10	5.5	3.1	1.0	1.7	1.9	3.5	1.8	0.7	1.1	4.4	4.0	1.1	1.5	1.7	2.7	1.5	1.2	2.7	12.4	13.3	20.5
	18:1	25.9±0.55	29.6	28.1	23.8	32.3	31.4	26.7	48.5	58.6	66.3	26.8	28.8	31.0	28.0	24.4	30.1	21.1	21.6	22.9	26.0	20.7	23.6
	24:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	5.4±0.00	4.6	3.6	2.8	10.4	13.0	7.8	9.2	6.4	6.0	21.2	18.2	27.1	10.0	5.5	11.1	31.7	23.2	32.4	9.1	4.3	6.6
	18:3	-	0.1	-	0.2	0.1	-	-	0.1	-	-	0.1	-	-	0.1	0.1	0.2	0.5	0.7	2.2	0.1	-	-
	20:2	1.9±0.05	0.2	0.3	-	0.3	0.3	0.3	1.0	2.5	1.5	0.9	0.2	0.3	0.3	0.3	0.1	1.1	0.2	-	0.4	0.6	0.5
	20:3	1.8±0.05	1.3	1.2	0.9	1.3	1.0	0.5	0.9	0.3	0.2	1.0	0.6	0.3	0.9	0.7	0.5	3.9	0.8	0.4	1.0	0.5	0.4
	20:4	3.5±0.10	4.7	3.9	2.7	5.5	5.6	2.8	2.7	0.6	0.6	2.5	1.4	0.8	4.6	1.7	1.4	3.2	1.4	1.1	3.4	0.9	1.0
	22:4	6.4±0.05	5.0	7.6	8.6	5.8	5.4	6.5	5.5	3.1	1.7	4.2	3.6	2.6	6.8	5.8	3.1	5.6	4.2	1.5	6.1	3.6	3.5
	22:5	1.2±0.10	0.1	1.0	0.1	-	0.3	-	-	0.5	-	0.1	0.6	0.1	-	0.6	-	-	0.7	0.1	0.1	0.4	0.1
	18:3	0.9±0.00	0.9	0.7	0.6	1.1	0.8	0.6	0.9	0.3	0.2	0.6	0.5	0.2	12.7	7.2	18.0	0.4	0.4	0.8	0.9	0.6	0.5
ω3	18:4	0.9±0.05	0.2	0.9	0.2	0.2	0.4	0.2	0.1	0.3	0.1	0.2	0.6	0.2	0.1	0.5	0.1	0.1	0.6	0.2	0.2	0.4	0.2
POLYS.	20:4	1.8±0.10	0.4	1.6	0.9	0.2	0.3	0.7	0.4	0.3	0.1	0.1	0.7	0.7	0.6	0.7	0.3	0.3	0.9	0.1	0.8	0.6	0.6
	20:5	0.4±0.00	0.5	0.9	0.4	1.0	0.6	0.2	0.2	0.1	-	0.1	0.1	-	0.8	0.2	0.4	0.3	0.3	0.1	3.8	1.1	1.6
	22:5	3.0±0.30	2.9	2.5	1.7	2.8	1.9	0.9	1.9	0.5	0.5	1.9	1.1	0.6	2.2	1.4	0.9	2.0	1.2	0.8	4.7	1.0	0.7
	22:6	2.5±0.30	2.9	2.4	1.4	2.8	2.1	0.7	1.8	0.4	0.3	1.7	0.6	0.4	1.9	1.0	0.7	2.0	0.9	0.6	2.5	0.6	0.6

Cells dosed with 20, 40 or 60mg/l p-MO contained 22.8%, 23.1% and 30.7% 16:0, respectively (controls 20.5%), but decreased amounts of 16:1ω9 (1.7% to 3.5% vs 3.8% in controls). 18:0 was decreased with 20 and 40mg/l p-MO (11.9% and 12.1%, respectively) and parallel to controls with 60mg/l p-MO (18.0%), while 18:1ω9 levels were greatest with 20 and 40mg/l p-MO (32.3% and 31.4%, respectively), and comparable to controls with 60mg/l p-MO (26.7%). 18:2ω6 uptake increased significantly over the range dosed (7.8% to 13.0% vs 5.4% in controls), and 5.5%, 5.6% and 2.8% 20:4ω6 was detected with 20, 40 and 60mg/l p-MO, respectively (3.5% in controls), yet other PDFA levels were not significantly increased.

A marked increase in 18:1ω9 proportions was found with 20, 40 or 60mg/l p-OO supplementation (48.5%, 58.6% and 66.3%, respectively vs 25.9% in controls). 16:0 levels were slightly decreased, but 16:1ω9 and 18:0 amounts were considerably reduced, compared to controls (20.5%, 3.8% and 18.6%, respectively). 6.0% to 9.2% 18:2ω6 was found (5.4% in control), with 2.5% 20:2ω6 detected with 40mg/l p-OO (1.9% in controls), but other PUFA levels were not significantly raised.

16:0 percentages were 23.1% and 25.8% with 20 and 40mg/l p-SSO, respectively, but only 17.8% with 60mg/l p-SSO (20.5% in controls). 16:1ω9 levels were slightly raised when incubated with 20mg/l p-SSO (4.4%), decreased with

60mg/l p-SSO (1.1%), and parallel to controls (3.8%) with 40mg/l p-SSO (4.0%). Decreased amounts of 18:0 were found (11.3% to 16.8% vs 18.6% in controls), but 18:1ω9 levels were increased (26.8% to 31.0% vs 25.9% in controls). Despite marked 18:2ω6 incorporation (5.4%, 21.2%, 18.2% and 27.1% with 0, 20, 40 and 60mg/l p-SSO, respectively), no significant increases in PDFA's were detected, and all ω3 PUFA levels were decreased.

16:0, 16:1ω9, 18:0 and 18:1ω9 percentages were parallel to controls or decreased with p-LO dosage, although increased 16:0 and 18:1ω9 were found with 40 and 60mg/l p-LO, respectively (32.3% and 30.1% vs 20.5% and 25.9% in controls, respectively). 18:2ω6 levels increased only with 20 and 60mg/l p-LO (10.0% and 11.1%, respectively vs 5.4% in controls), and 20:4ω6 was slightly increased with 20mg/l p-LO (4.6% vs 3.5% in controls). Incubation with p-LO raised cellular 18:3ω3 percentages markedly (7.2% to 18.0% vs 0.9% in controls), yet ω3 PDFA amounts were not significant increased.

The levels of the saturated and monoenoic FA's reported in Table 3.5.3.1. were parallel to controls or decreased with p-EPO supplementation, although increased 16:0 was detected with 40mg/l p-EPO (26.9% vs 20.5% in controls). A marked elevation in cellular 18:2ω6 occurred with all p-EPO concentrations dosed (23.2% to 32.4% vs 5.4% in controls), and 0.5%, 0.7% and 2.2% 18:3ω6 were detected with 20, 40 and 60mg/l p-EPO, respectively (no 18:3ω6 in

controls). 20:3ω6 levels increased approximately 2 fold with 20mg/l p-EPO (3.9% vs 1.8% in controls), but all other ω6 and ω3 PUFA percentages were decreased relative to controls.

Incubation with 20mg/l p-FO induced no significant change in 16:0 levels (19.5% vs 20.5% in controls), but amounts were dramatically raised with 40 and 60mg/l p-FO (41.1% and 33.5%, respectively). 16:1ω9 levels increased from 3.8% in controls to 12.4%, 13.3% and 20.5% with 20, 40 or 60mg/l p-FO, respectively, while 18:0 amounts were decreased with all concentrations dosed (6.1% to 10.2% vs 18.6% in controls), and 18:1ω9 percentages were generally parallel to control levels (25.9%). 18:2ω6 proportions ranged from 4.3% to 9.1% (5.4% in controls), and no significant increases were found for any other ω6 PUFA's. Significantly increased 20:5ω3 amounts were detected with 20, 40 and 60mg/l p-FO (3.8%, 1.1% and 1.6%, respectively vs 0.4% in controls), but with the exception of 4.7% 22:5ω3 detected with 20mg/l p-FO (3.0% in controls), no increases in other ω3 PUFA's were observed.

3.5.4 Effects of pseudo-Oils on Lipid Peroxide Formation.

Table 3.5.4.1. shows the lipoperoxides measured in rat brain cells and their respective growth media following incubation with 0, 20, 40 or 60mg/l p-oil. The values are shown as nmoles MDA/10^6 cells, but in the case of the spent media, this represents the nmoles of MDA in the volume of medium from which 10^6 cells were obtained.

0.4nmoles MDA/10^6 control cells was measured, while the amounts detected for dosed cells ranged from a low of 0.5nmoles MDA/10^6 cells (with 20mg/l p-EPO) to a high of 2.7nmoles MDA/10^6 cells (with 60mg/l p-MO). The cellular lipoperoxide amounts measured varied with the p-oil dosed and generally increased in a p-oil concentration dependent manner, but showed no correlation overall with the degree of p-oil unsaturation. Cells dosed with p-EPO contained only 0.5 to 0.8nmoles MDA/10^6 cells, whereas 0.9, 1.6 and 1.0nmole MDA/10^6 cells were detected with 20, 40 and 60mg/l p-SSO, and 0.7, 0.7 and 1.3nmoles MDA per 10^6 cells quantitated with p-LO, respectively. In comparison, cells incubated with 20, 40 or 60mg/l p-CO or p-OO contained 0.7, 1.1 and 1.8 or 0.8, 1.1 and 2.0 nmoles MDA/1x10^6 cells, respectively, and p-MO induced 0.6, 0.9 and 2.7nmoles MDA/1x10^6 cells. However, p-FO generated the largest cellular lipid peroxide amounts overall across the range dosed (1.4, 1.4 and 2.4nmoles

Table 3.5.4.1.

Lipoperoxide formation by rat brain cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	0.4			
CO		0.7	1.1	1.8
MO		0.6	0.9	2.7
OO		0.8	1.1	2.0
SSO		0.9	1.6	1.0
LO		0.7	0.7	1.3
EPO		0.5	0.6	0.8
FO		1.4	1.4	2.4
pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	2.7
MO		-	-	4.8
OO		-	-	3.4
SSO		-	-	3.3
LO		-	1.0	4.9
EPO		-	1.8	7.8
FO		-	2.9	12.3

MDA/10^6 cells, respectively).

Lipoperoxides were absent both in spent control medium, and in the spent media derived from cultures dosed with 20mg/l p-oil. Further, only p-LO, p-EPO and p-FO induced medium lipoperoxides when 40mg/l p-oil was incubated (1.0, 1.8 and 2.9nmoles MDA/1x10^6 cells, respectively). On the other hand, lipoperoxides were measured in all spent media derived from cultures supplemented with 60mg/l p-oil (2.7 to 12.3nmoles MDA/10^6 cells). Unlike the cells, however, the lowest concentrations of lipid peroxides in the spent media occurred with p-CO dosage, whereas larger MDA concentrations were quantitated with p-OO or p-SSO, followed by p-MO, p-LO, p-EPO or p-FO incubation, respectively.

3.5.5 Discussion.

This study showed that supplementation of primary cultures of normal rat brain with p-oils affected the viability of these cells (Figs. 3.5.1.2-3.5.1.8.), and that the p-oil-specific cell viability changes induced reflected the unique FA profile each p-oil exhibited (Table 2.3.3.2.). The FA compositions of p-OO, p-SSO and p-LO suggested that the effects induced with incubation of each p-oil were largely attributable to the high concentrations of 18:1ω9 (71%), 18:2ω6 (70%) or 18:3ω3 (63%) present, respectively. Since the capability to kill cultured rat brain cells increased markedly in the order p-LO, p-SSO and p-OO, it seemed possible that OA was the most, and ALA the least, cytotoxic of these moieties, and that such related inversely to the number of double bonds in these C18 FA's. This, however, was in direct contrast with a previous study in our laboratory which showed LD_{50} values of about 55, 50 and 35mg/l for rat brain primary cultures incubated with 18:1ω9, 18:2ω6 or 18:3ω3, respectively (Girao 1988). 18:3ω3 and 18:3ω6 were equally cytotoxic, whereas comparison of the FA profiles of p-EPO, p-LO, p-SSO and p-OO with the cell viability changes these p-oils induced suggested that 18:3ω6 exhibited greater cytotoxic capability than 18:3ω3, 18:2ω6 and 18:1ω9. Girao (1988) found that 18:0 induced cytotoxicity in the range 60 to 100mg/l and cytostasis overall in the range 0 to 60mg/l, although

amounts of 1, 25, 35 and 45mg/l 18:0 increased growth by 6% to 12%. Low concentrations of p-CO, p-OO, p-EPO and p-FO enhanced cell proliferation similarly by between 5% and 10% (Figs. 3.5.1.2., 3.5.1.4., 3.5.1.7., 3.5.1.8., respectively), but this was not found when individual C18 unsaturated FA's (18:1ω9, 18:2ω6, 18:3ω6 or 18:3ω3) were supplemented (Girao 1988). Others found that addition of 18:2ω6, 18:3ω3, 20:4ω6 or 22:6ω3 at seeding time to cultured fetal mouse brain cells increased [^{3}H]-thymidine incorporation into DNA, stimulated cell proliferation with optimal concentrations of 1.0mg/l FA, and were toxic with higher amounts (Bourre et al 1983). The finding that optimum cell growth was achieved with p-oil concentrations of 20mg/l p-CO, 40mg/l p-OO, 40mg/l p-EPO or 10mg/l p-FO, and cytotoxicity was induced only with significantly higher amounts of these compounds, clearly demonstrated that p-oils were less growth inhibitory than individual FA's.

The quantitative and qualitative differences described above with supplementation of individual FA's or p-oils were not surprising, but in fact supported the mediation of synergistic or antagonistic effects between FA's within a p-oil which may have enhanced or diminished the effects caused by individual FA's. This may indeed explain the different effects which p-SSO and p-EPO induced, despite the similar FA compositions these p-oils exhibited. Furthermore, this phenomenon more than

likely occurs *in vivo* when organisms consume FA mixtures as dietary oils, thus the modulation of cell viability reported with p-oil dosage probably reflects closer to the "real world" than studies using individual FA's.

Uptake of FA's by rat brain *in vivo* has been reported by Cook (1978a, 1978b, 1980). FA's must be in their activated state before being incorporated, and this requires plasma membrane acyl CoA synthetase, which has been demonstrated in rat brain synaptosomal plasma membranes in our laboratory (Davidson 1986). Thus, the potential exists within the brain to incorporate exogenous FA's, and there is no evidence that such is lost when cells are maintained in culture. Numerous studies have in fact shown rat brain cells in culture to be capable of exogenous FA incorporation. Yavin et al (1974) demonstrated that the rate of 12:0, 14:0, 16:0, 18:0, 18:1ω9, 18:2ω6 and 18:3ω3 uptake into cultured cells derived from rat cerebral tissue was greatest with 18:0 and decreased progressively with decreasing chain length, although no clear relationship was evident between uptake and degree of FA unsaturation. These cells have been reported to incorporate the above FA's into TAG's, PGL's and glycerolipids, although such moieties could also remain in the free form (Menkes 1972 and Yavin et al 1974). On the other hand, addition of 18:2ω6, 18:3ω3, 20:4ω6 or 22:6ω3 at seeding time to cultured fetal mouse brain cells resulted in these

FA's being incorporated predominantly into the PGL fraction (Bourre et al 1983). The site(s) of p-oil FA uptake were not investigated in the present study, but the data in Table 3.5.3.1. nevertheless supported the capability of cultured rat brain cells to incorporate exogenous FA's to varying extents. Hence, it was likely that membrane fluidity changes occurred. Brain membrane lipids have been reported to be highly unsaturated and conservative in nature (Crawford et al 1971), and any alteration in membrane stability induced with p-oil incubation may consequently have caused cell death. The cytotoxicity reported with p-CO and p-MO could well have related to a marked decrease in the unsaturation index of brain membrane lipids when these saturated FA-rich p-oils were dosed. This mechanism alone, however, could not fully explain why p-MO was more growth inhibitory than p-CO, despite the more saturated nature of the latter. The inclusion in p-MO of the PDFA and endoperoxide substrate, 20:4ω6, may be an explanation. Indeed, considerably more cytotoxicity was induced with incubation of p-OO, p-EPO or p-FO which also contained EFA desaturation and/or elongation products, than with p-SSO or p-LO which were EFA-rich and PDFA-deficient. In fact, the finding that p-FO was significantly more growth inhibitory than p-EPO correlated with the presence of almost 20% PDFA's in p-FO, compared to 10% in p-EPO. These moieties may have altered membrane

stability, either directly or indirectly, to such an extent that cell viability could not continue.

The cellular protein concentrations reported with p-oil dosage (Table 3.5.2.1.) generally paralleled the changes observed in cell viability (Figs. 3.5.1.2-3.5.1.8.). The slight increments in cellular protein levels found with 20mg/l p-CO, 40mg/l p-OO or 40mg/l p-EPO supplementation primarily reflected increased cell numbers with constant or slightly elevated protein per cell. Comparison of cellular protein concentrations with cell viability indicated that 20mg/l p-LO or p-EPO enhanced protein synthesis slightly, whereas such was suppressed with 20mg/l p-FO, 40 or 60mg/l p-SSO. On the other hand, the finding that dosed cells contained less protein than controls when cell viability was impaired correlated with decreased cell numbers with constant or decreased protein per cell; the latter may relate to the low turnover of proteins known to exist in the brain (Lehninger 1982). When Vignikin et al (1989) incubated primary cultures of newborn rat glial cells with single FA's at 30mg/l, they found that oleic acid induced a greater protein increment over a two week period than cells treated with linoleic, alpha-linolenic or especially arachidonic acid. This correlated with the mean protein concentrations reported in Table 3.5.2.1. for cells supplemented with 20 or 40mg/l p-OO, p-SSO, p-LO or p-MO, respectively, but the finding that such

largely reflected changes in cell viability extended the work of Vignikin et al (1989).

The results obtained from the analysis of FA's in control and p-oil dosed cells (Table 3.5.3.1.) were consistent with an overall lack or suppression both of desaturase and elongase expression in cultured rat brain. However, slightly increased levels of some PDFA's with dosage of certain p-oils supported the potential for limited desaturase cascade enzyme activity. 18:3ω3 incorporated with p-LO supplementation was not further metabolised, and such was consistent with impaired Δ6D expression. 18:2ω6 incorporated with p-SSO incubation was also not converted to PDFA's, thus 20:3ω6 formation with 20mg/l p-EPO incubation probably related only to elongation of incorporated 18:3ω6. The lack of further 20:3ω6 metabolism with p-EPO supplementation, however, was consistent with impaired Δ5D expression. Slightly increased 20:4ω6 and/or 20:5ω3 levels when certain concentrations of p-CO, p-MO or p-LO were supplemented reflected limited Δ5D expression or extramicrosomal retroconversion of cellular 22:4ω6 and 22:5ω3, respectively. No evidence for elongation of 20:4ω6 incorporated with p-MO dosage was found, although 22:5ω3 formation with 20mg/l p-FO supplementation supported elongation of incorporated 20:5ω3. However, the lack of significant increments in 22:5ω6 or 22:6ω3 with p-oil dosage indicated impaired Δ4D capability.

Literature reports on the capability of rat brain cells to desaturate and elongate FA's are contradictory. Menkes (1972) showed the capability of such cells in lipid-free culture medium to desaturate and elongate both [^{14}C]-16:0 and [^{14}C]-18:0 prior to incorporation into cellular lipids. The potential for 16:0 and 18:0 desaturation were also indicated in the present study, but neither these moieties nor their immediate desaturation products were further elongated (Table 3.5.3.1.). A possible explanation for the latter finding came out of the work of Yavin et al (1974) who found that incorporated FA's underwent elongation in cultured rat brain cells and that such was maximal for 12:0 and decreased with increased FA chain length. The same group demonstrated that these cells were also able to synthesise 20:4ω6 from 18:2ω6 and convert 18:3ω3 and 20:5ω3 to 22:6ω3 (Yavin et al 1974, 1975). Similar results were reported by Dhopeshwarkar et al (1971), Sinclair et al (1972) and Anding et al (1986) in neonatal and adult rat brain <u>in vivo</u>. Cook et al (1978a) found that developing rat brain had the capacity to desaturate both 18:0 and EFA's, but Δ6D activity clearly appeared a rate limiting step in suckling rats, and its activity was hardly above background in the adult. Hassam et al (1975) showed that when suckling rats were orally fed radiolabelled 18:2ω6 or 18:3ω6, most 18:2ω6 activity remained unchanged, while 18:3ω6 radioactivity

had been incorporated into 20:3ω6 and 20:4ω6, in brain lipids. Sinclair (1975b) also reported that desaturation of 18:2ω6 was very slow after birth in rat brain. The present study used newborn rat pups, hence this may partly account for the relatively low desaturase cascade enzyme activity reported overall (Table 3.5.3.1.). It was unlikely, however, that such activity was suppressed primarily as a result of the growth limitation or cytotoxicity induced with p-oil dosage, since the capability for PDFA formation was limited/impaired even when p-oils had little effect on, or stimulated, cell viability. Desaturase cascade enzyme suppression may rather be a characteristic of this cell type *in vitro* under the present culture conditions. Our results, nevertheless, supported the finding of Novelot et al (1986) that the liver is implicated in desaturating and elongating FA's *in vivo*, and packaging such moieties for transport to the brain.

MDA measurement in rat brain cultures both in the absence and presence of dosed p-oils (Table 3.5.4.1.) supported the ability of these cells to oxidise FFA's, as others have shown (Pitas et al 1987). This reflected intact cellular enzymic and/or non-enzymic mechanisms for lipoperoxide generation when the lines of protection against FA oxidation were saturated, impaired or absent. Control cell lipid peroxides implied oxidation of endogenous unsaturated FA's released during PGL

turnover, whereas the enhanced lipoperoxide amounts measured in dosed cultures were consistent with oxidation of unsaturated p-oil FA's.

The abundance of saturated FA's in new incubation medium (Table 2.3.3.3.) and the ability of these cells to incorporate exogenous FA's (Table 3.5.3.1.) supported the likelyhood that most peroxides found in the spent media of dosed cultures originated intracellularly. Such were released as a result of cell death with 60mg/l p-MO or p-FO dosage, whereas the ability of cellular lipoperoxides to alter membrane permeability (Mead 1976, Tappel 1980 and Frankel 1984) implied their leakage through the plasma membrane of intact cells with incubation of p-oils which did not induce cytotoxicity at the concentrations studied. Cellular FA's and peroxidising enzymes could also have been released into the culture medium in the above processes, thus the possiblity that enzymic and/or spontaneous FA oxidation occurred extracellularly, and contributed to the lipid peroxides measured in the spent medium, can not be ignored. Such may explain the greater lipoperoxide amounts shown in the spent medium of cultures dosed with 40mg/l p-LO, p-EPO, p-FO, or 60mg/l p-oil, than in the cells themselves.

The finding that lipoperoxide generation increased overall with the amount of p-oil supplemented (Table 3.5.4.1.) reflected increased FA availability for

oxidation. However, the numerical differences in the lipoperoxide amounts reported with p-oil supplementation at a particular concentration reflected the different p-oil FA compositions (Table 2.3.3.2.). This related not only to the amount of unsaturated FA's present in any p-oil, but also to their susceptibility to oxidation, dependent on the number and position of double bonds in a FA. The susceptibility of 20:5ω3 to oxidation may indeed explain why cultures dosed with p-FO exhibited the largest lipid peroxide amounts measured overall. The greater cellular lipoperoxide concentrations found with dosage of p-CO than with p-LO or p-EPO, for example, was surprising considering the significantly greater unsaturated FA content of the latter p-oils. This suggested that the substrates for lipoperoxidation in the p-LO and p-EPO were not so readily accessible to oxidation, possibly because they were present as components of complex membrane lipids. It may also reflect a slower rate of PGL turnover and therefore FA release into the cellular pool from membrane lipids with p-LO and p-EPO. Alternatively, low cellular lipoperoxide levels may reflect their release into the culture medium. This supported the higher lipid peroxide amounts found in the spent medium with p-EPO, compared to in the cells with p-SSO, dosage; these lipoperoxides probably related mainly to oxidation of free 18:2ω6. p-EPO and p-SSO both exhibited similar FA compositions, thus the greater total lipoperoxide amounts induced with the

former may reflect the presence of about 9% 18:3ω6 in p-EPO only. This PDFA merely requires chain elongation to be usable as a substrate for enzymatic peroxidation, whereas 18:2ω6 requires desaturation first. The lipid peroxides formed with p-MO or p-FO supplementation could similarly have related partly to the utilisation of exogenous 20:4ω6 and 20:5ω3, respectively as direct endoperoxide substrates.

The observation that the cell viability changes reported in Figs. 3.5.1.2-3.5.1.8. with p-oil incubation were generally inversely related to the MDA amounts produced (Table 3.5.4.1.) suggested lipoperoxide involvement in the modulation of cell proliferation. Support for this was the fact that 60mg/l p-MO induced the greatest increase in cellular lipoperoxide amounts and the most pronounced cytotoxicity. However, the finding that the greatest lipoperoxide levels in the spent incubation media did not correlate with the greatest cytotoxicity indicated that the modulation of cell viability also related to other mechanisms, such as alterations in membrane fluidity. Indeed, the lack of significant ability to desaturate or elongate FA's overall implied that these cells were largely unable to redress membrane lipid FA composition and fluidity altered with exogenous FA incorporation, and that such was involved in the modulation of growth limitation and inhibition reported in Figs. 3.5.1.2-3.5.1.8.

3.6 THE EFFECTS OF PSEUDO-OILS ON CELLS DERIVED FROM RAT LUNG.

3.6.1 Effects of pseudo-Oils on Cell Viability.

Microscopic examination of control cells before, during and after each experiment showed no morphological changes at any time, and p-oil supplemented cells were morphologically similar to controls, although cytoplasmic droplets were observed with p-oil incubation at high concentrations in some instances.

The results depicted in Fig. 3.6.1.1. showed that final medium albumin concentrations ranging from 0 to 250mg/l had no significant effect on rat lung cell viability. The effects induced with the supplemented p-oils were therefore a result of the exogenous FA's and not the albumin used as FA carrier.

The number of control cells, seeded at $10x10^4$/ml, at the end of the 24 hour post-trypsinisation recovery period was $13.1x10^4$/ml. This equated to about 60% of the final control cell number at the end of the 48 hour incubation period, and represented the cytostatic number. Only p-oil amounts reducing cell viability to significantly below 60% were therefore considered growth inhibitory.

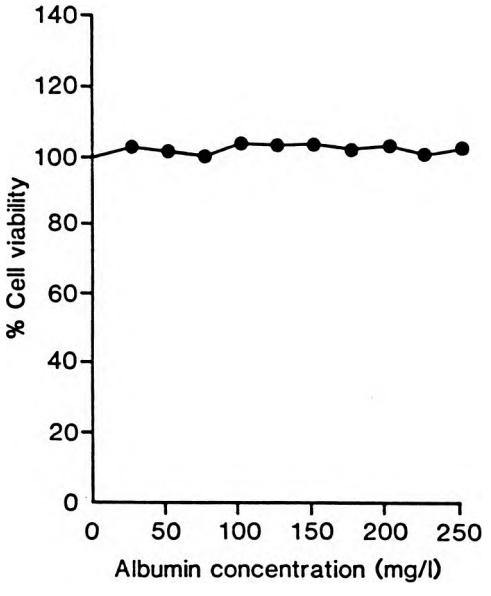
The results of the incubation of cells with p-oils are shown in Figs. 3.6.1.2-3.6.1.8. Overall, cell viability

Legend to Figs. 3.6.1.1-3.6.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.6.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.6.1.2-3.6.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.6.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.6.1.1.

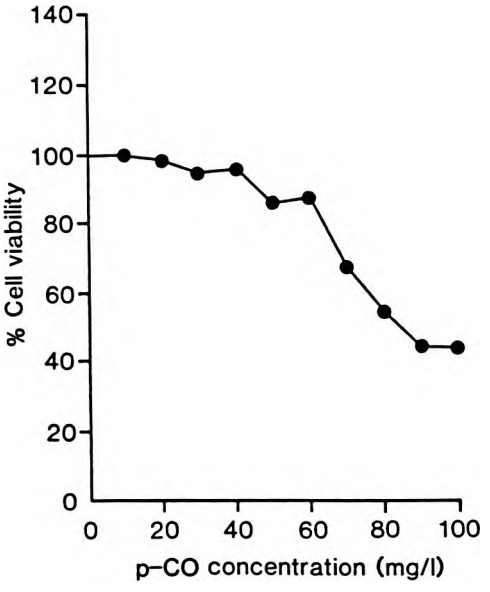
The percentage viability of cells derived from rat lung incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.6	12
25	103.2	4.0	12
50	101.1	5.3	12
75	100.0	4.9	12
100	104.7	5.5	12
125	103.3	3.8	12
150	104.5	3.9	12
175	102.1	3.1	12
200	104.7	4.2	12
225	100.9	2.3	12
250	103.7	5.1	12

Fig. 3.6.1.2.

