This is to certify that the

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A STUDY OF MALONALDEHYDE-AMINO ACID INTERACTIONS

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J. I. Gray
Major professor

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A STUDY OF MALONALDEHYDE-AMINO ACID INTERACTIONS

By

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ABSTRACT

A STUDY OF MALONALDEHYDE-AMINO ACID INTERACTIONS

By

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The formation of malonaldehyde by different methods and the reaction of malonaldehyde with glycine or its ethyl ester were studied. Acid hydrolysis of tetramethoxypropane resulted in the formation of malonaldehyde, 3,3-dimethoxypropionaldehyde, and polymerization products. The formation of malonaldehyde by steam distillation of malonaldehyde bis-bisulfite acid hydrolyzate resulted in a relatively pure malonaldehyde sample. Ethyl acetate extraction and thin layer chromatographic analysis of the products formed from the reaction of malonaldehyde and glycine ethyl ester indicated the presence of two fluorescent Schiff bases. Model systems containing autoxidized linolenic acid and glycine ethyl ester were also shown to produce similar fluorescent components. Fluorescence of these systems generally increased with thiobarbituric acid number. This fluorescence technique could possibly be developed as a means of monitoring lipid oxidation in biological systems.

Dedicated to

My Parents

Arthur and Mary Horenstein

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INTRODUCTION

Lipid autoxidation is one of the major degradative processes that occurs in foods and are responsible for changes in food quality. Autoxidation leads to the formation of short chain aldehydes, ketones, acids and polymers which cause unpleasant rancid flavors. In the autoxidation process, free radicals are produced which react with proteins and vitamins leading to possible nutrient loss. Bifunctional aldehydes, such as malonaldehyde, known to form during autoxidation may also react with proteins.

The changes produced during lipid autoxidation usually take place relatively slowly so that food quality deteriorates during prolonged storage. The importance of lipid oxidation in food deterioration has led to the search for reliable means to quantitate the amount of lipid oxidation that takes place in food systems. Two major classes of methods have been developed: chemical (peroxide value, thiobarbituric acid value, Kreis test, total and volatile carbonyl compounds, and oxirane determination) and physical (conjugated diene methods, infrared spectroscopy, infrared polarography, gas chromatography, and refractometry) (Gray, 1978). All of these methods have their limitations and thus the search for alternative methods continue. A

relatively sensitive technique has been developed utilizing the reaction of malonaldehyde, a lipid oxidation product, with amino compounds to form fluorescent Schiff bases (Fletcher et al., 1973).

The foremost element of this study was to investigate the potential of the malonaldehyde-amino acid interaction as a possible basis for monitoring the extent of lipid oxidation in a food system. Specific objectives of the proposed study were:

1. To investigate various procedures for the preparation of malonaldehyde since recent studies have shown that certain malonaldehyde preparations produce compounds other than malonaldehyde (Gutteridge, 1975; Marnett and Tuttle, 1980).

2. To study the reaction of malonaldehyde and an amino acid in order to characterize the products of the reaction. This reaction product was used as a prototype for the fluorescent malonaldehyde-amino compound reaction products that serve as monitors of lipid oxidation.

3. To evaluate the production of fluorescent Schiff bases in a dehydrated model system containing autoxidized linolenic acid and glycine ethyl ester as a means of monitoring lipid oxidation.

LITERATURE REVIEW

Lipid Oxidation

Lipids in biological systems can undergo oxidation, leading to deterioration of a given system. In food, these reactions can lead to rancidity (Gray, 1978) and the possible formation of toxic compounds (Cutler and Schneider, 1973, Cutler and Hayward, 1974). The oxidative deterioration of food lipids involves primarily autoxidative reactions which are accompanied by various secondary reactions having oxidative and non-oxidative character. The important lipids in oxidation are the unsaturated fatty acid moieties, oleic, linoleic, and linolenic. The rate of oxidation of these fatty acids increases somewhat geometrically with their degree of unsaturation (Labuza, 1971).

Mechanism of Lipid Autoxidation

The autoxidation of unsaturated fatty acids has been proposed to occur by a free radical chain mechanism involving three stages: (1) initiation, the formation of free radicals; (2) propagation, the free radical chain reaction; and (3) termination, the formation of non-radical products (Farmer and Sutton, 1943; Uri, 1961; Lundberg, 1962).

Initiation $RH + O_2 \rightarrow\rightarrow\rightarrow\rightarrow R\cdot + \cdot OOH$

Propagation $R\cdot + O_2 \rightarrow\rightarrow\rightarrow\rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow\rightarrow\rightarrow\rightarrow ROOH + R\cdot$, etc.

Termination $R\cdot + R\cdot \rightarrow\rightarrow\rightarrow\rightarrow RR$

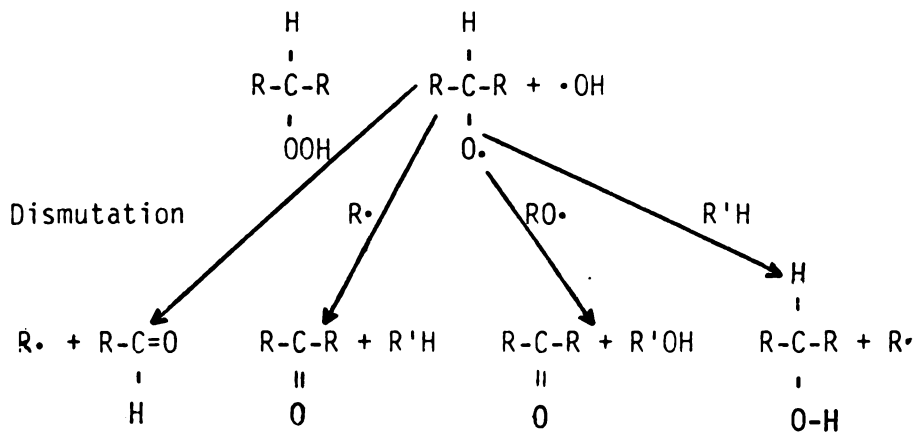
$R\cdot + ROO\cdot \rightarrow\rightarrow\rightarrow\rightarrow ROOR$

$ROO\cdot + ROO\cdot \rightarrow\rightarrow ROOR + O_2$

Other propagation and termination reactions are possible (Dugan, 1976).

Lipid oxidation can be initiated by a number of factors (Korycka-Dahl and Richardson, 1980). Singlet oxygen, formed by different chemical or enzymatic reactions, is one species that has been implicated as an initiator. Other activated oxygen species that could act as initiators are ozone and hydroxyl radicals. Superoxide anion and hydrogen peroxide can decompose to produce singlet oxygen or hydroxyl anions and thus could be considered initiators. Similarly, prooxidant metals such as iron and copper, and heme proteins can catalyze the decomposition of preformed hydroperoxides to initiate free radical chain reactions (Korycka-Dahl and Richardson, 1980).

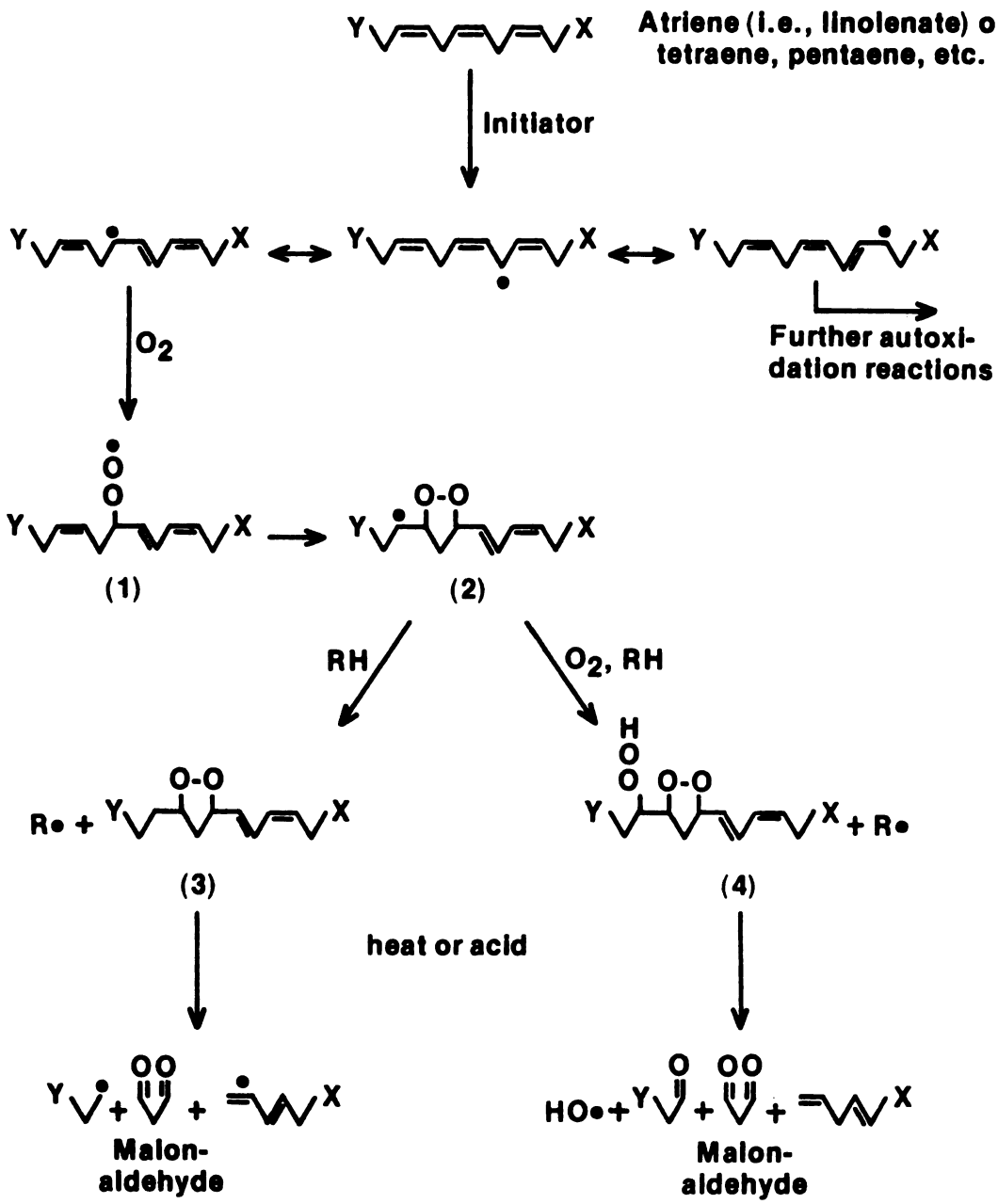
Free radicals produced during initiation react to form hydroperoxides, which break down to form other free radicals. This chain reaction continues and produces different types of free radicals. In termination, these free radicals react to form a wide variety of compounds including aldehydes, ketones, alcohols and acids (Dugan, 1976).



Formation Of Malonaldehyde In Autoxidation Reactions

Malonaldehyde is a three carbon dialdehyde which is produced during autoxidation of polyunsaturated fatty acids (Patton and Kurtz, 1951; Sinnhuber et al. 1959; Kwon and Olcott, 1966 a,b). Kenaston et al. (1955) reported that much more malonaldehyde was produced from autoxidized linolenate than from autoxidized linoleate, while autoxidized oleate produced essentially no malonaldehyde. These workers measured the malonaldehyde content by using the thiobarbituric acid test, the principle of which will be discussed later. Dahle et al (1962) reported that linoleate produced no malonaldehyde during the early stages of autoxidation They proposed that polyunsaturated fatty acids containing trienoic systems will undergo autoxidation resulting in the formation of a peroxide radical with unsaturation β,γ to the carbon bearing the peroxide group (Figure 1). This radical (compound 1) can cyclize to form a five membered cyclic peroxide radical (compound 2) which can abstract a hydrogen from an alkyl group to form compound 3, or undergo peroxidation and abstraction of a hydrogen to form compound 4. Compounds 3 and 4, when exposed to heat or acid as in the thiobarbituric

Figure 1. Mechanism for the formation of malonaldehyde from a triene system (Dahle et al., 1962).


Atriene (i.e., linolenate) or tetraene, pentaene, etc.
Initiator
O_2
Further autoxidation reactions
(1)
(2)
RH
O_2, RH
(3)
(4)
heat or acid
Malon-aldehyde
Malon-aldehyde

acid test, form malonaldehyde.

Pryor et al. (1976a) have criticized this mechanism on the basis that it does not adequately explain why dienes produce very little malonaldehyde as compared to trienes. According to these workers, Dahle et al. (1962) did not take into consideration initial hydrogen abstraction and subsequent peroxy radical formation at allylic positions on the ends of the diene system as shown in Figure 2. The abstraction of hydrogen from these positions would be statistically favored in a diene and would result in the formation of compound 5, a peroxy radical with unsaturation β,γ to the carbon bearing the peroxy radical. This radical could cyclize to form compound 6 and then undergo further reaction to produce compounds 7 or 8. These latter compounds, when treated with heat or acid, could produce malonaldehyde. Thus, according to Dahle et al. (1962) an autoxidized diene should produce a significant amount of malonaldehyde.

Pryor et al. (1976a) proposed a modification of the mechanism of Dahle et al. (1962) to more adequately explain the formation of malonaldehyde from polyunsaturated fatty acids (Figure 3). Compound 2, whose formation is shown in Figure 1, undergoes ring closure to produce the cyclic endoperoxide radical (compound 10) which subsequently undergoes peroxidation and abstraction of an alkyl hydrogen to produce compounds 11 and 12. When either of these compounds is subjected to heat or acid, malonaldehyde is formed. In a diene system, compound 6 would form the bicyclic endoperoxide radical 9 (Figure 2). This radical is about 10 kcal less stable than the allylic radical 10 (Figure 3). Thus, one would expect less endoperoxide formation from radical 9 than

Figure 2. Mechanism for the formation of malonaldehyde precursors in a diene system (Dahle et al., 1962).

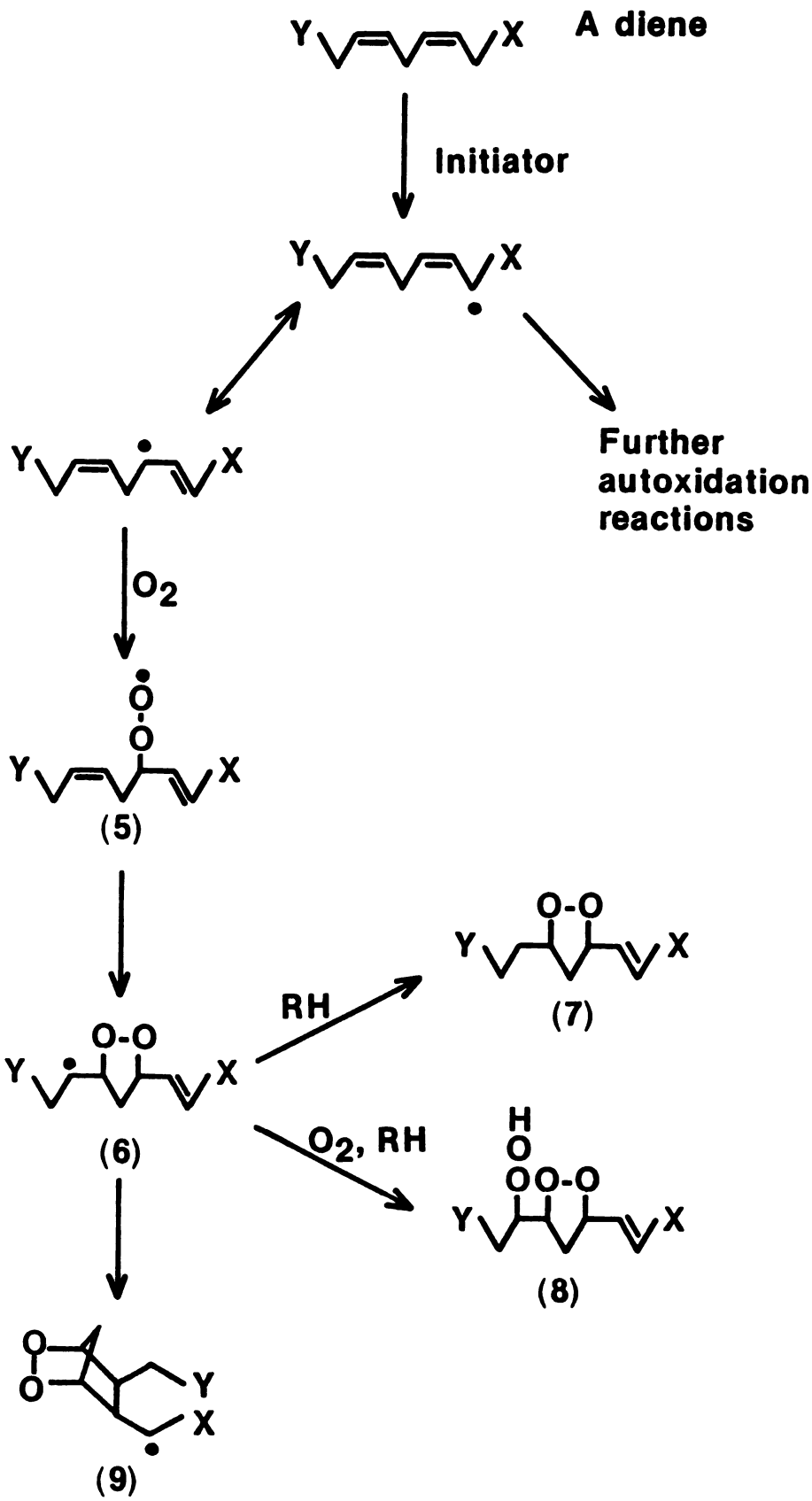
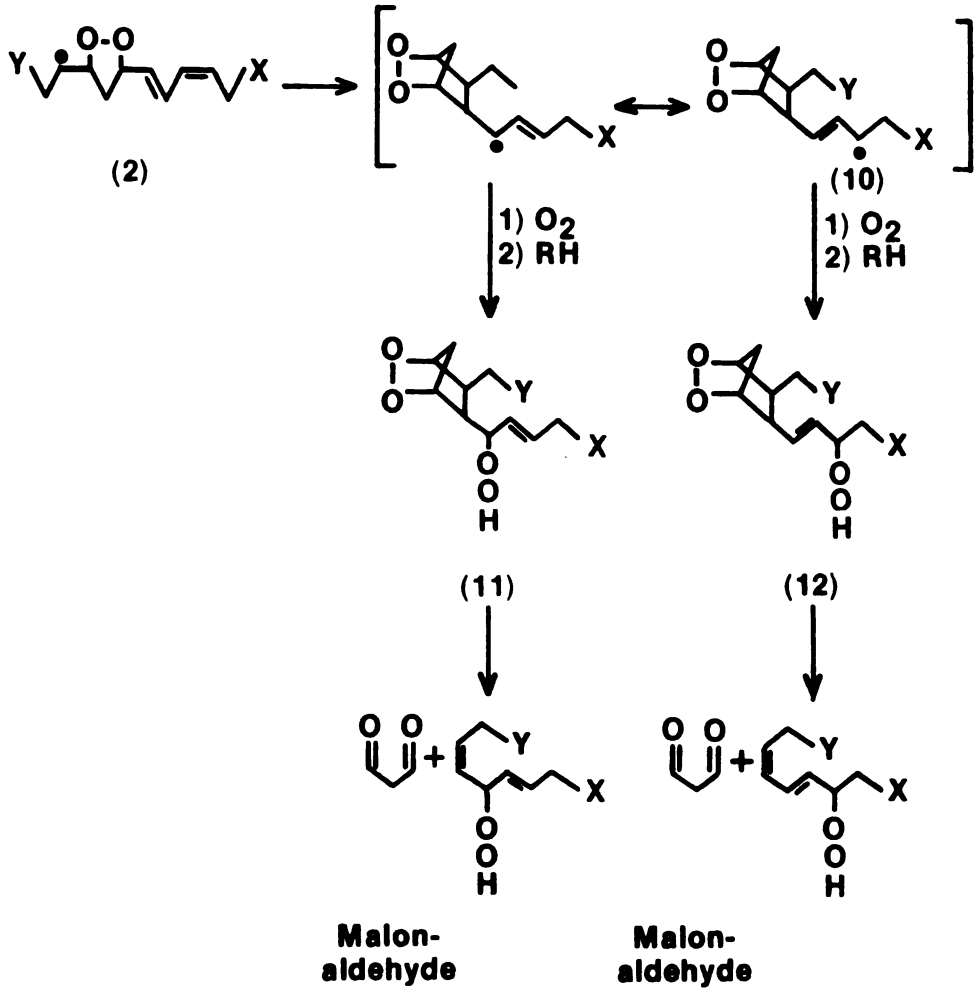

A diene
Initiator
Further autoxidation reactions
O_2
(5)
RH
(7)
(6)
O_2, RH
(8)
(9)

Figure 3. Prostaglandin-like endoperoxide mechanism for the formation of malonaldehyde in a triene system (Pryor et al. 1976a).


(2)
(10)
1) O_2
2) RH
1) O_2
2) RH
(11)
(12)
Malon-
aldehyde
Malon-
aldehyde

from radical 10 and consequently less malonaldehyde would be formed from the diene system.

