Arai, S., Noguchi, M., Yamashita, M., Kato, H., Fujimaki, M., *Agr. Biol. Chem.* **34**, 1569 (1970).
Arai, S., Suzuki, H., Fujimaki, M., Sakurai, Y., *Agr. Biol. Chem* **30**, 364 (1966a).
Arai, S., Suzuki, H., Fujimaki, M., Sakurai, Y., *Agr. Biol. Chem.* **30**, 863 (1966b).
Badenhop, A. F., Wilkens, W. F., *J. Amer. Oil Chem. Soc.* **46**, 179 (1969).
Bailey, L. H., Capen, R. G., LeClerc, J. A., *Cereal Chem.* **12**, 441 (1935).
Berczeller, L., U. S. Patent 1,509,076 (Sept 16, 1924).
Cotton, R. H., *J. Amer. Oil Chem. Soc.* **51**, 116A (1974).
Cowan, J. C., Rackis, J. J., Wolf, W. J., *J. Amer. Oil Chem. Soc.* **50**, 426A (1973).
Ehle, S. R., Jansen, G. R., *Food Technol.* **19**, 1435 (1965).
Eldridge, A. C., Kalbrener, J. E., Moser, H. A., Honig, D. H., Rackis, J. J., Wolf, W. J., *Cereal Chem.* **48**, 640 (1971).
Finney, K. F., Bode, C. E., Yamazaki, W. T., Swickard, M. T., Anderson, R. B., *Cereal Chem.* **27**, 312 (1950).
Fujimaki, M., Arai, S., Kirigaya, N., Sakurai, Y., *Agr. Biol. Chem.* **29**, 855 (1965).
Gardner, H. W., *J. Agr. Food Chem.* **23**, 129 (1975).
Gremli, H. A., *J. Amer. Oil Chem. Soc.* **51**, 95A (1974).
Grosch, W., Schwencke, D., *Lebensm. Wiss. Technol.* **2**, 109 (1969).
Haas, L. W. (to J. R. Short Milling Co.), U. S. Patent 1,957,334 (May 1, 1934).
Haas, L. W., Bohn, R. M. (to J. R. Short Milling Co.), U. S. Patent 1,957,333 (May 1, 1934).
Hand, D. B., Steinkraus, K. H., Van Buren, J. P., Hackler, L. R., el Rawi, I., Pallesen, H. R., *Food Technol.* **18**, 1963 (1964).
Hayes, L. P., Simms, R. P. (to A. E. Staley Mfg. Co.), U. S. Patent 3,734,901 (May 22, 1973).
Holman, R. T., Bergstrom, S., "The Enzymes, Chemistry and Mechanism of Action," Vol. II, Part 1, Academic Press, New York, N.Y., 1951, Chapter 60, pp 559–580.
Kalbrener, J. E., Eldridge, A. C., Moser, H. A., Wolf, W. J., *Cereal Chem.* **48**, 595 (1971).
Kalbrener, J. E., Warner, K., Eldridge, A. C., *Cereal Chem.* **51**, 406 (1974).
Kleinschmidt, A. W., Higashiuchi, K., Anderson, R., Ferrari, C. G., *Baker's* Dig. 37(5), 44 (1963).
Kon, S., Wagner, J. R., Guadagni, D. G., Horvat, R. J., *J. Food Sci.* **35**, 343 (1970).
Logan, J. L., Learmonth, E. M., *Chem. Ind. (London)*, 1220 (1955).
Mattick, L. R., Hand, D. B., *J. Agr. Food Chem.* **17**, 15 (1969).
Meyer, E. W., *J. Amer. Oil Chem. Soc.* **48**, 484 (1971).
Moser, H. A., Evans, C. D., Campbell, R. E., Smith, A. K., Cowan, J. C., *Cereal Sci. Today* **12**, 296 (1967).
Mustakas, G. C., private communication (1974).
Mustakas, G. C., Albrecht, W. J., Bookwalter, G. N., McGhee, J. E., Kwolek, W. F., Griffin, E. L., Jr., *Food Technol.* **24**, 1290 (1970).
Mustakas, G. C., Albrecht, W. J., McGhee, J. E., Black, L. T., Bookwalter, G. N., Griffin, E. L., Jr., *J. Amer. Oil Chem. Soc.* **46**, 623 (1969).
Nielsen, K., *J. Amer. Oil Chem. Soc.* **37**, 217 (1960).
Nelson, A. I., Wei, L. S., Steinberg, M. P., *Soybean Dig.* **31**(3), 32 (1971).
Ofelt, C. W., Smith, A. K., Evans, C. D., Moser, H. A., *Food Eng.* 24(12), 145 (1952).
Paulsen, T. W. (to Archer Daniels Midland Co.), U. S. Patent 3,100,709 (Aug 13, 1963).
Pringle, W., *J. Amer. Oil Chem. Soc.* **51**, 74A (1974).
Rhee, K. S., Watts, B. M., *J. Food Sci.* **31**, 664 (1966).
Sessa, D. J., Honig, D. H., Rackis, J. J., *Cereal Chem.* **46**, 675 (1969).
Sessa, D. J., Warner, K., Honig, D. H., *J. Food Sci.* **39**, 69 (1974).
Shemer, M., Wei, L. S., Perkins, E. G., *J. Food Sci.* **38**, 112 (1973).
Tsen, C. C., Hoover, W. J., *Cereal Chem.* **50**, 7 (1973).
Wilkens, W. F., Lin, F. M., *J. Agr. Food Chem.* **18**, 333 (1970).
Wilkens, W. F., Mattick, L. R., Hand, D. B., *Food Technol.* **21**, 1630 (1967).
Wolf, W. J., Cowan, J. C., *Crit. Rev. Food Technol.* **2**, 81 (1971).
Wood, J. C., *Food Mfr.* (Ingredient Survey), 11–15 (Jan 1967).

