STABILITY OF CURED VERSUS UNCURED FREEZE-DRIED BEEF

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ABSTRACT

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by Michael Victor Rathmill

The likelihood of increasing the stability of freeze-dried beef by curing was the major area of concern of this study. Experiments were set up to determine if cured freeze-dried beef does possess an increased stability over untreated controls. Freeze-dried beef was cured by using nitric oxide gas and by a sodium nitrite-sodium chloride solution. Samples were stored in polyethylene bags at two temperatures, 25°C (Study I) and 40°C (Study II).

The samples from Study I were subjected to the following tests, 2-thiobarbituric acid distillation method for malonaldehyde, 2, 4-dinitrophenylhydrazine estimation of carbonyls, N-methylbenzothiazolone hydrazone colorimetric test for carbonyls, measurement of nitric oxide heme, measurement of total pigment and an odor detection panel. The freeze-dried samples from Study II were subjected to the following tests, 2, 4-dinitrophenylhydrazine estimation of carbonyls, measurement of nitric oxide heme pigment, measurement of total pigment and a taste panel evaluation.

The studies showed that freeze-dried beef can be cured as such using either nitric oxide gas or a sodium nitrite-sodium chloride solution and that curing does increase the stability of freeze-dried beef under the conditions used. It was also found that the measurement of total pigment may be a useful measure of the stability of either untreated or cured, stored freeze-dried beef.

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By

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Dedicated to my mother.

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INTRODUCTION

Freeze-drying is a useful method for preserving beef. However, oxidation can take place readily in freeze-dried beef yielding products which are quite unacceptable. It is known that when the iron of meat pigment is present in the ferrous state, there is a lesser tendency for oxidative deterioration to occur (54, 61). Therefore, the possibility exists that if the iron in freeze-dried beef pigments could be kept in the ferrous state the stability of the freeze-dried beef would be increased. Curing, using nitric oxide gas or sodium nitrite, will yield the ferrous form of the pigment.

The likelihood of increasing the stability of freeze-dried beef by curing was the major area of concern of this study. Experiments were set up to determine if cured freeze-dried beef does possess an increased stability over untreated controls. Experiments were designed to study the stability of cured freeze-dried beef stored in polyethylene bags at 25°C. The feasibility of using nitric oxide gas as a curing agent was also studied. Beef was initially cured in two ways. One set was cured with nitric oxide gas. The second set of samples was cured with sodium nitrite-sodium chloride, freeze-dried again and then stored. A third set was left untreated and acted as a control.

Attempts were made to find an objective test that would correlate well with the organoleptic test used. As a result, samples from each treatment were subjected to a series of tests each week for two months. The 2-thiobarbituric acid distillation method for malonaldehyde, 2, 4-dinitrophenylhydrazine estimation of carbonyls, N-methylbenzothiazolone hydrazone colorimetric test for carbonyls, measurement of nitric oxide heme pigment, measurement of total pigment and an odor detection panel were used to evaluate the samples.

Additional experiments were made to study the stability of cured freeze-dried beef stored in polyethylene bags in an oven set at 40°C. A second objective was to determine if there was a difference in stability between freeze-dried beef samples which were initially cured before freeze-drying and then cured and those which were freeze-dried first, cured and then freeze-dried again. One set of samples was cured with sodium nitrite-sodium chloride and then freeze-dried. A second set was freeze-dried, cured with sodium nitrite-sodium chloride and then freeze-dried again. A third set, uncured, acted as a control.

In order to determine if an objective test could be found which would correlate with the organoleptic test used, each week for ten weeks the following tests were run on the samples; the 2, 4-dinitrophenylhydrazine estimation of carbonyls, measurement of nitric oxide heme pigment, measurement of total pigment and a taste panel evaluation.

REVIEW OF LITERATURE

Ocidative Rancidity of Lipids

The oxidative of fatty acids by molecular oxygen is known as autoxidation. This process is responsible for the development of rancidity in fat containing foods.

Many of the important concepts concerning the nature of the reactions occurring during autoxidation of fatty acids were developed in the early 1940's by Farmer and co-workers (12). Autoxidation is believed to follow a radical chain reaction process. According to this theory, oxidation takes place not at the double bond itself, but at a reactive methylene group adjacent to a double bond, with production of a hydroperoxide which still retains much of the original unsaturation. The reaction may be started by a photochemically activated oxygen molecule or by the direct attack of oxygen on the more reactive 1:4-pentadiene system (18). The attack occurs alpha to the double bond in a monoene system yielding four hydroperoxides. The 1:4-pentadiene system is often found in polycnes. The methylene group alpha to both double bonds is very labile in this system. The hydrogen on the methylene group can be abstracted with double bond shifts occurring. At the same time a resonance system is set up by the free radical which leads to the production of conjugated isomers. Geometric isomerization of the double bonds from cis to trans configurations also occurs. As a result the product of the first stage of autoxidation is a complex mixture of hydroperoxides with conjugated and trans unsaturation (12, 28).

Hydroperoxides are labile compounds which can readily undergo further reactions. The products of these secondary reactions are unpalatable and are responsible for the odors and flavors of rancid fat. Hydroperoxides can undergo fission forming two free radicals. The hydroxyl radical reacts with double bonds starting a series of reactions responsible for a decrease in unsaturation (18). Hydroperoxides can oxidize olefins to epoxides or glycols. Epoxides can undergo rearrangement to ketones. Hydroperoxides can also undergo dismutation and scission. This can account for primary products, initial secondary products and further secondary products. The scission products which develop during the latter stages of autoxidation are saturated and unsaturated carbonyl compounds and short chain acids. These compounds are largely responsible for the odors and flavors of rancid fat.

The rate of autoxidation of a lipid system is dependent, among other things, on the amount of unsaturation of the constituent fatty acids. Monoenes such as oleic acid autoxidize very slowly at room temperature after an induction period. Dienes such as linoleic acid react about twelve times as fast as monoenes. Linolenic acid, a triene, autoxidizes about twice as fast as linoleic acid with little induction period. Phospholipids autoxidize very rapidly. A major factor in this deterioration is the tendency for these compounds to contain highly unsaturated fatty acids (29). Very highly unsaturated fatty acids are quite widely distributed in small quantities, at least in animal tissues. They not only lead to an increase in the susceptibility to oxidation of the lipid containing them, but also appear to lead to the formation of strongly flavored decomposition products. This is true even at a relatively early stage of the autoxidative process (29). Watts has shown that the proteolipid fraction of cooked pork is responsible for the development of rancidity induced by heating muscle tissue (56). Later studies using aged lean beef and lean pork showed that the phospholipid extracts of these tissues developed rancid odors more quickly than the neutral fat extracts. Hornstein concluded that phospholipids in excessively lean meat could contribute to the development of poor flavor (22).

Carbonyls in Autoxidized Lipids

Carbonyl compounds are found in autoxidized lipids as a result of secondary degradation of unsaturated fatty acids (25, 29, 30). Many of these compounds are volatile and their flavors and odors are quite intense (7). A study by Lea and Swoboda in 1958, showed that the flavor threshold for aldehydes in water decreases from C_3 to C_{12} (31). The threshold rises sharply again for C_{14} aldehydes. The most potent members of the series (C_8-C_{12}) were detectable in weight concentrations of the order of 10^{-8} to 10^{-9}M.

The volatile odor constituents of reverted soybean oil have been fractionated into rancid and reversion components (24). The constituents apparently contained aldehyde groups since, after treatment with aldehyde reagents, they could no longer be detected organoleptically. Individual aldehydes were isolated and identified from the rancid and reverted fraction. Derivatives were formed using 2,4-dinitrophenylhydrazine. Acetaldehyde, propionaldehyde, 1-pentenal and hexanal were identified. Mookherjee and Chang found 21 monocarbonyl compounds in reverted but not in rancid soybean oil (36).

The carbonyl content of various vegetable oils has been measured at different stages of processing (3). The short-chain volatile carbonyls were found to be responsible for the oxidative off-flavors produced. The non-volatile carbonyls were not found to be responsible for these off-flavors but they could act as precursors of these compounds.

Monocarbonyls change progressively during oxidation (15). The compounds present and the proportions of each vary. Changes in the classes of monocarbonyls also occur when oxidized fat is heated at 165°C. Saturated aldehydes, minor amounts of low molecular weight methyl ketones, a mixed group of unknown class, 2-en-1-als and 2,4-dien-1-als have been found in oxidized fat. The proportion of saturated carbonyls increased when oxidized fat was heated.

Studies on the changes in the volatile carbonyl content of potato chips during storage were carried out (6). Ethanol, propenal, 2-propanone, n-butanal, 2-pentenal, 2, 3-butanedione, 2-hexanal, n-heptanal and 2-heptenal were tentatively identified (6). A relationship was found between monocarbonyl compounds and the flavor of potato chips (37). During storage there was an increase in the saturated aldehydes, most notable in hexanal and pentanal. An increase was found in the 2-alkanones, 2-pentanone and 2-propanone. There was also an increase in the 2-enals, 2-heptenal and 2-octenal. All of these carbonyl compounds were believed to arise from the autoxidation of oleic and linoleic acids. These acids are the main constituents of the triglycerides of cottonseed and corn oils, which are used for frying potato chips.

Lillard and Day found that 97 to 99% of the carbonyls in oxidized milk fat were non-volatile (33). Approximately 88% of the volatile monocarbonyls were saturated. The concentration of these volatile monocarbonyls at the flavor threshold was in parts per billion. The oxidized flavor, near the flavor threshold, was due to an additive effect of the carbonyl compounds. This tends to complicate the study of the effects that individual carbonyl compounds have on the oxidized flavor and odor of lipids.

Studies of the volatiles from unblanched frozen peas have revealed 12 carbonyl compounds (58). Ethanal, the major volatile carbonyl compound, accounted for 96% of the 2,4-dinitrophenylhydrazones isolated. These compounds could have resulted from either enzymic or autoxidative decomposition of the lipids in the peas. Enzymic oxidation would be favored at low temperatures.

Autoxidation of the lipids in meat and meat products is probably the most important single source of their deterioration. Sulzbacher and co-workers have also shown that carbonyl compounds are the principal contributors to the off-flavors of rancid meat (47). A complete qualitative determination of volatile saturated aldehydes in rancid pork fat was carried out. Propanal, hexanal, C_6 to C_{11} saturated carbonyls, 2-enals and 2,4-dienals with six to twelve carbons were identified. Studies with rancid lard established that 4.4% of the total determinable carbonyls were free monocarbonyls, 1.4% volatile monocarbonyls and 1.1% volatile dicarbonyls (16).

The compound n-deca-2,4-dienal was found to be a major component of the carbonyl compounds in deodorization distillates from beef tallow and lard (38). It is extremely potent as a flavor compound. Decadienal is probably a major contributor to the rancid flavors and odors of autoxidized fat in which it was found. Its flavor threshold value in water was determined to be 0.5 parts per billion.

Carbonyl compounds are formed when meat and meat products are subjected to gamma radiation for sterilization (2). These compounds increase in both meat and fat with increasing irradiation dosages (2). The volatile carbonyl compounds isolated from irradiated lard were found to be propanal, butanal, pentanal, hexanal, nonanal, decanal, acrolein, crotonal and 2,4-undecadienal (60). The alpha-beta unsaturated aldehydes are known to be important constituents of off-flavor and odor in autoxidized lipids. These same compounds probably contribute to the off-flavor and odor of irradiated meat in much the same way they do in autoxidized lipids (60).

The products of the autoxidation of pure unsaturated fatty acids have been studied. Using a system composed of only linoleic acid, Badings found that n-hexanal, 2-octenal and 2,4-decadienal were the major compounds formed by limited autoxidation of the fatty acid (1). The formation of these aldehydes was explained by the theories of autoxidation of unsaturated fatty acids and of the dismutation of the hydroperoxides formed. Mildly oxidized esters of oleic, linoleic and linolenic acids yielded the following volatile monocarbonyls, 7 n-alkanals, 8 n-alk-2-enals and 4 alk-2,4-dienals (9). Each unsaturated fatty acid produced three major characteristic aldehydes. The steam volatile monocarbonyl compounds from mildly autoxidized esters of oleic, linoleic and linolenic acids were identified (17). The major aldehydes from oleate and linoleate were the ones expected from the scission of reported monomeric hydroperoxide isomers. A predominance of hept-2,4-dienal and propanal in linolenate suggested that the major monomeric hydroperoxides were the 12 and 16-hydroperoxy conjugated dienoic isomers. The number of minor aldehydes increased with the degree of unsaturation of the fatty acids (17). Monocarbonyl compounds were isolated from autoxidized methyl arachidonate (10). Aldehydes from the scission of hydroperoxides of the pentadiene system farthest removed from the carboxyl group accounted for 80% of the total. These same compounds are characteristic of autoxidized linoleate. Hexanal was the major aldehyde and heptanal was second in quantity. Heptanal is not readily accounted for by conventional mechanisms of autoxidation. Two unsaturated aldehydes isolated were proposed to be alk-2,4,6-trienals.

Carbonyls are found in autoxidizing lipids which cannot be accounted for by the decomposition of theoretically predictable isomeric hydroperoxides. Lillard and Day (34) took a member from each of the monocarbonyl classes commonly observed in oxidizing lipids and oxidized them. Nonanal, non-2-enal, hepta-2,4-dienal, and oct-1-en-3-one were used. They found that nonanal was relatively stable and oxidized to nonanoic acid. Oct-1-en-3-one did not absorb oxygen. The unsaturated aldehydes oxidized at a faster rate than methyl linoleate or linolenate. The unsaturated aldehydes produced shorter chain mono- and dicarbonyls as oxidative degradation products. These products help to explain the presence of carbonyls found in oxidizing lipid systems which are not theoretically predicted from the decomposition of lipid hydroperoxides.

