Changes in Lysozyme due to Reactions with Volatile Products of Peroxidizing Methyl Linoleate

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Previous studies have shown that lysozyme undergoes polymerization, loss of biological activity, and other deteriorative changes when exposed to incubation in air with methyl linoleate in a freeze-dried model system. In the present study we demonstrated that similar effects can be achieved by exposing protein to the headspace over peroxidizing methyl linoleate or to vapors of the volatile products of linoleate peroxidation, 2,4-decadienal, *n*-hexanal, and 2-heptenal. Changes in solubility, enzymatic activity, and polymer formation were studied. We also demonstrated, through the use of electron spin resonance (ESR), that volatile reaction products generate protein-centered free radicals when lysozyme is exposed to these products by being incubated over oxidizing linoleate. Water activity had a significant effect on the volatile-initiated changes in the protein. Cross-linking, loss of enzyme activity, and insolubilization increased with increasing water activity. ESR signal intensity was greatly diminished at high water activity, probably because of rapid recombination of free radicals.

Free radicals, carbonyl compounds, and other reactive species formed during oxidation of unsaturated lipids may interact with proteins to cause various changes. Studies have demonstrated loss of enzyme activity (Bernheim et al., 1952; Chio and Tappel, 1969; Kanazawa et al., 1975; Kanner and Karel, 1976; Matsushita, 1975), changes in membrane structures (Tappel, 1973), decreased protein solubility because of complex formation and polymerization (Kanner and Karel, 1976; Pokorny and Janicek, 1968; Roubal and Tappel, 1966; Sundholm et al., 1978), polypeptide chain scission (Takahashi, 1970; Zirlin and Karel, 1969), accelerated formation of brown pigments (Pokorny et al., 1975; Venolia and Tappel, 1958), and destruction of amino acid residues (histidine, lysine, cysteine, and methionine) (Braddock and Dugan, 1973; Gardner, 1979; Roubal and Tappel, 1966). Reactions between free amino acids and peroxidizing lipids have also been demonstrated (Gardner, 1979; Karel et al., 1975; Schaich and Karel, 1976; Yong and Karel, 1979).

Electron spin resonance (ESR) signals from free radicals produced in proteins were observed in proteins exposed to peroxidizing methyl linoleate (ML) or to peroxides of ML and other unsaturated fatty esters (Karel et al., 1975; Schaich and Karel, 1975). We observed that, when proteins were lyophilized from emulsions containing both the protein and the lipid and then exposed to incubation in air, free radicals of proteins were formed readily.

Hydroperoxides, the initial products of lipid peroxidation, decompose to give a variety of compounds which are capable of reacting with other food constituents. Many volatile compounds that originate from oxidized fatty acids (Ellis, 1961; Gaddis, 1961), their esters (Cobbs and Day, 1965; Howalh, 1966), and triglycerides (Gaddis, 1958) have been identified. Of these components, volatile aldehydes and ketones have received the most attention, partly because these carbonyl compounds are readily detected via their potent odors. They also have a strong capacity for undergoing reactions with other food constituents, with deleterious effect on food quality. Hexanal and 2,4-decadienal are the two major aldehydes expected from decomposition of the conjugated 9- and 13-hydroperoxides, the initial products of linoleate autoxidation; octanal, nonanal, 2-decenal, and 2-undecenal are the major aldehydes expected from oxidation of oleate. Aldehydes other than those theoretically expected have also been found in relatively large quantities in several studies.

Composition of the volatile fraction varies with oxidation conditions. For example, Nawar et al. (1978) reported a remarkable effect of temperature on the polar components (alkanals, alkenals, and dienals) produced from vegetable oils. The amounts of the decadienals present were significantly reduced when the oils were heated at 250 °C compared to heating at 185 °C. It is possible that these compounds are decomposed further at the higher temperature. Kimoto and Gaddis (1969) found that when ML was oxidized under mild conditions (room temperature in a thin layer), hexanal was the dominant carbonyl compound, along with C_7, C_8, and C_9 alk-2-enals, and trace amounts of 2,4-decadienal. On the other hand, when linoleate was oxidized at higher temperatures in alkaline media, the amount of 2,4-decadienal increased tremendously. Limited data are available on the contribution of these volatile compounds to reactions with proteins (Franzen and Kinsella, 1974; Gamage and Matsushita, 1973; Kanazawa et al., 1975; Matsushita, 1975).

