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Inactivation of Ribonuclease and Other Enzymes by Peroxidizing Lipids and by Malonaldehyde*

K. S. Chio and A. L. Tappel

ABSTRACT: Quantitative enzyme inactivation by lipid peroxidation has been studied. Sulfhydryl enzymes are most susceptible to inactivation by lipid peroxidation intermediates. Oxidation products of polyunsaturated lipids also inactivate nonsulfhydryl enzymes, for example, ribonuclease A. Concomitant with the loss of ribonuclease A activity is the appearance of fluorescence in the enzyme–lipid reaction mixture. The inactivated RNase A shows fluorescent monomer, dimer, and higher molecular weight species in the Sephadex G-100 fractionation pattern. The fluorescence maximum is at 470 mμ; and the excitation maximum is at 395 mμ. Ribonuclease A, inactivated by malonaldehyde, has fluorescence and a gel filtration pattern similar to that of the enzyme inactivated by peroxidizing polyunsaturated lipids. Malonaldehyde is probably the agent responsible for the intra- and intermolecular cross-linking of ribonuclease A. The fluorescence produced from the cross-linking is attributed to the conjugated imine structure formed in protein between two ε-amino groups and malonaldehyde. There are marked similarities between the ribonuclease A–polyunsaturated lipid product and age pigment; cardiac age pigment is a protein–lipid complex and both have similar fluorescence characteristics.

Lipid peroxidation has been considered to be a damaging reaction in biological systems (Barber and Bernheim, 1967). Oxidation of polyunsaturated fatty acids is postulated as a mechanism of disruption of biological membranes and has been reviewed by Packer *et al.* (1967). Inactivation of sulfhydryl enzymes in mitochondria has been associated with lipid peroxidation (McKnight and Hunter, 1966). Tappel (1965) has summarized the properties of protein damage in peroxidation reaction systems and showed that there were considerable similarities between protein damage by lipid peroxidation and that caused by radiation. It is suggested that age pigments were derived from oxidized lipid constitutents of damaged membranes. In the study of the mechanism of damage of cytochrome *c*, a lipid peroxide–protein complex intermediate is reported (Desai and Tappel, 1963). Andrews *et al.* (1965) showed that in an autoxidized lipid–protein system, lipid intermediates reacted with the free amino groups of proteins. On the basis of trypsin hydrolysis and hydrogen fluoride solubility tests, they concluded that reactive lipid intermediates are produced which insolubilize proteins *via* a cross-linking reaction. By gel filtration and hydrogen fluoride solubility studies, Roubal and Tappel (1966) have shown that oxidized lipid–protein reaction products resulted in protein–protein cross-linked polymers which are formed by a free-radical chain polymerization mechanism.

Malonaldehyde, one of the many carbonyl compounds derived from oxidation of polyunsaturated lipids, was shown to react with bovine serum albumin (Kwon and Brown, 1965;

* From the Department of Food Science and Technology, University of California, Davis, California. *Received December 27, 1968.* This investigation was supported by U. S. Public Health Service Research Grant AM 09933 from the National Institute of Arthritis and Metabolic Diseases.

Crawford *et al.*, 1967) and with myosin (Buttkus, 1967). The reaction was interpreted as involving the free amino groups in the proteins with the free enol of malonaldehyde to form an enamine linkage. Crawford *et al.* (1967) further showed by viscometric measurements that malonaldehyde did not appear to participate in an intermolecular cross-linking reaction with gelatin sols. Menzel (1967) gave evidence that products of lipid oxidation were reactive and that malonaldehyde, one of the thiobarbituric acid reactive substances, reacted with ribonuclease and caused its polymerization. However, the mechanism by which malonaldehyde could cross-link the protein molecules is not known. The present study investigates the types of interactions that take place between oxidized lipid products and enzymes. The mechanism of the reactions between RNase A[1] and polyunsaturated lipids, and between RNase A and malonaldehyde to give yellow, fluorescent products, and the nature of the cross-linkage of polymerized RNase A are described.

