This is to certify that the

thesis entitled

Photooxidation of Cholesterol in Butter

presented by

Julie Marie Luby

has been accepted towards fulfillment
of the requirements for

Masters degree in Food Science

J. I. Gray
Major professor

Date 07/03/82

PHOTOOXIDATION OF CHOLESTEROL IN BUTTER

By

Julie Marie Luby

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1982

ABSTRACT

Photooxidation of Cholesterol in Butter

By

Julie Marie Luby

The effect of exposure to fluorescent light on the oxidative stability of cholesterol in fatty acid solutions and in butter was investigated. Aeration and greater unsaturation of the media accelerated cholesterol oxidation in fatty acid systems. Photooxidation of cholesterol in butter occurred, with known oxidation products detectable by thin layer chromatographic analysis after eight days of illumination. This oxidation appeared to occur via singlet oxygen attack as well as by free radical mechanisms to form 5-cholesten-3β,7α-diol, 5-cholesten-3β,7β-diol and possibly 6-cholesten-3β,5α-diol, as well as other unidentified compounds by day twenty. Packaging materials greatly influenced cholesterol stability in butter during illumination and this effect approximately paralleled their light barrier properties. Of the materials tested, only aluminum foil prevented cholesterol photooxidation after fifteen days. At ambient temperatures, exposure to daylight caused more oxidation of butter lipids, including cholesterol, than did exposure to roomlight.

To my family, Pat and Peg Luby, Jim, Mary Pat, and Bob Luby,
and, of course, Bill Orabone, Jr.

ACKNOWLEDGEMENTS

My sincere gratitude is extended to Dr. J.I. Gray for his guidance, input, and understanding as research chairman and major professor. The freedom granted to me during this study was very much appreciated.

The members of my guidance committee, Drs. P. Markakis, B.R. Harte, and L.R. Dugan, Jr. also deserve my appreciation for their critical review of this thesis.

My special thanks are extended to Tom Ryan for trailblazing in the area of cholesterol oxidation, to Susan Cuppett for technical assistance, and to Dr. B.R. Harte for help in obtaining packaging materials.

Further acknowledgements are also due to Ralston Purina Company and the Institute of Food Technologists for financial assistance during this study.

This degree would not have been so smoothly completed had it not been for the friendship, support, and comic relief given to me by Diane Fischer, Tom Custer, Sally Gates Phillips, Nancy King Theis, and all my friends from lab 311.

I am especially grateful to my family for all of the love, encouragement, and understanding they have shown me in all of my endeavors, and especially for bestowing on me a sense of humor.

And finally, my heartfelt appreciation is extended to Bill Orabone Jr. for his love, patience and sacrifices made during the course of my work at Michigan State University.

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INTRODUCTION

In recent years, there has been renewed interest in the role of cholesterol in diseases such as atherosclerosis and cancer. Increasing evidence seems to implicate cholesterol oxides specifically, rather than pure cholesterol, as the angiotoxic or carcinogenic agents (Taylor et al., 1979).

Cholesterol oxides may form when a system containing cholesterol is exposed to conditions such as heat or radiation (ionizing or non-ionizing). Because of the potential danger in ingesting these compounds, several researchers have stressed the need to investigate changes which cholesterol may undergo during processing and storage of foods (Kummerow, 1979; Taylor et al., 1979; Sheppard and Shen, 1980). To date, very little information is available on the photooxidation or autoxidation of cholesterol in foods (Smith, 1980).

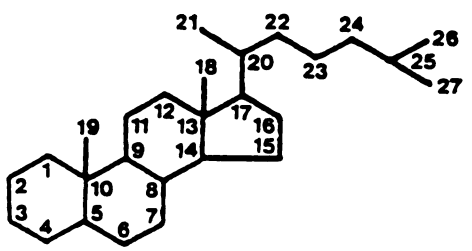
Animal products exposed to heat and/or light may be susceptible to cholesterol oxidation. It is conceivable that butter (0.2% cholesterol) stored in a fluorescently illuminated display case may be especially susceptible.

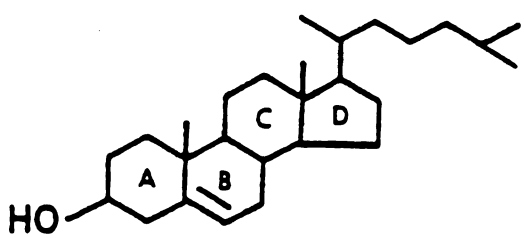
The major objective of this study was to determine if photooxidation of cholesterol occurs in butter and whether these changes take place under conditions to which butter is likely to be exposed during production and distribution. Secondary objectives include the determination of the mode of oxidation and identification of cholesterol oxidation products, as well as determination of the effect of various packaging materials in minimizing oxidation during illumination of butter.

REVIEW OF LITERATURE

Description of Cholesterol

Cholesterol (5-cholesten-3β-ol) is a relatively non-polar simple lipid with a chemical formula of $C_{27}H_{46}O$. The compound as shown below consists of a cyclopentanophenanthrene ring structure with an eight carbon side chain at C_7, angular methyl groups at the C_{10} and C_{13} positions, a double bond at the C_5-C_6 position and a hydroxyl group at C_3.





Cholesterol is widely distributed in animal tissues, with the highest concentrations found in the brain and nervous tissue. Cholesterol functions as a structural component in membranes, in the transport of blood lipids, and as a precursor to fecal sterols, bile acids, and steroid hormones.

Although cholesterol is mainly associated with animal tissues, it has also been detected in palm kernel oil and palm oil (Punwar and Derse, 1978).

Mechanism of Lipid Autoxidation

The reaction of unsaturated lipids with oxygen constitutes the major means by which lipids deteriorate, and unless it is mediated by other oxidants or enzyme systems, this oxidation proceeds via a free-radical chain reaction mechanism (Dugan, 1976). The reaction is often referred to as autoxidation due to its apparent self-catalytic properties.

Autoxidation of lipids occurs by oxygen attack and subsequent decomposition via the following steps (Farmer et al., 1942):

1. Initiation

$$RH + O_2 \longrightarrow R\cdot + \cdot OOH$$

2. Propagation

$$R\cdot + O_2 \longrightarrow ROO\cdot$$

$$RH + ROO\cdot \longrightarrow ROOH + R\cdot$$

$$ROOH \longrightarrow RO\cdot + \cdot OH$$

3. Termination

$$R\cdot + R\cdot \longrightarrow RR$$

$$R\cdot + ROO\cdot \longrightarrow ROOR$$

$$ROO\cdot + ROO\cdot \longrightarrow ROOR + O_2$$

RH refers to any unsaturated lipid in which the H is labile by reason of being on a carbon adjacent to a double bond. R· refers to a free radical formed by removal of a labile hydrogen. Hydroperoxides are the major initial autoxidation products and on further decomposition which may include bond scissioning, many oxygenated compounds and hydrocarbons may be formed.

After initiation of autoxidation in a lipid system, rapid uptake of oxygen will occur, as will an increase in the peroxide value.

Mechanism of Photooxidation

Chemical compounds can absorb the energy from light in different wavelength bands depending on molecular structure (Sattar and deMan, 1975). The absorption of radiant energy is known as the primary photochemical process and results in an activated molecule:

$$A + h\nu \longrightarrow A^* \qquad (1)$$

This is followed by secondary reactions in which the excited molecule can use the activation energy to emit light (Eq. 2) or heat (Eq. 3), form a new activated species (Eq. 4), form a bond (Eq. 5), or dissociate into ions (Eq. 6) or free radicals (Eq. 7).

$$A^* \longrightarrow A + h\nu \qquad (2)$$

$$A^* + A \longrightarrow A + A + \text{heat} \qquad (3)$$

$$A^* + B \longrightarrow A + B^* \qquad (4)$$

$$A^* + A \longrightarrow A\text{-}A \qquad (5)$$

$$A^* \longrightarrow A^+ + e^- \qquad (6)$$

$$AB^* \longrightarrow A\cdot + B\cdot \qquad (7)$$

The two most important types of reactions concerning light-induced lipid oxidation are 1) a photosensitized reaction in which the light absorbing species does not undergo permanent chemical change:

$$S + h\nu \longrightarrow S^* \qquad (8)$$

$$S^* + A \longrightarrow A^* + S \qquad (9)$$

$$A^* + B \longrightarrow AB \qquad (10)$$

and 2) a photoinduced reaction in which the reactive species produced by the radiant energy initiates another reaction (such as a free radical reaction):

$$I + h\nu \longrightarrow I^* \qquad (11)$$

$$I^* \longrightarrow I\cdot \qquad (12)$$

$$I\cdot + A \longrightarrow A\cdot + I \qquad (13)$$

Equation 9 is essentially equivalent to equation 4, and equation 10 is similar to equation 5.

Schenck (1963) has catagorized photosensitized oxidation reactions according to the intermediates formed. A "type I" sensitized reaction, which can proceed in the absence of oxygen, is one in which free radicals and electronically excited molecules are involved. Compounds which are readily oxidized or reduced favor this type of reaction. Substances such as olefins, dienes, or aromatic compounds, that are not easily oxidized or reduced by a sensitizer favor a "type II" reaction in which oxygen participates and which occurs only through electronically excited molecules as intermediates. Type II photooxygenation reactions in solution, and the effects of solvent polarity have been discussed by Gollnick (1968) and Young et al. (1971) respectively.

During a type II reaction either the oxygen (Eq. 16) or the acceptor, A (Eq. 14), may be activated before it enters the termination reaction to form the product, AO_2 (Eq. 15 & 17) (Gollnick, 1968).

$$S^* + A \longrightarrow S + A^* \qquad (14)$$

$$A^* + O_2 \longrightarrow AO_2 \qquad (15)$$

$$S^* + {}^3O_2 \longrightarrow S + {}^1O_2^* \qquad (16)$$

$${}^1O_2^* + A \longrightarrow AO_2 \qquad (17)$$

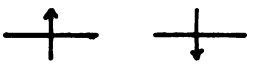
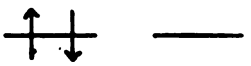
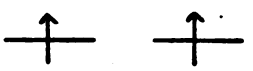
The latter scheme (Eq. 16 & 17) represents a pathway by which excited state singlet molecular oxygen ($^1O_2$) may be formed from ground state triplet oxygen ($^3O_2$), and subsequently oxidize an organic compound. This type of scheme has been implicated in the photooxygenation of aromatic hydrocarbons, some fatty acid esters, and oils (Foote and Wexler, 1964; Carlsson et al., 1976; Tereo and Matsushita, 1977).

Kearns (1971) has extensively reviewed the physical and chemical

properties of $^1O_2$, and its role in biological systems has been reviewed by Foote (1968), Bland (1976), and Krinsky (1977).

Oxygen may occur in one of two states, depending on the arrangement of electrons in the outer orbitals. Ground state triplet oxygen has two electrons in separate orbitals with opposed angular momentum and parallel spins. The first excited singlet state ($^1\Delta$) has both electrons in the same orbital with the same angular momentum and opposed spins. A second excited singlet state ($^1\Sigma$) has a very short lifetime and decays to the $^1\Delta$ state after 1×10^{-11} sec. There is no evidence that the $^1\Sigma$ state is formed in biological systems (Krinsky, 1977) and therefore the term singlet oxygen ($^1O_2$) shall refer to the $^1\Delta$ state in the remainder of this text. The relationship between the oxygen states is shown in Table 1.

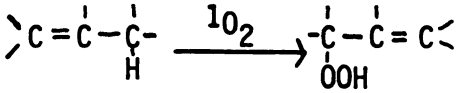
Table 1. Relationship between the three states of oxygen.[a]

State of O_2 Molecule	Symbol	Relative Energy	Occupancy of Highest Orbitals
Second excited	$^1\Sigma$	+37 kcal	
First excited	$^1\Delta$	+22 kcal	
Ground	$^3\Sigma$	0 kcal	

[a]Adapted from Foote,(1968).

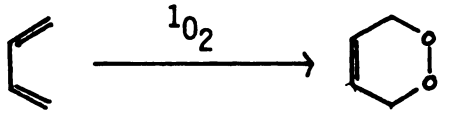
The $^1\Delta$ state has a lifetime of several μsec. and is highly electrophilic in nature since it seeks electrons to fill its vacant molecular orbital (Korycka-Dahl and Richardson, 1978). Therefore $^1O_2$ reacts readily with moieties containing high densities of electrons, such as double bonds. There are several reactions of $^1O_2$ which may be of importance in biological systems.

1) The "ene" mechanism occurs with olefins having at least two alkyl substituents and yields allylic hydroperoxides. Free radicals are not involved and there is no hydrogen abstraction prior to formation of the C-O bond. By this mechanism, lipid peroxides form when $^1O_2$ reacts with unsaturated fatty acids (Rawls and Van Santen, 1970).



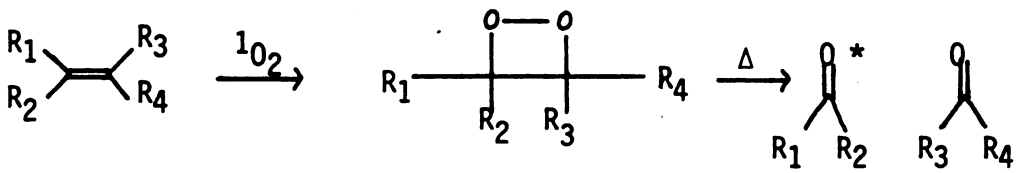
(18)

2) Endoperoxides may form by 1,4 addition of $^1O_2$ to 1,3-dienes. This bears a close relationship to the thermal Diels-Alder reactions (Gollnick, 1968).



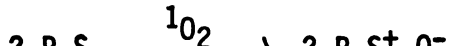
(19)

3) Dioxetane formation can occur when $^1O_2$ reacts with olefins or enamines. These often cleave to form excited carbonyls resulting in chemiluminescence. Dioxetanes may play a role in bioluminescence (Krinsky, 1977).



(20)

4) Exposure of molecules containing nitrogen or sulfur to $^1O_2$ can cause oxidation of these heteroatoms.



(21)

Singlet oxygen may be formed by photochemical, chemical, and biological systems by several mechanisms as reviewed by Krinsky (1977). In lipid systems, the combined action of sensitizer and light is perhaps the most important means of generating $^1O_2$, which may then cause photooxidation as described previously (Eq. 16 & 17). Most natural pigments

such as chlorophyll and hematoporphyrin act as sensitizers that tend to favor type II ($^1O_2$) mechanisms. Riboflavin and other flavins have been known to act as type I or II sensitizers, usually favoring the type I mechanism (Korycka-Dahl and Richardson, 1978).

Singlet oxygen quenchers have the opposite effect of sensitizers, and decrease the rate of photosensitized oxidation by either physically or chemically reacting with the $^1O_2$, thus deactivating it to the ground state:

$$^1O_2 + \text{quencher} \longrightarrow {}^3O_2 + \text{quencher}$$

β-Carotene has been shown to efficiently quench $^1O_2$ and therefore inhibit oxidation by $^1O_2$ (Foote and Denny, 1968; Foote et al., 1970b; Matsushita and Tereo, 1980). This quenching is believed to be the major mode of protection against photodynamic action in living organisms (Foote et al., 1970a). In methylene blue photosensitized oxygenations, one molecule of β-carotene was shown to quench up to 1000 molecules of $^1O_2$ before being consumed (Foote et al., 1970b), but it is also susceptible to photosensitized oxidation, forming mutachrome and aurochrome as major products (Seely and Meyer, 1971). Addition of δtocopherol increases the life of β-carotene in photosensitized systems (Matsushita and Tereo, 1980).

Photooxidation of Lipids

Exposure to light has long been known to accelerate oxidation in fats and oils (Coe and LeClerc, 1932; Coe, 1941). The effect of light may be promarily due to increased rates of hydroperoxide decomposition resulting in increased rates of autoxidation via the free radical chain mechanism. But singlet oxygen may also play a role in light-induced oxidation when sensitizers are present.

During autoxidation of unsaturated fatty acids via a free radical

mechanism, all hydroperoxides formed are conjugated (Khan et al., 1954a, 1954b). However, when methyl oleate and methyl linoleate were illuminated in the presence of chlorophyll, conjugated and non-conjugated hydroperoxides were formed, and the reaction was not inhibited by α-tocopherol, which effectively inhibits free radical autoxidation (Khan et al., 1954b). These observations indicated that during this photochemical oxidation, a mechanism unlike that responsible for autoxidation was involved.

Rawls and Van Santen (1970) have implicated $^1O_2$ as the reactive species in the photosensitized oxidation of fatty acids. Non-conjugated hydroperoxides which were absent in autoxidation reactions were formed from methyl linoleate during photosensitized oxidation and on exposure to externally generated singlet oxygen. Further proof for the involvement of $^1O_2$ in photooxidations was gained by showing that $^1O_2$ quenchers inhibited the reactions. Both $^1O_2$ quencher inhibition and presence of non-conjugated hydroperoxides are now commonly used to assess $^1O_2$ participation in oxidation reactions.

Rawls and Van Santen (1970) have identified $^1O_2$ as a likely source of hydroperoxides which initiate autoxidation in oils originally free of all hydroperoxides. Triplet oxygen cannot initiate the reaction since this would violate spin conservation laws (RH and ROOH are in singlet states) (Wigner, 1959, as cited by Labuza, 1971).

Evidence of $^1O_2$ participation in photooxidative deterioration of food lipids (Carlsson et al., 1976; Frankel et al., 1979), and photosensitized oxidation of fatty acid esters (Tereo and Matsushita, 1977), and micellar systems (Gorman et al., 1976) has also been presented.

Oxidation of Cholesterol

Autoxidation

Smith (1981) has reviewed the early history of cholesterol oxidation, the first accounts of which were recorded by Lifschutz who used acetic and sulfuric acids to detect sterols other than cholesterol in wool fat. In 1907, he recognized the deterioration of cholesterol as an oxidative process, the product of which he termed "oxycholesterol". He believed this to be a single chemical compound which could be derived by the action of oxidizing agents such as benzoyl peroxide, or isolated as a putative metabolite from some animal tissues. No pure "oxycholesterol" was ever described.

Blix and Lowenhielm (1928) expressed doubts about the purity of Lifschutz's oxycholesterol. They emphasized that oxygen was necessary for its formation and alkali soaps capable of dispersing cholesterol catalyzed this oxidation. Heilbron and Sexton (1928) dry distilled cholesterol at atmospheric pressure to form what they described as cholestenone and ψ-cholestene. All of this work was carried out prior to the assignment of the correct chemical structure to cholesterol in 1932.

Bergstrom and Wintersteiner (1941, 1942a, 1942b) studied the autoxidation of cholesterol in aerated colloidal aqueous solutions at 85°C and 37°C. After chromatographic separations and crystallization, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 7-ketocholesterol, and 7-keto-$\Delta^{3,5}$-cholestadiene were identified as products of the autoxidation, and were believed to be analogous to Lifschutz's "oxycholesterol" with the diols causing color formation in the Lifschutz reaction. Ferrous and zinc ions were found to accelerate the reaction, but did not increase the yields of the various cholesterol oxidation products. Susceptibility to oxygen attack was greatly diminished when cholesterol acetate, palmitate

or oleate esters were exposed to the same system at 85°C (Bergstrom and Wintersteiner, 1942c).

A mechanism of formation was postulated which involved formation of an unstable C_7 cholesterol hydroperoxide. Dehydration and decomposition of this hydroperoxide would allow for formation of the 7-keto and 7-hydroxy compounds, respectively. The 7-hydroperoxycholesterol was not isolated.

Adequate understanding of cholesterol oxidation mechanisms was dependent on the development and application of thin layer chromatography (TLC) to the complex mixture of products formed (Claude, 1966; Claude and Beaumont, 1966; Horvath, 1966; Fioriti and Sims, 1967; Smith et al., 1967). Gas liquid chromatographic (GLC) procedures have also aided cholesterol autoxidation investigations by providing a means for separating and identifying some oxidation products (Claude, 1966; Van Lier and Smith, 1968a), preparing pure sterol oxides (Van Lier and Smith, 1968b), and studying the thermal decomposition products and patterns of these oxides (Van Lier and Smith, 1970b; Teng et al., 1973a; Smith et al., 1973a).

Using these techniques, the pathways of cholesterol autoxidation have been elucidated, and reviewed by Smith (1980, 1981). There are two distinct autoxidation processes which appear to be free radical in nature. The major process (Eq. 22) involves formation of sterol hydroperoxides and the minor process (Eq. 23) involves formal alcohol dehydrogenation. This latter process is believed to be responsible for formation of small amounts of cholest-5-en-3-one (Ansari and Smith, 1978).

$$RH + O_2 \longrightarrow ROOH \qquad (22)$$

$$RCH(OH) + O_2 \longrightarrow RC{=}O + H_2O_2 \qquad (23)$$

The former reaction (Eq. 22), which has been outlined by Ryan (1982) (Figure 1), may be initiated by allylic C_7 hydrogen abstraction and reaction with ground state molecular oxygen to yield C_7 peroxy radicals. These are stabilized by hydrogen abstraction to yield the epimeric 7-hydroperoxides. The initial formation of these compounds was established by Smith and Hill (1972) using N,N-dimethyl-p-phenylenediamine- and N,N,N',N'-tetramethyl-p-phenylenediamine-dihydrochloride as developing reagents in TLC analysis.

