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F I N A L R E P O R T

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Title: Mechanism and Prevention of Lipid Peroxidation
 in Muscle Foods

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A. ABSTRACT

The work performed under BARD grant I-258-81 can be put into two categories. One deals with an evaluation of the prooxidants of muscle tissue in relation to their kinetic characteristics and mechanism of action. The second relates to the functioning of these and possibly other systems in the muscle tissue in situ and on means of preventing the subsequent lipid oxidation.

In the first category, we have delineated in both species - fish and poultry - the requirements of the muscle microsomal system for co-factors and response to environmental conditions. The absolute necessity for Fe^{+2} and the extremely low K_M for NADH ($1 \mu\text{M}$) in the presence of ADP are perhaps the most significant observations. We have also developed an improved procedure that gives a membrane preparation of a higher purity and greater stability. This latter characteristic has allowed us to solubilize and partially purify the membrane proteins responsible for the activity.

We have shown the effect of membrane lipid oxidation in vitro on its components, i.e. lipid and protein, as well as on its physiological ability to take up and hold Ca^{+2} . The oxidation in vitro has been shown to be related in some ways to changes in the membrane fraction as the fish gets older.

We have demonstrated that mitochondria have an enzymic lipid peroxidation capacity which at least is as great as that of the sarcoplasmic reticulum when expressed on a protein basis. It is hypothesized that the amount and type of iron available for the reaction in situ is a key feature of the stability of the muscle tissue.

Relative to what occurs in muscle tissue in situ, an important observation was made that the sarcoplasmic reticulum becomes more sus-

ceptible to oxidation with increasing age. This is expressed, in particular, as a greater susceptibility to an activator in the cytosolic fraction.

We have demonstrated in a model system that the peroxidizing microsomal fraction is capable of transmitting its oxidizing equivalents to an emulsified triglyceride. This may be important because it could indicate that initial oxidation occurs in the enzymic membrane system, and this initiates oxidation of other lipid components in the system. This would be especially important because we have demonstrated that at refrigerated temperatures there is a very large difference between the rate of enzymic and nonenzymic lipid oxidation, much more so than is observed at room temperature or higher temperatures.

Using a kinetic approach in minced muscle tissue, we have shown that the membrane lipids oxidize more rapidly than do the neutral lipids of fish tissue. This observation is consistent with the above model where oxidation occurs first in the membrane fraction. Using enzyme probes, we have demonstrated that lipid oxidation, as determined by chemical measurements, can be reduced by the addition of enzyme which will destroy cofactors, membranes, or remove active oxygen intermediates. The data are not easy to interpret, however, since the specific effect of the individual agents added depends on the method used to measure chemical lipid oxidation. Although lipid peroxidation has been found to occur in most all sarcoplasmic reticulum preparations of muscle tissues, the specific activity varies greatly with the source of membrane and appears to be related to the stability of the tissue to the development of lipid oxidation.

The probes used to elucidate the mechanism by which enzymatic membranal lipid peroxidation is initiated show that in fish it is de-

pendent on hydroxyl radicals, however, in turkey by ferryl ions.

It was first discovered a new non-enzymatic catalyst, H_2O_2 -activated metmyoglobin which could initiate membranal lipid peroxidation.

The interaction of hydrogen peroxide (H_2O_2) with metmyoglobin (MetMb) led very rapidly to the generation of an active species which could initiate lipid peroxidation. The activity of this prooxidant decreased rapidly during the first minutes, but 50% of its activity remained stable for more than 30 min. In this model system, it was found that small amounts of H_2O_2 (1-10 μM) could activate MetMb for significant lipid peroxidation. The incubation of the sarcosomal lipids with activated MetMb caused oxygen absorption. No absorption of oxygen was determined in the presence of membrane with MetMb or H_2O_2 alone. Methemoglobin (MetHb) was also found to be activated by H_2O_2 and to initiate lipid peroxidation. Membranal lipid peroxidation initiated by activated MetMb was inhibited by several reducing compounds and antioxidants. However, several hydroxyl radical scavengers and catalase failed to inhibit this reaction.

Heated MetMb maintained its capacity to be activated by H_2O_2 . The accumulation of thiobarbituric reactive substances (TBA-RS) and oxygen absorption showed a higher rate of lipid peroxidation by H_2O_2 -activated MetMb in microsomes separated from turkey than from chicken muscle tissues. Membranal lipid peroxidation initiated by activated MetMb was inhibited by low concentrations of either ascorbyl palmitate, α -tocopherol, or butylated hydroxytoluene (BHT). Inhibition was also observed by very low concentrations of ascorbic acid in the presence of EDTA. Only very high concentration of EDTA (1-10mM) inhibited significantly membranal lipid peroxidation by activated metmyoglobin.

The H_2O_2 -activated species which initiate membranal lipid peroxidation appear to be an oxo-ferryl radical, and not a hydroxyl radical.

Hydrogen peroxide (H_2O_2) and the superoxide radical (O_2^-) are normally present at low concentration as metabolites in aerobic cells.

The generation of H_2O_2 in turkey muscle tissues was determined by using (^{14}C) formate oxidation to $^{14}\text{CO}_2$ by the peroxidative activity of catalase.

The incubation of muscle tissues at 37°C shows H_2O_2 generation of 14.0 nmol/g of fresh weight/30 min. In the presence of added catalase we found that muscle tissues generate almost 26.6 nmol of H_2O_2 under the same conditions. Muscle tissues aging at 4°C increase the generation of H_2O_2 . It seems that endogenous generation of H_2O_2 plays an important role in the formation of the primary pool of biological catalysts in muscle tissues. It was demonstrated that lipid peroxidation in situ minced muscle foods dependent on oxygen concentration and could be inhibited by vacuum. Minced turkey muscle was inhibited by relatively low concentration of primary antioxidants. Ascorbyl-palmitate, an antioxidant which decomposes to natural products, was found to inhibit very efficiently in situ lipid peroxidation. The inhibition of relatively low concentration of EDTA (200 μM in raw or 400 μM in heated minced muscle) of in situ lipid peroxidation demonstrated that most of the initiation and stimulation of this process is dependent on "free" iron ions. Preliminary work, indeed, showed that catalytic "free" iron exists in muscle tissues.

B. INTRODUCTION

Lipid peroxidation is one of the primary mechanisms of quality deterioration in stored foods, especially in muscle tissues. The changes in quality can be manifested by deterioration in flavor, color, texture and nutritive value and the production of toxic compounds (Simic and Karel, 1980; Pearson et al., 1983; Benedetti et al., 1984).

With modern techniques of food formulation and the production of structured and pre-cooked products, problems with lipid oxidation will most likely be more acute than in the past. The use of mechanically de-boned meat and fish tissues in the production of restructured food products offers an entirely new approach to the development of food products. Minced tissue, however, is more readily subject to lipid oxidation changes, either because of the disruption of the normal structure in the cell or from the greater incorporation of air, and thus oxygen, into the tissue.

The mechanisms of lipid peroxidation in muscle food were studied in model systems containing membranal lipids extracted from poultry or fish muscle tissues.

During this study we have evaluated membranal lipid peroxidation by three possible pathways:

- a) enzymic microsomal lipid peroxidation dependent on NADPH, NADH;
- b) non-enzymic microsomal lipid peroxidation dependent on H_2O_2 activated metmyoglobin or methemoglobin;
- c) non-enzymic microsomal lipid peroxidation by a "redox cycle".

We have also studied accumulation of hydrogen-peroxide in situ muscle tissues and the effect of various antioxidants in the prevention and inhibition of the oxidative process.

C. OBJECTIVES OF THE RESEARCH PROPOSAL

The purpose of this study was to expand and deepen our knowledge in the role of the various factors affecting lipid oxidation in muscle foods.

- 1) To isolate and characterize water-soluble factors and membranes in poultry and fish muscles affecting lipid oxidation.
- 2) To determine the relation between enzymic and non-enzymic lipid peroxidation in muscle tissues of poultry and fish.
- 3) To compare the mechanisms of enzymic and non-enzymic lipid peroxidation in two types of tissues; evidence to date indicates that the enzymic systems in the fish and the poultry tissues have significant differences.
- 4) To study protective systems of lipid peroxidation in muscle poultry and fish and to establish means of inhibiting enzymic and non-enzymic peroxidation in the tissues.
- 5) To determine the utilization of natural and synthetic protective compounds in muscle foods in order to prevent quality deterioration of products associated with lipid peroxidation.
- 6) To determine what component of the fish system makes it more susceptible to lipid oxidation than chicken system, i.e. whether it is solely due to the higher content of fatty acids with 5 and 6 double bonds in fish muscle or whether there are also differences in other factors.

D. MATERIALS AND METHODS

See reports published by the authors of this research.

E. RESULTS

E.1 Characterization of lipid peroxidative activity of muscle microsomes from fish and poultry

Several measurements were used to follow the peroxidation of membrane lipids of isolated fish and turkey microsomes. These included lipid hydroperoxides (LOOH) or diene conjugated compounds, TBA-reactive substances expressed as malondialdehyde (MDA) and carbonyl (RCHO) compounds. Typical results are shown in Figs. 1, 2, 3. On a molar basis, approximately 6-fold (fish) and 3-fold (turkey) more hydroperoxides or diene conjugated compounds were produced than MDA or carboxyl compounds. The similarity in the kinetics of the production of all these, however, indicates that the measurements are equally good and imply that the rate-limiting step of the reaction occurs prior to the formation of the lipid hydroperoxide. It is most likely that this rate-limiting step is the initial abstraction of hydrogen from the unsaturated fatty acid molecule, as has been found to be the rate-limiting step in the oxidation of bulk lipids. The use of TBA-reactive substances test (MDA) was taken as the standard procedure because of its simplicity and speed.

In vitro, the membrane system showed an optimum (Figs. 4,5) at approximately pH 6.7 (fish) and pH 6.0 (chicken and turkey). Eighty-percent of the maximal activity was observed between the pH range of 5.5-6.7; thus, the pH activity curve shows very high activity over the whole range of pH values which one would be apt to find in postmortem fish and poultry muscles.

The lipid peroxidative specific activity was a function of the protein concentration in the reaction medium, decreasing as the protein

Fig. 1. Flounder muscle microsomal lipid peroxidation measured by three techniques. The reaction medium contained 0.5 mg protein per ml, 0.2 mM NADH, 0.2 mM ADP, 0.05 mM FeCl_3 , 0.12 M KCl and 5 mM histidine, pH 7.1. The reaction was carried out at 6°C.

Fig. 2 Turkey muscle microsomal lipid peroxidation. The reaction medium contained 0.5 mg protein per ml, 0.4 mM NADH or NADPH, 0.4 mM ADP, 0.01 mM FeCl_3 , 0.12 M KCl and 5 mM histidine, pH 7.3. The reaction was carried out at 25°C. A part of the microsomes were heated at 80°C for 5 min.

Fig. 3 The rate of conjugated dienes accumulated during turkey muscle microsomal lipid peroxidation. Conditions, see Fig. 2.

Fig. 4 The effect of pH on the activity of turkey muscle microsomal lipid peroxidation. Conditions, see Fig. 2.

Fig. 5 The effect of pH on the activity of chicken muscle microsomal lipid peroxidation. Conditions, see Fig. 2.

Fig. 6 The effect of protein concentration on the activity of turkey muscle microsomal lipid peroxidation. Conditions, see Fig. 2.

concentration increased (fish); however, in poultry the activity increases with increasing protein concentration. The specific activity of 1 mg of protein per ml of reaction medium was only 25% (fish) or 400% (turkey) of that observed at a protein concentration of 0.25 mg per ml (Fig. 6).

The effect of temperature on the production of MDA, as well as lipid hydroperoxides (LOOH) is shown in Fig. 7, 8 and 9. Again, both these measures of lipid oxidation follow roughly similar patterns with increasing activity with increasing temperature. The LOOH does reach a peak at 37⁰ (fish) which is probably due to the instability of the hydroperoxides. An important point to note is that the relative rate activity at low temperature (0 and 6⁰C) is still very considerable. In turkey, the activity at 5⁰C is only 15-20% of that from fish microsomes.

Stimulation of the microsomal (fish and poultry) lipid peroxidation by ADP and ATP, as well as its inhibition by high concentration, is illustrated in Figs. 10 and 11. The inhibition by high concentration of ADP was more significant in fish microsomes than in turkey microsomes. The ATP concentration in the muscle of freshly killed fish or poultry is approximately 4-5 mM. Thus, one would expect that immediately after postmortem these compounds would inhibit the activity in situ, but as they were consumed by the normal biochemical processes occurring in postmortem muscle, they would eventually stimulate the reaction.

We expect that the stimulation of microsomal lipid peroxidation is due to the formation of an ADP or ATP-Fe complex. Indeed, increasing of FeCl₃ concentration in the presence of ADP (0.2 mM) increases lipid peroxidation at any FeCl₃ concentration tested by us (Figs. 12, 13).

Fig. 7 Production of TBA-reactive substances (MDA and LOOH) by flounder microsomes as a function of temperature. The reaction conditions were similar to those described in Fig. 1 except the concentrations of ADP and NADH were 0.1 mM and the temperature was varied as indicated.

Fig. 8 Production of TBA-RS as MDA by turkey microsomes as a function of temperature. Reaction conditions were similar to Fig. 2.

Fig. 9 Arrhenius plot of the activity of turkey microsomes on lipid peroxidation.