We studied protein lysozyme (LYS) exposed to the volatile compounds produced during ML oxidation. We also investigated the presence of protein-free radicals formed through this interaction and the dependence of the above reactions on water activity.

MATERIALS AND METHODS

Overall Experimental Design. The experiments were designed to study the effects on LYS of exposure to oxidizing agents generated by lipid peroxidation, in one of the following conditions: (1) cooxidation in a system where LYS was mixed with ML; (2) exposure of LYS to volatile compounds produced by ML oxidation; and (3) exposure of LYS to vapor above pure aldehydes or above solutions of aldehydes in mineral oil.

Model Systems and Their Components. The lipid-free protein model system contained chicken egg white lysozyme (LYS) (three times crystallized; ICN Nutritional Biochemicals, Cleveland, OH) and was prepared by dispersing 1.5 mL of 1.34% (w/v) LYS in water (20 mg of LYS) on 12.5-cm ashless filter paper. Each application was followed by lyophilization in a Virtis 10 MRTR freeze-dryer for 24 h. The protein-free lipid system contained ML and was prepared by dispersing 1.5 mL of 50% (w/w) ML in hexane on similar filter papers and then vacuum-evaporating hexane in a desiccator over $CaSO_4$. Protein-

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lipid systems were prepared by emulsifying 5.33 g of ML with 1.34 g of LYS and 100 mL of water in a Sorval Omni-Mixer and then dispersing the emulsion (1.5 mL) on filter paper and lyophilizing. Hexanal, 2-heptenal, 2,4-decadienal, and mineral oil were used without further purification. LYS rods, used in studies involving ESR determinations, were prepared by placing approximately 0.15 mL of a solution of 40% LYS and 0.2% gelatin into 3 mm i.d. × 1.5 cm long polyethylene tubes and slow freezing (−20 °C) for 1 h. Once the solution froze, the rods were extruded into liquid N_2 and freeze-dried for 48 h. Each rod contained approximately 33.5 mg of LYS/cm and could be equilibrated to the desired water activity (a_w) by exposure over constant humidity solution. When we desired to place an ML layer on the LYS rods, the rods were immersed in a hexane solution of ML (0.5 g/mL) under vacuum and placed on tissues to remove excess surface solution. The solvent was removed by vacuum evaporation for 10 min. The lipid–protein rods contained approximately equal amounts of LYS and ML.

Incubation of Model Systems. To adjust their a_w values to desired levels, samples were equilibrated in desiccators at 37 °C over $CaSO_4$ ($a_w = 0$) or over saturated salt solutions, giving a_w values of 0.11 and 0.75. In preliminary experiments with aldehydes, LYS was exposed to the vapor phase in equilibrium with pure aldehyde. Lower vapor-phase concentrations of aldehydes were obtained by diluting pure aldehydes with mineral oil.

For experiments with volatile compounds formed in the ML oxidation, ML on filter paper and LYS on filter paper were placed in the same desiccator in such a way that the volatile compounds produced in the ML could reach the LYS only through the vapor phase. Effects of this exposure were compared to those of cooxidation in the LYS–ML system. All experiments were performed in the dark. In all cases, control samples of LYS system were incubated in desiccators containing no lipid, but maintaining the same temperature and a_w.

Determination of Incubation Effects on LYS. LYS systems were washed with hexane to eliminate lipid-derived volatile compounds absorbed in the paper. Samples exposed to the aldehydes were vacuum-treated at room temperature, to facilitate desorption of unreacted volatile carbonyl compounds, and washed again with hexane. Filter papers were cut in small pieces and the protein was extracted with distilled water (10 mL) during 4 h in the dark. The solution was filtered.

