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# Comparative Methyl Linoleate and Methyl Linolenate Oxidation in the Presence of Bovine Serum Albumin at Several Lipid/Protein Ratios

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The oxidation of methyl linoleate (LMe) and methyl linolenate (LnMe) in the presence of bovine serum albumin (BSA) in the dark at 60 °C was studied to analyze the role of the type of fatty acid and the protein/lipid ratio on the relative progression of the processes involved when lipid oxidation occurs in the presence of proteins. The disappearance of the fatty acid, the formation of primary and secondary products of lipid peroxidation, the loss of amino acid residues, the production of oxidized lipid/amino acid reaction products, and the development of color and fluorescence were studied as a function of incubation time in protein/lipid samples at 10:1, 6:1, and 3:1 w/w ratios. The incubation of LMe and LnMe in the presence of BSA at 60 °C rapidly produced lipid peroxidation and protein damage. Although reaction rates were much faster for LnMe than for LMe, both fatty acids had similar behaviors, and LnMe seemed to be only slightly more reactive than LMe for BSA by producing a higher increase of protein pyrroles in the protein and the development of increased browning and fluorescence. The protein/lipid ratio also influenced the relative progress of the reactions implicated. Thus, a lower protein/lipid ratio increased sample oxidation and protein damage. This also produced an increased browning, in accordance with the mechanisms proposed for browning production by oxidized lipid/protein reactions. On the contrary, browning of extracted lipids increased at higher protein/lipid ratios. This opposite tendency allowed evaluation of the overall significance of the different browning processes implicated in the final colors observed, concluding that color changes observed in BSA/lipid samples were mostly a consequence of oxidized lipid/protein reactions.

**KEYWORDS: Amino acid losses; carbonyl−amine reactions; lipid oxidation; nonenzymatic browning; oxidized lipid/protein reactions; protein damage; pyrrole amino acids**

## INTRODUCTION

Lipid oxidation is a major cause of deterioration in foods and feeds, both in those containing substantial amounts of fats, such as lard and edible oils, and in those where only minor amounts of lipids occur, as in several vegetable products (*1*−*9*). Lipids in foods can be oxidized by both enzymatic and nonenzymatic mechanisms, the latter being the main reaction involved in the oxidative deterioration of lipids. Nonenzymatic oxidation proceeds via typical free radical mechanisms, and hydroperoxides are the initial products. They are relatively unstable and enter into numerous complex reactions involving substrate degradation and interaction, which results in myriad compounds of various molecular weights, flavor thresholds, and biological significance (*10*). The enzymatic oxidation of lipids takes place sequentially. Lipolytic enzymes release polyunsaturated fatty acids that are then oxidized by either lipoxygenase or cyclooxygenase to form hydroperoxides or endoperoxides, respectively. The next sequence of events involves enzymatic cleavage or transformation of hydroperoxides and endoperoxides to yield a variety of breakdown products in addition to a broad series of oxidized long-chain fatty acids, which are often responsible for the characteristic flavors of natural products and for some important physiological functions (*11*).

When these reactions take place in the presence of other food components, oxidative reactions (both enzymatic and nonenzymatic) can be terminated by reactions with compounds other than those originating from oxidation of the lipid substrate, and this can influence reaction rates and produce significant consequences in the color, flavor, and texture of foods. Particularly, the reaction of lipid oxidation products with amines, amino acids, and proteins has long been related to both the browning observed in many fatty foods during processing and storage (*12*) and the progressive accumulation of age-related yellow-brown pigments (lipofuscins) in man and animals (*13*). In addition, a direct relationship between fluorescence and browning has been found in diverse studies (*14*, *15*).

In an attempt to fully understand both the different processes involved when lipid oxidation occurs in the presence of proteins and the relative progression of the several reactions implicated,

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a recent investigation studied the oxidation of methyl linoleate (LMe) in the presence of bovine serum albumin (BSA) as a function of incubation time (*16*). As a continuation of this study, the present investigation was undertaken to analyze the role of the type of fatty acid and the protein/lipid ratio on the relative progression of the reactions implicated. The disappearance of the fatty acid, the formation of primary and secondary products of lipid peroxidation, the loss of amino acid residues, the production of oxidized lipid/amino acid reaction products (OLAARPs), and the development of color and fluorescence were studied as a function of incubation time in mixtures of BSA and LMe or methyl linolenate (LnME) at three protein/lipid ratios.