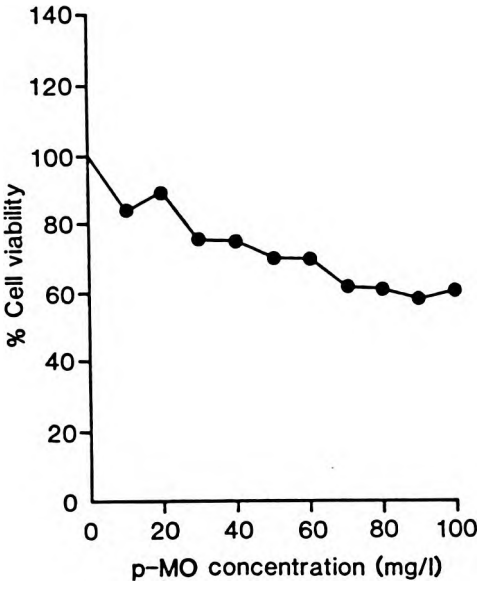
The percentage viability of cells derived from rat lung incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.7	6
10	100.0	2.5	6
20	97.8	2.3	6
30	94.2	2.9	6
40	95.6	2.9	6
50	85.4	2.5	6
60	87.3	2.7	6
70	67.2	2.2	6
80	54.3	1.2	6
90	44.3	1.6	6
100	43.3	1.5	6

Fig. 3.6.1.3.

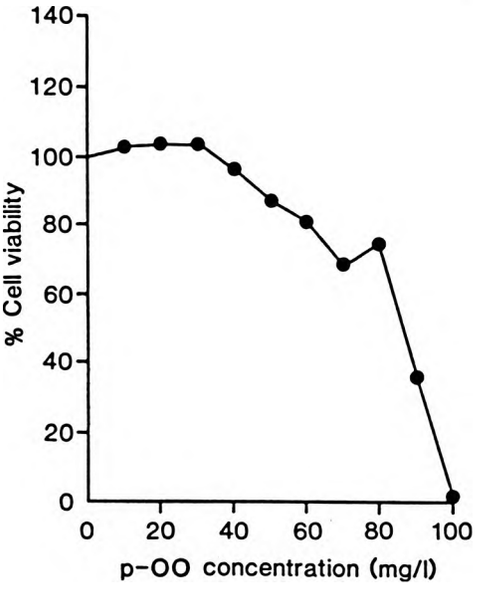
The percentage viability of cells derived from rat lung incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.3	6
10	84.0	4.1	6
20	89.3	2.5	6
30	75.7	1.4	6
40	74.7	4.7	6
50	69.6	4.0	6
60	70.4	2.9	6
70	61.4	4.7	6
80	61.1	2.9	6
90	58.3	1.1	6
100	60.6	1.4	6

Fig. 3.6.1.4.

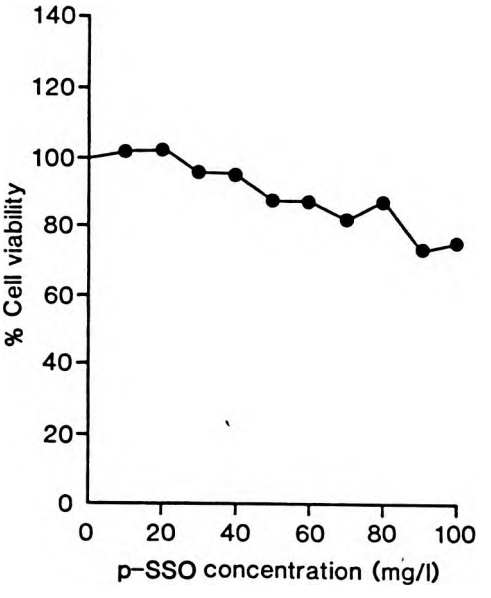
The percentage viability of cells derived from rat lung incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.8	6
10	102.2	3.9	6
20	103.8	1.8	6
30	103.4	5.6	6
40	96.3	2.4	6
50	86.8	7.4	6
60	81.1	4.7	6
70	68.5	2.4	6
80	74.6	5.6	6
90	35.9	2.9	6
100	0.8	0.5	6

Fig. 3.6.1.5.

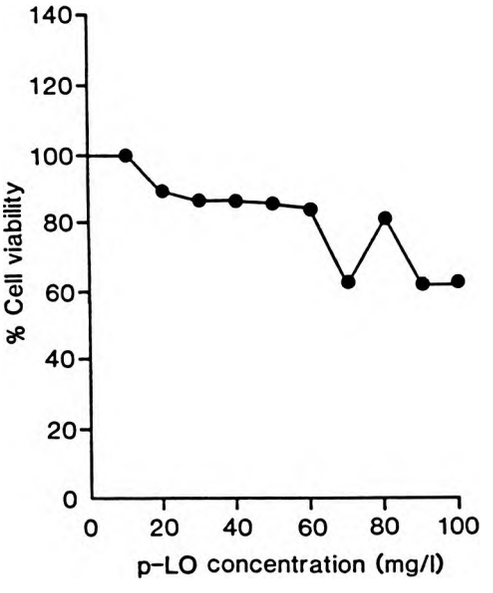
The percentage viability of cells derived from rat lung incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.8	6
10	101.8	4.4	6
20	102.4	2.8	6
30	94.6	4.1	6
40	93.7	3.4	6
50	87.1	4.2	6
60	86.5	4.0	6
70	80.7	4.7	6
80	86.7	5.9	6
90	72.9	4.7	6
100	74.5	2.4	6

Fig. 3.6.1.6.

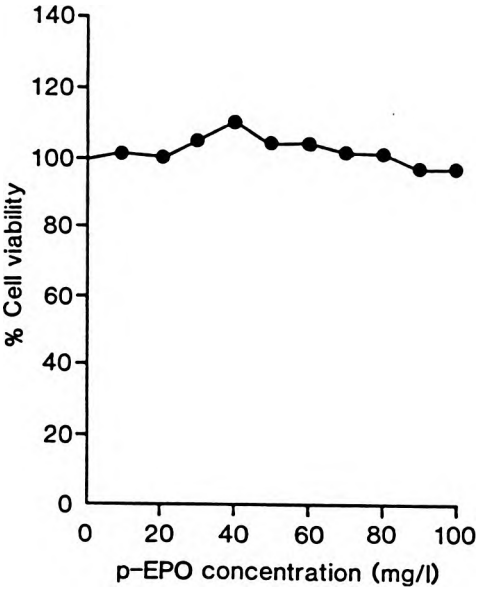
The percentage viability of cells derived from rat lung incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.5	6
10	99.8	1.5	6
20	89.4	2.2	6
30	86.3	2.1	6
40	87.1	1.3	6
50	86.1	2.0	6
60	83.5	2.2	6
70	63.0	1.9	6
80	80.8	2.9	6
90	62.1	3.0	6
100	62.6	2.5	6

Fig. 3.6.1.7.

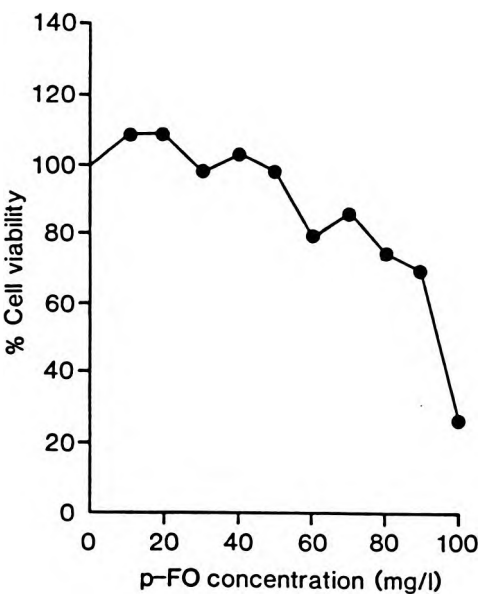
The percentage viability of cells derived from rat lung incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.9	6
10	101.4	1.5	6
20	99.5	1.8	6
30	105.1	2.8	6
40	110.4	2.7	6
50	104.2	1.8	6
60	105.0	1.9	6
70	101.1	2.2	6
80	101.2	1.3	6
90	96.6	1.6	6
100	97.7	1.7	6

Fig. 3.6.1.8.

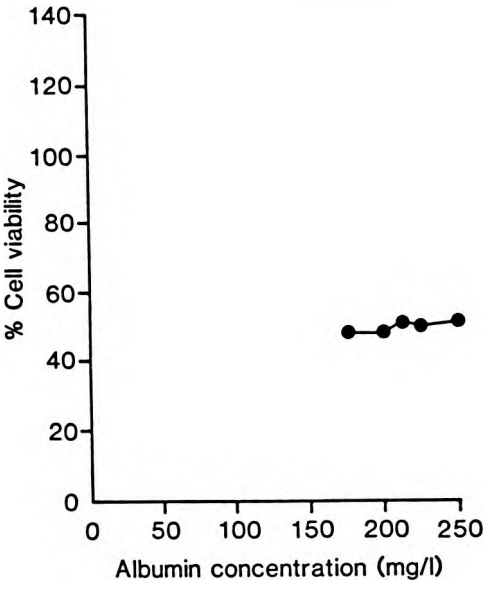
The percentage viability of cells derived from rat lung incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.1	6
10	111.0	4.7	6
20	109.6	4.2	6
30	98.3	4.3	6
40	104.4	5.8	6
50	98.5	3.9	6
60	80.4	3.6	6
70	87.9	5.8	6
80	75.4	4.1	6
90	69.8	4.3	6
100	26.2	4.6	6

Fig. 3.6.1.9.

The percentage viability of cells derived from rat lung incubated with albumin at the ID_{50} of p-00.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.6	6
175	49.1	2.8	6
200	48.4	2.1	6
212	51.6	3.2	6
225	50.5	4.6	6
250	52.6	2.7	6

was stimulated only with low amounts of p-EPO and p-FO, all p-oils besides p-EPO limited cell proliferation to a greater or lesser extent, and cytotoxicity was induced only with high concentrations of p-CO, p-FO and p-OO.

p-CO concentrations up to 40mg/l had little effect on cell viability, whereas 50 to 70mg/l p-CO limited cell proliferation, and greater amounts were cytotoxic (Fig. 3.6.1.2.). Half the cells were viable with 85mg/l p-CO (ID_{50}) and 43.3% with 100mg/l p-CO. On the other hand, p-MO induced concentration dependent growth limitation with amounts up to 60mg/l, and cytostasis with 70 to 100mg/l p-MO (Fig. 3.6.1.3.), therefore no ID_{50} could be calculated.

Concentrations up to 40mg/l p-OO induced no significant change in cell viability, but 50 to 80mg/l p-OO limited cell proliferation to a minimum of 68.5% with 70mg/l p-OO (Fig. 3.6.1.4.). 50% of cells were killed with about 86mg/l p-OO (ID_{50}), but higher amounts induced the greatest cytotoxicity observed and practically all cells took up Trypan blue with 100mg/l p-OO.

Dosage with up to 30mg/l p-SSO had little effect on cell proliferation, but greater amounts progressively limited the viable cell yield in an overall concentration dependent manner to 72.9% and 74.5% with 90 and 100mg/l p-SSO, respectively (Fig. 3.6.1.5.). Cell proliferation with 10mg/l p-LO mirrored controls, but ranged from

83.5% to 89.4% with 20 to 60mg/l p-LO (Fig. 3.6.1.6.). Higher p-LO concentrations induced cytostasis and were therefore slightly more growth limiting than p-SSO, although 80.8% viability occurred with 80mg/l p-LO.

30 to 60mg/l p-EPO stimulated cell proliferation to a maximum of 110.4% with 40mg/l, but other concentrations had little effect on cell viability, and no growth limitation was observed (Fig. 3.6.1.7.).

10 and 20mg/l p-FO enhanced cell proliferation to 111.0% and 109.6%, respectively, 30 to 50mg/l p-FO had little effect on cell viability, and higher amounts limited cell growth to a minimum of 69.8% with 90mg/l p-FO (Fig. 3.6.1.8.). Half the cells were viable with 95mg/l p-FO (ID_{50}), but only 26.2% with 100mg/l p-FO.

Rat lung cells were incubated with an amount of p-OO corresponding to its ID_{50} (approximately 86mg/l), and dosed with varying albumin concentrations to exclude the possibility that any effects induced with p-oil dosage were influenced by the amount of albumin bound to the FA's. Five points were chosen around which to vary the albumin concentration, keeping the p-oil concentration fixed, viz. two points above, two points below, and the ID_{50} of p-OO. The results are depicted in Fig. 3.6.1.9., and indicated that the p-OO concentration incubated reduced cell viability to approximately 50% of controls, and that such was not significantly influenced by

different albumin concentrations. Exclusion of the synergistic involvement of albumin thus implied that the effects reported in Figs. 3.6.1.2-3.6.1.8. were solely attributed to the exogenous FA's.

Cells were subsequently plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in amounts sufficient to permit all quantitative and qualitative analyses to be carried out. Cell viabilities relative to controls were compared upon harvesting and found to be statistically similar to those in Figs. 3.6.1.1-3.6.1.8. Hence, all further biochemical assays were performed on these samples.

3.6.2 Effects of pseudo-Oils on Total Protein.

The results derived from the quantitation of total protein in control and p-oil dosed cells are shown in Table 3.6.2.1.

176.5µg protein/10^6 control cells seeded was measured, while concentrations in dosed cells ranged from 158.5 to a maximum of 247.5µg protein/10^6 cells seeded (obtained with 60mg/l p-MO and 20mg/l p-OO, respectively). Cells contained protein amounts equal to, or greater than, controls when 20mg/l p-oil (except p-EPO) or 40mg/l p-oil were dosed (176.9 to 247.9µg/10^6 cells seeded), while concentrations were increased with 60mg/l p-EPO or p-OO (209.5 and 199.1µg/10^6 cells seeded, respectively), parallel to controls with 60mg/l p-SSO (176.8µg/10^6 cells seeded) and slightly decreased with 60mg/l p-CO, p-MO, p-LO or p-FO (158.5 to 165.6µg/10^6 cells seeded). The changes induced in total cellular protein were p-oil concentration dependent as protein levels increased in direct correlation with the amount of p-EPO dosed, but were inversely related to the p-CO, p-MO or p-LO concentration supplemented. With p-SSO or p-FO dosage, however, the highest protein levels measured were with a concentration of 40mg/l, but the reverse was true with p-OO supplementation.

Table 3.6.2.1.

The protein content of cells derived from rat lung, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	176.5			
CO		189.0	184.4	163.2
MO		187.1	182.0	158.5
OO		247.9	186.7	199.1
SSO		204.0	213.9	176.8
LO		186.7	176.9	160.0
EPO		167.9	203.5	209.5
FO		182.0	187.7	165.6

3.6.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Cells Derived From Rat Lung.

The FA spectra of cells incubated with 20, 40 or 60mg/l p-oil are shown in Table 3.6.3.1. in relation to undosed cells. The greatest proportion of any FA in controls was 16:0 (23.4%), but FA's contributing at least 5.0% to the total spectrum in controls included 18:0 (18.4%), 18:1ω9 (15.9%), 20:4ω6 (11.8%) and 22:4ω6 (9.0%). Significant proportions of these moieties were also detected in dosed cells.

In general, dosed cells contained 16:0 and 18:1ω9 proportions parallel to controls, but decreased 18:0 and more varied 16:1ω9 percentages. 18:2ω6, 20:2ω6, 18:3ω6, 22:4ω6, 18:3ω3 and 20:4ω3 levels were generally equal to, or greater than, controls, and 18:4ω3 amounts were consistently increased with 40mg/l p-oil. The relative percentages of 20:5ω3 and 22:5ω3 were more variable, but 22:5ω6 and 22:6ω3 levels were generally lower in dosed cells.

16:0 percentages were parallel to controls (23.4%) with p-CO incubation, 16:1ω9 was slightly increased with 20 and 40mg/l p-CO and decreased with 60mg/l p-CO (4.1%, 4.2% and 2.4%, respectively vs 3.5% in controls), while 18:0 levels were marginally reduced (15.8%, 15.2% and 13.8%, respectively vs 18.4% in controls). p-CO had no marked effect on 18:1ω9 and 18:2ω6 levels, but 20:4ω6

Legend to Table 3.6.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.6.3.1.

The fatty acid spectrum of cells derived from rat lung.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	23.4±0.15	23.8	20.9	25.1	23.1	20.3	27.1	18.3	20.3	22.2	23.4	18.7	12.5	24.6	19.6	26.5	22.4	18.9	26.8	26.8	20.4	23.2
	18:0	18.4±0.30	15.8	15.2	13.8	13.0	15.4	16.5	12.9	12.4	11.3	19.6	14.6	25.2	15.9	15.6	14.1	16.6	15.8	15.2	19.2	14.6	10.7
	20:0	0.1±0.00	-	0.1	-	-	-	-	-	-	-	-	0.2	3.2	-	-	-	-	-	-	-	0.1	-
	22:0	-	0.2	-	0.3	-	0.1	0.1	0.1	-	-	0.1	-	-	0.1	0.1	0.2	-	0.1	0.1	-	0.1	0.1
	24:0	0.2±0.00	-	0.7	-	-	-	-	-	-	-	-	0.2	-	-	0.6	-	-	0.8	-	-	0.7	-
ω9 MONOS.	16:1	3.5±0.05	4.1	4.2	2.4	11.2	2.9	-	0.9	1.0	3.8	4.6	3.0	1.1	6.6	1.4	4.6	4.9	1.4	4.8	6.8	5.9	8.3
	18:1	15.9±0.10	16.0	14.8	18.0	17.8	17.8	21.6	24.8	31.2	35.6	18.5	14.3	22.7	14.9	14.8	18.2	14.4	12.7	16.4	17.8	14.9	15.7
	24:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	4.0±0.10	5.2	3.7	6.3	7.3	7.7	9.3	8.0	7.4	8.7	14.1	15.4	20.3	7.5	8.1	9.1	16.3	19.3	15.3	7.4	5.8	7.5
	18:3	-	0.7	-	0.7	0.5	-	0.8	0.6	-	0.6	1.8	0.1	-	0.7	-	1.0	-	-	1.1	0.6	-	0.4
	20:2	-	-	-	-	-	-	0.1	0.3	1.1	0.9	0.2	0.9	1.2	-	-	-	-	0.8	0.1	-	0.3	0.3
	20:3	2.0±0.00	1.7	2.0	1.7	1.3	1.4	1.0	1.5	1.3	1.0	1.0	1.8	0.7	1.3	2.1	1.2	2.4	5.2	1.7	0.9	1.5	1.1
	20:4	11.8±0.20	8.2	9.7	7.3	6.8	8.6	6.2	8.5	6.6	3.9	5.0	6.5	2.0	5.3	7.3	4.5	6.7	5.7	3.7	5.2	6.7	3.8
	22:4	9.0±0.65	14.7	12.3	13.0	10.9	13.6	9.4	13.4	9.8	4.9	4.3	12.0	2.7	12.9	12.8	9.3	8.2	6.1	9.5	7.8	10.2	10.5
	22:5	1.3±0.10	0.2	1.5	0.2	0.2	1.5	0.2	0.1	0.8	-	0.4	1.0	0.2	0.1	1.4	0.2	0.8	0.8	0.1	0.2	0.8	0.1
ω3 POLYS.	18:3	0.5±0.05	0.8	0.6	1.0	0.7	0.6	1.0	1.2	1.0	1.3	1.3	0.6	4.0	2.2	4.9	6.2	-	0.5	0.9	0.5	0.5	0.7
	18:4	0.5±0.00	0.3	1.9	0.5	0.3	2.2	0.3	0.3	1.3	0.2	0.3	1.0	1.2	0.3	1.9	0.3	-	1.7	0.2	0.3	1.4	0.4
	20:4	0.7±0.00	1.0	1.0	1.7	1.4	0.7	1.3	1.1	0.4	0.6	0.1	1.0	0.4	1.4	0.7	1.4	0.3	0.8	1.0	0.6	1.0	1.9
	20:5	1.2±0.05	1.3	2.7	1.0	0.9	1.4	0.5	1.6	0.2	0.8	0.5	1.3	0.2	0.9	1.9	0.4	0.1	1.1	0.3	1.2	4.5	5.3
	22:5	3.1±0.15	2.6	3.7	2.9	2.1	2.6	2.1	2.8	2.4	1.8	2.2	3.4	1.2	2.4	3.2	1.5	2.9	3.8	1.3	2.9	7.8	7.5
	22:6	4.3±0.20	3.5	5.1	4.2	2.5	3.3	2.5	3.8	3.0	2.4	2.6	4.3	1.4	3.1	3.9	1.5	4.0	4.7	1.4	1.9	3.0	2.7

percentages were at least 2.0% lower than controls (11.8%), whereas 22:4ω6 levels were 3.0% to 6.0% greater than controls (9.0%). However, no significant increase in 22:5ω6 was found. 18:4ω3, 20:5ω3, 22:5ω3 and 22:6ω3 amounts increased only with 40mg/l p-CO (1.9%, 2.7%, 3.7% and 5.1% vs 0.5%, 1.2%, 3.1% and 4.3% in controls, respectively), but 20:4ω3 was increased slightly with all concentrations (1.0% to 1.7% vs 0.7% in controls).

20.3% to 27.1% 16:0 was detected with p-MO dosage (23.4% in controls), whereas 16:1ω9 levels were significantly increased with 20mg/l p-MO, but decreased with 40 and 60mg/l p-MO (11.2%, 2.9% and 0%, respectively vs 3.5% in controls). p-MO induced a slight decrease in 18:0 (13.0% to 16.5% vs 18.4% in controls), but an increase in 18:1ω9 (17.8% to 21.6% vs 15.9% in controls) and 18:2ω6 (7.3% to 9.3% vs 4.0% in controls), percentages. 20:4ω6 levels were lower than controls (11.8%), and a greater proportion of its elongation product, 22:4ω6, was found (up to 13.6% vs 9.0% in controls), but 22:5ω6 levels were parallel to controls (1.3%) or decreased. The only ω3 PUFA's that were significantly increased were 18:4ω3 (2.2% with 40mg/l p-MO vs 0.5% in controls) and 20:4ω3 (1.4% and 1.3% with 20 and 60mg/l p-MO, respectively vs 0.7% in controls).

18:1ω9 levels increased significantly with dosage of 20, 40 or 60mg/l p-OO (24.8%, 31.2% and 35.6%, respectively

vs 15.9% in controls), whereas 16:0, 18:0 and 16:1ω9 proportions were parallel to controls (23.4%, 18.4% and 3.5%, respectively), or decreased. Raised percentages of 18:2ω6 (7.4% to 8.7% vs 4.0% in controls) and 20:2ω6 (0.3% to 1.1% vs 0% in controls) were found, but 20:4ω6 levels were lower than controls (11.8%). However, 22:4ω6 was significantly raised with 20mg/l p-OO (13.4% vs 9.0% in controls), yet 22:5ω6 levels were significantly lower than controls (1.3%). 18:3ω3 increased at least 2 fold with p-OO dosage (1.0% to 1.3% vs 0.5% in controls), and 40mg/l p-OO raised 18:4ω3 percentages (1.3% vs 0.5% in controls), but other ω3 FA levels were only marginally raised, or decreased.

16:0 and 16:1ω9 percentages decreased inversely with the amount of p-SSO dosed (23.4% to 12.5% and 4.6% to 1.1% vs 23.4% and 3.5% in controls, respectively), but 18:0, 20:0 and 18:1ω9 proportions increased significanty with 60mg/l p-SSO (25.2%, 3.2% and 22.7% vs 18.4%, 0.1% and 15.9% in controls, respectively). p-SSO supplementation raised cellular 18:2ω6 amounts (14.1% to 20.3% vs 4.0% in controls), and induced small increments in 18:3ω6, 22:4ω6 and 20:2ω6 with concentrations of 20, 40 and 60mg/l, respectively (1.8%, 12.0% and 1.2% vs 0%, 9.0% and 0% in controls, respectively). Up to 4.0% 18:3ω3 and 1.2% 18:4ω3 were detected (0.5% each in controls), but other ω3 PUFA levels were parallel to controls, or decreased.

Cells dosed with p-LO exhibited 16:0 and 18:1ω9 levels similar to controls (23.4% and 15.9%, respectively), marginally decreased 18:0 (14.1% to 15.9% vs 18.4% in controls), and varied 16:1ω9 amounts (1.4% to 6.6% vs 3.5% in controls). A concentration dependent increase in 18:2ω6 incorporation (7.5% to 9.1% vs 4.0% in controls), and up to 1.0% 18:3ω6 (none in controls), were detected, and 22:4ω6 was increased with 20 and 40mg/l p-LO (12.9% and 12.8%, respectively vs 9.0% in controls). 18:3ω3 incorporation increased from 0.5% in controls to 2.2%, 4.9% and 6.2% when 20, 40 or 60mg/l p-LO were added to cultures, respectively. Increased 18:4ω3 and 20:5ω3 were detected with 40mg/l p-LO (1.9% each vs 0.5% and 1.2% in controls, respectively) and 20:4ω3 with 20 and 60mg/l p-LO (1.4% vs 0.7% in controls), yet 22:5ω3 and 22:6ω3 levels were parallel to controls, or reduced.

p-EPO dosage had no pronounced effect on cellular 16:0 and 18:1ω9 levels, but decreased 18:0 marginally (15.2% to 16.6% vs 18.4% in controls), and had a more variable effect on 16:1ω9 (1.4% to 6.6% vs 3.5% in controls). 18:2ω6 increased from 4.0% in controls to 16.3%, 19.3% and 15.3% with 20, 40 and 60mg/l p-EPO, respectively, and 1.1% 18:3ω6 was detected with 60mg/l p-EPO, but none in controls, with 20 or 40mg/l p-EPO. 20:3ω6 increased significantly with dosage of 40mg/l p-EPO (5.2% vs 2.0% in controls), but no such changes were shown for other ω6 PUFA's. With the exception of slight increments in

18:4ω3, 22:5ω3 and 22:6ω3 detected with 40mg/l p-EPO, ω3 FA levels were either parallel to controls or decreased.

16:0 and 18:1ω9 levels were not markedly altered with p-FO incubation, whereas 18:0 was decreased overall, and 16:1ω9 significantly increased (5.9% to 8.3% vs 3.5% in controls). 5.8% to 7.5% 18:2ω6 was detected (4.0% in controls), and up to 10.5% 22:4ω6 (9.0% in controls), but 22:5ω6 levels were decreased. A maximum of 1.4% 18:4ω3 was detected with 40mg/l p-FO and 1.9% 20:4ω3 60mg/l p-FO (0.5% and 0.7% in controls, respectively), whereas 20:5ω3 was significantly increased both with 40 and 60mg/l p-FO (4.5% and 5.3%, respectively vs 1.2% in controls). 22:5ω3 increased by at least two fold with 40 and 60mg/l p-FO (7.8% and 7.5%, respectively vs 3.1% in controls), yet 22:6ω3 was decreased (1.9% to 3.0% vs 4.3% in controls).

3.6.4 Incorporation of Radiolabelled C18 Fatty Acids into Cells Derived from Rat Lung.

The radioactivity recovered from cultures incubated with 2μCi of each of the radiolabelled C18 FA's is shown in Table 3.6.4.1.

The total counts recovered in the spent incubation media ranged from 2.4 to 3.0×10^6cpm, with only 0.5 to 0.8×10^6 cpm recovered from the pooled buffers after washing the cells; this accounted for non-specific binding. For both [^{14}C]-18:1ω9 and [^{14}C]-18:3ω3, the equivalent of 4.3×10^6cpm were dosed to the incubation medium. Total recovery of radiolabel from cultures dosed with these FA's was approximately 84% and 91%, respectively. For [^{14}C]-18:2ω6 the equivalent of 4.8×10^6cpm were dosed to the incubation medium, and total recovery of this radioisotope from the cultures was about 96%. The total amount of each of these radioisotopes incorporated into the cells was approximately 13% for [^{14}C]-18:1ω9, 15% for [^{14}C]-18:2ω6, and 7% for [^{14}C]-18:3ω3.

The percentage conversion of each of the incorporated radiolabelled C18 FA's above to ω9, ω6 or ω3 series products after 48 hours incubation with rat lung fibroblasts is shown in Table 3.6.4.2. As Δ6D represents the first enzyme in the desaturase cascade, total activity of this enzyme is reflected by the sum of all subsequent desaturase and elongase products.

Table 3.6.4.1.

The radioactivity (cpm) recovered from rat lung cultures incubated with radiolabelled C18 fatty acids, expressed as mean (±s.e.m.), n=3.

