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POLYUNSATURATED FATTY ACIDS, LIPID ACCUMULATION, AND OXIDANT STRESS IN CELLS IN CULTURE

The Ohio State University

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POLYUNSATURATED FATTY ACIDS, LIPID ACCUMULATION,
AND OXIDANT STRESS IN CELLS IN CULTURE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by
Victor Cruz Gavino, B.S. Chem., M.S.

The Ohio State University
1981

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to Grace and Linda
ACKNOWLEDGMENTS

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### PUBLICATIONS

Effect of low and high methional concentrations on prostaglandin biosynthesis in microsomes from bovine and sheep vesicular glands (1979), R.Y. Panganamala, V.C. Gavino and D.G. Cornwell. *Prostaglandins* 17:155-162


FIELDS OF STUDY

Major Field: Biochemistry

Studies on polyunsaturated fatty acid metabolism and lipid peroxidation in cells in culture. Dr. D. G. Cornwell

Studies on the effect of antioxidants and quinones on free radical reactions. Dr. D. G. Cornwell

Studies on the effect of dietary α-tocopherol on superoxide dismutase activity in mice tissues. Dr. A. S. Csallany

Studies on α-tocopherol oxidation by superoxide. Dr. A. S. Csallany
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LIST OF ABBREVIATIONS

AIBN ......................... azobis(isobutyronitrile)
AroH ......................... phenolic antioxidant
BHT ......................... 2,6-di-tert-butyl-4-methylphenol
CE ......................... cholesteryl ester
CHP ......................... cumene hydroperoxide
EDTA ......................... ethylenediaminetetraacetic acid
F ......................... flavin
FBS ......................... fetal bovine serum
FFA ......................... free fatty acid
FH' ......................... flavin semiquinone
I' ......................... free radical initiator
LL ......................... lyssolecithin
MDA ......................... malonaldehyde
PA ......................... phosphatidic acid
PC ......................... phosphatidyl choline
PE ......................... phosphatidyl ethanolamine
PI ......................... phosphatidyl inositol
PL ......................... phospholipid
PS ......................... phosphatidyl serine
Q ......................... quinone
R ......................... neutral lipid more polar than FFA
$R_f$.......................... relative mobility
RSSR.......................... organic disulfide
SLO............................ soybean lipoxidase
SOD............................ superoxide dismutase
Sph............................. sphingomyelin
$\alpha$-T.......................... $\alpha$-tocopherol
TBA............................. thiobarbituric acid
TG............................... triglyceride
TLC............................... thin layer chromatography
$\alpha$-TQ......................... $\alpha$-tocopherolquinone
$\alpha$-TQH'...................... $\alpha$-tocopherol semiquinone
$\alpha$-TQH$_2$.................... $\alpha$-tocopherol hydroquinone
INTRODUCTION

The importance of oxygen centered radicals and their reaction products with cellular components have become increasingly apparent over the years. As aerobic organisms, we can efficiently utilize the intrinsic energy in our foodstuff more so than anaerobic organisms can ferment theirs. We have to pay a price however, since oxygen has a tendency to go through a stepwise reduction to water and thus we have to deal with the superoxide radical, hydrogen peroxide, and perhaps the extremely reactive hydroxyl radical. Potentially, these species are highly damaging but we have evolved a set of defenses to modulate their effects. Thus we have enzymes that destroy superoxide and hydrogen peroxide and we incorporate antioxidants in cellular membranes that help prevent peroxidation of labile polyunsaturated fatty acids. Under normal conditions, there must exist an appropriate balance between oxygen centered radicals and our protective systems. It is easy to imagine what might happen if our defense mechanisms broke down. What is not so apparent is what might happen if we had an oversupply of antioxidants. The thrust of this thesis then is to investigate further the effects of polyunsaturated fatty acids, a source of oxidant stress, to cells in culture and to study how antioxidants modulate these effects.
OXYGEN

Oxygen in the ground state is a diradical (Pryor, W. A., 1966). It is a triplet in its lowest energy state having two unpaired electrons with parallel spins occupying its outermost shell as predicted by Hund's rule. This is fortunate because although most elements and compounds are thermodynamically unstable with respect to oxidation by molecular oxygen, oxygen being a triplet in the ground state provides a kinetic constraint to its reaction with other molecules (Collman, J. P., 1968; Hamilton, G. A., 1969, 1971; Hill, H. A. O., 1979).

Essentially all stable organic compounds are singlets (all electrons paired); however, the direct reaction of a triplet molecule with a singlet to give singlet products is a spin forbidden process and will not occur readily (Hamilton, G. A., 1974). For molecular oxygen to readily react with molecules in the singlet state, it must first undergo a spin inversion. This is a slow process compared to the lifetime of a collisional complex, hence ground state molecular oxygen is not as reactive as we might expect it to be. These kinetic constraints do not apply to the interaction of ground state molecular oxygen with single electrons, hydrogen atoms or molecules containing unpaired electrons (Hill, H. A. O., 1979) therefore we must concern ourselves with one step reductions of oxygen especially in a living cell.
The one step reductions of molecular oxygen to \( \text{H}_2\text{O} \) involve the formation of superoxide radical, hydrogen peroxide and the hydroxyl radical (eqn. 1) (Nishinaga, A., 1977).

\[
\begin{align*}
\text{O}_2 & \rightarrow \text{O}_2^- \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} \\
& \rightarrow ^\cdot\text{OH} + ^\cdot\text{OH} \rightarrow \text{H}_2\text{O}
\end{align*}
\]  

Hydrogen peroxide is produced when molecular oxygen goes through a two electron reduction. As early as 1957, the endoplasmic reticulum has been claimed to be a source of hydrogen peroxide (Gillette, J. R. et al., 1957). Sub-mitochondrial particles were also reported to produce hydrogen peroxide (Jensen, P. K., 1966; Hinckle, P. C. et al., 1967). With the aid of a newer and more sensitive assay for hydrogen peroxide (Boveris, A. et al., 1971), intact mitochondria in state 4 were shown to produce hydrogen peroxide at the rate of 0.5 nanomoles per minute per mg protein when succinate was the substrate. The same investigators also showed that hydrogen peroxide is produced when peroxisomes are supplemented with uric acid, and during the microsomal oxidation of both NADH and NADPH.

Perhaps one of the more controversial issues on oxygen centered radicals is the question of the existence of hydroxyl radicals in living systems (Willson, R. L., 1979). The difficulty lies in the situation that all the evidence that suggest its existence in vivo has so far been only circumstantial. This is quite understandable since the rate constants for the reaction of the hydroxyl radical with many compounds is on the order of $10^9$ mol$^{-1}$ sec$^{-1}$ (Dorfman, L. M. and G. E. Adams, 1973). Its lifetime in a biological medium will therefore be very short and it will probably react very close to where it is formed.

The formation of hydroxy radicals in vitro has been well known for many years. They can be formed by: 1) ionizing radiation in H$_2$O; 2) photolysis of hydrogen peroxide; 3) Fenton's reaction; 4) electron transfer to ozone; 5) radical-peroxide reactions (Willson, R. L., 1979). Of these, the photolysis of hydrogen peroxide is unlikely in vivo.
Fenton's reagent has attracted considerable attention over the years ever since it was proposed to form hydroxyl radicals (Haber, G. and J. Weiss, 1932) (eqns. 2 and 3). The so called Haber-Weiss reaction is now common knowledge (eqn. 4) and has generated much debate (Cohen, G., 1977). The ozonide ion is formed from a one-electron transfer to ozone (Willson, R. L., 1979) and will rapidly protonate or decay to give the hydroxyl radical (eqns. 5-8). Thus the hydroxyl radical may

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH} \\
\text{Fe}^{2+} + \cdot\text{OH} & \rightarrow \text{Fe}^{3+} + \cdot\text{OH} \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \rightarrow \text{O}_2 + \text{H}_2\text{O} + \cdot\text{OH} \\
\text{O}_3^- & \rightarrow \text{O}_2 + \cdot\text{O}^- . \\
\text{O}_2^- + \text{H}_2\text{O} & \rightarrow \cdot\text{OH} + \cdot\text{OH} \\
\text{O}_3^- + \text{H}_2\text{O} & \rightarrow \text{HO}_3 + \cdot\text{OH} \\
\text{HO}_3 & \rightarrow \cdot\text{OH} + \text{O}_2
\end{align*}
\]

have a role in ozone toxicity (Willson, R. L., 1977). The Haber-Weiss reaction (eqn. 4) is an example of a radical-peroxide interaction that might produce the hydroxyl radical. In cases where oxygen and radical concentrations are low, hydrogen peroxide might react with other
cellular components in preference to superoxide (Willson, R. L., 1979) (eqns. 9-12).

\[ \text{RSSR}^+ + \text{H}_2\text{O}_2 \rightarrow \text{RSSR} + \cdot\text{OH} + \cdot\text{OH} \] (9)

\[ \text{Q}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Q} + \cdot\text{OH} + \cdot\text{OH} \] (10)

\[ \text{FH}^+ + \text{H}_2\text{O}_2 \rightarrow \text{F}^+ + \cdot\text{OH} + \cdot\text{OH} \] (11)

\[ \text{OO}^- + \text{H}_2\text{O}_2 \rightarrow \text{OO}_2 + \cdot\text{OH} + \cdot\text{OH} \] (12)
Autoxidation is the slow oxidation of an organic compound by oxygen (Pryor, W. A., 1966). This process can be initiated by any free radical source. The most common autoxidation is that in which compounds with labile hydrogens react to form hydroperoxides (eqn. 13).

\[ \text{RH} + \text{O}_2 \rightarrow \text{ROOH} \]  

(13)

As discussed earlier, this reaction as written is spin forbidden, therefore the organic molecule must first be activated for autoxidation to proceed (eqn. 14). Molecular oxygen adds to the organic free radical (eqn. 15) forming a peroxy radical which can then react with another organic molecule to form a hydroperoxide and a free radical (eqn. 16). Equations 17-19 represent termination reactions. The contributions of

\[ \text{RH} + \text{I}^* \rightarrow \text{R}^* + \text{IH} \]  

(14)

\[ \text{R}^* + \text{O}_2 \rightarrow \text{ROO}^* \]  

(15)

\[ \text{ROO}^* + \text{RH} \rightarrow \text{ROOH} + \text{R} \]  

(16)

\[ 2\text{ROO}^* \rightarrow \text{non-radical products} \]  

(17)

\[ \text{ROO}^* + \text{R}^* \rightarrow \text{non-radical products} \]  

(18)
the three termination reactions depend on the partial pressure of oxygen (Pryor, W. A., 1966). At low $pO_2$, reaction 19 will predominate but at high $pO_2$, reaction 15 will be so fast that most of the free radicals will be peroxy radicals and reactions 17 and 18 will predominate as chain termination reactions.

It has been generally observed that autoxidizing polyunsaturated fatty acids produce a material which when allowed to react with thiobarbituric acid gives a red chromophore with absorbance maximum at 532 nm (Wilbur, K. M. et al., 1949). This material was later shown to be malondialdehyde (MDA) (Niehaus, W. G. Jr. and B. Samuelsson, 1968). The observation that MDA is volatile while the chromogen was not and that only fatty acids with at least three methylene interrupted double bonds gave positive thiobarbituric acid-tests led to the proposal that autoxidizing fatty acids result in the formation of cyclic endoperoxides which decompose under the conditions of the thiobarbituric acid-test to yield MDA (Pryor, W. A. and J. P. Stanley, 1975).

**NADPH-dependent lipid peroxidation**

The endoplasmic reticulum, isolated from mammalian liver as a microsomal fraction, is characterized by an NADPH-dependent electron transport system associated with the mixed function oxidase activity responsible for the hydroxylation of a large variety of drugs, steroids, carcinogens, and other lipid soluble compounds (Conney, A. H., 1967;
Gillette, J. R. et al., 1969). The microsomal fraction of the liver will also oxidize NADPH with an associated uptake of $O_2$ even in the absence of a substrate for mixed function oxidation (Fouts, J. R., 1961; Gillette, J. R. et al., 1957). Further studies on this system showed that in the presence of ferric ions and a chelator such as ADP or pyrophosphate, NADPH-dependent peroxidation of endogenous lipid will take place (Hochstein, P. and L. Emster, 1963; Hochstein, P. et al., 1964). The process results in oxidative chain scission of the 2-position polyunsaturated fatty acids in phosphatidylethanolamine and phosphatidylcholine and in the formation of transient phospholipid peroxides which appear to be intermediates in the chain cleavage process (May, H. E. and P. B. McCay, 1968; Tam, B. K. and P. B. McCay, 1969). Subsequent experiments showed that NADPH oxidation resulted in the generation of a component having the properties of a free radical as judged by its ability to cause rapid lysis of erythrocytes (Pfeifer, P. M. and P. B. McCay, 1971). This free radical species is apparently responsible for the peroxidation of endogenous phospholipid.

Previous experiments suggested that the cellular system that catalyzed NADPH-dependent lipid peroxidation was associated with the microsomal mixed function oxidase since drugs undergoing oxidative demethylation also inhibited the peroxidation (Orrenius, S. et al., 1964). Subsequent studies showed that antibodies specific to NADPH-cytochrome c reductase and which inhibited that reductase both in the purified form and in the intact microsome also inhibited NADPH-dependent lipid peroxidation in the presence of ferric ion chelated by ADP (Pederson, T. C. et al., 1973). The purified reductase will also catalyze NADPH
dependent lipid peroxidation of extracted microsomal lipid but only in
the presence of ferric ion chelated by EDTA. Furthermore, the optimum
conditions for the peroxidation are also those required for the optimal
reduction of EDTA-Fe(III) by the reductase. A microsomal enzyme,
NADH-cytochrome b5 reductase which also reduces EDTA-Fe(III) will also
catalyze an NADH-dependent lipid peroxidation. Although in intact
microsomes the peroxidation of endogenous lipids is specific for NADPH
in the presence of ADP-Fe(III), in the presence of both EDTA-Fe(III)
and ADP-Fe(III), NADH can substitute for NADPH (Pederson, T. C. et al.,
1973).

NADPH-dependent lipid peroxidation was proposed to occur in
two distinct sequential steps, initiation and propagation (Svingen, B. A.
et al., 1978a, 1978b). In the first step, ADP-Fe+3 is reduced to ADP-Fe+2
by NADPH-cytochrome P450 reductase after which molecular oxygen adds to
ADP-Fe+2 to form the perferryl ion (eqn. 20). The perferryl ion was

\[ \text{ADP-Fe}^{+2} + \text{O}_2 \rightarrow \text{ADP-Fe}^{+2-\text{O}_2} \text{ADP-Fe}^{+3-\text{O}_2} \]  \hspace{1cm} (20)

proposed to catalyze the rapid initial formation of lipid hydroperoxides.
This first step was found to be sensitive to superoxide dismutase (SOD)
and BHT but not to singlet oxygen traps (Svingen, B. A. et al., 1978b).

Other iron chelates besides ADP-Fe+2 such as EDTA-Fe+2 and
diethylenetriamine pentaacetic acid-Fe+2 was found to catalyze the
breakdown of lipid hydroperoxides in the second step of the peroxidation,
that is, in the propagation step (Svingen, B. A. et al., 1978b). The
iron-chelate catalyzed breakdown of lipid hydroperoxides appeared to
give rise to singlet oxygen and superoxide as well as other lipid free radicals also produced in autoxidation. In intact microsomes, ferricytochrome P₄₅₀ was shown to be an endogenous propagating agent; however, it was found to be destroyed irreversibly during the peroxidation. Singlet oxygen produced in the propagation step can react with other lipids to form more hydroperoxides. The lipid free radicals formed from the breakdown of the hydroperoxides can react with more lipid or go through chain termination reactions (see section on Autoxidation, p. 7). This step is sensitive to BHT.

**Soybean lipoxidase**

Soybean lipoxidase (EC 1.13.11.12) is present in a wide variety of plants particularly in legumes and catalyzes the oxygenation of a 1,4-cis,cis-pentadiene in a long chain fatty acid to a 1-hydroperoxy-2,4-trans,cis-pentadiene (Gibian, M.J. and R. A. Galaway, 1977) (eqn. 21).

\[
\begin{align*}
\text{H} & \text{C=CH}_2 \text{C=CH}_2 \\
\text{R}_1 & \text{CH} + \text{O}_2 \rightarrow \text{H} \\
\text{C=CH}_2 \text{C=CH}_2 & \text{R}_2 \\
\text{HO} & \text{H}
\end{align*}
\] (21)

The various sources, the early history and the metabolic role of the enzyme have been extensively reviewed (Tappel, A. L., 1963).
There are four isozymes of lipoxygenase in soybean, the major one having a pH optimum of 9 (Christopher, J. et al., 1972, Verhuc, W. and A. Francke, 1972; Yamamoto, H. et al., 1970). The molecular weight is close to 102,000 daltons and the amino acid composition shows four buried cysteines with 4 to 6 cystines (Christopher, J. et al., 1970, 1972; Stevens, F. et al., 1970; Yamamoto, H. et al., 1970). All four isozymes contain one iron per enzyme molecule; therefore if the iron plays a catalytic role, then there must be one active site per molecule of enzyme (Pistorius, E. K., and B. Axelrod, 1973).

Soybean lipoxidase (SLO) has a wide specificity but fatty acids are the best substrates and among the fatty acids, the best rates result when the unsaturation begins at the sixth carbon from the methyl end (ω) of the chain (Holman, R. et al., 1969).