Protein was determined by absorption at 282 nm in a Hitachi-Perkin Elmer Model 200 spectrophotometer and by comparison with a standard curve obtained with LYS solutions of known concentrations. Enzyme activity was determined by using the Worthing lysozyme reagent set as follows: (a) dissolve 10 mg of lyophilized *Micrococcus lysodeikticus* in 100 mL of distilled water, (b) pipet 3 mL of the resulting suspension into a glass cuvette, (c) add 0.3 mL of protein solution (after 1:70 dilution in water) to the cuvette, mix by immersion, and measure absorption at 550 nm (A_{550}) after 30 and after 60 s. Enzyme activity was expressed in terms of the difference in A_{550} after 30 and 60 s. After freeze-drying and dilution, the samples were separated by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis, performed according to the method of Weber and Osborn (1969). Three hundred and seventy-five microliters of protein solution of adequate concentration was incubated at 37 °C for 1 h with 0.24 g of urea and 125 μL of 0.04 M Tris/glycine buffer (pH 8.9), which also contained 4.8% $NaDodSO_4$ and 1.2% dithiothreitol. Separating gels [10% acrylamide–0.5% *N,N'*-methylenebis(acrylamide) (Bis)] were prepared in 1 M Tris/HCl buffer (pH 8.9) and stacking gels (3% acrylamide–0.3% Bis) in 0.15 M Tris/PO_4H_3 buffer (pH 6.4). The running buffer was 0.04 M Tris/glycine (pH 8.9) containing 0.1% $NaDodSO_4$. Protein samples (0.3 mg/0.1 mL) were applied to the gels and run at a constant current of 1 mA/tube until the tracking dye (bromophenol blue) had migrated approximately half the length of the gel.

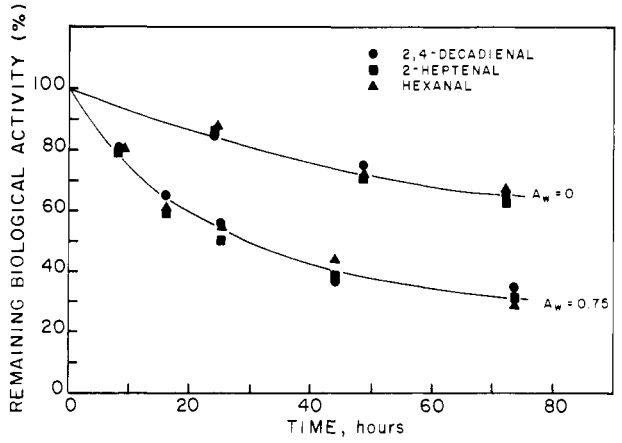

Figure 1. Loss of LYS biological activity during exposure to vapor over aldehydes, at two different water activities (a_w) at 37 °C.

The gels were removed from the tubes and proteins were fixed with MeOH/AcOH/H_2O (5:4:1) solution and stained with 1% Coomassie blue solution. The relative mobilities of the bands were calculated and compared to those of standard proteins to estimate the molecular weights of the fractions in each band. Destained gels were scanned colorimetrically at 550 nm in a Hitachi-Perkin Elmer UV-visual gel scanner. The areas were then integrated to obtain the percentages of each fraction. The insoluble fraction was determined by the difference between the amount of protein originally dispersed and that recovered after a quantitative extraction with distilled water. The reported percentage of each fraction was based on total protein content, including the insoluble fraction.

ESR Measurements. LYS rods were placed in desiccators which also contained either ML or pure aldehyde. The only contact between the rods and the lipid samples was through the vapor phase. All spectra were recorded by inserting the LYS rods without further treatment in the 3.5-mm i.d. quartz tubes, thus avoiding the problems of pulverizing solid samples, packing and unpacking powder, and variations in packing density. The ESR spectrometer was a Varian E-9 spectrometer operating in X band, equipped with Varian E-101 microwave bridge, E210A 100-kHz modulation unit, and E-231 multipurpose cavity TE_{102} (frequency, 9.45 GHz; microwave power, 10 mW; receiver gain, 8×10^3; detector current, 250 A; modulation amplitude, 12.5 G; time constant, 1 s; scan range, 100 G; and field set, 3370 G).