SPENT MEDIA (M)	POOLED WASHINGS (W)	TOTAL (M+W)	CELLS	TOTAL $\times 10^6$
Total 18:1 counts dosed to incubation medium = 4.3 x 10^6				
2350600 (±26420)	664370 (±14630)	3014970	548552 (±1989)	3.6
Total 18:2 counts dosed to incubation medium = 4.8 x 10^6				
3043260 (±18423)	784740 (±7191)	3828000	734574 (±1176)	4.6
Total 18:3 counts dosed to incubation medium = 4.3 x 10^6				
3044360 (±36020)	521690 (±5265)	3566050	304473 (±1779)	3.9

Table 3.6.4.2.

The counts detected for the radiolabelled fatty acids dosed and their resulting metabolites formed after incubation with cells derived from rat lung, expressed as a percentage of the total counts recovered upon GLC analysis.

ω9 MONOS.	[^{14}C]-18:1ω9	ω6 POLYS.	[^{14}C]-18:2ω6	ω3 POLYS.	[^{14}C]-18:3ω3
18:1	12.7	18:2	4.8	18:3	3.4
20:1	34.5	18:3	17.2	18:4	22.8
22:1	37.9	20:2	24.2	20:3	14.7
24:1	15.0	20:3	25.5	20:4	18.7
		20:4	14.7	20:5	19.0
		22:4	7.2	22:5	11.1
		22:5	6.5	22:6	10.3
TOTAL CPM	1107	TOTAL CPM	1865	TOTAL CPM	804

12.7% of the total [^{14}C]-18:1ω9 counts derived upon GLA analysis (1107cpm) remained as 18:1ω9, whereas 34.5%, 37.9% and 15.0% were elongated to 20:1ω9, 22:1ω9 and 24:1ω9, respectively.

Only 4.8% of the total [^{14}C]-18:2ω6-derived counts measured (1865cpm) remained unchanged, whereas 17.2% was shown to be 18:3ω6. 24.2% and 25.5% of the total counts were found as 20:2ω6 and 20:3ω6, respectively, compared to 14.7% 20:4ω6. Furthermore, 7.2% and 6.5% 22:4ω6 and 22:5ω6 were detected, respectively.

Only 3.4% of total counts (804cpm) remained as 18:3ω3, and 14.7% was found as the immediate elongation product, 20:3ω3. 22.8% 18:4ω3 was detected, compared to 18.7% 20:4ω3 and 19.0% 20:5ω3. 11.1% of total [^{14}C]-18:3ω3-derived counts were shown to be 22:5ω3 and 10.3% 22:6ω3, and these percentages were larger than those found for corresponding products in the ω6 series.

3.6.5 Effects of pseudo-Oils on Lipid Peroxide Formation.

Table 3.6.5.1. shows the lipoperoxides quantitated in dosed rat lung fibroblasts and their respective spent incubation media, compared to controls. The results are expressed as nmoles MDA/10^6 cells, but in the case of the spent media this represents the nmoles of MDA in the volume of medium from which $1x10^6$ cells were obtained.

Spent medium derived from cultures supplemented with 60mg/l p-SSO, 60mg/l p-FO, 40mg/l p-LO, or p-60mg/l p-LO contained 0.3, 0.6, 0.1 and 3.7nmoles MDA/10^6 cells, respectively, but MDA was absent from all other spent media. However, control and all dosed cells formed MDA. 1.1nmoles MDA/10^6 control cells was measured, whereas concentrations in dosed cells ranged from a low of 1.0 nmole MDA/10^6 cells (with 20mg/l p-SSO and p-EPO) to a maximum of 10.8nmoles MDA/10^6 cells (with 60mg/l p-EPO). Cellular lipoperoxide amounts varied with the p-oil supplemented, but increased in an overall concentration dependent manner. p-OO and p-SSO produced the smallest cellular lipoperoxide amounts overall, ranging from 1.0 to 1.3nmoles MDA/10^6 cells, whereas p-CO and p-MO generated 1.4 to 2.1nmoles MDA/10^6 cells. Cells dosed with 20mg/l p-LO, p-EPO or p-FO contained no more MDA than controls, but p-LO and p-FO generated more MDA than p-EPO with a concentration of 40mg/l (1.9, 2.1 and

Table 3.6.5.1.

Lipoperoxide formation by rat lung cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	1.1			
CO		1.4	1.4	1.5
MO		1.6	1.7	2.1
OO		1.2	1.2	1.3
SSO		1.0	1.1	1.3
LO		1.1	1.9	7.2
EPO		1.0	1.2	10.8
FO		1.1	2.1	2.6
pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	-
MO		-	-	-
OO		-	-	-
SSO		-	-	0.3
LO		-	0.1	3.7
EPO		-	-	-
FO		-	-	0.6

1.2nmoles MDA/10^6 cells, respectively). p-EPO, however, induced the greatest cellular lipoperoxide amounts with a concentration of 60mg/l, followed by p-LO and p-FO (10.8, 7.2 and 2.6 nmoles MDA/10^6 cells, respectively).

3.6.6 The Eicosanoid Profile of Cells Derived from Rat Lung.

Table 3.6.6.1. shows the eicosanoids positively detected in control and dosed rat lung fibroblasts. All data is presented as a percentage of the total area quantitated, but an indication of the actual amount found is given for controls in '( )' as pmoles total eicosanoids/10^6 cells.

Total eicosanoid production by control cells amounted to 727.6pmoles/10^6 cells, of which 22.0% correlated with the retention times of the prostanoid standards used. This fraction was shown to be composed of 11.9% PGE_1, 1.6% PGI_2, 3.0% TXB2, 4.2% PGF_α and 1.3% PGE2. Varying proportions of these prostanoids were found in dosed cells, but neither control nor dosed cells formed PGD_2.

The percentage of total prostanoids detected in dosed cells ranged from 15.8% (with 40mg/l p-MO) to a maximum of 28.5% (with both 60mg/l p-MO and p-LO), compared to 22.0% in controls, but no consistent correlation was evident between total eicosanoid percentages and p-oil concentration. p-Oil supplementation, however, altered the production of individual prostanoids in these cells. PGI_2 levels were lower in dosed cells than in controls (0.3% to 1.0% vs 1.6%, respectively), and no particular p-oil concentration consistently induced greater PGI_2 percentages than any other concentration. Cellular TXB_2

Legend to Table 3.6.6.1.

Values are tabulated as relative percent of the total area detected, and as pmoles total eicosanoids/10^6 control cells.

Table 3.6.6.1.

The profile of the eicosanoids detected in extracts of cells derived from rat lung.

pseudo-Oil (mg/l)		EICOSANOIDS (%)						
		TOTAL	PGI_2	TXB_2	$PGF_{\alpha(1+2)}$	PGE_2	PGE_1	PGD_2
CONTROL (727.6)		22.0	1.6	3.0	4.2	1.3	11.9	-
CO	20	25.4	0.9	14.6	8.5	1.2	0.2	-
	40	23.6	0.8	4.3	6.6	-	11.9	-
	60	24.3	0.5	14.9	7.4	1.2	0.3	-
MO	20	24.9	0.7	14.9	7.9	1.2	0.2	-
	40	15.8	1.0	3.2	4.2	-	7.4	-
	60	28.5	0.3	17.4	8.7	1.8	0.3	-
OO	20	21.0	0.7	11.0	8.1	1.1	0.1	-
	40	26.1	1.0	4.3	4.4	-	16.4	-
	60	26.5	0.3	14.9	9.7	1.1	0.5	-
SSO	20	26.6	0.3	15.5	9.1	1.5	0.2	-
	40	24.8	0.9	4.3	3.4	-	16.2	-
	60	27.5	0.4	15.9	8.0	2.1	1.1	-
LO	20	22.2	0.7	11.8	8.0	1.5	0.2	-
	40	18.1	0.7	3.7	4.3	-	9.4	-
	60	28.5	0.5	16.1	9.2	2.1	0.6	-
EPO	20	24.1	0.5	13.1	8.8	0.8	0.9	-
	40	20.1	0.7	5.1	3.1	-	11.2	-
	60	26.9	0.4	15.0	7.9	1.4	2.2	-
FO	20	28.3	0.9	17.0	8.7	1.5	0.2	-
	40	19.1	0.8	4.0	1.7	-	12.6	-
	60	20.7	0.8	12.3	5.7	1.2	0.7	-

percentages were comparable to controls (3.0%) with 40mg/l p-oil (3.2% to 5.1%), but were increased between 3 and 6 fold with 20 and 60mg/l p-oil (11.0% to 17.4%). PGF_{α} production was also enhanced with dosage of 20mg/l (7.9% to 9.1%) or 60mg/l (5.7% to 9.7%) p-oil, but more variable with 40mg/l p-oil (1.7% to 6.6%), compared to controls (4.2%). Incubation with 20 or 60mg/l p-oil had no profound effect on PGE_2 levels (0.8% to 2.1% vs 1.3% incontrols), but such was inhibited with 40mg/l p-oil. PGE_1 percentages, in contrast, were comparable in cells dosed with 0 or 40mg/l p-oil (11.9% vs 7.4% to 16.2%), and markedly decreased both with 60mg/l (0.3% to 2.2%) and 20mg/l (0.1% and 0.9%) p-oil.

3.6.7 Discussion.

It was apparent from lack of literature that very little is known regarding the effects of exogenous FA's on normal rat lung fibroblast growth. The present study indicated that p-oil supplementation affected the viability of such cells (Figs. 3.6.1.2-3.6.1.8.), and that this was dependent both on the p-oil concentration dosed and p-oil FA composition (Table 2.3.3.2.). The mediation of cytotoxicity only with p-OO, p-FO or p-CO dosage at concentrations greater than 80mg/l may relate partly to the relatively low population doubling time these cells exhibited (Table 2.3.1.1.). Furthermore, the ability of cultured rat lung fibroblasts to withstand large amounts of PUFA-rich p-oils without being killed may relate to the finding that this tissue has been implicated as the primary site for eicosanoid synthesis (Mathe et al 1977, Hyman et al 1978 and Harper et al 1984), the precursors of which are PUFA's.

OA, LA or ALA comprised about 71%, 70% and 63% of all FA's in p-OO, p-SSO and p-LO, respectively, thus it was likely that the cell viability changes induced with incubation of either p-oil related largely to these C18 FA's. Such moieties were all Δ6D substrates, but the variation in the number and position of their double bonds could explain the mediation of cytotoxicity with high p-OO concentrations, but not with p-SSO or p-LO.

The similar extent of growth limitation induced overall with p-SSO or p-LO dosage, however, implied that ALA and LA modulated similar effects in these cells, which may relate to the fact that both moieties are EFA's and exhibit a double bond at the ω6 position, unlike OA. On the other hand, the similar FA compositions of p-SSO and p-EPO suggested that the inability to limit cell viability with dosage of up to 100mg/l p-EPO related to the presence of 9% GLA only in this p-oil. Such may be important for optimal rat lung fibroblast proliferation, or may suppress the growth limiting capability of other FA constituents in p-EPO, particularly LA. The finding that the greatest enhancement of cell viability occurred with incubation of p-oils containing significant PDFA amounts (9% GLA in p-EPO and 18% EPA in p-FO) indeed implied involvement of these moieties in the modulation of rat lung fibroblast proliferation. Synergistic and antagonistic interations between FA's in any p-oil should, however, not be overlooked as mediators of the growth responses induced. This may explain why cell viability did not always relate inversely to the p-oil concentration dosed and why p-oils of similar FA composition induced different effects.

The data in Table 3.6.3.1. supported the ability of rat lung fibroblasts to incorporate exogenous FA's, which modified dosed cell FA compositions to a greater or lesser extent compared to controls. There is substancial

evidence to indicate that dietary FA supplementation induces changes in membrane physical properties (eg. King et al 1971, Ginsberg et al 1981, Simon et al 1982, Stubbs et al 1984 and Spector et al 1985). A possible mechanism whereby p-oils were involved in the reduction of cell viability reported may therefore relate to differential uptake of exogenous FA's and alterations in membrane fluidity, dependent on p-oil FA composition. Furthermore, incubation with low p-oil concentrations could have allowed the cells to maintain a similar degree of membrane fluidity to controls, whereas larger p-oil amounts could have led to more pronounced changes in the membrane unsaturation index such that cell viability was limited or inhibited.

The finding that the cellular protein concentrations reported with p-oil supplementation (Table 3.6.2.1.) did not always correlate with the cell viability changes induced (Figs. 3.6.1.2-3.6.1.8.) implied FA involvement in the modulation of protein synthesis. Increased protein levels when cell viability was parallel to controls or limited indeed indicated that protein synthesis was stimulated, whereas the finding that decreased cell numbers alone could not justify the lowest amount of protein measured with 60mg/l p-MO implied suppression of protein synthesis. Since the modulation of protein synthesis varied in magnitude with dosage of identical amounts of different p-oils, even

when such yielded similar cell numbers, it was suggested that the capability to enhance or suppress protein synthesis varied with FA, and was relatable to FA structure and concentration in any p-oil. Absolute changes in cellular protein with p-oil dosage may nevertheless relate to alterations in the production of structural membrane proteins and/or enzymes involved in FA metabolism.

The lung has been implicated as the primary site for eicosanoid synthesis (Mathe et al 1977, Hyman et al 1978 and Harper et al 1984), thus its ability to desaturate and elongate FA's to provide eicosanoid precursors is of major importance. Comparison of control cell FA spectra with those derived from p-oil dosed cells indicated the capability of cultured rat lung fibroblasts to express desaturase and elongase enzymes (Table 3.6.3.1.). The raised 16:1ω9 levels found in dosed cells implied 16:0 desaturation, and the possibility for 16:1ω9 elongation also existed. In addition, the significantly decreased 16:0 and increased 18:0, 20:0 and 18:1ω9 levels detected with 60mg/l p-SSO dosage supported the capability for elongase and Δ9D expression. Comparison of ω3 PUFA desaturase cascade substrates and products indicated Δ6D, subsequent elongase and Δ5D expression. 18:3ω6 and 20:3ω6 formation with 20mg/l p-SSO and 40mg/l p-EPO, respectively supported Δ6D and elongase capability. Further, raised 22:4ω6 levels in dosed cells suggested

rapid 20:3ω6 desaturation and 20:4ω6 elongation, and this could reflect a requirement for higher PDFA's to maintain membrane fluidity. 22:4ω6 accumulation may also reflect a lack of significant 22:5ω6 formation via Δ4D. Slightly increased 22:6ω3 levels with supplementation of 40mg/l p-CO suggested the capability to express Δ4D, although 22:6ω3 levels lower than controls with p-FO dosage implied suppression of Δ4D expression, despite substrate availability. It has been suggested that 22:5ω3 is the storage form of 20:5ω3 in rat lung (Swanson et al 1987). This could explain the preference to form 22:4ω6 and 22:5ω3 rather than 22:5ω6 and 22:6ω3, especially since the former moieties can undergo extra-microsomal retroconversion and further desaturation, thus giving the cells greater flexibility to utilise these PUFA's efficiently to form the products required, particularly for 2- and 3-series eicosanoid synthesis. Overall, the potential to express desaturase cascade enzymes implied that rat lung fibroblasts could control membrane fluidity, and this may explain why cytotoxicity was induced only with p-CO, p-OO and p-FO concentrations greater than 80mg/l.

Desaturation with individual radiolabelled C18 FA's was assessed to establish whether the pattern of p-oil desaturation reported in Table 3.6.3.1. was modulated with FA mixtures. 24:1ω9 detection when cultures were dosed with [^{14}C]-18:1ω9 was consistent with elongase

expression, although no evidence to suggest [^{14}C]-18:1ω9 desaturation was found (Table 3.6.4.2.). This probably reflects the inhibition of 18:1ω9 desaturation by EFA's, which has been well documented (Brenner et al 1966, 1969, Brenner 1977 and Holman 1986b). On the other hand, the pattern of radioactivity derived from cultures incubated with either [^{14}C]-18:3ω3 or [^{14}C]-18:2ω6 was consistent with significant Δ6D and subsequent elongase activity (Table 3.6.4.2.), and the significant amounts of [^{14}C]-18:3ω3 and [^{14}C]-18:2ω6 directly elongated to 20:3ω3 and 20:2ω6, respectively could have served as EFA storage products for retroconversion. Conversion of about 40% [^{14}C]-18:3ω3 and 28% [^{14}C]-18:2ω6 to post-Δ5D PUFA's supported Δ5D expression. 20:5ω3 and 20:4ω6 elongation was shown, but Δ4D activity was more limited. The finding that desaturase capability was greatest with EFA substrates and progressively decreased as more unsaturated PDFA's were formed nevertheless comfirmed the rate limiting nature of Δ6D in the cascade (Castuma et al 1972, Brenner 1974 and de Gomez Dumm et al 1976). Furthermore, the greater proportion of [^{14}C]-18:3ω3 converted to PDFA's than [^{14}C]-18:2ω6 supported the established preference for ω3, rather than ω6, series PUFA substrates for desaturation (Brenner et al 1966, Mead et al 1976 and Kanau et al 1977). This was consistent with the direct elongation of more [^{14}C]-18:2ω6 to 20:2ω6 than [^{14}C]-18:3ω3 to 20:3ω3 (Table 3.6.4.2.).

Comparison of the data in Tables 3.6.3.1. and 3.6.4.2. clearly indicated that the capability for desaturation and elongation were more limited with p-oil than with individual C18 FA incubation. Competitive interactions between p-oil FA's could well have suppressed desaturase cascade enzyme expression, particularly since the competitive nature of FA's for such enzymes have been well documented both in vivo and in vitro (Brenner et al 1966, Mohrhauer et al 1963a, Ullman et al 1971, de Schriver et al 1982, Holman 1986a, 1986b, Nassar et al 1986 and Cook et al 1987). Cells in vivo are exposed to FA mixtures rather than to individual FA's, hence the desaturase cascade enzyme capability reported with individual FA dosage may not necessarily reflect the situation in the "real world". On the other hand, p-oil FA composition mimicked that of dietary oils, thus enzyme expression reported with p-oil dosage probably reflects the capability of rat lung fibroblasts to desaturate and elongate dietary oils in vivo.

The cellular lipoperoxides measured both in the presence and absence of p-oil supplementation (Table 3.6.5.1.) supported enzymic and/or non-enzymic mechanisms for their production in rat lung fiboblasts. Control cells contained unsaturated FA's that were potential oxidation substrates (Table 3.6.3.1.), hence the lipid peroxides measured in such were not surprising. Control cell lipid peroxides could reflect the lack of sufficient

alpha-tocopherol to limit FA oxidation, or inefficient mechanisms to remove different species of activated oxygen that promote lipoperoxidation, such as catalase, peroxidase and superoxide dismutase (Lands et al 1971, Smith et al 1972, Tappel 1972, 1980 and Pryor 1976). The greater lipid peroxide amounts reported in certain dosed cultures than in controls implied oxidation of unsaturated p-oil FA's, and the numerical differences in lipoperoxide concentrations reported between dosed cultures reflected the different p-oil FA compositions (Table 2.3.3.2.). This related not only to the amount of unsaturated FA's present in any p-oil, but also to their susceptibility oxidation, which in turn related to the number and position of double bonds in a FA.

It was apparent that the small cellular lipoperoxide amounts formed with 60mg/l p-SSO incubation related largely to oxidation of free LA, but the greatest cellular lipoperoxide amounts induced with 60mg/l p-EPO incubation probably also reflected oxidation of GLA incorporated only with p-EPO dosage, as both p-oils otherwise exhibited similar FA compositions. It followed from comparison of the amount of C18 trienoic FA's present in p-EPO and p-LO (about 9% GLA and 63% ALA, respectively) with the lipid peroxides these p-oils induced that GLA was more susceptible to oxidation than ALA. This could relate to the easier production of endoperoxides from GLA than ALA because the former

merely requires chain elongation to be usable as an endoperoxide substrate, while ALA requires elongation and two desaturations first. On the other hand, the fact that incubation for 48 hours with 60mg/l p-EPO or p-LO induced markedly more lipoperoxides than p-FO, despite the presence of 18% EPA in the latter, may relate to FA availability for oxidation. The finding that lipoperoxide amounts approximated to controls with supplementation of certain unsaturated FA-rich p-oils (20mg/l p-LO, 20mg/l p-FO, 20 and 40mg/l p-EPO, and all concentrations of p-OO and p-SSO) indeed implied that the incorporated exogenous FA's were largely components of complex membrane lipids, and therefore not so readily accessible to oxidation. In fact, the ability of rat lung to peroxidise lipids <u>in vitro</u> has been shown to correlate with the presence of PUFA's in the TAG fraction (Kehrer et al 1978). It was also possible that dosage with different p-oils altered the rate of PGL turnover and thus the release of FA's from membrane lipids into the cellular pool. Such may explain the slightly greater cellular lipoperoxide amounts formed with supplementation of p-CO or p-MO than with certain more unsaturated FA-rich p-oils.

The significantly greater amounts of cellular than spent medium lipid peroxides (Table 3.6.5.1.) supported the ability of these cells to incorporate exogenous FA's (Table 3.6.3.1.). Hence it was unlikely that the lipid

peroxides measured in the spent medium of dosed cultures were formed primarily as a result of auto-oxidation of unincorporated p-oil FA's, particularly since these moieties were albumin-bound. The absence of cytotoxicity with dosage of 60mg/l p-oil (Figs. 3.6.1.2-3.6.1.8.) and the known ability of lipoperoxides to increase membrane permeability (Chio et al 1969 and Frankel 1984) rather suggested that the spent medium lipoperoxides originated intracellularly via passage through the plasma membrane. Cellular FFA's and lipoperoxidation enzymes could also have been released in this process, thus the possiblity that spontaneous and/or enzymic FFA oxidation occurred extracellularly can not be ignored as contributing to the spent medium lipid peroxides reported.

Growth inhibition was not induced with supplementation of 20, 40 or 60mg/l p-oil (Figs. 3.6.1.2-3.6.1.8.), thus no correlation between lipid peroxide formation (Table 3.6.5.1.) and cytotoxicity could be established. Lipid peroxides should, however, not be overlooked as a possible mechanism involved in the modulation of cytotoxicity with dosage of higher p-oil concentrations. It was nevertheless apparent that if lipid peroxides were directly involved in the inhibition of rat lung fibroblast proliferation, the concentrations required would have to be considerably greater than those reported. On the other hand, the inverse relationship generally found between rat lung cell viability and MDA

concentrations with p-oil incubation suggested lipid peroxide involvement in the modulation of growth limitation. However, the fact that the greatest cellular lipoperoxide concentrations induced with 60mg/l p-EPO correlated with the absence of growth limitation implied that the modulation of cell viability also related to other mechanisms, such as alterations in membrane fluidity. As the lung is continuously exposed to high oxygen levels in vivo, this tissue may be particularly resistant both to the formation and effects of lipid peroxides. The extent of lipoperoxidation in rat lung microsomes has indeed been shown to be considerably lower than in liver microsomes, and this was relatable to its markedly greater vitamin E content and affinity for the vitamin (Krishnamurthy et al 1963, Gallo-Torres et al 1971, Kelleher et al 1972, Kornbrust et al 1980 and Sevanian et al 1982).

Total eicosanoid production accounted for 66% of all cellular lipoperoxides measured in control cells (Tables 3.6.5.1. and 3.6.6.1.), and this supported studies which have shown the lung to be an important site for eicosanoid production (Mathe et al 1977, Hyman et al 1978 and Harper et al 1984). In the presence of exogenous AA, Pace-Asciak et al (1977) found that the primary eicosanoids formed by rat lung homogenates were 6-keto $PGF_{1\alpha}$, followed by TXB2, whereas such exhibited relatively little capacity to produce PGE_2, $PGF_{2\alpha}$ and

PGD_2. Others reported some PGE2, $PGF1_\alpha$ and $PGF2_\alpha$, but no PGD_2, formation in this tissue (Moore 1985). Under the conditions used in the present study, control rat lung fibroblasts similarly produced no PGD_2 and small amounts of PGE_2, PGI2, TXB2 and PGF_α, although marked PGE_1 formation was additionally shown (Table 3.6.6.1.). The preferential synthesis of PGE_1 may be the result of increased PGH_1-PGE_1 isomerase expression or relative decreases in the biosynthesis of other prostanoids. However, the finding that dosage with 20 or 60mg/l p-oil markedly enhanced TXB_2 and PGF_α, and suppressed PGE_1, production implied modulation of eicosanoid synthesis, probably at the level of the synthetase enzymes. Changes in the proportions PGI_2 and PGE2 were also found with p-oil dosage, although total eicosanoid production was not markedly altered compared to controls (Table 3.6.6.1.). Others showed that eicosanoid synthesis (6-keto $PGF_{1\alpha}$, TXB2 and PGE2) was not altered in this tissue when olive oil enriched diets were fed compared to controls (fed a lard enriched diet), despite displacement of AA with OA in membrane lipids (Lokesh et al 1988). Menhaden oil also displaced AA with ω3 PUFA's, although eicosanoid synthesis was decreased (Lokesh et al 1985). This implied that different FA components of these oils mediated eicosanoid production in a specific manner. It was apparent, however, that the modulation of eicosanoid production in our study did not relate primarily to incorporation of exogenous FA's per se.

since no clear differences in total or individual eicosanoid production was found between cells dosed with identical amounts of different p-oils, either containing or devoid of eicosanoid precursors. The production of the prostanoids reported was also not related to the degree of p-oil unsaturation (Table 2.3.3.2.) or the ability of these cells to form eicosanoid precursors with p-oil supplementation (Table 3.6.3.1.).

The inability to induce marked differences in the levels of the eicosanoids reported with dosage of the same concentration of different p-oils suggested utilisation of endogenous rather than exogenous PUFA precursors for eicosanoid production. The enhanced prostanoid levels reported in Table 3.6.6.1. could indeed relate to the presence of 20:3ω6 and particularly 20:4ω6 in undosed cells (Table 3.6.3.1.), which are usable by this tissue for prostanoid synthesis (Lokesh et al 1985). The eicosanoids detected nevertheless implied that the cyclooxygenase pathway was functional in these cells, although the exact mechanism by which prostanoid production was modulated with p-oil dosage was unclear. The possibility exists that incubation with different p-oil concentrations influenced eicosanoid precursor availability, or modulated various enzymic steps in the prostanoid biosynthetic pathway either by activating, suppressing or inhibiting their activity under the culture conditions used.

Eicosanoid profiles (Table 3.6.6.1.) did not correlate in any way with the changes reported in cell viability (Figs. 3.6.1.2-3.6.1.8.), total cellular protein (Table 3.6.2.1.), desaturation capability (Table 3.6.3.1.), or lipid peroxide production (Table 3.6.5.1.) with p-oil dosage. This implied that although p-oil supplementation influenced the overall production of the eicosanoids detected, or the balance between individual prostanoid groups, these endogenously synthesised eicosanoids were probably not directly involved in the modulation of the effects demonstrated with p-oil dosage. Indeed, this mechanism could not explain the effects of p-oils rich in saturated or monoenoic FA's, which can not be metabolised by this route. Membrane fluidity changes and lipoperoxides were proposed as alternative mechanisms.

3.7 THE EFFECTS OF PSEUDO-OILS ON CELLS DERIVED FROM RAT SKIN.

3.7.1 Effects of pseudo-Oils on Cell Viability.

Microscopic examination of rat skin fibroblasts before, during and after each experiment showed no morphological changes in control or dosed cells, although cytoplasmic droplets were observed with p-oil supplementation at high concentrations in some instances.

Final medium albumin concentrations ranging from 0 to 250mg/l had no significant effect on the viability of these cells (Fig. 3.7.1.1.). The effects observed with p-oil dosage were therefore a result of the exogenous FA's and not the albumin used as FA carrier.

The number of control cells, seeded at $10x10^4$/ml, at the end of the 24 hour post-trypsinisation recovery period was $10.8x10^4$/ml. This equated to approximately 72% of the final control cell number at the end of the 48 hour incubation period and represented the cytostatic number. Only p-oil concentrations reducing cell viability to significantly below the cytostatic number were hence considered cytotoxic.

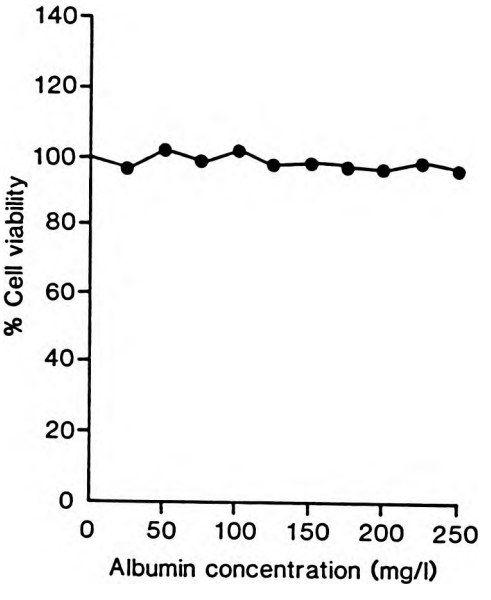
Figs. 3.7.1.2-3.7.1.8. show the data obtained from the incubation of cells with p-oils. Cell proliferation was significantly enhanced only with low concentrations of

Legend to Figs. 3.7.1.1-3.7.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where "n" is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.7.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.7.1.2-3.7.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.7.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.7.1.1.

