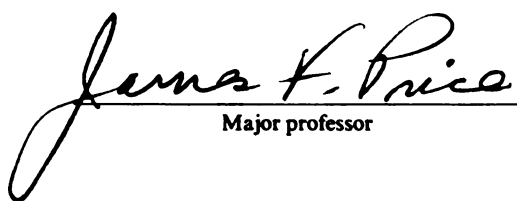This is to certify that the

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EFFECT OF LIPID OXIDATION ON FUNCTIONAL AND NUTRITIONAL

PROPERTIES OF CHICKEN MYOFIBRILLAR PROTEINS STORED AT

DIFFERENT WATER ACTIVITIES

BY

HAJAR (SHEILA) NOORMARJI

A DISSERTATION

Submitted to
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In partial fulfillment of the requirements
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ABSTRACT

EFFECT OF LIPID OXIDATION ON FUNCTIONAL AND NUTRITIONAL PROPERTIES OF CHICKEN MYOFIBRILLAR PROTEINS STORED AT DIFFERENT WATER ACTIVITIES

BY

HAJAR (SHEILA) NOORMARJI

Effects of lipid oxidation, lipid protein interaction, water activity and storage on functional and nutritional properties of freeze-dried chicken myofibrillar proteins were investigated using a model system. The effects of freezing and freeze drying on protein solubility and lipid oxidation were studied using chicken myofibrillar proteins as a model system.

Freezing and freeze drying decreased ($P < .05$) protein solubility and increased ($P < .05$) lipid oxidation. These effects were more drastic when methyl linoleate was added to myofibrillar protein at 15% of the dry protein weight. The effect of several different water activities on lipid oxidation and protein functionality in a freeze-dried chicken myofibrils (water activity environment of 0.43) was studied. It was demonstrated that the a_w 0.43 had a protective effect, while the model system, a_w 0.11 and a_w 0.85 (below BET monolayer value and far above BET value) showed prooxidant effects. Moreover, control myofibrillar protein had a lower monolayer value than the lipid treated counterparts.

Lipid oxidation increased ($P < .05$) and percent soluble proteins decreased ($P < .05$) with storage time. Proteins stored at a water activity close to their monolayer value were less suscep-

tible to lipid oxidation. Water-holding capacity and gel
strength were reduced ($P < .05$) by addition of lipid, storage and
increase in water activity. On the other hand, neither in vitro
digestibility nor the ability of myofibrillar proteins to support
growth of <u>Tetrahymena</u> <u>pyriformis</u> were affected by addition of
lipid, storage for 3 weeks or water activity. Thus, lipid oxida-
tion may affect the functional properties rather than the nutri-
tional quality of chicken myofibrillar proteins.

To my husband, MEHDI, for his love, support, hard work and patience during my doctoral studies. Without him I could never reach my goal.

To my children, MARYAM and HOOMAN, who have been the light of my eyes and the joy of spirit.

To my parents, who taught me the real values of life and encouraged me to pursue my education, words are totally inadequate.

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INTRODUCTION

Proteins are one of the most important components determining the functionality of food systems. The importance of proteins as functional components of food derives to a large extent from the following:

1. Proteins, along with polysaccharides, determine rheological properties of food which in turn determine food texture.

2. Proteins, along with polysaccharides, determine the water-holding properties of foods.

3. Proteins, often in combination with lipids, are the most important stabilizers of dispersed systems in foods.

4. Enzymes catalyze reactions that significantly affect food utility.

The properties of proteins in food systems depend mostly on the interactions of proteins with other components, such as water and lipids. Most proteins in foods are closely associated with lipids, and lipids are important to physical and chemical characteristic of muscle foods. Lipids are prone to autoxidation. Damage to proteins due to reaction with oxidized lipid is an important deteriorative mechanism in the processing and storage of foods (Melton, 1983; Matoba et al., 1984; Sikorski, 1978). Exposure of proteins to peroxidizing lipids or their secondary products may lead to undesirable changes in nutritional, biochemical and functional properties. Oxidizing lipids cause protein

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insolubization, polymerization, scission, poor digestibility, amino-acid and vitamin destruction (Funes et al., 1982; Funes and Karel, 1981; Pokorny, 1977; Nakhost and Karel, 1984). Functionality is the most important property for proteins in food items. Some proteins cannot be used because they lack such functional properties as gelation, solubility, water absorbability, foamability or emulsifiability (Kinsella, 1982, 1976).

There is an increasing interest in understanding the nature of lipid-protein interactions in biological and nonbiological systems. Since proteins play an important role in food systems, it is important to understand the effect of oxidized lipid-protein interactions on protein functionality. It is important to adjust the conditions of processing and storage of meat products in order to minimize, or at least to regulate, the changes due to lipid-protein interaction.

The purpose of this study was to examine the effect of lipid oxidation on functional and nutritional properties of myofibrillar protein.

The objectives of this research were:

1. To investigate the effect of lipid oxidation on protein functionality.

2. To determine how different conditions (water activities, storage time) affect protein-lipid interaction.

3. To investigate the effect of lipid-protein interactions on protein solubility, water-holding capacity and gelling properties.

4. To determine the effect of lipid-protein interaction on nutritional quality of myofibrillar proteins.

LITERATURE REVIEW

Role of Proteins in Food Systems

The importance of proteins, peptides and amino acids in all living tissue should be evident. Proteins function as structural components, enzymes, regulatory agents and transport factors. Proteins are the principal functional and structural components of processed meats and determine the characteristic handling, texture and appearance of these products (Hermansson et al., 1986; Hermansson, 1985). Proteins are used to fabricate and facilitate the engineering of new foods. To facilitate their use in foods and their conversion to desirable ingredients, they must have appropriate functional properties. Functional properties can be defined as physiochemical properties of a protein which determine their utility in foods, including its nutritional value, organoleptic properties, safety and response to processing and storage (Kinsella, 1982). Physicochemical properties are derived from a protein's amino acid composition, amino acid sequence, secondary structure, tertiary structure, and quaternary structure (Pour-El, 1981). Physiochemical properties include surface charge, sulfhydryl content, hydrophobicity, molecular weight and conformational stability (Wilding et al., 1984; Kinsella, 1982). Several typical classes of functional properties are listed in Table 1.

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Table 1: Typical functional properties performed by proteins in food systems (Kinsella, 1982).

Functional Property	Mode of Action	Food System Example
Solubility	Protein solvation	Beverages
Water absorption and binding	Hydrogen-bonding of water, Entrapment of water	Meats, Sausages, Breads, Cakes
Viscosity	Thickening, Water binding	Soups, Gravies
Gelation	Protein matrix formation and setting	Meats, Curds, Cheese
Cohesion-adhesion	Protein acts as adhesive material	Meats, Sausages, Baked goods, Pasta products
Elasticity	Hydrophobic bonding in gluten, Disulfide links in gels	Meats, Bakery
Emulsification	Formation and stabilization of fat emulsions	Sausages, Bologna, Soup, Cakes
Fat absorption	Binding of free fat	Meats, Sausages Donuts
Flavor-binding	Adsorption, entrapment, release	Simulated meats Bakery, etc.
Foaming	Form stable films to entrap gas	Whipped toppings, Chiffon desserts, Angel cakes

Different food applications require different characteristics, e.g.; in beverages, protein should be soluble, and in comminuted meats they should have emulsion stabilizing and gelling properties. In processed meat products, water binding, solubility, emulsifying capacity, viscosity and gelation are typical properties of proteins that determine their impact on the final quality (Hermansson, 1985; Acton et al., 1983; Kinsella, 1982).

As discussed earlier, the functional properties of proteins depend on such intrinsic physiochemical characteristics as amino acid composition and sequence, molecular weight, conformation and charge distribution on the molecules. The amount of hydrophobic amino acids affect conformation, hydration, solubility, gelation and denaturation (Kinsella, 1982). In general, the higher the polarity and the lower the hydrophobicity the higher the solubility (Nakai, 1983). Polar amino acids contribute to the water binding ability of a protein (Phillips and Beuchat, 1981). Myosin contains 38% polar amino acids with a large content of aspartic acid and glutamic acid residues which may bind 6-7 molecules of water each (Harrington, 1979). The gelation and emulsifying ability of proteins are affected by their structure. Voutsinas et al. (1983) showed a positive relation between gelation and hydrophobicity of the unfolded protein and sulfydryl content. Shimada and Matsumoto (1980) reported that proteins, e.g.; soybean, albumin, bovine serum containing 26-31% hydrophobic amino acids, formed gels upon heating, whereas proteins with 31% hydrophobic amino acids coagulated. Kato and Nakai (1983) re-

ported a close relationship between the hydrophobicity of pro-
teins and their emulsifying capacity.

Extrinsic factors which influence protein functionality
include pH, ionic strength (Kinsella, 1982), specific ions,
cooking temperature and frozen storage (Smith, 1988b; Whiting,
1988).

Muscle Proteins

Muscle proteins can be divided into three categories based
on their solubility characteristics: 1) water soluble proteins,
2) salt soluble proteins and 3) insoluble proteins (Forrest et
al., 1975). The water soluble proteins or sarcoplasmic proteins
are also soluble in salt solutions of low ionic strength (0.1).
These proteins compose about 30% of the total muscle protein or
about 5% of the weight of muscle (Lawrie, 1979) and include
oxidative, glycolytic and lysosomal enzymes, myoglobin and other
water soluble proteins (Kramlich, 1978). About 100 different
proteins are known to be present in the sarcoplasmic fraction
(Scopes, 1970). The sarcoplasmic proteins influence functionali-
ty, but differently than the myofibrillar proteins (Gillett,
1987a, 1987b; Sikorski et al., 1984). Myofibrillar or structural
proteins which are soluble in concentrated salt solutions (ionic
strength of 0.5 to 0.6) include myosin, actin, tropomyosin,
troponin, actinins and others. Connective tissue proteins or
insoluble proteins constitute 10-15% of the total muscle proteins
and are composed mostly of collagen (40-60%) and elastin (10-
20%).

Myofibrillar Proteins

The salt soluble proteins which compose the myofibrils within the muscle fibers are collectively defined as the myofibrillar proteins. The major proteins in this category are myosin, actin and regulatory proteins, which make up about 55%, 25% and 20% of the fraction, respectively. The myofibrillar proteins, myosin in pre-rigor and actomyosin in post-rigor muscle are generally considered to contribute the most functionality to processed meat products (Smith, 1988a).

Myosin is the major constituent of the thick filaments in the sarcomere. The myosin molecule is a large molecule consisting of two heavy chains with a molecular weight of about 205,000 daltons, and four light chains with an average molecular weight of 20,000 daltons. About 35% of the muscle proteins are myosin (Hanson and Lowey, 1964). Myosin has a high content of basic, acidic polar (70%) and sulfhydryl (3%) amino acids (Whiting, 1988). Myosin possesses adenosine triphosphatase (ATPase) enzymatic activity, which transfers the chemical energy of ATP into the contractions of the muscle.

Actin is the second most abundant protein in the contractile units. Actin forms the backbone of the thin filaments and accounts for 22% of the myofibrillar protein (Yates and Greaser, 1983). Actin can exist in two forms, as a monomer termed G-actin (globular), or as a polymer, termed F-actin (fibrous), depending

on environmental conditions (Steiner et al., 1952). At physio-
logical concentrations of salt, globular G-actin polymerizes to
form F-actin which can interact with myosin filaments to produce
mechanical energy for muscle contraction (Bandman, 1987; Pollard
et al., 1981). Actin monomers (G-actins) are relatively small,
having a molecular weight close to 46,000 dalton.

Solubility and Myofibrillar Proteins

Myofibrillar proteins are the most important functional
components in meat, and their structure, conformation and inter-
action affect the yield, juiciness and tenderness of food
products (Kinsella, 1982). Their presence is necessary for fat
binding, water binding and gel formation in meat products (Acton
et al., 1983).

The myofibrillar proteins must be solubilized in order to be
functional (Kinsella, 1976). Salt serves many functions in meat
systems, one of which is to solubilize myofibrillar proteins.
The addition of salt (NaCl) to minced and communited meats causes
solubilization and extraction of myofibrillar proteins. Salts
also contribute to flavor, influence shelf life and affect the
functional properties of muscle proteins (Olsen, 1982). Salt
facilitates protein extraction from the meat tissue through a
salting-in or solubilizing effect. The way in which salt ions
bind to proteins is primarily electrostatic due to the attraction
of the salt ions by the positively or negatively charged groups
of the proteins (Schellman, 1953). Factors which influence the
extractability of meat proteins are post-mortem age, pH, ionic

strength, temperature, and freezing (Bard, 1965). The extract-
ability of muscle proteins is generally reduced during the early
post-mortem period when pH is low and muscle temperature is still
high (Borchert and Briskey, 1965). Comminution physically dis-
rupts muscle tissue by damaging the sarcolema (Hamm, 1975),
endomyosin (Wilding et al., 1984) and the integrity of muscle
fibers. Comminution of muscle at ionic strengths above 0.6
causes swelling of muscle fibers (Wilding et al., 1984), depoly-
merization of myosin, solubilization of myosin and extraction of
myofibrils from the muscle fibers (Hamm, 1986).

Hamm (1973) reported that the effect of pH on the protein
solubility of a meat system is dependent on the presence of
other factors in the system. One of the most important factors
is the presence of salt. The presence of salt results in a
lowering of the isoelectric point of the proteins, thus signifi-
cantly increases the solubilization of protein. Meink et al.
(1972) studied the relationship between protein solubility, salt
concentration and pH. Their data indicated that myofibrillar
protein solubility increased with increasing salt concentration
at approximately pH 6. Protein solubility has been linked to
functionality by many researchers and is used as an index of
functionality (Kinsella, 1976).

Emulsification and Myofibrillar Proteins

When frankfurters, hot dogs or bologna are manufactured, the
meats are extensively chopped (comminuted) to produce small

particles. One can observe a heterogeneous mass of lean and fat transformed into a meat batter of homogeneous appearance. Meat batters are fluid and primarily composed of water, fat and protein. One key element in manufacturing these products is stabilizing the fat and moisture to prevent excessive losses or product failure. The meat proteins stabilize the fat, therefore, meat batters have historically been defined as meat emulsions (Foegeding, 1988). Meat batters are considered an oil-in-water emulsion and stability of this emulsion depends on the meat protein behavior. In all of the emulsion products, the type of meat protein used is important. Salt soluble myofibrillar proteins, actin, myosin and actomyosin are primarily responsible for emulsion formation and are recognized as the important emulsifiers and stabilizers in meat systems (Gaska and Regenstein, 1982). The physiochemical properties of myosin which may allow it to function as an emulsifier include: 1) a hydrophobic region which orients toward the fat globule, 2) a hydrophilic region which orients toward the continuous matrix and 3) molecular flexibility for unfolding at the interface to lower surface tension (Jones, 1984). Nakai et al. (1986) reported a good correlation between emulsification properties of salt extracted meat proteins and physiochemical protein properties of surface hydrophobicity, sulfhydryl group content, and solubility. Li-Chan et al. (1985) stated that solubility by itself is not a good predictor of emulsifying properties. They reported that both protein hydrophobicity and solubility were important parameters

affecting the emulsifying properties of meat proteins.   The sarcoplasmic proteins play a small role and are in fact poor participants in meat batter systems, possibly because they are not able to form a gel structure and/or they interfere with the gel (matrix) structure of myofibrillar proteins (Gaska and Regenstein, 1982).

Hegarty et al. (1963) reported that functionability of water soluble proteins, in the presence of salt, were enhanced, and the protein was capable of considerable emulsion stabilization at the pH of fresh meat, 5.6 - 5.8.   However, water soluble and salt soluble proteins reacted differently. The emulsification capacity of sarcoplasmic proteins increased with lower pH, but decreases with lower pH in the salt soluble fraction. This supports the work of Swift and Sulzbacher (1963) which showed that peak emulsification capacity was dependent upon both pH and salt concentration for both protein types. As the salt concentration increases, the emulsifying capacity also increases, due to the fact that salt serves to solubilize the myofibrillar proteins (Cunningham and Froning, 1972).

A great deal of work has been done concerning the effect of pH on the properties of muscle proteins (Froning and Janky, 1971; Froning and Neelakantan, 1971; Hwang and Carpenter, 1975; Swift and Sulzbacher, 1963). These researchers have all reported that as the pH of the system is moved away from the isoelectric point of muscle proteins, the emulsification properties of the protein are increased. This is probably due to improved solubility of the proteins at these pH levels.

The emulsifying capacity can vary among different types of poultry meat. May and Hudspetch (1966) reported that in various classes of poultry the amount of total protein that was salt soluble was greatest in hen white meat (40.67%), followed by broiler dark meat, turkey dark meat and hen dark meat (16.67%). However, they stated that in all classes of poultry, the dark meat proteins display greater emulsification capacity than did the white muscle proteins. McCready and Cunningham (1971) indicated that although dark meat was lower in total protein and salt soluble protein, its ability to emulsify oil was greater than that of broiler light meat, which was higher in total and salt soluble protein content. They stated that the pH of dark meat was higher than that of light meat and suggested that pH was more important to emulsification capacity than was the percentage of salt soluble protein in the meat tissue.

Gelation and Myofibrillar Proteins

In manufacturing of processed meat products, gelation, water holding and fat binding are the most important functional properties that influence product quality. In meat products that are chopped when raw, the heat processing transforms the highly viscous raw material into a solid, gel-like product. Muscle proteins denature and aggregate to form the gel matrix, hence the gelation properties of muscle proteins, particularly myosin and actomyosin, are important in determining their functional role in processed meats.

The presence of salt-soluble myofibrillar proteins has been shown to be necessary for binding in both emulsion and restructured meat products (Miller et al., 1980). Among the myofibrillar proteins, myosin is essential for gel formation. Myosin is one of the most important functional proteins in meat, and its states of aggregation have an impact on functional properties such as texture, fat and water holding of meat products (Acton et al., 1983). Actin does not form a gel, but coagulates on heating (Samejima et al., 1969). Nakayama and Sato (1971a, 1971b) stated that myosin and actomyosin were the proteins that produce the greatest gel strength, and therefore, were the most important in binding. Yasui et al. (1980) by using a model gelation system reported that the addition of myosin to actomyosin produced a gel that was much stronger than either myosin or actomyosin used separately.

Gelation, by the myofibrillar proteins, primarily myosin (pre-rigor) and actomyosin (post-rigor), is a heat-induced protein to protein interaction that leads to the formation of a three dimensional, well-ordered protein structural matrix (Acton and Dick, 1986; Hermansson et al., 1986). It is a two-step process which involves an initial heat denaturation of protein followed by the formation of three dimensional network of fibrous protein (Ferry, 1948).

Native protein ⟶ Denatured protein ⟶ Aggregated protein (gel matrix)

Thermal energy is the most important driving force in protein transition from the native state to the denatured state. Thermal transition temperatures represent points at which conformational changes (denaturation) occur in native structure of protein during heating. The sol to gel transformation results in formation of a three dimensional network produced from protein -- protein interaction (Samejima et al., 1969; Ishioroshi et al., 1979; Siegel and Schmidt, 1979). Meat and poultry muscle proteins undergo three thermal transitions at about $55 - 60^\circ C$, $65-67^\circ C$ and $80 - 83^\circ C$, depending on the species and test conditions (Xiong et al., 1987). Ishioroshi et al. (1979) reported that the transition from sol to gel by myosin begins at $30^\circ C$ and reaches a maximum of gel rigidity at $60^\circ C$. Quinn et al. (1980), using differential scanning calorimetry, showed that denaturation of meat (beef) proteins begins at about $50^\circ C$ and continues with increasing temperature up to $90^\circ C$. Grabowska and Sikorski (1976), using fish myofibrils, reported that the increase in gel strength started at $30^\circ C$ and continued up to temperature of $80^\circ C$.

The ability of a gel to exhibit viscosity, rigidity and elasticity seems to be a function of the types of protein, the temperature and time of heating, protein concentration, pH and ionic strength (Asghar et al., 1985; Acton and Dick, 1984; Hickson et al., 1980; Smith, 1988a). Smith et al. (1988) developed a generalized mathematical model to predict the combined effects of pH, protein concentration, processing time and endpoint cooking

temperature on the strength of chicken myofibril gels. Myosin formed an ordered, fine lacy strand network in 0.25M potassium chloride (KCL), pH 6.0, but a disordered, coarse aggregated or sponge-like gel structure was formed in 0.6M KCL, pH 6.0 (Hermansson et al., 1986). Intact heavy chains of myosin are necessary to obtain maximum gel strength, because the light chains are solubilized during heating (Samejima et al., 1984). Maximum gel strength in 0.6M KCL, pH 6.0, has been reported to occur at a free myosin-:-F-actin mole ratio of 27:1 which is equal to a weight ratio of 15:1 (Yasui et al., 1980; 1982). At this ratio, about 20% of the protein was actomyosin and 80% was free myosin. Gel strength is at a maximum in myofibrillar protein preparation prepared at pH 5.5 to 6.0 in 0.5 to 0.8 M salt solution (Yasui et al., 1980; 1982).