At pH 9 and 0°C, the oxygen was found to be inserted at position ω6 (Hamburg, M. and B. Samuelsson, 1967). The structural requirement for the cis,cis-1,4-pentadiene group was that the methylene should be at position ω8 of the fatty acid. The initial step in the catalyzed reaction was found to be the stereospecific removal of the pro-S hydrogen from position ω8 with a significant isotope effect (Hamburg, M. and B. Samuelsson, 1967). At pH 9, the resulting free radical then accepts oxygen at ω6 to form the hydroperoxide. Thus, 8,11,14-eicosatrienoic acid for example is converted to 15L-hydroperoxy-8(cis),11(cis),13(trans)-eicosatrienoic acid.

When the enzyme catalyzed reaction is carried out at another pH, other hydroperoxide isomers are produced. For example, 5,8,11-octatrienoate when reacted with SLO at pH 7 and 25°C forms both
ω6 and ω10 hydroperoxides at about equal amounts (Funk, M. O. et al., 1976). Subsequent experiments showed that soybean lipoxidase at pH 6.8 and at room temperature can catalyze the double oxygenation of arachidonic acid forming what appears to be 8,15-dihydroperoxy-5,9,11,13-eicosatetraenoic acid (Bild, G. S. et al., 1977a). Other fatty acids containing all-cis-1,4,7-octatriene moieties were later shown to also incorporate 2 moles of oxygen per mole fatty acid to produce bishydroperoxides (Bild, G. S., 1977b).

Mammalian lipoxygenase

The lipoxygenase found in platelets has a different specificity. The enzyme, found in the supernatant of broken platelets catalyzes the conversion of eicosapolyenoic acids with at least two cis double bonds at the ω8 and ω11 positions into L-12-hydroperoxy acids (Nugteren, D. H., 1975). The platelet lipoxygenase turned out to be much more complex than SLO as many other products were later identified mostly through gas chromatographic-mass spectrometric analyses. Among the "new" metabolites suggested are 8,11,12-trihydroxy-9,14-eicosadienoic acid, 8,9,12-trihydroxy-10,14-eicosadienoic acid, 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid, 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid, and 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (Bryant, R. W. and J. M. Bailey, 1079; Falardeau, P. et al., 1976; Jones, R. L. et al., 1978; Walker, I. C., 1979).
Free radical damage

It has not been easy to demonstrate a causal relationship between free radicals in vivo and the intracellular damage that they might bring about primarily because it has been difficult to demonstrate directly the existence of free radicals themselves in vivo (Mead, J. F., 1976). Moreover, attempts to demonstrate the existence of peroxides, both products and initiators of free radical processes, have been fraught with problems because too often, they are present in vivo in vanishingly small concentrations. However, good strong evidence has accumulated over the years that free radicals can exist in vivo and that they can induce cellular damage. Many experiments have been done where conditions were defined such that we might expect free radicals to form in vivo and these conditions are correlated with the appearance of new cellular components or with the modification of cell ultrastructure - events that are considered to have been brought about by free radical processes.

Diets deficient in antioxidants result in toxic symptoms in rats including peroxidation and discoloration of depot fat, destruction of vitamin A in the liver and brownish discoloration of the uterus (Christensen, F. et al, 1958; Dan, H. and H. Granados, 1945). These are all signs that a free radical chain reaction producing peroxides has taken place.

Carbon tetrachloride and ethanol toxicity are believed to be due to their ability to initiate free radical processes. Carbon tetrachloride is believed to undergo a homolytic cleavage involving electron capture in the microsome (eqn. 22) and the resulting free radicals would then initiate peroxidation of membrane lipid (Recknagel,

Atmospheric oxidants may also initiate free radical processes and cause damage in vivo. The toxicity of hyperbaric oxygen has been linked to the formation of peroxides (Gershcmann, R. et al., 1954; Haugaard, N., 1968). Ozone toxicity may also be partly due to its ability to form the hydroxyl radical (Willson, R. L., 1977). Other atmospheric pollutants like nitrogen dioxide and peroxyacylnitrates are also believed to induce biological damage because of their ability to form free radicals (Menzel, D. B., 1976; Mudd, J. B., 1976).

Other cellular effects of free radicals include changes in enzyme activities (Michelson, A. M., 1976), changes in membrane ultrastructure (Pasquale-Ronchetti, J., 1980), edema (Benedetti, A., 1980) and accumulation of lipofuscin (age) pigment (Miqluel, J., 1977).

SYSTEMS THAT COUNTERACT OXIDANT STRESS

Superoxide dismutase

Free radicals have long been thought to play a part in certain biological functions; however, not since the discovery that erythrocuprein, a blood protein previously thought to be only a storage form for copper,
had the ability to catalyze the dismutation of superoxide (eqn. 23)

\[ \frac{1}{2}O_2 + \frac{1}{2}O_2 + H^+ \stackrel{\text{H}_{2}O_{2} + O_2}{\longrightarrow} (23) \]

has there been so much interest generated on this topic (Fridovich, I., 1974a, 1974b, 1975).

As discussed earlier, superoxide is capable of interacting with hydrogen peroxide to form hydroxy radicals and perhaps singlet oxygen (see section on OXYGEN, p. 2). These free radicals can then initiate peroxidation of lipids. When the membrane itself contains some hydroperoxides, superoxide itself seems to be sufficient to start peroxidation of the lipids through, perhaps, initial formation of an alkoxide radical (Fridovich, I., 1979).

Intracellular SOD is increased by exposure to oxygen and this confers to the cell, a greater resistance to oxygen. This has been seen with Streptococcus faecalis (Gregory, E. M. and I. Fridovich, 1973a), Escherichia coli K12 (Hassan, H. M. and I. Fridovich, 1977), yeast (Gregory, E. M. et al, 1974) and rat liver (Crapo, J. D. and D. F. Tierney, 1974). The actual inducer of SOD may be molecular oxygen, superoxide or some derivative of one or the other; however, in E. coli, experiments done at constant pO\textsubscript{2} suggest that superoxide is the actual inducer of SOD synthesis (Fridovich, I., 1979).

Glutathione peroxidase and catalase

Hydrogen peroxide can be destroyed by two classes of enzymes - the catalases and the peroxidases. These enzymes catalyze the divalent
reduction of H$_2$O$_2$ to 2H$_2$O using H$_2$O$_2$ as the electron donor in the case of catalases or using a variety of reductants in the case of peroxidases (Fridovich, I., 1976). Catalases and peroxidases both contain hematin as the prosthetic group and both catalyze formally similar reactions. Catalase can act as a peroxidase when the concentration of H$_2$O$_2$ is kept low and other electron donors are present (Chance, B. and N. Oshino, 1973; Halliwell, B., 1974; Oshino, N. et al, 1973).

Respiring cells produce H$_2$O$_2$ which owing to its toxicity must not be allowed to accumulate. Although it is the most stable of the oxygen reduction intermediates (more stable than superoxide or the hydroxyl radical) it can interact with superoxide and/or transition metals in the cell that may result in the production of more reactive oxygen centered free radicals (see section on OXYGEN, p. 2). Catalases and peroxidases then serve the indispensable role of preventing the accumulation of H$_2$O$_2$.

Some mammals do lack catalase (acatalasemic) but this does not mean that catalase is unimportant (Aebi, H. and H. Suter, 1972). They exhibit sensitivity to H$_2$O$_2$ and cells in tissue culture derived from acatalesics do succumb to low levels of H$_2$O$_2$. Furthermore, there often is a higher level of glutathione and glutathione peroxidase in acatalasics that compensate for a lack of catalase.

Catalase efficiently destroys H$_2$O$_2$ at low or high concentrations in the former case acting as a peroxidase and in the latter case as a catalase. In the liver, the concentration of catalase is high in the peroxisomes where the steady state level of H$_2$O$_2$ is normally higher relative to that in the rest of the cell. Catalase is also high in the mitochondria where most of the oxygen reduction occurs (Fridovich, I.,
1976; Oshino, N. and B. Chance, 1976). This situation almost insures that catalase will be exposed to high concentrations of $\text{H}_2\text{O}_2$ and therefore act mostly in the catalatic mode.

Glutathione peroxidase appears to be very important in scavenging $\text{H}_2\text{O}_2$ (Cohen, G. and P. Hochstein, 1963; Mills, G. C., 1959). In addition, it can also act upon lipid hydroperoxides and thus counter the toxicity of a wide range of peroxides (Christopherson, B. O., 1968, 1969; Little, C., 1972; Little, C. and P. J. O'Brien, 1968). It converts various hydroperoxides to alcohols including ethyl hydroperoxide, t-butyl-hydroperoxide, cumene hydroperoxide, thymine hydroperoxide, fatty acid hydroperoxides and their corresponding esters, hydroperoxides of steroids and nucleic acids, and prostaglandin $\text{G}_2$ among others (Flohe, L. and W. A. Günzler, 1974; Flohé, L. et al, 1976).

The role of glutathione peroxidase as a biological defense mechanism against lipid peroxidation has been questioned (Burk, R. F. et al, 1978; McCay, P. B. et al, 1976). However there is considerable evidence that, although indirect, strongly suggests that the enzyme does indeed help in maintaining the integrity of labile cellular lipids. For example: 1) the enzyme can reduce esters of hydroperoxy fatty acids (Little, C. and P. J. O'Brien, 1968); 2) endogenous enzyme prevents lipid peroxidation and irreversible high amplitude swelling of rat liver mitochondria (Flohé, L. and R. Zimmerman, 1970); 3) purified glutathione peroxidase prevents the peroxidation of the phospholipids of isolated inner membrane mitochondria (Flohé, L. and R. Zimmerman, 1974); 3) bovine blood glutathione peroxidase added to illuminated chloroplasts inhibits swelling and MDA formation (Flohé, L. and N. Menzel, 1971);
4) *in vivo* inhibition of glutathione peroxidase by repeated administration of cadmium salts results in an accumulation of degradation products of unsaturated lipids in rat testes (Omaye, S. T. et al., 1975); 5) conditions requiring a high rate of lipid peroxide removal, such as the ingestion of lipid peroxides (Reddy, K. and A. L. Tappel, 1974) or exposure to ozone (Chow, C. K. and A. L. Tappel, 1972), lead to increased glutathione peroxidase activity; 6) in rats deficient in selenium and consequently in glutathione peroxidase, lipid peroxidation can be detected *in vivo* by monitoring the evolution of ethane. The effect can be inhibited partially by selenium alone and more consistently by a combined treatment with selenium and tocopherol (Hafeman, D. G. and W. G. Hoekstra, 1977); 7) low selenium and glutathione peroxidase levels were detected in Finnish children suffering from neuronal ceroid lipofuscinosis (Westermarck, T., 1977).

**Antioxidants**

As in the case of autoxidation, free radical reactions include a chain of events starting with: 1) initiation in which free radicals are first formed; 2) propagation in which the free radicals combine with other molecules to form the products and more free radicals; and 3) chain termination in which free radicals combine to form non-radical products (see section on *Autoxidation*, p. 7). Substances which can combine with atoms or free radicals and convert them into species incapable of participating in the chain-propagating steps are called radical traps or inhibitors (Roberts, J. D. and M. C. Caserio, 1965). Antioxidants as a group of compounds refer to substances which in small
quantities inhibit air oxidation of other compounds. These antioxidants include mono and polyalcohols or phenols and polyphenols (Aaes-Jørgensen, E., 1962; Chipault, J. R., 1962).

The first step in the mechanism for the inhibitory action of phenolic antioxidants is known to be hydrogen abstraction (eqn. 24) (Reich, L. and S. S. Stivala, 1969). Presumably, the free radical

\[
\text{ArOH} + \text{ROO}^+ \rightarrow \text{ArO}^+ + \text{ROOH} \tag{24}
\]

formed from the phenolic antioxidant is more stable than the initial free radical. The more stable phenolic free radical will eventually be converted to non-radical products. The more common phenolic antioxidants include propyl gallate, nordihydroguaiaretic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and the tocopherols (vitamin E group) (see Figure 1) (Chipault, J. R., 1962).

\( \alpha \)-tocopherol (\( \alpha \)-T) has been used as an antioxidant in fats and oils for many years. Its biological function however still remains to be precisely defined. Many believe that \( \alpha \)-T serves as a biological antioxidant (Tappel, A. L., 1962, 1972). This concept has been questioned on the basis of selenium and vitamin E effects and on the fact that vitamin E is not an efficient antioxidant in vitro (Green, J., 1972). The selenium question has largely been resolved upon the discovery that it is a component of the enzyme glutathione peroxidase (Hoekstra, W. G., 1973). It has also been observed that the efficiency of antioxidants in vitro do not parallel their biological activities (Pryor, W. A., 1976).
For example, it was demonstrated that \( \gamma \)-tocopherol was more effective than \( \alpha \)-T in delaying the autoxidation of monolayers of linoleic acid (Wu, G.-S. \textit{et al}, 1979) and both \( \gamma \) and \( \delta \) tocopherols were better antioxidants than \( \alpha \)-T in aqueous media (Cillard, J. and P. Cillard, 1980); however, it has been known for quite some time that \( \alpha \)-T has the greatest biological activity among all the other tocopherol isomers in the vitamin E group (Morton, R. A., 1967). Many other studies do show that \( \alpha \)-T seems to act as an antioxidant \textit{in vivo}. Rats that were deficient in vitamin E had higher liver lipid peroxides than do animals that were given a dietary supplement of \( \alpha \)-tocopheryl acetate (Bonnetti, E. \textit{et al}, 1975). Rats deficient in vitamin E also expire a greater concentration of ethane and pentane, products of lipid peroxidation, than do animals supplemented with \( \alpha \)-tocopheryl acetate (Dillard, C. J., \textit{et al}, 1977; Hafeman, D. G. and W. G. Hoekstra, 1977).

\( \alpha \)-T can go through a one or a two electron loss. When it loses one electron, a cation radical is formed which can lose a proton to form the phenoxy radical (eqn. 25). The phenoxy radical can decompose.

\[ \text{\vphantom{R}eqn. 25} \]
Figure 1. Some common phenolic antioxidants
to a carbon centered radical (eqn. 26) (Pryor, W. A., 1976). If this

\[ \text{happens, } \alpha-T \text{ may act as a prooxidant (Cillard, J. and P. Cillard, } 1980). \]

The carbon centered radical may add oxygen to form a peroxy radical which may then react with another molecule of \( \alpha-T \) to form the hydroperoxide and another phenoxy radical (eqns. 27 and 28) (Pryor, W. A., 1976). On

\[
\begin{align*}
\alpha-T' + O_2 & \rightarrow \alpha-TOO' \\
\alpha-TOO' + ArOH & \rightarrow \alpha-TOOH + ArO'
\end{align*}
\] (27) (28)

the other hand, the phenoxy radical may lose another electron to form the quinone methide (eqn. 29) or the phenoxonium ion (eqn. 30) when acting as an antioxidant. Under the proper conditions, the quinone methide may dimerize to form the a Spirodieneone ether while the phenoxonium ion may collapse to form \( \alpha \)-tocopherolquinone (\( \alpha \)-TQ) (Svanholm, U., et al., 1974; Patil, G. S. and D. G. Cornwell, 1978) (Figure 2).
a-\text{TQ} and its hydroquinone had previously been thought to be devoid of any biological activity (Columbia, C. and H. A. Mattill, 1940a, 1940b). Ten years later, it was convincingly demonstrated that this was not the case. The hydroquinone of a-TQ was as active as a-T when given intravenously (Mackenzie, J. B. et al, 1950) while a-TQ itself was less effective but still possessed some biological activity. Rabbits that were deficient in vitamin E responded to treatment with α-tocopheryl hydroquinone (α-TQH₂) as much as they did to the same amount of a-T. However, in contrast to the results obtained with a-T, increasing the
dosage of $\alpha$-TQH$_2$ did not prolong the response. Apparently, $\alpha$-TQH$_2$ or $\alpha$-TQ was not stored in the body as efficiently as $\alpha$-T was. It was also found that both $\alpha$-TQ and $\alpha$-TQH$_2$ when given orally to the animals were not as potent as when they were given intravenously. Presumably, the reduction in biopotency of both compounds when administered orally was due to poor absorption or to partial degradation before reaching the site of action. It was subsequently shown that $\alpha$-TQH$_2$ also prevented testicular degeneration in the hamster and in the rat (Mauer, S. I. and K. E. Mason, 1975) and fetal resorption in the rat (Rao, G. H. and K. E. Mason, 1975).

The biological activity of $\alpha$-TQ might be explained by its conversion to $\alpha$-TQH$_2$ in the body and the hydroquinone could then act as a phenolic antioxidant. Radiolabeled $\alpha$-TQH$_2$ given intraperitoneally resulted in the excretion of conjugated metabolites in the urine and in the feces (Chow, C. K. et al, 1967). The same investigators found that $\alpha$-TQ given the same way also resulted in the excretion of conjugated metabolites but there was also a significant amount of free $\alpha$-TQ found in the feces. Based on these observations, it was suggested that $\alpha$-TQ was reduced to $\alpha$-TQH$_2$ in the liver where it was conjugated for excretion into the bile. Recently, it was shown that liver mitochondria are able to reduce $\alpha$-TQ to $\alpha$-TQH$_2$ in the presence of $\beta$-hydroxybutyrate, pyruvate, succinate and NADH (Hughes, P. E. and S. B. Tove, 1980). Under strictly anaerobic conditions, it was found that equal amounts of $\alpha$-TQ and $\alpha$-TQH$_2$ were always isolated. It was suggested that the major form of $\alpha$-TQ in the liver was the semiquinone ($\alpha$-TQH'). In light of the previous observations that $\alpha$-TQH$_2$ can substitute for
vitamin E in the treatment of vitamin E deficiency diseases in certain animals, one might suppose that the interconversions of α-TQ to α-TQH' and α-TQH₂ form a redox system that might act as an antioxidant.

