Changes in Lysozyme Due to Reactions with Peroxidizing Methyl Linoleate in a Dehydrated Model System

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Lysozyme and peroxidizing methyl linoleate were allowed to react at 37 °C in a freeze-dried model system adjusted to controlled water activities. Changes in solubility, fluorescence, enzymatic activity, and gel electrophoresis patterns of the protein fraction were studied and compared with changes in lysozyme irradiated with γ rays in the absence of linoleate. Reactions with peroxidizing linoleate were found to produce lysozyme dimers and higher polymers. The polymerization was due to covalent bonds. The degree of cross-linking, protein insolubilization, and loss of enzyme activity increased with increasing water activity.

Damage to proteins due to reactions with peroxidizing lipids is an important deteriorative mechanism in the processing and storage of foods (Awad et al., 1968; St. Angelo and Ory, 1975; El-Lakany and March, 1974); it causes changes in biological materials and is a basic pathological process in vivo (Tappel, 1965; Packer et al., 1967).

Unsaturated fatty acids are peroxidized via a free-radical mechanism, and the major initial products are hydroperoxides. As oxidation progresses, hydroperoxide breakdown products accumulate in the system, including such reactive compounds as malonaldehyde.

A common manifestation of damage to proteins exposed to peroxidizing lipids is a decrease in protein solubility (Pokorny and Janicek, 1968). This loss of solubility may be caused by the formation of lipid–protein complexes, by protein aggregation initiated by free-radical reactions with the lipids, or by reactions with reactive decomposition products of the lipids.

Lipid–protein complexes are believed to be held together by either ionic attraction, hydrogen bonding, hydrophobic interactions, or ester-like bonds (Karel, 1973). Covalent bonds between lipids and proteins in naturally occurring systems are uncommon, but are reportedly present in lower organisms (St. Angelo and Ory, 1975). Peroxidized lipids and proteins have been shown to form both insoluble and soluble, polymeric lipid–protein complexes (Desai and Tappel, 1963; Roubal and Tappel, 1966; Gamage et al., 1973).

Using a system consisting of linoleic acid and egg albumin, Narayan and Kummerow (1958) concluded that there was no covalent bonding between oxidized lipids and proteins, and that hydrogen bonds were apparently responsible for complex formation. Formation of covalent bonds has been reported in reactions between protein –SH groups and linoleic acid hydroperoxides (Wills, 1961; Karel et al., 1975).

Proteins may also aggregate and polymerize as a result of reactions with lipid peroxides, but without forming lipid–protein complexes. The most widely reported mechanism involves reaction of proteins with malonaldehyde and other reactive products of hydroperoxide breakdown. Chio and Tappel (1969) and other authors (Shin et al., 1972; Gamage et al., 1973) have shown that malonaldehyde, one of the terminal products of lipid oxidation, forms intra- and intermolecular cross-linked products with proteins via Schiff base formation.

Desai and Tappel (1963) suggested that protein polymers can be formed through cross-linking by peroxy radical reactions. Cross-links such as P–OOLOO–P (where P is protein and L is lipid) result in this case. Other authors (Roubal and Tappel, 1966; Gamage et al., 1973) have suggested that peroxidized lipids can initiate protein cross-linking by a free-radical mechanism that yields polymers of the type $P-(P)_n-P$.

Recently it was proven (Schaich and Karel, 1975; Karel et al., 1975) that protein free radicals are actually formed during reactions with lipid peroxides. These authors believe that the process of free-radical transfer to the proteins occurs via complex formations of the following type:

$$PH + LOOH \rightarrow [PH\cdot\cdot\cdot LOOH] \rightarrow P\cdot + LOO\cdot + H_2O$$

where PH refers to protein and LOOH refers to lipid hydroperoxides. This hypothesis supports the possibility of cross-linking of proteins of the $P-(P)_n-P\cdot$ type.