Fig. 10 Effect of ADP concentration on flounder muscle microsomal lipid peroxidation at two concentrations of FeCl₃. Reaction conditions were similar to those described in Fig. 1 except the concentration of NADH was 0.01 mM in the presence of the NADH-regeneration system and the FeCl₃ concentration was as indicated.

Fig. 11 Effect of ADP concentration on turkey muscle enzymic microsomal lipid peroxidation. Conditions, see Fig. 2.

Fig. 12 Lipid oxidative activity of fish SR as a function of Fe²⁺ or Fe³⁺ concentration. Assay medium contained 0.1 mM NADH, 0.1 mM ADP, and 0.015 mg protein per ml. Temperature was 6°C.

Fig. 13 The effect of FeCl_3 concentration on turkey muscle enzymic microsomal lipid peroxidation. Conditions, see Fig. 2.

Fig. 14 Effect of various concentrations of NADH and NADPH on lipid oxidation of flounder microsomes.

NADH and NADPH show different effects on the lipid peroxidation system of the microsomes. The effect of NADH is much greater than that of NADPH in fish model systems, however the effect of NADPH was found to be higher than NADH in turkey model systems (Figs. 14,15). The effect of NADH on microsomal lipid peroxidation makes it unique in fish compared with other microsomal systems (rat liver, brain). NADPH and NADH at high concentrations were found to inhibit microsomal lipid peroxidation in turkey model systems (Figs. 14,15).

Microsomal lipid peroxidation was found to be inhibited only by high concentration of NADH and not by NADPH.

At low concentration of NADH and NADPH (0.1-0.2 mM) there were no problems with measuring the activity. However, at concentrations much lower than this an NADH oxidase present in the membrane interferes with the system by rapidly decreasing the amount of NADH available to the reaction. We overcame this problem by using a regeneration system of glucose-6-phosphate and glucose-6-phosphate dehydrogenase which maintained linearity in the reaction over the period of time that measurements were taken. Using this system it was possible to determine the K_m of the system for NADH. In the absence of ADP it was approximately 10 μM , and in the presence of 0.1 mM ADP it was about 1 μM . These values demonstrate that very low levels of NADH are required for the reaction. Even though this compound does decrease relatively rapidly postmortem, the decrease to values below the K_m takes place very slowly. We have determined that the NADH concentration in postmortem fish muscle remains above 5 μM after 2 weeks storage at 0°C and remained at this level after 3 weeks storage at -5°C. This is further evidence that the system might be important in postmortem fish muscle.

Fig. 15 The effect of NADPH and NADH concentration on the activity of turkey muscle enzymic microsomal lipid peroxidation. Conditions, see Fig. 2.

Fig. 16 Effect of phosphate on microsomal lipid oxidation.

Inorganic phosphate ion is an efficient inhibitor of microsomal lipid peroxidation (Fig. 16). It has been estimated that the inorganic phosphate concentration in living muscle tissue is somewhere between 3 and 7 mM. Thus, one would expect that it would be an efficient inhibitor in situ. However, when inorganic phosphate is added to minced fish muscle it does not have much of an effect on lipid peroxidation. We have also determined that when a press juice is prepared from fish muscle and separated into low and high molecular weight components, all of the measurable Fe is in the high molecular weight fraction. Our current working hypothesis is that unlike the in vitro system, iron in muscle is not free but is complexed to high molecular weight components, most likely proteins. Phosphate does not compete particularly well with the proteins for the iron. Thus, although phosphate is a very effective inhibitor in vitro, it is not an inhibitor in situ.

We have characterized the differences between the activity of the enzymic microsomal lipid peroxidation from chicken and turkey dark and light muscle. The microsomes prepared from chicken and turkey dark muscle were found to be more active in lipid peroxidation (Figs. 17,18, 19) than that of light muscle. Microsomal lipid peroxidation determined during activation of turkey dark microsomes were found between 6 and 10-fold higher than that of chicken dark microsomes.

E.2 Preparation of a stable sarcoplasmic reticulum

Preparation of a stable, more purified sarcoplasmic reticulum (SR) from fish. The microsomal preparation described in the previous section is relatively easy to prepare in large quantities and lends itself to a variety of kinetic studies. However, it was desirable to improve the stability of the isolated sarcoplasmic reticulum, particularly as this

Fig. 17 The rate of TBA-RS as MDA accumulation during turkey muscle enzymic microsomal lipid peroxidation. Conditions, see Fig. 2. $\square$, Dark muscle; $\circ$, Light muscle.

Fig. 18 The rate of conjugated dienes accumulation during turkey muscle enzymic microsomal lipid peroxidation. Conditions, see Fig. 2. $\square$, Dark muscle; $\circ$, Light muscle.

Fig. 19 The rate of TBA-RS as MDA accumulation during enzymic microsomal lipid peroxidation of chicken muscle. $\square$, Dark muscle; $\circ$, Light muscle.

Fig. 20 Stability of lipid peroxidative activity of purified sarcoplasmic reticular fraction from flounder muscle.

related to our desire to isolate and characterize the components of the membrane which are responsible for lipid peroxidation. Also, it was desirable to have a more purified preparation to study the physiological characteristics of the membrane and the effects of lipid oxidation on these characteristics. A procedure was developed for this purpose using a discontinuous sucrose gradient (Anal. Biochem. 137 (1984)58-65). This preparation gave greater stability than the previously used microsomal preparation, having over 80% of its activity remaining after 2 weeks of refrigerated storage in the presence of anti-microbial agents (Fig. 20). This was sufficient to allow us to proceed with the isolation work. The composition of the purified sarcoplasmic reticulum fraction of flounder muscle is shown in Table 1 and some properties of these membranes are listed in Table 2. We believe that at least part of the greater stability of the sarcoplasmic reticular fraction (SR) is due to the removal of some lysosomal impurities, the enzymes of which can subsequently degrade the SR.

Detergent solubilization of SR proteins and partial purification of the active components was conducted. The isolated SR was incubated with several detergents individually. After incubation, the membrane fraction was separated into supernatant and sedimented fractions by ultracentrifugation. The relative distribution of protein between the soluble and insoluble fractions was compared, as was the distribution of activity between the fractions and the total recovered activity. It was determined that Triton X-100 produced the best results in combining both effective solubilization without destruction of activity. A summary of the significant results with this detergent is shown in Table 3. At least 2.5 mg of the detergent were required per ml of suspension (when

the protein was present at a concentration of 1 mg per ml) to get good solubilization. This level also caused the majority of the activity to go into the supernatant fraction. It was necessary to remove excess Triton X-100 (with Amberlite) to regain maximal activity. Whether removal of the Triton X-100 removes a specific inhibitory effect or allows reformation of active membrane vesicles is not clear at this time.

The components responsible for activity in the membrane were estimated by solubilization of the membrane proteins by Triton X-100 followed by iso-electric focusing of the solubilized proteins. The proteins were reconstituted with SR phospholipid and the Triton X-100 was removed for the assay. Figure 21 shows the profile of the protein and activity patterns after iso-electric focusing of the solubilized SR proteins. There were two major peaks of activity, one including fractions 10 through 13, and a second containing fractions 19 through 22. The specific activity of the latter was higher, and it was, in fact, some 5 times greater than that of the original preparation. It is not known at this time whether the two active peaks represent two separate systems for lipid oxidation or whether they represent different aggregation states of the several proteins involved, with the more pure or more complete system being that of fractions 19-22. Further investigations will be carried out to elucidate this.

To determine the role of cytosolic factors on lipid peroxidative rates of the isolated SR membrane, a press juice was prepared from fish muscle by ultracentrifugation. This was then added into the final reaction medium at different concentrations, i.e. producing different dilutions. The results in Figure 22 show the effect of this cytosol on the rate of lipid peroxidation. Almost 80% of the lipid peroxidative activity of the SR is inhibited even when the cytosol is present at a con-

Table 1. Composition of sarcoplasmic reticular fraction of flounder muscle

	Percentage		
	Dry weight	Total lipid	Phospholipid
Protein	57(+2)	-	-
Lipid	43(+2)	-	-
Phospholipid fraction	-	60(+5)	-
Low polarity lipid fraction	-	32(+6)	-
Glycolipid fraction	-	8(+2)	-
Phosphatidylcholine	-	-	75(+7)
Phosphatidyl-ethanolamine	-	-	15(+4)
Phosphatidylinositol	-	-	8(+5)
Sphingomyelin	-	-	2(+2)

Table 2 . Some properties of sarcoplasmic reticular vesicles from flounder

Yield (mg protein/g muscle)	1.0+0.22
NADH-dependent lipid peroxidation (nmol MDA/mg protein/30 min)	99+15
ATPase (μ mol Pi/mg protein/min)	
Total ATPase (with Ca^{2+} -EGTA)	0.99+0.11
Basic ATPase (with EGTA)	0.04+0.03
Ca^{+2} -activated ATPase (by difference)	0.95
Acid phosphatase (μ mol pi/mg protein/min)	0
Succinate oxidase (nmol O_2 /mg protein/hr)	0.3+0
Succinate-cytochrome c reductase (nmol cytochrome c reduced/mg protein/min)	7.6+0.6

Fig. 21. Resolution by isoelectric focusing of lipid peroxidative activity and protein from sarcoplasmic reticulum of flounder after solubilization by Triton X-100.

Table 3. Solubilization of activity from SR by Triton X-100

(T X-100) mg/ml(mg)	% protein solubilized	Distribution of Activity, nmol MDA per 30'		
		Sed	Sup	Sup + Amber- lite
0	3	728	4	
0.2	12	706	11	2
0.5	17	669	20	6
1.0	35	515	94	92
2.5	78	61	177	362
5.0	85	15	148	685

Fig. 22 Inhibitory effect of cytosol on lipid peroxidation of flounder sarcoplasmic reticulum. The dilution factor is compared to estimated concentrations in situ.

centration which is equivalent to a 600-fold dilution of the concentration that would be found in situ. Heating the cytosol changes this only slightly, if at all. However, dialysis of the cytosol removes most of the inhibition. There does appear to be a slight activation as the concentration is increased, i.e. the dilution factor is decreased.

Since we had previously shown that inorganic phosphate was an effective inhibitor of SR lipid oxidation in vitro, we tested the possibility that the inorganic phosphate was the component of the cytosol which was responsible for the inhibition. By direct determination of inorganic phosphate in the cytosol and then testing the effect of this concentration of phosphate on the in vitro oxidation system, it was shown that inorganic phosphate could account for essentially all of the inhibition observed in the cytosolic fraction. As we had indicated earlier, however, this does not mean that inorganic phosphate is serving this role in situ because of the nature of the bound Fe.

An interesting observation was made when it was recognized that the inhibition of SR lipid peroxidation by the cytosolic fraction was a function of the age of the SR. This was true whether the SR was aged in vitro or whether the SR was prepared from fish which had been aged post-mortem (Fig. 23). We demonstrated that this effect was not related to the age of the cytosol, and we also determined that this change in the response of the aging SR was not due to its interaction with inorganic phosphate. It was, however, observed that the SR, aged in vitro or in situ, became more susceptible to the activating effects of a high molecular weight, thermolabile component of the cytosol (Fig. 24). It is apparently this greater sensitivity to this component which makes the aged SR more susceptible to oxidation. This may be an important obser-

Fig. 23 Loss of inhibitory response of sarcoplasmic reticulum to cytosolic fraction after aging of SR in vitro or in situ.

Fig. 24

Increased activating effect of high molecular weight thermolabile fraction on sarcoplasmic reticulum from flounder muscle aged in vitro or in situ. The activating factor was prepared by dialysis of a muscle press juice.

vation since it is known that fish aged prior to frozen storage will undergo greater lipid peroxidation than fresh fish. Preliminary evidence indicates that inhibitors and activators are present in the cytosolic fraction of the muscle cell, and their effect on SR lipid oxidation in vitro is different from lean fish, the white muscle of fatty fish, and the dark muscle of fatty fish.

E.3 Development of a enzymic model system containing emulsified lipids

In a first attempt to elucidate the role of the peroxidizing microsomal fraction in fish lipid oxidation, a model system utilizing the microsomal fraction along with emulsified lipids was tested. The purpose of this was to determine whether lipid peroxidation in the microsomal fraction would cause oxidation in a lipid emulsion in association with the microsomes. Results are shown in Figure 25. In the absence of the microsomal fraction there was no production of TBA-reactive substances. This would indicate that the lipid emulsion was not autoxidizing in the presence of NADH, ADP and iron. Likewise, when microsomes, ADP and iron were added but NADH was not, there was no oxidation indicating that nothing in the membrane preparation caused oxidation of the lipids of the emulsion. When the enzyme-catalyzed oxidation of the microsomal lipids was carried out in the absence of the lipid emulsion, oxidation was observed which levelled off after approximately 4 hr. The upper curve in the figure is the amount of MDA produced in the presence of both the peroxidizing microsomal fraction and the lipid emulsion. It can be seen that the amount of oxidation was much greater than that with the microsomal fraction alone. This indicates that the increase in lipid oxidation observed in the complete system was due to the oxidation of lipids in the emulsion. Since there was

no oxidation in the system when the microsomal fraction was not undergoing the enzyme-catalyzed oxidation of microsomal lipids, it was concluded that somehow enzymic peroxidation of microsomal lipids caused oxidation of the lipids in the emulsion.

E.4 The effect of storage on neutral and microsomal fatty acids composition

Although membrane lipid oxidation has been studied extensively in vitro, there has been little good information concerning the role of the lipid peroxidation system of the microsomal fraction in situ. In particular, nothing is known as to whether the membrane-catalyzed lipid oxidation was responsible for catalysis of the non-membrane lipids of fish muscle tissue during frozen storage.

