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STUDY OF SECONDARY DETERIORATION IN PARTIALLY OXIDIZED LIPIDS by Fred A. Andrews and David A. Thomson, Final Report, February 1963, 15 pp.-tables (Contract DA19-129-QM-1945) Project Nr. 7X84-13-002B, Unclassified Report

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CONTRACT RESEARCH PROJECT REPORT

QUARTERMASTER FOOD AND CONTAINER INSTITUTE FOR THE ARMED FORCES, CHICAGO QM Research and Engineering Command, U.S. Army, QM Research and Engineering Center, Natick, Massachusetts

Bjorksten Research Labs., Inc.
P.O. Box 265
Madison 1, Wisconsin

Project Nr.: 7X84-13-002B
Contract: DA19-129-QM-1945
Report Nr.: 4 (Final)
File Nr.: L-400
Period: 28 December 1961
27 December 1962
Initiation Date: 28 December 1961

Official Investigators:
Mr. Fred Andrews
Mr. David Thomson

Title of Contract: Study of Secondary Deterioration in Partially Oxidized Lipids

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INTRODUCTION

The primary objective of this program was to determine the nature of the interactions occurring between proteins and oxidative intermediates of lipids in dehydrated systems. It was reasoned that an understanding of protein reactions with lipid derived peroxides, epoxides, and free radicals should yield information which would lead to elimination of rancidity in field rations. This research also included studies on pro-oxidants and antioxidants, since such chemicals greatly influence the speed and course of autoxidation reactions.

Previously, through the use of model compounds, it was shown that an interaction occurred between the free amino groups of insulin and the aldehydes formed during autoxidative decomposition of methyl linoleate.* Studies involving collagen and gelatin indicated that aldehydes formed in autoxidized lipid cross-link the test proteins. Large excesses of oxygen were used in these studies to insure maximum bond scission of the lipid, and as a consequence the free radicals and peroxide interactions were minimized. In the present study, however, attempts were made to minimize bond scission of the lipid in most cases.

MATERIALS AND METHODS

A. Materials

Gelatin (312 Bloom) obtained from the Grayslake Gelatin Company, Grayslake, Illinois, was used exclusively in the foam studies. Bovine hemoglobin substrate obtained from Pentex Corporation, Kankakee, Illinois, was used in the pro-oxidant studies. Commercially available crystalline zinc insulin in acid solution (500 units/ml.) obtained from Eli Lilly & Co., Indianapolis, Indiana, was used in both the peroxide and free radical studies.

Hematin, amino acid controls, glutathione, 1-fluoro 2,4-dinitrobenzene, D(+) glucose, D(+) mannose, D(+) galactose, D(-) fructose, and the purified proteins were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. All reagents and solvents used in the hydrolysis and chromatographic phases were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

Methyl linoleate and olive oil peroxides were used throughout as model lipids. Both compounds were obtained from Hormel Foundation, Austin, Minnesota.

Anhydrous hydrogen fluoride was obtained from Matheson Company, Joliet, Illinois.

*Contract DA 19-129-QM-1549.

B. Methods

1. Pro-Oxidant Studies

a. Gelatin-Methyl Linoleate-Hematin Preparation: Five-gram samples of gelatin were dissolved in 100 ml. of distilled water containing 24 mg. of hematin. The solution was whipped to a stiff foam in a Sunbeam Mixmaster, with 2 gm. of pure methyl linoleate added during foaming. The foam was then shell-frozen and freeze-dried. Control foams (without M.L.*) were prepared in a similar fashion. Aliquots from both control and experimental foams were soxhlet extracted with ethanol-benzene azeotrope immediately upon removal from the freeze drier. The remainder of the foams were placed in an oven preset at 50°C. Portions of the foams were removed at various time intervals, and, after extraction, were subjected to trypsin hydrolysis. The degree of hydrolysis was followed using the standard Van Slyke α-amino nitrogen method.

b. Insulin-Methyl Linoleate-Hematin Preparation: The pro-oxidant properties of hematin were studied using an insulin-M.L. model system. In this study 50-mg. samples of zinc insulin crystals were dissolved in .02 N HCl containing 20 micrograms of hematin. The samples were freeze-dried, and to one sample was added 100 micrograms of M.L., dissolved in ether. The ether was removed under reduced pressure and both control and experimental (M.L.) samples were charged with O_2 and incubated at 50°C. At the end of six days both samples were ether extracted and then refluxed for 20 hours in 6 N HCl. Two dimensional paper chromatograms were prepared for both samples. The chromatograms were run first in butanol:water:acetic acid (250:250:60) followed by buffered m-cresol (pH 8.4) in the second direction. The chromatograms were developed with both ninhydrin and isatin according to standard procedure.