POLYUNSATURATED FATTY ACIDS, OXIDANT STRESS AND ATHEROSCLEROSIS

The accumulation of cholesterol and cholesteryl esters in aorta was observed as early as 1910 (Windaus, A., 1910) and has been repeatedly confirmed by other investigators (for review, see Cornwell, D. G. and R. V. Panganamala, 1981). Studies relating morphology and lipid composition demonstrate that there is no simple progression in either relative lipid composition or total lipid content from normal intima to fatty streaks to fibrous plaques; however, it is clear that the relative amount of cholesteryl ester increases with the appearance of intracellular lipid in the fatty streak lesion (Katz, S. S. et al, 1976; Panganamala, R. V. et al, 1974).

Cholesteryl esters of long chain fatty acids are only slightly soluble in other lipids (Small, D. M., 1970) and are not surfactants (Kwong, C. N., et al, 1971; Smaby, J. M. and H. L. Brockman, 1978). Thus, an increment in the relative and/or absolute cholesteryl ester content of the cell should result in the separation of cholesteryl ester droplets from other lipids. Indeed, anisotropic lipid droplets were observed in intima (Kaiserling, C. and A. Orgler, 1902; Stewart, G. T., 1961; Weller, R. O. et al, 1968) and were later shown to be almost pure cholesteryl ester (Hata, Y. et al, 1974). An extensive study of lipid phase behavior has demonstrated unequivocally that the cholesteryl
ester droplet is characteristic of the fatty streak lesion where it forms a relatively inert lipid pool (Katz, S. S. et al., 1976; Shipley, G. G., 1974).

The migration and proliferation of smooth muscle cells is an important event in atherosclerosis (Wissler, R. W., 1973; Ross, R. and J. Glomset, 1973; Stemerman, M. B., 1976). This, and the observation that the inert cholesteryl ester pool in the fatty streak lesion of the aorta contained relatively higher amounts of polyunsaturated fatty acids (Geer, J. C., et al., 1970; Cornwell, D. G. et al., 1975) led to the hypothesis that smooth muscle cell proliferation in atherosclerosis was due in part to the unavailability of polyunsaturated fatty acids that contribute to oxidant stress in the cell (Cornwell, D. G. et al., 1979; Cornwell, D. G. and R. V. Panganamala, 1980; Miller, J. S., 1980). This hypothesis was tested by the addition of specific polyunsaturated fatty acids to tissue culture media and the measurement of cell proliferation in guinea pig smooth muscle cell and human fibroblast cultures. Preliminary experiments in this laboratory showed that polyunsaturated fatty acids had highly specific effects on proliferation in both mammalian smooth muscle cell and human fibroblast cultures (Huttner, J. J. et al., 1977b; Huttner, J. J. et al., 1978). At close to 100 μM concentrations, both 8,11,14-eicosatrienoic acid (8,11,14-20:3) and 5,8,11,14-eicosatetraenoic acid (5,8,11,14-20:4) inhibited smooth muscle cell proliferation whereas 9,12-octadecadienoic acid did not, showing that the fatty acid effects were not simply due to non-specific detergent properties. Both 8,11,14-20:3 and 5,8,11,14-20:4 are well known precursors of prostaglandins. The prostaglandins derived from these fatty acids, PGE₁ and PGE₂ were
found to be even more potent inhibitors of smooth muscle cell proliferation (Huttner, J. J. et al, 1977b) suggesting that the fatty acids exerted their effects through their prostaglandin products. It was later shown in a series of experiments (Cornwell, D. G. et al, 1979) that:

1) 5,8,11-eicosatrienoic and 11,14,17-eicosatrienoic acids which by themselves are not prostaglandin precursors (Samuelsson, B. et al, 1975; Lands, W. E. M. et al, 1977) also inhibited smooth muscle cell proliferation; 2) the antioxidants α-tocopherol, 2,6-di-tert-butyl-4-methylphenol, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid all stimulated cell proliferation in the presence or in the absence of the triene or the tetraene fatty acids without inhibiting prostaglandin synthesis; and 3) indomethacin, a known inhibitor of prostaglandin biosynthesis (Samuelsson, B. et al, 1975; Lands, W. E. M. et al, 1977), and which does not inhibit the associated lipoxygenase pathway (Hamberg, M. and B. Samuelsson, 1974; Hammarström, S. et al, 1975) had no effect on cell proliferation and also did not reverse the inhibition caused by the triene or tetraene fatty acids. These elegant series of experiments forced the conclusion that polyene fatty acids through perhaps a lipoxygenase pathway were a source of oxidant stress for the cell and that oxidant stress was partly responsible for the control of cell proliferation.

**ANTIOXIDANTS, CELL PROLIFERATION AND OXIDANT STRESS**

A correlation between vitamin E and cell proliferation in tissue culture was first described in 1931 (Juhász-Schäffer, A., 1931).
This initial observation was subsequently confirmed by other investigators (Diplock, A. T. and A. S. M. Giasuddin, 1976; Huttner, J. J. et al, 1977b; Huttner, J. J. et al, 1978). It was observed in 1933 (Mason, K. E., 1933) that vitamin E was essential for tissues in which cell proliferation and differentiation were unusually rapid. Many other subsequent studies support this initial observation. For example: 1) the product of lipid peroxidation called age pigment or lipofuscin accumulates in postmitotic cells (Miquel, J. et al, 1977); 2) NADPH-dependent lipid peroxidation increases substantially with age (Player, T. J. et al, 1977); 3) α-T and α-TQ delay the appearance of lipofuscin and promote the life-span of nematodes (Epstein, T. and D. Gershon, 1972); 4) tissues with a high mitotic index such as regenerating liver, bone marrow, or intestinal mucosa show diminished lipid peroxidation when they are compared to other non-regenerating tissues (Wolfson, N. et al, 1956; Bernheim, F., 1963); 5) a number of early studies showed that lipid peroxides are low in tumor bearing animals and almost absent from the tumors themselves (Apffel, C. A., 1978); 6) tumors and tumor cell fractions are resistant to lipid peroxidation (Burlakova, E. B., 1967; Bartoli, G. M. and T. Galeotti, 1979; Utsumi, K. et al, 1970); 7) a tumor induced antioxidant deficiency has been described (Baumgartner, W. A. et al, 1978); and 8) α-T simulates the mitogenic response of murine lymphoid cells (Corwin, L. M. and J. Shloss, 1980).
OBJECTIVES

1. To study the uptake and metabolism of specific polyunsaturated fatty acids by smooth muscle cells in culture.

2. To investigate the potential for polyunsaturated fatty acids to act as a source of oxidant stress in the cell.

3. To investigate the effectiveness of antioxidants in counteracting oxidant stress in the cell.
MATERIALS AND METHODS

TISSUE CULTURE

Primary cultures of smooth muscle cells were established from guinea pig aorta obtained from prepubertal males and primary cultures of fibroblasts were established from neonatal foreskin. Details for our tissue culture procedure have been provided elsewhere (Cornwell, D. G. et al., 1979; Huttner, J. J. et al., 1977a; Miller, J. S., 1980). Subcultures were grown from 1 to 2 splits of confluent cells. Each 1 to 2 split grown to confluency was counted as one passage number (Schaeffer, W. I., 1979). Cells were used at passage number 2 to 6.

Growth Medium for establishing primary cultures and for subculturing cells was prepared from 1 X Eagle's minimal essential medium containing Hank's salts and 25 mM HEPES buffer (Grand Island Biological Company, Grand Island, New York), supplemented with 50 μg per ml of gentamycin sulfate (Schering Corporation, Kenilworth, New Jersey), 2 mM glutamine (GIBCO), 1 X nonessential amino acids (100X; Microbiological Associates, Inc., Bethesda, Maryland) and 2.2 mg sodium bicarbonate per ml. Growth Medium for smooth muscle cells contained in addition, 5 per cent fetal bovine serum (FBS) (Reheis Chemical Company, Phoenix, Arizona, Lot P34112) while Growth Medium for fibroblasts contained in addition, 10 per cent FBS.
The type of tissue culture flask used in each experiment is specified under the section that describes that particular experiment. Experimental Medium was used in all treatments except where noted. Experimental Medium contained 20 per cent FBS instead of the 5 or 10 per cent used in Growth Medium. Experimental Medium also contained 1 X essential vitamins (100X; Microbiological Associates), and 1 X essential amino acids (100 X; Microbiological Associates) in addition to the specified supplements for Growth Medium.

LABELLING OF CELLULAR LIPIDS

\[ \text{[1}^{14}\text{C]}-5,8,11,14\text{-Eicosatetraenoic acid (56 } \mu \text{Ci per mole)} \]

was purchased from New England Nuclear (Boston, Massachusetts) and diluted with cold 5,8,11,14-eicosatetraenoic acid (5,8,11,14-20:4) (Nu Chek Prep, Elysian, Minnesota) to a specific activity of either 0.6 \( \mu \text{Ci per } \mu \text{mole} \) or 1.1 \( \mu \text{Ci per mole} \). The labeled fatty acid was ascertained to be greater than 99 per cent pure by thin layer chromatography (TLC) on Whatman LK6D silica gel plates (Whatman, Inc., Clifton, New Jersey) run in TLC solvent system A (see section on Thin Layer Chromatography, p. 42) and scanned in a Packard model 7220/21 Radiochromatogram Scanner. The cold fatty acid was demonstrated to be free of peroxides by TLC on Whatman LK6D plates run in TLC solvent system C. Peroxides were detected by a starch-iodine spray consisting of a 4 per cent solution of potassium iodide in water followed by a 5 per cent solution of starch in 1 per cent acetic acid solution in water. Peroxides gave a purple color with this reagent (Huttner, J. J. et al,
The mixture of cold and labeled fatty acid was dissolved in 95 per cent ethanol to a final concentration of 60 mM.

α-Tocopherol (α-T) was purchased from Eastman Organic Chemicals (Rochester, New York), dissolved in 95 per cent ethanol to a final concentration of 25 mM and used without further purification.

Fatty acid labelling experiments were started by subpassaging a culture that had become confluent. The cells were seeded in LUX 75 cm² flasks and allowed to grow to confluency in 15 ml Experimental Medium. At confluency (4 - 5 days), the cells were again refed with 15 ml fresh Experimental Medium and treated with labeled fatty acid and α-T. The fatty acid, α-T or ethanol was first diluted 1 to 10 with Experimental Medium and then 1 to 50 when added to the cells. The final dilution was 1 to 500. The final fatty acid concentration in the medium in contact with the cells was 120, 1.6 or 0.6 μM and that of α-T was 10 μM. All cultures had the same final concentration of ethanol.

The incubation period was started as soon as the fatty acid was added to the cells. The cells were allowed to remain in contact with the fatty acid treatment for specified periods of time. A 0.5 ml aliquot was taken from the medium after the incubation period. The aliquots were mixed with 10.0 ml Aquasol (New England Nuclear) and counted in a Beckman LS100-C liquid scintillation spectrometer. Counts were corrected for quenching by the external standardization method and converted to disintegrations per minute (d.p.m.). The radioactivity that disappeared from the medium was assumed to have been taken up by the cell over the specified incubation period. This value was compared to the amount of label originally added to the culture and was expressed
as per cent uptake of fatty acid by the cell.

LIPID EXTRACTION

All solvents were glass distilled before use. Cellular lipids were extracted by a modification of a published procedure (Pong, S. S. et al., 1977). After the incubation period, media was withdrawn and each flask was rinsed three times with 5 ml portions of Growth Medium. The cells were quick frozen over dry ice. Five ml of 70 per cent methanol in water was added. Cells were detached with a rubber policeman and transferred to an Erlenmeyer flask. The LUX flask was rinsed two times with 5 ml portions of absolute methanol, and the rinses were combined with the cell suspension in the Erlenmeyer flask. Chloroform, 27 ml, was added and the mixture was allowed to stand at 4°C for at least 3 hours with periodic shaking. The suspension was then filtered through a glass wool plug and the organic phase which contained the cellular lipid was evaporated to a small volume in a Büchii evaporator. Traces of water were removed by adding 150 ml of ethanol-benzene (1:1, v/v) and evaporating the benzene-water azeotrope. The residue was dissolved in 2.0 ml chloroform. An aliquot from this was taken, mixed with 10 ml of Aquasol and counted in a Beckman LS100-C liquid scintillation spectrometer. Counts were converted to d.p.m. Extraction efficiency was expressed as the total radioactivity in the cell lipid extract relative to the radioactivity taken up by the cell over the incubation period. Over 99 per cent of the radioactivity was recovered from the cells by this extraction procedure.
LIPID ANALYSES

Major lipid classes

Neutral lipid fractions were resolved by TLC on Whatman LK6D plates using TLC solvent system A as the developing solvent. Neutral lipids were separated into cholesteryl esters (CE) ($R_f$, 0.80), triglyceride (TG) ($R_f$, 0.41), free fatty acids (FFA) ($R_f$, 0.17) and a more polar lipid fraction ($R$) ($R_f$, 0.10), while phospholipids (PL) remained in the preadsorbent area of the TLC plate. TLC MIX-1 (Applied Science Laboratories, State College, Pennsylvania) was used as the standard. The mixture contained oleic acid, triolein, cholesteryl oleate and methyl oleate. Ricinoleic acid (Nu Chek) was used as a standard for $R$. The $R_f$ value of the polar lipid fraction, $R$, was the same as the $R_f$ value for both hydroxy fatty acids and diglycerides. Bands were visualized with iodine vapors. Iodine was allowed to sublime away from the plate and bands corresponding to the different neutral lipid fractions were scraped into vials containing 10 mls of Thrift-Solv Scintillation Cocktail (KEW Scientific Company, Columbus, Ohio). These were counted in a Beckman LS100-C liquid scintillation spectrometer. The distribution of the labeled fatty acid in the different lipid fractions was calculated as per cent of the total label recovered on the TLC plate.

Major phospholipid classes were separated by TLC on Whatman LK5D plates using TLC solvent system B as the developing solvent. The phospholipids separated were lyssolecithin (LL) ($R_f$, 0.03), sphingomyelin (Sph) ($R_f$, 0.11), phosphatidyl choline (PC) ($R_f$, 0.24)
and phosphatidyl ethanolamine (PE) \( R_f, 0.48 \). Neutral lipids moved close to the solvent front in this system. The PC band also included phosphatidyl inositol (PI), phosphatidyl serine (PS) and phosphatidic acid (PA). TLC MIX-3 (Applied Science) was used as the phospholipid standard and contained PC, PE, Sph and LL. PI, PA and PS were also obtained from Applied Science and used as TLC standards. The standards on the TLC plate were visualized with iodine vapors. Bands were marked and the iodine allowed to sublime away from the plate. Bands corresponding to the different phospholipid fractions were scraped and counted as with the neutral lipids. The distribution of the labeled fatty acid in the different lipid fractions was also calculated as percent of the total label recovered on the TLC plate.

**Phospholipids**

Cell lipid extracts were fractionated into a neutral lipid and a phospholipid fraction by silicic acid column chromatography (Bills, T. K. et al, 1976). The lipids, dissolved in chloroform, were applied onto a glass column containing 0.5 g Unisil (Clarkson Chemical Company, Williamsport, Pennsylvania) previously washed with 50 mls chloroform. Neutral lipids were eluted with 10 mls chloroform followed by 5.0 mls 1 per cent methanol in chloroform. Phospholipids were eluted with 10 mls methanol and then with 10 mls 4 per cent water in methanol. Neutral lipids were further analyzed as described in the next section.

The phospholipid fraction from the silicic acid chromatography was further analyzed on various TLC systems. Part of the phospholipid sample was applied on an alumina TLC plate and developed in TLC solvent
system D (Rao, P. V. et al., 1968). PE, PC, PA, PS and PI were used as standards. In this system PA, PS and PI stay at the origin while PE has an $R_f$ of 0.33 and PC and $R_f$ of 0.65. Part of the phospholipid sample was also applied onto a Whatman LK5D silica gel plate and developed with TLC solvent system E. In this system, PA, PS and PI all have $R_f$ values of 0.19 while PC and PE have $R_f$ values of 0.52 and 0.68 respectively.

To further analyze the PA, PS and PI fraction separated by the TLC systems above, the phospholipid sample was applied to a Whatman preparative PLK5 plate and developed with TLC solvent system E. Standards PC, PE, PS, PI and PA were also run on one side of the plate not in contact with the sample. After the chromatography, the standards were visualized with iodine vapors taking care that the sample bands are not exposed to the vapors. This was done by putting iodine crystals in a Pasteur pipet and directing a gentle stream of air through the crystals and onto the standards on the plate while keeping the rest of the TLC plate covered with aluminum foil. The areas corresponding to the combined PA, PS and PI fractions and both the PE and PC fractions were each scraped from the plate and eluted with 200 mls of chloroform:methanol:water (60:30:6, v/v). Solvent was removed under reduced pressure in a Büchii rotary evaporator. The last traces of water was removed by adding benzene to the residue and evaporating the benzene-water azeotrope. The partially purified phospholipids were dissolved in 200 µl of chloroform:methanol (2:1, v/v). 10 µl aliquots from the combined PA, PS and PI sample and the PC + PE sample were counted. The PC + PE/PA + PS + PI ratio calculated from the radioactivity in the
partially fractionated phospholipid samples was 5.06. The same ratio calculated from the separation of the unfractionated phospholipid sample achieved on Whatman LK5D run in TLC solvent system E was 4.98.