The interaction between peroxidizing lipids and protein should also induce loss of enzymatic activity (Chio and Tappel, 1969; Matsushita, 1975), chain scission (Zirlin and Karel, (1969), and loss of specific amino acids (Roubal and Tappel, 1966; Roy and Karel, 1973).

The study of oxidation-induced changes in proteins is complicated by the variety of possible bonding mechanisms that exist even in the absence of added lipid peroxides. Chiang and Sternberg (1974) recently reported that prolonged storage of textured soy fibers induces changes at the molecular level. They have shown by treatments with buffers, urea, and mercaptoethanol that the protein structure is held together by hydrogen and hydrophobic bonds, as well as by disulfide bonds. Schaich (1974) observed that incubation of lysozyme with peroxidizing methyl linoleate resulted in both decreased solubility of the protein and in increased average molecular weight of the soluble fraction.

The present study was conducted on a model system containing lysozyme and peroxidizing methyl linoleate to identify the types of intermolecular links in the soluble protein fraction and to study the effect of water activity on the cross-linking, loss of solubility, and enzymatic activity of lysozyme.

MATERIALS AND METHODS

Model System. Lyophilized model systems of methyl linoleate (ML) (Nucheck Co.) and lysozyme (LYS) (Sigma Chemical Co.) were prepared by emulsifying 400 mg of lipid with 2 g of protein and 50 ml of water in a Sorval

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Omni-Mixer, freezing in liquid nitrogen, and freeze drying for 24 h in a Virtis Laboratory freeze drier. Control systems of lysozyme and water (without methyl linoleate) were prepared in the same manner.

Treatments. The lyophilized samples were equilibrated in desiccators at 37 °C over $CaSO_4$ or over saturated salt solutions at water activities of ~0, 0.11, 0.43, and 0.75 (Gal, 1967).

Other samples of lysozyme in water solution were exposed in air or nitrogen to 2 Mrads of γ radiation in a Gammacel ^{60}Co sauce with a dose rate of 9500 rads/min.

Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed on treated samples according to the method of Weber and Osborn (1969). Samples were dissolved in 0.01 M sodium phosphate (pH 7.0) at room temperature and homogenized with a tissue grinder.

Protein samples of 0.05 to 0.01 mg/0.1 ml were applied to the gels. Gel buffer diluted 1:1 with water was layered on top of each sample, and electrophoresis was performed at a constant current of 8 mA per tube until the tracking marker dye had migrated approximately half the length of the gel.

The gels were removed from the tubes, and the relative mobilities of the band were calculated. The standard proteins used for determining the molecular size of the polymerized products were lysozyme, trypsin, pepsin, and bovine serum albumin.

Fractionation of the Polymerized Products by Sephadex Column Chromatography. After incubation at a water activity (a_w) of 0.75 for 7 days, 50 mg of lysozyme/methyl linoleate was homogenized in 0.01 M phosphate buffer at pH 7.0. The homogenate was centrifuged for 10 min at 20000*g*. The soluble fraction was introduced onto a Sephadex G-150 column and run through two 2 × 30 cm columns. The columns were washed with 0.1 M phosphate buffer (pH 7.0). Three-milliliter fractions were collected and tested for protein by absorption at 280 nm, and for lysozyme activity, as described below.

Enzymatic Activity of Lysozyme. Lysozyme activity was assayed using lyophilized *M. lysodeikticus* (Sigma Chemical Co.) cells as substrate (Shugar, 1952). The test sample contained 2.5 ml of the *M. lysodeikticus* suspension in 0.066 M sodium phosphate buffer at pH 6.2. The optical density (OD) of this suspension at 450 nm was between 0.6 and 0.70. After the addition of 0.1 ml of enzyme solution, the decrease in absorbance at 450 nm was measured with a double-beam recording spectrophotometer. The initial rate of decrease in absorbance was computed from the recorded graph and converted into units of enzyme activity/milligram of protein.