c. Hemoglobin-Methyl Linoleate Foams: In another series of screening experiments on pro-oxidants, hemoglobin was used both as catalyst and model protein. Ten-gram samples of "bovine hemoglobin substrate" were dissolved in buffer solutions with grinding (mortar and pestle). During the grinding step one gram of M.L. was added in the hope that the lipid would be satisfactorily dispersed in the foam. In one series of tests 0.5 M. acetate buffer (pH 4.7) was used to dissolve the hemoglobin, while in another series the protein was dissolved in 0.5 M. $NaHPO_4$ (pH 9.2). The buffered solutions were shell-frozen and freeze-dried. The whipping step was omitted in this series because hemoglobin foams voluminously and is therefore very difficult to dry.

As in the gelatin studies, the dried hemoglobin-methyl linoleate mixtures were incubated at 50°C for various periods of time before removal of the lipid by extraction. As before, standard trypsin digests were prepared and the liberated amino nitrogen was measured in the Van Slyke apparatus.

*M.L. = methyl linoleate.

d. Enzyme Hydrolysis Procedure: Samples of defatted foams (0.5 g.) were mixed with 59 ml. of Sorenson's buffer (pH 7.7) containing 10 mg. of Difco trypsin (1:250) in 125 ml. Erlenmeyer flasks. Each sample was set up in duplicate with one being precipitated immediately upon mixing with 20 ml. of 20% trichloroacetic acid* (TCA). The second flask was shaken for 20 hours at 38°C and then precipitated with 20 ml. of TCA. Both samples were filtered and washed with 5% TCA in 100 ml. volumetric flasks. The filtrates were used for α-amino nitrogen determinations with standard Van Slyke apparatus.

2. Free Radical Studies Using Protein-Methyl Linoleate Models

Hemoglobin, gelatin, and insulin-methyl linoleate mixtures were used as models. Sixty mg. samples of the test proteins in aqueous solution were shell-frozen and freeze-dried in quartz interjoint tubes. One-tenth gram of M.L. dissolved in 5 ml. of dry ether was added to the experimental samples and the ether was then quickly removed in vacuo while the tubes were continually rotated to disperse the lipid uniformly. At this point the quartz tubes were placed on a vacuum line to obtain a pressure of less than 0.5 mm. of Hg. Both control and experimental samples were then placed under a "Hanovia" analytic ultraviolet lamp (without filter) set 17 inches above the tubes. To avoid air leakage the vacuum pumps were run during the full course of the irradiation which varied from 2-8 days' duration depending on the test. At the end of the irradiation both the control and experimental samples were washed quickly with anhydrous ether under nitrogen and then air-dried.

The gelatin samples were placed in a desiccator in preparation for HF solubility studies. Some hemoglobin and insulin samples were handled similarly, but were also used for paper electrophoretic study. In addition, several insulin samples were either hydrolyzed in 6 N HCl or reacted with 1-fluoro 2,4-dinitrobenzene** and then hydrolyzed. The hydrolysates were filtered, dried, and prepared in standard fashion for paper chromatographic analysis. In this work unidimensional chromatograms were used and the solvent mixture employed was butanol:water: acetic acid (250:250:60).

a. The Sanger Technique: In order to test the applicability of the FDNB technique, standard insulin-M.L. films were prepared by freeze-drying along with pure insulin controls in quartz tubes. After ultraviolet irradiation for 60 hours in vacuo, a 50% aqueous alcohol solution containing 770 mg. of FDNB and 500 mg. of $NaHCO_3$ was carefully added to the system so as to avoid the inclusion of air. After 4 hours the reaction mixture was removed from the tubes, and washed with alcohol and ether to remove M.L. and unreacted FDNB.

*TCA = trichloroacetic acid.
**FDNB = 1-fluoro 2,4-dinitrobenzene.