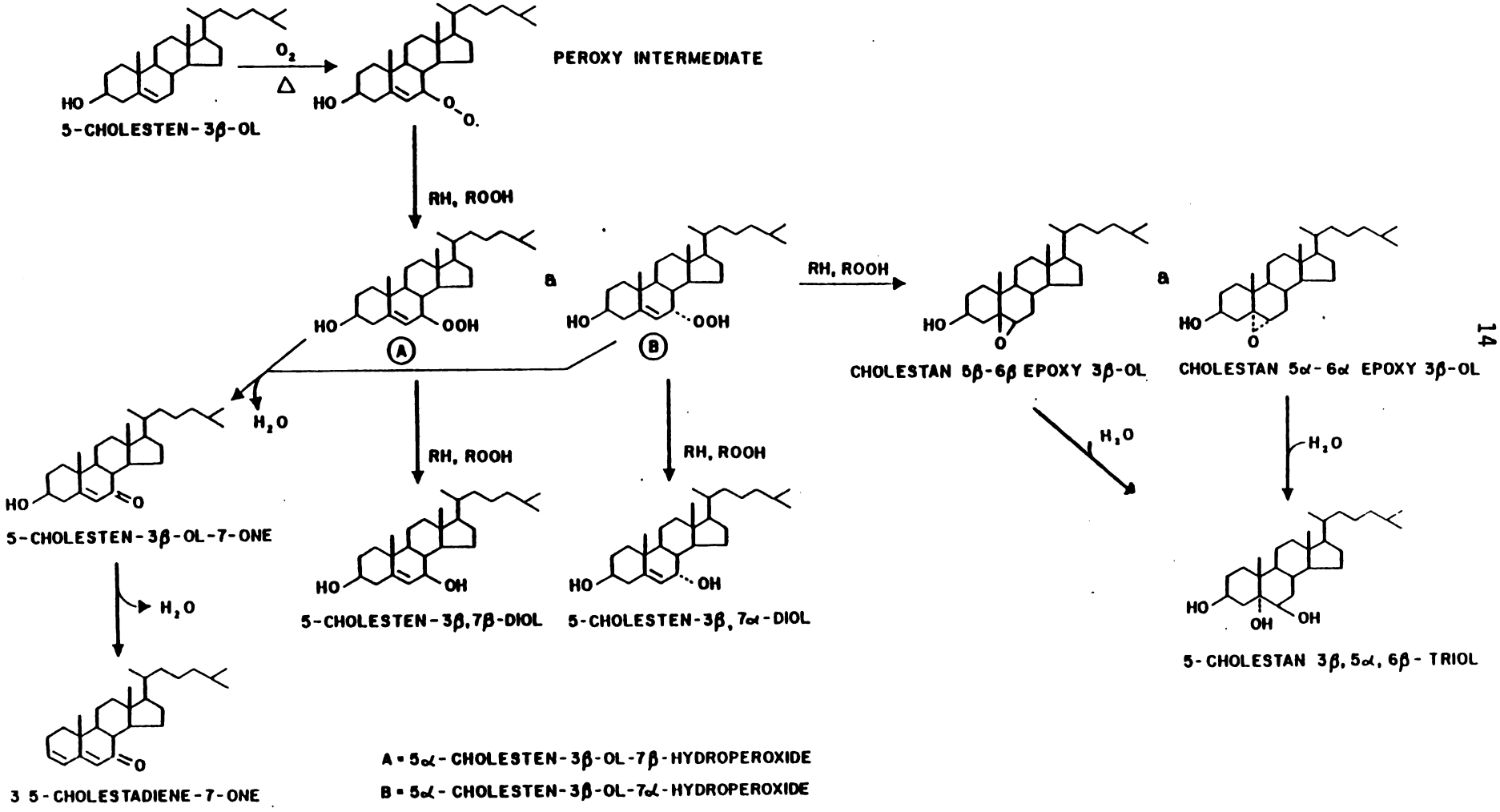
Both the cholesterol 7α- and 7β-hydroperoxides are formed with the more stable 7β-hydroperoxide predominating. The 7α epimer may epimerize to the 7β epimer in solution, solid state, or vapor state (Teng et al., 1973a, 1973b).

The 7α- and 7β-hydroperoxides then undergo formal reduction to form 5-cholesten-3β,7α-diol and 5-cholesten-3β,7β-diol respectively, or dehydration to yield 5-cholesten-3β-ol-7-one. The 7α-diol may epimerize to the 7β-diol, and the 7-ketone may undergo dehydration to form 3,5-cholestadien-7-one.

Cholesterol epoxidation may also occur as a secondary oxidation reaction (Figure 1). Attack on cholesterol by hydroperoxides (including cholesterol hydroperoxides as well as H_2O_2) yields both cholestan-5β,6β-epoxy-3β-ol and cholestan-5α,6α-epoxy-3β-ol with the β-epimer predominating (Smith and Kulig, 1975). Dehydration of either 5,6-epoxide yields cholestan-3β,5α,6β-triol, which may undergo further dehydrogenation to form cholestan-3β,5α-diol-6-one.

Smith (1980) reviewed other less common transformations of

Figure 1. Major pathways of cholesterol autoxidation.
(Adapted from Ryan, 1982)


5-CHOLESTEN-3β-OL
PEROXY INTERMEDIATE
RH, ROOH
A
B
CHOLESTAN 5β-6β EPOXY 3β-OL
CHOLESTAN 5α-6α EPOXY 3β-OL
5-CHOLESTEN-3β-OL-7-ONE
5-CHOLESTEN-3β,7β-DIOL
5-CHOLESTEN-3β,7α-DIOL
5-CHOLESTAN 3β,5α,6β-TRIOL
3 5-CHOLESTADIENE-7-ONE
A = 5α-CHOLESTEN-3β-OL-7β-HYDROPEROXIDE
B = 5α-CHOLESTEN-3β-OL-7α-HYDROPEROXIDE

cholesterol oxidation products occurring in the A and B rings which may explain the formation of compounds such as cholest-5-en-7α-ol-3-one, cholest-4-en-7α-ol-3-one, 4,6-cholestadien-3-one and cholestan-3β,5α,6β,-7α-tetraol.

Because of the geometry of its structure, cholesterol in the crystalline form is susceptible to side chain oxidation, involving reactions not known to occur with cholesterol in aqueous or organic solutions (Beckwith, 1958). Hydrogen abstraction, perhaps by cholesterol-7-peroxyl radicals, followed by reaction with $^3O_2$, yields cholesterol hydroperoxides in the C_{20}, C_{24}, C_{25} and C_{26} positions, with the tertiary C_{20} and C_{25} positions being most susceptible (Van Lier and Smith, 1970a, 1970b, 1971; Van Lier and Kan, 1972). These hydroperoxides undergo reduction and dehydration reactions as described for the 7-hydroperoxides, as well as β-scission of carbon-carbon bonds in the side chain. This latter mechanism is believed to be responsible for the formation of short chain volatile compounds during cholesterol autoxidation (Van Lier et al., 1975).

Cholesterol oxidation has been studied in many systems under various conditions and the findings have supported these proposed mechanisms. The instability of crystalline cholesterol exposed to air for periods up to 32 years has been reported by Mosbach et al. (1963), Fioriti and Sims (1967), Smith et al. (1967), and Van Lier and Smith (1970a). Over 30 autoxidation products have been detected in a 12 year old air aged cholesterol sample (Smith et al., 1967). Engel and Brooks (1971), to the contrary, have reported that a sample of highly purified cholesterol contained neglible amounts of impurities after exposure to air for over 30 years.

Aerated colloidally dispersed cholesterol systems have also been studied extensively (Norcia, 1958; Horvath, 1966; Fioriti and Sims, 1967; Chicoye et al., 1968b; Weiner et al., 1972, 1973; Norcia and Mahadevan, 1973; Kimura et al., 1976, 1979). Oxidation of cholesterol in monomolecular films has been investigated in hopes of simulating, in a crude way, occurrences in cell membranes (Norcia, 1961; Kamel et al., 1971; Weiner et al., 1972). The major products identified were 5-cholesten-3β-ol-7-one and the epimeric 5-cholesten-3β,7-diols.

Esterification of cholesterol can either increase or decrease the susceptibility of the cholesteryl moiety to oxidative attack, depending upon the degree of unsaturation of the fatty acyl moiety. Acetate, palmitate, and oleate esters in colloidally dispersed systems showed less susceptibility to oxidation than did unesterified cholesterol (Bergstrom and Wintersteiner, 1942c; Norcia and Janusz, 1965). This effect may be due to steric hindrance which interferes with the sensitive C_7 sterol position. Cholesteryl linoleate was found to be more susceptible to sterol moiety oxidative reactions than was cholesterol (Norcia and Janusz, 1965).

Norcia and Mahadevan (1973) reported that the linoleate, linolenate, and arachidonate cholesterol esters were all susceptible to oxidative attack in aqueous colloidal suspensions. All three underwent oxidation within two hours. The extent of oxidation differed and was greater in the linoleate system, followed by linolenate, then the arachidonate system. The differences were believed to be due to the extent and time course of free radical formation in the acyl chain with resultant intramolecular propagation of sterol oxidation. Conformational changes in the acyl chain, due to introduction of oxygen functional groups, may also have been responsible.

Cholesteryl acetate esters, spread as a monomolecular film on water were found to rapidly undergo hydrolysis which was followed by sterol oxidation (Kamel et al., 1971). Under these conditions, esterification was less effective in inhibiting cholesterol oxidation.

Photooxidation

The instability of cholesterol exposed to light and air was documented as early as 1926 when Shear and Kramer (1926), Hess et al., (1926) and Rosenheim and Webster (1926) irradiated cholesterol with a mercury vapor quartz lamp and sunlight, in hopes of finding an antirachitically active fraction. After recrystallization of the irradiated cholesterol and evaporation of the solvent from the mother liquor, a yellow oil remained. It was found that the presence of oxygen was necessary to form this "UV oil of cholesterol," which reacted positively to the Lifschutz test for oxycholesterol.

Stavely and Bergman (1937) further characterized cholesterol irradiated with a mercury vapor lamp. It was noted that during irradiation, the cholesterol increased in weight, gradually became more saturated, and a compound formed which decomposed rapidly in the presence of moisture to liberate H_2O_2. This was suspected to be a hydroperoxide or ozonide. It was also observed that the melting point was lowered, the optical rotation became less negative, and there was a decrease in the digitonin-precipitable material.

The products of light-induced oxidation under various conditions have been proposed. These products and mechanisms vary, depending on the state of the cholesterol, nature of solvents used, and presence or absence of a sensitizer.

Smith (1981) reported that the first photosensitized oxygenation of

cholesterol was performed by Schenck (1957), using pyridine as solvent, and hematoporphyrin as a singlet oxygen-producing sensitizer. This reaction yielded cholest-6-en-3β-ol-5α-hydroperoxide as the major product, and cholest-4-en-3β-ol-6α-hydroperoxide and cholest-4-en-3β-ol-6β-hydroperoxide as minor products (Kulig and Smith, 1973).

The 5α-hydroperoxide is only known to form by $^1O_2$ attack on cholesterol, and this is believed to occur via the cyclic ene mechanism in which oxygen attaches to one carbon of the double bond, followed by a shift of the double bond to the allyl position. The allyl hydrogen then migrates to the peroxy group. No free radicals are involved. Schenck (1957), as reported by Nickon and Bagli (1961) noted that no cyclic ene reaction occurred if the olefin lacked allylic hydrogens, or if the sensitizer, irradiation or oxygen was excluded. Free radicals were not involved in this initial reaction, and the epimeric cholesterol-7-hydroperoxides common to cholesterol autoxidation were not among the initial products (Kulig and Smith, 1973).

These hydroperoxidations have been shown to be stereospecific in that the new C-O bond formed is cis to the C-H bond broken, and both bonds prefer to be perpendicular to the olefinic plane (Nickon and Bagli, 1961). Quasiaxial hydrogens are favored over the quasiequatorial hydrogens in the cyclic reaction (Gollnick, 1968).

A similar mechanism has been proposed for the origin of the 6α- and 6β-hydroperoxides, but this has been the subject of controversy. Nickon and Mendelson (1965) reported that photooxygenation of steroidal olefins can be blocked when the C-O bond must develop into a 1,3-diaxial relationship to an alkyl substituent (as in the formation of the 6β-hydroperoxide). A free radical mechanism of formation was therefore implied.

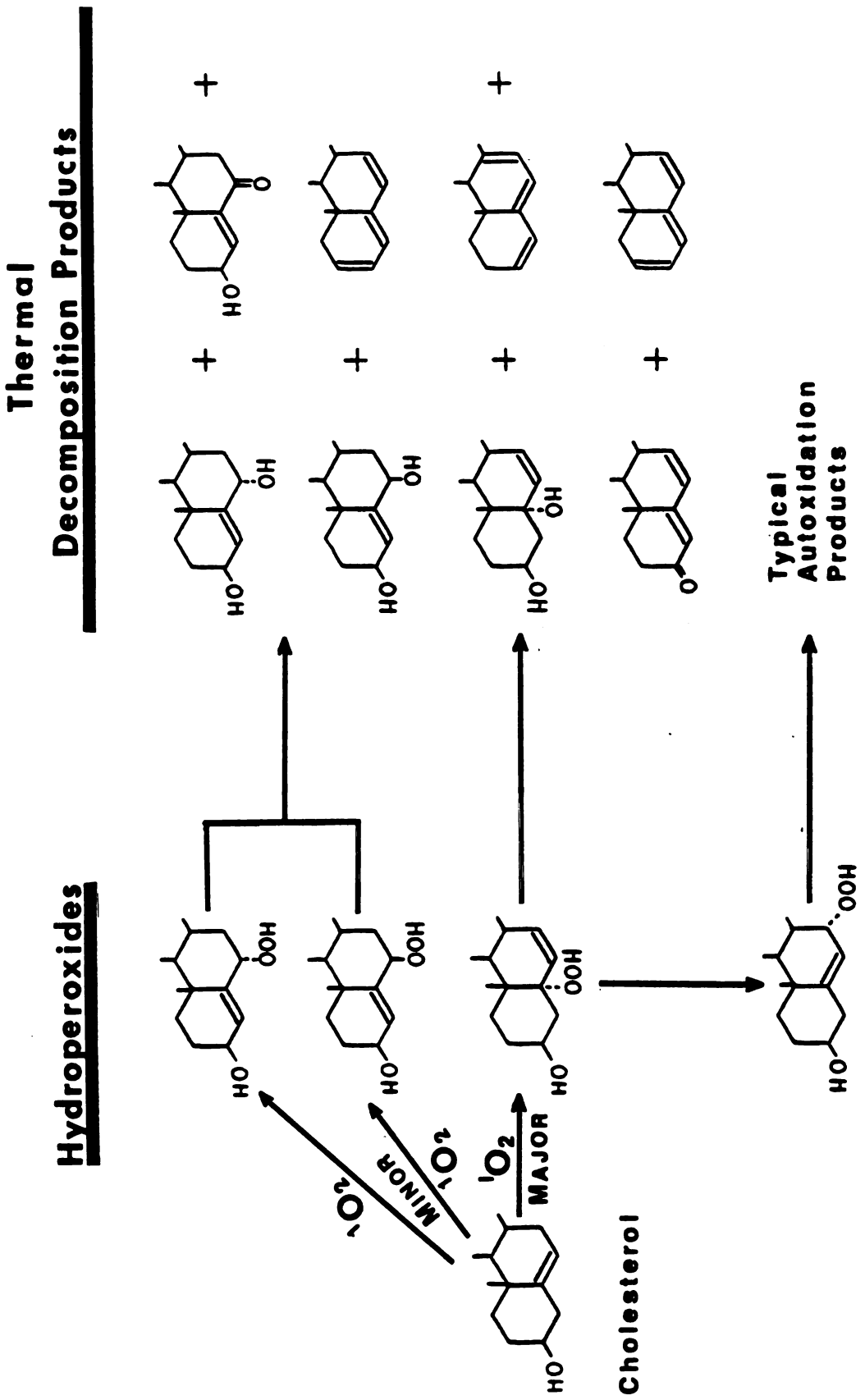
However, in photosensitized oxygenations of cholesterol by Kulig and Smith (1973), quasiaxial 6β-hydroperoxide formed in twice the yield of its 6α epimer, along with the major product, the 5α-hydroperoxide. This contradicts the free radical mechanism which would mandate formation of equal amounts of the epimers or possibly a higher yield of the more stable 6α-hydroperoxide. Furthermore, the 6-hydroperoxides did not form upon photolysis of oxygenated cholesterol solutions in cyclohexane in which the 5α-hydroperoxide was added as a radical initiator. Instead, traces of the 7α- and 7β-hydroperoxides, putative radical products, were detected.

In unsensitized photooxidations in pyridine, all three hydroperoxides formed in very low yield, but in equal (relative) proportions which suggests formation by the same mechanism. This was postulated to be the attack of $^1O_2$ on cholesterol with pyridine acting as a sensitizer of low efficiency. Thus, Kulig and Smith (1973) support $^1O_2$ attack as the mechanism of formation of the 6-hydroperoxides.

Further rearrangement and decomposition of the hydroperoxides may lead to the formation of a myriad of secondary products (Figure 2). This may include reaction pathways analogous to those discussed for cholesterol autoxidation, including dehydration, dehydrogenation and free radical mechanisms. The 5α-hydroperoxide is capable of undergoing rearrangement to form the 7α-hydroperoxide which is a characteristic intermediate of free radical autoxidations (Lythgoe and Trippet, 1959). Thus, photosensitized oxidation of cholesterol can lead to the formation of products commonly encountered in cholesterol autoxidation.

In the absence of a sensitizer, cholesterol may absorb light of sufficient energy to cause bond hemolysis, followed by radical propagation

Figure 2. Major pathways of photosensitized oxidation of cholesterol. (Partial structures shown.)


Thermal
Decomposition Products
Hydroperoxides
Typical
Autoxidation
Products
Cholesterol
MINOR
MAJOR

reactions involving $^3O_2$ and hydrogen abstraction leading initially to the 7α- and 7β-hydroperoxycholesterol species (Smith, 1981), with the 7β epimer predominating (Smith et al., 1973b). When irradiated in the crystalline form, 25-hydroperoxycholesterol may also be formed (Beckwith, 1958) by free radical processes previously discussed. Thus in the absence of a $^1O_2$ generating sensitizer, decomposition, reduction and dehydration reactions involving the initial hydroperoxides will yield products characteristic of free radical autoxidation of cholesterol.

These proposed mechanisms of light-induced cholesterol oxidation are supported by reports of oxidation products detected after irradiation of cholesterol-containing systems under a variety of conditions. Beckwith (1958), Fioriti and Sims (1967), and Smith et al. (1973b) exposed crystalline cholesterol to ultraviolet light and detected compounds such as 7β-hydroperoxycholesterol, 7α- and 7β-hydroxycholesterol, 7-keto-cholesterol, 25-hydroxycholesterol, 5-cholesten-3-one, and 1,4-cholesta-dien-3-one. Using fluorescent light, Claude and Beaumont (1966) as reviewed by Chicoye (1968a) detected 7α-, 7β-, and 25-hydroxycholesterol. In dilute aqueous cholesterol solution exposed to daylight, Hais and Myant (1965) detected substances more polar and less polar than cholesterol. The above observations most likely represent nonsensitized, free radical mechanisms.

Photosensitized oxidations of cholesterol have also been recorded. Schenck et al. (1957, 1958) as reviewed by Bergstrom and Samuelsson (1961) and Gollnick (1968) were the first to successfully detect 6-cholesten-3β-ol-5α-hydroperoxide in hematoporphyrin-sensitized pyridine solutions of cholesterol. The formation of the 3β,5α-diol was also noted. Smith and Hill (1972) and Kulig and Smith (1973) also detected

the 5α-hydroperoxide, with 4-cholesten-3β-ol-6α-hydroperoxide and the 6β-epimer detected in the latter study. Liposomal cholesterol in the presence of hematoporphyrin and light yielded the 5α-hydroperoxide as the major product along with lesser amounts of the epimeric 7-hydroperoxides (Suwa et al., 1977).

Acker and Greve (1963) detected "cholesteriline-hydroperoxyde" and "7-hydroxycholesterine" in egg-containing foods which had been exposed to daylight in a dry powder form. Chicoye et al. (1968a) detected 7-ketocholesterol, the epimeric 7-hydroxycholesterols, cholesterol-5β,6β-epoxide and cholestan-3β,5α,6β-triol in fluorescent or daylight exposed spray-dried egg yolk. The absence of cholesterol oxidized at the C_5 or C_6 positions suggests the absence of singlet oxygen participation in these oxidations.

Other Oxidation Systems

X-rays and γ-radiation have also been shown to promote cholesterol oxidation. Keller and Weiss (1950) isolated 5-cholesten-3β-ol-7-one and cholestan-3β,5α,6β-triol from irradiated aqueous cholesterol solutions. Hydroxyl radical (OH·) attack has been implicated in this process. When irradiated by X-rays, cholesterol in methanol yielded the above two products in addition to 5-cholesten-3β,7β-diol (Coleby et al., 1954). In acetone or dioxan, cholestan-5α,6α-epoxy-3β-ol, coprostan-5β,6β-epoxy-3β-ol, and the 3β,7β-diol were detected. Formation of the 3β,5α,6β-triol from the epoxide was not believed to be the major source of the triol because of the extreme slowness of this reaction. Formation of the 3β,7β-diol was believed to support the formation of the 7-keto compound by attack of two hydroxyl radicals on cholesterol. Truhaut (1947) as reviewed by Dauben and Payot (1956) found no oxidation of

cholesterol when it was X-irradiated in a non-polar solvent.

Ansari and Smith (1979) detected 7-ketocholesterol, the 3β,7-diols, the α- and β-epoxycholesterols and the 3β,5α,6β-triol in aqueous cholesterol dispersions exposed to ^{60}Co γ-radiation. Again, hydroxyl radical action was implicated, and sterol hydroperoxides were not detected when O_2 was absent. γ-Irradiation of crystalline cholesterol yielded 5-cholesten-3β-ol-7β-hydroperoxide as an initial product, and free radical processes, analogous to those for unsensitized UV-induced oxidation, were implicated (Smith et al., 1973b).

Radioactive ^{14}C-labeled cholesterol stored in air was found to be more susceptible to oxidation than similarly treated unlabeled cholesterol (Dauben and Payot, 1956). Products detected included 7-ketocholesterol, the epimeric 3β,7-diols, cholestan-3β,5α,6β-triol and cholestan-3β,5α-diol-6-one. The amount of decomposition depended on time and specific activity, and was postulated to proceed by a chain reaction. In the absence of air, no decomposition took place.

Aside from autoxidation and radiation-induced oxidation, other chemical and biological systems can promote the formation of common cholesterol oxides. Clemo et al. (1950) found that hydroxyl radicals produced by Fenton's reagent oxidized cholesterol to the 3β,5α,6β-triol and the 7-ketone under suitable conditions, the results being similar to those obtained by X-irradiation of aqueous cholesterol systems (Keller and Weiss, 1950). H_2O_2 promoted cholesterol oxidation by four mechanisms: epoxydation followed by hydration, formal hydration, free radical oxidation by $^3O_2$, and $^1O_2$ attack, the $^1O_2$ being formed by H_2O_2 disproportionation (Smith and Kulig, 1976). Superoxide radical anion ($O_2^{\cdot-}$) did not react with cholesterol (Smith et al., 1977), and the dioxygen cation

(O_2^+) yielded a complex mixture of oxidation products devoid of hydroperoxides (Sanche and Van Lier, 1976), but its occurrence in biological systems is not likely. Ozone is also known to react rapidly with cholesterol to form peroxidic and non-peroxidic products (Smith, 1980).