The combined PA + PS + PI sample was applied onto a TLC plate coated with silica gel H (E. Merck, Applied Science, Lot 441) that had been impregnated with 5 per cent NH₄NO₃. The plate was run in TLC solvent system F (Applied Science newsletter, 1973). PA, PS, PI and LL standards were applied to one side of the plate away from the sample lane. After the chromatography, the sample lane was divided into 0.5 cm sections along the full length of the solvent travel. Each section was scraped and mixed with 10 mls of ACS-II scintillation cocktail (Amersham Arlington Heights, Illinois) and counted in a Beckman LS100-C liquid scintillation spectrometer. The standards remaining on the plate were located with iodine vapors. In this system, LL had an Rf of 0.34, PI an Rf of 0.44, and PS an Rf of 0.62. PA moved to the solvent front.

Triglycerides

Triglycerides were isolated from the neutral lipid fraction obtained from silicic acid chromatography, by preparative TLC on Whatman PLK5 silica gel plates. TLC solvent system A was used as the developing solvent. Neutral lipid standards (TLC MIX-1) were also applied to one side of the preparative plate. These standards were visualized with iodine vapors taking care not to expose the sample to the vapors (see section on Phospholipids, p.37). The triglyceride spot was located and the area on the plate corresponding to where the triglycerides in the
sample would migrate was scraped and eluted with 150 mls diethylether stabilized with 0.001 per cent BHT (w/v). The ether was removed under reduced pressure using a Büchii rotary evaporator and the residue dissolved in chloroform.

The purified triglyceride sample was further analyzed by argentation-TLC. This was accomplished using TLC plates coated with silica gel H impregnated with 5 per cent silver nitrate. TLC solvent system G was used as the developing solvent. The standards used were triarachidonin, trilinolenin, and trilinolein all purchased from Nu Chek Prep (Elysian, Minnesota). These were applied to one side of the plate away from the sample. After the chromatography, the sample lane was divided into 0.5 cm sections all along the solvent travel. Each section was scraped and mixed with 10 mls of ACS-II liquid scintillation cocktail and counted in a Beckman LS-8100 liquid scintillation spectrometer. The extent of quenching was monitored by the external standard method and was found to be constant in all of the samples. The standards left behind on the plate were visualized with 2,7-dichlorofluorescein. In this system, triarachidonin had an $R_f$ of 0.11, trilinolenin an $R_f$ of 0.62 and trilinolein moved close to the solvent front.

The argentation-TLC described in the preceding paragraph was satisfactory as long as the acetic acid used in the developing solvent (TLC solvent system G) came from a freshly opened bottle. However, the separation achieved with that system deteriorated concurrently with the number of times the acetic acid bottle was opened. It was reasoned that exposing glacial acetic acid to the atmosphere allowed it to gain
moisture and that the moisture affected the separation. It was therefore decided to include 0.5 per cent water in the solvent system (TLC solvent system H) to make the moisture contribution of the acetic acid negligible, and to increase the silver nitrate content of the plates to 8 per cent. Although TLC solvent system H is more polar than TLC solvent system G because of the presence of water and the relatively greater proportion of methanol, the increased silver nitrate content of the plate was expected to compensate for this, therefore allowing a satisfactory and reproducible separation of highly unsaturated triglycerides. In this system, triarachidonin had an $R_f$ of 0.43, trilinolenin an $R_f$ of 0.54 and trilinolein an $R_f$ of 0.93.

In some experiments, cells were treated with cold 5,8,11,14-20:4 and extracted as described above. The triglycerides were applied on TLC plates coated with silica gel H impregnated with 8 per cent silver nitrate. Triarachidonin, trilinolenin and trilinolein were applied to one side of the plate as standards, away from the sample lane. The plate was developed in TLC solvent system H. After the chromatography, the triglycerides were made visible with 2,7-dichloro-fluorescein. The sample band opposite the triarachidonin standard was scraped and eluted as described previously (Litchfield, C., 1972). Briefly, the pink silver nitrate - 2,7-dichlorofluorescein color was discharged with a minimal amount of a saturated aqueous solution of sodium chloride before elution of the triglycerides with diethyl ether. The ether was removed under reduced pressure using a Büchi rotary evaporator and the residue treated with a sodium methoxide reagent. The resulting methyl esters from the triglycerides were analyzed by
gas-liquid chromatography (GLC). This will be described in detail later under a section on GLC (p.46).

THIN LAYER CHROMATOGRAPHY

Separation of major lipid classes

TLC plates used for separation of major lipid classes were purchased from Whatman, Inc., Clifton, New Jersey. Whatman LK6D plates were found to be suitable for separation of neutral lipids while Whatman LK5D plates were more suitable for the separation of phospholipids. The corresponding preparative plates were designated as FLK6 and FLK5 respectively. The preparative plates are 1.0 mm thick. All these plates have a preadsorbent area made of diatomaceous earth that does not have appreciable retention characteristics. Samples are applied on the preadsorbent area. As the developing solvent moves through this area, the solutes of interest are moved all at the same time to the interface between the preadsorbent area and the analytical gel. Separation commences at the interface. The preadsorbent area therefore acts like a stacking gel and makes possible a very sharp separation between solutes on the chromatogram. \( R_f \) calculations are made relative to the interface as the origin.

The Whatman TLC plates are prechromatographed in chloroform:methanol (2:1, v/v) before use. They are then dried at 70°C for two hours after which they are kept in a dessicator until right before they are used.
Argentation-TLC

The 20 x 20 cm glass plates used to prepare silver nitrate plates were first cleaned thoroughly with chromic acid cleaning solution before use. Silica gel H was slurried in a solution containing enough silver nitrate to make the ratio 6 or 8 parts silver nitrate to 100 parts silica gel H. The glass plates were coated with a 0.33 mm layer of this slurry. The plates were allowed to air dry and were stored in the dark. Prior to use, the plates were activated at 120°C for 2 hours and allowed to cool in a dessicator. Activated silver nitrate plates were always used under very low level light or under red lights.

Alumina-TLC

This technique has been described in detail elsewhere (Rao, P. V. et al, 1968). Briefly, 90 parts alumina (Woelm basic TLC, Alupharm Chemicals, St. Louis, Missouri) were mixed with 10 parts calcium sulfate binder (Applied Science) (w/w). 40 grams of this mixture were slurried in 60 mls water and was used to coat 5 TLC plates. The plates were air dried and activated at 150°C-160°C for 1 hour before use. The TLC tanks were lined with filter paper and allowed to equilibrate with TLC solvent system D 1 hour before use. Whatman precoated alumina K3F plates were also found to be suitable in this system; however, the fluorescent indicator in the commercial precoated plates interfered with liquid scintillation counting. There were no precoated plates available that did not contain the indicator.
Ammonium nitrate-TLC

Separation of PS, PI and PA was achieved using plates coated with silica gel H slurried in 0.62 M ammonium nitrate (Applied Science newsletter, 1973). Prior to use, the plates were activated for 1 hour at 100°C and allowed to cool in a dessicator.

TLC solvent systems

The following TLC solvent systems were used throughout this study:

A. hexane:ether:acetic acid, 85:15:1 v/v
B. chloroform:methanol:water, 60:30:5 v/v
C. heptane:ether:acetic acid, 50:50:0.8 v/v
E. chloroform:methanol:ammonia:water, 70:30:1:1 v/v
F. chloroform:propionic acid:n-propanol:water, 10:10:10:4 v/v
G. chloroform:methanol:acetic acid, 94:5:1 v/v
H. chloroform:methanol:acetic acid:water, 93:6:0.5:0.5 v/v

FATTY ACID PROFILE OF CELL PHOSPHOLIPIDS, TRIGLYCERIDES AND CHOLESTEROL ESTERS

Treatment of cells with fatty acid

Cells were used at passage numbers 3 to 5 (see section on TISSUE CULTURE, p.32). Cells that had been grown to confluency in Growth Medium were subpassaged and seeded in 150 cm² Falcon Petri dishes. Smooth muscle cells were fed with 25 mls of Experimental Medium and
allowed to grow to confluence (4 - 5 days). Fibroblasts were also seeded in 150 cm² Falcon Petri dishes but were fed with Growth Medium containing 10 per cent FBS and then allowed to grow to confluence.

8,11,14-Eicosatrienoic acid (8,11,14-20:3), 5,8,11,14-20:4, and 7,10,13,16-docosatetraenoic acid (7,10,13,16-22:4), purchased from Nu Chek (Elysian, Minnesota), were checked for purity by GLC (see section on GLC, p.47) and were shown to be peroxide free by TLC (see section on LABELLING OF CELLULAR LIPIDS, p.33). Fatty acids were dissolved in 95 per cent ethanol and diluted 1:10 then 1:50 to a final 1:50 dilution with medium containing either 10 per cent FBS (fibroblasts) or 20 per cent FBS (smooth muscle cells). The final fatty acid concentration was 120 μM. Medium on confluent cells was replaced by 25 mls of medium containing the fatty acid. Control cells were refed with medium that did not contain the fatty acid supplement. Cells in some flasks were rinsed with 0.02 per cent EDTA, detached with 0.1 per cent trypsin and counted in a hemocytometer. Cells in other flasks were detached mechanically after 24 hours and analyzed for lipids as described below. Cell DNA content per culture for confluent cells did not vary significantly between control and treatment groups. Cells from 2 Petri dishes were pooled and their fatty acid content reported either as nmoles per culture or nmoles per 10⁶ cells.

**Lipid extraction and fractionation**

After the 24 hour incubation period, medium was decanted from the Petri dishes and the cells rinsed 3 times with 10 ml portions of Dulbecco's phosphate buffered saline. Cells were detached from the Petri
dish and lipid extracted as previously described (see section on LIPID EXTRACTION, p.35). Triheptadecanoin, 200 µg, and cholesteryl heptadecanoate, 100 µg, were added as internal standards to the combined lipid extract from 2 Petri dishes. Non-lipid material was removed from this extract by chromatography on a Sephadex G-25 column (Rouser, G. et al, 1967). The purified lipid extract was then separated into neutral lipid and phospholipid fractions by silicic acid chromatography as described earlier (see section on LIPID ANALYSES, subsection on Phospholipids, p.37). Methyl heptadecanoate, 170 µg, was then added as an internal standard to the phospholipid fraction.

Triglycerides and cholesteryl esters were isolated from the neutral lipid fraction by preparative TLC on Whatman PLK6 precoated silica gel plates using TLC solvent system A as developing solvent (see section on THIN LAYER CHROMATOGRAPHY, p.42). The triglyceride and cholesteryl ester bands, located with 2,7-dichlorofluorescein were scraped onto glass Buchner funnels of medium porosity and eluted with 150 mls of diethylether stabilized with 0.001 per cent BHT. 2,7-dichlorofluorescein was removed from the eluate by washing once with 20 mls of 0.5 N ammonium hydroxide then twice with 20 ml portions of water. The ether solution was dried over anhydrous sodium sulfate and evaporated to dryness in a Büchii rotary evaporator. The residue was dissolved in chloroform.

Preparation of samples for GLC

Phospholipids, triglycerides, and cholesteryl esters were converted to methyl esters using a sodium methoxide reagent (Glass, R. L.,
1971). The sodium methoxide reagent was prepared by dissolving 2.3 g of metallic sodium in 100 mls of a mixture consisting of 60 mls of anhydrous methanol, 40 mls of reagent grade benzene and 15 mg phenolphthalein.

The lipid samples dissolved in chloroform were placed in Teflon lined screw cap vials. Chloroform was removed with a gentle stream of prepurified argon. 0.2 ml of sodium methoxide reagent was added and allowed to react with the lipids for 20 minutes at room temperature. The reaction mixture was then neutralized with methanolic HCl until the pink color of the phenolphthalein was discharged.

The completeness of the methanolysis was ascertained by subjecting a radioactive cell lipid extract to the same conditions. The reaction products were analyzed by TLC on Whatman LK6D plates run in TLC solvent system A. The radiochromatogram showed only one peak corresponding to the methyl ester band (Figure 3).

Prior to GLC, the fatty acid methyl esters (FAME) were applied onto a silicic acid column previously washed with chloroform (see section on LIPID ANALYSES, subsection on Phospholipids, p.37). The FAME were eluted with 10 mls chloroform. Chloroform was removed under reduced pressure using a Büchii rotary evaporator and the residue dissolved in carbon disulfide, ready for GLC.

**Gas liquid chromatography (GLC)**

Retention time data were obtained by GLC using a Varian Aerograph model 1740 instrument equipped with a 6-ft, 2 mm i.d. glass column packed with 15 per cent EGSS-X on 100/120 Gas Chrom P (Applied
Figure 3

Radioscan of fatty acid methyl esters derived from a total lipid extract from smooth muscle cells treated with $[1^{-14}O]^{-5,8,11,14-20:4}$. The lipid extract was treated with sodium methoxide for 20 minutes at room temperature and the reaction products were chromatographed alongside standards that included free fatty acid (FFA), triglyceride (TG), fatty acid methyl ester (ME) and cholesterol ester (CE).
Science). The column temperature was 180°C. Helium was the carrier
gas and the flow-rate was 20 ml per min. Peaks were identified by
comparison of the relative retention times with those of FAME standards.

FAME standards were purchased from Nu Chek (Elysian, Minnesota).
Methyl 5,8,11,14,17-eicosapentaenoate was kindly provided by Dr. H.
Sprecher, Department of Physiological Chemistry, Ohio State University.
Retention times of the fatty acids were all expressed relative to the
retention time for methyl octadecanoate. A plot of the logarithm of
the relative retention times against the carbon number was made (Figure
2) and a family of straight lines was produced. Each straight line
included fatty acids with the same number of double bonds which were
always in the same positions relative to the methyl end of the fatty
acid chain. No FAME standards could be obtained for 10,13,16-docosa-
trienoic acid (10,13,16-22:3) or 9,12,15,18-tetracosatetraenoic acid
(9,12,15,18-24:4). The relative retention times for these two fatty acids
were predicted from the plot of the logarithm of relative retention times
against carbon number (Figure 4) and through calculation of separation
factors as described previously (Geer, J. C. et al., 1970).

Quantitative data were obtained by GLC using a Packard model
428 instrument equipped with a flame ionization detector and a 6-ft, 2 mm
i.d. glass column packed with 10 per cent Alltech CS-10 on 100/120 mesh
temperature was programmed from 167°C to 205°C. Nitrogen was the carrier
gas and the flow-rate was 20 ml per minute. Peak areas were calculated
electronically by a Spectra Physics System I computing integrator.
Figure 4

Logarithm of relative retention times of fatty acids as a function of chain length. ▼—▼: saturated fatty acids; ▼—▼: monoenes, ω9; ○—○: dienes, ω6; ●—●: trienes, ω6; ◆—◆: trienes, ω3; ▲—▲: tetraenes, ω6; ⋆—⋆: pentaenes, ω3.
Figure 4
TREATMENT OF CELLS WITH FATTY ACID, ANTIOXIDANTS AND QUINONES

8,11,14-20:3, 5,8,11,14-20:4 and 7,10,13,16-22:4, purchased from Nu Chek (Elysian, Minnesota) were checked for purity by GLC and were shown to be peroxide free by TLC (see section on LABELLING OF CELLULAR LIPIDS, p.33). These were dissolved in redistilled 95 per cent ethanol to a final concentration of 60 mM. 

α-Tocopherol (α-T) and α-tocopherolquinone (α-TQ) were purchased from Eastman Organic Chemicals (Rochester, New York) and were dissolved in 95 per cent ethanol to a final concentration of 25 mM. Menadione (Sigma Chemical Company, St. Louis, Missouri) and indomethacin (Merck, Sharp and Dohme, Rahway, New Jersey) were each dissolved in 95 per cent ethanol to a final concentration of 25 mM.

Confluent cells were subpassaged (see section of TISSUE CULTURE, p.32) and grown to confluency in Corning T-25 flasks with 4 mls of Experimental Medium. This was done with both smooth muscle cells and fibroblasts. At confluency, the cells were refed with 4 mls of Experimental Medium that contained the treatments. Fatty acids, dissolved in ethanol, were diluted 1:6:100 then 1:8 to a final dilution of 1:500 with Experimental Medium. Antioxidants, quinones and indomethacin, all dissolved in ethanol, were diluted 1:6:100 then 1:40 to a final 1:2500 dilution with Experimental Medium. Control cultures were treated with Experimental Medium containing the same amount of 95 per cent ethanol.
Malonaldehyde assay

After a specified time period of exposure of the cells to the treatments, the cells were killed and disrupted by the addition of 2 mls of 20 per cent trichloroacetic acid to the medium in the flask. Four mls of 0.67 per cent thiobarbituric acid (TBA) were added to the flask and this mixture was incubated for 20 minutes at 97°C. The flask contents were decanted and centrifuged at 12,000 x g for 10 minutes at 4°C. The absorbance of the supernatant was measured at 532 nm (Wilbur, K. M., 1949) in a Beckman Acta II spectrophotometer. Tissue culture media contains a pH indicator phenol red which contributes a background absorbance at 532 nm. The TBA absorbance from a culture without added fatty acid was subtracted from the TBA absorbance from a culture treated with fatty acid. Absorbance was converted to nmoles malonaldehyde (MDA) from a standard curve generated with 1,1,3,3-tetramethoxypropene (Aldrich Chemical Company, Milwaukee, Wisconsin) (Figure 5). Lipid peroxidation was reported as nmoles MDA per culture. Lipid peroxidation was not observed when the media and fatty acid were incubated without cells. A small amount of MDA was observed when the media and cells were incubated without added polyunsaturated fatty acid.