## EXPERIMENTAL PROCEDURES

**Materials.** LMe, LnMe, and essentially fatty-acid-free BSA were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and were purchased from reliable commercial sources.

**Sample Preparation.** Different mixtures of the fatty ester and BSA were triturated in a mortar until a homogeneous powder was obtained. The mixtures were prepared using three different protein/lipid weight/weight ratios: 10:1, 6:1, and 3:1. Triplicate mixtures (1 g) at each ratio were placed in Petri dishes and incubated for 6 days under air in the dark at 60 °C.

**Analytical Measurements.** At different periods of time, 100-mg samples were removed for analytical measurements. Lipids were extracted twice with 2 mL of chloroform/methanol (2:1), and lipid extracts were combined, evaporated, diluted with methanol to 1% solution, and analyzed for fatty ester disappearance, lipid oxidation, and color changes. Delipidated proteins were analyzed for color and fluorescence development, amino acid losses, and OLAARP formation.

Fatty acid analysis was carried out by capillary GC (*17*) using the methanolic solution and methanolic methyl heptadecanoate, which was added as internal standard.

Three determinations were used to evaluate lipid peroxidation: the absorbances at 234 and 270 nm and the thiobarbituric acid-reactive substances (TBARS) assay. Absorbances at 234 and 270 nm were measured using 0.01% solutions in methanol. Values are expressed as specific extinctions, conventionally indicated as $K_{234}$ and $K_{270}$ (*18*). The TBARS assay was carried out according to the method of Kosugi et al. (*19*) using the 0.01% solutions in methanol.

Protein damage was evaluated by determining amino acid losses and OLAARP formation. Amino acid analysis was carried out by hydrolyzing the delipidated proteins overnight in the presence of 6 N HCl. The produced amino acids were derivatized with diethyl ethoxymethylenemalonate and fractionated by HPLC using a previously described gradient (*20*, *21*). OLAARP formation was determined spectrophotometrically after derivatization of protein pyrroles produced with *p*-dimethylaminobenzaldehyde (*22*, *23*). Pyrrole determination is a good measurement of OLAARPs because these compounds have been shown to be produced both in the reaction of protein amino groups with many lipid oxidation products, including 4,5-epoxy-2-alkenals (*21*), 4-hydroxy-2-alkenals (*24*, *25*), unsaturated epoxyoxo fatty acids (*26*), and lipid hydroperoxides (*27*), and in the oxidation of fish microsomes in the presence of reactive oxygen species (*28*, *29*).

Color changes were determined spectrophotometrically in both lipid extracts and delipidated proteins. Lipid extracts were prepared at 1% in methanol, and delipidated proteins were solubilized in 6 M guanidine−HCl with 20 mM potassium phospate/trifluoroacetic acid, pH 2.3 (3 mg/mL). Colors in protein/lipid nonextracted mixtures were determined using a Minolta CR200 chromameter (Minolta Camera Co., Osaka, Japan). Yellowness index (YI) was expressed according to Francis and Clydesdale (*30*) as follows:

$$\mathrm{YI} = 142.86 \times (b^*/L^*)$$

Fluorescence spectra were recorded on a Perkin-Elmer LS-5 fluorescence spectrometer using 1 mg/mL solutions of delipidated proteins in 6 M guanidine−HCl with 20 mM potassium phosphate/trifluoroacetic acid, pH 2.3. A slit width of 5 nm was used, and the instrument was standardized with quinine sulfate (0.1 $\mu$M in 0.1 N $H_2SO_4$) to give a fluorescence intensity of 100 at 450 nm, when excitation was carried out at 350 nm (*31*).