RESULTS

LYS dispersed on filter paper was incubated over pure *n*-hexanal, 2-heptenal, and *trans,trans*-2,4-decadienal at 37 °C, at a_w's of ~0 and 0.75. Figure 1 shows the effect of incubation on loss of LYS enzyme activity. The results are expressed as percent of the activity of lysozyme incubated in the absence of aldehyde vapor. Losses of activity of lysozyme incubated in the absence of the aldehydes were negligible during this experiment. The aldehydes dramatically reduced the biological activity. At the higher a_w, we observed extensive development of brown color, as is expected in the well-known carbonylamino nonenzy-

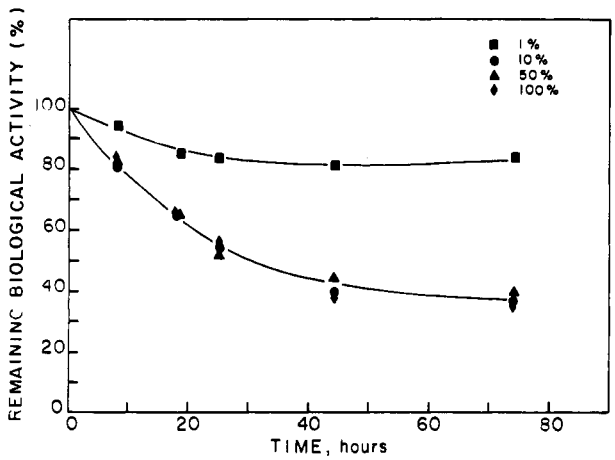

Figure 2. Loss of LYS biological activity during exposure to vapor over solutions of 2,4-decadienal in mineral oil at 37 °C. (Percentages refer to concentration of the aldehyde in mineral oil.)

Table I. LYS Aggregates Formed during LYS System Incubation at 37 °C Over a 1% Solution of 2,4-Decadienal in Mineral Oil

protein fraction	relative concentration, % incubation time and a_w						
	1 day		2 days		3 days	8 days	
	~0	0.75	~0	0.75	0.75	~0	0.75
monomer	66	59	50	47	44	50	36
dimer	12	20	20	22	24	20	29
trimer	6	8	6	10	13	9	10
tetramer		4	2	4	5	4	2
pentamer				2	4	1	
insoluble fraction	12	9	20	16	11	16	19

matic browning. At the low a_w, the loss of LYS biological activity occurred without apparent nonenzymatic browning and was qualitatively similar to that observed in LYS incubated in mixtures containing lipid hydroperoxides (Kanner and Karel, 1976). Results were quantitatively similar for each of the three aldehydes, despite differences in their vapor pressures and, hence, in their headspace concentrations.

In another experiment, we incubated the LYS system over different vapor-phase concentrations of 2,4-decadienal achieved by diluting the aldehyde with mineral oil. Figure 2 shows LYS enzymatic activity as a function of incubation time. Incubation over concentrations of 10% and 50% of the aldehyde had the same effect as incubation over the pure compound, but the effect was greatly reduced after incubation over a 1% solution. At the high concentrations, the reaction apparently is limited by factors other than aldehyde concentration in the vapor phase.

We also determined the increase in LYS cross-linking during incubation at a_w's of ~0 and 0.75 over a 1% solution of 2,4-decadienal in mineral oil. Table I gives those results and the percentages of different protein fractions determined by $NaDodSO_4$ gel electrophoresis. The percentage of the insoluble fraction was determined by weight; those of other fractions were estimated from the optical density of gels at 550 nm, as outlined in the Materials and Methods section. In the experiments reported in this table, the amount of LYS per sample was 200–300 μg/gel. We failed to detect any dimer in nonincubated controls at this concentration.

2,4-Decadienal from the vapor phase is capable of cross-linking lysozyme. The degree of cross-linking increases with increasing water activity. Similar results were

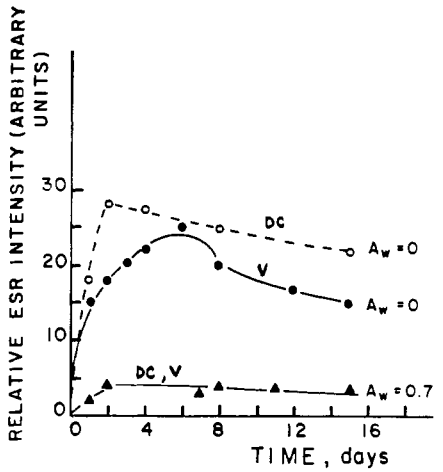

Figure 3. Changes in free-radical concentration in LYS rods incubated over peroxidizing ML, $(ML + LYS)_v$, or in direct contact with ML, $(ML + LYS)_{DC}$.

obtained by Kanner and Karel (1976) with linoleate hydroperoxides reacting with lysozyme, in which case the mechanism was attributed to formation of covalent bonds by a free-radical mechanism. Because of the similarity of effects, we studied free radicals in lysozyme exposed to the aldehyde.

