STUDIES ON THE NATURE OF THE 2-THIOBARBITURIC ACID REACTIVE MATERIAL IN RANCID FAT

Thesis for the Degree of M. S.
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John Robert Quinn

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By

John Robert Quinn

AN ABSTRACT

Submitted to the College of Agriculture
Michigan State University of Agriculture and
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the requirements for the degree of

MASTER OF SCIENCE

Department of Animal Husbandry

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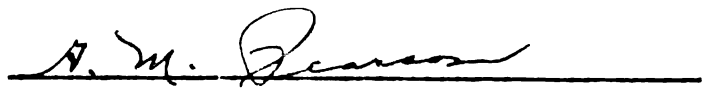
Approved: 

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AN ABSTRACT

The purpose of this research project was to characterize the thiobarbituric acid (TBA) reactive material occurring in rancid samples. Since the TBA reactive material could not be extracted from rancid samples with the common fat solvents, it was decided to specifically study the TBA test positive, solvent resistant lipid-protein complex formed from a linoleic acid-soluble pork protein model system.

Because the lipid-protein complex could not be broken without changing the structure of the lipid fraction, the original purpose of the project was abandoned. Efforts to characterize the TBA reactive material were hence centered on the study of the acetone extracts from the crude complex and on linoleic acid emulsions oxidized in the absence of protein.

Removal of the aldehydes and methyl ketones from these oxidized lipid solutions by addition of excess sodium bisulfite and collection of the carbonyl-bisulfite addition product formed, resulted in only a small decrease in the TBA value of the solutions. Thus it was indicated that the TBA reactive material in rancid products is mostly due to a class of compound other than an aldehyde or methyl ketone.

To determine whether or not the TBA reactive carbonyl in the bisulfite addition product was malonaldehyde, the carbonyls were liberated from the bisulfite compound. Volatile carbonyls were collected and analyzed for TBA value. The results obtained indicated that there was an insignificant amount, if any, of malonaldehyde present.

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TABLE OF CONTENTS

INTRODUCTION

The thiobarbituric acid test has found extensive use in the past few years as a measure of fat oxidation. Patton and Kurtz (1951) in a preliminary study found that the thiobarbituric acid (TBA) test is a more sensitive measure of oxidative deterioration in pure milk fat than conventional tests such as iodine number and the Kreis test. Dunkley (1951) demonstrated that the TBA test was closely correlated with numerical flavor scores of milk samples having oxidized flavor of varied intensity. Biggs and Bryant (1953) on the basis of studies on butter, whole milk powder and cheese stated that the TBA test was capable of measuring the degree of oxidation below the level of organoleptic sensitivity. Sidwell et al. (1955) have shown a direct relationship between flavor scores and TBA values of dried milk products. Working with frozen pork, Turner et al. (1954) reported that the method gave a more reliable index of rancidity than any other chemical test, and showed a significant correlation between taste acceptability scores of weiners and pork patties and the TBA value of the pork used. Sidwell et al. (1954) demonstrated that the test differentiates cottonseed and soybean oils on the basis of stability more effectively than does either the total carbonyl or peroxide tests. The oxidation of fat in a wide variety of fishery products was satisfactorily determined by the TBA method as modified by Yu and Sinnhuber (1957). Ryan and Stansby (1959) correlated TBA values and organoleptic scores of oxidizing fresh fish.

Other workers have used the test to measure rancidity in lard (Romero and Gonzalez-Quijano, 1957), in cooked oyster tissue (Schwartz and Watts, 1957), and in cereal and baked goods (Caldwell and Grogg, 1955).

Although the TBA test does appear to give a reliable index of fat oxidation for some food products, it has not as yet been accepted as an index for others. The reason why the test gives a satisfactory indication of rancidity in some products and not in others remains obscure. Also obscure is the identity of the TBA reactive compound as it exists in rancid samples. It is certainly possible that identification of this compound would result in an explanation for the apparent inconsistency of the test. Obviously, its identification would also throw some light on the area of the fatty acid oxidative breakdown process which is still largely unresolved. Thus, the purpose of this research project, was to further study the TBA reactive material present in oxidized fat.