In her review (Smith, 1988a) points out the following: "Several researchers have observed differences in fat and water-binding ability and textural properties between light and dark meat when used in further processed products (Froning and Norman, 1966). Chicken white muscle myosin (Asghar et al., 1984), chicken white muscle actomyosin (Brekke et al., 1987) and beef white muscle myosin (Fretheim et al., 1986) exhibited greater gel strength than the corresponding protein from red muscle. White muscle myosin from beef had higher water-holding capacity and solubility below pH 5.7 than red muscle myosin (Fretheim et al., 1986). Acton and Dick (1986) reported that broiler breast actomyosin had a lower transition temperature than thigh actomyosin during heat-induced aggregation."

Water-holding Capacity and Myofibrillar Proteins

The ability of meat, particularly processed meat, to retain water is called water-holding capacity (WHC), (Hamm, 1960). Water-holding capacity is an important factor in determining meat quality and acceptability because of its close relation to taste, color, tenderness and juiciness (Lawrie, 1979; Forrest et al., 1975).

Water is held by protein in two forms: 1) free water and 2) bound water. The free form or biologically active form of water is that water which is held by surface forces (Fennema, 1976; Forrest et al., 1975). The bound water also known as the structural or protective form is tightly bound as water of hydration by functional groups of the protein in the form of mono- and multimolecular layers (Wismer-Pedersen, 1978). About 4% of muscle water exists in a bound form, being bound to the hydrophilic groups on proteins (Lawrie, 1979; Forrest et al., 1975). The bound water of hydration in meat is not readily released except under severe conditions such as protein denaturation, rigor mortis and change in muscle pH.

Myofibrillar proteins play an important role in water-holding capacity of meat products. Generally as the amount of soluble protein increases, water-holding capacity also increases. Honikel et al. (1981) demonstrated that a close relationship between the change in solubility of myofibrillar proteins induced by postmortem metabolism and WHC of salted beef tissue homogenates. Cook (1967) using a filter paper moisture absorption

technique found a highly significant correlation (r = -0.51) when comparing salt-soluble nitrogen and moisture released by pressing. All of these studies indicate that myofibrillar proteins are required for binding and water-holding capacity.

Water-holding capacity can be affected by many factors such as pH, species of animal, sex, age and salt concentration (Hamm, 1960). The effect of pH on WHC is well known (Hamm, 1986; Hamm, 1960). As pH values are increased away from the isoelectric point of proteins, water-holding capacity and protein solubility increases (Honikel et al., 1981). Kaufman et al. (1986) stated that the water-holding capacity is increased at pH levels considerably above or below the isoelectric point of a muscle. With the reference to animal species, pork meat has the highest water-holding capacity followed by beef and poultry.

A number of different methods are used to measure water-holding capacity. Two major types of water-holding capacity methods, water binding potential (WBP) and expressible moisture (EM) respond to different properties of flesh. Water binding potential (WBP) refers to the ability of a protein system to hold water present in the system and under the influence of an external force. It, therefore, represents potential maximum water retention of a protein system under the measurement condition (Regenstein et al., 1979).

Expressible moisture refers to the amount of liquid squeezed from a protein system by the application of force, and measures the amount of loose water released under the measurement conditions (Regenstein, 1984).

LIPID OXIDATION

Lipid oxidation is one of the major causes of deterioration in the quality of meat and meat products (Asghar et al., 1988). It can result in reduction of quality, nutritional value and safety of foods (Frankel, 1984; Pearson et al., 1983).

The important fatty acids involved in oxidation are unsaturated oleic, linoleic and linolenic acid (Pearson et al., 1983). Younathan and Watts (1960) and Pearson et al. (1977) reported that chicken fat is high in oleic and linoleic fatty acids; and because of this unsaturated nature, poultry meat is more susceptible to rancidity than the red meats. Labuza (1971) reported that the rate of oxidation increases geometrically with the degree of unsaturation. Generally speaking, the higher the proportion and degree of unsaturation of fatty acids, the more labile the lipid system is to oxidation (Dawson and Gartner, 1983).

Mechanism of Lipid Oxidation

The oxidation of fatty acids proceeds by a free radical chain reaction mechanism which involves three stages, as shown in the following scheme (Frankel, 1984; Khayat and Schwall, 1983; Dugan, 1976; Labuza, 1971; Lundberg, 1962):

Initiation: RH $\xrightarrow{\text{initiator(s)}}$ R. + H.

RH + O $\xrightarrow{\hspace{3cm}}$ ROO. + H.

Propagation: R. + O $\xrightarrow{\hspace{3cm}}$ ROO.

ROO. + RH $\xrightarrow{\hspace{3cm}}$ ROOH + R.

Termination: ROO.R + R. $\xrightarrow{\hspace{3cm}}$ ROOR

R. + R. $\xrightarrow{\hspace{3cm}}$ R-R

ROO. + ROO. $\xrightarrow{\hspace{3cm}}$ ROOR + O
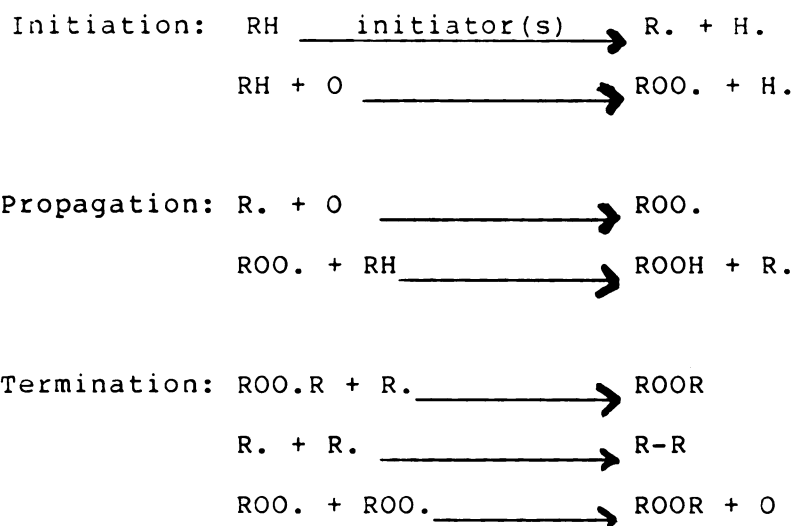
Where RH is an unsaturated fatty acid, R. is an alkylradical, ROO. is a lipid peroxyradical and ROOH is a hydroperoxide.

Initiation takes place when a labile hydrogen is removed from a carbon atom adjacent to a double bond in an unsaturated fatty acid with the formation of a free radical. Once initiated, the reduction is propagated by the level of hydroperoxides produced due to their ability to decompose to free radicals. Termination begins when the concentration of free radical is sufficiently high to begin interacting to form non-free radical products. Free radical inhibitors include antioxidants that may form inert end products as a termination step. Fig. 1 shows the overall mechanism of lipid oxidation.

Lipid hydroperoxides are the first relatively stable intermediates that are colorless, odorless and do not contribute to the off-flavor associated with lipid oxidation (Watts, 1954; Sato et al., 1973). Hydroperoxides are quite stable at low temperatures, however, they can be thermally or catalytically decomposed

Fig. 1 - Mechanisms of Lipid Oxidation

Unsaturated Fatty Acids
and Triglycerides

Free Radicals

$RH \longrightarrow R\cdot + H\cdot$

$RH + O \longrightarrow ROO\cdot + H\cdot$

Hydroperoxides

oxidation of pigments,
flavors and vitamins

$ROO\cdot + R\cdot$

Insolubilization of proteins

$ROOH + R\cdot$

Polymerization dark
color may be toxic

Breakdown Products
off-flavor compounds
hydrocarbons
alcohols, ketones
aldehydes, acids
epoxides, OH-glycerides
Di-OH-glycerides

H_2O

keto-glycerides
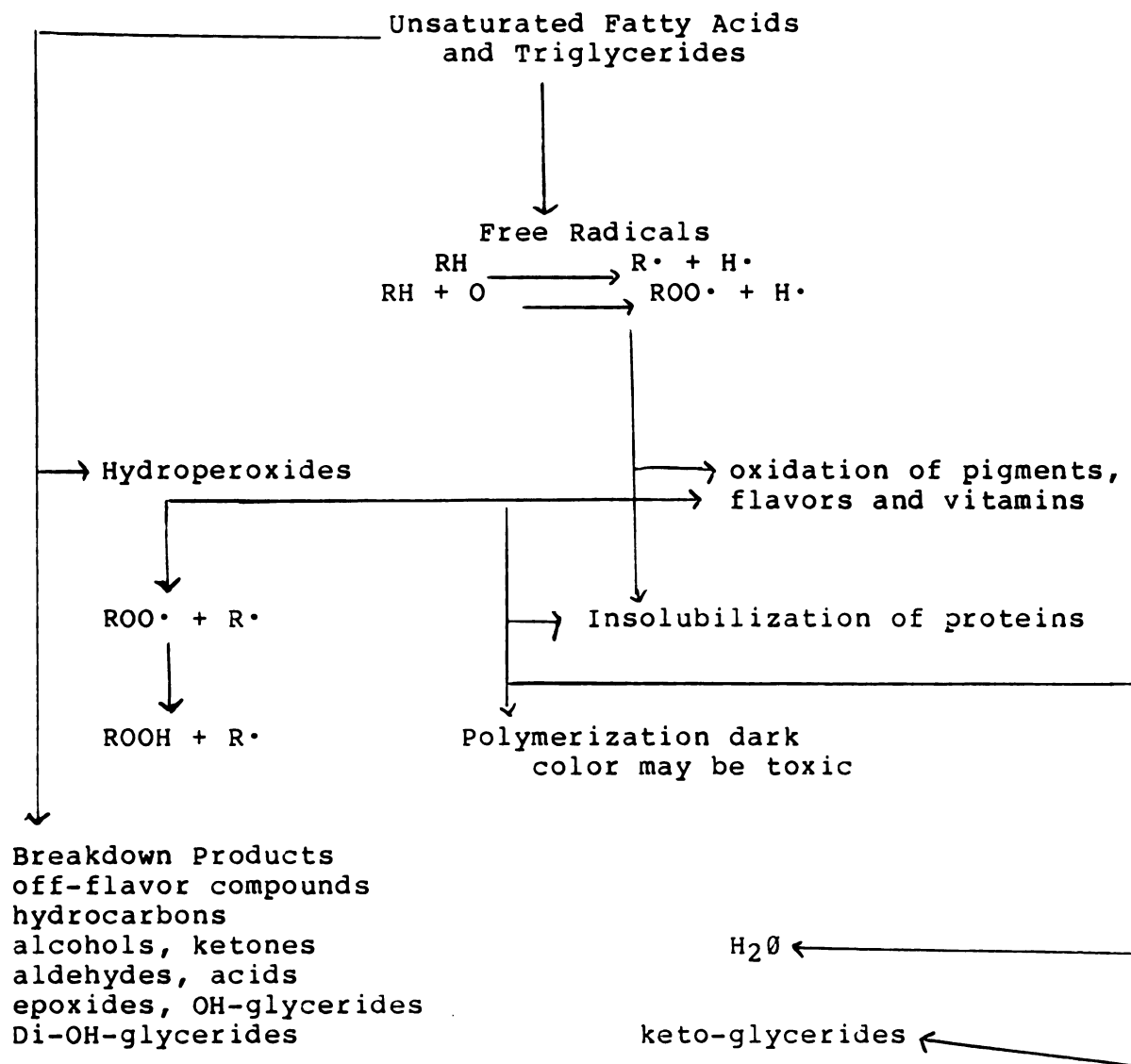
leading to the formation of short chain aldehydes, ketones, alcohols, lactones, acids and unsaturated hydrocarbons (Labuza, 1971; Frankel, 1984). These secondary products of lipid oxidation are responsible for the off-flavor of oxidative rancidity in red meats, poultry and fish (Sato et al., 1973; Pearson et al., 1983). Malonaldehyde was reported by Frankel (1985) and Igene et al. (1985b) to be the most important breakdown product of lipid oxidation.

Factors such as light, heat, oxygen, fatty acid composition, radiation and catalysts are important initiators of lipid oxidation (Khayat and Schwall, 1983; Labuza, 1971). The content and composition of muscle lipids differ within an animal and influence oxidation potential (Dawson and Gartner, 1983). The variability in content and composition of muscle lipids depend upon the muscle function (Allen and Foegeding, 1981). Turkey breast muscle contains about half as much total lipid as thigh muscle (Wilson, 1974). Table 2 shows the proximate composition of chicken meat indicating the considerable variability in fat composition. Total saturated, mono-, di-, and polyunsaturated fatty acids in beef and in both chicken white and dark meat triglycerides and phospholipids are presented in Table 3. These data demonstrate that the phospholipid fraction contains about 15 fold higher levels of polyunsaturated fatty acids than the triglyceride fraction. Thus, the phospholipids are responsible for the susceptibility of the membranes to oxidation during cooking, flaking or other processes that cause membrane disruption. Phospholipids are much more unsaturated than triglycerides

Table 2 – PROXIMATE COMPOSITION
of chicken meat (raw)

| Composition | Types of Meat | |
	Breast	Thigh
Moisture (%)	74.30	75.48
Fat (%)	1.63	3.61
Protein (%)	22.20	18.74
Ash (%)	1.10	1.04

Source: Data adapted from USDA, 1979.

in beef and in white and dark chicken meat (Igene et al., 1981). There are virtually no polyunsaturates in beef triglycerides and only 1.4% in chicken dark meat triglycerides. In contrast, polyunsaturates make up 15.5% of beef phospholipids and 22.7% of chicken dark meat phospholipids. Since most phospholipids are found in the membranes, they become exposed to oxygen and subject to oxidation during any processing step that disrupts membrane integrity (Pearson et al., 1977).

Hemoproteins (Tappel, 1955) and free transition metals (Wills, 1965) are powerful oxidation catalysts of unsaturated fatty acids in muscle and model systems. Hemoproteins have been reported to be the major catalysts of lipid oxidation in beef, chicken, turkey, fish (Tappel, 1952; Lee and Toledo, 1977). Igene et al. (1985a), Sato et al. and Love and Pearson (1974) conclude that non-heme iron was the principle catalyst of lipid oxidation in cooked meat. Tappel (1955) reported that hematin compounds catalyze the oxidation of unsaturated fatty acids, and that iron is the active factor in catalytic activity. The ferric form of heme is the active catalyst of lipid oxidation in muscle (Younathan and Watts, 1960). In a 1975 review, Green and Price concluded either Fe^{2+} or Fe^{3+} hemes might function as catalysts of lipid oxidation; but the Fe^{3+} hemes may be necessary for rapid catalysis.

TABLE 3 - Comparison of the Saturated, Mono-, Di-, and Polyunsaturated Fatty Acid Content in Beef and in Chicken White and Dark Meat Triglyceride and Phospholipid Fractions[a]

Type of fatty acid	Beef		Chicken dark meat		Chicken white meat	
	Triglycerides	Phospholipids	Triglycerides	Phospholipids	Triglycerides	Phospholipids
Saturated (%)	45.2	32.9	30.5	33.7	26.5	28.9
Monosaturated (%)	51.7	39.7	47.4	22.0	49.0	24.8
Disaturated (%)	3.1	12.0	20.6	21.5	22.6	16.7
Polyunsaturated (%)	0.0	15.5	1.5	22.7	2.0	29.6
Total unsaturated (%)	54.8	67.1	69.5	66.2	73.5	71.1

Source: Data adapted from Igene et al. (1981).
[a] Values are percentages of total fatty acids in each fraction.

Lipid Oxidation in Muscle Foods

Lipid oxidation is one of the major causes of deterioration in the quality of muscle foods. Table 4 shows the unsaturated fatty acid composition of the major muscle foods. The differing ranges of unsaturated fatty acids in this table show why poultry and fish lipids are so sensitive to lipid oxidation. Poultry meat and fish muscle are known to have a higher content of phospholipids than the red meats (Igene et al., 1981; Acosta et al., 1966; Younathan and Watts, 1960). There is also good evidence that more active muscles contain a greater proportion of phospholipids than less active muscles (Igene et al., 1981; Acosta et al., 1966). Also, there are species-to-species differences in the degree of unsaturation of the fatty acids in the triglycerides, with the proportion of polyunsaturated fatty acids (PUSFAs) being much higher in poultry, meat and fish than in beef and pork (Igene et al., 1981; Acosta et al., 1966; Zisper et al., 1964). This explains why development of oxidative rancidity and/or warmed-over flavor occur faster in fish and poultry than in beef (Igene et al., 1981; Acosta et al., 1966; Zisper et al., 1964). Decker et al. (1986) reported that beef and chicken phospholipids contain equal amounts of unsaturated fatty acids, but the fatty acids of chicken triglycerides are over 69% unsaturated compared to 55% unsaturation in beef triglycerides. The large percentage of unsaturatged fatty acids in the lipids of poultry muscle results in products susceptible to oxidative deterioration.

Fish fats are deposited primarily as phospholipids and as triglycerides (Stansby, 1973). The phospholipids in fish contain the greatest proportion of PUFAs which are more susceptible to oxidative change and interaction with proteins (Moreck and Ball, 1974). Wilson et al. (1976) showed that phospholipids were the major contributors to rancidity in beef and lamb, but the total lipids were more important than phospholipids in pork. Igene et al. (1980), using a beef and poultry model system, reported that during frozen storage both the triglycerides and the phospholipids contributed to development of rancidity in meat products. The effect of frozen storage on the overall quality of fish has been investigated. Fish phospholipids can oxidize during storage. This has been reported in a variety of species, including cod (Lovern et al., 1969), trout (Jonas and Bilinski, 1976), herring (Bosund and Garnot, 1969), fresh water whitefish (Awad et al., 1969), salmon (Botta et al., 1973), silver hake (Hiltz et al., 1976), carp, red sea beam (Toyomizu et al., 1977) and capelin (Botta and Shaw, 1978).

It was shown that susceptibility to lipid oxidation catalyzed by metmyoglobin is in the following order in muscle system: fish $>$ turkey $>$ chicken $>$ pork $>$ beef $>$ lamb. Salih et al. (1989a) and Salih et al. (1989b) reported that lipid oxidation is higher in dark meat than light meat of chicken and turkey.

**Table 4 - Unsaturated Fatty Acid Content of Lipids
in Some Muscle Foods**

Melton, 1983

Fatty acid	Content (%)				
	Lamb	Beef	Pork	Chicken	Fish
C18:1	9.51	33.44	12.78	20.25	19.59
C18:2	18.49	10.52	35.08	14.20	5.88
C18:3	0.43	1.66	0.33	0.90	8.07
C20:2	0.34	0.69	----	----	0.20
C20:3	0.62	2.77	1.31	1.30	0.36
C20:4	13.20	8.51	9.51	11.60	3.75
C20:5	----	0.76	1.31	1.55	7.16
C22:4	----	0.88	0.98	2.10	0.65
C22:5	----	0.92	2.30	5.75	2.39
C22:6	----	----	2.30	5.75	2.39

Measurement of Lipid Oxidation

Many techniques, ranging from sensory evaluation to chemical and physical methods, are available for assessing the extent of oxidation in lipid-containing foods. However, the most widely used test for measuring the extent of oxidative deterioration of lipids in fat containing foods, especially muscle foods, is the 2-thiobarbituric acid test or TBA test (Melton, 1983; Gray, 1978; Rhee, 1978). The TBA test is a colorimetric analytical technique in which the absorbance of a pink chromogen formed between TBA and malonaldehyde (MA) is measured (Gray, 1978; Tarladgis et al., 1960). The red pigment obtained in the reaction occurs as a consequence of the condensation of 2 mole of TBA with 1 mole of MA (Sinnhuber and Yu, 1958). The intensity of color is a measure of malonaldehyde concentration (Tarladgis et al., 1960, 1964) and has been correlated organoleptically with rancidity (Zisper et al., 1964). However, the TBA procedure should be used mainly to assess the extent of lipid oxidation rather than to quantitate malonaldehyde, as malonaldehyde may only contribute a part of the total color complex. Kakuda et al. (1981) suggested that the TBA reagents may react with a variety of compounds, other than malonaldehyde, present in oxidized foods and thus lead to production of various colored compounds. Jacobson et al. (1964) has shown that other products of lipid oxidation, such as alka-2,4-dienals, also react with TBA to form a red complex with the same absorption maximum (532 nm) as the malonaldehyde-TBA complex. However,

Igene et al. (1985b) reported that the major TBA-reactive substances in the distillate of cooked chicken was malonaldehyde. Kosugi and Kikugawa (1985) reported that there were several kinds of TBA reactive substances (TBARs) in the autoxidized chicken fat and methyl linoleate: the TBARs at 532 nm which were unstable in autoxidation process and slowly liberated malonaldehyde; and TBA-reactive substances at 455 nm which liberate other aldehydyes unstable after reaction with TBA. The result of the TBA test is expressed as the malonaldehyde content of foods in mg/kg of sample, or TBA.number.