Experimental Section

Materials and Methods

Quantitative Determination of Damage to Enzymes by Lipid Peroxidation. The reaction system consisted of 5 mg of enzyme (RNase A, 2 mg) in 0.5 ml of 0.05 M sodium phosphate buffer (pH 7), unless otherwise stated, and 100 mg of methyl linolenate (Hormel Institute). Peroxidation of the linolenate was measured by the conventional Warburg manometric method in an oxygen atmosphere at 37°. At intervals, 20-μl aliquots were withdrawn and their enzyme activities were determined. Control reaction systems did not contain methyl linolenate. Papain (twice crystallized, Sigma Chemical Co.) was activated by the method of Soejima and Shimura (1961) and assayed by the method of Kirsch and Igelström (1966). α-Chymotrypsin (salt-free, three-times crystallized) was from Mann Research Laboratories and was assayed by the method of Schwert and Takenaka (1955). Creatine kinase (in 0.05 M glycine, pH 7), lysozyme, yeast alcohol dehydrogenase, carboxypeptidase A (in 10% LiCl), and lactate dehydrogenase were obtained from Worthington Biochemical Corp. and their activities were determined by methods given in the data sheets published by Worthington (1967). Urease (type IV, Sigma Chemical Co.) was assayed by the method of Sumner (1955). Ribonuclease (five-times crystallized, salt-free) was from Mann Research Laboratories and ribonuclease A was purified by the method of Taborsky (1959) and assayed as described by de Duve *et al.* (1955). The substrate for ribonuclease was yeast ribonucleic acid (Nutritional Biochemical Corp.), 3 mg/2 ml of 0.1 M sodium acetate buffer (pH 5), which had been dialyzed against the same buffer for 48 hr at 4° with three changes of buffer. In addition to the manual assay, an automated method was used to determine RNase A activity (Barrera *et al.*, 1969).

Inactivation of Ribonuclease A by Lipid Peroxidation. Ethyl arachidonate (200 mg), which was a generous gift of Hoffman-LaRoche Inc., or methyl linolenate was reacted with 50 mg (or 5 mg) of RNase A dissolved in 2.5 ml of 0.01 M sodium phosphate buffer (pH 7) in a 25-ml erlenmeyer flask. The buffers used in all experiments contained 5 ppm of Myprozine (American Cyanamid Co.) as a fungicide. The flask was covered with parafilm and shaken at 37° in a Metabolyte waterbath shaker (New Brunswick Scientific Co.) at 100 rpm. Aliquots, 20 μl each, were taken at intervals and diluted to determine the RNase A activity. Measurements were also made of the thiobarbituric acid value and the fluorescence intensity at 470 mμ with an excitation at 390 mμ using an Aminco-Bowman spectrophotofluorometer. The amount of malonaldehyde formed was calculated using an extinction coefficient of 158,000 at 532 mμ (Sawicki *et al.*, 1963). The solution containing the inactivated RNase A was extracted with chloroform and then with ether to remove excess lipid and oxidized lipid products, and then dialyzed against distilled water at 4° for 48 hr with three changes of water. The dialyzed RNase A was lyophilized. Protein concentration was determined by the method of Miller (1959). The chloroform and ether extracts were combined and evaporated to dryness, and the peroxide value of the residue was measured by the method described by Swern (1961).

Amino Acid Analyses. After hydrolyzing 5 mg of protein with 6 N HCl in an evacuated, sealed tube at 110° for 20 hr, ninhydrin analyses were performed as described by Moore and Stein (1963). An aliquot of hydrolysate equivalent to about 0.5 mg of protein was used for chromatographic analysis, which was carried out with a Technicon amino acid analyzer.