Role of Meat Pigments in Autoxidation of Lipids

Myoglobin, the muscle pigment, and hemoglobin, the blood pigment, are the heme pigments of meat. Myoglobin is the principal pigment, but hemoglobin will comprise 20 to 30% or more of the total pigment (13). These two pigments are identical in their reactions involving meat color. However, they have different rates in such important reactions as autoxidation, reaction with nitrite and denaturation. Therefore, it seems probable that quantities and reactivities of both hemoglobin and myoglobin would have to be considered when correlating color changes with pigment concentration (13).

Myoglobin and hemoglobin are known to catalyze the oxidation of unsaturated fatty acids (4, 19, 35, 46, 49, 50, 54, 56, 57, 61). Watts noted in 1947 that decomposition of hemoglobin, with a resulting discoloration of the meat, was accompanied by development of rancidity (56). Watts pointed out that oxidation of the fat, resulting in rancidity, and oxidation of the heme pigments, resulting in discoloration, are closely related and can in fact accelerate each other. Each reaction can also proceed independently of each other (57). It has been found that autoxidative reactions are greater in tissues containing large quanitities of lipids and heme pigments than in tissues containing a lesser amount (63). The degree of oxidation is also a function of the lipid composition since the greater the quantity of highly unsaturated fatty acids the greater the degree of oxidation (63).

The action of ferrous and ferric iron on the oxidation of linoleate was studied by Smith and Dunkley (45). Both ferrous and ferric iron catalyzed the oxidation reaction, but the rate was greater with ferrous iron.

Derivatives of hemoglobin and myoglobin such as methemoglobin and metmyoglobin, imidazole hemoglobin hemichrome and imidazole myoglobin hemichrome, carbon monoxide hemoglobin, oxyhemoglobin and nitric oxide hemoglobin were used as catalysts for the oxidation of unsaturated lipids (4). All of the above were active catalysts. Some of the ferrous forms of the above mentioned compounds were found to have induction periods.

Studies on cured versus uncured pork were carried out (61). Younathan and Watts found that the ferric form of the pigment is the active catalyst. The ferrous nitric oxide hemochromogen is not the active catalyst. Loss of color of the cured meat pigment may result from oxidation of the ferrous pigment to the active ferric form or porphyrin ring destruction of the heme group. Both of these reactions are involved in the fading of the pink cured meat color.

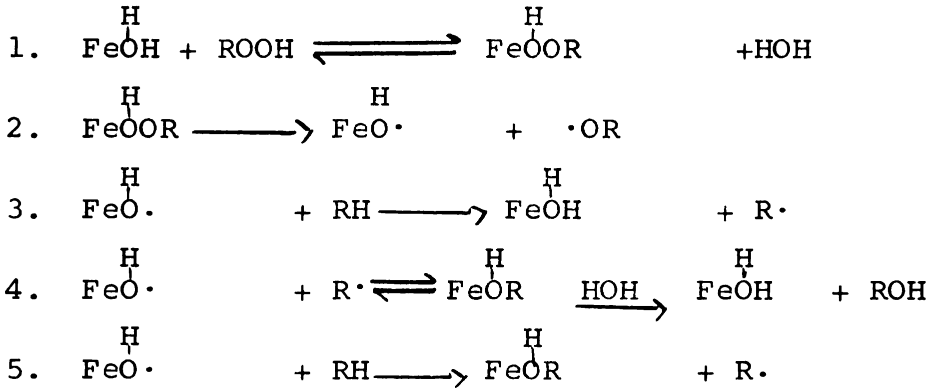
Mechanisms have been proposed for the heme catalyzed oxidation of lipids (35, 49, 50, 54). Tappel has suggested that hematin catalysis involves the formation of a lipid peroxide-hematin compound (50). This compound then decomposes to free radicals which in turn propagate the chain reaction oxidations. The reactions of free radical intermediates can account for the fragmentation of fatty acids and the concurrent destruction of the catalyst.

Tarladgis (54) believed that the mechanism of heme catalyzed lipid oxidation involved the initial donation of an electron by ferrous iron to a hydroperoxide with the iron being oxidized to the ferric state. The decomposition products of the hydroperoxides in turn reduce the iron back to the ferrous state. A catalyst could only accelerate the chain reaction of oxidation at the initial removal of an electron from a labile site of the molecule and accelerate the decomposition of the hydroperoxides. This is accomplished by a reversible acceptance and donation of electrons to the fatty acid and the hydroperoxides. Heme compounds can be reversibly oxidized and reduced. Therefore, they are ideally suited for a role as catalysts of the oxidation of unsaturated fatty acids.

Tarladgis came to the conclusion that the ferric heme compounds initiated lipid oxidation, while the ferrous heme compounds were inactive in the absence of preformed peroxides (54).

The strong catalytic activity of cooked meat pigments for the lipid oxidation in animal tissues was explained by Tarladgis (55). Hemoglobins may be regarded as essentially square-planar iron-porphyrins, further coordinated to one strong-field ligand (histidine) and one weak-field ligand (water) (11). It is the presence of water as a ligand that agrees with the strong catalytic activity of cooked meat pigments (55)

The products of the hematin-hydroperoxide reaction in unsaturated fatty acids have been studied by Maier and Tappel (35). The initial product was the alkoxy free-radical produced by the homolytic cleavage of the O-OH bond of the hydroperoxide. The secondary products were found to be composed mainly of oxirane, hydroxyl and carbonyl compounds. Cleavage of the carbon chain, loss of conjugated double bonds and polymerization also took place. The following reactions were suggested as the mechanism for the hematin compound catalyzed decomposition of hydroperoxides in the absence of oxygen (35).



1. $\mathrm{Fe\overset{H}{O}H} + \mathrm{ROOH} \rightleftharpoons \mathrm{Fe\overset{H}{O}OR} + \mathrm{HOH}$
2. $\mathrm{Fe\overset{H}{O}OR} \longrightarrow \mathrm{Fe\overset{H}{O}\cdot} + \cdot\mathrm{OR}$
3. $\mathrm{Fe\overset{H}{O}\cdot} + \mathrm{RH} \longrightarrow \mathrm{Fe\overset{H}{O}H} + \mathrm{R\cdot}$
4. $\mathrm{Fe\overset{H}{O}\cdot} + \mathrm{R\cdot} \rightleftharpoons \mathrm{Fe\overset{H}{O}R} \xrightarrow{\mathrm{HOH}} \mathrm{Fe\overset{H}{O}H} + \mathrm{ROH}$
5. $\mathrm{Fe\overset{H}{O}\cdot} + \mathrm{RH} \longrightarrow \mathrm{Fe\overset{H}{O}R} + \mathrm{R\cdot}$

The pigments of cured meats are not expected to initiate fat oxidation in the absence of preformed peroxides. The iron in cured meats exists in the ferrous state. Lipid oxidation is delayed in cured meats, but eventually does occur with pigment fading and destruction. These pigment changes may be attributed to the oxidation of ferrous iron to the ferric state and by the donation of an electron for the decomposition of preformed peroxides. Nitric oxide at same time dissociates from the heme complex. When the decomposition products of the hydroperoxides build up, ring destruction results. The removal of an electron and the opening at the methene bridge occurs, with the subsequent appearance of green or gray colors (54).

Hemoglobin is also an important catalyst for copolymer formation (51). Large amounts of protein can be incorporated into copolymers. Physical and chemical properties of the proteins are altered to some extent. The copolymers also contain a relatively large amount of oxygen which was removed from the air during unsaturated fat oxidation. The amount can vary over a wide range. The mechanism of copolymer formation probably involves active aldehyde browning concurrent with unsaturated fat oxidation.

Stability of Freeze-Dried Beef

Freeze-drying is a process whereby ice crystals in a frozen product are removed by sublimation in a high vacuum. When beef is freeze-dried, oxymyoglobin is deoxygenated to myoglobin. The myoglobin is readily oxidized to metmyoglobin, during storage and subsequent rehydration. This oxidation of myoglobin is primarily responsible for the early development of an undesirable brown color during storage of freeze-dried beef (52).

The most deteriorative oxidation reaction in freeze-dried beef, according to Tappel (52), involves the oxidation of the protein fraction. Tappel has found that the oxidation of the protein fraction of freeze-dried beef can account for 50 to 100% of the total oxygen absorbed. Storing freeze-dried beef in an inert atmosphere or at a high vacuum may help prevent the oxidation of the protein fraction. However, this will not prevent the oxidation of myoglobin in freeze-dried beef (52).

Precooked freeze-dried beef has been shown by Tappel to be stable for six months' storage at 21°C and 38°C (53). Samples stored at 54°C began to deteriorate after two months. Rehydration in 100°C water at atmospheric pressure was found to be poor.

METHODS AND PROCEDURES

Extraction and Measurement of Meat Pigments

Hornsey's method for the estimation of nitric oxide-heme pigment and total pigments was used to follow pigment loss in freeze-dried beef (21).

The nitric oxide-heme of cured meat was extracted as a nitric oxide-heme-acetone complex. This was achieved using an acetone/water solvent. Other meat pigments were not extracted under the conditions used. A ratio of 4:1 acetone to water was found to be critical. The method could be adapted to measure the total pigments by adding hydrochloric acid to the solvent (21).

Extraction of Nitric Oxide-Heme

Ten grams of freeze-dried beef were weighed into a Virtis flask. A 10 ml portion of a mixture of 40 ml of acetone and distilled water was added. The sample was allowed to absorb the solution before the remainder was added. The Virtis "45" homogenizer was set at 55 and run for two minutes. The slurry that formed was placed in fluted filter paper. The absorbance of the clear filtrate was read on a Beckman Model DU spectrophotometer at 540 mu. The absorbancies were used as a comparison for each test period. An 80% acetone/water solution was used as a blank.

Extraction of Total Pigments

A ten gram sample of freeze-dried beef was weighed into a Virtis flask. A 10 ml portion of a mixture of 40 ml acetone; 9 ml distilled water and 1 ml concentrated hydrochloric acid was added. The sample was allowed to absorb the solution before the remainder was added. The Virtis "45" homogenizer was set at 55 and run for two minutes. The flask was covered with aluminum foil and placed in the dark for one hour. The sample was then filtered using fluted filter paper. The absorbance of the clear filtrate was read on a Beckman Model DU spectrophotometer at 640 mu. The absorbancies were used as a comparison for each test period. An 80% acetone/water solution was used as a blank.

Measurement of the Carbonyl Content of Freeze-dried Beef

Two methods were used to evaluate the carbonyl content of the various freeze-dried beef samples. The first method employed the use of 2,4-dinitrophenylhydrazine to estimate carbonyl compounds. The second method used N-methylbenzothiazolone hydrazone in a colorimetric test for carbonyls.

Carbonyls were estimated using 2,4-dinitrophenylhydrazine (2,4-DNPH) in a spectrophotometric assay. Up to 5.0 umoles per milliter of the higher (C_6 to C_{11}) ketones as their 2,4-DNP hydrazones were recovered quantitatively by Lawrence (28). He demonstrated further that although the 2,4-DNP hydrazones of volatile carbonyls in acid solution are not stable in open systems, under closed conditions the reaction of 2,4-DNPH with carbonyls up to C_{11} was quantitative and rapid. The reaction was found to be complete in 30 minutes.

N-methylbenzothiazolone hydrazone was used in a colorimetric procedure for the determination of carbonyl compounds (39). N-methylbenzothiazolone hydrazone (MBTH) reacts with various carbonyl compounds. Azine and "osazine" derivatives are formed under the conditions of the test. These derivatives have characteristic spectra which permit the identification of saturated and unsaturated aldehydes and ketones, keto acids and many other related compounds in a simple spectrophotometric assay. The azines of aldehydes can be further reacted with the oxidized form of MBTH to give tetra azopentamethine cyanine dyes.

Extraction of Carbonyls Using Benzene and Ethanol

Six grams of freeze-dried beef were weighed into a Virtis flask. Twenty ml of carbonyl-free ethanol was added to the flask. After the ethanol had been absorbed by the sample, 40 ml of carbonyl-free benzene was added. The carbonyl-free reagents were prepared according to Schwartz and Parks (43). The mixture was blended using the Virtis "45" homogenizer for two minutes at a setting of 55. The flask was then cooled in an ice bath for several minutes.

The slurry was filtered using a Büchner funnel. Forty ml of benzene-ethanol (2:1, v/v) was used to wash the flask.

Estimation of Carbonyls Using 2,4-Dinitrophenylhydrazine

A reagent of 2,4-DNPH was prepared by adding two grams of 2,4-DNPH per liter of 2N hydrochloric acid.

One ml of the filtrate from the above extraction was pipetted into a glass stoppered test tube. Two ml of the 2,4-DNPH reagent was added. The test tube was stoppered and allowed to stand for 30 minutes. Next, twenty ml of carbonyl-free hexane was added, and the tube was shaken for 40 seconds. Two layers formed. Some of the upper hexane layer was pipetted into a cuvette. Its absorbance was read at 345 mu on a Beckman Model DU spectrophotometer against a reagent blank. The values obtained for each test period were used as a comparative measure of the carbonyl content.