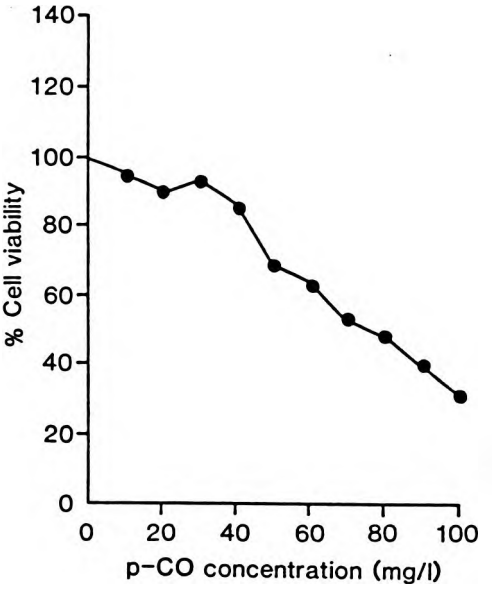
The percentage viability of cells derived from rat skin incubated with albumin.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.8	12
25	96.2	3.2	12
50	101.1	3.2	12
75	97.9	1.1	12
100	101.4	3.5	12
125	96.6	2.4	12
150	97.5	2.3	12
175	97.2	3.1	12
200	96.4	3.0	12
225	98.4	3.2	12
250	96.3	5.4	12

Fig. 3.7.1.2.

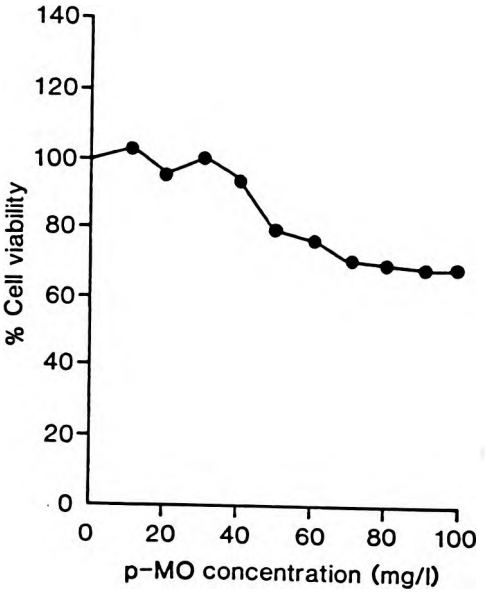
The percentage viability of cells derived fron rat skin incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.6	6
10	94.9	2.8	6
20	90.3	4.5	6
30	94.2	1.1	6
40	86.5	5.5	6
50	69.4	4.6	6
60	63.8	6.1	6
70	53.1	4.4	6
80	49.0	2.4	6
90	40.3	2.3	6
100	30.9	3.3	6

Fig. 3.7.1.3.

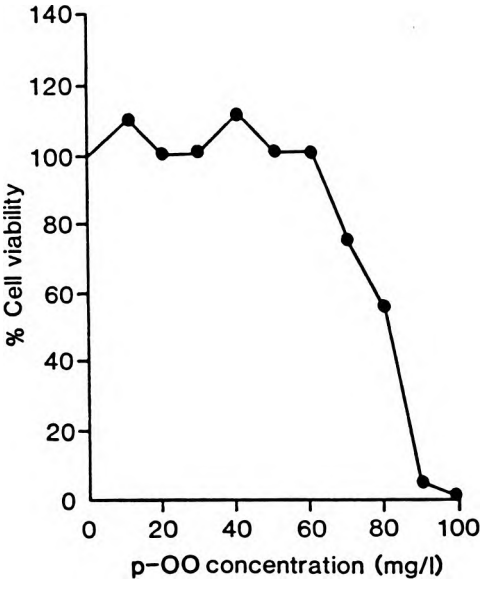
The percentage viability of cells derived from rat skin incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.2	6
10	103.3	2.7	6
20	96.2	1.8	6
30	100.3	1.9	6
40	93.8	2.4	6
50	80.0	1.7	6
60	77.8	2.0	6
70	70.5	2.0	6
80	69.7	2.1	6
90	68.8	2.0	6
100	69.7	1.8	6

Fig. 3.7.1.4.

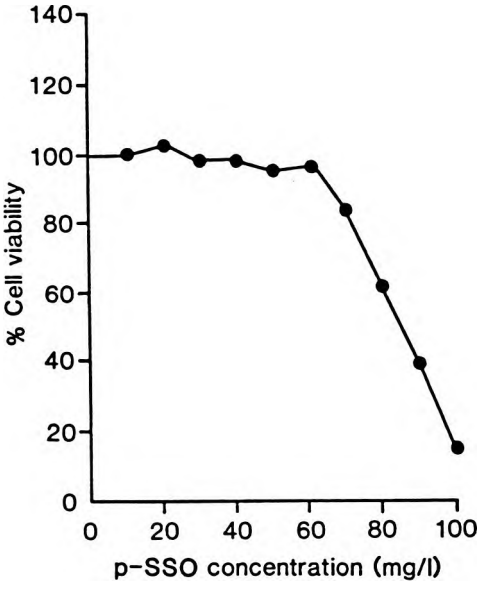
The percentage viability of cells derived from rat skin incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.8	6
10	110.6	2.9	6
20	100.4	2.0	6
30	101.8	4.7	6
40	112.4	2.7	6
50	101.9	3.1	6
60	101.4	5.0	6
70	76.9	3.0	6
80	56.9	3.4	6
90	4.8	0.8	6
100	0.0	0.0	6

Fig. 3.7.1.5.

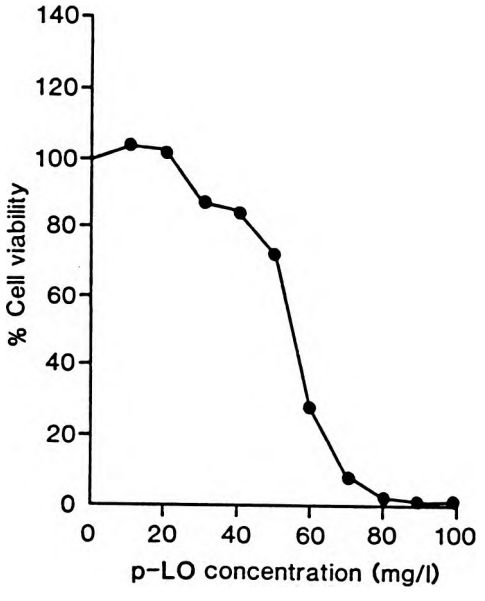
The percentage viability of cells derived from rat skin incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.1	6
10	100.7	2.0	6
20	103.4	2.3	6
30	98.8	2.2	6
40	99.0	1.8	6
50	95.8	1.8	6
60	97.6	2.2	6
70	83.9	2.4	6
80	61.6	3.1	6
90	39.1	1.9	6
100	14.9	1.2	6

Fig. 3.7.1.6.

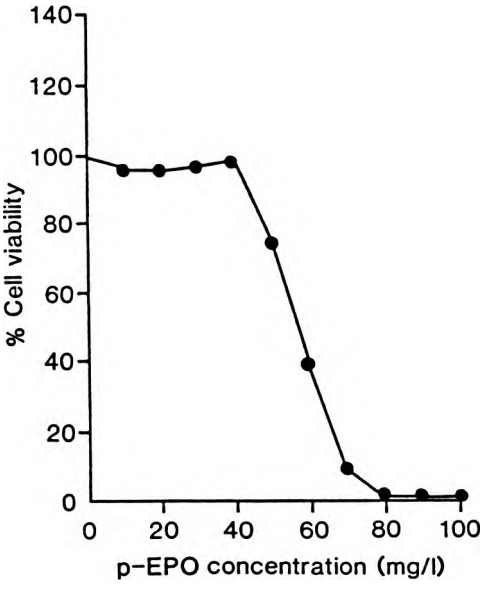
The percentage viability of cells derived from rat skin incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.4	6
10	103.9	4.9	6
20	102.7	3.9	6
30	88.2	4.4	6
40	86.0	2.9	6
50	74.0	4.7	6
60	29.4	5.0	6
70	9.2	1.9	6
80	2.1	1.0	6
90	0.5	0.3	6
100	0.3	0.3	6

Fig. 3.7.1.7.

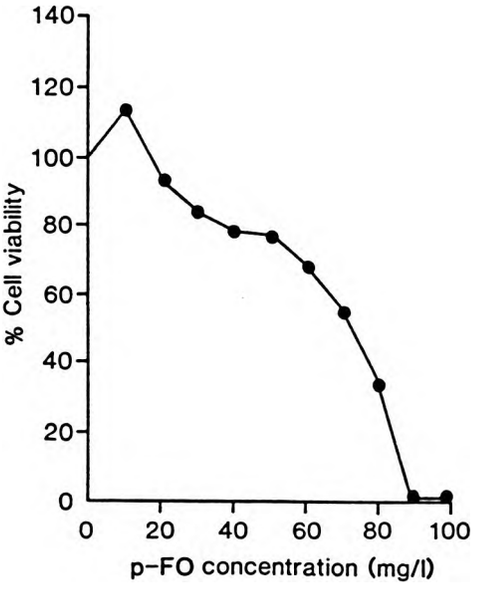
The percentage viability of cells derived from rat skin incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.7	6
10	95.9	2.1	6
20	96.3	6.0	6
30	97.6	2.8	6
40	98.9	2.7	6
50	74.3	4.6	6
60	38.9	4.3	6
70	8.9	1.1	6
80	0.9	0.6	6
90	0.0	0.0	6
100	0.0	0.0	6

Fig. 3.7.1.8.

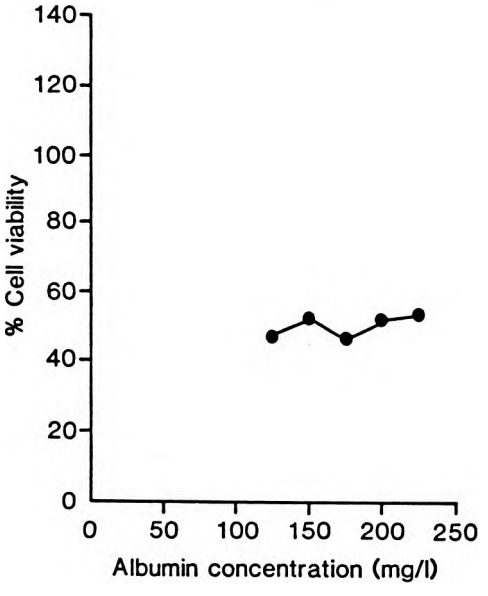
The percentage viability of cells derived from rat skin incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.3	6
10	114.8	2.5	6
20	94.1	2.5	6
30	84.5	2.9	6
40	79.4	3.4	6
50	78.7	4.0	6
60	68.9	3.4	6
70	56.0	2.3	6
80	35.5	2.5	6
90	0.0	0.0	6
100	0.0	0.0	6

Fig. 3.7.1.9.

The percentage viability of cells derived from rat skin incubated with albumin at the ID_{50} of p-FO.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.7	6
125	47.9	2.5	6
150	53.5	1.4	6
175	47.1	3.5	6
200	52.8	1.1	6
225	54.0	3.7	6

p-OO or p-FO, whereas all p-oils limited, and with the exception of p-MO all p-oils inhibited, cell viability to a greater or lesser extent, dependent on the p-oil and concentration supplemented.

Dosage with increasing amounts of p-CO reduced cell viability in an overall concentration dependent manner (Fig. 3.7.1.2.). Cytostasis was induced with 50mg/l p-CO, whereas half the cells were viable with 76mg/l p-CO (ID_{50}) and 30.9% with 100mg/l p-CO.

Enrichment of the culture medium with up to 30mg/l p-MO had little effect upon cell viability, but such was progressively reduced to 77.8% with 60mg/l p-MO (Fig. 3.7.1.3.). Cell viability plateaued and ranged from 68.8% to 70.5% with dosage of 70 to 100mg/l p-MO. This approximated to the cytostatic number, thus no ID_{50} could be calculated.

Cell viability was similar to controls with dosage of up to 60mg/l p-OO or p-SSO (Figs. 3.7.1.4. and 3.7.1.5., respectively), although proliferation was stimulated to 110.6% with 10mg/l p-OO and 112.4% with 40mg/l p-OO. Growth limitation occurred with 70mg/l p-OO or p-SSO, but greater amounts were increasingly cytotoxic. Both p-OO and p-SSO exhibited comparable ID_{50} values (about 82 and 85mg/l, respectively), but no viable cells were found when cultures were enriched with 100mg/l p-OO, compared to 14.9% with 100mg/l p-SSO.

Dosage with up to 20mg/l p-LO or 40mg/l p-EPO had little significant effect on cell viability, but growth was limited to a point of cytostasis with 50mg/l p-LO or p-EPO (Figs. 3.7.1.6. and 3.7.1.7., respectively). These were the most effective p-oils to inhibit rat skin fibroblast proliferation, and this correlated with the lowest ID_{50} values calculated (about 55mg/l for p-LO and 57mg/l p-EPO). More than 90% of the cells took up Trypan blue with 70mg/l p-LO or p-EPO, and this increased to practically 100% with concentrations of 90 or 100mg/l.

Cell proliferation was enhanced to a maximum of 114.8% with 10mg/l p-FO, and was progressively limited with concentrations of 20 to 50mg/l p-FO such that cytostasis occurred with 60mg/l p-FO and cytotoxicity with higher amounts (Fig. 3.7.1.8.). Half the cells were viable with approximately 73mg/l p-FO (ID_{50}) compared to 35.5% with 80mg/l p-FO, but 100% cell death was observed with 90mg/l and 100mg/l p-FO.

To eliminate the possibility that any of the effects induced with p-oil dosage were influenced by the amount of albumin bound to the FA's, these cells were incubated with a fixed amount of p-FO corresponding with its ID_{50} (approximately 73mg/l) and dosed with varying amounts of albumin. Five concentrations were chosen around which to vary the amount of albumin, viz. two points above, two points below and the ID_{50} of p-FO. The results shown in

Fig. 3.7.1.9. indicated that the amount of p-FO dosed reduced cell viability to approximately 50% of controls, and that such was not affected by the amount of albumin used as FA carrier. Synergistic effects of albumin could hence be excluded, and any effects induced by the p-oils could be attributed solely to the exogenous FA's.

Fig. 3.7.1.10. depicts the growth of cells obtained from rat skin in culture medium supplemented with 10% serum derived from its own species compared to commercially available fetal calf serum (FCS) in order to establish whether FCS modulated cell growth in any way. These cells grew from 10.0 to 13.1×10^4 over a 72 hour period when culture medium was supplemented with 10% FCS. Plating efficiency was low in the presence of 10% rat serum (RS), however, resulting in an initial decrease in the number of viable cells at 24 hours to 7.0×10^4. The cells grew in a linear manner over the next 48 hours, although only 8.2×10^4 viable cells were found at the end of the incubation period. Thus, the routine use of 10% FCS did not limit cell growth compared to 10% RS, and promotion of growth limitation and cytotoxicity were attributed solely to the exogenous FA's incorporated.

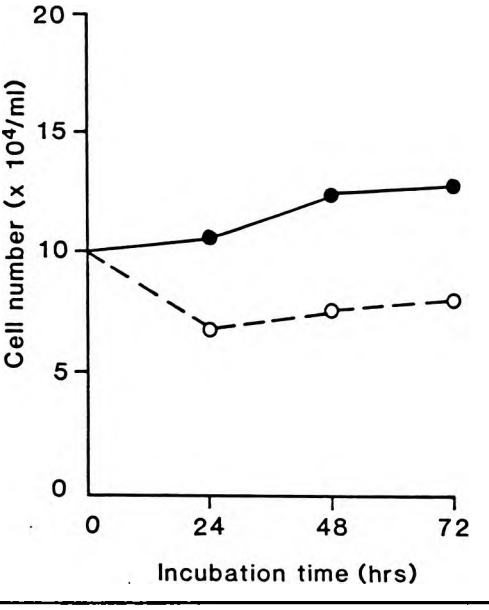
Rat skin fibroblasts were subsequently plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient amounts for all quantitative and qualitative analyses to be carried out. Upon harvesting, cell numbers were compared and found to be statistically

Legend to Fig. 3.7.1.10.

The results are expressed as mean cell number±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The cell numbers given are $x10^4$/ml growth medium. Fig. 3.7.1.10. shows mean cell numbers versus the incubation period (hours).

Fig. 3.7.1.10.

The growth of cells derived from rat skin in DMEM containing 10% foetal calf serum compared with 10% adult rat serum.



Incubation Time (hrs)	Mean Cell Number (x10^4/ml)	±s.e.m.	n
Foetal calf serum:			
24	10.8	0.3	9
48	12.7	0.2	9
72	13.1	0.4	9
Adult rat serum:			
24	7.0	0.2	9
48	7.7	0.3	9
72	8.2	0.3	9

similar to those obtained in Figs. 3.7.1.1-3.7.1.8. All biochemical assays were thus performed on these samples.

3.7.2 Effects of pseudo-Oils on Total Protein.

Table 3.7.2.1. shows total protein content of rat skin fibroblasts at each of the 3 p-oil concentrations dosed compared with controls, expressed as µg total protein per 10^6 cells seeded.

Control fibroblasts exhibited 374.7µg total protein/10^6 cells seeded, whereas p-oil dosed fibroblasts contained 269.4 to 579.6µg protein/10^6 cells seeded (obtained with 60mg/l p-LO and 40mg/l p-OO, respectively). Protein levels were higher than controls in every instance with incubation of 20mg/l p-oil (392.2 to 473.6µg protein/10^6 cells seeded). This was also true with dosage of 40mg/l p-oil (436.1 to 579.6µg protein per 10^6 cells seeded), although cells exhibited less protein than controls with addition of 40mg/l p-CO (327.4µgprotein/10^6 cells seeded). Enhanced protein levels were found with 60mg/l p-OO supplementation (445.4µg protein/10^6 cells seeded), but amounts were decreased with incubation of other p-oils at this concentration (269.4 to 366.8µg protein per 10^6 cells seeded). Cellular protein levels decreased inversely with the amount of p-CO, p-SSO, p-LO or p-FO supplemented, whereas the greatest and lowest amounts measured with p-MO, p-OO or p-EPO dosage occurred with a concentration of 40 and 60mg/l, respectively.

The spent incubation media derived from these cultures were also analysed for total protein. The values for all

Table 3.7.2.1.

The protein content of cells derived from rat skin, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	374.7			
CO		392.2	327.4	305.9
MO		422.5	449.3	366.8
OO		471.2	579.6	445.4
SSO		444.9	436.1	361.4
LO		473.6	466.3	269.4
EPO		436.1	468.2	348.4
FO		458.1	440.5	366.9

samples were summed, and a mean protein concentration of $46.8 \pm 0.35 mg/10^6$ cells seeded obtained (n=22). However, statistical comparison of individual samples by analysis of variance showed no significant differences in the spent media protein levels between control and dosed cultures ($F=136.5$, $p=0.001$).

3.7.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Cells Derived From Rat Skin.

Table 3.7.3.1. lists the FA spectra of cells incubated with 0, 20, 40 or 60mg/l p-oil.

Control cells exhibited a FA spectrum in which 18:0 comprised 19.1% of all FA's detected. 16:0, 18:1ω9, 20:4ω6 and 22:4ω6 contributed 18.3%, 16.5%, 13.3% and 9.9% to the total spectrum, respectively, while other FA's contributed less than 5% each to the total.

Dosed cells generally contained variable proportions of 16:0, less 18:0 and 16:1ω9, and more 18:1ω9 and 18:2ω6, than control cells. 20:4ω6 and 22:5ω6 percentages were decreased in dosed cells, although 22:4ω6 was markedly increased with supplementation of 20mg/l p-oil. Dosed cells generally contained less 18:4ω3 and 20:4ω3 than controls, while the relative amounts of 18:3ω3, 20:5ω3, 22:5ω3 and 22:6ω3 were more variable.

Dosage with 20mg/l p-CO had little effect on 16:0 levels (17.3% vs 18.3% in controls, respectively), but this was increased with 40 and 60mg/l p-CO (25.9% and 27.7%, respectively). Similar changes occurred in 18:1ω9 levels (16.5%, 15.1%, 19.3% and 23.3% with 0, 20, 40 and 60mg/l p-CO, respectively), while 18:0 and 16:1ω9 proportions were decreased compared to controls (19.1% and 4.2%, respectively). p-CO had little effect on 18:2ω6 levels

Legend to Tables 3.7.3.1. and 3.7.3.2.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.7.3.1.

The fatty acid spectrum of cells derived from rat skin.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	18.3±0.00	17.3	25.9	27.7	17.0	20.8	24.3	14.6	15.9	12.0	19.2	16.5	9.5	14.2	16.7	21.2	20.9	16.5	14.7	15.4	19.0	15.0
	18:0	19.1±0.05	12.0	13.7	16.7	14.3	13.4	16.6	8.6	7.1	15.2	11.6	11.0	21.7	12.1	16.1	9.9	11.5	10.1	16.2	11.7	12.2	19.7
	20:0	0.4±0.00	-	0.2	-	-	0.2	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	0.1	-
	22:0	0.2±0.00	0.4	0.2	0.2	0.2	0.2	0.1	0.1	0.2	-	0.2	-	0.1	0.2	0.1	0.1	-	-	0.3	-	0.1	0.1
	24:0	0.3±0.00	0.7	0.8	0.2	-	0.8	-	0.3	0.1	-	0.1	0.1	-	-	-	-	0.2	1.0	-	-	0.3	-
ω9 MONOS.	16:1	4.2±0.10	2.3	2.7	3.9	0.8	1.2	2.0	1.1	1.4	1.6	4.0	1.1	1.1	1.1	0.8	3.5	4.3	1.7	2.2	5.4	11.1	11.0
	18:1	16.5±0.10	15.1	19.3	23.3	15.6	19.0	28.5	30.9	41.8	55.1	13.6	18.2	22.5	14.1	16.8	16.1	9.8	12.5	17.9	15.9	18.7	22.6
	24:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	3.2±0.00	3.2	3.4	2.7	6.2	7.8	8.2	6.4	8.0	5.8	15.3	25.9	36.3	8.0	9.2	11.2	15.2	23.1	28.9	5.3	6.8	15.8
	18:3	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.1	3.1	-	-	-
	20:2	0.1±0.00	-	-	-	-	0.3	0.1	1.0	1.5	1.6	1.2	1.8	0.6	-	0.3	-	0.8	1.1	1.2	0.1	0.5	0.3
	20:3	1.6±0.05	1.4	1.4	1.1	1.5	1.6	0.9	1.1	1.1	0.4	1.2	1.4	0.5	1.1	1.1	0.7	3.5	3.8	2.4	1.1	1.0	0.4
	20:4	13.3±0.05	11.1	10.1	6.5	13.2	11.8	7.5	8.3	5.7	2.2	6.9	6.3	1.8	10.8	8.9	5.1	7.4	4.8	2.5	8.7	6.4	2.5
	22:4	9.9±0.35	26.6	11.3	9.1	21.1	14.3	6.3	20.3	10.0	3.0	20.4	10.7	3.4	26.0	11.5	8.0	20.0	17.2	4.2	23.6	8.0	2.8
	22:5	1.8±0.03	-	1.3	-	-	0.3	0.2	-	0.8	-	0.1	0.9	0.1	-	1.3	0.1	0.2	1.6	0.1	0.1	1.1	0.1
ω3 POLYS.	18:3	0.3±0.00	0.4	0.6	0.4	0.7	0.6	0.4	1.1	1.2	0.5	0.4	0.5	0.1	4.0	9.1	19.5	0.2	0.3	2.4	0.4	0.5	0.3
	18:4	1.6±0.05	0.3	1.7	0.8	0.5	1.1	0.3	0.2	0.8	0.3	0.3	0.9	0.3	0.2	2.1	0.2	0.2	1.1	1.2	0.3	1.2	0.5
	20:4	4.3±0.05	1.7	2.0	2.6	2.3	2.3	1.4	1.2	1.8	1.2	1.6	1.6	1.2	1.5	2.9	1.5	1.9	3.1	1.2	2.1	1.4	1.5
	20:5	0.7±0.00	1.4	1.2	1.0	0.4	0.5	0.2	0.2	0.2	-	0.2	0.3	-	2.0	0.5	0.3	0.1	0.3	0.1	2.3	4.1	4.1
	22:5	3.1±0.03	2.9	2.7	1.7	3.2	2.5	1.4	2.4	1.8	0.5	2.0	2.1	0.5	2.5	2.0	1.1	2.0	1.4	0.6	6.1	6.8	2.7
	22:6	1.2±0.10	3.1	1.7	2.3	3.2	1.4	1.6	2.4	0.9	0.7	1.9	0.6	0.6	2.3	0.6	1.4	1.7	0.5	0.8	1.6	0.8	0.8

(2.7% to 3.2% vs 3.2% in controls), but decreased 20:4ω6 amounts from 11.1% to 10.1% and 6.5% with 20, 40 and 60mg/l, respectively (13.3% in controls). Significantly increased 22:4ω6 was found with 20mg/l p-CO (26.6% vs 9.9% in controls), but this did not raise 22:5ω6 levels. On the other hand, increased 20:5ω3 and 22:6ω3 reflected decreased 18:4ω3, 20:4ω3 and 22:4ω3 proportions.

Decreased 18:0 and 16:1ω9 levels were found when cells were incubated with p-MO, but 16:0 and 18:1ω9 increased significantly with 60mg/l p-MO (24.3% and 28.5% vs 18.3% and 16.5% in controls, respectively). 18:2ω6 increased from 6.2% to 7.8% and 8.2% with 20, 40 and 60mg/l p-MO, respectively (3.2% in controls), and 20:4ω6 decreased from 13.2% to 11.8% and 7.5%, respectively (13.3% in controls), whereas the elongation product, 22:4ω6, was markedly increased with 20mg/l p-MO (21.1% vs 9.9% in controls). ω3 PDFA levels were parallel to controls or decreased, although 22:6ω3 was significantly increased with 20mg/l p-MO (3.2% vs 1.2% in controls).

The percentages of 18:1ω9 detected in cells dosed with 0, 20, 40 or 60mg/l p-OO increased from 16.5% to 30.9%, 41.8% and 55.1%, respectively, whereas 16:0, 16:1ω9 and 18:0 levels were decreased. Significant increments in 18:2ω6 (5.8% to 8.0% vs 3.2% in controls) and 20:2ω6 (1.0% to 1.6% vs 0.1% in controls) were detected, but 20:3ω6 and 20:4ω6 were decreased. However, 22:4ω6 increased about two fold with 20mg/l p-OO (20.3% vs 9.9%

in controls). With the exception of a two fold increase in 22:6ω3 with 20mg/l p-OO (2.4%), ω3 PDFA levels were reduced with p-OO dosage.

16:0, 16:1ω9, 18:0 and 18:1ω9 percentages were either parallel to controls or decreased in cells dosed with p-SSO, although 18:1ω9 was increased with 60mg/l p-SSO (22.5% vs 16.5% in controls). The provision of p-SSO resulted in concentration dependent 18:2ω6 incorporation from 3.2% in controls to 15.3%, 25.9% and 36.3% with concentrations of 20, 40 or 60mg/l, respectively. 20:2ω6 levels were raised (0.6% to 1.8% vs 0.1% in controls), and 22:4ω6 increased approximately two fold with 20mg/l p-SSO (20.4% vs 9.9% in controls), but other ω6 PDFA levels were parallel to controls or decreased. With the exception of a slight increase in 22:6ω3 with 20mg/l p-SSO (1.9% vs 1.2% in controls), ω3 PDFA levels were also decreased.

p-LO generally had little effect on cellular 16:0 and 18:1ω9 levels, but lowered the amount of 16:1ω9 and 18:0. 18:2ω6 increased from 3.2% in controls to 8.0%, 9.2% and 11.2% with 20, 40 and 60mg/l p-LO, respectively and 26.0% 22:4ω6 was detected with 20mg/l p-LO (9.9% in controls). Other ω6 PDFA levels, however, approximated to controls or were decreased. The percentage of 18:3ω3 increased from 4.0% to 9.1% and 19.5% when 20, 40 or 60mg/l p-LO was incubated with the cells, respectively

(0.3% in controls). 18:4ω3 was enhanced with 40mg/l p-LO (2.1% vs 1.6% in controls), 20:5ω3 with 20mg/l p-LO (2.0% vs 0.7% in controls) and 22:6ω3 with 20mg/l p-LO (2.3% vs 1.2% in controls), while other concentrations of p-LO had little effect on, or lowered ω3 PDFA levels.