Fractionation of Inactivated RNase A by Gel Filtration on Sephadex G-100. The lyophilized inactivated RNase A (10 mg) was dissolved in 0.4 ml of 0.1 M sodium acetate–0.2 M NaCl (pH 6). This solution was heated at 65° for 20 min prior to chromatography to prevent aggregation of ribonuclease (Crestfield *et al.*, 1962). The sample was then chromatographed on a 120 × 1.5 cm column of Sephadex G-100 (Pharmacia Fine Chemicals) in the same sodium acetate–NaCl solution at a flow rate of 6 ml/hr. Fractions of 2.1 ml were collected. The protein concentration and the fluorescence intensity at 470 mμ with excitation at 390 mμ of the eluent were measured. The molecular weight of the fractionated RNase A was determined by the method of Whitaker (1963), using bovine serum albumin, pepsin, α-chymotrypsin, and cytochrome *c* as protein standards.

Reaction of RNase A with Methyl Linoleate and Methyl Oleate. The reaction system was the same as for the arachidonate and linolenate esters. Methyl linoleate or oleate (Hormel Institute; 200 mg) was added to 2.5 ml of 0.01 M sodium phosphate buffer (pH 7) containing 5 mg of RNase and shaken at 37° for 120 hr. Aliquots were withdrawn at intervals for enzyme activity and fluorescence intensity measurements. Peroxide and thiobarbituric acid values were determined at the start of the experiment and after 120 hr. The enzyme solutions were separated from the lipid layers, dialyzed, lyophilized, and chromatographed as previously described on the Sephadex G-100 column.

Inactivation of RNase by Malonaldehyde. Malonaldehyde was prepared from its methyl acetal, 1,1,3,3-tetramethoxypropane (Aldrich Chemical Co.), by HCl hydrolysis (Kwon and Watts, 1963). RNase A (40 mg) was dissolved in 3 ml of 2×10^{-2} M malonaldehyde (pH 7) or 10 mg of RNase A was dissolved in 3 ml of 4×10^{-3} M malonaldehyde (pH 7), and the reaction systems were treated as with the fatty acid esters. Samples were taken to determine RNase A activity and fluorescence. After inactivation, dialysis, and lyophilization, protein hydrolysis and chromatography on Sephadex G-100 were done on the inactivated enzyme by the methods previously

[1] Abbreviation used: RNase A, bovine pancreatic ribonuclease A.

TABLE I: Quantitative Enzyme Inactivation by Lipid Peroxidation.

Enzyme	Moles of Enzyme Inactivated/Mole of Oxygen Uptake
Papain	1.75×10^{-1}
Creatine kinase	2.32×10^{-2}
Yeast alcohol dehydrogenase	1.98×10^{-2}
α-Chymotrypsin	1.48×10^{-2}
Carboxypeptidase A	1.82×10^{-3}
Lactate dehydrogenase	1.48×10^{-3}
Lysozyme	8.94×10^{-4}
Ribonuclease A	5.34×10^{-4}
Urease	2.31×10^{-4}

described. The absorption and fluorescence spectra of the main peaks of the eluate were taken.

Preparation of the Fluorescent Compound Derived from Malonaldehyde and α-N-Acetyl-L-lysine. Malonaldehyde methyl acetal (2.5 g, 0.015 mole) was dissolved in 1.35 ml of 1 N HCl at 40°. After cooling, the solution was added to 1.9 g (0.01 mole) of α-*N*-acetyl-L-lysine (Cyclo Chemical Corp.) in 2 ml of H_2O and stirred for 1 hr. The excess malonaldehyde was extracted from the solution four times with equal volumes of ether. The yellow, fluorescent (under ultraviolet light, 366 mμ) solution (2.5 ml) was chromatographed on a Bio-Gel P-2 (200–400 mesh, Bio-Rad Laboratories) column (100 × 2.5 cm) with deionized distilled H_2O as eluent at a flow rate of 68 ml/hr using a Technicon proportioning pump. The absorption of the eluate (6.8 ml/fraction) was measured at 400 mμ and the peak was found at 305 ml. The main fractions around the peak were pooled and lyophilized (300 mg, 45% yield). After rechromatography, the derivative was found to decompose at 54–58°. Its absorption and fluorescence spectra were measured.