Enzyme systems such as soybean lipoxygenase, horseradish peroxidase (Teng and Smith, 1973), and those found in rat liver microsomes (Johansson, 1971) were also found to convert cholesterol to oxidation products commonly associated with autoxidative mechanisms.

Biological Consequences of Cholesterol Oxidation

Angiotoxicity and Atherosclerosis

Induction of atherosclerosis as a result of cholesterol fed to rabbits was first reported in 1912 by Anitschkow (Smith, 1981), and has been repeated in several species. Cholesterol preparations commonly used for the dietary induction of atherosclerosis are of USP grade, which normally contain companion sterols as well as autoxidation products. Following reports (as reviewed by Smith et al., 1967) of the existence of several cholesterol oxidation products such as 3,5-cholestadien-7-one, 4,6-cholestadien-3-one, cholestan-3β,5α,6β-triol, 5-cholesten-3β,7α-diol, 5-cholesten-3β,7β-diol, 5-cholesten-3β-ol-7-one and 5-cholesten-3β,26-diol in atherosclerotic human aorta samples, and cholestan-5α,6α-epoxy-3β-ol, 4-cholesten-3-one, and 4,6-cholestadien-3-one in human serum (Gray et al., 1971), studies were designed to elucidate the roles of cholesterol oxidation products, USP grade cholesterol contaminants, and highly purified cholesterol in eliciting arterial damage.

MacDougall et al. (1965) tested nine steroids on rabbit aorta cultures and found cholestan-3β,5α,6β-triol, 5-cholesten-3β,26-diol, cholestanol and lathosterol to be highly toxic. 5-Cholesten-3-one,

5-cholesten-3β,7β-diol, 5-cholesten-3β,25-diol and cholesterol were moderately toxic, while 5-cholesten-3β,7α-diol showed no toxic effects.

Impurities from USP-grade cholesterol were concentrated and orally administered to rabbits (Imai et al., 1976). Twenty four hours after gavage at 250 mg/kg, the concentrate caused increased frequency of dead aortic smooth muscle cells (an index of acute angiotoxicity) and induced focal intimal edema. New and five-year old cholesterol produced the same effects. The concentrate, administered at a dose of 1 g/kg/seven weeks induced intimal diffuse fibrous lesions which are arteriosclerotic features distinct from those induced by conventional cholesterol feedings. Purified cholesterol showed no increase in degenerated cells after 24 hours and no effect after seven weeks.

In a similar experiment (Imai et al., 1980), impurities from USP-grade cholesterol were separated into three fractions and injected into rabbits in saline solution. The fraction identified as the sterol hydroperoxides showed no angiotoxicity. The concentrate of impurities from USP-grade cholesterol, as well as several synthesized cholesterol oxidation products were also injected in rabbits. Two weeks after the injections, cell death and inflammation were observed with intimal fibromuscular thickening apparent after 10 weeks. Freshly purified cholesterol induced no changes in major or minor pulmonary artery branches while cholestan-3β,5α,6β-triol and 5-cholesten-3β,25-diol caused cell death within 24 hours, followed by necrosis, inflammation and fibromuscular thickening after 10 weeks. Cholestan-5α,6α-epoxy-3β-ol and 5-cholesten-3β-ol-7-one produced lesions in minor pulmonary artery branches.

In vitro studies of the effects of TLC bands of impurities separated from USP-grade cholesterol, utilizing cultured rabbit aortal smooth

muscle cells showed the 5-cholesten-3β,25-diol band to be the most potent angiotoxic agent, followed by the 3β,5α,6β-triol (Peng et al., 1979; Taylor et al., 1979).

Compounds necrogenic to arterial smooth muscle cells have also been isolated from powdered whole eggs and dried whole milk (Taylor et al., 1977).

The aforementioned studies all support the theory that autoxidation products of cholesterol, rather than pure cholesterol, may play the primary role in arterial wall injury and lesion development in atherosclerosis. This may also explain the observations of Chaikoff et al. (1948) as discussed by Taylor et al. (1977), that endogenously-induced hypercholesterolemia caused only a meager amount of spontaneous atherosclerosis in chickens whereas hypercholesterolemia induced by a 2% cholesterol diet caused numerous, grossly visible atheromata. In light of its instability, it is highly likely that the cholesterol added to the diets was accompanied by angiotoxic oxidation products.

Several cholesterol oxides have been shown to inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Chen et al., 1974; Kandutsch and Chen, 1978) which is a regulatory enzyme in the synthetic pathway leading to cholesterol. It has been proposed that this inhibition plays a key role in the mechanism involved in the angiotoxic effects of cholesterol oxidation products, because suppression of HMG-CoA reductase can lead to many alterations and disorders in cell function (Kandutsch, 1979).

The inhibited cholesterol synthesis leads to a decline in the concentration of cholesterol in the plasma membrane which in turn increases the membrane fluidity and permeability. It has also been shown that

when the cycle of sterol synthesis is blocked by an oxysterol, DNA synthesis and cell division do not occur (Kandutsch et al., 1978). Cells depleted of cholesterol are unable to carry on pinocytosis, or to divide, and eventually die (Kandutsch, 1979).

An additional mechanism for the cytotoxicity of cholesterol oxidation products was proposed to explain why cholestan-3β,5α,6β-triol was more toxic than 5-cholesten-3β,25-diol, but was much less effective in HMG-CoA reductase inhibition. Peng et al. (1979) suggested that cholesterol oxides, such as the triol, may be able to replace cholesterol in membranes. Because of their amphiphilic nature, membrane entry would be possible, followed by cell disfunction.

Mutagenicity and Carcinogenicity

Several cholesterol oxidation products have been long suspected as carcinogens. However, conflicting and uncertain data have been reported in several cases. Fieser (1954) believed 5-cholesten-3-one to be carcinogenic, but this was later rejected by Bischoff (1969).

Early studies by Fieser et al. (1955) and Bischoff (1963, 1969) implicated several cholesterol oxides as carcinogens but these studies involved administration in oily vehicles which are now believed to have cocarcinogenic activity. Only cholestan-5α,6α-epoxy-3β-ol was found to induce local fibrosarcomas in rats and mice when injected in an aqueous medium (Bischoff, 1963). The β-epoxide which is invariably present with the α-epoxide may also act as a cocarcinogen (Bischoff, 1969). Another problem plaguing early studies was the use of supersaturated solutions for injection. At the site of injection, the oxides crystallized out, which led to solid state carcinogenesis (Bischoff, 1969). Unsaturated solutions of the same compounds showed no effect.

As cited by Black and Lo (1971), Roffo (1931, 1933) suggested that steroids in the skin may be converted to carcinogens photochemically. Black and Lo (1971) isolated cholestan-5α,6α-epoxy-3β-ol and 5-cholesten-3β-ol-7-one in UV-irradiated skin, and in addition found cholestan-3β,5α,6β-triol and the cholesterol 3β,7-diols in skin incubated with ^{14}C-cholesterol prior to UV irradiation (Lo and Black, 1972). Effects due to the radioactivity of the cholesterol were not assessed. Cholesterol α-epoxide also formed in the skin of hairless mice after UV irradiation (Black and Douglas, 1972).

Further studies suggested that the known carcinogen (the α-epoxide) was involved in the etiology of UV-induced skin cancer (Black and Douglas, 1973). The α-epoxide concentrations in the skin of UV-irradiated hairless mice peaked just prior to the formation of squamous cell carcinomas and the concentration was related to the UV light dose. The α-epoxide was formed in highest levels in the epidermis, from which low levels appeared to diffuse out (Chan and Black, 1976).

The feeding of cholesterol oxidation products has also been reported to promote cancer. Leduc (1980) found an increase in the incidence of liver tumors in mice after a brief feeding of cholestan-5α,6α-epoxy-3β-ol, but not after similar feeding of cholestan-3β,5α,6β-triol. Dehydrated egg and milk powders, in which the cholesterol may be susceptible to oxidation, also were found to induce liver tumors in mice.

Cholesterol and its oxidation products have also been examined for their mutagenic effects on Salmonella typhimurium strains (Smith et al., 1979). Purified cholesterol was nonmutagenic in all strains tested, but gave mutagenic responses after heated or γ-irradiated in air. Naturally air-aged commercial preparations of USP or reagent grade cholesterol

also exhibited mutagenic responses, although some differences were noted between apparently similar samples to two bacterial strains. This was attributed to fine distinctions in sample compositions not evident by chromatographic analysis. In an effort to identify one or more specific compounds present in oxidized cholesterol samples, 5-cholesten-3β-ol-7-one, 3,5-cholestadien-7-one, 5-cholesten-3-one, cholestan-3β,5α,6β-triol, cholestan-3β,5α-diol-6-one, cholestan-5α,6α-epoxy-3β-ol, and cholestan-5β,6β-epoxy-3β-ol were found to be nonmutagenic at levels as high as 6 mg/plate. 5-Cholesten-3β,25-diol exhibited toxicity in the assays, and conclusions as to its mutagenicity were not drawn.

Cholesterol Oxidation Products in Foods

Increased awareness of the instability of cholesterol under various conditions and of the adverse biological effects of some cholesterol oxidation products has caused concern over the possible formation and presence of these compounds in cholesterol-containing foods. It has been suggested that foods containing cholesterol oxides may be hazardous for human consumption (Sheppard and Shen, 1980).

Wilson (1976) considers that the consumption of old powdered whole egg or whole milk may be a significant risk factor in human atherosclerosis. Kummerow (1979) suggests that consumption of large doses of vitamin D during infancy may initiate arterial smooth muscle cell death which would be aggravated and accelerated in later years by consumption of foods containing cholesterol oxidation products. Ingestion of dehydrated egg and milk powder caused a high incidence of tumors in mice (Leduc, 1980). Necrogenic compounds have also been isolated from these foods (Taylor et al., 1977).

Several researchers have stressed the need to investigate changes which cholesterol may undergo during processing and storage of foods.

Those foods which have been singled out include fats used to fry chicken and fish as well as potatoes fried in beef tallow (Kummerow, 1979), egg batters spread thin and exposed to high frying temperatures (Taylor et al., 1979), any dried or powdered foods containing cholesterol and stored at room temperature in air (Taylor et al., 1979), as well as cheese, smoked meat, fish and sausage (Taylor et al., 1979).

Closer control over storage and processing of cholesterol-containing foods may need to be instituted (Taylor et al., 1979). It has been suggested that these foods be stored in well-sealed containers, preferably under vacuum or nitrogen, at refrigerator or freezer temperatures. Addition of antioxidants such as glutathione may also help prevent cholesterol oxidation in foods (Taylor et al., 1979).

Very little information is currently available regarding the levels of cholesterol oxides in foods, and it has been suggested that a systematic analytical study be initiated (Smith, 1980). Table 2 contains a list of foods found to contain cholesterol oxidation products. These data cannot be regarded as definitive since only the most recent works were conducted with adequate analytical methods (Smith, 1981).

The only literature reports of detection of light-induced cholesterol oxidation products in foods are those of Acker and Greve (1963) and Chicoye et al. (1968a). In the latter study, spray-dried egg yolk was irradiated with fluorescent light or summer sunlight. The major cholesterol oxidation products are listed in Table 2. Fresh yolk and unirradiated spray-dried yolk did not contain significant amounts of cholesterol oxidation products.

Egg-containing foods exposed to light were found to contain "cholesterine-hydroperoxyde" and "7-hydroxycholesterine" (Acker and Greve, 1963).

Table 2. Cholesterol Oxidation Products in Foodstuffs[a]

Foodstuff	Sterol
Egg yolk	Cholestan-3β,5α,6β-triol
	3,5-Cholestadien-7-one
Egg dough	5-Cholesten-3β,7-diols
	Cholesterol hydroperoxides
Spray-dried egg	5-Cholesten-3β,7-diols
	5-Cholesten-3β-ol-7-one
	Cholestan-5β,6β-epoxy-3β-ol
	Cholestan-3β,5α,6β-triol
Heat-dried egg	Cholestan-5α,6α-epoxy-3β-ol
	Cholestan-5β,6β-epoxy-3β-ol
Anhydrous milk fat	4-Cholesten-3-one
	3,5-Cholestadien-7-one
Nonfat dry milk	4-Cholesten-3-one
	3,5-Cholestadien-7-one
Butterfat	5α-Cholest-7-en-3-one
Pork fat	5-Cholesten-3β-ol-7-one
Baker's yeast	3,5-Cholestadien-7-one
Beef	3,5-Cholestadien-7-one
Heated tallow[b]	5-Cholesten-3β,7-diols
	3,5-Cholestadien-7-one
	Cholestan-3β,5α,6β-triol
French fried potatoes[b] (tallow fried)	5-Cholesten-3β,7-diols
	3,5-Cholestadien-7-one
	Cholestan-3β,5α,6β-triol

[a]Adapted from Smith (1980).

[b]From Ryan (1982).

Oxidation was more severe in the powdered than in the unground form.

Butter

Composition

Butter, churned from cow's milk, contains at least 80% milk fat as well as water, protein, vitamins and trace minerals. It may be salted or unsalted.

The lipid makeup of butter is very complex. Over five hundred fatty acids have been detected, the major ones being oleic, palmitic, stearic and myristic, in that order (Swern, 1979). Triglycerides comprise 97-98% of all lipids present along with lesser amounts of diglycerides (0.28 - 0.59%), monoglycerides (0.016 - 0.038%), free fatty acids (0.10 - 0.44%), phospholipids (0.2 - 1.0%), and free sterols (0.22 - 0.41%) (Swern, 1979). Cholesterol is the major steroid component in milk fat (Flannagan and Ferretti, 1974) and has been measured at a level of 0.19% by Punwar and Derse (1978).

Oxidation

Because of its delicate flavor and high lipid content, butter is susceptible to deterioration resulting from desiccation, absorption of taints, microbial growth and oxidation, which result in flavor and/or color defects (Pont et al., 1958).

Autoxidation of the lipids in butter will occur very slowly in the dark. Factors which typically enhance this oxidation include storage at elevated temperatures, metal ions, and light exposure.

Hamm et al. (1968) autoxidized butter at -27, -10, 4, 21, 35 and 50°C. Autoxidation flavors were more intense at -27°C than at -10°C and intensity also increased as the temperature was raised above -10°C.

At the higher temperatures the peroxide value (PV) correlated significantly with flavor values. Pont and Gunnis (1958) found that storing butter at -23.3, -17.8, or -11.1°C had no significant effect on butter grade after three and six months. Dixon and Rochford (1973) observed similar results, but butter at -6.5°C experienced a serious decrease in grade over eight months, and the PV indicated chemical changes which paralleled the grade loss. Thus, it appears that butter stored between -10 and -27°C should be least susceptible to autoxidative deterioration.

Iron and copper levels in butter are greatly influenced by manufacturing methods and machinery since these metals can migrate into butter from water or equipment. McDowell (1964) has reported that if the copper content of the butter exceeds 0.080 ppm, the storage life of butter decreases, and an iron content of 0.50 ppm will cause off-flavor in the butter. Both metals are capable of catalyzing autoxidative deterioration in butter. Maloney and Armstrong (1969) surveyed the iron and copper contents of Alberta butter and found mean values to be under the levels prescribed, but samples were found which contained up to 0.176 ppm copper and 1.400 ppm iron.

When exposed to ultraviolet (UV) radiation, unsalted butter underwent less oxidation than salted butter based on flavor score and chemical analysis (Wilster, 1957). This was attributed to the ability of the chlorides of Mg, Ca, Al, Zn, and Be, found in some salts, to accelerate butterfat oxidation.

It has long been known that exposure to light accelerates deteriorative reactions in oils or fat-containing foods, leading to rancidity (Coe, 1941; Chahine and deMan, 1971). Light-induced oxidation of butter may occur when inadequately protected butter is illuminated. The severity

of deterioration depends upon factors such as the light source, wavelength of light, time of exposure, temperature of butter, distance of butter from light source and salt and β-carotene content of the butter.

Koo and Kim (1971), as cited by Sattar et al. (1976b), observed that sunlight has the strongest prooxidant activity followed by UV radiation, fluorescent and incandescent lights. In a study cited by Pont (1960), after short exposures, fluorescent light was more damaging to butter than was light from filament lamps, but with exposures of 4 hr or more, there was little difference. Cool-white, daylight, and gold fluorescent lamps had similar effects on milk fat oxidation (Du and Armstrong, 1970).

Only light which is absorbed can produce chemical changes (Coe, 1941). Not all wavelengths of light are equally absorbed, nor do they appear to promote oxidation of butter to the same extent. When the intensity of light was the same, Coe (1941) found that wavelengths below 490 nm promoted rancidity in oil-bearing foods most, while regions at 540 nm and above 740 nm promoted it light.

When butter was irradiated with a cool white fluorescent tube, the curve of relative radiant flux resembled the PV curve, except for the near UV region where the light energy showed an unusually large effect in promoting oxidation (Du and Armstrong, 1970). The other PV peaks were at 576 nm which corresponded to the maximum energy emission of the lamp and at 446 nm which corresponds to the region of maximum carotene absorption. Scott (1962) suggested that the range of wavelengths from 250-450 nm was most detrimental to butter and the use of filters which blocked wavelengths below 550 nm prevented pronounced off-flavors after 48 hr illumination. Similar results were reported by Sattar et al. (1976a) during irradiation of milk fat.

As would be expected, the longer the exposure to light, the more severe the butter deterioration (Scott, 1962; deMan et al., 1965; Gilchrist et al., 1968; Downey and Murphy, 1968). PV's and flavor scores vary widely for a given exposure time, and are dependent on other factors such as light source, distance, and temperature. After extinguishing the light source, the oxidation rate drops significantly (Sattar et al., 1976a, 1976b).

Chahine and deMan (1971) reported that at freezer temperatures the oxidative deterioration of illuminated corn oil was greatly delayed, but the earliest stage was not greatly affected. Light absorption, resulting in the primary oxidation events, is not temperature dependent (Livingston, 1961), but it is possible that the faster secondary stages of oxidation at higher temperatures are due to greater peroxide instability under these conditions, thus accelerating the free radical chain reaction. After the first 48 hours of illumination, the oxidation of milk fat was more rapid at 30°C than at 15°C (Sattar et al., 1976b), however, the effect was less obvious with butter (Gilchrist et al., 1968). The range of storage temperatures used in the industry probably has little effect on light-induced oxidation (Armstrong, 1967; Gilchrist et al., 1968).

As the distance from a light source increases, the intensity of light decreases (Gilchrist et al., 1968). Downey and Murphy (1968) state that the distance only affects the initial oxidation rate, with no effect on off-flavor intensity after prolonged exposure. When butter was illuminated in a display case 10, 20, or 30 inches from a fluorescent tube for up to two weeks, no significant differences in flavor were noted (deMan et al., 1965).

As mentioned previously, salt has a prooxidant effect on illuminated butter, presumably because of catalytic contaminants in the salt (Wilster, 1957). β-Carotene acts as an inhibitor of photooxidation in oils, and after the photo-bleaching of this pigment in butterfat, there is a rapid increase in PV (Sattar et al., 1976a, 1976b). Butterfat depigmented with charcoal showed an oxidation pattern at various wavelengths very different from that of untreated butterfat (Du and Armstrong, 1970). Oxidation occurred only with wavelengths below 410 nm. Addition of β-carotene to the depigmented butterfat did not alter this pattern except that it intensified oxidation in the near-UV range. Therefore, it appeared that unknown components of butter which may be removed by activated charcoal treatment, must promote oxidation in butterfat exposed to visible light (Du and Armstrong, 1970).

Prevention of Photooxidation by Packaging

Inhibition of light-induced deteriorative reactions may be facilitated through the use of packaging materials. This protective effect has been shown with oil-containing foods (Coe and LeClerc, 1932), such as potato chips (Sacharow, 1969), milk (Sattar and deMan, 1973), egg dough products (Acker and Greve, 1963), edible oils (Moser et al., 1965), as well as butter.

Butter prints were traditionally packaged in parchment which offered minimal light protection (Gilchrist et al., 1968) and often contained prooxidant substances such as copper, iron, or sulfuric acid (Pont, 1961). Random samples of parchment-wrapped butter from supermarkets were found to have undergone noticible oxidation (deMan et al., 1965).

Several studies have been initiated to determine the best butter

wrapping material. Charlton and Delong (1956) observed that parchment-wrapped butter, overwrapped in a waxed sulfite sheet developed rancidity in a dairy display cabinet in one week. Parchment-wrapped butter in waxed paperboard cartons showed no off-flavor after seven weeks.

By monitoring flavor scores, aldehyde value and PV, Wilster (1957) examined butter in several packaging systems which had been exposed to fluorescent light. Parchment, parchment overwrap, pliofilm, and parchment plus pliofilm-wrapped butters were very tallowy within one week. Parchment plus cellophane-wrapped butter was tallowy by four weeks and samples wrapped in foils, paraffined cartons and "Mullinix wrap" were protected from light. Aldehyde values and PV's also demonstrated these effects and increased rapidly as flavor scores decreased.

Downey and Murphy (1968) tested parchment, moisture-proof white paper, moisture-proof metal-pigmented paper and an aluminum foil laminate for their protection against light-induced oxidation. Only those wrappers which transmitted no light (foil) were completely satisfactory. When the transmission value approached 0.5%, extensive oxidation took place after three days of exposure.

Emmons and coworkers (1981) exposed butter to fluorescent light of different intensities for up to six days and subsequently stored them in the dark at $5^{o}C$ for 1, 4 or 8 weeks. Overall, the aluminum foil laminated to bleached wet strength sulfite offered the best protection. Metallized aluminum on wet strength sulfite and vacuum metallized aluminum on parchment sheet were considered marginally acceptable based on oxidized flavor scores and peroxide values. The others including parchment, overwaxed yellow bleached sulfite, and several high density polyethylene wraps containing mica and/or TiO_2 were considered

unacceptable. Butter wrapped in these materials experienced significant oxidized flavor development and PV increases.