The TBA test can be performed on: 1) the whole food followed by extraction of the red pigment formed (Sinnhuber and Yu, 1958), 2) distillate of the food (Tarladgis et al., 1960) and 3) extract of the food (Witte et al., 1970). The distillation method is the most popular one of the three methods (Rhee, 1978), but that fact does not necessarily mean that it is the most accurate or reproducible method. Salih et al. (1987) reported that TBA values determined by the distillation method were twice as large as those determined by extraction. High correlations between the two methods were observed by these authors.

However, several possibilities for the differences between the two TBA methods were suggested by Witte et al. (1970). The heat of distillation may increase the quantities of aldehyde and disrupt certain carbonyl compounds formed by reaction between malonaldehyde and amino acids or proteins (Buttkus, 1967). Heat during distillation is used to free malonaldehyde from its bound state with protein, whereas per chloric acid is used to release

malonaldehyde in the extraction method. Heat used in distilla-
tion may also speed up the oxidation process (Witte et al.,
1970). In the extraction method, the filtration may have given
incomplete extraction of malonaldehyde, since no heat was in-
volved in the method. The TBA test has been used by many re-
searchers to follow lipid oxidation in cooked beef, pork and
poultry (Melton, 1983; Huang and Greene, 1978; Igene et al.,
1979), during refrigerated and frozen storage of beef, pork and
poultry (Drerup et al., 1981), in freeze-dried beef and pork
(Chipault and Hawkins, 1971) and in fish (Lee and Toledo, 1977)
as well as to study lipid deterioration in relation to warmed-
over flavor of beef and poultry (Greene and Cumuze, 1981; Igene
et al., 1980; Sato and Hegarty, 1971; Sato et al., 1973; Wilson
et al., 1976).

Kakuda et al. (1981) used a high performance liquid chromat-
ographic technique (HPLC) to measure the extent of lipid oxida-
tion in the distillate from freeze-dried chicken samples. These
investigators reported that there was a linear relationship with
a simple correlation coefficient of 0.946 between the TBA absorb-
ance at 532 nm and HPLC peak height of the MA. Csallany et al.
(1984) also used a HPLC method to measure free malonaldehyde in
rat liver and beef, pork and chicken muscle. They found that the
malonaldehyde level measured by the TBA assay generally were
four- to five-fold higher than those estimated by the HPLC meth-
od.

The determination of peroxides to assess lipid oxidation in muscle foods has been applied by many researchers. Melton (1983) has reviewed the application of the peroxide assay as a measure of lipid oxidation in pork, beef and poultry products. She reported that the peroxide assay is capable of detecting lipid oxidation in muscle foods, although it may not be useful for ground meat samples stored for prolonged periods. Apparently, the relationship of peroxide value (PV) to oxidized flavor in meat systems varies with the type of meat and the manner in which it has been processed.

Jermiah (1980) used PV to investigate the lipid oxidation in frozen pork in different types of packaging wrap. He concluded that PV increased for up to 140 days of frozen storage for fresh pork cuts, but only up to 56 days for cured meat products. Sinnhuber and Yu (1977) reported a linear relationship between peroxide value and malonaldehyde concentrations during oxidation of various classes of polyunsaturated acids.

Significant ($P < .05$) correlation coefficients of r= -0.57 and r = -0.51 were found between TBA values and sensory scores for beef and chicken white meat model systems, respectively (Igene and Pearson, 1979; Salih et al., 1987). Igene et al. (1985b) studied the relationship between TBA numbers and panel scores for WOF in cooked chicken white meat and dark meat with and without chelators and/or antioxidants. They reported a correlation coefficient of 0.87 between the two measurements, which was statistically significant ($P < .01$). This showed that the TBA numbers were closely related to warmed-over flavor (WOF) panel

scores.

Turner et al. (1954) observed that pork patties made with ground raw pork having a TBA number of 0.46 were judged to be of "borderline" quality, while those with a TBA value over 1.20 were found unacceptable by a test panel.

Among the other methods for following lipid oxidation in foods is to quantitate the carbonyl compounds formed by the degradation of lipid hydroperoxides (Gray, 1978).

One of the most sensitive chemical assays for lipid hydroperoxides that is of use to biochemists is the measurement of conjugated dienes. In the formation of a hydroperoxide from a 1,4 non-conjugated diene during autoxidation, the diene moiety rearranges into conjugation. Four isomeric conjugated diene hydroperoxides can be formed on rearrangement. Chan and Levett (1977a, b) used HPLC to separate the mixture of four isomers that are formed in the air oxidation of methyl linoleate. Both cis-trans and trans-trans isomers were isolated and showed absorption maxima at 236 and 223 nm.

A typical procedure to measure conjugated dienes would be to extract the lipids from the samples to be tested using 2:1 (v/v) chloroform/ methanol and evaporate this extract to dryness under a stream of nitrogen at ambient temperature (Buege and Aust, 1985). The dried extract is then redissolved in hexane or cyclo-hexane of spectroscopic quality, and its absorbance at 234 nm measured against the solvent blank or by difference spectroscopy versus nonperoxidized lipid.

This sensitive assay can be used to measure lipid peroxidation both in vivo and in vitro. When studying model systems comprised of aqueous suspensions of purified lipid, it is often possible to analyze for conjugated dienes directly without the need to extract the lipid into organic solution (Pryor et al., 1976).

Antioxidants

Antioxidants are substances capable of slowing the rate of oxidation in fat or fat containing foods. Their action may be due to donation of hydrogen or an electron which reacts with free radicals to form inert products terminating the chain reaction mechanisms (Anonymous, 1986). Labuza (1971) divided the antioxidants into three categories: Type I: free radical chain terminators, which donate hydrogen to the free radical and thus stop the chain reaction. This group is composed primarily of phenolic-type compounds, like butylated hydroxyanside (BHA), butylated hydroxytoluene (BHT) and tocopherol; Type II: radical production preventers, mostly water soluble chelating agents, like citric acid, ascorbic acid and ethylenediaminetetraceticacid (EDTA); and Type III: environmental factors such as temperature, water activity regulators and packaging materials which affect the partial pressure of O_2 and affect the rate of oxidative reactions.

Desirable features of an antioxidant are that they (1) be effective at low concentration, (2) do not react with food compo-

nents, (3) be safe and inexpensive and (4) have a good carry-through property. The term carry-through refers to the ability to survive baking and frying operation. Pearson and Gray (1983) pointed out that antioxidants are of two types, naturally occurring and synthetic ones.

Application of various antioxidants to meat and other food systems have been investigated by several researchers. The synthetic phenolic antioxidants, BHA and BHT, have been widely used in meat systems and in general have been shown to be effective in retarding lipid oxidation. Butylated hydroxyanisol has proven to be effective in a number of foods.

A combination of antioxidants is frequently used in fat containing foods. Numerous combinations of BHA, BHT, n-Propyl-gallate and citric acid are available commercially for use in fat and fatty foods. Chen et al. (1984b) reported that Tenox 4, which contains BHA, citric acid and propylene glycol, coated on salt was an effective inhibitor of lipid oxidation. Pikul et al. (1984) also have shown that addition of BHT to chicken samples during sample homogenization, distillation or extraction step of the TBA assay prevent sample autoxidation. Smith (1987) reported that Tenox II which contains BHA, PG and citric acid prevented lipid oxidation and subsequent oxidized-lipid-protein interaction which caused myofibrillar protein denaturation during frozen storage.

Acidic compounds such as citric acid, ascorbic acid and EDTA delay the onset of oxidative rancidity by inactivation of proxi-dant metals, and are often used in combination with phenolic

antioxidants. These chelating agents exhibit synergistic effects which increase the effectiveness of many antioxidants (Anonymous, 1986; Dugan, 1960).

The naturally occurring antioxidants also can be added to foods to retard oxidation. Plant or vegetable proteins are known to contain natural antioxidants. Rhee and Ziprin (1981) studied the effectiveness of various oil seed proteins in retarding lipid oxidation in stored cooked meats when they are used as an ingredient of gravy or sauce for these products. Younathan et al. (1980) also showed that rancidity in turkey was effectively controlled by hot-water extracts of eggplant tissue and peelings of yellow onions and potatoes.

Lipid-Protein Interactions

One of the most common concerns associated with muscle lipids is their lack of stability to oxidation and associated effects on meat odor, flavor and the functionality of the muscle proteins. Oxidation of unsaturated fatty acids leads to the formation of hydroperoxides and tneir secondary breakdown products, such as carbonyl compounds, acids, etc. Exposure of proteins to peroxidizing lipids or their secondary breakdown products can produce changes in proteins including insolubiliza- tion, polymerization, decomposition to lower molecular weight products, browning, production of toxic compound, poor digesti- bility and damage to specific amino acid residues and formation of lipid-protein complexes (Nakhost and Karel, 1983, 1984; Funes and Karel, 1981; Funes et al., 1982; Matoba et al, 1984; Yanagita

et al., 1973; Shimada and Matsushita, 1978; Pokorny, 1977). These chemical changes contribute to the deterioration of food proteins during processing, storage and cooking. Proteins can react with either the free radicals, hydroperoxides or secondary products of lipid oxidation (Schaich and Karel, 1975).

Lipid-protein interactions alter the functional properties of meat. Loss of solubility due to protein-lipid interaction may be caused by the formation of lipid-protein complexes, by protein aggregation or by reactive decomposition products of lipids. Polymerization can occur by protein-protein and protein-lipid crosslinking (Roubal and Tappel, 1966). The most widely reported mechanism involves reaction of proteins with malonaldehyde. Gamage et al. (1973) have shown that malonaldehyde can react with proteins through free radical and nonradical mechanisms to form intra- and inter-molecular cross-linked products via Schiff base formation. In nonradical reaction, malonaldehyde reacts with the amino group of histidine, arginine and the epsilon-amino group of lysine in protein to form polymerized products (Shin et al., 1972). The reaction of malonaldehyde with protein is not the only process which can lead to denaturation. Olley and Duncan (1965) reported that proteins in solution have a strong affinity for fatty acids and detergents. The effect of fatty acids and detergents on proteins is complex and can lead to precipitation, disaggregation as well as to stabilization.

The interactions between protein and lipid can occur by hydrogen bonding, ionic attraction or hydrophobic interaction

(Karel, 1973). And bonds can be affected by factors such as temperature, water activity, presence of catalysts, storage time and reactant concentration (Nielsen et al., 1985b; Funes et al., 1982).

Reaction of Amino Acids with Oxidized Lipids and Subsequent Effect on Nutritional Quality of Proteins.

Most foods are cooked, industrially-processed or stored before consumption. During such treatments protein can react with other food components or chemical additives (Hurrell, 1984). These reactions can lead to flavor and color formation, loss of nutritional value and occasionally to potentially toxic compounds. For many years, nutritional research has concentrated on the stability of amino acids during food processing and storage.

There have been several publications concerning the damage to amino acids by oxidized lipids. Many investigators have studied the reactions of proteins with oxidizing lipids and other food components such as reducing sugars and food additives. The reaction of proteins with oxidizing lipids may occur during the storage of cereal and fish products (Harrison et al., 1976; Khayat and Schwall, 1983) leading to serious losses in digestibility and bioavailability of some essential amino acids (Nielson et al., 1985a). Due to the presence of reducing sugars in some foods, Maillard reactions have been reported to occur in such foods (Hurrell and Finot, 1983). Food additives (alkaline materials) which are used to improve protein solubility or other physical characteristics (Tannenbaum et al., 1970; Provansal et al., 1975) may also react with food proteins. These reactions

are also reported to cause changes in protein digestibility and bioavailability of some essential amino acids (Nielsen et al., 1985a). These changes in turn may lead to severe losses in the quality of proteins.

Modification of amino acid side chains can result from lipid-protein interaction. The main sites of peroxide reactions are in the sulfur containing amino acids, cysteine and methionine. Cysteine can be oxidized or undergo additional reactions with malonaldehyde and hydroperoxides (Gardner et al., 1977). Methionine can undergo oxidation to methionine sulfoxide, or it can react with a carbonyl compound in a Maillard reaction (Tuft and Wartheson, 1979). The epsilon-amino group of lysine forms Schiff's base condensation products with aldehydes during reactions with reducing sugars (Mauron, 1981). Histidine is also susceptible to reaction with lipid hydroperoxides (Matoba et al., 1984). Since methionine and lysine are the most limiting amino acids in certain foods, their oxidation can affect the nutritive value of foods. A few reports have indicated, however, that degradation of tryptophan during food processing and storage could be of nutritional importance (Kanazawa et al., 1975). These authors found large losses of tryptophan on reaction of protein with oxidizing lipids. Tryptophan has also been shown to be sensitive to oxidation (Nielsen et al, 1985a).

Methods to Measure Nutritional Quality of Proteins.

Even though the amino acid profile is important in evaluating the nutritive quality of a protein, the digestibility of that

protein is also important for determination of protein quality. The digestibility and the amino acid profile both can be measured by using rat bioassay, but this is an expensive and time consuming procedure.

Several in vitro methods for the measurement of protein digestibility have been developed. Akeson and Stahmann (1964) found that a pepsin-pancreatic enzyme system gave a reasonably accurate approximation of protein digestibility. Saunders et al. (1973) developed a papain-trypsin system, which correlated well with in vivo digestibility (r = 0.91). Hsu et al. (1977) developed a multienzyme system consisting of trypsin, chymotrypsin

and peptidase which correlated well with in vivo digestibility (r = 0.90) also.

The concept of biological availability as applied to amino acids in food proteins has been studied and a number of biological, chemical, microbiological and enzymic methods for determining amino acid availability have been proposed.

Numerous in vivo and in vitro methods for determining amino acid availability have been evaluated. One such method was described by Stott and Smith (1966) who developed a procedure, based on the method of Fernell and Rosen (1956), which uses Tetrahymena pyriformis W for measuring the availability of lysine, methionine, arginine and histidine in intact protein sources.

Nutritional labeling requirements are concerned with nutrient retention during storage, and the effect of processing on the

nutritive quality of protein products have created a need for an assay method to measure the quality of protein which is less time consuming and less expensive than official bioassay for protein efficiency ratio (PER) (AOAC, 1970). The Tetrahymena pyriformis bioassay appears to be the most suitable since it is simple, rapid and inexpensive, and the amino acid requirements of the organism are in reasonable agreement with human (Rolle, 1975) and rat (Kidder and Dewey, 1961) requirements. Tetrahymena assays have also been shown to correlate well with rat-PER bioassay of commercial foods, $r = 0.90$ (Evancho et al., 1977).

Effect of Freezing and Frozen Storage on Protein Functionality

Freezing and frozen storage are widely used for meat preservation. By freezing and in frozen storage many undesirable changes, such as microbial growth and metabolic processes, are inhibited. However, some chemical changes still can occur which affect the quality of meat products. Technological problems induced by freezing include drip loss, and with prolonged storage, toughening of the muscle tissue causing reduced acceptability and economic loss (Sikorski, 1978; Warrier et al., 1975).

Denaturation of muscle proteins, especially myofibrillar proteins, play a major role in the deterioration in functionality of meats of beef, pork, poultry and fish during frozen storage. Functional changes during frozen storage have been related to protein insolubilization in the intact muscle of chicken (Khan and Van den Berg, 1967; Yamamoto et al., 1977), beef (Wagner and Anon, 1986), turkey (Smith, 1987) and fish (Matsamoto, 1980;

Shenouda, 1980). Wagner et al. (1986) reported that the denatu-
ration of myofibrillar protein occurred during freezing and
frozen storage of beef when the myosin head region unfolds,
followed by a weakening of the actin-myosin interaction as indi-
cated by mg^{2+} and Ca^{2+} ATPase activity losses. These changes
caused protein aggregation and decrease solubility of protein.
Changes in ATPase activity and water absorption capacity due to
freezing rate were also observed by these authors.

Decrease in water-holding capacity during freezing is due to
the fact that during freezing water-protein associations are
replaced by protein-protein association or other interactions
(Fennema, 1976; Hamm, 1975). Protein denaturation during freez-
ing and frozen storage have been shown to influence gel proper-
ties in protein from chicken (Smith, 1987), beef (Wagner and
Anon, 1986) and fish (Kim et al., 1986).

Protein denaturation during frozen storage of meat may be
caused by one or more of the following factors: 1) formation of
ice crystals (it has been shown that freezing causes the forma-
tion of inter- and intra-cellular ice crystals which damage the
cells and rupture the membranes), 2) enzymatic activity, 3)
dehydration of protein molecules (i.e., migration of the water
molecules of hydration to form ice crystals which results in a
disruption of the hydrogen bonding system as well as the exposure
of a hydrophoic or hydrophilic surface of protein molecule, and
consequently would leave these regions unprotected), 4) an in-
crease in solute concentration in the unfrozen water phase and 5)

reaction of proteins with oxidizing lipids (Matsamato, 1980; Shenouda, 1980). Lipid oxidation occurs extensively in the refrigerated and frozen storage of meat products (Dawson and Gartner, 1983) and may be one cause of myofibrillar protein denaturation (Sikorski, 1978; Buttkus, 1967). Lipid-protein interactions alter the functional properties of meat and may cause deleterious changes in final product quality (Sikorski, 1978).

Igene et al. (1979) reported that frozen storage increased malonaldehyde concentration in chicken meat. Pikul et al. (1984) also reported a significant increase in malonaldehyde concentration, when they stored frozen chicken at $-18^{\circ}C$.

Freeze-drying of Meat and Meat Products

Freeze-drying is the mildest method known for drying meats (Gooding et al., 1957; Regier and Tapple, 1956) and has become an important process for the preservation of foods, because the process involves a minimal structural change in the food (Anonymous, 1980). But even this process causes undesirable changes in meat quality. The texture of rehydrated freeze-dried meat is drier and tougher than the original meat. The decrease of tenderness and juiciness is caused by reduction in water-holding capacity of the muscle proteins (Hamm and Deatherage, 1960). These undesirable changes possibly result from denaturation of muscle protein. The possibility of denaturation is indicated by the fact that the ATPase activity of actomyosin is partially reduced by freeze-drying of meat (Hunt and Matheson, 1959). They

found that 20 to 60% of the ATPase activity and some of the contractility had been lost during drying.

In freeze-dried food, loss of protein quality due to the interaction of protein with oxidizing lipid is a serious problem. Lipid oxidation is one of the most important problems in dehydrated food, because of the highly porous nature of the product, making the lipid more accessible to oxygen and because of the low moisture content, which tend to promote oxidation. Relatively small increases in the water content of dehydrated foods often retard oxidation (Maloney et al., 1966).

Funes et al. (1982) reported that freeze-drying promotes protein polymerization in aqueous emulsions containing lyzozyme and peroxidizing methyl fatty acid esters and decreases lipid hydroperoxides and malonaldehyde concentration.

Water Activity and Its Influence on Food Product Quality and Stability

The water activity (a_w) of many foods is an important thermodynamic property which can be used to predict the state and relative stability of food with respect to physical properties, rate of deteriorative reactions and microbial growth (Labuza, 1980). Water activity (a_w) is defined as the ratio of the vapor pressure (P) of water in the food to the vapor pressure of water (Ps) at the same temperature (Brockman, 1970). The state of water in foods has direct effect on quality and stability through its effects on chemical and enzymatic reactions. Most bacteria do not grow below a_w = 0.90, and most mold and yeast strains are

inhibited between 0.88 and 0.80 (Scott, 1957; Troller and Christian, 1978). The occurrence of enzymatic reactions in low moisture foods when the enzymes have not been activated by heating has been the subject of extensive studies (Acker, 1969; Multon and Guilbot, 1975). It has been shown that there is a correlation between the activity of enzymes and the water content of foods. Nonenzymatic browning and lipid oxidation are the major chemical deteriorative mechanism that occur in most dehydrated and intermediate-moisture foods. Oxidation is often at minimum when a food surface is covered by a monolayer of water (Labuza, 1971). Oxidation may increase to a maximum rate in an intermediate moisture range, which corresponds to a water activity range of 0.55 to 0.85.