REVIEW OF LITERATURE

The Thiobarbituric Acid Test Reaction

Kohn and Liversedge (1944) observed that various animal tissues after incubation under aerobic conditions produced a compound which formed a color complex when reacted with TBA. They suggested that the TBA reactive material was a carbonyl compound since the reaction was blocked by semicarbazine or phenylhydrazine. Bernheim *et al.* (1947) concluded that the color obtained by Kohn and Liversedge was due to an oxidation product of unsaturated fatty acids, particularly linolenic acid. They reported the isolation of an impure red TBA pigment and suggested that the reactive compound was a 3-carbon chain containing an aldehyde or ketone group. Patton and Kurtz (1951) tested a number of substances and concluded on the basis of spectral measurements that the compound responsible for the TBA reaction was malonaldehyde and that it existed in rancid milk fat. In a paper regarding the Kreis color reaction for rancid fats, Patton *et al.* (1951) presented evidence which suggested that epihydrin aldehyde was not necessarily responsible for the Kreis test. Malonaldehyde was shown to give a positive reaction in the Kreis test and the resulting color was demonstrated to be spectrally similar to the Kreis colors obtained with epihydrin aldehyde, rancid lard, and oxidized milk fat. The characteristics of a water soluble, low molecular weight, Kreis positive, carbonyl compound from oxidized milk fat were observed to be very similar to those expected of malonaldehyde, being strongly acidic, enolic, and relatively stable to heating with dilute mineral acids.

Kurtz et al. (1951) tentatively identified the compound in oxidized milk fat that is responsible for the results of the TBA test as malonaldehyde and postulated that the reaction with TBA occurred by attack of the monoenolic form of malonaldehyde on the active methylene groups of TBA, followed by ring closure.

Kohn (1945) reported that TBA was a specific reagent for the sulfonamide drug, sulfadiazine (2-sulfanilamidopyrimidine). He suggested that the color-forming reaction involved the amino pyrimidine moiety. Shepherd (1948) later established that the color reaction involved the pyrimidine ring specifically, providing there were no substituents in the 4-, 5-, or 6- position. Jennings et al. (1955) noted that although the pigments prepared from TBA and oxidized milk fat, malonaldehyde, and sulfadiazine exhibited the same spectral characteristics in the visible range, this fact did not justify the conclusion that the compounds were identical. A spectrophotometric technique suggested that the malonaldehyde pigment was formed as an equi-molar reaction without loss of water.

Sinnhuber and Yu (1958) described a method for the quantitative determination of malonaldehyde with TBA using 1, 1, 3, 3-tetraethoxypropane (TEP) as a standard. Acid hydrolysis of TEP yields malonaldehyde quantitatively which then reacts with TBA. In a more recent study, Sinnhuber et al. (1958) prepared crystalline TBA pigment from rancid salmon oil, sulfadiazine and malonaldehyde. The results obtained by elemental analysis, absorption spectrophotometry, and paper chromatography all suggested the pigments to be identical. The data indicated that the crystalline pigment was a condensation

product of one molecule of malonaldehyde with two molecules of TBA and the probable elimination of two molecules of water. Structural and empirical formulas were proposed. No explanation as to the mechanism of the cleavage of the pyrimidine ring of sulfadiazine to yield a 3-carbon fragment was offered.

Existence of TBA Reactive Material in Rancid Samples

Solvent extraction of the TBA reactive material from rancid samples prior to reaction in the TBA test has been attempted. Patton and Kurtz (1951) found that the milk fat residue left after exhaustive extraction with hot water still gave a strong positive TBA test. An attempt was made by Yu and Sinnhuber (1957)to extract the reactive material from oxidized fish meal using methanol-benzine, ethyl ether-petroleum ether, and acetone solvent systems. Because only very little of the reactive substance was extracted by any of these solvent systems, they suggested that it had reacted with protein or itself to form a compound resistent to solvent extraction.

Sinnhuber et al. (1958) have pointed out the following four points for consideration. (1) The oxidative origin of malonaldehyde in rancid fats or actual proof of this carbonyl as the free compound still remains uncertain. (2) Powick in 1928 explained the Kreis test as due to the reaction between phloroglucinol and epihydrin aldehyde, and suggested that the unstable aldehyde probably existed in fat as an acetal compound since he was unable to isolate the free compound. (3) Lea in 1939 stated it was possible that the

aldehyde did not exist, even as an acetal in the fat, but was formed from some precursor, possibly a peroxide, under the influence of the acid used in the Kreis test. (4) Patton et al. (1951) concluded that malonaldehyde, not epihydrin aldehyde, was the compound responsible for both the Kreis test and the TBA test.