Received for review June 11, 1974. Accepted July 20, 1974. Presented at the Symposium on Effects of Oxidized Lipids on Food Proteins and Flavor, Division of Agricultural and Food Chemistry, 167th National Meeting of the American Chemical Society, Los Angeles, Calif., April 1974. Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Effects of Lipoperoxides on Proteins in Raw and Processed Peanuts

Allen J. St. Angelo* and Robert L. Ory

Oxidative degradation of unsaturated lipids in peanuts produces hydroperoxides and their subsequent breakdown products, acids, alcohols, aldehydes, and ketones. These compounds have been reported by others to damage proteins, enzymes, and amino acids. In the present investigation, lipid–protein interaction was examined in deoiled meals and in the proteins extracted from raw and roasted whole peanuts and peanut butter. Polyacrylamide electrophoresis was used as the principal technique to compare proteins before and after storage under conditions designed to promote peroxidation of lipids. Disc gels of deoiled residues from peanuts were stained for protein and lipid. The Sudan stains, which are used extensively for detecting liproproteins in mammalian tissues, were not sensitive enough to detect the small amount of lipid bound to peanut proteins, but Rhodamine 6G and Oil Red O were satisfactory. Details of these procedures and observations on the effects of peroxidized lipid–protein interactions on electrophoretic mobility and on solubility of various protein fractions are discussed.

Peroxidation of fatty acids has long been a concern to academia and food industry because lipid peroxides are involved in the development of rancidity in foods containing unsaturated fatty acids, the production of "off" odors and flavors, and the production of toxic or physiologically active compounds that can damage proteins, enzymes, and amino acids. Lipid peroxidation involves a free-radical mechanism, initiated by autoxidation, that can be catalyzed by either metalloproteins or enzymes, to form fatty acid hydroperoxides. Once initiated, the reaction is self-propagating, forming more hydroperoxide and more free radicals and/or breakdown products, depending upon the conditions. The products formed can complex with amino acids, proteins, or enzymes.

Lipid–protein complexes are believed to be held together either by electrostatic (ionic) attractions, as reported by Green and Fleicher (1963), by hydrogen bonding, van der Waals interactions, or hydrophobic interactions, considered by Nêmethy (1967) to be the main type of bonding between lipids and proteins *in vivo*. Covalent bonds between lipids and proteins in natural systems are uncom-

Southern Regional Research Center, one of the facilities of the Southern Region, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70179.

mon. On the other hand, Tappel (1965) and Roubal and Tappel (1966) have reported that peroxidized or autoxidized lipids can form rather stable complexes with mammalian proteins, to ultimately yield insoluble lipid-protein complexes. Many more studies on the formation of autoxidized lipid-protein complexes have been reported in mammalian systems than in plant systems.

Enzymatic catalysis by lipoxygenase has been confirmed only in plant systems. (Reports of lipoxygenase in animal tissues have been confused by the presence of heme-type catalysis.) Legumes are a major source of lipoxygenase although it has been reported in numerous plant sources (Pinsky *et al.*, 1971). We have reported partial purification and some properties of the enzyme in peanuts (St. Angelo and Ory, 1972a,b), but the effects of lipid peroxidation on peanut proteins were not examined. The present work was undertaken to determine: (1) if lipid peroxides and their breakdown products combine with specific proteins in raw and roasted peanuts, or if they combine randomly with all proteins; (2) if the addition of oxidized lipids or their breakdown products cause changes in the major proteins, arachin and conarachin, detectable by disc gel electrophoresis; and (3) if these oxidized lipids alter the physical properties of the proteins (*e.g.*, solubility, electrophoretic mobility).

MATERIALS AND METHODS

Peanut Samples. Virginia, Spanish, and Runner peanuts (1972 crop) were obtained from various commercial suppliers. Peanut butters were commercial brands. For roasted samples, Spanish and Runner peanuts were roasted in a laboratory forced-draft oven at 150–175° for 25–30-min periods and stored as whole nuts.

Extraction Procedures. Fractionation of oil and proteins and solubilization of proteins from deoiled meals were performed as follows. Accurately weighed (to 0.1 mg) samples of raw or roasted peanuts or peanut butters were deoiled with hexane in a 1:5 (w/v) ration, then centrifuged at 14,000*g* for 30 min. The hexane fraction was saved and the meal was reextracted with hexane and centrifuged. The supernatants were combined and used for determination of conjugated diene hydroperoxide (CDHP) contents in the oils. After drying, the deoiled meals were extracted with pH 7 buffered 10% sodium chloride (NaCl) in a 1:10 (w/v) ratio for 1 hr under constant stirring and then centrifuged as before. The salt-soluble protein extracts were dialyzed against deionized water and then lyophilized to obtain the proteins free of salt and small molecules. The salt-insoluble residues were resuspended in water, dialyzed, and lyophilized to obtain the salt-free residue consisting of protein, cell wall debris, etc. Samples of the deoiled meal, the salt-soluble proteins, and the salt-insoluble residues were analyzed by macro-Kjeldahl for nitrogen. Protein is expressed as N × 5.46.