Determination of Carbonyl Compounds with N-Methylbenzothiazolone Hydrazone

Vacuum Distillation

Fifty grams of freeze-dried beef were weighed into a Virtis flask. Two-hundred ml of distilled water was added. The Virtis "45" homogenizer was set at 70 and run for two minutes. The slurry formed was transferred to a one liter two neck round bottom flask. The flask was connected to the distillation apparatus. Nitrogen gas was used to sweep the volatile compounds through the system. Two traps were used to remove any carbonyl compounds which might have been present in the nitrogen. The first trap contained a saturated solution of 2,4-DNPH in 2N sulfuric acid. The second trap contained glass wool. A vacuum of approximately two centimeters was used. The volatile carbonyl compounds, which were swept through the system from the freeze-dried beef samples were collected in two traps connected in series. The traps contained 40 ml of a one per cent solution of MBTH to which was added 160 ml of distilled water. The vacuum distillation was allowed to run for 20 hours. The MBTH colorimetric procedure was run on aliqnots from the traps.

Determination of Carbonyl Compounds - MBTH Colorimetric Procedure

One ml from each collecting trap was pipetted into glass stoppered test tubes. The tubes were stoppered and heated in a boiling water bath for three minutes. The tubes were cooled to room temperature and then 2.5 ml of ferric chloride solution was added. After five minutes, 6.5 ml of reagent grade acetone was added. The solution was mixed and its absorbance was read at 670 mu on a Beckman Model DU spectrophotometer against a reagent blank. The values obtained for each test period were used as a comparative measure of the carbonyl content. The ferric chloride solution (0.2% ferric chloride in distilled water) was made up fresh each time.

The 2-Thiobarbituric Acid Distillation Method (53)

Reagents

Hydrochloric Acid Solution - one part concentrated hydrochloric acid to two parts water.

TBA Reagent- 0.02M 2-thiobarbituric acid in distilled water.

Dow Antifoam A Spray.

Procedure

Ten grams of freeze-dried beef were weighed into a Virtis flask. Fifty ml of distilled water was added. The Virtis "45" homogenizer was set at 50 and run for two minutes. The slurry was transferred quantitatively to a 500 ml Kjeldahl flask containing boiling chips. A spatula and 47.5 ml of distilled water were used to transfer the homogenized sample. Next, 2.5 ml of the hydrochloric acid solution was added. Dow Antifoam A was sprayed into the neck of the flask. The Kjeldahl distillation apparatus was set at the highest heating rate. Frequent agitation was needed to prevent the sample from burning. It took approximately 25 minutes to collect 50 ml of the distillate.

A five ml sample of the distillate was pipetted into a 50 ml glass stoppered test tube. Five ml of the 0.02M TBA solution was then added. A reagent blank was also prepared. The tubes were stoppered and placed in a boiling water bath for 35 minutes. They were then cooled under tap water for five minutes. The absorbance of the solution was read at 538 mu on a Beckman Model DU spectrophotometer.

Preparation of Nitric Oxide Gas

A. A mixture of sodium nitrite, potassium nitrate, chromium oxide, and ferric oxide was made. The amounts of the above reactants respectively were 6.37 g, 2.52 g, 7.60 g, and 12,0 g. The Teflon lined muffin pan was used to mix together the above mentioned compounds. A little distilled water was added to make a stiff paste. The pan was then placed in an oven at 100°C overnight to dry. The mixture was stored in a desiccator until used (40).

B. A second method involved the use of ferric sulfate and sodium nitrite. Ferric sulfate (17.5 g) and sodium nitrite (8.3 g) were mixed together in a 250 ml Florence flask. Evolution of nitric oxide was immediate if some distilled water was added. The nitric oxide produced by this method was utilized in flushing air out the apparatus prior to production of nitric oxide gas using method A (48).

C. Evolution of Nitric Oxide for Experimental Use

A combination of the above two methods was employed to obtain the nitric oxide gas for use in the various experiments. According to one report (40), the combination of sodium nitrite, potassium nitrate, chromium oxide, and ferric oxide yields nitric oxide containing only a fraction of a per cent impurities (99.78% pure). As previously mentioned, the ferrous sulfate-sodium nitrite method was used to flush the apparatus. Water displacement was employed to collect the nitric oxide gas, since it does not dissolve readily in cold water. The water was distilled and then boiled to remove dissolved oxygen. Nitric oxide is a colorless gas which combines with oxygen immediately upon exposure to air to form dark nitrogen dioxide. This fact was used as a simple means of determining if any oxygen was in the system. A one liter polyethylene graduated cylinder was utilized to collect the nitric oxide gas by displacement. Ten grams of the sodium nitrite, potassium nitrate, chromium oxide and ferric oxide mixture yielded 800 ml of nitric oxide gas.

Curing of Freeze-dried Beef

Nitric Oxide Gas Curing

The freeze-dried beef samples were placed in a metal desiccator fitted with a vacuum gauge. Approximately 200 g of meat were treated at a time. The desiccator was purged with nitrogen gas several times and a vacuum was placed on the desiccator. A system made up of glass and plastic tubing, traps and clamps was also purged with nitrogen several times to insure the removal of air. This was done by putting a vacuum on the system and breaking it with the nitrogen gas. After the desiccator containing the meat samples was connected to the above mentioned system, the clamps were opened and the nitric oxide was allowed to come in contact with the sample. The freeze-dried beef samples were kept in contact with the nitric oxide overnight. A vacuum was once again placed on the desiccator and broken with nitrogen gas. This was done to remove any of the nitric oxide remaining in the reaction vessel. In order to check the effectiveness of the cure, a small piece of the treated freeze-dried beef was rehydrated in boiling water for 10 minutes. If the pink color typical of cured meat developed, the samples were considered cured. The treated samples were then placed in polyethylene bags for storage.

Conventional Curing

Freeze-Drying Followed by Curing Then Freeze-Drying Again

Samples of freeze-dried beef were cured using sodium nitrite and sodium chloride. The sodium nitrite was added at the rate of 1.12 g per 473.2 ml of pickle. The amount of pickle was based on the freeze-dried beef gaining water until 70% of its fresh weight was achieved upon rehydration with water. Sodium chloride was added at a rate of 2.5% based on the weight of the freeze-dried beef used. The meat was cured in a two liter beaker having a weighted top to insure complete immersion of the beef in the pickle. The samples were left in the pickle under refrigeration until rehydration was complete (approximately three days). The cured rehydrated beef was then quick frozen at -34°C, freeze-dried once more, and stored in polyethylene bags.

Curing Followed by Freeze-drying

Raw wet beef cubes were cured with sodium nitrite and sodium chloride. The sodium nitrite was added at the rate of 1.12 g per 473.2 ml of pickle. Sodium chloride was added at a rate of 2.5% based on the weight of raw beef cubes used. The meat was cured in a two liter beaker having a weighted top to insure complete immersion of the beef in the pickle. The samples were left in the pickle under refrigeration for 24 hours.

Organoleptic Procedures (23)

Odor Detection Panel

The object of the odor detection panel was to determine if there was a significant difference between a standard sample and the room temperature stored samples. The preference of the panel for the various samples was also determined. Eleven persons with no previous training were chosen for the panel.

The different freeze-dried beef samples were rehydrated in warm distilled water and blended in a Virtis "45" homogenizer for two minutes. The slurry was then transferred to brown glass bottles, the caps of which were lined with aluminum foil. The bottles containing the various samples were placed in an oven set at 60°C until presented to the panelists. Five such bottles were prepared. They were numerically coded from one to four. The fifth bottle was marked with an "S" representing the word standard.

The following samples were prepared: freeze-dried beef cured with the nitric oxide gas; freeze-dried beef conventionally cured using sodium nitrite-sodium chloride; an untreated control; and two sets of nitric oxide gas cured samples which were stored at -34^{o}C in nitrogen gas. One of the cold stored samples was used as the standard and the other as a numbered sample to check the panelist's reliability.

Each panelist received a sample marked "S", which was the standard. They also were presented with four numerically coded samples and were asked to rate each sample on a zero-to-four scale, zero indicating no difference and four indicating a great difference from the standard. The panelists were also asked to indicate whether they felt that the odor of the coded samples was "better than" the reference standard. As previously mentioned one of the coded samples was identical to the reference standard and served as a check on the reliability of the individual panel member.

The data from the preference portion of this organoleptic test was recorded as the per cent of panelists who checked each category.

The difference scores were analyzed by statistical methods. The fiducial limits for the control sample were calculated. This was used to determine whether or not the panel's average score for the test samples was significantly different from the average score of the control sample which was identical to the standard or "S" sample. The fiducial limits represent a range of average scores (above and below average score for a particular sample is not significantly different from the control. Average scores above or below the fiducial limits are significantly different from the control sample.

Taste Panel

The taste panel was used to evaluate samples of freeze-dried beef which had been stored in polyethylene bags in an oven set at 40°C. Three sets of samples were prepared. One set was cured with sodium nitrite-sodium chloride while still fresh and then freeze-dried. The second set was freeze-dried initially, then cured with a rehydrating solution of sodium nitrite-sodium chloride, and finally freeze-dried. The third set was used as a control.

The object of the taste panel was to determine if there was a significant difference between a standard sample and the stored samples. The panel's preference for the various samples was also determined. Eight persons were picked for the panel, all of whom had participated in the odor detection panel.

The samples presented to the panelists were cooked in boiling water for twenty minutes. Sodium chloride at the rate of 0.3% by volume of liquid was added to the cooking water of the control and standard samples. This was found necessary since the treated samples had been cured with sodium chloride and their salty taste could easily be detected.

Each panelist received a sample marked "S", which was the standard. This standard sample was untreated freeze-dried beef, which had been stored in polyethylene bags, under nitrogen at -34°C. They were also presented with four numerically coded samples. One of the coded samples was identical to the reference standard, one was the untreated control, one was the cured, freeze-dried sample, and one was the freeze-dried, cured, freeze-dried treated sample. These samples were placed on aluminum cups and served hot.

The taste panel was conducted in exactly the same manner as the odor detection panel. All samples were compared with the reference standard and the results were treated statistically in the same way as the odor detection panel.

Preparation of the Beef

Beef (commercial grade round) was obtained from the Michigan State University Food Stores. The previous history of the meat was unknown. The beef had been cut into one-half inch slices before being received. It was trimmed of visible fat and connective tissue. The beef was cut into three-quarter inch squares and frozen in a single layer on aluminum foil trays at $-34^{o}C$.

Freeze-Drying

The beef was freeze-dried in a Stokes Freeze-Dryer, Model 2003-F2 (F. J. Stokes Machine Company, Philadelphia, Pa.). The trays of frozen beef were placed on the upper two shelves of the freeze-dryer. The minimum absolute pressure observed using a Stokes-McLeod gauge was 0.1 mm of mercury. A temperature of approximately $35^{o}C$ was maintained on the heating shelves. Under these conditions it took 24 hours to dry the beef cubes. The vacuum was broken with nitrogen.

Study I

A total of 2.1 kg of freeze-dried beef was prepared. The meat was divided into three equal sets. One set was cured with nitric oxide gas. The second set of samples was cured with sodium nitrite-sodium chloride and freeze-dried again. The third set was left untreated and acted as a control. All of the samples were stored in polyethylene bags at room temperature in a drawer. The temperature remained fairly constant and averaged about 25°C.

Each week for a period of two months samples from each treatment were subjected to a series of tests. These included TBA, 2,4-DNPH estimation of carbonyls, MBTH colorimetric test for carbonyls, nitric oxide heme pigment, total pigment, and an odor detection panel. After the fifth week the tests were performed every other week. This gave a total of seven test periods. Approximately 100 grams from each treatment was used per test period.

Study II

Approximately 500 grams of freeze-dried beef was prepared for this experiment. Three sets of samples were made up. One set was cured with sodium nitrite-sodium chloride before freeze-drying. The amount of sodium nitrite and sodium chloride needed was based on the volume of the pickle.

In this case 1425 ml was used. The sodium nitrite was added at the rate of 0.85 grams per 475 ml or 2.55 grams total. The sodium chloride was added at the rate of 2.5% or 28 grams. The second set of samples was cured after being freeze-dried with sodium nitrite-sodium chloride as explained previously. The third set of beef samples was left untreated and used as the control. The samples were all stored in polyethylene bags in an oven set at 40°C.

Each week for ten weeks the following tests were performed on the samples; 2,4-DNPH estimation of carbonyls; measurement of nitric oxide heme; measurement of the total pigments; and a taste panel evaluation.

RESULTS

All of the chemical tests used gave qualitative rather than quantitative measurements. The absorbance values obtained from the various chemical determinations were used to compare the effectiveness of the different curing procedures.

Study I

The first study was designed to investigate the stability of freeze-dried cured beef stored in polyethylene bags at 25°C. The meat was initially cured in two ways. One set was cured with nitric oxide gas. The second set of samples was cured with sodium nitrite-sodium chloride, freeze-dried again and then stored. A third set was left untreated and acted as a control.

Each week for two months samples from each treatment were subjected to a series of tests. TBA, 2,4-DNPH estimation of carbonyls, MBTH colorimetric test for carbonyls, nitric oxide heme pigment, total pigment and an odor detection panel were used to evaluate the samples.

The TBA distillation method (Figure 1) gave results which increased during the first three weeks and then decreased. The cured samples gave zero readings after five weeks.