Thus, it is possible that the TBA reagent on contact with rancid fat could hydrolyze an acetal or decompose a precursor to give the reactive compound malonaldehyde. Sinnhuber et al. (1958) attempted to answer the question of the presence or absence of free malonaldehyde in rancid fat. In their experiment, rancid salmon oil was held at room temperature while a stream of nitrogen carried any volatile material through a reflux condenser and into a flask containing a solution of TBA. Results indicated that a small amount of malonaldehyde (2.2% of the total amount) probably was present as the free carbonyl. When acid was added to the rancid oil, large quantities of volatile TBA reactive material were produced. This observation indicated to the authors that the reactive material was present as a larger molecule before the acid was added, and again could be explained by the acetal theory of Powick or the precursor theory of Lea.

Lipid - Protein Complexes

Proteins are known to form complexes with a wide variety of substances such as gossypol, phospholipids, sterols, and various other lipid materials. Most of these complexes are labile and can be easily ruptured to yield the constituent molecules in their original

form by use of simple techniques, such as extraction with a suitable solvent system. Folch et al. (1951) have reported the existence of a strongly bound lipoprotein in brain tissue which is resistent to solvent action.

Tappel (1955) observed that unsaturated fatty acids could react with proteins forming stable complexes which could not be ruptured by digestion with papain or by use of ethanol, xylene or ethyl ether solvent treatments. He formed these complexes by agitating a solution of protein with an excess of linoleic acid in the presence of a hematin catalyst at 37°C. According to Tappel, the stability of these complexes was caused by a chemical union between the reactive amino groups of the protein and the aldehydes produced during oxidation of the lipid. Later publications by Narayan and Kummerow (1958) and Venolia and Tappel (1958) showed that the active carbonyl-amino browning mechanism was not responsible for the stability of the complexes. Narayan and Kummerow formed stable complexes using egg albumin and oxidized linoleic acid. Little or no complex could be formed from unoxidized linoleic acid, oleic acid, or lauric acid. Pure solutions of acetaldehyde, nonaldehyde, and lauryl aldehyde were found to complex with the albumin, but only to a very small extent. A possible structure on the basis of a large number of hydrogen bonds was proposed for the lipid-protein complex. Presumably, the hydroxy, hydroperoxy, and carbonyl groups of the oxidized lipid contributed to the hydrogen bonding.

Specificity of the TBA Test

Wilbuy et al. (1949) concluded that in the absence of ultraviolet light, the TBA test might be considered primarily as an index of the amount of oxidized linolenic acid present and perhaps still unidentified C_{20} acids. They found that oxidized linoleic acid produced much less color than the oxidized linolenic acid, that oxidized methyl arachidonate produced color only after exposure to ultraviolet light, and that oxidized oleic and stearic acids were inactive when reacted with TBA. In a later paper Kenaston et al. (1955) reported the TBA test to be relatively insensitive to the oxidation products of oleic acid, but the most sensitive of all the chemical methods used for the oxidation products of linolenic and linoleic acids.

From the literature presented, the following observations seem significant.

(1) The TBA test is specific for an oxidation product of the polyethenoic acids linoleic and linolenic.

(2) The TBA reactive material cannot be removed from rancid samples by solvent extraction.

(3) Oxidation products of at least one polyethenoic acid are responsible for the formation of solvent resistant, lipid-protein complexes.

The TBA reactive material, malonaldehyde, may then exist in rancid samples in a highly polymerized, solvent resistant form and/or in a combination with protein. It may exist in these forms as itself, as its oxidation precursor, or as an acetal. In order to characterize

the form in which the reactive material is present in oxidized samples an investigation of the lipid-protein complex would appear to be the logical field of study.

EXPERIMENTAL PROCEDURE

Preparation of the Lipid-Protein Complex

The Protein Fraction

Water-soluble pork protein was used in the formation of all complexes. Samples of ground pork were removed from freezer storage and allowed to thaw in distilled water. When thawed, the samples were thoroughly dispersed in the water by use of a Waring blendor. The viscous suspension was then poured into 250 ml. centrifuge tubes and centrifuged at 2300 r.p.m. for 10 minutes. The fat layer was removed from the top of the tubes with a spatula and the liquid was filtered. The bright red filtrate was stored at approximately 2°C. for a very short time before use. The residue was again extracted with distilled water and the resulting filtrate added to the first. No special precautions were taken to avoid denaturation.