ANTIOXIDANTS

Assay for antioxidant property in an organic phase

Antioxidants are assayed most easily by their ability to interrupt a free radical reaction. In the organic phase, this can be done by measuring the rate of the azoiso(butynitrile)(AIBN) initiated
Figure 5

The thiobarbituric acid assay for malonaldehyde. Absorbance at 532 nm as a function of malonaldehyde concentration.
Figure 5
Formation of cumene hydroperoxide (CHP) from cumene at 60°C and studying the effect of antioxidants on this process (Arneson, R. M. et al, 1978).

AIBN is an azo compound that undergoes thermal homolysis with an activation energy of 31 kcal/mole to give free radicals and nitrogen (Pryor, W. A., 1966) (eqn. 31). The free radicals produced induces the formation of CHP from cumene. CHP is assayed by reacting it with ferrous ion. The ferric ion produced by this reaction is measured spectrophotometrically through its complexation with thiocyanate ion to give a chromophore with an absorption maximum at 490 nm.

AIBN (Aldrich) was recrystallized from methanol. Cumene (Aldrich) was passed through a column of basic alumina (Woelm, Gr. I, Chemical Samples, Columbus, Ohio) to remove hydroperoxides.

The entire procedure for the assay was carried out under red lights. The reaction mixture consisted of 50 micromoles cumene, 500 nanomoles AIBN and the indicated amount of antioxidant in a total volume of 250 µl benzene. The reaction mixtures were contained in air tight Teflon lined screw cap vials and kept in ice before the incubation period. At time zero, the vials were placed in a 60°C water bath.
After the specified incubation time, the vials were promptly placed back in the ice. To each of these vials were added 1.8 mls of 6.7 per cent ammonium thiocyanate in methanol (w/v), and 10 l of 0.44 per cent aqueous FeCl₂. The vials were allowed to stand for 5 minutes at room temperature after which the absorbance at 490 nm was measured in a Beckman Acta II spectrophotometer.

Assay for antioxidant property in an aqueous phase

Hydrogen peroxide and organic hydroperoxides can act as initiators of free radical reactions when they undergo homolytic cleavage. This can happen when they are reacted with iron salts or when they are subjected to light and heat (Pryor, W. A., 1966).

22 µl CHP (Matheson, Coleman and Bell, Norwood, Ohio) was dissolved in 10 mls water. 6,9,12-octadecatrienoic acid (6,9,12-18:3) (Nu Chek) was dissolved in 95 per cent ethanol to a final concentration of 88 mM. The reaction mixture contained 880 nmoles 6,9,12-18:3, 250 nmoles FeCl₂ and the indicated amount of antioxidant in 1.0 ml of 0.1 M phosphate buffer at pH 7.4. The reaction was started by the addition of 100 µl CHP solution. The mixture was allowed to stand for 10 minutes at room temperature after which 2.0 mls TBA reagent was added. The TBA reagent was made up of 15 per cent trichloroacetic acid and 0.375 per cent thiobarbituric acid in 0.25 N HCl (Buege, J. A. and S. D. Aust, 1977). The reaction mixture was placed in a boiling water bath for 15 minutes after which it was cooled in ice. Three mls of chloroform: acetic acid (2:1, v/v) were added and the mixture vortexed at slow speed to separate the phases. The absorbance of the upper phase was read at 532 nm (Asakawa, T. and S. Matsushita, 1980).
Incorporation of [1-^{14}C]-5,8,11,14-eicosatetraenoic acid in major lipids of smooth muscle cells in culture

Introduction

Polyunsaturated fatty acids inhibit the proliferation of guinea pig aortic smooth muscle cells and human skin fibroblasts in culture while antioxidants such as BHT, α-naphthol and α-T stimulate the proliferation of these same cells (Huttner, J. J. et al., 1977b; Huttner, J. J. et al., 1978; Cornwell, D. G. et al., 1979). BHT, α-naphthol and α-T but not indomethacin overcame the inhibitory effect of polyunsaturated fatty acids on cell proliferation (Cornwell, D. G. et al., 1979). The same investigators observed that certain polyenes that are not precursors for prostaglandins also had inhibitory properties on cell proliferation that are overcome by antioxidants. This led to the hypothesis that polyunsaturated fatty acids were a source of oxidant stress in the cell and that oxidant stress was responsible in part for the control of cell proliferation.

This section describes results of experiments designed to investigate further the metabolism of exogenous 5,8,11,14-20:4 by cells in culture and the effect of α-T on these cellular processes.
Separation of cell lipid extract into major lipid classes

Figure 6 shows a radioscan of a cell lipid extract analyzed by TLC in a system that would separate neutral lipids into various fractions. In this system, phospholipids stay at the preadsorbtent area of the plate. Figure 7 shows the radioscan of the same cell lipid extract analyzed by TLC in a system that would separate major phospholipids. Neutral lipids migrate close to the solvent front in this system.

Uptake of \([1^{-14}C]-5,8,11,14-20:4\) by smooth muscle cells in culture

The time course for the uptake of fatty acid by smooth muscle cells treated with 0.6 \(\mu\)M \([1^{-14}C]-5,8,11,14-20:4\) is shown in Figure 8. The distribution of the incorporated fatty acid into the neutral or phospholipid fractions are also shown in the same figure. Although it would seem that fatty acid incorporation into cells treated with \(\alpha\)-T proceeded faster at 1 and 6 hours of incubation than that in control cells, it must be noted that each point in the graph represents only two replications so that it is not possible to make any statistical inferences from these data. The differences between incorporation into phospholipids and triglycerides is so marked however that we may perhaps safely conclude that in smooth muscle cells treated with 0.6 \(\mu\)M fatty acid, there is preferential incorporation of the exogenous fatty acid into the phospholipid fraction.

Figure 9 shows the time course for the uptake of fatty acid by smooth muscle cells treated with 120 \(\mu\)M \([1^{-14}C]-5,8,11,14-20:4\). There were no significant differences in rates of fatty acid uptake by cells treated with 10 \(\mu\)M \(\alpha\)-T with those of control cells. In contrast to cells
Figure 6

Radioscan of a thin layer chromatogram of lipids isolated from smooth muscle cells in culture treated with 120 μM \[1^{14}C\]-5,8,11,14-eicosatetraenoic acid. Standards that were co-chromatographed with the lipid sample included cholesteryl ester (CE), triglyceride (TG), free fatty acid (FFA) and ricinoleic acid (R). In this system, phospholipids stay at the preadsorbent area.
Figure 6
Figure 7

Radioscan of a thin layer chromatogram of a lipid extract from smooth muscle cells in culture showing separation of major phospholipids. Standards that were co-chromatographed with the sample included triglyceride (TG), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), sphingomyelin (Sph) and lysolecithin (LL).
Figure 7
Figure 8. Time course for the uptake of fatty acid by smooth muscle cells in culture treated with 0.6 μM [1-14C]-5,8,11,14-eicosatetraenoic acid. Open symbols represent cells that were also treated with 10 μM α-T and closed symbols represent controls. Triangles represent the uptake of fatty acid, circles represent the incorporation of fatty acid into phospholipids and diamonds represent incorporation of the fatty acids into triglycerides.
Figure 9

Time course for the uptake of fatty acid by smooth muscle cells in culture treated with 120 μM [1-\(^{14}\)C]-5,8,11,14-eicosatetraenoic acid. Open symbols represent cells that were also treated with 10 μM α-T and closed symbols represent controls. Triangles represent the uptake of fatty acid, circles represent the incorporation of fatty acid into triglycerides, and diamonds represent the incorporation of fatty acid into phospholipids.
Figure 9

FATTY ACID (NANOMOLES)

INCUBATION PERIOD (HOURS)
treated with 0.6 μM fatty acid, cells treated with 120 μM fatty acid incorporated the radiolabel preferentially into the triglycerides.

**Distribution of [1-^14C]-5,8,11,14-20:4 in major lipids**

The distribution of the radiolabeled fatty acid in the various lipid classes are shown in Table 1. Most of the radioactivity was found to be associated with the phospholipids and the triglycerides. There was very little unesterified radiolabeled fatty acid and very little incorporation into the cholesterol esters. The radioactivity in the phospholipid fraction was mostly associated with PE and PC. The radioactivity that was retained at the origin of the phospholipid TLC plate remain unidentified.

Table 1 shows the same relationships between exogenous fatty acid concentration and incorporation into phospholipids and triglycerides as that shown in Figure 8 and 9. The PL to TG ratio decreased as the concentration of the fatty acid treatment was increased from 0.6 μM to 120 μM. At 24 hours, the PC to PE ratio appeared to be the same for cells treated with 0.6 μM or 1.6 μM fatty acid and was up slightly for cells treated with 120μM fatty acid.

The results of a fatty acid pulse experiment is also shown in Table 1. Confluent cells were treated with 0.6 μM fatty acid for 24 hours after which the cells were rinsed with incomplete medium and refed with Experimental Medium. 24 hours after refeeding, the cells were analyzed for lipid. The PL to TG ratio was up and the PC to PE ratio was down in these cells relative to those cells that were treated with fatty acid for 24 hours and then immediately analyzed for lipid.
Table 1. Uptake and lipid distribution of different concentrations of [1-\(^{14}\)C]-5,8,11,14-eicosatetraenoic acid by smooth muscle cells in culture

<table>
<thead>
<tr>
<th>Lipid Fraction</th>
<th>Initial Concentration of FFA, (\mu M^a)</th>
<th>0.6 (\mu M) FFA pulse (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>% Uptake (^c)</td>
<td>86.3, 89.2</td>
<td>83.2, 82.0</td>
</tr>
<tr>
<td>TLC system A (^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>59.4, 62.3</td>
<td>43.9, 55.2</td>
</tr>
<tr>
<td>FFA + R</td>
<td>0.6, 0.5</td>
<td>0.7, 0.5</td>
</tr>
<tr>
<td>TG</td>
<td>38.4, 36.7</td>
<td>54.9, 43.2</td>
</tr>
<tr>
<td>CE</td>
<td>0.5, 0.5</td>
<td>0.5, 1.0</td>
</tr>
<tr>
<td>FL/TG</td>
<td>1.6, 1.7</td>
<td>0.8, 1.3</td>
</tr>
<tr>
<td>TLC system B (^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Or</td>
<td>0.3, 0.5</td>
<td>0.3, 0.3</td>
</tr>
<tr>
<td>IL</td>
<td>0.4, 0.4</td>
<td>0.3, 0.2</td>
</tr>
<tr>
<td>Sph</td>
<td>0.4, 0.4</td>
<td>0.4, 0.3</td>
</tr>
<tr>
<td>FC</td>
<td>35.8, 36.8</td>
<td>27.2, 33.9</td>
</tr>
<tr>
<td>PE</td>
<td>19.5, 22.3</td>
<td>14.8, 18.7</td>
</tr>
<tr>
<td>NL</td>
<td>43.6, 39.7</td>
<td>57.0, 46.5</td>
</tr>
<tr>
<td>FC/PE</td>
<td>1.8, 1.7</td>
<td>1.8, 1.8</td>
</tr>
</tbody>
</table>

\(^a\)Medium on confluent cells was replaced with Experimental Medium containing the fatty acid treatment.

\(^b\)Cells were treated with FFA for 24 hours, rinsed 5x with incomplete medium then refed with Experimental Medium. Lipids were extracted 24 hours after refeding.

\(^c\)Uptake was calculated from the difference in total label in medium before and after incubation with cells divided by the amount of label originally put in.

\(^d\)TLC system A: 90:20:1, hexane:ether:acetic acid (v/v)

B: 60:30:5, chloroform:methanol:water (v/v)

\(^e\)Data reported as per cent of total radioactivity on plate.
120 μM polyunsaturated fatty acids inhibit cell proliferation and α-T overcomes this inhibition (Cornwell, D. G. et al, 1979). In confluent smooth muscle cells treated with 120 μM 5,8,11,14-20:4 however, α-T had no effect on either the uptake or the distribution of the radiolabeled fatty acid in the different lipid classes (Figure 9 and Table 2).

At short incubation periods with fatty acid, smooth muscle cells had relatively more PC than PE than in cells that were exposed to fatty acid for longer periods of time (Figure 10 and 11). α-T had no effect on the distribution of radiolabeled fatty acid in the PE and PC fractions of cells treated with 120 μM fatty acid at any time period (Figure 10). Although it would seem that α-T had an effect on the PC to PE ratio of cells treated with 0.6 μM fatty acid at the 1 minute incubation period (Figure 11), it must be pointed out that there was not enough data points in that particular experiment to make any statistical inference.

Cells at low density (200 cells/cm²) treated with 120 μM 5,8,11,14-20:4 took up much less fatty acid than confluent cells (Table 3). The distribution of radiolabeled fatty acid into the different lipid classes however, was not different from that in confluent cells. Again, α-T had no effect on the uptake of fatty acid or on the distribution of the fatty acid into the different lipid classes.

The PC fraction separated by TLC on Whatman LK5D plates using chloroform:methanol:water (60:30:5, v/v) (TLC solvent system B) also contains PS, PI and PA. To resolve this particular phospholipid fraction, the cell lipid extracts were separated into neutral lipid and phospholipid
Table 2. Uptake and lipid distribution of 120 µM \([1-^{14}C]-5,8,11,14-20:4\) by confluent smooth muscle cells\(^a\)

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Relative Distribution of Label +E(^b)</th>
<th>-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Uptake(^c)</td>
<td>80.7 ± 9.3</td>
<td>81.4 ± 12.2</td>
</tr>
<tr>
<td>PL</td>
<td>17.5 ± 1.6</td>
<td>17.3 ± 3.7</td>
</tr>
<tr>
<td>R</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>FFA</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>TG</td>
<td>81.1 ± 1.6</td>
<td>81.2 ± 3.5</td>
</tr>
<tr>
<td>CE</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>TLC system A(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Or</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LL</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sph</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>PC</td>
<td>10.9 ± 0.8</td>
<td>11.0 ± 2.5</td>
</tr>
<tr>
<td>PE</td>
<td>5.2 ± 0.7</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td>NL</td>
<td>83.8 ± 1.5</td>
<td>83.5 ± 3.8</td>
</tr>
</tbody>
</table>

\(^a\) Cells were grown to confluence with Experimental Medium. At confluence, the medium was replaced with Experimental Medium containing the fatty acid treatment.

\(^b\) Cells had either 10 µM α-T (+E) or the ethanol vehicle alone (-E).

\(^c\) Calculated from the difference in total label in the medium before and after incubation with cells relative to the label originally in the medium.

\(^d\) TLC system A: hexane:ether:acetic acid, 85:15:1 (v/v)

\(^b\) TLC system B: chloroform:methanol:water, 60:30:5 (v/v)

\(^e\) Data reported as mean ±s.d. of per cent of total label on plate.
Table 3. Uptake and lipid distribution of 120 µM [1-\(^{14}\)C]-5,8,11,14-20:s4 by smooth muscle cells at low density\(^a\)

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Relative distribution of label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+E(^b)</td>
</tr>
<tr>
<td></td>
<td>-E</td>
</tr>
<tr>
<td>% uptake(^c)</td>
<td>23.3 ± 9.5</td>
</tr>
<tr>
<td></td>
<td>29.5 ± 7.5</td>
</tr>
<tr>
<td>PL</td>
<td>21.0 ± 3.7(^g)</td>
</tr>
<tr>
<td></td>
<td>18.3 ± 0.3</td>
</tr>
<tr>
<td>R</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>FFA</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>TG</td>
<td>75.0 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>80.6 ± 0.4</td>
</tr>
<tr>
<td>CE</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Or</td>
<td>0</td>
</tr>
<tr>
<td>LL</td>
<td>0</td>
</tr>
<tr>
<td>Sph</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>PC</td>
<td>10.3 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>PE</td>
<td>7.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>6.3 ± 0.9</td>
</tr>
<tr>
<td>NL</td>
<td>82.0 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>84.6 ± 1.9</td>
</tr>
</tbody>
</table>

\(^a\)Cells were seeded at 200 cells/cm\(^2\).
\(^b\)Cells had either 10 µM o-T (+ E) or the ethanol vehicle alone (-E).
\(^c\)Calculated from the difference in total label in medium before and after incubation with cells relative to the label originally in medium.
\(^d\)TLC system A: hexane:ether:acetic acid, 85:15:1 (v/v)  
B: chloroform:methanol:water, 60:30:5 (v/v)
\(^g\)Data reported as mean ± s. d. of per cent of total label on plate.
Figure 10. The PC to PE ratio of confluent smooth muscle cells incubated with 120 μM [1-14C]-5,8,11,14-eicosatetraenoic acid over a time period. Open symbols represent cells that were also treated with 10 μM α-T while closed symbols represent controls.
Figure 11. The PC to PE ratio of confluent smooth muscle cells incubated with 0.6 μM [1-14C]-5,8,11,14-eicosatetraenoic acid over a period of time. Open symbols represent cells that were also treated with 10 μM α-T while closed symbols represent controls.
fractions by silicic acid chromatography. The purified phospholipid extract was subjected to various TLC systems the object being to separate PS, PI and PA from PC and then to resolve the PS, PI and PA subfraction. Figure 12 shows the radioscan obtained when the phospholipid fraction was analyzed by alumina TLC (Rao, P. V. et al, 1968). PA, PS and PI remain at the origin in this system. PE runs behind PC in contrast to silica gel TLC where PE "normally" runs ahead of PC. This serves as a good check for the identity of the phospholipid fractions. Figure 13 shows a radioscan of the same phospholipids on a Whatman LK5D plate developed in chloroform:methanol:ammonia:water (70:30:4:1, v/v) (TLC solvent system E). PA, PS and PI remained close to the origin in this system and were separated from PC and PE. PA, PS and PI were recovered from the silica gel plate and analyzed by TLC on NH₄NO₃ impregnated plates. Separation of standard PI, PS and PA samples obtained from Applied Science is shown in Figure 14. The separation of the radioactive sample is shown in Figure 15. Most of the radioactivity is associated with the PI fraction. Phospholipid fractions isolated from silicic acid chromatography were analyzed this way (Table 4). At 1 minute incubation periods of smooth muscle cells with fatty acid, the PI content was high relative to the other phospholipids. The relative content of PI decreased as the incubation period with fatty acid increased.