From the discussion above, it is apparent that the mechanism of Pryor et al. (1976a) explains more adequately why oxidized polyunsaturated fatty acids produce much more malonaldehyde than fatty acids with two or less double bonds. This mechanism is similar to the one proposed for the synthesis of prostaglandins, in which malonaldehyde is a side product.

While the above mechanism explains the formation of malonaldehyde from its precursors as a result of acid or heat treatment, it does not explain the observations of Kwon and Olcott (1966a,b) who reported that free malonaldehyde, as produced in Figure 1, existed in autoxidized linolenate and squalene systems. Pryor et al. (1976b) determined by driving off thiobarbituric acid reactive material from an autoxidizing linolenate system with helium that about 10% of malonaldehyde was volatile and presumably free, while the remaining 90% of malonaldehyde was non-volatile and thus presumably formed according to the Pryor mechanism.

Malonaldehyde In Food And Biological Systems

Malonaldehyde in Food

While malonaldehyde is considered to be associated with oxidative rancidity, it has also been shown to occur in non-rancid foods including meats (Bidlack et al., 1972), herring (Kuusi et al., 1975), vegetable oil (Arya and Nirmala, 1971) and orange juice essence (Braddock and Petrus, 1971). Shamberger et al. (1977) determined the malonaldehyde

content of foods purchased in supermarkets in the Cleveland area. They found that fresh, fresh-frozen, or canned fruits and vegetables contained little or no malonaldehyde, possibly as a result of air-tight cans, natural antioxidants, or skin preventing oxidation. Vegetable oil and corn oil margarine also had no detectable malonaldehyde, possibly as a result of the antioxidant effect of the vitamin E present. Levels of malonaldehyde in some meats are listed in Table 1. Cooking generally increased the malonaldehyde content of most meat samples, especially chicken. Other differences in meat samples may have been due to variations in the handling time of the animals before slaughter and aging time. For example, beef had higher malonaldehyde levels than pork, a meat with more unsaturated fatty acids and thus greater susceptibility to autoxidation. The authors attributed this observation to the fact that beef was aged for up to several weeks while pork was probably handled faster during processing. Thus, beef was more prone to oxidation than pork. Pork, on the other hand, was probably handled faster in meat processing plants because of its increased susceptibility to lipid oxidation.

Siu and Draper (1978) also made a survey of malonaldehyde content in various meats obtained from selected Ontario supermarkets (Table 1). Overall, the malonaldehyde levels were lower than those reported by Shamberger et al. (1977). They found that processed and cured meats had low malonaldehyde levels. Among fresh meats, chicken and pork contained less malonaldehyde than beef and veal. Cooked chicken, pork and beef roasts had the highest values which was attributed to their longer cooking times. Malonaldehyde content in relation to cooking time was

Table 1. Malonaldehyde Content of Raw and Cooked Meat

Meat Sample	Malonaldehyde (μg/g) Shamberger et al.[1], Siu and Draper[2]	
Sirloin Steak		
Uncooked	13.7	1.69
Cooked	11.0	1.78
Round Steak		
Uncooked	7.2	2.39
Cooked	3.7	2.32
Roast, Sirloin tip		
Uncooked	9.4	1.00
Cooked	27.0	2.92
Veal Sirloin		
Uncooked	13.9	2.89
Cooked	1.3	3.21
Frankfurters		
Uncooked	0.5	0.44
Cooked	0.5	0.43
Pork Chops		
Uncooked	1.2	0.50
Cooked	0.4	1.59
Chicken		
Uncooked	7.7	0.61
Cooked	39.0	2.75

[1]Shamberger et al. (1977)
[2]Siu and Draper (1978)

investigated and it was found that cooked beef and pork showed the greatest increase in malonaldehyde levels between 90 and 150 min of cooking time. These authors also attributed differences in beef samples to handling, aging and other variations. The observation of Kuusi et al. (1975) that frozen fish contained more malonaldehyde than fresh fish was confirmed. This was attributed to the highly unsaturated character of the fatty acids in fish.

Malonaldehyde in Biological Systems

Lipid oxidation, as described in the previous section can also occur in biological systems, since both oxygen and unsaturated fatty acids are present. Prostaglandin endoperoxides, intermediates in prostaglandin biosynthesis, were shown to be produced by platelets to induce platelet aggregation (Hamburg et al. 1974). They were then found to metabolize rapidly to non-prostaglandin-like compounds, a major one being sodium malonaldehyde. Malonaldehyde was formed from these endoperoxides by the action of the enzyme, thromboxane synthetase (Diczfalusy et al., 1977; McMillan et al., 1978).

Fogelman et al. (1980) reported that malonaldehyde and blood platelets modified low density lipoproteins (LDL), producing cholesterol ester accumulation in human monocyte microphages. Since this accumulation was found within cells of the atherosclerotic reaction, these workers proposed that malonaldehyde released from blood platelets or produced by lipid peroxidation at the site of arterial injury may be a modifier of LDL in vivo.

Quantitation of Malonaldehyde In Biological Systems

Since lipid oxidation can cause detrimental effects in biological systems, much research has been devoted to the development of methods for quantifying the extent of lipid oxidattion in a given system. The many physical and chemical methods have recently been received by Gray (1978) and Logan and Davies (1980).

The thiobarbituric acid test

One of the most popular procedures to assess the extent of lipid oxidation in a biological system is the 2-thiobarbituric acid (TBA) method. In this procedure, the system or an extract of the system to be evaluated is mixed with acid (usually acetic or trichloroacetic acid combined with hydrochloric acid) and TBA reagent and heated in boiling water for a given time (Sinnhuber and Yu, 1977). The absorbance of the red complex that is produced is measured at 532 nm.

Kohn and Liversedge (1944) showed that TBA reacted with aerobically-incubated brain tissue samples to give an orange-red product with an absorption maximum at 532 nm. They suggested that the compound that reacted with TBA was a carbonyl compound since the reaction was blocked by semicarbazine and phenylhydrazine. Bernheim et al. (1948) found TBA reactive material in oxidized phospholipids and fatty acids systems and postulated that the compound contained three carbon atoms and one oxygen atom. Wilber et al. (1949) reported that autoxidized linolenate reacted with TBA to produce the characteristic colored compound. Patton and Kurtz (1951) observed that oxidized milk fat and malonaldehyde produced identical colored compounds having absorption maxima at 532 nm when heated with TBA and trichloroacetic acid for 10 minutes. Similar

spectra were obtained by these workers when compounds produced by the reaction of TBA and oxidized lipid containing unsaturated fatty acids were analyzed. Thus, malonaldehyde was implicated as the oxidation product that reacted with TBA. Sinnhuber et al. (1958) provided further evidence that malonaldehyde was indeed the TBA reactive compound by demonstrating that TBA, when mixed with either rancid salmon oil or 1,1,3,3-tetramethoxypropane (a malonaldehyde precursor), produced the same pigment. These authors also found that only two percent of the TBA reactive material could be extracted from the oxidized salmon oil. The rest of the material reacted with TBA only after acid had been added. The mechanism proposed by Pryor et al. (1976a) took this latter observation into account.

While the theories of Dahle et al. (1962) and Pryor et al. (1976a) explained malonaldehyde production from oxidized triene systems, other systems have shown positive TBA tests. These include highly oxidized oleate and linoleate (Biggs and Bryant, 1953), oxidized 2-alkenals and 2,4-decadienals (Patton and Kurtz, 1955; Patton et al., 1959; Tarladgis and Watts, 1960; Lillard and Day, 1964; Haase and Dunkley, 1969; Marcuse and Johansson, 1973), a 1-amino-3-iminopropene system (Buttkus and Bose, 1972), a mixture of sucrose and some woodsmoke compounds (Dugan, 1955), and a mixture of acetaldehyde and sucrose (Baumgartner et al., 1975). Lillard and Day (1964) suggested that 2-nonenal formed hydroperoxides, one of which on degradation yielded malonaldehyde. Formation of TBA-reactive substances has been shown to correlate with other lipid oxidation measurement methods including peroxide value (Dahle et al., 1962; Yu and Sinnhuber, 1967), oxygen uptake (Tarladgis and Watts,

1960), and diene conjugation (Corliss and Dugan, 1970).

The TBA test has been subject to some criticism (Yu and Sinnhuber, 1977; Gray, 1978). Yu and Sinnhuber (1964) pointed out the importance of using uncontaminated reagents for the test in order to prevent side reactions that would give misleading results. Certain compounds react with TBA to give colored complexes with absorption maxima between 450-460 nm (Yu and Sinnhuber, 1977; Gray, 1978). The color intensity can be such that it would interfere with absorption at 532 nm, causing a higher than normal value. Buttkus and Bose (1972) found that malonaldehyde-aromatic amine reaction products gave lower TBA values than expected because they were relatively stable to acid and heat, causing less hydrolysis to malonaldehyde. Dillard and Tappel (1971) reported that in peroxidizing microsomes the TBA value stabilized while oxygen absorption continued. Wills (1966) demonstrated that TBA values increased much more than oxygen uptake in the presence of ascorbate and postulated that partially oxidized fatty acids in a tissue homogenate could breakdown without further oxygen absorption to give additional TBA reacting products. Since more TBA reactive material is formed from fatty acids of greater unsaturation (Kenaston et al., 1955), TBA values can vary between different oils with the same peroxide number (Sidwell et al., 1954).

Other Methods of Quantitating Malonaldehyde in Biological Systems

Several other methods have been used to quantitate malonaldehyde, Kwon and Watts (1963) found that malonaldehyde in dilute aqueous solution had a different absorbance maximum at pH 3.0 (245 nm) than at pH 7.0 (267 nm). These workers proposed that the absorption differences

could possibly be utilized to measure malonaldehyde content in the presence of other compounds in this spectral region. They used this method to assay malonaldehyde in distillates from rancid foods and found it to be simpler but less sensitive than the TBA test.

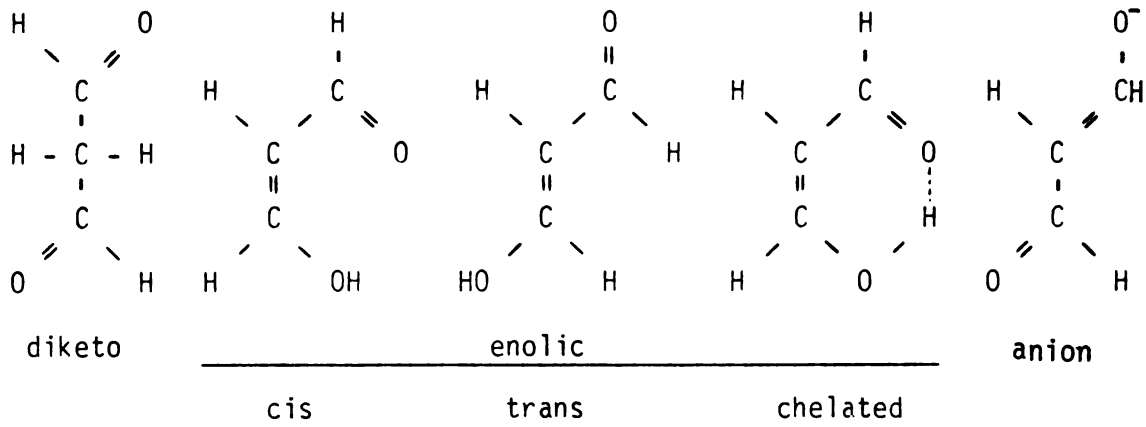
Gutteridge (1975), using gas liquid chromatography, found that malonaldehyde produced from its bis-bisulfite salt or from hydrolysis of tetraalkoxypropanes gave a characteristic peak. Thus, this method could be used to determine if malonaldehyde was present in a preparation.

Shamberger et al. (1977) silylated the TBA-malonaldehyde complex to make it soluble in chloroform and then used gas liquid chromatography for quantitation. The gas chromatogram showed one major peak and two minor peaks. They found that a TBA-beef extract complex gave the same chromatogram and that peak heights of this complex correlated with TBA test results.

Properties Of Malonaldehyde

Structure of Malonaldehyde

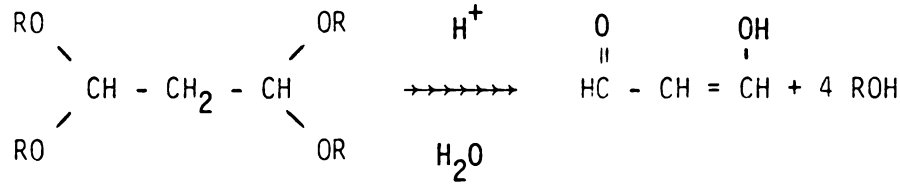
Malonaldehyde is a three carbon dialdehyde and was first reported to exist as a hydrolysis product of aqueous β-ethoxyacrolein diethylacetal (Clausen, 1903). It was first prepared in its crystalline form by Huttel (1941). It can exist in five forms, namely, the diketo form, three enolic forms (cis, trans, and chelated), and an enolate anion (Kwon and Watts, 1964). The five forms are shown on next page.



Malonaldehyde exists in its chelated enol form below pH 2.9 and in its enolate anion form above pH 6.5 (Kwon et al, 1965). Data are unclear for the pH values in between. In methylene chloride solutions, the diketo form prevails (Kwon and Van de Veen, 1968).

Preparation of Malonaldehyde

Being volatile and hygroscopic, malonaldehyde is not commercially available. However, it is readily produced by acid hydrolysis of its acetal derivative, tetraalkoxypropane (Huttel, 1941). Consequently, acid hydrolysis of tetramethoxypropane (TMP) has become the common method of obtaining the compound. The hydrolysis reaction is shown below:



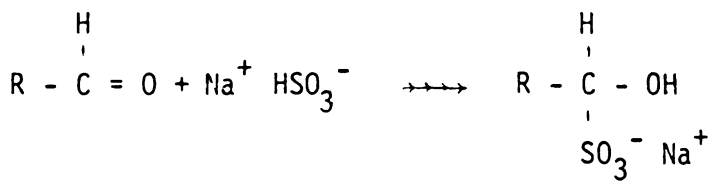
TMP or TEP Malonaldehyde

R = CH_3(TMP) or C_2H_5(TEP)

Though hydrolysis procedures vary, they all have the same basic steps. The TMP or TEP, either pure or as an aqueous solution, is mixed with hydrochloric acid and held for approximately one hour at room temperature or above.

Recently, TMP and TEP acid hydrolysis has been studied in more detail revealing hydrolytic intermediates (Marnett and Tuttle, 1980). Hydrolysis of TMP, for example, can lead to the formation of β-methoxyacrolein (BMA) and 3,3-dimethoxypropionaldehyde (DMP) as well as malonaldehyde (Figure 4).

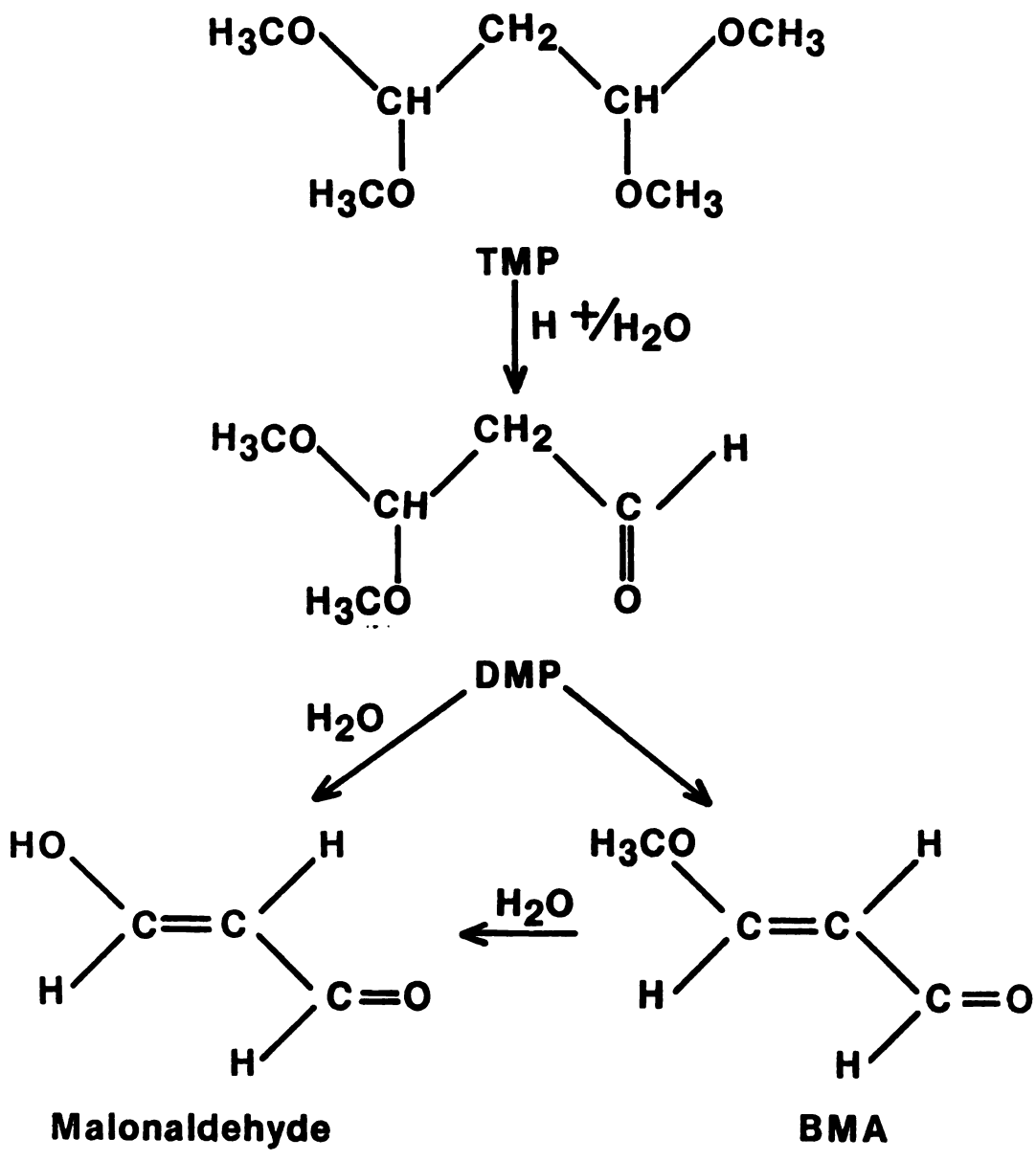
Other ways of synthesizing malonaldehyde have been described in the literature. Malonaldehyde bis-bisulfite salt can be formed by reacting the TEP acid hydrolyzate with sodium bisulfite (Saslaw and Waravdekar, 1957). This reaction is a nucleophilic addition in which the SO_3^{2-} anion adds to a carbonyl carbon as shown below:



Since malonaldehyde exists in its diketo form in this reaction (Kwon and Watts, 1964), both carbonyl groups react with bisulfite ion to produce the malonaldehyde bis-bisulfite salt.

Protopopovna and Skoldinov (1958) formed the sodium salt of malonaldehyde by hydrolyzing TEP and adding sodium hydroxide until the mixture was alkaline. The salt was precipitated out of solution by adding alcohol and acetone. Modifications of this procedure, in which

Figure 4. Probable pathway of hydrolysis of tetramethoxypropane (TMP) to malonaldehyde with the formation of reaction intermediates 3,3-dimethoxypropionaldehyde (DMP) and β-methoxyacrolein (BMA) (Marnett and Tuttle, 1980).


H3CO
CH2
OCH3
CH
CH
H3CO
OCH3
TMP
H +/H2O
H3CO
CH2
H
CH
C
H3CO
O
DMP
H2O
HO
H
C=C
H
C=O
H
H2O
H3CO
H
C=C
H
C=O
H
Malonaldehyde
BMA

unhydrolyzed tetraalkoxypropane was mixed with Dowex 50, have also been described (Brooks and Klamerth, 1968; Marnett et al., 1980). Evidently, the tetraalkoxypropane must at least partially hydrolyze and form a charged species whose cation can at least partially exchange with the exchange resin to form the sodium salt of malonaldehyde.

Summerfield and Tappel (1978) prepared malonaldehyde enzymatically from 1,3-propanediol using alcohol dehydrogenase. The substrate had a K_m value of 1.7 mM. and optimum synthesis occurred at an ionic strength between 0.02 and 0.1 and at pH 9. Borate, cyanide, azide and thiol solutions inhibited the reaction. The malonaldehyde produced was adjusted to pH 3.0, volatilized by an air stream, and condensed at -78^{o}C. Malonaldehyde was the only lipid oxidation product produced.

Polymerization of Malonaldehyde

Polymerization of malonaldehyde was first proposed to explain why the concentration of malonaldehyde in aqueous solutions decreased on standing at room temperature (Mashio and Kimura, 1960). Kwon and Watts (1964) observed that, over time, dilute aqueous solutions of malonaldehyde turned from colorless to yellow at room temperature. They theorized that dimerization of two enolic malonaldehyde tautomers held together by hydrogen bonding was responsible for the change in the absorption of light, thus causing color change.