The Protein-Lipid Reaction

Between 25 and 30 grams of 75% linoleic acid (Nutritional Biochemicals Corporation) were added to the protein solution while stirring. The protein concentration of the reaction solution varied between 1.0 and 1.5 grams per 100 mls. The mixture was stirred either by means of a mechanical stirrer or with a magnetic stirrer and was exposed to air during the entire reaction period. Heat was applied either by a hot water bath or with an electric oven. The temperature of the mixture during the reaction period varied from 30 to 50°C. At least 24 hours were allowed for development of the crude complex.

The conditions of temperature, pH, time, lipid and protein concentration and state of protein denaturation have been thoroughly studied by Narayan (1957). No attempt was made in this project to control and study these conditions. On the contrary, formation of the complexes under randomized conditions was deemed advisable for this study.

Solvent Extraction

As mentioned previously, the TBA reactive material in rancid samples cannot be extracted with common fat solvents. Hence, it was desirable that as much of the fat be removed from the complex as possible using ordinary fat solvents. The most complete removal of the weakly bound lipid was accomplished by the method of Narayan and Kummerow (1958). The crude complex was separated from the reaction mixture by filtering with a Buchner funnel. It was then dispersed in acetone and stored at room temperature for 24 hours with occasional agitation. The product was separated by filtration, then extracted in a Goldfisch apparatus for 24 hours with acetone, followed by an additional 24 hour extraction with ethyl ether. In most cases, the ether extraction was unnecessary. The residual lipid-protein complex was dried in a 37°C. oven and stored in a dry, cool place.

Attempts to Break the Lipid-Protein Complex

It was observed on testing the complexes with TBA that a relatively high value was obtained, although all samples contained less than 10% lipid. Thus, it was apparent that the lipid fraction of

the complex was rich in the TBA reactive material. With the aim of studying this fraction and the eventual isolation of the reactive material, attempts were made to split the lipid fraction from the protein in such a way as not to damage or rearrange the lipid structure.

Narayan and Kummerow (1958) have postulated hydrogen bonding as the complex binding force. Thus, a series of solvents not commonly used for fat extraction but exhibiting special hydrogen bond breaking properties were used in an attempt to split the complex. These solvents included ethylene glycol, dimethylformamide, acetonitrile, nitromethane, 1-4 dioxane and an aqueous solution of urea. The complex was agitated for 24 hours in the chosen solvent using a magnetic stirrer. The complex was removed by filtration and stirred another 24 hours with a solution of 30 parts ethyl alcohol and 70 parts ethyl ether.

To eliminate the possibility that the hot air drying step may have had an effect on the solvent resistancy of the complexes, a freeze-dehydration technique was used in some instances. The complexes were dispersed and frozen in water immediately after the 24 hour ethyl ether extraction period. They were later thawed and refrozen in layers in a 250 ml. centrifuge tube to await freeze-dehydration. The dried samples were then tested with the various solvents under study as described previously.

Analytical Methods

The TBA Test

The procedure used was the same as that of Yu and Sinnhuber (1957) with only slight modification.

Reagents:

Trichloroacetic acid (TCA) solution. 20 g. of reagent grade TCA were dissolved in distilled water and made up to 100 mls.

Pyridine hydrochloride solution. 30 ml. of pyridine, reagent grade, were mixed with 70 ml. of 6N HCl.

2-Thiobarbituric acid reagent. The reagent was prepared according to Kohn and Liversedge (1944) except that the charcoal treatment was omitted. A 1% TBA solution was obtained by mixing 2 g. of TBA (Eastman product), 193 ml. water and 6.6 ml. of 2N NaOH. The mixture was stirred until the TBA dissolved, then was placed in a hot water bath for several minutes and 0.7 ml. 4N HCl was added. The completed reagent was made by mixing 2 parts of TBA solution with 1 part of a citrate buffer. The citrate buffer contained 29.5 g. reagent sodium citrate and 25 ml. concentrated HCl in 200 ml. solution. The pH of the completed reagent was adjusted to 26.

HCl-TCA-pyridine reagent. 650 ml. of 0.6N HCl were mixed with 50 ml. of the TCA solution. 50 ml. of pyridine solution were then added.