Determination of Peroxide Content in Extracted Oils. Peroxidation in oil samples from all peanut products was determined by the CDHP (conjugated diene hydroperoxide measured at 234 nm) method described earlier (St. Angelo *et al.*, 1972b; St. Angelo and Ory, 1973).

Gel Electrophoresis of Proteins. Salt-soluble protein samples were dissolved in 0.1 *M* Tris-HCl buffer (pH 7.2) and examined by polyacrylamide gel electrophoresis according to Weber and Osborn (1969) and Cherry *et al.* (1970), but with four modifications: (a) each fraction was examined at protein concentrations from 0.3 to 0.8 mg/gel to determine major and minor protein bands; (b) protein samples electrophoresed were extracted from deoiled meal instead of from homogenized and centrifuged whole seeds; (c) three drops of a 40% sucrose solution were added to each tube instead of capping the upper gel with a thin strip of lower gel; (d) the glass gel tubes were 86 mm long × 5 mm i.d.; the running gel was 76 mm long.

Salt-insoluble residues were dissolved in Tris-HCl buffer containing 1% sodium dodecyl sulfate (SDS) and examined by polyacrylamide gel electrophoresis according to Shapiro *et al.* (1967) and Cherry (1974), but with slight variations: (a) 7.5% acrylamide gels were used; (b) neither gels nor solutions contained β-mercaptoethanol; (c) samples were electrophoresed for about 3.5 hr (or until the Bromophenol Blue tracking dye had migrated to the end of each tube); (d) prior to staining, gels were fixed with a solution containing 25% isopropyl alcohol–15% trichloroacetic acid for 16 hr at room temperature; (e) Tris-HCl buffer was used instead of phosphate.

Staining Techniques. For protein banding patterns, gels were stained overnight with Amido Black. For detecting lipids, either Oil Red O or Rhodamine 6G was used. Oil Red O was prepared by suspending 500 mg in 500 ml of 70% methanol and warming to 50° with stirring. This solution was then made to 15% (w/v) with trichloroacetic acid and stored at 37°. Rhodamine 6G was prepared by dissolving 10 mg in 100 ml of 0.05 *M* sulfurous acid and then stored at room temperature. The gels were stained by incubating them in either solution overnight at 37°. All gels, except those stained with Rhodamine 6G, were destained by soaking with frequent changes in 7% acetic acid.

RESULTS AND DISCUSSION

Enzymatically catalyzed peroxidation in raw peanuts is a more specific process than the hemoprotein or autoxidation processes that appear to take place in roasted nuts. St. Angelo *et al.* (1972a) showed that in raw peanuts, lipoxygenase attacks linoleic acid specifically at the C-13 position. Owing to this specificity, the types of peroxidized end products bound to proteins in raw peanuts should be less complex than those found in roasted peanuts or in mammalian systems catalyzed by autoxidation or metalloproteins. The linoleic acid hydroperoxide formed by peanut lipoxygenase will cleave at the C-13 position to produce hexanal, a carbonyl compound that can also combine with certain protein functional groups.

In our long-term storage studies on whole raw undamaged peanuts, we found that the rate of peroxidation was too slow to show significant changes. Therefore, in order to promote peroxidation of lipids in raw peanuts and to enhance any possible reaction with the protein, raw Virginia peanuts were ground in a food blender, sampled, and stored under different conditions. Sample 1 was analyzed immediately after grinding in a food blender; sample 2 was stored in an open jar at 30°; sample 3 was ground, then manually blended with 10% (w/v) rancid vegetable oil and stored in an open jar at 30°. (The open jars were covered with cheesecloth and kept in a closed metal cabinet.) The data in Table I are averages of five experiments in which weights varied from 5 to 12 g.

Peanuts assayed prior to storage formed less total hydroperoxide (CDHP) than those stored at 30°, and less than those blended with rancid oil. In samples 1, 2, and 3, the percentage of deoiled meal recovered increased from 53.90 to 57.28 after 3 months storage, as peroxidation increased. While this increase is not statistically significant, this trend was apparent in all experiments, suggesting that the weights of defatted meals increase with the increased peroxidation of the oil. Recoveries of salt-insoluble residues from these meals decreased slightly while salt-soluble material increased. Per cent protein of the salt-soluble fraction was virtually unchanged, despite an increase in total recovered material (Table I, part B). Roubal and Tappel (1966) reported that free radicals formed from lipid peroxides can induce protein–protein interaction. This might explain the apparent increase in solubilized protein from the salt-insoluble residue, but the increases in total material recovered and in the salt-soluble fraction suggest that protein being solubilized may be

Table I

Sample	% oil removed	% deoiled meal	CDHP,[a] A units	% protein[b] in meal
(A) Effects of Lipid Peroxidation on Protein Solubility in Raw Peanuts after Storage for 3 Months				
1(control)	46.10	53.90	0.53	46.64
2	45.09	54.91	1.21	44.47
3	42.72	57.28	1.89	44.16

				Protein content, %	
Sample	NaCl-insol. residue, %	NaCl-sol. fraction, %	Total recovd, %	Salt insol.	Salt sol.
(B) Recoveries after NaCl Extraction of Deoiled Meals[c]					
1(control)	37.48	31.60	69.08	22.99	88.48
2	35.80	36.55	72.35	18.96	87.86
3	34.30	39.17	73.47	17.29	88.01

[a] Absorbency at 234 nm. [b] N × 5.46. [c] Values shown are those after dialysis and lyophilization.

combining with small amounts of nonprotein material (*e.g.*, peroxides or their breakdown products).