On several occasions the inadequacy of parchment or paper as a light barrier has been noted, along with recommendations for wrapping butter in opaque materials, as is the practice in several European countries (Charlton and Delong, 1956; Wilster, 1957; Scott, 1962; deMan et al., 1965; Sattar et al., 1976a; Paquette et al., 1981). However, for reasons which perhaps include economics or consumer appeal, many butter manufacturers have ignored such advice.

MATERIAL AND METHODS

Butter

A sixty-eight pound block of freshly churned salted butter was purchased from Michigan Milk Producers Association (Ovid, MI), stored at -20°C and used for the duration of the butter study to avoid variations among butter samples.

Standard Cholesterol Oxidation Products

Standard 5-cholesten-3β-ol, 5-cholesten-3β,7α-diol, 5-cholesten-3β,7β-diol, 5-cholesten-3β-ol-7-one, 3,5-cholestadien-7-one, 5-cholesten-3β,25-diol, cholestan-5α,6α-epoxy-3β-ol, cholestan-3β,5α,6β-triol, 4-cholesten-3-one, 5-cholesten-3-one, 5-cholesten-3β,4β-diol, 5α-cholestan-3,6-dione, cholestan-3β,5α-diol-6-one, and 5α-cholestan-3β-ol-6-one were purchased from Steroloids Inc., Wilton, N.H.

Other Chemicals

Oleic acid was purchased from Eastman Kodak (Rochester, N.Y.) while linoleic acid was obtained from Fisher Inc. (Pittsburgh, PA) for use in the model system study. Redi/Plate (0.25 mm thick) precoated Silica Gel G plates (20 X 20 cm), were obtained from Fisher Inc. (Pittsburgh, PA). All other reagents and chemicals used were reagent grade.

Edible Materials

The unsalted soda crackers used in sensory panels, and all cookie ingredients were national brand name items which were purchased at Meijers Inc., Okemos, MI.

Packaging Materials

Heavy duty aluminum foil (0.96 mil) was purchased from Reynolds Metal Co. (Richmond, VA). Paper based materials were donated by West Carrollton Parchment Co. (W. Carrollton, Ohio). These include: 1) a 27 lb (basis weight) "margarine wrap" (2.4 pt) which is a paper made of a highly refined pulp containing hardwood and softwood fibers; 2) a 20 lb wet strength dry wax paper (2.4 pt) which has a tissue paper base with wax incorporated into the paper; 3) a 27 lb opaque parchment (2.2 pt) which is made by acid treatment on good quality chemical pulp. A high density-low density polyethylene coextruded film (0.038 lb/rm, 2.2 mil), impregnated with butylated hydroxyanisole on the high density side, was obtained from Crown Zellerbach (Greensburg, IN). Low density polyethylene (2.0 mil) was obtained from Arco Polymers (Philadelphia, PA). These materials shall be referred to in this study as foil, margarine wrap (MW), dry wax paper (WSDW), opaque parchment (OP), BHA-PE, and PE respectively.

Light Sources

A General Electric 20 watt cool white fluorescent tube (23 inches) was used as a light source in all investigations except the packaging study in which a Sears 20 watt cool white fluorescent tube was used.

1. Model System

A. Sample Preparation and Illumination

Solutions of cholesterol in linoleic and oleic acids were prepared by adding 0.12 g cholesterol to 600 g of the fatty acid. Each was divided into three 200 g portions which were placed in 400 ml beakers. For each set of three beakers, one was wrapped in foil and stored at 5°C, one was illuminated at 5°C as described below, and one was illuminated at 5°C while aerated with a slow stream of air which was filtered through a plug of glass wool.

During illumination, samples were placed in an aluminum foil-lined box (34 X 64 X 22 cm). The fluorescent tube was suspended approximately 17 cm above the bottom of the box where the light intensity measured approximately 1500 lux (Luna Pro by Gossen, W. Germany). The box was kept in a dark room at 4°C, to eliminate light from other sources.

Samples of the illuminated oils were taken every four days until day 20, and at 30, 41, and 61 days. Dark samples were taken at 20, 41, and 61 days. The peroxide value was measured and a portion of the lipid was saponified for cholesterol analysis.

B. Peroxide Value

Peroxide values of lipid samples were determined using the AOCS Official Method Cd 8-53. A 3 g rather than a 5 g sample size was used.

C. Saponification and Extraction of Non-saponifiable Material

The saponification and extraction procedures of Itoh et al. (1973) were performed to separate the cholesterol and any oxidative derivatives from the oil samples. A 7.5 g sample of lipid and 75 ml of 1.0 N alcoholic KOH were refluxed for 1 hr followed by multiple extractions

with isopropyl ether. The ether extracts were combined and washed with distilled water, dried with sodium sulfate, and evaporated to dryness at 40°C in a Buchi Rotovapor R rotary evaporator (Buchi Inc., Switzerland). This non-saponifiable material was then dissolved in a known volume of ethyl acetate, nitrogen flushed, and stored at -20°C until analysis.

D. Thin Layer Chromatographic Analysis of Cholesterol and Oxidation Products

Precoated analytical plates were activated at 110°C for 1 hour. One-dimensional thin layer chromatograms were made by spotting 30 µl of the non-saponifiable solutions on the plates and developing three times in a heptane-ethyl acetate (1:1, v/v) solvent system, with air drying between irrigations. The plates were then sprayed with 50% H_2SO_4 and heated at 110°C for 10 min for color development. R_f values and spot colors were recorded. Thin layer chromatograms of standard cholesterol oxidation products were run under identical conditions.

Two-dimensional thin layer chromatography was performed by spotting 30 µl of the non-saponifiable solution on a 20 X 20 cm plate. Triple irrigation in the first dimension using heptane-ethyl acetate (1:1, v/v) was followed by a double irrigation in the second dimension using a benzene-ethyl acetate (3:2, v/v) solvent system. Visualization by 50% H_2SO_4 was performed as previously described.

E. Gas Chromatographic Analysis of Cholesterol Oxidation Products

The ethyl acetate solutions of non-saponifiable fractions were analyzed by packed column gas-liquid chromatography using a Hewlett Packard Model 5840A gas chromatograph equipped with a flame ionization detector and a 2 m X 4 mm i.d. glass column packed with 3% SP-2100 on 100-120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). The

chromatograph was operated isothermally at 260°C with a nitrogen flow rate of 25 ml/minute. The temperatures of the injection port and detector were maintained at 280°C and 350°C, respectively. Sample volume was 1 µl.

2. Butter Systems

A. Sample Preparation, Illumination, and Sampling Schedule

1. Unpackaged Blocks. Butter was cut into blocks measuring approximately 65 X 25 X 20 mm. These blocks were arranged in parallel rows, on the bottom of the previously described illumination box, so that one 65 X 25 mm side faced the light, and with at least 20 mm between blocks on all sides. Temperature and light intensity were identical to those used for the model system. At least six blocks were randomly selected every four days. Two blocks were analyzed immediately, with the remainder stored in the dark at -20°C. Two of the stored samples were analyzed after two months, and the remaining two samples after four months.

2. Unpackaged Surface Layers. To investigate changes specifically at the surface of the butter, twelve blocks of butter measuring 60 X 70 X 15 mm were cut, wrapped in wax paper and foil and stored in the dark at 4°C until illuminated. Every four days, two samples were placed in the illumination box under conditions described previously (Part 1A). After 20 days, samples which had been illuminated for 0, 4, 8, 12, 16, and 20 days were removed for analysis. Using a stainless steel cheese wire, a 5 mm layer was removed from the exposed face (60 X 70 mm) of the butter block for further analysis.

3. Packaged Slices. Butter slices measuring 150 X 65 X 5 mm were wrapped in aluminum foil, opaque parchment, margarine wrap, dry wax

paper, polyethylene and BHA-impregnated polyethylene, so that the 150 X 65 mm face exposed to the light was in close contact with one layer of packaging material. The butter wrapped in the BHA-PE film was wrapped so that the low density side was in contact with the butter. No attempt was made to make packages air-tight. Samples were placed in the bottom of the foil-lined box and illuminated at 5°C with two Sears fluorescent tubes at an intensity of approximately 1500 lux. Butter samples wrapped in each material were analyzed at 0, 5, 10, and 15 days.

4. Simulated Home-use Study. To simulate possible light exposure at the consumer level, blocks of butter (70 X 40 X 15 mm) were placed on plastic weigh boats and exposed to six different time-temperature-light source combinations. Two were stored in the dark at 4°C or 22°C. Two were exposed to fluorescent room light on the benchtop at 22°C, one for 8 hr/day (5 day/wk), and the other for two 1½ hr periods per day (5 day/wk). The last two were held at 22°C on a north-facing windowsill and exposed to August daylight (no direct sunlight) for 8 hr or two 1½ hr periods per day (5 days/wk). Samples were analyzed at 0, 1, and 2 weeks.

B. Preparation and Analysis of Butterfat

Butter samples were melted in 250 ml beakers in a 40°C water bath, followed by separation of lipid from non-lipid layers in an IEC Clinical Centrifuge Model CL (Damon, Needham Hts, MA) set at speed 4 for 3 minutes. The lipid layer was decanted following removal of any foam on top. Peroxide values were determined and solutions of non-saponifiable materials were prepared as previously described. Thin layer and gas-liquid chromatographic analyses were also performed on the non-saponifiable fractions as discussed before (Parts 1D, 1E) with the following exceptions:

1) For unpackaged surface layers, only the day 20 sample was analyzed by two-dimensional TLC. The solvent system employed for the second dimension was benzene-ethyl acetate (3:2, v/v).

2) For packaged butter slices, ethyl acetate solutions of the non-saponifiable material were concentrated five-fold prior to TLC analysis.

3) GLC results are reported only for the unpackaged surface layer samples.

C. Sensory Analysis--Hedonic Scores

Sensory analysis of the unpackaged butter blocks was performed after 0, 4, and 8 days of illumination. The butter blocks were uniformly blended and approximately 1.5-2.0 g butter was spread on an unsalted soda cracker and refrigerated until served. Untrained panelists tasted one sample at a time and marked the score sheet (Appendix I) to indicate how well they liked the sample. The order of sample presentation was randomized and rinse water was provided between samples. Sensory scores were analyzed using the t-by-difference test (ASTM, 1968).

D. Fatty Acid Analysis

Fatty acid analyses were performed on the butter taken from the surface layers immediately after illumination. Butterfat samples were methylated by the boron trifluoride-methanol method of Morrison and Smith (1964) utilizing the preparative conditions for triglycerides. Analysis of fatty acid methyl esters was carried out with a Hewlett Packard Model 5840A gas chromatograph using a glass column (2 m X 4 mm i.d.) packed with 15% diethylene glycol succinate on Chromosorb W 80/100 mesh (Supelco Inc., Bellefonte, PA). The analysis was performed isothermally at 190^{o}C with injection port and flame ionization detector

temperatures at 210°C and 350°C, respectively. Nitrogen carrier gas flow rate at the detector was 30 ml/minute. Peak retention times were compared to those of a standard mixture of fatty acid methyl esters.

E. BHA Analysis

Analyses were performed on duplicate butter samples wrapped in BHA-PE film after various periods of illumination. Butter was dissolved in HPLC-grade chloroform (MCB, Inc., Cincinnati, OH) and passed through a sodium sulfate column to remove any moisture. BHA analyses were performed using a Waters Associates ALC 201, 202 High Pressure Liquid Chromatograph equipped with a Model 440 absorbance detector at 284 nm. The column (30 cm X 3.9 mm) was packed with μ-Porasil and the injector system was a Model U6K. The carrier solvent was chloroform. The flow rate was set at 1.0 ml/min with a retention time of 4.5 minutes. A sample of 100 μl was injected for each analysis.

F. Analysis of Packaging Materials

1. Thickness. The thickness of the six packaging materials was determined by averaging at least five values as measured with a micrometer (Model 549M, Testing Machines Inc., Amityville, NY).

2. Light Transmission. Using a Beckman DK-2A Ratio Recording Spectrophotometer (Palo Alto, CA), the light transmission characteristics of packaging materials were determined over the scanned range of 230-700 nm using tungsten and hydrogen light sources. The sample consisted of a quartz cuvette wrapped in such a way that the light beam penetrated one thickness of the packaging material. The reference beam passed through a quartz cuvette only.

3. Analysis of Cookies Made from Illuminated Butter

A. Formulation and Baking

Butter (455 g) was mixed with 400 g sugar, 2.5 ml vanillin and 5 ml water, followed by the addition of 500 g all-purpose flour, 3.6 g salt, 2.6 g cream of tartar, and 2.6 g baking soda. The dough was formed into one inch balls and flattened on an ungreased baking sheet with a beaker bottom dipped in sugar. The cookies were baked 14 min at 350^{o}F in a Rotary Hearth Test Baking Oven (National Manufacturing Co., Lincoln, NE).

Fresh butter and unpackaged butter slices (5 mm) which had been illuminated as described previously, for 10 days, were used in the making of cookies. Cookies were stored at 22^{o}C for 0, 1, 2 weeks and at -20^{o}C for 1 and 2 months before analysis.

B. Lipid Extraction and Analysis

The lipids in the uncooked dough and baked cookies were extracted by blending 80 g of cookies or dough with two aliquots (200 ml followed by 150 ml) of hexanes-diethyl ether (95:5, v/v) in a Virtis homogenizer Model 45 for 2 minutes. Combined extracts were filtered and dried over anhydrous sodium sulfate overnight. The solvent was removed in a Buchi rotary evaporator. Ethyl acetate solutions of non-saponifiable material were obtained by saponification and extraction methods described in parts 1B and 1C. One-dimensional TLC was performed as previously described.

The two butter samples used in the dough formulation were also analyzed for the presence of cholesterol oxidation products as in part 2B.

Lipids were extracted from 200 g of all-purpose enriched bleached flour using the cookie extraction procedure. The non-saponifiable material was analyzed by one-dimensional TLC following saponification and extraction.

4. Oxidation of Cholesterol in Solution

Photosensitized oxidation of cholesterol was carried out with hematoporphyrin or riboflavin as sensitizers, using a procedure adapted from Kulig and Smith (1973). Riboflavin (17.2 g) or hematoporphyrin (17.8 g) was added to solutions of 1.0 g cholesterol in 150 ml pyridine. The solutions were bubbled with a slow stream of air and irradiated with fluorescent and UV (366 nm) light for 24 hours. The pyridine solutions were analyzed by TLC using triple irrigation in ethyl acetate-heptane (1:1, v/v) or ethyl acetate-benzene (1:1, v/v) solvent systems. The solutions were also reduced by spotting sodium borohydride in ethanol solution over the sample spot on the TLC plate prior to irrigation or by adding excess sodium borohydride to the pyridine solutions in a test tube. This was followed by TLC analysis in the solvent systems mentioned previously on plates with illuminated butter samples.

Sterol hydroperoxides were visualized on TLC plates by the method of Smith and Hill (1972) using a N,N-dimethyl-p-phenylenediamine dihydrochloride spray. This was followed by spraying with 50% H_2SO_4 and heating at 110^{o}C for 10 min to visualize all compounds present.

An ethyl acetate solution of cholesterol was oxidized at room temperature in the dark by addition of cumene hydroperoxide. This solution was analyzed by TLC before and after sodium borohydride reduction as discussed above.

RESULTS AND DISCUSSION

I. Fluorescent Illumination of Fatty Acid Solutions of Cholesterol

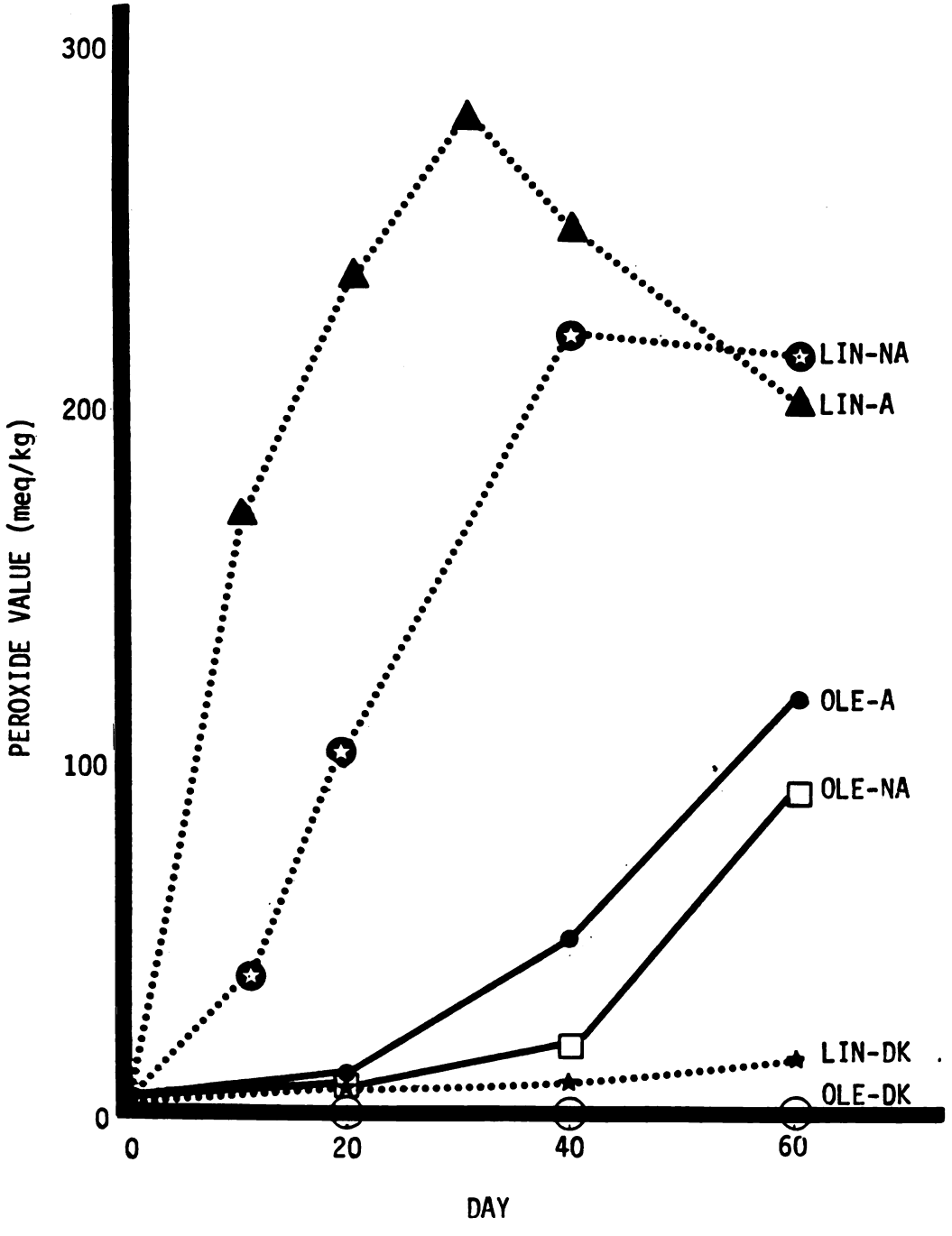
A. Effects of illumination, aeration, and degree of unsaturation of system on peroxide value

The changes in PV over time of oleic acid and linoleic acid solutions of cholesterol after exposure to light under various conditions are represented in Figure 3. None of the solutions were free from peroxides on day 0. After relatively slow initial PV increases, of varying time lengths, the rate of peroxide formation increased in the illuminated samples. The PV leveled off or began to decline after reaching very high levels, in the illuminated linoleic acid samples. These patterns are consistent with those expected during the initiation, propagation, and termination reactions of autoxidation of fatty acids since the PV test measures the concentration of primary products of lipid oxidation (Holman, 1954, as cited by Gray, 1978). After 61 days, the PV of the samples stored in the dark increased slowly, with the PV of the LIN-DK samples increasing at a greater rate than that of the OLE-DK samples. Neither reached the level at which the illuminated samples experienced rapid peroxide formation.

On all days of analysis, the linoleic acid solution had a greater PV than the similarly treated oleic acid solution. Susceptibility to and rate of fatty acid oxidation increase in a geometric fashion relative to degree of unsaturation (Labuza, 1971). Initial differences in PV may also have contributed to the observed differences in the samples during storage.

Figure 3. Effect of illumination, aeration, and degree of unsaturation of media on peroxide value of linoleic and oleic acid solutions of cholesterol.

LIN - Linoleic acid
OLE - Oleic acid
A - Aerated and illuminated
NA - Illuminated, not aerated
DK - Held in dark, not aerated


300
200
100
0
PEROXIDE VALUE (meq/kg)
0
20
40
60
DAY
LIN-NA
LIN-A
OLE-A
OLE-NA
LIN-DK
OLE-DK

Exposure to light appeared to be responsible for higher PV's in both linoleic and oleic acid systems, especially after long exposure times. This may have been due to enhanced peroxide decomposition which would promote further free radical chain reactions (Carlsson et al., 1976), and/or to photosensitized oxidation if appropriate sensitizers were present as contaminants of the fatty acids or the cholesterol.

Bubbling air through the systems in the presence of light enhanced oxidation, possibly by increasing the surface area exposed to oxygen, which is necessary for peroxide formation (Dugan, 1976). In both systems, the increased oxygen availability appeared to accelerate the autoxidation cycle by shortening the induction period or increasing the rate at which the peroxides formed. The LIN-A system showed erratic PV's at the very high levels, indicative of the transitory nature of peroxides (Gray, 1978). The apparent decrease in PV in the last analyses may indicate formation of secondary lipid oxidation products from the primary peroxides (Holman, 1954, as cited by Gray, 1978).

B. Effects of illumination, aeration, and degree of unsaturation of system on cholesterol oxidation

Solid cholesterol is relatively stable to air oxidation, but is readily oxidized when present in lipid films, where cholesterol stability is closely linked to the stability of the lipid to autoxidation (Norcia, 1961). It has been shown that in corn oil, added cholesterol oxidized before the PV reached 100 and continued well beyond the point of secondary decomposition of peroxides, indicating that the cholesterol was oxidized by propagative reactions (Norcia, 1961).

