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Studies of lipid peroxidation in microsomes isolated from beef and pork muscles: and, Bioluminescent bacterial mutagenesis test for fatty acid derivatives, heated oils, mycotoxins and heterocyclic amines

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Studies of lipid peroxidation in microsomes isolated from beef and pork muscles and bioluminescent bacterial mutagenesis test for fatty acid derivatives, heated oils, mycotoxins and heterocyclic amines

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Studies of lipid peroxidation in microsomes isolated from beef and pork muscles and Bioluminescent bacterial mutagenesis test for fatty acid derivatives, heated oils, mycotoxins and heterocyclic amines

by

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DEDICATION

TO MY MOTHER AND MY WIFE

AND

IN THE MEMORY OF MY FATHER

GENERAL INTRODUCTION

Lipid peroxidation in muscle foods is a major cause of spoilage and can lead to changes in texture, flavor, and nutritive properties of the products. The extent of lipid peroxidation can be affected by several factors including availability of oxygen, storage temperature, the composition of lipids, presence of metal ions and heme iron, and exposure to ionizing or ultraviolet radiation.

Since lipid peroxidation is an important problem in muscle foods, such as beef, pork, chicken, turkey, and fish; there has been a great deal of interest in identifying the catalysts that promote the oxidation of muscle lipids. Many studies concerning the effect of heme iron and nonheme iron on lipid peroxidation have been conducted using muscle homogenates or fatty acid emulsions as model systems (Hirano and Olcott, 1971; Schaich, 1980; Sato and Hegarty, 1971; Love and Pearson, 1974; Rhee et al., 1987). There is general consensus that heme iron in the 3+ state and nonheme iron in the 2+ state are the most active catalysts of lipid peroxidation in meats and that nonheme iron plays a more important role than heme iron.

For a long time, lipid peroxidation in muscle foods has been commonly regarded as nonenzymatic in nature. There is evidence now for the presence of enzymatic lipid peroxidation

systems associated with microsomes isolated from fish, beef and poultry muscles (Lin and Hultin, 1976; Slabyj and Hultin, 1982; Rhee et al., 1984; Kanner and Harel, 1985). The enzymatic systems require NADPH or NADH, ADP, and ferric or ferrous iron.

The present study was designed to: (1) study the enzymatic systems in microsomes isolated from beef and pork muscles; (2) examine the effect of storage time and storage temperature on enzymatic activity; (3) investigate the activity of nonenzymatic systems using heme iron, nonheme iron, and Fenton reagent in microsomes isolated from beef and pork muscles; and (4) determine the effect of BHT, EDTA, α-tocopherol, and ascorbic acid on both enzymatic and nonenzymatic lipid peroxidation in muscle microsomes.

The human population is exposed to an ever increasing number of food additives, drugs, cosmetics, and chemical substances which are essential for life and are of great social and economic benefits. In conjunction with the introduction of each new chemical, an equally great probability exists for the incidence of adverse effects upon the population. Substances that are not natural to a food may also become a part of the food by several routes and consumed by humans and thus raise questions of safety. Toxicity testing has become a major concern nationally because of the extensive legislation that now mandates such testing as well

as internationally because of the need for mutually acceptable products as a prerequisite to economic cooperation in the marketing of foods and chemicals.

Mutagenicity testing is one of the important branches of toxicity testing systems. There are now many mutagenicity tests available that employ diverse organisms from bacteria to mammalian cells in culture. Mutagens are being discovered in many foods that form important parts of our diet, such as cooked meats and fried foods. Some contaminates such as aflatoxin B_1 that can be identified in the human diet or animal feeds have also been recognized as mutagens. The Ames test, also called *Salmonella*/mammalian microsome test, has been widely used as a screening test by food scientists and environmentalists (Ames et al., 1975). Recently, Dr. Ulitzur and his colleagues developed a mutagenicity test using a bacterial system and bioluminescent technique. The bioluminescence test for mutagens used dark mutants of luminous bacteria to determine the ability of the tested agent to restore luminescence by inducing mutation. The restored luminescence can be measured by using photometric instrumentation (Ulitzur, 1986).

The current study was conducted to validate the Mutatox® test (commercial name for the bioluminescent bacterial mutagenicity test) in our laboratory, to utilize the Mutatox®

test to screen some chemical compounds that may be found in foods or feeds, to confirm the reliability of the Mutatox® test, and to contribute to a data base for the Mutatox® test.

Explanation of Dissertation Format

This dissertation is composed of two sections. The first section reports the studies of enzymatic and nonenzymatic lipid peroxidation in microsomes isolated from beef and pork muscles. This section of the study was supervised by Dr. Jane Love. The second section reports the results of the bioluminescent bacterial mutagenicity test for autoxidized fatty acids, chlorinated fatty acids, heated oils, mycotoxins and heterocyclic amines. This section of the study was supervised by Dr. Henry Stahr.

SECTION I

STUDIES OF ENZYMATIC AND NONENZYMATIC LIPID PEROXIDATION IN MICROSOMES ISOLATED FROM BEEF AND PORK MUSCLES

INTRODUCTION

Mechanism of Lipid Peroxidation

Lipid peroxidation plays a major role in food deterioration. Oxidation of unsaturated lipids not only produces offensive odors and flavors which render lipid-containing foods unacceptable or reduces their shelf life but also decreases the nutritional quality of foods. It is becoming increasingly evident that exposure of biological membranes to oxidative stress results in progressive degeneration of membrane structure and loss of activity. The biological consequences that arise from in vivo lipid peroxidation reactions or from ingestion of foods containing oxidized lipids have attracted the attention of biochemists and food scientists. Lipid peroxidation products are implicated in the disruption of biological membranes, the inactivation of enzymes, the damage of proteins, carcinogenesis, and mutagenesis (Logani and Davies, 1980; Kappus and Sies, 1981; Pearson et al., 1983; Frankel, 1984; Kanner et al., 1986). The active role of lipid peroxidation in atherosclerosis has also been indicated by the results of several workers. There appears to be a close relationship between lipid peroxidation and aging as indicated by the accumulation of lipofuscin pigments and the development of

atherosclerosis (Yagi, 1982; Goto, 1982; Csallany and Isayaz, 1976; Jain and Hochstein, 1980; Kumar et al., 1977). Reviews by Addis (1986) and Addis and Park (1989) expressed concern about the occurrence of lipid oxidation products such as lipid peroxides, malonaldehyde, and cholesterol oxidation products in foods and their role in coronary artery disease (CAD), mutagenesis, and carcinogenesis.

In general, the reaction of oxygen with unsaturated lipids (LH) involves free radical initiation, propagation, and termination processes. Initiation takes place in the presence of factors, such as metals, pigments, enzyme systems, light, or heat, that have sufficient reactivity to abstract a hydrogen atom from a methylene group. This results in an unpaired electron on the carbon. The lipid free radical (L•) tends to be stabilized by molecular rearrangement to produce a conjugated diene, which then easily reacts with an oxygen molecule to give a peroxy radical (LOO•). Peroxy radicals can abstract a hydrogen atom from other lipid molecules and thus create more L•, this constitutes the propagation stage of lipid peroxidation. The LOO• combines with the abstracted hydrogen atom to form lipid hydroperoxide (LOOH), the primary product of lipid peroxidation. In actuality, a variety of termination processes may compete with propagation. Termination involves two alkyl radicals, two peroxyl radicals, or combinations of these reactions to form non-radical species.

Initiation	LH	——>	L• + H•
Propagation	L• + O_2	——>	LOO•
	LOO• + LH	——>	LOOH + L•
Termination	L• + L•	——>	Nonradical product
	LOO• + LOO•	——>	Nonradical product
	L• + LOO•	——>	Nonradical product

Decomposition of lipid hydroperoxides is a very complicated process and produces a multitude of materials that may have biological effects and cause deterioration in lipid containing foods. Decomposition proceeds by homolytic cleavage of lipid hydroperoxides to form alkoxy radicals (LO•). These radicals undergo carbon-carbon cleavage to form breakdown products including aldehydes, ketones, alcohols, hydrocarbons, esters, furans, and lactones. Lipid hydroperoxides can react with oxygen to form such secondary products as epoxyhydroperoxides, ketohydroperoxides, dihydroperoxides, cyclic peroxides, and bicyclic endoperoxides. These secondary products can in turn decompose like monohydroperoxides to form volatile breakdown products. Lipid hydroperoxides also can condense into dimers and polymers which can decompose and produce volatile materials

(Frankel, 1984; Nawar, 1985).

Factors that Affect Lipid Peroxidation

Due to the economic consequences of lipid peroxidation in foods and to the toxic implications of lipid peroxidation to aerobic life, extensive research has been done not only to identify the products of lipid peroxidation and the conditions that influence their production, but also to study the mechanisms involved. In biological systems, including foods, the extent of lipid peroxidation can be affected by factors including oxygen, temperature, the composition of lipids, enzyme systems (e.g., lipolytic enzymes, xanthine oxidase, or cytochrome P450 reductase), metal ions (e.g., iron or copper), heme iron, ionizing or ultraviolet radiation, reducing agents, and photo-activated pigments (Girotti, 1985; Nawar, 1985).

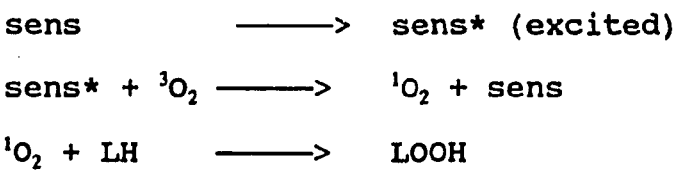
Oxygen and active oxygen derivatives

Molecular oxygen is the major biological oxidant, yet its direct reaction with most organic compounds is spin forbidden. The system of nomenclature used for spin states has its origins in the number of spectral lines, which is always one more than the number of unpaired spins in the state, i.e. "singlet" for no unpaired spins, "doublet" for one unpaired spin, "triplet" for two unpaired spins, etc. The electronic

structure of oxygen has two unpaired electrons, each located in a different π antibonding orbital in the triplet state. These two electrons have the same spin quantum number and if oxygen attempts to oxidize another molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin so as to fit into the vacant spaces in the π orbitals. A pair of electrons in a molecule would, however, have antiparallel spins in the orbital, in accordance with Pauli's principle. The reaction of oxygen, therefore, is spin forbidden with ground state molecules of singlet multiplicity such as polyunsaturated fatty acids (Kanner et al., 1986; Halliwell and Gutteridge, 1984). Therefore, oxygen must be activated in order for oxidation of these compounds to occur. Activation of oxygen can proceed through many different processes. Among them are activation to singlet oxygen, reduction by organic chemicals, complexation or reduction by transition metals, and activation via enzyme systems (Aust et al., 1985).

Singlet oxygen Conversion of triplet oxygen to singlet oxygen can be facilitated by sensitizers (sens) such as chlorophyll and myoglobin (Privett and Blank, 1962; Rawls and van Santen, 1970; 1971). There are two excited states of singlet oxygen: singlet O_2 $^1\Delta g$, the most important in

biological systems, has no unpaired electrons and is not a radical; and singlet O_2 $^1\Sigma g$, which usually decays to the $^1\Delta g$ state before it react with any molecule. The initiation of unsaturated fatty acid peroxidation by singlet oxygen proceeds by a mechanism (photosensitized reaction) different than the free-radical process.



Singlet oxygen reacts with unsaturated fatty acid by the "ene" reaction. The oxygen molecule is joined onto one end of a double bond simultaneously as it abstracts an allylic proton and a new double bond is formed between the allylic position and the other end of what was the carbon-carbon double bond. Attack of singlet oxygen can take place at either end of the double bond so that for a molecule such as methyl oleate the products will be 9-hydroperoxide isomers and 10-hydroperoxide isomers (Chan and Coxon, 1986; Frankel, 1985).

Superoxide radical The spin restriction of oxygen may also be circumvented by the univalent reduction of oxygen (O_2) to form the superoxide anion radical ($O_2\bullet^-$):

$$O_2 + e^- \longrightarrow O_2\bullet^- \text{ (superoxide)}$$

The radical is unstable, with a lifetime of milliseconds at neutral pH. It is in equilibrium with its conjugate base, the perhydroxyl radical ($HO_2\bullet$), which is a considerably more powerful oxidant than superoxide itself (Kanner et al., 1986). Under biological conditions, significant amounts (0.1 to 1 μM) of superoxide radical can be generated by subcellular organelles such as mitochondria and chloroplasts and by a number of enzymes including xanthine oxidase and NADPH-oxidase (Loschen et al., 1974; Asada and Kiso, 1973; McCord and Fridovich, 1968; Massey et al., 1969). Superoxide anion is also generated by autoxidation of certain molecules such as hemoglobin and myoglobin (Misra and Fridovich, 1972; Gotoh and Shikama, 1976). The oxidation of reduced metals such as Fe^{2+} by oxygen can also produce $O_2\bullet^-$ (Cohen and Sinet, 1980; Fee and Valentine, 1977):

$$Fe^{2+} + O_2 \longrightarrow O_2\bullet^- + Fe^{3+}$$

Several studies have demonstrated that the perhydroxyl radical or superoxide radical can initiate the chain oxidation of linoleic and arachidonic acids (Bielski et al., 1983;

Gebicki and Bielski, 1981; Sutherland and Gebicki, 1982). Thomas et al. (1982) also suggested that superoxide or perhydroxyl radicals can decompose lipid hydroperoxides and thus prolong the chain reaction of lipid peroxidation:

$$LOOH + O_2\bullet^- \longrightarrow LO\bullet + OH^- + O_2$$

$$LOOH + HO_2\bullet \longrightarrow LO\bullet + H_2O + O_2$$

Superoxide radicals can also indirectly inactivate catalase, peroxidases, and α-tocopherol and via this mechanism, impair the protective effects of these systems against lipid peroxidation (Kono and Fridovich, 1982; Hayashi and Yamazaki, 1979; Nishikimi et al., 1980; Fukuzawa and Gebicki, 1983).

Hydrogen peroxide Addition of a second electron to superoxide gives the peroxide ion, O_2^{2-}, which has no unpaired electrons. Usually, peroxide ion formed at physiological pH will immediately be protonated to give hydrogen peroxide (H_2O_2). Hydrogen peroxide is normally present as a metabolite at low concentrations in aerobic cells. The amount of hydrogen peroxide is maintained at approximately 10^{-9} to 10^{-7} M by superoxide dismutase and glutathione peroxidases (Oshino et al.,1973; Tyler, 1975). A study by Harel and Kanner (1985) indicated that hydrogen peroxide could be generated in turkey

muscle tissues incubated at either 37°C or 4°C. Mitochondria, microsomes, peroxisomes, and cytosolic enzymes have all been recognized as effective hydrogen peroxide generators when fully supplemented by appropriate substrates (Boveris et al., 1972; Hildebrandt and Roots, 1975; Chance et al., 1979). Hydrogen peroxides may be produced nonenzymatically from oxidation of flavins, thiols, myoglobin, hemoglobin, and other reducing compounds or produced enzymatically from the action of superoxide dismutase on superoxides (Fridovich, 1983; Satoh and Shikama, 1981; Wallace et al., 1982). The overall reaction can be written as:

$$2O_2\bullet^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide is a strong oxidizing agent but as is true in the case of oxygen, its direct reaction with many organic compounds occurs only very slowly. There is no evidence to prove the direct reaction between hydrogen peroxide and polyunsaturated fatty acids. However, the activity of hydrogen peroxide in biological tissues is due to the capability of this compound to be reduced to the potent oxidizing agent hydroxyl radical (OH•).

Hydroxyl radical Hydrogen peroxide will rapidly react with transition metals such as ferrous iron to form an oxidant capable of reacting with organic molecules. The most cited reaction is Fenton's reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \bullet OH$$

The oxidizing agent generated in this reaction was postulated to be hydroxyl radical (•OH) (Dixon and Norman, 1962; Lai and Piette, 1978; Rosen and Rauckman, 1981). Fenton's reaction is actually a redox chain reaction occurring as follows (Walling, 1975):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$
$$\bullet OH + LH \longrightarrow H_2O + L\bullet$$
$$L\bullet + Fe^{3+} \longrightarrow \text{Product} + Fe^{2+}$$

Another mechanism for the generation of hydroxyl radical in the presence of superoxide and hydrogen peroxide is called the Haber-Weiss reaction:

$$O_2\bullet^- + H_2O_2 \longrightarrow O_2 + OH^- + \bullet OH$$

Koppenol et al. (1978) found that this reaction does occur but

has a very low rate constant and is too slow to be an efficient source of hydroxyl radical. Catalysis by transition metals, metal chelates, or hemoproteins allows the Haber-Weiss reaction to occur at a much faster rate:

$$O_2\bullet^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow HO\bullet + OH^- + Fe^{3+}$$

$$\text{Sum: } O_2\bullet^- + H_2O_2 \longrightarrow HO\bullet + OH^- + O_2$$

This has been called the iron-catalyzed Haber-Weiss reaction or superoxide-driven Fenton reaction (Kanner et al., 1986; Aust et al., 1985; Grisham and McCord, 1986). Naturally occurring iron-containing compounds active in catalyzing this reaction include transferrin, ADP-Fe^{3+}, hemoglobin, and ferritin (Motohashi and Mori, 1983; Tien and Aust, 1982; Sadrzadeh et al., 1984; Thomas et al., 1985). A similar mechanism has been proposed for a Cu(I)-stimulated reaction (Rowley and Halliwell, 1983):

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + \bullet OH + OH^-$$

The hydroxyl radical is the most reactive free radical produced in biological systems and reacts with an extremely high rate constant with almost every type of molecule found in

living cells. This type of reaction initiates lipid peroxidation according to the following reaction scheme (Halliwell and Gutteridge, 1984):

$$LH + \bullet OH \longrightarrow H_2O + L\bullet$$

$$L\bullet + O_2 \longrightarrow LOO\bullet$$

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet$$

Lipid peroxidation in vitro has been studied by many researchers. Fridovich and Porter (1981) found that a mixture of xanthine oxidase and its substrate ethanal oxidizes arachidonate. This reaction was inhibited by mannitol (an •OH scavenger) which suggested that peroxidation was stimulated by hydroxyl radicals formed by an iron-catalyzed Haber-Weiss reaction. Gutteridge (1984a) further proved the involvement of hydroxyl radical in the initiation of lipid peroxidation.

Transition metals

Biological systems It is well accepted that transition metals such as iron and copper play a crucial role in many lipid peroxidation reactions (Girotti, 1985). These metal complexes include simple complexes of iron salts with phosphate ion or phosphate esters such as ADP. Also effective

are heme, hemoproteins such as hemoglobin, myoglobin, cytochrome P450, and other cytochromes, and nonheme iron proteins (Kaschnitz and Hatefi, 1975; Aust and Svingen, 1982). As mentioned above, the transition metals can be involved in the metal-catalyzed Haber-Weiss reaction in the presence of superoxide and hydrogen peroxide and thus stimulate lipid peroxidation. Another mechanism has also been proposed, specifically, that the addition of an iron complex to lipid hydroperoxide can stimulate peroxidation by a peroxide-decomposition reaction. With a ferrous iron compound an alkoxy radical (LO•) will be formed, while with a ferric iron compound a peroxy radical (LOO•) will form. Both alkoxy and peroxy radicals can stimulate the chain reaction of lipid peroxidation by further abstracting hydrogen atoms. This mechanism has been categorized as lipid hydroperoxide-dependent initiation (Halliwell and Gutteridge, 1984; Girotti, 1985):

$$LOOH + Fe^{2+}\text{-complex} \longrightarrow Fe^{3+}\text{-complex} + OH^- + LO\bullet$$

$$LOOH + Fe^{3+}\text{-complex} \longrightarrow Fe^{2+}\text{-complex} + H^+ + LOO\bullet$$

$$LO\bullet + LH \longrightarrow LOH + L\bullet$$

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet$$

Food systems Since lipid peroxidation is an important problem in muscle foods such as beef, pork, chicken, turkey, and fish; there has been a great deal of interest in identifying the catalysts that promote the peroxidation of muscle lipids. Heme pigments have been found to catalyze peroxidation of muscle tissue lipids. Younathan and Watts (1960) reported that the Fe^{3+} hemes were the active lipid peroxidation catalysts in pork muscle. When Brown et al. (1963) reacted unsaturated fatty acids with heme compounds, they found no difference in the rate of catalysis between the Fe^{2+} and Fe^{3+} compounds. Hirano and Olcott (1971) found similar results in a study of the effect of heme concentration on linoleate peroxidation. Considerable differences of opinion have been expressed among researchers regarding the importance of the state of iron to the catalytic activity of heme. Greene and Price (1975) suggested that heme pigments may be more active lipid peroxidation catalysts when iron is in the 3+ state. Kaschnitz and Hatefi (1975) found that the Fe^{3+} forms of hemoprotein were the most active catalysts of lipid peroxidation. Schaich (1980) also concluded that Fe^{3+} forms of hemoprotein and hematin are the most active in increasing lipid peroxidation.

There are also many studies concerning the effect of nonheme iron on lipid peroxidation. In beef homogenate, both

types of catalysts, hemoprotein and nonheme iron, may be active catalysts of lipid peroxidation (Liu, 1970a; 1970b; Liu and Watts, 1970). Sato and Hegarty (1971) presented evidence that in meat nonheme iron may be the catalytic agent rather than heme iron. Studies by Love and Pearson (1974), Igene et al. (1979), and MacDonald et al. (1980) further confirmed the observation of Sato and Hegarty (1971) that nonheme iron rather than heme iron was an effective prooxidant in meat. Tichivangana and Morrissey (1985) showed that both metal ions and heme pigments catalyzed lipid peroxidation in raw and cooked meats and fish muscle. However, nonheme iron was proposed to be the major prooxidant. Rhee et al. (1987) also concluded that metmyoglobin alone had little or extremely low catalytic activity while nonheme iron played a major role in the catalysis of lipid peroxidation in beef muscles.

Enzymatic lipid peroxidation

Biological systems Lipid peroxidation may also be driven enzymatically. Important examples of enzymatically-driven lipid peroxidation are the xanthine oxidase-induced reaction (Kellogg and Fridovich, 1975) and the NADPH-cytochrome P450 reductase-dependent reaction (Aust and Svingen, 1982).

Xanthine oxidase occurs in high concentrations in the fatty particles of milk and in large amounts in mammalian

liver, kidney, jejunal mucosa cells, heart, and lung (Bray, 1976). It can react with different kinds of substrates, such as acetaldehyde, salicylaldehyde, and different purines, but its main substrates in vivo are hypoxanthine and xanthine (Kanner et al., 1986). Molecular oxygen can serve as an efficient oxidizing substrate leading to the production of both superoxide and hydrogen peroxide. In a typical reaction, xanthine oxidase generates superoxide and hydrogen peroxide during the conversion of hypoxanthine to xanthine and then to uric acid:

$$\text{Hypoxanthine} + H_2O + O_2 \longrightarrow \text{xanthine} + H_2O_2 \text{ or } O_2\bullet^-$$

$$\text{Xanthine} + H_2O + O_2 \longrightarrow \text{uric acid} + H_2O_2 \text{ or } O_2\bullet^-$$

The relative proportions of hydrogen peroxide and superoxide produced vary with pH and P_aO_2 (Kellogg and Fridovich, 1975). As mentioned above, the production of active oxygen derivatives may play a crucial role in lipid peroxidation. Grisham (1985) demonstrated that myoglobin, in the presence of hypoxanthine and xanthine oxidase, catalyzes the peroxidation of arachidonic acid.

Extensive studies have been done by using rat liver microsomes to explain the NADPH-cytochrome P450 reductase-dependent lipid peroxidation. Lipid peroxidation in isolated

liver microsomes requires NADPH, cytochrome P450 reductase, and molecular oxygen and is markedly stimulated by ADP-Fe^{3+} (Hochstein and Ernster, 1963; Hochstein et al., 1964; May and McCay, 1968; Pederson et al., 1973). The role of NADPH-cytochrome P450 reductase in lipid peroxidation was established by the ability of an antibody to the reductase to inhibit NADPH-dependent microsomal lipid peroxidation (Pederson et al., 1973). Other investigations demonstrated that NADPH-dependent lipid peroxidation could be reconstituted utilizing a purified protease solubilized NADPH-cytochrome P450 reductase (Pederson and Aust, 1972). Buege and Aust (1978) also presented evidence that in the absence of iron or NADPH, no lipid peroxidation occurs in rat liver microsomes. In their study they also indicated that the chelation of ferric iron by ADP greatly stimulated activity while addition of both ADP-Fe^{3+} and EDTA-Fe^{3+} resulted in maximal activity. However, EDTA-Fe^{3+} alone can not catalyze NADPH-dependent lipid peroxidation, it can enhance the rate of lipid peroxidation only in the presence of ADP-Fe^{3+}. Numerous studies with EDTA have demonstrated the complexity which it imparts upon the reactivity of iron. Gutteridge et al. (1979) and Winston et al. (1984) suggested that the chelate to iron ratio can markedly affect the mechanism of initiation of lipid peroxidation by EDTA-Fe^{3+} complexes. At EDTA : iron ratios of

less than one, maximal rates of peroxidation were noted which were suggested to be due to optimum hydroxyl radical generation resulting from a rapidly autoxidizing pool (EDTA-Fe^{2+}) and the slower oxidizing free Fe^{2+}. At a strict 1 : 1 ratio, it has been suggested that hydroxyl radical could be formed to initiate lipid peroxidation; but at ratios greater than one, lipid peroxidation was inhibited (Gutteridge, 1984b; Kornbrust and Mavis, 1980; Tien et al., 1982). The observation by Buege and Aust (1978) about the ineffective catalysis of EDTA-Fe^{3+} in lipid peroxidation might be due to the ratio of EDTA to ferric iron.

Many studies have been conducted to identify the primary free radicals that initiate NADPH-dependent lipid peroxidation. Fong et al. (1973) suggested that the hydroxyl radical is the species involved in the reaction and proposed that it is the radical generated by reducing hydrogen peroxide in the iron-catalyzed Haber-Weiss reaction. Lai and Piette (1978) further confirmed the presence of hydroxyl radical by using Electron Spin Resonance (ESR) analysis. In contrast, Pederson and Aust (1975) suggested that microsomal lipid peroxidation was not inhibited by either superoxide dismutase or catalase and questioned the involvement of hydroxyl radical. Morehouse et al. (1984) supported the finding by Pederson and Aust (1975) and suggested that hydroxyl radical

formation may not adequately account for the initiation of microsomal lipid peroxidation. Tien and Aust (1982) concluded that an oxygen bridged di-iron complex (Fe^{2+}-O_2-Fe^{3+}) intermediate may be involved in the mechanism. More study might be necessary to validate this assertion.