The following set of experiments was designed to provide some insight as to the possible role of the SR lipid peroxidation system in lipid oxidation in frozen stored fish muscle. The basic approach in these studies was to determine the kinetics of lipid oxidation in the SR fraction from fish muscle and compare this to the kinetics of oxidation of the neutral lipids of the flesh. If the neutral lipid fraction oxidized faster than the SR lipid component, it would indicate that the membrane-catalyzed system was not the rate-limiting factor in lipid oxidation. On the other hand, if the lipids of the SR oxidized faster than the neutral lipids, this would be consistent with the hypothesis that oxidation of the SR lipids plays an important role in initiating lipid oxidation in the tissue.

The tissue chosen for this study was herring (Clupea harengus). This is a fatty fish which contains a variable amount of lipid depending on the season. Herring that we used contained on the average 8.6% total

lipid. The outline of the procedure to be followed is shown in Figure 26. Minced muscle was stored at -20°C . At given periods of time, the SR were isolated and the lipid was extracted and analyzed. Fatty acids were determined by a gas chromatographic technique after methylation, and oxidation was evaluated by a determination of the change in the ratio of C22:6 to C16:0. At the same time, total lipid was extracted from the fish flesh and extracted and analyzed both for its fatty acid composition (C22:6 to C16:0 ratio) as well as its content of TBA-reactive substances. The total lipid was used as an approximation for the neutral lipid since this fraction would make up by far the greatest part of the total lipid fraction and should introduce no more than 10% error, a value which we felt would be within our ability to measure oxidation.

For competitive purposes, flounder muscle, which does not carry storage lipids, i.e. neutral lipids, in its muscle tissue, was examined. The difference between the two fish would be that with flounder, the total lipid should represent mostly membrane (polar) lipid with little neutral lipid.

The content of the principal fatty acids found in both the total lipid fraction and the SR lipid fraction of Atlantic herring (Table 4) and winter flounder (Table 5) is given. As is commonly found in fish and other muscle tissues as well, the membrane lipids of herring had considerably more polyunsaturated fatty acids than did the total lipids which are made up of the greatest part by triacylglycerols. The contrast between total lipids and SR lipids was not quite as great in the winter flounder, but nevertheless indicated the relatively highly unsaturated nature of the SR lipids.

Fig. 25 Peroxidation of lipid emulsion by herring light muscle microsome fraction. Complete assay system (O), no lipid emulsion (▽), no NADH (Δ), and no microsomes (○).

Fig. 26 Scheme for following oxidation in SR and total lipids of minced fish muscle.

Table 4 Fatty acid composition of Atlantic herring

Fatty Acid	% Fatty Acids	
	Total Lipids	SR Lipids
14:0	6.0	1.0
16:0	14.4	10.2
18:0	1.0	1.4
Saturated	21.4	12.5
16:1	6.0	1.0
18:1	12.0	3.0
20:1	9.9	2.6
22:1	9.2	3.3
Monoenoic	37.1	9.4
20:4	-	4.2
20:5	14.5	10.1
22:4	1.2	3.3
22:5	0.6	5.9
22:6	14.4	33.2
Polyenoic	30.7	57.2
Others	10.8	20.9
Total lipid = 8.6%.		

Table 5 Fatty acid composition of winter flounder

Fatty Acid	% Fatty Acids	
	Total Lipids	SR Lipids
14:0	1.2	1.5
16:0	14.4	13.9
18:0	2.9	3.5
Saturated	18.5	18.9
16:1	2.7	1.6
18:1	6.8	4.8
20:1	2.3	1.5
22:1	18.7	1.7
Monoenoic	30.5	9.7
20:4	-	3.2
20:5	4.4	20.2
22:4	2.6	2.1
22:5	4.6	5.2
22:6	22.4	20.7
Polyenoic	34.0	51.4
Others	17.0	20.0
Total lipid = 1.2%.		

The results for MDA production and the ratio of C22:6 to C16:0 in both the tissue (total) and the SR lipids for herring is given in Figure 26a. The results are reported per g of lipid for easier comparison between the two types of lipids, as well as the comparison between the fatty and lean species of fish. There was an almost linear increase in MDA with storage for 8 months at -20°C in the whole tissue. Likewise, the ratio of the highly polyunsaturated C22:6 to the saturated C16:0 decreased in the isolated SR fraction. Assuming that the C16:0 fatty acid would be relatively resistant to oxidation, this would be an effective measurement of the loss of C22:6 fatty acid. It was reported as the ratio rather than the direct value for C22:6 to normalize everything to account for differences in sampling techniques, degree of methylation among experiments, and any other factors. On the other hand, the ratio of C22:6 to C16:0 for the total tissue lipids did not change over the 8-month storage period. This indicates that the SR lipids oxidize more rapidly than the total lipids of the tissue; the latter can be taken as a measure of the triacylglycerols as a first approximation.

Similar results are given for flounder muscle in Figure 27. Again, we see an increase in the malondehyde (MDA) value for the tissue as a function of storage. The increase is somewhat higher for the flounder, a lean fish than it was for the fatty herring. This is perhaps related to the fact that in the lean fish most of the lipids are membrane lipids and thus contain a very high proportion of the polyunsaturated fatty acids. There is again a decrease in the C22:6 to C16:0 ratio in the SR fraction, but it is not as great as it was in the case of the herring. This is consistent with the observation that the activity of the SR fraction of the herring is some 3 to 12 times greater than that of the flounder (depending on the ratio of light to dark herring tissue taken).

Months at -20°

Fig. 26 a Change in tissue MDA and ratio of C22:6/C16:0 in SR and tissue lipids in frozen, minced herring muscle.

Months at -20°

Fig. 27 Change in tissue MDA and ratio of C22:6/C16:0 in SR and tissue lipids in frozen, minced flounder muscle.

The change in this case is only about 20% of that in the herring. Unlike the herring tissue, however, the C22:6 to C16:0 ratio in the flounder tissue lipids changed more than the same ratio in the SR. (In the herring it did not change at all). This is probably related to the fact that there is little neutral lipid in the flounder tissue. It would indicate that there are membranes in the flounder which undergo oxidation at a faster rate than do the lipids from the SR.

A word of caution must be expressed concerning these results. The amount of SR that was isolated as a function of time of storage at -20°C was not constant but decreased rather rapidly with storage. We did not expect this since our earlier studies had indicated that fish could be frozen for a considerable period of time with no loss in the quantity of isolated SR. However, these earlier studies utilized a storage temperature of -80°C . Obviously, the higher temperature makes a difference. Although we cannot be sure, we suspect that the recovered SR probably has lipid of lower oxidation than the unrecovered SR and that one of the reasons for lack of recovery of SR might be the extreme oxidation of the lipids in the membrane causing it either to disintegrate or to be bound to structural components of the muscle and making it unable to be extracted.

Although these data do not definitely show that the primary oxidation occurs in the membrane fraction, they are nevertheless consistent with this concept. These are considered preliminary experiments only which will help us define some of the conditions at which we want to look. They also provide us with knowledge of the factors that have to be controlled in order to definitively indicate the role of the SR in lipid oxidation in muscle tissue in situ.

We have also determined the fatty composition of SR of turkey and chicken. The same SR were tested for enzymatic lipid peroxidation. The composition of the fatty acids from turkey and chicken was very similar, however turkey microsomes tend to oxidize more rapidly (Table 6, Fig. 28). It seems that the more rapid oxidation of the turkey microsomes derived from a higher activity of NADPH-cytochrome reductase (Table 7). It was found that NADPH-cytochrome reductase is almost 3-fold higher in turkey microsomes than in chicken microsomes.

EDTA, which inhibits the lipid peroxidation during the test of NADPH-cytochrome reductase, demonstrates that almost 60-70% of NADPH consumed during enzymic microsomal lipid peroxidation interacts with oxidized lipids.

We have also determined the oxidation of microsomal turkey muscle in situ. Minced turkey muscle with and without EDTA was stored at 4°C. At given periods of time, the SR were isolated and the lipids were extracted and analyzed for conjugated dienes. At the same time the content of TBA-reactive compound from the muscle were analyzed.

The content of TBA-RS as MDA (Fig. 29) and conjugated dienes from microsomal lipid (Fig. 30) is given. There was significant increase in the amount of TBA-RS in the minced muscle incubated without EDTA. EDTA inhibit completely TBA-RS accumulation in stored minced turkey muscle. Very similar results were obtained by determining changes in microsomal lipids by the accumulation of conjugated dienes.

A similar experiment was designed with unminced turkey muscle stored in the same condition. The accumulation of TBA-RS in the muscle was almost similar with those of minced muscle, however the accumulation of conjugated dienes in microsomes were very low (Fig. 31, Table 8).

Table 6. The composition of microsomal fatty acids from dark muscles of turkey and chicken.

Fatty acid		µg/mg protein	
		Turkey	Chicken
Palmitic acid	(16:0)	134.0	130.5
Stearic acid	(18:0)	109.6	106.8
Oleic acid	(18:1)	103.5	100.8
Linoleic acid	(18:2)	121.8	118.6
Arachidonic acid	(20:4)	67.0	56.4
Docosahexaenoic acid	(22:6)	39.1	43.4

Fig. 28 Changes on the composition of fatty acid during microsomal lipid peroxidation.

Table 7. The enzymic activity of NADPH cytochrome P-450 reductase from turkey and chicken microsomes.

Treatment	Decomposition of NADPH $\mu\text{M}/\text{mg protein}/10 \text{ min}$	% from control
Chicken	28.5	100.0
Chicken + EDTA, 100 μM	11.7	41.1
Turkey	88.3	100.0
Turkey + EDTA, 100 μM	28.4	32.2

Fig. 29 Lipid peroxidation of microsomal (endoplasmic reticulum) fraction during storage of minced turkey muscles at 4°C. $\square$, Control without EDTA; $\circ$, minced muscles in the presence of 200 μM EDTA.

Fig. 30 Lipid peroxidation of the microsomal (endoplasmic reticulum) fraction during storage of minced turkey muscles at 4°C.
□, control without EDTA; ○, minced muscles in the presence of 200 μM EDTA.

Fig. 31 Lipid peroxidation of the microsomal (endoplasmic reticulum) fraction during storage of sliced turkey muscles at 4°C.

E.5 Lipid peroxidation in muscle cells other than microsomes

Earlier results had indicated that there was a membrane fraction in fish muscle which was undergoing lipid oxidation during frozen storage more rapidly than was the sarcoplasmic reticulum. It seems likely that this membrane might be mitochondrial. This could be very important in the rate of lipid oxidation of fish muscle as well as other muscle foods that contain much red muscle since it has been estimated that 20-25% of the volume of red muscle fiber is composed of mitochondria. There had been research which indicated that lipid oxidation did occur in mitochondria and one or two studies had suggested that perhaps this lipid oxidation was enzymic in nature. There was, however, no attempt in these reports to quantitatively assess the potential contamination of the mitochondria with other membrane fractions such as the endoplasmic reticulum.

Standard procedures of mitochondrial preparation were worked out for adaptation to fish muscle.

Mitochondria were initially prepared and characterized from mackerel. However, it became apparent that recovery of mitochondria from frozen fish was unsatisfactory. During frozen storage the yield of mitochondria decreased very rapidly. Thus, it was necessary to utilize a fish that could be kept alive at our facilities for preparation of mitochondria from the freshly killed animal. We decided to use rainbow trout for these studies. Mitochondria are prepared on a discontinuous sucrose gradient and are collected at the interface between 45 and 35% sucrose solutions. They are then further washed and isolated by differential centrifugation.

Detection of contaminating sarcoplasmic reticulum in mitochondrial preparations is difficult because the muscle SR does not contain the

standard marker enzyme, glucose-6-phosphatase, which is present in the endoplasmic reticulum of most other cells. A summary of the results with fish mitochondria and SR is shown in Table 9. It appears from the data that the maximum contamination of the mitochondrial preparation by SR could be no more than 15-20%. We were particularly concerned that a small amount of highly active SR could confuse the results with mitochondria. This turned out, in fact, not to be a problem since it was shown that the specific activity of the mitochondrial lipid peroxidation system was greater than that of the sarcoplasmic reticulum. From the same muscle tissue we observed a specific activity of about 34 nmol malondialdehyde (MDA) per mg of protein per 30 min for SR, whereas the mitochondria had a specific activity of 42 nmol MDA per mg of protein-30 min. Thus, in red muscle, mitochondrial could contribute more to lipid peroxidation than the SR based both on the total amount of membrane as well as its higher activity.

Some characteristics of the enzymic lipid oxidation system of fish muscle mitochondria are shown in Table 10. There are several things to note about these data. First of all, the nonenzymic catalyzed oxidation of mitochondria (13 nmol of MDA per mg of protein-30 min) is very much higher than is observed with the sarcoplasmic reticulum. This could be due to the presence of high concentrations of iron compounds in the mitochondria. Mitochondria may also contain more the necessary cofactors such as NADH than SR. Fe^{+3} and ADP give very little stimulation alone or together. NADH and NADPH, however, do give a significant stimulation, again probably because of the presence of iron compounds in the mitochondria. NADH is the preferred cofactor in the fish muscle mitochondria, as it is in the sarcoplasmic reticulum. Heating the mito-

Table 8. The effect of storage of minced turkey muscle at 4°C on the microsomal fatty acids with and without 400 M EDTA.