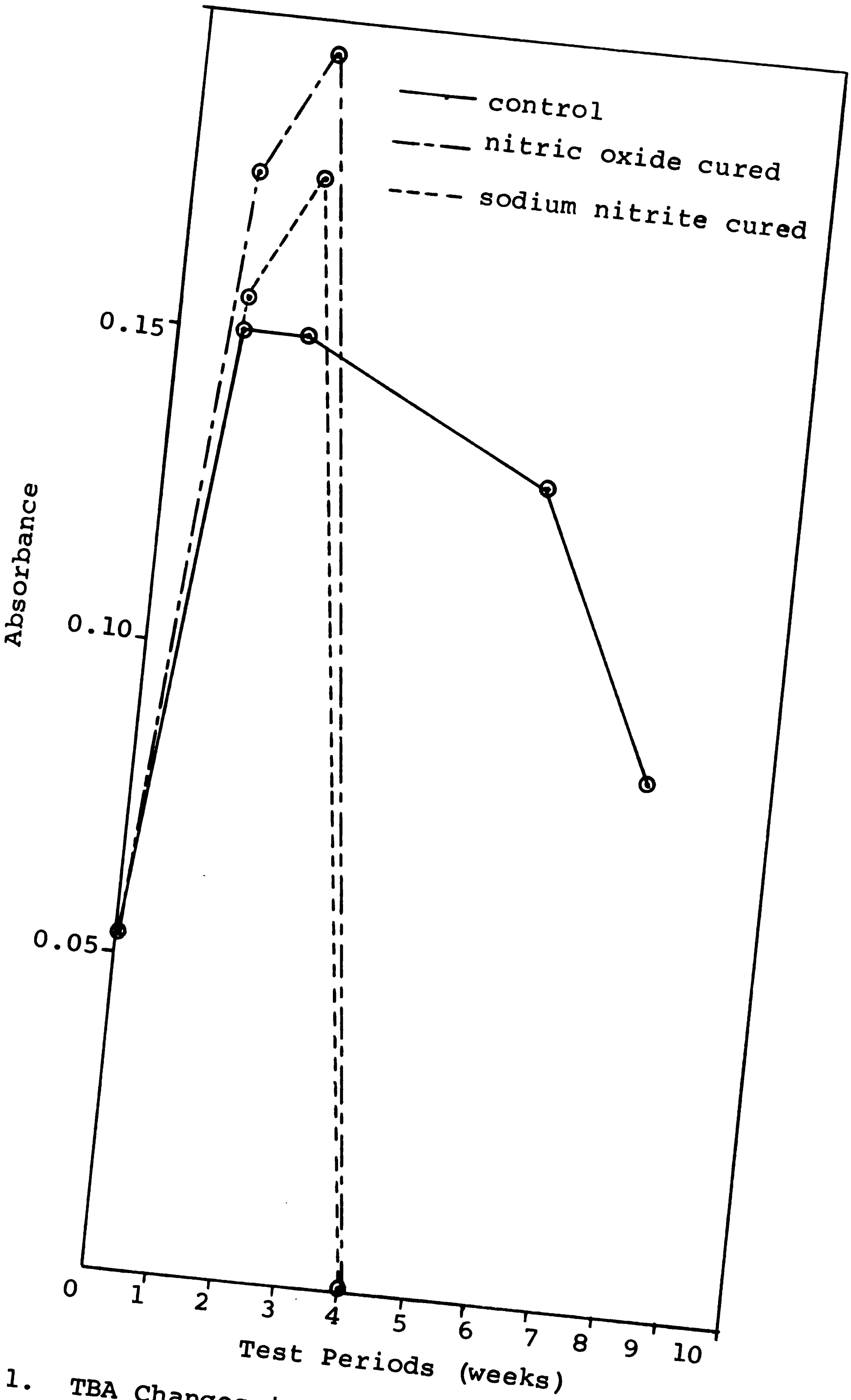


Figure 1. TBA Changes in Absorbance of Freeze-Dried Beef Stored at Room Temperature

The 2,4-DNPH estimation of carbonyls (Figure 2) generally gave erratic results. There was an initial increase in values during the first three test periods. The values became irregular after this point. The control and the sodium nitrite cured samples ended up with higher carbonyl values than their initial values. The nitric oxide treated samples, however, had lower carbonyl values at the termination of the experiment than initially.

The MBTH colorimetric test for carbonyls (Figure 3) gave results somewhat different from the 2,4-DNPH procedure. The control remained fairly constant throughout the storage time. The nitric oxide treated samples increased gradually during the entire period. The sodium nitrite cured samples decreased as a general trend during the storage period.

The extraction and measurement of nitric oxide heme (Figure 4) was carried out on the cured samples only. The values obtained for the nitric oxide cured samples decreased during the first seven weeks. A 300 per cent drop occurred during the first week of storage. Following this, a gradual decrease in values was experienced. The sodium nitrite treated samples were initially much lower, 0.170 to 0.6.0 Abs., (absorbance units) than the nitric oxide treated samples. Their values, however, did not decrease as rapidly or to as great an extent as the nitric oxide gas cured samples did. The last two test periods reversed this trend and increased.

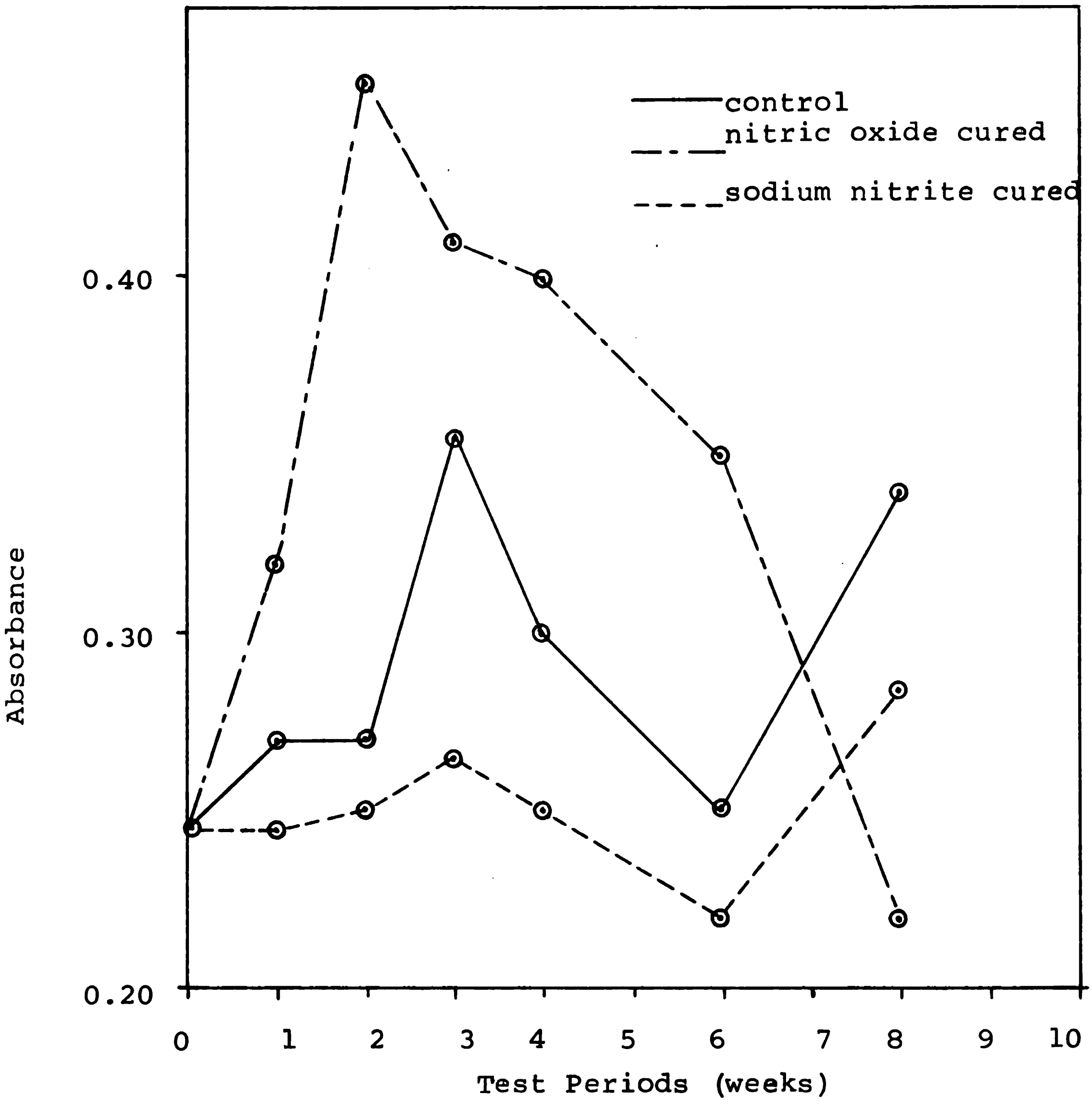


Figure 2. Colorimetric Determination of Carbonyls in Freeze-Dried Beef Stored at Room Temperature

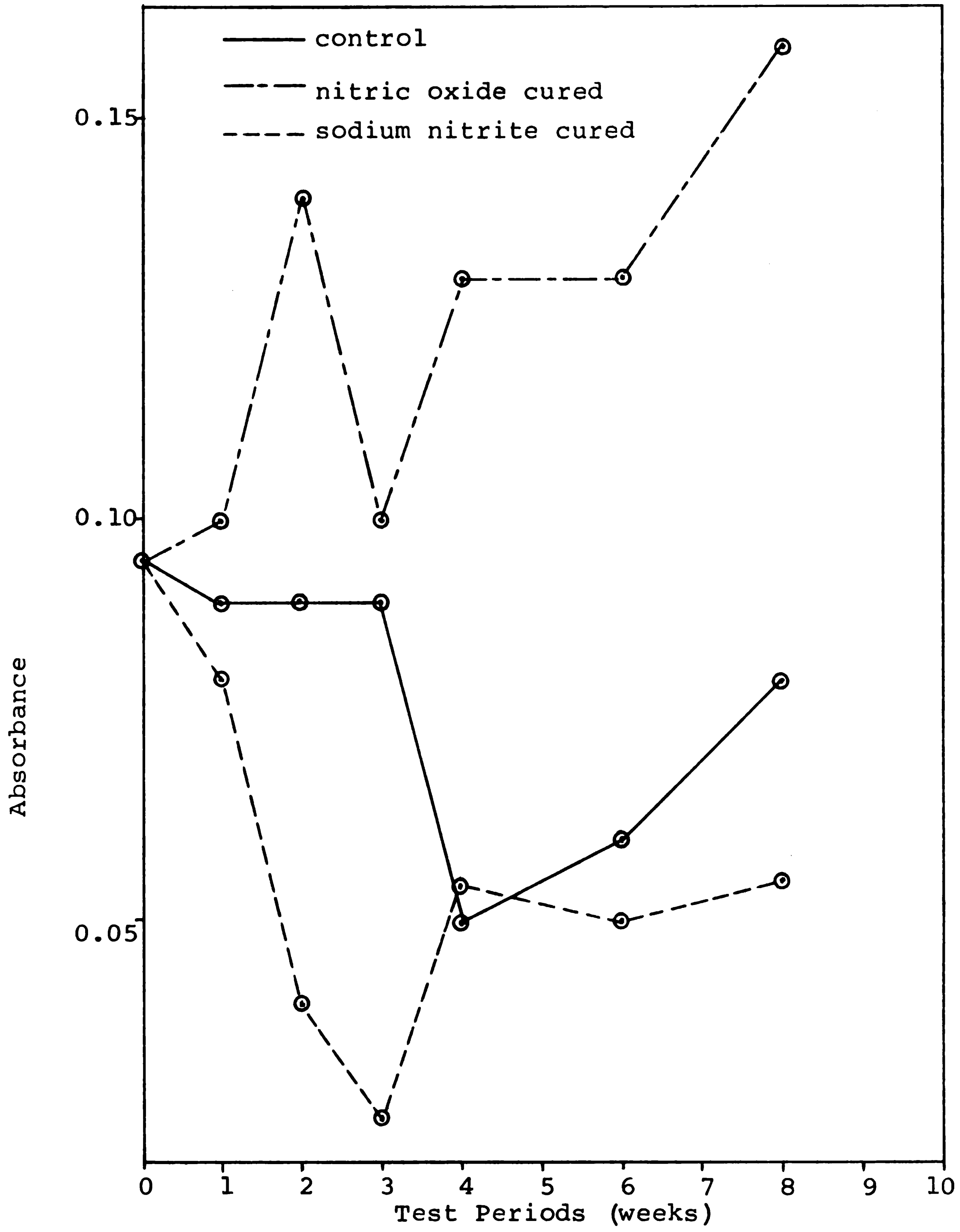


Figure 3. MBTH Colorimetric Values for Carbonyls in Freeze-Dried Beef Stored at Room Temperature