Protein Determination. Loss of protein solubility in incubated lysozyme/methyl linoleate was measured on samples solubilized in 0.01 M phosphate buffer (pH 7). The homogenate was centrifuged for 10 min at 20000*g*, and the amount of protein in the supernatant tested by the Lowry method (Lowry et al., 1951) after precipitation with 10% Cl_3CCOOH and solubilization by 0.1 N NaOH.

Disruption of Noncovalent Bonds. After centrifugation, the soluble fraction was adjusted to pH 2 with 0.1 M HCl and, after 2 h at room temperature, neutralized with 0.1 M NaOH to disrupt hydrophobic bonds. To prevent these bonds from re-forming, the whole procedure was carried out in the presence of 0.1% sodium dodecyl sulfate (Rosen, 1959). To disrupt other noncovalent bonds, dry urea was added to the soluble fraction and dialyzed against 8 M urea.

Table I. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Bands in Lysozyme after Incubation with Methyl Linoleate

Treatment	Incubation time, days	Mobility					
Lysozyme	0	0.80					
Lysozyme	7	0.81					
Lysozyme/methyl linoleate	0	0.80					
Lysozyme/methyl linoleate	1	0.80	0.54	0.41			
Lysozyme/methyl linoleate	3	0.81	0.54	0.41	0.30	0.22	
Lysozyme/methyl linoleate	7	0.79	0.56	0.39	0.28	0.20	0.02
Lysozyme/methyl linoleate	14	0.79	0.53	0.40	0.27	0.19	0.02

Disruption of Disulfide Bonds. Disulfide bonds in protein were disrupted by 2-mercaptoethanol (2-ME) in 8 M urea as described by Sela et al. (1959). The reaction was stopped by the addition of a large quantity of iodoacetate, and the resulting solution was dialyzed against 8 M urea and 0.5% 2-mercaptoethanol.

Disruption of Ester-Like Bonds. Hydroxylamine was used to disrupt ester-like bonds. Before and after incubation with ML, samples were treated as described by Gallop and Feney (1959): the solution, 15 mg/ml of protein, was mixed with an equal volume of 2 M hydroxylamine-HCl and adjusted to pH 10 with 1 M NaOH. After 90 min incubation at 37 °C the solution was transferred into a cellophane sack and exhaustively dialyzed against water, and then against 8 M urea.

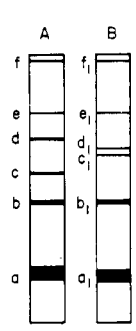
Other Treatments. Sodium dodecyl sulfate gel electrophoresis was also performed on incubated samples after treatment with solvents (ethanol, benzene, and hexane), 1% sodium dodecyl sulfate, and 2-mercaptoethanol.

Fluorescence. A Perkin-Elmer fluorescence spectrophotometer MPF-3 was used to measure fluorescence of soluble fractions of control samples (lysozyme) and of the lysozyme/methyl linoleate model system after homogenization and centrifugation at 20000*g*. Quinine sulfate (0.2 μg/ml) was used for intensity calibration and as a check on wavelength calibration of the spectrophotofluorometer. Fluorescence excitation was at 350 nm and emission at 460 nm.

RESULTS

The soluble protein fraction recovered from a lyophilized emulsion of lysozyme and methyl linoleate after incubation at 37 °C at a water activity of 0.75 was subjected to sodium dodecyl sulfate electrophoresis. The results indicated lysozyme cross-linking (Figure 1 and Table I). Two bands were found after 1 day of incubation. The mobility of the first band was identical with that of the native lysozyme, whereas the mobility of the second band corresponded to the molecular weight of the dimer. After 7 days of incubation it was possible to observe four other bands. A longer incubation period increased the number of polymers in each band, but not the number of bands.