Water activity of a food has various effects on lipid oxidation. Water affects the rate of lipid oxidation in at least three ways: 1) an antioxidant effect due to hydration of metal catalysts, which decreases their catalytic action, 2) an antioxidant effect due to bonding of hydroperoxides, which reduces their activity and 3) a proxidant effect due to increasing the mobility of reactants and catalysts (Labuza, 1971). Labuza (1969) and Labuza et al. (1971, 1972) reported that as both water content and water activity (a_w) increase well above the Brunau-Emmet-Teller monolayer (BET), oxidation rate increases due to mobilization of catalysts. At very low water activity levels, food containing unsaturated fats and exposed to atmospheric oxygen are highly susceptible to development of oxidative rancid-

ity. This high oxidative activity occurs at water activity levels below the so-called monolayer level of moisture. As water activity is increased, both the rate and the extent of autoxidation decrease until an a_w in the range of 0.3 - 0.5 is reached, depending upon the system investigated, then the rate of oxidation increases through the intermediate moisture food (IMF) range.

Malony et al. (1966) reported that water had an inhibitory effect on the oxidation of a freeze-dried model system consisting of micro-crystalline cellulose and methyl linoleate, varying with water activity up to 0.5, and then at intermediate moisture levels, presumably a_w 0.5 to a_w 0.75; the lipid oxidation of model systems becomes accelerated (Labuza et al., 1970, 1969).

Moisture Sorption Isotherm

Water is a key component in foods, not only because it is the constituent in the highest concentration, but also because it strongly influences the physical structure, palatability and technical handling capability of the food material. Even more important, almost all deteriorative processes taking place in foods are in some way influenced by the concentration and mobility of water.

The graphical relationship between water content of a food product and its corresponding water activity at a given temperature is expressed by a sorption isotherm (Labuza, 1968). Changes in temperature affect the relationship markedly (Wolf et al., 1973). Moisture adsorption isotherms have been used to calculate

the Brunaue, Emmet and Teller (BET) monomolecular layer value, and they have been used to predict the optimum moisture content of numerous foods (Rockland, 1969). The practical importance of sorption isotherms results from their usefulness in various areas of food technology. The knowledge of the water-vapor sorption isotherm of food products enables the food technologist to calculate the sorption and desorption enthalpies, to determine "bound water", to facilitate the calculation and operation of drying, mixing and packaging processes, to evaluate the physical, chemical and microbiological stability of the product and finally to predict its shelf life (Stamp et al. 1984; Labuza, 1980; Iglesias et al., 1979). Many investigators (Taylor, 1961; Rockland, 1969; Rockland and Nishi, 1980) have described the procedure for obtaining water vapor isotherms for foods.

In one general method the dehydrated food material is placed in a vacuum desiccator containing a specific saturated salt solution known to provide a definite equilibrium relative humidity necessary for determination of the isotherms. Typical isotherms in foods are S shaped, as shown in Figure 2. According to the theory of Brunaue et al. (1938), water is bound (strongly adsorbed) in a monomolecular layer in the region of the first slope of a moisture adsorption isotherm, up to point a, Figure 2. Above point a, in the linear section, bi- or multimolecular adsorption occurs. From about point b on, water is condensed in capillaries with increasing water activity (Acker, 1969). The bound water in food is strongly bound to hydroxyl groups of polysaccharides, the carbonyl and amino groups of proteins, and
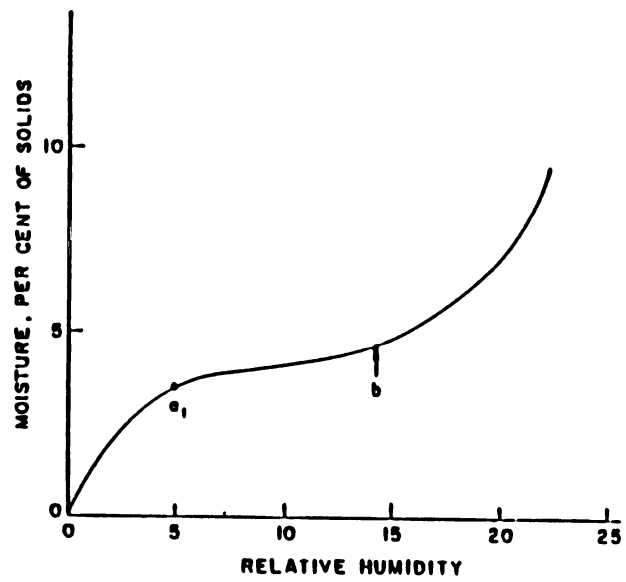
Figure 2. Moisture isotherm curve of food products.

others on which water can be held by hydrogen bonding or by stronger interactions. The most effective way of estimating the contribution of adsorption at specific sites to total water binding is the use of BET. This mathematical relation is useful as an estimate of the "monolayer value", which can be considered as equivalent to the amount of water held adsorbed on specific sites. The amount of water which represents a monomolecular layer according to the BET theory may be regarded as a protective film which protects the food particles from attack by oxygen. The statistical monomolecular layer may not in fact represent a continuous film but rather corresponds to the number of available reactive adsorption sites in the protein, carbohydrate and fat components of the food. When the amount of water is adequate for combining with these functional group, they are protected from reaction with oxygen. In other terms, the relative humidity or moisture vapor pressure at this point represents a partial pressure of water vapor, which is competitive with the oxygen partial pressure to the extent of being protective.

The equilibrium moisture content of a food sample depends on sorption mode, temperature and composition. With reference to composition, the sorption characteristic of food sample may be modified by the relative protein and fat contents. Proteins adsorb much more water at low a_w's than do fatty materials (Labuza, 1968), and subsequently the presence of fat may depress the sorptionability of a protein sample (Hermansson, 1977). The presence of fat modifies the water sorption capacity of foods;

i.e., the higher the fat content, the lower the equilibrium moisture content for a specific water activity (Saravacos, 1969; VanTwisk, 1969; Heldman et al., 1965). For this reason and to allow a comparison between similar foods but differing in fat content, moisture content should be given on a percentage non-fat dry basis or grams of water per 100 gram of non-fat dry material. This procedure is based on the assumption that fat does not adsorb water.

For the reasons discussed, it is clear that there is not a single isotherm for a given product. Pretreatment, composition and chemical changes may all somehow influence the shape of the isotherm. For this reason, the researchers should select the sorption data that most closely resembles their particular interest.

EXPERIMENTAL PROCEDURE

Experimental Design

This study was conducted in four experiments, the first of which was designed to investigate the effect of processing (freezing and freeze drying) on lipid oxidation and protein solubility of chicken breast myofibrillar proteins.

Three groups of samples were prepared as follows: 1) control sample with no methyl linoleate, 2) sample + 15% fresh methyl linoleate and 3) sample + 15% oxidized methyl linoleate (methyl linoleate was air oxidized for 24 hrs). The degree of freshness and rancidity in methyl linoleate was measured by the conjugated diene method (Pryor et al, 1976). The samples were frozen in liquid nitrogen. Frozen blocks were freeze dried for 24 and 48 hrs. Freeze dried samples were analyzed for solubility and lipid oxidation.

The second experiment was designed to study the moisture sorption isotherm and calculate the monolayer value of freeze-dried chicken protein under different water activities.

Freeze-dried chicken myofibrillar proteins were prepared as described subsequently herein. Desiccators equilibrate to six different water activities, a_w 0.08, a_w 0.11, a_w 0.22, a_w 0.43, a_w 0.65, a_w 0.85 were used for this study. Freeze dried chicken proteins were humidified in vacuum desiccators each of which contained a specific saturated salt solution for the desired water activities (See table 5).

The third experiment was conducted to investigate the effect of lipid oxidation, water activity and storage time on protein solubility.

Freeze-dried chicken myofibrillar proteins were exposed to five different water activities a_w 0.11, a_w 0.22, a_w 0.43, a_w 0.65, a_w 0.85 in this experiment. All freeze-dried samples were stored for three weeks in desiccators containing saturated salt solutions to give the above water activities.

The fourth experiment was conducted to study the effect of lipid oxidation, storage time and water activity on gelling, water-holding capacity and nutritional quality of protein.

For this experiment only, two water activities, a_w 0.43 and a_w 0.85, were prepared for this study. All freeze-dried chicken protein samples were stored for three weeks in desiccators containing desired saturated salt solutions.

Materials

The materials used in the experiment and their sources are listed below:

1. Chicken meat was purchased from local retail establishments (East Lansing, MI).

2. Methyl linoleate (methyl ester of linoleic acid) (Sigma Chemical Company. St. Louis, MO).

3. Amino acids and other chemicals used in protein digesti
    bility test (Sigma).

4. Enzymes used in the in vitro digestibility study
    (Sigma). They were porcine pancreatic trypsin (Type IX)
    with 14,190 BAEE units per mg protein; bovine pancreatic
    chymotrypsin (Type II) 60 units per mg powder; and
    porcine intestinal peptidase (Grade III), 40 units per g
    powder.

5. Tetrahymena Pyriformis (American Type Culture Collec-
    tion, Rockville, MD).

6. Liquid Nitrogen (MSU campus)

7. Double Beam absorption spectrophotometer (Lambda 4B,
    Perkin-Elmer, Norwalk, CT).

8. All ingredients used for specific water activities
    (Sigma).

9. Automatic refrigerated centrifuge (Sorval Co. RC2-B)

10. Electronically Speed-Controlled Stirrer (Heller GT-21)
    equipped with a LM Jiffy Mixer Stirrer Shaft (Thomas
    Scientific Apparatus, Cat. 8634-S20).

11. Instron Universal Testing Machine Model 4202, (Instrom,
    Canton, OH)

12. Virtis pilot plant freeze-drier (Model FF D42 WS)

13. Brinkman Polytron (Brinkman Instrument, Westbury, NY).

Methods

Meat Sample Preparation

Fresh unfrozen chicken breast was skinned, hand deboned and most of the fatty tissue and connective tissue were physically removed prior to grinding the muscle. The muscle was minced twice through a chilled grinder (Kitchenaid stand mixer KSA with a plastic food grinder attachment [Model FG-A]) and stored in a cold room (2°C) for further processes.

Isolation of Myofibrillar Proteins

Myofibrillar proteins were extracted in a 2°C cold room following a procedure described by Eisele and Brekke (1981) and Smith et al. (1988) with some modifications. The ground muscle was blended for 30 sec in a Warning blender at maximum speed with four volumes of 0.1M KCL and 0.05M K phosphate buffer, pH = 7.1. The suspension was stirred for 30 min with a motorized propeller, but without foaming. Any connective tissue which accumulated on the propeller was discarded, and the suspension was transferred to 250 ml centrifuge plastic bottles and centrifuged at 6000 x g for 10 min at 0°C. The supernatant, containing fat and sarcoplasmic protein, was discarded and the pellet resuspended in the original volume of fresh buffer and stirred for 60 min. The suspension was centrifuged and pellet resuspended in two volumes of chilled distilled water and stirred for 30 min. The suspension was strained through two layers of cheese cloth to remove excess connective tissue. The extraction procedure of stirring, centrifugation and resuspension was repeated two times with

chilled distilled water.

The volume of final pellet was measured, and the pellet was analyzed for protein by Micro-Kjeldahl procedure (AOAC, 1984). A flow diagram of the extraction sequence is shown in Figure 3.

Model System

Myofibrillar proteins and 15% methyl linoleate (as percentage of drip protein weight) were mixed with a motorized propeller for 2 min. The resultant slurry was then distributed in equal amounts in aluminum pans and placed in an insulated box on a perforated suspended floor at which liquid nitrogen was maintained at a level of 2 cm below to 1/2 cm above in order to freeze samples within 3 min. The pans were covered with filter paper. Frozen blocks in the pans were transferred to a Virtis laboratory freeze-dryer (Model FF D42 WS). A control batch of myofibrillar protein and water was prepared in the same manner but without methyl linoleate. Figure 4 shows the experimental flow diagram for preparation of the model system.

All freeze-dried samples were resuspended in 0.6M KCL prior to analysis. Saturated salt solutions which were used in adjusting water activity are presented in Table 5. Saturated salt solutions were placed in bottom of the desiccators which were held at room temperature in the dark.

Figure 3 - Flow Diagram of Myofibrils Extraction

Chicken breast
skinned, hand deboned
Grind twice through
a 4.8 mm plate

↓

Blend with 4 volumes
fresh, chilled buffer
(.1 M KCl, .05 K_2PO_4)
15 seconds

↓

Stir 30 min

↓

| supernatant (sarcoplasmic protein and fat) | ← Centrifuge 6000 X g 10 min |

Discarded

|
pellet

↓

Resuspend in 4 vol
fresh buffer
stir for 1 hr

↓

| Supernatant | ← Centrifuge 6000 X g 40 min |

Discarded

|
pellet

↓

Resuspend in
distilled diionized dd H_2O Stir 30 min

↓

Strained

|

Centrifuge
6000 X g 10 min

↓

pellet

↓

Resuspend in ddH_2O two times

↓

Washed myofibrillar protein

Figure 4 - Model System Flow Chart

Washed myofibrillar protein

↓

Methyl linoleate added
15% based on protein content

Experiment I

Frozen in Liquid Nitrogen

Freeze dried for
24 and 48 hr

Freeze dried for 48 hrs

*EXP 3   *EXP 4

*EXP 1

Stored for 2 weeks in
desiccators containing
saturated salt solutions
a_w = 0.08, 0.11, 0.22,
0.43, 0.65, 0.85

Stored for 3 weeks in
desiccators containing
saturated salt solutions
a_w = 0.43, 0.85

Stored for 3 weeks in
desiccators containing
saturated salt solution
a_w = 0.11, 0.22, 0.43, 0.65, 0.85

*EXP - experiment

Table 5 - Relative Humidities of Saturated Solutions at 25°C

Saturated Salt solution	Relative Humidity (%) by Reference at 25°C
Sodium Hydroxide (NaOH)	8
Lithium Chloride (LiCl)	11
Potassium Acetate $(KC_2H_3O_2)$	22
Potassium Carbonate (K_2Co_3)	43
Cobalt Chloride $(CoCl_2)$	65
Potassium Chloride (KCL)	85

Source: Data adapted from Labuza, 1984.

Protein Solubility

Protein solubility of freeze-dried chicken protein was measured following a procedure described by Morr et al. (1985) with small modifications. About 500 mg of freeze-dried protein were weighed accurately in a beaker and mixed with 20 ml of 0.6M KCl, .05M K phosphate, pH 7.0. The mixture was homogenized with a Brinkman polytron (Brinkman Instrument, Westbury, NY) for 5 sec. Additional 0.6M KCl solution was added to bring the total volume of the dispersion to about 40 ml. The beaker was placed on a magnetic stirrer and dispersion was stirred overnight in 2°C cold room. The pH of dispersion was determined and adjusted to 7.0 with NaOH solution. The dispersion was quantitatively trans- ferred into a 50 ml volumetric flask diluted to the volume with additional 0.6M KCl solution and mixed by inverting and swirling, and then centrifuged 30 min at 27300 x g. The protein content of the supernatant was determined by Micro-Kjeldahl (AOAC, 1980). The solubility of the protein was calculated as:

$$\text{Protein solubility } \% = \frac{\text{supernatant protein (mg/ml) x 50}}{\text{Sample wt (mg) X decimal } \% \text{ protein in sample}} \quad 100$$

Gelation Preparation of Myofibril Gels

Gels were prepared by adjusting a suspension of freeze-dried chicken myofibrillar proteins to 4.0% (w/v) protein, 0.6M KCl, pH 6.0. The suspension was mixed with a Brinkman polytron (Brinkman Instruments, Westbury, NY) for 5 sec and stirred overnight in 2°C cold room. The resultant protein suspension was transferred to 16 x 100 mm disposable culture tubes (approximately 10 g per tube) and placed in a 80°C water bath for 10 min. Heat-treated protein solutions were transferred to an ice-water bath, permitted to cool for 30 min and kept at room temperature for one hour.

Water-holding Capacity

Water-holding capacity of the gels were measured following a procedure described by Jauregui et al. (1981) with small modifications. One and a half gram of gel were added to preweighed filter paper folded into a 50 ml conical centrifuge tube. The weight of the sample and filter paper was recorded. The sample was then centrifuged at 30000 x g for 15 min. The gel was removed from the filter paper, and the paper was reweighed. The expressible moisture was measured according to the following formula and reported as percent weight lost from original sample.

$$\text{Expressible moisture (\%)} = \frac{Pw - P}{Ps - P} \times 100$$

When:
   Pw is the weight of filter paper after centrifugation and
      after removing the gel
   P = is the weight of paper
   Ps = is the weight of paper and sample before centrifuga-
      tion.

Gel Strength

The work required to penetrate the gel was evaluated as the back extrusion apparent viscosity using an Instron Universal Testing Machine (Model 4202, Canton, OH) equipped with a 50N load cell and coupled with a microcomputer (Hewlett-Packard 86B)(Lever, 1988). The speed of the plunger was 20 mm/min, travel distance 30mm, load calibration cell 50N. Distance-force data were used by the computer to calculate the back extrusion apparent viscosity and the apparent elasticity using the procedure described by Hickson et al. (1982).

Thiobarbituric Acid Test (TBA)

The TBA distillation method of Tarladgis et al. (1960) was used to measure lipid oxidation with minor modification. Two grams of freeze-dried protein was used instead of the prescribed 10 grams of fresh meat. This amount was used based on estimation of 20% protein in chicken meat. TBA numbers were expressed as mg malonaldehyde/g protein.

Apparent In vitro Protein Digestibility

The digestibility of freeze-dried chicken protein, casein, navy bean protein and treated casein was measured following the in vitro procedure described by Hsu et al. (1977). This procedure utilizes a multienzyme system consisting of trypsin, chymotrypsin and peptidase.

Fifty ml portions of aqueous protein suspension (6.25 mg protein/ml) were adjusted to pH 8.0 with 0.1N HCl or NaoH, while

stirring in a 37°C water bath. The multienzyme solution (1.3 mg trypsin, 3.3 mg chymotrypsin and 0.52 mg peptidase/ml) was maintained in an ice bath and adjusted to pH 8.0 with 0.1N NaoH and/or HCl. Five ml of the fresh prepared multienzyme solution were then added to the protein suspension which was being stirred at 37°C. The pH drop was recorded over a 10 min period using a pH meter. Percent digestibility was expressed relative to casein.

$$\text{Digestibility (\%)} = \frac{\text{pH sample}}{\text{pH casein}} \times 100$$

The samples were allowed to continue to digest for three hours and the three hour digests were then used to determine protein quality as assessed by the Tetrahymena bioassay. The digests were adjusted to pH 7.1, and the total nitrogen content of the digest was measured by using Micro-Kjeldahl procedure. The digest was diluted to contain 1 mg nitrogen/ml with distilled water.

Tetrahymena Bioassay of Protein Quality

All samples analyzed for protein digestibility were further assayed for protein quality.

The protein quality was measured by the Tetrahymena bioassay procedure as described by Stott and Smith (1966) and Shorrock (1976) (described in detail in Appendix B). Preliminary tests were conducted to verify that the Tetrahymena bioassay procedure would detect differences in protein quality (amino acid profile and amino acid availability) and that Tetrahymena growth was

proportional to nitrogen concentration. Casein (high nitrogen, United States Biochemical Corporation, Cleveland, OH) and glucose were mixed (1:1 ratio) with 20 ml water, then heated in an oven (oven dry) at 90°C for 2 hr. Casein was also incubated with 100 ml of .15M NaOH and autoclaved at 120°C for 4 hr. The pH was adjusted to 7.0 with HCl. Casein, navy bean protein, heated casein plus glucose and heated casein plus NaOH were assayed at 0.025, 0.05, 0.075, 0.10 and 0.20 milligram nitrogen/ml. Growth was proportional to nitrogen concentration from 0.025 through at least 0.1 mg nitrogen/ml. Therefore, all samples were assayed at a concentration of 0.1 mg nitrogen/ml. Percent protein utilization was calculated according to the following formula:

$$\text{Protein utilization (\%)} = \frac{\text{OD sample} - \text{sample blank}}{\text{OD casein} - \text{casein blank}} \times 100\%$$

Determination of Moisture Sorption Isotherms

Moisture sorption isotherms for freeze-dried chicken protein were obtained as described by Labuza, (1984). Freeze-dried chicken proteins were mixed uniformly by mortar and pestle and humidified in vacuum desiccators each of which contained a specific saturated salt solution for desired water activity. All samples were stored for two weeks and the moisture content of freeze-dried samples was determined on duplicate samples by the method described in AOAC (1980). After data had been collected, a plot of moisture on the Y axis vs a_w on the X axis was made, and tnen the isotherm was used to calculate the BET mono-

layer.