After exposure of LYS rods to the volatile aldehydes, we detected ESR signals similar to those produced by exposure to γ radiation in air, to ML peroxides, and to high temperature. Exposure to volatile compounds formed by ML oxidation also produced similar signals. The ESR signals obtained were single lines with g values of 2.005 ± 0.005 with line width of 11 ± 3 G. We detected some signals at a_w = 0.75, a condition under which good ESR signals are usually difficult to obtain because of rapid recombination of free radicals. We believe the presence of ESR signals and their apparent rapid disappearance at a_w = 0.75 indicate a free-radical mechanism for LYS cross-linking.

Figure 3 shows changes in the intensity of ESR spectra of LYS rods exposed to vapor phase over oxidizing ML (curve labeled V). Similar changes in LYS rod oxidized in direct contact with ML are shown in curves labeled DC. The signal intensity changes with time and this change depends on water activity. Only extremely weak signals were detected at a_w = 0.75, probably because of rapid recombination of radicals. When those LYS rods exposed to volatile compounds at a_w = 0 were transferred to a_w = 0.75, the ESR signals disappeared rapidly, with concomitant rapid polymerization. When the rods were maintained at a_w = 0, ESR signals were detected after 3 months of storage at room temperature, and also after 15 days at 60 °C. Under these conditions, we were unable to detect any polymerization in these LYS rods. Having shown that pure aldehydes attack LYS, with effects similar to those in reactions between LYS and linoleate hydroperoxides, and that exposure to vapor over oxidizing ML produces ESR signals in LYS, we studied other effects on LYS of exposure to vapor over oxidizing linoleate. Figure 4 shows loss of LYS enzyme activity caused when oxidizing lipid is in intimate contact with the protein (curve DC) and when protein and lipid are connected only by the vapor phase (curve V). The curves labeled R show the relative contributions of the volatile compounds (expressed as percentages) to protein damage. They were obtained by considering the extent of change when ML and LYS are in the same filter paper as 100% change. The contributions of volatile compounds to enzyme damage increase

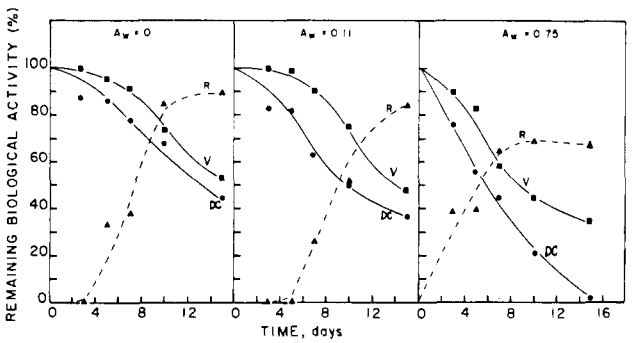

Figure 4. Comparison of effects on LYS biological activity of exposure at 37 °C to vapor over peroxidizing ML (curves labeled V) and direct contact with peroxidizing ML (curves labeled DC). Curves labeled R show the ratio (percent) of activity loss due to volatiles (vapor phase) to total activity loss (direct exposure).