Food systems While lipid peroxidation in muscle foods has been commonly regarded as nonenzymatic in nature, there is evidence for the presence of enzymatic lipid peroxidation systems associated with muscle microsomes. Lin and Hultin (1976; 1977) and Player and Hultin (1977) demonstrated the presence of an enzymatic system for the peroxidation of microsomal lipids in the microsomal fraction of chicken muscle. Incubation of the microsomal fraction isolated from chicken muscles in the presence of NADPH, ADP, and ferric iron was shown to result in lipid peroxidation. NADH was able to replace NADPH as the source of reducing equivalents but was less efficient. However, for enzymatic lipid peroxidation in fish muscle microsomes, NADH was much more efficient than NADPH. Other requirement for this system in fish muscle were the same as those of chicken muscle (McDonald et al., 1979; Slabyj and Hultin, 1982). Enzymatic lipid peroxidation in microsomes isolated from beef muscle has been demonstrated by Rhee et al. (1984). The reaction required NADPH or NADH, ADP,

and ferrous or ferric iron. The rate of peroxidation was higher with NADPH than with NADH and also higher with ferrous iron than with ferric iron. Rhee et al. (1986) indicated that microsomal enzymatic lipid peroxidation activity may play an important role in production of TBA-reactive substance in beef muscle. Hultin (1980) reported that heating of a microsomal preparation to 80°C for 5 minutes eliminated much of the enzyme-catalyzed lipid peroxidation. He suggested that the relative amount of enzymatic vs. nonenzymatic peroxidation in muscle microsomes is strongly dependent upon temperature, while the extent of the nonenzymatic reaction being greater as temperature increases. At present, the relationship between the effects observed in the in vitro experiments and results obtained with muscle is not clear and needs further exploration.

Activated heme proteins

The ability of heme proteins, such as myoglobin and hemoglobin, to promote lipid peroxidation has been demonstrated by many researchers. The postulated mechanism of lipid peroxidation by heme compounds is based on homolytic scission of preformed fatty acid hydroperoxides to free radicals and is not based on promoted initiation processes (Kendrick and Watts, 1969; Kaschnitz and Hatefi, 1975).

However, it was discovered that the interaction of hydrogen peroxide with metmyoglobin (MetMb) or methemoglobin (MetHb) led to the generation of active species which initiate lipid peroxidation (Shiga and Imaizumi, 1975; McCarthy and White, 1983; Sadrzadeh et al., 1984). In their study, Sadrzadeh et al. (1984) found the following: (1) In the presence of a superoxide anion-generating system, hypoxanthine and xanthine oxidase, hemoglobin promotes hydroxyl radical formation in a dose-dependent fashion; (2) Hydroxyl radical forms readily upon the addition of hydrogen peroxide to hemoglobin; and (3) Hemoglobin also increases hypoxanthine/xanthine oxidase-driven peroxidation of polyunsaturated fatty acids such as arachidonic acid. Thus, they concluded that hemoglobin may be biologically hazardous, in part because it acts as a "Fenton reagent", having the potential to catalyze hydroxyl radical generation in the presence of superoxide or hydrogen peroxide. Grisham (1985) also demonstrated that myoglobin,in the presence of hypoxanthine and xanthine oxidase, catalyzes the peroxidation of arachidonic acid. They proposed that the reactivity of this initiator is similar to that of ferryl iron or hydroxyl free radical.

By using the microsomal fraction from chicken muscle, Kanner and Harel (1985) and Harel and Kanner (1985) found that microsomal lipid peroxidation initiated by hydrogen peroxide-activated MetMb or MetHb was inhibited by several reducing

compounds and antioxidants. However, several hydroxyl radical scavengers and catalase failed to inhibit this reaction. These results led them to hypothesize that the activated heme protein which initiates lipid peroxidation appears to be a porphyrin cation radical, $P^{+}-Fe^{4+}=O$, which is a ferryl species and not a hydroxyl radical. They proposed two possible routes, lipid hydroperoxide independent and lipid hydroperoxide dependent; for porphyrin cation radical to stimulate lipid peroxidation:

(1) $P^{+}-Fe^{4+}=O + LH \longrightarrow P-Fe^{4+}=O + L\bullet + H^{+}$

$L\bullet + O_2 \longrightarrow LOO\bullet$

$LOO\bullet + LH \longrightarrow LOOH + L\bullet$

(2) $P^{+}-Fe^{4+}=O + LOOH \longrightarrow P-Fe^{3+} + LOO\bullet + OH^{-}$

$LOO\bullet + LH \longrightarrow LOOH + L\bullet$

Hemoglobin and myoglobin play an essential role in maintaining aerobic metabolism and are present in abundant amounts in human and other animal tissue (Stryer, 1981). Autoxidation of oxyhemoglobin or oxymyoglobin results in the formation of methemoglobin or metmyoglobin, respectively (Satoh and Shikama, 1981; Ribarov and Bochev, 1982). Several of the studies discussed above indicated that active oxygen derivatives, such as superoxide and hydrogen peroxide, may be

present in biological systems. From all these information one can speculate that active oxygen derivatives and heme proteins may be a focus for initiation of lipid peroxidation in vivo and in vitro in biological tissues and muscle foods (Kanner et al., 1986).

Biological Defense Systems

Because of the potentially harmful effects initiated by reactive oxygen derivatives, nature has provided several antioxidant enzymes and scavengers to protect biological tissues from lipid peroxidation. They include superoxide dismutase (SOD), catalase, glutathione peroxidase, α-tocopherol, ß-carotene, glutathione (GSH), and ascorbic acid (Halliwell and Gutteridge, 1985; Grisham and McCord, 1986; Kanner et al., 1986).

Superoxide dismutases (SOD)

There are two forms of the enzyme SOD which catalyzes superoxide dismutation: a Mn-containing protein in mitochondria, and a Cu-Zn-containing protein in the cytoplasm of most mammals (Steinmen, 1982). These enzymes prevent the initiation of lipid peroxidation by the superoxide radical. They catalyze the dismutation of superoxide to hydrogen peroxide, thereby preventing the iron redox cycling reaction

and iron-catalyzed Haber-Weiss reaction (Halliwell and Gutteridge, 1985; Kanner et al., 1986):

$$SOD\text{-}Cu^{++} + O_2\bullet^- \longrightarrow SOD\text{-}Cu^{+} + O_2$$

$$SOD\text{-}Cu^{+} + O_2\bullet^- \longrightarrow SOD\text{-}Cu^{++} + H_2O_2$$

Catalase

Although SOD enzymes are protective, they produce hydrogen peroxide which is the precursor of hydroxyl radical and the activator of hemeproteins. Hydrogen peroxide must be eliminated from tissues to prevent further damage. Acting in concert with SOD to remove hydrogen peroxide generated from either the dismutation of superoxide or from the two-electron reduction of oxygen is the hemoprotein catalase. Catalase occurs in almost all animal tissues. The enzyme is widely distributed and the highest activity is found in red cells and liver. This hemoprotein catalyzes the decomposition of hydrogen peroxide to yield oxygen and water (Grisham and McCord, 1986):

$$H_2O_2 + \text{catalase} \longrightarrow \text{catalase} + H_2O + O_2$$

Catalase is specific for hydrogen peroxide and will not decompose organic hydroperoxides, thereby distinguishing it

from other hydrogen peroxide decomposing enzymes such as glutathione peroxidase.

Glutathione peroxidase

Glutathione peroxidase is found in the cytosol and mitochondria of animal cells. The enzyme is a selenium-containing protein that catalyzes the decomposition of hydrogen peroxide or organic peroxides. Glutathione (GSH), the substrate for the enzyme, is to maintain the cell in a proper oxidation/reduction state. Glutathione peroxidase is activated by variety of peroxides, including hydrogen peroxide, cumene hydroperoxide, and lipid hydroperoxide; at the expense of GSH to form oxidized glutathione (GSSG) and water or the organic alcohol (Grisham and McCord, 1986).

One may view the enzyme systems as the first line of defense for the cell against lipid peroxidation because these enzymes function to decompose precursors such as superoxide and hydrogen peroxide before they can interact to form more oxidants. A second line of defense is composed of the nonenzymatic scavengers such as phenolic compounds, carotenoids, and sulfhydryl compounds that scavenge free radicals that escape decomposition by enzyme systems.

Tocopherols (Vitamin E)

Vitamin E refers to at least eight tocopherol structures possessing vitamin E activity; α-tocopherol predominates in most species and is significantly more potent than any other naturally occurring tocopherols known. The primary role of α-tocopherol is to inhibit free radical reactions such as lipid peroxidation by interfering with the propagation reactions. Alpha-tocopherol (ArOH) reacts with L• or LOO• to interfere with the propagation of lipid peroxidation (Halliwell and Gutteridge, 1985):

$$L\bullet + ArOH \longrightarrow LH + ArO\bullet$$

$$LOO\bullet + ArOH \longrightarrow LOOH + ArO\bullet$$

The ArOH may be regenerated by the reaction with GSH to GSSG, which in turn is reduced by GSH reductase (Pryor, 1976):

$$ArO\bullet + GSH \longrightarrow ArOH + GS\bullet$$

$$GS\bullet + GS\bullet \longrightarrow GSSG$$

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+}$$

Vitamin E has also been shown to be capable of scavenging singlet oxygen (Grams and Eskins, 1972). However, this property of vitamin E may be secondary to its function as a

free radical chain breaker. Catignani et al. (1974) demonstrated that vitamin E deficiency produces a marked increased in liver xanthine oxidase in rabbits and rats by increasing its in vivo synthesis. As mentioned above, xanthine oxidase plays a role in the generation of superoxide radicals, vitamin E may thus protect the cells from oxidative damage by inhibiting the generation of free radicals via decreased synthesis of xanthine oxidase.

Beta-carotene

Beta-carotene has been shown to be an effective quencher of singlet oxygen and an effective radical trapping antioxidant (Foote and Denny, 1968; Burton and Ingold, 1984). The importance of this compound has been demonstrated in studies in which carotene deficient bacterial mutants are much more sensitive to oxidative damage than are carotene replete bacteria (Krinsky, 1974). Kellogg and Fridovich (1975) found that ß-carotene can inhibit lipid peroxidation initiated by xanthine oxidase. Prior treatment of ß-carotene has also been shown to reduce lipid peroxidation of guinea pigs injected with CCl_4 as measured by ethane and pentane (Kunert and Tapple, 1983). Recently, considerable epidemiological and laboratory evidence exists suggesting that ß-carotene and retinols may be natural protective agents against cancer development (Peto et

al., 1981; Ames,1983; Cutler, 1984).

Glutathione (GSH)

Glutathione (GSH) is a tripeptide of cysteine, glycine, and glutamic acid and is the principal nonprotein sulfhydryl compound in the tissues (Stryer, 1981). The role of GSH as a substrate for the hydrogen peroxide removing enzyme glutathione peroxidase has already been discussed. In addition GSH is a scavenger of hydroxyl radicals and singlet oxygen. GSH is also necessary for the stability of sulfhydryl containing enzymes and protects hemoglobin and many cofactors from peroxidation. Recently, it has been shown to be involved in the regeneration of vitamin E. Deficiency of GSH has been shown to aggravate the toxicity of a number of chemicals and environmental agents, probably due to increased oxidative damage (Reddy et al., 1982; Niki et al., 1982; Larsson et al., 1983).

Ascorbic acid (Vitamin C)

Ascorbic acid (Vitamin C) is a reducing agent and an important water-soluble vitamin for humans and certain species of animals. Ascorbic acid functions to maintain sulfhydryl compounds, including GSH, in a reduced state and participates in many redox reactions. It has also been shown to be capable of scavenging free radicals and singlet oxygen and has a

synergistic effect on the antioxidant function of α-tocopherol (Chou and Khan, 1983; Hruba et al., 1982). However, like superoxide, ascorbic acid can reduce ferric iron to ferrous iron and, in the presence of hydrogen peroxide, can stimulate hydroxyl radical formation by the Fenton reaction. It has been suggested that the concentration and subcellular distribution are important in determining the antioxidant or prooxidant functions of ascorbic acid (Halliwell and Gutteridge, 1985).

Antioxidants in Food Systems

Antioxidants are substances that can delay the induction period or decrease the rate of lipid peroxidation in lipid-containing foods. No single antioxidant can offer the ultimate solution to oxidative deterioration for all food products. The selection of certain antioxidants depend upon their compatibility with and effectiveness in certain foods. The effectiveness of an antioxidant also depends on many factors, including solubility in the fat or water phase of the product, its dispersibility throughout the product, and its stability during processing of the product (Dziezak, 1986; Nawar, 1985; Pokorny', 1987). Dziezak (1986) categorized the antioxidants into four groups: (1) those compounds that terminate the free radical chain in lipid peroxidation (free-

radical terminators) such as butylhydroxytoluene (BHT), butylhydroxyanisole (BHA), tertiary-butylhydroquinone (TBHQ), propyl gallate (PG), and tocopherol; (2) those compounds that bind metal ions which catalyze lipid peroxidation (chelating agents, such as citric acid, ethylene diaminetetraacetic acid (EDTA), and polyphosphate); (3) those compounds that can inactivate active oxygen derivatives thus inhibit lipid peroxidation (oxygen scavengers, such as ascorbic acid and ascorbyl palmitate); and (4) secondary antioxidants which break down the lipid hydroperoxides into stable end products thus prevent further chain reactions (such as thiodiopropionic acid and dilauryl thiodiopropionic acid).

Free-radical terminators

Antioxidants are believed to interfere with the free-radical chain of peroxidation reactions by contributing hydrogen from the phenolic hydroxyl groups. The major synthetic phenolic compounds in common foods use are BHA, BHT, TBHQ, and PG. Tocopherols are well known natural antioxidants.

BHA BHA exists as white waxy crystals, insoluble in water but soluble in fats and oils. Most commercial preparations are isomeric mixtures of 3-tertiary butyl-4-hydroxyanisole and 2-tertiary butyl-4-hydroxyanisole with the

commercial component comprising a minimum 90% of the 3-tertiary BHA isomer. It is very active for stabilizing lard but is less suitable for vegetable oils, where it should be combined with other antioxidants (Pokorny', 1987; Dziezak, 1986).

BHT BHT is a white crystalline solid with good solubility in oil and negligible solubility in water. Because of its good carry through activity it may be used for the stabilization of fats in baked or fried products. BHT is very active in lard, but provides relatively poor protection for vegetable oils (Pokorny', 1987).

TBHQ The most suitable antioxidant for vegetable oils is TBHQ. TBHQ is a white crystalline solid slightly soluble in water and moderate soluble in oils but sufficient for applications as an antioxidant (Sherwin, 1976). TBHQ was more active than BHA and BHT in refined palm oil and stabilized snacks fried in palm oil and was recommended as the most suitable antioxidants for frying shortenings based on hydrogenated coconut and palm oils (Fritsch et al., 1975).

PG Propyl Gallate (PG) is available as a white, crystalline powder and is very slightly water soluble. PG functions well in stabilizing animal fats and vegetable oils,

but to a lesser extent than TBHQ in vegetable oils. Also PG is useful in inhibiting oxidation in meat products, including fresh and fresh frozen pork sausage, species, and snacks. With melting point of 148°C, PG loses its effectiveness under heat conditions and is therefore unsuitable for frying applications (Pokorny', 1987; Dziezak, 1986).

Tocopherols Tocopherols are the most widely used natural antioxidants. They belong to the vitamin E group as a blend of alpha, beta, gamma, and delta homologs. In general, tocopherols with high vitamin E activity are less effective as antioxidants than those with low vitamin E activity (Nawar, 1985). A relatively high proportion of the tocopherols present in crude vegetable oils survive the oil processing steps and remain in sufficient quantities to provide oxidative stability in the finished product. As antioxidants, tocopherols have been found to be effective in number of products, including bacon, baked foods, butterfat, lard, margarine, rapeseed oil, safflower oil, and sunflowerseed oil (Dugan, 1980). Tocopherols exert their maximum effectiveness at relatively low levels, the most effective concentrations are ascribed to the range of 0.01 to 0.02%. If used at a very high concentration, they may actually act as prooxidants (Cort, 1974).

Chelating agents

Chelating agents are often added to fats or foods to transform trace metal compounds into inactive complexes in order to improve the stability of lipids. Any molecule or ion with an unpaired electron can coordinate or form complexes with metal ions. Therefore, compounds containing two or more functional groups can chelate metals in a favorable environment. Citric acid and its derivatives, various phosphates, and salts of ethylene diaminetetraacetic acid (EDTA) are the most popular chelating agents used in foods (Pokorny', 1987).

Citric acid Citric acid is a highly effective sequesterant. In the seafood industry, citric acid is used synergistically with ascorbic acid in the form of a dipping solution to chelate prooxidants of rancidity and to inactivate certain enzymes that lead to deterioration (Dziezak, 1986). Combined with other oxidants, citric acid can also be used in dry sausage, fresh pork sausage, and dried meats at the usage levels of 0.003%, 0.01%, and 0.01%, respectively, which are allowed by USDA (Dziezak, 1986).

EDTA EDTA dissolves to only a limited extent and is not effective in pure fat systems. The EDTA salts, however, are very effective antioxidants in emulsion systems, such as

salad dressing and mayonnaise because they can function in the aqueous phase (Nawar, 1985). In a study by Igene et al. (1979), the increased rate of lipid peroxidation in cooked meat was found to be due to the release of nonheme iron during cooking. The addition of 2% EDTA effectively chelated the nonheme iron and thus significantly reduced lipid peroxidation.

Polyphosphate The activity of phosphoric acid is well known, the acid being added during deodorization of edible oils to increase oxidative stability. Watts (1950) found that phosphates are nearly inactive in meat while oligophosphates have good metal-chelating activity (rising from diphosphate to hexametaphosphate with increasing number of phosphate residues). Sato and Hegarty (1971) examined the effects of polyphosphates on precooked ground beef stored at 4°C for 2 days and indicated that hexametaphosphate was more efficient than triphosphate in inhibiting lipid peroxidation. Matlock et al. (1984) also found that triphosphate reduced the development of off-flavor and rancidity during extended periods of frozen storage of pork sausage patties.

Oxygen scavengers

The level of available oxygen may be controlled in foods by reducing compounds which function by transferring hydrogen to the system and scavenge oxygen thus preventing the involvement of oxygen in lipid peroxidation.

Ascorbic acid (Vitamin C) Ascorbic acid (Vitamin C) occurs widely in the vegetable world. Ascorbic acid and its salts are virtually insoluble in fats and oils and consequently cannot in practice be used as antioxidants in this medium. Being water soluble, ascorbic acid can be used as an antioxidant in products such as beer, soft drinks and fruit juices (Coppen, 1983). Ascorbic acid and its sodium salt are suggested to regenerate phenolic antioxidants by contributing hydrogen atoms to phenoxyl radicals provided by lipid peroxidation. When used in combination with other antioxidants, it functions as a synergist by promoting their antioxidative effects (Lindsay, 1985).

Ascorbyl palmitate Ascorbyl palmitate is a synthesized compound and has a greater solubility in fats and oils than that of ascorbic acid. However, its solubility is still very low when compared with other fat-soluble antioxidants and in order to dissolve ascorbyl palmitate it is usually necessary to use it in combination with a solubilizing

agent such as monoglyceride (Coppen, 1983). Ascorbyl palmitate appreciably increases the shelf life of vegetable oils when used at a level of 0.01%. According to Cort (1974), ascorbyl palmitate at 0.01% is more effective than BHA and BHT at 0.02% in its inhibitory action on rancidity.

MATERIALS AND METHODS

Materials

The beef muscle was purchased from the Tama Packing House in Tama, Iowa and the Meat Laboratory of Iowa State University. The pork muscle was purchased from the IBP, Inc. in Perry, Iowa and the Meat Laboratory of Iowa State University. The semitendinosus muscle from both beef and pork was used in the experiments. The muscle was obtained within 4-6 hours after slaughter. All muscles were trimmed of external fat, excessive marbling fat and visible connective tissue. Each muscle sample, weighing approximately 150 g, was placed in a polyethylene bag, wrapped in aluminum foil and immediately stored in an ultralow freezer (-70°C). On a given day for an assay, each muscle sample was taken out and used immediately.

Hydrogen peroxide (30% solution), L-ascorbic acid, trichloroacetic acid, potassium chloride, and disodium ethylene-diamine tetraacetate (EDTA) were purchased from Fisher (Fairlawn, NJ). Myoglobin type I from equine skeletal muscle, L-histidine-free base, thiobarbituric acid (TBA), bovine serum albumin (BSA), ferric chloride, ferrous chloride, ß-nicotinamide adenine dinucleotide phosphate disodium salt

reduced form type X (NADPH), ß-nicotinamide adenine dinucleotide disodium salt reduced form (NADH), adenosine 5'-diphosphate salt (ADP), α-tocopherol, and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

Preparation of microsomes

Isolation of the microsome fraction from either beef or pork muscle tissues was done by a procedure described by Apgar and Hultin (1982) with some modifications. On the day of microsome preparation, the muscle sample was removed from the freezer. The muscle was ground in a Sunbeam Oskar food processor with a plastic work bowl and a stainless steel blade for 60 seconds. Seventy grams of ground muscle were mixed with 280 ml 0.12 M KCl: 5 mM histidine buffer (pH 6.8) and were then homogenized for 90 seconds with Tekmar SDT tissue homogenizer. The homogenate was centrifuged at 4°C at 17,600 xg for 30 minutes in Beckman J2-21 M/E centrifuge. The supernatant fraction was filtered through eight layers of cheesecloth and centrifuged at 2°C at 105,000 xg for 60 minutes in a Beckman L3-50 ultracentrifuge to recover the crude microsome fraction. The sediment was then suspended in 0.6 M

KCl: 5 mM histidine buffer (pH 6.8) to solubilize contaminating myofibrillar protein. This was centrifuged again at 2°C at 105,000 xg for 60 minutes. The final sediment was resuspended in 0.12 M KCl: 5 mM histidine buffer (pH 6.8) to a volume of 2 ml. The resulting mixture was homogenized for 30 seconds using a Tekmar SDT tissue homogenizer. To preserve the microsomal activity, all the microsome preparation procedures were conducted in a 4°C cold room and all the equipment, glassware, and reagents were also prechilled and stored in the 4°C cold room. Except when indicated otherwise, microsomal fractions were used on the day of preparation.

Determination of protein content

Protein content of microsome fractions was determined immediately after preparation by the modified Lowry procedure (Markwell et al., 1978), using bovine serum albumin (BSA) as a standard.

Reaction mixture

The reaction mixtures for lipid peroxidation assays were incubated in air in a water bath shaker (Reichert Scientific Instruments) at 37°C. Basically, each reaction mixture contained 0.6 mg to 1 mg microsomal protein per ml buffer

solution (0.12 M KCl: 5 mM histidine, pH 6.8) with different additives according to the experimental design described in the following section.

Experimental design

In this study, the same experimental design was assigned to both beef and pork muscles. Each treatment in each design was triplicated using the same microsome preparation. Each experimental design was repeated at least twice using muscle tissues from different carcasses of beef or pork.

Enzymatic study This study was undertaken to determine if an enzyme-catalyzed lipid peroxidation system exists in microsomal fractions of beef and pork muscles. The additives include 0.2 mM NADPH, 0.2 mM NADH, 0.2 mM ADP, 15 μM $FeCl_2$, and different combinations of these which will be described in detail in the Results section.

Storage study The effects of storage conditions on the enzymatic activity of the microsomal fractions isolated from beef and pork muscles were examined. The reaction mixture included 0.2 mM NADPH, 0.2 mM ADP and 15 μM $FeCl_2$. Muscles were ground and randomly divided into several portions. These portions were stored at 4°C and -70°C for up

to 10 days and 4 weeks, respectively. Microsomes were prepared on each designated day the enzymatic activity test was conducted. Microsomal fractions were also prepared on day 0 then stored at 4°C and -70°C for up to 10 days and 4 weeks, respectively. Aliquots of microsome fractions were removed from the freezer on each designated day the enzymatic activity test was conducted.

Non-enzymatic study Two experiments were conducted to evaluate the effect of non-enzymatic systems on lipid peroxidation in microsomal fractions isolated from beef and pork muscles. The two experiments were: (1) Activity of a hydrogen peroxide-metmyoglobin system: the concentrations of both H_2O_2 and MetMb ranged from 0 to 100 μM and (2) activity of a hydrogen peroxide-ferrous chloride system: the concentrations of H_2O_2 ranged from 0 to 100 μM while that of $FeCl_2$ ranged from 0 to 250 μM.

Effects of antioxidants The effect of different types of antioxidants on both enzymatic and non-enzymatic lipid peroxidation in microsomes isolated from beef and pork muscles was examined. The antioxidants were (1) a chelating agent: EDTA (0 - 200 μM), (2) free-radical terminators: BHT (0 - 200 μM) and α-tocopherol (0 - 500 μM), and (3) an oxygen

scavenger: ascorbic acid (0 - 500 μM).

2-Thiobarbituric acid test (TBA test)

The quantity of thiobarbituric acid-reactive substance, expressed as malonaldehyde (MA), that was produced during the incubation was determined by the procedure described by Buege and Aust (1978). The MA concentration of the reaction mixture was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Microsomal lipid peroxidation activity was expressed as nanomoles of MA per milligram of microsomal protein.

Statistical analysis

For all statistical analyses, an analysis of variance was carried out and the Duncan's multiple-range test and Tukey's studentized range test were used to test treatment means. Statistical significance was accepted at $p < 0.01$ level.