16:0, 16:1ω9, 18:0 and 18:1ω9 levels were parallel to controls or decreased in cells incubated with p-EPO, but 18:2ω6 increased markedly (15.2%, 23.1% and 28.9% with 20, 40 or 60mg/l, respectively vs 3.2% in controls). 18:3ω6 was absent from controls, but up to 3.1% was found in dosed cells. Significant increments in 20:2ω6 (0.8% to 1.2% vs 0.1% in controls) and 20:3ω6 (2.4% to 3.8% vs 1.6% in controls) were found, but 20:4ω6 levels decreased from 13.3% to 7.4%, 4.8% and 2.5% with 0, 20, 40 and 60mg/l p-EPO, respectively. 22:4ω6 percentages were significantly raised both with 20 and 40mg/l p-EPO (20.0% and 17.2%, respectively vs 9.9% in controls), but this did not induce an increase in 22:5ω6. Incubation of cells with 20mg/l p-EPO caused a slight increase in 22:6ω3 (1.7% vs 1.2% in controls), but other ω3 PDFA levels were parallel to controls or decreased.

Cells supplemented with p-FO exhibited 16:0 and 18:0 percentages either approximating to controls (18.3% and 19.1%, respectively) or decreased, raised 16:1ω9 levels (5.4% with 20mg/l, 11.1% with 40mg/l and 11.0% with 60mg/l, respectively vs 4.2% in controls), and increased 18:1ω9 with 40 and 60mg/l p-FO (18.7% and 22.6% vs 16.5%

in controls). 18:2ω6 increased significantly from 5.3% with 20mg/l p-FO to a maximum of 15.8% with 60mg/l p-FO (3.2% in controls), and 22:4ω6 increased more than two fold with 20mg/l p-FO (23.6% vs 9.9% in controls), but no increment in 22:5ω6 was detected. 20:5ω3 percentages increased with p-FO dosage (2.3% with 20mg/l and 4.1% each with 40 and 60mg/l vs 0.7% in controls), and its elongation product, 22:5ω3, increased approximately two fold with 20 and 40mg/l (6.1% and 6.8%, respectively vs 3.1% in controls). Yet, 22:6ω3 was only slightly raised with 20mg/l p-FO (1.6% vs 1.2% in controls).

The FA spectra of the spent growth medium removed from each of the above cultures is shown in Table 3.7.3.2. The medium in which control cells had been grown contained 96.6% 14:0, 2.0% 17:0 and less than 0.5% each of 16:0, 16:1ω9, 18:0, 18:1ω9 and 22:4ω6. Spent media obtained from cultures dosed with p-oils similarly contained at least 96% 14:0 and 0.4% to 2.7% 17:0, but only trace amounts of the major FA components present in the p-oils remained in the medium following the 48 hour incubation period.

3.7.4 Incorporation of Radiolabelled C18 Fatty Acids into Cells Derived from Rat Skin.

Table 3.7.4.1. shows the radioactivity recovered from cultures incubated with 2μCi [^{14}C]-18:1ω9, [^{14}C]-18:2ω6 or [^{14}C]-18:3ω3.

Total counts recovered from the spent incubation media ranged from 2.0 to 2.8x10^{6}cpm. Only 0.3 to 0.5x10^{6}cpm were recovered from the pooled buffers after washing the cells, and this accounted for non-specific.binding. For both [^{14}C]-18:1ω9 and [^{14}C]-18:3ω3 the equivalent of 4.3x10^{6}cpm were dosed to cultures, and total recovery of each was approximately 77% and 84%, respectively. For [^{14}C]-18:2ω6 the equivalent of 4.8x10^{6}cpm were dosed to the incubation medium, and the total recovery of this radioisotope amounted to about 88%. However, the total amount of each of these radioisotopes incorporated into the cells approximated to 22% for [^{14}C]-18:1ω9, 23% for [^{14}C]-18:2ω6 and 10% for [^{14}C]-18:3ω3.

Table 3.7.4.2. shows the percentage of each incorporated radiolabelled C18 FA converted to ω9, ω6 or ω3 series products after incubation with rat skin fibroblasts.

15.5% of the total [^{14}C]-18:1ω9 counts derived upon GLC analysis (1151cpm) remained unchanged, while the proportions of 20:1ω9, 22:1ω9 and 24:1ω9 detected were 35.3%, 39.1% and 10.2%, respectively.

Table 3.7.3.2.

The fatty acid spectrum of the spent culture medium of cells derived from rat skin.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	12:0	-	0.1	0.2	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14:0	96.6±0.38	98.3	97.7	98.1	98.5	98.1	96.3	97.9	96.5	97.8	97.4	97.1	96.6	97.5	98.2	97.9	97.8	97.7	96.1	98.3	97.9	97.9
	16:0	0.4±0.09	0.2	0.2	0.2	0.2	0.2	0.7	0.2	0.2	0.2	0.2	0.4	0.7	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	17:0	2.0±0.06	0.6	0.9	0.5	0.4	1.1	1.4	0.8	2.2	1.0	1.2	1.3	1.5	1.5	0.6	0.7	0.9	0.9	2.7	0.4	0.7	0.6
	18:0	0.2±0.08	0.2	0.2	0.2	0.1	-	0.4	0.2	-	-	0.2	0.1	-	-	0.1	0.2	0.2	0.2	-	0.2	0.1	0.2
	20:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24:0	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MONO.	16:1	0.1±0.03	-	-	-	-	-	0.2	-	-	-	-	0.1	0.1	-	-	-	-	-	-	0.1	0.2	0.3
	18:1	0.4±0.05	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.6	0.6	0.3	0.4	0.3	0.3	0.2	0.2	0.2	0.3	0.4	0.3	0.3	0.3
ω6 POLYS.	18:2	-	-	-	-	-	-	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.2	0.3	0.3	0.1	0.1	0.1
	18:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20:2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20:4	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-
	22:4	0.3±0.03	0.2	0.4	0.3	0.4	0.2	0.4	0.4	0.3	0.2	0.4	0.3	0.4	0.3	0.2	0.4	0.4	0.3	0.2	0.3	0.4	0.3
	22:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω3 POLYS.	18:3	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	0.3	-	-	-	-	-	-
	18:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20:4	-	-	-	-	0.1	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	-	0.1	0.1	-	-	-	-	-
	20:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.7.4.1.

The radioactivity (cpm) recovered from rat skin cultures incubated with radiolabelled C18 fatty acids, expressed as mean (±s.e.m.), n=3.

SPENT MEDIA (M)	POOLED WASHINGS (W)	TOTAL (M+W)	CELLS	TOTAL $\times 10^6$
Total 18:1 counts dosed to incubation medium = 4.3×10^6				
1946880 (±15780)	421260 (±3240)	2368140	959907 (±5226)	3.3
Total 18:2 counts dosed to incubation medium = 4.8×10^6				
2524200 (±22620)	528840 (±2520)	3053040	1121016 (±3027)	4.2
Total 18:3 counts dosed to incubation medium = 4.3×10^6				
2831960 (±11900)	344100 (±5040)	3176060	407862 (±3507)	3.6

Table 3.7.4.2.

The counts detected for the radiolabelled fatty acids dosed and their resulting metabolites formed after incubation with cells derived from rat skin, expressed as a percentage of the total counts recovered upon GLC analysis.

ω9 MONOS.	[^{14}C]-18:1ω9	ω6 POLYS.	[^{14}C]-18:2ω6	ω3 POLYS.	[^{14}C]-18:3ω3
18:1	15.5	18:2	6.7	18:3	7.0
20:1	35.3	18:3	16.1	18:4	17.1
22:1	39.1	20:2	16.7	20:3	16.8
24:1	10.2	20:3	18.3	20:4	12.2
		20:4	23.2	20:5	23.2
		22:4	10.9	22:5	10.6
		22:5	8.2	22:6	13.1
TOTAL CPM	1151	TOTAL CPM	2689	TOTAL CPM	1011

On the other hand, only 6.7% of the total [^{14}C]-18:2ω6-derived counts measured (2689cpm) remained unchanged. 16.7% and 16.1% of total counts recovered were found as 20:2ω6 and 18:3ω6, respectively, compared to 18.3% 20:3ω6, 23.2% 20:4ω6, 10.9% 22:4ω6 and 8.2% 22:5ω6.

The total counts derived following GLC analysis of cells incubated with [^{14}C]-18:3ω3 were 1011cpm, of which only 7.0% was detected for 18:3ω3. The percentage for 18:4ω3 (17.1%) was comparable with that of 18:3ω6 (16.1%), as were those for the immediate elongation products of 18:3ω3 and 18:2ω6 (16.8% 20:3ω3 and 16.7% 20:2ω6). 12.2% 20:4ω3 was detected, while the levels of the subsequent products of desaturation and elongation (23.2% 20:5ω3 and 10.6% 22:5ω3) were almost identical to those found for corresponding products in the ω6 series. However, a larger proportion of total [^{14}C]-18:3ω3-derived counts were shown as 22:6ω3 (13.1%) than for the corresponding Δ4D product in the ω6 series (8.2% for 22:5ω6).

Δ6D represents the first enzyme in the desaturase enzyme cascade, thus total activity of this enzyme is reflected by the sum of all subsequent desaturase and elongase products.

3.7.5 Effects of pseudo-Oils on Lipid Peroxide Formation.

The lipoperoxides quantified in dosed cells and their respective growth media are presented in Table 3.7.5.1. in relation to controls. Values are reported as nmoles MDA/10^6 cells, but in the case of the spent incubation media this represents the nmoles of MDA in the volume of medium from which $1x10^6$ cells were derived.

Medium derived from cultures incubated with 60mg/l p-LO or p-FO for 48 hours contained 1.0 and 0.9nmoles MDA/10^6 cells, respectively, but MDA was absent from all other spent media. On the other hand, MDA was measured both in control and all dosed cells. The MDA amounts quantitated in dosed rat skin fibroblasts ranged from 0.1 to 2.6 nmoles MDA/10^6 cells (with 20mg/l p-MO or 60mg/l p-FO incubation, respectively), compared to 0.2nmoles MDA/10^6 control cells. Little lipoperoxide formation occurred when cells were dosed with 20, 40 or 60mg/l p-CO, p-MO, p-OO or even p-SSO since only 0.1 to 0.4nmoles MDA/10^6 cells were measured. Cells supplemented with 20mg/l p-LO, p-EPO or p-FO induced similar lipoperoxide amounts (0.4 to 0.5nmoles MDA/10^6 cells), but p-EPO formed more lipoperoxides than p-LO or p-FO at quantities of 40mg/l. (1.3, 0.4 and 0.5nmoles MDA/10^6 cells, respectively). However, p-FO induced the greatest quantity of cellular lipoperoxides with a concentration of 60mg/l, followed

Table 3.7.5.1.

Lipoperoxide formation by rat skin cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	0.2			
CO		0.2	0.2	0.4
MO		0.1	0.4	0.2
OO		0.2	0.2	0.4
SSO		0.2	0.2	0.2
LO		0.4	0.4	0.7
EPO		0.5	1.3	2.1
FO		0.4	0.5	2.6
pseudo-Oil	SPENT INCUBATION MEDIUM			
(mg/l)	0	20	40	60
Control	-			
CO		-	-	-
MO		-	-	-
OO		-	-	-
SSO		-	-	-
LO		-	-	1.0
EPO		-	-	-
FO		-	-	0.9

by p-EPO and p-LO (2.6, 2.1 and 0.7nmoles MDA/10^6 cells, respectively).

3.7.6 The Eicosanoid Profile of Cells Derived from Rat Skin.

The eicosanoids positively detected in control and dosed rat skin fibroblasts are presented in Table 3.7.6.1. as a percentage of the total area found. However, an indication of the total eicosanoid amounts quantitated is given for controls in '( )', expressed as pmoles/10^6 cells.

Total eicosanoid formation amounted to 165.7pmoles/10^6 control cells, of which 15.4% could be positively identified with the prostanoid standards available. Approximately half of this was TXB_2 (7.3%) and one third PGF_α (5.2%), compared to 1.3% PGI2, 1.0% PGE2 and 0.6% PGE_1. Varying proportions of these prostanoids were also found in dosed cells, whereas PGD_2 was not produced by control, or dosed, rat skin fibroblasts.

Total percentages for the prostanoids detected in dosed cells varied significantly with p-oil concentration as such approximated to controls (15.4%) with 40mg/l p-oil (9.5% to 15.1%), but were significantly increased both with 20mg/l (22.9% to 29.4%) and 60mg/l (24.1% to 33.7%) p-oil. These differences were reflected by appropriate changes in the amount of individual prostanoids. PGI_2 levels generally approximated to controls (1.3%) with supplementation of 40mg/l p-oil, but none was detected with 20 or 60mg/l p-oil. Cellular TXB_2 percentages were

Legend to Table 3.7.6.1.

Values are tabulated as relative percent of the total area detected, and as pmoles total eicosanoids/10^6 control cells.

Table 3.7.6.1.

The profile of the eicosanoids detected in extracts of cells derived from rat skin.

pseudo-Oil (mg/l)		EICOSANOIDS (%)						
		TOTAL	PGI_2	TXB_2	$PGF_{\alpha(1+2)}$	PGE_2	PGE_1	PGD_2
CONTROL (165.7)		15.4	1.3	7.3	5.2	1.0	0.6	-
CO	20	29.0	-	17.5	9.3	1.9	0.3	-
	40	13.9	0.9	6.2	6.5	0.3	-	-
	60	33.7	-	18.2	15.2	0.3	-	-
MO	20	22.9	-	13.0	8.4	1.5	-	-
	40	15.1	0.6	5.5	8.8	0.2	-	-
	60	33.4	-	20.6	12.5	0.3	-	-
OO	20	27.5	-	16.6	8.8	1.7	0.4	-
	40	13.0	0.8	6.8	5.1	0.3	-	-
	60	29.2	-	18.0	11.1	0.1	-	-
SSO	20	23.7	-	13.3	8.9	1.2	0.3	-
	40	11.2	0.4	7.4	3.1	0.3	-	-
	60	24.1	-	13.4	10.0	0.1	0.6	-
LO	20	24.3	-	14.9	7.8	1.4	0.2	-
	40	9.7	1.9	4.5	3.1	0.2	-	-
	60	24.5	-	13.5	10.4	0.1	0.5	-
EPO	20	27.6	-	16.9	9.1	1.4	0.2	-
	40	14.8	1.0	8.8	4.4	0.6	-	-
	60	27.2	-	16.3	10.3	0.1	0.5	-
FO	20	29.4	-	17.7	9.9	1.5	0.3	-
	40	9.5	0.9	5.3	3.1	0.2	-	-
	60	33.2	-	20.3	12.3	0.6	-	-

comparable to controls (7.3%) with 40mg/l p-oil dosage (4.5% to 8.8%), but these increased 2 to 3 fold with incubation of 20 or 60mg/l p-oil (13.0% to 20.6%). Control PGF_{α} proportions (5.2%) were also parallel to those found in cells supplemented with 40mg/l p-oil (3.1% to 8.8%), but levels increased slightly with 20mg/l p-oil (7.8% to 9.9%) and 2 to 3 fold with 60mg/l p-oil (10.0% to 15.2%). Incubation with 20mg/l p-oil enhanced PGE_2 production only slightly (1.2% to 1.9% vs 1.0% in controls), but decreased PGE_2 levels were detected with 40 and 60mg/l p-oil (0.1% to 0.6%). p-Oil supplementation, however, had little effect on PGE_1 production as trace amounts ($\leq$0.6%) of this compound were found both in control and dosed cells.

3.7.7 Discussion.

Supplementation of cultured rat skin fibroblasts with p-oils affected the viability of such cells. The finding that the p-oils used exhibited varying abilities to modulate cell proliferation reflected the different FA compositions of these compounds (Table 2.3.3.2.). The greater potential to inhibit cell viability with high concentrations of p-OO, p-SSO, p-LO, p-EPO and p-FO than with p-CO and p-MO (Figs. 3.7.1.2-3.7.1.8.) implied that monoenoic and polyenoic FA's were more effective growth inhibitory agents than saturated FA's. The greater cytotoxic potential of p-EPO compared to p-SSO could relate to the presence of approximately 9% GLA in the former only as both p-oils contained similar proportions of other FA's. It seemed possible therefore, that induction of the greatest degree of cytotoxicity with p-EPO or p-LO supplementation related to the GLA and ALA concentrations in these p-oils, respectively, especially since both p-oils contained similar complements of saturated and monounsaturated FA's. However, in the light of the similar effects these p-oils induced, and the fact that the proportion of GLA in p-EPO was much lower than ALA in p-LO (9% vs 63%), it was apparent that GLA exhibited greater growth inhibitory potential than ALA. This probably relates to the different double bond positions in these C18 trienoic FA's. It should be borne in mind, however, that synergistic or antagonistic

interactions between different p-oil FA's could also have played a role in the modulation of cell viability. This phenomenon may explain the enhanced cell growth induced only when certain concentrations of p-OO or p-FO were dosed, and why cell viability changes did not always correlate linearly with the amount of p-oil supplemented.

The finding that only trace amounts of the major FA components present in the p-oils remained in the spent medium following the 48 hour incubation period (Table 3.7.3.2.) indicated the capability of these cells to incorporate exogenous FA's, even in the presence of high 14:0 levels derived from 90% DMEM+10% FCS itself (Table 2.3.3.3.). Support for p-oil FA uptake from the culture medium was also shown in Table 3.7.3.1. Modifications of the membrane PGL FA composition have been reported to induce changes in membrane physical properties (Stubbs et al 1984 and Spector et al 1985). A mechanism whereby p-oils affected cell viability may therefore relate to differential exogenous FA uptake and alterations in membrane stability, dependent on p-oil FA composition and concentration dosed. Pronounced shifts in membrane fluidity would indeed support the limitation and inhibition of cell proliferation shown when large quantities of saturated or unsaturated FA-rich p-oils were fed (Figs. 3.7.1.2-3.7.1.8.).

p-Oil supplementation induced concentration changes in total cellular protein (Table 3.7.2.1.) which did not always parallel the changes found in cell viability (Figs. 3.7.1.2-3.7.1.8.). In fact, cell numbers could not justify the marked protein increase found with 40mg/l p-OO dosage, nor the increased cellular protein measured when p-oil dosage induced little effect on, or limited, cell viability. This, however, indicated p-oil FA involvement in the stimulation of protein synthesis, which may reflect enhanced protein turnover or enzyme expression to metabolise the incorporated p-oil FA's. Numerical differences in the protein concentrations measured when supplementation with identical amounts of different p-oils yielded similar cell numbers suggested that the ability to modulate protein synthesis related to differences in FA structure and probably also to FA synergism and antagonism. On the other hand, the protein amounts quantified in the spent medium of dosed cells were not statistically different from controls (section 3.7.2), thus it was unlikely that medium protein itself contributed significantly to the cellular protein changes demonstrated (Table 3.7.2.1.).

Cultured rat skin fibroblasts had little capability to desaturate or elongate 16:0 when incubated with p-oils, although decreased 18:0 and enhanced 18:1ω9 levels in dosed cells suggested the potential for Δ9D expression (Table 3.7.3.1.). The fact that all p-oils contained

18:1ω9 (Table 2.3.3.2.), however, could have contributed to the enhancement of cellular 18:1ω9 levels, although 16:1ω9 elongation could possibly be excluded in this regard since 16:1ω9 incorporated with p-FO dosage did not produce a concommitant increase in 18:1ω9. The lack of 18:1ω9 desaturation or elongation may nevertheless relate to competitive inhibition by PUFA's (Brenner et al 1966, 1969, Brenner 1977 and Holman 1986a, 1986b).

Cultured rat skin fibroblasts exhibited the potential to desaturate and chain elongate PUFA's in the presence of p-oil supplementation (Table 3.7.3.1.). No significant 18:3ω6 production from 18:2ω6 was apparent with p-oil dosage, although elevated 20:3ω6 levels with incubation of p-EPO, providing 18:3ω6, suggested that delta-6-desaturation of 18:2ω6 was slow, probably due to the rate limiting nature of this enzyme (eg. Stoffel 1961, Holloway 1963, Marcel et al 1968, Brenner 1971, 1974, 1977, 1982 and de Gomez Dumm et al 1976). Detection of small amounts of 20:2ω6 supported the capability for limited 18:2ω6 elongation, while decreased 20:3ω6/20:4ω6 and increments in 22:4ω6 levels, particularly with 20mg/l p-oil dosage, suggested Δ5-desaturation of 20:3ω6 and rapid subsequent elongation. However, the inability to form 22:5ω6 via Δ4D was consistent with 22:4ω6 accumulation. The finding that the immediate products of desaturation were lower than those following elongation supported documentation implicating the desaturases as

the rate limiting steps in the desaturase enzyme cascade (eg. Stoffel 1961, Holloway 1963, Marcel et al 1968 and Brenner 1971, 1977, 1982). A similar pattern of slow Δ6- and Δ5-desaturation was found with ω3 PUFA substrates. Despite 22:5ω3 formation by elongation of 20:5ω3 incorporated with p-FO dosage, only a small increase in 22:6ω3 production occurred via Δ4D with 20mg/l p-FO, suggesting suppressed expression of this enzyme, possibly because membrane unsaturation index was increased. Detection of low levels of long chain more unsaturated PDFA's when PUFA-rich p-oils were dosed could indeed relate to the known inhibitory effect of such moieties on desaturase enzymes, particlarly when product levels are sufficient for maintaining cell functions and membrane fluidity (eg. Garcia et al 1965, Brenner et al 1966, 1967, Brenner 1971 and Sprecher 1981). On the other hand, the greatest increase in relative 22:6ω3 percentages found when saturated or monoenoic FA-rich p-oils were dosed implied an increased need for highly unsaturated PDFA's to re-establish membrane fluidity altered with exogenous FA uptake, for example. However, increased detection of higher PDFA levels with p-CO, p-MO or p-OO dosage suggested enhanced desaturation and/or elongation of endogenous PUFA's as these p-oils contained relatively little, or no, PUFA's. The degree of FA unsaturation attained in the cells with p-oil dosage was therefore a factor involved in control

of desaturase cascade enzyme expression, particularly since modifications of the membrane PGL FA composition have been shown to induce changes in the activity of membrane bound enzymes (eg. Brenner et al 1965a, 1965b, 1966, 1967, 1969, Stubbs et al 1984 and Spector et al 1985). This could explain why the capability for desaturation and elongation were generally greatest with addition of low p-oil concentrations, despite increased cellular uptake of major p-oil FA components with the amount dosed (Table 3.7.3.1.).

To assess whether the pattern of p-oil desaturation described above was modulated by the presence of FA mixtures, p-oil desaturation was compared with that of individual radiolabelled C18 FA's. Only the pathway for elongation was shown with [^{14}C]-18:1ω9 dosage, whereas the pattern of radioactivity derived from cells enriched with either [^{14}C]-18:3ω3 or [^{14}C]-18:2ω6 was consistent with both desaturation and elongation of these moieties (Table 3.7.4.2.). Conversion of approximately 76% of incorporated [^{14}C]-18:3ω3 or [^{14}C]-18:2ω6 to PDFA's supported Δ6D expression, and the formation of 20:3ω3 and 20:2ω6 by elongation could have served as storage substrates for retroconversion or further desaturation. Conversion of about 47% of incorporated [^{14}C]-18:3ω3 and 42% [^{14}C]-18:2ω6 to post-Δ5D FA's was evidence for Δ5D activity, but Δ4D expression was more limited. Chapkin et al (1984) demonstrated a lack of conversion of 18:2ω6

to 18:3ω6 and 20:3ω6 to 20:4ω6 in isolated microsomes of rat epidermis, even when different amounts of 18:2ω6 or 20:3ω6 were fed as Δ6D or Δ5D substrates, respectively. They suggested that 20:4ω6 in rat skin was synthesised elsewhere, presumably the liver, and transported to the skin. Others reported that Δ6D and Δ5D activities were suppressed in the skin of EFA-deficient rats only, and that significant Δ6D and Δ5D expression occurred in the skin when the diet of normal rats was supplemented with 18:2ω6 (Lowe et al 1978). The variations in desaturase activity described above may relate to different experimental designs, but could also reflect the ability of this tissue to selectively regulate PDFA production.

The finding that desaturase cascade enzyme expression was more limited with p-oil compared to individual FA supplementation (Tables 3.7.3.1. and 3.7.4.2.) supported the mediation of competitive interactions between p-oil FA's for these enzymes. However, as organisms consume mixtures rather than individual FA's in the diet, the desaturase capability shown with p-oils probably relates closer to the situation <u>in vivo</u> than that shown with single FA's.

MDA quantitation showed that rat skin fibroblasts exhibited the capability to produce lipoperoxides (Table 3.7.5.1.). This could occur either enzymatically or by auto-oxidation of free monoenoic and/or polyenoic FA's. Measurement of relatively low MDA concentrations in

control and most dosed fibroblasts, however, indicated efficient intracellular mechanisms of protection against free radical formation. Indeed, it is recognised that aerobic organs are protected from oxygen toxicity by an array of defence systems, including preventative anti-oxidants such as vitamin E, and anti-oxidant enzymes such as peroxidase, superoxide dismutase and catalase which remove different species of activated oxygen that promote lipoperoxidation (Lands et al 1971, Smith et al 1972, Tappel 1972, 1980 and Pryor 1976). Alternatively, FA oxidation could have been suppressed by limited substrate availability. If FA's were present within the membranes as components of complex lipids they would not have been so readily accessible to oxidation, although the rate of PGL turnover could have influenced the release of FA's from membrane lipids into the cellular pool. These factors probably accounted for the greater cellular lipoperoxide amounts p-EPO induced compared to p-SSO, despite the similar FA compositions of these p-oils, although such could also relate to the presence of 18:3ω3 in p-EPO only (Table 2.3.3.2.) and the greater capability to form 20:3ω6 with p-EPO incubation (Table 3.7.3.1.). This could also explain the greater amounts of total lipoperoxides generated with p-EPO than p-LO supplementation (despite 63% 18:3ω3 in p-LO and only 9% 18:3ω6 in p-EPO), which probably related to the easier production of endoperoxides from 18:3ω6 and 20:3ω6 than

from 18:3ω3. The suppression/inhibition of microsomal PG biosynthesis or oxygenase activity by ω3 series PUFA's (Morisaki et al 1982b) could probably be excluded in this regard since dosage with 60mg/l p-FO induced the greatest cellular lipid peroxide amounts measured, which was probably largely attributable to the susceptibility of 20:5ω3 to oxidation.

Lipoperoxides have been implicated in the disruption of biological membranes (Mead et al 1976, Tappel et al 1980 and Frankel 1984), thus the small MDA amounts found in the spent medium of cultures dosed with 60mg/l p-FO or p-LO may have arisen from the release of cellular lipid peroxides through the membrane of intact cells or as a result of cell lysis. This could also have resulted in the escape of cellular FFA's and enzymes responsible for peroxidation into the medium, thus extracellular auto-oxidation should not be overlooked as a contributor to these medium lipoperoxide levels. On the other hand, the absence of MDA from all other spent media supported cellular uptake of dosed p-oil FA's.

Low cellular lipoperoxide levels generally correlated with high cell yields, whereas the significant increases in cellular and/or medium lipoperoxides levels found only with p-LO, p-EPO or p-FO dosage (Table 3.7.5.1.) correlated inversely with cell viability (Figs. 3.7.1.6-3.7.1.8.). It was apparent, however, that lipoperoxides were only partly responsible for the modulation of cell

viability since the greatest cytotoxicity induced with 60mg/l p-LO or p-EPO did not correlate with the highest cellular lipoperoxide amounts found. Skin *in vivo* is continuously exposed to oxygen in air, thus this tissue may be particularly resistant both to the generation and effects of lipoperoxides, and the changes induced in cell viability with p-oil dosage probably involved other mechanisms, such as alterations in membrane stability.

The finding that total eicosanoid formation by control rat skin fibroblasts comprised 83% of all lipoperoxides quantified (Tables 3.7.5.1. and 3.7.6.1.) was not surprising considering endogenous 20:4ω6 levels and the capability of these cells to form 1-, 2- and 3-series eicosanoid precursors via desaturation and elongation (Tables 3.7.3.1. and 3.7.4.2.). The observation that the prostanoids studied comprised a fraction of the molar amount quantitated indicated that these cells were largely involved in the production of other eicosanoids, but nevertheless supported functional cyclooxygenase activity in rat skin fibroblasts. Under the experimental conditions employed, the enzymic pathways for TXB_2 and PGF_{α} synthesis were greatly favoured over those involved in PGI_2, PGE_2 and PGE_1 production, and it was possible that PGH_2-PGD_2 isomerase was inhibited rather than absent as PGD_2 formation has been reported in rat skin *in vivo* (Ujihara et al 1988).