Results

The inactivation of enzymes as a function of lipid peroxidation, as measured by oxygen uptake, is shown in Figure 1. A linear relationship between log of enzyme activity remaining and oxygen uptake is observed. As this relationship is similar to that for enzymes inactivated by radiation, the ratios of mole of enzyme inactivated per mole of damaging species formed were calculated on similar principles (McDonald, 1955). Quantitative determinations of enzyme inactivation were calculated for the first 10% enzyme damage and are given in Table I. It is noted that sulfhydryl enzymes are most susceptible to inactivation by lipid peroxidation.

Using RNase A to study the mechanism of enzyme inactivation and the nature of the cross-linking agent, the correlation of loss of RNase A activity with malonaldehyde production and the appearance of fluorescence is shown in Figure 2. With 5 mg of RNase A in 5 ml of 0.05 M sodium phosphate buffer (pH 7) containing 200 mg of ethyl arachidonate, the enzyme was completely inactivated in about 22 hr. Dialysis of the inactivated enzyme against the sodium phosphate buffer

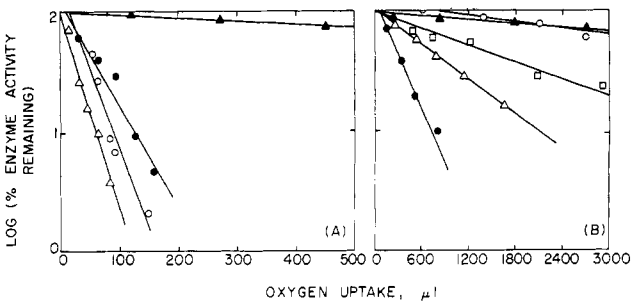


FIGURE 1: Inactivation of enzymes by methyl linolenate peroxidation as measured by oxygen uptake. (A) ▲, Carboxypeptidase A; ●, creatine kinase; ○, yeast alcohol dehydrogenase; and Δ, papain. (B) ○, Ribonuclease A; ▲, lysozyme; □, lactate dehydrogenase; Δ, urease; and ●, α-chymotrypsin.

or against distilled H_2O, after ether and chloroform extractions, did not restore the enzyme activity. The inactivated enzyme is yellow in color and fluoresces in the visible region. The peroxide value of the ethyl arachidonate at the end of the reaction period was 820 mequiv/kg.

When RNase A was completely inactivated by oxidation of the ethyl arachidonate, the results, given in Table II, show that of the component amino acids only lysine and histidine were considerably decreased and tyrosine was slightly diminished.

The gel filtration and elution pattern of the arachidonate-inactivated RNase A on Sephadex G-100 is shown in Figure 3a. It is noted that fluorescence peaks correspond with protein peaks, and the arachidonate-inactivated RNase A product contains aggregate (peak D), trimer (peak C), dimer (peak B), and monomer (peak A) of the enzyme, which are all inactive toward yeast ribonucleic acid hydrolysis. The reaction products of RNase A and methyl linolenate also are fluorescent and give the same gel filtration elution pattern as that of arachidonate-inactivated RNase A. The fluorescence spectra of all the peaks have the same characteristics with emission maximum at 470 mμ and excitation maximum at 395 mμ, as shown in Figure 4. In the visible region of the absorption spectrum, only a shoulder was observed at 390 mμ. This indicates that more than one reaction product is obtained in the reaction mixture.