Storage (days)	Dark muscle	Light muscle	EDTA	A ₂₃₃ nm	A ₂₁₅ nm	A ₂₃₃ A ₂₁₅
0	+	-	+	0.13	1.10	0.12
0	+	-	-	0.14	0.75	0.19
0	-	+	+	0.14	1.10	0.13
0	-	+	-	0.12	0.80	0.15
7	+	-	+	0.13	0.73	0.18
7	-	+	-	0.13	0.63	0.21
13	+	-	+	0.13	0.68	0.19
13	+	-	-	0.15	0.60	0.25
13	-	+	+	0.13	0.63	0.21
13	-	+	-	0.15	0.80	0.19
22	+	-	+	0.17	0.08	2.14
22	-	+	+	0.20	0.11	1.82

Table 9 Data for evaluation of purity of mitochondrial preparation

	SR	Mito
Succinate oxidase, $\mu\text{mol O}_2/\text{mg prot/hr}$		
-cyt.c	0.19	1.79
+cyt.c	--	1.99
Inosine diphosphatase, $\mu\text{mol Pi/mg prot/hr}$	1.99	0.44
NADH-cytochrome <u>c</u>		
reductase, unit/mg prot/min		
-rotenone	0.038	0.088
+rotenone	0.024	0.010
Lipid oxidative activity,		
nmol MDA/mg prot/30 min	33.9	41.5

Table 10 Enzymic lipid oxidation in fish muscle mitochondria

Mitochondria plus	nmol MDA per mg protein-30 min
-	13.0
Fe^{+3}	12.4
ADP	11.0
Fe^{+3} , ADP	16.5
NADH	24.0
NADPH	26.1
Fe^{+3} , ADP, NADH	54.9
Fe^{+3} , ADP, NADPH	32.3
Boiling, Fe^{+3} , ADP, NADH	15.7
SR plus	
-	2.5
Fe^{+3} , ADP, NADH	36.4
Fe^{+3} , ADP, NADPH	11.7

Concentrations used were: Fe^{+3} , 0.015 mM; ADP, 0.1 mM; NADH, 0.1 mM; NADPH, 0.1 mM.
Incubation was 6°C.

chondria completely destroys the NADH-catalyzed activity, but there still remains what appears to be a significant non-enzymic lipid oxidation.

The response of the fish muscle mitochondria to NADH and NADPH is similar to that which has been observed for the SR, i.e. NADH is the preferred cofactor, and high concentrations of NADH inhibit the reaction whereas the same levels of NADPH do not. The effect of pH on mitochondrial enzymic lipid peroxidation is also similar to that of the SR although the peak is not quite so broad. Nevertheless, it does have a maximal activity somewhere around pH 6.5, indicating that it could be an important reaction in postmortem fish muscle.

As is the case with fish SR, Fe^{+2} gives greater lipid peroxidation than Fe^{+3} , particularly at low concentrations. It appears that saturation is reached at rather low concentrations of Fe^{+2} which is not observed with the SR. As with the SR, the reaction appears to be sigmoidal but unlike the SR, there is a faster rate of reaction at 20°C than there is at 37°C . We have shown that the mitochondria loses approximately 1/3 of its activity after incubation for 45 min at 37°C . It appears, therefore, that the decrease in activity at 37°C is due to denaturation of the enzymes involved in the reaction.

The effect of protein concentration on mitochondrial lipid oxidation is shown in Table 11. The reaction rate is linear up to 0.2 mg of protein per ml of incubation medium. After that, there is a decrease similar to that observed with the SR. The effect of ionic strength as achieved by sodium chloride and potassium chloride is shown in Table 12. There appears to be no significant difference between sodium and potassium ions. An inhibitory effect is observed which is very similar to accumulation of fluorescent age pigments (FAP) in muscle tissue from older

Table 11 Effect of mitochondrial protein concentration on rate of lipid peroxidation

Mitochondrial protein in assay medium, mg/ml	nmol MDA per mg protein-30 min
0	0
0.0125	98.5
0.025	99.2
0.05	98.9
0.1	102.1
0.2	110.2
0.4	59.6

Mitochondria were incubated with 0.1 mM NADH, 0.1 mM ADP, 0.015 mM Fe^{+3} , 0.12 M KCl, and 5 mM histidine, pH 7.3 at 6°C.

Table 12 Effect of ionic strength on mitochondrial lipid oxidation

Ionic strength	nmol MDA per mg protein-30 min	
	NaCl	KCl
0	90.8	90.8
0.1	63.8	66.9
0.2	58.5	43.1
0.3	36.2	35.4
0.4	32.3	36.9

Mitochondria (0.005 mg protein per ml) were incubated with 0.1 mM ADP, 0.1 mM NADH, 0.015 mM Fe^{+3} and 5 mM histidine, pH 7.3, with NaCl or KCl added as indicated. Temperature of assay was 6°C.

fish compared to younger. However, this difference was not observed in the isolated SR. This was somewhat surprising since FAP is thought to accumulate in the membrane. However, this might be due to incomplete recovery of the SR, i.e. the SR which has undergone the most oxidation and has the most FAP is destroyed by the isolation procedure. We did observe that there was a significant increase in FAP in SR that was allowed to undergo enzymic lipid oxidation in vitro.

Older fish had a higher potential for lipid peroxidation in their SR than younger fish (Table 13). This was not observed in the content of FAP but was strongly noticed in the production of TBA-reactive substances (MDA). There was also an increase in high molecular weight proteins in the SR isolated from fish of increasing age. These appeared to be the same protein polymers that were formed by lipid oxidation in vitro.

No differences in fatty acid compositions were observed in the SR of fish aged from 2 to 8 years old. However, a consistently higher content of polyunsaturated fatty acids were found in fish caught in the winter compared to those caught in the summer (Table 14). It was found that the Ca^{+2} uptake ability of SR was affected by captive stress. That is, the membrane became less efficient in vitro in taking up Ca^{+2} with the length of time that the fish were in storage tanks. Although it appeared that the deterioration of the membrane from older fish was greater than that of the younger fish, both in absolute terms and as a percentage, the results serve as a warning that in studies such as these, the functioning of membranes isolated from the tissue may be very drastically affected by antemortem conditions.

TABLE 13

FLUORESCENT AGE PIGMENT (FAP) PRODUCTION IN SR DURING INCUBATION WITH PEROXIDATION SYSTEM

SR (10 mg/ml) were incubated with 0.08 mM NADH, 0.08 mM ADP and 0.01 mM Fe^{+3} at 3°C. The values were the average of 3 assays. Old fish: 44.2 cm, 1338 g, 9 years old. Young fish: 22.8 cm, 155 g, 1 year old. 0.1 ppm of quinine sulfate gave a deflection of 100 U. Units for FAP and MDA are U/mg protein and nmol/mg protein, respectively.

1-YEAR-OLD FISH									
9-YEAR-OLD FISH									
Incubation time	Control			Sample			Control		
(h)	FAP	MDA		FAP	MDA		FAP	MDA	Sample
0	8.8±0.3	4.0		8.8±0.3	4.0		8.8±0.3	3.8	8.3±0.3 3.8
1	8.9±0.2	4.0		8.8±0.3	12.7		8.1±0.2	3.8	7.5±0.5 6.8
3	8.9±0.3	4.0		10.5±0.2	19.2		8.0±0.3	3.8	11.3±0.5 10.4
19	8.7±0.2	4.0		12.3±0.6	51.3		8.2±0.2	3.8	10.7±0.5 27.0
32	8.9±0.3	4.0		11.7±0.6	72.0		8.4±0.1	3.8	11.7±0.5 26.2
44	8.8±0.2	4.0		13.8±0.8	72.5		8.2±0.2	3.8	12.5±0.5 26.1

TABLE 14

LIPID COMPOSITION IN SR MEMBRANE IN WINTER FLOUNDER OF VARIOUS AGES

$$\text{Ratio} = \frac{16:0 + 18:0 (\%)}{20:5 + 22:6 (\%)}$$

Sample	Scale	Annuli	16:0	18:0	18:1	20:4	20:5	22:5	22:6	Ratio
(fish caught in winter)										
#1	8	21:1	3.4	3.4	8.9	3.6	26.0	4.9	32.0	0.42
#2	5+	23.0	5.0	5.0	7.4	3.6	24.1	5.5	32.9	0.49
#3	4+	20.1	7.7	7.7	10.8	4.2	22.7	4.4	30.1	0.53
#4	3+	21.7	5.2	5.2	10.3	4.1	23.3	4.5	31.8	0.49
#5	2+	22.2	4.9	4.9	9.8	3.7	23.0	5.0	30.9	0.50
(fish caught in summer)										
#6	5+	21.8	6.9	6.9	12.1	4.2	22.0	6.0	27.2	0.58
#7	5+	23.3	6.2	6.2	10.6	3.6	23.9	3.6	28.7	0.57
#8	5+	19.3	6.2	6.2	8.8	5.3	28.8	5.6	26.2	0.46
#9	3+	21.9	5.2	5.2	10.3	4.1	23.3	4.5	26.0	0.55
#10	2+	20.6	8.2	8.2	12.1	4.6	24.6	5.3	25.8	0.57
#11	2+	18.7	6.9	6.9	9.0	6.6	29.6	6.9	23.6	0.48

Fig 32 Concomitant production of MDA and loss of Ca^{+2} sequestering ability in fish muscle SR in vitro.

Fig. 33 Decrease in fatty acids during lipid oxidation of SR normalized to value of C16:0.

○ -16:0	□ -20:5
▲ -18:0	▼ -22:5
■ -18:1	△ -22:6
● -20:4	

E.6 Inhibition of enzymatic lipid peroxidation

The effect of various compounds added to the incubation medium in order to elucidate microsomal enzymic lipid peroxidation in fish and chicken or turkey muscles are presented.

The inhibitory role of catalase would indicate that H_2O_2 is involved in the reaction while the inhibitory activity of superoxide dismutase would indicate a role for the superoxide ion. Enzymic microsomal lipid peroxidation was inhibited partially by catalase and by superoxide dismutase. Thiourea was an effective inhibitor of membrane lipid peroxidation as were dimethyl sulfoxide, mannitol and benzoate, all of these are hydroxyl free radical scavengers. The lipid free radical nature of the reaction is indicated by the inhibitory effect of propyl gallate (Table 15). In contrast to fish microsomal lipid peroxidation which was determined at $6^{\circ}C$, those of turkey enzymic lipid peroxidation were determined at $37^{\circ}C$. Similar compound added to elucidate fish microsomal lipid peroxidation was also tested in the model containing turkey microsomes. Catalase and superoxide dismutase were not found to inhibit enzymatic lipid peroxidation in turkey. Hydroxyl radical scavengers were also not found to inhibit enzymatic lipid peroxidation in turkey (Table 16). The only compound which was found to inhibit this reaction in turkey was caeruloplasmin, an enzyme which catalyzed the oxidation of ferrous ions to ferric ion. Caeruloplasmin work also as iron chelators (Table 17). Several other iron chelators were found to inhibit the reaction such as EDTA, desferrioxamine, thiourea. Lipid peroxidation was inhibited also by several primary antioxidants such as BHT and ascorbyl palmitate (Figs. 35, 36, 37).

Fig. 34 Increase in high molecular weight protein fraction (Peak A) and decrease in $\text{Ca}^{+2}, \text{Mg}^{+2}$ -ATPase protein as a function of time of oxidation.

Fig. 35 The effect of EDTA concentration on the activity of turkey muscle enzymic microsomal lipid peroxidation. $\square$, after 10 min; $\circ$, after 20 min.

Table 15 Inhibitors of microsomal lipid peroxidation

Compound	% of control, no additive
catalase, 500 units/ml	64
superoxide dismutase, 300 units/ml	11
thiourea, 20 mM	55
diphenylfuran, 0.2 mM	0
propyl gallate, 0.02%	0
Na tripolyphosphate, 0.2 mM	0
p-hydroxymercuribenzoate, 1.0 mM	0
reduced glutathione, 0.2 mM	132

Table 16. The effect of hydroxyl radical scavengers on enzymic
membranal lipid peroxidation. Conditions, see Fig. 2.

Treatment	mM	TBA-RS nmole	activity %
Control	-	28.5±0.16	100.0
Ethanol	400	35.5±0.31	124.5
Salicylic acid	10	40.0±0.28	140.5
Sodium formate	50	29.8±0.23	104.4
Methional	1	28.9±0.28	136.6
Mannitol	100	38.7±0.27	135.7
DMSO	200	32.4±0.25	113.8
Histidine	25	34.1±0.29	119.8

Table 17. The effect of several enzymes on the enzymic membranal lipid peroxidation. Conditions, see Fig. 2.

Treatment	Units of activity	TBA-RS nmole/mg protein	Activity %
Control	-	24.0±0.21	100.0
BSA	0.05%	18.0±0.13	78.4
Catalase	100	24.5±0.27	102.0
Catalase	300	27.3±0.17	113.6
Catalase	1000	20.7±0.06	86.1
SOD	300	30.8±0.23	128.4
Catalase with SOD	100)	31.6±0.16	
"	300)		
Caeruloplasmin	1	0	0
"	10	0	0

Fig. 36 The effect of BHT concentration on the activity of turkey muscle enzymic microsomal lipid peroxidation. ▲, after 10 min. ◻, after 20 min.

Fig. 37 The effect of ascorbyl palmitate concentration on the activity of turkey muscle enzymic microsomal lipid peroxidation. ◻, after 10 min; ○, after 20 min.