The reason why bands c and d migrate to c_1 and d_1 after treatment with mercaptoethanol is not known. One possibility is that the opening of some disulfide bonds changes the physical migration properties. It is also



Band	Mobility	Molecular Weight Range	
a, a_1	0.81-0.79	14,500	15,300
b, b_1	0.52-0.54	27,000	28,600
c	0.39-0.40	37,000	38,000
c_1	0.35-0.36	42,000	43,000
d	0.28-0.30	48,000	49,000
d_1	0.34-0.35	43,000	44,000
e, e_1	0.20-0.22	57,000	59,000
f, f_1	0.02	over 90,000	

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soluble lysozyme/methyl linoleate (incubated 7 days at 37 °C; water activity, 0.75): (A) sodium dodecyl sulfate gel electrophoresis of incubated lysozyme/methyl linoleate without treatment with 2-mercaptoethanol; (B) sodium dodecyl sulfate gel electrophoresis of incubated lysozyme/methyl linoleate treated with 2-mercaptoethanol.

possible that we observe trimers because of reactions between a monomer and a dimer, as well as from the degradation of a polymer containing six monomers.

Cross-linking, as observed by sodium dodecyl sulfate electrophoresis, was most extensive at water activities of 0.43 and 0.75. In systems incubated at a very low water activity (0.11) the cross-linking was very limited, and only the dimer was identified. No cross-linking was found in a system incubated at a water activity of approximately zero until day 20 of incubation. No cross-links formed in the incubated controls, although some aggregation may have occurred at high water activities after 20 days of incubation, as indicated by the reduced mobility of the native band.

Effects of Treatments to Break Down Noncovalent Bonds on the Mobility of the Bands. Samples of untreated lysozyme, irradiated lysozyme, and the protein recovered from the model system after incubation periods of 0, 7, or 15 days (at a water activity of 0.75) were solubilized with 0.01 M phosphate buffer (pH 7.0) and centrifuged. A few samples were treated with ethanol, benzene, and hexane before the solubilization.

After centrifugation the soluble fraction was subjected to treatments designed to disrupt hydrogen, hydrophobic, ester-like, and disulfide bonds. The treatment with 2-mercaptoethanol to disrupt disulfide bonds changed the mobility of bands c and d (Figure 1) but did not change the number of bands. All of the other treatments had no effect on the number or mobility of the bands.

After gel electrophoresis, samples of lysozyme irradiated with 2 Mrads of γ rays showed only two bands, which corresponded to the monomer and the dimer. None of the treatments for breakdown of noncovalent bonds disrupted the dimer band.

Separation of Soluble Polymeric Fraction from Incubated Lysozyme/Methyl Linoleate by Sephadex G-150. The lysozyme control and the lysozyme/methyl linoleate model systems were incubated for 7 days at a water activity of 0.75. Samples were dissolved and centrifuged as described above. The soluble fraction was introduced onto a Sephadex G-150 column and eluted with 0.1 M phosphate buffer (pH 7.0). The control lysozyme appeared as a sharp single peak. The soluble fraction from lysozyme/methyl linoleate separated into a plateau and two peaks (Figure 2). The plateau near the first peak contains the high polymers and did not show any enzymatic activity. After sodium dodecyl sulfate gel electrophoresis, the mobility of the second peak corresponded to the dimer and contained trace amounts of the monomer.

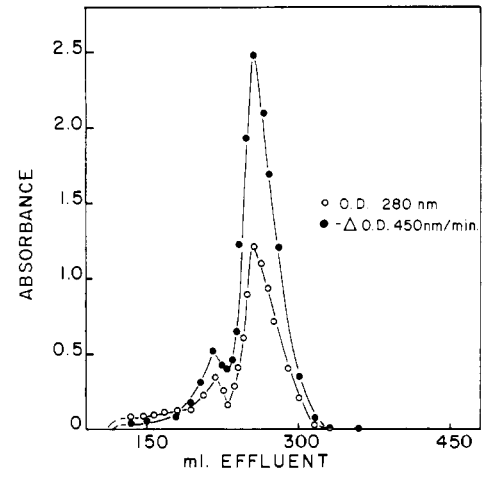


Figure 2. Separation on Sephadex G-150 of buffer-soluble fraction of lysozyme after 7 days of incubation with methyl linoleate.