Triglycerides were isolated from cells that were treated with 120 µM [1-¹⁴C]-5,8,11,14-20:4. These were further fractionated by argentation-TLC on silica gel plates impregnated with 6 per cent silver nitrate. Figure 16 shows the radioactivity profile of the triglyceride
Figure 12

Radioscan of an alumina thin layer chromatogram of a phospholipid sample isolated from smooth muscle cells treated with $[1^{-14}C]^{-5,8,11,14}$-eicosatetraenoic acid. Standards that were co-chromatographed with the sample included phosphatic acid (PA), phosphatidyl serine (PS), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC).
Figure 12
Figure 13

Radioscan of a thin layer chromatogram of a phospholipid sample isolated from smooth muscle cells treated with [1-¹⁴C]-5,8,11,14-eicosatetraenoic acid. The silica gel plate was developed in a basic solvent system made up of chloroform:methanol:ammonia:water (70:30:4:1, v/v). Standards that were co-chromatographed with the sample included phosphatidic acid (PA), phosphatidyl serine (PS), phosphatidyl inositol (PI), phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE).
Figure 13

SOLVENT FRONT

PRE-ADSORBENT AREA  PA+PI+PS  PC  PE
Figure 14

Thin layer chromatogram of phosphatidic acid (PA), phosphatidyl serine (PS), phosphatidyl inositol (PI) and lysolecithin (LL) standards on a silica gel plate impregnated with NH₄NO₃. The plate was developed in chloroform:propionic acid:n-propanol:water (10:10:10:4, v/v).
Figure 14
Figure 15. Radioactivity profile of an NH$_4$NO$_3$ impregnated thin layer chromatogram of a phospholipid sample isolated from smooth muscle cells treated with [1-14C]-5,8,11,14-eicosatetraenoic acid. Standards that were co-chromatographed with the sample included lysolceithin (LL), phosphatidyl inositol (PI), phosphatidyl serine (PS) and phosphatidic acid (PA).
Table 4. The incorporation of exogenous 120 μM \(^{14}C\)-5,8,11,14-eicosatetraenoic acid into phospholipids by smooth muscle cells in culture

<table>
<thead>
<tr>
<th>Phospholipid Fraction</th>
<th>Incubation period with fatty acid (in minutes)</th>
<th>1</th>
<th>30</th>
<th>60</th>
<th>360</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-E (^a)</td>
<td>+E</td>
<td>-E</td>
<td>+E</td>
</tr>
<tr>
<td>% Uptake</td>
<td>trace (^b)</td>
<td>trace</td>
<td>6.3, 5.8 (^c)</td>
<td>2.4</td>
<td>5.8, 12.4</td>
</tr>
<tr>
<td>% Phospholipid (^d)</td>
<td>34.1</td>
<td>25.7</td>
<td>29.2, 33.5</td>
<td>29.8</td>
<td>30.9, 32.0</td>
</tr>
<tr>
<td>PI</td>
<td>60.2</td>
<td>46</td>
<td>31.7, 31.5</td>
<td>26.8</td>
<td>30.4, 27.2</td>
</tr>
<tr>
<td>PC</td>
<td>33.6</td>
<td>37</td>
<td>59.4, 53.0</td>
<td>45.1</td>
<td>57.2, 54.8</td>
</tr>
<tr>
<td>PE</td>
<td>3.0</td>
<td>12.9</td>
<td>9.0, 11.8</td>
<td>15.0</td>
<td>12.4, 18</td>
</tr>
<tr>
<td>SF</td>
<td>3.1</td>
<td>3.9</td>
<td>0 , 3.6</td>
<td>13.1</td>
<td>0 , 0</td>
</tr>
</tbody>
</table>

\(^a\)Smooth muscle cells were also treated with 10 M α-T (+E) or with the ethanol vehicle alone (-E).

\(^b\)Uptake was less than 0.1 per cent.

\(^c\)Calculated from the difference in total label in medium before and after incubation with cells relative to the label originally in medium.

\(^d\)Calculated from the TLC analysis of major lipids (see Figures 4 and 7).

PI: phosphatidyl inositol; PC: phosphatidyl choline; PE: phosphatidyl ethanol amine;
SF: radioactivity in the solvent front of the TLC system.
Figure 16. Radioactivity profile of a silver nitrate impregnated thin layer chromatogram of a triglyceride sample isolated from smooth muscle cells treated with [1-14C]-5,8,11,14-eicosatetraenoic acid. The separation of the standards triarachidonin, trilinolenin and trilinolein achieved with the system is indicated by bars below the figure.
Figure 17. Separation of triarachidonin, trilinolenin and trilinolein standards on a silica gel plate impregnated with silver nitrate (8:100, silver nitrate:silica gel, w/w). The chromatogram was developed in chloroform:methanol:acetic acid:water (93:60:0.5:0.5, v/v).
sample. A major portion of the radioactivity co-migrated with a triarachidonin standard while another major radioactive band migrated just ahead of a trilinolenin standard. A small portion of the radioactivity migrated close to the solvent front.

Triglycerides were also isolated from cells that were treated with 120 μM cold 5,8,11,14-20:4. These were fractionated on silica gel plates that were impregnated with 8 per cent silver nitrate. A more polar solvent system was used to develop the plate (see section on Triglycerides, p. 39). Separation of triarachidonin, trilinolenin and trilinolein standards is shown in Figure 17. The triglycerides isolated from the region on the plate where triarachidonin would migrate was analyzed by GLC and was found to contain 96 per cent 5,8,11,14-20:4 and 4 per cent 7,10,13,16-22:4.

Discussion

Cells in culture have long been known to accumulate intracellular lipid when exposed to serum (Lewis, M. R and W. H. Lewis, 1911). The serum factor responsible for this event was later identified as albumin bound fatty acid (Moskowitz, M. S., 1967; MacKenzie, C. G. et al, 1970). Apparently, free fatty acids are transferred reversibly from albumin to binding sites at or near the cell surface by an energy independent process (Spector, A. A. et al, 1965; Spector, A. A., 1975) so that uptake of fatty acid is mainly dependent on the fatty acid to albumin ratio in the medium to which the cells are in contact with.

Smooth muscle cells treated with 5,8,11,14-20:4 incorporate the exogenous fatty acid into intracellular esterified lipid as shown
in Figures 8 and 9 and in Tables 1 to 3. This is accompanied by the formation of intracellular lipid droplets that stain with oil red 0 (Miller, J.S. et al., 1980b). These lipid droplets are probably triglyceride containing a high percentage of triarachidonin. Similar results have been reported for strain L fibroblasts (Geyer, R. P., 1967). These cells also accumulated intracellular lipid droplets when they were exposed to unesterified fatty acid in the medium. The lipid droplets were found to be almost pure triglyceride. 90 per cent of the fatty acids found in these triglyceride droplets were found to be the same as the substrate fatty acid.

Strain L fibroblasts when exposed to exogenous unesterified fatty acid have been reported to continually incorporate the fatty acid into triglycerides until the cells cease dividing, round up, and disintegrate (Geyer, R. P., 1967). It has been suggested since then that triglyceride buildup can itself reduce the growth rate of cells in culture (Rosenthal, M. D. and R. P. Geyer, 1978; Spector, A. A. et al., 1979). Recently however, it was shown that the growth of smooth muscle cells are not affected by triglyceride droplet buildup in the cell (Miller, J. S. et al., 1980b). 120 μM polyunsaturated fatty acid in the medium inhibits smooth muscle cell proliferation. e-T reverses this inhibition; however, e-T does not prevent the formation of these lipid droplets (Miller, J. S. et al., 1980b). This is a situation, therefore, where cells are growing normally even if they are loaded with lipid droplets.

The data on radiolabeled fatty acid incorporation into PC and PE (Figures 10 and 11) suggest that at short incubation periods, there
is preferential incorporation of the exogenous fatty acid into PC. As the incubation period is lengthened, incorporation into the PE fraction builds up and this results in a decrease in the PC to PE ratio. It might be that there is a transfer of radiolabeled fatty acid from the PC fraction to the PE fraction or that the synthesis of PE gradually accelerates during the course of the incubation thereby competing more effectively for fatty acid equivalents in the medium. In pulse chase experiments with fibroblasts however, an actual transfer of fatty acid equivalents from PC to PE has been demonstrated (Aeberhaard, E. E. et al., 1978).

At very short incubation periods with fatty acid, smooth muscle cells seem to incorporate a large proportion of the exogenous fatty acid into the phosphoinositides. These data suggest that PC and PI form a labile pool which rapidly incorporates fatty acid. These fatty acid equivalents are subsequently transferred to other more biochemically stable lipids. Indeed, platelets labeled with [1-\(^{14}\)C]-5,8,11,14-20:4 when challenged with thrombin respond with a large decrease in PC and PI radioactivity (Bills, T. K. et al., 1976). This event is associated with the release of radiolabeled prostaglandins in the medium.

The results of the pulse experiments (Table 2) showed that there is a transfer of fatty acid equivalents from TG to PL. This is not the only explanation for the decrease in TG to PL ratio in pulsed cells. It might be that triglyceride is lost from the cell through oxidation to CO\(_2\) or by exchange with the serum. Experiments with strain L fibroblasts however have shown that lipid droplets and other cellular lipids are metabolically stable under certain conditions (Geyer, R. P., 1967). Pulse experiments with these cells also suggest that there is a transfer of
fatty acid equivalents from triglycerides to phospholipids.

In conclusion, these experiments show that smooth muscle cells behave towards exogenous fatty acid in a manner similar to other cell lines that have been studied previously. a-T had no effect on any of these processes. However, a-T has been shown to have profound effects on the proliferation of smooth muscle cells (Cornwell, D. G. et al, 1979). The effect of a-T on fatty acid metabolism in these cells must then be on a small pool that cannot be detected by the analytical methods described in this section. This small pool might be made up of hydroperoxy fatty acids that exert oxidant stress through formation of oxygen centered radicals.
POLYUNSATURATED FATTY ACID ACCUMULATION IN THE LIPIDS OF CULTURED FIBROBLASTS AND SMOOTH MUSCLE CELLS

Introduction

Triglycerides and cholesteryl esters accumulate as intracellular lipid droplets in several tissues (Bojesen, I. 1974; Brown, M. S. et al, 1979; Comai, K. et al, 1975; Cornwell, D. G. et al, 1975; Danon, A. et al, 1975; Geer, J. C. et al, 1970; Fowler, S. et al, 1980; Vahouny, G. V. et al, 1979). The triglyceride droplets in the renal medulla contain large amounts of 8,11,14-20:3 and 7,10,13,16-22:4 (Bojesen, I. 1974; Comai, K. et al, 1975; Danon, A. et al, 1975). The cholesteryl droplets in the adrenal and the fatty streak lesions of the aorta also contain large amounts of 8,11,14-20:3 and 7,10,13,16-22:4 (Cornwell, D. G. et al, 1975; Geer, J. C. et al, 1970; Vahouny, G. V. et al, 1979). These fatty acids are synthesized from 9,12-octadecadienoic acid (9,12-18:2) by a microsomal desaturation-chain elongation pathway (Sprecher, H. 1977). The microsome is also the site of the acyl CoA synthetase (Groot, P. H. E. et al, 1976) required in the microsomal biosynthesis of triglycerides and cholesteryl esters from acyl CoA intermediates. It has been proposed (Cornwell, D. G. et al, 1975) that this unusual fatty acid composition of triglyceride and cholesteryl ester droplets is characteristic of tissues in which microsomes are stimulated to shunt fatty acyl CoA intermediates into neutral lipid droplets.
In this section, results of experiments designed to test the hypothesis that fatty acyl-CoA intermediates are shunted into neutral lipid droplets during accelerated lipid accumulation are presented. Smooth muscle cells and fibroblasts in culture were challenged to accumulate triglyceride droplets by the addition of specific polyunsaturated fatty acids to the medium (Figure 18). Cellular lipids were then isolated and analyzed. If fatty acyl-CoA intermediates are shunted into lipid droplets, triglycerides should accumulate fatty acids characteristic both of microsomal desaturation-chain elongation (Sprecher, H., 1977) (Figure 18) and of extramicrosomal retroconversion (Kunau, W. H., 1977) (Figure 18). Furthermore, these fatty acyl groups should exchange into the phospholipid fraction without a net increase in the total fatty acid content of this fraction (Figure 18).

Fatty acid accumulation in the lipids of fibroblasts

Fibroblasts were treated with 6000 nmoles of 7,10,13,16-22:4 per culture and were examined by dark-field microscopy (Miller, J. S. et al, 1980). The cells contained lipid droplets in the perinuclear region of the cytoplasm. These lipid droplets were not as numerous as the lipid droplets found when smooth muscle cells were treated with the same amount of fatty acid (Miller, J. S. et al, 1980).

Twenty five per cent (1530 nmoles) of the 7,10,13,16-22:4 added to the medium of fibroblast cultures was taken up in cellular lipids as the fatty acid and its proximate derivatives (chain elongation and retroconversion products in Figure 18) (Table 5). Triglyceride, phospholipid and cholesteryl ester fractions all contained unusually
Figure 18. Outline of polyunsaturated fatty acid metabolism in cultured cells showing fatty acid accumulation (heavy arrow) in neutral lipid and fatty acid equilibration in phospholipid when cells are challenged by the addition of fatty acid to the medium. The microsomal desaturation-chain elongation and extramicrosomal retroconversion products are outlined for each fatty acid. Microsomal lipoxygenase and microsomal cyclooxygenase products of the fatty acids are shown as metabolites in the diagram.
Table 5. Effect of adrenic acid on the lipid composition of fibroblasts

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Triglyceride</th>
<th>Phospholipid</th>
<th>Cholesteryl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 2214</td>
<td>Control 2214</td>
<td>Control 2214</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids and conversion retroconversion products</td>
<td>6,9,12-18:3</td>
<td>3.0 ± 1</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>8,11,14-20:3</td>
<td>5.3 ± 0.6</td>
<td>20 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>5,8,11,14-20:4</td>
<td>8.7 ± 3.5</td>
<td>117 ± 28</td>
</tr>
<tr>
<td></td>
<td>7,10,13,16-22:4</td>
<td>7.0 ± 2.6</td>
<td>1030 ± 215</td>
</tr>
<tr>
<td></td>
<td>9,12,15,18-24:4</td>
<td>n.d.</td>
<td>35 ± 30</td>
</tr>
<tr>
<td>Other fatty acids</td>
<td>16:0</td>
<td>25 ± 16</td>
<td>92 ± 13</td>
</tr>
<tr>
<td></td>
<td>9-16:1</td>
<td>7.7 ± 3.5</td>
<td>28 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>18:0</td>
<td>15 ± 6.1</td>
<td>40 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>9-18:1</td>
<td>61 ± 16</td>
<td>164 ± 36</td>
</tr>
<tr>
<td></td>
<td>9,12-18:2</td>
<td>7.7 ± 3</td>
<td>30 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>7,10,13,16,19-22:5</td>
<td>22 ± 3.6</td>
<td>33 ± 14</td>
</tr>
<tr>
<td></td>
<td>4,7,10,13,16,19-22:6</td>
<td>5.7 ± 4.2</td>
<td>9 ± 3</td>
</tr>
</tbody>
</table>

*Cultures in confluency were incubated with fatty acid (6000 moles/culture) in media containing 10 per cent FBS for 24 hours.

Cultures (cells from 2 Petri dishes) contained 119 ± 20.7 ug DNA (13.8 pg DNA/cell).

Fatty acid not detectable (less than 1 mole/culture).

Data reported as mean ± s. d.

Fatty acid not detectable in other members of the experimental group.

Patty acid treatment group differed from control group at P 0.05* and P 0.005** levels.
large amounts of $7,10,13,16-22:4$ but only the triglyceride fraction increased significantly in total fatty acid content (Table 5). The polyunsaturated fatty acid and its proximate derivatives exchanged with other phospholipid fatty acids which then showed statistically significant decreases in concentration (Table 5). The fatty acids which decreased in the phospholipid fraction accumulated in significant amounts in the triglyceride fraction (Table 5).

Microsomal chain elongation was demonstrated by the appearance of $9,12,15,18-24:4$ in triglyceride, phospholipid and cholesteryl ester fractions (Table 5). Fibroblast microsomes contained very little $\Delta^4$-desaturase since $4,7,10,13,16-22:5$ was not found when cells were treated with $7,10,13,16-22:4$ (Table 5). Extramicrosomal retroconversion was shown by the statistically significant accumulation of $5,8,11,14-20:4$ in the triglyceride fraction (Table 5).