Infrared spectra of two different concentrations of malonaldehyde in methylene chloride as solvent were compared and found to be completely different (Kwon and Van der Veen, 1968). The more dilute sample had the characteristic absorption band of carbonyl groups. The more concentrated sample showed a smaller absorption band due to carbonyl groups as

well as other bands which were attributed to a C=C-C=O structure. They proposed that this structure existed in the solvent because of the phenomenon of aggregation due to intermolecular hydrogen bonding. This solution was light brown as opposed to clear and contained a precipitate, suggesting polymerization of malonaldehyde. Interestingly, when the more concentrated sample was diluted, the characteristic carbonyl band increased while the other bands decreased. This suggested the prevalance of diketo malonaldehyde in more dilute methylene chloride solutions.

Interest in malonaldehyde polymerization prompted Buttkus (1975) to study what happened when the enolic salt of malonaldehyde was allowed to react with itself in a concentrated semisolid film formed by the evaporation of solvent. The substance produced had an infrared spectrum similar to the one of the concentrated sample of Kwon and Van der Veen (1968). Buttkus postulated that malonaldehyde was undergoing aldol condensation, resulting in the formation of polymeric substances, as shown below:

$$X - OCH = CHCHO$$

$$\downarrow 2H^+$$

$$HOCH = CHCH(OH)CH(CHO)CHO$$

$$\downarrow -H_2O$$

$$HOCH = CHCH = C(CHO)CHO$$

$$\downarrow -H_2O$$

$$HOCH = CHCH(C(CHO)CH)_{n-2} = C(CHO)CHO$$

Gutteridge (1975) investigated TMP and TEP acid hydrolysis using gas

liquid, thin layer, and gel filtration chromatography. He showed that complete hydrolysis of TEP and TMP, using a standard hydrolysis method, took 90 min and 4 hr respectively. Gas liquid chromatographic analysis of the hydrolysis products of both mixtures showed that compounds other than the starting material and malonaldehyde were present, the concentrations of some of these compounds increasing with increasing hydrolysis time. Thin layer chromatography of a TEP hydrolysis product indicated nine distinct bands. Gel filtration separation of TMP hydrolysis products showed five distinct zones. Gutteridge explained these results by hypothesizing that polymerization of malonaldehyde took place.

Malonaldehyde: Toxicity, Mutagenicity, and Carcinogenicity Studies

The presence of malonaldehyde in food and other biological systems has prompted investigation into its possible harmful effects in animals. Crawford et al. (1965) showed that malonaldehyde, as its sodium salt and as TEP, was toxic in rats with LD_{50} levels of 632 mg/kg and 527 mg/kg, respectively. This indicated that malonaldehyde was more toxic than formaldehyde or glyoxal. They found that the more concentrated the dose of TEP, the lower the toxicity. They attributed this unusual relationship to the fact that the predominant toxic effect was due to something other than malonaldehyde, possibly formed during TEP hydrolysis.

Deoxyribonucleic acid (DNA) has been shown to react with malonaldehyde (Brooks and Klamerth, 1968). The preferential reaction sites on the DNA molecule were the guanine and cytidine bases and malonaldehyde was postulated to crosslink with the amino groups of these bases.

Evidence for structural changes in DNA consisting of loss of DNA template activity correlating with an increase in malonaldehyde reaction products was presented (Reiss et al., 1972). This same evidence plus a decreased melting point, decreased hyperchromicity, and partial resistance to deoxyribonuclease hydrolysis was shown in DNA when it was reacted with oxidized arachidonic acid (Reiss and Tappel 1973).

Shamberger et al. (1974) applied 12 mg of malonaldehyde to the shaved backs of mice and after daily treatment with 0.1% croton oil, 52% of the mice had tumors at thirty weeks. Malonaldehyde caused mutagenesis in certain strains of Salmonella typhimurium bacteria (Mukai and Goldstein, 1975; Shamberger et al., 1979). The latter investigators used levels as low as 13.85 μmoles and found that antioxidants reduced mutagenesis in many of the strains tested. Yau (1979) studied possible mutagenicity and cytotoxicity of malonaldehyde in mammalian lymphoma cells and found that 24 hr exposure to as little as 20 μmoles of malonaldehyde caused mutagenicity and cytotoxicity. The induced mutation frequency was dose-dependent within the range of 10-1000 μmoles of malonaldehyde. The latter three investigations were based on the belief that malonaldehyde crosslinked with DNA, in these cases leading to frameshift mutation of the error-prone repair mechanism.

Marnett and Tuttle (1980), as previously discussed, characterized two intermediates in the hydrolysis of TMP and TEP. They showed that β-alkoxyacrolein and 3,3-dialkoxypropionaldehyde, were much more mutagenic than the malonaldehyde monomer itself. These compounds were found in significant amounts in the hydrolysis product. Hydrolysis of TEP under standard conditions yielded 25% malonaldehyde and 13%

β-ethoxyacrolein. These results indicated that these hydrolytic intermediates were probably responsible for significant overestimation of the actual mutagenicity and carcinogenicity of malonaldehyde reported in previous studies.

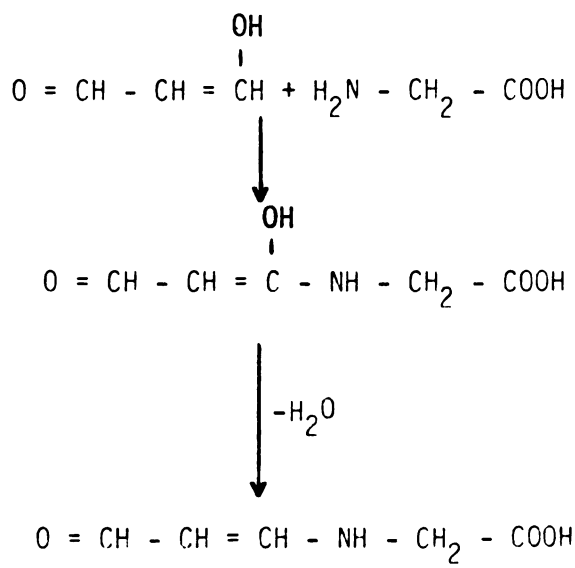
Reaction of Malonaldehyde with Amines

Lipid oxidation products can react with proteins and amino acids to cause severe damage in food and other biological systems (Gardner, 1979). Andrews et al. (1965) found that the reaction between autoxidized linoleate and two different proteins, insulin and gelatin, in model systems resulted in modifications of the proteins. When these proteins were subjected to trypsin hydrolysis, the gelatin system underwent less hydrolysis than the insulin system. These workers inferred that the reacted gelatin was involved in a crosslinking reaction. The amino groups that reacted with the oxidation products were the ε-amino group of lysine and N-terminal amino groups.

Since malonaldehyde was found to be a product of lipid oxidation (Sinnhuber and Yu, 1958, Kwon and Olcott, 1966a,b), it was logical to investigate how it affected proteins and amino acids when allowed to react with them. Kwon et al. (1965) postulated that near the neutral pH of most food systems, malonaldehyde, being in its anion form would not volatilize because of intermolecular hydrogen bonding and hydration of the center carbon atom through electrostatic forces. It could thus react with other constituents of a food system. These workers found that malonaldehyde formed a bond with tuna muscle that could only be broken by using acid and heat. This complex gave a positive TBA test.

Malonaldehyde has been shown to react with bovine serum albumin (Kwon and Brown, 1965) and cytochrome C (Kwon and Olcott, 1966c). The latter authors suggested that the reactive sites of the cytochrome molecule were the free amino groups. Crawford et al. (1967) reported that malonaldehyde reacted with bovine plasma albumin at ε-amino groups of lysine and the N-terminal aspartic acid group, while Buttkus (1967) demonstrated that malonaldehyde reacted with myosin at the ε-amino groups of lysine and other free amino groups. Kuusi et al. (1975) showed that malonaldehyde formed during cold storage of herring reacted with protein such that an increase in TBA value correlated with a decrease in free amino groups.

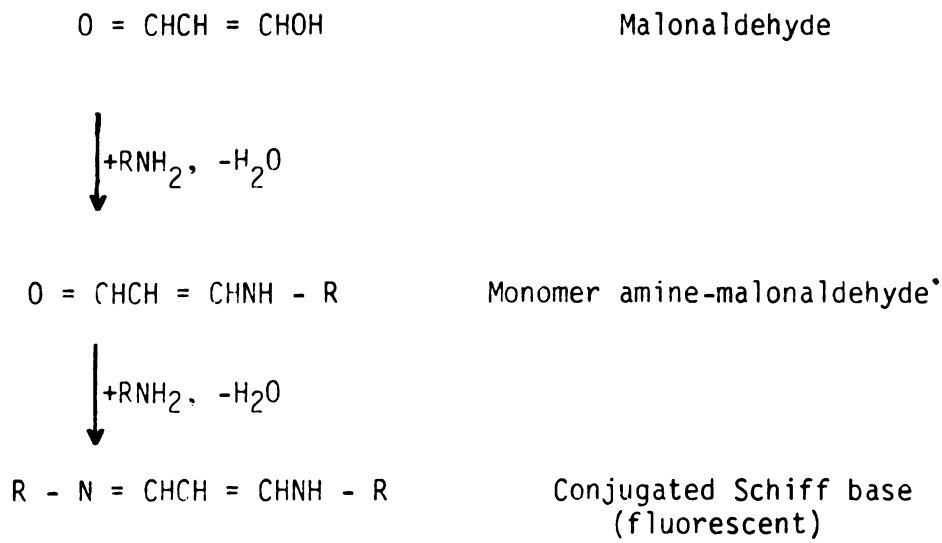
Crawford et al. (1966) reacted malonaldehyde with glycine to form an enamine, N-prop-2-enal aminoacetic acid. This occurred by nucleophilic addition of the amine nitrogen of glycine to the enol carbon of malonaldehyde, as shown below:



Menzel (1967) found that when oxidized linolenic acid or malonaldehyde were allowed to react with ribonuclease, polymerization of the enzyme molecule occurred. The author attributed the polymerizing action to malonaldehyde and other unspecified lipid oxidation products. Shin et al. (1972) observed that malonaldehyde reacted with ribonuclease and bovine serum albumin to give crosslinked polymeric forms of both proteins, while Davidkova et al. (1975) showed that malonaldehyde caused the crosslinking of collagen by reacting with ε-amino groups of lysine.

Malonaldehyde-Amine Fluorescent Reaction Products

Fluorescence has long been known to occur in non-enzymatic browning systems in which sugars and amines react to form Maillard browning products (Friedman and Kline 1950; Pearce, 1950; Burton et al.. 1962). This reaction between carbonyl and amino compounds to form fluorescent products prompted investigators to determine whether fluorescent products would occur when malonaldehyde reacted with amines. Sawicki et al. (1963) found that a fluorescent product was formed when aromatic primary amines reacted with malonaldehyde. These workers postulated that the electrons of the conjugated Schiff base were delocalized with aromatic rings. Chio and Tappel (1969a) characterized compounds formed by the reaction of leucine, valine, or glycine with malonaldehyde. They found that two amino acid molecules reacted with one molecule of malonaldehyde to form a conjugated Schiff base by the mechanism shown on next page.



This compound had fluorescent excitation and emission maxima at 370 nm and 450 nm, respectively. Trombly and Tappel (1975) repeated this study and reported fluorescent excitation and emission maxima of 375 nm and 450 nm, 375 nm and 445 nm, 390 nm and 470 nm for N,N-dileucinyl-1-amino-3-iminopropene, N,N-divalinyl-1-amino-3-iminopropene, and N,N-diglycinyl-1-amino-3-iminopropene, respectively. These workers also observed an excitation maximum of 265-275 nm. Buttkus and Bose (1972) reported that glycine, methionine, and ammonia reacted with malonaldehyde to give products with fluorescent excitation and emission maxima of 395 nm and 470 nm, respectively. These workers also showed that aniline did not produce a fluorescent substance when it reacted with malonaldehyde. Kaya (1977) observed fluorescence with excitation and emission maxima of 390 nm and 460 nm respectively for a malonaldehyde-valine mixture. Kaya also reacted acrolein and crotonaldehyde with valine and produced no compounds that fluoresced significantly when compared with the malonaldehyde-valine product. Formaldehyde and acetaldehyde did not produce fluorescent compounds when reacted with valine.

The observations of Chio and Tappel (1969a) led to other investigations regarding fluorescence of carbonyl-amine reaction products. Chio and Tappel (1969b) reacted malonaldehyde and ribonuclease and produced inactivated fluorescent enzyme dimers and trimers, which they said were caused by malonaldehyde forming a crosslink with two ε-amino groups of the enzyme. The reaction product had fluorescence characteristics identical to the reaction products of malonaldehyde mixed with α-N-acetyl-l-lysine and of autoxidized arachidonate mixed with ribonuclease A. All three reaction products had excitation and emission maxima of 395 nm and 460 nm, respectively. The authors proposed a N,N^1-disubstituted 1-amino-3-iminopropene structure as being responsible for the fluorescence. Reiss et al. (1972) found that the amino groups of the bases of DNA reacted with malonaldehyde to form a product that fluoresced with excitation and emission maxima at 390 nm and 460 nm, respectively. It was also observed that increase in fluorescence correlated linearly with a decrease in DNA template activity. Adhikari and Tappel (1973) showed that glucose reacted with glycine at $100^{o}C$ to give fluorescent excitation and emission maxima of 350 nm and 430 nm, respectively, and attributed this fluorescence to a 1-amino-3-iminopropene system formed from glycine and glucose degradation products.

Since malonaldehyde was known, as previously discussed, to be a product of lipid autoxidation, investigators sought to find out if fluorescent compounds similar to those discussed above were produced in biological systems in which autoxidized lipids and amine compounds were both present. Investigators were also interested in whether

fluorescence due to these compounds could be used to monitor the degree to which lipid autoxidation had taken place. When rat liver mitochondria, lysosomes, and microsomes were allowed to undergo lipid peroxidation, fluorescence occurred (Chio et al. 1969). All reaction products had emission maxima at 460 nm. In the mitochondrial and microsomal systems the increase in fluorescent intensity correlated with an increase in TBA values. Malonaldehyde added to mitochondrial systems reacted with mitochondrial membranes and increased oxygen uptake, and TBA reactants of peroxidizing rat mitochondria and microsomes were affected by the amount of lipid oxidation products that were formed. They found that fluorescent compounds produced by these systems had excitation and emission maxima at 360 and 430 nm, respectively. Fluorescence, oxygen uptake, and TBA reactants produced during the initial phase of oxidation were shown to increase concurrently. These three parameters had an inverse relationship with diet α-tocopherol levels. Since α-tocopherol is an antioxidant, the three parameters paralleled the occurrence of lipid oxidation. As expected, fluorescence and oxygen uptake increased with greater diet polyunsaturated fatty acid (PUFA) levels since PUFAs undergo peroxidation to a greater degree than do other fatty acids (Labuza, 1971).

Fletcher et al. (1973) developed a reproducible assay for measuring the extent of lipid peroxidation that had taken place in a biological system by measuring the fluorescence of the system. In this method, tissue which had been allowed to undergo lipid peroxidation damage was extracted with a chloroform-methanol solvent system and the fluorescent characteristics of the extracts measured.

Phosphatidyl ethanolamine (PE), which contains polyunsaturated fatty acids and a free amino group, is a natural component of biological membranes. Corliss and Dugan (1970) observed a decrease in the amino content of PE during lipid peroxidation, indicating reaction of TBA reactive substances with the amino group. Fletcher et al. (1973) mixed varying amounts of oxidized PE with biological tissue and found that fluorescence intensity increased with increased PE content. Rao and Dugan (1972) reacted nonanal, oleyl aldehyde, 10-undecene-1-al, 2-hexene-1-al, 2,4-hexadienal, glucose, and malonaldehyde with PE in model systems and reported fluorescence in all systems. The malonaldehyde system had excitation maxima at 340 and 410 nm and an emission maximum at 455 nm. The other systems had excitation maxima between 350-400 nm and emission maxima between 425-475 nm.

When PE and phosphatidyl serine were allowed to peroxidize, fluorescent chromophores were obtained with excitation maxima between 365-370 nm and emission maxima between 435-440 nm (Bidlack and Tappel, 1973). These workers also reacted PE with oxidizing methyl arachidonate and malonaldehyde and reported fluorescent excitation and emission maxima for the two systems at 370 nm, 445 nm and 400 nm, 475 nm, respectively. The difference in fluorescence characteristics was postulated as being due to fewer fluorescent chromophores forming in the latter system because fewer peroxidation products were present. Bidlack and Tappel (1973) showed that fluorescence formation correlated with the TBA test in the reactions discussed above, at least during the early stages of peroxidation. The malonaldehyde-PE reaction produced the greatest amount of fluorescent product. When PE was mixed with

peroxidizing methyl linoleate, there was little fluorescence or TBA reactive substances produced. Fluorescence, TBA values, and oxygen uptake were shown to correlate in PUFA-PE and phenylalanine-PE reaction systems (Dillard and Tappel, 1973). Fluorescent characteristics were the same as in the Bidlack and Tappel study.

Shimasaki et al. (1977) showed that the formation of fluorescent substances in an aqueous emulsion containing autoxidizing methyl linoleate and glycine correlated linearly with a decrease in diene conjugation and an increase in TBA values. Kaya (1977) reported that malonaldehyde extracted from autoxidized rape seed oil and reacted with valine produced a compound that fluoresced with excitation and emission maxima of 390 nm and 460 nm, respectively.

The correlations between fluorescence, the TBA test, and oxygen absorption discussed above show fluorescence to be a valid lipid oxidation measurement technique. The fluorescence method compares favorably to the TBA test because detection by fluorescence is ten to one hundred times more sensitive than detection using a colorimetric method such as the TBA test. Also, while the TBA test does not parallel oxygen absorption values after the early stages of oxidation the fluorescence method does. Finally, in vivo systems can be measured using the fluorescence method (Dillard and Tappel, 1971).

Malshet and Tappel (1973) defined the structural requirement for a fluorescent conjugated Schiff base, R-N=C-C=C-XR, by specifying that the Schiff base must be conjugated with an olefin attached to a fairly strong electron donating group, denoted here as X. In the reaction of malonaldehyde with an amine compound, X would be -NH-. If X were -OH,

with no R' group, the compound would also fluoresce, but since the hydroxyl group is less electron donating, the compound would fluoresce less than if an amine group were in its place. Unsaturated aldehydes, such as 2,4-hexadienal, when reacted with an amine compound, would not fluoresce since X would be $-CH_2-$ group, which is not electron donating. Buttkus and Bose (1972) found that the nature of the R groups attached to the nitrogens of the 1-amino-3-iminopropene was also a factor in determining whether a compound fluoresced. If both R groups were phenyl groups, fluorescence did not occur.

Malonaldehyde-amine fluorescent reaction products have been shown to occur in certain biological processes. The fluorescence characteristics of the 1-amino-3-iminopropene compounds were similar to those of lipofuscin age pigments (Hendley et al., 1963). Lipofuscin pigments are formed from lipid peroxidation of polyunsaturated lipids of subcellular membranes (Tappel, 1973). These lipid-protein complexes accumulate with age in animal tissues such as the brain, heart, and testes. Tappel (1973) believed that the fluorescence of lipofuscin pigments was due to the conjugated Schiff bases previously discussed. Trombly et al. (1975) reported that polyenoic acids made up about 40% of the total lipid of human testes. These acids are more susceptible to lipid peroxidation and thus more likely to form aldehyde-amine reaction compounds. These workers separated testes fluorescent pigments using column and thin layer chromatography and several of these compounds exhibited fluorescence characteristics similar to the conjugated Schiff base fluorophores. When components of human brain lipofuscin pigment were purified and characterized, the amount of conjugation was found not to

be great, but the fluorescence spectrum indicated that the fluorochrome possessed some type of Schiff base structure (Taubold et al., 1975). A small amount of material may have been responsible for the very intense fluorescence. These workers found that the lipofuscin pigment consisted of polymeric lipids, phospholipids, and amino acids either bound to lipids or attached to protein molecules.

Fluorescence typical of a malonaldehyde-amine system was used to detect spinal cord damage in cats (Seligman et al., 1977). Spinal cord injury was produced in cats given ethanol, a substance that enhances lipid free radical reactions. Fluorescent extracts of injured spinal cord gave excitation and emission maxima of 360nm and 440nm, respectively. TBA reactive compounds increased up to 24 hr after the injury was induced and fluorescence occurred between one and three days after injury. The authors proposed that the fluorescence was due to the malonaldehyde produced from the peroxidizing fatty acids of membrane phospholipids forming crosslinks with amine-containing compounds present in the system. The fluorescence would thus have resulted from the formation of conjugated Schiff bases.