Similarly, as peroxidation increased during storage of peanut butter, recovery of meal after hexane extraction increased slightly (Table II). As anticipated, salt extraction of these deoiled meals yielded large amounts of insoluble residue and a very low amount of soluble materials. The total weights of materials recovered again showed slight increases in total yield with increased peroxidation. In contrast, the per cent meal removed from whole roasted peanuts and the salt-soluble fractions decreased in each case. The reason for this decrease is not known. It could be that the roasted peanuts were not homogenized as were the raw nuts and peanut butters, but were stored as whole seeds. Comparing the yields of total protein in salt extracts of deoiled meals from peroxidized raw peanuts (Table IB) with those from roasted peanuts (Table IIB), the per cent protein was 88% for raw peanuts and 17% for roasted. Protein contents for salt-insoluble residues were 19% for raw peanuts and 56% for roasted.

The weights of salt-insoluble residues from deoiled meals of whole roasted peanuts also decreased with increased oxidation (similar to that for raw peanuts) as did the per cent protein, but the yield of salt-soluble extract remained about the same. However, the per cent protein in salt-soluble fractions showed a very large increase. These results from roasted peanuts and peanut butters suggest that the greater changes caused by peroxidized lipids may be found in the salt-insoluble material.

These proteins were examined further by gel electrophoresis. Initial attempts to use the Sudan dyes (developed for staining mammalian lipoproteins) to detect lipid–protein bands in the gels proved to be inadequate for peanut products, so that other lipid stains were examined. The most promising were Rhodamine 6G and Oil Red O.

Rhodamine 6G has been used as a histochemical counterstain for lipids in mixtures of plasmalogens on paper chromatograms (Hack and Helmy, 1967). Although it is a nonspecific lipid stain that produces a pink-red color under visible light, Rhodamine 6G stained lipids were best visualized under ultraviolet (uv) illumination (Helmy *et al.*, 1967). Because of the minor quantities of lipid bound to peanut proteins, the procedure of Hack and Helmy (1967) was modified by incubating the gels in the staining solution for 16 hr at 37° and then observing the bands under both visible and uv light. The gels were not destained since only lipid bands absorbed the dye.

When diffuse light was placed under the gels, easily distinguishable bright red bands were seen and the background had a much lighter pink color. However, under uv light, the background appeared brilliant yellow and lipid bands were a bright fluorescent red.

Table II

Sample	Months stored	Hexane extraction: % oil removed[a]	Hexane extraction: % meal removed	CDHP units[b]
(A) Effects of Lipid Peroxidation on Protein Solubility in Roasted Peanut Products				
Peanut butter	0	49.46	50.54	7.272
Peanut butter	1	48.95	51.05	11.377
Whole peanuts	0	49.21	50.79	6.536
Whole peanuts	12	51.25	48.75	38.930

		NaCl extraction			% protein[d]	
Sample	Months stored	% NaCl residue	% NaCl soluble	% total recovd	NaCl res.	NaCl sol.
(B) Recoveries after NaCl Extraction of Deoiled Meals[c]						
Peanut butter	0	53.6	13.8	67.4		
Peanut butter	1	53.3	15.5	68.8		
Whole peanuts	0	61.23	17.22	78.45	55.80	78.08
Whole peanuts	12	55.80	17.06	72.86	53.51	90.20

[a] Calculated by differences; original sample weight less meal yield. [b] Millimoles of conjugated diene hydroperoxide/gram of peanut butter or peanuts. [c] Values shown are those after dialysis and lyophilization. [d] N × 5.46.

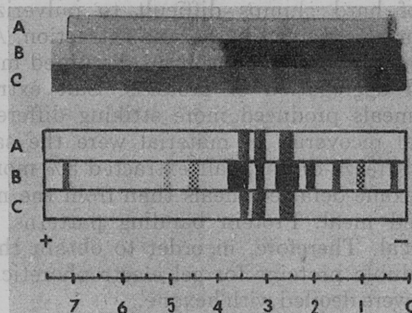

Figure 1. Polyacrylamide gel electrophoretic patterns of salt extracts from raw peanut meal stained with (A) Oil Red O, (B) Amido Black, and (C) Rhodamine 6G.

Figure 1 shows three stained gels photographed under visible light. Approximately 0.6 mg of protein extracted from raw peanuts (sample 3, Table I) was applied to each gel prior to electrophoresis. Figure 1A represents the gel stained with Oil Red O; gel B, Amido Black; gel C, Rhodamine 6G. Gel B shows four very dense protein bands and at least six minor bands. Gels A and C had only three bands indicating that at least three of the proteins in gel B were combined with lipid in some manner. These results also indicate that Rhodamine 6G is similar to Oil Red O in staining ability for peanut lipid–protein complexes viewed under visible light and that Rhodamine 6G stained gels do not necessarily have to be viewed under uv light. When viewed under uv light, however, the bands in gel C did fluoresce.