RESULTS

Enzymatic Study

The presence of enzymatic lipid peroxidation systems in microsomal fractions of beef and pork muscles was demonstrated in this study. Malonaldehyde (MA) values for the pork muscle microsomes incubated with the enzyme cofactors and ferric chloride ($FeCl_3$) are shown in Table 1. There was no MA produced when the microsomes were incubated with ADP or ferric iron alone. The addition of NADPH alone only showed a slightly increased level of MA. The amount of MA produced by the reaction of microsomes with ADP plus ferric iron was no more than that produced by the reaction of microsomes with ADP or ferric iron alone but was slightly higher than that of the microsome control. When the NADPH was combined with ADP or/and ferric iron, the extent of lipid peroxidation became much more apparent. The effect of NADPH with ferric iron on lipid peroxidation in microsomes was much greater than that of NADPH with ADP. Lipid peroxidation reached its maximum when ADP, ferric iron, and NADPH were incubated together. These results indicated that NADPH was essential for enzymatic lipid peroxidation in microsomes isolated from pork muscle. Analogous experiments were performed using beef muscle

TABLE 1. Effect of the addition of NADPH, ADP, and $FeCl_3$ on malonaldehyde (MA) production in pork muscle microsomes

Treatment	MA (nmol/mg microsomal protein)
Microsome control	0.00[f]
Control + ADP	0.28[e,f]
Control + $FeCl_3$	0.28[e,f]
Control + ADP + $FeCl_3$	0.44[d,e]
Control + NADPH	0.66[c,d]
Control + ADP + NADPH	0.92[c]
Control + $FeCl_3$ + NADPH	3.17[b]
Control + ADP + $FeCl_3$ + NADPH	10.12[a]

[a,b,c,d,e,f]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f.

microsomes (see Table 2). The effects of NADPH, ADP and ferric iron on MA production were similar to those found for pork muscle microsomes. For maximum microsomal lipid peroxidation, the presence of NADPH, ADP, and ferric iron was necessary.

In the same study reported in Table 1, the enzyme system with ferrous chloride was examined in the pork muscle microsomes. The data from these experiments are presented in Table 3. The activity of ferrous iron alone is different from that of ferric iron alone. As shown in Table 1, no lipid peroxidation was measured by adding ferric iron alone, however the addition of ferrous iron alone dramatically increased MA production (see Table 3). The presence of ADP seems to have an inhibitory effect on ferrous iron in MA production, while NADPH with ferrous iron further increased MA production. Lipid peroxidation in microsomes of pork muscle reached its maximum only when NADPH, ADP and ferrous chloride were added to the reaction mixture. A similar result was also obtained using the microsomes of beef muscle (see Table 4).

In Tables 5 and 6, paired comparisons of the effect of ferric and ferrous iron are reported for pork and beef muscle microsomes, respectively. These comparisons clearly demonstrate that with any kind of combination the effect of

TABLE 2. Effect of the addition of NADPH, ADP, and $FeCl_3$ on malonaldehyde (MA) production in beef muscle microsomes

Treatment	MA (nmol/mg microsomal protein)
Microsome control	0.00^{f}
Control + ADP	$0.06^{e,f}$
Control + ADP + $FeCl_3$	$0.09^{e,f}$
Control + $FeCl_3$	$0.12^{d,e}$
Control + NADPH	0.23^{d}
Control + ADP + NADPH	0.38^{c}
Control + $FeCl_3$ + NADPH	0.98^{b}
Control + ADP + $FeCl_3$ + NADPH	3.57^{a}

^{a,b,c,d,e,f}Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f.

TABLE 3. Effect of the addition of NADPH, NADH, ADP, and $FeCl_2$ on malonaldehyde (MA) production in pork muscle microsomes

Treatment	MA (nmol/mg microsomal protein)
Microsome control	0.00[h]
Control + ADP	0.28[g,h]
Control + NADPH	0.66[f,g]
Control + ADP + NADPH	0.92[f]
Control + ADP + $FeCl_2$	2.30[e]
Control + ADP + NADH + $FeCl_2$	4.22[d]
Control + $FeCl_2$	6.12[c]
Control + NADPH + $FeCl_2$	7.50[b]
Control + ADP + NADPH + $FeCl_2$	11.90[a]

[a,b,c,d,e,f,g,b]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

TABLE 4. Effect of the addition of NADPH, NADH, ADP, and $FeCl_2$ on malonaldehyde (MA) production in beef muscle microsomes

Treatment	MA (nmol/mg microsomal protein)
Microsome control	0.00[h]
Control + ADP	0.06[h]
Control + NADPH	0.23[g]
Control + ADP + NADPH	0.38[f]
Control + ADP + $FeCl_2$	0.77[e]
Control + ADP + NADH + $FeCl_2$	1.24[d]
Control + $FeCl_2$	1.89[c]
Control + NADPH + $FeCl_2$	2.31[b]
Control + ADP + NADPH + $FeCl_2$	4.48[a]

[a,b,c,d,e,f,g,h]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

TABLE 5. Paired comparison of the effect between $FeCl_2$ and $FeCl_3$ on Malonaldehyde (MA) production in pork muscle microsomes

Treatment	MA (nmol/mg microsomal protein)
Comparison 1	
ADP + $FeCl_3$	0.44[b]
ADP + $FeCl_2$	2.30[a]
Comparison 2	
$FeCl_3$	0.28[b]
$FeCl_2$	6.12[a]
Comparison 3	
NADPH + $FeCl_3$	3.17[b]
NADPH + $FeCl_2$	7.50[a]
Comparison 4	
ADP + NADPH + $FeCl_3$	10.12[b]
ADP + NADPH + $FeCl_2$	11.90[a]

[a,b]Means of each comparison followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b.

TABLE 6. Paired comparison of the effect between $FeCl_2$ and $FeCl_3$ on Malonaldehyde (MA) production in beef muscle microsomes

Treatment	MA (nmol/mg microsomal protein)
Comparison 1	
ADP + $FeCl_3$	0.09[b]
ADP + $FeCl_2$	0.77[a]
Comparison 2	
$FeCl_3$	0.12[b]
$FeCl_2$	1.89[a]
Comparison 3	
NADPH + $FeCl_3$	0.98[b]
NADPH + $FeCl_2$	2.31[a]
Comparison 4	
ADP + NADPH + $FeCl_3$	3.57[b]
ADP + NADPH + $FeCl_2$	4.48[a]

[a,b]Means of each comparison followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b.

ferrous iron was always higher than that of ferric iron on microsomal lipid peroxidation in both pork and beef muscles.

The effect of the addition of NADPH or NADH on the extent of enzymatic lipid peroxidation in microsomes isolated from beef and pork muscles was also examined and reported in Table 3 and Table 4. Both beef and pork muscle microsomes utilized either NADPH or NADH, but NADPH was utilized more effectively. The rates of oxidation in the presence of NADH were 35% and 28% of the rates in the presence of NADPH for pork and beef muscle microsomes, respectively.

Storage Study

The study investigated the effect of storage time and storage temperature on microsomal enzymatic activity of both pork and beef muscles. The optimum combination of cofactors for the enzymatic system was used to conduct the storage study. Specifically 0.20 mM NADPH, 0.20 mM ADP, and 15 μM ferrous chloride were added to microsome preparations. When the intact pork muscle was stored at 4°C for 8 days, the enzyme activity of the isolated microsomes decreased gradually over the test period (see Table 7). The data also indicated that the intact muscle still possessed about 76% of the original enzyme activity (day 0) after 8 days of storage at

TABLE 7. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from pork muscle stored at 4°C

Days of Storage	MA (nmol/mg microsomal protein)
0	9.08[a]
2	7.95[b]
4	7.62[c]
8	6.96[d]

[a,b,c,d]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d.

4°C. Table 8 shows the results of the second approach used to study the stability of enzyme in the pork muscle. Microsomes were prepared freshly on the day of slaughter and stored at 4°C for 8 days. The enzyme activity of the isolated microsomes gradually decreased and microsomes retained around 77% of the original enzyme activity (day 0). Similar experiments were conducted by using beef muscle and the results are shown in Tables 9 and 10. The data demonstrated the same trends seen in the pork muscle. After 8 days of storage at 4°C, the enzyme activity was about 64% and 39% of initial values for intact muscle and isolated microsomes, respectively.

Effects of prolonged storage (up to 4 weeks) at -70°C on the enzyme activity of microsomes from pork and beef muscles were also studied (see Tables 11 to 14). For either muscles or microsomes at the ultralow temperature (-70°C), the microsomal enzyme activity did not change significantly up to 4 weeks of storage for either pork and beef muscles.

Non-enzymatic Study

This study was conducted to examine the effect of non-enzymatic systems on microsomal lipid peroxidation of pork and beef muscles. Two non-enzymatic systems were designed and

TABLE 8. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from pork muscle on day 0 and stored at 4°C

Days of Storage	MA (nmol/mg microsomal protein)
0	12.33[a]
2	11.70[b]
4	10.89[c]
6	10.14[d]
8	9.52[e]

[a,b,c,d,e]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e.

TABLE 9. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from beef muscle stored at 4°C

Days of Storage	MA (nmol/mg microsomal protein)
0	2.69[a]
2	2.66[a]
4	1.97[b]
8	1.73[c]

[a,b,c]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c.

TABLE 10. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from beef muscle on day 0 and stored at 4°C

Days of Storage	MA (nmol/mg microsomal protein)
0	3.31[a]
2	3.17[b]
4	2.82[c]
6	2.47[d]
8	1.29[e]

[a,b,c,d,e]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e.

TABLE 11. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from pork muscle stored at -70°C

Days of Storage	MA (nmol/mg microsomal protein)
0	9.03[a]
28	9.28[a]

[a]Means followed by the same letter are not significantly different at $P<0.01$.

TABLE 12. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from pork muscle on day 0 and stored at -70°C

Days of Storage	MA (nmol/mg microsomal protein)
0	8.57[a]
28	8.54[a]

[a]Means followed by the same letter are not significantly different at $P<0.01$.

TABLE 13. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from beef muscle stored at -70°C

Days of Storage	MA (nmol/mg microsomal protein)
0	3.57[a]
28	3.52[a]

[a]Means followed by the same letter are not significantly different at P<0.01.

TABLE 14. Effect of storage time on enzymatic lipid peroxidation in microsomes isolated from beef muscle on day 0 and stored at -70°C

Days of Storage	MA (nmol/mg microsomal protein)
0	3.57[a]
28	3.70[a]

[a]Means followed by the same letter are not significantly different at P<0.01.

used in our study: (1) a hydrogen peroxide (H_2O_2)-metmyoglobin (MetMb) system and (2) a H_2O_2-ferrous chloride (Fe^{2+}) system.

Microsomal lipid peroxidation in pork and beef muscles, initiated by the H_2O_2-MetMb system, is shown in Figures 1 and 2. Metmyoglobin in the presence of H_2O_2 initiated microsomal lipid peroxidation, whereas no peroxidation occurred with MetMb or H_2O_2 alone regardless of the concentration (10 - 100 μM) used. At 10 μM, 30 μM, 50 μM, or 100 μM MetMb, the lipid peroxidation increased as the concentration of H_2O_2 increased (10 -100 μM), 10 μM H_2O_2 had no effect on lipid peroxidation caused by 10 μM MetMb. For 10 μM, 30 μM, 50 μM, or 100 μM H_2O_2, maximum lipid peroxidation was always reached when the concentration of MetMb was 50 μM. Further increase in the concentration of MetMb up to 100 μM caused lipid peroxidation to decline for 50 μM and 100 μM H_2O_2 while it remained the same for 10 μM and 30 μM H_2O_2. The results for both pork and beef muscles were quite similar.

The results of the H_2O_2-Fe^{2+} system as shown in Figures 3 and 4 were quite different from that of H_2O_2-MetMb system. The addition of ferrous chloride alone within certain concentration ranges increased the microsomal lipid peroxidation significantly. As shown in Figures 3 and 4, from 10 μM to 50 μM ferrous chloride stimulated lipid peroxidation.

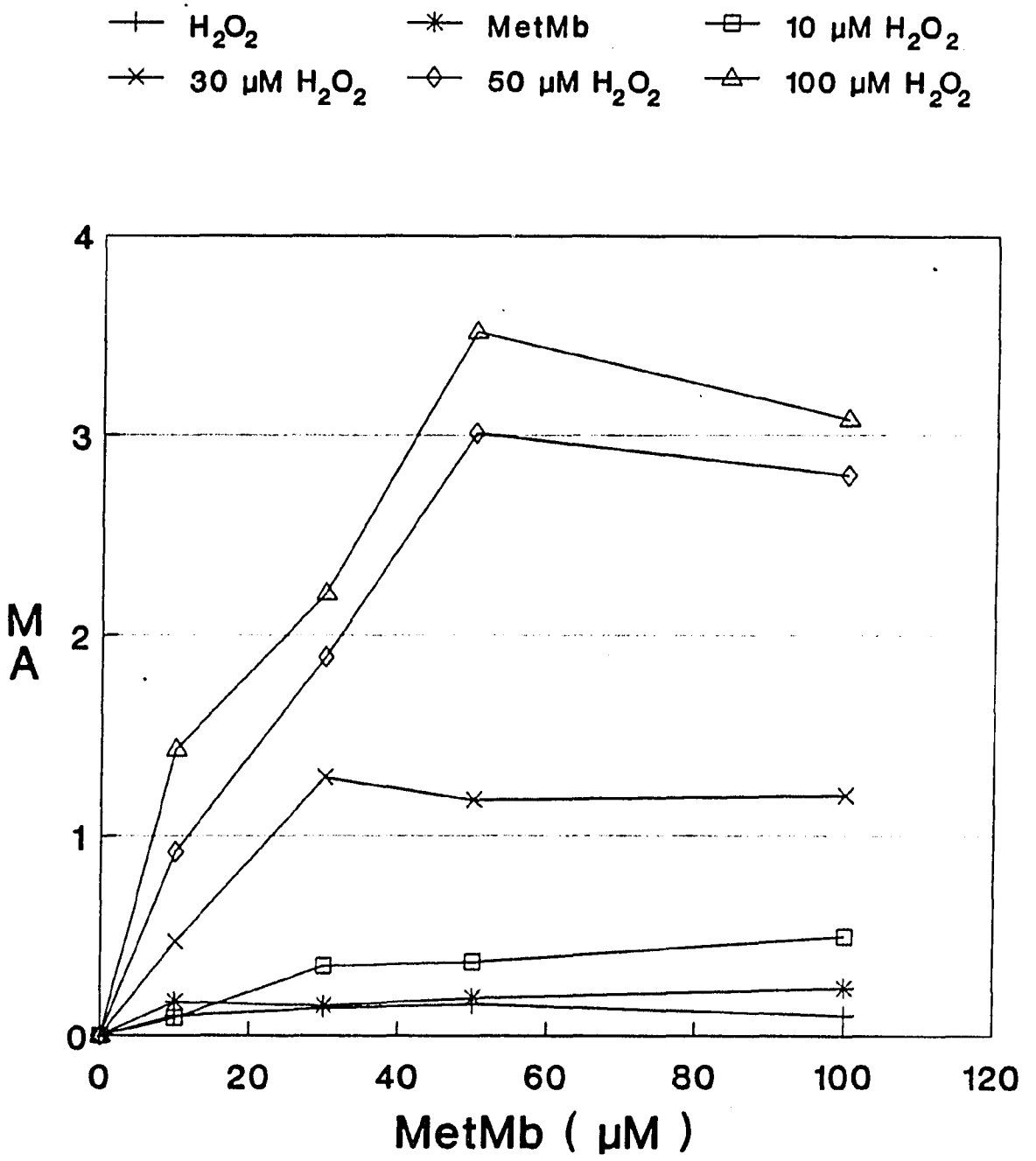


Figure 1. Effect of different concentration combinations of metmyoglobin and hydrogen peroxide on lipid peroxidation in microsomes isolated from pork muscle

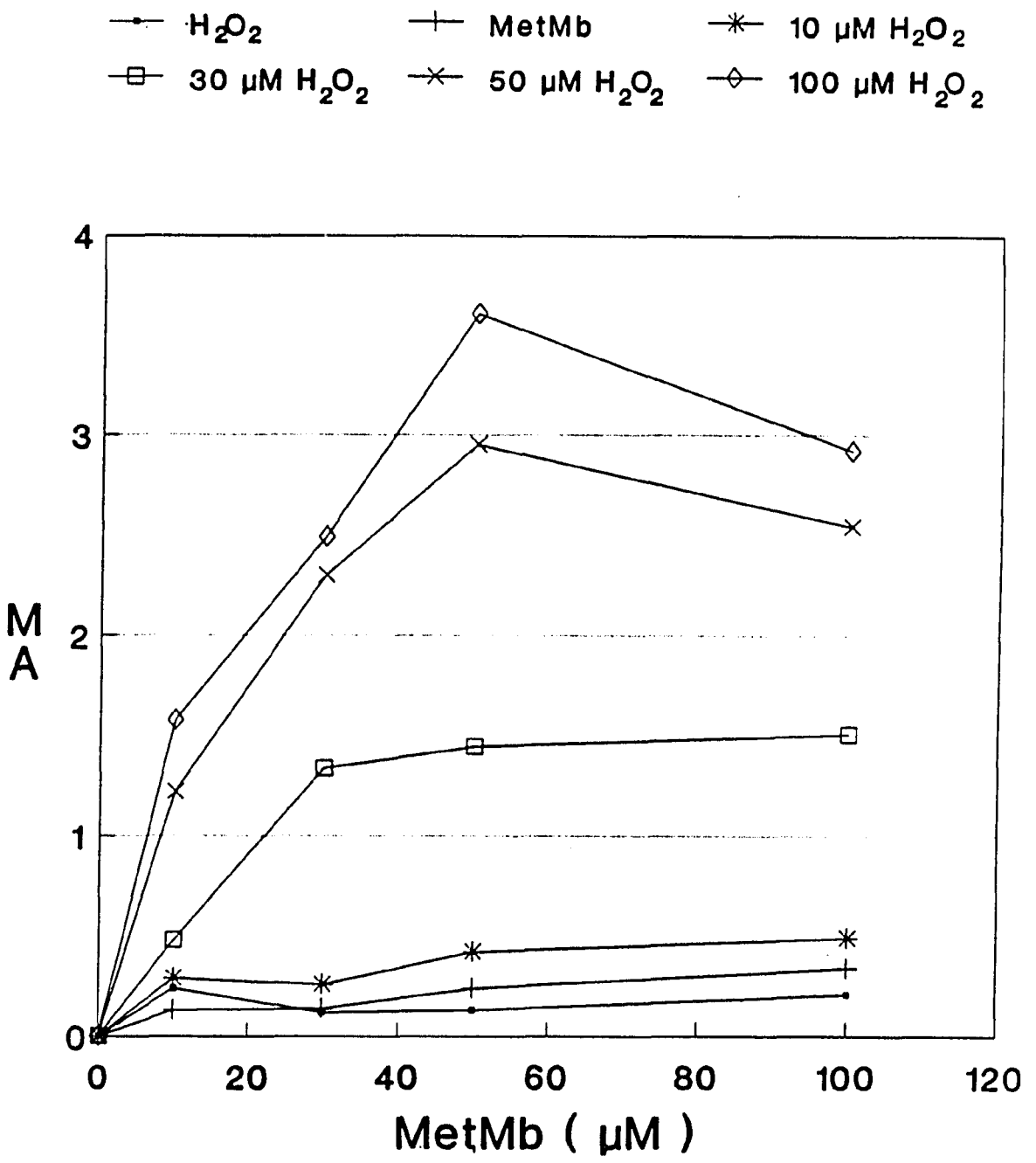


Figure 2. Effect of different concentration combinations of metmyoglobin and hydrogen peroxide on lipid peroxidation in microsomes isolated from beef muscle

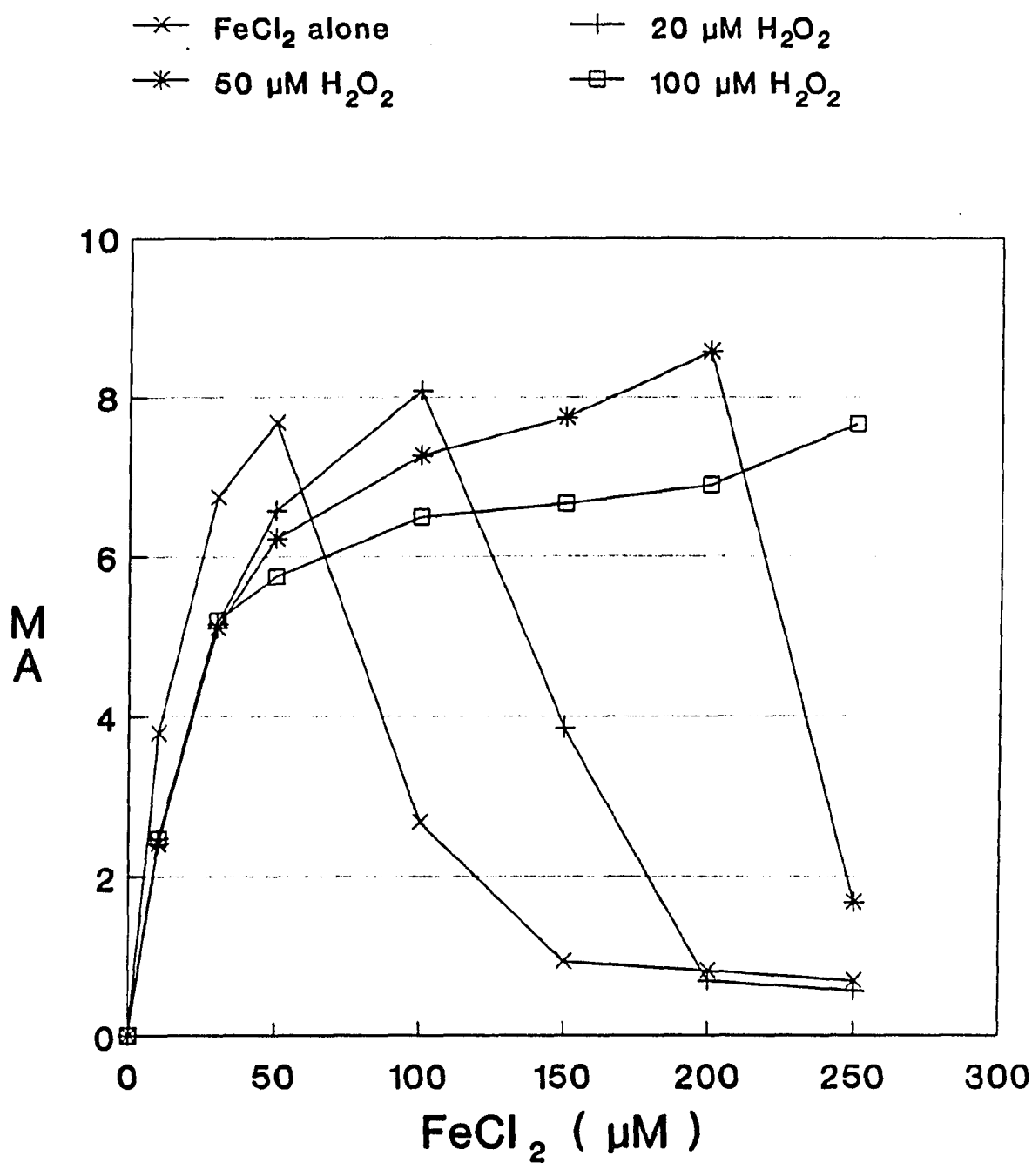


Figure 3. Effect of different concentration combinations of ferrous chloride and hydrogen peroxide on lipid peroxidation in microsomes isolated from pork muscle

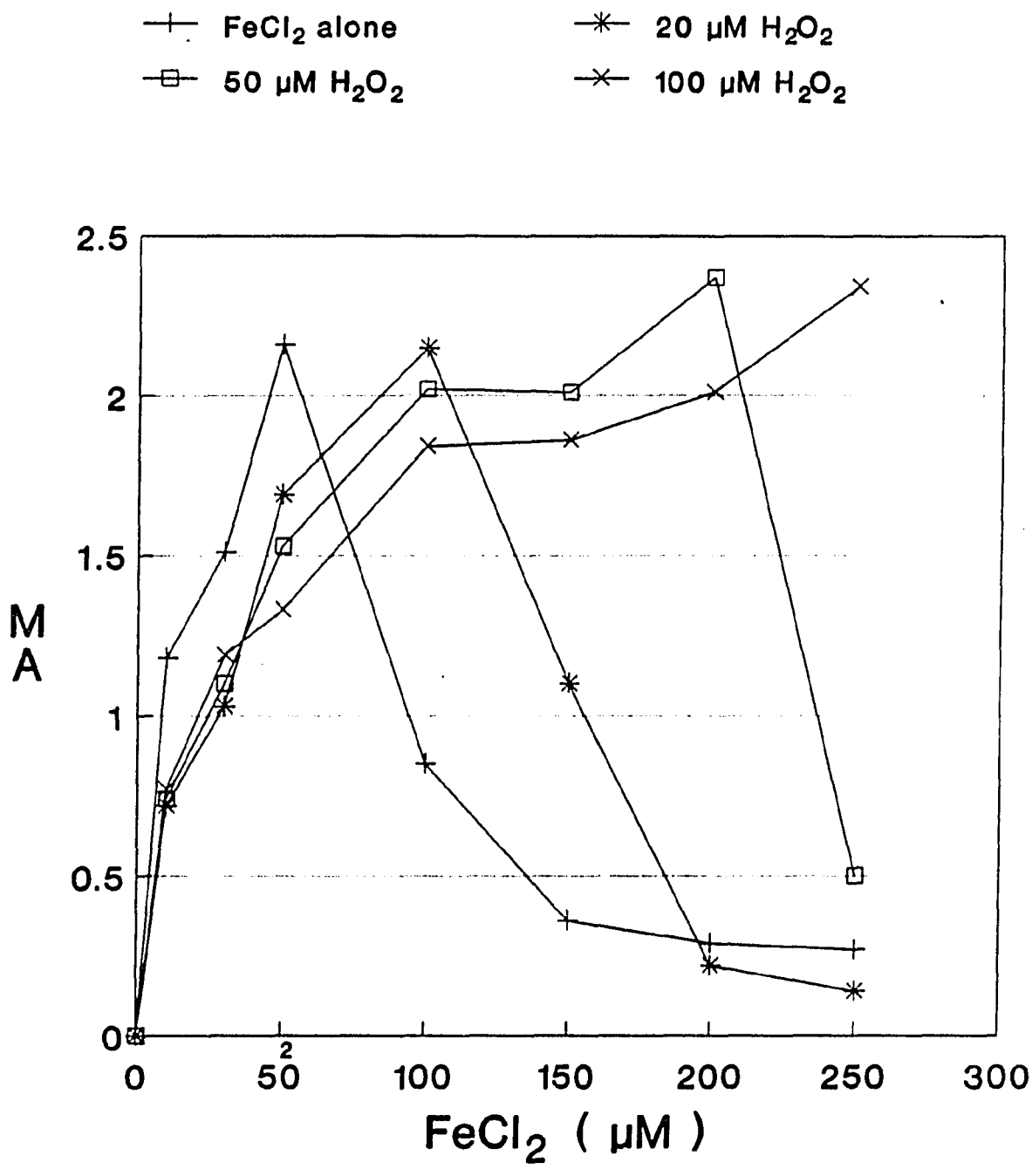


Figure 4. Effect of different concentration combinations of ferrous chloride and hydrogen peroxide on lipid peroxidation in microsomes isolated from beef muscle

Within this concentration range of ferrous chloride (10 - 50 μM), the incorporation of H_2O_2 decreased the extent of lipid peroxidation at all levels of H_2O_2 ranging from 20 μM to 100 μM. On the other hand, increasing ferrous chloride beyond 100 μM decreased microsomal lipid peroxidation dramatically. No MA production was measured beyond 150 μM up to 250 μM of ferrous chloride. Depending on the concentration, within 100 μM to 250 μM ferrous chloride, the addition of H_2O_2 from 20 μM to 100 μM may have different effects on microsomal lipid peroxidation. When 100 μM ferrous chloride and 20 -100 μM H_2O_2 were incubated together, MA production was much higher than that with 100 μM ferrous chloride alone. The enhanced effect was highest for 20 μM H_2O_2, 50 μM H_2O_2 was second highest and 100 μM H_2O_2 followed. This order changed when the concentration of ferrous chloride was increased to 150 μM. At this level, 20 - 100 μM H_2O_2 increased lipid peroxidation with the order of 50 μM > 100 μM > 20 μM. At 200 μM $FeCl_2$, the presence of 20 μM H_2O_2 did not enhance the effect, while 50 μM and 100 μM enhanced MA production with 50 μM having greater effect. The microsomal lipid peroxidation was increased significantly by 100 μM H_2O_2 when the concentration of $FeCl_2$ was 250 μM. The results imply that either the ferrous iron itself at low concentration ($\leq$ 50 μM) or a higher concentration of ferrous iron (>100 μM) and H_2O_2 (20 - 100 μM) are non-enzymatic

systems capable of initiating microsomal lipid peroxidation.