E.7 Non-enzymatic lipid peroxidation

Accumulation of H_2O_2 in muscle tissues

Hydrogen peroxide and the superoxide radical are normally present at low concentration as metabolites in aerobic cells. Hydrogen peroxide was determined by the peroxidative activity of catalase which is based on the presence of a specific electron donor compound to catalase compound I. Formate is a substrate which donates two electrons and decomposes to CO_2 by the following reaction

Formate oxidation by the peroxidative activity of catalase was found to be high at low H_2O_2 concentration. At high concentration of H_2O_2 the catalatic activity of catalase started to dominate and formate oxidation decreased. Figure 38 shows that high levels of H_2O_2 are competitive to the interaction of compound I with formate. The generation of H_2O_2 in muscle tissues was tested by using formate(^{14}C) oxidation to $^{14}CO_2$.

The incubation of ground muscle tissues

The incubation of ground muscle tissues led to the generation of 14.0 nmol of H_2O_2 /g of fresh wt/30 min. The oxidation of formate in this homogenate seemed to be activated by endogenic catalase. In the presence of added catalase, muscle homogenate generated almost 26.6 nmol of H_2O_2 /g of fresh wt/30 min, in a 4-mL reaction mixture. This amount is equal to 5.3 μM H_2O_2 in the reaction mixture. The generation of H_2O_2 for the first minute was 0.3 μM . As the reaction mixture contained 4 mL of aqueous solution in the presence of 1 g of muscle tissues, the rise in H_2O_2 concentration in situ of 1 g of muscle tissues was almost 5-fold

Figure 38. Formate peroxidation by catalase as affected by H₂O₂ concentration. Catalase (425 U) in the presence of formate Na 1 mM contained 0.5 μCi (¹⁴C) in buffer acetate, 0.05 M, pH 5.6, and was incubated at 37 °C. The reaction was initiated by various concentrations of H₂O₂ and stopped after 1 min by the addition of 0.5 mL of acetic acid 1 N. ¹⁴CO₂ was collected and determined as described in Materials and Methods.

Table 18 Production of H₂O₂ by Ground Muscle Tissues Incubated at 37 °C, pH 5.6

treatment	production of H ₂ O ₂ nmol/g of fresh wt/30 min
ground dark muscle	14.0
ground dark muscle plus catalase (425 U)	26.6
ground dark muscle plus sodium azide (1 mM)	3.8
ground dark muscle plus catalase plus azide	3.8

higher, about $1.5 \mu\text{M H}_2\text{O}_2$. From Figure 39, it is possible to see that catalase (425 U) reacts with formate until $3\text{--}4 \mu\text{M H}_2\text{O}_2$ at a stoichiometric rate of 1:1. We found also that in a model system glucose oxidase, which generated $1 \mu\text{M H}_2\text{O}_2/\text{min}$, could activate metmyoglobin for a significant membranal lipid peroxidation (Harel and Kanner, 1985). Azide (1 mM) inhibited almost 85% of the formate oxidation (Table 18). The significant increase in formate oxidation by the addition of catalase indicates clearly that this oxidation is activated by the presence of H_2O_2 .

Hydrogen peroxide accumulated in dark muscle homogenate at a rate of 1.5 nmol/min/g of fresh wt (for the first minute) at 37°C and pH 6.5. The generation of H_2O_2 decreased significantly after 30 min of incubation. Similar results were obtained by the incubation of light muscle tissue under the same conditions.

The incubation of dark muscle homogenate at 60°C increased the rate of H_2O_2 generation. Azide inhibition of formate oxidation at high temperature showed that the oxidation of formate is produced by catalase and not affected by the heating itself (Fig. 39).

Increasing the incubation temperature to 100°C permitted the accumulation of 24 nmol/g of fresh wt of H_2O_2 for the first 10 min, but after this time catalase was completely inhibited (results not shown) and this also inhibited formate oxidation. Heating of (^{14}C)formate does not elaborate $^{14}\text{CO}_2$.

Muscle tissues were aging at 4°C for a period of 5 days, and the generation of H_2O_2 was determined. The results in Figure 40 show an increase in H_2O_2 production of almost 2-3-fold during a storage of 5 days.

E.8 Muscle membranal lipid peroxidation initiated by H_2O_2 -activated metmyoglobin

The nature of the reactive heme-oxygen species complexes differs depending on their oxidation states. Methemoglobin (MetHb) and Metmyo-

Figure 39. Generation of H_2O_2 by dark muscle incubated at 37 and 60 °C in the presence of added catalase (425 U): ↓, cooled to 37 °C and catalase added once more; □, in the presence of added catalase and sodium azide (1 mM).

Figure 40 The effect of storage time on H_2O_2 generation by incubated dark muscle at 37 °C: □, without added catalase; ▨, with added catalase.

globin (MetMb) in the presence of H_2O_2 form a complex which absorbs near 545 nm. Spectrophotometric determination of this compound in our model system showed a maximum concentration after 3 min of incubation at 25°C (Fig. 41). The specific spectra of activated Metmyoglobin changed rapidly with the addition of ascorbic acid. Simultaneously, ascorbic acid was oxidized (results not shown).

Of great interest is the finding that activated MetMb could initiate membranial lipid peroxidation. Lipid peroxidation was determined by accumulation of malondialdehyde (MDA) and conjugated dienes. The results from both methods show a significant increase in oxidized lipids. Lipid peroxidation by the activated MetMb was not inhibited by catalase (Table 19). We found that MetMb could also bleach β -carotene (Fig. 42), and this could be inhibited by ascorbic acid (results not shown).

The peroxidation of the unsaturated fatty acids and the destruction of β -carotene in the presence of activated MetMb indicates a possible attack on one of the double bonds, or on an allylic hydrogen and the production of a hydroperoxide. The abstraction of an electron from this position changes the specific absorption of β -carotene.

Our work shows that activated MetMb also has the ability to produce ethylene from methional. The conversion of methional to ethylene was dependent on the amount of H_2O_2 and gave an excellent yield of 70-80% (Fig. 43). Using the same technique, Pryor and Tang reported yields of 42% and 17% for $RO\cdot$ (alkoxyl) and $\cdot OH$ (hydroxyl) radicals, respectively.

The generation of ethylene from methional originally was proposed as an assay for hydroxyl radicals. However, Pryor and Tang showed that a variety of organic radicals lead to the production of ethylene from methional.

FIG 41 Spectra changes of metmyoglobin (30 μ M) incubated in the presence of H_2O_2 (30 μ M) and after the addition of ascorbic acid (100 μ M).

FIG 43. The effect of hydrogen peroxide concentration on the generation of ethylene from methional by activated metmyoglobin (30 μ M). Reaction mixture contained buffer phosphate (1 mM), EDTA (1 mM) at pH 7.0 and 25 C. Control H_2O_2 (60 μ M) without the addition of metmyoglobin = 34 nmole ethylene.

FIG 42. Carotene oxidation by activated metmyoglobin. A, metmyoglobin (30 μ M); B, H_2O_2 (30 μ M); C, metmyoglobin (30 μ M) and H_2O_2 (30 μ M) incubated for 3 min before addition.

FIG 44. Formate oxidation by catalase and metmyoglobin affected by H_2O_2 concentration. $\circ$, catalase; $\square$, metmyoglobin. Reaction mixture contained catalase (425 U) or metmyoglobin (30 μ M), formate-Na 1 mM contained 0.5 μ Cl (14 C) in buffer acetate (0.05 M) pH 6.5, for 10 min at 37 C.

Of all the molecules tested by us, only sodium formate was stable and not decomposed in the presence of activated MetMb. Formate (^{14}C) oxidation to $\text{CO}_2(^{14}\text{C})$ by the peroxidative activity of catalase in the presence of low H_2O_2 concentration was determined as a positive control treatment (Fig. 44). As is well known, formate could be oxidized to CO_2 by catalase compound I or by hydroxyl radicals. The redox potential of hydroxyl radical at pH 7.0 ($\cdot\text{OH}/\text{H}_2\text{O}$) is about +2.3 V, high enough to oxidize almost all bio-molecules; however, activated MetMb was found to have a redox potential of only +1.4 V.

Metmyoglobin activated by H_2O_2 initiated membranal lipid peroxidation, whereas no oxidation occurred in the presence of MetMb or H_2O_2 alone.

The interaction of H_2O_2 with MetMb led very rapidly to the production of an activated species which initiated lipid peroxidation. The activity of this catalyst as a prooxidant decreased rapidly during the first 5 min, and within 10 min was down to 50% of the fresh preparation. However, this activity remained very stable for a long period of time (Fig. 45). Small amounts of H_2O_2 could activate MetMb for significant lipid peroxidation. Figure 46 shows a linear increase in the rate of lipid peroxidation by elevation of MetMb of H_2O_2 concentration to approximately 30 μM . Increasing MetMb concentration (keeping H_2O_2 at 30 μM) beyond 120 μM was found to decrease the rate of lipid peroxidation. A much slower decline was obtained when H_2O_2 concentration was elevated (keeping MetMb at 30 μM) beyond 200 μM . Analogous experiments were performed with MetHb (Fig. 47). Here, the maximum was reached at 5-7 μM , with a much more rapid decline with further increase of MetHb concentration. As the MetHb molecule contains four heme groups, it seems that the prooxidant effect is obtained when the ratio between heme groups and H_2O_2

FIG 45 The effect of time of incubation of metmyoglobin (30 μ M) in the presence of H₂O₂ (30 μ M), on the initiation of membranal lipid peroxidation. $\circ$, MetMb - H₂O₂; $\square$, MetMb or H₂O₂ alone.

FIG 47 Membranal lipid peroxidation by activated methemoglobin with H₂O₂ (30 μ M).

FIG.46, The effect of metmyoglobin and H₂O₂ concentration on membranal lipid peroxidation. $\circ$, H₂O₂ (30 μ M) in the presence of different MetMb concentrations; $\triangle$, MetMb (30 μ M) in the presence of different amounts of H₂O₂. (The mixtures were preincubated for 3 min before addition to microsomes.)

Fig. 48 Membranal lipid peroxidation by activated MetHb and MetMb as affected by pH. (Reaction mixture described in Materials and Methods, acetate and phosphate buffers, 0.05 M).

is almost 1:1. Lipid peroxidation by activated MetMb + H₂O₂ (30 µM of each) was found to be dependent on membrane concentration, the rate increasing linearly up to 1.5 mg protein/ml (results not shown).

The pH activity curve of activated myoglobin shows a maximal lipid peroxidation at pH 6.5 and a sharp drop in activity at pH 5.0. (Fig. 48). Methemoglobin activated by H₂O₂ shows a maximal lipid peroxidation at pH 7.3. Both compounds were found to show maximum activity similar to that of the pH in situ of muscle tissues (6.5) and blood (7.3). Arrhenius plots for the rate of MDA accumulation by activated MetMb during 30 min as a function of temperature are linear between 37 and 4°C. The apparent activation energy for lipid oxidation by activated MetMb was 25.1 kcal/mol.

Oxygen absorption increased during incubation of the microsomal membrane with activated MetMb. No oxygen was found to be absorbed in the presence of the membrane with MetMb or H₂O₂ alone. Maximum oxygen absorption was found during incubation of activated MetMb with 30-40 µM H₂O₂ (Fig. 49). Similar results were obtained when determining MDA accumulation during incubation of sarcosomes with MetMb (30 µM) and 30-40 µM H₂O₂ (Fig. 46).

The difference between the amount of O₂ uptake and MDA accumulation arose since not all the unsaturated fatty acids hydroperoxides produce, by breakdown, thiobarbituric-acid-reactive substances.

We have also activated MetMb by glucose oxidase/glucose system which generates H₂O₂ continuously.

The glucose oxidase system was adjusted for H₂O₂ generation of 1 nmol/mL/min (results not shown). Initiation of membranal lipid peroxidation occurred only in the presence of both the glucose oxidase system

and MetMb, but not with only one of either compounds. Catalase (40 units) very effectively inhibited the activation of MetMb and the initiation of lipid peroxidation (Fig. 50). Lipid peroxidation was initiated also by activation of MetMb (30 M) with H_2O_2 (30 M) for 3 min before the addition to the membranal suspension.

Lipid peroxidation of turkey and chicken (dark and light) muscle microsomes, initiated by activated MetMb, is shown in Figures 51 and 52. Thiobarbituric acid reactive substances accumulated and oxygen was absorbed at higher rates by the turkey than by the chicken muscle. Dark muscle microsomal lipids peroxidized faster than light muscle microsomal lipids. Heated MetMb maintained its capacity to be activated by H_2O_2 (Table 20).

The relative activity of several H_2O_2 -activated hemoproteins toward membranal lipid peroxidation is presented (Table 21). Cytochrome C which contains iron chelated to all six coordinations does not initiate membranal lipid peroxidation in the presence of H_2O_2 . The effect of H_2O_2 -activated MetMb on sarcoplasmic lipid peroxidation from flounder fish is described in Table 22.

E.9 Muscle membranal lipid peroxidation initiated by an "iron redox cycle" system

Ascorbic acid is a strong two-electron reducing agent, which is readily oxidized in one electron steps by metal ions and metal complexes in their higher valence states.

The incubation of turkey muscle membranes with ascorbic acid in the presence of iron initiates very rapidly membranal lipid peroxidation.

Lipid peroxidation was determined by the accumulation of thiobarbituric acid reactive compounds (TBA-RS as malondialdehyde) and conjugated

FIG.49 Oxygen absorption during membranal lipid peroxidation of microsomes by metmyoglobin (30 μ M) with different concentrations of H_2O_2 . O, MetMb (30 μ M) after 3 min incubation with H_2O_2 ; Δ , H_2O_2 alone.