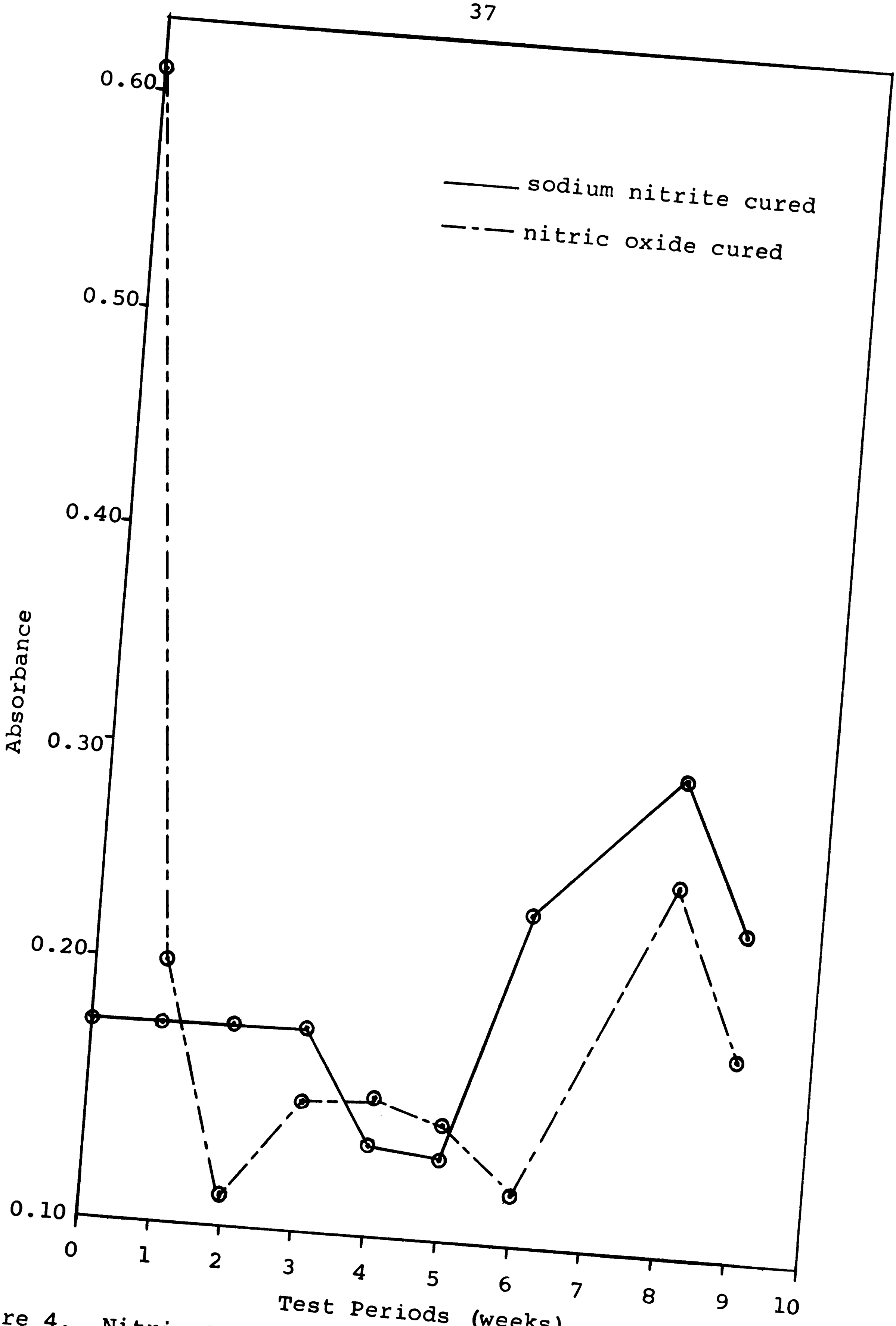


Figure 4. Nitric Oxide Heme Pigment of Freeze-Dried Beef Stored at Room Temperature

The extraction and measurement of the total pigment (Figure 5) was carried out on all samples. A gradual decrease in values was experienced for all treatments. The pigments in the untreated control began to decrease more rapidly after six weeks than that in the cured samples.

The odor detection panel was used to determine the time required for a significant difference to develop between a standard sample (stored in nitrogen gas at -34°C) which was untreated and the various stored samples. After two weeks, the panel found a significant difference between the standard and the control. This difference continued for the remainder of the storage period. A significant difference was found between the standard and the nitric oxide cured samples after three weeks. However, there was no differences the following week. There was a significant difference on the fifth week between the stored samples and the standard. This difference was erratic until the last two test periods at which time all of the stored samples were significatnly different from the standard.

The odor detection panel was also set up to measure preference. The percentage of the panel checking three preferences levels was determined. Each sample was rated as being more acceptable, comparable, or less acceptable than the standard. The majority of the panel felt that the control was less acceptable than the standard throughout the entire storage period. The nitric oxide cured samples ended up being rated less acceptable by 50% of the panel, while the sodium nitrite-sodium chloride cured samples ended up being rated less acceptable by 40% of the panel.

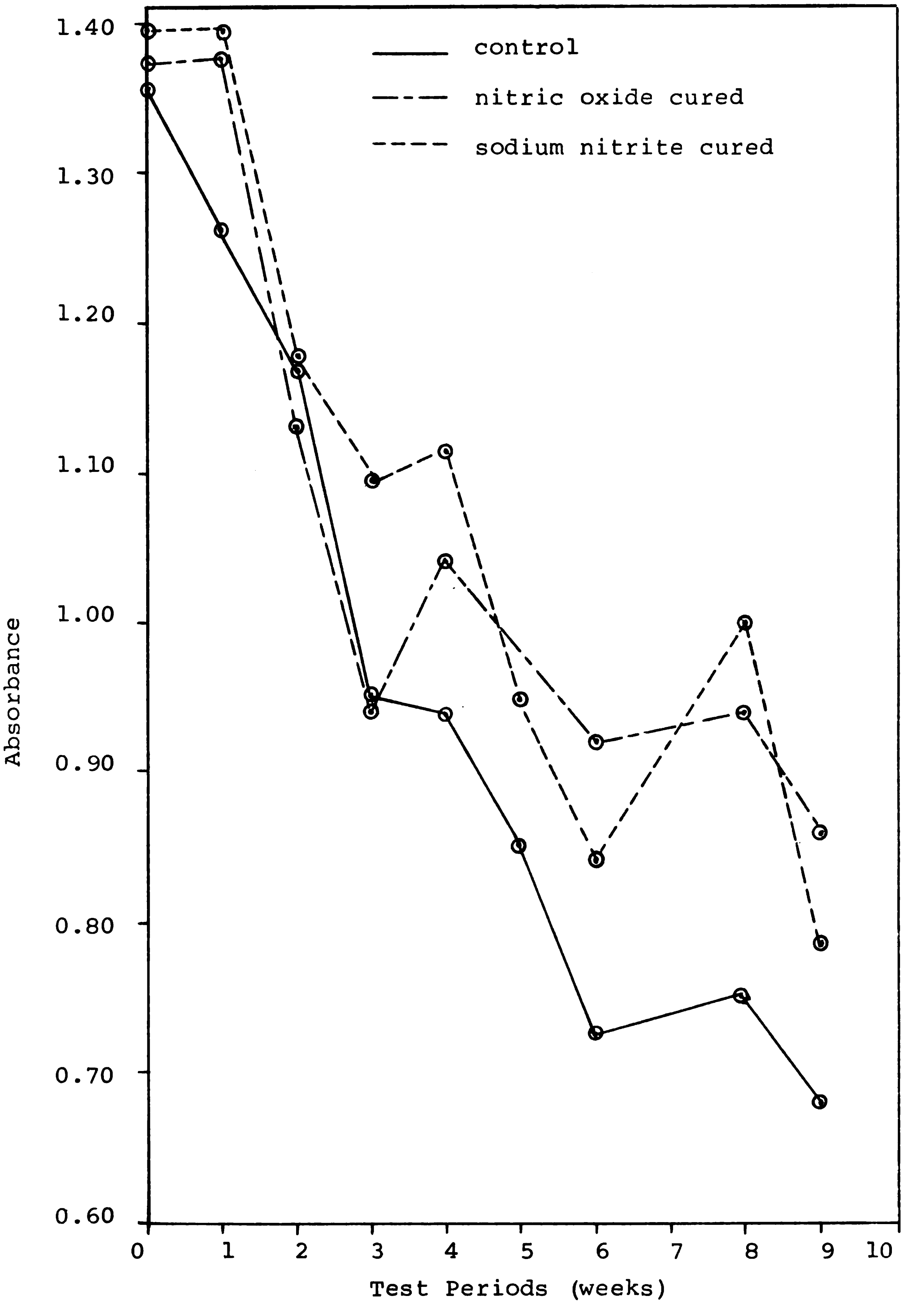


Figure 5. Measurement of Total Pigment in Freeze-Dried Beef Stored at Room Temperature

Graphs were plotted for each set of stored samples (Figures a, b, and c) using the data obtained from the total pigment measurement and the average difference scores of the odor detection panel. The average scores for the standard samples were subtracted from the average scores for each set of stored samples. This difference was then plotted against the total pigment for that particular sample. An inverse relationship could be seen. As the pigment values decreased the difference scores increased.

Study II

The second study was designed to investigate the stability of freeze-dried cured beef stored in polyethylene bags in an oven set at 40°C. The meat was initially cured in two ways. One set of samples (conventionally cured I) was cured with sodium nitrite-sodium chloride and then freeze-dried. A second set (conventionally cured II) was freeze-dried, cured with sodium nitrite-sodium chloride and then freeze-dried again. A third uncured set acted as a control.

Each week, for ten weeks, the following tests were run on the samples; 2,4-DNPH estimation of carbonyls, measurement of the total pigment and a taste panel evaluation.

The 2,4-DNPH estimation of carbonyls, measurement of nitric oxide heme, measurement of the total pigment and a taste panel evaluation.

The 2,4-DNPH estimation of carbonyls (Figure 7) showed an upward trend for five weeks. A sharp decrease occurred followed by a leveling off during the last four

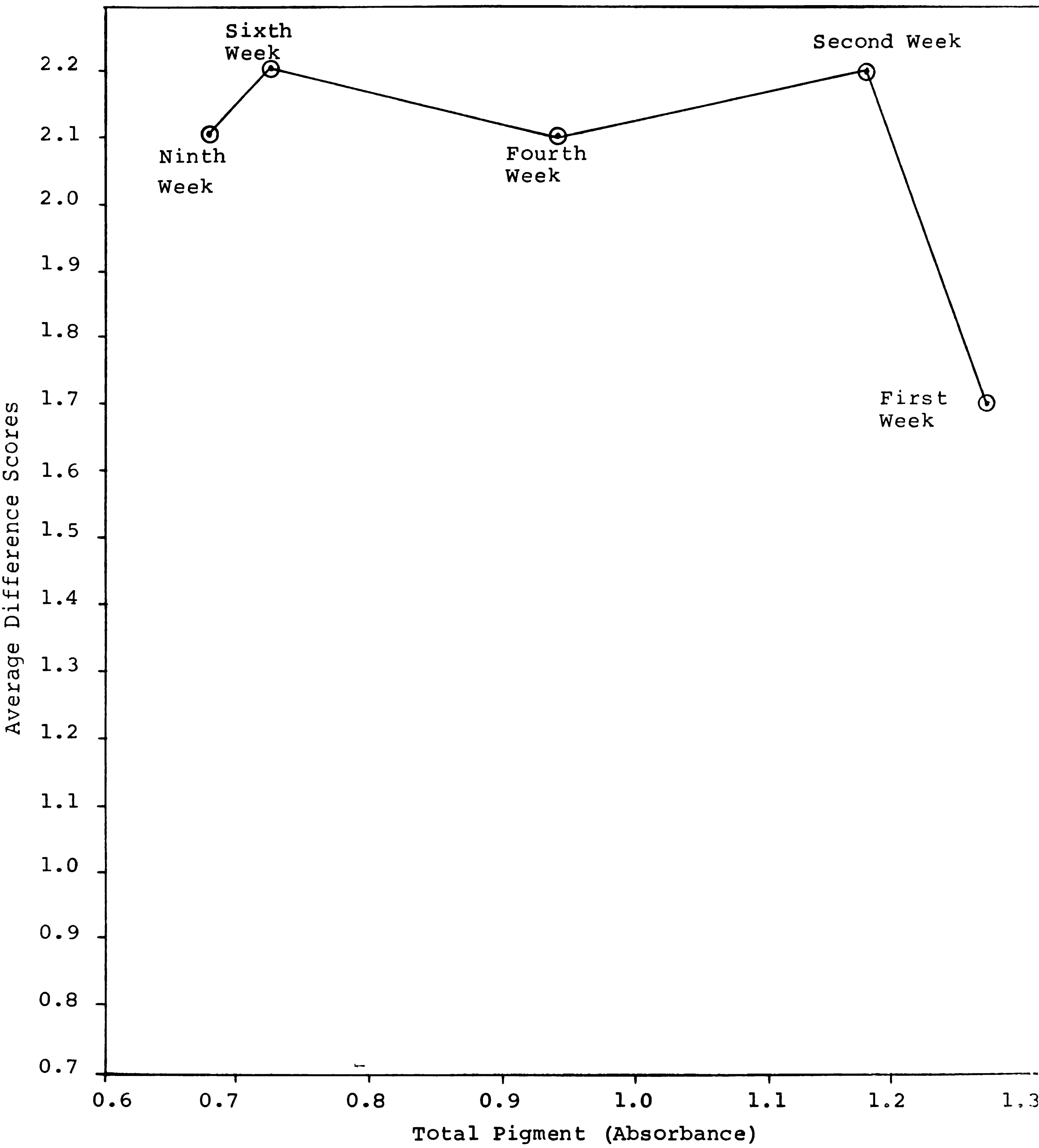


Figure 6. Relationship Between Changes in Total Pigment in Stored Freeze-Dried Beef and Taste Panel Evaluation