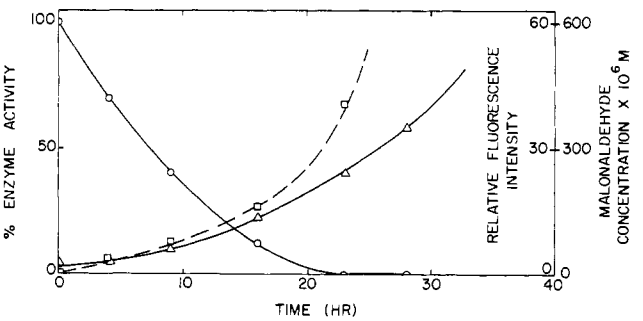


FIGURE 2: Correlation of disappearance of RNase A activity with malonaldehyde and fluorescence production. The reaction system consisted of 5 mg of RNase A in 5 ml of 0.05 M sodium phosphate buffer (pH 7) containing 200 mg of ethyl arachidonate. (○) RNase A activity; (□) fluorescence at 470 mμ when excited at 390 mμ; (Δ) malonaldehyde concentration.

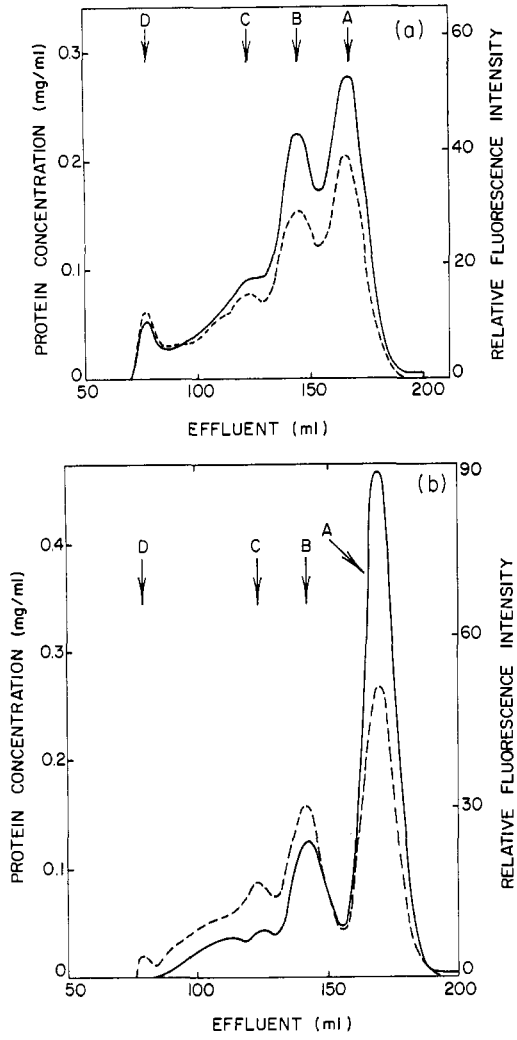


FIGURE 3: Sephadex gel filtration of (a) ethyl arachidonate inactivated RNase A and (b) malonaldehyde-inactivated RNase A. The inactivated enzyme (10 mg) was used for chromatography. A (14,000), B (27,000), C (40,000), and D refer to monomer, dimer, trimer, and aggregate of RNase A, respectively; numbers in parentheses refer to approximate molecular weights. (——) Relative protein concentration measured by the method of Miller (1959). (———) Relative fluorescence at 470 mμ when excited at 390 mμ.

RNase A was fully active even after 120-hr oxidation with methyl linoleate or methyl oleate, which developed peroxide values of 300 and 80 mequiv per kg, respectively. The thiobarbituric acid values were zero in both cases, showing that malonaldehyde was not produced during that period of oxidation. Separate chromatography on the Sephadex G-100 column showed that each enzyme sample was eluted only at peak A, as in Figure 3a; no other peaks were observed.

Figure 5 shows the rate of inactivation of RNase by malonaldehyde and the appearance of fluorescence. The increase in fluorescence at the beginning was low and then rose to a maximum when about two-thirds of the RNase activity was lost. The malonaldehyde-inactivated RNase A is yellow and has visible fluorescence. The amino acid composition of the malonaldehyde-inactivated RNase A is shown in Table II. About 40% of the tyrosine residues is destroyed together with 25% destruction of each of the amino acids, methionine and lysine.

TABLE II: Amino Acid Composition[a] of Native and Ethyl Arachidonate and Malonaldehyde-Inactivated RNase A.