Effect of Antioxidants

This study was conducted to evaluate the effect of EDTA, BHT, α-tocopherol, and ascorbic acid on enzymatic and non-enzymatic lipid peroxidation in microsomes isolated from pork and beef muscles. In this study, the enzymatic system included 15 μM $FeCl_2$, 0.2 mM ADP, and 0.2 mM NADPH, while the non-enzymatic system included 150 μM $FeCl_2$ and 50 μM H_2O_2.

Tables 15 and 16 show the effect of EDTA on enzymatic lipid peroxidation in microsomes isolated from pork and beef muscles, respectively. In both cases, when the concentration of EDTA was lower than 15 μM, the presence of EDTA either slightly increased or did not change the extent of enzymatic lipid peroxidation. Once the concentration of EDTA exceeded 15 μM, enzymatic lipid peroxidation was significantly inhibited. At 30 μM EDTA, the reduction rates of MA production were 94% and 97% for pork and beef muscles, respectively. These observations suggest that the ratio of EDTA to ferrous iron plays a very important role in enzymatic lipid peroxidation. When the ratio is smaller than one, EDTA may act as a prooxidant. On the other hand, EDTA acts as an antioxidant when the ratio is larger than one.

TABLE 15. Effect of EDTA concentration on enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[h]
Enzymatic System	13.09[d]
Enzymatic System + EDTA	
1 μM	14.26[c]
5 μM	15.83[a]
10 μM	14.98[b]
15 μM	14.16[c]
30 μM	0.77[e]
60 μM	0.69[e,f]
100 μM	0.45[f,g]
200 μM	0.53[e,f,g]
500 μM	0.35[g]
1 mM	0.25[g,h]

[a,b,c,d,e,f,g,h]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

TABLE 16. Effect of EDTA concentration on enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[h]
Enzymatic System	2.83[c]
Enzymatic System + EDTA	
1 μM	3.09[d]
5 μM	3.86[a]
10 μM	3.50[b]
15 μM	3.36[c]
30 μM	0.21[f]
60 μM	0.20[f]
100 μM	0.12[f,g]
1 mM	0.09[g,h]

[a,b,c,d,e,f,g,h]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

The data presented in Table 17 and 18 show that EDTA at a concentration higher than 150 μM inhibits non-enzymatic lipid peroxidation almost completely. As the amount of ferrous iron used in non-enzymatic system was 150 μM, the results support the theory that EDTA accelerates lipid peroxidation up to a 1:1 ratio of EDTA to ferrous iron.

At concentrations as low as 10 - 20 μM, BHT significantly inhibited (90%) both enzymatic and non-enzymatic lipid peroxidation in microsomes isolated from pork and beef muscles (see Tables 19 to 22). The concentrations of α-tocopherol required to reduce lipid peroxidation significantly were much higher than that for BHT (see Tables 23 to 26). Results suggest that in order to obtain 90% inhibition of lipid peroxidation, the concentration of α-tocopherol has to go as high as 1 mM. The effectiveness of ascorbic acid as an antioxidant fell somewhere between BHT and α-tocopherol. Around 90% inhibition of both enzymatic and non-enzymatic lipid peroxidation was achieved with 500 μM ascorbic acid (see Tables 27 to 30).

TABLE 17. Effect of EDTA concentration on non-enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[f]
Non-enzymatic System	10.60[c]
Non-enzymatic System + EDTA	
1 μM	10.09[d]
5 μM	10.83[c]
10 μM	10.74[c]
15 μM	10.60[c]
30 μM	11.91[b]
100 μM	12.34[a]
150 μM	11.58[b]
200 μM	0.93[e]

[a,b,c,d,e,f]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f.

TABLE 18. Effect of EDTA concentration on non-enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[e]
Non-enzymatic System	6.29[b,c]
Non-enzymatic System + EDTA	
1 μM	6.22[b,c]
5 μM	6.37[b,c]
10 μM	6.23[b,c]
15 μM	6.12[c]
30 μM	6.50[b]
100 μM	7.09[a]
150 μM	6.50[b]
200 μM	0.48[d]

[a,b,c,d,e]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e.

TABLE 19. Effect of BHT concentration on enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[e]
Enzymatic System	3.86[a]
Enzymatic System + BHT	
1 μM	1.88[b]
5 μM	0.64[c]
10 μM	0.32[d]
15 μM	0.29[d]
30 μM	0.07[e]
50 μM	0.06[e]

[a,b,c,d,e]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e.

TABLE 20. Effect of BHT concentration on enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[e]
Enzymatic System	2.49[a]
Enzymatic System + BHT	
1 μM	2.44[a]
5 μM	1.26[b]
10 μM	0.46[c]
15 μM	0.19[d]
30 μM	0.18[d]
50 μM	0.07[e]

[a,b,c,d,e]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e.

TABLE 21. Effect of BHT concentration on non-enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[g]
Non-enzymatic System	9.09[a]
Non-enzymatic System + BHT	
1 μM	4.93[b]
5 μM	1.64[c]
10 μM	0.77[d]
20 μM	0.64[d,e]
50 μM	0.47[e,f]
100 μM	0.32[f]

[a,b,c,d,e,f,g]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g.

TABLE 22. Effect of BHT concentration on non-enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[g]
Non-enzymatic System	4.24[a]
Non-enzymatic System + BHT	
1 μM	3.14[b]
5 μM	1.36[c]
10 μM	0.91[d]
20 μM	0.44[e]
50 μM	0.35[e]
100 μM	0.22[f]

[a,b,c,d,e,f,g]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g.

TABLE 23. Effect of α-tocopherol concentration on enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[i]
Enzymatic System	11.90[a]
Enzymatic System + α-tocopherol	
1 μM	12.06[a]
5 μM	11.71[a]
10 μM	11.99[a]
15 μM	11.88[a]
20 μM	11.29[b]
40 μM	10.85[c]
60 μM	10.17[d]
100 μM	8.15[e]
200 μM	3.39[f]
500 μM	1.39[g]
1 mM	0.80[h]

[a,b,c,d,e,f,g,h,i]Means followed by the same letter are not significantly different at P<0.01, and means decreased in the order of a>b>c>d>e>f>g>h>i.

TABLE 24. Effect of α-tocopherol concentration on enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[h]
Enzymatic System	2.49[a,b]
Enzymatic System + α-tocopherol	
1 μM	2.47[a,b]
15 μM	2.51[a]
30 μM	2.42[b]
60 μM	2.14[c]
100 μM	1.57[d]
200 μM	1.44[e]
500 μM	0.64[f]
1 mM	0.22[g]

[a,b,c,d,e,f,g,h]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

TABLE 25. Effect of α-tocopherol concentration on non-enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[h]
Non-enzymatic System	8.08[a]
Non-enzymatic System + α-tocopherol	
1 μM	7.68[b,c]
10 μM	7.78[b]
30 μM	7.57[c]
100 μM	6.42[d]
200 μM	5.23[e]
500 μM	1.74[f]
1 mM	0.61[g]

[a,b,c,d,e,f,g,h]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

TABLE 26. Effect of α-tocopherol concentration on non-enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[h]
Non-enzymatic System	4.24[a]
Non-enzymatic System + α-tocopherol	
1 μM	4.15[a,b]
5 μM	4.05[b]
10 μM	4.09[b]
30 μM	3.57[c]
100 μM	3.02[d]
200 μM	2.17[e]
500 μM	1.12[f]
1 mM	0.81[g]

[a,b,c,d,e,f,g,h]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h.

TABLE 27. Effect of ascorbic acid concentration on enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[i]
Enzymatic System	10.13[a]
Enzymatic System + Ascorbic Acid	
1 μM	9.69[b]
5 μM	9.55[b]
10 μM	9.32[c]
15 μM	9.16[c]
30 μM	9.18[c]
60 μM	8.70[d]
100 μM	3.24[e]
200 μM	1.83[f]
500 μM	0.67[g]
1 mM	0.26[h]

[a,b,c,d,e,f,g,h,i]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h>i.

TABLE 28. Effect of ascorbic acid concentration on enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[i]
Enzymatic System	3.07[b,c]
Enzymatic System + Ascorbic Acid	
5 μM	3.15[a,b]
10 μM	3.13[a,b]
30 μM	3.04[c]
60 μM	2.81[d]
100 μM	2.44[e]
200 μM	1.27[f]
500 μM	0.57[g]
1 mM	0.08[h]

[a,b,c,d,e,f,g,h,i]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g>h>i.

TABLE 29. Effect of ascorbic acid concentration on non-enzymatic lipid peroxidation in microsomes isolated from pork muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[g]
Non-enzymatic System	10.60[a]
Non-enzymatic System + Ascorbic Acid	
1 μM	10.06[b]
5 μM	10.20[b]
10 μM	4.42[c]
30 μM	2.24[d]
100 μM	1.99[d]
200 μM	1.49[e]
500 μM	0.90[f]

[a,b,c,d,e,f,g]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f>g.

TABLE 30. Effect of ascorbic acid concentration on non-enzymatic lipid peroxidation in microsomes isolated from beef muscle

Treatment	MA (nmol/mg microsomal protein)
Microsome Control	0.00[f]
Non-enzymatic System	4.50[a]
Non-enzymatic System + Ascorbic Acid	
1 μM	4.40[a]
5 μM	3.56[b]
10 μM	2.32[c]
30 μM	0.64[d]
100 μM	0.70[d]
200 μM	0.36[e]

[a,b,c,d,e,f]Means followed by the same letter are not significantly different at $P<0.01$, and means decreased in the order of a>b>c>d>e>f.

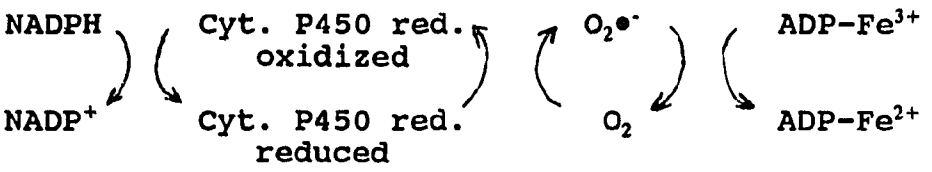
DISCUSSION

Because lipid peroxidation is an important problem in muscle foods such as beef, pork, chicken, turkey, and fish, there has been a great deal of interest in identifying the catalysts that promote the peroxidation of muscle lipids. Lin and Hultin (1976; 1977) and Player and Hultin (1977) demonstrated the presence of an enzymatic system for the peroxidation of microsomal lipids in the microsome fraction of chicken muscle. Incubation of the microsome fraction isolated from chicken muscle in the presence of NADPH, ADP, and ferric iron was shown to result in maximum lipid peroxidation. NADH was able to replace NADPH as the source of reducing equivalents but was less efficient. However, for enzymatic lipid peroxidation in fish muscle microsomes, NADH was much more efficient than NADPH. Other requirement for this system in fish muscle microsomes were the same as those of chicken muscle microsomes (McDonald et al., 1979; Slabyj and Hultin, 1982).

Enzymatic lipid peroxidation in microsomes isolated from beef muscle has also been demonstrated by Rhee et al. (1984). The reaction required NADPH or NADH, ADP, and ferrous or ferric iron. The rate of peroxidation was higher with NADPH than with NADH and also higher with ferrous iron than with ferric iron.

Results shown in Table 2 and Table 4 confirm the occurrence of an enzymatic system for lipid peroxidation in the microsomes of beef muscle. Similar to findings by Rhee et al. (1984), the rate of peroxidation was higher with ferrous iron than with ferric iron. The effect of NADH on microsomal lipid peroxidation was also examined (see Table 3 and Table 4). The rate of oxidation in the presence of NADH was 28% of the rate in the presence of NADPH for the microsomes of beef muscle. Our study is the first investigation of microsomal lipid peroxidation in pork muscle. The enzymatic reaction required NADPH or NADH, ADP, and ferrous or ferric iron, but NADPH and ferrous iron were utilized more effectively. The extent of lipid peroxidation in the presence of NADH was 35% of the rate in the presence of NADPH.

Based on these findings, it appears that the microsomal enzymatic lipid peroxidation system from fish muscle is more dependent on the presence of NADH while the systems from mammalian and avian muscles have a preference for NADPH. Ferrous iron consistently plays a more active role than that of ferric iron in the microsomal enzymatic systems from all muscle studies. In a review paper, Kanner et al. (1986) proposed a schematic outline of electron flow in microsomal NADPH-dependent lipid peroxidation, shown as follows:



This scheme indicates that NADPH is necessary to initiate the reaction. The role of cytochrome P450 reductase in membrane lipid peroxidation was established by Pederson et al. (1973). In the presence of NADPH, reduction of oxygen to produce active oxygen species ($O_2\bullet^-$) via cytochrome P450 reductase can be triggered. Through a redox reaction, the $O_2\bullet^-$ can further reduce the ADP-Fe^{3+} to form ADP-Fe^{2+}. Ferrous chelates could undergo several reactions which might lead to formation of an initiator for the microsomal lipid peroxidation processes.

When the enzymatic system with ferric chloride was used (see Tables 1 and 2), the addition of NADPH, ADP, ferric chloride, and microsomes initiated the enzymatic reaction of cytochrome P450 reductase by producing active compounds such as ferrous iron. Then these active compounds can initiate lipid peroxidation processes through different kinds of reactions which may involve non-enzymatic means. The term "enzymatic system" used in our study should be defined as the enzymatic reaction which produces the active compound such as ferrous chelates.

The scheme mentioned above can also explain the different effects observed between ferric and ferrous chloride on the

extent of microsomal lipid peroxidation (see Tables 5 and 6). Microsomal lipid peroxidation was always higher with ferrous iron than that with ferric iron. One possible reason is that the direct addition of ferrous iron eliminates the lag transition phase which is needed for ferric iron. Also, when NADPH, ADP, ferrous chloride, and microsomes are incubated together, there are two possible reactions that occur simultaneously: (1) NADPH triggers the enzymatic reaction of cytochrome P450 reductase and (2) ferrous iron triggers other reactions to initiate microsomal lipid peroxidation.

The role of iron in initiating lipid peroxidation reactions within biological membranes has been examined in a variety of systems. Ferric iron or ferric chelates can initiate lipid peroxidation reactions provided that a reducing agent is present to reduce Fe^{3+} to Fe^{2+}. Initiation of lipid peroxidation both in vitro and in vivo by Fe^{3+} and $O_2\bullet^-$ has been hypothesized to occur via production of •OH arising through the iron-catalyzed Haber-Weiss reaction (Halliwell, 1978):

$$Fe^{3+} + O_2\bullet^- \longrightarrow Fe^{2+} + O_2$$

$$O_2\bullet^- + HO_2\bullet \longrightarrow O_2 + H_2O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

Certain iron chelates, particularly those with ADP, are known

to accelerate the reaction and the formation of •OH. The •OH produced is highly reactive and can initiate lipid peroxidation by hydrogen abstraction (Gutteridge et al., 1979):

$$LH + {}^{\bullet}OH \longrightarrow L^{\bullet} + H_2O$$

The addition of 15 μM $FeCl_3$ alone (see Tables 1 and 2) or of 10 - 100 μM MetMb (Fe^{3+}) alone (see Figures 1 and 2) to microsomal fractions of pork and beef muscles resulted in no stimulation of lipid peroxidation. The results are expected because there is no reducing agent in the incubated system to initiate the Haber-Weiss reaction.

Ferrous iron may also initiate lipid peroxidation reactions provided an oxidant to oxidize Fe^{2+} to Fe^{3+}. In most cases, the oxidant is either O_2 or H_2O_2:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2{}^{\bullet-}$$

$$O_2{}^{\bullet-} + O_2{}^{\bullet-} \xrightarrow{H^+} H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

$$LH + {}^{\bullet}OH \longrightarrow L^{\bullet} + H_2O$$

The combination of Fe^{2+} and H_2O_2, known as Fenton's reagent, is often used to initiate lipid peroxidation reaction. Fenton's reagent can produce highly reactive •OH which can initiate the lipid peroxidation reaction (Aust et al., 1985).

The decomposition of H_2O_2 by Fe^{3+} also occurred but it was considerably slower than Fenton's reaction. Like the metal catalyzed Haber-Weiss reaction, it also appeared to be a redox chain reaction (Barb et al., 1951; Walling and Weil, 1974):

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HO_2\bullet + H^+$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

$$\bullet OH + H_2O_2 \longrightarrow H_2O + HO_2\bullet$$

$$HO_2\bullet + Fe^{3+} \longrightarrow Fe^{2+} + H^+ + O_2$$

$$HO_2\bullet + Fe^{2+} \xrightarrow{H^+} H_2O_2 + Fe^{3+}$$

$$\bullet OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^-$$

From this series of reactions it is implied that the incubation of both Fe^{3+} and H_2O_2 may also initiate lipid peroxidation reactions.

In this study, the effect of the H_2O_2-MetMb system and H_2O_2-Fe^{2+} system on microsomal lipid peroxidation in pork and beef muscles was also examined. Figures 1 and 2 show the results of H_2O_2-MetMb system. No MA production was found in the presence of microsomes with MetMb or H_2O_2 alone. Under these conditions, there were no other substrates present with which either MetMb or H_2O_2 could react to produce active compounds and result in initiation of lipid peroxidation. However, the incubation of H_2O_2 with MetMb led to increasing

lipid peroxidation. This finding was similar to that of Kanner and Harel (1985). In their study, they used the microsomes of turkey dark muscles as a model system; and further suggested that the interaction between MetMb and H_2O_2 can produce an active compound; porphyrin cation radical, $P^+ - Fe^{4+} = O$; and lead to the initiation of lipid peroxidation. Our study with the H_2O_2-MetMb system indicates that at certain concentrations of MetMb, lipid peroxidation increased as the concentration of H_2O_2 increased. Also, for a certain concentration of H_2O_2, the lipid peroxidation always reached its maximum when MetMb was 50 μM. These results suggested that excessive amounts of H_2O_2 over MetMb may be necessary to stimulate the redox chain reaction of H_2O_2 with Fe^{3+} as proposed above, while excessive amounts of MetMb may somehow inhibit the redox chain reaction.

This study was the first one to use the H_2O_2-Fe^{2+} system in microsomes of muscle foods. The findings were much more complicated than that of the H_2O_2-MetMb system (see Figures 3 and 4). At low concentrations (10 to 50 μM), the presence of Fe^{2+} alone increased microsomal lipid peroxidation. Within this concentration range, Fe^{2+} with oxygen (O_2) can initiate the reaction:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2\bullet^-$$

and subsequent redox chain reactions. Active compounds such as H_2O_2, $O_2\bullet^-$, and $\bullet OH$ can be produced from these reactions. Directly or indirectly, these active compounds may be involved in the initiation of lipid peroxidation. At the low concentrations of Fe^{2+}, the addition of 20 - 100 μM H_2O_2 slightly decreased the MA production at each level of Fe^{2+}; 10 μM, 30 μM, and 50 μM. One possible reason is that under these conditions (relatively high amount of H_2O_2) H_2O_2 may also be involved in some reactions other than Fenton's reaction. Borg and Schaich (1988) suggested that increasing concentrations of H_2O_2 can also convert these same strong oxidants to less reactive species by way of the following reactions:

$$H_2O_2 + \bullet OH \longrightarrow HO_2\bullet + H_2O$$

$$H_2O_2 + LO\bullet \longrightarrow HO_2\bullet + LOH$$

As the amount of Fe^{2+} increased from 100 μM to 250 μM, MA production sharply decreased. Borg and Schaich (1988) proposed an explanation for this phenomenon. At high concentrations, metals shift from being repeatedly cycled catalysts to serving as stoichiometric reactants, and they may also exhibit dominant chain termination behavior, which is not seen at low concentration. An important chain-terminating reaction of ferrous iron is shown in the following:

$$Fe^{2+} + LOO\bullet + H^{+} \xrightarrow{fast} Fe^{3+} + LOOH$$

Other antioxidant actions of high levels of ferrous iron may include the following:

$$Fe^{2+} + \bullet OH + H^{+} \xrightarrow{fast} Fe^{3+} + H_2O$$

$$Fe^{2+} + LO\bullet + H^{+} \xrightarrow{fast} Fe^{3+} + LOH$$

These reactions inhibit initiation reactions and chain branching thus preventing the onset of lipid peroxidation. When 20 - 100 μM H_2O_2 was added with the "antioxidant" concentrations of Fe^{2+}, the MA production increased again. It is possible that H_2O_2 competes with the radical compounds and reacts with the excessive amount of Fe^{2+} to produce a Fenton's reaction.

EDTA, a good sequestering agent, has been studied extensively in recent years in many model systems of lipid peroxidation. Numerous studies with EDTA have demonstrated the complexity it imparts upon the reactivity of iron. Gutteridge et al. (1979) and Winston et al. (1984) suggested that the chelate to iron ratio can markedly affect the mechanism of initiation of lipid peroxidation by EDTA-Fe^{3+} complexes. At EDTA:iron ratios of less than one, maximal rates of peroxidation were noted which were suggested to be

due to optimum •OH generation resulting from a rapidly autoxidizing pool (EDTA-Fe^{2+}) and the slower oxidizing free Fe^{2+}. At a strict 1:1 ratio, it has been suggested that •OH could be formed to initiate lipid peroxidation but at ratios greater than one, lipid peroxidation was inhibited. Similarly, lipid peroxidation initiated by Fenton's reaction using EDTA-Fe^{2+} is dependent upon the EDTA:Fe^{2+} ratio (Gutteridge, 1984b; Kornbrust and Mavis, 1980; Tien et al., 1982). The results from this study concerning the effect of EDTA on either enzymatic system or H_2O_2-Fe^{2+} system were consistent with this theory.

The effectiveness of other antioxidants such as BHT, α-tocopherol, and ascorbic acid has also been examined in our study. Based on the results, BHT was the most effective antioxidant, followed by ascorbic acid and α-tocopherol. The action of BHT and α-tocopherol was to terminate the free radical chain which may be involved in iron-catalyzed redox reactions or initiation and propagation of lipid peroxidation processes. Ascorbic acid, however, acts through the inactivation of active oxygen such as O_2•$^-$ and •OH and thus inhibits lipid peroxidation.

Lipid peroxidation is a major cause of quality deterioration in stored foods, especially in muscle foods. The mechanism of lipid peroxidation in muscle foods has been

studied by several researchers utilizing different model systems such as linoleate emulsion system (Liu, 1970a,b; Tay et al., 1983), water-washed muscle system (Love and Pearson, 1974; Rhee et al., 1987), and the muscle microsome system (Lin and Hultin, 1976; Rhee et al., 1984; Harel and Kanner, 1985). The fatty acid emulsion system can be used to investigate the general problem of lipid peroxidation but not to simulate lipid peroxidation in muscle foods, while the water-washed muscle system and muscle microsome system are closer to the actual situation in muscle foods. This study was the first investigation of microsome lipid peroxidation in pork muscle and the first examination of the effect of H_2O_2-Fe^{2+}, Fe^{2+}, Fe^{3+}, and antioxidants on lipid peroxidation in microsomes isolated from beef and pork muscles. This research was conducted in order to extend knowledge of the initiation and stimulation of lipid peroxidation in muscle tissues.

There has been a great deal of interest in identifying the catalysts that promote the peroxidation of muscle lipids. Considerable differences of opinion have been expressed among researchers regarding the importance of the state of iron to catalytic activity in lipid peroxidation. It is more convincing to believe that the Fe^{3+} state of the heme compound and the Fe^{2+} state of the non-heme iron are the most active in increasing lipid peroxidation (Schaich, 1980; Love and

Pearson, 1974; Rhee et al,. 1987). These observations in the non-enzymatic studies suggested that under certain circumstance, the ferrous iron is much more active than ferric free iron and MetMb. Furthermore, H_2O_2 plays a very important role in activating MetMb and ferrous free iron to stimulate lipid peroxidation. It was also found that the concentration of H_2O_2, heme iron, and non-heme iron should be taken into account when dealing with activating or deactivating questions. The contradicting reports with regard to the roles of heme iron and non-heme iron in lipid peroxidation of muscle foods may be explained by the conditions of the meat samples, especially the amount of heme iron, non-heme iron and H_2O_2 present in different studies. While lipid peroxidation in muscle foods has been commonly regarded as non-enzymatic in nature, there is evidence for the presence of enzymatic lipid peroxidation systems associated with muscle microsomes. The presence of this enzymatic system in pork muscle microsomes was further confirmed.

All the factors, such as H_2O_2, heme iron, non-heme iron, NADPH, NADH, and ADP, that were examined in this study may be present in some amount in muscle foods. From these observations some assumptions can be made. The enzymatic system and non-enzymatic system are equally important. Once the enzymatic system has been initiated, the enzymatic and

non-enzymatic lipid peroxidation may occur simultaneously.