A. Control Samples (Study I)

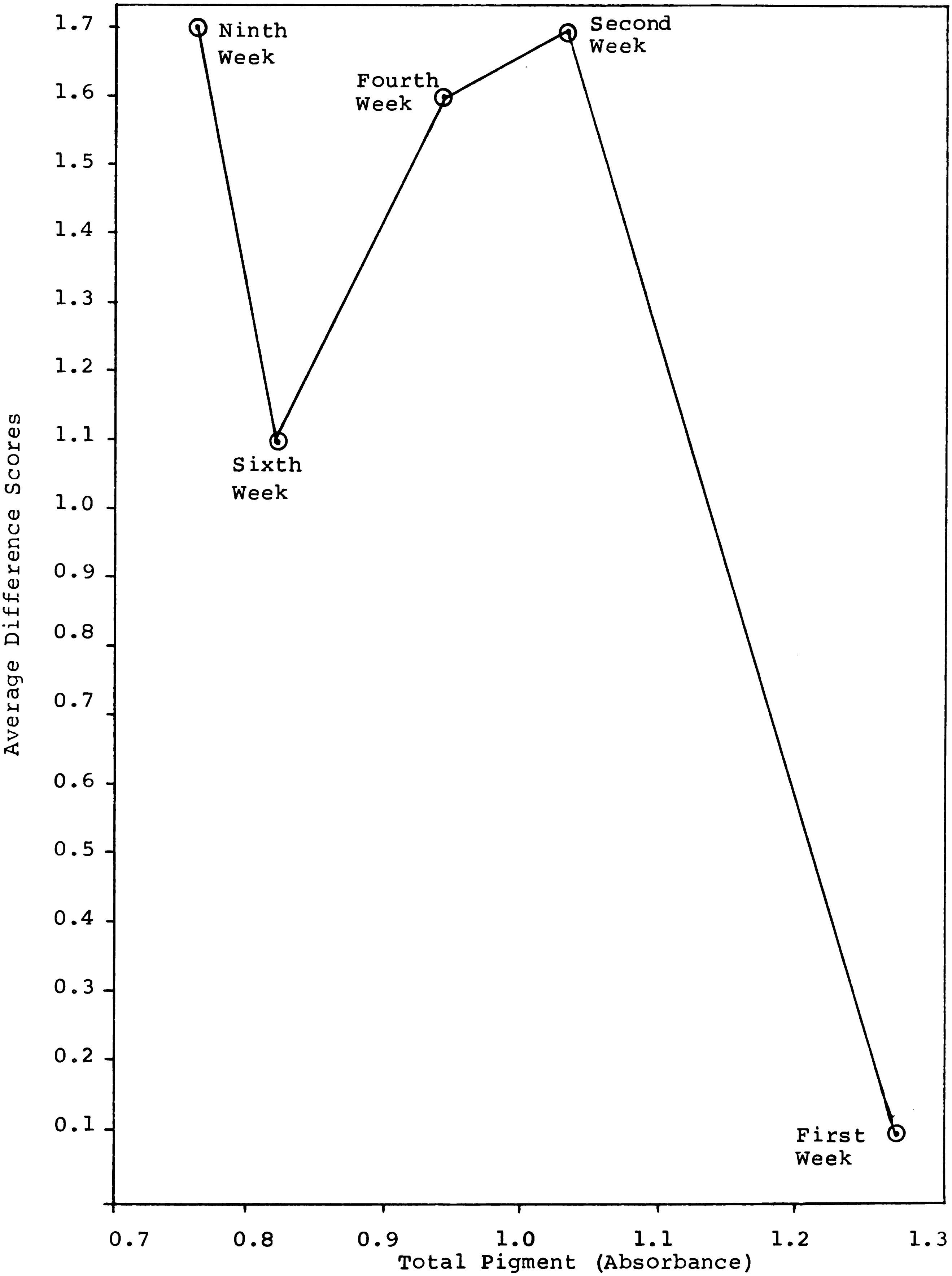


Figure 6. Relationship Between Changes in Total Pigment in Stored Freeze-Dried Beef and Taste Panel Evaluation

B. Nitric Oxide Cured Samples (Study I)

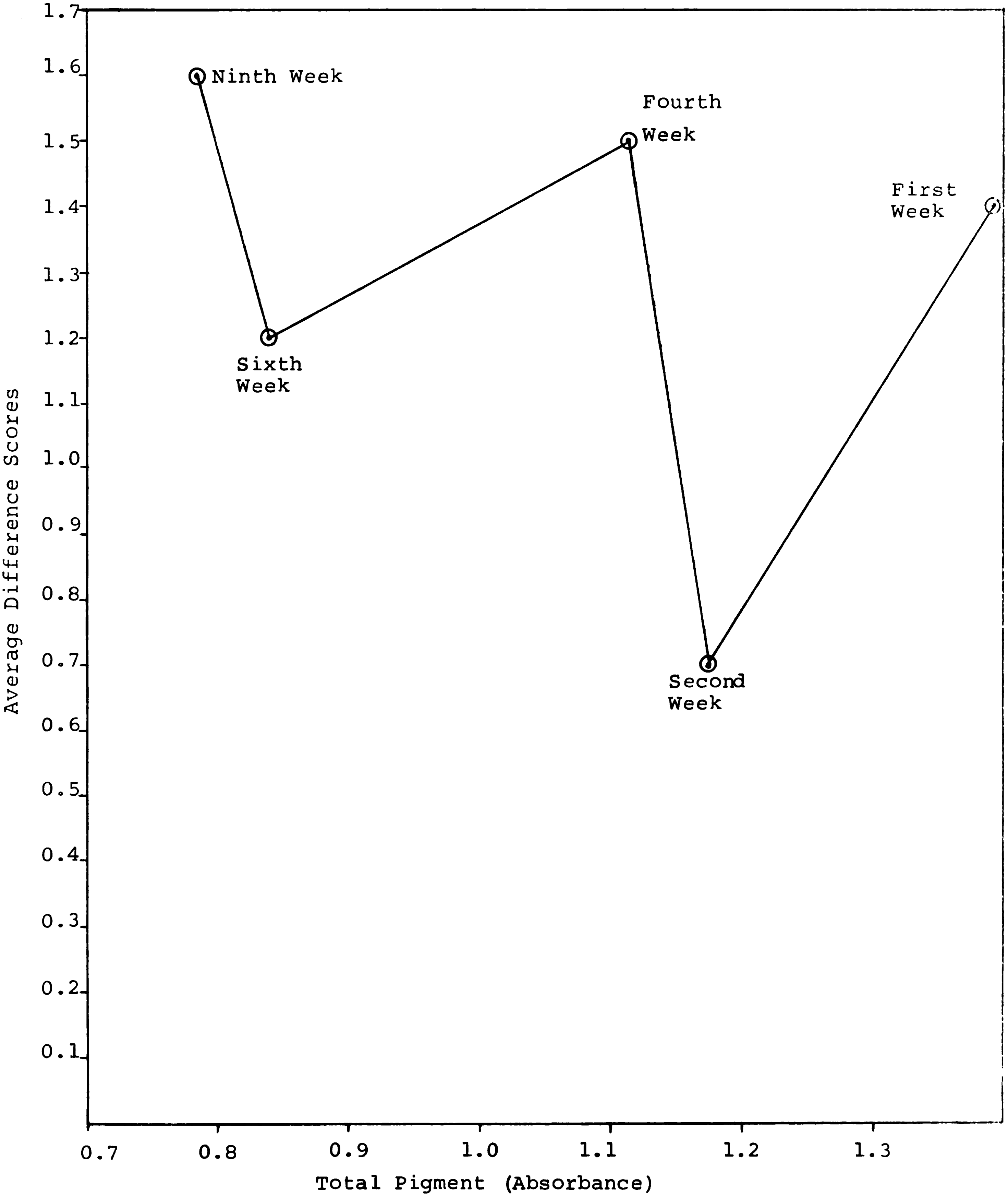


Figure 6. Relationship Between Changes in Total Pigment in Stored Freeze-Dried Beef and Taste Panel Evaluation

C. Sodium Nitrite Cured (Study I)

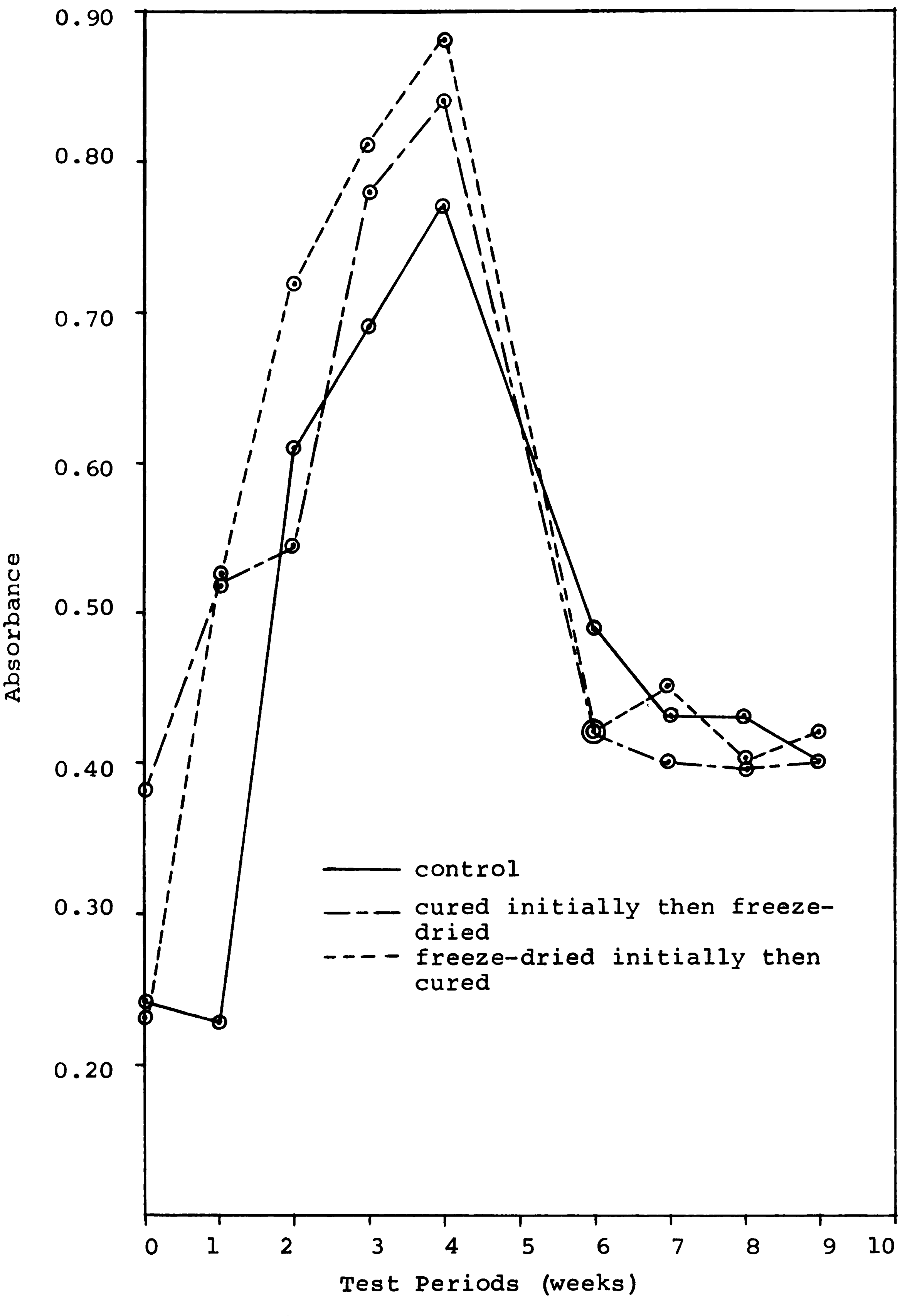


Figure 7. Colorimetric Determination of Carbonyls in Freeze-Dried Beef Stored at 40°C

weeks. This occurred with all of the sets of samples.

The nitric oxide heme measurement (Figure 8) decreased during the storage time. The samples which were cured first and then freeze-dried had initial pigment values almost three times those of the freeze-dried, cured and freeze-dried samples. However, after five weeks the values for both treatments were comparable.

The extraction and measurement of the total pigment (Figure 9) showed a gradual decrease in pigment in all samples. The untreated control began to decrease more rapidly after three weeks than the treated samples.

A taste panel was used to determine the time required for a significant difference to develop between a standard (stored in nitrogen gas at $-34^{o}C$) untreated sample and the stored samples.

The panel's preference for the various samples was also measured.

All of the stored samples were found to be significantly different from the standard sample after two weeks of storage. The panel consistently preferred the standard sample over the stored samples. It also preferred the untreated control over each of the cured samples. However, at the termination of the experiment the panel found all of the stored samples unacceptable.