In these protein extracts the major storage protein, arachin, appears to be the principal protein that interacts with lipids. Neucere and Ory (1968) observed that the lipid layer of a peanut homogenate after centrifugation contained some arachin, suggesting that arachin could have the characteristics of a lipoprotein. If this were true, polar solvents used to deoil peanut might selectively rup-

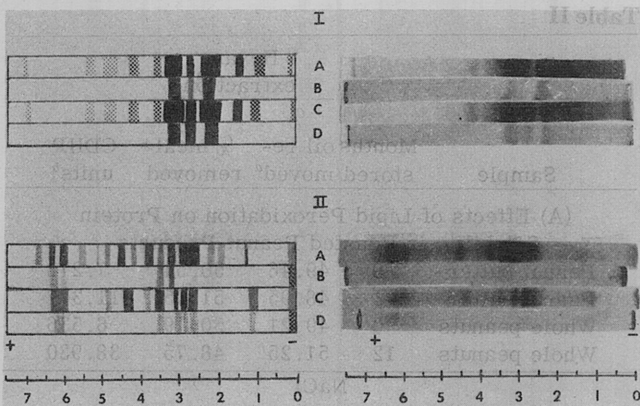

Figure 2. Photograph and diagrammatic sketches of polyacrylamide gel electrophoretic patterns of salt-soluble (I) and insoluble (II) proteins from raw peanut meals. Samples A and B represent controls; samples C and D are peanuts that were stored for 4.5 months. Gels were stained either for protein (A and C) or for lipid (B and D). Migration was toward the anode.

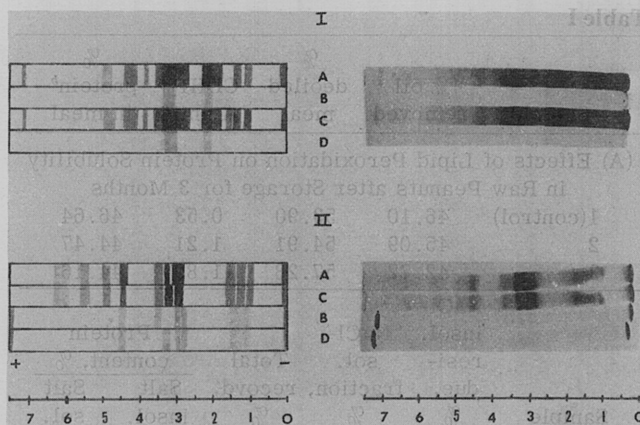

Figure 3. Photograph and diagrammatic sketches of polyacrylamide gel electrophoretic patterns of salt-soluble (I) and insoluble (II) proteins from roasted peanut meals. Samples A and B are freshly roasted peanuts; samples C and D are roasted peanuts that were stored for 1 year. Gels were stained either for protein (A and C) or for lipid (B and D). Migration was toward the anode.

ture weak bonds between the lipid and protein to release subunits with various degrees of polarity. Hexane, on the other hand, should cause less damage to the proteins but might not completely remove all lipid from the meals, leaving small amounts bound to the arachin. For comparison, two raw peanut samples were extracted, one with hexane and one with a mixture of 3:1 chloroform–methanol. At least 2% more lipid material was removed with chloroform–methanol than with hexane, but the product consisted of hard clumps difficult to pulverize, not a smooth flour as obtained by hexane extraction. Apparently, the chloroform–methanol solvent disrupted membranes and caused aggregation of proteins. Salt extraction of these two meals produced more striking differences. Although total recoveries of material were the same after dialysis and freeze-drying, salt extracted 5% more protein from the hexane-defatted meals than from the mixed solvent-defatted meal. Protein banding patterns, however, were identical. Therefore, in order to obtain the highest yields of soluble proteins for gel electrophoretic analysis, all samples were deoiled with hexane.

Lipid–protein associations in the salt-soluble protein extracts of raw peanuts are presented in Figure 2, part I. Sample 1 is the control while sample 3 represents rancid peanuts stored in an open jar at 30° for 4.5 months. In each case, one set of gels was stained for protein and the corresponding set for lipid with Oil Red O. Results showed four major and several minor protein bands that were similar in control and peroxidized samples. Protein bands in the 25–30-mm region of the gel were somewhat broader in the peroxidized sample but other bands appeared to be identical. After staining with Oil Red O, at least three proteins were associated with lipid in the control sample, I-B. Two of these, in the 20–30-mm region, seemed to have equal amounts of lipid, while the middle band, at 27 mm, had much less. In the 4.5-month old sample, I-D, the two heavy lipid bands were also present, but the middle band was noticeably denser and broader than the corresponding band in the control. This suggests that one of the major salt-soluble proteins may undergo a change in composition by becoming associated with more lipid as rancidity progresses, but the amount of lipid did not alter its electrophoretic mobility.

The salt-insoluble proteins of raw peanuts were solubilized overnight in SDS and then electrophoresed. Figure 2, part II, indicates that major proteins of controls did not differ markedly from those of the 4.5-month-old samples, but differences were observed in minor banding patterns. Lipid bands also appeared to have a few minor differences. The SDS-solubilized proteins contained much less lipid than did the salt-soluble proteins, suggesting that peroxidized lipids had greater affinity for the large peanut globulins than for proteins present in cell wall matrices and in cellular membranes.