SUMMARY

The current study was conducted to: (1) determine whether enzymatic systems which could induce lipid peroxidation are present in microsomes isolated from beef and pork muscles; (2) investigate the effect of storage time and storage temperature on enzymatic activity; (3) examine the effect of non-enzymatic systems using heme iron (Metmyoglobin), non-heme iron (ferrous iron), and Fenton reagent (hydrogen peroxide and Metmyoglobin or ferrous iron) on lipid peroxidation in microsomes isolated from beef and pork muscles; and (4) evaluate the effect of BHT, EDTA, α-tocopherol and ascorbic acid on both enzymatic and non-enzymatic lipid peroxidation.

The presence of an enzymatic lipid peroxidation system in microsomal fractions of beef or pork skeletal muscle has been demonstrated in this study. The enzymatic reaction required ADP, NADPH or NADH, and ferrous or ferric iron. The extent of lipid peroxidation was higher with NADPH than with NADH and also was higher with ferrous iron than with ferric iron. The enzyme activity of the isolated microsomes decreased when muscle was stored at 4°C for 8 to 10 days. The same result was also found when isolated microsomes were stored at 4°C for 8 to 10 days. But either intact muscles or isolated microsomes could be stored at -70°C for up to a month without a decrease

in the enzyme activity of the microsomes.

A hydrogen peroxide - MetMb (heme iron) system and a hydrogen peroxide - ferrous iron (non-heme iron) system also caused lipid peroxidation in muscle microsomes. Metmyoglobin in the presence of hydrogen peroxide initiated microsomal lipid peroxidation, whereas no oxidation occurred in the presence of MetMb or hydrogen peroxide alone. At certain concentrations of MetMb, lipid peroxidation increased as the concentrations of hydrogen peroxide increased (10 to 100 μM). For certain concentrations of hydrogen peroxide, the maximum lipid peroxidation was always reached when MetMb was 50 μM. When the concentration of MetMb was elevated up to 100 μM, lipid peroxidation began to decline.

The results of the hydrogen peroxide - ferrous iron system were quite different from those of hydrogen peroxide - MetMb system. With 10 μM to 50 μM ferrous iron alone, there was significant stimulation of microsomal lipid peroxidation. Within this concentration range, the addition of hydrogen peroxide decreased the extent of lipid peroxidation regardless of the amount of hydrogen peroxide (20 - 100 μM). On the other hand, when the concentrations of ferrous iron were further increased to 100 μM - 250 μM range, the ferrous iron alone caused little or no lipid peroxidation. Depending on the concentrations of ferrous iron within this range, the addition of hydrogen peroxide (20 to 100 μM) may have

different effects on microsomal lipid peroxidation. The following combinations enhanced lipid peroxidation:

20 μM	hydrogen peroxide -	100 and 150 μM ferrous iron
50 μM	hydrogen peroxide -	100, 150, and 200 μM ferrous iron
100 μM	hydrogen peroxide -	100, 150, 200, and 250 μM ferrous iron

For the enzymatic and non-enzymatic systems, under the same conditions, the extent of microsomal lipid peroxidation of pork muscles was always higher than that of beef muscles.

Antioxidants such as BHT, EDTA, and ascorbic acid in either enzymatic or non-enzymatic systems inhibited microsomal lipid peroxidation. At concentrations as low as 10 μM and 20 μM, BHT totally inhibited enzymatic and non-enzymatic lipid peroxidation, respectively. The ratio between EDTA and iron plays a crucial role in determining whether it increased or decreased lipid peroxidation. When the ratio was smaller than one, the presence of EDTA either slightly increased or did not change lipid peroxidation. However, EDTA totally inhibited lipid peroxidation when the ratio was larger than one. The concentration of ascorbic acid and α-tocopherol that effectively inhibited peroxidation was 500 μM and 1 mM, respectively, for both enzymatic and non-enzymatic lipid peroxidation.

SECTION II

BIOLUMINESCENT BACTERIAL MUTAGENESIS TEST FOR AUTOXIDIZED FATTY ACIDS, CHLORINATED FATTY ACIDS, HEATED OILS, MYCOTOXINS AND HETEROCYCLIC AMINES

INTRODUCTION

Safety Evaluation of Chemicals

It has been estimated that approximately 25,000 to 55,000 chemicals are in significant production in the United States and about 50,000 to 65,000 are believed to be in everyday use. In the United States alone, 300-700 to as many as 3,000 new industrial chemicals are introduced annually into economic use (Fishbein, 1987). The human population is exposed to an ever increasing number of food additives, drugs, cosmetics, and chemical substances which are essential for life and are of great social and economic benefit. In conjunction with the introduction of each new chemical, an equally great probability exists for the incidence of adverse affects upon the population. Not only the manufacturer but the consumer and subsequently various legislative bodies are increasingly aware of the need for establishing not only the effectiveness of such chemicals but their safety as well.

The evaluation of safety or hazard must take into account the conditions of use or possible predictable misuse of the chemicals. Toxicity testing has become a major concern nationally because of the extensive legislation that now mandates such testing as well as internationally because of the need for mutually acceptable products as a prerequisite to

economic cooperation in the marketing of foods and chemicals (Fishbein, 1987; Hall and Taylor, 1989; Joselow, 1983). To establish safety or acceptable risk, one must develop a decision-making process. The accuracy of decision whereby safety is established depends on the adequacy, accuracy, and validity of investigations used to profile a substance's biological effects.

The initial step in the process of evaluating the safety of a particular substance consists of the collection of certain factual information regarding the substance, its toxicity, and the exposure of the public to the substance (Middlekauff, 1984). The safety decision tree (see Figure 5), developed some years ago by the Food Safety Council, is an agreed-upon system for testing in toxicology (Hayes and Campbell, 1986). In the first step, the test chemical is defined by physical and chemical properties. The more complex the substance, the more difficult it is to establish suitable specifications. The next step undertaken in the evaluation of the safety of a particular chemical consists of a determination of the concentration to which the human population is exposed. This step includes many complexities relating to the nature of the use of the chemical, the levels of use, the population which is exposed to the chemical, the degree of exposure, and the conditions of exposure.

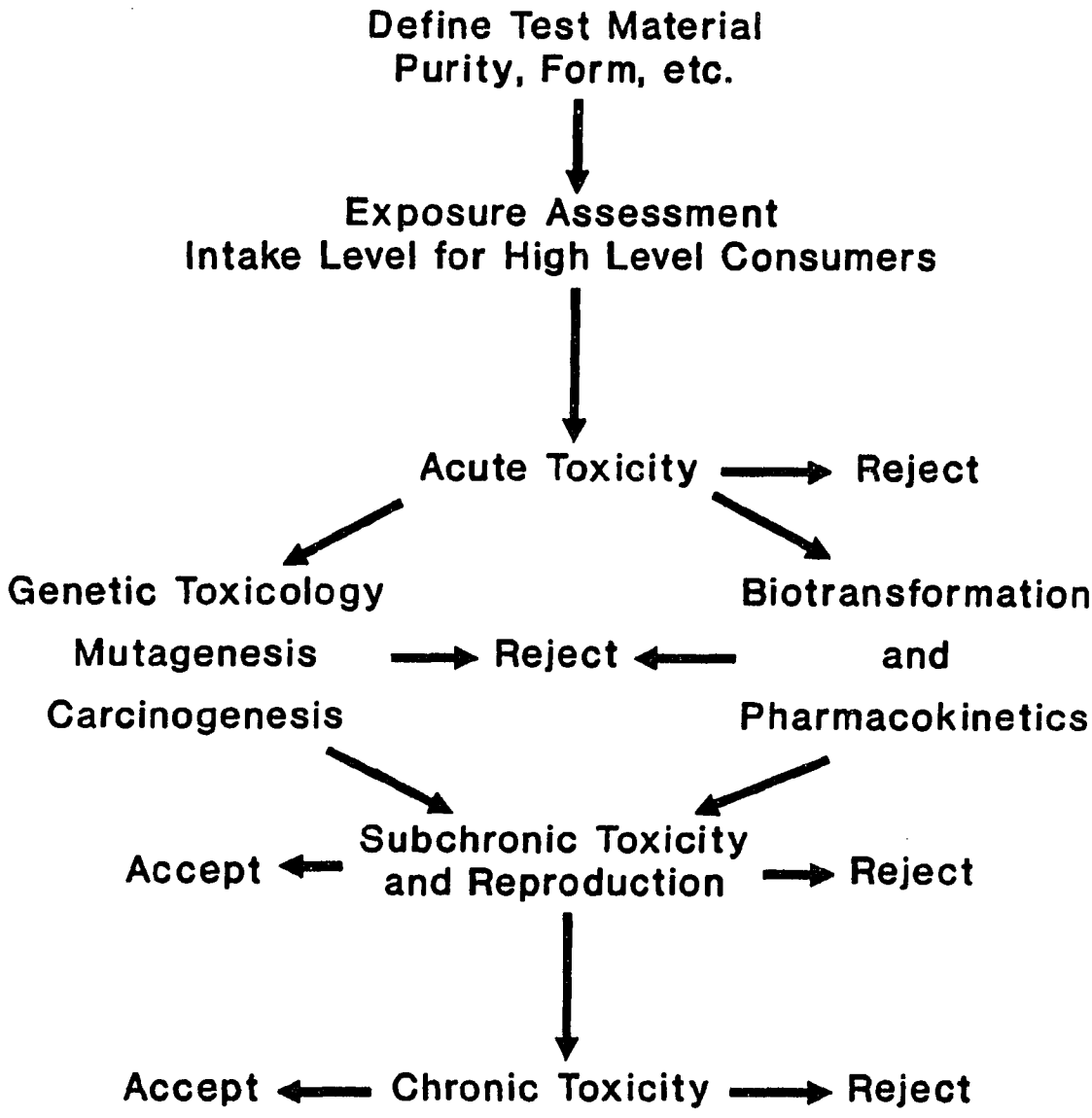


Figure 5. Safety decision tree

Basically, there are three types of tests, acute tests, subchronic tests, and chronic tests performed on laboratory animals to evaluate safety. These tests differ primarily in their durations and objectives. Tests that use only a single dose of the chemical, administered on one occasion, are referred to as acute tests. Longer tests, in which the chemical is given at least once daily, for periods of up to three months, are referred to as subchronic tests. Even longer tests, involving the administration of a chemical daily for periods of one to two years to simulate life-time exposure, are referred to as chronic tests. While other tests for specific toxic effects have also been developed, these basic tests remain the backbone of current toxicity testing procedures. Recent additions to toxicity testing procedures may require the determination of genetic effects (teratogenesis, mutagenesis, carcinogenesis, and reproduction), as well as determination of the biotransformation of the chemical and pharmacokinetical information about the chemical. Each of these activities has become an important sub-branch of toxicity testing (Ebert,1989; Middlekauff, 1984; Hayes and Campbell, 1986). The following literature review will focus on mutagenicity testing.

Mutation

This section of the literature review is based on comprehensive study of the reviews by Martin (1983), Oppenheimer (1985), Stryer (1981), and Venitt and Parry (1984).

To live and to multiply, organisms depend on the information encoded in their genes which are composed of deoxyribonucleic acid (DNA). A DNA molecule consists of two long strands wrapped around one another, each in the form of a helix. Each strand is a chain made of alternating units of a phosphate group and a 5-carbon sugar (deoxyribose); to each deoxyribose is attached one of four different organic bases. The base on one chain forms hydrogen bonds to the base on the other chain. The four bases in DNA are: adenine (A), thymine (T), guanine (G), and cytosine (C). Adenine on one chain can pair only with thymine on the other chain, and guanine on one chain can pair only with cytosine on the other chain. The sequence of bases in each strand of DNA and the specific pairing of bases between DNA strands accounts for the ability of DNA to serve as genetic material. The information stored in the base-pair sequence of DNA is translated into the composition of enzymes and structural proteins of the cell by the protein synthesizing machinery. The sequence of amino acids in every protein molecule is determined by the sequence

of bases in a particular segment of DNA. The relation between the base sequence in DNA and the amino acid sequence of a protein is straight forward: triplets of adjacent bases, such as CAC, TGG, AAC, etc., correspond to particular amino acids. Thus, simply by reading the bases in order, the amino acid sequence of the protein product can be determined directly. The set of all relations between base triplets and amino acids is called the genetic code. Each of the 64 possible triplets is called a codon. A single gene usually is defined as the length of DNA that codes for a single protein molecule. A chromosome, which is a large discrete macromolecule of DNA, contains many different genes in sequence along its length.

A heritable change in the properties of a cell is called a mutation; all mutations involve changes in the base sequence of DNA. There are three general types of mutation: gene mutation, chromosomal mutation, and genomic mutation.

Gene mutations are changes in the nucleotide sequence in one or a few codons, and can occur by: (1) the substitution of one base pair for another or of several pairs; (2) the deletion of one or more base pairs; and (3) the insertion of one or more base pairs. Insertion or deletion changes the reading frame of the DNA and is known as frameshift mutation. There are two kinds of single base-pair substitutions. A transition is the replacement of one purine by another purine or of one pyrimidine by another pyrimidine. In contrast, a

transversion is the replacement of a purine by a pyrimidine or of a pyrimidine by a purine. The amino acid sequence of a protein is determined by the base sequence of a gene. Furthermore, the properties of a protein molecule are determined almost exclusively by its sequence of amino acids. A change in the position or identity of a single amino acid can completely destroy the biological function of the protein which may have deleterious effects including carcinogenesis.

Chromosomal mutations are recognized as morphological alterations in the gross structure of chromosomes, and are usually detected by microscopic examination. Chromosomal mutation consists of deletion (loss of long rows of base pairs), duplication (repeat of long rows of base pairs), inversion (simple reversal of segments of DNA within a single chromosome), transposition (relocation of a long DNA sequence within a single chromosome), and translocation (relocation of a long DNA sequence from one chromosome to another). The loss of DNA or its repositioning during these processes may have dramatic consequences for gene expression, and in many cases is lethal to the affected cell or individual.

Genomic mutations are changes in the number of chromosomes in the genome. The normal diploid genome is euploid, and contains a complete set of chromosomes from each parent. Polyploidy occurs where the diploid genome is double or tripled. Loss or gain of a single chromosome is known as

aneuploidy, and may occur as a result of non-disjunction during cell division. Addition of one chromosome is trisomy, deletion of one chromosome is monosomy. The gene imbalance resulting from these processes usually leads to death.

Mutagens

Mutation is induced mainly by radiation and chemicals. Radiation that can caused mutation includes ionizing radiation, ultraviolet light, and sometimes visible light (when it excites a chemical that transfers this energy to DNA). Rinkus and Legator (1980) grouped the mutagenic chemicals into about 40 chemical classes. General speaking, there are two categories of chemical mutagens; direct-acting mutagens and indirect-acting mutagens.

Of chemicals known to be mutagenic, some are intrinsically reactive and can form DNA adducts directly, either in vivo or in vitro. These are called direct-acting mutagens and include many alkylsulphonic esters, epoxides, aromatic N-oxides, aromatic nitro-compounds, lactons, alkylnitrosoureas, and alkylnitrosamides. These are all electrophilic to a greater or lesser extent, i.e., they acquire electrons during chemical reactions. DNA-adduct formation occurs mainly by the reaction of electrophiles with nucleophilic centers in DNA (Venitt and Parry, 1984).

It was generally believed that all drugs and other environmental chemicals were pharmacologically active, toxic, carcinogenic, or mutagenic in their parent (nonmetabolized) form. The function of drug-metabolizing enzymes was therefore regarded as detoxification, i.e., inactivation of the active parent compound. Now it is evident that, although some chemicals are indeed active in their nonmetabolized form, most are inactive until they are metabolized; this process is called toxification (Goldstein et al., 1974; Miller and Miller, 1979). Chemicals which require metabolic activation before they are mutagenically active are called indirect-acting mutagens. These mutagens are not electrophilic but are converted to electrophiles by the activity of cellular enzyme systems. These enzyme systems, which are principally in the liver, but probably also present to some degree in virtually all tissues of the body; are usually divided into two groups: phase I and phase II. During phase I metabolism, one or more water-soluble groups (such as hydroxyl) are introduced into the lipid-soluble parent compound, thus allowing a position for the phase II conjugating enzymes to attack. Many phase I products, but especially the conjugated phase II products, are sufficiently water-soluble for these chemicals to be readily excreted from the body. Unfortunately, this activity is occasionally perverted to a more sinister course when a chemically inert and mutagenically inactive molecule is

converted to an electrophilic metabolite capable of reacting with DNA. The inactive compound is termed the pro-mutagen, its intermediate metabolites are proximate mutagens, and the electrophilic metabolite which actually reacts with DNA is the ultimate mutagen. Most phase I oxidations are performed by cytochrome P-450 which is a multigene family of hemoproteins with catalytic activity toward thousands of substrates. Active preparations of cytochrome P-450 are easily prepared by homogenizing tissues rich in these enzymes (e.g., the liver) and retaining the supernatant left after centrifuging the homogenate at about 9,000 xg. This supernatant contains endoplasmic reticulum and microsomes, soluble enzymes and endogenous co-factors, and is known as the S-9 fraction. The S-9 fraction has been widely used in mutagenicity testing to mimic the metabolism systems in mammals (Lu, 1985; Venitt and Parry, 1984).

Mutagenicity Testing

Because mutagens can cause heritable genetic damage or cancer in human, considerable effort has been devoted to developing and validating methods for detecting mutagens. A brief overview of major assay systems used in mutagenicity testing is presented in this section.

Mutagenicity testing using bacterial systems

As with most fields of genetics and biochemistry, genetic toxicology has derived much of its initial success from experiments with bacterial systems. Genetic toxicologists are attracted to bacterial tests because they are rapid and relatively inexpensive. In general, bacterial mutagenicity tests rely on one of three mutational schemes: reverse mutation, forward mutation, and DNA damage.

Reverse-mutation assays are the most widely used of all methods for determining the mutagenicity of chemicals. The bacterial strains used in these assays lack the enzymes necessary for the metabolism of pro-carcinogen or pro-mutagen to ultimate carcinogen or mutagen, respectively. Therefore, rat liver S-9 fraction, usually induced with Aroclor 1254, is added as a crude surrogate of the mammalian metabolism system. These assays are run both with and without rat liver S-9 fraction, to detect chemicals that are already mutagenic and might be inactive, as well as those which require activation to become mutagenic. In this method, bacterial strains are used which carry base substitution or frameshift mutations in operons coding for synthesis of specific amino acids. Therefore these mutants can not synthesize all their required amino acids from inorganic sources of nitrogen. The principle of these tests is to detect the point mutation and measure reverse mutation from amino acid auxotrophy to prototrophy

(Ames et al., 1975).

Mutagenicity testing using fungal systems

The main reason for using fungi for screening potential mutagens is that some mutagens that can not be detected with bacterial systems might be revealed with fungal system. Being eukaryotes, fungi possess many genetic properties that do not have counterparts in bacteria and have their own metabolic activation systems. In addition to endogenous activation, these assays can also be designed to incorporate the rat liver S-9 fraction. The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the molds *Neurospora Crassa* and *Aspergillus nidulans* have been used most extensively to screen chemical and physical agents for mutagenicity (Zimmermann et al., 1983; Loprieno et al., 1983; Brockman et al., 1983; Scott et al., 1982).

It is generally agreed that fungal test systems are less sensitive than bacterial test system: in most cases, a higher dose of the agent being tested is required to elicit a response in the fungal systems. Recent advances in recombinant-DNA technology may permit the construction of yeast strains that have mammalian components and mutagenic response equivalent to those of mammalian cells.

Mutagenicity testing using cultured mammalian cells

The obvious benefit from adapting mammalian cell systems to detect mutagens is their biochemical, structural and genetic similarity to the target cells at risk in the whole animal. Several mammalian mutagenesis systems are used widely to detect environmental mutagens by measuring different end points.

In DNA repair tests the occurrence of DNA damage is inferred from the induction of DNA repair by the test agent. The most widely used indicator of repair is the incorporation of radiolabelled nucleotides during gap-filling after excision repair (Waters, 1984).

The mammalian systems can also be used to detect gene mutation. In these assays, point mutation (usually from drug sensitivity to drug resistance) is measured in cultured mammalian cells. Human cells may be employed, allowing induction of point mutation to be assessed in a system which is more closely related to human genetic toxicology (Cole and Arlett, 1984).

Another assay enables the detection of chromosomal mutations by direct microscopical examination of cultured mammalian cells. These tests can be applied to a variety of cell types, including human cells. The most widely used are cell lines in which the capacity for metabolic activation of indirect-acting mutagen has been lost and the S-9 fraction is

therefore added in much the same way as in bacterial assays (Dean and Danford, 1984).

Mutagenicity testing in Drosophila

Drosophila breeding techniques for detecting physical and chemical mutagens have been developed for the rapid and easy detection of mutations, chromosomal rearrangements, and aneuploidy. The major advantage in using Drosophila as a test system for mutagens are that it is a sexually reproducing eukaryotic organism with male and female germ cell stages equivalent to those found in mammals, with a generation time of about 12 days and production of hundreds of offspring by a single pair of parents, and that more is known about the genetics of Drosophila than any other higher eukaryote. Drosophila geneticists have constructed a wide array of tester stocks that detect all classes of gene and chromosomal mutations that are also likely to occur in man. Drosophila has a very active mixed-function oxidase system that is comparable to that of the rat (Mitchell and Combes, 1984).

Mutagenicity testing in mammals

The organism most closely related to man for which any germinal-mutation rates have been measured is the mouse. Laboratory rodents are dosed with the test chemical by an appropriate route and, after a given time, suitable tissues

are sampled and chromosome spreads are prepared for examination. The most widely used somatic tissue is bone marrow, since this is a rich source of rapidly dividing cells. Germ cell tissue, usually from the tests, is also used. The specific-locus test detects recessive gene mutations and small deletions at seven specific gene loci; some of those have dominant effects on vital characters. Another mouse test uses dominant mutations that affect the skeleton, many of which mimic serious human disabilities. Also, there are direct tests for chromosomal mutations, based either on direct microscopic observation of germ cells or on partial sterility of the progeny of treated parents (Adler, 1984; Anderson, 1984).

Choice of Test

The series of tests summarized above provide a wide range of assays for the detection of genotoxic activity. All have an extensive scientific literature to support their use. The choice of a test necessarily represent a compromise; relevance, sensitivity, cost, and other considerations must be balanced against each other.

In the choice of tests to be incorporated into a screening system, the following criteria are of importance (NRC, 1983):

1. The number of chemicals that have been tried in a given test.
2. Concordance of results with chemicals previously subjected to other tests.
3. The genetic end points detected by the test.
4. The expense of the test and the speed with which it can be performed.
5. The number of laboratories that have performed the test and the reproducibility of results among laboratories.
6. The sensitivity and specificity of the test.

Ames Test

The most widely used bacterial test for detecting gene mutations is that developed by Dr. Bruce Ames and usually called by his name (Ames et al., 1975). The Ames test, also called the salmonella/mammalian microsome test, uses the mutated strains of the bacterium *Salmonella typhimurium* which carry mutations in the gene governing the bacteria's ability to synthesize the amino acid histidine. The strains are thus unable to produce their own histidine and cannot grow unless histidine is added to their culture medium. The assay measures the ability of a tested substance to correct this genetic defect (back mutate) of the strains to the wild-type state in which they can grow in a histidine-free medium.

Unlike mammals, these bacteria strains lack the necessary oxidative enzyme systems for metabolizing some compounds to electrophilic metabolites capable of reacting with DNA. Under some circumstances, the bacteria are therefore treated with the test compound in the presence of a post-mitochondrial supernatant (S-9 or microsome fraction) prepared from the liver of mammals (usually rats). The metabolic activity of the S-9 fraction (predominantly mono-oxygenase activity mediated via the cytochrome P-450 system) is enhanced by treating the rats with a potent inducer of drug-metabolizing enzymes before they are killed and their livers are removed. A mixture of polychlorinated biphenyls, Aroclor 1254, is the most commonly used inducer.

Currently, five strains of *Salmonella* are employed in the test: TA 98, TA 100, TA 1535, and TA 1538. Strains TA 98, TA 1637, and TA 1538 carry frameshift mutations and therefore are reverted by agents that will produce new frameshifts. Strains TA 100 and TA 1535 carry missense mutations and are reverted by agents that cause base-pair substitutions. Aside from the expression of histidine mutations, other properties have been built into the *Salmonella* strains by mutation to increase their sensitivity. The increased sensitivity probably is due to the failure to remove some DNA adducts that could lead to mutation. The strains also possess a mutation that removes part of the lipopolysaccharide barrier of the bacterial cell

wall and thereby makes the cells more permeable to some chemicals. Finally, strains TA 98 and TA 100 contain the R-factor plasmid, which increases sensitivity, probably by increasing the activity of an error-prone DNA-repair system.

The assay procedure is very simple and has been described in detail by Ames et al. (1975). The *Salmonella* strains are grown overnight in a fairly rich medium to product a concentrated cell suspension. A small aliquot of this suspension containing about 10^8 cells and test compound is added to molten, dilute agar (top agar) which is then mixed and poured onto the surface of a minimal-agar plate containing glucose (bottom agar) and allowed to solidify. The plates are incubated at 37°C for two to three days. The top agar also contains just enough histidine to allow the cells to divide a few times. This minimal amount of cell division is necessary because the activity of test mutagens only can be detected with replicating DNA in growing cells. If desired, an extract of rat liver, S-9 fraction, may be added to the top agar as well, to provide mammalian metabolizing enzymes.

The assay is measured by simply counting the number of colonies on each plate. A certain background number of colonies appear on control plates not exposed to the test compound, and these represent the spontaneous reversions occurring in the population. The plates exposed to mutagens

show a significant increase in the colony count compared to the control plates. Usually, a colony count of at least double the control colony count is taken as an indication of mutagenesis.

The Ames test is a valuable test in predicting the carcinogenic potential of a test compound. About 300 carcinogens and non-carcinogens of a wide variety of chemical types have been tested over a wide dose range with and without the S-9 fraction. A high correlation between carcinogenicity and mutagenicity was observed: 90 percent of the known carcinogens tested were mutagenic, 87 percent of the non-carcinogens tested were non-mutagenic (McCann et al., 1975; McCann and Ames, 1976). The Ames test has several advantages: it is easy to perform and may be carried out in a minimally equipped bacteriology laboratory, it requires inexpensive materials, and it gives results in two days.