Graphs were plotted for each set of stored samples (Figures 10a and b) using the data obtained from the total

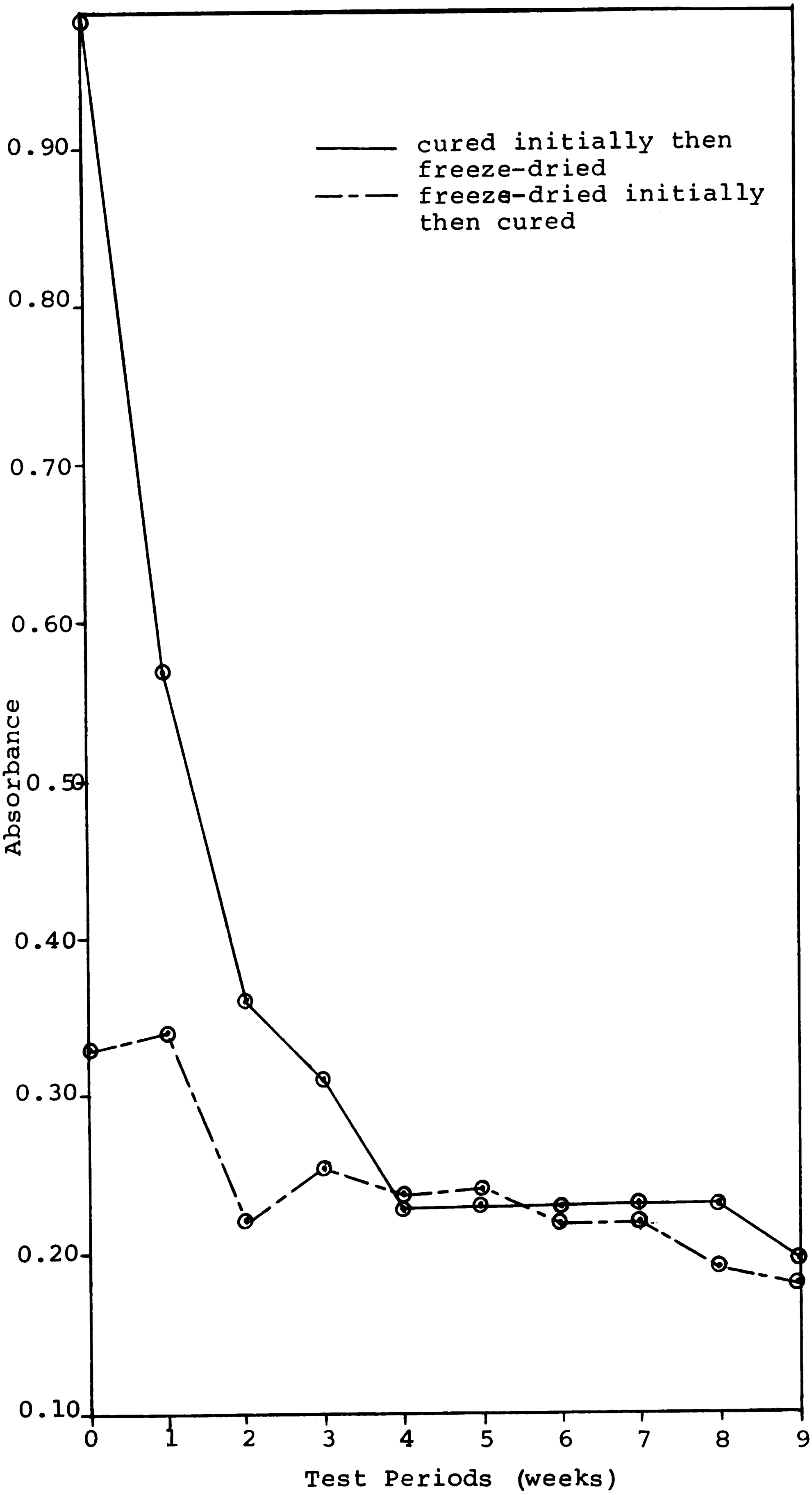


Figure 8. Nitric Oxide Heme Pigments in Freeze-Dried Beef Stored at 40°C

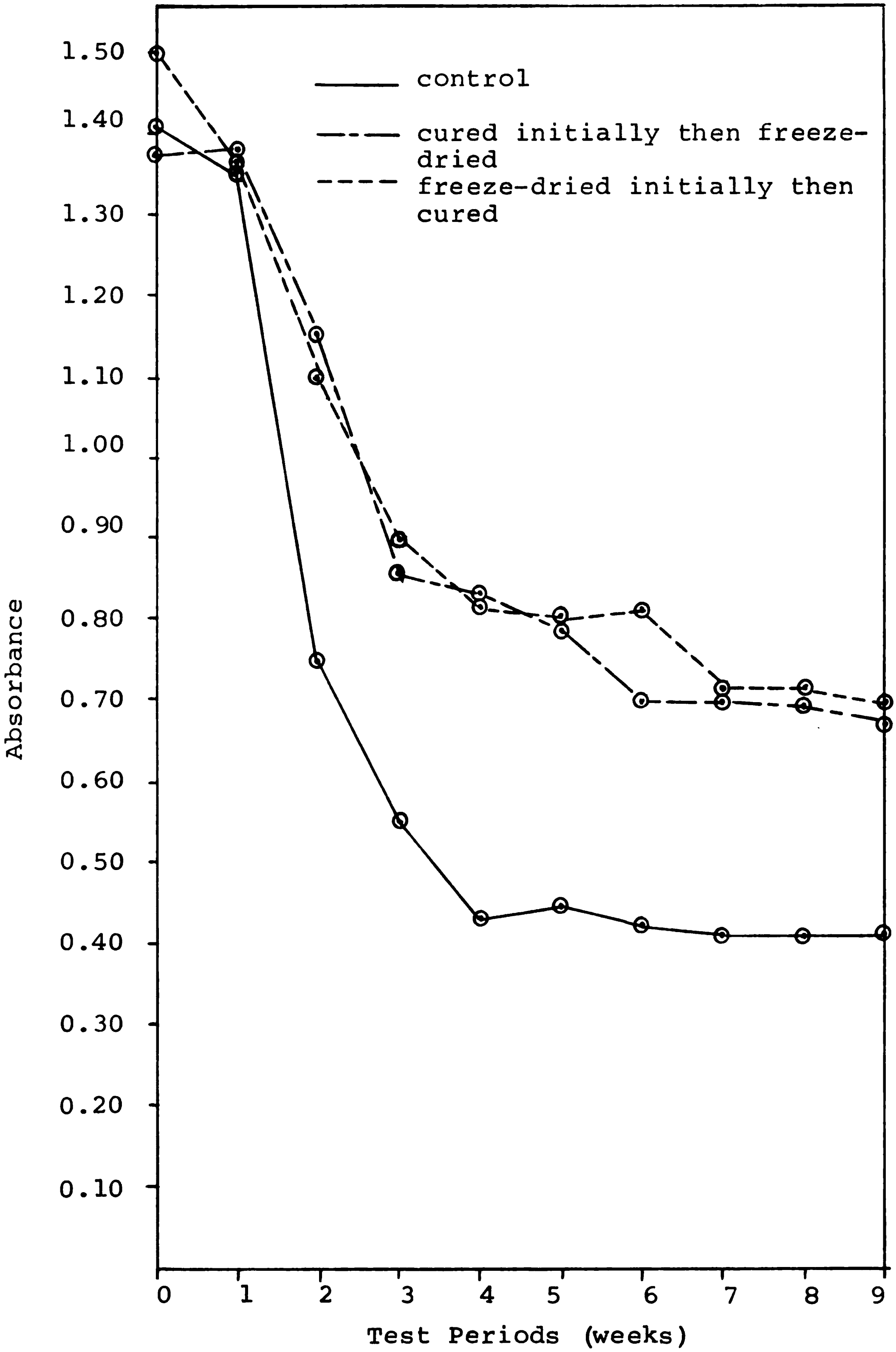


Figure 9. Total Pigments in Freeze-Dried Beef Samples Stored at 40°C

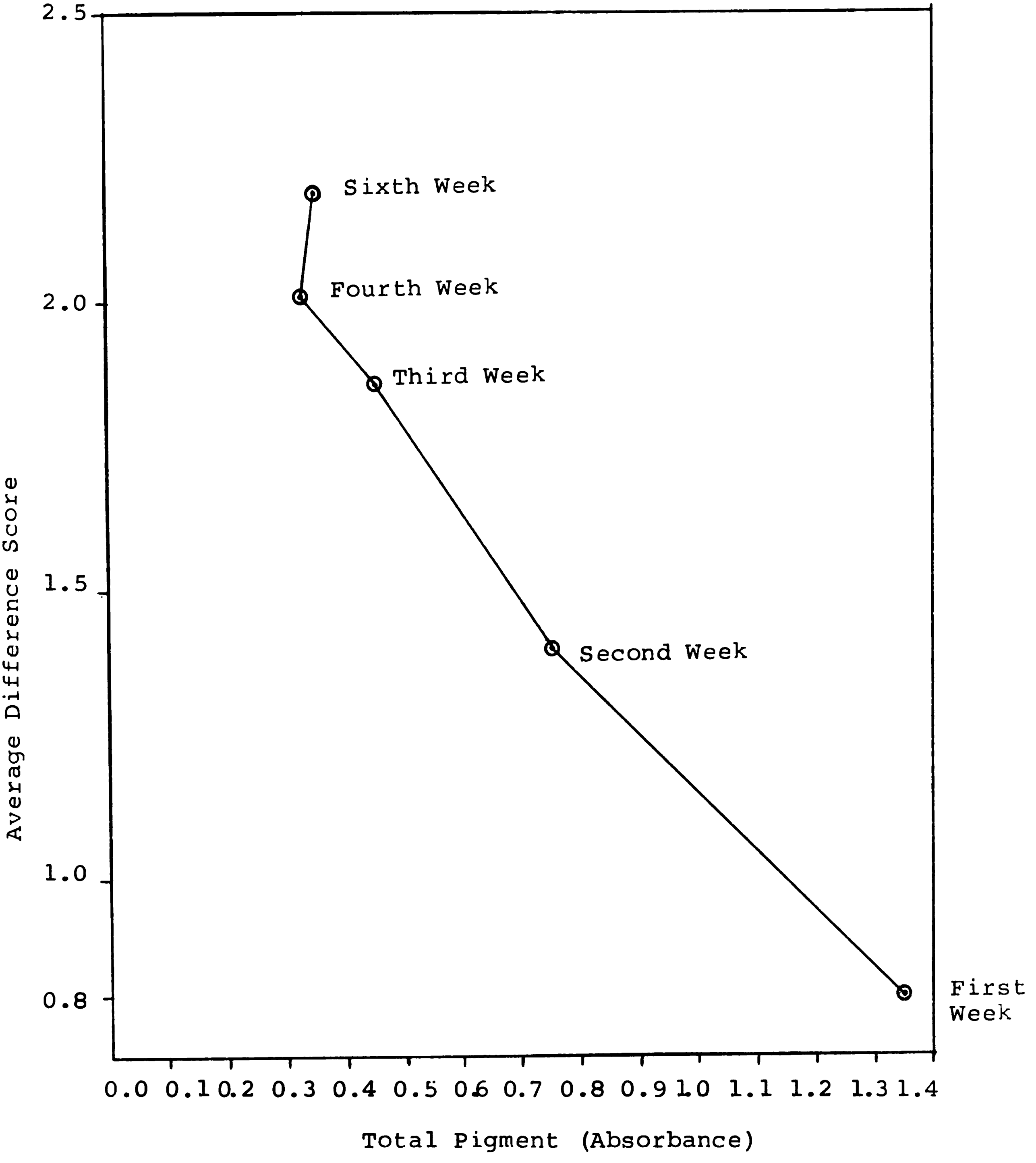


Figure 10. Relationship Between Changes in Total Pigment in Stored Freeze-Dried Beef and Taste Panel Evaluation
A. Control Samples (Study II)

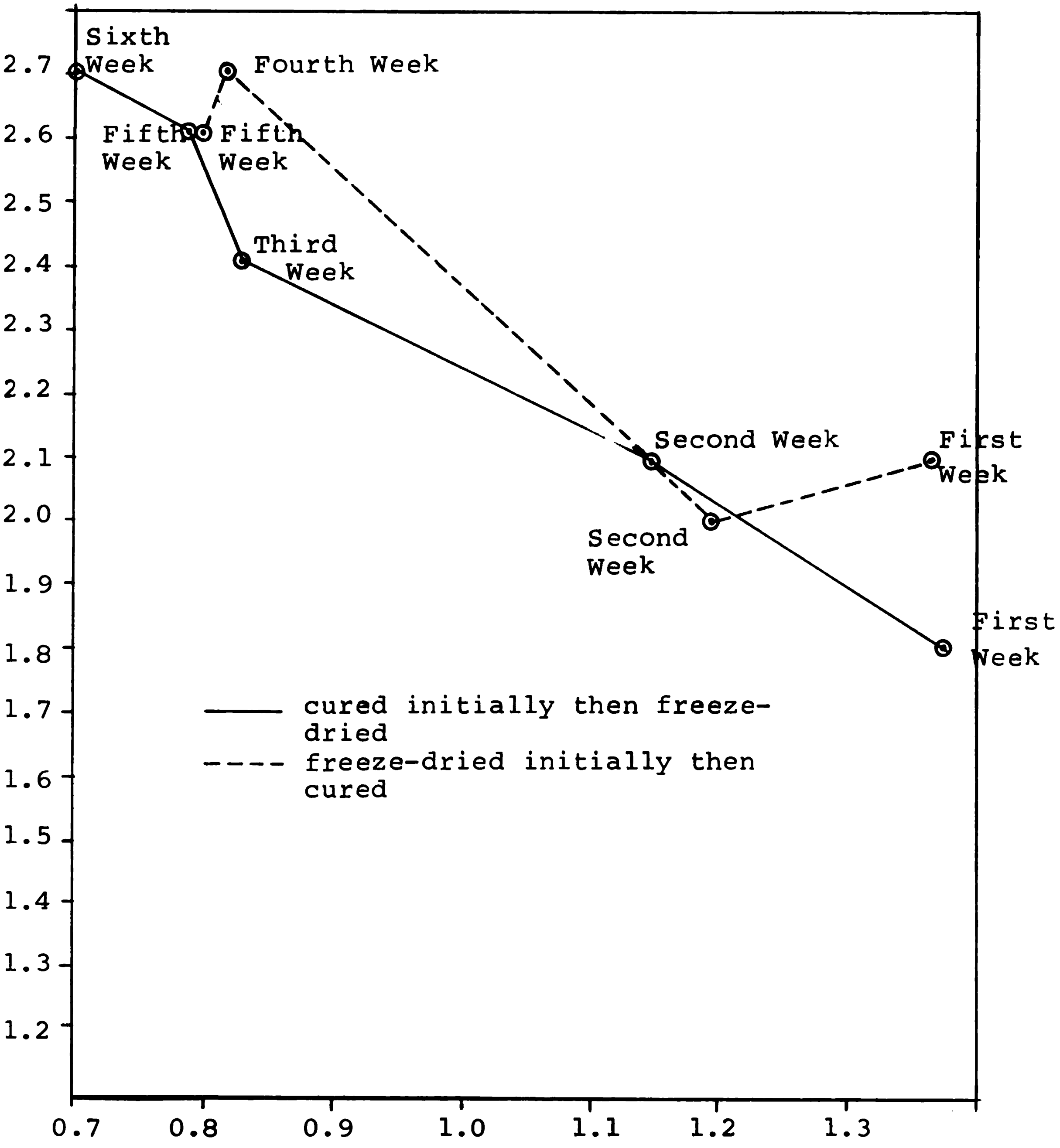


Figure 10. Relationship Between Changes in Total Pigment in Stored Freeze-Dried Beef and Taste Panel Evaluation

B. Cured Initially then Freeze-Dried Samples and Freeze-Dried Initially then Cured Samples (Study II) pigment measurement and the average difference scores of the taste panel. The average scores for the standard samples were subtracted from the average scores for each set of stored samples. This difference was then plotted against the total pigment for that particular sample. An inverse relationship was shown to exist between the pigment values and the difference scores. As the pigment values decreased the difference scores increased.