Procedure:

Less than 1 gram of sample was weighed into a 250 ml. round bottom boiling flask. 3 ml. distilled water, 10 ml. TCA solution,

5 ml. pyridine solution and 6 ml. TBA reagent were added without shaking the flask. The flask was connected to a cold water condenser and placed in a vigorously boiling water bath. In exactly 30 minutes, 75 ml. HCl-TCA-pyridine solution were added through the top of the condenser. The flask was shaken several times and was allowed to reflux an additional 10 minutes. The flask was disconnected from the condenser and placed in a cold water bath until it attained room temperature. About 40 ml. of the solution was centrifuged for 10 minutes at 2500 r.p.m. 15 ml. of the clear solution was measured into another centrifuge tube and 10 ml. of petroleum ether was added. The tube was shaken vigorously for $\frac{1}{2}$ minute and then centrifuged for 5 minutes at 2500 r.p.m. 5 ml. of the colored aqueous layer was drawn off using a pipette and placed in a cuvet. Color density was determined in a Bausch and Lomb Spectronic 20 spectrophotometer at 535 mμ, using a reagent blank as reference. The results were expressed in milligrams of malonaldehyde by using the straight line relationship between optical density and tetraethoxypropane concentration ascertained by Sinnhuber and Yu (1958).

Peroxide Determination

Three aliquots of the lipid material dissolved in ethyl alcohol and water were removed from the stoppered flask. One of the aliquots was placed in a previously dried and weighed beaker. The solvent was evaporated off in a 37°C. oven and the beaker was then placed in a 100°C. oven for 30 minutes. The weight of this sample was used in the calculations. The other two aliquots were placed in 250 ml. Erlenmeyer flasks. 10 ml. glacial acetic acid and 1 ml. of saturated

potassium iodide were added. The flask was swirled for exactly 1 minute. 30 ml. distilled water were then added and the contents titrated against a standardized sodium thiosulfate solution using starch indicator. The peroxide content of the sample was expressed as milliequivalents of peroxide per 1000 grams fat

(equals $\frac{\text{ml. } Na_2S_2O_3 \text{ X N}}{\text{weight of sample}}$ X 1000 where N is the normality of the $Na_2S_2O_3$ solution).

Estimation of lipid content in the complexes

The lipid content of the complex was estimated according to the procedure of Narayan and Kummerow (1958). 2 to 3 g. of the product were hydrolyzed in a 10% aqueous solution of potassium hydroxide for 10 hours. The contents were then acidified with HCl and extracted two or three times with ethyl ether. The residue obtained upon evaporation of the ether was taken as indicative of the amount of complexed fatty acid.

The Separation of Carbonyls from Other Oxidation Products

The acetone extracts from the crude complex also contain considerable TBA reactive material. In an attempt to separate the various oxidation products into general classes and thereby characterize the reactive material, sodium bisulfite was added. Aldehydes and some methyl ketones form addition products with the bisulfite and can be separated from a solution of alcohols, hydrocarbons, carboxylic acids and esters by adding an excess of the bisulfite and collecting the crystalline salt formed.

Distilled water and ethyl alcohol were added to the acetone extracts. Since acetone forms the bisulfite addition compound, it was evaporated off from the mixture in a 37°C. oven. The resulting solution had a peroxide value of 528.3. Excess sodium bisulfite was added while the extract was stirred with a magnetic stirrer. After 1 hour the salt formed on adding the bisulfite was allowed to settle and was collected by filtration. The salt was purified by washing twice in a large volume of 30 parts ethanol and 70 parts ethyl ether. The addition compound was then dried in a 37°C. oven.

Separation of the aldehyde and methyl ketone fraction from an oxidized emulsion of linoleic acid was also attempted. The emulsion contained 24 g. 75% linoleic acid and 130 mg. ascorbic acid, to catalyze oxidation, in 500 ml. of KH_2PO_4 - NaOH buffer at pH 6.0. 150 ml. of 95% ethanol were added to emulsify the mixture. The emulsion was heated at approximately 45°C. for about 3 days and had a final peroxide value of 519.3. Excess sodium bisulfite addition and collection, purification and drying of the addition product followed the procedure outlined.

The dried addition products were tested for TBA value and an estimate made of the percent of TBA reactive material removed from solution in the bisulfite compound.