Bioluminescence Test (BLT)

Since 1980, Dr. Ulitzur and his colleagues (Technion-Israel Institute of Technology, Haifa, Israel) have initiated and developed a new mutagenicity test by using bacterial system and bioluminescent technique. The bioluminescence test (BLT) for mutagens uses dark mutants of the luminous bacteria *Photobacterium* to determine the ability of the tested agent to

restore luminescence by inducing mutation. The restored luminescence can be measured by using photometric instrumentation (Ulitzur, 1986). In the dark mutant the transduction of the luminescence operon is under continuous repression probably by an intercistronic repressor, consequently any mutation which affects the regulatory genes can restore the luminescence. Three groups of genotoxic agents have been shown to be active in the BLT: (1) direct mutagens being either base-substitution or frameshift agents, (2) DNA-damaging agents and DNA synthesis inhibitors, and (3) DNA-intercalating agents. Restoring the luminescence of the repressed dark mutant can theoretically be achieved by three independent events: (1) blocking the formation of the repressor; altering its or the operator site's structure, (2) inactivating the repressor of the luminescence system, and (3) changing the physical configuration of the DNA, thus allowing unrepressed transcription of the luciferase operon. Present evidence supports the possibility that all these agents act through their ability to trigger the "SOS function" that involves the inactivation of the luminescence system's repressor (Weiser et al., 1981).

The BLT system can also incorporate the S-9 activating enzyme systems for the indirect-acting mutagens. More than 100 chemicals, with and without the S-9 fraction, representing twelve different chemical classes have been assayed using this

test. The evidence for mutagenicity produced with the BLT correlates well with the results of the Ames test and with evidence of carcinogenicity. The BLT provides a rapid, general screening test which can be used to assay large numbers of pure and complex compounds including environmental samples and volatile compounds. The test can be run automatically and up to 50 chemicals can be tested in one day. Sterility of the assay mixture is not required. By testing serial dilutions of a compound it is possible to gather dose response data and concurrently determine the toxic levels of the compound (Ulitzur, 1986). The details of this test will be discussed in the Materials and Methods and Results sections.

Toxicological Aspects of Foods

Substances that are not natural to a food, xenobiotics, may become a part of the food by several routes. They may be deliberately added as food or color additives; they may result from migration of additives from wrappings into the food; they may result from the chemical changes of food components during processing or storage of the food; they may also result from the incorporation of environmental pollutants into the food while that food is growing or maturing. All such xenobiotics inevitably raise questions of safety (Joselow, 1983).

Toxicity of lipid peroxidation products

The problem of oxidative deterioration is of great economic importance in the production of lipid-containing foods. Oxidation of unsaturated lipids not only produces offensive odors and flavors but can also decrease nutritional quality and safety by the formation of peroxidation products in foods after cooking and processing (Frankel, 1980). Many reviews have appeared on the biological effects of lipid oxidation products and their relevance to human diseases. Lipid peroxidation products are implicated in the disruption of biological membranes, the inactivation of enzymes, the damage of proteins, the formation of age pigments, carcinogenesis, and mutagenesis (Logani and Davies, 1980; Kappus and Sies, 1981; Pearson et al., 1983; Frankel, 1984). The possibility that consumption of heated and/or oxidized fats may be detrimental to health has stimulated extensive research.

The feeding of highly oxidized fats, in the form of oxidized cod liver oil which contains high concentrations of C_{20}-C_{22} polyunsaturated fatty acids, to animals has produced a wide spectrum of injurious effects including diarrhoea, growth retardation, hemolytic anemia and accumulation of peroxides in adipose tissue (Sanders, 1983). Toxicity studies in animals have been conducted on fats oxidized for very long period at

very high temperatures. Fat subjected to such extreme processing conditions caused severe irritation of the gastrointestinal tract, growth retardation and death in experimental animals (Kubow, 1990). Some researchers have also found elevated liver and kidney weights, cellular damage in various organs, and altered fatty acid composition of tissue lipids after administration of heated oils and fats subjected to normal usage (Alexander et al., 1987). There has been considerable concern over the mutagenic potential of continually re-used fats. Studies by Taylor et al. (1983), Hageman et al. (1988) and Saleh et al. (1986) indicated that repeatedly used frying fats were shown to be mutagenic in the Ames test.

Recently, there are indications that dietary lipid peroxidation products may play a role in atherosclerosis (Addis and Park, 1989). Fatty acid hydroperoxides have been shown to accelerate all three phases of atherosclerosis: endothelial injury (initiation), accumulation of plaque (progression) and thrombosis (termination). Higher serum levels of lipid peroxides are observed in animals and patients with atherosclerosis than in those with no clinical evidence of atherosclerosis (Yagi, 1988). Giani et al. (1985) also found that the consumption by rats of a diet containing mildly oxidized oils results in changes in prostaglandin levels and

thromboxane A_2 production, which consequently increase the risk of atherosclerosis.

The primary products of autoxidation, fatty acid hydroperoxides, are very toxic to animals. An early study by Horgan et al. (1957) demonstrated the toxicity of a number of hydroperoxides when injected intraperitoneally in mice. Cortesis and Privett (1972) found that methyl linoleate hydroperoxide, when injected intravenously in the rat at a level of 30 mg/100 g body weight, killed the animal within 24 hours. An investigation (Cutler and Schneider, 1973) was made of the embryotoxicity in mice of purified linoleic acid, oxidized linoleic acid or purified linoleic acid hydroperoxide applied directly to the ovaries. After the treatment with linoleic acid hydroperoxide an increase in fetal malformations occurred in litters of the first generation, and second-generation litters showed an increase in embryonic resorptions. The incidence of malformations after treatment with linoleic acid was similar to that occurring in the group of untreated controls. A number of studies have demonstrated that lipid hydroperoxides can initiate free radical oxidation of carcinogens to the ultimate active form. For example, benzo(a)pyrene was oxidized to the highly mutagenic 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo (a) pyrene in the presence of 13-hydroperoxylinoleic acid and the catalyst,

hematin (Dix and Marnett, 1981). A review by Pryor (1985) suggested that lipid hydroperoxides either in foods or produced endogenously can be a source of free radicals in vivo and indicated some evidence for free radical involvement in some types of chronic disease, such as emphysema, atherosclerosis, cirrhosis, and diabetes. Oxidized linoleic and linolenic acids, their purified monohydroperoxides, and secondary oxidation products have been shown to be weakly mutagenic in the Ames test (Yamaguchi and Yamashita, 1979; 1980; MacGregor et al., 1985). However, two other studies by Scheutwinkel-Reich et al. (1980) and Gardner et al. (1983) did not support the mutagenic role of fatty acid hydroperoxides.

Not only are lipid hydroperoxides themselves toxic, but their decomposition products also can be toxic. Malondialdehyde (MA), a three-carbon dialdehyde, which is produced during oxidative decomposition of polyunsaturated fatty acids has received much attention. Surveys confirmed the presence of MA in supermarket samples of meat, poultry, and fish, which constitute the main source of MA in the North American diet (Shamberger et al., 1977; Siu and Draper, 1978). The presence of MA in foods has prompted investigation into its possible harmful effects in animals. A study by Bird and Draper (1980) indicated that MA reduced the mitotic index, inhibits DNA and RNA synthesis, and induced micronucleation and DNA repair synthesis in cultured rat skin fibroblasts.

Two investigations into the subchronic and chronic toxicity of MA in mice have been published (Bird et al., 1982; Siu et al., 1983). These studies suggested that the most pronounced histopathological changes were found in the liver, where both the severity and the incidence of the lesions appeared to be dose-dependent. Mukai and Goldstein (1976) reported that the mutagenesis of MA was observed in frameshift mutants with normal excision repair and did not occur in those base-pair substitution mutants in the Ames test. Yau (1979) also found that MA was highly mutagenic and cytotoxic in mammalian cells and therefore may be a potent carcinogen in humans. However, the methodology used to determine the content of MA have been criticized by several investigators. Addis (1986) suggested that the method overestimated the amount of MA present by at least twofold. As a consequence of such technical difficulties, there is insufficient evidence to indicate that MA, per se, is a risk factor in foods.

In recent years, much attention has been focused on the cholesterol peroxidation products. Newer and more powerful chromatographic methods, combined with mass spectrometry, nuclear magnetic resonance spectroscopy and infrared spectroscopy determinations, have facilitated the definition and quantification of cholesterol oxidation products in foods and biological tissues (Addis, 1986). Substantial amounts of cholesterol oxidation products have been detected in deep-

fried foods, dehydrated milk, and egg products (Addis, 1986; Nourooz-Zadeh and Appelqvist, 1987). Hubbard et al. (1989) conducted both in vivo and in vitro studies with products of oxidized cholesterol. They found that the oxidation products of cholesterol are highly toxic to cultured aortic smooth muscle cells of the rabbit and can induce arterial injury with 24 hours of administration by gavage to rabbits. An early investigation of the effect of feeding oxysterols at levels estimated to be average U.S. dietary intakes have been conducted by Jacobson et al. (1985). The effect of oxysterol feeding in White Carneau pigeons was a five times increase in coronary artery atherosclerosis after three months, compared with birds given pure cholesterol. Oxidation products of autoxidized cholesterol and cholesterol hydroperoxides have been shown to be weakly mutagenic in the Ames test (Ansari et al., 1982).

Mycotoxins

Mold spores are distributed in nature, and they easily germinate and grow on foods and feeds, especially under moisture conditions. Mold growth generally results in unpleasant flavors and other undesirable changes in products. Another possible consequence of mold growth is that poisonous substances may be produced and toxic symptoms of various kinds may occur when foods or feeds containing the molds are eaten

by humans or animals. These poisonous substances are referred to generally as mycotoxins, and the toxicity syndromes produced by them are called mycotoxicoses (Wogan and Marletta, 1985). Mycotoxins can survive heat treatment and are persistent in processed foods. They posses a spectrum of biological activities due to the diversity of their chemical structures and can produce acute, subacute, and chronic toxicities; some of them are also carcinogenic, mutagenic, and teratogenic (Bullerman, 1979; Stoloff, 1982; Hsieh, 1987). Today great attention is paid to the problems associated with mycotoxins, especially aflatoxins, ochratoxin A, and fumonisins.

Aflatoxins The aflatoxins are a group of structurally related toxic compounds produced by certain strains of *Aspergillus flavus* and A. *parasiticus* and are frequently encountered due to outgrowth of these bacterial strains on stored commodities, especially under conditions prevailing in many tropical areas. The most important source of human exposure in the United States in corn and peanuts. The major aflatoxins of concern are referred to as aflatoxins (AF) B_1, B_2, G_1, G_2 and M_2. The first four toxins are usually found together on various foods in varying proportions, however, AFB_1 is normally predominant in amount and toxicity. Aflatoxin M_1

is a toxic metabolite of AFB_1. It is excreted in the milk of dairy cattle and other mammalian species that have consumed aflatoxin-contaminated food (Pohland and Wood, 1987).

The broad range of biological effects of the aflatoxins probably relates to their reaction with cell nucleoproteins and nucleic acids and the ultimate effect of these reactions on protein synthesis and cellular integrity. Most laboratory animals respond to the toxic and carcinogenic effects of AFB_1. The Fisher strain of rat is probably the most sensitive mammal; 1 ppb in the diet can elicit a carcinogenic response (Wogan et al., 1974). The rainbow trout is the most sensitive fish known; less than 4 μg/Kg in the diet will produce a significant incidence of liver cancer (Palmgren and Hayes, 1987). Susceptibility varies with the species, age, and strain; among individuals within a group; and with the effect of various factors on the liver enzyme systems. Depending on the amount of toxin consumed and animal's susceptibility to aflatoxin, the biological effects that may occur are: (1) acute and clinically obvious disease, (2) chronic, less clinically apparent, impairment of health and productivity, and (3) impairment of resistance and immune responsiveness that becomes clinically apparent as an infectious disease that the diagnostician would not readily associate with aflatoxin consumption (Diener, 1982).

A comparison of the mutagenicity of the aflatoxins in the Ames test with in vivo carcinogenicity data for the naturally occurring aflatoxins and various metabolites showed a remarkable correlation between bacterial mutagenicity of those compounds and their in vivo carcinogenicity. Wong and Hsieh (1976) reported that AFB_1, is a very potent mutagen when a microsomal activation system is used in the Ames test. They also found that AFB_2, AFG_1, AFG_2, and AFM_1 were inactive in the Ames test.

Ochratoxin A Ochratoxins were first isolated by South African workers from cultures of *Aspergillus ochraceus* obtained from domestic legumes and cereal products. A chlorine-containing metabolite, designated as ochratoxin A, was isolated as a major toxic substance together with its less toxic analogue, ochratoxin B (van der Merwe et al., 1965). Ochratoxin A, the most toxic ochratoxin, is also produced by other aspergilli and penicillia and is frequently encountered as a natural contaminant of feeds and foods. It is considered as a probable causative agent of diseases in human, pigs and poultry. The toxic effects of ochratoxin A have been studied over several years by many investigators. Different species vary in their susceptibility to acute poisoning by ochratoxin A. The peroral LD_{50} were 5.9, 21.4 and 30.3 mg/Kg for turkey,

female rat, and male rat, respectively (Krogh, 1987). Ochratoxin A is a potent nephrotoxin; it is implicated in nephrotoxic syndromes in pigs in Denmark and humans in Balkan counties (Ueno, 1985). Kanisawa and Suzuki (1978) reported that oral administration to mice induced both liver and kidney tumors. Embryotoxicity and teratogenicity of ochratoxin A have been observed using the chicken embryo (Choudbury and Carlson, 1973). Ochratoxin A was not mutagenic to *Salmonella typhimurium* TA 1535, TA 1537, TA 1538, TA 98 or TA 100 at doses of up to 500 μg per plate, with or without exogenous metabolic activation (Kuczuk et al., 1978; Wehner et al., 1978).

Fumonisins *Fusarium moniliforme* Sheldon is one of the most prevalent fungi associated with basic human and animal diet components such as corn. Culture material of F. moniliforme MRC 826, isolated from corn intended for human consumption in Transkei, southern Africa, proved to be highly toxic to a variety of experimental animals, was shown to cause leukoencephalomalacia in horses and to be hepatocarcinogenic in rats, and have cancer-promoting activity in a short-term cancer initiation-promotion model in rats (Jaskiewicz et al., 1987a;b; Kriek et al., 1981; Marasas et al., 1981; 1984) Gelderblom et al. (1988) isolated and purified two new

mycotoxins, fumonisins B_1 and B_2, from culture material of F. moniliforme MRC 826. Fumonisin B_1 has been shown to have cancer-promotion activity in rats and to cause leukoencephalomalacia in horses. The principal pathological change in rats treated with fumonisin B_1 was a progressive toxic hepatitis similar to that induced by toxic culture material of F. moniliforme MRC 826. Alberts et al. (1990) found that fumonisin B_1 was heat stable, as there was not reduction in the fumonisin B_1 concentration after boiling culture material of F. moniliforme MRC 826. The fumonisins have recently been found to occur naturally in corn in South Africa and the United States (Sydenham et al., 1990; Voss et al., 1989). These findings emphasize the importance of screening human and animal foodstuffs for the presence of fumonisins. The fumonisins may be of major importance in the etiology of several animal and human diseases.

Toxic components in heated foods

The appearance of carcinogenic polynuclear aromatic hydrocarbons (PAH) on the surface of charcoal broiled beef steaks was first reported by Lijinski and Shubik (1964). Benzo(a)pyrene and other hydrocarbons were formed when fat from the meat dripped onto hot coals and was incinerated. The smoke from the burned fat containing the hydrocarbons was then

adsorbed onto the surface of the meat. Benzo(a)pyrene is a carcinogen of moderate potency (Scribner and Suss, 1978) and is one of the strongest mutagens detected by using Ames test (McCann et al., 1975). Sugimura and Nagao (1979) suggested that the generation of mutagens on the surface of fish and beef subjected to open flame broiling could be accounted for by their benzo(a)pyrene content. These studies led to the discovery of several other new mutagens produced during pyrolysis of amino acids and proteins. Kasai et al. (1980a,b) first isolated and identified mutagenic aminoimidazoquinoline (IQ) and aminomethylimidazoquinoline (MeIQ) from broiled sun-dried sardines. The involvement of Maillard reaction products and creatinine in meat in the formation of these compounds was proposed by Jägerstad et al. (1983). Other types of heterocyclic amines were isolated from pyrolysates of amino acids and protein. They are Trp-P-1:3-amino-1,4-dimethyl-5H-pyrido (4,3,-b) indole and Trp-P-2:3-amino-1-methyl-5H-pyrido (4,3,-b) indole from a tryptophan pyrolysate reported by Sugimura et al. (1977). The presence of Trp-P-1 and Trp-P-2 in broiled foods has been reported (Yamaizumi et al., 1980).

Hepatocellular carcinomas and adenomas and adenocarcinomas and adenomas in the intestine were found in mice given 0.04% MeIQ (Ohgaki et al., 1985). Matsukura et al. (1981) found that liver tumors were induced in a high incidence between experimental days 402 and 621 in mice given

Trp-P-1 and Trp-P-2 at a concentration of 0.02%. Trp-P-1 and Trp-P-2 were also found to induce liver tumors in rats (Sugimura et al., 1989).

MATERIALS AND METHODS

Mutatox® Mutagen Test

The Mutatox® test, which was developed by Microbics Corporation (Carlsbad, CA), is the commercial version of the bioluminescent bacterial test (BLT) described by Ulitzur (1986). The Microbics Corp. has lyophilized the bacteria culture, modified the assay medium and optimized the assay protocol to provide a simple and reliable system for the detection of genotoxic agents. The Microbics Corp. provided all the reagents that were needed for the Mutatox® test which included the bacterial culture, assay medium, 3.6-diaminoacridine (proflavin), and N-methyl-N'-nitro-nitrosoguanidine (MNNG).

Bacterial culture

A dark mutant, M 169, of *Photobacterium phosphorium* was prepared by Dr. Ulitzur. The Microbics Corp. has lyophilized the bacteria culture which is stable at -20°C for one year. Two kinds of bacteria culture have been developed for different purposes: (1) for direct assay: the test includes the tested chemical without the S-9 activation and (2) for S-9 assay: the test includes the tested chemical with the S-9

activation. Prior to each test the lyophilized culture is rehydrated by addition of 1 ml prechilled deionized water into the culture vial then mixing gently to obtain a homogenous suspension. The rehydrated culture is stable for 2 hours at 4°C. The rehydrated culture was always used immediately after it was prepared.

Assay medium

The Mutatox® test was performed in the growth medium designated as M 169 growth medium which also available as lyophilized form prepared by Microbics Corp. The powdered medium is also very stable at -20°C. For each test, the proper amount of lyophilized assay medium was weighed out and dissolved into a premeasured amount of prechilled deionized water. The concentration of the assay medium in the deionized water was 3% (W/V). There are also two kinds of assay medium, one for the direct assay and the other for the S-9 assay. The assay medium was prepared freshly for each test.

S-9 preparation

The S-9 preparation contained rat liver microsomes induced by Aroclor 1254 and was purchased from Molecular Toxicology Inc. (Annapolis, MD). The S-9 preparation is stable for one year if kept in a ultralow freezer (-70°C). On

the day the S-9 assay was conducted, the S-9 preparation was thawed and used immediately. For the S-9 assay, the concentration of S-9 preparation in the assay medium was 0.5% (V/V).

S-9 cofactor

In addition to S-9 preparation, when S-9 assay was conducted the S-9 cofactor was also needed. The S-9 cofactor included a stock solution of NADP and glucose-6-phosphate (G-6-P). For the S-9 assay, the concentration of both NADP and G-6-P was 0.25 mM in the assay medium. The stock solution was kept in the freezer at -20°C.

Sample preparation

Different concentration ranges of tested chemicals were prepared to determine in which range the mutagenesis occurs with the Mutatox® test. Usually, the concentration ranges prepared were 0.01 µg/µl, 0.1 µg/µl, 1 µg/µl, and 10 µg/µl. Depending on the response of the tested chemical the concentration ranges can be adjusted. For water insoluble chemicals, the solvent systems that are compatible with the Mutatox® test are acetone, acetonitrile, dimethyl sulfoxide (DMSO), ethanol, and methanol. The solvent systems are compatible with both direct and S-9 assay except that DMSO is

not recommended for the S-9 assay.

Control preparation

For each Mutatox® test, concurrent positive and negative controls should be used in the same system as that used for the tested chemical. Both a solvent and an untreated (blank) control should be used as negative controls. MNNG or proflavin was used as a positive control for the direct assay while benzo(a)pyrene (B(a)P) or aflatoxin B_1 (AFB_1) was used as a positive control for the S-9 assay.

Luminescence determination

The photometer used for the bioluminescence determination in the Mutatox® test is the Tri-Carb® liquid scintillation spectrometer (Model 2420, Packard). The scintillation counter was operated without coincidence at the ^3H setting. Each sample preparation should be read at least once for each hour up to 24 hours. The test requires new clean scintillation glass vials and equipped with polyethylene lined plastic cap.

Test protocol

Steps in the test protocol are as follows:

1. Add 2 ml of assay medium into scintillation vials.

1'. For the S-9 assay: prepare S-9 preparation and S-9

cofactor mixture in the assay medium then add 2 ml of the assay medium mixture into scintillation vials.

2. Add desired concentrations of the tested chemical(s) into scintillation vials and shake gently.

3. Add 20 μl of the rehydrated bacterial culture into each scintillation vial.

4. Cap the scintillation vials and shake the vials for 20 to 30 minutes.

5. Measure light emission by using Scintillation Counter hourly for 24 hours.

6. Prepare for each Mutatox® test, the blank control, concurrent solvent control, and positive control.

7. Prepare each tested chemical in triplicate.

Analysis of results

The best way to evaluate the activity of the chemical in question is to determine the maximal luminescence value that has been reached at any time during the test. One can define a significant genotoxic activity when the maximal luminescence developed due to the chemical in question is 3 times higher than the maximal luminescence of the corresponding control (Ulitzur, 1986).

Experimental design

In this study, each tested chemical was prepared in triplicate in each Mutatox® test. Each tested chemical was examined for mutagenicity three times by using Mutatox® test. Results reported represent one typical result from three tests for each tested chemical.

Preparation of Autoxidized Fatty Acids

Methyl linoleate (18:2) and methyl linolenate (18:3) (95%) were purchased from Sigma Co. (St Louis, MO). Methyl linoleate and linolenate (5 grams of each) were autoxidized in a water bath at 37-40°C in the dark by bubbling air into the fatty acids for 144 and 72 hours, respectively. The autoxidized fatty acid sample was fractionated on a silica gel column (2 X 15 cm) with diethyl ether/hexane eluants into five different fractions. Fraction one was eluted with 200 ml 1:9 diethyl ether/hexane. The following oxidation products were then eluted with 100 ml portions of diethyl ether/hexane mixtures of the volume proportions indicated: fraction 2 (2:8), fraction 3 (3:7), fraction 4 (5:5), and fraction 5 (7:3). The remaining oxidation products were eluted with 100% diethyl ether. The eluted solvent mixture was removed by vacuum evaporator. Different concentration ranges of the

fractionated autoxidized fatty acid samples were prepared by dissolving in methanol. Samples were stored at -20°C until tested for mutagenesis.

Thin-layer Chromatography

The fractionated autoxidized fatty acids were further analyzed by thin-layer chromatography (TLC), using Merck pre-coated silica gel 60 plates (20 X 20 cm) with fluorescent 254 nm indicator (Darmstadt, Germany). TLC was conducted with diethyl ether/hexane/acetic acid (50:50:1) as the development solvent.

Heated Oil Samples

Crisco soybean oil was purchased from a local retail grocery store in Ames, Iowa. The cooking oil (100 ml) was heated at 180-190°C in a beaker on the hot plate for 8 hours each day on five consecutive days. Aliquots of the heated cooking oil were taken out at the end of heating on each day. Different concentration ranges of the heated cooking oil were prepared by dissolving in acetone and storing at -20°C until they were tested for mutagenesis. For each mutagenicity test, a fresh oil sample without heating was always prepared as a

blank control.

A different experiment was also conducted by using the same cooking oil. One percent (W/V) pure crystalline cholesterol (Sigma, Grade I) was dissolved in the cooking oil and then used in the same experimental design as that for the cooking oil.

RESULTS

The success of a mutagen assessment programs depends strongly on the efficiency and reliability of the screening system, for most of the decisions regarding individual chemicals must be made on the basis of short-term screening tests. The bioluminescence bacterial test (BLT) for mutagen has been developed by Ulitzur (1986) and further commercialized and designated as Mutatox® test by Microbics Corp., CA. Ben-Issak et al. (1985) suggested that unlike the Ames test the BLT is not affected by the presence of amino acids or other nutrients; thus it is possible to assay complex organic matter such as foodstuffs and biological fluids. From their study, Ulitzur (1986) found that the BLT detects all the 46 tested chemicals that are known to be active in the Ames test. Moreover, quite a large number of carcinogenic agents that are not active in the Ames test are detected by the BLT. Ulitzur also suggested that being a general, sensitive, simple, and cheap assay, the BLT can be applied as a prescreening test for carcinogenic agents. The current study was conducted to validate the Mutatox® test in our laboratory, to utilize the Mutatox® test to screen some chemical compounds that might be found in foods or feeds, to further confirm the reliability of Mutatox® test, and to contribute to a data base for the Mutatox® test.

There are two systems that have been designed for the Mutatox® test: (1) direct assay: tested chemicals without the activation by S-9 fraction and (2) S-9 assay: tested chemicals with activation by S-9 fraction. These two systems were also adapted in our study. From the study by Ulitzur (1986), the positive controls selected for the direct assay was 3,6-diaminoacridine (proflavin) or N-methyl-N'-nitro-nitrosoguanidine (MNNG or NTG) while for the S-9 assay was benzo(a)pyrene (B(a)P) or aflatoxin B_1 (AFB_1).