Lowe et al (1978) showed that 18:2ω6 supplementation of the diet of normal mice resulted in at least a two fold increase in cutaneous PGE and PGF levels compared to controls, but had little effect in EFA-deficiency. p-Oil supplementation also influenced the ability of rat skin fibroblasts to produce the prostanoids studied (Table 3.7.6.1.), but this was not related to the degree of p-oil unsaturation, the ability of the p-oils to provide direct eicosanoid precursors (Table 2.3.3.2.), or the capability of the cells to form eicosanoid precursors via the desaturase cascade (Table 3.7.3.1.). Indeed, the detection of increased levels of the 1-series eicosanoid precursor, 20:3ω6, only when cells were dosed with p-EPO induced no marked effect upon eicosanoid production compared to other p-oils at identical concentrations. Even p-LO and p-FO yielded prostanoid profiles similar to those derived from cells incubated with corresponding amounts of other p-oils, despite the known inhibitory actions of 18:3ω3 and 20:5ω3 on 20:4ω6 metabolism via cyclooxygenase and therefore 2-series prostanoid production (Lands et al 1971, 1973, Dyerberg et al 1978, Culp et al 1979, Hwang et al 1980, Hamazaki et al 1982, Marshall et al 1982 and Nassar et al 1987). The lack of significant differences in PGI_2, TXB_2, PGF_α, PGE_2 and PGE_1 levels found with dosage of different p-oils at the same concentration implied that exogenous FA's <u>per se</u> induced little effect on the biosynthesis of

the eicosanoids studied, thus enhancement of prostanoid production related to utilisation of membrane 20:3ω6 and 20:4ω6, which were detected in rat skin fibroblasts (Table 3.7.3.1.). However, the finding that 40mg/l p-oil had little effect on or suppressed prostanoid formation, whereas 20 and 60mg/l p-oil significantly stimulated eicosanoid production primarily by enhancing TXB_2 and PGF_{α} synthesis, implied p-oil concentration involvement in prostanoid synthesis. This could have influenced eicosanoid precursor availability, or modulated the expression of enzymes involved in the formation of the prostanoids studied, particularly at the PGH level.

Numerous studies have implicated PG involvement in the modulation of cell division in skin when such compounds were provided exogenously (Ziboh et al 1972, Bem et al 1974, Lupulescu 1976, Lowe et al 1977 and McCullough et al 1978). PGE compounds in particular, have been associated with increased epidermal proliferation *in vivo* (Lowe et al 1977), and similar results have been obtained in culture (Bem et al 1974). Others, however, found that the rate of epidermal proliferation increased 5 fold over controls in EFA-deficient rats with impaired PGE_2 synthesis, and that topical PGE2 application daily for one week restored the slower, normal rate of growth (Ziboh et al 1972 and McCullough et al 1978). `The present study, in contrast, could not establish any correlation between the eicosanoid profiles (Table

3.7.6.1.) and cell viability changes (Figs. 3.7.1.2-3.7.1.8.) induced with p-oil supplementation, and this implied that the endogenously synthesised prostanoids studied were not directly involved in the modulation of cell proliferation, despite alterations in overall prostanoid production or the balance between individual prostanoid groups. The involvement of lipoperoxides and alterations in membrane physical properties were proposed as likely mechanisms.

3.8 THE EFFECTS OF PSEUDO-OILS ON CELLS DERIVED FROM RAT ADIPOSE TISSUE.

3.8.1 Effects of pseudo-Oils on Cell Viability.

No morphological changes were found between control cells before, during and after each experiment, and dosed cells were morphologically similar to controls, except for cytoplasmic droplets observed with p-oil incubation at high concentrations in some instances.

The data depicted in Fig. 3.8.1.1. showed that final medium albumin concentrations ranging from 0 to 250mg/l induced no significant changes in cell viability. This excluded the possibility that albumin as FA carrier had any effect on cell viability, hence the effects induced with the p-oils were a result of the exogenous FA's.

11.4×10^4 control cells/ml culture medium were found at the end of the 24 hour post-trypsinisation recovery period. This equated to 70% of the final control cell number at the end of the 48 hour p-oil incubation period and represented the cytostatic number. Growth inhibition was therefore induced only with p-oil concentrations which reduced cell viability to significantly below 70%.

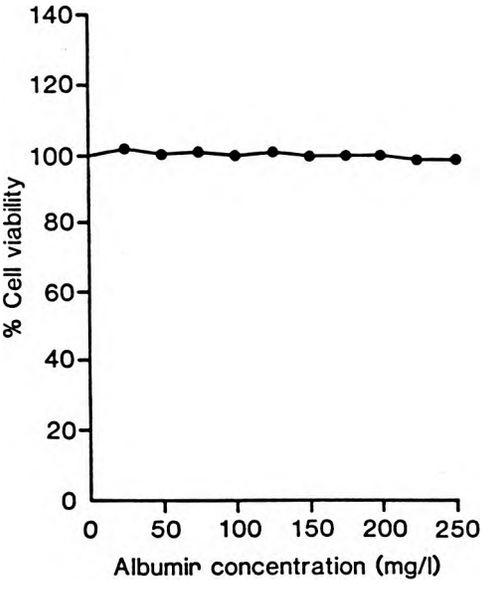
The cell viability changes induced following incubation with 0 to 100mg/l p-oil for 48 hours are shown in Figs. 3.8.1.2-3.8.1.8. All p-oils enhanced cell proliferation

Legend to Figs. 3.8.1.1-3.8.1.9.

The results are expressed as mean percent (%) cell viability±standard error of the mean (s.e.m.), where 'n' is the number of experiments. The concentrations given are as mg albumin or pseudo-oil per litre of growth medium. Fig. 3.8.1.1. shows the mean percent cell viability versus the albumin concentration (mg/l), and Figs. 3.8.1.2-3.8.1.8. depict the mean percent cell viability versus the pseudo-oil concentration (mg/l). Fig. 3.8.1.9. shows the mean percent cell viability versus the albumin concentration (mg/l) for cells dosed with the amount of pseudo-oil which limits cell viability to 50% of the controls.

Fig. 3.8.1.1.

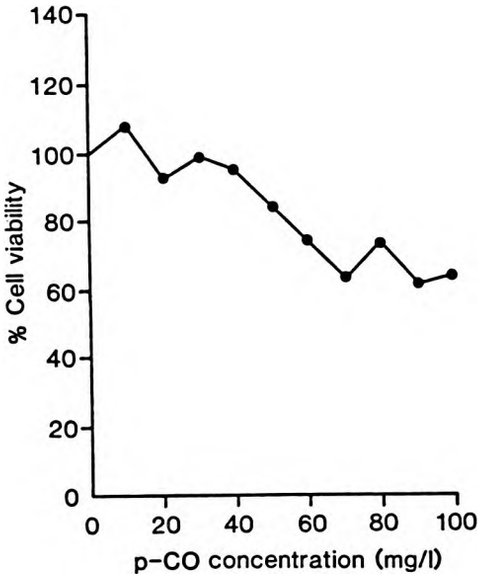
The percentage viability of cells derived from rat adipose tissue incubated with albumin.



Albumin Concentration (mg/l).	Mean	±s.e.m.	n
0	100.0	2.3	12
25	102.8	2.0	12
50	100.7	2.6	12
75	101.6	2.1	12
100	99.3	2.0	12
125	101.1	2.1	12
150	99.8	3.1	12
175	99.9	3.6	12
200	100.3	2.8	12
225	98.8	3.7	12
250	98.1	2.1	12

Fig. 3.8.1.2.

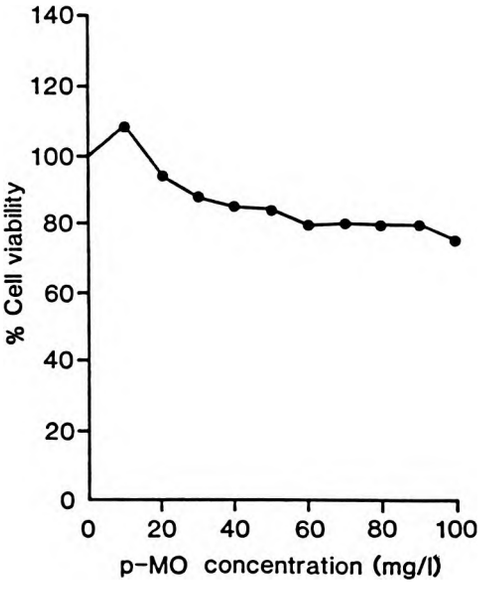
The percentage viability of cells derived from rat adipose tissue incubated with p-CO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.6	6
10	108.8	5.6	6
20	93.4	4.5	6
30	99.5	5.5	6
40	96.0	3.9	6
50	84.8	3.6	6
60	74.4	2.6	6
70	63.0	1.8	6
80	73.3	5.1	6
90	61.5	3.4	6
100	64.4	3.3	6

Fig. 3.8.1.3.

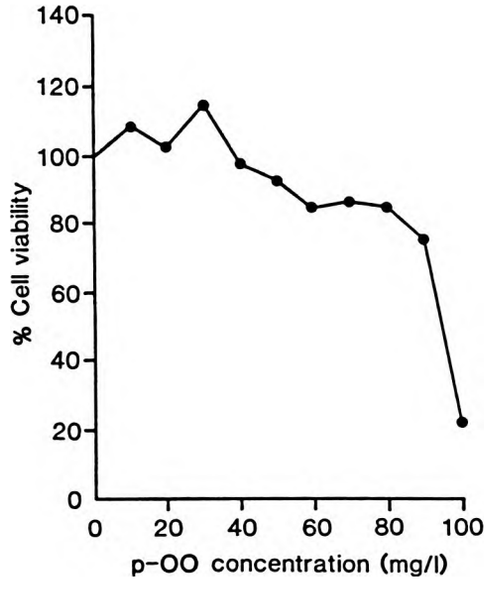
The percentage viability of cells derived from rat adipose tissue incubated with p-MO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.1	6
10	108.6	4.2	6
20	95.1	5.7	6
30	88.3	4.9	6
40	86.0	5.9	6
50	84.8	2.1	6
60	80.5	5.0	6
70	80.9	3.8	6
80	80.3	5.3	6
90	82.3	2.0	6
100	76.9	2.7	6

Fig. 3.8.1.4.

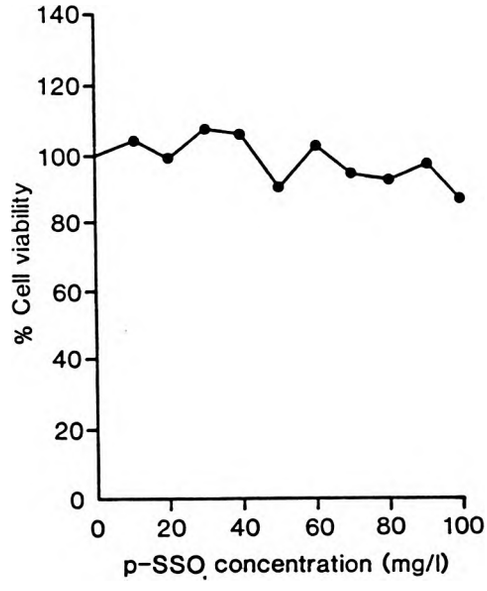
The percentage viability of cells derived from rat adipose tissue incubated with p-OO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.7	6
10	110.1	5.2	6
20	102.2	6.4	6
30	114.1	6.1	6
40	97.8	1.9	6
50	93.4	3.2	6
60	85.1	4.3	6
70	87.0	2.7	6
80	85.1	7.0	6
90	75.9	3.4	6
100	21.9	2.1	6

Fig. 3.8.1.5.

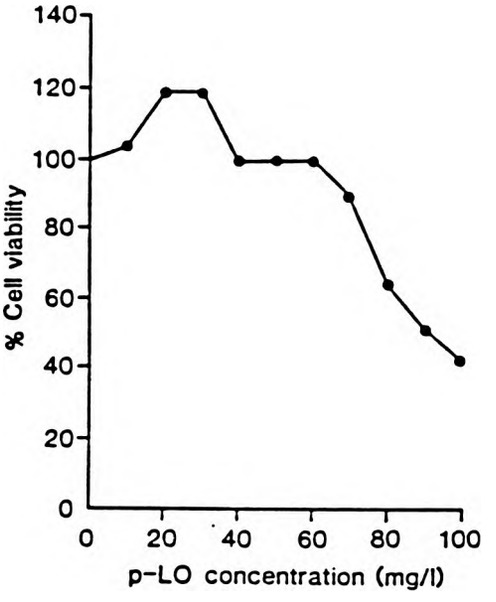
The percentage viability of cells derived from rat adipose tissue incubated with p-SSO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	1.6	6
10	104.7	4.7	6
20	98.9	4.3	6
30	107.0	3.6	6
40	105.4	2.2	6
50	90.4	2.2	6
60	102.8	3.7	6
70	93.7	2.5	6
80	92.2	4.7	6
90	97.0	3.6	6
100	87.0	3.6	6

Fig. 3.8.1.6.

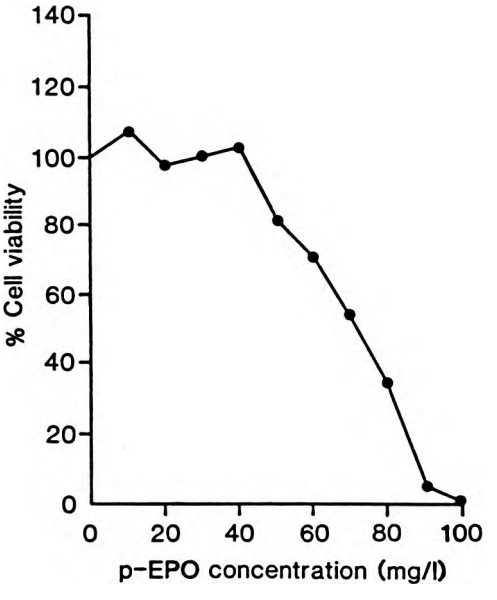
The percentage viability of cells derived from rat adipose tissue incubated with p-LO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	4.0	6
10	104.0	5.2	6
20	120.5	3.9	6
30	120.3	4.0	6
40	99.9	4.0	6
50	100.9	2.4	6
60	99.8	5.1	6
70	89.1	5.8	6
80	64.4	5.4	6
90	51.8	4.4	6
100	42.0	3.6	6

Fig. 3.8.1.7.

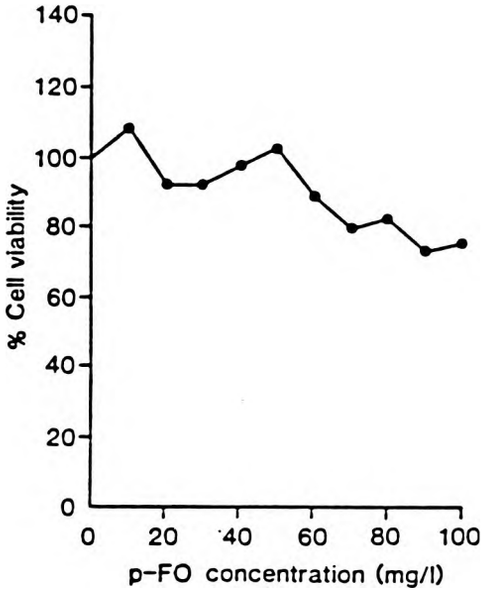
The percentage viability of cells derived from rat adipose tissue incubated with p-EPO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	5.6	6
10	108.1	2.6	6
20	97.8	4.3	6
30	100.5	4.8	6
40	103.3	4.6	6
50	80.9	5.4	6
60	71.2	5.4	6
70	53.9	1.9	6
80	35.2	1.9	6
90	4.7	1.2	6
100	0.3	0.3	6

Fig. 3.8.1.8.

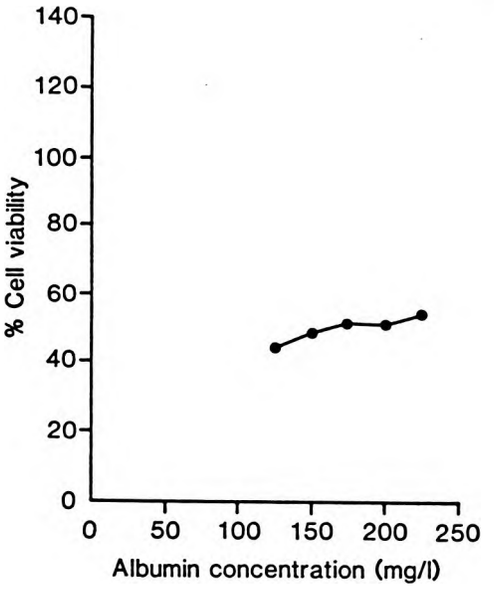
The percentage viability of cells derived from rat adipose tissue incubated with p-FO.



pseudo-Oil Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	2.8	6
10	107.8	7.1	6
20	91.7	4.3	6
30	92.2	3.7	6
40	97.5	4.5	6
50	102.4	4.4	6
60	88.5	4.8	6
70	79.1	3.5	6
80	81.9	5.6	6
90	72.6	4.4	6
100	75.2	5.0	6

Fig. 3.8.1.9.

The percentage viability of cells derived from rat adipose tissue incubated with albumin at the ID_{50} of p-EPO.



Albumin Concentration (mg/l)	Mean	±s.e.m.	n
0	100.0	3.2	6
125	45.0	4.5	6
150	49.3	2.4	6
175	52.0	4.1	6
200	51.0	3.1	6
225	54.1	3.1	6

slightly with low concentrations and limited growth to a greater or lesser extent with higher amounts dosed, although p-EPO, p-LO and p-OO were cytotoxic at high concentrations.

p-CO enhanced cell viability to 108.8% with 10mg/l, had little effect with 20 to 40mg/l, and limited growth to 84.8% with 50mg/l (Fig. 3.8.1.2.). Cell viability ranged from 61.5% to 74.4% with 60 to 100mg/l p-CO, relative to the cytostatic number of 70%, thus cytotoxicity was not marked and no ID_{50} was found. Cell proliferation was also stimulated with 10mg/l p-MO to 108.6%, and growth limitation increased progressively with higher amounts dosed to a minimum of 76.9% with 100mg/l p-MO (Fig. 3.8.1.3.), thus no ID_{50} could be calculated either.

Cell proliferation increased to 110.1% and 114.1% with 10 and 30mg/l p-OO, respectively, but approximated to controls with 20 and 40mg/l p-OO (Fig. 3.8.1.4.). Higher concentrations progressively limited cell growth to 85.1% with 60mg/l and 75.9% with 90mg/l p-OO, whereas 50% of cells were viable with 95mg/l p-OO (ID_{50}), but only 21.9% with 100mg/l p-OO.

Incubation with 10 to 90mg/l p-SSO had little effect on cell proliferation, although such was increased to 107.0% and 105.4% with 30 and 40mg/l p-SSO, respectively (Fig. 3.8.1.5.). This was the least effective p-oil to impair cell growth as 87.0% viability occurred even with

100mg/l p-SSO.

p-LO stimulated cell proliferation by 20% with 20 and 30mg/l and had little effect with 40 to 60mg/l, but limited growth to 89.1% with 70mg/l (Fig. 3.8.1.6.). p-LO induced cytotoxicity with concentrations of 80mg/l, or more, and half the cells were viable with 92mg/l p-LO (ID_{50}), compared to 42% with 100mg/l p-LO.

Cell viability was 108.1% with 10mg/l p-EPO, but in line with controls following 20 to 40mg/l p-EPO dosage (Fig. 3.8.1.7.). Cell proliferation was limited to 80.9% with 50mg/l p-EPO and parallel to the cytostatic number with 60mg/l p-EPO, but higher amounts induced concentration dependent cytotoxicity such that half the cells were killed with 73mg/l p-EPO (ID_{50}) and practically all with 90 and 100mg/l p-EPO. This was the most effective p-oil to impair the proliferation of these cells.

Cell viability remained within 10% of controls with dosage of up to 50mg/l p-FO, and was merely limited with higher concentrations to a minimum of 72.6% with 90mg/l p-FO (Fig. 3.8.1.8.). p-FO thus induced similar effects to p-MO (Fig. 3.8.1.3.) and p-SSO (Fig. 3.8.1.5.).

Cells were incubated with a fixed amount of p-EPO to correspond with its ID_{50} (70mg/l) and supplemented with varying amounts of albumin to eliminate the possibility that any effects induced with p-oil dosage were related to the amount of albumin used as FA carrier. The albumin

concentration was varied at 2 points above and below the ID_{50} of p-EPO, and the results obtained following the 48 hour incubation period are depicted in Fig. 3.8.1.9. This indicated that the concentration of p-EPO dosed reduced cell viability to about 50% of controls, and that such was not significantly influenced by different albumin concentrations. Exclusion of the synergistic involvement of albumin therefore implied that the effects induced with the p-oils were attributed solely to the exogenous FA's.

Subsequent to these studies, cells derived from rat adipose tissue were plated and dosed appropriately with 0, 20, 40 or 60mg p-oil/l culture medium in sufficient amounts to permitt all quantitative and qualitative analyses to be carried out. Upon harvesting, cell yields were compared and found to be statistically similar to those in Figs. 3.8.1.1-3.8.1.8. All further biochemical assays were thus performed on these samples.

3.8.2 Effects of pseudo-Oils on Total Protein.

The total protein concentrations determined for cells supplemented with 0, 20, 40 or 60mg/l p-oil are shown in Table 3.8.2.1. as µg total protein/10^6 cells seeded.

134.7µg total protein per 10^6 control cells seeded was quantitated, whereas concentrations in dosed cells were increased and ranged from 142.8 to 205.5µg protein/10^6 cells seeded (found with 60mg/l p-EPO and 20mg/l p-LO, respectively). Cellular protein concentrations decreased over the range dosed in the presence of p-CO, p-SSO or p-LO, but increased with the amount of p-MO added. On the other hand, more cellular protein was measured with 40mg/l than with 20 or 60mg/l p-EPO and p-FO, but the converse was found with p-OO.

Table 3.8.2.1.

The protein content of cells derived from rat adipose tissue, expressed as µg total protein/10^6 cells seeded.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	134.7			
CO		162.4	161.1	157.5
MO		158.3	165.7	178.8
OO		167.4	149.4	155.3
SSO		164.5	165.6	143.9
LO		205.5	175.5	156.2
EPO		164.6	172.8	142.8
FO		172.4	196.9	167.4

3.8.3 Effects of pseudo-Oils on the Fatty Acid Spectrum of Cells Derived From Rat Adipose Tissue.

The FA spectra for cells incubated with 20, 40 or 60mg/l p-oil are shown in Table 3.8.3.1. in relation to undosed cells. The greatest proportion of any single FA in the controls was 18:1ω9 (23.4%), but similar percentages were found for 16:0 (22.3%) and 18:0 (21.8%). Other FA's contributing >5.0% to the total spectrum in control cells included 18:2ω6 (9.0%) and 22:4ω6 (5.8%). Varying amounts of these moieties were present in dosed cells.

In general, dosed cells contained less 18:0 and 18:1ω9, variable proportions of 16:0 and 16:1ω9, and more 18:2ω6 and 18:3ω6, compared to controls. ω6 PDFA levels were generally parallel to controls or increased in dosed cells, although 22:5ω6 was decreased. 18:3ω3 and 20:4ω3 levels were decreased, but 20:5ω3, 22:5ω3 and 22:6ω3 amounts were generally increased in dosed cells.

16:0 levels increased from 22.3% and 21.4% to 28.1% and 31.6% when cells were supplemented with 0, 20, 40 and 60mg/l p-CO, respectively. On the other hand, 16:1ω9 was significantly raised only with 40mg/l p-CO (4.0% vs 1.5% in controls), while 18:0 and 18:1ω9 levels were parallel to controls or decreased. Dosed cells contained less 18:2ω6 than controls (9.0%), but 20:4ω6 was increased at least 2 fold (3.6% to 5.3% vs 1.6% in controls). 22:4ω6

Legend to Table 3.8.3.1.

All values are tabulated as relative percent total area. Control values are reported as mean±s.e.m., where 'n' is the number of experiments. '[p-Oil]' refers to the pseudo-oil concentration used.

Table 3.8.3.1.

The fatty acid spectrum of cells derived from rat adipose tissue.

FATTY ACID SPECTRUM (%)		CONTROLS (n=3)	[p-CO] (mg/l) 20	40	60	[p-MO] (mg/l) 20	40	60	[p-OO] (mg/l) 20	40	60	[p-SSO] (mg/l) 20	40	60	[p-LO] (mg/l) 20	40	60	[p-EPO] (mg/l) 20	40	60	[p-FO] (mg/l) 20	40	60
SATURATES.	16:0	22.3±0.55	21.4	28.1	31.6	19.3	21.9	23.2	17.7	16.0	19.1	17.5	17.6	19.8	26.1	14.3	23.6	11.0	24.4	23.5	18.5	20.6	21.2
	18:0	21.8±0.00	15.7	15.7	18.7	15.6	18.1	15.1	10.8	13.6	6.7	14.6	17.2	10.8	15.4	13.9	11.6	21.6	20.9	11.7	13.8	11.2	8.1
	20:0	0.8±0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.1	-	1.0	-	-	-	-
	22:0	0.2±0.00	0.2	0.6	0.1	0.1	1.0	0.1	-	-	-	-	-	-	0.1	1.8	-	-	0.1	0.1	-	0.1	0.1
	24:0	0.3±0.03	-	0.5	0.1	0.1	0.6	0.4	-	-	-	-	0.2	0.3	-	0.2	-	-	0.3	0.4	-	-	-
ω9 MONOS.	16:1	1.5±0.05	0.9	4.0	1.8	0.5	0.4	2.2	0.7	0.4	2.3	0.4	0.7	2.7	4.1	3.1	3.8	0.4	0.7	0.9	4.6	11.7	15.9
	18:1	23.4±0.75	20.7	18.1	25.3	20.0	19.1	23.4	31.6	42.2	48.8	19.4	19.1	19.7	15.8	17.1	18.3	22.3	21.3	18.8	19.6	17.4	18.1
	24:1	-	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ω6 POLYS.	18:2	9.0±0.85	8.4	3.4	5.6	13.0	10.3	14.3	11.1	11.8	11.6	24.7	26.7	31.6	11.1	11.9	11.7	31.1	20.7	33.7	11.5	7.8	9.2
	18:3	-	0.9	0.6	0.3	0.9	0.2	0.9	0.6	-	0.4	1.8	-	1.1	0.7	1.4	0.9	4.6	0.9	1.6	1.4	0.2	0.3
	20:2	1.4±0.05	-	3.9	-	-	3.7	0.3	0.9	3.7	1.7	0.9	2.0	1.3	-	1.7	-	0.5	0.5	-	-	0.9	0.5
	20:3	1.0±0.05	1.9	2.3	1.3	1.6	2.7	1.4	1.4	0.6	0.7	1.2	1.4	1.0	1.0	1.9	0.9	4.1	1.0	0.4	1.2	1.4	0.9
	20:4	1.6±0.05	5.3	4.0	3.6	5.0	4.6	5.6	3.5	1.5	1.7	2.7	2.3	1.9	3.7	3.2	2.7	1.6	1.5	0.6	3.5	2.7	2.3
	22:4	5.8±0.90	10.5	7.7	4.1	10.8	7.2	5.5	11.4	4.0	2.1	8.0	4.3	5.0	8.4	6.6	7.4	1.0	4.4	6.5	8.5	5.4	4.9
	22:5	2.0±0.05	0.2	1.9	0.2	0.2	1.5	0.2	0.1	0.6	0.1	0.3	1.1	0.1	0.1	1.7	0.1	0.1	0.7	-	0.2	1.0	0.1
ω3 POLYS.	18:3	0.2±0.00	1.0	0.4	0.6	1.1	0.3	0.9	1.3	0.6	1.2	1.8	0.5	1.1	5.1	10.7	15.7	-	0.2	0.4	1.4	0.5	0.7
	18:4	2.9±0.20	0.4	-	0.6	0.7	0.2	0.2	0.3	1.0	0.1	0.4	1.5	0.2	0.3	1.5	0.2	0.1	0.9	0.2	0.3	0.2	0.3
	20:4	3.0±0.15	2.2	2.2	0.5	1.9	1.9	0.7	1.0	1.5	0.1	1.1	1.5	0.4	1.8	2.6	0.6	-	0.5	0.3	0.7	1.7	1.2
	20:5	0.4±0.00	1.3	1.4	0.7	1.5	1.2	0.6	1.4	0.2	0.4	0.3	0.4	0.4	1.6	0.6	0.3	0.2	-	-	4.3	5.8	9.5
	22:5	1.1±0.00	3.9	2.3	2.2	3.6	2.4	2.3	2.9	1.0	1.4	2.5	1.8	1.4	2.3	4.3	1.1	0.8	0.3	0.5	7.7	9.1	4.9
	22:6	1.3±0.10	4.6	3.0	2.8	4.1	2.9	2.7	3.2	1.3	1.6	2.5	1.9	1.4	2.6	1.5	1.0	0.6	0.2	0.4	3.0	2.3	2.1

percentages were raised with 20 and 40mg/l p-CO (10.5% and 7.7%, respectively vs 5.8% in controls), yet 22:5ω6 levels were parallel to controls (2.0%) or decreased. p-CO induced a marked reduction in 18:4ω3 (0% to 0.6% vs 2.9% in controls), but 20:5ω3, 22:5ω3 and 22:6ω3 levels increased 2 fold or more in relation to controls (0.4%, 1.1% and 1.3%, respectively).

p-MO dosage had little effect on 16:0 and 18:1ω9 levels, but reduced 18:0 and 16:1ω9 amounts, although the latter was slightly raised with 60mg/l p-MO (2.2% vs 1.5% in controls). Up to 14.3% 18:2ω6 and 2.7% 20:3ω6 were found (9.0% and 1.0% in controls, respectively), 20:4ω6 levels ranged from 4.6% to 5.6% (1.6% in controls), and 22:4ω6 was increased almost 2 fold with 20mg/l (10.8% vs 5.8% in controls), yet 22:5ω6 amounts were decreased. p-MO induced a marked reduction in 18:4ω3 and 20:4ω3, but up to 1.5% 20:5ω3, 3.6% 22:5ω3 and 4.1% 22:6ω3 were found (0.4%, 1.1% and 1.3% in controls, respectively).