Peanuts that are processed for particular products, such as candy or peanut butter, are roasted for a specified time at a rather high temperature. This denatures the proteins and decreases protein solubility (Table II). To examine the effects, if any, of peroxidation on proteins from roasted peanuts, whole roasted peanuts were stored in glass jars at 4° for 1 year and analyzed monthly. As indicated in Table II, the CDHP values of these peanuts increased, but the protein patterns were not significantly different (Figure 3). Comparing the patterns for salt-soluble and salt-insoluble proteins extracted with SDS (Figure 3, parts I and II), the only difference appeared in the salt-soluble proteins. The 1-year-old sample had a protein band in the 35-mm region and the control did not (Figure 3, part I, A and C).

Because of denaturation, protein patterns from roasted peanuts had fewer bands than those from raw peanuts. There were only two bands for major components in roasted peanuts, compared to four in raw peanuts. Another major difference was the presence of only two lipid bands in roasted peanuts (three in raw peanuts). No differences were noticed between protein patterns of control and 1-year-old peanuts.

In gel patterns for SDS-solubilized proteins, no difference between freshly roasted and 1-year-old samples was apparent; both had two major and several minor protein bands. Lipid bands also were similar, but the two major lipid bands stained deepest with Oil Red O.

Insoluble protein fractions differed somewhat between raw and roasted peanuts, but in both, the two major protein bands and some minor constituents were unchanged after electrophoresis in SDS gels. The two most significant changes occurred in the protein bands at the origin and toward the end of the gel. Roasted peanut extracts had very few fast-moving proteins but had a slow-moving band in the 10–16-mm region, whereas raw peanut proteins, with very few components at 10–16 mm, had a very dense area (too dispersed to call one band) in the 58–64-mm region. Proteins in the 10–16-mm range are probably the ones most affected by heat denaturation. In both of the salt-insoluble fractions some lipids were attached (probably membrane lipid). In raw peanuts, the heaviest concentration of lipid was found at 58–64 mm, whereas in roasted peanuts, most lipid was associated with those proteins in the 10–16-mm region.

Cherry *et al.* (1973) reported that lipid appeared to be

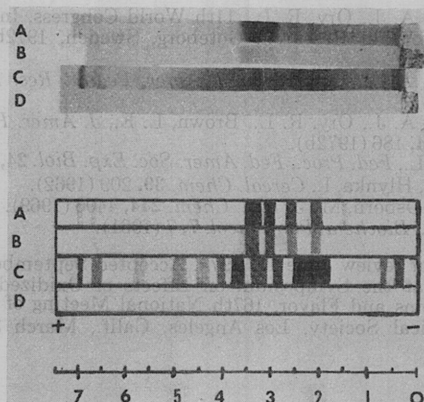

Figure 4. Photograph and diagrammatic sketches of polyacrylamide gel electrophoretic patterns of ammonium sulfate fractions from protein extracts from raw peanuts. Samples A and B are patterns from the 40% precipitated fraction, arachin. Samples C and D are patterns from the 85% precipitated fraction, conarachin. Gels were stained for protein (A and C) or for lipid (B and D). Migration was toward the anode.

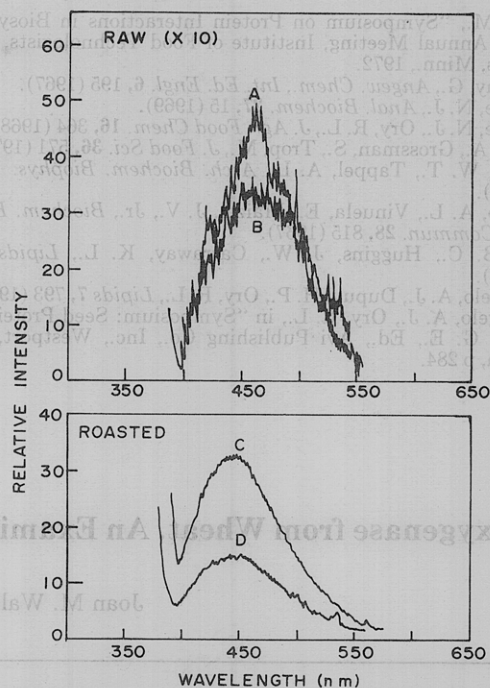

Figure 5. Fluorescence spectra of salt-soluble proteins: (A) extracts from fresh raw peanuts; (B) extracts from raw rancid peanuts stored 4.5 months at 30°; (C) extracts from freshly roasted peanuts; (D) extracts from roasted peanuts stored for 12 months at 4°.

associated with arachin rather than with other proteins in extracts of Virginia peanuts. To verify this, the salt-soluble proteins from 4.5-month-old raw peanuts were fractionated with ammonium sulfate into the classical 40% saturated (arachin) and 85% saturated fractions (conarachin). The results confirmed that only the arachin fraction is associated with lipid (Figure 4).

Although the foregoing investigations using dual staining of gels provide some information on the effects of peroxidized lipids on peanut proteins, they do not provide any information on possible conformational changes in the protein. Therefore, we analyzed two sets of soluble proteins (fresh and peroxidized samples) by fluorescence spectroscopy. Chio and Tappel (1969) characterized the fluorescent product formed by the reaction of malonaldehyde with amino acids. These conjugated Schiff bases fluoresced at 401–404 nm. Fluorescent scanning of the salt-soluble proteins from peanuts showed that peanut protein solutions (0.1% NaCl, Figure 5A,B) have natural fluorescence. For raw peanuts, the maximum was at 460 nm and that for roasted peanuts (curves C and D) was at 440 nm. Also, instead of increased fluorescence, as found by Chio and Tappel (1969), peroxidized lipid–protein complexes in peanuts showed a decrease (curves B and D). While malonaldehyde is one product of lipid peroxidization, this apparent quenching effect suggested that other nonfluorescing products bound to peanut proteins overcame any effects of malonaldehyde–amino acid reaction. In future investigations, fluorescence spectroscopy will be employed for examination of interactions between peroxidized lipids and peanut proteins.