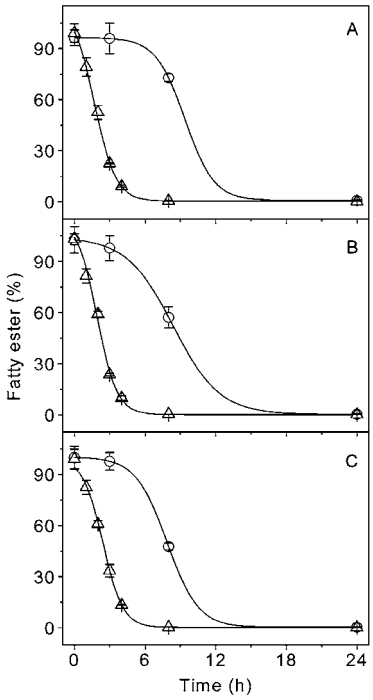


**Figure 1.** Time course of fatty ester disappearance in the lipid extracts isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

**Statistical Analysis.** All results are expressed as mean values ± standard deviations (SD) of three experiments except those for zero time, which are mean values of six experiments. Statistical comparisons among different groups were made using ANOVA. When significant *F* values were obtained, group differences were evaluated according to the Student−Newman−Keuls test (*32*). All statistical procedures were carried out using *Primer of Biostatistics: The Program* (McGraw-Hill, Inc., New York). Significance level is $p < 0.05$ unless otherwise indicated.

## RESULTS

**Lipid Peroxidation in the Protein/Lipid Model Systems.** As encountered in the previous study (*16*), the method described under Experimental Procedures allowed quantitative extraction of all lipid compounds present in the different incubations. Thus, after extraction, the lipids were 9.3 ± 0.4, 14.1 ± 0.5, and 24.2 ± 0.4% of the incubation mixture for the three lipid/protein ratios studied, respectively, and these initial values increased slightly with incubation time as a consequence of fatty acid oxidation.

Fatty acid oxidation took place very rapidly as could be observed by both fatty ester disappearance and production of oxidized derivatives. **Figure 1** shows the time course of fatty ester disappearance as determined by GC. The observed decreases for LMe and LnMe concentrations at the three lipid/protein ratios studied could be adjusted by using the Boltzmann equation (Microcal Origin, v. 4.10, Microcal Software, Northampton, MA)