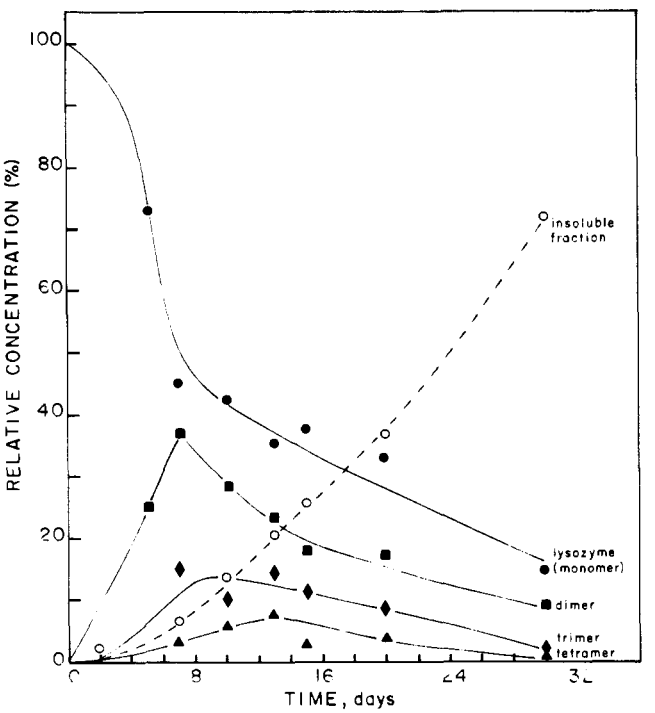

Figure 5. Effect of exposure at 37 °C and a_w = 0.75 to vapor over peroxidizing ML on formation of different cross-linked LYS fractions.

with time, as expected, considering their origin from hydroperoxide breakdown, which accelerates during oxidation.

Figure 5 shows the progress of LYS cross-linking as a function of time of exposure to volatiles over peroxidizing ML (incubation at 37 °C; a_w = 0.75). The different fractions were estimated by scanning gels after electrophoretic separation. The insoluble fraction was estimated gravimetrically. The relative concentration of each fraction is expressed in percent. In calculating the percentages, we assumed that the optical density of each soluble fraction was proportional to its weight, with the proportionality constant for all soluble fractions.

Figure 6 compares the course of cross-linking at three different water activities. The cross-linking is progressive and higher water activities promote it. The loss of enzyme activity and the increase in molecular weight show the same time pattern, but the loss of LYS monomer is not equal to loss of biological activity. Table II compares the retention of enzyme activity with the sum of the concentrations of monomer and dimer fractions. The biological activity per unit concentration of these fractions decreases,

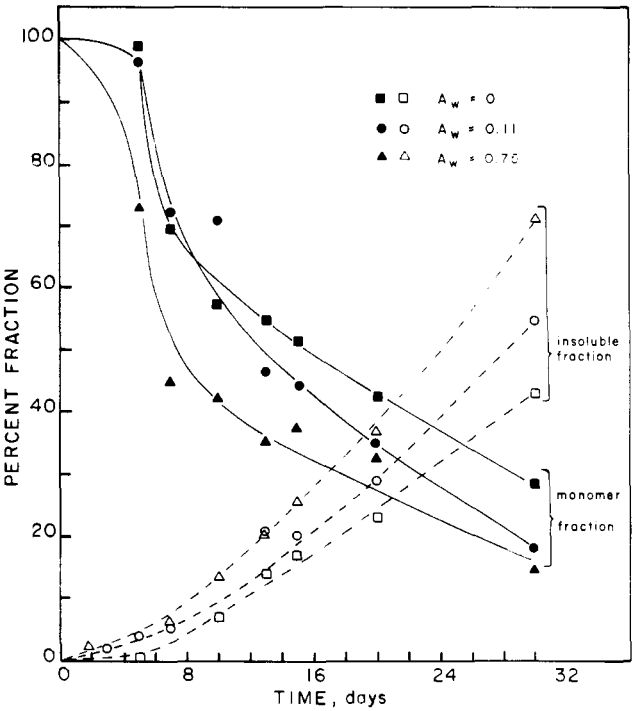

Figure 6. Effect of water activity on decrease in LYS monomer fraction and increase in LYS insoluble fraction during exposure to vapor over peroxidizing ML.

Table II. Retention of Enzyme Activity (A) and of Monomer Plus Dimer Fractions (B) during LYS Incubation Over Oxidizing ML at 37 °C

time, days	incubation at a_w = 0.75			incubation at a_w = 0		
	A, %	B, %	A/B	A, %	B, %	A/B
0	100	100	1.00	100	100	1.00
5	83.3	98.5	0.85	100	99.3	1.01
7	57.8	74.9	0.77	95.4	91.7	1.04
10	45.8	71.5	0.64	73.4	83.4	0.88
15	35.0	53.8	0.65	53.2	77.4	0.69
20	13.0	49.2	0.26	36.7	59.0	0.62

especially at the high water activity. Kanner and Karel (1976) reported that their results were consistent with a retention in dimer fraction of approximately 60% of enzyme activity as compared with the monomer. The data in Table II indicate, however, that a simple correlation between aggregation reactions and reactions leading to loss of enzyme activity is not justified by these results.