First, the efficiency of the chosen positive controls was examined. Figure 6 represents a typical result of the Mutatox® test when comparing the effects of proflavin and a blank control. In the presence of 5 μg/ml proflavin, the restored luminescence reached its maximum around 12 hours. In the blank control without proflavin, the spontaneous luminescence reached its maximum around 5 hours. According to the criteria for decision making, one can define a significant genotoxic activity when the maximal luminescence developed within 24 hours due to the chemical in question is at least 3 times higher than the maximal luminescence of the corresponding control. The luminescence increased for the proflavin treatment is at least 20 times higher than that of blank control. From Figure 6, the mutagenic activity of proflavin has been confirmed. In Figure 7, the response of

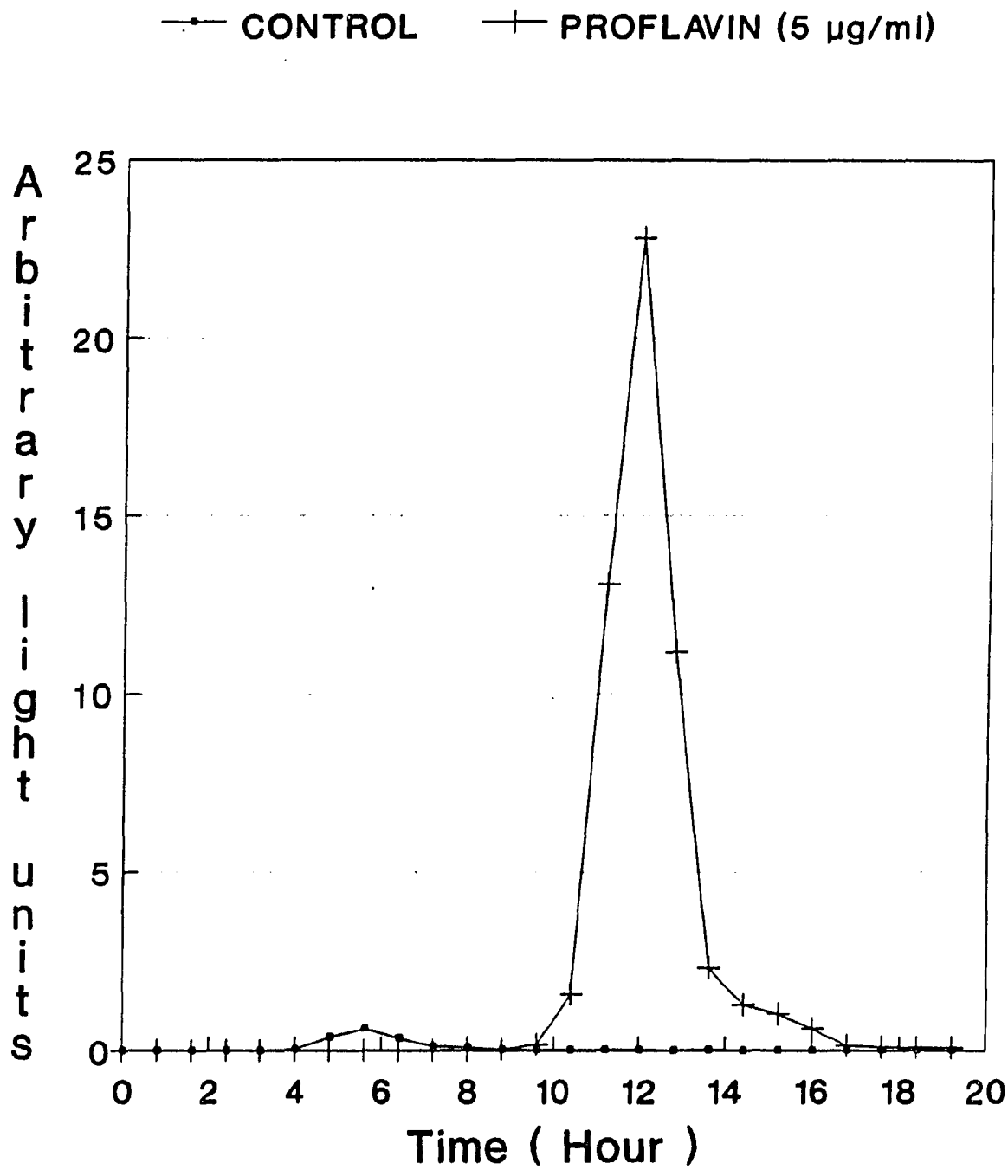


Figure 6. Typical Mutatox® test for proflavin

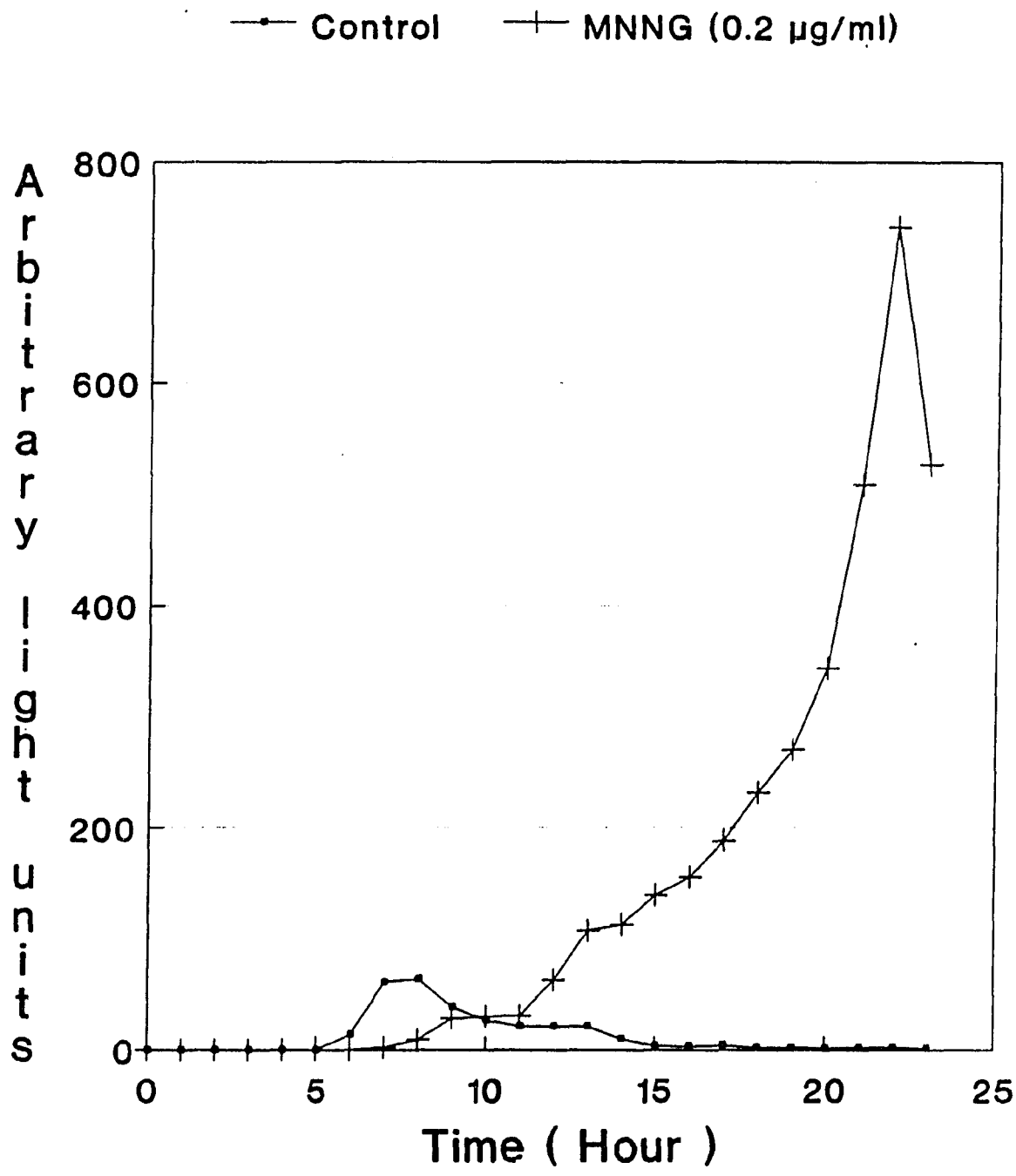


Figure 7. Typical Mutatox® test for MNNG

MNNG in the Mutatox® test was also examined. Again, a positive response of mutagenesis has been confirmed for MNNG at concentration of 0.2 μg/ml media.

The other advantage of the Mutatox® test is that not only a positive or negative result for mutagenesis can be obtained but also the dose-response within certain concentration range of the tested chemical can be obtained. Figures 8 and 9 show the dose-response of proflavin and MNNG, respectively. From 0.4 μg/ml up to 1.5 μg/ml of proflavin, the luminescence increased as the concentration increased (see Figure 8). For MNNG, the concentration range between 0.01 μg/ml to 0.2 μg/ml also shows dose-response (see Figure 9). One more observation from our study (see Table 31) was that when the concentrations of proflavin and MNNG were further increased beyond the dose-response range up to 10 μm/ml and 0.3 μg/ml, respectively, the presence of proflavin and MNNG totally eliminated the bacterial growth. The concentration of certain tested chemical at which will inhibit the bacterial growth is defined as the toxic concentration.

Next, the S-9 assay was conducted by using B(a)P and AFB_1. Figure 10 shows the result of B(a)P at 5 μg/ml media. In the presence of S-9 activation, the luminescence of B(a)P treatment increased dramatically after 17 hours while without S-9 activation the maximal luminescence of B(a)P treatment was

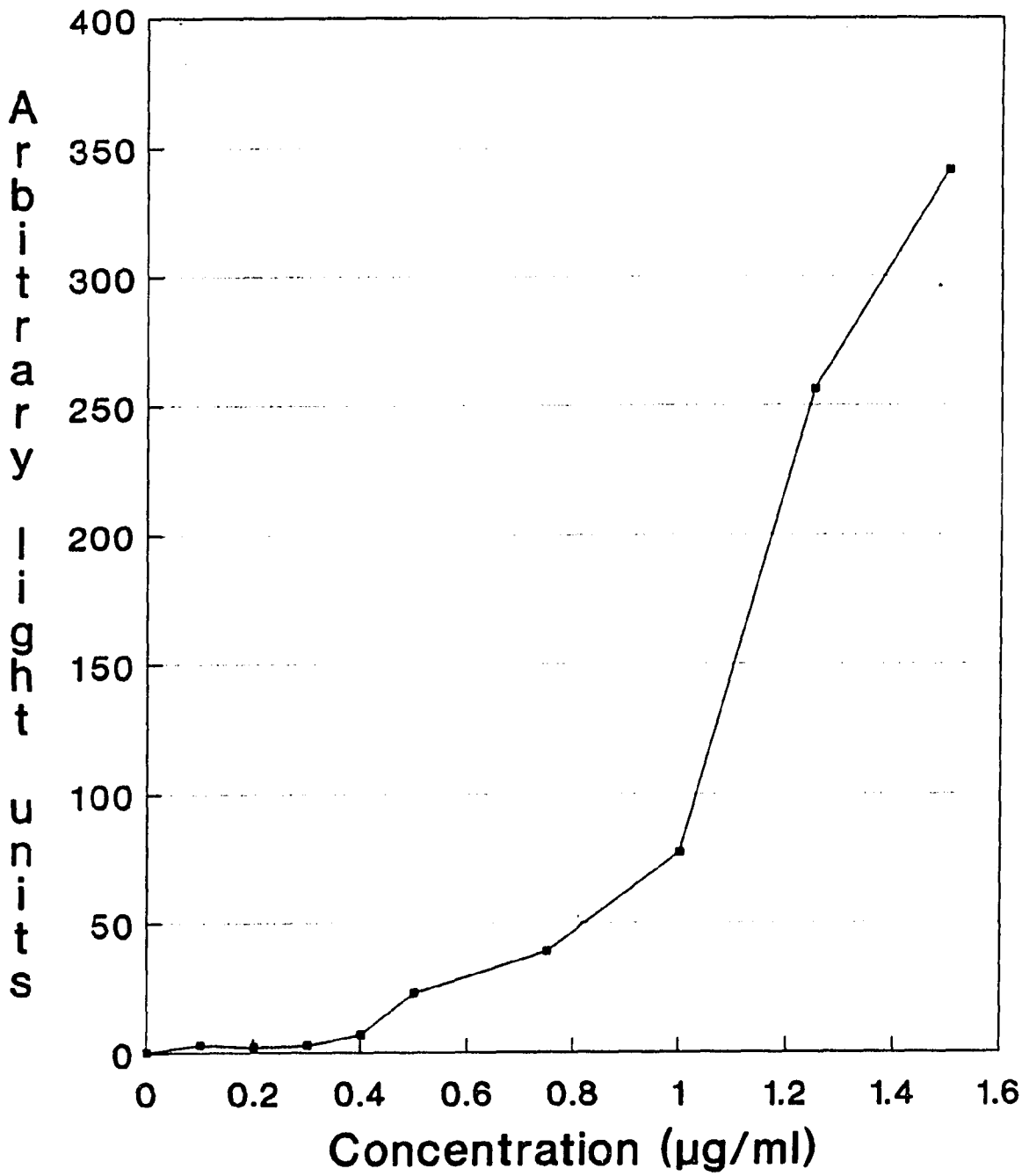


Figure 8. Typical dose-response of proflavin by using Mutatox® test

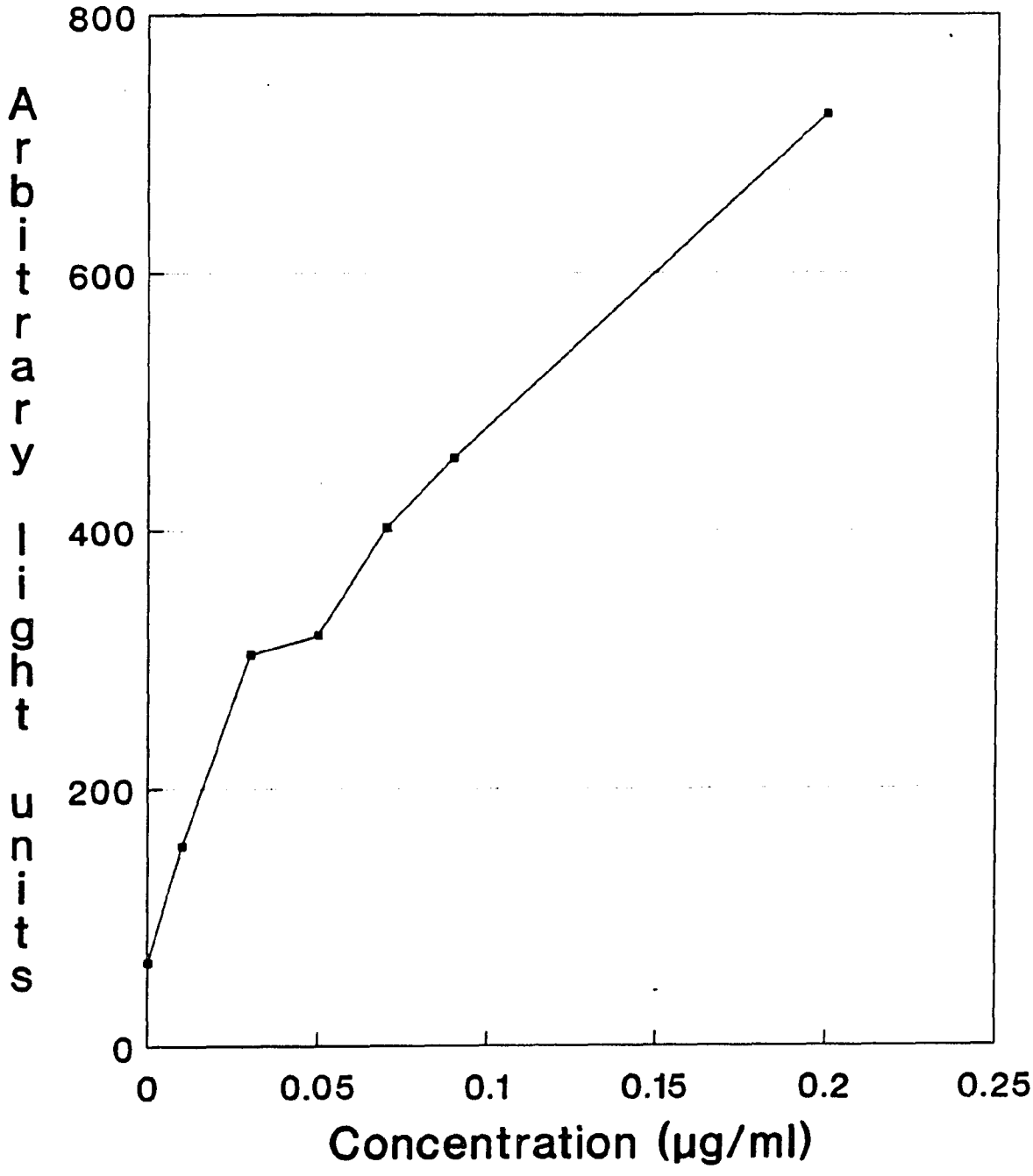


Figure 9. Typical dose-response of MNNG by using Mutatox® test

Table 31. Mutagenicity of proflavin, MNNG, B(a)P, and aflatoxin B_1

Chemical	Direct assay	S-9 assay	Concentration range for positive response (μg/ml media)	Toxic Concentration (μg/ml media)
Proflavin	+	N.T.[a]	1.00 - 7.00	10.0
MNNG	+	N.T.	0.03 - 0.25	0.3
B(a)P	-	+	1.00 - 9.00	15.0
AFB_1	-	+	1.00 - 5.00	20.0

[a]N.T. = Not Tested.

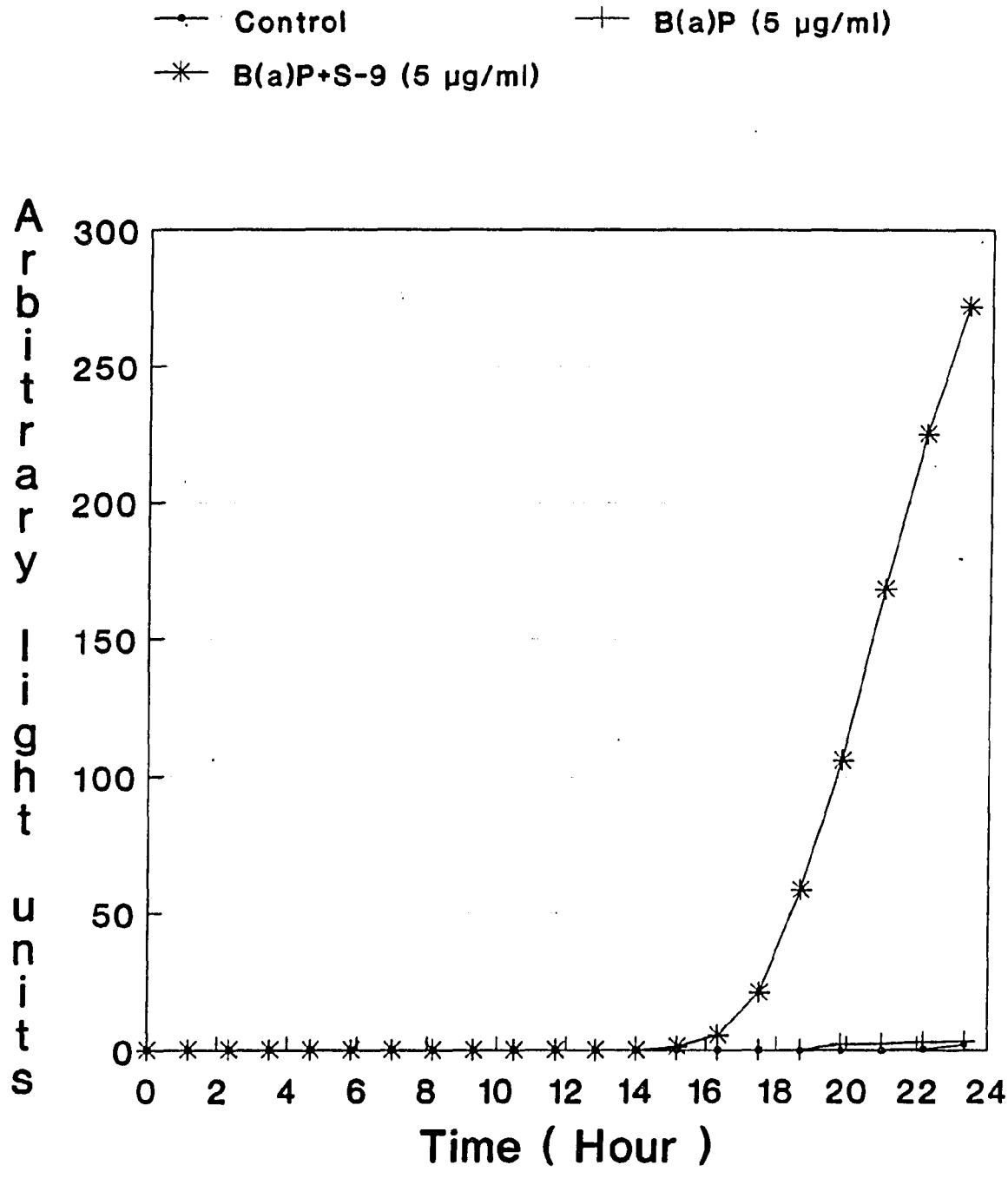


Figure 10. Typical Mutatox® test for Benzo(a)pyrene

similar to that of the blank control. These results suggest that S-9 activation is necessary for the B(a)P to exhibit mutagenic activity. As shown in Figure 11, the S-9 activation is also necessary for the AFB_1 to show a positive response in the Mutatox® test. Similar to the direct assay, the S-9 assay can also get different kinds of information concerning certain chemical such as mutagenicity response, dose-response and toxic concentration.

From the experiments with proflavin, MNNG, B(a)P and AFB_1, the positive controls were established for further study with other chemicals to be tested. Table 31 summarizes the results of all the positive controls. In Figures 6 to 11, it is indicated that: (1) the onset times for the maximal luminescence differed from chemical to chemical, (2) the mutagenic or toxic concentration differed from chemical to chemical, and (3) for all chemicals tested, a confirmed result can be obtained within 24 hours.

The autoxidized methyl linoleate and linolenate were fractionated by silica gel column. Different fractions were collected and concentrated under reduced pressure. Basic TLC was conducted to identify the polarity of different fractions. As shown in Figures 12 and 13, from fraction 1 to fraction 5 the polarity increased as fraction number increased. The whole autoxidized and unoxidized (pure) methyl linoleate and

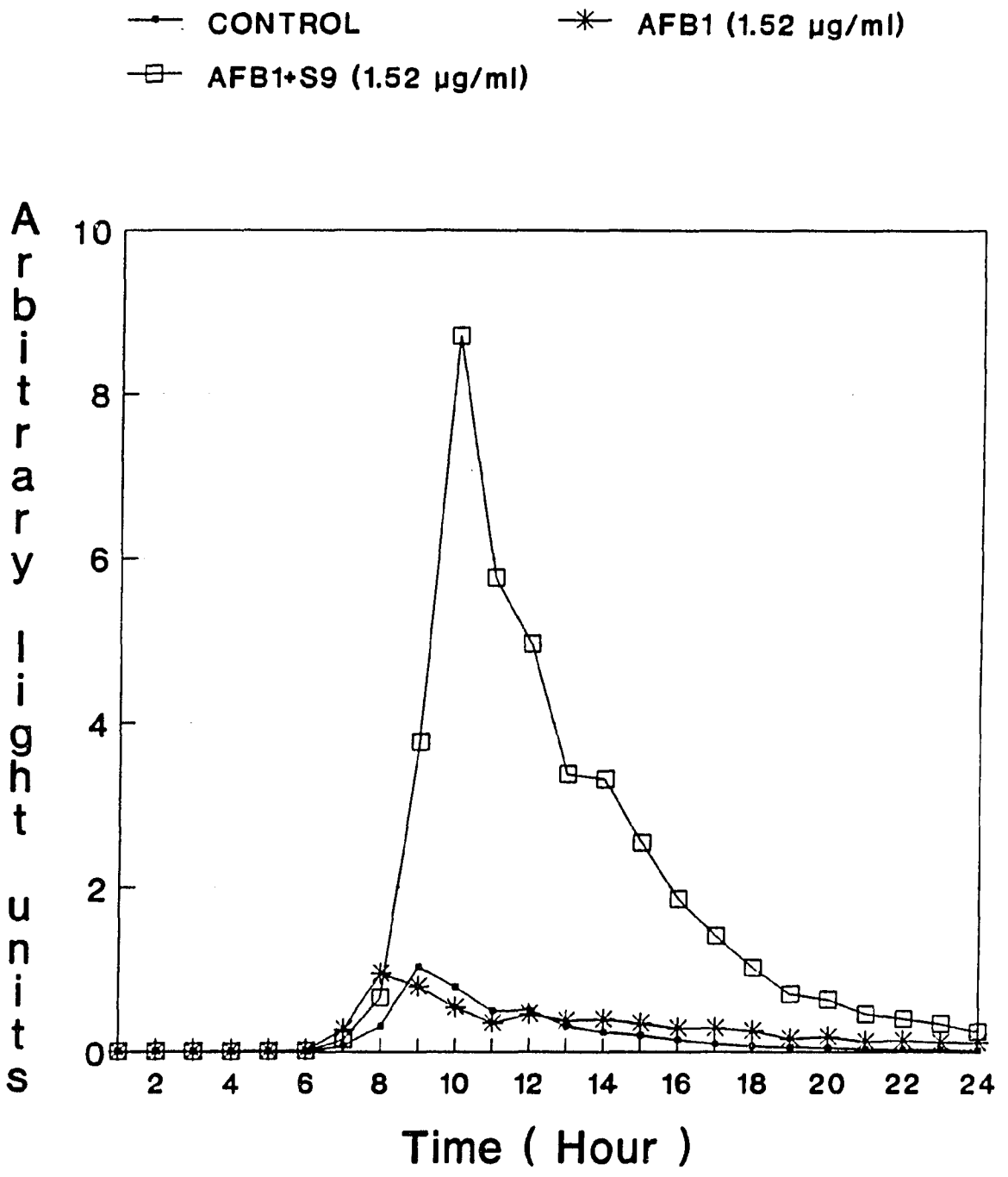


Figure 11. Typical Mutatox® test for Aflatoxin B_1

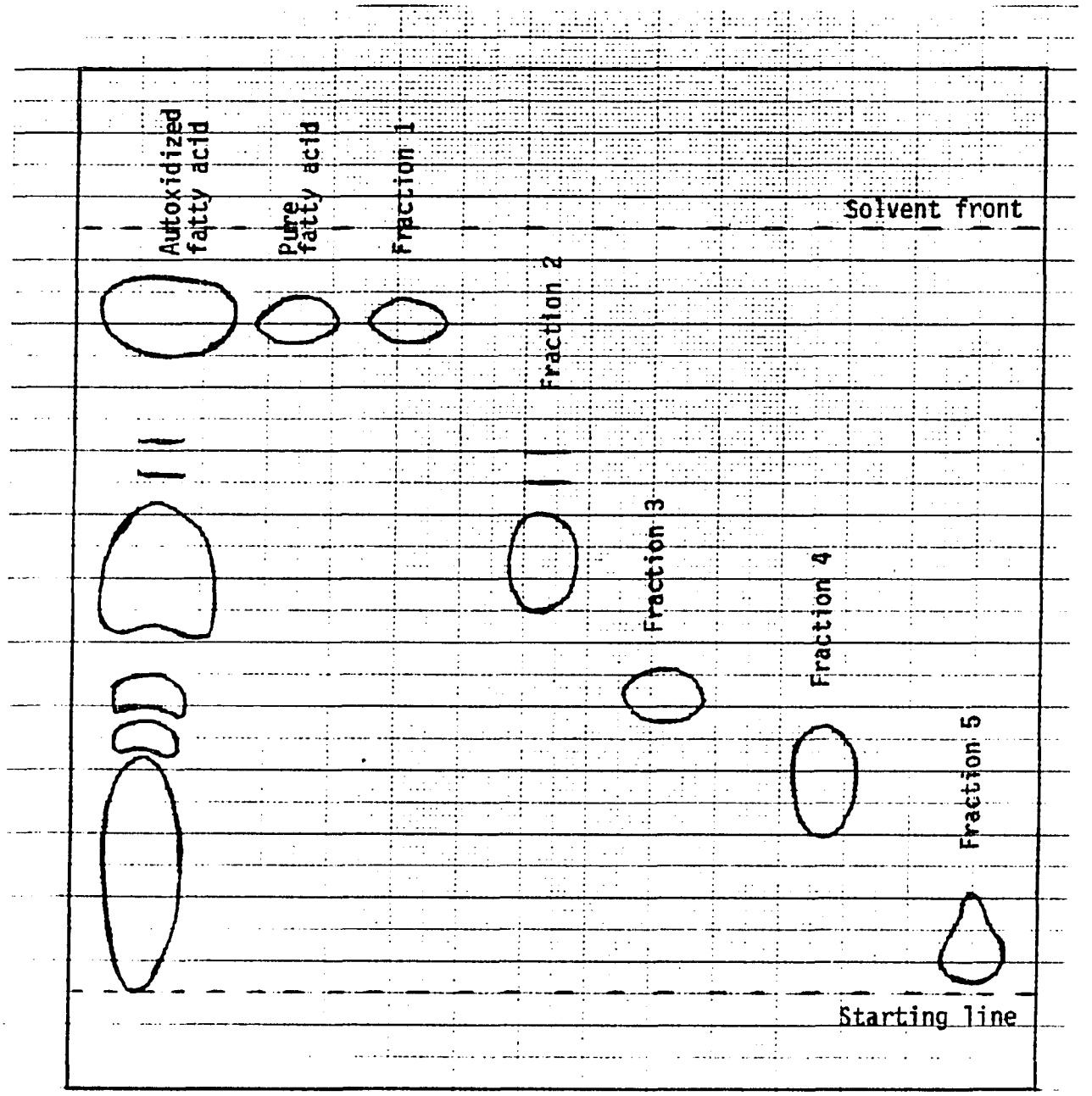


Figure 12. Thin-layer Chromatography of column fractions from autoxidized methyl linoleate