Cholesterol oxidized in all illuminated linoleic and

oleic acid systems. No cholesterol oxidation was apparent in the unilluminated systems. The illuminated systems differed in the number of oxidation products formed, the time of appearance of these products, as well as their relative concentrations after 61 days. Cholesterol in the aerated, illuminated linoleic acid (LIN-A) system was most susceptible to oxidation as indicated by the number of apparent oxidation products separated by thin layer chromatography (Table 3).

In the LIN-A system, at least 16 compounds formed after 61 days and are presumed to derive from cholesterol (R_{f1} = 0.85) or the linoleic acid non-saponifiable component at R_{f_1} = 0.98. This contaminant may very likely be a carotenoid compound. In a previous experiment, β-carotene was found to produce no TLC-visible compounds following photooxidation. Thus, it is very likely that all 16 newly formed compounds derive from cholesterol.

The LIN-NA system exhibited less oxidative deterioration than the LIN-A system; 8 compounds were formed at much lower levels than in LIN-A. By day 61 only four compounds were present in the OLE-A and OLE-NA systems with greater concentrations observed in the aerated sample. All oxides present in OLE samples were also present in LIN samples indicating that the same oxidative mechanism was probably occurring in both fatty acid systems.

The mode of cholesterol oxidation in these systems appears to be a free radical chain mechanism. The mobilities and color of the blue spots at R_{f1} = 0.39 and 0.45 matched those of 5-cholesten-3β,7α-diol and its 7β epimer which are common products of cholesterol oxidation by a free radical mechanism (Smith, 1980). The apparent absence of 6-cholesten-3β,5α-diol suggests that singlet oxidation of cholesterol

Table 3. TLC analysis in two solvent systems of non-saponifiable material from illuminated and illuminated + aerated fatty acid solutions of cholesterol.

Spot Color	R_{f1}[a]	R_{f2}[b]	Intensity With Time[c]	First Day of Appearance[de]			
				LIN-A	LIN-NA	OLE-A	OLE-NA
Purple	38	24		61			
Blue	39	24	increase	20	41	61	61
Blue	45	32	increase	20	41	61	61
Purple	51	42		61			
Blue	58	45		61	61		
Blue	62	48		61			
Yellow	64	49	increase	20	61	61	61
Blue	67	52		61			
Purple	70	56	increase	41			
Not Distinct	74	58		61	61		
Purple	79	63	increase	41	61		
Purple	81	67		61			
Magenta	85	73	no change	0	0	0	0
Purple	90	79	increase	41			
Purple	93	82	increase	41			
Not Distinct	95	86	increase	30	61		
Purple	98	93	decrease	0	0	0	0
Brown-Red	99	93		61	61	61	61
Standard Reference Compounds							
5-Cholesten-3β,7α-diol	39	24					
5-Cholesten-3β,7β-diol	45	32					

[a] Solvent system: ethyl acetate - heptane 1:1 (v/v); R_f reported X 100
[b] Solvent system: Benzene - ethyl acetate 3:2 (v/v); R_f reported X 100
[c] For those appearing before day 61
[d] LIN = linoleic acid solution; OLE = oleic acid solution; A = aerated and illuminated; NA = illuminated only (no aeration)
[e] Samples analyzed on days 0, 2, 4, 8, 12, 16, 20, 30, 41, and 61

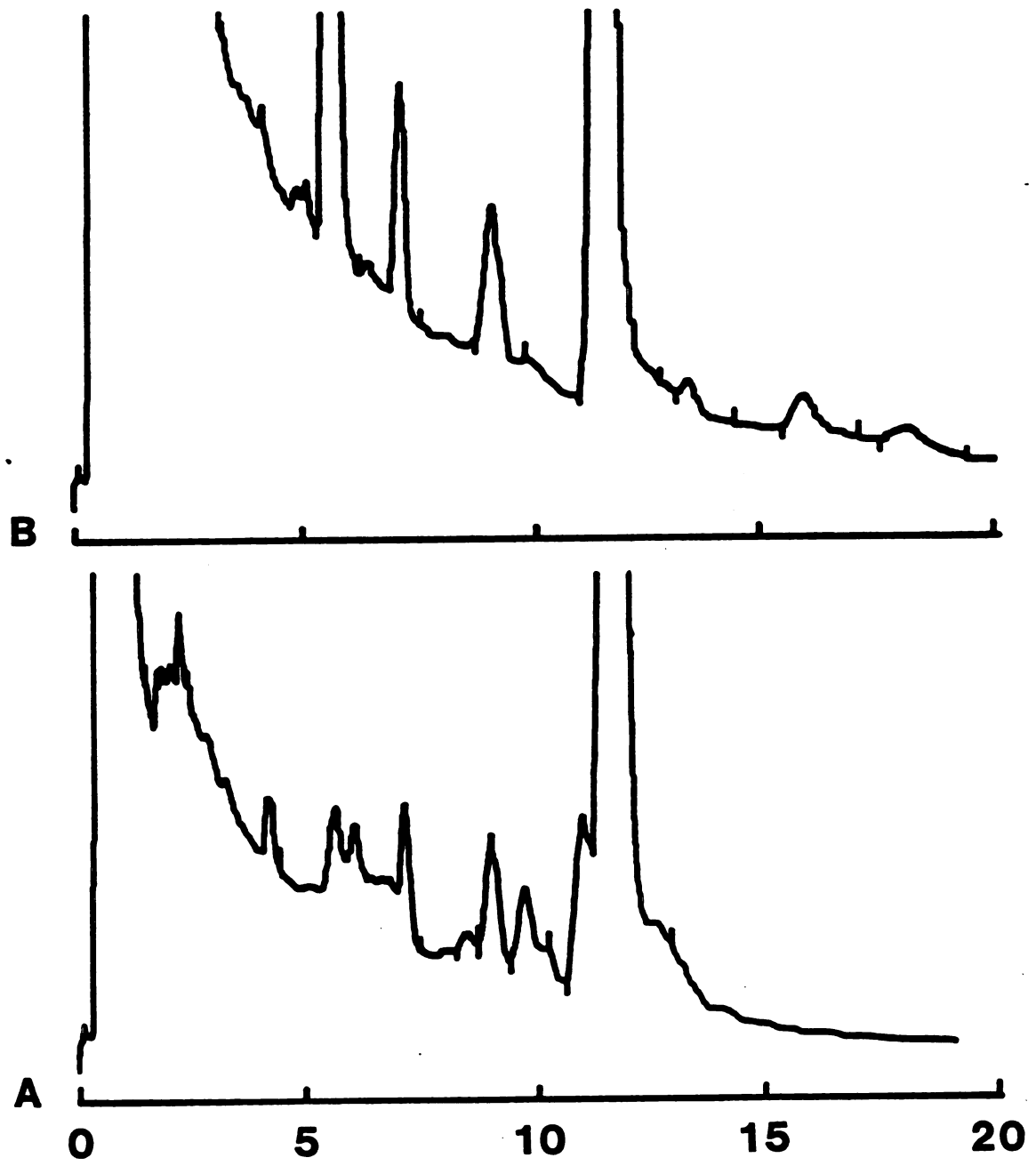
is not occurring (Smith, 1980). Lack of known sensitizers also supports this hypothesis.

Because no cholesterol oxidation was observed in the systems held in the dark, it is obvious that light accelerates this reaction. This is believed to be due to accelerated fatty acid oxidation. It is possible that free radicals formed in this process must reach a certain concentration before they will begin to attack the cholesterol. This "threshold" level of oxidation appears to depend on the degree of unsaturation of the fatty acid since cholesterol oxidation did not begin in linoleic acid systems below PV = 170 while it occurred in oleic acid systems below PV = 100. Since cholesterol is monounsaturated, it is possible that it competes more easily with oleic rather than linoleic acid as a substrate during free radical autoxidation.

The presence of bubbled air also enhanced cholesterol oxidation. This effect is probably also due to the acceleration of oxidation of the lipid system as evidenced by peroxide values.

As discussed in the literature review, there are many problems and ambiguities associated with the monitoring of cholesterol oxidation by gas-liquid chromatography (GLC). However, the appearance and disappearance of some compounds in illuminated aerated versus unilluminated quiescently-stored linoleic acid systems after 61 days is shown in Figure 4. Those peaks forming with retention times greater than 11.5 min. represent compounds more polar than cholesterol which would include any oxygenated cholesterol derivatives.

Figure 4. Gas chromatograms of the non-saponifiable material in linoleic acid solutions of cholesterol after 61 days (A) in the dark, not aerated (LIN-DK) and (B) illuminated and aerated (LIN-A).


B
A
0
5
10
15
20

II. Fluorescent Illumination of Unpackaged Butter at 5°C

Butter contains 0.4% non-saponifiable material, approximately half of which is cholesterol (Swern, 1979). During its production and distribution, butter is exposed to light in creameries (0.4 - 2.4 $\mu W/cm^2$) (Gilchrist et al., 1968) and in retail display cabinets (3.0 - 15 $\mu W/cm^2$, 10 - 5400 lux) (Gilchrist et al., 1968; Paquette et al., 1981) usually in the form of fluorescent light.

The radiant flux of a cool white fluorescent lamp is shown in Figure 5 (Du and Armstrong, 1970). Light between 300 and 700 nm from fluorescent sources is detrimental to butter, especially in the 500 to 600 nm wavelength range (Emmons et al., 1981). Ultraviolet radiation is most destructive at any given intensity, but cool white fluorescent lamps emit relatively small amounts (Coe, 1941; Paquette et al., 1981).

Cholesterol undergoes oxidative changes when exposed to fluorescent light in a model lipid system. Under certain oxidizing conditions, cholesterol may form angiotoxic (Imai et al., 1976; Peng et al., 1978) or carcinogenic compounds (Bischoff, 1969; Smith and Kulig, 1975). Taylor and coworkers (1979) indicated a need for an investigation of cholesterol-containing foods which may be exposed to such oxidizing conditions. Exposure to fluorescent light has been known to cause deteriorative reactions in butter (Scott, 1962; deMan et al., 1965; Armstrong, 1967; Sattar and deMan, 1975) but the oxidative state of cholesterol after such exposure has never been investigated.

Comparisons were made of PV and relative concentration of cholesterol oxidation products between entire butter blocks and surface layers (5 mm) of butter blocks exposed to fluorescent light at 5°C. At all time intervals, the PV's were higher in the surface layer (Figure 6).

Figure 5. Radiant flux of a cool white fluorescent lamp. (From Du and Armstrong, 1970.)

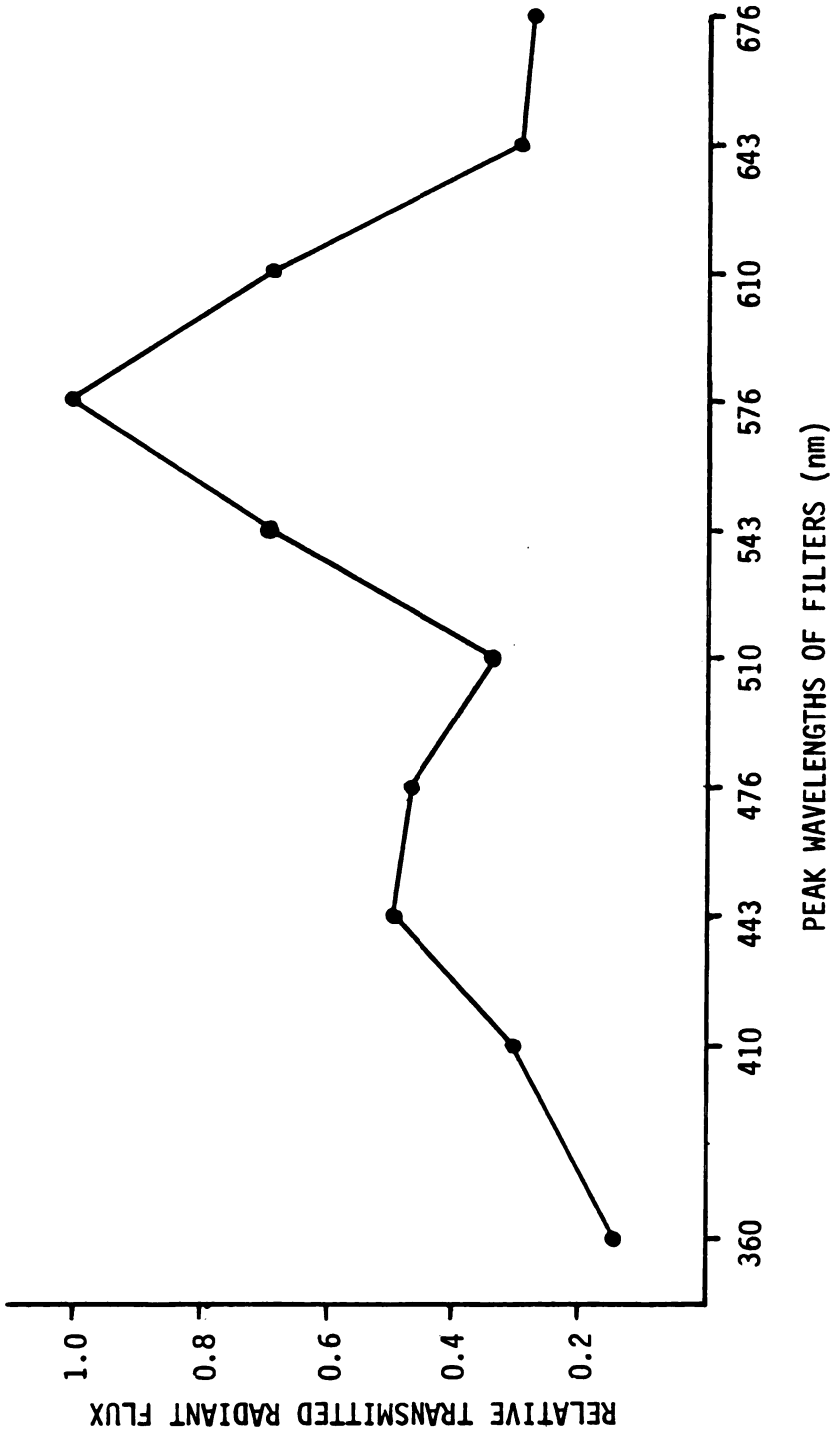

RELATIVE TRANSMITTED RADIANT FLUX
1.0
0.8
0.6
0.4
0.2
360
410
443
476
510
543
576
610
643
676
PEAK WAVELENGTHS OF FILTERS (nm)

Figure 5. Radiant flux of a cool white fluorescent lamp. (From Du and Armstrong, 1970.)

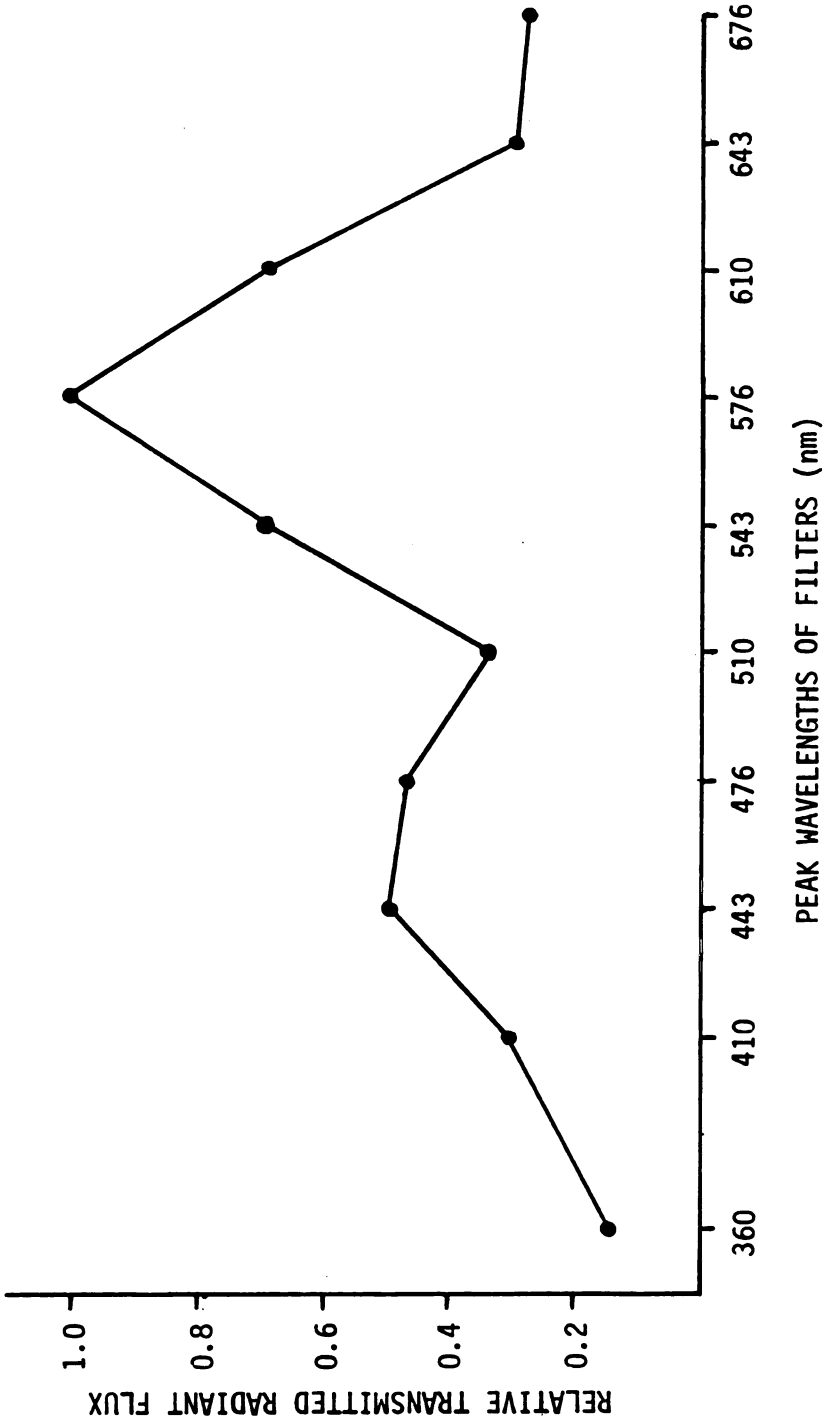
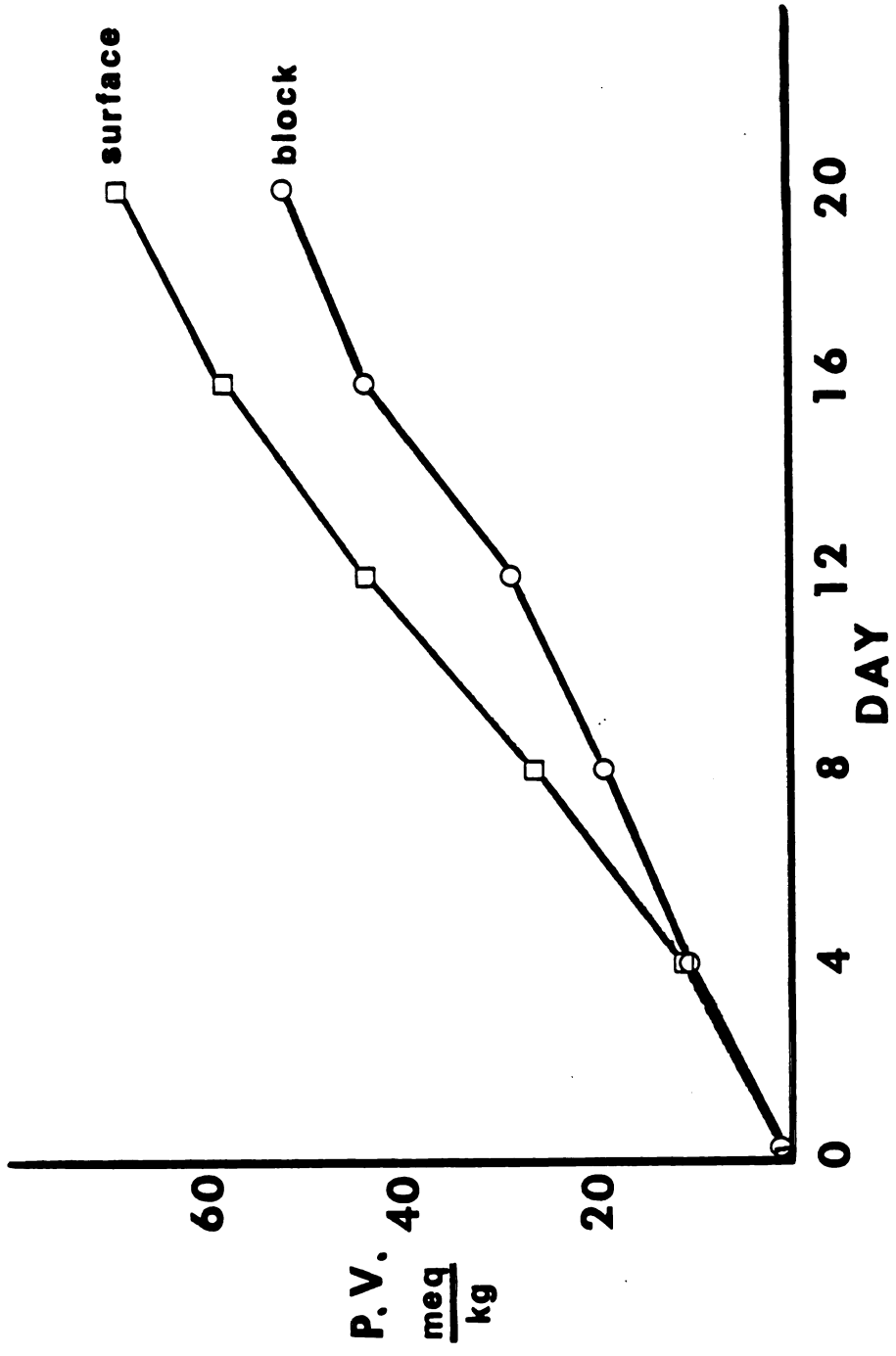

RELATIVE TRANSMITTED RADIANT FLUX
1.0
0.8
0.6
0.4
0.2
360
410
443
476
510
543
576
610
643
676
PEAK WAVELENGTHS OF FILTERS (nm)

Figure 6. Effect of illumination on peroxide value of entire butter blocks and surface layers (5 mm) of butter.


surface
block
60
40
20
P.V.
meq/kg
0
4
8
12
16
20
DAY

Similarly, it was found that cholesterol oxides were more concentrated in the surface layer than throughout the entire block. These effects were not due to oxygen availability differences since churned butter contains 5% air by volume (Pont, 1961), a level which permits autoxidation in the interior to occur at the same rate as at the surface. It can be concluded, therefore, that the observed differences were light-induced, and further studies were performed on the surface layer samples.