$$y = [(A_1 - A_2)/(1 + e^{(x-x_0)/dx})] + A_2$$

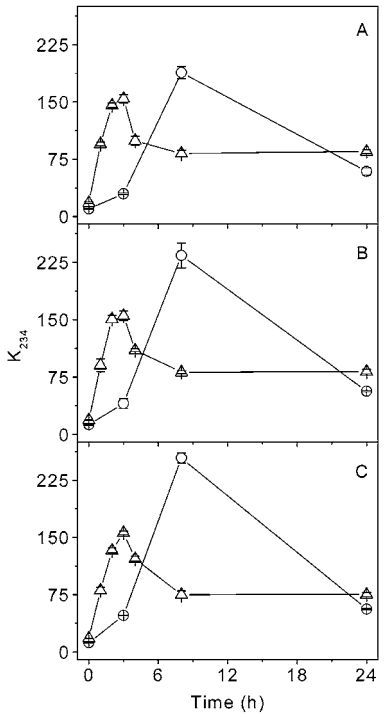


**Figure 2.** Time course of $K_{234}$ in the lipid extracts isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

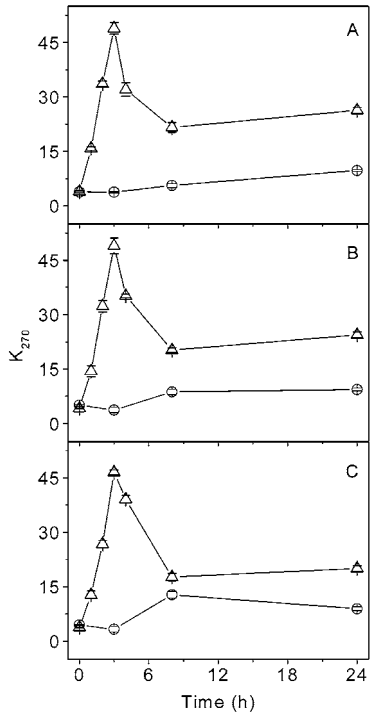


**Figure 3.** Time course of $K_{270}$ in the lipid extracts isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

where $A_1$ is the initial $y$ value, $A_2$ is the final $y$ value, $x_0$ is the $x$ value at $y_{50}$, and d$x$ is the width. This function produces a sigmoidal curve that adjusted very well the obtained values [$r = 1.0$, 1.0, and 1.0, respectively, for LMe at the three protein/lipid ratios studied (10:1, 6:1, and 3:1) and $r = 0.9998$, 0.998, and 0.9998, respectively, for LnMe at the same protein/lipid ratios]. The times for the disappearance of half of the fatty ester ($x_0$) calculated from the adjusted curves were 9.38, 8.38, and 7.86 h, respectively, for LMe at the three protein/lipid ratios studied (10:1, 6:1, and 3:1) and 1.67, 1.92, and 2.42 h, respectively, for LnMe at the same protein/lipid ratios. As expected, LnMe disappeared much more rapidly than LMe, but only small differences were observed among the different protein/lipid ratios assayed. In addition, the different protein/lipid ratios assayed seemed to affect oppositely LMe and LnMe. Thus, LMe disappeared more rapidly when a higher concentration of the lipid was present, and the shortest half-life of LnMe was observed for the 10:1 BSA/LnMe ratio.

Fatty ester disappearance was a consequence of fatty acid oxidation, which, in a first step, produced fatty acid hydroperoxides. **Figure 2** shows the time course of hydroperoxide formation and decomposition by determining $K_{234}$. For both fatty esters and at the three protein/lipid ratios assayed, $K_{234}$ increased for the first few hours to arrive at its maximum value and then decreased rapidly. Considering an adsorptivity for the LMe hydroperoxide of 26000 (*33*), the results obtained indicated the presence after 3 h of 3.7, 5.1, and 6.0% of hydroperoxides, respectively, for the 10:1, 6:1, and 3:1 BSA/LMe ratios assayed, and 23.6, 29.3, and 31.9% of hydroperoxides, respectively, after 8 h for the same BSA/LMe ratios. These values were analogous to the losses of LMe observed by GC for the 10:1 BSA/LMe ratio (27.2% after 8 h) but were lower than the data obtained by GC after 8 h for the 6:1 and 3:1 BSA/LMe (42.8 and 52.4%, respectively), suggesting that the maximum $K_{234}$ values for these last two BSA/LMe ratios should be achieved between 3 and 8 h and confirming that LMe oxidation was produced slightly more quickly at lower BSA/LMe ratios. $K_{234}$ values were lower for the BSA/LnMe incubations. Considering an adsorptivity for the LnMe hydroperoxide of 24600 (*33*), the results obtained indicated the presence after 1 h of 12.5, 11.9, and 10.6% of hydroperoxides, respectively, for the 10:1, 6:1, and 3:1 BSA/LnMe ratios assayed; 19.2, 19.8, and 17.5% of hydroperoxides, respectively, after 2 h for the same BSA/LnMe ratios; and 20.3, 20.4, and 20.6% of hydroperoxides, respectively, after 3 h for the same BSA/LMe ratios. All of these values were lower than LnMe losses observed by GC for the three times, suggesting that these hydroperoxides are very unstable and are decomposed very rapidly. Therefore, they were not present in any incubation at high concentration, and a lower concentration of hydroperoxides in BSA/LnMe incubations than in BSA/LMe incubations was found.

Fatty acid hydroperoxide decomposition was parallel to the increase observed in both $K_{270}$ and TBARS values. **Figure 3** shows the time course of $K_{270}$ during protein/lipid incubations. This figure shows only the changes produced during the first 24 h. After that time, most hydroperoxides were already decomposed and $K_{270}$ always increased more likely as a consequence of the formation of the conjugated products responsible for the brown color production in these reactions (*16*). For the first 24 h, $K_{270}$ was not lineal and increases and decreases with time were observed for both BSA/LMe and BSA/LnMe incubations, although they was more clearly observed in BSA/LnME incubations. These last incubations showed an almost linear increase of $K_{270}$ for the first 3 h [$r = 0.997$, $p = 0.0027$; $r = 0.994$, $p = 0.0061$; and $r = 0.986$, $p = 0.014$, respectively, at the three protein/lipid ratios studied (10:1, 6:1, and 3:1)], followed by a decrease during the next 5 h. After that time, a slow increase was initiated. This behavior was less clearly observed in BSA/LMe incubation mixtures, except for the 3:1 BSA/LMe ratio.