Liberation of Carbonyls from the Bisulfite Addition Product and Testing for Malonaldehyde

A 2-necked 500 ml. boiling flask containing TBA reagent and 0.6 N HCl was connected to 2 condensers (water temperature 15°C.)

and placed in a boiling water bath. One end of a length of plastic tubing was inserted down through one of the condensers and into the TBA solution. The other end was connected to a 1000 ml. Erlenmeyer flask containing 120 ml. distilled water. A dry mixture of the bisulfite addition compound and Na_2CO_3 in the ratio 1 mole to $\frac{1}{2}$ mole was added to the Erlenmeyer flask which was then tightly stoppered but leaving openings for a stream of nitrogen to pass into the flask and carry any volatile carbonyls over into the TBA solution. The flask containing the liberated carbonyls was held at room temperature. The process was allowed to continue for 1 hour after which both the TBA solution and the solution containing the reactants were analyzed for TBA value.

To check the apparatus and method for efficiency in determining the presence of malonaldehyde a standard was run. Tetraethoxypropane was hydrolyzed with 0.6 NACl. The solution was brought to nearly neutral pH with NaOH and an excess of bisulfite was added. After stirring for 1 hour, the addition compound was collected, dried and run through the same process as described.

RESULTS AND DISCUSSION

Relationship Between the Lipid-Protein Complex and TBA Values

In the linoleic acid-soluble pork protein model system used in these studies, very little of the TBA reactive material (roughly 3%) was so firmly bound to the protein that it could not be removed with ordinary fat solvents. This was somewhat surprising since Yu and Sinnhuber (1957) had been able to extract only a relatively small amount of the reactive material from oxidized fish meal using the common fat solvents. The aqueous portion filtered from the crude linoleic acid-protein complex contained roughly 28% of the reactive material and the acetone extracts contained approximately 69%. However, on the basis of lipid content, some of the complexes were relatively rich in the reactive material. It is possible that much of the TBA reactive material in the acetone extracts could have been formed during the extraction procedure.

Narayan and Kummerow (1958) found that under no conditions could the amount of complexed linoleic acid in the complex be increased to more than 8%. Estimated lipid contents and TBA values of several of the complexes formed in this study are presented in the following table. There appears to be no relationship between these factors.

Table 1
Lipid Content of Complexes and TBA Values

Complex	Estimated Lipid Percentage	TBA Value (per gram of complex)
1	2.72	2.10×10^{-2}
2	2.79	2.36×10^{-2}
3	7.08	1.27×10^{-2}
4	9.77	1.76×10^{-2}

Attempts to Break the Lipid-Protein Complex and Incorporation of Bisulfite Ion

The lipid-protein complexes were found to be extremely stable, the lipid being unextractable with both polar and non-polar solvents. Narayan (1957) extracted an egg albumin-linoleic acid complex in a Soxhlet apparatus with ether for 10 days without success. He also used benzene, Skelly F. methanol, absolute alcohol and dioxane for 24 hours with the same result. Heating the complex with distilled water on a steam bath again resulted in his failure to break the complex.

In this experiment various solvents with hydrogen-bond breaking properties were tested. However, all of the solvents tested proved inadept at breaking the complex. The freeze-drying technique used to replace oven drying of the complexes did not result in making them less resistant to breakage.

At this point attempts to break the lipid-protein complex by solvents were discontinued. Both acid and base hydrolysis will break the complex, but in the first case, malonaldehyde would be formed from the possible precursor or possibly from acetal, and in the second case, the lipid structure would be rearranged from the naturally-occurring form.

Venolia and Tappel (1958) introduced the sulfite ion into a lipid-protein emulsion to make the active carbonyl groups of the oxidizing lipid unavailable for reaction with the protein. They stated that the addition of the sulfite ion did not inhibit formation of the complex. If carbonyls are present in the complex and

if they have been tied up by addition of the sulfite ion, they perhaps they would be stabilized during alkaline hydrolysis and could be characterized. To test this possibility, 5% sodium bisulfite was added to a lipid-protein emulsion. On KOH hydrolysis of the complex formed (TBA value of 2.39×10^{-2}/g. and lipid content of 8.22%) however, the hydrolyzate gave a negative TBA test (see Figure 1B). This negative result indicated that the TBA reactive material was not a carbonyl, or that the carbonyl in the complex was not tied up by bisulfite, or that bisulfite did not stabilize the carbonyl during alkaline hydrolysis. No further attempts were made to characterize the TBA reactive material present in the lipid-protein complex.

Observed Spectral Characteristics of TBA Solutions Studied

On reacting tetraethoxypropane or oxidized samples with TBA a clear red color is obtained. In this study a brownish color was observed in some of the TBA test solutions. The absorption spectra of 3 of the TBA test solutions studied are presented in Figure 1.

(A) was obtained from an acetone extract. The color of the TBA test solution was dark orange-red.