The BET monomolecular layer value of freeze-dried chicken proteins (control and treated samples) was calculated from the moisture adsorption isotherm data by the following procedure:

BET equation:

$$\frac{a}{(1-a)m} = \frac{1}{m_O} + \frac{(c-1)\,a}{m_a c}$$

Where
    m: Grams of water per 100 g of dry matter at a water activity a and temperature T.

    c: Constant related to the heat of adsorption.

    m_O = Monolayer value (grams of water equivalent to monolayer absorbed on 100 g of dried solids.

    a = water activity (a_w)

This equation can be rearranged to give:

$$\frac{a}{(1-a)m} = I + (S.a)$$

Where
    I = Intercept and S = slope. Thus, a plot of a/(1-a)m vs a gives a straight line. Then the monolayer from this plot is $m_O = \dfrac{1}{I + S.}$

Statistical Analysis:

Data for protein solubility and lipid oxidation (TBA numbers) in experiment 1 were analyzed as 3x4 factorial including: 1) control,+ 15% ML, + 15% oxidized ML; 2) either fresh, frozen, freeze dried for 24 hr or freeze dried for 48 hr. (The exercise repeated twice with three observations per subsample).

Results for the moisture sorption isotherms (experiment 2) were analyzed as 2x6 factorial. The factorial design

includes: 1) control vs ML treated myofibrillar proteins and 2) water activities of 0.08, 0.11, 0.22, 0.43, 0.65 and 0.85.

For experiment 3, data on protein solubility and lipid oxidation were analyzed as a non-symmetrical 3 factor factorial which includes: 1) control vs ML treated myofibrillar proteins; 2) water activities of 0.11, 0.22, 0.43, 0.65 and 0.85; and 3) three weeks of storage.

Results on WHC, gelation and the nutritional data (digestibility and microbiological assay) in experiment 4 were analyzed as a nonsymmetrical 3 factor factorial. The factorial design includes: 1) control vs ML treated myofibrillar proteins; 2) water activities of 0.43 and 0.85; and 3) three weeks of storage.

The significance between treatments was determined using either the Tukey test or Bonferroni t-test for comparison analysis, after a significant F was determined (Gill, 1978; Woolf, 1968). Graphs were plotted using Harvard Graphic.

RESULTS AND DISCUSSION

Effect of Freezing and Freeze Drying on Chicken Myofibrillar Protein.

Extracted myofibrillar protein slurries from chicken breast were allotted into three portions: 1) a control or untreated portion without added methyl linoleate, 2) a portion with 15% fresh methyl linoleate and 3) one with 15% oxidized methyl linoleate. Aliquots of each of the above lots were frozen in liquid nitrogen and freeze dried for 24 and 48 hrs in a freeze drier. Freeze-dried samples were analyzed for moisture, protein solubility and lipid oxidation.

The percent solubility of chicken breast proteins during different stages of freeze drying is illustrated in Table 6. It is evident from the data that both freezing and freeze drying decreased ($P < .05$) protein solubility. Percent protein solubility dropped from 96.9% to 82.3%, 81.2% and 67.2% when the fresh isolate was frozen in liquid nitrogen, freeze dried for 24 hr and 48 hr, respectively.

Freezing the samples in liquid nitrogen decreased solubility significantly, while freeze drying for 24 hr caused no further decrease in protein solubility in the control. However, freeze drying for 48 hr decreased ($P < .05$) protein solubility further as compared to freezing alone or freeze drying for 24 hrs. It seemed that the time of freeze drying had a significant impact on protein solubility. Similar results were obtained for the group of samples that

Table 6: Percentage solubility of chicken breast myofibrils during different stages of freeze drying

Treatment	Percent Solubility in 0.6M NaCl, pH 7.0		
	Control	+ 15% Methyl Linoleate	+ 15% Oxidized Methyl Linoleate
Fresh isolate	96.9[a]	96.9[a]	96.9[a]
Frozen in LN$_2$	82.3[b]	82.0[b]	79.6[b]
Freeze dried for 24 hr	81.2[b]	81.9[b]	71.6[c]
Freeze dried for 48 hr	67.2[c]	33.8[c]	17.8[d]

[a,b,c,d] Means in the same column followed by a common superscript do not differ (P<0.05).

All values are tne average of triplicate determinations.

were treated with 15% fresh methyl linoleate, but the results for the samples treated with oxidized methyl linoleate were different. In the presence of 15% oxidized methyl linoleate, protein solubility dropped from 96.9 to 79.6, 71.6 and 17.8% for frozen, freeze dried for 24 and 48 hrs. In this case, the decrease in solubility attributable to the first 24 hrs of freeze drying was also significant but smaller than that seen with an additional 24 hr of freeze drying. The drastic change in protein solubility after 48 hr in the samples treated with oxidized methyl linoleate may be attributed to the presence of oxidized lipid material itself and/or the duration of freeze drying. It is speculation that a greater amount of protein denaturation may have occurred during the latter stages of freeze drying. Also, the presence of unoxidized methyl linoleate may have been oxidized during freeze drying and led to insolubilition of chicken proteins.

Lipid oxidation of chicken breast myofibrillar proteins during different stages of freeze drying is shown by the results of TBA tests in Table 7. It is clear from the data in Table 7 that rapid freezing in liquid nitrogen had no significant effect on lipid oxidation in the control or methyl linoleate treated samples. Freeze drying for 24 hr increased (P<.05) lipid oxidation only in the presence of methyl linoleate. However, freeze drying for 48 hr increased (P<.05) lipid oxidation in the control and methyl linoleate treated samples. It is evident that the presence of methyl linoleate and the longer period of freeze drying increased lipid oxidation as measured by TBA numbers

Table 7. Lipid Oxidation of Chicken Breast Proteins During Different Stages of Freeze Drying

Treatment	TBA Number		
	Control	+ 15% Methyl Linoleate	+ 15% Oxidized Methyl Linoleate
Fresh isolate	0.20[a]	0.23[a]	1.01[a]
Frozen in LN_2	0.23[a]	0.31[a]	1.06[a]
Freeze-dried for 24 hr	0.29[a]	0.73[b]	1.69[b]
Freeze-dried for 48 hr	0.52[b]	1.30[c]	6.70[c]

[a,b,c] Means in the same column followed by a common superscript do not differ ($P < 0.05$)

Thus, the interaction of oxidized lipid with protein may be the reason for the decrease in the protein solubility mentioned above. Freeze drying for 48 hrs in the presence of oxidized methyl linoleate led to a 5-fold increase in lipid oxidation as compared to freeze drying for 24 hrs. This clearly indicates that both the presence of oxidizable lipid during extended freeze drying and lipid oxidation per se cause decreases in protein solubility. The presence of fresh methyl linoleate during the longer drying period effected a 50% reduction in solubility, while the use of already oxidized material resulted in a 74% decrease in protein solubility as compared to control samples.

The results on protein solubility correspond to those reported by Wagner and Anon (1986). These authors found that freezing and frozen storage decreased protein solubility, altered the structure of myofibrillar proteins and rheological behaviour of the myofibrillar proteins. They also reported that freezing of muscle had a denaturation effect on myofibrillar proteins. The changes in muscle proteins during freezing or frozen storage have been regarded by many investigators as a denaturation phenomenon (Matsumoto, 1980; Wagner and Anon, 1985, 1986; Fennema et al., 1973; Shenouda, 1980 and Park et al., 1987). These changes are manifested mainly by a decrease in solubility or extractability of the myofibrillar fraction. The decrease in the amount of liquid water available to the proteins at freezing temperatures, the mechanical damage at various muscle structures caused by ice crystals and the induced increase in concentration of tissue

salts and other extractives have been regarded as major causes of protein denaturation in frozen foods (Sikorski, 1978). Other investigators also related the loss of protein solubility during freezing to the denaturation of proteins (Dyer and Morton, 1956 and Love, 1958). According to Dyer (1951), the proteins present in the sol form in the fresh tissue are converted to gel form, which is denaturated by salts in the muscle at the eutectic point. It, therefore, appears that the dehydration of muscle cells during frozen storage is the main factor favoring the condition for denaturation.

The results obtained showing the effect of freeze-drying on lipid oxidation and protein solubility are in close agreement with the data of Kuo and Ockerman (1984) which showed that freeze drying caused undesirable changes in muscle proteins. Hamm and Deatherage (1960) reported that decrease of tenderness and juiciness in freeze-dried post-rigor meat was caused by a loss of water-holding capacity and solubility of muscle proteins. They concluded that these undesirable changes resulted from denaturation of muscle proteins. The possibility of denaturation is indicated by the fact that the adenosinetriphosphatase (ATPase) activity of actomyosin is partially reduced by freeze drying of meat (Hunt and Matheson, 1959).

The results on lipid oxidation agree with those reported by many investigators (Kanner and Karel, 1976; Schiach and Karel, 1975 and Funes and Karel, 1981). Kanner and Karel (1976) reported similar changes in lysozyme due to reaction with peroxidizing

methyl linoleate in a dehydrated model system. Kazuki et al. (1987) reported a big loss in solubility and protein digestibility when they incubated casein with linoleic acid. Funes et al. (1982) reported that freeze drying promotes protein polymerization in the presence of peroxidizing lipids. This effect may be due to concentration of protein and lipids by removal of the solvent, which results in a higher rate of protein-free radicals formation and subsequent protein polymerization.

Moisture Sorption Isotherms

The relation between water activity and moisture content of freeze-dried chicken myofibrillar proteins can be precisely described by the moisture adsorption isotherm depicted in Figure 5. These isotherms show the typical sigmoidal shape of a general moisture adsorption isotherm reported by other investigators (Konstance et al., 1983; Labuza, 1984; Mittal and Usborne, 1985). The curve for the methyl linoleate treated myofibrillar proteins is higher ($P<.01$) than that for the control (no methyl linoleate added). Thus, this data indicates that methyl linoleate increased the water sorption at any given water activity. These results are, however, contrary to the general concensus that increasing the fat content of foods decreases their sorption isotherms (VanTwisk, 1969; Hermansson, 1977; Konstance et al., 1983; Mittal and Usborne, 1985). These investigators have worked on air dried model systems. Hence, their proteins were probably native and made the most contribution to the sorption properties of their model systems.
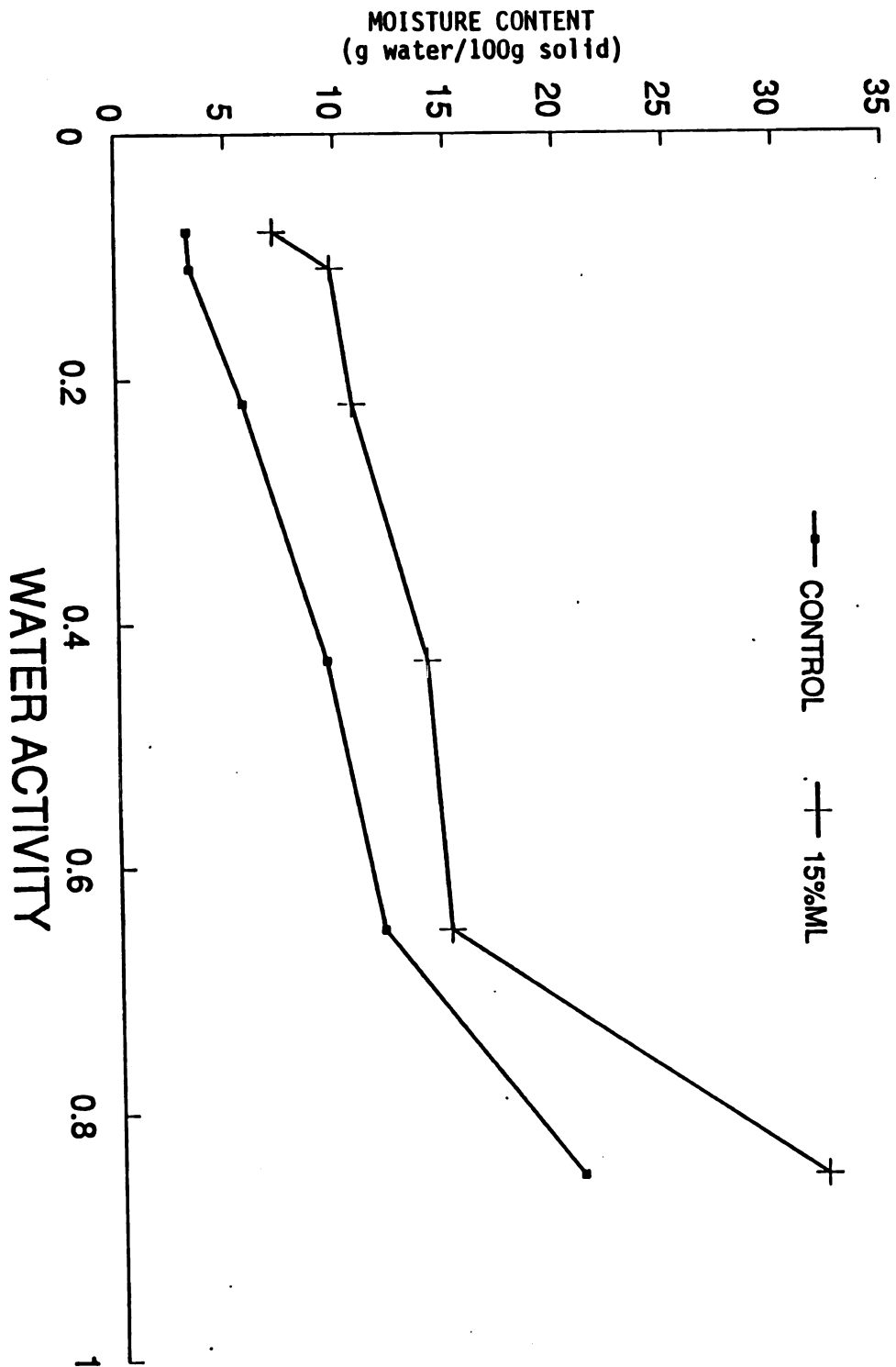
Figure 5. Moisture adsorption isotherm of the control and treated (+ 15% methyl linoleate) freeze-dried chicken myofibrillar proteins at 25°C.

Freeze drying of myofibrillar proteins in this study may have denatured the proteins thereby greatly reducing their contribution to the sorption properties of the model system studied. However, Iglesias and Chirife (1977) have demonstrated that fat contributes to the sorption properties of foods. Although the contribution of fat was negligible in their study due to the presence of native proteins, it could be of greater importance in this study since the proteins were denatured by freeze drying. Another plausible explanation for these observations is that the methyl linoleate treated samples always had higher moisture content immediately after freeze drying than the control samples. In preliminary work, the moisture content of control and methyl linoleate treated samples immediately after freeze drying was found to be 7.55% and 10.15%, respectively. Thus, starting at a higher moisture content may have caused the methyl linoleate treated samples to equalibrate at a higher moisture content at all water activities when compared to control samples.

The BET monomolecular layer value of freeze-dried chicken protein (control and 15% methyl linoleate) was calculated from the moisture adsorption isotherm data (Figure 6).

The BET monolayer values of freeze dried myofibrillar proteins are presented in Table 8. The monolayer values are calculated by linear regression analysis for the control and methyl linoleate treated samples from the lower portion of the isotherms (i.e. a_w 0.08, a_w 0.11, a_w 0.22). These values indicate that methyl linoleate treated samples adsorb more moisture at lower
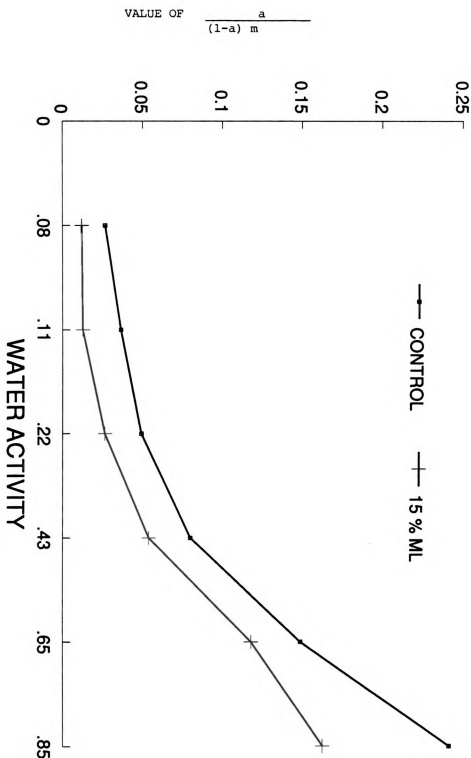
Figure 6. The BET monolayer plot of the freeze-dried chicken breast myofibrillar proteins at 25°C. a = water activity; m = moisture content; ML = methyl linoleate

water activities than control samples. The BET monolayer values for the control and methyl linoleate treated samples calculated on fat free basis correspond to water activities of 0.22 and 0.11, respectively.

Table 8. Monolayer values (m_o) of freeze-dried chicken breast myofibrillar proteins calculated using the BET equation.

Treatment	m_o (gH$_2$O/100g) solids	
	Total Solids Basis	Fat Free Basis
Control	6.5	6.5
+ 15% Methyl Linoleate	7.7	9.0

Protein Solubility

In this experiment, extracted myofibrillar protein slurries were divided into two portions: 1) control samples (no methyl linoleate) and 2) treated samples (sample with 15% methyl linoleate). Aliquots of the two sample groups were frozen in liquid nitrogen and freeze dried for 48 hrs. After freeze drying, samples were placed in environments designed to have five different water activities (0.11, 0.27, 0.43, 0.65, 0.85) for three weeks at room temperature in a dark area. Samples were analyzed for protein solubility and lipid oxidation every week.

The effect of storage time and water activity on protein solubility of the control and the methyl linoleate treated samples is shown in Figure 7. It was evident from the plot of the data that at zero day, protein solubility was significantly higher ($P < .05$) than that of all other storage times. Protein solubility was significantly reduced ($P < .05$) by the storage time at each water activity. The decrease in protein solubility resulting from one or more weeks of storage (55.8% reduction, Figure 8) may be attributed to protein denaturation, lipid oxidation or the interaction of these factors.

The protein solubility was also significantly lower at week II and III as compared to week I, but it seems that there was less change in protein solubility between week II and III than between week I and II. Thus, it appears that most of the reduction in protein solubility occurs in the earlier part of the storage period at room temperature.
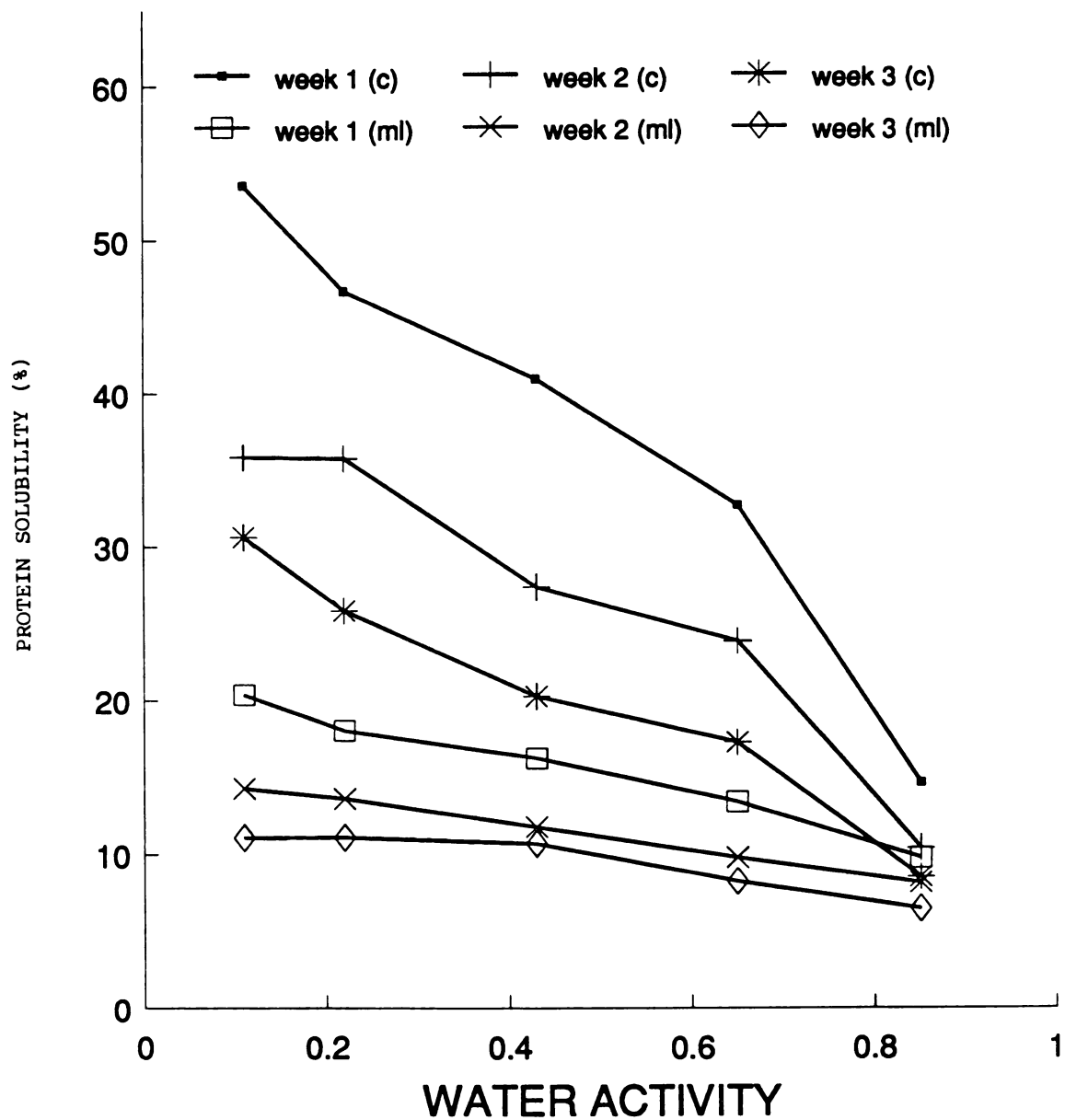
Figure 7. Effect of storage time and water activity on protein solubility
of control and treated freeze-dried chicken myofibrillar proteins.