**Table 1.** Amino Acid Composition of Delipidated Proteins[a]

| | | $t = 24$ h | | | | | |
|---|---|---|---|---|---|---|---|
| | | BSA/LMe (ratio) | | | BSA/LnMe (ratio) | | |
| amino acid | $t = 0$ h | 10:1 | 6:1 | 3:1 | 10:1 | 6:1 | 3:1 |
| Ala | 0.70 ± 0.01 | 0.68 ± 0.01 | 0.71 ± 0.01 | 0.70 ± 0.01 | 0.72 ± 0.02 | 0.71 ± 0.01 | 0.72 ± 0.02 |
| Arg | 0.33 ± 0.01 | 0.34 ± 0.01 | 0.32 ± 0.01 | 0.33 ± 0.01 | 0.33 ± 0.01 | 0.34 ± 0.01 | 0.34 ± 0.01 |
| Asx[b] | 0.71 ± 0.04 | 0.67 ± 0.04 | 0.69 ± 0.03 | 0.68 ± 0.05 | 0.68 ± 0.04 | 0.70 ± 0.03 | 0.71 ± 0.02 |
| cystine | 0.25 ± 0.04 | 0.24 ± 0.03 | 0.23 ± 0.04 | 0.25 ± 0.02 | 0.26 ± 0.02 | 0.27 ± 0.02 | 0.26 ± 0.02 |
| Glx[b] | 1.08 ± 0.04 | 1.03 ± 0.03 | 1.05 ± 0.03 | 1.03 ± 0.04 | 1.04 ± 0.04 | 1.06 ± 0.03 | 1.08 ± 0.02 |
| Gly | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.27 ± 0.01 | 0.26 ± 0.01 |
| His | 0.25 ± 0.01 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.25 ± 0.01 | 0.25 ± 0.01 | 0.25 ± 0.01 | 0.25 ± 0.01 |
| Ile | 0.21 ± 0.01 | 0.22 ± 0.01 | 0.22 ± 0.01 | 0.22 ± 0.01 | 0.20 ± 0.01 | 0.20 ± 0.01 | 0.20 ± 0.01 |
| Leu | 0.89 ± 0.02 | 0.88 ± 0.01 | 0.89 ± 0.01 | 0.88 ± 0.01 | 0.88 ± 0.01 | 0.88 ± 0.01 | 0.89 ± 0.01 |
| Lys | 0.76 ± 0.01 | 0.73 ± 0.01 | 0.74 ± 0.02 | 0.72 ± 0.02 | 0.73 ± 0.02 | 0.73 ± 0.01 | 0.73 ± 0.01 |
| Met | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 |
| Phe | 0.42 ± 0.01 | 0.42 ± 0.01 | 0.42 ± 0.01 | 0.42 ± 0.01 | 0.42 ± 0.01 | 0.42 ± 0.01 | 0.43 ± 0.01 |
| Ser | 0.42 ± 0.01 | 0.41 ± 0.01 | 0.41 ± 0.01 | 0.42 ± 0.01 | 0.43 ± 0.01 | 0.43 ± 0.01 | 0.42 ± 0.01 |
| Thr | 0.50 ± 0.01 | 0.49 ± 0.01 | 0.49 ± 0.01 | 0.49 ± 0.01 | 0.50 ± 0.01 | 0.50 ± 0.01 | 0.51 ± 0.01 |
| Tyr | 0.30 ± 0.01 | 0.29 ± 0.01 | 0.29 ± 0.01 | 0.30 ± 0.01 | 0.30 ± 0.01 | 0.30 ± 0.01 | 0.31 ± 0.01 |
| Val | 0.52 ± 0.03 | 0.54 ± 0.02 | 0.54 ± 0.02 | 0.53 ± 0.02 | 0.51 ± 0.01 | 0.50 ± 0.02 | 0.51 ± 0.02 |

[a] Values are mean ± SD for three experiments (18 experiments for $t = 0$ h) and are given in micromoles per milligram of protein. [b] Asx, aspartic acid + asparagine; Glx, glutamic acid + glutamine.