Amino acid	Theor Value[b]	Native RNase A	RNase Inactivator: Ethyl Arachidonate	RNase Inactivator: Malonaldehyde
Aspartic acid	15	15.4	15.1	15.2
Threonine	10	9.8	9.7	9.2
Serine	15	12.8	12.4	12.0
Glutamic acid	12	12.3	12.2	12.4
Proline	4	3.9	4.0	4.1
Glycine	3	3.1	3.3	3.2
Alanine[c]	12	12.0	12.0	12.0
Valine	9	8.8	8.5	8.7
Half-cystine	8	7.1	6.9	6.7
Methionine	4	3.8	3.6	2.7
Isoleucine	3	2.2	2.1	2.1
Leucine	2	2.0	2.1	2.0
Tyrosine	6	5.7	4.8	3.2
Phenylalanine	3	2.9	2.8	2.9
Amide NH_3	17	16.3	15.7	15.8
Lysine	10	10.2	6.6	7.8
Histidine	4	4.0	2.6	3.8
Arginine	4	4.1	4.2	3.9

[a] Values correspond to 21-hr hydrolysis and no corrections are made for the destruction of labile amino acids during acid hydrolysis. The values shown represent the average of two determinations. [b] From Hirs *et al.* (1956). [c] Used as basis of calculation.

When the malonaldehyde-inactivated RNase A was chromatographed on the Sephadex G-100 column, the elution pattern as shown in Figure 3b, was about the same as that of the arachidonate-inactivated RNase A products. The effluent has a maximum absorption in the visible region at 395 mμ. The fluorescence spectra show an emission maximum at 470 mμ and an excitation maximum at 392 mμ, which is quite similar to that of Figure 4.

The yellow, fluorescent product obtained from the reaction of malonaldehyde and α-*N*-acetyl-L-lysine was presumed to be the *N,N'*-disubstituted 1-amino-3-iminopropene, RNHCH=CHCH=NR, where R = $CH_3C(=O)NHCH(COOH)(CH_2)_4$. This derivative has its absorption maximum at 395 mμ, and the fluorescence spectrum shows a maximum at 470 mμ when excited at 395 mμ. The derivative has the same spectral characteristics as those of the *n*-hexylamine malonaldehyde reaction product and identification is based on our recent study (Chio and Tappel, 1969).

Discussion

The linear relationship between log of enzyme activity remaining and lipid peroxidation, indicates the similarity between inactivation of enzyme by lipid peroxidation and ionizing radiation damage to enzymes, proteins, and amino acids. For comparison with the values of enzyme inactivated in Table

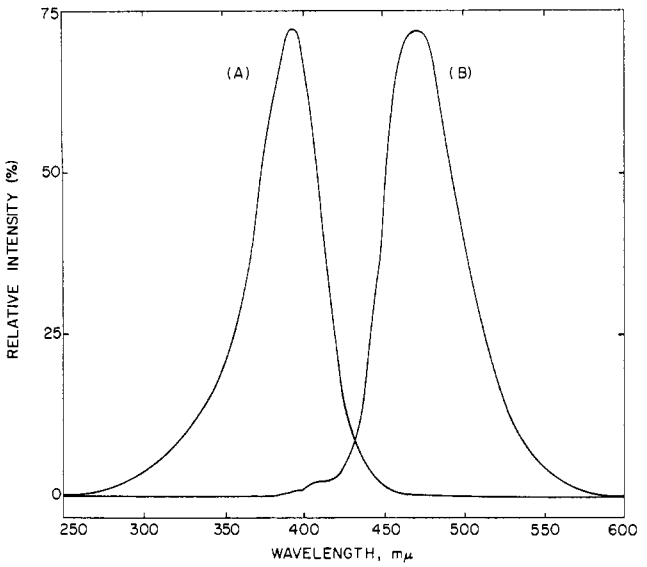


FIGURE 4: Excitation (curve A) and fluorescence (curve B) spectra of RNase A inactivated by ethyl arachidonate.