DISCUSSION

Previous studies have demonstrated that free radicals are involved in various lipid-peroxidation-initiated changes in proteins. The degree of contact necessary for the reactions to occur has never been firmly established, but it has been postulated that complex formation between radical-producing species (hydroperoxides) and proteins may precede the transfer of free radical from lipid to protein. Such complex formation would require intimate contact between the phase containing the lipid hydroperoxides (lipid phase) and the phase containing the proteins (aqueous phase). In fact, it has been suggested that most of such interactions would occur at interfaces. Membrane phospholipids are therefore particularly important for such interactions.

The present study shows that, in addition to protein damage from direct contact with lipid hydroperoxides, there is an important, and under some conditions dominant, mechanism of damage by volatile products of per-

oxidation which can be transported through the vapor phase (and, in the case of water-soluble compounds, undoubtedly also through the aqueous phase). Furthermore, these breakdown products can react through either the well-known nonenzymatic browning reactions or a free-radical mechanism analogous to that postulated for hydroperoxides (Schaich and Karel, 1976).

Some possible reactions, in addition to those known for nonenzymatic browning between proteins and carbonyl compounds, resulting from lipid oxidation, may include those indicated below.

$$V\cdot + PH \rightarrow P\cdot + VH \quad (1)$$

or

$$VH + PH \rightarrow [V\cdots PH] \rightarrow P\cdot + V\cdot + NR \quad (2)$$

or

$$VOOH + PH \rightarrow [VOOH\cdots PH] \rightarrow P\cdot + VO\cdot + H_2O \quad (3)$$

where VH = volatile breakdown product of hydroperoxides, PH = protein, and NR = nonradical fragments.

The protein cross-linking may involve direct incorporation of volatile (eq 4) or recombination of protein free

$$V\cdot + 2P\cdot \rightarrow P\text{-}V\text{-}P \quad (4)$$

radicals (eq 5). The mechanism in eq 5 seems most

$$P\cdot + P\cdot \rightarrow P\text{-}P \quad (5)$$

plausible, given the similarity of products observed when LYS is irradiated with γ rays in the *absence* of lipids to those obtained in the presence of either hydroperoxides or their products.

As expected, high water activities promote cross-linking by facilitating free-radical recombination.

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Lysinoalanine Formation in Yeast Proteins Isolated by Alkaline Methods

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The treatment of disrupted yeast cells with high concentration of alkali at elevated temperatures for the extraction of proteins with low nucleic acid content caused the destruction of amino acids and the formation of lysinoalanine. The generation of lysinoalanine was greater (i.e., 3.59 g/16 g of nitrogen) during the isolation of yeast proteins by a high-alkali, low-temperature (pH 12.5, 65 °C, 2 h) process than when isolated by a low-alkali, high-temperature (pH 10.5, 85 °C, 4 h) process (i.e., 0.49 g/16 g of nitrogen).

Because of their productivity on a variety of substrates, ease of production, and high protein content, microbial sources, particularly yeast, provide attractive supplementary sources of food protein (Tannenbaum and Wang, 1975; Kinsella and Shetty, 1978). However, it is not possible to exploit these sources in significant amounts unless the rigid indigestible cell wall is removed and the nucleic acid content is reduced to permissible levels (Kihlberg, 1972; Edozien et al., 1970; Miller, 1968; Waslien et al., 1970). Several methods have been proposed to reduce the nucleic acid content in yeast (Kinsella and Shetty, 1978). The method commonly recommended for the preparation of yeast protein isolate low in nucleic acid requires the treatment of ruptured cells with alkali at temperatures >60 °C (Cunningham et al., 1975; Hedenskog and Mogren, 1973; Lindblom, 1974; Newell et al., 1975; Vananuvat and Kinsella, 1975a). While this treatment is effective in reducing the nucleic acid, it results in denaturation of proteins and impairs functional properties (Vananuvat and Kinsella, 1975b). Furthermore it may

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