Other Fluorescent Compounds Derived From Malonaldehyde

There is evidence to support the formation of fluorescent compounds in malonaldehyde systems other than those derived from reaction with amino compounds. Fluorescent products have been shown to form from malonaldehyde. Shin et al. (1972) reported that malonaldehyde solutions stored at 4^{o}C and pH 5.0 developed fluorescence with excitation and emission maxima at 400 and 550nm, respectively. Potassium β-oxyacrolein undergoes self-condensation to form a polymeric product with excitation

maxima at 390 and 468 nm, and an emission maximum at 550 nm (Buttkus, 1975). When leucine ethyl ester was added to the salt solution, fluorescence intensity increased 25-fold and maxima shifted to those characteristic of conjugated Schiff base systems. Malonaldehyde polymers were separated by gel filtration and fractions had excitation maxima between 365-395 nm and emission maxima between 460-490 nm and 550-555 nm (Gutteridge et al. 1977). The malonaldehyde monomer produced from its bis-bisulfite salt exhibited no fluorescence. These workers suggested that malonaldehyde polymers could be fluorescent artifacts in measuring Schiff bases produced by malonaldehyde-amine reactions. When methyl arachidonate and methyl linoleate were allowed to autoxidize on a silica gel TLC plate, fluorescence occurred with excitation and emission maxima similar to those of conjugated Schiff base compounds (Shimasaki et al., 1977). The linoleate sample and a sample of autoxidized linoleate-glycine reaction product showed identical fluorescence maxima, but the intensities could not be compared. The arachidonate sample had a greater fluorescence intensity than the linoleate sample. Fluorescence was shown to increase with the amount of oxidation that took place in a system. These results and those of Gutteridge et al. (1977) indicated that lipid peroxidation products could cause fluorescence similar to malonaldehyde-amine reaction products.

Yagi (1976) reported that the malonaldehyde-TBA complex exhibited a characteristic fluorescence with excitation and emission maxima of 510 and 553 nm, respectively. This technique was used to measure TBA reactive substances in blood plasma (Yagi, 1976; McMillan et al., 1977) and was shown to be more sensitive than the colorimetric measurement

technique (McMillan et al., 1977).

MATERIALS AND METHODS

Materials

Solvents--Distilled-in-glass HPLC grade methanol, ethyl acetate, and chloroform were purchased from Burdick and Jackson Laboratories Incorporated, Muskegon, Michigan. Deuterated chloroform (minimum isotopic purity 99.8 atom % d) was purchased fom MSD Isotopes, Merck, Sharp and Dohme of Canada Limited, Montreal, Canada. The other solvents used in the study were reagent grade.

Chemicals--Glycine hydrochloride was purchased from Sigma Chemical Company, St. Louis, Missouri. Glycine ethyl ester hydrochloride and malonaldehyde bis-(dimethylacetal) were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Linolenic acid (technical grade) and sodium dodecyl sulfate were purchased from Eastman Kodak Company, Rochester, New York and Pierce Chemical Company, Rockford, Illinois, respectively. Carboxymethylcellulose, type 7HF, was obtained from Hercules, Incorporated, Wilmington, Deleware. All other chemicals used in the study were reagent grade.

Methods

Gel Filtration Chromatography of Malonaldehyde-Glycine Reaction Products

Malonaldehyde-glycine reaction product(s) was/were prepared and

analyzed using gel filtration chromatography as described by Chio and Tappel (1969a). Malonaldehyde, formed by heating 24.6g of tetramethoxypropane (TMP) and 13.5 ml of 1N hydrochloric acid (HCl) at 40°C until they were miscible, was added to 7.5 g of glycine in 20 ml of water and stirred for 1 hr at room temperature. The yellow solution was stored at 4°C for an additional hour, after which the precipitate was removed by filtration. The yellow filtrate was extracted four times with diethyl ether to remove excess malonaldehyde and lyophilized in a Virtis Super freeze dryer with a 10-117A Macro Trap and 10-136 Super stainless steel trap. Two hundred mg of the yellow-brown product were dissolved in 3 ml of water, filtered through a Gelman Metricel DM-450 0.2 µm membrane filter and applied to a Bio-Gel P-2 glass column (90 x 2.5cm). The malonaldehyde-glycine reaction product was eluted from the column with distilled water at a flow rate of 1 ml per min using a Technicon proportionating pump. Pump elution was used to insure a stabilized flow rate of a large eluant volume (Bio-Rad Laboratories, 1975). Six ml fractions were collected and scanned for fluorescence at 450 nm when excited at 370 nm using a Varian SP 330 spectrofluorimeter. The major fluorescent fractions were pooled and lyophilized, and the process of chromatography and lyophilization was repeated. The yellow fluorescent fractions were stored at -18°C in a desiccator.

Preparation of Malonaldehyde

1. Hydrolysis of the malonaldehyde bis-bisulfite salt

Malonaldehyde bis-bisulfite salt was prepared according to the method of Saslaw and Waravdekar (1957). A fifty ml aliquot of TMP was mixed with 27.5 ml of 1N HCl and shaken for one min in a 60°C water

bath to effect miscibility. The resulting faint yellow solution was stored overnight at room temperature and then cooled at 4°C. Two hundred ml of a freshly prepared saturated sodium bisulfite solution chilled in an ice bath were added to the cold TMP acid hydrolyzate and the mixture stored at 4°C for 5 hours. The orange-yellow precipitate formed was collected by filtration and washed successively with 75 ml of a cold saturated sodium bisulfite solution and 75 ml of a cold 50% ethanol solution. The precipitate was dissolved in water to give a saturated solution and cooled at 4°C. This solution was kept cool in an ice bath as cold absolute ethanol was gradually added until it made up a quarter of the solution volume. The resulting precipitate was obtained by filtering through Whatman No. 1 paper. A second precipitate was obtained by bringing the alcohol concentration of the filtrate to approximately 50%. The combined precipitates were washed successively with 75 ml aliquots of cold 70% ethanol and diethyl ether. The resulting crystalline white precipitate contained the sodium salt of malonaldehyde bis-bisulfite.

The malonaldehyde monomer was obtained from the bis-bisulfite salt by acid hydrolysis and steam distillation. A 5 g aliquot of the salt was solubilized in 30 ml of water and added to a steam distillation apparatus, in which the water had been allowed to boil. Seven ml of concentrated HCl were added to the salt solution and the distillate collected for 15 min in a receiving flask immersed in an ice bath. On addition of the acid, the salt solution turned from colorless to yellow to orange to brown.

2. Preparation of malonaldehyde by acid hydrolysis of TMP

Malonaldehyde was also prepared by different TMP acid hydrolysis procedures. Studies were carried out using a modification of the procedure of Chio and Tappel (1969a) in which 24.6 ml of TMP were mixed with 6 ml of 1N HCl and sonicated at room temperature with occasional stirring until there was miscibility.

The procedure of Buttkus (1969) was also used in which 5.9 ml of TMP were hydrolyzed with 3 ml of 1N HCl at 50°C for 20 minutes. A 2 ml aliquot of water was then added and the hydrolysis mixture stored at room temperature for an additional 20 minutes.

Analysis of Malonaldehyde

1 Gas liquid chromatography (GLC) analysis

Analysis of the malonaldehyde preparations by GLC was carried out using a Hewlett Packard HP 5830A gas chromatograph equipped with a flame ionization detector. The column and operating conditions used were similar to those described by Gutteridge (1977). The column was a 1.83 m x 4 mm i.d. glass column packed with a stationary phase of 6% Carbowax 20M on Chromosorb W (80-100 mesh) (Chromatographic Specialities, Brockville, Ont.). The operating conditions for all analyses were: column temperature, 165°C; injector temperature; 175°C; FID temperature, 215°C; and nitrogen flow rate, 45 ml per minute.

The malonaldehyde samples were prepared differently for GLC analysis. Various volumes (0-10 µl) of malonaldehyde bis-bisulfite acid hydrolysis steam distillate were injected directly into the gas chromatograph immediately after the collection of the distillate. A 2

ml aliquot of malonaldehyde prepared according to Buttkus (1969) was extracted with 8 ml of chloroform and 1 µl of the chloroform extract was injected into the gas chromatograph. Malonaldehyde, prepared according to Chio and Tappel (1969a), was subjected to two studies in order to determine the stability of malonaldehyde in solution with time. In one study, 2 ml of a TMP solution that was hydrolyzed for 18 hr were extracted with 8 ml of chloroform, and 1 µl of the chloroform extract was analyzed by GLC. In the second study, 1 ml aliquots of the hydrolysis mixture were taken after 5,15,25,35,45,55,65,75,90,120 min of hydrolysis, extracted with 9 ml of chloroform, and analyzed by GLC.

2. Thin layer chromatography (TLC) of the steam distillate of the malonaldehyde bis-bisulfite acid hydrolyzate

The steam distillate of the acid-hydrolyzed malonaldehyde bis-bisulfite salt was analyzed by TLC using a modification of the method of Gutteridge (1975). A 1 ml aliquot of the steam distillate was extracted with an equal volume of chloroform and 10 µl of the chloroform phase were applied to TLC plate.

TLC analysis was carried out using 20 x 20 cm Silica Gel G plates (0.6mm) prepared with a Desaga variable thickness applicator (Brinkman Industries, Westbury, N.Y.). Plates were stored in a desiccator and activated at 110°C for 1 hr before use. The plates were developed for approximately 15 cm using a hexane: diethyl ether: butanol: ethanol (60:40:1:1) solvent system. The detection reagent used was a TBA solution consisting of 4 volumes of a 1% aqueous TBA solution and 1 volume of 28% trichloroacetic acid. Color development was allowed to take place at room temperature.

3. GLC-mass spectrometry of TMP acid hydrolyzate

The TMP acid hydrolyzate prepared for GC-mass spectrometry analysis was extracted three times with equal volumes of diethyl ether. A 1 μl aliquot of the ether extract was injected into a gas chromatograph containing the same column as described previously. The gas chromatograph was operated with a temperature program of 155°C for 2 min, followed by an increase of 2°C per min until the temperature of 165°C was attained. Helium was the carrier gas. The GLC mass spectrometer system used was a Hewlett Packard 5985A GC-MS unit with the following parameters: Repeller (V)=7.35; Drawout (V)=10.5 Ion focus (V)=28: ENT lens (MV/AMV)=91; X-Ray (V)=112; Emission (VA)=300; Electron energy (EV)=70; Lag amp offset=102; Mass axis gain=1.00536; Mass axis offset=2.722E-02; Ions: Positive; Actual source temperature, 200°C.

Reaction of Malonaldehyde with Glycine Ethyl Ester

The steam distillate of the malonaldehyde bis-bisulfite acid hydrolyzate was mixed with 5 g of glycine ethyl ester in a stoppered flask using a vortex mixer until the glycine ethyl ester was solubilized. The solution was held at room temperature for 5 hr and gradually turned from clear to yellow.

The malonaldehyde-glycine ethyl ester was also prepared by the Chio and Tappel (1969a) method previously discussed except that an equivalent molar amount of glycine ethyl ester was used instead of glycine.

A similar experiment was also carried out to study the reactivity of the malonaldehyde preparation under the same experimental conditions as above.

Analysis of the Malonaldehyde-Glycine Ethyl Ester Reaction Products

1. TLC analysis

TLC plates were prepared and developed as previously described. The solvent used for the analysis of the reaction product was a butanol: acetic acid:water (80:20:20) mixture. After development, the TLC plates were examined under UV light (366 nm).

Both malonaldehyde-glycine ethyl ester mixtures previously discussed were prepared for TLC analysis in the same way. The reaction mixture was extracted three times with equal volumes of ethyl acetate. The aqueous fraction was lyophilized as previously described, solubilized in 5 ml of methanol, filtered through a 0.2 µm membrane, and an aliquot of the filtrate was applied to the TLC plate. The ethyl acetate fraction was evaporated to dryness at 25°C using a rotary evaporator, resolubilized in 1 ml of ethyl acetate, filtered through an 0.45 µm membrane, and an aliquot of the filtrate was applied to a TLC plate. Filtration was used to obtain 100% solution of the resolubilized product. Two types of analyses were run: analytical plates, in which one µl of sample was spotted on a plate, and preparative plates, in which 25 to 500 µl of sample were streaked across the plate. TLC analyses of the unreacted steam distillate control were also conducted above, except that only the ethyl acetate fraction was utilized.

2. Fluorometric Analysis

TLC spots and bands corresponding to the products of the two malonaldehyde-glycine ethyl ester reactions were either scraped off the TLC plates or removed using a Pasteur pipet packed with glass wool that was attached to a vacuum pump. The spots and bands were extracted with

3 ml of water or methanol and mixed using a vortex mixer. The precipitate of the mixture was allowed to settle and the solvent decanted and filtered through a 0.2 µm membrane. The spots removed using a Pasteur pipet were extracted by passing solvent through the pipet. A 3 ml aliquot of the sample solution was fluormetrically scanned over relevant wavelength light ranges in a one cm^2 Varian quartz cuvette using the fluorimeter previously described.

3. GLC analysis

The ethyl acetate fraction of the malonaldehyde-glycine ethyl ester reaction product separated by TLC was analyzed by GLC using the same column and conditions as the malonaldehyde preparation studies. TLC bands were scraped off the plates and each one was extracted with 1 ml of ethyl acetate. The extracts were filtered through an 0.45 µm membrane and 1 to 7 µl aliquots of the filtrate were analyzed by GLC.

4. Infrared spectroscopy analysis

The fluorescent bands of the TLC streak plates were collected, stored at -18°C and prepared for infrared spectroscopic analysis. The fluorescent bands were extracted three times with 10 ml of ethyl acetate. The combined extracts were filtered through an 0.45 µm membrane, evaporated to dryness using a rotary evaporator and resolubilized in deuterated chloroform. A portion of the chloroform solution was added to an NaCl cell and infrared spectroscopic analysis was carried out with a Perkin Elmer-167 grating infrared spectrophotometer. The extract corrresponding to the major fluorescent band on the TLC plate was reduced by sodium borohydride (Dugan and Rao, 1972) and again analyzed by infrared spectroscopy. The deuterated chloroform

sample of this band was evaporated to dryness at 25°C using a rotary evaporator and resolubilized in 10 ml of methanol. One hundred mg of sodium borohydride were added slowly to the alcoholic solution along with glass beads and the resulting mixture refluxed for 3 hours. The mixture was cooled at -18°C, filtered through a 0.2 µm membrane, evaporated to dryness at 25°C using a rotary evaporator, and analyzed as a deuterated chloroform solution by infrared spectroscopy as described above.

5. GLC-mass spectrometry analysis

Two fluorescent components derived from the steam distillate-glycine ethyl ester reaction were analyzed by mass spectrometry. These components were isolated by TLC, scraped from the plate, and extracted three times with 10 ml of ethyl acetate each. The ethyl acetate extracts were combined and filtered through 0.45 µm membrane, evaporated to dryness at 25°C using a rotary evaporator, and resolubilized in 1 ml of ethyl acetate. This mixture was filtered as above and one µl of the filtrate was used for GLC-mass spectrometry studies as previously described.

Model System Studies

1. Preparation of model systems

Autoxidized linolenic acid-glycine ethyl ester model systems were prepared to ascertain if fluorescent compounds similar to those produced in the malonaldehyde-glycine ethyl ester reaction were formed. An inert matrix was prepared according to the method of Bishov et al (1960). Two g of carboxymethylcellulose and 200 ml of borate buffer (pH 7.0) were

blended at high speed for 1 min in a Waring blender. Linolenic acid (0.1g), glycine ethyl ester (2 g), and sodium dodecyl sulfate (1g) were added and the mixture blended at high speed for 1 minute. The model system was frozen overnight at -18°C and freeze dried for 24 hr in a Virtis RePP, Model 42 freeze dryer at a pressure of 5μ , a platen temperature of 40°C, and a condenser temperature of -60°C. The freeze dried sample was stored at 40°C in a desiccator.

In a similar study, 5 g of linolenic acid, 2 g of glycine ethyl ester, and 5 g of sodium dodecyl sulfate were used in place of the quantities listed above.

2. Analysis of model systems

The model systems were analyzed by fluorescence and subjected to TBA tests. The low linolenic acid systems were analyzed after 0,3,6,9,12,15 days of storage at 40°C, and the high linolenic acid systems were analyzed after 0,7,14 days of storage at 40°C.

The freeze-dried model systems were prepared for fluorescence analysis by grinding them up into a fine powder, transfering them to a 500 ml Erlenmeyer Flask, and extracting them with 100 ml of ethyl acetate with vigorous shaking for 15 minutes. The mixture was filtered through Whatman No. 1 filter paper and the filter paper and its contents were washed three times with 25 ml of ethyl acetate added slowly with stirring. A 50 ml aliquot of the filtrate was evaporated to dryness at 25°C using a rotary evaporator and resolubilized in 3 ml of water. This aqueous solution was filtered through an 0.2 μm membrane, diluted 1:1000 with water, and its fluorescence measured as previously described. The excitation and emission maxima used were 382 nm and 468

nm, respectively.

The low level linolenic acid model systems were analyzed by TLC using the procedure as described for the analysis of the malonaldehyde-glycine reaction products. A 3 µl aliquot of each ethyl acetate extract and one 1 of the ethyl acetate fraction of the malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction mixture were applied to the TLC plate.

Both types of model systems were subjected to the TBA test according to Tarladgis et al (1960). A model system was transferred to a 500 ml flat bottom flask, to which 4 glass beads and 2.5 ml of 4N HCl had been added. A 122.5 ml aliquot of water was then added to the flask and a distillation was carried out until 50 ml of distillate had been collected. Two 5 ml aliquots of distillate were transferred to stoppered test tubes, to which were added 5 ml of TBA reagent consisting of 0.02 M 2-thiobarbituric acid in 90% distilled glacial acetic acid. The tubes were immersed in a boiling water bath for 35 min, cooled immediately in a cold water bath, and the contents applied to a Whatman CF-1 cellulose powder column (25 x 1 cm) in order to remove a yellow pigment with an absorbance maximum at 450 nm (Caldwell and Grogg, 1955). The column was washed with 10 ml of water to elute the yellow fraction. The adsorbed red fraction was eluted with aqueous pyridine, exactly 10 ml being collected. Nitrogen was used to force each applied liquid through the column because the measured pigment degrades after one hour. The eluant % transmission was determined at 532 nm. This value was converted to absorbance and multiplied by 7.8 to obtain the TBA number expressed as mg malonaldehyde per kg sample (Tarladgis et

al., 1960).

RESULTS AND DISCUSSION

Gel Filtration Chromatography of Malonaldehyde-Glycine Reaction Products

The products of the malonaldehyde-glycine reaction were separated into different components by gel filtration and analyzed by fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 450 nm. However, after fluorimetrically scanning the gel filtration fractions, it was found that excitation and emission wavelengths of 390 and 468 nm, respectively, were more reflective of the fluorescent maxima of the samples. Chio and Tappel (1969a) reported that compounds formed from the reaction of malonaldehyde with glycine, leucine, or valine had excitation and emission maxima of 370 and 450 nm, respectively. Buttkus and Bose (1972) reported that malonaldehyde reacted with glycine to give a product that had excitation and emission maxima of 395 and 470 nm, respectively, whereas Trombly and Tappel (1975) reported values of 390 and 470 nm for the same product. Kaya (1977) reported that the malonaldehyde-valine reaction product fluoresced with excitation and emission maxima of 390 and 460 nm, respectively. In the Trombly and Tappel (1975) study the reaction product was subject to purification procedures, but from the maxima values obtained it did not seem to

affect the fluorescence characteristics. The differences between the results of Chio and Tappel (1969a) and those of Trombly and Tappel (1975) both of whom used the same reaction procedure, were never explained.

The fluorescence characteristics of the gel filtration fractions of the present study were in agreement with the results of all of the other studies except those of Chio and Tappel (1969a). More than one fluorescent compound was present in the malonaldehyde-glycine reaction. When the gel filtration column was irradiated with UV light of 366 nm, eight fluorescent bands were observed, two of which exhibited the blue fluorescence characteristics of conjugated Schiff bases (Chio and Tappel, 1969a), and two others which exhibited green fluorescence. Analysis by fluorescence showed that three major fluorescent bands eluted from the column and that all of these bands eluted in more than one 6 ml fraction. The fractions representative of the fluorescent species which exhibited the most intense fluorescence and their excitation and emission maxima are listed in order of decreasing fluorescence intensity in Table 2. While all the fractions had fluorescent characteristics of conjugated Schiff bases, fractions 16 and 35 had additional excitation and emission maxima that were similar. These were of higher wavelengths than maxima characteristic of conjugated Schiff bases (Chio and Tappel, 1969a). Two other compounds exhibited green fluorescence. From the analysis of the 6 ml fractions of the column eluant three major fluorescent species were observed. The presence of more than one fluorescent species and the two different excitation and emission maxima for each

of two of the species showed that there was more than one fluorescent compound produced in the malonaldehyde-glycine reaction. Chio and Tappel (1969a) did not report the existence of more than one fluorescent compound.

Table 2. Fluorescence characteristics of the major malonaldehyde-glycine reaction products separated by gel filtration chromatography. Fractions and maxima are listed in order of relative intensities

Fraction Number	Excitation Maximum (nm)	Emission Maximum (nm)
68	390	460
16	390-396	470
	448-450	514-517
35	398-402	470-471
	442	514-515

Difficulties were encountered in separating the products of the malonaldehyde-glycine reaction by gel filtration chromatography and measuring their fluorescence. There are several possible reasons why this procedure was not altogether successful. The use of fluorescence to analyze fractions is subject to criticism since many of the fractions eluted in the present study had a yellow color of varying intensity. The measurement of fluorescence could have been affected because absorption influences fluorescence measurement (Udenfriend, 1962). As absorption of a sample increases, the light from the fluorimeter light source penetrates less through the sample. The closer a molecule is to the cuvette surface, the more light it absorbs and consequently, the less light a molecule further

away from the surface absorbs. The lack of uniform absorption thoughout the solution means that more excitation occurs at the surface nearest to the light source and thus less light is emitted to the fluorimeter detector. A more dilute solution, one in which light can pass through uniformly, gives a greater amount of emission. Thus an eluate fraction with a high concentration of conjugated Schiff base derived from the reaction of glycine and malonaldehyde could have less fluorescence than an more dilute fraction, even though there is more fluorescent product present in the concentrated sample (Udenfriend, 1962).