Table II. Effect of Water Activity and Incubation Time on Lysozyme/Methyl Linoleate Solubility

Water act.	Loss of protein solubility, %,[a] at incubation time (days)				
	1	3	8	15	26
0	n.s.[b]	n.s.	16.1	14.6	14.8
0.11	n.s.	n.s.	11.5	10.2	10.5
0.43	n.s.	5.5	20.3	17.8	18.2
0.75	n.s.	13.7	40.4	67.5	76.0

[a] Measured by the Lowry method (Lowry et al., 1951). [b] No significant difference from control at zero time. (A difference of 3.6% represents significant difference at the 95% level.)

It was shown that the enzymatic activity of the dimer was about 60% of that of the monomer on an equal protein basis. The third peak corresponded to the mobility of the monomer and had an enzymatic activity of 85% of the native lysozyme (Figure 2). It is interesting to note that the lysozyme dimer formed by very high doses of γ radiation (60 Mrads) has been reported to lose almost all of its enzymatic activity (Stevens et al., 1970).

The Effect of Water Activity on the Solubility of Protein in the Incubated Model System. The solubility of the protein decreased during the incubation of the lysozyme/methyl linoleate model system. Samples incubated at a water activity of 0.75 showed the greatest change in solubility, losing 76% after 26 days (Table II). The data in Table II are based on two experiments at each set of conditions, with triplicate samples in each experiment. (At lower water activities, solubility decreased somewhat during the first 8 days of incubation.)

The Effect of Incubation at Different Water Activities on the Enzymatic Activity of Lysozyme Incubated with Methyl Linoleate. Samples from the incubated lysozyme/methyl linoleate model system were homogenized with 0.01 M phosphate buffer (pH 7.0). The enzymatic activity was measured before centrifugation. The loss of protein solubility correlated well with the loss of enzymatic activity, which was rapid in samples incubated at a water activity of 0.75. The loss before centrifugation may have been due to steric hindrance and, especially, to loss of activity in higher polymers. At lower water activities enzymatic activity decreased until the 8th day of incubation; subsequent losses were not significant (Figure 3). After 26 days of incubation, control samples of lyophilized lysozyme showed a 2–3% enzymatic activity loss at a water activity of zero, 5% loss at water activity

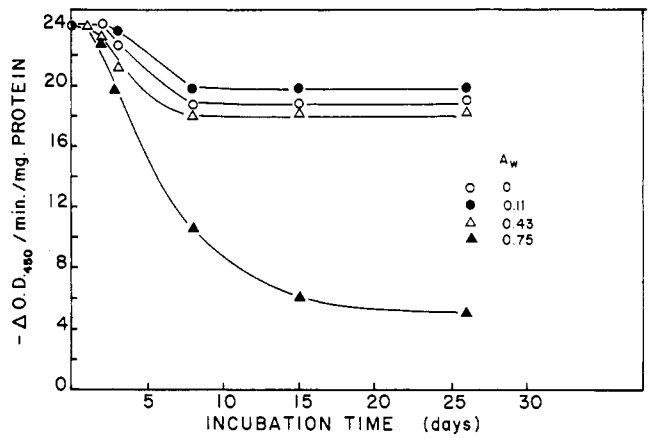


Figure 3. Effect of water activity on enzymatic activity of total protein homogenate obtained from lysozyme after incubation with methyl linoleate: (○) water activity ≃ 0; (●) water activity = 0.11; (△) water activity = 0.43; (▲) water activity = 0.75.