A concentration dependent increase in cellular 18:1ω9 occurred with dosage of 0, 20, 40 or 60mg/l p-OO (23.4% to 31.6%, 42.2% and 48.8%, respectively), but 16:0 and 18:0 levels were decreased and increased 16:1ω9 was found only with 60mg/l p-OO (2.3% vs 1.5% in controls). p-OO had little effect on 18:2ω6 levels, but 20:2ω6 was increased with 40mg/l (3.7% vs 1.4% in controls). 20:4ω6 and 22:4ω6 increased about 2 fold with 20mg/l p-OO (3.5%

and 11.4% vs 1.6% and 5.8% in controls, respectively), but no increase in 22:5ω6 was found. 18:4ω3 and 20:4ω3 amounts were decreased with all concentrations, but 20:5ω3, 22:5ω3 and 22:6ω3 were significantly increased only with 20mg/l p-OO (1.4%, 2.9% and 3.2% vs 0.4%, 1.1% and 1.3% in controls, respectively).

p-SSO supplementation decreased cellular 16:0, 18:0 and 18:1ω9 amounts, and 16:1ω9 was significantly increased only with 60mg/l p-SSO (2.7% vs 1.5% in controls). 18:2ω6 percentages increased from 9.0% to 24.7%, 26.7% and 31.6% with 0, 20, 40 and 60mg/l p-SSO, respectively, and up to 1.8% 18:3ω6 was found (none in controls). 20:3ω6 levels were parallel to controls (1.0%), but 20:4ω6 amounts were increased (1.9% to 2.7% vs 1.6% in controls). 22:4ω6 levels were increased only with 20mg/l p-SSO (8.0% vs 5.8% in controls), and decreased 22:5ω6 was found. p-SSO decreased cellular 18:4ω3 and 20:4ω3 amounts, but 22:5ω3 and 22:6ω3 were increased about two fold with 20mg/l p-SSO (2.5% each vs 1.1% and 1.3% in controls, respectively).

16:0 levels ranged from 14.3% to 26.1% with p-LO dosage (22.3% in controls), 22:0 increased significantly with 40mg/l p-LO (1.8% vs 0.2% in controls), 16:1ω9 increased at least two fold (1.5% in controls), whereas 18:1ω9 and 18:0 percentages were decreased. p-LO had little effect on 18:2ω6 levels, 20:4ω6 and 22:4ω6 were increased (2.7% to 3.7% and 6.6% to 8.4% vs 1.6% and 5.8% in controls,

respectively), although 22:5ω6 was decreased. 18:3ω3 percentages increased from 0.2% to 5.1%, 10.7% and 15.7% with 0, 20, 40 or 60mg/l p-LO dosage, respectively, yet decreased 18:4ω3 and 20:4ω3 were found. Increments were, however, detected for 20:5ω3 with 20mg/l p-LO (1.6% vs 0.4% in controls), 22:5ω3 with 20 and 40mg/l p-LO (2.3% and 4.3%, respectively vs 1.1% in controls) and 22:6ω3 with 20mg/l p-LO (2.6% vs 1.3% in controls).

16:0, 16:1ω9, 18:0 and 18:1ω9 percentages approximated to controls or were decreased with p-EPO incubation, but 18:2ω6 proportions were greatly increased (31.1%, 20.7% and 33.7% with 20, 40 and 60mg/l, respectively vs 9.0% in controls). Cells contained 0%, 4.6%, 0.9% and 1.6% 18:3ω6 with 0, 20, 40 and 60mg/l p-EPO, respectively, and 4.1% 20:3ω6 was detected with 20mg/l p-EPO (1.0% in controls). 20:4ω6 levels, however, were not increased, and the percentage of 22:4ω6 was raised only with 60mg/l p-EPO (6.5% vs 5.8% in controls). On the other hand, ω3 PDFA levels were parallel to controls or decreased.

p-FO incubation increased cellular 16:1ω9 levels to 4.6% with 20mg/l, 11.7% with 40mg/l and 15.9% with 60mg/l (1.5% in controls), reduced 18:0 percentages from 21.8% in controls to 13.8%, 11.2% and 8.1%, respectively, but had no marked effect on 16:0, 18:1ω9 and 18:2ω6. Raised amounts of 20:4ω6 were found with 20, 40 and 60mg/l p-FO (3.5%, 2.7% and 2.3%, respectively vs 1.6% in controls),

and 22:4ω6 with 20mg/l p-FO (8.5% vs 5.8% in controls), but 22:5ω6 percentages were decreased. Dosage with 20, 40 or 60mg/l p-FO greatly increased the levels of 20:5ω3 (4.3%, 5.8% and 9.5%, respectively vs 0.4% in controls), and 22:5ω3 (7.7%, 9.1% and 4.9%, respectively vs 1.1% in controls), but the increment in 22:6ω3 was smaller (3.0%, 2.3% and 2.1%, respectively vs 1.3% in controls).

3.8.4 Incorporation of Radiolabelled C18 Fatty Acids into Cells Derived from Rat Adipose Tissue.

The radioactivity recovered from cultures incubated with 2μCi [^{14}C]-18:1ω9, [^{14}C]-18:2ω6 or [^{14}C]-18:3ω3 is shown in Table 3.8.4.1.

For both [^{14}C]-18:1ω9 and [^{14}C]-18:3ω3 the equivalent of 4.3×10^6cpm were dosed to the cultures, and total recovery approximated to 77% and 91%, respectively. For [^{14}C]-18:2ω6 the equivalent of 4.8×10^6cpm were dosed to the growth medium, and the total recovery of this radioisotope after 48 hours incubation amounted to 85%. The counts recovered from the spent incubation media ranged from 1.8 to 2.8×10^6cpm, whereas only 0.4 to 0.5×10^6cpm were recovered from the pooled buffers after washing the cells, which accounted for non-specific binding. The amount of each of these radioisotopes incorporated into the cells themselves equated to 22% for [^{14}C]-18:1ω9, 29% for [^{14}C]-18:2ω6 and 14% for [^{14}C]-18:3ω3.

The percentage conversion of incorporated [^{14}C]-18:1ω9, [^{14}C]-18:2ω6 and [^{14}C]-18:3ω3 to ω9, ω6 and ω3 series products, respectively after 48 hours incubation with cells derived from rat adipose tissue are shown in Table 3.8.4.2.

29.9% of the total [^{14}C]-18:1ω9 counts derived upon GLC

Table 3.8.4.1.

The radioactivity (cpm) recovered from rat adipose cultures incubated with radiolabelled C18 fatty acids, expressed as mean (±s.e.m.), n=3.

SPENT MEDIA (M)	POOLED WASHINGS (W)	TOTAL (M+W)	CELLS	TOTAL $\times 10^6$
Total 18:1 counts dosed to incubation medium = 4.3×10^6				
1838100 (±10480)	528000 (±7440)	2366100	938754 (±771)	3.3
Total 18:2 counts dosed to incubation medium = 4.8×10^6				
2235400 (±14720)	508380 (±4020)	2743780	1368009 (±5787)	4.1
Total 18:3 counts dosed to incubation medium = 4.3×10^6				
2841400 (±11940)	419400 (±3360)	3260800	613707 (±2496)	3.9

Table 3.8.4.2.

The counts detected for the radiolabelled fatty acids dosed and their resulting metabolites formed after incubation with cells derived from rat adipose tissue, expressed as a percentage of the total counts recovered upon GLC analysis.

ω9 MONOS.	[^{14}C]-18:1ω9	ω6 POLYS.	[^{14}C]-18:2ω6	ω3 POLYS.	[^{14}C]-18:3ω3
18:1	29.9	18:2	2.8	18:3	6.7
20:1	28.1	18:3	18.3	18:4	26.9
22:1	30.4	20:2	17.6	20:3	11.4
24:1	11.7	20:3	16.3	20:4	11.4
		20:4	20.3	20:5	21.1
		22:4	12.8	22:5	8.2
		22:5	11.9	22:6	14.2
TOTAL CPM	1237	TOTAL CPM	2745	TOTAL CPM	1485

analysis (1237cpm) were unchanged. Similar percentages were detected for 20:1ω9 (28.1%) and 22:1ω9 (30.4%), but only 11.7% was found as 24:1ω9.

Only 2.8% of the total [^{14}C]-18:2ω6-derived counts detected following GLC (2745cpm) remained unaltered, whereas 18.3% was found as 18:3ω6 and 17.6% as 20:2ω6. 20:3ω6 and 20:4ω6 accounted for 16.3% and 20.3% of the total, respectively, compared to 12.8% 22:4ω6 and 11.9% 22:5ω6.

1485cpm were derived upon GLC analysis of cells dosed with [^{14}C]-18:3ω3, of which only 6.7% remained as 18:3ω3 and 11.4% found for its immediate elongation product, 20:3ω3. The percentage for 18:4ω3 (26.9%) was almost 10% higher than that of the corresponding ω6 series product, 18:3ω6 (18.3%), while 11.4% 20:4ω3 was found. 21.1% of the total counts were shown to be 20:5ω3 compared to 8.2% 22:5ω3, and the level of the subsequent product of delta-4-desaturation was similar to that found for the corresponding product in the ω6 series (14.2% 22:6ω3 vs 11.9% 22:5ω6).

Δ6D is the first and rate limiting enzyme in the desaturase cascade, thus total activity of this enzyme is reflected by the sum of all subsequent desaturase and elongase products.

3.8.5 Effects of pseudo-Oils on Lipid Peroxide Formation.

Table 3.8.5.1. shows the MDA concentrations measured in cells dosed with 0, 20, 40 or 60mg/l p-oil, as well as their respective growth media. The data is expressed as nmoles MDA/10^6 cells, but in the case of the spent media this represents the nmoles of MDA in the volume of medium from which $1x10^6$ cells were obtained.

0.5nmoles MDA/10^6 control cells were quantified, while concentrations in dosed cells ranged from 0.6 to 4.2 nmoles MDA/10^6 cells. MDA generation varied with p-oil, but generally increased with the concentration of p-oil supplemented. Cells incubated with 20, 40 or 60mg/l p-OO induced the lowest MDA amounts overall (0.6, 0.6 and 0.9 nmoles MDA/10^6 cells, respectively), concentrations were similar with p-CO, p-MO or p-FO supplementation (0.8 to 1.5nmoles MDA/10^6 cells), while PUFA-rich p-oils induced the largest MDA amounts. Cellular levels increased from 0.6 to 0.8 and 4.2nmoles MDA/10^6 cells in the presence of 20, 40 or 60mg/l p-LO compared to 1.1, 1.1, 2.1nmoles MDA/10^6 cells with p-SSO and 1.6, 1.6, 2.2nmoles MDA/10^6 cells with p-EPO, respectively. Medium derived from cultures incubated with 60mg/l p-EPO contained 2.1nmoles MDA/10^6 cells, but none was found in other spent media.

Table 3.8.5.1.

Lipoperoxide formation by rat adipose cultures incubated with p-oils, expressed as nmoles MDA/10^6 cells.

pseudo-Oil (mg/l)	CELLS			
	0	20	40	60
Control	0.5			
CO		0.9	1.3	1.3
MO		1.0	1.2	1.5
OO		0.6	0.6	0.9
SSO		1.1	1.1	2.1
LO		0.6	0.8	4.2
EPO		1.6	1.6	2.2
FO		0.8	1.2	1.5

pseudo-Oil (mg/l)	SPENT INCUBATION MEDIUM			
	0	20	40	60
Control	-			
CO		-	-	-
MO		-	-	-
OO		-	-	-
SSO		-	-	-
LO		-	-	-
EPO		-	-	2.1
FO		-	-	-

3.8.6 The Eicosanoid Profile of Cells Derived from Rat Adipose Tissue.

Table 3.8.6.1. shows the eicosanoids detected positively in control and dosed cells. All data is presented as a percentage of the total area found, but an indication of the total eicosanoid amounts quantified is given for controls in "( )", expressed as pmoles total eicosanoids per 10^6 cells.

Total eicosanoid production by control cells amounted to 215.9pmoles/10^6 cells, of which 35.9% correlated with the retention times of the prostanoid standards used. This was composed of 16.6% PGF_{α}, 8.2% TXB2, 7.7% PGE2 and 3.4% PGI_2, and no PGD2 was shown in control cells. Varying proportions of these prostanoids were found in dosed cells, but neither control nor dosed cells formed PGE_1.

The percentage of total prostanoids detected in dosed cells varied significantly with p-oil concentration, as such was decreased with supplementation of 60mg/l p-oil (20.9% to 26.7%) and particularly 40mg/l p-oil (11.8% to 19.7%), but was more variable with 20mg/l p-oil (22.8% to 41.4%). Such changes related to alterations in the production of individual prostanoids in these cells. PGI_2 levels were increased with dosage of 40mg/l p-oil (3.8% to 5.8% vs 3.4% in controls), generally decreased with 60mg/l p-oil (1.9% to 3.4%), and more variable with

Legend to Table 3.8.6.1.

Values are tabulated as relative percent of the total area detected, and as pmoles total eicosanoids/10^6 control cells.

Table 3.8.6.1.

The profile of the eicosanoids detected in extracts of cells derived from rat adipose tissue.

pseudo-Oil (mg/l)		EICOSANOIDS (%)						
		TOTAL	PGI_2	TXB_2	$PGF_{\alpha(1+2)}$	PGE_2	PGE_1	PGD_2
CONTROL (215.9)		35.9	3.4	8.2	16.6	7.7	-	-
CO	20	22.8	4.8	11.0	7.0	-	-	-
	40	11.8	5.3	3.3	2.2	0.4	-	0.6
	60	20.9	2.5	10.3	6.1	2.0	-	-
MO	20	27.8	3.9	13.1	8.2	2.6	-	-
	40	17.2	5.3	5.4	4.0	1.9	-	0.6
	60	22.7	2.9	11.2	7.0	1.6	-	-
OO	20	36.3	2.5	15.3	18.5	-	-	-
	40	17.8	3.9	6.1	4.6	2.3	-	0.9
	60	26.7	2.0	14.6	7.7	2.4	-	-
SSO	20	41.4	3.1	23.0	9.3	6.0	-	-
	40	19.6	3.8	6.3	4.5	3.1	-	1.9
	60	24.3	2.1	12.5	7.8	1.9	-	-
LO	20	29.2	2.6	18.1	7.5	1.0	-	-
	40	15.5	5.8	4.4	3.6	1.5	-	0.2
	60	23.5	1.9	12.8	7.0	1.8	-	-
EPO	20	36.4	4.7	17.8	13.0	0.9	-	-
	40	19.7	5.5	7.1	4.5	1.8	-	0.8
	60	21.2	3.4	9.1	5.7	3.0	-	-
FO	20	30.1	4.1	13.5	8.5	4.0	-	-
	40	14.5	5.5	3.1	2.0	3.3	-	0.6
	60	22.2	2.3	12.2	6.8	0.9	-	-

20mg/l p-oil (2.5% to 4.8%). TXB_2 amounts were increased in relation to controls (8.2%) with 20mg/l (11.0% to 23.0%) and 60mg/l (9.1% and 14.6%) p-oil, but decreased with 40mg/l p-oil (3.1% to 7.1%). p-Oil supplementation suppressed the production of PGF_{α}, particularly with a concentration of 40mg/l (2.0% to 4.6%), although amounts were comparable to controls (16.6%) in the presence of 20mg/l p-OO (18.5%). Dosed cells also formed less PGE_2 than controls (0% to 6.0% vs 7.7%), and small amounts of PGD_2 were detected only with incubation of 40mg/l p-oil (0.6% to 1.9% vs 0% in controls).

3.8.7 Discussion.

p-Oil supplementation affected the viability of cultured rat adipose cells (Figs. 3.8.1.2-3.8.1.8.). The effects induced, however, were not consistent with the degree of p-oil unsaturation, hence it could not be established whether PUFA-rich p-oils exhibited greater potential to modulate cell proliferation than p-oils abundant in monounsaturated or saturated FA's. The p-oil-specific effects induced could rather be explained in terms of p-oil FA composition (Table 2.3.3.2.) and concentration dosed, although the possibility also existed that effects were enhanced or deminished by p-oil FA's operating either in concert with each other, or antagonistically. The data supported FA involvement in the enhancement of cell proliferation with incubation of low p-oil concentrations. However, the finding that cell proliferation was stimulated over a wider concentration range with incubation of p-OO, p-SSO or p-LO than with other p-oils implied greater involvement of 18:1ω9, 18:2ω6 and 18:3ω3, respectively in this process than other FA's. This may relate to the chain length of these moieties and the fact that 18:1ω9, 18:2ω6 and 18:3ω3 are Δ6D substrates, whereas the different number and position of double bonds in these C18 FA's may explain why 18:3ω3 was a more effective growth promoting agent than 18:2ω6 and 18:1ω9. This indicated that FA structure was an important determinant of cell viability. p-SSO

and p-EPO exhibited similar FA compositions with a rich supply of 18:2ω6 (about 70%), thus the cytotoxicity induced with p-EPO, but not p-SSO, dosage probably related to the presence of 9% 18:3ω6 in p-EPO only. This implied, therefore, that 18:3ω6 was a more potent growth inhibitory agent than 18:2ω6. The cell death p-LO and p-OO induced at high concentrations also indicated that 18:3ω3 and 18:1ω9 exhibited greater cytotoxic potential than 18:2ω6, but it was apparent that these C18 FA's were less effective than 18:3ω6. On the other hand, it seemed that 18:3ω3 was more effective than 18:3ω6 in stimulating cell proliferation. Such effects could have related to the presence of a double bond at the ω12-position in 18:3ω6.

No reference has been found in the literature in which the effects of FA's on rat adipose cell growth has been investigated, although the influence of exogenous FA's has been studied with rat mammary cells in culture (Wicha et al 1979). Mammary tissue is largely modified adipose tissue and is thus another cell type specialised for handling large amounts of FA's. Like adipose tissue, mammary tissue is actively involved in FA biosynthesis and is affected by dietary lipids (Lehninger 1982). In the presence of de-lipidised FCS, insulin, progesterone, estrogen and prolactin, Wicha et al (1979) showed that normal mammary epithelial cell proliferation in culture was inhibited with all concentrations of palmitic and

stearic acids tested (0.1-10mg/l), whereas unsaturated FFA's induced both stimulatory and inhibitory effects. Linoleic and alpha-linolenic acids were most stimulatory and resulted in a doubling of the growth rate with concentrations of 1.0 and 0.1mg/l, respectively, whereas both oleic and arachidonic acids were vastly less stimulatory. Linoleic acid could not induce cytotoxicity with any concentration dosed (0.1-10mg/l), but this was induced with greater than 0.5mg/l arachidonic acid and 5mg/l oleic and α-linolenic acids (Wicha et al 1979). Comparison with our data clearly indicated that the effects individual FA's induced were more dramatic than with p-oil dosage, and this supported the mediation of antagonistic effects between FA's, which probably occurs in vivo when FA mixtures are consumed as dietary oils. On the other hand, it was possible that the individual FA effects Wicha et al (1979) reported on cell growth were mediated by the EFA-deficient culture conditions used, or modifications of membrane-bound hormone receptors as a result of hormonal supplementation. Wicha et al (1979) solubilised the FA's used in ethanol such that the final ethanol concentration in the culture medium was 1.0%. Our studies, however, have shown this concentration to be toxic both to normal and transformed cells (Davidson et al 1987a, 1987b, 1988a, 1988b, Giangregorio et al 1988b and Girao 1988), thus their data should be interpreted with caution. This could also explain discrepancies with our data (Figs. 3.8.1.2-

3.8.1.8.), whereas similar trends between the two studies may reflect similarities in these lipid storing tissues. Both studies nevertheless established that FA's played a role in cell growth, and it was possible that the response of the cells to the FA's reflected their physiological requirements in vivo.

We presented evidence which supported the ability of rat adipose cells to incorporate the exogenous FA's dosed (Table 3.8.3.1.). It was mechanistically possible, therefore, that the p-oil effects on cell growth were the result of changes in cell membrane fluidity and/or modifications of membrane-bound enzymes, as evidence suggests that dietary supplementation with FA's induces changes in membrane physical properties and membrane-associated cell functions (King et al 1971, Ginsberg et al 1981, Simon et al 1982, Spector et al 1985 and Stubbs et al 1984). On the other hand, the finding that growth inhibition occurred only with p-OO, p-LO or p-EPO dosage at high concentrations could reflect the ability of these cells to tolerate relatively large changes in FA concentration as adipose tissue is primarily involved in TAG storage and release (Lehninger 1982).

p-Oil dosage caused changes in the content of total cellular protein (Table 3.8.2.1.), but this did not always parallel the cell viability changes induced (Figs. 3.8.1.2-3.8.1.8.). The greatest increase in total protein correlated with the greatest stimulation of cell

viability with 20mg/l p-LO dosage. However, as protein increased approximately 50% and cell viability increased about 20%, compared to controls, increased protein did not appear to relate to increased cell numbers alone. In fact, increased cellular protein was found even when p-oil dosage induced growth limitation. It was apparent, therefore, that while total protein quantitation could reflect cell growth changes in terms of actual cell numbers, p-oil FA's were involved in the stimulation of protein synthesis. The finding that the extent of protein stimulation varied with dosage of identical concentrations of different p-oils, even when such yielded similar cell numbers, suggested that FA's of different chain lengths and degrees of unsaturation vary in their capability to modulate protein synthesis. Whether this related to specific FA's in any p-oil, or to synergistic effects induced between p-oil FA's, was unclear, although a combination of such effects seemed most likely. The enhancement of protein biosynthesis with p-oil supplementation may, nevertheless, reflect the extremely active metabolism of adipose tissue <u>in vivo</u> (Lehninger 1982). The involvement of this tissue in processes such as FA synthesis, TAG storage and release, as well as its ability to rapidly respond to metabolic changes/stimuli (Allman et al 1965, Fain et al 1966 and Lehninger 1982) suggested that the increased production of cellular proteins related to the enhanced expression

of lipid metabolising enzymes, such as lipases and those involved in FA synthesis and oxidation.

Comparison of control cell FA spectra with those derived from p-oil supplemented cells indicated the ability of cultured rat adipose cells to desaturate and elongate PUFA's (Table 3.8.3.1.). This correlated with the active involvement of adipose tissue in FA biosynthesis in vivo (Allman et al 1965, Fain et al 1966 and Lehninger 1982). Formation of small 18:3ω6 amounts and no increment in 18:4ω3 in dosed cells, even when significant EFA levels were present as substrate, supported the rate limiting nature of Δ6D (Stoffel 1961, Holloway 1963, Marcel et al 1968 and Brenner 1971, 1977, 1982). Slightly increased 20:2ω6 levels indicated the capability for limited 18:2ω6 elongation, whereas increments in 20:3ω6 and 20:4ω6 implied rapid 18:3ω6 elongation and subsequent Δ5-desaturation. On the other hand, the decreased 18:4ω3/20:4ω3 and increased 20:5ω3 levels found even with incubation of p-oils deficient in ω3 PUFA's implied elongation and Δ5-desaturation of endogenous 18:4ω3. 22:5ω3 and 22:6ω3 formation with p-FO dosage supported elongation of incorporated 20:5ω3 and subsequent Δ4-desaturation, while production of these moieties also with incubation of p-oils deficient in ω3 PDFA's implied elongation and desaturation of endogenous ω3 PDFA's. However, despite 22:4ω6 formation via elongation, this moiety was not further desaturated, hence explaining the

accumulation thereof. Such implied greater Δ4D activity with 22:5ω3 than 22:4ω6, and this supported the greater activity of desaturase cascade enzymes with ω3 than ω6 PUFA's (Brenner 1966, Mead et al 1976 and Kanau et al 1977) and the potential of ω3 PDFA's to suppress/inhibit ω6 PDFA desaturation (eg. Garcia et al 1965 and Sprecher 1981).

The raised 16:1ω9 levels found in p-oil dosed cells (Table 3.8.3.1.) implied 16:0 desaturation, but it was apparent that 16:1ω9 elongation was inhibited/suppressed since no increment in 18:1ω9 was found, despite 16:1ω9 uptake with p-FO incubation. 18:0 desaturation to 18:1ω9 via Δ9D was not demonstrated with p-oil supplementation (Table 3.8.3.1.), although Gellhorn et al (1964) found that rat adipose tissue was able to desaturate 18:0. Lack of Δ9D expression in our study, however, could have related to PUFA inhibition of the enzyme, which has been well documented in the rat (Ullman et al 1971, Mahfouz et al 1984, Pugh et al 1984 and Garg et al 1988b). This could also explain the lack of 18:1ω9 desaturation and elongation found, even when large amounts of 18:1ω9 were incorporated with p-OO dosage (Table 3.8.3.1.).

Dietary-induced alterations in microsomal FA composition and competitive interactions between cellular FA's of the same and different families modulate desaturase cascade enzyme expression (Garcia et al 1965, Brenner et al 1966, Nervi et al 1968, Ullman et al 1971, Kurata et

al 1980, de Schriver et al 1982, Garda et al 1984, 1985, Nassar et al 1986 and Garg et al 1988a, 1988b, 1988c). Such factors therefore contributed to the pattern of desaturation and elongation reported in Table 3.8.3.1. with p-oil dosage. In vitro studies using microsomes isolated from rat liver, known to exhibit desaturase activity, indicated that saturated FA's inhibited PUFA desaturation (Brenner et al 1966). This, however, did, not appear to be the case in cultured adipose cells, as detection of increased PDFA levels in cells dosed with saturated FA-rich p-oils (p-MO and p-CO in particular) implied that a relative EFA/PDFA deficiency enhanced desaturase cascade enzyme expression, probably in an attempt to restore membrane fluidity. PDFA formation with dosage of p-oils abundant in PUFA's was consistent with the availability of desaturase cascade enzyme substrates, although such moieties could suppress enzyme expression by negative feedback reactions once optimal PUFA levels were established to maintain normal membrane fluidity and cellular functions. This could explain the slightly greater cellular 22:6ω3 amounts found with supplementation of p-CO or p-MO than with other p-oils. The amount of FA synthesised in adipocytes has, however, been shown to decline with age in the rat (Henderson et al 1979 and Gandemer et al 1985), suggesting that PDFA availability and lipid deposition in adipose tissue of older rats is more dependent on exogenous lipid than on

de novo biosynthesis (Etherton et al 1980 and Jamdar et al 1986). The ability of these cells to desaturate and elongate FA's nevertheless reflected a mechanism to control their FA composition, and this could partly account for the lack of growth inhibition found with most p-oils and concentrations dosed (Figs. 3.8.1.2-3.8.1.8.).

The capability of these cells to desaturate and elongate individual C18 FA's was assessed to establish whether the pattern of p-oil desaturation found (Table 3.8.3.1.) was modulated in any way by the presence of FA mixtures. The pattern of radioactivity derived from cells enriched with [^{14}C]-18:1ω9 was consistent only with elongation of this moiety, but the finding that these cells converted 80% of incorporated [^{14}C]-18:2ω6 and [^{14}C]-18:3ω3 to PDFA's indicated Δ6D expression (Table 3.8.4.2.). Δ6D activity was also shown in rat mammary tissue microsomes by Cunnane et al (1981), although Brenner (1971) found that microsomes isolated from epididymal fat of young rats exhibited no potential to convert 18:2ω6 to 18:3ω6. This discrepancy may relate to the fact that Δ6D is extremely sensitive and easily inhibited by slight changes in the metabolic and physical environments (Cook 1979 and Brenner 1982), and could have been damaged upon microsomal isolation or inhibited by the experimental incubation conditions used. Enzyme activity expressed in whole cells is nevertheless more realistic and

relevant than microsomal assays since the former more closely parallels the situation in the intact organism. [^{14}C]-18:2ω6 and [^{14}C]-18:3ω3 elongation to 20:2ω6 and 20:3ω3, respectively (Table 3.8.4.2.) supported the capability for extramicrosomal retroconversion (Stearns et al 1967) and/or Δ8-desaturation. The presence or absence of Δ8D has not been reported in adipocytes, but such has been found in rat testes (Albert et al 1977). The capability for Δ8D expression in the present study could therefore not be ruled out as a second pathway for 20:3ω6 and 20:4ω3 synthesis. The formation of about 45% post-Δ5D FA's from incorporated [^{14}C]-18:2ω6 and [^{14}C]-18:3ω3 supported Δ5D expression, whereas the detection of 22:5ω6 and 22:6ω3 was consistent with Δ4D activity (Table 3.8.4.2.).