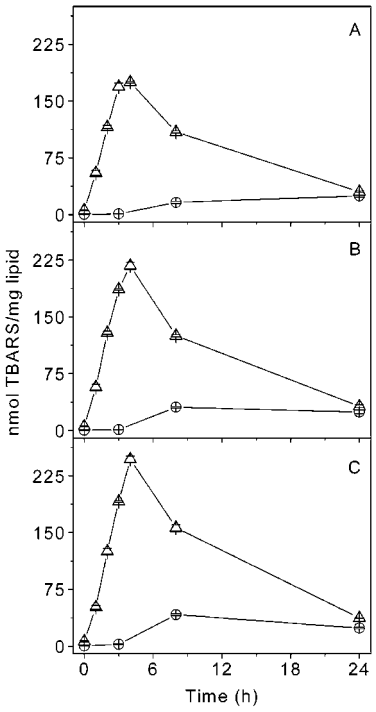


**Figure 4.** Time course of TBARS formation in the lipid extracts isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

**Figure 4** shows the time course of TBARS formation. Analogously to $K_{270}$ values, TBARS increased at the same time that lipid hydroperoxides were decomposed to arrive at a maximum value after which a more or less rapid decrease was observed. These highest values were observed after 8−24 h in BSA/LMe mixtures and after 3−4 h in BSA/LnMe mixtures. BSA/LMe mixtures exhibited a lag period for the first 3 h, after which time a rapid increase was observed to arrive at the maximum TBARS value. These values were higher at lower BSA/LMe ratios. The lag period was not observed in BSA/LnMe mixtures, more likely as a consequence of the reaction rate. However, similarly to BSA/LMe mixtures, TBARS values were also higher at lower BSA/LnMe ratios. In addition, TBARS values determined with BSA/LnMe mixtures were much higher than values obtained with BSA/LMe mixtures.

**Protein Damage in the Protein/Lipid Model Systems.** As expected, hydroperoxide decomposition not only produced the formation of secondary lipid oxidation products but also induced protein damage. Thus, the number of some amino acid residues recovered after acid hydrolysis decreased at the same time that OLAARP production was observed. **Table 1** shows the amino acids recovered after acid hydrolysis of the delipidated proteins. The main changes were observed in recovered lysine residues that decreased from 0.76 to 0.73 $\mu$mol/mg of BSA after 24 h of incubation, and no significant changes were observed for the two fatty esters or the three protein/lipid ratios assayed. Some of these losses of lysine residues can be explained by their transformation into pyrrole amino acid residues, according to the mechanism described for these reactions (*21*, *31*). **Figure 5** shows the time course of formation of pyrrole rings in the lysine residues of the protein. Protein pyrroles were produced very rapidly in BSA/LnMe mixtures, exhibiting the maximum values after 4 h, and more slowly in BSA/LMe mixtures, where the maximum values were observed after 24 h. For both BSA/LMe and BSA/LnMe incubation mixtures the pyrrole content found increased slightly with the decrease of protein/lipid ratio, and the increase of pyrrole derivatives observed during protein incubation was 0.3−0.4 nmol/mg of protein.

Simultaneously to the formation of pyrrole groups in the lysine residues, the protein developed fluorescence. **Figure 6** shows the time course of the development of fluorescence in the protein. Thus, fluorescence increased very rapidly in BSA/LnMe mixtures and more slowly in BSA/LMe mixtures. These time courses were similar to those obtained for protein pyrrolization. In fact, there was a correlation among pyrrole and fluorescence data [$r = 0.976$, $p = 0.024$; $r = 0.920$, $p = 0.080$; and $r = 0.969$, $p = 0.031$, respectively, for BSA/LMe at the three assayed protein/lipid ratios (10:1, 6:1, and 3:1); and $r = 0.864$, $p = 0.012$; $r = 0.857$, $p = 0.014$; and $r = 0.899$, $p = 0.0059$, respectively, for BSA/LnMe at the same assayed ratios]. This is in agreement with the mechanism proposed for fluorescence production in these reactions (*21*, *31*, *34*).