DISCUSSION

Study I

The results obtained from the TBA test (Figure 1) increased and then decreased. It was felt, therefore, that the TBA test could not be used as a measurement of the stability of a freeze-dried beef system. The zero readings obtained for the cured samples could not be completely explained. It is possible that no malonaldehyde was formed after three weeks of storage. The possibility also exists that the malonaldehyde was removed through typical carbonyl-amino reactions (20). Malonaldehyde is known to be a very reactive compound. These reactions could possibly tie up the malonaldehyde, thus preventing its detection by the TBA test. Watts and co-workers have shown that added malonaldehyde disappeared from cooked beef in sealed cans, suggesting further reaction of this compound (58).

The 2,4-DNPH estimation of carbonyls and the MBTH colorimetric procedure for carbonyls (Figures 2 and 3) gave results which were quite erratic. Carbonyl compounds as a group are very reactive. Their presence in a system undergoing oxidation could therefore be quite variable. An oxidizing system is not static by any means. There is most likely a continual turnover of products. As carbonyl compounds are formed, many of them enter into various reactions

which prevent their detection. Carbonyl-amino reactions, common browning reactions, involve reactions of aldehydes and ketones with amines, amino acids, peptides and proteins (20). Some of these reactants are found in freeze-dried beef undergoing oxidation. The common browning reactions possibly provide a significant pathway for the removal of free carbonyls.

The preceding suppositions may help to explain the erratic nature of the results achieved by both the 2,4-DNPH and MBTH procedures. However, the possibility of sampling errors cannot be overlooked. It is somehwat difficult to obtain a representative sample for the test procedures used. After freeze-drying, the cubes were mixed and randomly placed in polyethylene bags. The cubes of freeze-dried beef were randomly picked from each lot for each test. However, there is some doubt, due to biological variations, that representative samples were always used. This type of sampling error was probably responsible, at least in part, for the variations in results obtained from the various test procedures.

The extraction and measurement of nitric oxide heme gave results that decreased during the storage period of both the nitric oxide gas cured and the sodium nitrite cured samples. Apparently there was much greater conversion to nitric oxide heme with the use of nitric oxide gas than with sodium nitrite. Evidently, however, the nitric oxide heme produced by nitric oxide gas must not be as stable to oxidation as that produced by sodium nitrite (Figure 4). There was an increase in values during the last two test periods.

Since there could not be a rise in nitric oxide heme, this increase may be explained by the formation of colored polymers from carbonyl-amino reaction (20). These browning reaction products might have interfered with the measurement of nitric oxide heme. Some measurements were made on the control samples. The absorbance of these samples was zero for the first six weeks. The acetone extract was clear. After eight weeks though, the acetone extract became yellow and the absorbance readings increased. This most likely happened with the cured samples and resulted in an increase in values.

There was a variation in pigment of both the nitric oxide gas cured and sodium nitrite cured samples from test period to test period. Although the general trend was toward a decrease in values, there also were increases. These variations can best be explained by the failure to obtain representative samples for extraction and measurement of nitric oxide heme.

A gradual decrease in pigment was obtained from the extraction and measurement of total pigment. The untreated control decreased more rapidly than the treated samples after six weeks (Figure 5). Evidently the meat pigments in the untreated samples oxidized at a faster rate during the last four weeks than those of the cured samples. This would tend to indicate that curing does lend a measure of protection against rapid oxidation of the pigments in freeze-dried beef.

The absorbancies of the total extracted pigments were plotted against the average difference scores of the odor detection panel. The average difference scores were used because they gave more consistent values than the average scores themselves. The preference scores and the average scores did not follow any sort of pattern. The average difference scores, however, did fall into a pattern. They generally increased with storage time. Subtracting the average scores for the standard sample (stored in nitrogen at -34°C) from the average scores for the test samples seemed to even out the variations from panel evaluation to panel evaluation. This enabled the results to be graphed. The curves obtained from these graphs (Figures 6a, b and c) decreased from left to right for all three sets of samples. It appears that an inverse relationship might exist between the average difference scores and the total pigment measurement. As the average difference scores increased, the total pigment decreased. The higher the average difference scores, the greater the difference between the standard sample and the test samples. The decrease in total pigment in conjunction with the increase in the average difference scores indicates that the meat pigment is oxidizing and that undesirable odors are being produced. The freeze-dried beef system is deteriorating. This usually meant that the standard sample was preferred over the stored samples, especially when the average difference scores were greater than 1.5.

The results from the TBA test, the 2,4-DNPH estimation of carbonyls, the MBTH colorimetric test for carbonyls and the extraction and measurement of nitric oxide heme were erratic and did not show any general trends. Only the measurement of total pigment showed a trend. This indicates that the measurement of total pigment may prove to be a good indicator of the quality of freeze-dried beef.

Study II

The 2,4-DNPH estimation of carbonyls gave results which increased for five weeks and then decreased. A leveling off occurred during the last four weeks. This phenomenon happened with all three sets of samples (Figure 7). Evidently, curing the freeze-dried beef made no difference, since the cured samples followed the same pattern as the control samples. There probably was a buildup of carbonyl compounds to a certain critical level at which point they reacted with other oxidation products of lipids and/or with proteins present (20). The carbonyl compounds could even have been oxidized to acids (18, 34). Short chain fatty acids C_2 to C_{10} are quite flavorful and could be partly responsible for the off-flavors and odors in oxidized freeze-dried beef. All of these reactions were most likely accelerated by the $40^{\circ}C$. temperature, as compared to the $25^{\circ}C$. storage temperatures of the samples in Study I.

The extraction and measurement of nitric oxide heme gave results that decreased during the storage time. The decrease or disappearance of nitric oxide heme pigment was probably due to the oxidation of the pigment, the ferrous iron being oxidized to the ferric form and ring destruction occurring. The fading of the pigment color is a gradual process and both of the above mentioned reactions may proceed simultaneously (61).

The conventionally cured samples I, those that were cured first and then freeze-dried, had a much greater conversion to nitric oxide heme than the conventionally cured samples II, those that were freeze-dried, cured and then freeze-dried again. The same level of sodium nitrite was used in both cases. There are several explanations possible. It is possible that some heme pigment was oxidized after removal from the freeze-drier and before curing. If this was a significant amount, the conversion to nitric oxide heme would not be as great in samples freeze-dried first and then cured. It is doubtful, however, that this was significant. Uncured freeze-dried beef was not left exposed to the atmosphere for more than 10 minutes. The rate of dehydration of the freeze-dried beef by the curing pickle may have been the cause of the difference between the two sets of samples. The conventionally cured samples I (cured before freeze-drying) took approximately 24 hours to be cured. The conventionally cured samples II (freeze-dried before curing) were left in the curing pickle for three days. It took at least this long for the samples to rehydrate. Since it was not possible to check every cube of meat in the curing pickle, the possibility exists that some cubes did not fully rehydrate. If this occurred, then the sodium nitrite could not have reacted to form nitric oxide heme throughout the entire cube of beef. The combination of these two events might help explain some of the difference in conversion to nitric oxide heme between the two curing methods.

Although the conventionally cured samples I had initial values of almost three times that of the conventionally cured samples II, with regard to nitric oxide heme, after five weeks the values for both treatments were comparable. Figure 8 indicates that the nitric oxide heme disappeared at a faster rate in the conventionally cured samples I than in the conventionally cured samples II, for the first three weeks. After this time the rates were about equal.

The extraction and measurement of the total pigment showed a gradual decrease in values with all samples. This decrease was most noticeable in the untreated control samples, especially after three weeks of storage. This tends to substantiate the results from Study I concerning the oxidation of total pigment in freeze-dried beef. Curing does lend a measure of protection against rapid oxidation of meat pigments in freeze-dried beef. There was no difference in pigment loss between the two treated sets of samples. Evidently, it does not matter, as far as pigment stability is concerned, whether the beef is cured before or after freeze-drying.

The values obtained from the measurement of total pigment were plotted against the average difference scores of the taste panel. Once again, as in Study I, it was found that only the average difference scores followed a pattern. They generally increased with storage time. The curves obtained from these graphs (Figures 10a and b) decreased from left to right for all three sets of samples. It appears that an inverse relationship might exist between the average difference scores and the total pigment measurement. This possibility was also found in the results of Study I. The average difference scores increased while the total pigment decreased. The higher the average difference scores the greater the difference between the standard sample and the test samples. Since the taste panel consistently preferred the standard sample (stored in nitrogen gas at -34^{o}C.) to the experimental samples, this difference was a numerical measure of the panel's dislike of the stored samples.

The results of the 2,4-DNPH estimation of carbonyls and the extraction and measurement of nitric oxide heme were too erratic to indicate any general trends. Only the measurement of total pigment showed a trend. As previously discussed, the total pigment decreased with increasing storage time. This measurement also correlated with the organoleptic procedures employed.

Destruction of Meat Pigments

El-Gharbawi found that in cooked freeze-dried beef the total soluble nitrogen, soluble protein nitrogen and soluble non-protein nitrogen decreased with increasing storage time and the initial concentration of oxygen. There was also a decrease in free amino groups (8). The cooked freeze-dried beef was stored in air-tight cans. Koch has reported some reactions of oxidized lipids in systems physically similar to freeze-dried foods. Freeze-dried protein foams containing added methyl linoleate were heated at 50°C. A gelatin-linoleate foam lost 22% of its a-amino nitrogen, when heated for six days in air. There was no loss in the absence of oxygen (27).

Hemoglobins may be regarded as essentially square-planar iron-porphyrins, further coordinated to one strong-field ligand (histidine) and one weak-field ligand (water) (11). It is known that transient free-radicals are produced in peroxidizing lipid-protein reaction systems (41). Freeze-dried beef can be considered to incorporate such a system. The damage to proteins, induced by these free-radicals, consists of denaturation and destruction of constituent amino acids (5, 41). Tappel found that histidine was among the most labile of amino acids (41). Tappel also found that freeze-dried beef stored at 38°C reacted with relatively large amounts of atmospheric oxygen. Approximately from 50 to 100% of the oxygen absorbed could be accounted for by the oxidation of the protein fraction of freeze-dried beef (52).

Therefore, the destruction of freeze-dried beef pigment, which occurred at 25°C in Study I and 40°C in Study II, can be attributed to the presence of free radicals contributed by the peroxidizing lipids present. These free radicals may destroy the histidine coordinated to the heme pigments, allowing separation from the protein portion of the molecule. Further attack by free radicals may result in porphyrin ring destruction. A close relationship exists between protein structure, porphyrin ring and electronic configuration of the iron. Even minor alterations of the protein structure, such as the breaking of a few hydrogen bonds in the neighborhood of the heme group could profoundly affect linkage and interaction between the heme and oxygen or peroxide bound to it (32). It is probably safe to assume, therefore, that a major event, such as the breaking of the histidine bond of the hemoglobin molecule could have such a deleterious effect as to readily open up the heme portion to destruction by free radicals.

CONCLUSIONS

1. The 2-thiobarbituric acid distillation method is not a useful tool for measuring the stability of either untreated or cured stored freeze-dried beef.

2. The 2,4-dinitrophenylhydrazine estimation of carbonyls and the N-methylbenzothiazolone hydrazone colorimetric procedure for carbonyls were found not to correlate with the organoleptic procedures used. Therefore, it is felt that these tests cannot be used to measure the stability of either untreated or cured stored freeze-dried beef.

3. The measurement of nitric oxide heme also did not correlate with the organoleptic procedures used. It is felt that this test is of little value in measuring the stability of stored freeze-dried beef.

4. The measurement of total pigment may be useful as a measure of the stability of either untreated or cured stored freeze-dried beef, since it did not correlate with the organoleptic procedures employed.

5. The results from Study I, using both the chemical and organoleptic tests, seem to indicate that the sodium nitrite cured samples have a greater measure of stability, under these particular storage conditions, than either the nitric oxide gas cured samples or the control samples. The increase in carbonyl content, the apparently faster rate of pigment oxidation, and the lower acceptance by the odor detection panel of the nitric oxide gas cured and the control samples, were the criteria used.

6. There appears to be no difference in the stability of sodium nitrite cured beef whether it is cured first and then freeze-dried or freeze-dried first and then cured during rehydration.

7. Study I showed that freeze-dried beef can be cured as such, using either nitric oxide gas or conventional methods.

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