Protein solubility of the control was also significantly affected (P<.05) by water activity as is shown in Figure 7. Water activity had a significant effect on protein solubility at each storage time. The loss of protein solubility was greater at the higher water activities in the range of 0.11 to 0.85. In general as water activity increased, the protein solubility decreased in the control sample set. A water activity of 0.85 caused a loss in protein solubility of 88.8% after 21 days of storage.

For the treated samples (treatment with methyl linoleate), it is clear from the data plot that both storage time and water activity had a significant effect (P<.05) on protein solubility. Protein solubility was significantly affected (P<.05) by the storage time at each water activity. As was discussed earlier, the reduction in protein solubility resulting from one or more weeks of storage may be attributed to one or combination of several factors. The effect of water activity on protein solubility in treated samples was similar to that of control samples, i.e., the higher the water activity, the lower the protein solubility. A water activity of 0.85 caused a loss in protein solubility of 87.5% and 88.8% after 21 days of storage in treated and control, respectively. These results are in close agreement with Kanner and Karel (1976) who observed a decrease in protein solubility during incubation of a lysozyme/methyl linoleate model system. They showed that samples incubated at a water activity of 0.75 showed the greatest change in solubility, losing 76% of their solubility after 26 days of storage.

The effect of methyl linoleate on protein solubility at each of the storage periods is shown in Figure 8. Samples which were incubated with methyl linoleate had a lower protein solubility than those that were not treated with methyl linoleate. The presence of methyl linoleate had a very consistent effect on protein solubility. Protein solubility of the control sample at zero day was 71.6%, while the protein solubility of treated sample was 55%. This reduction in protein solubility can be attributed to the presence of methyl linoleate and lipid oxidation and protein denaturation during storage.

As discussed earlier, the presence of methyl linoleate, water activity and storage time each had a significant effect $(P<.05)$ on protein solubility in both control and treated samples (Figure 7). The water activity of 0.85 resulted in greater loss in protein solubility than was the case at lower water activity levels. Furthermore, the lower the water activity, the higher the protein solubility despite the rapid lipid oxidation at a very low water activity. These findings are in close agreement with Kanner and Karel (1976) who showed that the water activity of the model system plays an important role in controlling the cross-linking and insolubilization of protein. They showed that at a water activity of approximately zero, the losses in solubility were lower than those at a water activity of 0.75, despite the rapid oxidation of the lipid at the lower water content. Schaich and Karel (1975), who worked with a similar model, have shown that at a water activity of approximately zero, the protein
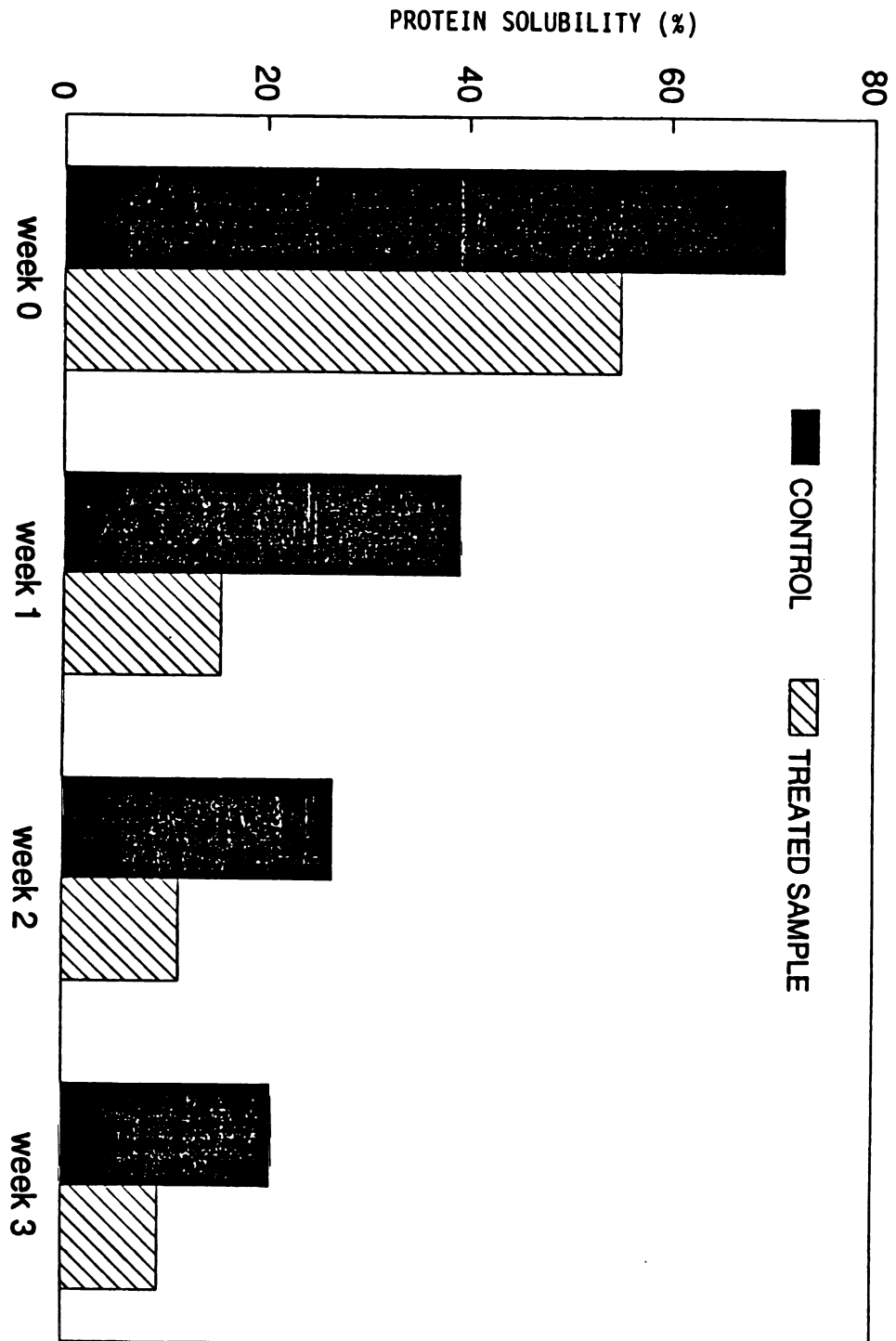
Figure 8. Effect of methyl linoleate on protein solubility of control and treated freeze-dried chicken myofibrillar proteins.

radical concentration is higher than at a water activity of 0.75. Water is an important mediator for protein and lipid interaction. At very low water activity, there is not enough water available to carry fat toward protein molecules. Therefore, even if the lipid oxidation is high at lower water activity, the protein solubility may also remain high due to the fact that fat could not get to protein to cause any denaturation. Increasing the mobility of the radicals promotes cross-linking and insolubilization. Low water activity not only prevents the recombination of protein radicals but also prevents the cross-linking involving soluble intermediates, such as malonaldehyde. The low solubility which was observed in this work may be related to protein denaturation which occurs during processing.

Fish actomyosin has been found to undergo protein aggregation and conformational changes leading to a decrease in solubility (Matsamoto, 1980). With increasing storage time, there is a progressive decrease in the amount of protein extractable by neutral salt from frozen flesh. Solubility has been linked to functionality by many researchers, although other factors also play a role in functionality (Nakai, 1983).

Lipid Oxidation

Oxidative deterioration of freeze-dried meats has been considered a major factor limiting the shelf-life of such meat products (Nakhost and Karel, 1984). The extent of lipid oxidation in this study was measured by the 2-thiobarbituric acid test.

The effects of storage time and water activity on lipid oxidation (TBA values) for the control and methyl linoleate treated samples are presented in Figures 9 and 10. Figure 9 shows the TBA values for the control, and Figure 10 shows those of the methyl linoleate treated samples. An analysis of variance of the data revealed that storage time significantly increased ($P < .05$) lipid oxidation in both control and treated samples. The effect of water activity on lipid oxidation is similar for the control and treated samples. For the control samples (Figure 9), myofibrillar proteins stored at a water activity of 0.11 have higher TBA values than those stored at a water activity of 0.22 or 0.43. It is evident that lipid oxidation was decreased as the water activity was increased from 0.11 to 0.43. At higher water activities of 0.65 and 0.85, lipid oxidation was higher than at 0.11. These results are in conformity with the results of other investigators who showed a similar trend (Martinez and Labuza, 1968; Labuza, 1972).

The decrease of lipid oxidation at a_w 0.43 compared to a_w 0.11 may be explained by the hypothesis of Labuza et al. (1966), and Maloney et al. (1966). They indicated that hydrogen bonding of hydroperoxide with water alters the decomposition rate of hydrogen peroxide. Another explanation by Chung and Pfost (1967) can be described as part of this phenomenon. As the moisture content increases up to a certain level, the water will bond to the polar units such as -OH, -COOH, + -NH and exclude oxygen from adsorption from these sites. Still another possible explanation could be the inactivation of metal catalysts by hydration.
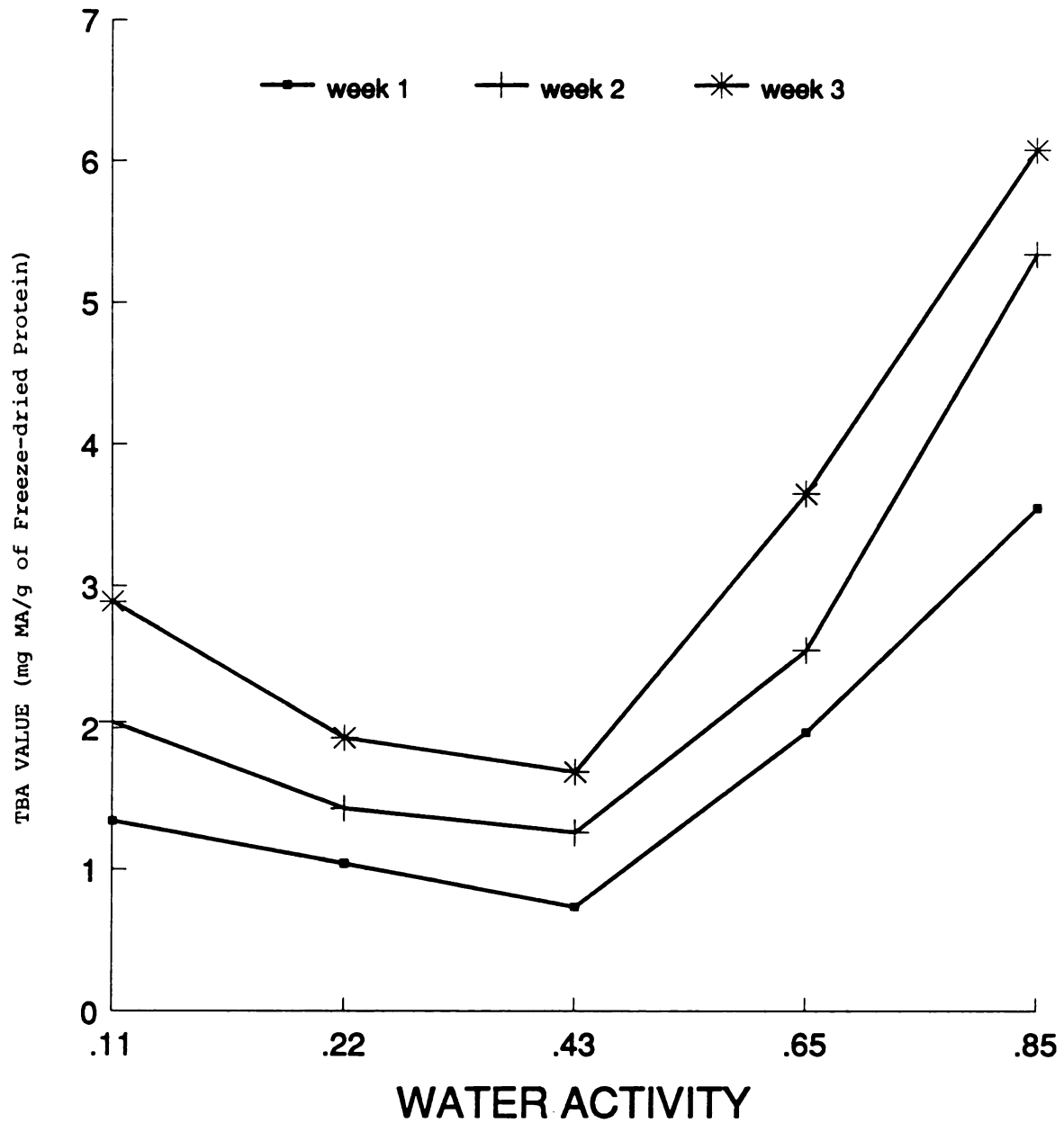
Figure 9. Effect of water activity and storage time on lipid oxidation of control freeze-dried chicken myofibrillar proteins.
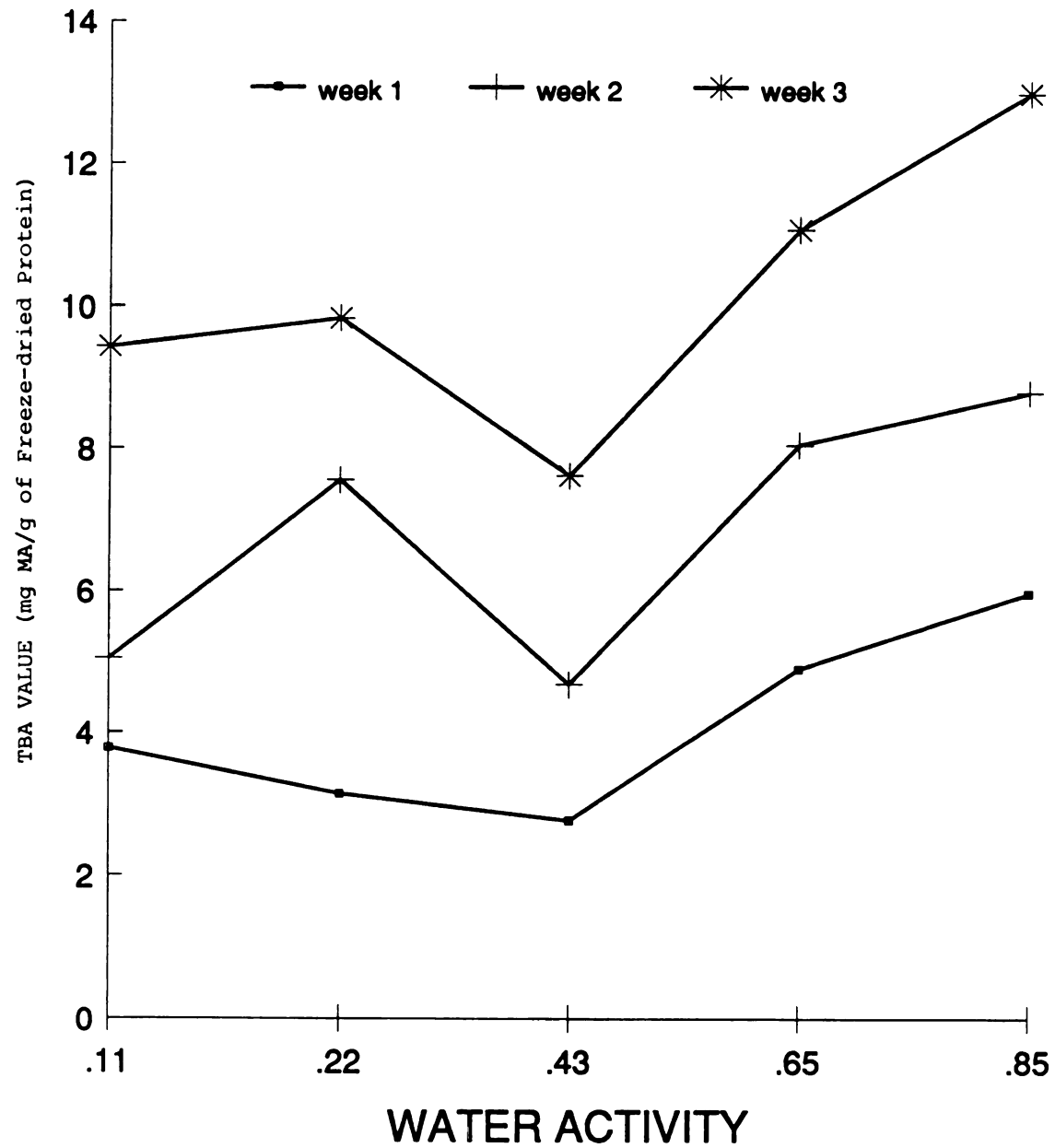
Figure 10. Effect of water activity and storage time on lipid oxidation of treated freeze-dried chicken myofibrillar proteins.

At the water activity of 0.65 and 0.85, a pro-oxidant effect has been observed. This effect may be explained by the hypotheses of Labuza et al. (1970), Labuza (1971) and Heidelbaugh et al. (1971) which suggest that the effect is due to increasing the mobility of reactants and catalysts possibly to swelling of porous matrix which allows for more contact with atmospheric oxygen.

Labuza (1972) reported that as both water content and water activity increase well above the BET monolayer, oxidation rate increases due to mobilization of catalysts. Figure 9 and 10 showed the rate of lipid oxidation to be lowest at water activity of 0.43, which can be considered as the monolayer value of the products in this experiment and then increased with increasing water activity.

For the methyl linoleate treated samples (Figure 10), the effect of water activity on lipid oxidation follows a similar trend as that for the control. However, for weeks 2 and 3, mean TBA values are slightly higher for the water activity 0.22 as compared to those for 0.11.

Figure 11 shows the effect of methyl linoleate on lipid oxidation at different storage periods. It is evident that methyl linoleate increased (P$<$.05) lipid oxidation at each storage time. Moreover, methyl linoleate greatly augmented the effect of storage time on lipid oxidation.