**Fatty acid accumulation in the lipids of smooth muscle cells**

Smooth muscle cells were treated with 6000 nmoles per culture of either $8,11,14-20:3$, $5,8,11,14-20:4$ or $7,10,13,16-22:4$. The mean fatty acid uptake for the three $9,12-18:2$ metabolites and their proximate fatty acid derivatives, $83 \pm 10$ per cent, was the same as the uptake of labeled $5,8,11,14-20:4$, $81 \pm 9$ per cent, obtained with smooth muscle cells in a previous study (Table 2). Fatty acid uptake in smooth muscle cells correlated with the increased number of lipid droplets in these cells compared to fibroblasts (Miller, J. S. *et al.*, 1980).

Microsomal chain elongation was demonstrated by the net accumulation (difference between treatment and control for the sum of fatty acid in triglyceride and phospholipid fractions) of $10,13,16-22:3$ (98 nmoles)
after treatment with 8,11,14-20:3, the net accumulation of 7,10,13,16-
22:4 (772 nmoles) after treatment with 5,8,11,14-20:4, and the net
accumulation of 9,12,15,18-24:4 (141 nmoles) after treatment with 7,10,13,
16-22:4 (Table 6). Smooth muscle cell microsomes had little desaturase
activity since 5,8,11,14-20:4 did not accumulate after treatment with
8,11,14-20:3 and 4,7,10,13,16-22:5 was not found after treatment with
7,10,13,16-22:4 (Table 6). Extramicrosomal retroconversion was confirmed
by the net accumulation of 6,9,12-18:3 (114 nmoles) after treatment with
8,11,14-20:3 and the net accumulation of 5,8,11,14-20:4 (392 nmoles)
after treatment with 7,10,13,16-22:4 (Table 6).

Smooth muscle cells, like fibroblasts, showed a significant
increase in the total fatty acid content of the neutral lipid or
triglyceride fraction (Table 6). The triglycerides were unusually rich in
the polyunsaturated fatty acid and its proximate derivatives (Table 6).
Argentation TLC separated a major triglyceride molecular species from
cells treated with 5,8,11,14-20:4 which was almost pure triarachidonin
since it contained 96.2 per cent 5,8,11,14-20:4 and 3.8 per cent 7,10,13,

The total fatty acid content of the phospholipid fraction did
not increase even though there were very large increases in the
polyunsaturated fatty acid and its proximate derivatives (Table 6). These
fatty acids accumulated in the phospholipid fraction at the expense of
other unsaturated fatty acids. Fatty acid uptake and distribution was
examined by treating each related polyunsaturated fatty acid (a 9,12-18:2
metabolite) individually and by calculating mean values for other unrelated
fatty acids (Table 6). This analysis showed that phospholipid 9-16:1,
Table 6. Effect of polyunsaturated fatty acids on the lipid composition of smooth muscle cells

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Triglyceride</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>8,11,14-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20:3</td>
</tr>
<tr>
<td>6,9,12-18:3</td>
<td>n.d.</td>
<td>98</td>
</tr>
<tr>
<td>8,11,14-20:3</td>
<td>8 ± 2</td>
<td>4560</td>
</tr>
<tr>
<td>5,8,11,14-20:4</td>
<td>20 ± 4</td>
<td>256</td>
</tr>
<tr>
<td>7,10,13,16-22:4</td>
<td>20 ± 4</td>
<td>78</td>
</tr>
<tr>
<td>9,12,15,18-24:6</td>
<td>n.d.</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
</tr>
<tr>
<td>9-16:1</td>
</tr>
<tr>
<td>18:1</td>
</tr>
<tr>
<td>9-18:1</td>
</tr>
<tr>
<td>9,12-18:2</td>
</tr>
<tr>
<td>7,10,13,16,19-22:5</td>
</tr>
<tr>
<td>4,7,10,13,16,19-22:6</td>
</tr>
</tbody>
</table>

| Total fatty acid | 607 ± 42 | 5880 ± 640 | 3410 ± 881 | 2760 ± 255 |

* Cultures in confluence were incubated with fatty acid (6000 n mole/culture) in media containing 20 per cent FBS for 24 hours (see Materials and Methods).
* Cultures (cells from 2 Petri dishes) contained 143 ± 31 µg DNA (10.8 µg DNA/cell).
* Fatty acid not detectable (less than 1 n mole/culture).
* Data reported as mean ± s.d.
* Fatty acid treatment group differed from control group at P 0.025* and P 0.01** levels.
9-18:1, 9,12-18:2, 7,10,13,16,19-22:5 and 4,7,10,13,16,19-22:6 all decreased significantly when cells were treated with polyunsaturated fatty acid (Table 6). These fatty acids all increased significantly in the triglyceride fraction (Table 6). The total amount of each fatty acid was conserved. Thus the amount of each fatty acid in a combined triglyceride-phospholipid fraction did not change when control and treatment groups were compared (Table 7). Finally, the cholesteryl ester fraction in smooth muscle cells was too small for fatty acid analysis.

Discussion

The lipid composition of cells in tissue culture depends both on the composition of the growth medium and the cell line. Human fibroblasts cultured in Experimental Medium containing 20 per cent fetal bovine serum (Gavino, V. C. et al, 1981) contained twice as much triglyceride and cholesteryl ester as fibroblasts cultured in Growth Medium containing 10 per cent fetal bovine serum (Table 5). The increase in neutral lipid undoubtedly reflects the additional free fatty acid, calculated as 700 nmoles from free fatty acid data (Cornwell, D. G. et al, 1979), supplied when 20 per cent fetal bovine serum is used in place of 10 per cent fetal bovine serum.

Human fibroblasts contain much less triglyceride and phospholipid than guinea pig smooth muscle cells (Tables 5 and 6). Total lipid values per cell for the two cell lines are compared in Table 8. Fibroblasts which are challenged with a polyunsaturated fatty acid take up a much smaller amount of this fatty acid as triglyceride than smooth muscle cells (Table 8). Nevertheless, fibroblasts and smooth muscle cells both show a
Table 7. General conservation of unsaturated fatty acid\(^a\) in smooth muscle cells treated with specific polyunsaturated fatty acids

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>Treatment(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/culture</td>
<td></td>
</tr>
<tr>
<td>9-16:1</td>
<td>120 ± 16(^c)</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>9-18:1</td>
<td>1300 ± 330</td>
<td>1100 ± 87</td>
</tr>
<tr>
<td>9,12-18:2</td>
<td>190 ± 42</td>
<td>180 ± 19</td>
</tr>
<tr>
<td>7,10,13,16,19-22:5</td>
<td>200 ± 32</td>
<td>170 ± 13</td>
</tr>
<tr>
<td>4,7,10,13,16,19-22:6</td>
<td>170 ± 30</td>
<td>120 ± 22</td>
</tr>
</tbody>
</table>

\(^a\)Sum of fatty acid in triglyceride and phospholipid fractions (see Table 6).

\(^b\)Cells treated with 6000 nmoles polyunsaturated fatty acid per culture.

\(^c\)Mean ± s. d. Control and treatment groups did not differ significantly.
Table 8. The amount of lipid per cell for fibroblasts and smooth muscle cells in control and treatment cell populations

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Triglyceride</th>
<th>Phospholipid</th>
<th>Cholesteryl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles fatty acid/10^6 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblasts^a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>193</td>
<td>8</td>
</tr>
<tr>
<td>Treatment</td>
<td>179</td>
<td>154</td>
<td>14</td>
</tr>
<tr>
<td>Smooth muscle cells^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>259</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>443</td>
<td>205</td>
<td></td>
</tr>
</tbody>
</table>

^a Calculated from data in Table 9.
^b Calculated from data in Table 7.
marked increase in lipid droplets and the accumulation of neutral lipid when they are challenged with polyunsaturated fatty acids. These results confirm a number of studies which have shown that triglycerides accumulate when cells are challenged with excess free fatty acid (Miller, J. S. et al., 1980; Moskowitz, M. S., 1967; Schneeberger, E. E. et al., 1971; Spector, A. A. et al., 1979).

In the present investigation, we show that a polyunsaturated fatty acid added to the medium, its microsomal chain elongation products, and its extramicrosomal retroconversion products all accumulate in cellular phospholipids and cellular triglycerides. These fatty acids accumulate in phospholipids by exchanging with fatty acyl groups already present in this lipid fraction. These fatty acids accumulate in triglycerides through a net increase in the concentration of this lipid fraction. The data confirm classic lipid studies (Terroine, E. F. and P. Belin, 1927) which characterized cellular phospholipids as the élément constant and cellular triglycerides as the élément variable. The data support the hypothesis that microsomal fatty acyl-CoA intermediates are shunted into neutral lipids which are unusually rich in chain elongation and retroconversion products such as 8,11,14-20:3 and 7,10,13,16-22:4 when excess lipid stimulates neutral lipid accumulation (Figure 18). These studies show that precursor shunting into neutral lipid droplets may control fatty acid availability for the microsomal biosynthesis of hydroperoxides (lipoxygenase pathway) and endoperoxides (cyclooxygenase pathway).
Introduction

The experiments that led to the hypothesis that oxidant stress plays a role in the control of cell proliferation was reviewed earlier (pp. 27-30). Experiments by other investigators that showed the association of vitamin E and other antioxidants with rapidly proliferating cells was also reviewed in the same section. In this section, the relationship between lipid peroxidation and cell proliferation is further examined. This section contains results of experiments performed by Dr. James S. Miller (Miller, J. S., 1980) that are pertinent to the discussion. These experiments are so identified when presented.

Lipid peroxidation

Malondialdehyde (MDA), a product of both lipid peroxidation (Wilbur, K. M. et al, 1949) and of prostaglandin biosynthesis (Flower, R. J. et al, 1973), was formed when smooth muscle cells were incubated with either 8,11,14-20:3, 5,8,11,14-20:4 or 7,10,13,16-22:4 (Table 9). The incubation was repeated in the presence of sufficient indomethacin to block prostaglandin biosynthesis in a smooth muscle cell culture (Huttner, J. J. et al, 1977b). More MDA was formed when 8,11,14-20:3 and 5,8,11,14-20:4 were incubated with indomethacin than without
Table 9. Lipid peroxidation in confluent smooth muscle cells incubated for 52 hours with a polyunsaturated fatty acid, with or without indomethacin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles MDA/culture</td>
<td></td>
</tr>
<tr>
<td>8,11,14-20:3 (120 μM)</td>
<td>9.9 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.1 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5,8,11,14-20:4 (120 μM)</td>
<td>9.9 ± 0.4</td>
<td>14.5 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7,10,13,16-22:4 (120 μM)</td>
<td>11.1 ± 1.6</td>
<td>11.4 ± 3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data reported as mean ± s. d.
<sup>b</sup>Significant difference (p<0.05) from incubation with fatty acid alone.
<sup>c</sup>Significant difference (p<0.005) from incubation with fatty acid alone.

Indomethacin. Indomethacin had no effect on MDA formation in cells treated with 7,10,13,16-22:4.

MDA formation was time dependent in both fibroblast and smooth muscle cell cultures. Fibroblasts (Table 10) generated small amounts of MDA at 6 hours. The MDA content of fibroblast cultures increased from 24 to 52 hours. Smooth muscle cells (Table 11) generated more MDA from 8,11,14-20:3 than fibroblasts (Table 10) at every time interval studied (p<0.005). The MDA in smooth muscle cell cultures reached a maximum in 24 hours and did not change when the incubation period was extended to 52 hours. These data are consistent with fatty acid uptake data (see section on POLYUNSATURATED FATTY ACID ACCUMULATION IN THE LIPIDS OF CULTURED FIBROBLASTS AND SMOOTH MUSCLE CELLS, pp. 99-100) which showed that confluent smooth muscle cells accumulated 83 per cent of added
Table 10. Lipid peroxidation in confluent fibroblasts incubated for specified times with a polyunsaturated fatty acid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (hrs)</th>
<th>nmoles MDA/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>8,11,14-20:3 (120 µM)</td>
<td>0</td>
<td>0.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5,8,11,14-20:4 (120 µM)</td>
<td>0</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>7,10,13,16-22:4 (120 µM)</td>
<td>0</td>
<td>0.9 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data reported as mean ± s. d.

120 µM fatty acid in 24 hours while confluent fibroblasts accumulated only 25 per cent of added 120 µM fatty acid in 24 hours.

α-T and α-TQ have been shown to stimulate cell proliferation and overcome the inhibitory effect of polyunsaturated fatty acids on cell proliferation (Miller, J. S., 1980). MDA formation was significantly inhibited at 24 and 52 hours when 8,11,14-20:3 and either α-T or α-TQ were added at the same time to the incubation mixture (Table 11). MDA formation at 6 hours was not changed significantly by either α-T or α-TQ that was added at the same time as the fatty acid (Table 11).

Menadione has been shown to inhibit cell proliferation at greater than 1 µM concentrations (Miller, J. S., 1980). This quinone had no effect on the formation of MDA from 8,11,14-20:3 even at 52 hours (Table 11).

The initial studies with α-T and α-TQ showed that these compounds had little effect on MDA formation at early incubation times (Table 11).
Table 11. Lipid peroxidation in confluent smooth muscle cells incubated for specified times with 8,11,14-20:3 with or without the concurrent addition of α-T, α-TQ, or menadione

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (hrs)</th>
<th>nmoles MDA/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>8,11,14-20:3 (120 μM)</td>
<td>0.7 ± 0.3a</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>&quot; + α-T (10 μM)</td>
<td>0.4 ± 0.6</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>&quot; + α-TQ (10 μM)</td>
<td>0.8 ± 0.1</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>&quot; + menadione (10 μM)</td>
<td>0.5 ± 0.3</td>
<td>3.8 ± 1.2</td>
</tr>
</tbody>
</table>

aData reported as mean ± s. d.

bSignificant difference (p<0.005) from incubation with fatty acid alone.
These observations suggested that the cellular uptake of the antioxidant was not rapid enough for inhibition at early incubation times. Smooth muscle cells were, therefore, preincubated for 24 hours with media containing α-T or α-TQ. 8,11,14-20:3 was then dissolved in alcohol and added to the preincubated media. MDA formation was inhibited at 2 and 6 hours when cultures were treated in this way (Table 12). It is interesting that more MDA was formed when the fatty acid was added to preincubated media (control in Table 12) than when the fatty acid was added with fresh media (control in Table 11).

Recent studies (Pryor, W. A. et al, 1976; Svingen, B. A. et al, 1979) on the mechanism of lipid peroxidation suggest that the peroxidation reaction occurs in two sequential steps, initiation (formation of peroxy radicals or lipid hydroperoxides) and propagation (breakdown of lipid hydroperoxides or cyclic endoperoxides to reactive intermediates). BHT inhibits both initiation and propagation steps (Svingen, B. A. et al, 1979). It was attempted to separate these steps of the peroxidation reaction by preincubating smooth muscle cells with 8,11,14-20:3 for 24 hours then adding an antioxidant and continuing the incubation to 52 hours (Table 13). In preliminary experiments, BHT had no effect on absorbance when it was added directly to MDA in the generation of a standard curve. However, BHT had an immediate effect on MDA formation when BHT was added to the incubation systems (Table 13). Neither α-T nor α-TQ had an immediate effect on MDA formation (Table 13). These compounds both inhibited MDA formation when incubations were continued for an additional 28 hours (Table 13). α-TQ was a more effective inhibitor than α-T in this system (p<0.005).
Table 12. Lipid peroxidation in confluent smooth muscle cells preincubated for 24 hours with either α-T or α-TQ and then incubated for specified times with 8,11,14-20:3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (hrs)</th>
<th>2</th>
<th>6</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,11,14-20:3 (120 μM)</td>
<td>2.6 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3 ± 0.4</td>
<td>9.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>&quot; + α-T (10 μM)</td>
<td>0.6 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>&quot; + α-TQ (10 μM)</td>
<td>0.2 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Data reported as mean ± s. d.

<sup>b</sup>Significant difference (p<0.05) from incubation with fatty acid alone.

<sup>c</sup>Significant difference (p<0.005) from incubation with fatty acid alone.
Table 13. Lipid peroxidation in confluent smooth muscle cells preincubated for 24 hours with 8,11,14-20:3 and then incubated for specified times with either α-T or α-TQ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (hrs)</th>
<th>nmoles MDA/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,11,14-20:3 (120 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; + α-T (10 μM)</td>
<td>10.5 ± 0.8</td>
<td>9.9 ± 1.8</td>
</tr>
<tr>
<td>&quot; + α-TQ (10 μM)</td>
<td>11.3 ± 1.5</td>
<td>7.5 ± 0.8</td>
</tr>
<tr>
<td>&quot; + BHT (10 μM)</td>
<td>10.8 ± 0.5</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1.9 ± 0.2</td>
<td>1.5 ± 0.5</td>
</tr>
</tbody>
</table>

aIncubation times with antioxidant represent 24 and 52 hour incubation times with fatty acid.
bData reported as mean ± s. d.
cSignificant difference (p<0.01) from incubation with fatty acid alone.
dSignificant difference (p<0.005) from incubation with fatty acid alone.

Discussion

The studies described in this section are consistent with our hypothesis that cell proliferation is controlled in part by oxidant stress supplied through lipid peroxidation (Cornwell, D. G. et al., 1979; Cornwell, D. G. and R. V. Panganamala, 1980; Miller, J. S. et al., 1980). The polyunsaturated fatty acids that inhibit cell proliferation are peroxidized (generate MDA) when they are incubated with smooth muscle cells (Table 9) and fibroblasts (Table 10). Furthermore, the fatty acids that function as good substrates for both cyclooxygenase and lipoxygenase, 8,11,14-20:3 and 5,8,11,14-20:4 generate more MDA when they are incubated
with a cyclooxygenase inhibitor (Table 9). The fatty acid that inhibits
cyclooxygenase (Cagen, L. M. and P. G. Baer, 1980), 7,10,13,16-22:4,
is unaffected by this treatment (Table 9). Diminished growth has been
found in tumors treated with indomethacin (Hial, V. et al., 1976). The
MDA data reported in Table 9 show that this effect could reflect the
accumulation of lipid peroxides through precursor shunting from the
cyclooxygenase to the lipoxygenase pathway.