(B) was obtained from the KOH hydrolyzed complex as previously mentioned. It was brown in color.

(C) was obtained from a lipid-protein complex. The TBA test solution exhibited the characteristic clear red color of the TBA-malonaldehyde color compound. It is not known what the brown substance with maximum absorption at 460 mμ is but it obviously does not interfere with the TBA value.

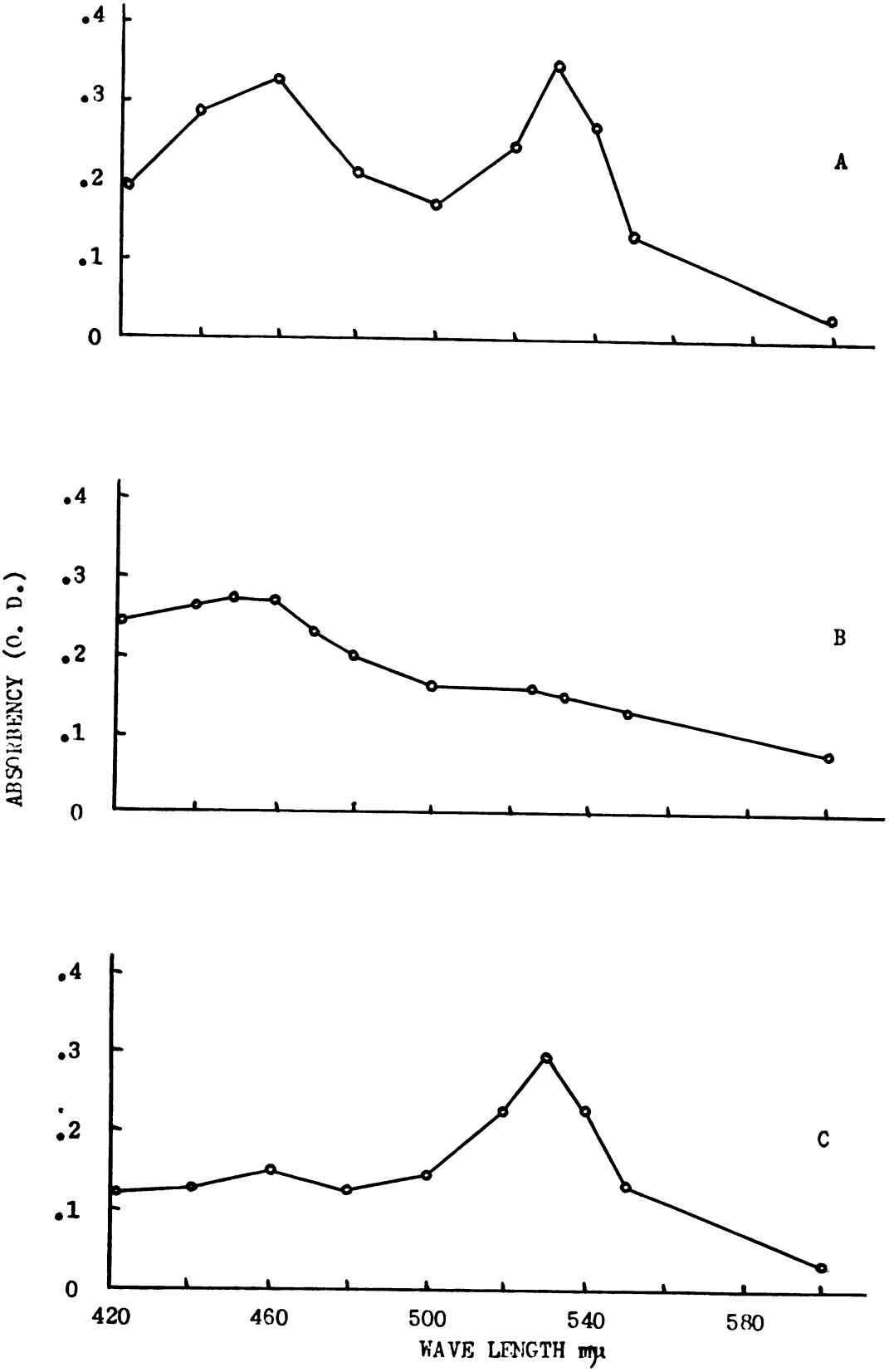

Figure 1.