Figure 11. Lipid oxidation of control and methyl linoleate treated freeze-dried chicken myofibrillar proteins.
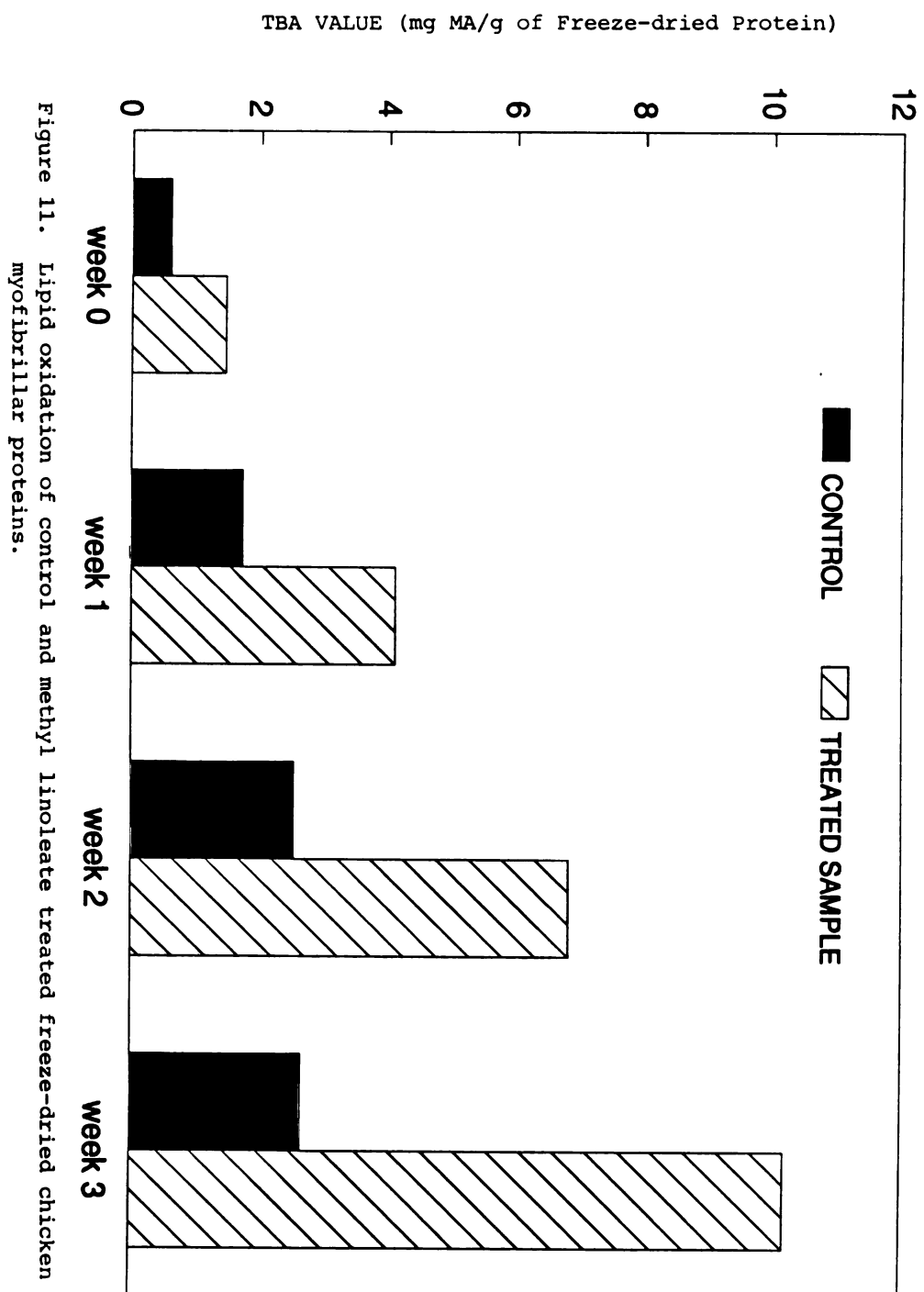
Water-Holding Capacity

The water-holding capacity (WHC) of the rehydrated freeze-dried myofibrillar proteins was assessed as the expressible moisture percentage, which is inversely related to water-holding capacity. Analysis of variance indicated a significant effect $(P < .05)$ for methyl linoleate addition, water activity and storage time on water-holding capacity (Appendix A). The two factor interaction involving methyl linoleate and water activity was also significant.

These data are illustrated in Table 9. The dramatic effect of methyl linoleate on water-holding capacity is clearly shown by the much higher expressible moisture % for the methyl linoleate treated samples as compared to control samples. After equilibrating samples to water activity levels of either 0.43 or 0.85 and holding for three weeks, the control samples held at a_w of 0.43 had a lower expressible moisture than the control samples held at a_w of 0.85 or the methyl linoleate treated samples at either a_w level. Water activity alone had a small but significant effect $(P < .05)$ on water-holding capacity. Thus, increasing a_w level from 0.43 to 0.85 reduced $(P < .05)$ water-holding capacity of the samples.

Table 9. The Effects of Methyl Linoleate and Water Activity on Water-holding Capacity of Rehydrated Freeze-dried Myofibrillar Proteins.[a]

Water Activity	Treatments		Mean a_w
	C	ML	
.43	76.96[b]	89.23[c]	83.11[b]
.85	89.24[c]	88.64[c]	88.9[c]
Mean Treatment	83.1[b]	88.9[c]	

[a] Values are expressible moisture %.
[b,c] Means having different superscript are significantly different (P<0.5).

The influence of storage time on WHC is shown in Table 10. Expressible moisture percentage increased (P$<$.01), (i.e.; WHC decreased) with storage regardless of water activity or treatment with methyl linoleate. The rate of change in WHC seemed to be related to the initial WHC. The higher the initial WHC, the greater decrease in WHC over time. Another way of interpreting this would be that as the expressible moisture level approaches its theoretical maximum (100%), the extent of the influence of storage time and a_w is obscured or less apparent. This is illustrated in Figure 12 which shows the effect of storage time on WHC for the control and methyl linoleate treated samples. The initial WHC (week 0) is greater for the control and so is the decrease in WHC over time (increase in expressible moisture percentage of 27% as compared to a 16% increase for the methyl linoleate treated samples).

These results suggest that both methyl linoleate and higher water activity caused significant changes in the myofibrillar proteins resulting in a dramatic loss in water-holding capacity.

Table 10. The Main Effect of Storage Period on the Water-Holding Capacity of Rehydrated Freeze-Dried Myofibrillar Proteins.

Weeks	Expressible Moisture Percent
1	80.94[a]
2	85.84[b]
3	91.27[c]

[a,b,c] Means having different superscripts are significantly different (P$<$.05).
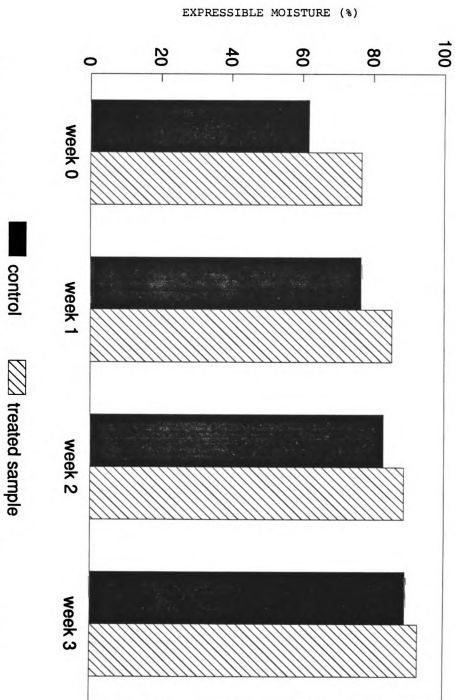
Figure 12.   Effect of methyl linoleate on storage time on water-holding capacity of rehydrated freeze-dried chicken myofibrillar proteins.

Heat-Induced Gelation.

Myofibril gels were prepared using 4% (w/v) protein in 0.6M KCl, pH 6.0, by heating for 10 min at 70°C. These conditions were reported to maximize gel strength (Yasui et al., 1980).

The effect of storage time, water activity and treatment with methyl linoleate on the gel strength of myofibrillar proteins is shown by data in Table 11. For the control samples, it is evident that the gel strength measurement of myofibrillar proteins was significantly reduced ($P<.05$) by storage time and water activity. As the water activity increased from 0.43 to 0.85, the gelling ability of myofibrillar protein dropped at each storage time. For the water activity of 0.43, the gelling property was reduced 12% from its initial value by one week of storage. For the other level of water activity 0.85, the gel strength was reduced 79% from its initial value by one week of storage. This illustrates the interaction between storage time and water activity. Further storage reduced ($P<.05$) the gelling properties by 21% and 25% at week 2 and week 3, respectively, for the water activity level of 0.43. However for the 0.85 level, the gel strength was further reduced by 29% at week 2 with no further reduction when the control samples were stored to week 3. Apparently the full detrimental effect of the a_w 0.85 environment was realized within 2 weeks of holding.

Treatment with methyl linoleate reduced ($P<.05$) the gelling property of myofibrillar proteins. The initial value dropped 58% when the samples were treated with methyl linoleate.

Table 11. Effect of Methyl Linoleate, Water Activity and Storage
Time On Gel Strength (Poise) of Myofibrillar Proteins.

Time (weeks)	Treatments			
	C		ML	
a_w	0.43	0.85	0.43	0.85
initial	7890		3323	
1	6931	1608	1049	1632
2	5482	1136	766	872
3	4088	1184	993	969

Again, storage for one week reduced the gelling properties by 68% and 51% for the water activity levels of 0.43 and 0.85, respectively. On week two, the gel strength was further reduced by 27% and 46% for the two levels of water activity, respectively. Further storage to week three had no additional effect on gel strength of the treated samples. So, the impact of methyl linoleate in reducing gel strength was realized within two weeks.

In the case of methyl linoleate treated samples, the effect of the water activity was reversed at least for the first two weeks of storage. Increasing the water activity from 0.43 to 0.85 slightly increased the gelling property of the treated samples. This may be due to the fact that the gel rigidity of these samples on week one is probably at, or near, the threshold of the sensitivity of the method used.

The effect of the interaction between treatment with methyl linoleate and storage time is shown in Figure 13. It is evident that at all storage periods, gels made from the control samples are more rigid than gels made from treated samples (ML). In addition, the effect of storage time is manifested in the dramatic reduction in gel rigidity by one week of storage. Further storage reduced the gel rigidity at a lower rate for both control and treated samples.

These data and those on solubility, lipid oxidation and water-holding capacity of myofibrillar proteins clearly show the effect of peroxidizing lipids on the deterioration of the functionality of proteins in food systems. Protein-peroxidizing lipid interaction can take place through two mechanisms: 1)
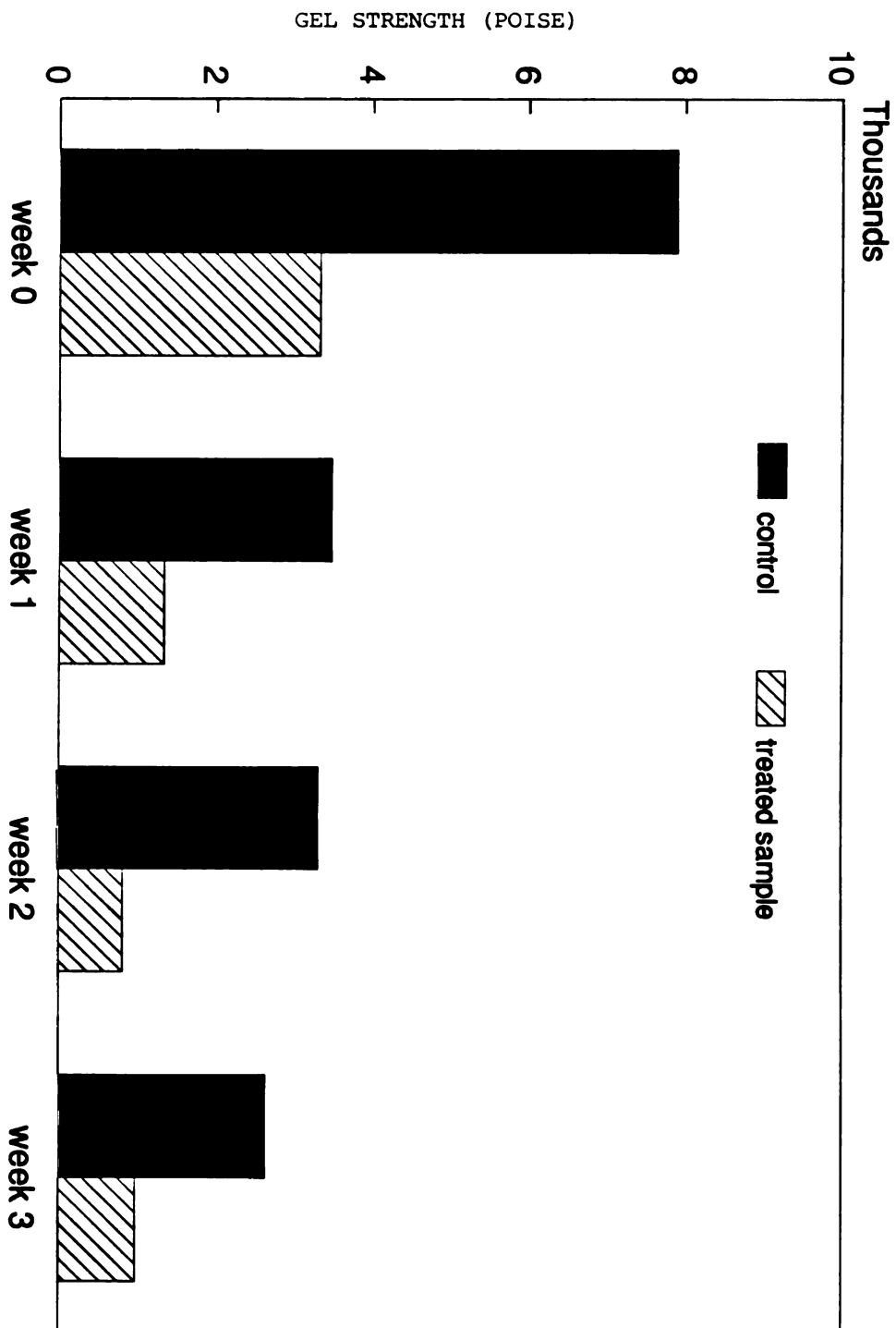
Figure 13. Effect of methyl linoleate and storage time on gel strength of rehydrated freeze-dried chicken myofibrillar proteins.

protein-amino condensation reaction involving lipid peroxidation breakdown products; 2) reaction of protein with lipid oxidation products (e.g., lipid-free radicals) resulting in the formation of protein-centered free radicals (Funes et al., 1982). Thus, exposure of proteins to peroxidizing lipids or their secondary breakdown products can produce serious changes in proteins. These changes decrease the nutritional and organoleptic quality of protein containing foods (Funes et al., 1982).

It is clear from these results and the work of other investigators (Labuza, 1972; Kanner and Karel, 1976; Funes et al., 1982) that lipid oxidation with the concomitant production of secondary products and free radicals, is increased by storage and water activity levels above 0.43 (Labuza, 1972). These lipid oxidation products interact with proteins and produce deteriorative changes in these proteins (Funes et al., 1982). These changes are clearly manifested in the loss of solubilization, water-holding capacity and rigidity of the gels formed by these proteins. Gelation of the myofibrillar protein is considered to contribute substantially to the binding between meat particles and sectioned muscles (Acton et al., 1983). Loss of this property would have a great impact on many meat products. Moreover, these losses in protein functionality seriously decrease the organoleptic quality of protein-containing foods.

Effect of lipid oxidation on nutritional quality of protein.

This experiment was conducted in two parts. The first of which was to verify the Tetrahymena bioassay. In preliminary work, casein and navy bean protein were included as controls since digestibility and quality is known for these proteins. Casein digestibility and quality was reduced by heating casein in the presence of glucose or sodium hydroxide. An in vitro apparent protein digestibility hydrolysis, using a multi-enzyme system of trypsin, chymotrypsin and peptidase was used prior to each tetrahymena bioassay for two purposes: first, to obtain in vitro digestibility, and second, to predigest the protein for enhanced tetrahymena growth.

Percent protein digestibility for these four samples is shown in Figure 14. It is clear from the results that protein digestibility decreased (P$<$.05) from 100% for casein to 72.41, 97.9 and 26.77% for navy bean, heated casein + glucose, and heated casein + NaOH, respectively.

Results from the tetrahymena bioassay (Figure 15) also revealed significant decreases (P$<$.05) in percent protein utilization among treatments. As is shown in this figure, the percent protein utilization by tetrahymena is reduced significantly in the presence of lower quality protein and altered casein.

The results correspond to those reported by Nielson et al. (1985a). These authors found that the treatment of casein with NaOH resulted in losses of serine (36%), lysine (33%), cysteine (25%), methionine (19%), arginine (15%), threonine (14%) and tryptophan (10%). When they fed the rats the alkali-treated
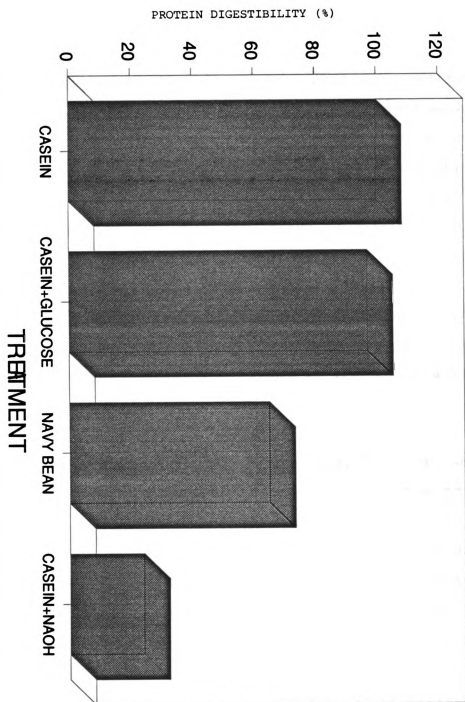
Figure 14. Digestibility of protein from casein, navy bean and adulterated casein.

casein as the only protein source, extensive weight losses oc-curred.

Incubation of casein with glucose resulted in poor digestibility. This result is in close agreement with Nielson et al. (1985a). Maillard reactions between protein and reducing sugars are probably the most important reactions which occur in food proteins during processing and storage. Finot and Magnent (1981) reported that half the lysine in casein which had under-gone Maillard reaction with glucose was extracted in faeces of rats. In addition, in vitro enzymatic release of lysine after Maillard reaction has been found to be especially low (Scarbieri et al., 1973). Navy bean protein, as a source of lower quality plant protein, showed reduced digestibility compared to casein control.

The second part of the experiment was done on the freeze dried chicken proteins. The extracted myofibrillar proteins were divided into two groups: 1) control group with no methyl linoleate and 2) treated group with 15% methyl linoleate. All samples were frozen in liquid nitrogen and freeze dried for 48 hrs. The freeze-dried chicken protein was placed in two different water activities (0.43, 0.85) for three weeks, and samples were analyzed for protein digestibility and protein quality. Casein was used as a positive control.

Figure 16 shows the percent protein digestibility of control and treated samples at different water activities. It was clear from the data that in vitro protein digestibility did not change
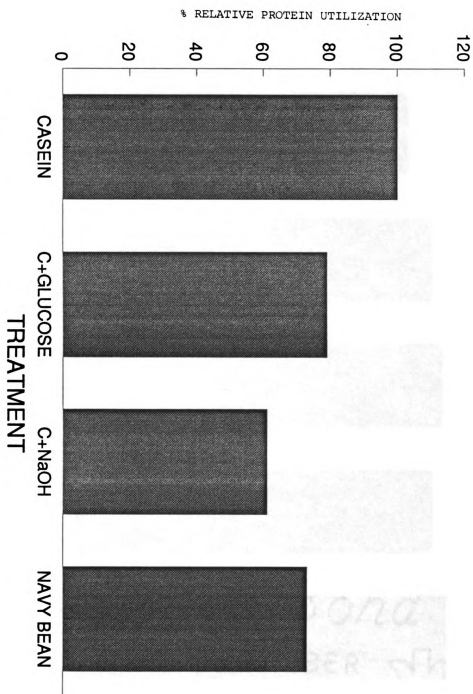
Figure 15. Percent Protein Utilization by Tetrahymena Pyroformis as Affected by Different Protein Sources.
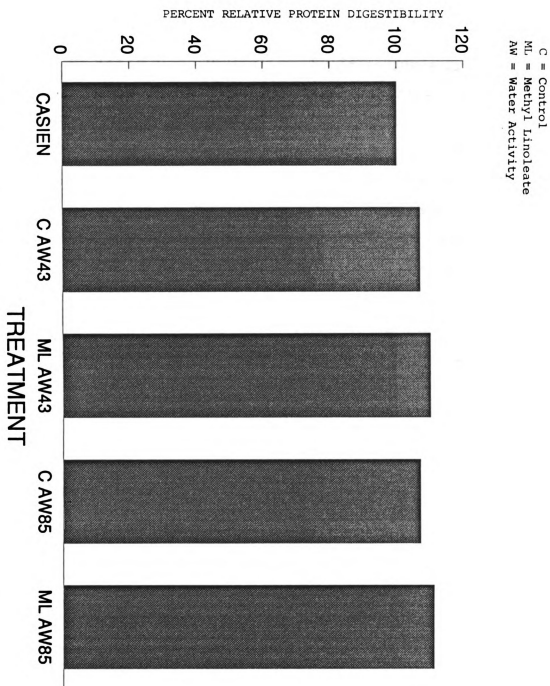
FIGURE 16. Digestibility of Protein as Affected by Treatment and Water Activity.

due to the presence of methyl linoleate or storage at detrimental water activities.