A. Effect of illumination on the stability of butter lipids in the surface samples

Carotenoid compounds in the butterfat interfered with the end-point in the PV determinations in samples exposed for short periods of time, but some trends were observable. Figure 6 shows an almost linear rate of peroxide development between days 4 and 16 in the surface samples, with lower rates before and after this time. Exposure of fat to UV radiation was found to greatly decrease the induction period of oxidative reactions (Holm et al., 1927, as cited by Sattar and deMan, 1975). PV has been found to indicate the extent of exposure of fats and oils to light since the concentration of the primary products, hydroperoxides, is approximately proportional to total light absorbed in the early stages (Sattar et al., 1976a). Other researchers have also observed the PV to increase after exposure of butter to light (Gilchrist et al., 1968; Du and Armstrong, 1970).

The fatty acid composition of the butter samples was determined before and during illumination. The distribution of six major fatty acids in the butter surface samples before and after 20 days of illumination is shown in Table 4. It can be seen that the fresh butter used

in these experiments had a higher palmitic acid and a lower linolenic acid content than those reported in the literature (Swern, 1979). Sampling procedures or animal feed differences may account for this. All other values fell within literature ranges.

Table 4. Fatty acid analysis of butter surface layers (5 mm) before and after fluorescent illumination for 20 days.

	Fatty Acid Composition (%)		
Fatty Acid	Day 0	Day 20	Swern, 1979
Palmitic acid	30.0	29.6	22 - 25
Palmitoleic acid	2.6	3.1	2.3 - 3.0
Stearic acid	12.7	12.5	11 - 14
Oleic acid	28.4	27.4	25 - 30
Linoleic acid	2.8	2.9	1.2 - 2.8
Linolenic acid	1.3	1.0	2.3 - 2.6
$\frac{\text{Saturated}}{\text{Unsaturated}}$ (ratio)	1.22	1.23	

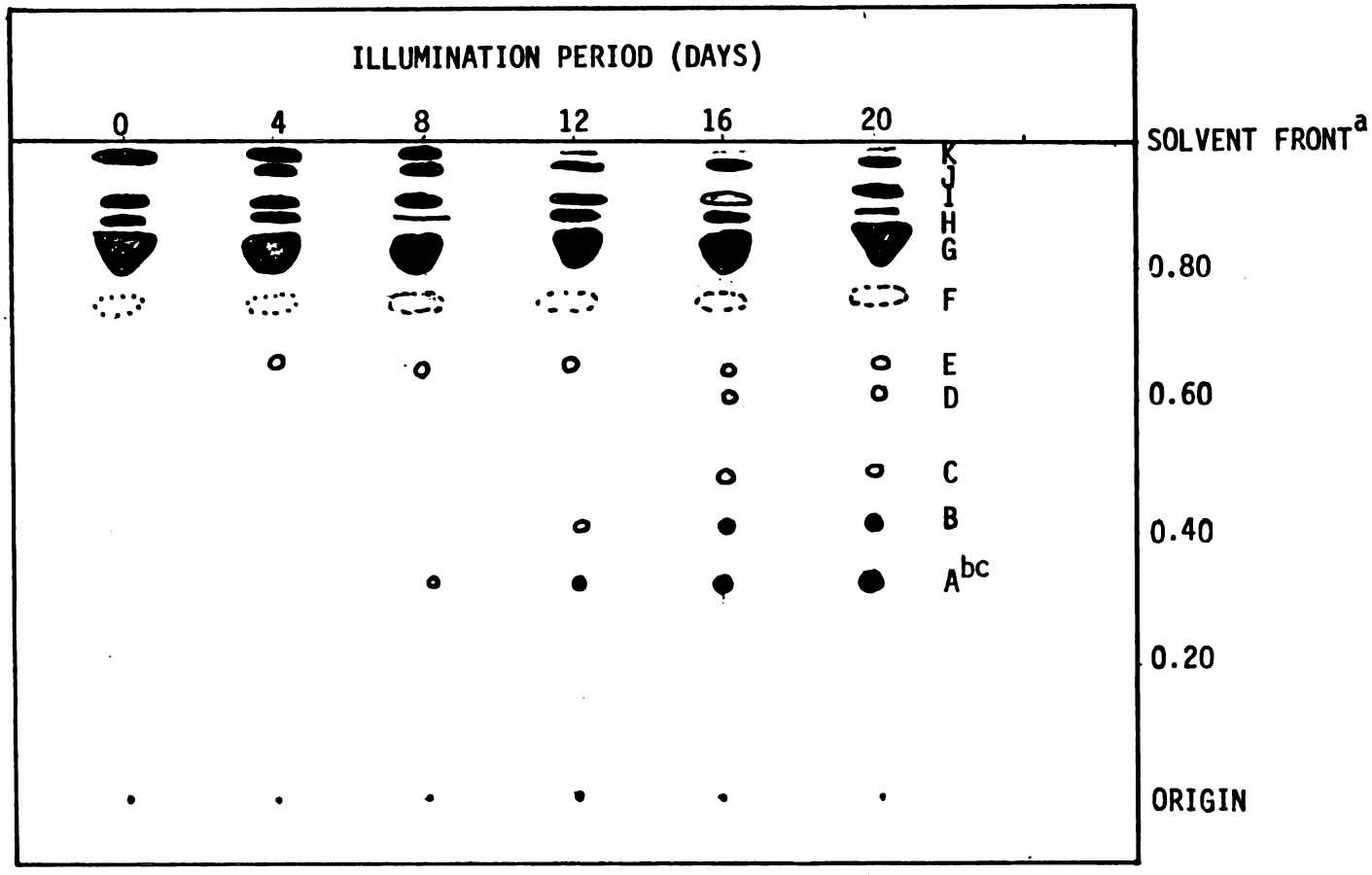
During autoxidation, the more unsaturated fatty acids undergo oxidation at a faster rate due to a lower activation energy, than the more saturated ones (Gunstone and Hilditch, 1945, as cited by Sattar et al., 1976b). However, when Sattar and coworkers (1976b) studied the photooxidation of several oils and fats they observed that the rate of oxidation did not depend entirely on the degree of unsaturation or on the ratios of linolenic to linoleic acid or linoleic to oleic acid. It was assumed that spontaneous autoxidation and photooxidation proceeded by different mechanisms, as suggested by Lundberg (1962). Thus, butter, with a relatively low level of polyunsaturated fatty acids may be expected to undergo photooxidation.

During the course of this photooxidation of butter, only the linolenic acid percentage appeared to drop, while the relative concentrations of the other fatty acids did not follow any particular pattern related to their degree of saturation. It is probable that during photooxidation, the primary attack was more or less random with respect to the fatty acyl groups involved. The linolenic acyl groups may have been more susceptible to further attack once the chain reaction was initiated, thus leading to its greater loss.

Although no formal determination of β-carotene levels was made, a loss of yellow color was observed with time, during the illumination period. A spot which was colored yellow prior to H_2SO_4 treatment on the TLC plates also decreased in intensity with time. β-Carotene may have strong protective properties in some fats and oils exposed to photooxidative conditions (Foote and Denny, 1968; Sattar et al., 1976b; Matsushita and Tereo, 1980). However, oxidation of β-carotene is stimulated in the presence of an oxidizing unsaturated fat (Holman, 1949) or during photooxidation (Seely and Meyer, 1971). In the latter case, as the β-carotene breaks down, oxidation products are produced which may generate free radicals (Seely and Meyer, 1971). This may account for the rapid increase in PV after photobleaching of β-carotene (Sattar et al., 1976b), and may accelerate oxidation of many lipid components in photobleached butterfat.

TLC analysis indicated that cholesterol in butter underwent oxidative changes during butter illumination. The non-saponifiable matter extracted from butter after various illumination periods is represented in Figure 7. In the original butter (day 0), five compounds were present: cholesterol (R_f 0.84, G), carotenoids (R_f 0.98, K), a possible

Figure 7. TLC analysis of the non-saponifiable material in unpackaged butter (5 mm surface layer) samples after fluorescent illumination.



[a] Solvent System: ethyl acetate - heptane 1:1 (v/v)
[b] Colors after H_2SO_4 treatment: A, B, C - blue, F - yellow, G - magenta (cholesterol), H, I, K - red to purple, J - brown, D, E - undistinguishable
[c] Open circles denote lower concentrations than closed circles on all TLC plate representations

contaminant (R_f 0.76, F) found also in a blank, and two unidentified compounds (R_f 0.89, H, and 0.91, I) which are probably other butter hydrocarbons or steroids, less polar than cholesterol.

During the illumination period, at least five compounds, more polar than cholesterol, were visible on the plate (A-E), and their intensities increased with time. The compounds C, D, and E were very faint and colors were not discernable for the latter two. One compound, less polar than cholesterol appeared at R_f 0.96 (J). Insufficient resolution and trailing of the compounds less polar than cholesterol generally obscured these spots.

The spot apparently corresponding to the carotenoids decreased in intensity over time, as would be expected due to photobleaching of this pigment. In a separate experiment, no TLC-visible compounds formed when β-carotene underwent photooxidation. Thus, it was concluded that compounds which appeared during butter illumination were not oxidation products of β-carotene.

No change in intensity over time was observed for the cholesterol spot as was the case when cholesterol oxidized in heated tallow (Ryan, 1982). This was probably due to the fact that only a relatively small amount of the cholesterol originally present underwent oxidation.

Two dimensional TLC analysis was performed on the day 20 surface sample to further characterize the cholesterol oxides formed during exposure of butter to fluorescent light (Table 5). Standard reference compounds were analyzed under identical conditions. Impurities present in the standard cholesterol oxidation products hindered identification of the steroids during both TLC and GLC analyses. However, 5-cholesten-

Table 5. Two dimensional TLC analysis of cholesterol oxide standard references and non-saponifiable material in butter surface sample after 20 days of fluorescent illumination.

Sample	Color	R_{f1}[a]	R_{f2}[b]	Probable Identity
Day 20 Butter Surface	Blue	0.30	0.19	5-Cholesten-3β,7α-diol
	Blue	0.39	0.26	5-Cholesten-3β,7β-diol
	Blue	0.48	0.33	
	Not Distinct	0.58	0.42	
	Magenta	0.85	0.69	Cholesterol
	Blue	0.85	0.74	
	Purple	0.90	0.81	
	Purple	0.92	0.83	
	Purple	0.94	0.96	
Standard Reference Compounds				
5-Cholesten-3β,7α-diol[c]	Blue	0.29	0.20	
5-Cholesten-3β,7β-diol	Blue	0.39	0.27	
5-Cholesten-3β-ol-7-one	Yellow	0.37	0.34	
	Yellow	0.19	0.18	Contaminant
5-Cholesten-3β-ol-7-one (Saponified)	Brown	0.74	0.68	
	Brown-Gold	0.89	0.86	
	Purple	0.90	0.94	
Cholestan-5α,6α-epoxy-3β-ol[c]	Gold	0.52	0.40	
Cholestan-3β,5α,6β-triol	Brown	0.08	0.06	
3,5-Cholestadien-7-one[c]	Gold-Brown	0.91	0.86	
	Gold-Brown	0.83	0.79	
	Yellow	0.70	0.66	

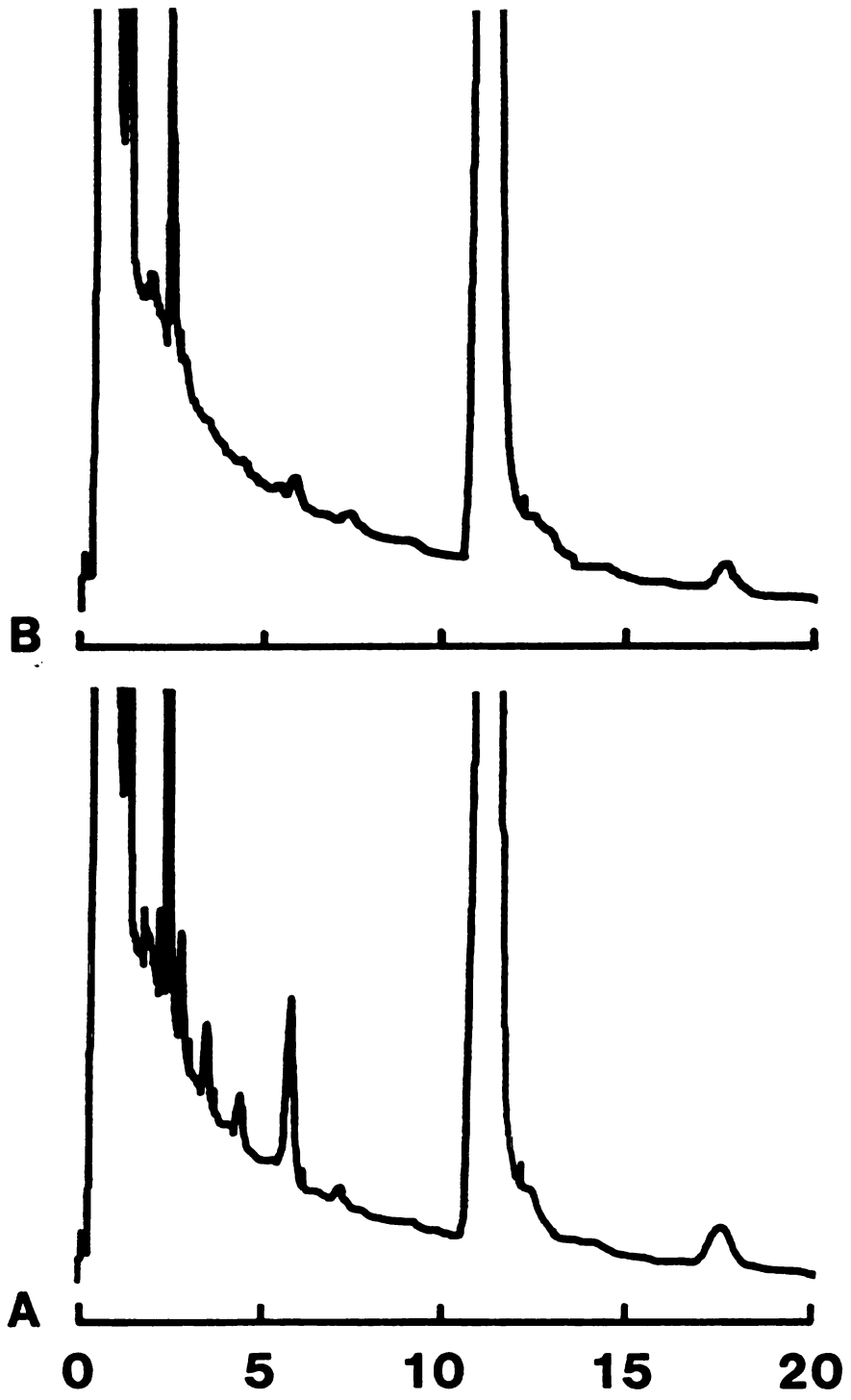
[a]Solvent system: ethyl acetate - heptane 1:1 (v/v)
[b]Solvent system: benzene - ethyl acetate 3:2 (v/v)
[c]Other minor components were present.

3β,7α-diol and 5-cholesten-3β,7β-diol were identified in the illuminated butter samples.

GLC analysis of non-saponifiable material from illuminated butter provided very little information (Figure 8) due to poor resolution and small relative quantities of the oxides. In general, there was a decrease in peak heights and number of peaks with retention times lower than that of cholesterol (R_t 10.8 min) and a possible increase in material eluting after cholesterol. The peak at R_t 17.2 min is believed to be an impurity.

In the absence of appropriate and pure standard reference compounds for all spots encountered on TLC plates, the cholesterol oxides in the butter were compared to those formed before and after reduction of products from hematoporphyrin-sensitized photooxidation of cholesterol in pyridine and cumene hydroperoxide-oxidized cholesterol in ethyl acetate (Figure 9). When sprayed with the N,N-dimethyl-p-phenylenediamine dihydrochloride, a major hydroperoxide was detected at R_f 0.71 with minor positive responses at R_f 0.66, 0.82, and 0.91 in the hematoporphyrin sample. Reduction of these revealed no hydroperoxides, but did indicate the presence of a major blue spot having an R_f value of 0.54, minor spots at R_f 0.36, 0.46, 0.75, and 0.79 and an intense magenta spot at R_f 0.85. Hydroperoxides were visible in the cumene hydroperoxide-oxidized sample at R_f 0.72, 0.75, and 0.94. Reduction led to an intense blue spot at R_f 0.47 and faint blue and purple spots at R_f 0.37 and 0.92 respectively. The butter sample taken after 20 days revealed blue spots with R_f values of 0.37, 0.48, and 0.54 after spraying with H_2SO_4; however, no positive hydroperoxide spots were observed.

Figure 8. Gas chromatograms of the non-saponifiable material in butter surface layers (5 mm) after (A) 0, and (B) 20 days of fluorescent illumination.

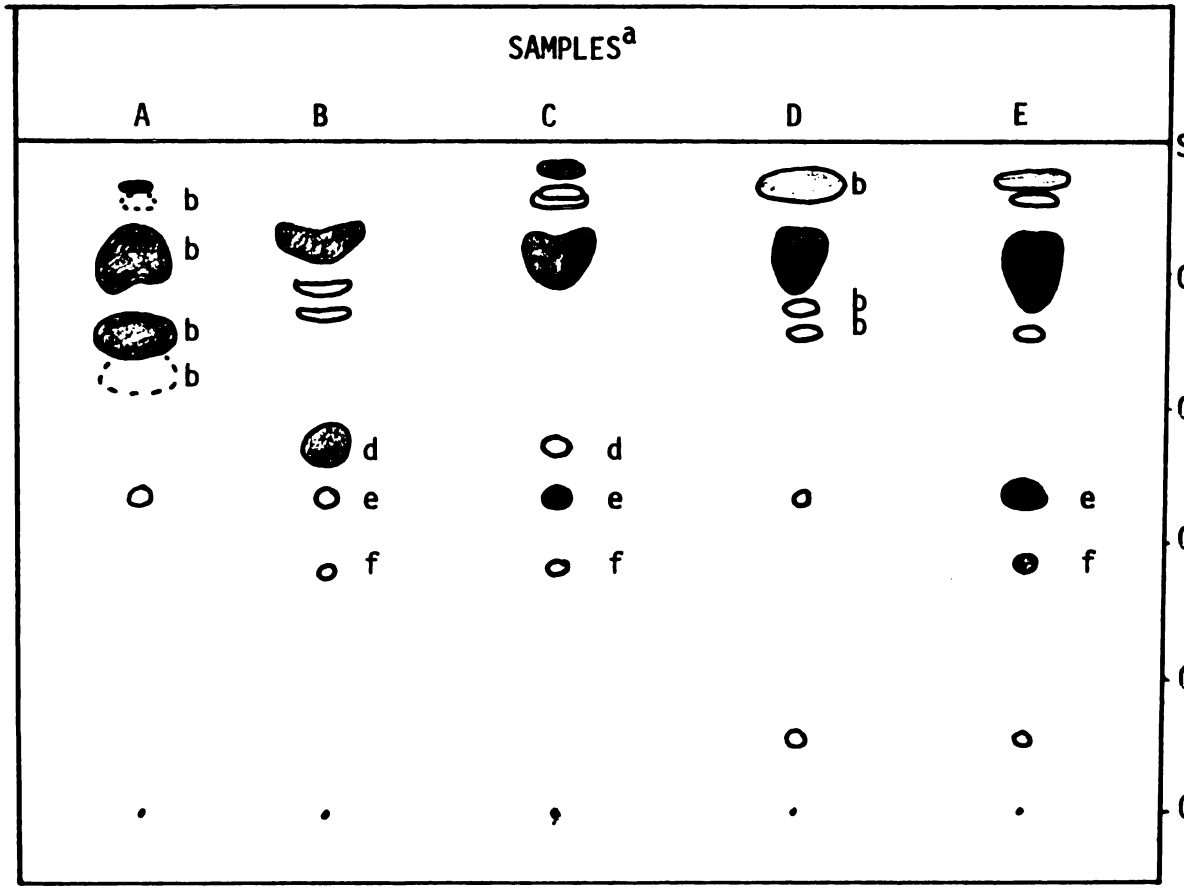

B
A
0
5
10
15
20

As discussed in the literature review, cholesterol is susceptible to oxidation when exposed to heat, radiation, or metal catalysts in the presence of oxygen. This study indicated that cholesterol in butter oxidized when exposed to fluorescent light. The array of products formed was similar to, but not as extensive as that reported in UV-irradiated cholesterol (Fioriti and Sims, 1967; Smith et al., 1967; Smith et al., 1973b), irradiated egg yolk solids (Chicoye et al., 1968a), and irradiated foods containing eggs (Acker and Greve, 1963).

When cholesterol undergoes photosensitized oxidation in pyridine, 6-cholesten-3β-ol-5α-hydroperoxide is the major product (Kulig and Smith, 1973). This may be reduced to 6-cholesten-3β,5α-diol with sodium borohydride, thus the spot at R_f 0.54 (Figure 9) is strongly suspected to be the 3β,5α-diol. Cholesterol autoxidations involving radical processes (radiation or cumene hydroperoxide induced) yield 5-cholesten-3β-ol-7β-hydroperoxide and the 7α-hydroperoxide as the major and minor products,respectively. The 5α-hydroperoxide has not been encountered except in sensitized photooxidations (or other reactions involving $^1O_2$), but once formed, it may rearrange to the 7α-hydroperoxide (Smith, 1980).

Hydroperoxides are the primary products of light induced oxidations and can decompose to form alcohols (among other secondary products) in the presence of radiation, heat, metals or enzymes (Dugan, 1976). The presence of the suspected 3β,5α-diol (R_f 0.54, Figure 9) indicates that the 5α-hydroperoxide may have been present in the illuminated butter, and that the butter must have undergone at least partial oxidation by a sensitized $^1O_2$ mechanism similar to that of photosensitized oxidation of cholesterol in pyridine. The 3β,7α-diol may have been formed via rearrangement of the 5α-hydroperoxide to the 7α-hydroperoxide followed by formal dehydration to the diol (Smith, 1980) and/or direct

Figure 9. TLC analysis of cholesterol oxidized by cumene hydroperoxide and hematoporphyrin + light, and the non-saponifiable material from butter after 20 days of fluorescent illumination.