**Color Changes in the Protein/Lipid Model Systems.** As a consequence of the above-described processes, the formation of brown color was observed. Both lipid and protein developed

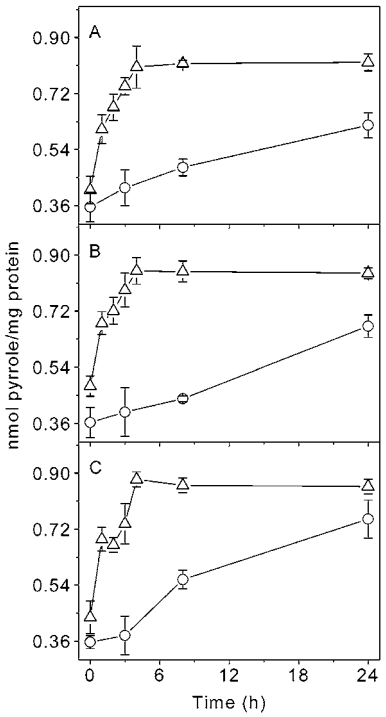


**Figure 5.** Time course of protein pyrroles determined in the delipidated proteins isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

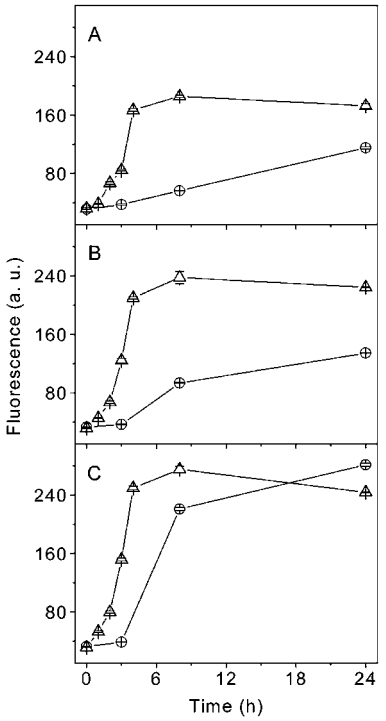


**Figure 6.** Time course of fluorescence development in the delipidated proteins isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

color changes that were parallel but were oppositely influenced by the protein/fatty ester ratio. **Figure 7** shows the time course of YI for the lipid extracts as a function of the incubation time. Because color changes were more slowly produced than either

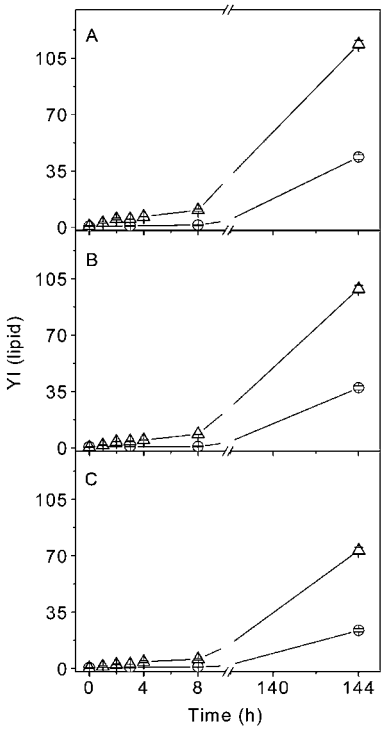


**Figure 7.** Time course of YI in the lipid extracts isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

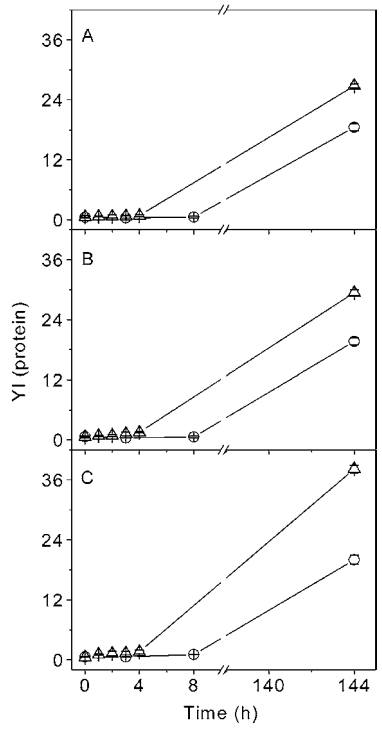


**Figure 8.** Time course of YI in the delipidated proteins isolated from BSA/LMe (○) and BSA/LnMe (△) mixtures incubated in the dark at 60 °C and using (A) 10:1, (B) 6:1, and (C) 3:1 protein/lipid (w/w) ratios. Data are mean values ± SD of three independent experiments.