In the presence of Chio and Tappel (1969a) the products of the malonaldehyde-glycine reaction were chromatographed two times on a gel filtration column. In the present study, the reaction mixture was passed through a Bio-Gel P-2 column only once since it was observed that the sample adsorbed irreversibly to the column packing. Washing of the column with 30 volumes of water, 0.5 M sodium sulfate solution, 15% methanol, and sodium hydroxide solution (pH 8.5) did not elute all of the adsorbed material. The column washings fluoresced at wavelengths identical to the reaction product and the column packing had a faint yellow color. Since Bio-Gel can adsorb aromatic compounds (Bio-Rad Laboratories, 1975) it is possible that the conjugated Schiff base was also adsorbed. The reaction product also adsorbed to Sephadex G-10, which was investigated as a possible alternative to Bio-Gel P-2. Marnett et al. (1980) observed that polymerization products formed from the acid hydrolysis of TEP, adsorbed to a Sephadex G-10 column packing. The

presence of many bands when the malonaldehyde-glycine reaction products were eluted from the Bio-Gel P-2 column could possibly have been due to polymerization of malonaldehyde with subsequent adsorption of these polymers onto the column packing.

Analysis of Malonaldehyde Preparations

Gas-liquid chromatography was used to study the products of the acid hydrolysis of TMP. Analysis of the hydrolyzate prepared according to Buttkus (1969) indicated the presence of four major components (Figure 5). The major component had a retention time of 1.55 min, but the other components were present in substantial quantities.

When TMP was hydrolyzed for 18 hr at room temperature following the procedure of Chio and Tappel (1969a) many more products were obtained (Figure 6). In general, the chromatograms were qualitatively similar, but there were some quantitative differences in the sizes of the major peaks. These two chromatograms were similar to the one reported by Gutteridge (1975) for the acid hydrolysis of TMP.

Mass spectrometry was used in the identification of several of the compounds present in the hydrolyzate. The first major peak (Figure 5, retention time 1.55 min), the mass spectrum of which is shown in Figure 7, corresponded to malonaldehyde. The major ion masses were m/e 72, m/e 71, and m/e 43. Since malonaldehyde was found to be present in its diketo form in methylene chloride (Kwon and Vander Veen, 1968), it is probably present in this form in ethyl

Figure 5. Gas chromatogram of the acid hydrolysis products of tetramethoxypropane prepared according to Buttkus (1969). Numerical values shown are retention times in minutes and, in parentheses, the peak area percentages.

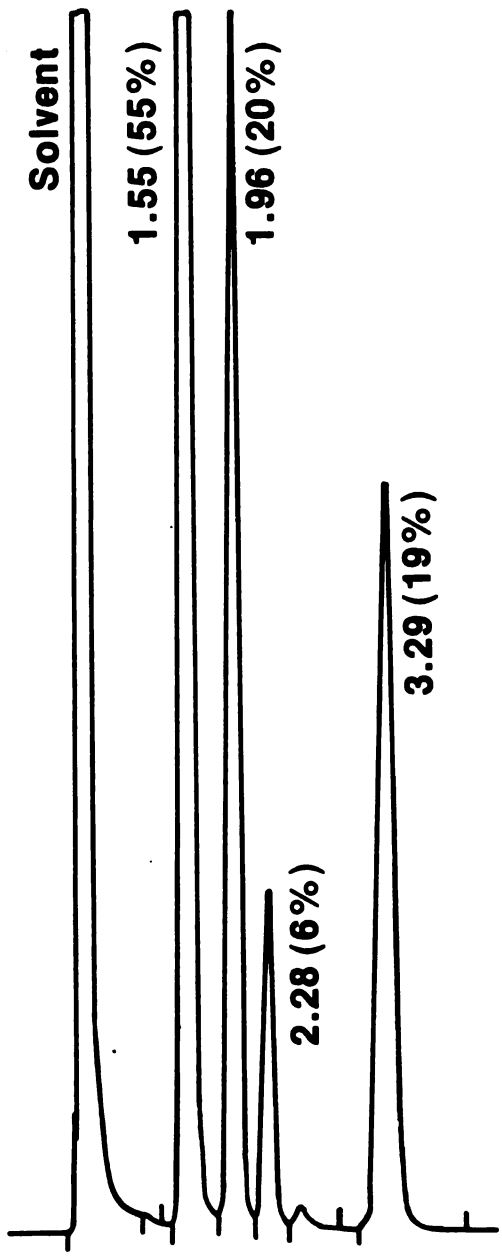

Solvent
1.55 (55%)
1.96 (20%)
2.28 (6%)
3.29 (19%)

Figure 6. Gas chromatogram of an 18 hr acid hydrolyzate of tetramethoxypropane prepared according to Chio and Tappel (1969a). Numerical values are peak retention times in minutes and, in parentheses, the peak area percentages, excluding solvent.

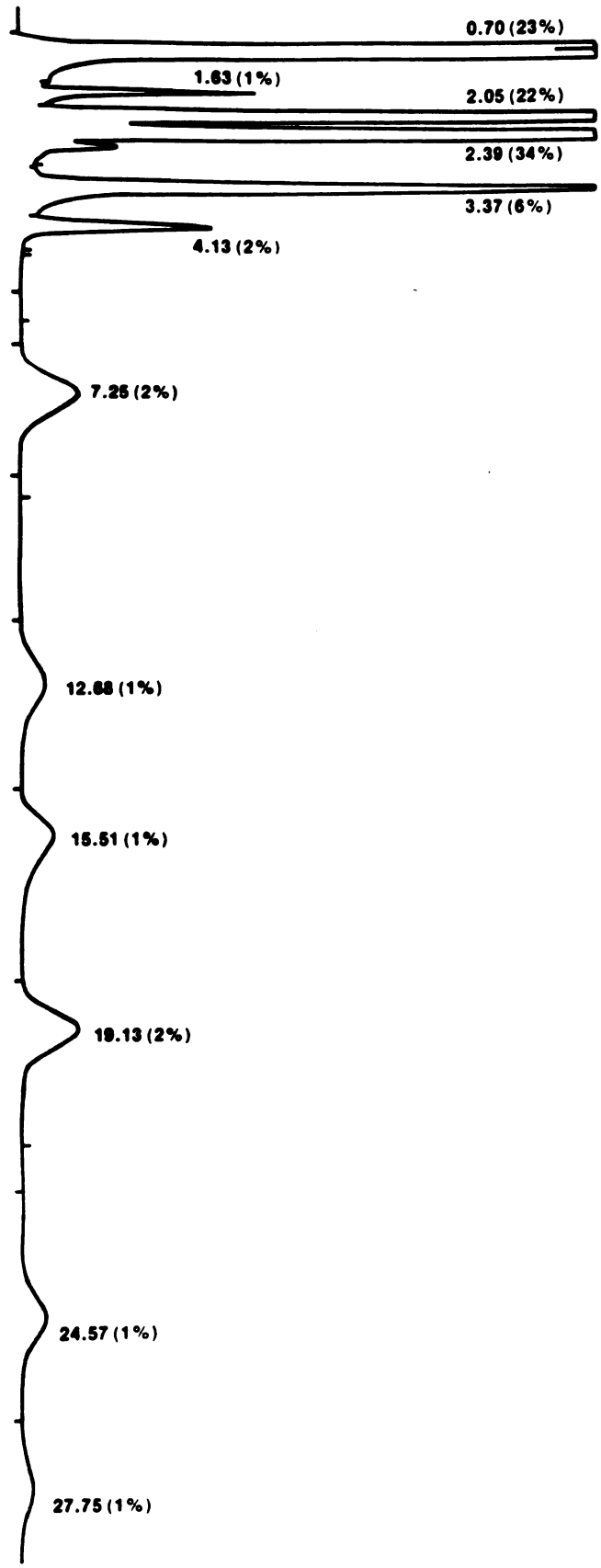
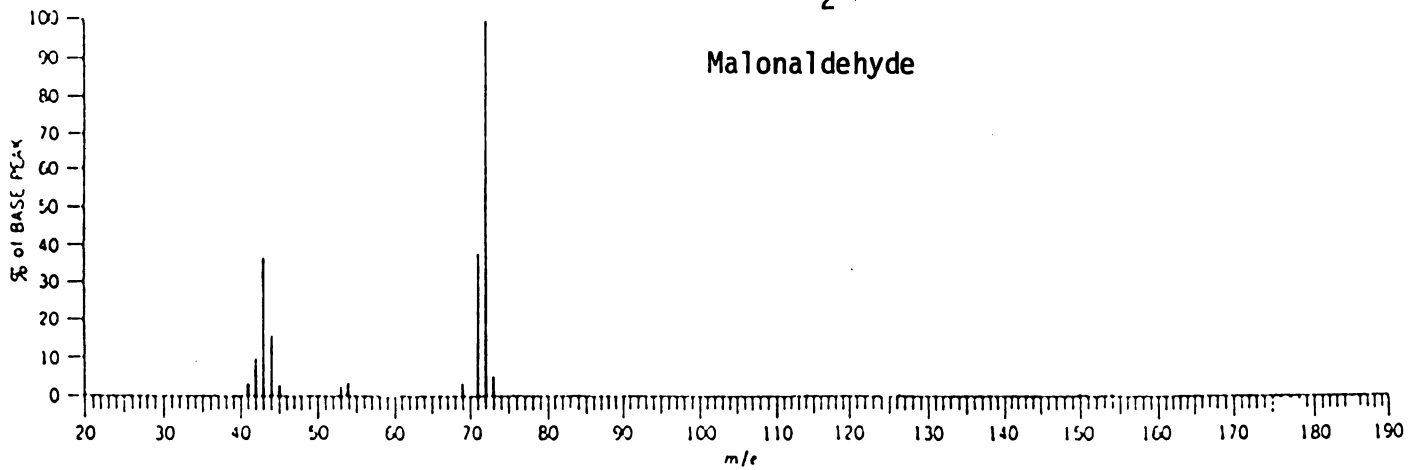

0.70 (23%)
1.63 (1%)
2.05 (22%)
2.39 (34%)
3.37 (6%)
4.13 (2%)
7.25 (2%)
12.68 (1%)
15.51 (1%)
19.13 (2%)
24.57 (1%)
27.75 (1%)

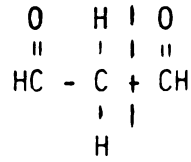
Figure 7. Mass spectrum of malonaldehyde.



O O
‖ ‖
HC - CH_2 - CH

Malonaldehyde

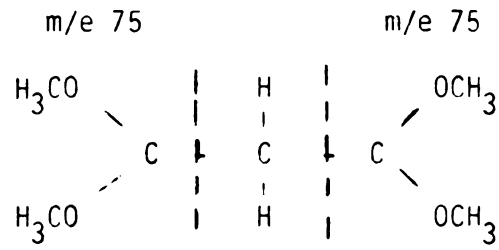
ether. The m/e 72 ion, the molecular ion, was formed by abstraction of an electron from the molecule. The m/e 71 ion was formed by loss of a hydrogen atom from the malonaldehyde molecule. The m/e 43 ion was formed by homolytic cleavage of a carbon-carbon bond as shown below



m/e 43

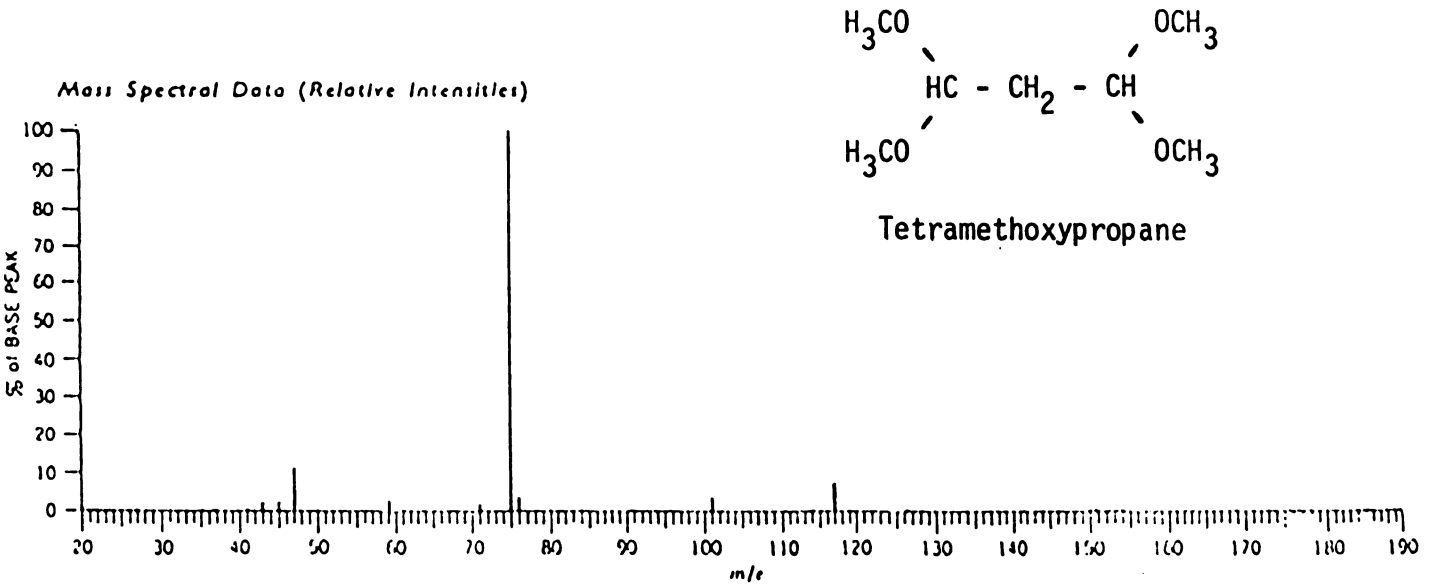
The mass spectrum did not include any ions below a molecular weight of 40 because of resolution problems below this molecular weight.

The third major peak on Figure 5, having a retention time of 2.28 min was identified as unhydrolyzed TMP by mass spectrometry (Figure 8). The m/e 75 ion was probably formed by homolytic cleavage of the bonds between the 1 and 2 carbons and 2 and 3 carbons as shown below:



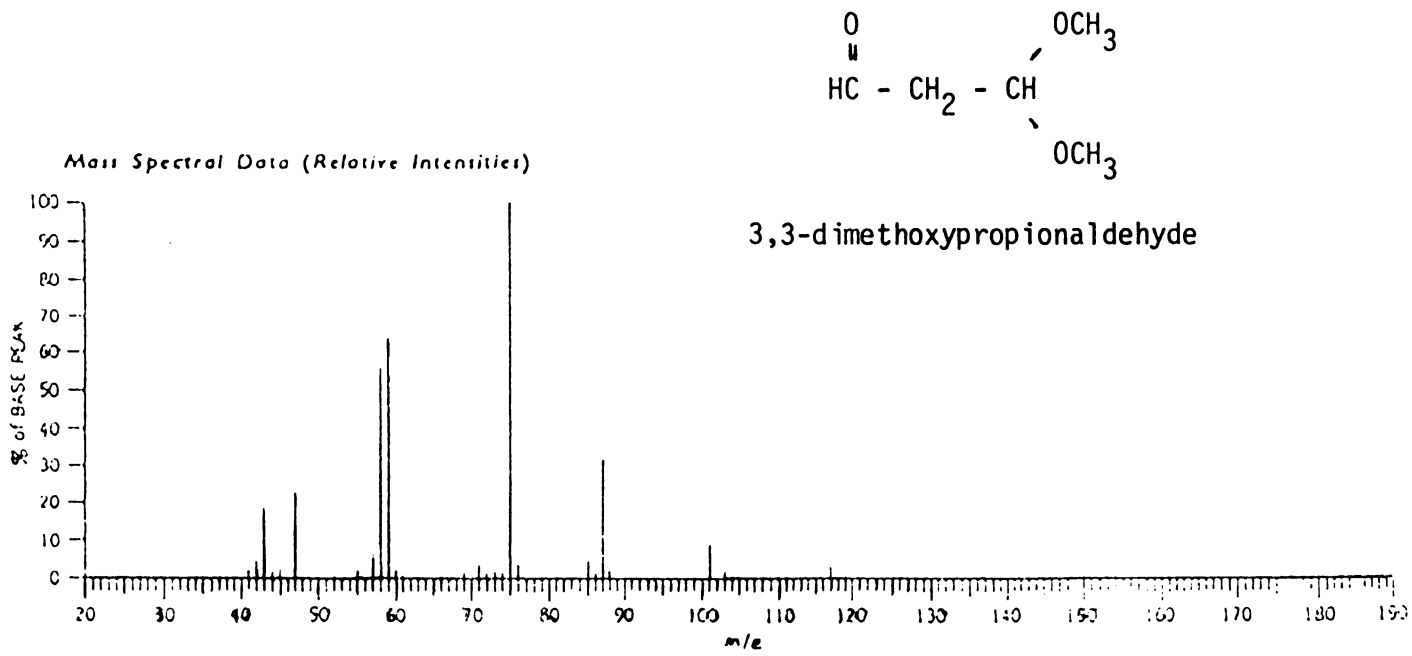
The compound having a retention time of approximately 2 min (Figure 5) was postulated to be an intermediate in the acid hydrolysis of TMP. The mass spectrum of this compound exhibited similar ion masses (m/e 47, 101, and 117) to that of TMP, thus

Figure 8. Mass spectrum of tetramethoxypropane.

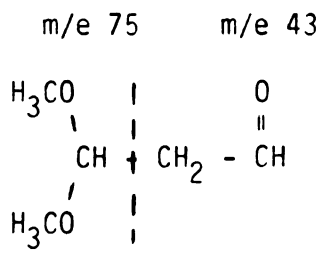


Tetramethoxypropane

Figure 9. Mass spectrum of 3,3-dimethoxypropionaldehyde.

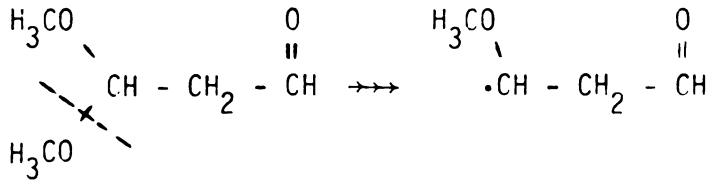

Mass Spectral Data (Relative Intensities)
% of BASE PEAK
m/e
3,3-dimethoxypropionaldehyde

indicating that the unknown compound was probably structurally related to TMP (Figure 9). As already discussed, two intermediates of the hydrolysis reaction, β-methoxyacrolein (BMA) and 3,3-dimethoxypropionaldehyde (DMP) have been characterized by Marnett and Tuttle (1980). The mass spectrum shown in Figure 9 was indicative of DMP. The ions m/e 43 and 75 arose from homolytic cleavage products as follows:



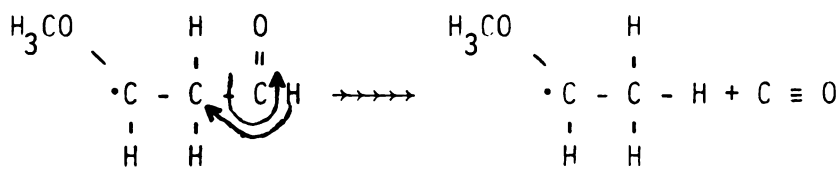
DMP (MW = 118)

The peak at m/e 87 probably represented an ion formed by the loss of a methoxy group from DMP.



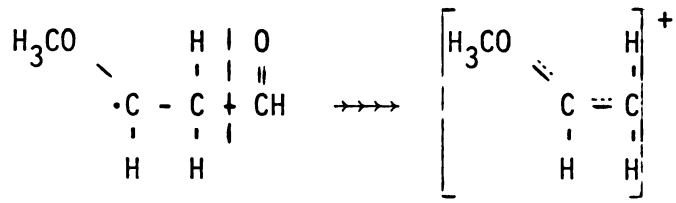
m/e 87

The m/e 59 ion could have been formed by a homolytic cleavage of the bond between carbons 1 and 2, transfer of the carbon-1 hydrogen to the 2 carbon and subsequent loss of carbon monoxide.