[a] Samples: A - hematoporphyrin-photosensitized cholesterol; B - Sample A reduced with $NaBH_4$; C - Day 20 butter surface sample; D - cumene hydroperoxide-oxidized cholesterol; E - Sample D reduced with $NaBH_4$

[b] Positive reaction for hydroperoxides

[c] Solvent system: benzene - ethyl acetate 1:1 (v/v)

[d] Suspected 6-cholesten-3β,5α-diol (blue color after H_2SO_4 treatment)

[e] 5-Cholesten-3β,7β-diol (blue color after H_2SO_4 treatment)

[f] 5-Cholesten-3β,7α-diol (blue color after H_2SO_4 treatment)

free radical attack by hydrogen abstraction at C_7 followed by reaction with ground state oxygen, further hydrogen abstraction and formal dehydration. The 3β,7β-diol is also formed by the latter mechanism, and usually in higher yield than the 7α-epimer due to greater stability of the corresponding hydroperoxides (Kulig and Smith, 1973). Since all three diols were present in the sample taken from the butter surface layer after 20 days of illumination, it appears that both $^1O_2$ attack, and free radical attack by oxidizing lipids, were responsible for cholesterol oxidation in the butter.

Singlet oxygen is formed during photochemical reactions in the presence of a sensitizer (Krinsky, 1977). Thus, some component of the butter must act as a sensitizer in the presence of fluorescent light. Riboflavin may have the potential to sensitize in this manner although it is often associated with Type I photooxidation reactions (Korycka-Dahl and Richardson, 1978). Other butter components may also be involved.

Since β-carotene acts as a $^1O_2$ quencher (Foote et al., 1970b) it is possible that substantial bleaching of the carotenoid pigments might occur prior to singlet oxygen attack on cholesterol. This may contribute to the late appearance of the suspected 3β,5α-diol (Figure 7).

Δ^7-Cholesten-3-one is the only cholesterol oxide reported to be present in butter (Parks et al., 1966), but there are suggestions that it may be an artifact (Smith, 1980). Data in this study indicated the presence of the 3β,7-diols, and possibly the 3β,5α-diol. 5-Cholesten-3β-ol-7-one was not detected in the sample. Saponification procedures may cause some destruction of the compound (Chicoye et al., 1968b), and products similar to those of saponified 7-ketocholesterol were detected

in the day 20 sample (Table 5). Therefore the presence or absence of 7-ketocholesterol prior to saponification is uncertain. Neither cholestan-5α,6α-epoxy-3β-ol, its β-epimer, nor the hydration product, cholestan-3β,5α,6β-triol were detected although Smith and Kulig (1975) reported that both epoxides may form from cholesterol in the presence of its own primary oxidation products, the 7α-, 7β-, or 5α-hydroperoxides. It is unclear whether 3,5-cholestadien-7-one was present due to impurities in the commercial standard. If present, it may have arisen from the destruction of 7-ketocholesterol during saponification since conditions during illumination were probably not severe enough to cause the dehydration reaction.

Compounds undetectable by the methods of this study may form, albeit in very small quantities, in the butter. Quantitation was not an objective of this study, but it was found by comparative coloration that less than 1.1 μg 5-cholesten-3β,7α-diol was present per gram of butter. The detection limits on TLC plates of the 3β,7α-diol and the 3β,7β-diol, which are the most easily detectable oxidation products, are 0.01 to 0.025 μg, and 0.025 to 0.05 μg for 7-ketocholesterol (Smith and Price, 1967).

Two dimensional TLC analysis appeared to be the most useful method for detection and identification of cholesterol oxidation products in this study. This has been confirmed by Smith (personal communication to Ryan, 1982) who indicated that R_f values and spot coloration after acid treatment adequately confirm the identities of sterols when compared to standard references. The difficulty of obtaining pure standards is a major deterrent to further identification studies (Sheppard and Shen, 1980).

No single GLC system or derivatization procedure has been found that provides complete resolution of all important compounds arising from cholesterol oxidation (Van Lier and Smith, 1968a; Sheppard and Shen, 1980). Several products, especially the 3β,7-diols and the 3β,5α-diol undergo on-column decomposition and have somewhat similar breakdown patterns (Smith et al., 1973a). Conversion of 5-cholesten-3β-ol-7-one to 3,5-cholestadien-7-one via on-column dehydration has also been observed (Van Lier and Smith, 1968a). Impure standards, decomposition, and lack of resolution make quantitation by GLC virtually impossible, even in derivatized forms (Chicoye et al., 1968a; Van Lier and Smith, 1968a). Identification of some oxides by mass spectrometry has also been limited by these problems (Ryan, 1982).

B. Effect of illumination on sensory qualities of butter

Results of hedonic scaling, by 12 untrained panelists, of butter (served on crackers) after 0, 4, or 8 days of fluorescent light exposure are presented in Table 6. At the 99% confidence level, the day 0 sample was significantly different from the day 4, and day 8 samples; there was no significant difference between the latter two. Data indicated that only 3 out of 12 panelists rated the samples hedonically in order of PV, and 5 of 12 panelists scored the fresh (unilluminated) butter lower than at least one illuminated sample. An unpleasant aftertaste was noted by three panelists for the illuminated samples. (See Appendix II.)

It appears that off-flavors in butter eaten in this manner were not extremely disliked by untrained panelists, even when butter had an extremely high PV. Conflicting opinions surround the level of

peroxides acceptable in butter. A PV of 1.0 is considered critical as a point at which oxidized flavor is easily recognized (deMan et al., 1965; Armstrong, 1967). Butter with a PV of "nearly 2" is no longer edible (Sattar et al., 1976a) and Downey and Murphy (1968) claim that butter is distinctly oxidized at a PV of 2.5 to 3.5. All of these opinions were based on butter graded by trained taste panels and not by untrained consumers.

Table 6. Hedonic sensory scores[1] and peroxide values for butter blocks after 0, 4, and 8 days of fluorescent illumination.

	Sample		
	Day 0	Day 4	Day 8
Mean[2]	34.25[a]	64.92[b]	56.42[b]
Std. dev.	26.8	26.2	26.8
Range	7 - 85	14 - 97	11 - 99
Peroxide value	0	10.4	19.0

[1] 0 = like extremely; 50 = neither like nor dislike; 100 = dislike extremely

[2] Means not showing common letters are significantly different at ($p<0.01$) using the t-by-difference method (ASTM, 1968).

Hamm et al. (1968) stated that PV and flavor do not always correlate well. It seems that this may be especially likely when flavor is judged by consumers who eat butter infrequently, and the butter may be of questionable quality when purchased. Ghita et al. (1977) stated that rancid flavor is so commonplace that many consumers quite likely accept it as normal butter flavor, and at low levels it is tolerated and perhaps liked by some people.

The panel used in this study did not overwhelmingly reject the butter flavor at PV levels at which trained graders most likely would have. It is possible that tasting the butter plain would have produced different results, but this would have been an unlikely eating situation for most people. These results may indicate that rancid butter flavor is less noticable or more acceptable when eaten with other foods, rather than alone.

In butter exposed to light, flavor defects are often especially noted at the surface (deMan et al., 1965; Armstrong, 1967). It is extremely likely that if the surface layer had been rated, rather than the entire block, both hedonic scores and PV's would have been different.

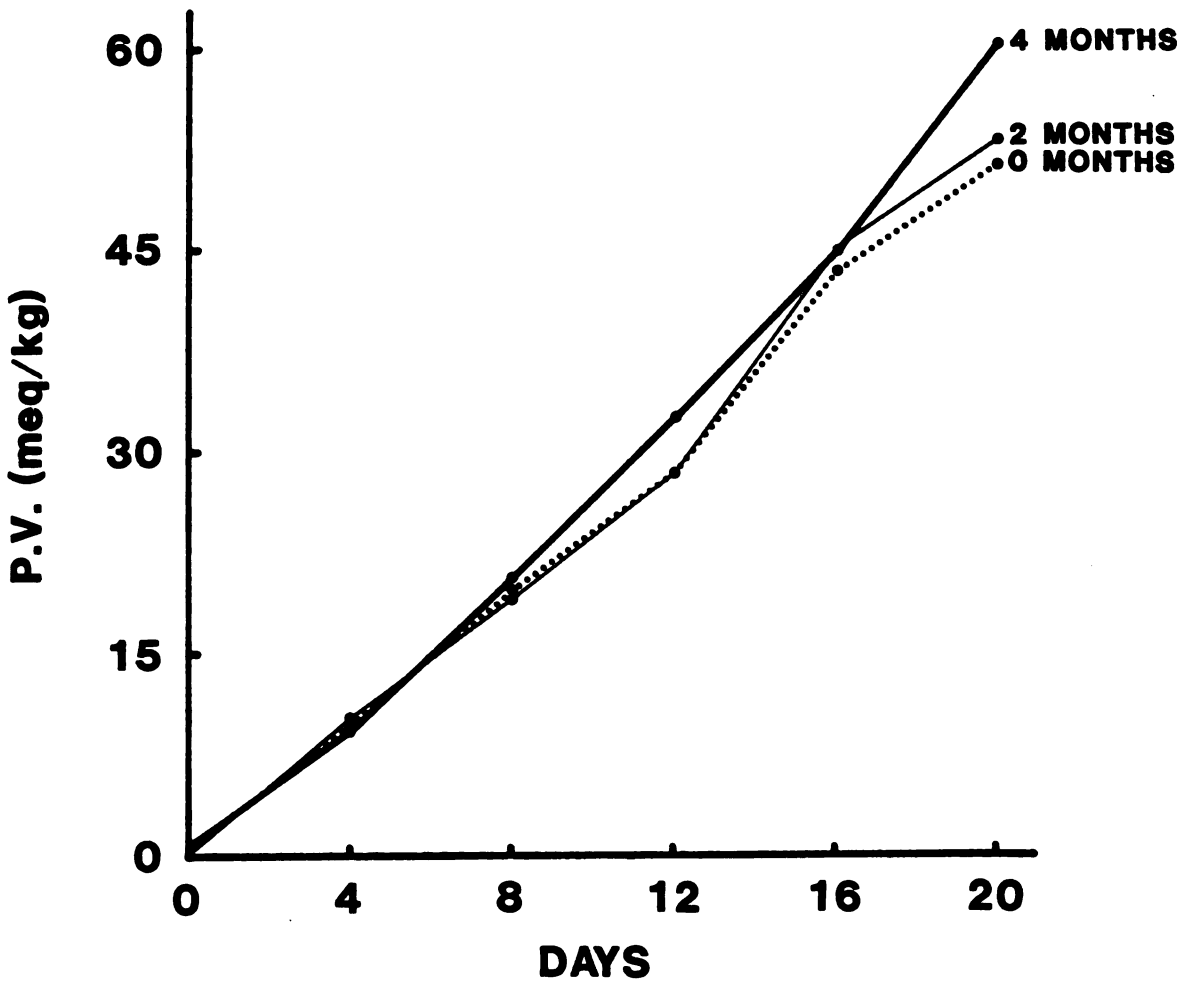
Referring to Figure 7, it can be seen that cholesterol oxidation products are present in detectable levels by day 8. If consumers do not reject the butter due to flavor defects by this time, they may ingest some cholesterol oxidation products. Biological consequences of such ingestion or ingestion of undetectable amounts after shorter exposure times remain to be evaluated.

C. Effect of frozen storage after light exposure on butter blocks

The PV of butter, illuminated fluorescently for up to 20 days, after 0, 2, and 4 months of dark frozen storage is represented in Figure 10. Data indicate that the PV increases during dark frozen storage only when it is extremely high prior to freezing (20 day sample). There is a greater PV increase after 4 months than after 2 months in the day 20 sample.

Comparison of cholesterol oxidation products on TLC plates showed no observable differences in number of compounds or appearance or

Figure 10. Effect of storage at -20°C following 0 to 20 days of fluorescent illumination on peroxide value of unpackaged butter blocks.


60
45
30
15
0
P.V. (meq/kg)
0
4
8
12
16
20
DAYS
4 MONTHS
2 MONTHS
0 MONTHS

disappearance trends. The only difference noted was a slightly increasing concentration of the 5-cholesten-3β,7-diol epimers over the 4 months in the day 20 sample. This trend was not obvious in the samples illuminated for shorter time periods.

Davies (1931) as cited by Coe (1941) stated that once autoxidation was initiated, subsequent dark storage will not arrest rancidity development in shortening. However, light-induced oxidation of butterfat occurred only during direct light exposure and the extent was proportional to the energy from the light source (Gilchrist et al., 1968). The PV of these samples exposed to low intensities of light remained "reasonably stationary" after 3 months at -17.8°C following exposure. Similar results were observed by Sattar et al. (1976b).

It appears that at low peroxide levels, propagating reactions which lead to increased PV and cholesterol oxidation were retarded at freezer temperatures. As the peroxide and free radical levels increased, further oxidation during frozen storage was observable in butter, albeit at a rate much lower than during illumination at 5°C. This resulted in further peroxide formation and cholesterol oxidation by free radical processes. As anticipated, there was no increase in concentration of cholesterol oxidation products unique to photosensitized oxidation pathways during dark frozen storage.

III. Fluorescent Illumination of Packaged Butter at 5°C

A. Effect of various packaging materials on peroxide value and cholesterol stability

Results discussed in the previous section indicated that butter lipids, including cholesterol, were susceptible to oxidative changes when exposed to fluorescent light. Protection from potential toxicological and sensory problems may be achieved by light barrier packaging materials. With butter, this is especially necessary in retail distribution rather than during export (MacBean, 1974). In addition to light barrier properties, Wilster (1957) suggested that butter wrappers be sealable, odor and water impermeable, reclosable and slightly rigid. Parchment was traditionally used because of its wet strength and grease resistance; however, it is generally ineffective in protecting butter from even low light intensities (Gilchrist et al., 1968).

Light transmission properties for the materials used in this study are shown in Table 7. Only the low density polyethylene (PE) transmitted greater than 10% of the light at any wavelength. The BHA-impregnated polyethylene (BHA-PE) showed the next highest transmission, followed by the margarine wrap (MW). The opaque parchment (OP), wet strength dry wax paper (WSDW) and aluminum foil transmitted less than 0.1% at all wavelengths. Downey and Murphy (1968), Sacharow (1969), Sattar and deMan (1973, 1975) and Emmons et al. (1981) observed similar transmission characteristics for similar materials.

The data for PV determinations of butter wrapped in these materials are presented in Table 8. There was essentially no increase in the PV of the foil and dark samples after 15 days, which indicated that the foil offered excellent light protection. Similar results with foil or foil laminates have been reported by Wilster (1957), Downey and Murphy,

Table 7. Percent light transmission at various wavelength ranges for packaging materials.

Material[c]	% Transmission[a] Wavelength Range (nm) 225-249	250-299	300-399	400-499	500-599	600-700
PE	67	73	77	75	78	79
BHA-PE	5	7	9	9	10	10
MW	*[b]	0.2	0.3	0.2	0.2	0.2
WSDW	*	*	*	*	*	*
OP	*	*	*	*	*	*
Foil	*	*	*	*	*	*

[a] Highest Value in range.
[b] * = less than 0.1% transmission.
[c] PE = polyethylene BHA-PE = BHA-impregnated polyethylene; MW = margarine wrap; WSDW = wet strength dry wax paper; OP = opaque parchment

Table 8. Peroxide values of packaged butter after fluorescent illumination.

Material[d]	Peroxide Value (meq/kg)[a] Illumination Time (days) 0	5	10	15
No Wrap	0.18[c]	19.4	29.2	42.8
PE		13.1	24.5	49.7
BHA-PE		11.0	29.8	36.8
MW		16.2	25.9	45.2
WSDW		11.2	19.7	38.0
OP		10.8	18.2	22.7
Foil		0.2	0.0	0.2
Dark[b]		0.1	0.0	0.3

[a] Average of two measurements.
[b] "Dark" samples not exposed to light.
[c] Peroxide value of all starting samples.
[d] PE = polyethylene; BHA-PE = BHA-impregnated polyethylene; MW = margarine wrap; WSDW = wet strength dry wax paper; OP = opaque parchment

(1968) and Emmons et al. (1981). OP appeared to offer the next best protection against peroxide formation. With the exception of BHA-PE which showed relatively good protection during the first five days, the order of inhibition of peroxide development approximately paralleled the order of decreasing light transmission characteristics of the packaging materials.

Butylated hydroxyanisole (BHA) is a free radical scavenger which can inhibit autoxidative reactions in foods (Dugan, 1976). Trace amounts of BHA were present in butter samples after the exposure period and most likely accounted for the initial low PV in the BHA-PE wrapped butter sample. After migrating from the film into the butter, the BHA probably inhibited propagation of oxidation during the early illumination period, resulting in PV's which are lower than expected for such a light permeable material. Free radical scavengers such as BHA are not effective at inhibiting photosensitized singlet oxygen attack which forms primary hydroperoxides (Khan et al., 1954b). Eventually, as the oxidation chain reaction greatly accelerated, the BHA in the butter was not able to effectively inhibit it and may have undergone destruction in the process.

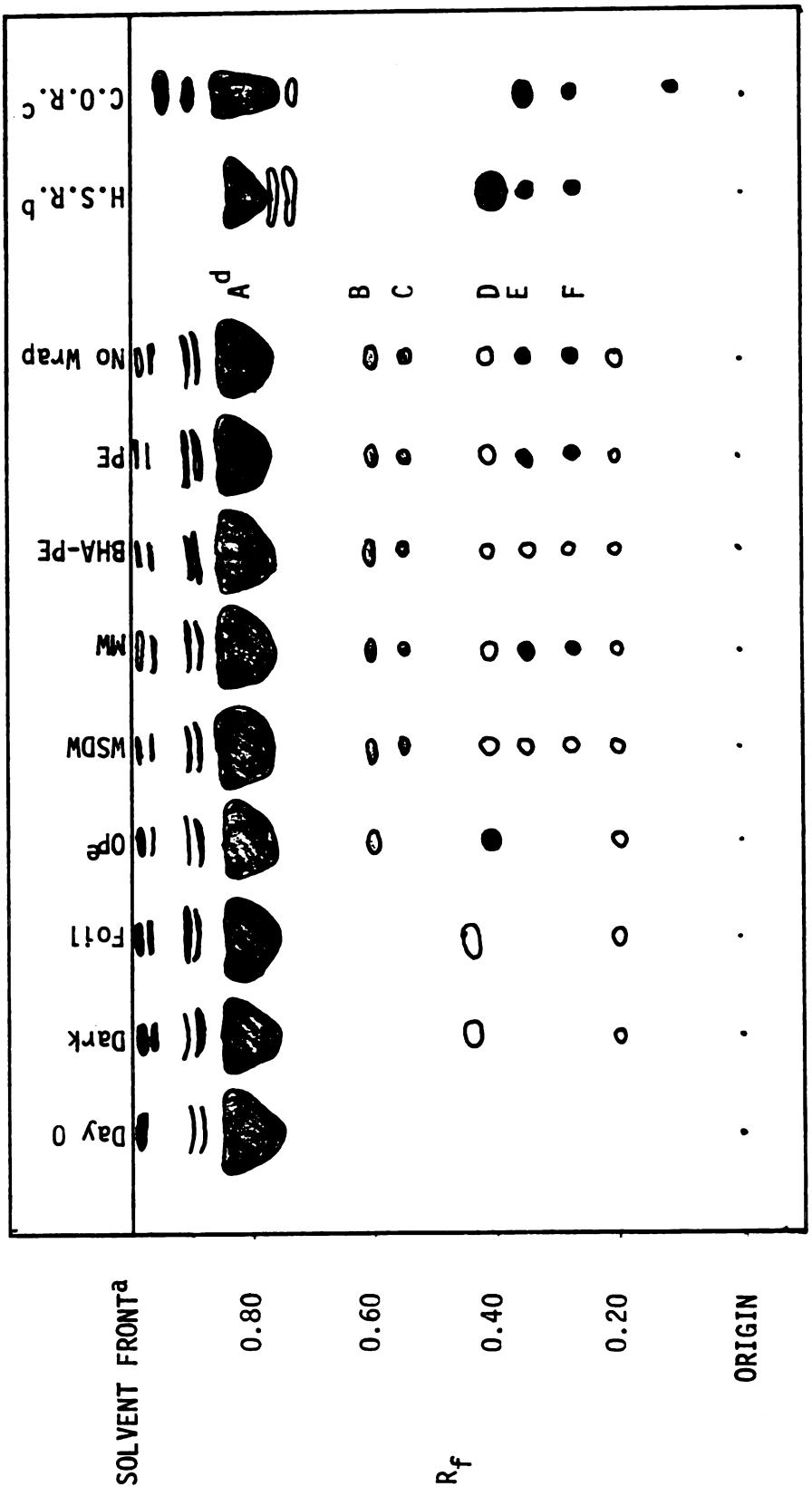
The cholesterol stability in packaged butter was also investigated (Table 9, Figure 11). In general, the concentration of cholesterol oxidation products was lower in the packaged samples. Therefore it was necessary to concentrate the non-saponifiable solutions five-fold to allow better color visibility on the TLC plates. This concentration step decreased the resolution of the compounds less polar than cholesterol. However, it did permit excellent separation and visualization of the more polar oxidation products.

Table 9. TLC analysis of the non-saponifiable material more polar than cholesterol in fluorescently illuminated packaged butter.