It was evident from comparison of the data in Tables 3.8.3.1. and 3.8.4.2. that desaturase cascade enzyme expression was more limited with p-oil than with [^{14}C]-18:1ω9, [^{14}C]-18:2ω6 or [^{14}C]-18:3ω3 incubation. This implied suppressed enzyme expression as a result of competitive interations between p-oil FA's, particularly in the light of the competitive nature of FA's for such enzymes (Brenner et al 1966, 1967, Mohrhauer et al 1963a, Ullman et al 1971, de Schriver et al 1982, Holman 1986a, 1986b, Nassar et al 1986 and Cook et al 1987). Desaturase cascade enzyme expression reported with p-oil supplementation may nevertheless be a better indication

of such capability in rat adipose tissue in vivo than that reflected with individual FA's as cells are exposed to mixtures rather than single FA's in the "real world".

The presence of MDA in cultured rat adipose cells (Table 3.8.5.1.) supported the capability for enzymatic and/or spontaneous FFA oxidation when the protective mechanisms against free radical formation were saturated. Control cells contained unsaturated FA's which could have served as potential oxidation substrates (Table 3.8.3.1.), and the greater MDA amounts found in dosed cells reflected oxidation induced by unsaturated p-oil FFA's. Adipocytes are largely concerned with TAG storage (Lehninger 1982), and it is well known that the FA composition and level of dietary fat influence the FA composition of rat adipose tissue TAG's (Stein et al 1962, Knittle et al 1965, Awad 1981, Becker et al 1986 and Lhuillery et al 1988). Thus, the cellular lipoperoxides formed may well reflect oxidation of highly unsaturated FFA's in the TAG's.

The numerical variations in MDA concentrations between dosed cells (Table 3.8.5.1.) reflected the different FA compositions of the p-oils (Table 2.3.3.2.). This was a function of the amount of unsaturated FA's present in any p-oil and also their susceptibility oxidation, which related to the number and position of double bonds in a FA. It was also possible that supplementation with different p-oils altered the rate of PGL turnover in the

cells, which would have influenced FA availability for oxidation by affecting FA uptake and release. Indeed, FA's would not be so readily accessible to oxidation if they were components of the plasma membranes. This could explain the lowest cellular lipoperoxide amounts formed with p-OO dosage, despite significant OA incorporation (Table 3.8.3.1.) and a recognised mechanism for oleate auto-oxidation (Frankel 1984). The fact that p-CO and p-MO contained predominantly saturated FA's and induced larger cellular lipoperoxide amounts than p-OO, however, suggested the availability of endogenous polyenoic FA's for oxidation. Lipid peroxide formation with p-FO dosage reflected the more even balance of saturated, monoenoic and polyenoic FA's present in this p-oil, whereas the preference of polyenoic FA substrates for oxidation was reflected with p-SSO, p-LO or p-EPO dosage, particularly with a concentration of 60mg/l. Formation of more MDA with addition of 20 or 40mg/l p-EPO than p-SSO probably related to the availability of GLA present in p-EPO only, whereas the largest cellular lipoperoxide amounts formed with 60mg/l p-LO reflected the abundance of ALA in this p-oil.

The cytostasis induced with dosage of 60mg/l p-EPO and the known ability of lipoperoxides to increase membrane permeability (Chio et al 1969, Tappel 1975, Mead 1976 and Frankel 1984) suggested that the spent medium lipid peroxides originated intracellularly via passage through

the plasma membrane and/or as a result of extracellular oxidation of released cellular FFA's. On the other hand, the absence of MDA in all other spent growth media (Table 3.8.5.1.) supported p-oil FA incorporation and correlated with the lack of cytotoxicity 20, 40 or 60mg/l p-oil induced (Figs. 3.8.1.2-3.8.1.8.). As a result of the latter finding, it was unclear whether lipoperoxides were directly involved in the modulation of cytotoxicity, although this was a possible mechanism with dosage of higher p-oil concentrations. The inverse correlation generally found between cell viability and MDA concentrations with p-CO, p-MO, p-OO, p-EPO or p-FO dosage was suggestive of lipoperoxide involvement in the modulation of growth limitation. However, as 60mg/l p-LO induced the greatest cellular lipoperoxide amounts, but no growth limitation, it was proposed that the control of cell viability also related to other mechanisms, such as membrane fluidity changes.

The finding that total molar eicosanoid formation accounted for 43% of the lipid peroxides quantitated in control cells (Tables 3.8.5.1. and 3.8.6.1.) reflected the eicosanoid requirement of these cells and rate of biosynthesis under the experimental conditions employed. It was possible, however, that endogenous eicosanoid production was suppressed/limited as a result of the small 20:3ω6, 20:4ω6 and 20:5ω3 proportions found in control cells (Table 3.8.3.1.) as 1-, 2- and 3-series

eicosanoid precursors, respectively. Negrel et al (1981a 1981b) found that the rate of PGE_2 and 6-keto-$PGF_{1\alpha}$ biosynthesis in mouse epidermal adipocytes was inversely related to cell density, and decreased considerably after confluence. Hammarstrom (1977) also described this phenomenon, which could well explain the total molar eicosanoid amount found and the relative proportions to which the prostanoids studied contributed to the total molarity (Table 3.8.6.1.).

Prostanoid detection supported cyclooxygenase expression in these cells, but it was apparent that PGH_1-PGE_1 and PGH_2-PGD_2 isomerases were inhibited/suppressed since no significant PGE_1 and PGD_2 formation was shown in control or dosed cells (Table 3.8.6.1.), despite receptors for such compounds reported in rat adipocytes (Moore 1985). Alternatively, it was possible that these PG's were degraded at a faster rate than other eicosanoids by cytoplasmic enzymes such as 15-hydroxy PG dehydrogenase. On the other hand, the finding that the rate of PGF_α production was twice as great as that of TXB_2 and PGE_2, but considerably greater than PGI_2, synthesis in control cells may have been the result of increased enzyme expression for PGF_1 and/or PFG_2 formation, or relative decreases in the biosynthesis of other prostanoids. Lipinski et al (1978) similarly found that the <u>in vitro</u> production of $PGF_{2\alpha}$ was approximately two fold greater than PGE_2 in rat adipocytes. This supported the findings

of Lambert et al (1976), although earlier studies found very low $PGF_{2\alpha}$ levels in such cells (Dalton et al 1974). Others reported that isolated fat cells do not produce detectable amounts of TXB_2 (Fredhold et al 1976), unlike the present study (Table 3.8.6.1.). Possible reasons for such discrepancies were diet fed, incubation conditions and method of assay.

The eicosanoid profiles obtained from dosed cells (Table 3.8.6.1.) were consistent with the ability of the p-oils to modulate prostanoid synthesis. This, however, did not correlate in any way with degree of p-oil unsaturation, the ability of the p-oils to provide direct eicosanoid precursors (Table 2.3.3.2.), or the capability of the cells to form eicosanoid precursors via the desaturase cascade (Table 3.8.3.1.). Lipinski et al (1978) showed that changing the amount of dietary polyunsaturated fat altered PG production in rat adipocytes stimulated with norepinephrine <u>in vitro</u>. As the soyabean oil to beef tallow (P/S) ratio increased in the diet, so increased $PGF_{2\alpha}$ and PGE_2 formation. However, basal PGE_2 production was not altered in unstimulated adipocytes, even with increments in the P/S ratio, and $PGF_{2\alpha}$ increased only slightly. This implied hormonal involvement in the modulation of prostanoid biosynthesis, and the absence of hormonal supplementation in the present study could explain the findings we described. The modulation of prostanoid production could only be related to the p-oil

concentration dosed (Table 3.8.6.1.). This could have influenced intracellular calcium ion availability, which has been implicated as an essential factor regulating phosholipase activity (Moore 1985), and thus the release of FA's from membrane lipids for eicosanoid production. This phenomenon could explain why prostanoid synthesis was decreased overall with p-oil dosage. It was also possible that different p-oil concentrations influenced the expression of enzymes involved in the formation of the prostanoids studied from PGH to a greater or lesser extent and accounted for the alterations found. However, the fact that no correlation could be found between the eicosanoid profiles (Table 3.8.6.1.) and cell viability changes (Figs. 3.8.1.2-3.8.1.8.) induced with p-oil dosage was <u>in vitro</u> evidence against the concept that the endogenously synthesised prostanoids studied played a direct role in the modulation of cell proliferation. It was more likely such related to alterations in membrane physical properties and the generation of lipid peroxides.

3.9 General Discussion.

This chapter presented comprehensive data with regard to the effect of p-oil supplementation on the viability of cultured normal rat cell types, the capability of such cells to incorporate, desaturate and elongate FA's, the formation of lipoperoxides and eicosanoids and possible roles these compounds played in the modulation of cell viability. Furthermore, the data obtained from the parameters investigated permitted valid comparisons to be made between different rat cell types under standard experimental conditions.

p-Oil supplementation influenced rat cell viability, but the effects induced and magnitude thereof varied with rat cell type. The finding that cell viability was increased when particular p-oils and concentrations were dosed to nucleated rat cell types correlated with the work of Launay et al (1968, 1969, 1981), who showed that LA enrichment of rat diets increased cell numbers and the DNA content in liver and adipose tissue. The present study demonstrated that both saturated and unsaturated FA-rich p-oils exhibited the capability to promote cell viability, but such was usually induced with low and/or intermediate concentrations in the range dosed. This nevertheless implied FA involvement in the modulation of proliferation, dependent on rat cell type, p-oil FA composition and concentration dosed. Such factors also

determined the capability of the p-oils to reduce cell viability. All p-oils were cytotoxic to blood cells, but the fact that erythrocytes were more susceptible to the inhibitory effects of unsaturated FA-rich p-oils than lymphocytes (sections 3.1.1 and 3.2.1) may reflect the more limited metabolic capability of erythrocytes due to a lack of organelles. p-Oils were nevertheless far more effective in killing suspension cultures of blood cells than adherent cells, which could have related to the ability of the later to divide in culture without prior stimulation. Adherent cells exhibited different rates of proliferation (Table 2.3.1.1.), thus the growth limiting range established with p-oil incubation was broader with faster than with slower growing cells. This range should always be defined with dividing cells to ensure valid expression of the data as it clearly distinguishes between p-oil concentrations which induce mere growth limitation or cytostasis from those promoting cell proliferation or cytotoxicity. In this regard, rat endothelium, skeletal muscle, lung and adipose cell viabilities were generally limited with p-oil dosage and cytotoxicity induced only with high concentrations of certain p-oils (sections 3.3.1, 3.4.1, 3.6.1 and 3.8.1, respectively), whereas brain and skin cells were more susceptible to growth inhibition (sections 3.5.1 and 3.7.1, respectively). Hence, it was clear that the threshold for cytotoxicity varied with rat cell type, although no clear correlation could be established

between the rate of cell proliferation (Table 2.3.1.1.) and extent of growth inhibition induced. The different cell viability changes shown with p-oil dosage between these rat cell types may well relate to the different physiology and functions of such tissues in vivo. Extrapolation of cell viability results from one rat cell type to another should thus be avoided to ensure valid interpretion of data.

Evidence was presented in this chapter which supported the ability of rat cells to incorporate exogenous FA's to a greater or lesser extent. This modified the overall FA composition of the cells, suggesting that a mechanism whereby cell viability was reduced, particularly with supplementation of high p-oil concentrations, related to differential uptake of exogenous FA's and alterations in membrane fluidity.

Considerable variations were demonstrated with regard to the total protein concentrations of the different rat cell types studied (sections 3.1.2, 3.2.2, 3.3.2, 3.4.2, 3.5.2, 3.6.2, 3.7.2 and 3.8.2). Amounts were lowest in control lymphocytes and erythrocytes, intermediate in adipose, lung and endothelium, and greatest in skin, muscle and brain (6.5, 13.0, 134.7, 176.5, 181.1, 374.7, 401.7 and 455.5$\mu g/10^6$ cells seeded, respectively). The fact that control adherent cells contained considerably more total protein than control erythrocytes or lymphocytes may once again have related to the ability

of the former to replicate under the culture conditions employed without prior stimulation. Control cell protein levels, however, showed no clear correlation with the proliferation rate of these cells (Table 2.3.1.1.), and probably reflected the morphological and physiological differences of each cell type in vivo, as well as variations in the rate of total protein turnover with cell type (Lehninger 1982). The protein content of p-oil supplemented cells reflected the cell viability changes induced, although it was apparent that exogenous FA's had the capability to modulate protein synthesis in nucleated cells; the magnitude thereof varied with rat cell type, p-oil FA composition and concentration dosed. The enhancement or suppression of absolute cellular protein levels was suggested to relate to alterations in membrane protein synthesis and/or enzyme expression involved particularly in FA metabolism.

This chapter provided in vitro evidence to support the presence or absence of desaturase cascade enzyme capability in a range of normal rat cell types exposed to exogenous FA's. The lack of desaturase cascade enzyme activity was confirmed in erythrocytes (Table 3.1.3.1.) (Lehninger 1982), and these cells could thus be regarded as non-enzymatic controls within the species. Similarly, lymphocytes exhibited no significant Δ6D, Δ5D, Δ4D or elongase activity, but the potential to express Δ8D was suggested (Table 3.2.3.1.). Rat brain cells had limited

PUFA elongase, but no significant desaturase, capability (Table 3.5.3.1.), whereas skeletal muscle cells showed some Δ6D and Δ5D capability with PUFA substrates and Δ4D activity was implicated with ω3 PUFA's (Table 3.4.3.1.). On the other hand, Δ6D, Δ5D, Δ4D and elongase activities were found in rat endothelial cells (Table 3.3.3.1.). Rat lung and skin fibroblasts showed Δ6D, Δ5D and elongase activities both with ω3 and ω6 PUFA substrates, whereas Δ4D activity was found only with ω3 PUFA's and this was slightly greater in skin cells (Tables 3.6.3.1. and 3.7.3.1.). Δ6D and Δ5D were particularly active with both ω3 and ω6 PUFA substrates in rat adipose cells, although Δ4D activity was expressed only with ω3 PUFA's (Table 3.8.3.1.). These cells, however, exhibited the greatest capability for desaturation and elongation in relation to the rat cell types studied. Δ6D, Δ5D, Δ4D and elongase expression were also indicated when rat lung, skin and adipose cells were incubated with [^{14}C]-18:2ω6 or [^{14}C]-18:3ω3 (Tables 3.6.4.2., 3.7.4.2. and 3.8.4.2.). Such supported the slightly greater capability to form PDFA's with rat adipose, followed by skin and lung cells, as shown with p-oil dosage (Tables 3.6.3.1., 3.7.3.1. and 3.8.3.1.), which was consistent with the greater incorporation of [^{14}C]-18:2ω6 and [^{14}C]-18:3ω3 into adipose than skin or lung cells (Tables 3.6.4.1., 3.7.4.1. and 3.8.4.1.).

The above findings clearly demonstrated that desaturase

enzyme capability varies between different rat tissues. A study in young rats has indeed shown that the percentage desaturation of LA to GLA via Δ6D was 20% adrenals, 14% in liver, 6% in testes and 2% each in heart, kidney and brain (Brenner 1971). No desaturation was detected in lung or epididymal fat (Brenner 1971), but this may have related to the fact that desaturation was assessed in isolated microsomes, which may not necessarily reflect the situation in a genetically entire cell. In vivo studies have indeed shown PDFA formation from LA in rat adipose tissue, heart, brain and liver (Mohrhauer et al 1963a, 1963b, 1963c, 1963d, 1963e). Little comparative data is available regarding the tissue distribution of other desaturases in the rat and the relative activities thereof. Δ5D, Δ4D and Δ9D activities have nevertheless been reported in several rat tissues, including adrenals, testes, liver, adipose, heart and brain (Mohrhauer et al 1963a, 1963b, 1963c, 1963d, 1963e, Gellhorn et al 1964, Brenner et al 1967, Bridges et al 1970, Takayasu et al 1970, Ayala et al 1973, Dannon et al 1975, Anon 1979, Stone et al 1979, Blond et al 1981 and Garg et al 1988b, 1988c). On the other hand, Δ8D activity has been found in rat testes (Albert et al 1977), but not in liver or brain (Ullman et al 1971b, Sprecher et al 1975 and Dhopeshwarkar et al 1976). There is evidence, however, that desaturase activity varies with tissue development as Cook (1978a)

detected limited Δ6D activity in fetal rat brain, which subsequently fell to very low levels 4 weeks after birth and was hardly detectable in the adult. In contrast, liver levels were very low after birth, but rose sharply after weaning (Cook 1978a). It was apparent, therefore, that desaturase activity relates both to the type and developmental state (age) of a tissue. Such factors warrant some consideration in the interpretation of the capability of different tissue types to desaturate FA's and in the comparison of the desaturase capabilities we reported with that of other workers.

The finding that desaturase enzyme capability was more limited with p-oil than individual FA dosage in all rat cell types studied supported competitive interations between p-oil FA's for such enzymes. Numerous studies have indeed indicated that desaturation can be modulated by FA's in dietary oils (eg. Brenner 1971, de Gomez Dumm et al 1983, Kirstein et al 1983, Barzanti et al 1986, Mosconi et al 1988, Garg et al 1989, Dang et al 1989). Δ9D, Δ6D and Δ5D expressions were enhanced in rat liver with corn oil, and suppressed with coconut oil, feeding (Pugh et al 1984), whereas others showed that hepatic Δ9D, Δ6D and Δ5D activities were not significantly altered by supplementing fat-free rat diets with 10% hydrogenated coconut oil, but were depressed with 10% safflower oil (de Schriver et al 1983). When rats were fed diets enriched with either hydrogenated beef tallow,

linseed or fish oils, Δ9D, Δ6D and Δ5D activities were inhibited by the latter two oils in liver microsomes, although the inhibition was greater with fish oil (Garg et al 1988a, 1988b, 1988c). Other investigations showed that hepatic Δ6D activity was greatest when rat diets were supplemented with linseed rather than safflower oil (Lee et al 1988), consistent with the fact that ALA is desaturated more efficiently than LA (Jeffcoat et al 1984). Linseed oil supplemenation of rat diets has also been shown to suppress/inhibit ω6 PUFA desaturation (Brazanti et al 1986), whereas fish oil administration decreased both Δ6D and Δ5D expression (Kirstein et al 1983). These studies indicated that the desaturase capabilities of the rat cell types we studied related to the overall balance of FA's both of the same and different series, as well as the cellular demand and/or requirement for longer chain more unsaturated FA's. Complex mechanisms control FA desaturation (eg. Holman 1964, Brenner et al 1966, 1967, 1969, Sprecher 1981 and de Schriver et al 1982), but maximum enzyme potential is genetically determined for every tissue type within a species.

The variations we reported in desaturation capability between the rat cell types studied may well relate to the different physiology of these tissues <u>in vivo</u>. However, the fact that desaturase capability was not distributed evenly between the rat tissues implied <u>in</u>

vivo dependence of some tissues on others for a supply of PDFA's. The fact that desaturase capability varied with rat cell type studied nevertheless indicated that extrapolations in this regard to other rat cell types should be avoided.

Comparative data obtained from the measurement of lipid peroxides indicated that the capability to produce these compounds varied with rat cell type. Lipoperoxides were absent from control erythrocytes (section 3.1.4), but were formed by all nucleated control cells, although relative concentrations were low in skeletal muscle and skin (0.1 to 0.2nmoles MDA/10^6 cells), intermediate in lymphocytes, brain, adipose and aortic endothelium (0.4 to 0.7nmoles MDA/10^6 cells), and greatest in lung (1.1nmoles MDA/10^6 cells) (sections 3.4.4, 3.7.5, 3.2.4, 3.5.4, 3.8.5, 3.3.4 and 3.6.5, respectively). This implied that the efficiency of the mechanisms of protection against free radical formation and rates of FFA oxidation varied between rat tissues. The finding that control cell lipid peroxide levels showed no clear correlation with the proliferation rates (Table 2.3.1.1.) or total unsaturated FA contents of these cells, however, probably reflected the physiological differences of each cell type in vivo, as well as variations in the rate of PGL turnover with cell type.

Supplementation of cultures with p-oils induced the formation of lipoperoxides from exogenous FA's, although

the concentrations varied numerically between rat cell types. Lipoperoxide concentrations greater than 10nmoles MDA/10^6 cells were rarely demonstrated in dosed adherent cells or their spent incubation media (Tables 3.3.4.1., 3.4.4.1., 3.5.4.1., 3.6.5.1., 3.7.5.1. and 3.8.5.1.), whereas a significantly wider range was found (0 to 103 nmoles MDA/10^6 cells) for erythrocyte and lymphocyte suspension cultures supplemented with p-oil (Tables 3.1.4.1. and 3.2.4.1.). Cellular lipoperoxide formation increased in an overall concentration dependent fashion when rat cultures were enriched with increasing amounts of p-oil, and this supported exogenous FA incorporation from the growth medium. Mouri et al (1984) similarly showed that TBARM levels increased in serum and liver when rats were fed increasing amounts of a marine oil diet containing a total of 15% ω3 PUFA's. Kobatake et al (1983) demonstrated that serum and liver lipoperoxide levels were more elevated in rats fed 5% marine oil (containing 75% ω3 PUFA's) compared to 5% oleate or linoleate supplementation, consistent with the greater susceptibility of ω3 PUFA's to oxidation. Others showed that lipid peroxidation decreased by 90% in rat liver microsomes when a 16% coconut oil diet was fed for 3 days, whereas normal lipid peroxidation was subsequently restored within 10 days of feeding a 16% safflower oil diet (Lokesh et al 1981). They suggested that diets rich in saturated fat reduced microsomal lipid peroxidation

by decreasing the availability of PUFA's in substrate PGL's. The findings we presented in this chapter indicated that p-oil-induced lipoperoxidation related to the amount of unsaturated FA's present in any p-oil (Table 2.3.3.2.) and their susceptibility to oxidation. This was dependent not only on the number and position of double bonds in a FA, but also the availability of these moieties. The possibility also existed that synergistic or antagonistic interations between p-oil FA's influenced lipoperoxide formation to a greater or lesser extent.

The absence of lipoperoxides in spent control media from all rat cultures reflected the abundance of saturated FA's present in new incubation medium (Table 2.3.3.3.), and the retention of intracellular lipoperoxides. This implied that the p-oils induced the spent medium lipid peroxides found occasionally in dosed cultures. The ability of the rat cell types studied to incorporate exogenous FA's and the presence of albumin as p-oil FA carrier in the culture medium supported the likelyhood that most spent medium lipid peroxides originated intracellularly, and were released as a direct result of cell lysis or via leakage through the plasma membrane. Both intracellular and extracellular lipoperoxides could nevertheless have induced cellular damage, and the involvement of these compounds in the modulation of cell viability was proposed.

The desaturation capability of rat lung, skin and adipose cells supported the production of DGLA, AA and EPA as precursors for 1-, 2- and 3-series eicosanoids, which correlated with the ability of these cells to form eicosanoids (Tables 3.6.6.1., 3.7.6.1. and 3.8.6.1.). Total molar eicosanoid production varied considerably between these different rat cell types, however, being greatest for control lung fibroblasts, whereas adipose and skin cells formed considerably smaller amounts (727.6, 215.9 and 165.7pmoles total eicosanoids per 10^6 cells, respectively). This correlated with reports implicating the lung as a primary site of eicosanoid biosynthesis (Mathe et al 1977, Hyman et al 1978 and Harper et al 1984). These variations in eicosanoid production may relate partly to the different eicosanoid requirements of such cells, rather than the ability to solely provide eicosanoid precursors via desaturation. Low eicosanoid production may also reflect greater FA incorporation into the TAG rather than the PGL pool. This could explain the lower molar eicosanoid levels generated by adipose than lung cells, despite the greater capability for desaturation with the former. The finding that the total eicosanoid:lipoperoxide molar ratio's for control skin (Tables 3.7.5.1. and 3.7.6.1.), lung (Tables 3.6.5.1. and 3.6.6.1.) and adipose (Tables 3.8.5.1. and 3.8.6.1.) cells ammounted to 83%, 66% and 43%, respectively, nevertheless implied a greater

preference to synthesise eicosanoids in skin than in lung or adipose cells under the experimental conditions employed. This probably related to the different physiology and requirements of these tissues in vivo.

The finding that control rat lung, skin and adipose cells differed in the relative amounts of PGI_2, TXB_2, PGF_{α}, PGE_2 and PGE_1 they produced signified that the expression and activity of enzymes involved in eicosanoid synthesis varied between rat tissues and that such was tissue-specific. This supported the work of Pace-Asciak et al (1977), who showed considerable tissue specificity in PGE_2, $PGF_{2\alpha}$, PGD_2, TXB_2 and 6-keto $PGF_{1\alpha}$ production in rat lung, liver, spleen, stomach, kidney and heart homogenates in the presence of AA. These tissues contained low PGE_2, $PGF_{2\alpha}$ and PGD_2 levels, although PGE_2 was detected in greater amounts than $PGF_{2\alpha}$ or PGD_2 in all tissues. 6-keto $PGF_{1\alpha}$ was specifically formed by stomach, although lung also formed significant amounts, whereas TXB_2 was mostly directed to spleen, with lesser amounts in lung. We showed that PGE_1 was the major prostanoid detected in control rat lung cells and this correlated with the considerably lower levels of other prostanoids reported (Table 3.6.6.1.). This could relate to the fact that PGE_1 is partly responsible for the control of cAMP levels, a potent inhibitor of AA release (Feinstein et al 1977 and Minkes et al 1977). High PGE_1 would have resulted in high cAMP levels, and

thus decreased AA mobilisation. In contrast, control skin and adipose cells produced little, or no, PGE_1, and such could explain the synthesis of 2-series prostanoids (Tables 3.7.6.1. and 3.8.6.1.). In the light of this phenomenon, it was possible that a greater proportion of the PGF_{α} fraction detected was composed of $PGF2_{\alpha}$ rather than $PGF_{1\alpha}$.

p-Oil supplementation modulated the production of the prostanoids studied by influencing the balance between individual prostanoids to a greater or lesser extent (Tables 3.6.6.1., 3.7.6.1. and 3.8.6.1., respectively), although total prostanoid production was largely unaltered in lung fibroblasts, decreased in adipose cells and more varied in skin fibroblasts. These changes, however, could not be related to variations in p-oil FA composition. Lee et al (1988) similarly found no significant difference in TXB_2 and PGI2 production between rats fed safflower or palm oils, despite the large difference in P/S ratio. The reason for these findings were unclear, but could have related to sufficient endogenous PUFA's in the precursor pool to produce an adequate level of prostanoids, or the relative unavailability of incorporated exogenous FA's for the production of these prostanoids. The possibility that dosage with different p-oils induced variations in other eicosanoid levels, however, could not be excluded. The finding that the modulation of prostanoid production

related to the p-oil amount dosed suggested a preference for certain p-oil concentrations to selectively modulate enzyme activity involved in the synthesis of these compounds. However, the fact that rat lung, skin and adipose cell prostanoid profiles showed no p-oil concentration dependent correlation with cell viability, desaturation capability or lipid peroxide production implied that the endogenously synthesised prostanoids studied were not directly involved in, or responsible for, the effects induced with p-oil supplementation. Durant et al (1989) showed that exogenous prostanoids ($PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ and PGE2) could alter endogenous PG synthesis/release and increase fibroblast proliferation, whereas endogenous PG's alone were not involved in the control of cell proliferation. This supported the findings we presented, particularly with regard to p-oil induced cytotoxicity. We extended the work of Durant et al (1989) by proposing lipoperoxides and alterations in membrane physical properties as alternative mechanisms involved in the modulation of cell proliferation. The role of eicosanoids in the regulation of cell functions, however, remains undisputed.

In summary, this chapter provided comparative data on a range of cell parameters for different cultured rat tissues. Control cells from different tissues exhibited different growth rates, FA and prostanoid profiles, contained different concentrations of total protein, and

produced different molar amounts of lipoperoxides and eicosanoids. Variations in cell viability, total protein and lipoperoxide formation, FA and eicosanoid profiles, and desaturation capability were also demonstrated between different rat cell types when p-oils were supplemented. These findings implied variations in FA metabolism between different rat tissues, and that these differences were important in the modulation of the effects reported. The data indicated that different rat tissues *in vivo* probably also respond differently when fed identical dietary oils due to inherent variations in cell physiology. Careful consideration should thus be given to the selection of rat tissues for experimental purposes, and extrapolation of experimental findings between rat cell types should be avoided to ensure valid interpretation of results.