Many previous studies have related vitamin E and other anti-
oxidant levels to cell proliferation (Baumgartner, W.A. et al., 1978;
Corwin, L. M. and J. Shloss, 1980; Diplock, A. T. and A. S. M. Giasuddin,
Other studies have correlated lipid peroxidation with diminished cell
proliferation (Apffel, C. A., 1978; Burlakova, E. B., 1967; Bernheim, F.,
1963; Bartoli, G. M. and T. Galeotti, 1979; Miquel, J. et al., 1977;
Player, T. J. et al., 1977; Utsumi, K. et al., 1970; Wolfson, N. et al.,
1956). The tissue culture experiments described here strongly support
a direct relationship between antioxidants, lipid peroxidation and cell
proliferation. α-T and α-TQ was found to stimulate cell proliferation
and overcome the inhibitory effect of polyunsaturated fatty acids on
cell proliferation (Miller, J. S., 1980). These closely related compounds
also diminish MDA formation in cells challenged with a polyunsaturated
fatty acid (Tables 11 and 12).

Intracellular lipid peroxidation may occur either through the
lipoxygenase pathway or the NADPH-linked pathway (Slater, T. F., 1979).
The antioxidant data presented in this section support the lipoxygenase
pathway. α-T, a compound that inhibits both plant (Panganamala, R. V.
et al, 1977) and mammalian (Gwobu, E. T. et al, 1980) lipoxygenase, stimulates cell proliferation (Miller, J. S., 1980) and inhibits lipid peroxidation (Tables 11 and 12). NADPH-linked lipid peroxidation is not inhibited by a 1,4-benzoquinone (Slater, T. F., 1979) yet α-TQ is more effective than α-T in inhibiting lipid peroxidation (Tables 11-13). NADPH-linked lipid peroxidation is inhibited by a 1,4-naphthoquinone (Slater, T. F., 1979) yet menadione does not stimulate cell proliferation (Miller, J. S., 1980) or inhibit MDA formation (Table 11).

Antioxidants may affect both the initiation step (formation of peroxyl radicals or lipid hydroperoxides) and the propagation step (breakdown of lipid hydroperoxides or cyclic endoperoxides to reactive intermediates) in lipid peroxidation (Brownlee, N. R. et al, 1977; Mead, J. F., 1976; Pryor, W. A., 1976; Pryor, W. A. et al, 1976; Slater, T. F., 1979; Svingen, B. A. et al, 1979; Witting, L. A., 1980). Several observations suggest that antioxidants act at both steps in the tissue culture system described in this section. BHT has an immediate effect on MDA formation in cells that are preincubated with a polyunsaturated fatty acid (Table 13). These data are most readily explained by inhibition at a propagation step involving an MDA precursor which may be cyclic endoperoxide (Pryor, W. A. et al, 1976) (eqn. 32).

\[ \text{Precursor} \xrightarrow{\text{BHT}} \text{MDA} \]

(32)

α-TQ functions as effectively as BHT but only after an incubation period (Table 13). α-T does not function as effectively as α-TQ when cells are preincubated with fatty acid (Table 13). However, α-T and α-TQ
are equally effective when they are added before or at the same time as the fatty acid (Tables 11 and 12). These data are consistent with the suggestion (Mead, J. F., 1976; Witting, L. A., 1980) that a-T inhibits the initiation step in lipid peroxidation (eqn. 33).

\[
\begin{align*}
\text{Fatty acid} & \xrightarrow{a-T} \text{Peroxy radical or Hydroperoxide} \\
(33)
\end{align*}
\]

Although lipid peroxidation has a direct effect on the properties of synthetic bilayers and monolayers (Van Zutphen, H. and D. G. Cornwell, 1973), there is increasing evidence that events in biological systems such as hemolysis are not the direct result of the peroxidation of membrane lipids (Brownlee, N. R. et al., 1977). The concept is developing (Recknagel, R. O. et al., 1977) that the breakdown of relatively stable lipid peroxides may explain how oxidant injury occurs through, "action at a distance". Antioxidant studies in cultures preincubated with a fatty acid (Table 13) provide evidence for the accumulation of relatively stable peroxides which decompose rapidly to form MDA in the thiobarbituric acid assay.

Several studies have shown that 1,4-benzoquinones such as ubiquinone-4 (Folkers, K., 1974; Smith, J. L., 1964) and a-TQ (Epstein, T. and D. Gershon, 1972) possess vitamin E activity. These observations have been confirmed and extended by showing that a-TQ was more effective than a-T in stimulating cell proliferation (Miller, J. S., 1980) and inhibiting MDA formation (Tables 11-13). The question of why a-TQ possesses vitamin E activity will be addressed in the following section.
It has been demonstrated previously however, that the hydroquinone of α-TQ, (α-TQH₂) has vitamin E activity (Mackenzie, J. B. et al., 1950; Mauer, S. I. and K. E. Mason, 1975; Rao, G. H. and K. E. Mason, 1975). Recent studies on biohydrogenation show that α-TQ participates in a quinone-hydroquinone redox cycle (Hughes, P. E. and S. B. Tove, 1980b) and that liver mitochondria are able to synthesize α-TQH₂ (Hughes, P. E. and S. B. Tove, 1980a). It is possible that the vitamin E like activity of α-TQ is due to its conversion to the hydroquinone or semiquinone in the body.
THE ANTIOXIDANT PROPERTIES OF \(\alpha\)-T, \(\alpha\)-TQ AND BHT

Introduction

The antioxidant properties of \(\alpha\)-T and BHT are well known. \(\alpha\)-TQ, an oxidation product of \(\alpha\)-T (Svanholm, U. et al., 1974), can substitute for \(\alpha\)-T in the treatment of certain vitamin E deficiency diseases (for review, see pp. 25-27 and pp. 108-111). This section presents results of experiments designed to test whether \(\alpha\)-TQ, like \(\alpha\)-T and BHT, can also act as an antioxidant in vitro.

The antioxidant properties of \(\alpha\)-T, \(\alpha\)-TQ and BHT in a non-aqueous system

The ability of \(\alpha\)-T, \(\alpha\)-TQ and BHT to interrupt a free radical reaction involving the AIBN-initiated formation of hydroperoxide or peroxide from cumene or from 8,11,14-20:3 was tested (Arneson, R. M. et al., 1978) and the results are presented in Table 14. As discussed earlier (pp. 54-58), AIBN undergoes thermal homolysis to give cyano-propyl radicals which initiate the formation of hydroperoxides from cumene or from 8,11,14-20:3, processes which by themselves are also mediated by free radicals. The hydroperoxides formed during the course of the reaction are assayed by their ability to oxidize \(\text{Fe}^{2+}\) to \(\text{Fe}^{3+}\). The \(\text{Fe}^{3+}\) is estimated by the absorbance of its isothiocyanate complex at 490 nm. Over a 30 minute incubation period, the formation of hydroperoxides was linear with respect to time (Figure 19). Antioxidants inhibited
Table 14. The effect of α-T, α-TQ and BHT on the AIBN-initiated generation of hydroperoxide from cumene or 8,11,14-20:3

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Nanomoles test compound per ml</th>
<th>12</th>
<th>60</th>
<th>120</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rate of formation of fatty acid hydroperoxide expressed as per cent of control&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-T</td>
<td></td>
<td>76.85</td>
<td>74.60</td>
<td>50.61</td>
<td>54.54</td>
</tr>
<tr>
<td>α-TQ</td>
<td></td>
<td>116.102</td>
<td>105 ± 9</td>
<td>92.109</td>
<td>95.99</td>
</tr>
<tr>
<td>BHT</td>
<td></td>
<td>116.98</td>
<td>109.82</td>
<td>78.62</td>
<td>49.48</td>
</tr>
<tr>
<td></td>
<td>rate of formation of cumene hydroperoxide expressed as per cent of control&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-T</td>
<td></td>
<td>58</td>
<td>34</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>α-TQ</td>
<td></td>
<td>108</td>
<td>108</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>BHT</td>
<td></td>
<td>98</td>
<td>82</td>
<td>39</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup>The reaction mixture contained 500 nmoles AIBN, 58 µmoles cumene or 333 nmoles 8,11,14-20:3 in a total volume of 0.25 ml benzene.

<sup>b</sup>The formation of hydroperoxide was linear up to 30 minutes from the start of the reaction.
Figure 19. Time course for the AIBN-initiated formation of cumene hydroperoxide from cumene. Each reaction mixture contained 500 nmoles AIBN and 58 μmoles cumene (circles) or 58 μmoles cumene plus 60 nmoles α-T (triangles) in a total volume of 0.25 ml benzene.
the reaction (Figure 19). Reaction velocities were calculated from the slopes of the lines. The reaction velocity for AIBN and cumene or fatty acid alone was taken as the control. The reaction velocity for a system with added antioxidant was expressed as per cent of the control and listed in Table 14. Both a-T and BHT were effective in inhibiting the formation of hydroperoxide although a-T appeared to be more effective than BHT at low concentrations. a-TQ had no effect on the formation of hydroperoxides. Thus a-TQ was not an antioxidant in this system.

The antioxidant properties of a-T, a-TQ and BHT in an aqueous medium

The results of experiments designed to test the antioxidant properties of a-T, a-TQ and BHT in an aqueous medium are presented in Table 15. The reaction between CHP and 5,8,11-18:3 results in the formation of a minimal amount of MDA (Table 15). MDA is believed to arise from an MDA precursor under the conditions of the thiobarbituric acid test (Pryor, W. A. and J. P. Stanley, 1975; Pryor, W.A. et al, 1976). The same investigators have shown that the MDA precursor is formed from peroxidizing fatty acids with at least 3 methylene interrupted double bonds. The peroxidation of fatty acids is a free radical process (Pryor, W. A., 1966). In this system, a-T, a-TQ and BHT all inhibited the formation of MDA from 5,8,11-18:3 (Table 15) and are thus all antioxidants. Again, a-T seems to be more effective than either BHT or a-TQ at low concentrations (Table 15).

Discussion

In many instances, a-TQ mimics a-T effects in biological systems. Like a-T, it has been found to reverse the dystrophy caused
Table 15. Effect of α-T, α-TQ and BHT on the CHP initiated MDA production from 5,8,11-18:3

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Concentration of test compound (nmoles/ml)</th>
<th>nmoles MDA/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Control</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>α-T</td>
<td>0.33 ± 0.03</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>α-TQ</td>
<td>0.56 ± 0.017</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>BHT</td>
<td>0.46 ± 0.10</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 880 nmoles 5,8,11-18:3, 250 nmoles FeCl₃ and antioxidant in 1.0 ml 0.1 M phosphate buffer pH 7.4. To this was added 100 μl CHP solution (see Materials and Methods).

Antioxidants were added from ethanolic 3 mM stock solutions. The control had corresponding amounts of ethanol. Ethanol by itself did not affect MDA production.

Results are reported as mean ± s. d. (n= number of experimental units).

by vitamin E deficiency in rabbits (Mackenzie, J. B. et al, 1950), decrease lipofuscin pigment accumulation and increase the lifespan of nematodes (Epstein, T. and D. Gershon, 1977), stimulate cell proliferation (Miller, J. S., 1980) and diminish lipid peroxidation (Tables 9-13) in smooth muscle cells and fibroblasts in culture. It has been proposed that α-TQ is reduced to the hydroquinone in the body (Chow, C. K. et al, 1967) which then, like α-T, acts as an antioxidant and gives vitamin E like effects.
The formation of $\alpha$-TQH$_2$ from $\alpha$-TQ was in certain cases shown to occur in living systems (Hughes, P. E. and S. B. Tove, 1980a, 1980b). Results presented in this section show that this process does not have to be enzyme mediated (Table 15). Apparently, under the conditions listed in Table 15, $\alpha$-TQ is converted into a species capable of interrupting free radical mediated reactions. This does not occur under non-aqueous conditions as shown in Table 14.

The Fenton reaction (eqn. 34) has been known for many years to be a source of a highly reactive oxygen centered radicals. This would be either the hydroxyl radical or a metal-oxo compound with properties similar to the hydroxyl radical (Fee, J. A. and J. S. Valentine, 1977). This oxygen centered radical is represented by "OH" in eqns 34 and 35.

The Fenton reaction and eqns 35 to 38 have been invoked to explain the iron catalysis of H$_2$O$_2$ decomposition (Fee, J. A., 1980).

\[
\begin{align*}
\text{Fe}^{+2} + \text{H}_2\text{O}_2 & \xrightleftharpoons[H^+] \text{"OH"} + \text{Fe}^{+3} + \text{H}_2\text{O} \quad (34) \\
\text{"OH"} + \text{H}_2\text{O}_2 & \rightarrow \text{O}_2^- + \text{H}_2\text{O} + \text{H}^+ \quad (35) \\
\text{Fe}^{+3} + \text{O}_2^- & \rightarrow \text{Fe}^{+2} + \text{O}_2 \quad (36) \\
\text{Fe}^{+3} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{+2} + 2\text{H}^+ + \text{O}_2^- \quad (37) \\
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (38)
\end{align*}
\]

Equation 37 shows the production of superoxide from H$_2$O$_2$. This reaction has been described elsewhere (Pryor, W. A., 1976) and has been

\( \text{O}_2^- \) has a heat of solution that is close to -100 kcal/mole (Fee, J. A. and J. S. Valentine, 1977) and therefore under the conditions described in Table 14, \( \text{O}_2^- \) would be strongly solvated and might be expected to be a weak nucleophile, its nucleophilicity being similar to \( \text{F}^- \) in aqueous solution (Fee, J. A., 1980). One might envision, therefore, a reaction between CHP and \( \text{Fe}^{+3} \) at 98°C (aqueous) producing \( \text{Fe}^{+2} \), \( \text{O}_2^- \) and cumene alcohol (2-hydroxy-2-phenylpropane). As part of the driving force of the reaction, \( \text{O}_2^- \) might be oxidized to \( \text{O}_2 \) by \( \text{Fe}^{+3} \) (eqn 36) or by a quinone if it is present (eqn, 39). The rate constant for the

\[
\text{Q} + \text{O}_2^- \xrightarrow{H^+} \text{QH}^+ + \text{O}_2 \tag{39}
\]

reaction between duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) and \( \text{O}_2^- \) has been estimated to be \( 5 \times 10^6 \) \( \text{M}^{-1}\text{sec}^{-1} \) (Fee, J. A. and J. S. Valentine, 1977). One might expect \( \alpha\text{-TQ} \) to have a similar rate constant for its reaction with \( \text{O}_2^- \).

A series of reactions leading to the production of MDA might be envisioned for the experiment described in Table 15. It is apparent

\[
\text{Fe}^{+3} + \text{CHP} \xrightarrow{\text{H}_2\text{O}} \text{Fe}^{+2} + \text{O}_2^- + \text{cumene alcohol} \tag{40}
\]

\[
\text{Fe}^{+3} + \text{O}_2^- \rightarrow \text{Fe}^{+2} + \text{O}_2 \tag{36}
\]

\[
\text{Fe}^{+2} + \text{CHP} \rightarrow \text{Fe}^{+3} + "\text{OH}" + \text{cumene alcohol} \tag{41}
\]
"OH" + FFA $\rightarrow$ peroxides and hydroperoxides \hspace{1cm} (42)

peroxides and hydroperoxides $\rightarrow$ $\rightarrow$ MDA \hspace{1cm} (43)

from eqns 36, 40-43 that the peroxidation of FFA under the conditions defined in Table 15 is dependent on the reduction of Fe$^{3+}$ to Fe$^{2+}$. Fe$^{3+}$ is reduced to Fe$^{2+}$ by CHP (eqn 40) or by O$_2^-$ produced from CHP (eqn 36). Aqueous O$_2^-$ itself is stable relative to most organic compounds (Fee, J.A., 1980) and its role in peroxidation is linked to its ability to reduce transition metal ions. The transition metal ions would then interact with peroxides to give extremely reactive hydroxy radicals (Fee, J.A., 1980).

An antioxidant will cause the net disappearance of a chemical species that contributes to the propagation of the free radical mediated reaction (Pryor, W.A., 1976). It is possible that α-TQ might react with O$_2^-$ to produce the semiquinone α-TQH* thus taking away one chemical species, O$_2^-$, that contributes to the propagation of the reaction. α-TQH* might trap "OH" by donating a hydrogen atom thus destroying another chemical species that contributes to the propagation.

The α-TQ/α-TQH* redox pair may be responsible for the antioxidant activity of α-TQ in aqueous systems. In non-aqueous systems, the redox potential for the O$_2$/O$_2^-$ couple is so low that O$_2^-$ is probably not formed (Fee, J.A. and J.S. Valentine, 1977). α-TQ does not act as an antioxidant in non-aqueous systems (Table 14). In biological systems, α-TQ has vitamin E activity probably because of its reduction to the
semiquinone or the hydroquinone. This process does not have to be enzyme mediated as shown in Table 15. It is interesting to note that α-TQ exists mainly in the form of the semiquinone in liver microsomes (Hughes, P. E. and S. B. Tove, 1980a).
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