Spot Color	R_f[a]	First Day of Appearance[b]							
		DK[c]	Foil	OP	WSDW	MW	BHA-PE	PE	No Wrap
Purple	0.59			5	5	5	5	5	5
Yellow	0.54				10	10	10	10	10
Yellow	0.44	15	15						
Blue	0.41			10	10	10	10	10	10
Blue	0.35				15	10	10	10	10
Blue	0.27			5	5	5	10	10	10
Not Distinct	0.20	5	5	5	5	5	5	5	5

[a] Solvent system: ethyl acetate - heptane 1:1 (v/v)
[b] Samples analyzed on days 0, 5, 10, 15.
[c] DK = dark; OP = opaque parchment; WSDW = wet strength dry wax paper; MW = margarine wrap; BHA-PE = BHA-impregnated polyethylene; PE = polyethylene

Figure 11. TLC analysis of the non-saponifiable material from packaged butter samples after 15 days of fluorescent illumination.



a Solvent System: ethyl acetate-heptane 1:1 (v/v)
b Hematoporphyrin-photosensitized cholesterol reduced with $NaBH_4$
c Cumene hydroperoxide-oxidized cholesterol reduced with $NaBH_4$
d Colors: A - magenta, B - purple, C - yellow, D, E, F - blue
e For abbreviations, see text.

The compound suspected to be 6-cholesten-3β,5α-diol was present in low concentrations in the MW, WSDW, BHA-PE, PE, and unpackaged 15 day samples, with a higher concentration present in the OP sample (Figure 11). The 3β,7-diols were present in greater concentrations in the unpackaged, PE and MW samples with lesser amounts in the WSDW and BHA-PE samples. They were not present in the OP, foil and dark samples. Unidentified purple and yellow spots with R_f values 0.60 and 0.55 were also detected in the 20 day unpackaged butter study. Other spots corresponding to R_f values 0.44 and 0.20 may be contaminants.

The severity and extent of cholesterol oxidation in packaged butter approximately paralleled light transmission characteristics of the packaging materials. Less oxidation was observed in butter samples wrapped in materials with high light barrier properties. This may have been due to two effects. Initiation of photo-induced oxidation of cholesterol would be inhibited since less light energy would reach the butter. Additionally, lower peroxide concentrations (due to less photo-oxidation of other lipids) would lead to less attack on cholesterol by products of propagative reactions of lipid oxidation. Acker and Greve (1963) observed no changes in cholesterol when egg dough products were stored in light protected packages after two years.

It also appears that the BHA in the BHA-PE sample caused inhibition of cholesterol oxidation in addition to that expected due to the light barrier properties of the film. Concentrations of the 3β,7-diols, which are formed by free radical attack on cholesterol, were lower in the BHA-PE than in the MW sample even though the BHA-PE transmitted more light. There was no observable difference in the suspected 3β,5α-diol concentration, the formation of which should not be greatly affected

by a free radical scavenger.

The absence of the 3β,7-diols in the OP sample is a phenomenon worth noting. Only the suspected diol which is unique to photosensitized oxidation was present. An explanation is unavailable at this time.

Downey and Murphy (1968) found that only wrappers that transmitted no light prevented oxidation in butter. When transmission approached only 0.5%, oxidation took place. This appears to be true of cholesterol oxidation as well. If cholesterol oxidation is to be prevented because of the potential health risks, only one of the packaging materials studied, the aluminum foil, could be recommended. It should be noted that no cholesterol oxidation product known to be carcinogenic was isolated and identified in the illuminated butter. However, the potential for their formation may exist (Smith and Kulig, 1975), and possibly could not have been detected due to the limitations of the analytical procedure. Foil or foil laminates have been recommended for butter packaging and are used widely in foreign countries (Sacharow, 1969). Findings of this study support that recommendation that foil, or any other material with zero light transmission, be used for packaging butter.

IV. Exposure of Butter to Roomlight and Daylight at Ambient Temperatures

A. Effects of light source and exposure period on peroxide value and cholesterol stability

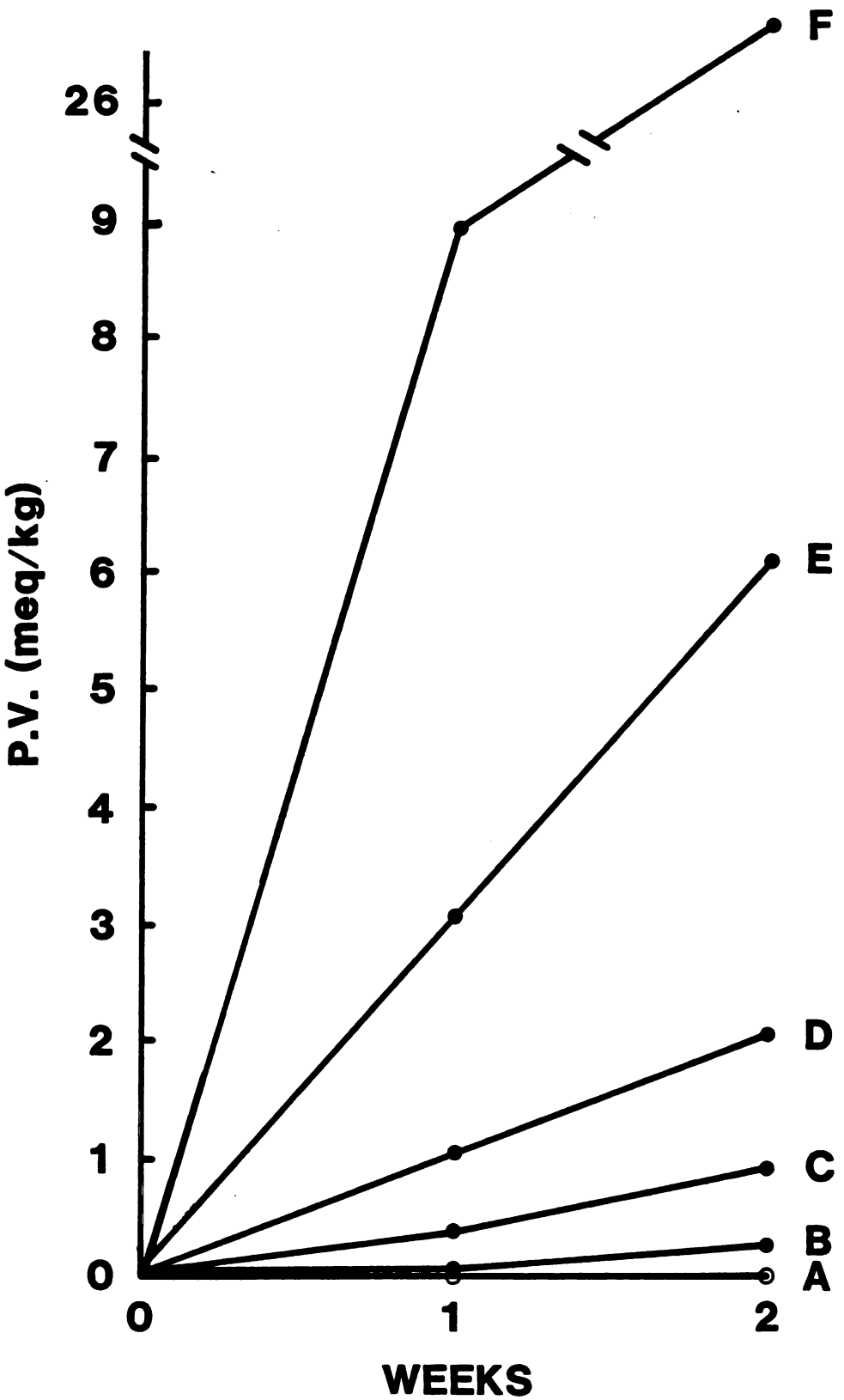
Since chemical reactions proceed more rapidly as temperature increases, a study was made of butter held at room temperature (22°C) and exposed to diffuse daylight (no direct sunlight) from a north window, or roomlight (fluorescent). Trials were designed to simulate unrefrigerated butter in a consumer's eating area either left out all day (8 hr), or for two meals (2 X 1½ hr), in a well lighted room, or near a window.

The extent of oxidation of the samples, as measured by PV, is presented in Figure 12. When stored in the dark, oxidation did not proceed any faster at 22°C than at 4°C until the second week. In the case of each light source, the longer the exposure, the higher the PV. Three hr/day exposure to daylight caused more peroxide formation than eight hr/day exposure to roomlight. PV increases were nearly linear over two weeks for samples D and E (Figure 12), but rose slightly faster during the second week for samples F and C.

A small difference in PV was noted in butter stored in the dark from 5 to 50°C for 120 hr (Gilchrist et al., 1968). Oxidation as measured by PV generally occurs at a rate proportional to the light energy which falls on the sample (Sattar et al., 1976a). It may be assumed that since the samples exposed to the daylight had higher PV's than those exposed to roomlight, the wavebands that accelerate peroxide formation in butter are more intense in the daylight. Light in the range of 300 to 700 nm has been reported to cause oxidation in butter (Sattar and deMan, 1975), with the shorter wavelengths causing the most deterioration (Moser et al., 1965). Thus, even weak artificial light will have an effect, and daylight is probably more conducive to oxidation

Figure 12. Effects of illumination, light source, exposure period, and storage temperature on the peroxide value of butter.

(A) - Stored in dark at 4°C
(B) - Stored in dark at 22°C
(C) - Exposed to roomlight 3 hr/day at 22°C
(D) - Exposed to roomlight 8 hr/day at 22°C
(E) - Exposed to daylight 3 hr/day at 22°C
(F) - Exposed to daylight 8 hr/day at 22°C


F
E
D
C
B
A
26
9
8
7
6
5
4
3
2
1
0
P.V. (meq/kg)
0
1
2
WEEKS

since it contains higher intensities in the lower wavelength range (Emmons et al., 1981; Paquette et al., 1981).

Trends in cholesterol oxidation were not obvious because low concentrations prevented adequate color development following acid treatment of the TLC plates (Table 10). Tentative identification was made for the 3β,7-diols based on comparison to reference standards. No compound was detected with the same R_f value as the suspected 3β,5α-diol, which indicated that cholesterol oxidation was largely due to free radical attack rather than singlet oxidative mechanisms. Cholesterol oxidation did not occur to the same extent in the roomlight and daylight exposed samples, as in the previously reported fluorescent light exposed samples at 5°C (Figure 7). Light intensities were lower, which led to less photosensitized oxidation of the cholesterol. Additionally, less overall lipid oxidation occurred, as evidenced by lower PV's, and this meant less free radical attack on the cholesterol.

Table 10. TLC analysis of the non-saponifiable material in butter exposed to roomlight, daylight, or no light for various time periods.

Spot Color	R_f[c]	Samples												
		Week	0	1	2	1	2	1	2	1	2	1	2	1	2
		Light[a]	-	Dk	Dk	Dk	Dk	Rm	Rm	Rm	Rm	Da	Da	Da	Da
		Hr/Day[b]	-	-	-	-	-	3	3	8	8	3	3	8	8
		Temp. (°C)	-	4	4	22	22	22	22	22	22	22	22	22	22
Purple	0.98		X[f]	X	X	X	X	X	X	X	X	X	X	X	X
Brown	0.95							X						X	X
Purple	0.90		X	X	X		X	X	XX	X	XX	X	XX	X	XX
Magenta	0.84		X	X	X	X	X	X	X	X	X	X	X	X	X
Yellow	0.66			X	XX	X	XX	X	XX		XX		XX	X	XX
Not Distinct	0.55						X						X		X
Not Distinct[d]	0.48				?	?	X	X		X	X		X	X	X
Not Distinct[e]	0.33				?	?	X			X	X	X	X	X	X

[a] Light source: Dk = no light; Rm = roomlight; Da = Daylight
[b] Hr/Day = time of exposure to light source
[c] Solvent system: ethyl acetate - heptane 1:1 (v/v)
[d] Same mobility as 5-cholesten-3β,7β-diol
[e] Same mobility as 5-cholesten 3β,7β-diol
[f] In a given row, XX represents greater concentration than X; "?" means presence is questionable.

V. Use of Illuminated Butter in Sugar Cookies

A. Cholesterol stability in butter during baking

This study was undertaken to determine whether cholesterol in butter oxidized during baking and whether exposure of butter to light prior to incorporation into cookie dough had an effect on cholesterol stability during baking or during subsequent storage of cookies.

Table 11 represents spots present on TLC plates for the fresh and illuminated butter as well as doughs and cookies made from each. The non-saponifiable matter from flour is also represented. The compounds in the cookies and doughs at R_f values 0.08, 0.87, 0.90, 0.96, and 0.98 are indistinguishable from those in the flour. Spots with R_f values of 0.98, 0.90, and 0.82 were observed in both the butter and flour samples. The spot at R_f 0.98 is believed to be due to carotenoid compounds since this spot is more intense in all fresh samples than in illuminated samples. As a food from a non-animal source, flour is not expected to contain cholesterol. Therefore, the compound at R_f 0.82 most likely represents a major plant sterol. Phytosterol standards were not used to determine its identity. Compounds with R_f values of 0.08 and at the origin in the cookie and dough extracts, are presumed to be introduced from the flour.

There were no observable cholesterol oxides more polar than cholesterol in the fresh or illuminated butter before dough incorporation. Therefore, the compounds detectable at R_f 0.59 and 0.27 must have formed in the dough, before and after baking, respectively, or were present in the illuminated butter at undetectable levels. Since these were only observed in the samples containing illuminated butter, it

Table 11. TLC analysis of the non-saponifiable material from flour, fresh and illuminated butter, and cookies and dough made from each butter and analyzed after various storage periods.

Samples			TLC Spots (R_f X 100)[a]									
Butter Condition[b]	Product Code[c]	Storage (weeks)	98	96	90	87	82	75	59	27	8	2
F	B	0	X		X		X	X				
I	B	0	X	X	X		X	X				
F	D	0	X	X	X	X	X	X			X	
I	D	0	X	X	X	X	X		X		X	
F	C	0	X	X	X	X	X				X	
I	C	0	X	X	X	X	X	X	X	X	X	
F	C	1	X	X	X	X	X				X	
I	C	1	X	X	X	X	X	X	X	X	X	
F	C	2	X	X	X	X	X	X			X	
I	C	2	X	X	X	X	X	X	X	X	X	
F	C	4[d]	X	X	X	X	X	X			X	
I	C	4[d]	X	X	X	X	X	X			X	
F	C	8[d]	X	X	X	X	X				X	X
I	C	8[d]	X	X	X	X	X		X		X	X
-	F	-	X	X	X	X	X	X			X	X

[a] Solvent system: ethyl acetate - heptane 1:1 (v/v)
[b] F = Fresh; I = Illuminated
[c] B = Butter; D = Dough; C = Cookie; F = Flour
[d] The 4 and 8 week samples were in frozen storage (-20°C), all others were at 22°C.

appears that this illumination did affect sterol stability. Identities of these two compounds are inconclusive due to absence of color at such low concentrations, although the R_f 0.27 spot has a mobility very similar to 5-cholesten-3β,7α-diol. This compound may be an hydroxylated cholesterol or phytosterol compound. These compounds were not detected in the cookies held in frozen storage. Degradation may have occurred during the long storage times.

Bleaching agents used in flour, and heat, may have been responsible for sterol oxidation under these conditions. Cholesterol was found to undergo oxidation within 1 hr in a non-aerated aqueous dispersion at 85°C (Kimura et al., 1979). When hydroperoxides were initially present, this oxidation occurred more rapidly. The cookies were exposed to temperatures of 177°C for 14 min and although internal temperatures were not that high, this heating could have affected the cholesterol in the cookies containing illuminated butter since hydroperoxides were also present.

SUMMARY AND CONCLUSIONS

The effect of fluorescent illumination on the oxidative stability of cholesterol in fatty acid solutions and in packaged and unpackaged butter was investigated. Cholesterol in linoleic acid solution underwent greater oxidative changes than did cholesterol in oleic acid solution. Continuous aeration also accelerated cholesterol oxidation during light exposure. At least 16 compounds were formed after 61 days of aeration and illumination of a linoleic acid solution of cholesterol.

In unpackaged butter, known cholesterol oxidation products were detectable by TLC analysis after 8 days of illumination, and their intensities increased with time. 5-Cholesten-3β,7α-diol and 5-cholesten-3β,7β-diol, products of free radical attack on cholesterol, were detected among the oxidation products, along with a compound suspected to be 6-cholesten-3β,5α-diol. This latter compound is only known to form following singlet oxygen attack on cholesterol.

Frozen storage of butter for up to 4 months following illumination did not result in further substantial oxidation of cholesterol, except in samples which were illuminated 20 days prior to freezing.

Packaging materials greatly influenced cholesterol stability in butter during illumination. This effect approximately paralleled the light barrier properties of the materials. Only aluminum foil prevented cholesterol oxidation after 15 days of illumination. Cholesterol in butter samples wrapped in margarine wrap and polyethylene oxidized at a rate similar to that of the unpackaged butter. The opaque parchment, dry wax paper, and BHA-impregnated polyethylene delayed formation of some

oxides. In general, the same oxidation products formed in packaged butter as in unpackaged butter, but at lower concentrations. These products included 5-cholesten-3β,7α-diol, 5-cholesten-3β,7β-diol and the suspected 6-cholesten-3β,5α-diol.

Daylight elicited oxidation in butter at 22°C at a greater rate than did roomlight. Cholesterol oxidation products were detectable in some samples after two weeks, but concentrations were very low and restricted identification of the compounds.

It appeared that heat and/or other cookie components caused oxidative changes in cholesterol during formulation and baking of cookies made from butter which had been subjected to illumination. Very low levels of cholesterol oxidation products were detectable in cookies stored at room temperature. Similar changes were not apparent in cookies made from fresh butter.

The conclusions drawn from this study are summarized as follows:

1. Photooxidation of cholesterol in butter does occur although the oxidation products are detectable only after prolonged exposure to light, which would be more severe than that expected during production and distribution.

2. Cholesterol oxidation in illuminated butter is a light-induced surface phenomenon, with cholesterol oxides more concentrated at the surface than throughout the entire butter block.

3. During the illumination of butter, cholesterol appears to undergo oxidation via singlet oxygen attack, as well as by free radical mechansims to form the 5-cholesten-3β,7-diol epimers and possibly 6-cholesten-3β,5α-diol.

4. Packaging butter in a high light barrier material can minimize

cholesterol oxidation under adverse storage conditions.

5. Frozen storage of butter does not result in substantial oxidation of lipids, including cholesterol, except when the butter is extremely oxidized prior to freezing.

6. Daylight was more detrimental to butter lipids than fluorescent roomlight. Consumers wishing to minimize lipid oxidation in butter should store butter covered and at refrigerator temperatures, and especially avoid extended uncovered storage near windows.

7. Cholesterol oxidation may occur in butter exposed to prooxidant conditions (e.g. heat) following exposure to light.

8. The oxidation products of cholesterol which were identified in illuminated butter have not been implicated as having carcinogenic or atherosclerotic properties. However, their presence indicates the potential for more hazardous compounds to form, perhaps at levels undetectable by the methods used in this study.

PROPOSALS FOR FURTHER RESEARCH

During the course of this study, several questions were raised that may be worthy of further research.

1) An investigation of lipid and cholesterol oxidation as a function of distance from the surface of the illuminated butter using thinner layers than were employed in this study.

2) The monitoring of β-carotene levels in the illuminated butter, and correlation of these levels to cholesterol and lipid oxidation.

3) A study of the effects of specific wavelengths (or wavelength ranges) of light on cholesterol oxidation in the butter system.

4) Identification of butter component(s) that may act as singlet oxygen-producing sensitizers during butter illumination.

5) Cholesterol oxidation in illuminated butter appeared to be accelerated by other oxidizing lipids. An investigation of the effect of using α-tocopherol-coated salts in butter manufacturing, on cholesterol and lipid oxidation may provide a means of slowing down photooxidative deterioration in butter in the absence of adequate protection from light.

6) Improvement of the quality of standard reference compounds would prove beneficial to the identification of cholesterol oxides in foods. Application of gas chromatography-mass spectrometry may also provide additional information as to the chemical nature of some compounds which are isolated, but for which no standards are available.

7) Further work needs to be done to establish whether cholesterol oxides, introduced into the body through foods, pose a true health hazard.

APPENDIX

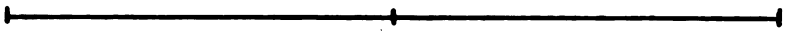
Appendix I. Sensory Score Sheet.

BUTTER

PANELIST _____ PRODUCT CODE _____

DATE _____

TASTE THE SAMPLE AND PUT A MARK ON THE LINE TO REPRESENT HOW YOU LIKE THE PRODUCT.



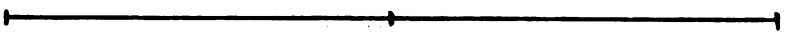
LIKE EXTREMELY — NEITHER LIKE NOR DISLIKE — DISLIKE EXTREMELY

BUTTER

PANELIST _____ PRODUCT CODE _____

DATE _____

TASTE THE SAMPLE AND PUT A MARK ON THE LINE TO REPRESENT HOW YOU LIKE THE PRODUCT.



LIKE EXTREMELY — NEITHER LIKE NOR DISLIKE — DISLIKE EXTREMELY

Appendix II. Data and Statistics for Sensory Analysis.

Scores			Differences					
Day 0	Day 4	Day 8	D_{4-0}	$(D_{4-0})^2$	D_{8-0}	$(D_{8-0})^2$	D_{4-8}	$(D_{4-8})^2$
78	48	94	-30	900	16	256	-46	2116
42	96	56	54	2916	14	196	40	1600
22	86	74	64	4096	52	2704	12	144
37	67	69	30	900	32	1024	-2	4
16	14	45	-2	4	29	841	-31	961
7	67	67	60	3600	60	3600	0	0
8	64	22	56	3136	14	196	42	1764
14	49	11	35	1225	-3	9	38	1444
35	97	30	62	3844	-5	25	67	4489
85	64	99	-21	441	14	196	-35	1225
56	95	53	39	1521	-3	9	42	1764
11	32	57	21	441	46	2116	-25	625
TOTALS			368	23024	266	11172	102	16136

$$t_{4-0} = \frac{368}{\left(\frac{12(23024)-(368)^2}{11}\right)^{\frac{1}{2}}} = 3.25^* \qquad t_{4-8} = \frac{102}{\left(\frac{12(16136)-(102)^2}{11}\right)^{\frac{1}{2}}} = 0.75$$

$$t_{8-0} = \frac{266}{\left(\frac{12(11172)-(266)^2}{11}\right)^{\frac{1}{2}}} = 3.51^*$$

d.f. = 11

Critical t values: $t_{(p < 0.05)} = 2.20$ $t_{(p < 0.01)} = 3.11$

* indicates significant difference at $p < 0.01$

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