The percent protein utilization by <u>Tetrahymena</u> <u>pyrigotmis</u> of freeze-dried myofibrillar protein is shown in Figure 17. Casein was utilized better than freeze-dried myofibrillar proteins. The effects of methyl linoleate treatment and storage at different water activities are not significant (P$<$.05) and do not follow any specific pattern. These results seem surprising considering the fact that these treatments increased lipid oxidation, decreased water-holding capacity and decreased solubility of myofibrillar proteins as has been discussed earlier. These data indicate that severe deterioration in protein functionality can occur before there is any loss in the nutritive value of the protein.
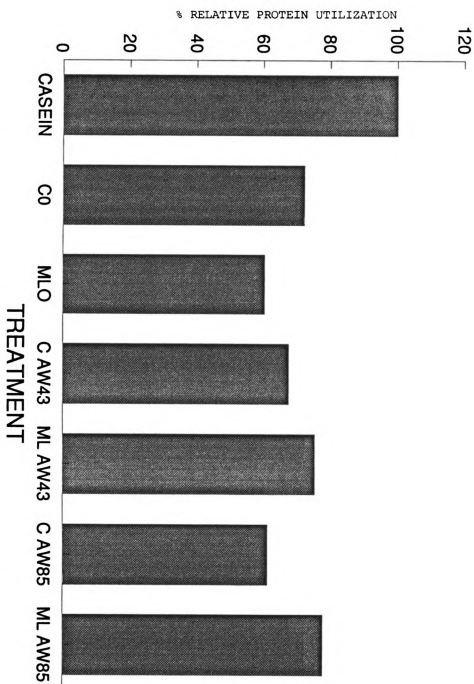
Figure 17. Percent Protein Utilization by Tetrahymena pyroformis Affected by Treatment & Water Activity.

SUMMARY AND CONCLUSIONS

A series of experiments were conducted to investigate the effects of lipid oxidation, lipid protein interaction, water activity and storage time on the functional properties as well as the nutritional quality of chicken myofibrillar proteins. Effects of processing (freezing vs freeze drying) on protein solubility and lipid oxidation were also assessed.

Both freezing and freeze drying decreased (P$<$.05) protein solubility and increased (P$<$.05) lipid oxidation of chicken myofibrillar proteins. This effect was more dramatic in samples that contained methyl linoleate (15%) as opposed to control samples (no methyl linoleate). Calculation of monolayer values revealed that myofibrillar proteins with no lipid had a lower monolayer value than methyl linoleate treated myofibrillar proteins at any water activity.

Myofibrillar proteins that contained methyl linoleate had higher (P$<$.05) TBA numbers and lower (P$<$.05) percent soluble proteins than the control at any water activity or storage time. Moreover, lipid oxidation increased (P$<$.05) and percent soluble proteins decreased (P$<$.05) with storage time up to 3 weeks. Proteins stored at a water activity closer to their monolayer moisture content were less susceptible to lipid oxidation than proteins stored at water actvities on either side of their mono-layer water activity.

The intrinsic properties of myofibrillar proteins (water-holding capacity and gelling properties) were also affected by addition of methyl linoleate, water activity and storage time.

Water-holding capacity and gel strength were reduced ($P < .05$) by the addition of methyl linoleate and increases in storage time after processing up to three weeks. An increase in water activity from 0.43 and 0.85 decreases the gel strength of myofibrillar proteins with no lipid added while it did not affect the gel strength of myofibrillar proteins treated with methyl linoleate.

In vitro digestibility of myofibrillar proteins was not affected ($P < .05$) by addition of methyl linoleate (15%) or two weeks of storage after processing. Also, storage at water activities of 0.43 or 0.85 had no adverse effect ($P < .05$) on the in vitro digestibility of myofibrillar proteins. Furthermore, addition of methyl linoleate, storage for two weeks and storage at water activities of 0.43 or 0.85 did not hinder the growth of Tetrahymena pyriformis on a broth containing myofibrillar proteins. Thus, it is concluded that an increase in lipid oxidation adversely affects the functional properties of myofibrillar proteins but does not extensively damage their nutritional quality.

APPENDICES

APPENDIX A.   Statistical Procedures

Table A.1. Analysis of Variance for Protein Solubility of Freeze-dried Chicken Myofibrillar Proteins

Source	DF	Sum of Squares	Mean Square	F Value	PR F
WEEK	2	4187.37454	2093.93727	568.17	0.0001
AW	4	7166.03382	1791.50845	486.11	0.0001
TR	1	11669.92464	11669.92464	3166.50	0.0001
WEEK*AW	8	527.44905	65.93113	17.89	0.0001
AW*TR	4	2549.53483	637.38371	172.95	0.0001
WEEK*TR	2	958.41693	479.20847	130.03	0.0001
WEEK*AW*TR	8	184.04384	23.00548	6.24	0.0001
ERROR	150	552.81517	3.68543		

Table A.2.   Analysis of Variance for Lipid Oxidation

Source	DF	Sum of Squares	Mean Square	F Value	PR F
WEEK	2	433.6937940	216.8468970	230.96	0.0001
AW	4	341.9658459	85.4914615	91.06	0.0001
TR	1	935.7530404	935.7530404	996.67	0.0001
WEEK*AW	8	19.6190972	2.4523871	2.61	0.0105
AW*TR	4	18.7669479	4.6917370	5.00	0.0008
WEEK*TR	2	156.6225782	78.3112891	84.41	0.0001
WEEK*AW*TR	8	11.5528785	1.4441098	1.54	0.1485
ERROR	150	140.831714	0.938878		

Table A.3. Analysis of Variance for Water-holding Capacity
of Freeze-dried Chicken Myofibrillar Proteins

Source	DF	Sum of Squares	Mean Square	F Value	Pr F
TR	1	203.8751042	203.8751042	20.81	0.0007
ST	2	427.4167583	213.7083792	21.81	0.0001
AW	1	204.5752042	204.5752042	20.88	0.0006
TR*ST	2	32.6837583	16.3418792	1.67	0.2295
TR*AW	1	248.5197042	248.5197042	25.36	0.0003
ST*AW	2	13.6055583	6.8027792	0.69	0.5184
TR*ST*AW	2	54.3171583	27.1585792	2.77	0.1024
ERROR	12	117.576450	9.798038		

Table A.4. Analysis of Variance for Gelation of
Freeze-dried Chicken Myofibrillar Proteins

Source	DF	Sum of Squares	Mean Square	F Value	Pr F
TR	1	50055389.17	50055389.17	342.81	0.0001
ST	2	6429581.70	3214790.85	22.02	0.0001
AW	1	35449123.67	35449123.67	242.78	0.0001
TR*ST	2	2515111.21	1257555.60	8.61	0.0015
TR*AW	1	43816809.40	43816809.40	300.09	0.0001
ST*AW	2	1313375.69	656687.84	4.50	0.0219
TR*ST*AW	2	3436170.64	1718085.32	11.77	0.0003
ERROR	24	3504328.3	146013.7		

TABLE A.5. Summary statistic for Tetrahymena Bioassay

Source	Number	Average[1]	SE±[2]
control sample (initial)	12	.59	.026
treated sample (15% ML) initial	12	.49	.032
control sample a_w = .43	12	.55	.02
treated sample a_w = .43	12	.61	.037
control sample a_w = .85	12	.50	.032
treated sample a_w = .85	12	.63	.032
casein	12	.81	.017

ML = Methyl Linoleate
[1] = Average optical density
[2] = Standard error estimate

TABLE A.6. One way analysis of variance for Tetrahymena Bioassay

	Degrees of Freedom	Sum of Squares	Error Mean Square	F-Value	Prob
Between	6	0.8586	0.14	16.43	.001
Within	77	0.6708	0.01		
Total	83	1.5294			

TABLE A.7. Statistical analysis of the linear regression for casein, casein + glucose, casein + NaOH and navy bean protein

Treatment	Regression Coefficient	Standard Error	Correlation	Error Mean Square
Casein	2.45	$\pm$.064	.990	.00046
Casein + Glucose	1.94	$\pm$.014	.999	.000018
Casein + NaOH	1.43	$\pm$.036	.993	.00012
Navy bean protein	1.78	$\pm$.011	.999	.000047

NOTE: Computer program Plotit (Scien Progr. Enterprices, Haslett, MI, ver. 1.5, 1989) used to do linear regression. The regression model forced the line through the origin.

TABLE A.8. T-Value for differences in regression coefficient

Treatment Comparison	T-value	$T_{.01}$, DF = 2.76
Casein vs navy bean	223	Highly significant (P<.01)
Casein vs Ca + NaOH	300	Highly significant (P<.01)
Casein vs Ca + Glucose	164	Highly significant (P<.01)

$$\text{T-value for difference in regression coefficient} = \frac{\text{Regression coefficent of casein} - \text{Regression coefficient of sample}}{\sqrt{\frac{\text{EMS (casein)}}{(n-2)} + \frac{\text{EMS (sample)}}{(n-2)}}}$$

Where:

   n = number of observations used for linear regression

Woolf, 1968.

APPENDIX B. TETRAHYMENA BIOASSAY PROCEDURE, ASSAY MEDIUM AND FIGURES FOR EXPERIMENT IV.

Appendix B.1. Tetrahymena Bioassay Procedure
(Shorrock; 1976)

B. 1. The test digests were assayed at dose levels of 0.1 ml.

2. Two milliliters of basal medium providing minerals, nucleic acids, vitamins and dextrin were added to each test tube.

3. One tenth milliliter of protein digest plus 7.9 ml of distilled water was added to each test tube to give a total of 10 ml per tube.

4. The tubed culture medium was autoclaved at $110^{\circ}C$ for 10 min.

5. Centrifuge the 3 day culture of Tetrahymena at 1000 xg for 15 minutes with phosphate buffer saline (.15M NaCl, 10 milimole K phosphate pH 7.2).

6. After cooling,the tubes were inoculated with 0.5 ml of culture and incubated for 96 hours at $25^{\circ}C$ in a shaker and using large size test tubes for proper aeration.

7. The growth was stopped by adding 2-3 drops of formaldehyde solution (36%).

8. All culture tubes were vortexed for 15 seconds.

9. Growth was assessed by measuring turbidity with a Beckman DU Single Beam Absorption Spectrophotometer at 580 nm.

10. Optical density was measured as follows:

$$\text{Protein utilization (\%)} = \frac{\text{OD sample}}{\text{OD casein}} \times 100$$

Appendix B.1. Assay Medium

Basal medium (10 times final strength)

Stock Sol. A	2 ml + 3 ml distilled (d.) water
Stock Sol. B	2 ml + 3 ml d. water
Stock Sol. C	5 ml
Stock Sol. D	5 ml
Glucose	3 g

Dissolve the followings in 10 ml d. water and add the above compounds. Make up to 40 ml. Adjust the pH to 7.2 with NaOH.

Guanylic Acid (sodium salt)	30 mg
Adenosine-2' (3')-phosphoric acid monohydrade	20 mg
Cystidylic Acid	25 mg
Uracil	10 mg

NOTE: Stock solutions were sterilized by filtration before storage.

Stock Solution A (100 times final strength)

	mg/200 ml
Calcium pantothenate........	12.5
Nicotinamide................	12.5
Pyridoxine hydrochloride....	125.0
Pyridoxal hydrochloride.....	12.5
Pyridoxamine hydrochloride..	12.5
Riboflavin..................	12.5
Folic acid..................	1.25
Thiamine hydrochloride......	125.0
Inositol....................	12.5
Choline chloride............	125.0
p-Aminobenzoic acid.........	12.5
Biotin......................	1.25
DL-a-Lipoic acid............	0.4

Stock Solution B (100 times final strength)

	g/200 ml
$MgSO_4.H_2O$.............	2.8
$Fe(N_4)_2(SO_4)_2.6H_2O$.	1.25
$MnCl_2/4H_2O$............	0.025
$ZnCl_2$..................	0.0025

Stock Solution C (100 times final strength)   g/200 ml

    $CaCl_2$... 600
    $CuCl_2.2H_2O$... 60
    $FeCl_3.6H_2O$... 15

Stock Solution D (100 times final strength)   g/500 ml

    KH_2PO_4... 1.36
    K_2HPO_4... 1.74

Appendix B.2. Maintenance of Tetrahymena Pyriformis W

Tetrahymena pyriformis W cultures were maintained in tetrahymena medium (5 g proteose peptone, 5 g tryptone, 0.2 g K_2HPO_4, 2 g yeast extract, 10 g dextrose, 8 g NaCl, 1000 ml distilled water). The pH of the medium was adjusted to 7.2 and autoclaved for 15 min at $110^{\circ}C$. The organisms were transferred regularly at 4 - 5 day intervals into 10 ml of sterile medium and grown in the dark at room temperature. Immediately prior to inoculation of the sample culture in the assay procedure, a 3-day broth culture of the organism was centrifuged, washed once and resuspended in phosphate buffered saline, pH 7.2.

APPENDIX B.3.   Figures 1 and 2.

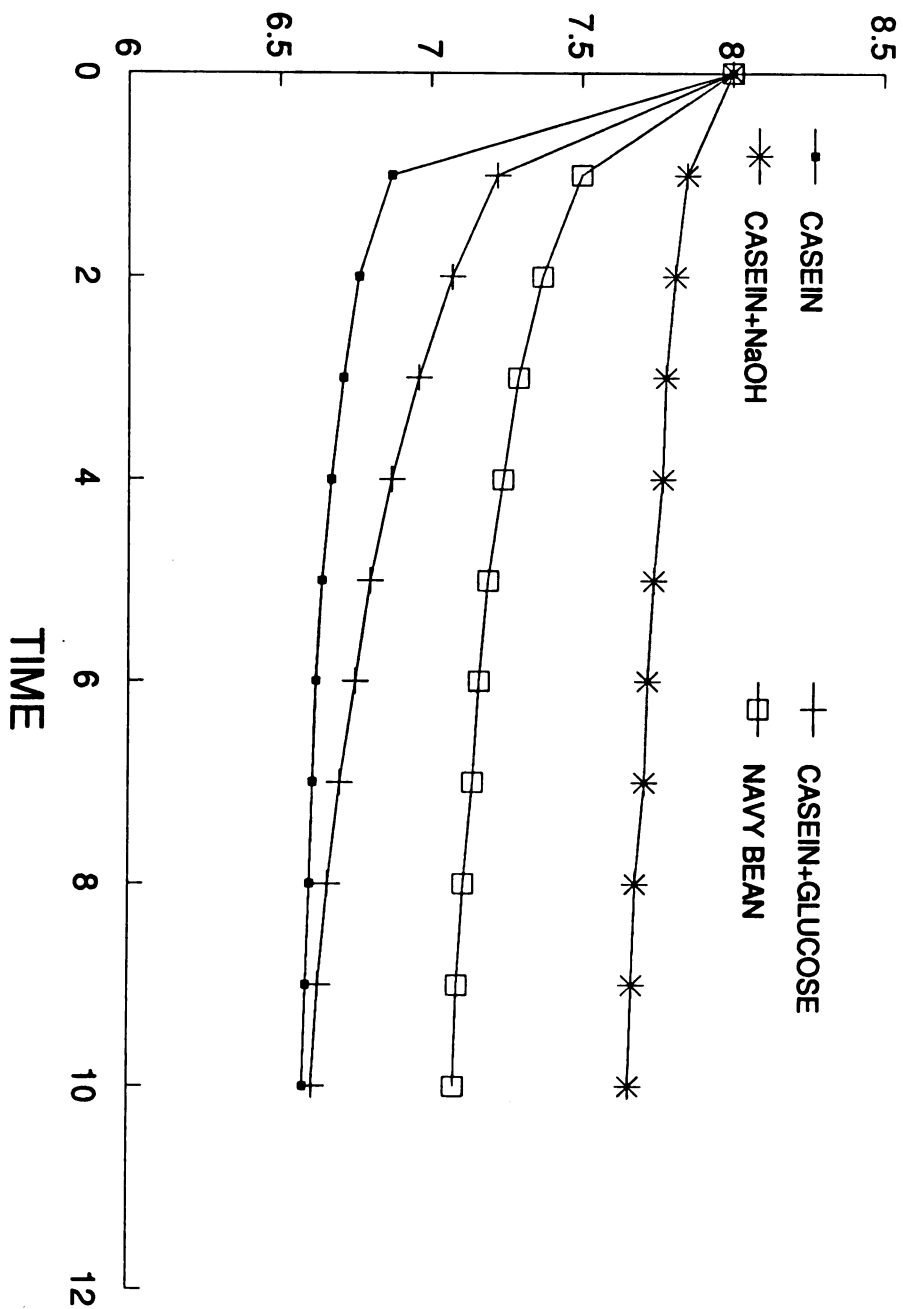Figure 1. Change in pH over time of incubation for four different treatments. The pH vs time curves obtained by incubating casein, navy bean protein, and adultrated casein with the multienzyme system.
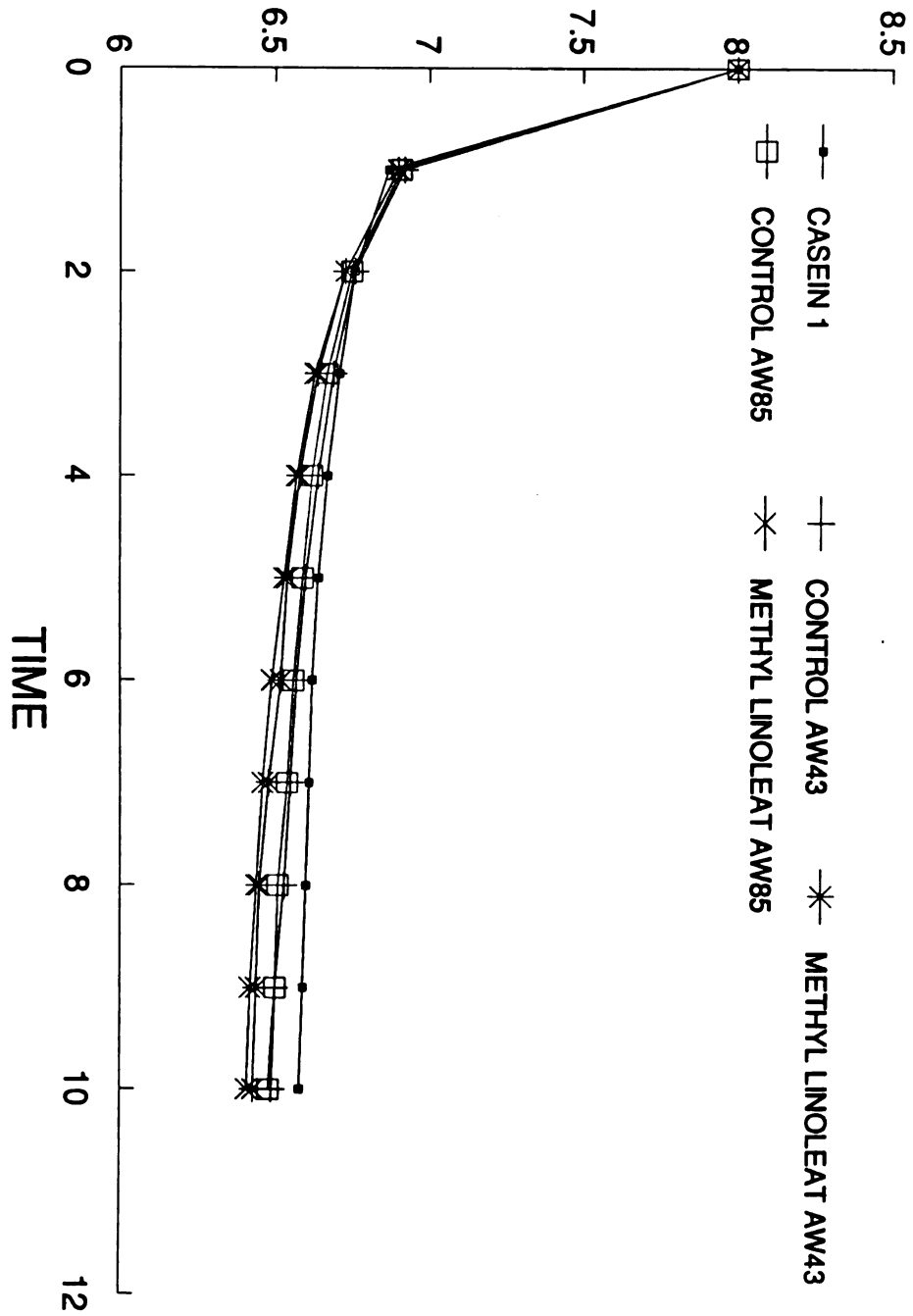
Figure 2. Change in pH over time of incubation for five different treatments. The pH vs time curves obtained by incubating control and treated sample at different water activities with multienzyme system.

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