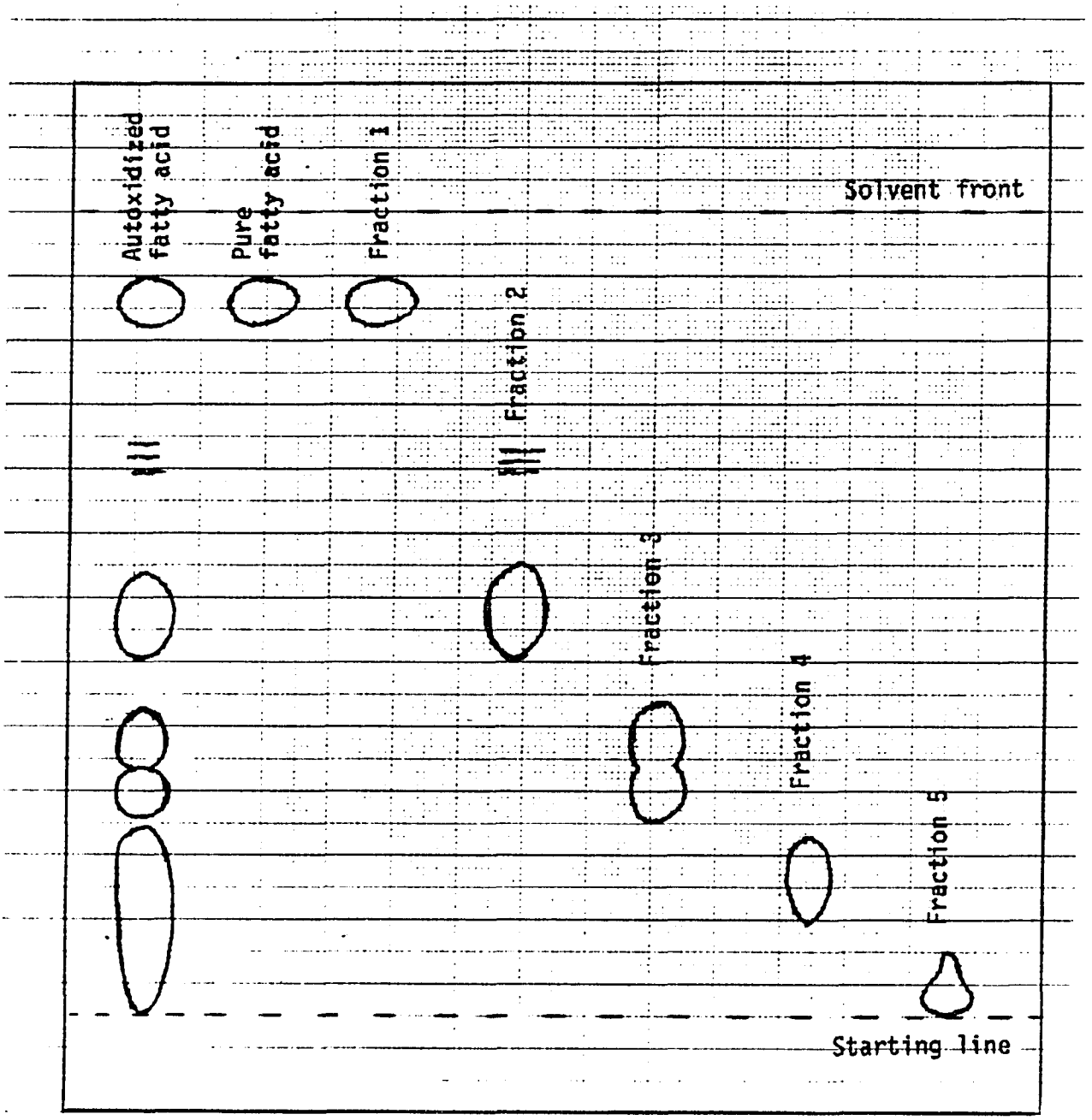


Figure 13. Thin-layer chromatography of column fractions from autoxidized methyl linolenate

linolenate as well as five different fractions from each autoxidized fatty acid were used to conduct the Mutatox® test. None of the compounds or fractions tested exhibited mutagenic activity for either direct or S-9 assay (see Tables 32 and 33). Because of the lack of mutagenicity, we did not further identify specific compounds from these two autoxidized fatty acids. But, according to Neff et al. (1981), the possible compounds that might be present in each fraction are as follow: fraction 1 - unoxidized methyl linoleate or linolenate; fraction 2 - epoxy compounds; fraction 3 - hydroperoxide mixture; fraction 4 - mixture of hydroperoxides and hydroperoxy-cyclic compounds; and fraction 5 - unidentified polar compounds.

Chlorinated fatty acid, another fatty acid derivative, is produced during food processing. Heikes and Griffitt (1979) identified different chloroethyl fatty acid compounds in different products such as French dressing, frankfurters, dried peppers and cheese crackers. Dr. Heikes provided us 2-chloroethyl caprate, laurate, palmitate, and linoleate for the Mutatox® test. As shown in Table 34, none of these four chlorinated fatty acids were mutagenic in the direct assay. However, in the presence of S-9 activation, all these samples show mutagenic activity within the concentrations between 10 μg/ml and 100 μg/ml. Also, the toxic concentrations for both

Table 32. Mutagenicity of autoxidized products of methyl linoleate

Chemical	Direct assay	S-9 assay	Concentration range used (μg/ml media)	Toxic Concentration (μg/ml media)
Whole autoxidized fatty acid	-	-	0.001 - 50	15
Pure fatty acid	-	-	0.001 - 50	N.D.
Fraction 1	-	-	0.001 - 50	N.D.[a]
Fraction 2	-	-	0.001 - 50	15
Fraction 3	-	-	0.001 - 50	10
Fraction 4	-	-	0.001 - 50	10
Fraction 5	-	-	0.001 - 50	10

[a]N.D. = Not determined within the concentration range used.

Table 33. Mutagenicity of autoxidized products of methyl linolenate

Chemical	Direct assay	S-9 assay	Concentration range used (μg/ml media)	Toxic Concentration (μg/ml media)
Whole autoxidized fatty acid	-	-	0.001 - 50	15
Pure fatty acid	-	-	0.001 - 50	N.D.[a]
Fraction 1	-	-	0.001 - 50	N.D.
Fraction 2	-	-	0.001 - 50	15
Fraction 3	-	-	0.001 - 50	10
Fraction 4	-	-	0.001 - 50	10
Fraction 5	-	-	0.001 - 50	10

[a]N.D. = Not determined within the concentration range used.

Table 34. Mutagenicity of chlorinated fatty acids

Chemical	Direct assay	S-9 assay	Concentration range for positive response (μg/ml media)	Toxic Concentration (μg/ml media)
2-chloroethyl caprate	-	+	10 - 100	150
2-chloroethyl laurate	-	+	10 - 100	150
2-chloroethyl palmitate	-	+	10 - 100	150
2-chloroethyl linoleate	-	+	10 - 100	150

direct and S-9 assays for all those chlorinated fatty acids were 150 μg/ml.

Frying in oil is one of the most commonly used procedures for the manufacture and preparation of foods. During frying the oil is continuously or repeatedly used at elevated temperatures in the presence of air and moisture. Heating of oil under these conditions leads to the formation of both volatile breakdown products and nonvolatile oxidation products. The nonvolatile oxidation products accumulate in the heated oil and are subsequently ingested into the human body when they are absorbed by the fried foods. Table 35 summarizes the results of Mutatox® test of heated oil. The fresh soybean oil without any heating gave a negative response for both direct and S-9 assay. For 0.01 to 150 μg/ml fresh oil, the toxic concentration could not obtained. After 32 hours and 40 hours of heating, the soybean oil showed a positive response for mutagenic activity for both direct and S-9 assays. In these cases, the concentration range for positive response was between 1 μg/ml media and 75 μg/ml media, while the toxic concentration was 100 μg/ml.

Recently, the toxicity of cholesterol oxidation products has drawn considerable attention. Ansari et al. (1982) suggested that oxidation products of autoxidized cholesterol and cholesterol hydroperoxides were mutagenic in the Ames

Table 35. Mutagenicity of heated cooking oil

Day(s) of heating	Direct assay	S-9 assay	Concentration range used (μg/ml media)	Toxic Concentration (μg/ml media)
0	-	-	0.01 - 150	N.D.[a]
1	-	-	0.01 - 150	N.D.
2	-	-	0.01 - 150	N.D.
3	-	-	0.01 - 150	120
4	+	+	0.01 - 150 (1 - 75)[b]	100
5	+	+	0.01 - 150 (1 - 75)[c]	100

[a]N.D. = Not determined within the concentration range used.

[b,c]The concentration range responded positive.

test. As shown in Table 36, after only 16 hours of heating the soybean oil with 1% cholesterol responded positively to both direct and S-9 assays in the Mutatox® test.

Table 37 presents the results of tests with some of the mycotoxins selected for the Mutatox® test. As indicated in the positive control study (see Figure 11), AFB_1 responded positively for mutagenic activity in the presence of S-9 activation. Within the concentration range between 0.01 μg/ml and 50 μg/ml, AFB_2 did not show mutagenic activity for both direct and S-9 assays. The toxic concentration for AFB_2 was 50 μg/ml. Epoxide AFB_1, a derivative of AFB_1, was synthesized according to the method described by Baertschi et al. (1988) and evaluated for mutagenic activity. For the direct assay, epoxide AFB_1 exhibited mutagenesis between 2 μg/ml and 10 μg/ml while at 20 μg/ml, the epoxide AFB_1 played a inhibitory role for bacterial growth. In the Mutatox® test, ochratoxin A was not mutagenic with or without the activation by S-9 fraction. The toxic concentration for ochratoxin A was 50 μg/ml. Fumonisin B_1 showed mutagenic activity without the activation of S-9 fraction. The concentration range which respond positively was between 5 μg/ml and 20 μm/ml, while the toxic concentration was 40 μg/ml.

Heterocyclic amines such as MeIQ, Trp-P-1, and Trp-P-2 were kindly provided by Dr. Fiddler and were tested for

Table 36. Mutagenicity of heated cooking oil with addition of 1% cholesterol

Day(s) of heating	Direct assay	S-9 assay	Concentration range used (μg/ml media)	Toxic Concentration (μg/ml media)
0	-	-	0.01 - 150	N.D.[a]
1	-	-	0.01 - 150	N.D.
2	+	+	0.01 - 150 (0.5 - 40)[b]	100
3	+	+	0.01 - 150 (0.5 - 40)[c]	75
4	+	+	0.01 - 150 (0.5 - 40)[d]	75
5	+	+	0.01 - 150 (0.5 - 40)[e]	75

[a]N.D. = Not determined within the concentration range used.

[b,c,d,e]The concentration range responded positive.

Table 37. Mutagenicity of some mycotoxins

Mycotoxin	Direct assay	S-9 assay	Concentration range used (μg/ml media)	Toxic Concentration (μg/ml media)
AFB_1	-	+	0.01 - 50 (1 - 5)[b]	20
AFB_2	-	-	0.01 - 50	50
Epoxide AFB_1	+	N.D.[a]	0.10 - 50 (2 - 10)[c]	20
Ochratoxin A	-	-	0.01 - 50	50
Fumonisin B_1	+	N.D.	0.01 - 50 (5 - 20)[d]	40

[a]N.D. = Not determined within the concentration range used.

[b,c,d]The concentration range responded positive.

Table 38. Mutagenicity of some heterocyclic amines

Chemical	Direct assay	S-9 assay	Concentration range for positive response (μg/ml media)	Toxic Concentration (μg/ml media)
MeIQ	-	+	0.5 - 10.0	30
Trp-P-1	-	+	0.5 - 7.5	40
Trp-P-2	-	+	0.5 - 7.5	40

mutagenicity and toxicity in the Mutatox® test. All three compounds responded negatively for the direct assay and positively for the S-9 assay (see Table 38). The concentration ranges that gave a positive response were 0.5 μg/ml to 10 μg/ml, 0.5 μg/ml to 7.5 μg/ml, and 0.5 μg/ml to 7.5 μg/ml for MeIQ, Trp-P-1 and Trp-P-2, respectively. The toxic concentration for MeIQ was 30 μg/ml and for both Trp-P-1 and Trp-P-2 was 40 μg/ml.

DISCUSSION

In recent years, there have been thousands of chemicals tested for genotoxic activity. This has been brought about by the development of rapid, relative inexpensive genetic screening tests. There are now many these tests available that employ diverse organisms (from bacteria to mammalian cells in culture). Mutagens are being discovered, at a still increasing rate, in many foods that form an important part of our diet. Mutagens are also present in the water we drink and the air we breath; indeed it appears that contact with mutagens is a daily event. The Ames test, also called the Salmonella/mammalian microsome test, has been widely used as a screening test by food scientists and environmentalists to evaluate the mutagenic activity of chemical compounds. Our study is the first one using the Mutatox® test system to determine the mutagenicity of chemical compounds that may be found in foods and feeds. The Mutatox® test is a good alternative to the Ames test. The Mutatox® test is much cheaper and easier to conduct. Minimum personnel training and laboratory facility requirements are other advantages. In the Ames test, results are obtained by counting the colonies of bacteria. Any unexpected contamination by other bacteria may affect the outcome significantly thus aseptic technique is necessary. The dark mutant of luminous bacteria used in the

Mutatox® test, however, only responds to a mutagen and then luminescence is restored. Unless the contamination contains mutagen(s), the luminescence won't be altered and thus affect the outcome of the Mutatox® test. The Mutatox® test provides a rapid, general screening test which can be used to assay large numbers of pure chemicals and complex samples. The test can be performed in one day, and by serially diluting the compound, dose response data plus toxicity data can be generated for a number of samples simultaneously.

A direct mutagenic effect of lipid hydroperoxides has been tested in several studies with varying results. The Ames test has been utilized to demonstrate weak mutagenicity of both peroxidized fatty acids and isolated hydroperoxides of methyl linoleate. Because cumene hydroperoxide and t-butyl hydroperoxide were also found to be mutagenic, while peroxides, peracids and hydrogen peroxide were not, the mutagenicity was attributed to the hydroperoxide group (Yamaguchi and Yamashita, 1979; 1980). However, Scheutwinkel-Reich et al. (1980) failed to find mutagenicity for linoleic acid hydroperoxide. Contradictory results have also been observed when examining the mutagenicity of fatty acid epoxides by using the Ames test. Gardner et al. (1983) suggested that the epoxide fatty esters were not mutagenic at concentrations up to 2,000 μg/test plate even though these esters had structural characteristics similar to certain

potent mutagens. On the other hand, MacGregor et al. (1985) found that these epoxide fatty esters were weakly mutagenic in strains TA97 and/or TA100. From investigations conducted so far, mutagenic properties of lipid peroxidation products of fatty acids have not been conclusively demonstrated. The Mutatox® test was used to examine the mutagenic activity of lipid peroxidation products. None of the fractions tested positively for mutagenicity.

The 2-chloroethyl esters of fatty acids have been identified in spice and food samples by gas-liquid chromatography-mass spectrometry by Heikes and Griffitt (1979). The formation of 2-chloroethyl esters from the reaction of 2-chloroethanol and fatty acids was predicted to occur quite readily. Chloroethanol (ethylene chlorohydrin) has been found in whole and ground spices after they were fumigated with ethylene oxide. The fumigant combines with moisture and natural inorganic chloride to form the corresponding chlorohydrin (Wesley et al., 1965). It has been postulated that the 2-chloroethyl esters were the product of the reaction of 2-chloroethanol and the natural fatty acids. Twenty-four spice samples were analyzed for the 2-chloroethyl esters of fatty acids, and the esters of capric, lauric, palmitic and linoleic acids have been identified at levels up to 1,400 ppm. 2-Chloroethyl linoleate was the most abundant ester in all samples. Several foods such as French dressing,

frankfurters, dehydrated onions, and dried peppers analyzed by the same procedures showed levels of 2-chloroethyl linoleate as high as 35 ppm (Heikes and Griffitt, 1979). Dr. Heikes isolated the 2-chloroethyl esters of capric, lauric, palmitic and linoleic acids and provided them for the Mutatox® test. This was the first study conducted to evaluate the toxicity of these esters. Table 4 indicates that from 10 μg/ml up to 100 μg/ml all chloroethyl esters are mutagenic in the presence of S-9 activation. Without metabolic activation, all chloroethyl esters were not mutagenic. For all these esters the toxic concentration was 150 μg/ml.

When cooking oils are heated, numerous non-volatile oxidation products are formed that may be absorbed by the fried food and subsequently eaten. Feeding experiments with laboratory animals have shown that thermally oxidized oils may cause growth retardation, increases in liver and kidney weights and damage to the liver, thymus and testes (Alexander et al., 1987). There has also been considerable concern over the mutagenic potential of continually heated oils. It has been found by Taylor et al. (1983) that severely abusive frying conditions, such as repeated use of frying oil, are necessary to produce appreciable mutagenic activity in French-fried potatoes or in fish fillets. More recent studies, however, have detected moderate mutagenic activity in the polar fat fractions of deep-frying fat samples from

restaurants and oils treated according to normal frying practices (Saleh et al., 1986; Hageman et al., 1988). Hageman et al. (1988; 1990) suggested that mutagenic compounds other than lipid peroxidation products, such as heterocyclic amines and other pyrolysis products may be formed in foods during deep frying and migrate into the frying fat. These products may have been responsible for part of the mutagenic activity of the polar fraction of the frying fat. Table 5 shows that after 32 hours of heating, the oil became mutagenic in the Mutatox® test both with and without S-9 activation. The study indicated that the lipid peroxidation products of heated oil may be responsible for the mutagenicity. It can be concluded that overused and abused oils undoubtedly contain oxidized material that, if chronically consumed, could pose a human health risk.

Cholesterol oxidation products have attracted much attention in recent years. A substantial amount of cholesterol oxidation products have been detected in deep-fried foods, dehydrated milk, and egg products (Addis, 1986). Several cholesterol autoxidation products have been found to possess undesired biological effects, which were related to feedback inhibition of cholesterol biosynthesis, cytotoxicity, angiotoxicity, mutagenicity, and carcinogenicity (Smith, 1981; Finocchiaro and Richardson, 1983; Maerker, 1987) Smith et al. (1979) found that when pure cholesterol, which is not

mutagenic, was subjected to autoxidation by heating in air, it became mutagenic towards the bacterial strains used in the Ames test. A study by Ansari et al. (1982) indicated that the peroxidation products of autoxidized cholesterol and cholesterol hydroperoxides were mutagenic in the Ames test. Watanabe et al. (1988) also examined the mutagenic activities of degradation products from pure crystalline cholesterol that had been subjected to heat-treatment in air. They found that heating at 225°C for periods of over 5 hours or at temperatures above 150°C for 7 hours were required for mutagens to be detected. One percent crystalline cholesterol was incorporated into the vegetable oil and heated at 180° for 8 hours each day on 5 consecutive days. After 16 hours of heating, the oil showed mutagenic activity. Compared to the oils without cholesterol after 32 hours of heating, the presence of cholesterol seems to have a significant effect on mutagenicity. This study suggests that the peroxidation products of cholesterol may play an important role for the mutagenicity.

Mycotoxins are intimately associated with almost every food or feed and can be produced by contaminating fungi at every stage of harvesting, production, and storage of food. The widespread presence of mycotoxins creates serious environmental and economic problems. AFB_1 is one of the most

potent naturally occurring mutagens. It can induce mutations in bacterial, algal, fungal, and insect species and in mammalian cell culture (Ong, 1975), Metabolism of aflatoxins is considered a prerequisite for carcinogenicity and mutagenicity. It is well established that the chemical site implicated as responsible for the biological activity of aflatoxins is the C_2-C_3 double bond in the dihydrofurofuran moiety of these molecules (Stark, 1980). Reduction of this bond, yielding 2,3-dihydro AFB_1 (AFB_2) results in a 500-fold decrease in mutagenicity and 150-fold decrease in carcinogenicity (Wogan et al., 1971; Wong and Hsieh, 1976). The ultimate carcinogen and mutagen in the metabolic activation of aflatoxin has been postulated to be the AFB_1-2,3-epoxide. This compound has been proposed to be a highly active metabolite that reacts with nucleophilic sites of macromolecules (Essigmann et al., 1979). Metabolic activation by the S-9 fraction was necessary for the mutagenic activity of AFB_1 in the Mutatox® test, while AFB_1-epoxide exerted mutagenicity in the direct Mutatox® assay. AFB_2 did not show mutagenic activity in either direct or S-9 assays of Mutatox® test. The results with ochratoxin A were similar to that of Kuczuk et al. (1978) and Wehner et al. (1978), who indicated that ochratoxin A was not mutagenic with or without S-9 activation. The mutagenicity of fumonisin B_1 has been

demonstrated for the first time in the work reported here. Results indicated that fumonisin B_1 is a direct mutagen for which the metabolic activation system is not necessary.

Heterocyclic amines are not themselves mutagenic to *Salmonella typhimurium* strains. But they exert mutagenic activity in the presence of S-9 fraction (Sugimura et al., 1986). Kato and Yamazoe (1987) found that synthetic hydroxyamine derivatives of heterocyclic amines were mutagenic in the absence of the S-9 activation system and suggested that cytochrome P450s in the S-9 fraction may convert the heterocyclic amines to their hydroxyamine derivatives and thus exert mutagenicity. Our study further confirmed the necessity of the S-9 fraction activation system for heterocyclic amines, such as MeIQ, Trp-P-1 and Trp-P-2, to exhibit mutagenicity in the Mutatox® test.

Quantitative data on heterocyclic amines in various cooked foods have been reported by Sugimura et al. (1986) and are shown in Table 39. As present, it is very difficult to obtain precise quantitative information about heterocyclic amines. But on the basis of the limited information available, Sugimura et al. (1989) recommended that exposure to smoke produced by broiling fish and meat and a high intake of food containing significant amount of heterocyclic amines should be avoided.

Table 39. Amount of heterocyclic amines in cooked foods (ng/g cooked food)

Compound	Broiled Sun-dried Sardine	Broiled Beef
MeIQ	72.0	
Trp-P-1	13.3	53.0
Trp-P-2	13.1	1.6

SUMMARY

Mutatox® test is a very good alternative to the Ames test. The Mutatox® test is much cheaper and easier to conduct than the Ames test. Minimum personnel training and laboratory facility requirements are other advantages. The test provides a rapid, screening test which can be used to assay large numbers of pure and complex compounds. The test is performed in one day, and by serially diluting the compound, dose response data plus toxicity data can be generated for a number of samples simultaneously.

The Mutatox® test system was used to determine the mutagenicity of a spectrum of chemical compounds that might be present in foods and feeds. Five different fractions isolated from either autoxidized methyl linoleate or autoxidized methyl linolenate were tested for mutagenic activity by the Mutatox® test. None of these fractions were mutagenic with or without activation by S-9 (microsomal) fraction. Chlorinated fatty acids including chloroethyl caprate, laurate, palmitate and linoleate were mutagenic in the presence of the S-9 activation system. Without metabolic activation, chloroethyl esters were not mutagenic.

The mutagenic activity of heated cooking oils was also examined. Results showed that after heating at 180 °C for 32

hours (8 hours a day on 5 consecutive days) the soybean oil exhibited mutagenic activity with or without the metabolic activation system. The addition of cholesterol into the soybean oil increased the onset of the mutagenicity in the soybean oil.

AFB_1 was mutagenic in the Mutatox® test in the presence of S-9 activation, while AFB_1 epoxide and Fumonisin B_1 showed direct mutagenic activity. AFB_2 and Ochratoxin A were not found to be mutagenic by using Mutatox® test. MeIQ, Trp-P-1, and Trp-P-2, three important chemical compounds isolated from cooked meats, were mutagenic in the presence of S-9 activation.

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