Table III. Specific Enzymatic Activity of Soluble Lysozyme/Methyl Linoleate as Affected by Water Activity[a]

Water act.	Enzymatic act., $-\Delta OD_{450}$/min per mg of protein for incubation time (days)					
	0	1	3	8	15	26
0	24.1	24.0	22.5	20.5	20.3	20.0
0.11		23.8	22.8	21.5	21.0	21.3
0.43		23.8	22.0	20.9	20.5	20.9
0.75		23.7	21.6	20.6	19.5	18.0

[a] Based on the protein in the soluble fraction.

of 0.11, and 9% loss at water activity of 0.75.

Enzymatic activity was also measured in the soluble fraction after centrifugation. The loss in specific activity of the enzyme in the incubated lysozyme/methyl linoleate system after homogenization and centrifugation varied from 12% at a water activity of 0.11 to 25% at water activity of 0.75. The specific activity was based on enzyme activity per unit of protein as measured by the Lowry method (Lowry et al., 1951) (Table III).

Comparative analyses for protein in the incubated samples using the Lowry and Kjeldahl methods showed that the Kjeldahl method yielded higher values. Samples incubated at a water activity of 0.75 showed a difference of 15% between the two methods; in other samples the difference was less. Results obtained by the Lowry method are dependent on the reaction between copper ions and the protein, and on the reaction between this complex and the reagent. During incubation the chelation capability of the protein was apparently reduced, probably because of changes in the amino acids, especially tyrosine and tryptophan. It is possible, therefore, that the specific activity losses reported in Table III are underestimated for the samples incubated at high water activities.

DISCUSSION

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of lysozyme incubated with oxidizing linoleate shows extensive cross-linking when incubation was conducted at high water activities. Our current results, which show that proteins polymerize during incubation with linoleate, agree with those of Schaich and Karel (1975), who studied a dehydrated lysozyme–linoleate model system, and with those of other authors (Roubal and Tappel, 1966; Gamage et al., 1973), who studied aqueous systems. It seems that polymerization is a gradual process

Table IV. Fluorescence of Lysozyme Incubated with Methyl Linoleate in a Dry Model System[a]

Water act.	Rel fluorescence for incubation time (days)		
	0	3	15
0	1.8[b]	2.0	5.2
0.75	2.2	14.0	88.4

[a] Fluorescence is given in optical density units per 0.2 mg of soluble protein/ml. [b] Control lysozyme samples without methyl linoleate gave a relative fluorescence of 2.0.

that proceeds from the monomer to the dimer, and on to higher polymers. Such a mechanism is consistent with the free-radical mechanism suggested by Roubal and Tappel (1966), and was anticipated by the work of Zirlin and Karel (1969) and Schaich and Karel (1975).

Treating the polymers with reagents that disrupt noncovalent bonds shows that covalent bonds cause the polymerization of lysozyme by peroxidizing methyl linoleate or by γ irradiation. Noncovalent bonds also might be involved, but their disruption did not break the polymers down into the monomer.

These results support the observations of Roubal and Tappel (1966) and Schaich and Karel (1975) suggesting that proteins allowed to react with lipid peroxides polymerize via a $P-(P)_n-P\cdot$ type of reaction. In our model, the cross-linking during the first incubation period is probably due to interaction between carbon radicals on the protein (Schaich and Karel, 1975). Disulfide bonds do not appear to be involved in these polymers; this observation is consistent with the absence of ESR signals due to sulfur-centered radicals in the incubated lysozyme/methyl linoleate model system (Schaich, 1974). After a long incubation period peroxide breakdown products accumulate, and reactions with these products might also initiate polymerization, as Shin et al. (1972) demonstrated with malonaldehyde-initiated polymerization.

Most recently, El-Zeany et al. (1975) reported formation of dimers and trimers of egg albumin during incubation in the dry state (water activity not controlled) with a mixture of polyunsaturated fatty esters. In the experiments reported by El-Zeany et al. (1975) protein solubility also decreased substantially. In contrast to the results for lysozyme reported here, the polymerization of egg albumin occurred both in the presence and absence of added lipid, but proceeded more rapidly when peroxidizing lipids were present. El-Zeany et al. (1975) attribute the polymerization to oxidative formation of disulfide bridges.