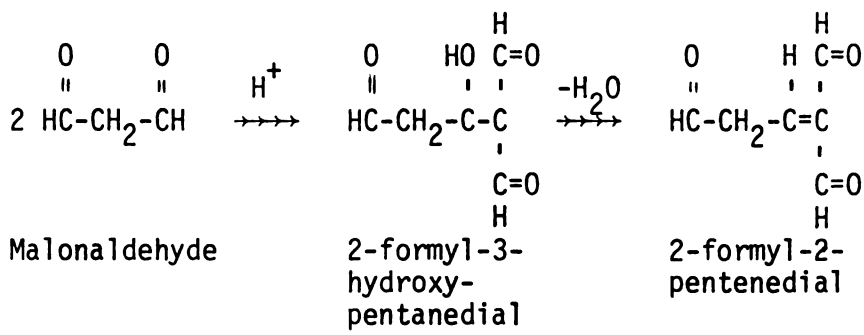
m/e 87 m/e 59

The m/e 58 ion could have been formed by the heterolytic cleavage of a formyl group from the m/e 87 ion.



m/e 87 m/e 58

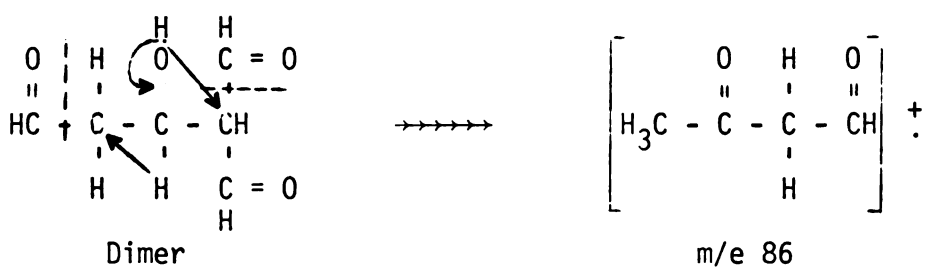
The compound having a retention time of 3.29 min (Figure 5) gave a mass spectrum as shown in Figure 10. This compound was identified as being a dimer of malonaldehyde that could have been formed by the mechanism proposed by Buttkus (1975) as follows:



Malonaldehyde 2-formyl-3-hydroxy-pentanedial 2-formyl-2-pentenedial

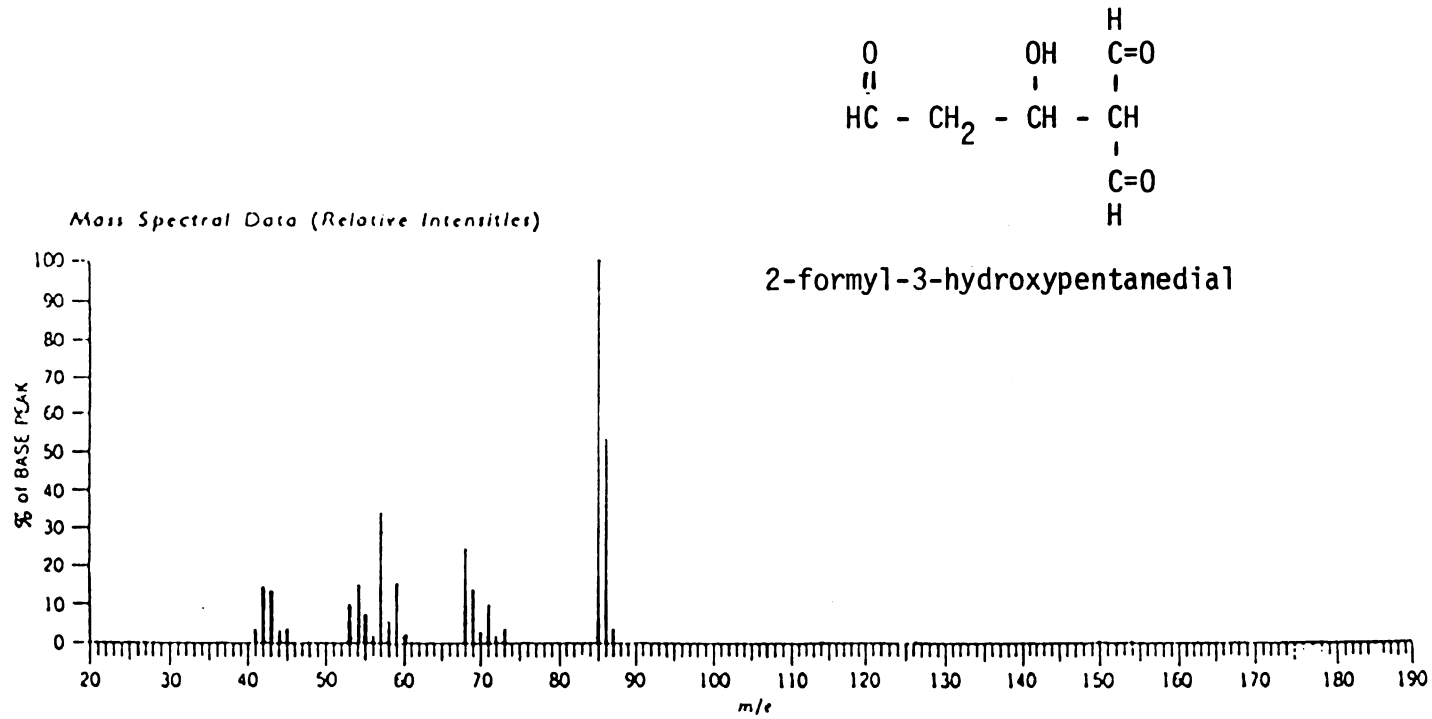
Gutteridge (1975) also reported the formation of polymeric substances arising from the acid hydrolysis of TMP.

The mass spectrum shown in Figure 10 was due to the 2-formyl-3-hydroxypentanedial form of the dimer. The m/e 86 ion resulted from the loss of two formyl groups followed by hydrogen rearrangement, carbonyl formation, and the loss of an electron.



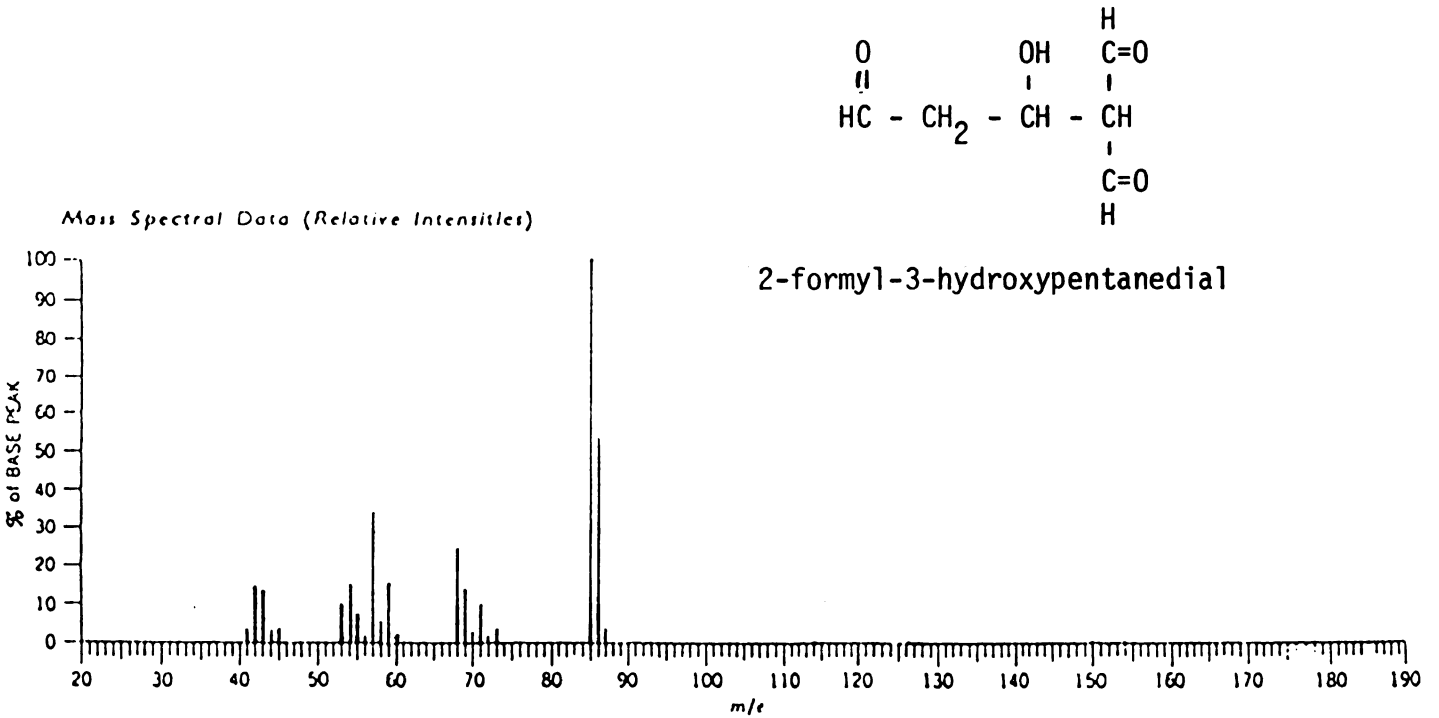
Dimer m/e 86

Figure 10. Mass spectrum of 2-formyl-3-hydroxypentanedial.

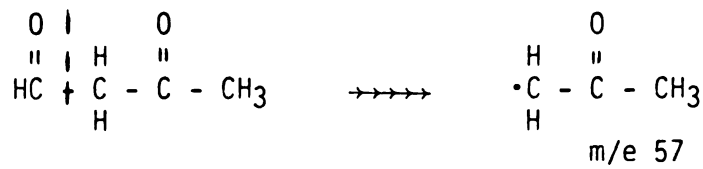


2-formyl-3-hydroxypentanedial

Figure 10. Mass spectrum of 2-formyl-3-hydroxypentanedial.


Mass Spectral Data (Relative Intensities)
% of BASE PEAK
m/e
2-formyl-3-hydroxypentanedial

The m/e 85 ion was formed by the loss of a hydrogen atom instead of an electron. The m/e 57 ion was due to a cleavage of the bond of the m/e 86 fragment between the 1 and 2 carbons as shown below:



The products of the hydrolysis of TMP were monitored over a 2 hr period by GLC (Table 3. Figure 11) As expected, the peak corresponding to TMP (2.33 min) decreased with hydrolysis time while all of the other peaks increased over the same period. The DMP peak (1.99 min) and an unidentified peak at 0.69 min were the predominant peaks formed. A peak having a retention time of 4.04 min was also observed during the hydrolysis reaction. Gutteridge (1975), who obtained a similar chromatogram for the acid hydrolysis of TMP, reported that this compound was probably due to the formation of a malonaldehyde polymerization product. This worker used higher temperatures and longer hydrolysis times and thus further promoted the formation of malonaldehyde polymerization products. The presence of additional GLC peaks with retention times greater than 4 min in the samples hydrolyzed for 18 hr possibly indicated that more polymerization products could have been formed as hydrolysis time increased (Figure 6).

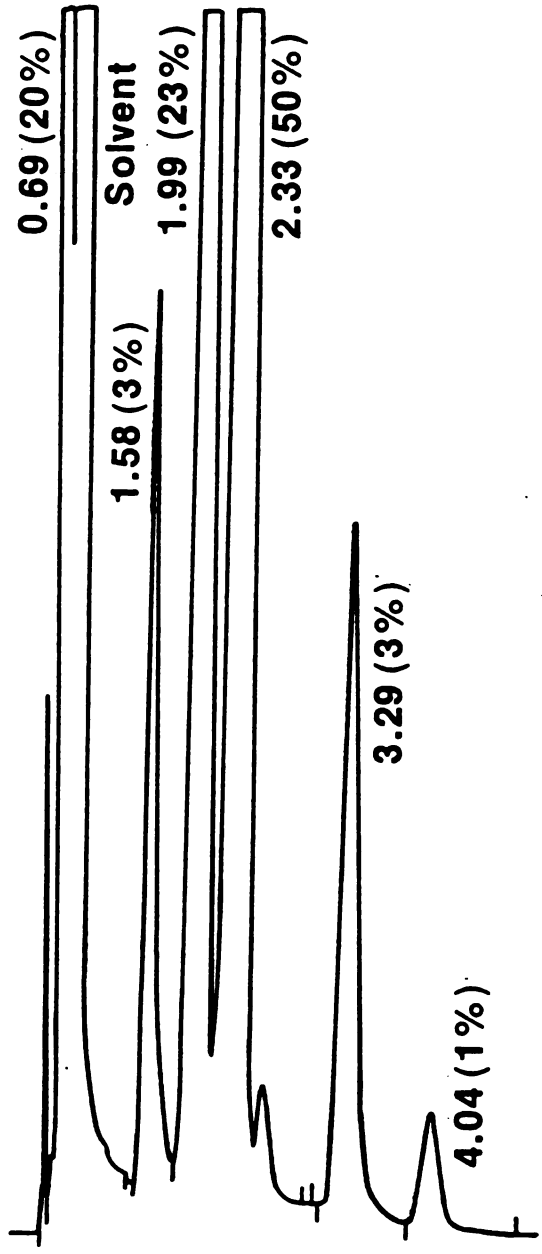
The most commonly used procedure for preparing malonaldehyde is the acid hydrolysis of TMP. The results of the present hydrolysis study indicate that compounds other than malonaldehyde are produced. It is thus possible that this procedure could cause substantial artifacts in experiments where the TMP acid hydrolyzate is assumed to

Table 3. GLC data for the acid hydrolysis of tetramethoxypropane prepared according to Chio and Tappel (1969a)

Time of Hydrolysis (Min)	Peak Area Percent					
	Peak Retention Time (min)					
	0.7	1.6	2.0	2.3	3.3	4.0
5	9.8	0.4	9.0	79.1	0.6	0.1
15	11.9	1.0	14.9	69.5	1.5	0.2
25	11.3	1.7	17.8	66.2	1.8	0.2
35	14.4	2.0	19.7	60.6	2.1	0.2
45	16.0	2.4	21.1	56.4	2.6	0.4
55	17.6	2.4	22.5	52.7	3.2	0.6
65	19.5	2.7	22.9	50.2	3.2	0.7
75	21.3	2.4	23.8	45.4	4.9	1.2
90	23.2	2.4	23.9	42.3	5.3	1.4
120	25.1	3.5	25.4	39.0	4.9	1.1

[a]Malonaldehyde
[b]3,3-Dimethoxypropionaldehyde
[c]Tetramethoxypropane
[d]2-Formyl-3-hydroxypentanedial

Figure 11. Gas chromatogram of the acid hydrolyzate of tetramethoxypropane after 65 min of hydrolysis prepared according to Chio and Tappel (1969a). Numerical values are the peak retention times and, in parentheses, the percent of total peak area of each peak.


0.69 (20%)
Solvent
1.58 (3%)
1.99 (23%)
2.33 (50%)
3.29 (3%)
4.04 (1%)

be pure malonaldehyde. For this reason, an alternative method of malonaldehyde preparation was studied.

The gas chromatogram of the malonaldehyde bis-bisulfite acid hydrolysis steam distillate indicates that malonaldehyde was the major product (Figure 12). This was in contrast to the other methods employed in the synthesis of malonaldehyde. Two minor components having retention times of 0.62 and 0.73 min had similar retention times to methanol and formaldehyde, respectively. However, their identities could only be considered tentative since mass spectrometric conformation was not achieved.

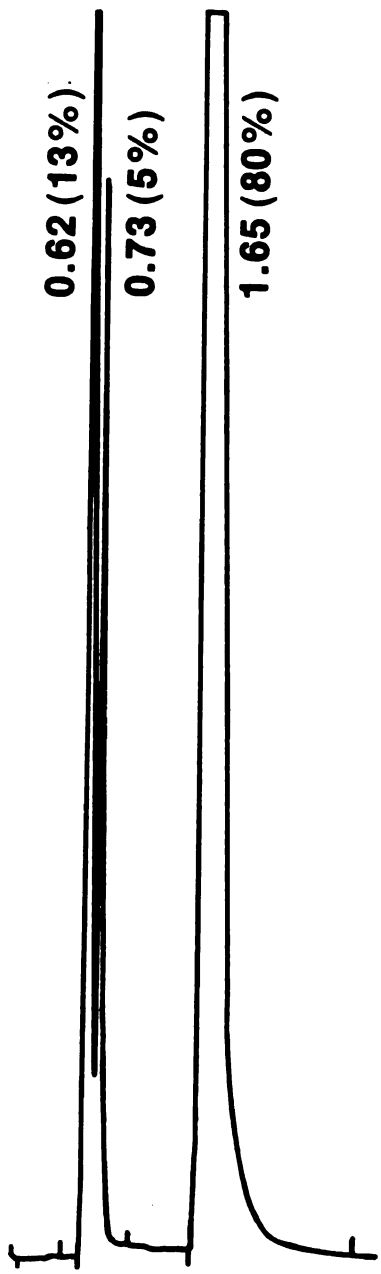
The steam distillate of malonaldehyde bis-bisulfite acid hydrolyzate was analyzed by TLC using TBA reagent as the detection system. One major red spot was observed on the chromatogram. This result agreed with the findings reported by Gutteridge (1975).

Analysis of Malonaldehyde-Glycine Ethyl Ester Reaction Products

Fluorescence Characteristics

The products of the reaction between two different malonaldehyde preparations and glycine ethyl ester were separated by TLC and the fluorescent characteristics of the individual components determined. Both reaction products were separated into polar and ethyl acetate fractions for analysis. The TLC solvent system used (butanol:acetic acid:water, 80:20:20) did not satisfactorily separate the polar fraction of either reaction product; however, it did separate the ethyl acetate fractions. TLC analysis of the products of the reaction between malonaldehyde prepared by the steam distillation of the acid

Figure 12. Gas chromatogram of the steam distillate of the malonaldehyde bis-bisulfite acid hydrolyzate. Numerical values are peak retention times and, in parentheses, the percent of total peak area of each peak.


0.62 (13%)
0.73 (5%)
1.65 (80%)

hydrolyzate of malonaldehyde bis-bisulfite salt, and glycine ethyl ester revealed the presence of 9 distinct components (Figure 13). The R_f values and fluorescence characteristics of the components are listed in Table 4. The fluorescent components all had similar excitation and emission maxima. Only component 4, a minor fluorescent component, had fluorescence characteristics similar to those of the malonaldehyde-glycine reaction product prepared by Chio and Tappel (1969a) i.e. an excitation maximum of 370 nm and an emission maximum of 450 nm. All the other components had emission maxima similar to the 460-470 nm maxima reported by Buttkus and Bose (1972), Trombly and Tappel (1975), and Kaya (1977).

The data in Table 4 show that three excitation maxima were common to eight of the components. Trombly and Tappel (1975) reported that conjugated Schiff base fluorophores had a second excitation maximum in the 260-280 nm range due to the electronic transition induced by radiation of this wavelength from the singlet ground state S_0 to the second excited singlet state S_2*. The decay of this excited state involved an S_2* to S_1* transition, followed by an S_1* to S_0 transition. The latter transition was the same as the transition of the excited state induced by 350-390 nm light, thus causing the emission maxima of both excitation maxima ranges to be the same. Components 1,3,4,6,8 and 9 had excitation maxima that fell within this range, while component 7 had an excitation maximum closer to the lower end of the range.

The most intense fluorescence for all of the components except component 8 was due to excitation at between 221-233 nm. Excitation

Figure 13. TLC analysis of the fluorescent malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction products, ethyl acetate fraction. The numbers correspond to fluorescent components.

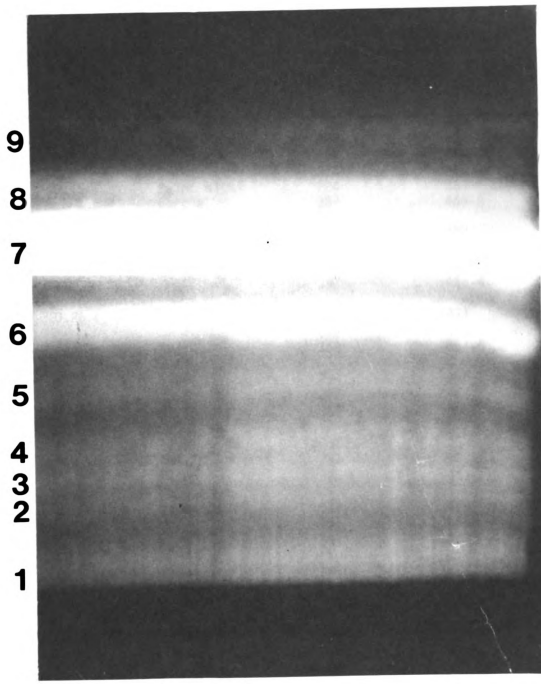

9
8
7
6
5
4
3
2
1

Table 4. TLC and fluorescence characteristics of the reaction products of malonaldehyde bis-bisulfite acid hydrolysis steam distillate and glycine ethyl ester [a,b].