One of the important problems being investigated concerns the mechanisms of lipid–protein interactions. Permanent covalent binding of lipids to proteins is not common, but there have been reports of the natural occurrence in lower organisms of lipids covalently bound to either amino acids or peptides. Covalent bonds have been reported to form in reactions between protein-SH groups and linoleic acid hydroperoxide (Wills, 1961; Tsen and Hlynka, 1962; Bloksma, 1963; Karel, 1972), and between linoleic acid hydroperoxide and cysteine (Gardner *et al.*, 1973). Desai and Tappel (1963) and Roubal and Tappel (1966) proposed two kinds of cross-linking reactions for such reactions. Andrews *et al.* (1965), Shin *et al.* (1972), and Chio and Tappel (1969) described inter- and intramolecular cross-linked proteins with lipids *via* a Shiff's base reaction with malonaldehyde. However, Gamage and Matsushita (1973) indicated that no general rule can be made for a single mechanism to describe the reaction of autoxidized lipids with different proteins. They have shown that both radical intermediate and nonradical products of peroxidized linoleic acid can polymerize with bovine pancreatic ribonuclease (Gamage *et al.*, 1973). While great strides have been made in this area of research during the past decade, much more information is needed before lipid–protein interactions are completely understood.

Arachin might be a good model for studying lipid–protein interaction in oilseeds. However, arachin has been shown to be very low in sulfur amino acids (Neucere, 1969). Therefore, if covalent bonding is involved in the arachin–lipid complex, it is presumably through an amino acid other than cysteine. With raw and roasted peanuts, there is definitely more interaction between the peroxidized lipids or their degradation products and arachin. The location(s) of the binding site in this complex is presently under investigation.

ACKNOWLEDGMENT

The authors are grateful to A. F. Fayette and J. J. Bergquist for the photography and J. H. Wall for the fluorescence spectroscopic analysis.

LITERATURE CITED

Andrews, F., Bjorksten, J., Trenk, F. B., *J. Amer. Oil Chem. Soc.* 42, 779 (1965).
Bloksma, A. H., *J. Sci. Food Agr.* 14, 529 (1963).
Cherry, J. P. (1974), *J. Soc. Exp. Biol. Med.* **147**, in press.
Cherry, J. P., Dechary, J. M., Ory, R. L., *J. Agr. Food Chem.* **21**, 652 (1973).
Cherry, J. P., Katterman, F. R. H., Endrizzi, J. E., *Evolution* **24**, 431 (1970).
Chio, K. S., Tappel, A. L., *Biochemistry* **8**, 2827 (1969).
Desai, I. D., Tappel, A. L., *J. Lipid Res.* **4**, 204 (1963).
Gamage, P. T., Matsushita, S., *Agr. Biol. Chem.* **37**, 1 (1973).
Gamage, P. T., Mori, T., Matsushita, S., *J. Nutr. Sci. Vitaminol.* **19**, 173 (1973).
Gardner, H. W., Kleiman, R., Inglett, G. E., 64th Annual Spring Meeting, American Oil Chemists Society, New Orleans, La., 1973, Abstract 52.
Green, D. E., Fleicher, S., *Biochim. Biophys. Acta* **70**, 554 (1963).
Hack, M. H., Helmy, F. M., *Acta Histochem. Bd.* **27**, 74 (1967).
Helmy, F. M., Hack, M. H., Yeager, R. G., *Comp. Biochem. Physiol.* **23**, 565 (1967).

Karel, M., "Symposium on Protein Interactions in Biosystems," 32nd Annual Meeting, Institute of Food Technologists, Minneapolis, Minn., 1972.
Némethy, G., *Angew. Chem., Int. Ed. Engl.* **6**, 195 (1967).
Neucere, N. J., *Anal. Biochem.* **27**, 15 (1969).
Neucere, N. J., Ory, R. L., *J. Agr. Food Chem.* **16**, 364 (1968).
Pinsky, A., Grossman, S., Trop, M., *J. Food Sci.* **36**, 571 (1971).
Roubal, W. T., Tappel, A. L., *Arch. Biochem. Biophys.* **113**, 150 (1966).
Shapiro, A. L., Vinuela, E., Maizel, J. V., Jr., *Biochem. Biophys. Res. Commun.* **28**, 815 (1967).
Shin, B. C., Huggins, J. W., Carraway, K. L., *Lipids* **7**, 229 (1972).
St. Angelo, A. J., Dupuy, H. P., Ory, R. L., *Lipids* **7**, 793 (1972a).
St. Angelo, A. J., Ory, R. L., in "Symposium: Seed Proteins," Inglett, G. E., Ed., Avi Publishing Co., Inc., Westport, Conn., 1972a, p 284.
St. Angelo, A. J., Ory, R. L., 11th World Congress, International Society for Fat Research, Goteborg, Sweden, 1972b, Abstract 215.
St. Angelo, A. J., Ory, R. L., *J. Amer. Peanut Res. Ed. Ass.* **5**, 128 (1973).
St. Angelo, A. J., Ory, R. L., Brown, L. E., *J. Amer. Peanut Res. Ed. Ass.* **4**, 186 (1972b).
Tappel, A. L., *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **24**, 73 (1965).
Tsen, C. C., Hlynka, I., *Cereal. Chem.* **39**, 209 (1962).
Weber, K., Osborn, M., *J. Biol. Chem.* **244**, 4406 (1969).
Wills, E. D., *Biochem. Pharmacol.* **7**, 7 (1961).