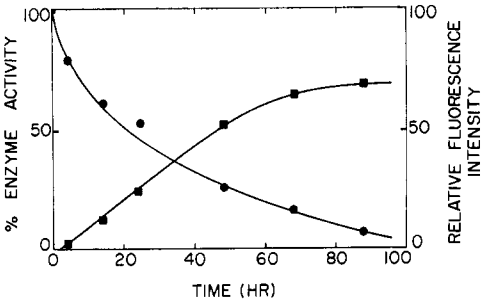


FIGURE 5: Rate of loss of activity of RNase A in the presence of malonaldehyde. RNase A (10 mg) was dissolved in 3 ml of 4 × 10^{-3} M malonaldehyde (pH 7); at intervals, aliquots were removed and diluted to determine (●) enzyme activity and (■) fluorescence at 470 mμ when excited at 390 mμ.

I are yield values of moles of enzymes inactivated per ion pair from radiation as follows: 0.05, 0.03, 0.10, and 0.48 for invertase, catalase, cytochrome *c*, and ribonuclease, respectively (Setlow and Pollard, 1962); 0.20 for trypsin (McDonald, 1955); 0.01, 0.02, and 0.10 for aldolase, alcohol dehydrogenase, and ribonuclease, respectively (Romani and Tappel, 1959). Lipid peroxidation damage is quantitatively similar to radiation damage for sulfhydryl enzymes. For nonsulfhydryl enzymes it is considerably less than radiation damage. Lower levels of lipid peroxidation damage can be related to the biphasic lipid reaction system where many of the peroxy free radicals would not come into contact with the enzymes. On a quantitative basis, sulfhydryl enzymes are more readily inactivated by methyl linolenate than nonsulfhydryl enzymes. The free-radical intermediates such as peroxy radicals formed during peroxidation could account for the destruction of the SH groups in the enzymes. Wills (1961) has shown that peroxides produced from oxidation of unsaturated fatty acid emulsions inhibit sulfhydryl enzymes, and lipid peroxides cause oxidation of sulfhydryl groups in enzymes (Lewis and Wills, 1962).

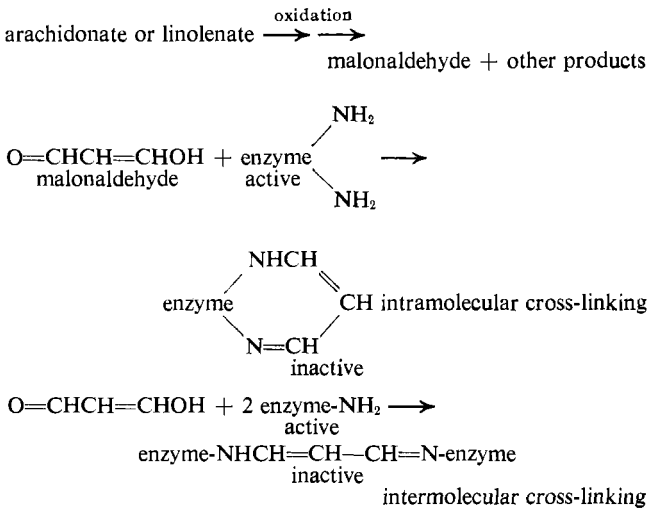
RNase A was chosen for the study of the mechanism of inactivation of nonsulfhydryl enzyme by the oxidation of unsaturated fatty acid esters. As shown in Figure 2, the first 50% of the RNase activity was inactivated in about 7 hr. Inactivation at the beginning can be related to specificity of the oxidation products of ethyl arachidonate for certain groups in the enzyme. Loss of tyrosine, lysine, and histidine residues in arachidonate-inactivated RNase could account for the loss of enzyme activity. Findlay *et al.* (1962) showed the general acid-base catalysis of two imidazole residues in the enzymic mechanism; and a lysine residue was reported to be essential for structural integrity and enzymic activity (Hirs, 1962).