Studies on the Existence of TBA Reactive Aldehydes and/or Methyl Ketones in Lipid Solutions

Attention was focused on studying the character of the reactive material in the acetone extracts of the crude complex and in an emulsion of linoleic acid oxidized in the absence of protein. If malonaldehyde, or an aldehyde or methyl ketone precursor to malonaldehyde exists in these samples, then the TBA reactive material should be removed by bisulfite addition and salting out. As can be seen from table 2, very little of the TBA reactive material was removed from the rancid sample.

Table 2
Percent TBA Reactive Material Removed by Bisulfite

Lipid Solution	TBA value in total volume of solution	TBA value in total weight of Bisulfite addition compound	% TBA reactive material removed from solution
Acetone extracts	2.55	1.68×10^{-1}	6.59
Oxidized linoleic acid solution	1.70	8.62×10^{-2}	5.07

These results indicate that if malonaldehyde, or an aldehyde or methyl ketone precursor does occur in oxidized samples, it occurs in small quantities.

In order to determine whether or not the TBA reactive material removed by bisulfite addition was malonaldehyde, the bisulfite was neutralized by Na_2CO_3 to liberate the carbonyls. Malonaldehyde, if present, would then volatilize off at room temperature. Table 3 indicates that possibly a very small amount of malonaldehyde was present in the bisulfite addition compound.

Table 3

Presence of Malonaldehyde in Bisulfite Addition Compound

Bisulfite Compound formed from	TBA Value in: amount bisulfite compound tested	TBA Value in: volatiles collection flask	TBA Value in: $NaHSO_3 - Na_2CO_3$ reaction flask	% TBA Reactive Material Volatilized	pH of reaction flask
malonaldehyde	1.53×10^{-3}	1.76×10^{-3}	0.00	115.00	4.8
acetone extract (1)	1.07×10^{-1}	1.27×10^{-3}	----	1.19	9.1
acetone extract (2)	4.84×10^{-2}	too little to measure	----	0.00	8.4
oxidized linoleic acid solution	3.45×10^{-3}	---	2.82×10^{-3}	18.27	8.6

Contrary to expectation, the addition of HCl after 1 hour to acidify the solution containing the non-volatile carbonyls did not increase the TBA value in the flask used for collection of the volatiles. No explanation other than a possible inaccurate reading is offered for the 115% recovery of the malonaldehyde control.

None of the percentage results are conclusive but are merely indicative. Not only does there seem to be some error involved in the method, but also it is to be expected that no two samples oxidized under even slightly different conditions would yield exactly the same results.

However, it is fairly obvious that malonaldehyde does not occur in any significant amount in oxidized samples. It is also apparent that either an aldehyde or a methyl ketone is a precursor of malonaldehyde, but not the only precursor present in rancid samples.

According to Knight and Swern (1949) "some carbonyl is invariably formed in the reduction of purified peroxides" by the sodium bisulfite method. Although it is not known what proportion of this carbonyl group is comprised of aldehydes and methyl ketones, possibly the reduction of some of the peroxides present in the oxidized solutions during the bisulfite treatment resulted in the formation of the TBA reactive carbonyls, which were then later salted out by the bisulfite. Both of the oxidized lipid solutions used in this study had very high peroxide values. It is then possible that a peroxide is the naturally-occurring malonaldehyde precursor in rancid samples.

No evidence is presented that malonaldehyde does not exist as an acetal in oxidized fat. However, by this theory, it is possible to explain the presence of the TBA reactive material in the bisulfite addition compound, only if it is assumed that malonaldehyde occurs in more than one form in oxidized fat.

SUMMARY AND CONCLUSIONS

In the model system used for these studies, it was found that only a small amount of the TBA reactive material was present in a solvent unextractable, lipid-protein bound form. Although relatively rich in the reactive material, the lipid portion of the lipid-protein complex was unavailable for study due to failure to break the complex.

There appears to be no relationship between lipid contents and TBA values of the complexes formed under the randomized conditions employed.

Results of this study indicate fairly definitely that malonaldehyde occurs in insignificant quantities, if present at all, in rancid samples as the free carbonyl.

Also eliminated is the possibility that malonaldehyde occurs chiefly as an aldehyde or short-chain methyl ketone precursor in oxidized fat.

Probably the malonaldehyde occurs as a peroxide precursor and/or in more than one form in rancid fat samples. However, neither of these possibilities was investigated in this study.

A great deal of work must yet be done to characterize and eventually identify the naturally-occurring TBA reactive material in oxidized food products.

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