Received for review June 11, 1974. Accepted September 27, 1974. Presented at the Symposium on Effects of Oxidized Lipids on Food Proteins and Flavor, 167th National Meeting of the American Chemical Society, Los Angeles, Calif., March 31–April 4, 1974.

Lipoxygenase from Wheat. An Examination of Its Reaction Characteristics

Joan M. Wallace* and Edward L. Wheeler

Four lipoxygenase fractions were separated from wheat germ extract by DEAE-cellulose chromatography. The activity *vs.* pH curves for all four fractions varied as a function of linoleic acid substrate concentration. This behavior is interpreted as demonstrating a pH-dependent substrate inhibition of lipoxygenase. Evidence is presented that, in addition to linoleic acid hydroperoxide, an unidentified product was formed in the aerobic reaction of wheat germ lipoxygenase with linoleic acid.

Isoenzymes of lipoxygenase have been isolated from soybeans (Christopher *et al.*, 1970, 1972), peas (Eriksson and Svensson, 1970; Anstis and Friend, 1974), and alfalfa (Ben-Aziz *et al.*, 1971). The aerobic reaction of lipoxygenase with the *cis,cis*-1,4-pentadiene system of unsaturated fatty acids such as linoleic acid generally has been found to produce, as primary products, C-13 and/or C-9 conjugated diene hydroperoxides, depending on the source of the enzyme or on the isoenzymes under study. Graveland (1970a) demonstrated that unsaturated trihydroxy compounds were formed as secondary products of the aerobic reaction in wheat doughs but only if lipoxygenase was bound to glutenin during the reaction. Garssen *et al.* (1971, 1972) showed that a mixture of various dimers and unsaturated carbonyl monomers, as well as pentane, could be produced in an anaerobic reaction of soybean lipoxygenase in the presence of both linoleic acid and linoleic acid hydroperoxide. This paper describes preliminary results of the partial purification of four lipoxygenase fractions from wheat germ by DEAE-cellulose chromatography. All four fractions produced an unidentified early reaction product in addition to linoleic acid hydroperoxide during the aerobic reaction with linoleic acid.

EXPERIMENTAL SECTION

Materials. Linoleic acid (99+% pure) and Absorbosil 5 TLC plates were purchased from Applied Science Laboratories, Inc. Soybean lipoxygenase was obtained from Mann Research Laboratories and used without further purification. Sephadex G-200 was from Pharmacia Fine Chemicals, Inc., and DE-32 anion exchange cellulose from H. Reeve Angel, Inc.

Methods. To prepare linoleic acid substrate, 100 ml of sodium borate buffer (pH 9.0) containing 2.5×10^{-4} *M* EDTA was deaerated, after which 1 g of linoleic acid was added, under a stream of argon, to give a concentration of 36 m*M*. The mixture was sonicated under an argon atmosphere, transferred to storage vials, flushed three times with argon, and stored at −10°. For lipoxygenase activity measurements, this stock linoleate was diluted with phosphate buffer (pH 6.9) to give concentrations ranging from 0.06 to 3.6 m*M*. A solvent system of hexane–ethyl ether–glacial acetic acid (80:20:1) was used for tlc separations. Hydroperoxide concentrations were calculated from the absorbance at 234 nm, measured with a Perkin-Elmer M202 spectrophotometer, using a molar extinction coefficient of 25,000 l. mol^{-1} cm^{-1} (Johnston *et al.*, 1961).

Lipoxygenase activity was measured as the initial rate of O_2 uptake determined with a Clark oxygen electrode in a volume of 3 ml containing linoleic acid in concentrations up to 3.6 m*M*, and buffered with 0.025 *M* sodium phosphate (pH 6.9). One unit of activity is defined as the uptake of 1 μmol of O_2/min at 25°. The initial O_2 concentration in the reaction mixture was 0.24 m*M*, as determined by the method of Robinson and Cooper (1970). The method of Lowry *et al.* (1951) was used for protein determinations with bovine serum albumin for standardization.

Defatted wheat germ (50 g) was extracted for 1 hr at 2° with 5 vol of 0.12 *M* sodium phosphate buffer (pH 6.9) (all succeeding operations were carried out at 2°). Insoluble material was separated by centrifugation (31,000*g*, 25 min). The supernatant (210 ml) was adjusted to 35% saturation with $(NH_4)_2SO_4$, stirred 1 hr, and then centrifuged to remove the inactive precipitate. The resulting supernatant was brought to 55% saturation with $(NH_4)_2SO_4$ and the active precipitate was collected by centrifugation. This final supernatant contained negligible activity. The precipitate was dissolved in 0.12 *M* sodium phosphate buffer (pH 6.9) to a volume of 20.5 ml and dialyzed against the same buffer. This was accomplished by passing the buffer through the fibers of a Bio-Fiber 80 beaker (Bio-Rad Laboratories, Richmond, Calif.) at the rate of 16

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710.