There are similarities between the effect of ethyl arachidonate and malonaldehyde on the activity of RNase A, such as the rate of inactivation, loss of the amino acids tyrosine and lysine, and the gel filtration pattern. The loss of methionine has not been related to loss of enzyme activity. The ethyl arachidonate and malonaldehyde-inactivated RNase A also show similar fluorescence characteristics with emission maximum at 470 mμ and excitation maxima at 395 and 392 mμ, respectively.

The derivative of malonaldehyde and α-*N*-acetyl-L-lysine has the same fluorescent spectrum as those of arachidonate- and malonaldehyde-inactivated RNase A. This indicates that malonaldehyde reacts with the ε-amino groups of RNase A to form conjugated imines. That there is intramolecular cross-linking is evident in Figure 3b, which shows that peak A, corresponding to RNase A monomer, has relatively high fluorescence intensity as compared with that of peak B, a dimer which is formed as a result of intermolecular cross-linking. The losses of enzymic activity observed in the cross-linked enzymes are most likely related to alteration of functional groups, directly affecting the enzymic activity, but other possibilities such as restricted diffusion for substrate have not been eliminated.

The gel filtration pattern of ethyl arachidonate inactivated RNase A as shown in Figure 3a also suggests that malonaldehyde produced from the oxidation of the polyunsaturated ester could be the cross-linking agent as judged by their fluorescence characteristics. Since methyl linolenate inactivated RNase A also has the same fluorescence pattern, it can be deduced that the same oxidation product of both esters, that is, malonaldehyde, reacts with ε-amino groups in RNase A to give the conjugated imines. Malonaldehyde is the major water-soluble product in the oxidation of unsaturated lipids (Kwon and Olcott, 1966a,b). That malonaldehyde is the necessary cross-linking agent and that it inactivates RNase A is further indicated by the experiment with methyl oleate and methyl linoleate which did not inactivate the enzyme or produce fluorescence or polymerization of the enzyme. These experiments also ruled out the possibility that inactivation of RNase A was caused by lipid–protein interaction. The inactivation and cross-linking of RNase A by ethyl arachidonate and methyl linolenate is shown in Scheme I. Since intra- and intermolecular cross-linking are of the same chemical nature, it is not surprising that all the peaks in Figure 3a,b have the same fluorescence characteristics. Likewise, any proteins reacting with malonaldehyde would have the same spectral properties as that derived from α-*N*-acetyl-L-lysine and malonaldehyde, since in proteins, mostly the ε-amino groups will react with the aldehyde. Thus, malonaldehyde can be included

SCHEME I



as a bifunctional reagent for protein modification, a review of which was written by Wold (1967). Ceroid and lipofuscin or "age pigment" are generally considered as reaction products of lipid oxidation. Harman (1957) attributed the interaction of autooxidized lipid with protein as a possible factor in aging. Human cardiac age pigment was isolated and purified by Hendley *et al.* (1963), and lipid extracts of the heart homogenate showed fluorescence peaks at 450–470 mμ when excited at 365 mμ. The age pigment showed an inflection in the region 360–400 mμ of the absorption spectrum. Hydén and Lindström (1950) also found peaks of absorbance at 375 mμ and fluorescence at 440–460 mμ. These spectral properties are similar to those of the products obtained here and from the reaction of glycine, valine, or leucine with malonaldehyde (Chio and Tappel, 1969).

Some of the chemical properties of lipofuscin age pigment are given by Strehler and Mildvan (1962). The fluorescent component of the age pigment particles that are extractable with chloroform–methanol had chemical, infrared, and chromatographic properties suggesting that it was identical with or closely related to autooxidized cephalin. This present study could explain their observations. Oxidation of the polyunsaturated lipid of cephalin would result in the formation of malonaldehyde which could then react with the primary amino groups of the bases leading to the formation of the yellow, fluorescent compounds. Since membrane structures are rich in lipid materials, especially phospholipids, products of lipid peroxidation can cause extensive damage to cell structures and result in the formation of age pigments.

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