the lipid oxidation or the protein damage, the incubation mixtures were maintained for 6 days at 60 °C. During the whole incubation period, lipid fractions obtained from BSA/LnMe samples always were darker than those obtained from BSA/LMe samples. In addition, the darker lipid samples were

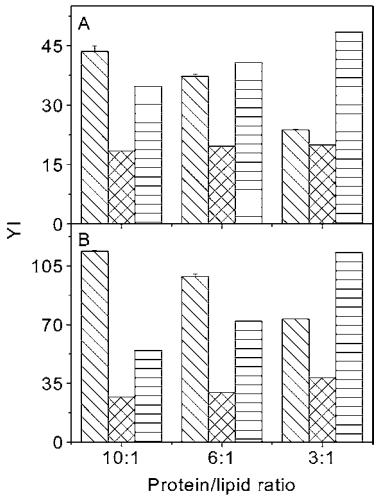


**Figure 9.** YI of lipid extracts (slashed bars), delipidated proteins (cross-hatched bars), and nonextracted samples (horizontally striped bars) of (A) BSA/LMe and (B) BSA/LnMe mixtures incubated for 6 days in the dark at 60 °C. Data are mean values ± SD of three independent experiments.

obtained for the higher protein/lipid ratios. A similar behavior, but opposite results for protein/lipid ratios, was obtained for the delipidated protein (**Figure 8**). In this case the higher YI values were obtained for the lower protein/lipid ratios.

This different behavior as a function of the protein/lipid ratio allowed the color produced as a consequence of the polymerization reactions of lipid oxidation products to produce brown-colored oxypolymers (*35*) to be distinguished from the color produced by reactions between lipid oxidation products and the protein (*36*). **Figure 9** shows the YI obtained after 6 days in the extracted lipids, the delipidated proteins, and the nonextracted samples as a function of the protein/lipid ratio. Analogously to the delipidated protein, YIs in the nonextracted samples were higher at lower protein/lipid ratios.

## DISCUSSION

The incubation of LMe and LnMe in the presence of BSA at 60 °C rapidly produced lipid peroxidation and protein damage. As expected, LnMe was much more easily oxidized than LMe, and all of the processes were accelerated when compared with the reactions produced by the latter fatty ester. In addition, LnMe gave lower $K_{234}$ and higher TBARS values than LMe. Both results are not necessary contradictory. The former indicated only that LnMe hydroperoxides never achieved the concentration of LMe hydroperoxides, more likely because of the higher instability of LnMe hydroperoxides. In addition, the big differences found in TBARS values between BSA/LnMe and BSA/LMe reactions may be a consequence of a higher fluorescence quantum yield of TBARS derived from LnMe oxidation products. In fact, TBARS are easily produced during LnMe oxidation and much less easily from LMe oxidation (*37*). By analyzing protein pyrrolization and fluorescence development results, LnMe seemed to be only slightly more reactive than LMe for BSA, because no large differences were found among the results obtained with the two fatty esters.

The protein/lipid ratio also influenced the relative progress of the reactions implicated. Thus, a lower protein/lipid ratio increased sample oxidation, which produced higher TBARS values in the extracted lipids and higher pyrrole contents and fluorescence development in the delipidated proteins. This tendency, which is likely a consequence of the antioxidative activity described for the proteins (*38*, *39*), was also observed in nonextracted sample colors and in the color of the delipidated proteins. All of these results are in accordance with the mechanisms proposed for these reactions by which a higher oxidation implies a higher protein damage and, therefore, a higher color and fluorescence development (*36*).

In contrast to delipidated proteins, the YI of extracted lipids increased at higher protein/lipid ratios, suggesting that colors developed in lipids are not uniquely related to oxidative processes. Furthermore, this opposite tendency allowed evaluation of the overall importance of the different browning processes implicated in the final colors observed. The results obtained in this study suggest that the changes produced in the color of protein/lipid samples were mostly a consequence of oxidized lipid/protein reactions, which are responsible for the color changes produced in the protein, and not a consequence of polymerization of lipid oxidation products.

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