Figure 50 Effect of glucose oxidase system (GOxS) on membranal lipid peroxidation by activated MetMb. The reaction mixture contained glucose oxidase, 0.04 units, glucose, 400 μ M, microsomes, 1 mg protein, KCl (0.12 M), EDTA (0.1 mM), and MetMb (30 μ M) in 1 mL of histidine buffer (5mM), pH 7.3, at 37 $^{\circ}$ C. Error bars denote standard deviation ($n = 3$): O, MetMb + GOxS; O, MetMb + catalase (40 units) and GOxS; Δ , MetMb or GOxS alone.

Figure 51 Turkey and chicken (from dark or light muscles) microsomal lipid peroxidation initiated by activated MetMb/H₂O₂ (30 μ M each). The reaction mixture contained microsomes, 1 mg protein, KCl (0.12 M), and EDTA (0.1 mM) in 1 mL of histidine buffer (5 mM), pH 7.3, and was incubated at 37 °C.

TABLE 19

Microsomal Lipid Peroxidation Initiated by Activated MetMb^a

Treatments	Malondialdehyde nmole/mg protein/30 min	Conjugated dienes A 233 nm/30 min
Microsomes alone	0	0.16
Microsomes + H ₂ O ₂ ^b	0	0.15
Microsomes + MetMb ^b	5.4 $\pm$ 2.7	0.16
Microsomes + MetMb/H ₂ O ₂ ^c	35.0 $\pm$ 2.4	0.31
Microsomes + MetMb + H ₂ O ₂	32.4 $\pm$ 3.2	0.30
Microsomes + catalase + MetMb/H ₂ O ₂	6.5 $\pm$ 3.0	0.15
Microsomes + catalase + MetMb + H ₂ O ₂	32.0 $\pm$ 1.8	0.28

^aMetMb + H₂O₂ the same conc. incubated for 3 min before addition.

^bH₂O₂ or MetMb at a conc. of 30 μ M each.

^cMetMb/H₂O₂ the same conc. not incubated for 3 min.

Figure 52. Oxygen absorption during membranous lipid peroxidation by activated MetMb/H₂O₂ (30 μ M each). The reaction mixture (see Figure 2) was incubated at 37 °C.

Table 20 Effect of Heating on Membranous Lipid Peroxidation Initiated by Activated MetMb^a

treatment	TBA-RS as MDA, nmol/30 min	activity, %
control	10.8	100
MetMb heated for 1 min	9.2	85
MetMb heated for 3 min	8.6	80
MetMb heated for 5 min	8.6	80

^a MetMb (30 μ M) heated for several minutes at 98 °C and cooled before the interaction with H₂O₂ (30 μ M) for 3 min.

Table 21 INITIATION OF MICROSOMAL LIPID PEROXIDATION BY H₂O₂ ACTIVATED HAEMO-PROTEINS

Compound	TBA-RS as MDA nmole/mg protein/30 min
Metmyoglobin	15.0 $\pm$ 0.8
Methaemoglobin	8.6 $\pm$ 0.5
Peroxidase (HR)	3.8 $\pm$ 0.8
Lactoperoxidase	2.5 $\pm$ 0.6
Hemin	2.3 $\pm$ 0.4
Cytochrome C	0 -

dienes. A high correlation was achieved between the accumulation of TBA-RS and conjugated dienes. The amount of TBA-RS and conjugated dienes that accumulated in the model system containing ferric chloride and microsomes was found to be dependent on the concentration of ascorbic acid. Maximal TBA-RS was accumulated at a concentration of 200 μ M ascorbate. A high concentration of either compound inhibits the production of TBA-RS and conjugated dienes (Fig. 53).

The characteristics of cysteine-activated peroxidation of the fish microsomes are shown in Figures 54 and 55. Although cysteine probably takes part in the non-enzymic catalyzed reaction, the data indicate that some of the effect of cysteine is on the enzyme-catalyzed reaction since heating destroys much of this activity. NADH stimulates lipid peroxidation over and above that which is caused by cysteine. On the other hand, the addition of ADP which stimulates enzymic oxidation completely eliminates the stimulation caused by cysteine. It is well known that cysteine in the presence of Fe causes a rapid breakdown of LOOH. Our data indicate that this compound also increases the rate of formation of the lipid hydroperoxides (Fig. 55).

Ascorbate catalyzes a non-enzymic oxidation of the membrane lipids. A typical experiment is shown in Figure 56. Unlike the results with cysteine, the addition of NADH or ADP has no significant effect on the rate of the reaction. We interpret these data to indicate that either the ascorbate or the NADH can maintain the iron in a reduced state, and that with the concentrations used in these experiments, either was sufficient by itself to do so. The addition of the second component had no added effect.

Table 22 Effect of H_2O_2 and myoglobin on lipid oxidation of sarcoplasmic reticulum from flounder (Pseudopleuronectes americanus)

H_2O_2 Mb		Standard cofactors ‡	nmol MDA per mg protein - 30 min
uM			
24°			
0	0	-	0
0	0	+	89
50	50	-	71
200	200	-	47
*50	50	-	67
6°			
0	0	+	44
50	50	+	12
50	50	-	17
*50	50	-	17
* 0	0	+	1

‡Standard cofactor concentrations were NADH, 0.1 mM; ADP, 0.1 mM; $FeCl_3$, 0.015 mM.

*Microsomal fractions were heated for 5 min in boiling water before incubation.

The pH of the reaction medium was 7.3, and 0.1 mg of sarcoplasmic reticular protein per ml of reaction medium was used.

Figure 53 Membrane lipid peroxidation as affected by ascorbate concentration (Fe^{3+} constant—10 μ M).

E.10 Inhibition of membranal non-enzymic lipid peroxidation

Membranal lipid peroxidation initiated by activated MetMb was inhibited by glutathione, NADPH, salicylic acid, and low concentrations of BHT (Table 23). We found, in our model system, traces of approximately 3-5 μM free iron. Ascorbic acid in the absence of MetMb promoted lipid peroxidation. EDTA and desferrioxamine inhibited this initiation. It is well known that reducing agents in the presence of trace amounts of transition metals act as prooxidants. Several hydroxyl radical scavengers were also tested as potential inhibitors of activated MetMb, including ethanol, DMSO, formate, benzoate, and histidine, but they failed to inhibit the reaction. This experiment consisted of sarcosomes incubated with activated MetMb in KCl (without histidine), titrated to pH 7.0 (Table 24).

The addition of catalase to MetMb solution before activation with H_2O_2 eliminated the activation of MetMb by H_2O_2 ; however, if catalase was added 3 min after the interaction between MetMb and H_2O_2 , no inhibition occurred.

Activated MetMb dependent lipid peroxidation was inhibited by several antioxidants. Ascorbic acid at a low concentration enhanced lipid peroxidation but inhibited at a very high concentration.

The prooxidative effect of ascorbic acid was eliminated by the addition of a very low concentration of EDTA (Fig. 57). In the presence of 10 μM EDTA, 10 μM ascorbic acid inhibited the initiation of lipid peroxidation by activated MetMb. Activated MetMb dependent lipid peroxidation was not inhibited by a low concentration of EDTA (10-100 μM) but 50% inhibition was obtained with 10 mM EDTA (Fig. 58).

Membranal lipid peroxidation initiated by activated MetMb was partially inhibited by a very low concentration of either ascorbyl palmitate,

Fig. 54 Characteristics of cysteine-catalyzed lipid oxidation. The reaction medium contained 0.2 mM cysteine, 0.015 mM FeCl_3 , 0.1 mM NADH as indicated, 0.1 mM ADP where indicated and 0.5 mg protein per ml in 0.12 M KCl, 5 mM histidine buffer, pH 7.1 at 6°C. The components in the system labeled "heated control" were identical to the control except that the microsomes were heated at 85°C for 5 min prior to incubation.

Fig. 55 Effect of cysteine on the production of lipid hydroperoxides during oxidation of flounder muscle microsomes. The assay medium of the control system contained 0.015 mM FeCl_3 , 0.1 mM NADH, 0.1 mM ADP and 0.5 mg protein per ml in 0.12 M KCl, 5 mM histidine buffer, pH 7.1 at 6°C. The system designated by the dotted line was the same as the control but also contained 2 mM cysteine.

Fig. 56 Characteristics of ascorbic acid catalyzed lipid oxidation. The reaction medium contained 0.2 mM ascorbate, 0.015 FeCl_3 , 0.1 mM NADH where indicated, 0.1 mM ADP as indicated and 0.5 mg protein per ml in 0.12 M KCl, 5 mM histidine buffer, pH 7.1 at 6°C.

TABLE 23

THE EFFECT OF SEVERAL REDUCING COMPOUNDS ON
MEMBRANAL LIPID PEROXIDATION
BY ACTIVATED MetMb^a

Treatment	MDA (nmol/30 min)	
	-EDTA	+EDTA ^c
Control	17.5 (100) ^b	17.3 (98)
Ascorbic acid (200 μM)	21.5 (122)	1.2 (6)
Cysteine (200 μM)	17.5 (100)	1.5 (8)
Glutathione (200 μM)	11.5 (65)	1.4 (8)
NADPH (200 μM)	5.0 (28)	1.0 (5)
Salicylic acid (10 μM)	9.5 (54)	—
BHT (50 μM)	0.0 (0)	—

^a MetMb/ H_2O_2 (30 μM each) incubated for 3 min before addition to microsomes (1 mg protein/ml).

^b Activity, %.

^c EDTA, 30 μM .

Figure 57 Membranal lipid peroxidation initiated by activated MetMb/H₂O₂ (30 μM each) as affected by ascorbic acid (○) and ascorbic acid in the presence of EDTA (10 μM) (○). The reaction mixture (see Figure 2) was incubated at 37 °C.

Figure 58 Membranal lipid peroxidation by activated MetMb/H₂O₂ (30 μM each) as affected by EDTA concentration. The reaction mixture (see Figure 2) was incubated at 37 °C. Error bars denote standard deviation (n = 3).

TABLE 24
THE EFFECT OF HYDROXYL RADICAL SCAVENGERS ON
MEMBRANAL LIPID PEROXIDATION
BY ACTIVATED MetMb^a

Treatment	MDA (nmol/30 min)	Activity (%)
Control	17.7	100
Ethanol (400 mM)	18.8	107
DMSO (200 mM)	17.8	101
Benzoate (10 mM)	16.5	94
Formate (10 mM)	20.0	114
Histidine (5 mM)	17.5	98
Catalase ^b (40 U)	17.0	96
Catalase ^c (40 U)	0	0
Bovine serum albumin (0.5 mg/ml)	17.5	98

^a MetMb/H₂O₂ (30 μM each) incubated for 3 min before addition to microsomes (1 mg protein/ml) in 0.12 M KCl (without histidine) titrated to pH 7.0.

^b Catalase added to sarcosomes before activated MetMb.

^c Catalase added to sarcosomes and MetMb before the addition of H₂O₂.

Table 25 Effect of Several Antioxidants on Membranal Lipid Peroxidation Initiated by Activated MetMb^a

treatment	TBA-RS as MDA, nmol/30 min	inhi- bition, %
control	11.5	0
BHT 5 μM	8.0	30
ascorbyl palmitate 5 μM	6.9	40
α-tocopherol 5 μM	4.0	65
each antioxidant 15 μM	0	100

^a MetMb/H₂O₂ (30 μM each) preincubated for 3 min before the addition to microsomes (1 mg/mL of protein) in a 1-mL buffer of histidine, pH 7.3, at 37 °C, which contained 0.86 M ethanol. The antioxidants were solubilized in ethanol to a final concentration in the mixture reaction of 0.86 M ethanol.

α -tocopherol, or BHT (Table 25) and completely inhibited by all anti-oxidants at a concentration of 15 μ M.

"Iron redox cycle" initiation was found to be inhibited at very low concentrations of EDTA.

E.11 Fish muscle lipid peroxidation in situ

Total thiobarbituric acid-reactive substances (TBA-RS) were used to measure total lipid peroxidation in fish and poultry tissues. In addition, lipids were extracted using chloroform-methanol procedure of Bligh and Dyer (1959). These lipids were determined for conjugated dienes before and after separation into neutral and polar lipids by silicic acid chromatography. In addition, the fatty acid content of the two fractions were identified by gas chromatographic procedure after saponification and methylation of the fatty acids. Samples stored at -18°C or at 4°C were analyzed at specific intervals.

Some of the preliminary results of the TBARS of the herring flesh are shown in Table 26. The results are not easily interpreted since they do not show a general linear increase with time. Further work will be required to be able to make sound judgements concerning these results. A potential problem in utilizing this method of measurement of lipid oxidation is that the reaction product may be interacting with components of the tissue on prolonged storage. This could account for some of the peaks in the values of TBARS with respect to time. Results with the extracted lipids and the other measurement techniques may give a better indication of which samples are undergoing oxidation. Thus, results obtained by measuring TBARS after the first month or two may be a better indication of what is working. If this were the case, samples containing phospholipase A_2 and/or the nucleotide pyrophosphatase would

Table 26 Production of TBA-reactive substances in minced herring muscle stored at -18°C .