Component	R_f Value	Emission Color	Maxima (nm) Excitation	Emission
1	0.05	Faint blue	221	454,493
			264	460-1
			374	460-1
2	0.15	Faint blue-purple	224	461
			387	466-8
3	0.20	Blue purple	224	457
			242	494,467
			269	468
			397	465-6
4	0.26	Blue	230	471
			269	455
			369	454-5
5	0.37	Blue	231	466
			247	462
			383	465
6	0.48	Light blue	231	468
			247	462
			383	465
7	0.62	Light blue	229	469
			257	466
			391	468
8	0.72	Blue	385	465
			233	463
			262	464
9	0.82	Blue	222	459
			260	465
			376	465

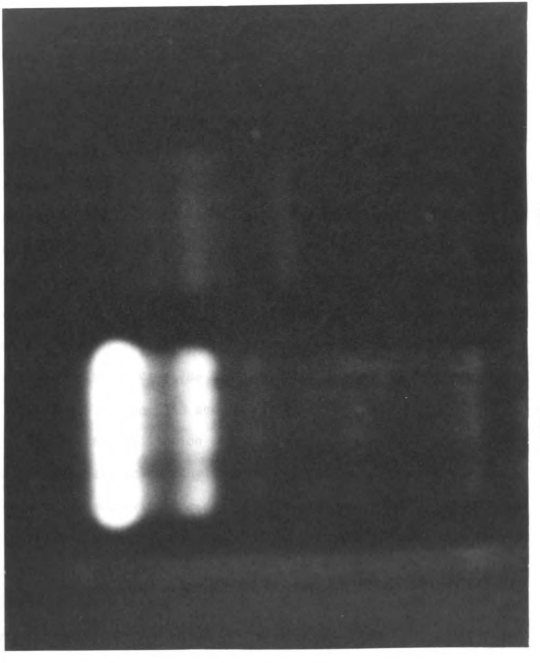
a. Emission color when illuminated by 366nm light.
b. Fluorescence characteristics are listed in order of decreasing fluorescence intensity.

within this wavelength range was due to an electronic transition induced by this wavelength radiation from the singlet ground state S_0 to the third excited singlet state S_3*. The decay of this excited state involved an S_3* to S_1*, transition and then an S_1* to S_0 transition (Trombly and Tappel, 1975).

Some observations could not be readily explained by the previous discussion. Components 3 and 4 had emission maxima for the three excitation wavelengths of more than 10 nm apart, which may have been due to the possible presence of more than one compound in the band. Component 3 had a fourth excitation maximum which had an emission maximum much different from the other emission maxima and was probably due to more than one compound being present. Components 1-7 all had similar GLC retention times. This was probably due to the breakdown of these components to similar compounds at the temperatures used in this analysis.

TLC separation and fluorescent detection of unreacted malonaldehyde bis-bisulfite acid hydrolysis steam distillate showed that fluorescent components were also present (Figure 14). The amounts of steam distillate in this sample and the reacted sample were the same, thus making the TLC plates quantitatively comparable. The unreacted steam distillate separated into six faint fluorescent components, three of which had R_f values similar to the fluorescent components of the steam distillate-glycine ethyl ester reaction products. The major component had an R_f value similar to component 6 of the reaction product. It took a longer time for fluorescence to develop in the unreacted sample. Gutteridge (1977) reported that an aqueous solution

Figure 14. TLC analysis of the fluorescent malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction products (A) and steam distillate reaction products (B), ethyl acetate fractions.


A
B

and chloroform extract of malonaldehyde bis-bisulfite salt did not fluoresce. As previously discussed, Shin et al. (1972), Buttkus (1975) and Gutteridge (1977) all reported fluorescence of the acid hydrolyzate of TMP that had been allowed to stand for varying amounts of time. The latter worker used gel filtration to separate out six fluorescent polymers in the hydrolyzate. Therefore, the fluorescent bands of the unreacted distillate samples were probably due to polymerization.

TLC analysis also indicated that the mode of preparation of malonaldehyde had an effect on the malonaldehyde-glycine ethyl ester reaction products that were formed (Figures 15 and 16, Table 5). The two major blue fluroescent components of the TLC plates of both products had similar R_f values and fluorescence characteristics. A comparison of Figures 13, 15 and 16 reveals that the two products did not share other common components, such as the yellow fluorescent component with an R_f value of 0.36, which exhibited the most intense fluorescence on the Chio and Tappel (1969a) sample plate.

The steam distillation and Chio and Tappel methods of preparing malonaldehyde-glycine ethyl ester reaction products were qualitatively compared by observing the relative fluorescence of the two major blue fluorescent components in contrast to the other fluorescent components present in each sample (Figure 17). The two major blue fluorescent components were more predominant in the steam distillate-glycine ethyl ester reaction product than in the Chio and Tappel method reaction product. As already noted, the latter product contained a yellow fluorescent component that exhibited more intense fluorescence than the two major blue fluorescent components.

Table 5. TLC and flourescent characteristics of the reaction products of the acid hydrolyzate of TMP and glycine ethyl ester.

Band	R_f Value	Color at Excitation of 366nm
1	0.07	Faint yellow
2	0.17	Purple
3	0.27	Faint yellow
4	0.36	Bright yellow
5	0.42	Light blue
6	0.48	Blue
7	0.57	Yellow
8	0.62	Blue
9	0.68	Yellow
10	0.73	Purple

Figure 15. TLC analysis of the fluorescent malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction products (A) and TMP acid hydrolyzate-glycine ethyl ester reaction products (B), ethyl acetate fractions.

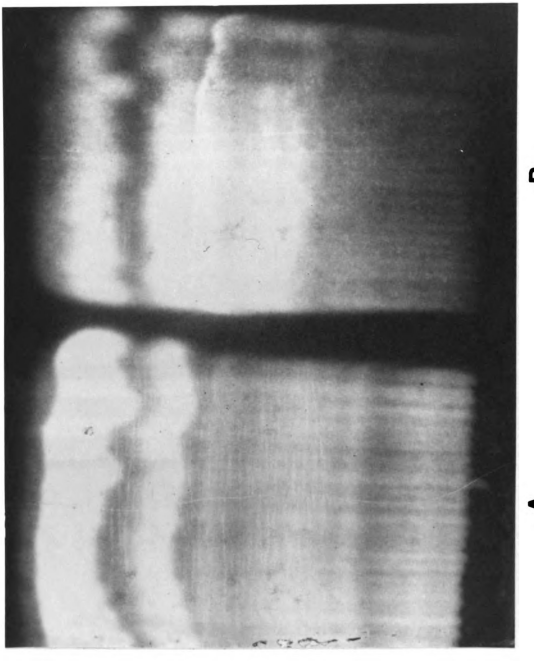

B
A

Figure 16. TLC analysis of the fluorescent malonaldehyde-glycine ethyl ester reaction products prepared according to Chio and Tappel (1969a), ethyl acetate fractions. The numbers correspond to fluorescent components.

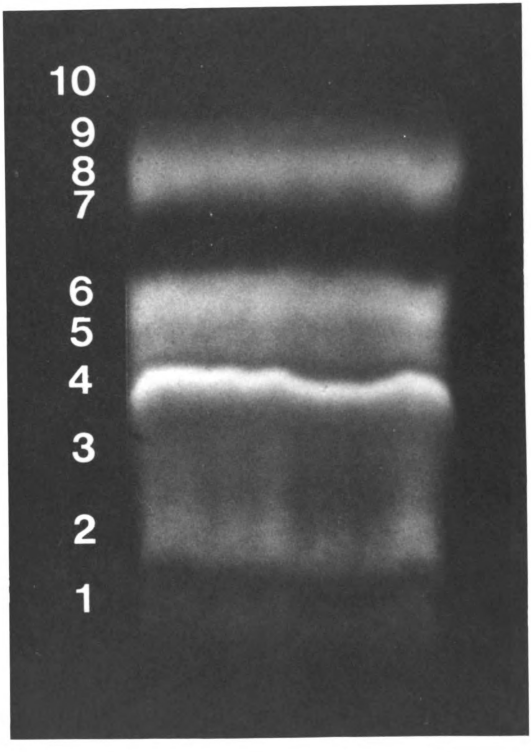
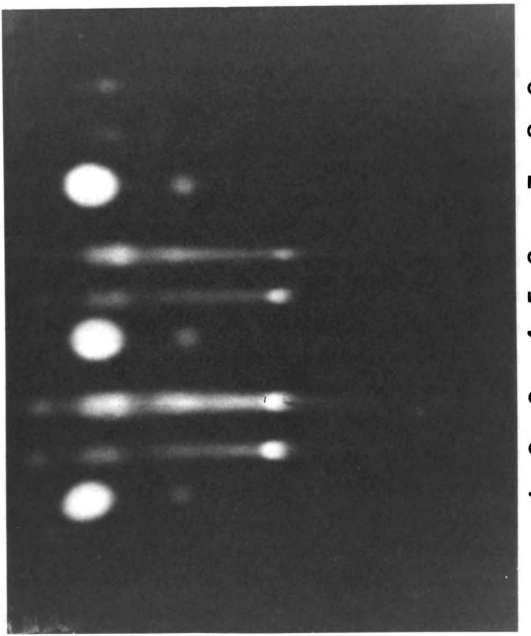

10
9
8
7
6
5
4
3
2
1

Figure 17. TLC analysis of the fluorescent malonaldehyde-glycine ethyl ester reaction products, ethyl acetate fractions, prepared from steam distillate and glycine ethyl ester (spots 1,4, and 7; 1 µl samples) and according to Chio and Tappel (1969a)(spots 2 and 3, 5 and 6, 8 and 9; 5 µl, 2 µl, and 1 µl samples respectively).


1 2 3 4 5 6 7 8 9

Infrared Spectroscopy of Fluorescent Components

The infrared spectra of components 6 and 7 of the steam distillate-glycine ethyl ester reaction product and the infrared spectrum of component 7 that had been subject to sodium borohydride reduction are shown in Figures 18, 19, and 20 respectively. The spectrum of component 6 displayed a band at 1663 cm^{-1} typical of a C=N bond and a band at 1620 cm^{-1} typical of a C=C bond (Figure 18). The spectrum of component 7 displayed a band at 1655-1663 typical of a C=N bond and a band at 1620-1625 cm^{-1} typical of a C=C bond (Figure 19). The reduced sample did not show a band in either region (Figure 20).

The spectra of the present study are similar to those reported in the literature. Chio and Tappel (1969a) reported infrared spectra bands for conjugated Schiff bases of valine ethyl ester-malonaldehyde and leucine ethyl ester-malonaldehyde reaction products of 1650-1655 cm^{-1} and 1610-1620 cm^{-1} and attributed these bands to C=N and C=C bonds, respectively. Dugan and Rao (1971) reported infrared spectra bands for a Schiff base, derived from the reaction of malonaldehyde and PE, of 1675 cm^{-1} and 1628 cm^{-1}. These were attributed to C=N and C=C bonds, respectively.

From the results above, it can be concluded that the two major fluorescent components of the malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction product both contained the conjugated Schiff base group (R-N=C-C=C-N-R) that is necessary for fluroescence to occur (Malshet and Tappel, 1973). Sodium borohydride reduction resulted in the loss of double bond character of

Figure 18. Infrared spectrum of component 6 of the malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction product, ethyl acetate fraction.

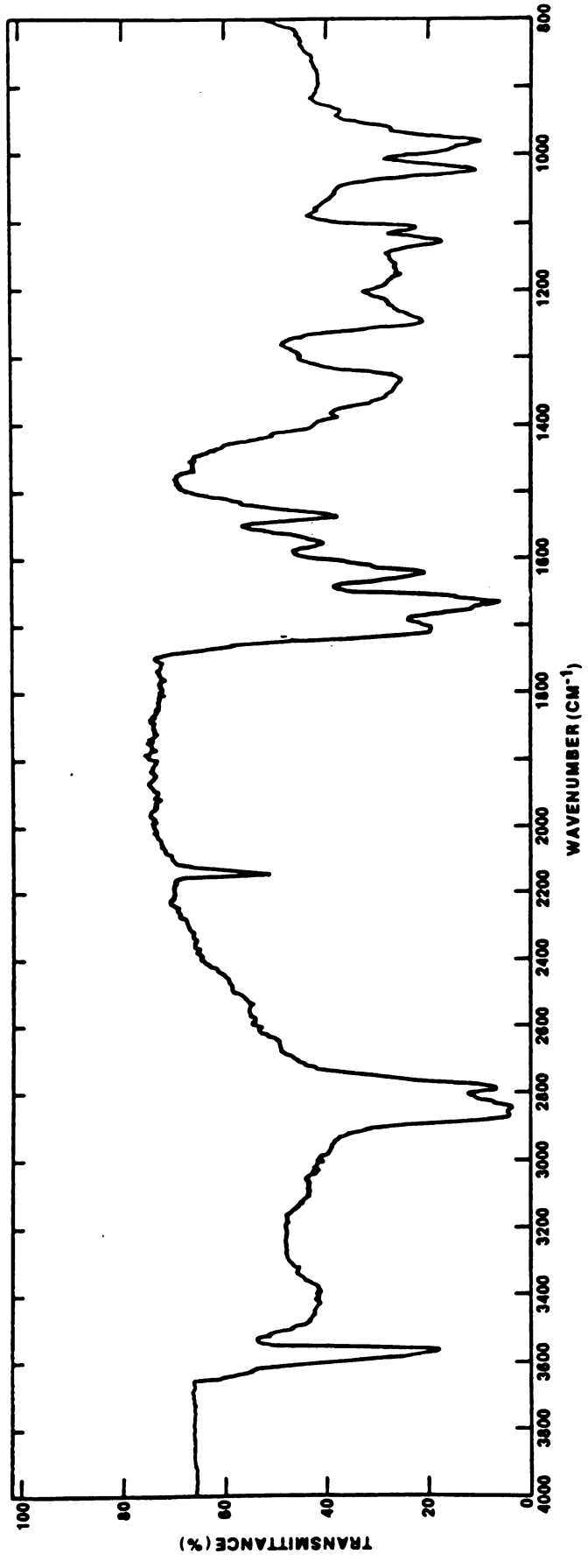

TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
WAVENUMBER (CM^{-1})

Figure 19. Infrared spectrum of component 7 of the malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction product, ethyl acetate fraction.

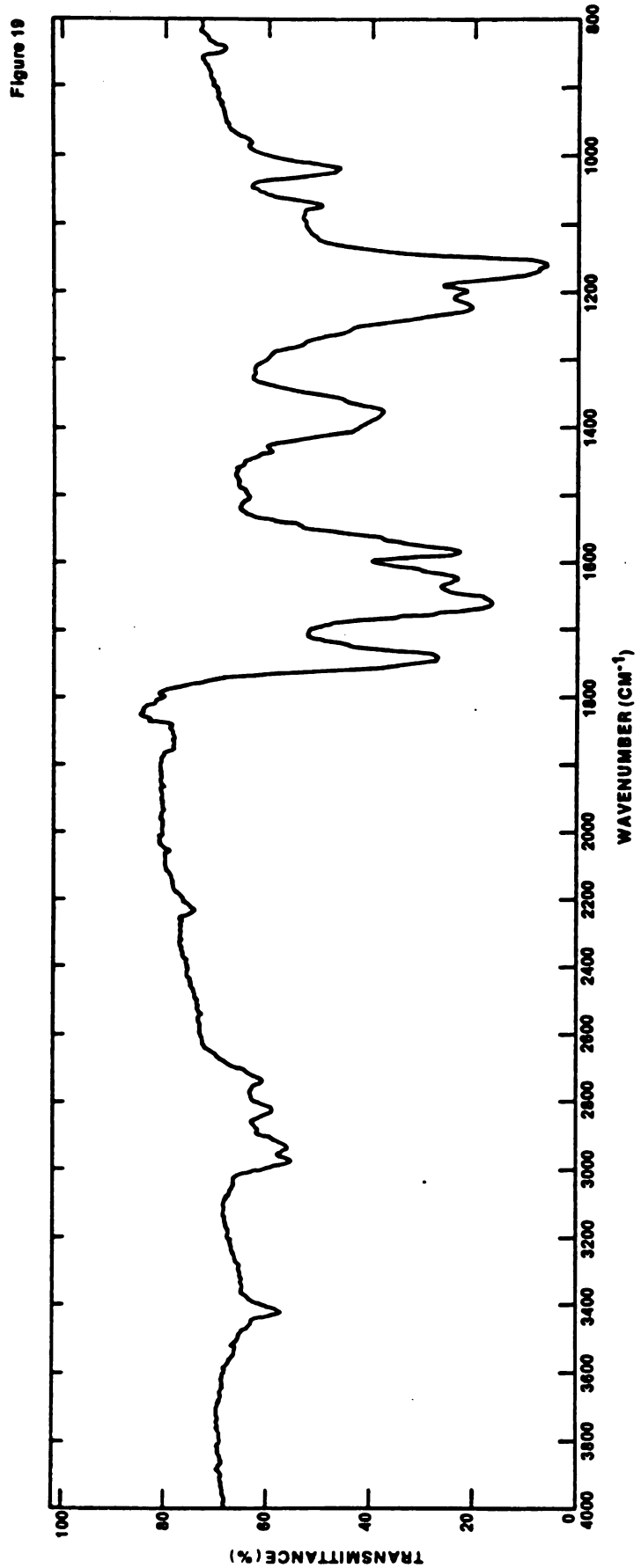

TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
WAVENUMBER (CM⁻¹)

Figure 19

Figure 20. Infrared spectrum of reduced component 7 of the malonaldehyde bis-bisulfite acid hydrolysis steam distillate-glycine ethyl ester reaction product, ethyl acetate fraction.

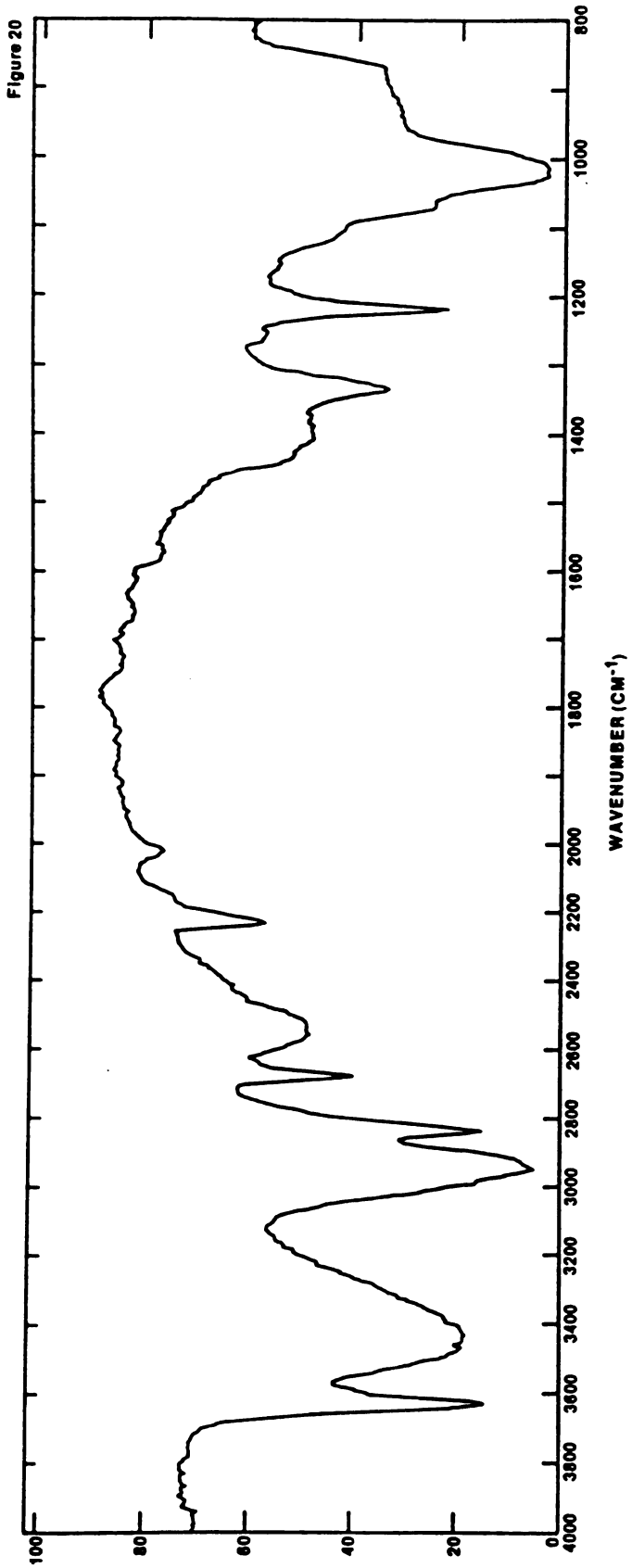


Figure 20
100
80
60
40
20
0
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
WAVENUMBER (CM-1)

the Schiff base and also loss of the carbonyl group represented by the 1740 cm^{-1} band. Chio and Tappel (1969a) reported a similar band in a spectra for the Schiff base derived from malonaldehyde and leucine ethyl ester; however, this band was present in the reduced sample.

GLC-Mass Spectrometry Analysis of Malonaldehyde-Glycine Ethyl Ester Reaction Products

The two major fluorescent TLC bands of the malonaldehyde-glycine ethyl ester reaction product and the major fluorescent band of the TMP hydrolyzate were analyzed by electron impact and chemical ionization mass spectrometry. Unfortunately, the mass spectra produced were insufficient to derive specific chemical structures. The mass spectra for all three samples were similar, however, indicating that the three samples probably consisted of compounds with similar chemical structures.

Model System Studies

Model systems containing linolenic acid and glycine ethyl ester were stored at 40°C for zero, seven and fourteen days and then subjected to the TBA test and fluorescence measurement at excitation and emission wavelengths of 382 nm and 468 nm, respectively. Results are shown in Table 6. The TBA value and fluorescence intensity both increased with time and thus, with increasing linolenic acid autoxidation.