The differences in results obtained with the two systems are not surprising, since Karel et al. (1975) showed that lipid peroxides are capable of producing sulfur-centered radicals in dehydrated model systems with egg albumin, but not in model systems with lysozyme.

Water activity of the model system plays an important role in controlling the cross-linking process, insolubilization of the protein, and the loss of enzymatic activity. At a water activity of approximately zero the losses in solubility and enzymatic activity were lower than those in water activity of 0.75, despite the rapid oxidation of the lipid at the low water content. The mobilization function of water, which allows radical recombination, is apparently more important to the stability of the lysozyme than the ability of the water to slow the rate of lipid oxidation. Schaich and Karel (1975), who worked with a similar model, have shown that at a water activity of approximately zero, the protein radical concentration is higher than at a water activity of 0.75. Water activity of 0.75 increased the

mobility of the radicals, which promotes cross-linking, insolubilization, and loss of enzymatic activity.

Low water activity not only prevents the recombination of protein radicals, but also prevents cross-linking involving soluble intermediates, such as malonaldehyde. A lesser degree of visually observable browning occurred at low water activities (LeRoux, 1969), and the fluorescence of samples recovered from incubated model systems also was least at the low water activities (Table IV).

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Structural Changes in Actomyosin Induced by Ca^{2+} Ions

Ryo Nakamura

To study the structural changes of actomyosin induced by Ca^{2+} ions, viscometric and light scattering experiments were made on solutions of F-actin, natural actomyosin, and synthetic actomyosin. The viscosity of both F-actin and actomyosin decreased with the addition of Ca^{2+} ions. The amounts of Ca^{2+} ions needed to decrease the viscosity of actomyosin, however, were much smaller than that of F-actin. Although the particle weight of actomyosin did not change at all, its root-mean-square radius $(r^2)^{1/2}$ increased with the addition of small amounts of Ca^{2+} ions such as 100 μM. Both viscosity and the $(r^2)^{1/2}$ value of actomyosin were not affected by the addition of 1 mM Mg^{2+} ions. Discussions are included about these Ca-induced changes of actomyosin and the possible role of Ca^{2+} ions in the meat tenderization phenomenon during post-mortem aging.

Recently Ca^{2+} ions have been shown to have an important role in the meat tenderization phenomenon during postmortem aging (Davey and Gilbert, 1969; Busch et al., 1972a,b; Nakamura, 1972). Although some workers prefer to consider that Ca^{2+} ions activate a muscle protease and cause the meat tenderization phenomenon (Busch et al., 1972b; Penny, 1974; Penny et al., 1974), there is a possibility that Ca^{2+} ions bind to some muscle proteins, change their properties, and increase the tenderness of meat. In a previous work (Nakamura, 1974), to study the effect of Ca^{2+} ions on the properties of muscle proteins, Ca-induced change of viscosity was studied about F-actin which is one of the main components of myofibril. The result obtained from the work was that the viscosity of F-actin decreased largely with the addition of small amounts of Ca^{2+} ions and this phenomenon was explained by the decrease in the hydration of F-actin induced by Ca binding. Structural changes induced by Ca^{2+} ions were also reported about F-actin (Yanagita et al., 1974) and thick filaments (Morimoto and Harrington, 1974).

The purpose of this paper is to study the structural changes of actomyosin induced by Ca^{2+} ions. The differences in Ca-induced changes between actomyosin and F-actin are also studied.

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MATERIALS AND METHODS

Preparation of F-actin, Myosin, and Actomyosin. All muscle proteins used in this experiment were prepared from chicken breast muscle immediately after death. F-actin was prepared as described previously (Nakamura, 1974); G-actin was extracted from the acetone powder and further purified by the method of Spudich and Watt (1971). Myosin was prepared according to the method of Perry (1955). Synthetic actomyosin was made by mixing 1 part of F-actin to 4 parts of myosin by weight in 0.6 M