Addition	Relative production of TBARS, O.D.									
	Month	1	2	3	4	5	6	7	8	9
None		0.51	0.54	0.70	1.16	0.71	0.58	0.45	0.56	0.50
TBHQ		0.22	0.56	0.89	0.68	0.72	0.61	0.43	0.56	0.69
NADase		0.47	0.66	0.79	0.99	0.93	0.82	0.78	1.01	0.87
Nucleotide pyrophosphatase (NPPase)		0.32	0.30	0.72	0.40	0.74	0.53	0.46	0.46	0.45
Catalase & superoxide dismutase		0.71	0.67	0.65	0.78	1.24	0.61	0.50	0.81	0.53
Phospholipase A_2 (PLase A_2)		0.22	0.38	0.49	0.56	0.48	0.57	0.66	0.50	0.48
Apyrase		0.44	0.64	0.66	0.70	0.77	0.55	0.60	0.61	0.56
NPPase & PLase A_2		0.26	0.28	0.34	0.40	0.46	0.41	0.41	0.51	0.45
NADase & PLase A_2		0.32	0.32	0.56	0.46	0.60	0.54	0.58	0.51	0.51

The 0 time sample (applicable to all treatments) was 0.25.

Fig. 59 The accumulation of TBA-RS products during storage at 4°C of minced turkey muscles as affected by oxygen concentration.

appear to potentially have some effectiveness. This work will be continued.

The preliminary work shows that minced Atlantic herring (Clupea harengus) during storage at -18°C undergoes lipid peroxidation. The total TBA-RS increase during the first 4 months. During the same time, lipid peroxidation was inhibited by TBHQ, a well-known primary anti-oxidant.

In order to elucidate the mechanism of lipid peroxidation, we included in the minced fish several enzymes. The enzymes added included those capable of destroying NAD (NADase) and nucleotide pyrophosphatase, apyrase to breakdown ATP and ADP, phospholipase A_2 (PLA $_2$) to disrupt membrane integrity, catalase and superoxide dismutase to reduce O_2^- and H_2O_2 , and a combination of some of the above. It was found that only phospholipase A_2 significantly decreased lipid peroxidation.

E.12 Poultry muscle lipid peroxidation in situ

Minced turkey muscle was stored at 2°C in a package with and without air (vacuum). Lipid peroxidation was determined by accumulation of TBA-RS and conjugated dienes. It was found that the TBA-RS increased very rapidly in minced turkey muscle stored in the presence of air. Minced turkey muscles stored in vacuum keep a low TBA-RS, however if the package was opened to air the tissues accumulate rapidly TBA-RS. The same experiment was prepared with heated minced muscles which accumulated much more TBA-RS in packages with air, and still keep a low TBA-RS in vacuum packages (Figs. 59, 60). We obtained the same results using conjugated dienes as a test for lipid peroxidation in situ minced muscles (Fig. 61).

In situ, minced turkey muscles stored in air at 2°C were inhibited by high concentration of ascorbic acid ($\sim 10 \text{ mM}$) or by a concentration

of only 300 μ M ascorbyl palmitate or BHT which act as primary antioxidants. Ascorbic acid at low concentration did not affect the rate of in situ lipid peroxidation in minced or sliced turkey muscles (Fig. 62). The effect of ascorbyl concentration on in situ lipid peroxidation in minced turkey muscle is presented in Figure 63. It was also found that EDTA inhibits very efficiently both raw and heated minced muscle tissues. The inhibition by EDTA was found with a concentration of 200-400 μ M which is 1/10th of that which inhibits membranal lipid peroxidation initiated by H_2O_2 -activated metmyoglobin (Fig. 64).

Fig. 60 The accumulation of TBA-RS products during storage at 4°C of heated minced turkey muscles as affected by oxygen concentration.

Fig. 61 The accumulation of conjugated dienes during storage at 4°C of heated minced turkey muscles as affected by oxygen concentration.

Fig. 62 The accumulation of TBA-RS products during storage at 4°C of turkey muscle as affected by the addition of ascorbic acid and ascorbyl palmitate and BHT. A, minced muscles; B, sliced muscles; O, control; O, 3 mM ascorbic acid; Δ, 300 μM ascorbyl palmitate; Δ, 300 μM BHT.

Fig. 63 The effect of ascorbyl palmitate concentration on the TBA-RS and conjugated dienes accumulation in minced turkey muscles. 0, control; ○, 150 μM ascorbyl palmitate; □, 300 μM ascorbyl palmitate; Δ, 600 μM ascorbyl palmitate; ▽, 300 μM BHT.

Fig. 64 The effect of EDTA (400 μ M) on the accumulation of TBA-RS as MDA during storage of minced (raw and heated) turkey muscles at 4°C.

Fig. 65 The effect of EDTA (400 μ M) on the accumulation of conjugated dienes during storage of minced raw turkey muscles at 4 $^{\circ}$ C.

F. DISCUSSION

Lipid peroxidation in fatty fish and poultry, especially turkey, is one of the more important factors responsible for quality loss of both refrigerated and frozen products.

The discovery of an NADPH-dependent lipid peroxidation system in rat liver microsomes (Hochstein and Ernster, 1963) has stimulated numerous researchers to study this topic. The system is characterized by a requirement of an iron source and stimulation by ADP, which chelates the iron, making it more available for the reaction. Initially it was thought that the peroxidation system was absent in muscle microsomes until an NADPH-dependent lipid peroxidation system in chicken muscle microsomes was described (Player and Hultin, 1977). A similar lipid-peroxidation system was found in fish muscle which was more active with NADH than NADPH.

Lipid peroxidation by microsomes isolated from skeletal muscle of herring required NADPH, which when replaced with NADH reduced the activity by >90% in both the light and dark microsomes. Microsomes isolated from red hake and winter flounder also showed preferences for NADH, with minimal activity detected when NADPH was used.

However, microsomes isolated from skeletal muscle of turkey required NADPH and when replaced with NADH the activity decreased by 60%. Membranal lipid peroxidation was affected by NADH and NADPH concentration. Increasing the concentration of these compounds until 0.3-0.4 mM enhanced the reaction, however higher concentration inhibited lipid peroxidation. We assume that at high concentration, both compounds act as electron donors for lipid free radicals, and by this inhibit the chain reaction. Enzymic lipid peroxidation with fish microsomes was found

to be more sensitive to nucleotide concentration than those of turkey microsomes. High concentration of ADP (1.0 mM) inhibited fish microsomal lipid peroxidation, but did not affect significantly turkey microsomal lipid peroxidation.

The addition of ferrous ions gives greater lipid peroxidation than ferric ions, particularly at low concentration. It appears that saturation is reached at rather low concentrations of Fe^{+2} which is not observed with Fe^{+3} . Similar results were obtained with both species - fish and poultry. Lipid peroxidation initiated by enzymic activity were found to be dependent on pH, showing maximum activity similar to that of the pH in situ of muscle tissues. The energy of activation of peroxidation was similar for both light (4.7 kcal per mole) and dark (4.2 kcal per mole) muscle microsomes from herring. In comparison, the energy of activation were higher for lipid peroxidation of microsomes obtained from red hake or turkey.

Our results demonstrated that the higher lipid peroxidation of dark turkey or chicken microsomes compared with those of light microsomes seems to derive from a higher cytochrome P 450 reductase activity.

The content of the principal fatty acids in flounder muscle, which does not carry storage lipids and those of herring was compared. The amount of the lipid in herring was almost 7-fold higher than that in flounder. As is commonly found in fish and other muscle tissues, the membrane lipids had considerably more polyunsaturated fatty acids than did the total storage lipids which are made up of the greatest part of the triacylglycerols.

The content of the principal fatty acids in turkey microsomes showed a high concentration of linoleic acid and relatively low con-

concentration of arachidonic (20:4) and docosahexaenoic acid (22:6).

In both species, arachidonic acid and docosahexaenoic were more susceptible to oxidation. During storage of minced herring muscle in -20°C , the ratio of the highly polyunsaturated C22:6 to the C16:0 decrease in the isolated microsomal fraction. On the other hand, the ratio of C22:6 to C16:0 for the total tissue lipids did not change over the 8-month storage period. This indicates that the microsomal lipids oxidize more rapidly than the total lipids of the tissue.

In flounder, which is a lean fish, almost the same decrease was found in the C22:6 to C16:0 in the microsomal fraction and in the other tissue lipids. These results indicate that in flounder seems to be other membranes which undergo oxidation at a rapid rate.

Minced turkey muscles stored at 4°C undergo lipid oxidation, as was determined by TBA-RS. This oxidation was inhibited by EDTA.

From the same minced turkey muscles, we isolated microsomes which were determined for changes on lipid conjugated dienes. The changes in conjugated dienes were very similar with that of the TBARS in total tissues and were also inhibited by EDTA.

In an attempt to elucidate the role of the peroxidizing microsomal fraction in fish lipid oxidation, a model system utilizing the microsomal fraction along with emulsified lipids, was tested. The results demonstrated that lipid peroxidation in the microsomal fraction could cause oxidation in the lipid emulsion. The amount of oxidation was much greater than that with the microsomal fraction alone. Since there was no oxidation in the system when the microsomal fraction was not undergoing the enzyme-catalyzed oxidation of microsomal lipids, it was concluded that these changes are completely enzymic-dependent.

Preliminary evidence indicates that natural inhibitors and activators are present in the cytosolic fraction of muscle cell; however, we do not have any results which identify them.

The enzymic-catalyzed lipid peroxidation system of fish and poultry skeletal muscle microsomes has been characterized functionally in vitro. Based on our observation with fish model system, we suggest that during the enzymic reaction oxygen is activated by ADP-Fe^{+2} producing superoxide (O_2^-) which dismutates to hydrogen peroxide (H_2O_2). Hydrogen peroxide is decomposed by reduced iron producing the hydroxyl free radical. This radical reacts with a lipid molecule (LH) to produce lipid free radicals, and lipid hydroperoxides (LOOH). The LOOH may then breakdown in the presence of ADP-Fe^{+2} to free radicals, which would keep the chain reaction.

In contrast with the results of fish model systems, the results with turkey model systems demonstrated no accumulation of free superoxide or hydrogen peroxide in the system. Catalase and superoxide dismutase did not inhibit turkey microsomal lipid peroxidation. Likewise, hydroxyl radical scavengers such as formate, mannitol or DMSO did not inhibit enzymic membranal lipid peroxidation. Turkey enzymic membranal lipid peroxidation was inhibited only by caeruloplasmin (ferroxidase I), EDTA and several primary antioxidants. It seems to us that in turkey microsomal model systems membranal lipid peroxidation derived by the activation of iron to a ferryl ion which initiates lipid peroxidation. Our results fitted well with those supported by the Aust group on the initiation of liver microsomal lipid peroxidation (Tien and Aust, 1982).

Hydrogen peroxide and the superoxide radical are normally present at low concentration as metabolites in aerobic cells (Ramasarma, 1982).

The amount of superoxide radical, O_2^- , and hydrogen peroxide, H_2O_2 , is maintained at approximately 10^{-12} - 10^{-11} M and 10^{-9} - 10^{-7} M, respectively, by superoxide anion dismutase and glutathion peroxidase (Oshino et al., 1973; Tyler, 1975).

Mitochondria (Loschen et al., 1971; Boveris et al., 1972), microsomes (Thurman et al., 1972; Hildebrandt and Roots, 1975), peroxisomes (Boveris et al., 1972) and cytosolic enzymes (Chance et al., 1979) have all been recognized as effective H_2O_2 generators when fully supplemented by their substrates. Phagocytic leukocytes during phagocytosis generate large amounts of O_2^- and H_2O_2 which are released into the medium surrounding the phagocytes (Root et al., 1975).

Hydrogen peroxide is generated also by non-enzymatic reactions, such as oxidation of oxymyoglobin, oxyhemoglobin and several redox compounds in the presence of transition metals.

The incubation of muscle tissues at $37^{\circ}C$ shows H_2O_2 generation of 14 nmole/g fresh weight/30 min. In the presence of added catalase, which increases the sensitivity of the method toward H_2O_2 , we found that muscle tissues generate almost 26 nmole of H_2O_2 under the same conditions. Muscle tissues aging at $4^{\circ}C$ increase the generation of H_2O_2 .

Hydroxyl radicals also oxidize formate, however we had increased formate oxidation by about 2-fold with the addition of exogenous catalase. These results indicate that most of the formate oxidation is via H_2O_2 /catalase complex (compound I) and not by $HO\cdot$ radicals. We assume that endogenous generation of H_2O_2 plays an important role in the formation of the primary pool of biological catalysts in situ in muscle tissues.

More recently, it was first discovered by us that the interaction of hydrogen peroxide with metmyoglobin (MetMb) or methemoglobin (MetHb)

very rapidly led to the generation of active species which promote membranal lipid peroxidation (Kanner and Harel, 1985). Activated MetMb could be described as true initiators of lipid peroxidation.

Peroxidation of PUFA is a naturally occurring free-radical chain of reaction that has been implicated as a mechanism of tissue damage (Pryor, 1980). The exact nature of the predominant forms of iron catalysts in muscle tissues which are involved in the initiation of lipid peroxidation in situ is lacking (Love, 1983). Scientists often refer to added metal salts and their complexes as "initiators" of lipid peroxidation. However, in most cases, the salts cause the decomposition of preformed lipid hydroperoxides. All commercially available unsaturated fatty acids contain traces of hydroperoxides (Halliwell and Gutteridge, 1984), thus most of the reports on the oxidation of lipids in model muscle foods studied the propagation stage. Nonenzymic catalysts for the propagation of lipid oxidation in muscle tissues have been studied and reviewed by many researchers (Pearson et al., 1977; Love, 1983; Khayat and Schwall, 1983). Hultin and co-workers (Lin and Hultin, 1976; Shewfelt and Hultin, 1983) developed a muscle model for the determination of lipid peroxidation initiated by an enzymic microsomal NAD-(P)H dependent system, in which lipid peroxidation was initiated by iron salts. Our muscle model system for the initiation of lipid peroxidation contains endogenous compounds found in muscle tissues, such as membranal lipids, myoglobin and H_2O_2 .