The ethyl acetate extracts of the low level linolenic acid-glycine ethyl ester model systems were separated by TLC as shown in Figure 21. The samples had major blue fluorescent spots with R_f values similar

to the TLC spots of the ethyl acetate fraction of the steam distillate-glycine ethyl ester reaction product, indicating that conjugated Schiff bases were probably formed from degradation products of autoxidized linolenic acid reacting with the amine group of glycine ethyl ester. Other blue fluorescent components were also present in the model system samples.

Table 6. TBA numbers and relative fluorescence intensities of model systems stored at 40 C.[a]

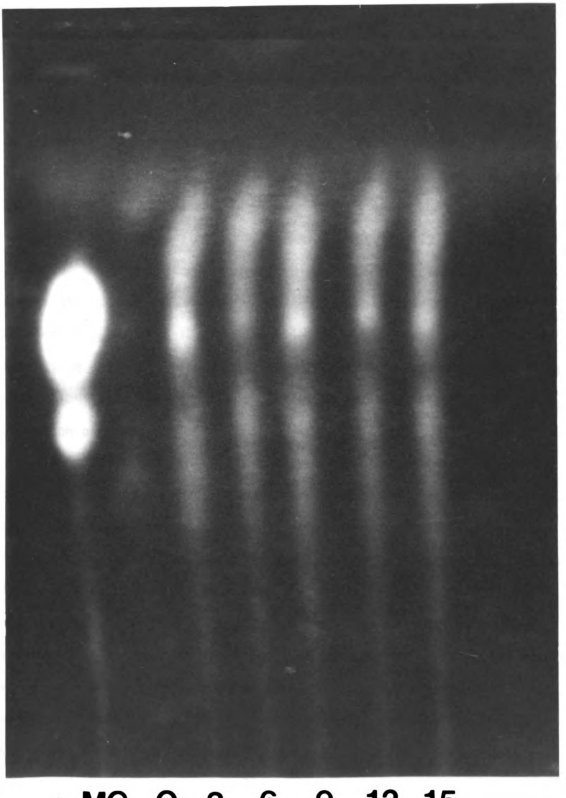
Storage Time (days)	TBA Number (mg malonaldehyde per Kg sample)	Relative Fluorescence Intensity
0	0	0.24
7	1.91	2.12
14	4.03	3.03

a. The relative fluorescence intensities were measured at an excitation wavelength of 382 nm and emission wavelength of 468 nm.

General Discussion

This study was designed to investigate the formation of conjugated Schiff bases from the reaction of malonaldehyde and glycine, and to determine whether fluorescence measurement of these compounds could be correlated to other parameters of lipid oxidation as proposed by Fletcher et al. (1973). Gutteridge (1975) and Gutteridge et al. (1977) showed that the commonly accepted method of preparing malonaldehyde from TMP (Kwon and Watts, 1963) yielded many compounds besides malonaldehyde, some of which fluoresced. Chio and Tappel (1969a),

Figure 21. TLC analysis of ethyl acetate extracts of linolenic acid-glycine ethyl ester model systems stored at 40°C. The MG sample is the steam distillate-glycine ethyl ester sample. The numbers represent days of storage of model systems. The plate was illuminated by 366 nm radiation.


MG 0 3 6 9 12 15

Dugan and Rao (1972), Buttkus and Bose (1972), Buttkus (1975), Trombly and Tappel (1975), and Kaya (1977) used similar methods to prepare malonaldehyde that was reacted with amino acids to produce fluorescent conjugated Schiff base products. Problems were encountered in this study with the separation of malonaldehyde-glycine fluorescent reaction products by gel filtration chromatography, especially adsorption of the sample to Bio-Gel P-2 gel filtration chromatography packing. The findings of Gutteridge (1975) and Gutteridge et al. (1977) that acid hydrolysis of TMP produced polymers, along with the observation of Marrett and Tuttle (1980) that malonaldehyde polymerization products adsorbed to Sephadex G-10 gel filtration chromatography packing, could possibly be applied to the malonaldehyde-glycine reaction product. The preparation of malonaldehyde was studied in hopes of clarifying this adsorption problem.

Acid hydrolysis of TMP, the most common method of forming malonaldehyde, was shown to produce compounds other than malonaldehyde. Gas-liquid chromatography and mass spectrometry indicated the presence of 3,3-dimethoxypropionaldehyde in the hydrolyzate. Marnett and Tuttle (1980) have proposed that this compound is partially responsible for the carcinogenic action originally attributed to malonaldehyde. Gas liquid chromatographic and mass spectrometric analysis of the TMP acid hydrolyzate products also indicated the presence of 2-formyl-3-hydroxypentanedial, a dimer of malonaldehyde, and other compounds that were probably polymerization products. These latter products may have adsorbed to the Bio-Gel P-2 column packing and been responsible for the residual fluorescence of the column packing.

The fact that malonaldehyde is not the only compound produced during the acid hydrolysis of TMP is reason to reconsider some of the effects attributed to malonaldehyde. For example, the findings of Yau (1979) that malonaldehyde was both carcinogenic and mutagenic in mammalian cells were based on malonaldehyde produced by the acid hydrolysis of TMP. Marnett and Tuttle (1980) showed that 3,3-dimethoxypropionaldehyde, an intermediate in the acid hydrolysis of TMP, was more carcinogenic than malonaldehyde, thus calling into question the findings of Yau (1979). Other investigators (Shamberger et al., 1974; Mukai and Goldstein, 1976) have also reported the mutagenicity and carcinogenicity of malonaldehyde. In these studies, malonaldehyde was prepared by shaking TMP with Dowex 50, a procedure which also involves acid hydrolysis. However, these authors did not report the formation of any compounds other than malonaldehyde. It is quite possible that mixing TMP with Dowex 50 would produce 3,3-dimethoxypropionaldehyde and other compounds.

Gas liquid chromatography results suggested the possible formation of formaldehyde during acid hydrolysis of TMP. Kurechi et al. (1980) prepared malonaldehyde by TMP acid hydrolysis and found that it increased N-nitrosamine formation at pH values between 4 and 7 in model systems containing nitrite and secondary amines. Of all other compounds tested in this study, only formaldehyde possessed the ability to catalyze the N-nitrosation reaction. If formaldehyde were formed during the acid hydrolysis of TMP, then the effects that Kurechi et al. (1980) attributed to malonaldehyde could, in fact, be at least partially due to formaldehyde. Additional studies are necessary in

order to confirm the presence of formaldehyde as a product of the hydrolysis reaction.

Since TMP is not a precursor of malonaldehyde in biological systems, it is questionable whether observations made concerning the acid hydrolyzate of TMP, such as the presence of 3,3-dimethoxypropionaldehyde and malonaldehyde polymerization products, are relevant when applied to biological systems. The use of malonaldehyde prepared from malonaldehyde bis-bisulfite acid hydrolysis steam distillate, which contains less impurities, would probably reflect biological phenomena more accurately.

The reaction between malonaldehyde and glycine ethyl ester produced fluorescent conjugated Schiff bases regardless of the mode of sample preparation. Two major blue fluorescent components were formed with similar TLC, GLC, fluorescence, infrared spectroscopy, and mass spectrometry characteristics. These data suggest that the two components had similar structures. Further investigation is required for complete characterization, such as the use of nuclear magnetic reasonance analysis to determine whether these two components are geometrical isomers.

The TLC separation of the malonaldehyde-glycine ethyl ester reaction product prepared according to Chio and Tappel (1969a) also revealed compounds that appeared yellow on fluorescence, including component 4 (Figure 16, Table 5), the most intense fluorescent component of the product. This yellow fluorescence indicated that these components were not conjugated Schiff bases, but further investigation is needed in order to discern their specific structures.

Tne TLC analysis of the acid hydrolysis steam distillate-glycine ethyl ester reaction mixture indicated the presence of seven components in addition to the two previously mentioned. These compounds possessed fluorescence characteristics similar to those of the two major compounds and thus characteristic of conjugated Schiff base systems. Since the only compound in the steam distillate that could react with glycine ethyl ester to produce fluorescent compounds was malonaldehyde, either more than two fluorescent compounds could have formed by the reaction of the two compounds, or the malonaldehyde in the reaction mixture could have, beside reacting with glycine ethyl ester, undergone condensation reactions to form fluorescent polymerization products.

Since malonaldehyde is a bifunctional aldehyde it could possible react with one or two molecule of glycine. The 1:1 malonaldehyde: glycine reaction product proposed by Crawford et al. (1966) and Chio and Tappel (1969a), N-prop-2-enal aminoacetic acid ($OHC-CH=CH-NH-CH_2-COOH$), was synthesized by reacting TMP acid hydrolyzate and glycine for one hour at room temperature. The mixture was then filtered and the precipitate contained the 1:1 product, while the filtrate contained the 2:1 product. This manner of isolating the N-prop-2-enal aminoacetic acid does not seem satisfactory since the presence of three polar groups in the molecule seems to indicate that the compound is at least slightly soluble in aqueous solutions.

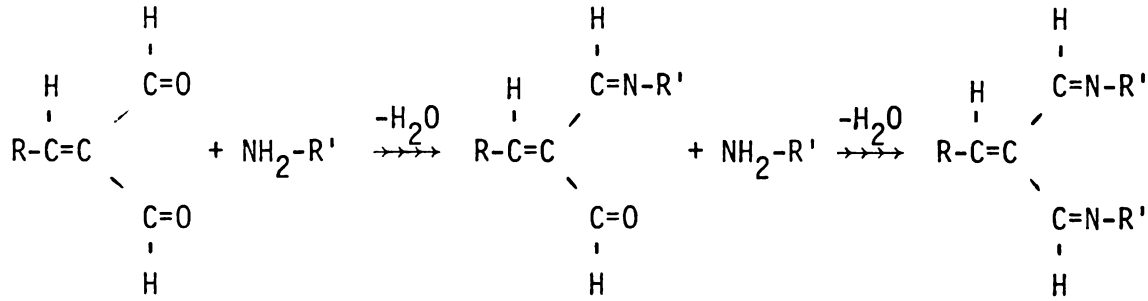
N-Prop-2-enal aminoacetic acid is not a conjugated Schiff base and thus would not fluoresce. Buttkus and Bose (1972) repeated the Crawford et al (1966) study and produced a fluorescent 1:1 reaction product for which they proposed the following structure,

HO-CH=CH-CH=N-CH_2-COOH. This structure had an electron donating group attached to an alkene carbon of a conjugated Schiff base and thus satisfied the criteria for conjugated Schiff base fluorescence later proposed by Malshet and Tappel (1973). Adhikari and Tappel (1973) proposed a similar type of structure for the fluorescent reaction product formed from glucose degradation products and malonaldehyde. The proposed 1:1 reaction product structures of Buttkus and Bose (1972) and Adhikari and Tappel (1973) were different from those of Crawford et al. (1966) and Chio and Tappel (1969a). The Tappel group has not explained the discrepancies of their proposed structures. Crawford et al. (1966) reported infrared spectroscopic evidence of an N-H group in the 1:1 reaction product, but the proposed conjugated Schiff base structures of the 1:1 reaction product (Buttkus and Bose, 1972; Adhikari and Tappel, 1973) do not contain this group. Since clarification of these conflicts in the literature requires further research, no conclusion can be made regarding the presence of a 1:1 fluorescent reaction product in either malonaldehyde-glycine ethyl ester reaction preparations.

The presence of fluorescent compounds in the non-reacted steam distillate of the malonaldehyde bis-bisulfite acid hydrolyzate showed that fluorescent polymerization products were probably formed. The major fluorescent polymer had the same R_f value as one of the two major fluorescent compounds of the steam distillate-glycine ethyl ester reaction product. However, the latter compound had an infrared absorption band characteristic of a C=N bond. The polymerization products could have been responsible for the malonaldehyde-glycine

ethyl ester reaction fluorescent components other than 6 and 7 but only one of the bands had an emission maximum similar to the major fluorescent emission maxima of the malonaldehyde polymerization product gel filtration bands separated by Gutteridge et al. (1977).

Polymers formed according to Buttkus (1975) could have reacted with glycine ethyl ester to produce conjugated Schiff bases as shown below:



Compound 1 Compound 2

Both compounds 1 and 2 fail to satisfy the structural criteria of Malshet and Tappel (1973) for fluorescence of conjugated Schiff bases but the resonance structures of these compounds were not considered in developing the criteria. The amount of pi bond character in these structures suggests that there is a good possibility that they would fluoresce and thus be responsible for some of the fluorescent compounds of the steam distillate-glycine ethyl ester reaction product. Further characterization of components 1-5 and 8 and 9 (Figure 13) is required.

Even if polymer-amine reaction products were responsible for the fluorescent compounds, the relevance of these type of fluorescent compounds to biological systems would be questionable because, as

already pointed out, it is not known if malonaldehyde produced in biological systems polymerizes. The existence of more than two fluorescent spots on the TLC plates of the autoxidized linolenic acid glycine ethyl ester model systems studies, however, indicated that 2:1 amine-malonaldehyde reaction compounds are not the only fluorescent compounds that could be formed in biological systems.

The structural criteria for fluorescent conjugated Schiff bases proposed by Malshet and Tappel (1973) have been questioned by the results of the present study and the previously discussed results of Rao and Dugan (1972) and Buttkus and Bose (1972). Further clarification of what exactly must be present in a Schiff base structure in order for it to exhibit its characteristic fluorescent properties is of increasing importance as these types of compounds may be useful indicators of biological phenomena.

SUMMARY AND CONCLUSIONS

Malonaldehyde-glycine reaction products were separated by Bio-Gel P-2 gel filtration chromatography into eight fluorescent bands, three of which had fluorescent excitation and emission maxima at 390 nm and 468 nm respectively. Monitoring of the column eluant by fluorescence proved unsatisfactory since eluant fractions with the highest fluorescence intensity probably did not contain the highest concentration of fluorescent product. The reaction product was found to adsorb to the column packing, thus making this separation technique unsatisfactory.

Malonaldehyde was prepared by TMP acid hydrolysis and the hydrolyzate was analyzed by gas-liquid chromatography and mass spectrometry. The hydrolyzate was found to contain malonaldehyde, TMP, 3,3-dimethoxypropionaldehyde, and 2-formyl-3-hydroxypentanedial. An unidentified component in the hydrolyzate was found to have a GLC retention time greater than the dimer and was probably a trimer derived from malonaldehyde. All of the above GLC peaks increased in area with hydrolysis time except the TMP peak. When TMP acid hydrolysis was allowed to take place at 25^{o}C for 18 hr, other GLC peaks with retention times greater than the ones already mentioned were obtained from the hydrolyzate sample. These peaks were possibly formed from polymerization of malonaldehyde.

Malonaldehyde was also prepared from its bis-bisulfite salt by acid hydrolysis of the salt, followed by steam distillation. The steam distillate was analyzed by GLC and shown to contain three components, the major one being due to malonaldehyde and the other two tentatively identified as methanol and formaldehyde. The steam distillate was also separated by silica gel G thin layer chromatography and shown to contain one TBA reactive spot.

The acid hydrolyzate of TMP and malonaldehyde bis-bisulfite acid hyrolysis steam distillate were both reacted with glycine ethyl ester and the ethyl acetate fractions of the reaction products were analyzed by thin layer chromatography. Both reaction products were found to contain two major blue fluorescent components, the structures of which were determined to be conjugated Schiff bases by their fluorescence characteristics and their infrared absorption bands characteristic of C = C and C = N bonds. The steam distillate derived sample also contained seven other fluorescent components, all with fluorescent characteristics similar to conjugated Schiff bases. The acid hyrolyzate derived sample contained eight other components, many of which fluoresced yellow and thus were probably not due to conjugated Schiff bases. Steam distillate was found to form fluorescent products when stored at 25^{o}C for five hours. These were probably malonaldehyde polymerization products.

Model systems containing linolenic acid and glycine ethyl ester stored at 40^{o}C for two weeks were analyzed after zero, seven and fourteen days by fluorescence and the TBA test. These two parameters increased with time of storage. Fluorescent components of the model

system were separated by thin layer chromatography and two of the major components had R_f values similar to the two major fluorescent components of the steam distillate-glycine ethyl ester reaction product.

The conclusions reached as a result of this study are summarized below:

1. TMP acid hydrolyzate-glycine reaction product adsorbs to Bio-Gel P-2 filtration packing, thus making gel filtration separation of this product unsatisfactory.

2. TMP acid hydrolysis produces malonaldehyde, 3,3 dimethoxy-propionaldehyde, 2-formyl-3-hydroxypentanedial, and other compounds that can be separated by GLC.

3. Malonaldehyde bis-bisulfite acid hydrolysis steam distillate is a purer source of malonaldehyde than TMP acid hydrolyzate.

4. Steam distillate-glycine ethyl ester reaction product can be separated by TLC into nine distinct components, all having fluorescence properties characteristic of conjugated Schiff base compounds.

5. Seven of the components have three excitation maxima 221-233 nm, 257-269 nm, and 369-397 nm.

6. The two major fluorescent components are conjugated Schiff bases.

7. Fluorescence and TBA value both increase with increased autoxidation of linolenic acid-glycine ethyl ester model system emulsions.

8. Model systems containing autoxidized linolenic acid and glycine ethyl ester produce fluorescent compounds, two of which are the same as the two major fluorescent compounds of the steam distillate glycine ethyl ester reaction product.

PROPOSALS FOR FUTURE RESEARCH

This study has raised many questions. Some relevant possible research topics that have evolved from the results and observations of this study are discussed below.

1. Malonaldehyde bis-bisulfite salt acid hydrolysis steam distillate was used in this study as a source of malonaldehyde for the malonaldehyde-glycine ethyl ester reaction. Summerfield and Tappel (1978) developed an enzymatic method for making malonaldehyde, using propanediol as the substrate and alcohol dehydrogenase as the enzyme. It would be interesting to prepare malonaldehyde in this manner and analyze it by gas-liquid chromatography to determine the purity of the preparation.

2. It would also be interesting to separate the steam distillate-glycine ethyl ester reaction products by gel filtration chromatography according to Chio and Tappel (1969a). Since the steam distillate did not contain malonaldehyde polymerization products, less of these would probably be produced in the steam distillate-glycine reaction product than if the procedure of Chio and Tappel (1969a) were used. As the present study showed, in the latter procedure there were probably malonaldehyde polymerization products present in the malonaldehyde used for conjugated Schiff base production. If there were less

malonaldehyde polymers present in the reaction product sample, there would possibly be no adsorption to the gel filtration column packing. If gel filtration chromatography could be used with no adsorption to the column packing more reaction product could be separated than with TLC because of the greater sample capacity of the former technique. Also, the molecular weight of the compounds could be estimated if gel filtration chromatography could be used successfully.

Fluorescent bands separated by gel filtration chromatography should not be detected by monitoring the eluant by fluorescence measurement at specific wavelengths. The separating column should be illuminated by 366 nm radiation and fluorescent bands collected as they are eluted out. If the absorption maxima of the fluorescent bands could be determined, the eluate could possibly be monitored by absorption as done by Chio and Tappel (1969a). Absorption is not as much affected by concentrated colored solution as fluorescence (Udenfriend, 1962).

3. Since the steam distillate-glycine ethyl ester reaction product was separated by TLC, the use of a column containing the adsorbent Silica Gel G or silicic acid should be investigated. A column of this type eluted with butanol: acetic acid: water (80:20:20) would probably separate the reaction product.

4. The fluorescent conjugated Schiff bases were not successfully characterized by mass spectrometry. Improvements in the isolation and sample preparation of these compounds are needed. Purification by the techniques already discussed could be attempted. Since larger amounts of product could probably be obtained using these techniques, nuclear magnetic resonance analysis could possibly be used to determine if

geometrical isomers are present. Samples could be prepared for mass spectrometry analysis by sodium borohydride reduction of the double bonds of the conjugated Schiff bases. The ions produced by these saturated compounds could possibly fragment into ions that could be better reconstructed to discern structure.

5. A solvent system should be found that would better separate the aqueous fraction of the malonaldehyde-glycine ethyl ester reaction product so that the entire reaction product could possibly be characterized and quantitated. The system used would have to be determined by trial and error. One would probably use a less polar system than the one used in this study because it would probably have a better partitioning effect on a polar sample.

6. Kwon and Watts (1964) extracted cooked fish samples with an aqueous solution of sodium bisulfite and analyzed the malonaldehyde content of the sample using the TBA test. These workers found that excess free sodium bisulfite in the extract reduced the amount of TBA chromogen and could not find a simple way to overcome this problem. This problem could possibly be solved by hydrolyzing the extract solution with acid and collecting the malonaldehyde by steam distillation of the acidified solution. The distillate could be analyzed by GLC to determine its components and the relative amount of malonaldehyde present could be compared with the relative amount present in other samples. Alternatively, if the only TBA reactive compound in the steam distillate were malonaldehyde, a TBA test could be run. Thus, the TBA test could possibly be used to quantitate the amount of malonaldehyde, but not malonaldehyde precursors and any other TBA

reactive material, present in a meat extract sample. Possibly other food systems could be subjected to this procedure.

7. Malonaldehyde preparations should be analyzed by high resolution mass spectrometry, which enables detection of specific ion masses below m/e 40, to determine whether methanol and formaldehyde are present in TMP acid hydrolyzate.

8. Model systems should be prepared using linolenic acid levels between the levels used in this study. The intervals between sample storage times should be shortened in order to obtain more detailed data regarding the relationship between fluorescence and TBA value.

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