Hemoglobin, and especially myoglobin, are very abundant in muscle tissues (Livingston and Brown, 1981) and play essential roles in maintaining aerobic metabolism. During cutting, chopping, or grinding, oxyhemoglobin and oxymyoglobin are oxidized. The autooxidation of oxyhemo-

globin and oxymyoglobin leads to the formation of MetMb or MetHb and O_2^- which dismutate to H_2O_2 (Brown and Meline, 1969; Misra and Fridovich, 1972; Satoh and Shikama, 1981; Wallace et al., 1982). Oxymyoglobin, which generates H_2O_2 during autooxidation, could activate its own molecule; however, endogenous H_2O_2 could be generated in muscle tissues also by other systems (Harel and Kanner, 1985).

The interaction of MetMb with H_2O_2 was reported to produce a free radical (King and Winfield, 1963) and later confirmed (Yonetani and Schleyer, 1967; Shiga and Imaizumi, 1975). This compound was found to oxidize a series of phenols (Shiga and Imaizumi, 1975) and uric acid (Ames et al., 1981) and to cause protein cross-linking (Rice et al., 1983).

The glucose oxidase system demonstrated that small amounts of H_2O_2 , generated continuously, could activate MetMb and lipid peroxidation more efficiently than incubation of large amounts of H_2O_2 with MetMb. Activated MetMb species were not affected by catalase, but catalase could prevent their formation (Kanner and Harel, 1985).

The results demonstrated that the interaction of MetMb with H_2O_2 generated a new catalyst, unaffected by catalase. Lipid peroxidation initiated by activated MetMb was found to be dependent on pH, showing maximal activity similar to that of the pH in situ of muscle tissues. It was also found that the activity of the prooxidant decreased during the first minutes, but 50% of its activity remained stable for more than 30 min. Small amounts of H_2O_2 (1-10 μ M) could activate MetMb for significant lipid peroxidation, however high concentrations of MetMb or methemoglobin inhibited the reaction (Kanner and Harel, 1985).

Lipid peroxidation of turkey muscle microsomes by activated MetMb was more rapid than that of chicken muscle microsomes. These differences

were obtained by using both the accumulation of TBA-RS and oxygen absorption methods. The results in model system are closely correlated with those reported by others for muscle tissues (Wilson et al., 1976; Shagalovich and Kanner, 1981). From our findings, however, the reason for these differences is not clear (research on this topic is in progress).

As compared to the relative stability of fresh meats, cooked meats more rapidly develop off flavors (warmed-over flavor) (Pearson et al., 1977). Catalase, which is present in uncooked meat, might prevent partially the activation of metmyoglobin (Harel and Kanner, preceding paper), whereas, heated meat would have greatly reduced catalase.

Of great interest is the finding that heated MetMb retained its capacity to be activated by H_2O_2 . This indicates that heating of muscle foods, which was shown to accelerate the production of H_2O_2 (Harel and Kanner, 1985), does not prevent the possible initiation of lipid peroxidation by activated MetMb.

The prevention and control of lipid peroxidation in muscle foods is an important goal for many industries. Ascorbic acid is a known antioxidant, but in the presence of metal ions it was shown to act also as prooxidant (Kanner et al., 1977b). Similar results were obtained by using ascorbic acid in muscle tissues (Deng et al., 1978).

In the presence of activated MetMb and microsomes, a low concentration of ascorbic acid accelerated lipid peroxidation and only a high concentration - 5 mM - inhibited this reaction almost completely. Traces of iron (equivalent to 3-5 μM $FeCl_3$), which were found in our model system, could explain this behavior of ascorbic acid.

A low concentration of EDTA (10 μM) enhanced the antioxidative effect of ascorbic acid by 500-fold. Only at a very high (10 mM) concentration could EDTA alone inhibit lipid peroxidation initiated by activa-

ted MetMb. The antioxidative effect of EDTA seems to stem from two different mechanisms. The first arises from its metal chelating capacity, which inhibits metal "redox cycle" initiation of lipid peroxidation (Kanner, 1974,1976; Searle and Wilson, 1983). The second inhibitory activity of EDTA occurs at high concentration by its characteristics to act as a weak electron donor (Frisell et al., 1959; Holmstrom, 1964).

Several other antioxidants, such as ascorbyl palmitate, α -tocopherol and BHT, at a concentration range of 1-10 μ M inhibit initiation of lipid peroxidation by activated MetMb. This high efficiency of the antioxidants seems to derive from the ability of phenols and reducing compounds to act as electron donors, deactivating the catalyst, as well as suppressing lipid peroxy radicals and preventing propagation of lipid peroxidation.

When our data and those from the literature were used, a comprehensive reaction for initiation and autooxidation of lipids by myoglobin (heme proteins) has been drawn up (Kanner and Harel, 1975); these include the following: (a) Autooxidation and oxygen activation of oxymyoglobin to MetMb and H_2O_2 ; (b) Catalyst activation of MetMb by H_2O_2 , like peroxidases, to porphyrin cation radical, $P^+-Fe^{IV}=O$; (c) Initiation of lipid peroxidation by $P^+-Fe^{IV}=O$ via two-electron reduction of the catalyst, based on the results of uric acid oxidation (Howell and Wyngaarden, 1960) and titration of the catalyst with $K_4(Fe-(CN)_6)$ (Kremer, 1981).

(LH = unsaturated fatty acid; L· = allyl radical; LOO· = peroxy radical; LOOH = hydroperoxide); (d) Hydroperoxides which are generated during lipid peroxidation could also activate MetMb (Jones et al., 1977; Kalyanaraman et al., 1982); (e) Reducing compounds and the antioxidants deactivate the catalyst and inhibit lipid peroxidation by the following reactions:

We assume that in muscle tissues one of the first steps leading to the initiation of lipid peroxidation is the generation of endogenous H_2O_2 (Harel and Kanner, 1985). This could occur via the superoxide anion or directly and be the precursor of the first catalysts which appears to initiate lipid peroxidation - the hydroxyl radicals and activated MetMb (heme proteins).

The studies described above relate to the functioning of various prooxidants and antioxidants in vitro. Studies of this type are necessary to understand the mechanisms and pathways involved in the chemical reactions. It is difficult, however, to extrapolate the results to what happens in stored muscle tissue because of the greater complexity in the latter case.

One of the problems in studying any membrane preparation such as the SR in vitro is isolation from the other components of the cell which might influence its activity. Certainly a major fraction to be considered is the cytosolic or soluble fraction since it is likely that this

cellular fraction contains many components which will have free access to the membrane. As we had described earlier, we found a potent inhibitor in the low molecular weight fraction of flounder muscle cytosol which appeared to be identical to inorganic phosphate. It was also observed that there was a high molecular weight thermolabile component in the cytosol that enhanced lipid oxidation. An interesting observation was that the post mortem age of the SR was important from the point of view of its response to this high molecular weight activator (9). With increasing age, the membrane fraction became more susceptible to the activating effects of this cytosolic component. This activation was observed whether the isolated SR was aged or whether the muscle tissue was aged before the preparation of the SR. More recent work has allowed us to partially purify the high molecular weight activating factor, and its identification is being pursued. We have also shown that there are high molecular weight inhibitors in the cytosol although their effect is not as significant as the low molecular weight inhibitor nor the high molecular weight activator. This work is being prepared for publication. One of the questions which we addressed was if peroxidizing microsomal fractions could initiate lipid peroxidation in a triglyceride fraction. This was taken as the first step in developing a model system more closely related to what might occur in muscle tissue where triglyceride would exist as droplets in the muscle tissue. It was shown that the muscle microsomal fraction isolated from Atlantic herring (Clupea harengus) could oxidize the lipids of an emulsion only under conditions where the microsomal membrane itself was undergoing enzymic catalyzed lipid peroxidation. There is apparently some mechanism by which the oxidation components can initiate oxidation of the triglycerides of the emulsified fish lipids. This work has been described (Slabyj and Hultin, 1984).

Enzymes were added to minced Atlantic herring muscle to determine the mechanism of lipid peroxidation in the tissue as well as exploring the possibility of using added enzymes to reduce lipid peroxidation. Enzymes were added that were capable of destroying essential components of the lipid peroxidation reaction, such as NADase and nucleotide pyrophosphatase to destroy NAD, enzymes known to disrupt membrane integrity, such as phospholipase A_2 , and enzymes capable of reducing active oxygen intermediates such as catalase and superoxide dismutase. Samples were evaluated that contained no additives; TBHQ was added to others for comparative purposes.

For measurement of lipid deterioration, TBA-reducing substances, free fatty acids, absorbance at 232 nm, and quantitation of fatty acids by gas chromatographic analysis were utilized. Results were difficult to interpret since different analytical methods produced different indications of lipid deterioration. TBA-reactive substances increased with increased time of storage. Nucleotide pyrophosphatase, a combination of catalase and superoxide dismutase (SOD), and t-butylhydroquinone (TBHQ) all gave indications of reduced oxidation over a 10-month storage period. The enzyme NADase, however, had no inhibitory effect on lipid oxidation and may, in fact, have stimulated it. This result would not be unexpected since it is known that high concentrations of NADH can inhibit lipid oxidation, and this enzyme may remove part of that inhibition.

However, when adsorbance at 232 nm was used as a measure of oxidation, i.e. production of conjugated dienes, it was observed that all of these treatments, including NADase, decreased the oxidation. In fact, NADase worked better than did the other treatments.

The content of free fatty acid also increased with storage time. NADase and nucleotide paraphosphatase appeared to have some effect on slowing down the rate of free fatty acid production, but the combination of catalase and SOD and the antioxidant, TBHQ, slowed down the production of free fatty acids even more. Rationale as to why free fatty acid production might be inhibited by substances which inhibit lipid oxidation is that it has been recently shown that phospholipase preferentially hydrolyzes fatty acids that have undergone some oxidation. There were decreases in the content of C22:6 and C20:5 fatty acids with time of storage. This change was most pronounced in the polar fraction of the lipids. No differences were observed, however, amongst the various treatments in the loss of these unsaturated fatty acids.

Minced turkey muscles were protected from lipid peroxidation by ascorbyl palmitate and by other primary antioxidants. Ascorbyl palmitate is a very safe compound, because it is deesterified in human organisms to ascorbic acid and palmitic acid. The inhibition of minced turkey muscles by a relatively low concentration of EDTA seems to indicate that lipid peroxidation in situ in turkey muscles are not initiated by H_2O_2 -activated metmyoglobin, but by a free catalytic iron. As was shown by us more recently, EDTA at low concentrations inhibits microsomal lipid peroxidation initiated by NADPH/cytochrome P-450 reductase or by redox-cycle compounds (Kanner et al., 1986; Chasan and Kanner, 1986). Our work demonstrated that the initiation and probably the stimulation of muscle tissues in situ is dependent on "free" iron ions and reducing compounds or system which could cycle ferric iron to ferrous state.

G. REFERENCES

See reprints published by the authors of this research.

H. DESCRIPTION OF COOPERATION

Dr. J. Kanner and Prof. H. Hultin visited the lab. in Israel and U.S.A. During this period the researchers exchanged ideas on the results accepted during the work and planned new experiments. The principal investigators prepared several progress reports for one another to keep each of them conversant in what the other had done, was doing, and was planning.

Ideas were received at Volcani Center from Prof. H. Hultin on experiments with enzymatic microsomal lipid peroxidation. Dr. J. Kanner transferred results on the methodology of the measurement of H_2O_2 in muscle tissues and on the role of H_2O_2 activated metmyoglobin in generating lipid peroxidation in membrane system, on the role of leukocytes in lipid peroxidation in muscle food, and in iron redox cycle non-enzymatic oxidation processes in general. Information on different species, particularly chicken, turkey and fish, were also of considerable use.

These communications, both personal and written, have allowed the groups at both countries to design better experiments, it has given direction for our research efforts, and it has saved much time in the development of analytical procedures. The results accepted led both researchers to write a new BARD project on the same subject which was approved by BARD administration.

I. EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL

1. Water soluble factors which affect lipid peroxidation of membranes from muscle chicken and fish were evaluated.
We found that metmyoglobin, free iron and the generation of H_2O_2 could affect lipid peroxidation in model systems.
2. The relation between enzymic and non-enzymic lipid peroxidation in muscle tissues of chicken and fish were evaluated.
3. Preliminary study was done to determine what components of the fish system make it more susceptible to lipid peroxidation than the chicken system.
4. Natural protective systems of lipid peroxidation in muscles of chicken and fish were determined.
5. The utilization of natural and synthetic protective compound was tested in order to prevent quality deterioration of products associated with lipid peroxidation.

J. BENEFIT TO AGRICULTURE

For both Israel and the U.S.A. the production, local distribution and overseas exporting of poultry and fish products is of major economic importance and involves large financial investments. For Israel there is a large possibility to export frozen "Kosher" muscle foods to Europe and other developed countries if the quality of the product is high. This research yielded a better understanding of the mechanism of lipid oxidation in muscle foods and a better control of this process. The research led to the development of several by-products from poultry, such as a frozen sausage produced without nitrite. The quality improvements of frozen muscle foods will extend the distribution of these products to local markets and especially will increase the exporting of poultry and fish - two factors of major agricultural and economic importance in both countries.

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