The air-dried DNP-insulin* was acid hydrolyzed (6 N HCl) and ether extracted to separate DNP-amino acids as described elsewhere.**

Both the ether and aqueous phases of the hydrolysates were chromatographed on Whatman #1 paper with butanol:water:acetic acid (250:250:60). The ether extract (containing DNP-amino acids) was chromatographed after hydrolysis with 14% $Ba(OH)_2$.

b. Solubility Studies - Anhydrous Hydrogen Fluoride

(1) Sample Preparation: For the sake of brevity the details concerning the preparation of formaldehyde, epoxide and quinone cross-linked proteins is annotated to the tables of data.

(2) Solubility Test Procedure: Approximately 20 mg. of each native protein, as well as of the substances which had been cross-linked or irradiated, were carefully dried in a desiccator and placed in threaded tubes of transparent polytrifluoroethane, "Kel-F", having a bore of 8 mm. diameter and a depth of 60 mm. The Kel-F tubes containing the samples were dried in a vacuum for 16 hours at a temperature of 25°C over $CaCl_2$ as desiccant. The tubes containing the samples were then cooled to -78°C and attached each in turn to a cylinder of anhydrous hydrogen fluoride by means of threaded refrigerator connectors. The HF line was swept free of moisture with dry nitrogen prior to attachment of the samples. Approximately 1 ml. of anhydrous hydrogen fluoride was condensed into the tubes containing the substances under test. The tubes were then removed from the lines, quickly capped, and allowed to warm gradually to about 25°C in 2 hours. All of the control, cross-linked and irradiated compounds were observed for solubility after 2 hours.

3. Lipid Hydroperoxide Studies

Four 50 mg. samples of both glutathione and insulin were weighed into small test tubes; 100 lambda of oxidized olive oil was added without solvent to two of the tubes, while nothing was added to the controls. A second set of samples was also prepared but high purity methyl linoleate (100 λ) was used instead of the olive oil. Duplicate samples from each combination of ingredients were heat-sealed under oxygen and nitrogen and reacted at 25°C for one year. The tubes were opened and washed with ether several times. The extracted samples were then hydrolyzed with 6 N HCl at 110°C for 20 hours and prepared as described earlier for paper chromatographic separation.

Chromatograms were run on each of the four sets of samples using butanol:water:acetic acid (250:250:60) as the developing solvent, and ninhydrin was used for color development.

*DNP-insulin = dinitrophenyl-insulin.

**Fourth Quarterly Report, Contract DA 19-129-QM-1549, October 15, 1960 - January 15, 1961.

RESULTS AND DISCUSSION

In previous lipid-protein interaction studies gelatin foams and insulin films containing methyl linoleate (M.L.) were used to good advantage to demonstrate the aldehyde cross-linking reaction resulting from autoxidation. The simplicity and success of this approach to the study of interactions suggested that it could be extended to include studies on lipid pro-oxidants and other lipid intermediates as well.

A. Effect of Pro-Oxidants on Lipid-Protein Interactions

Gelatin foams containing M.L. when incubated at 50°C in the presence of excess oxygen become increasingly resistant to hydrolysis by trypsin. When liberated amino nitrogen data from hydrolysates are plotted versus autoxidation time, an S-shaped curve results with maximal reaction occurring within five days. It was reasoned that the addition of pro-oxidants would materially alter the slope of the curve and accordingly new knowledge of the interaction would result.

The influence of hematin as a pro-oxidant on gelatin-methyl linoleate is shown in Table 1 below.

TABLE 1

Liberation of α-Amino Nitrogen in Methyl-Linoleate-Gelatin-Hematin Foams After Autoxidation and Trypsin Hydrolysis

Gelatin Foams Containing:	Reaction Time (Hrs.)	Mg. of N_2 Lib./g. Gelatin Corrected	Reduction in Hydrolysis Rate (%)
Hematin	72	1.45	0
Methyl Linoleate	72	1.11	-23.5
Hematin + M.L.	72	1.33	-8.3

Although it was expected that hematin would accelerate the gelatin-M.L. cross-linking interaction (expressed as a reduction in hydrolysis rate in this type of experiment), such is not the case. From this data it would appear that hematin inhibits the carbonyl addition to ε-amino groups of lysine in the gelatin. Neither the data nor the method of analysis, however, are accurate enough to warrant such a simple interpretation. It seems quite likely that two or more reactions are competing for the protein reactive groups in this system, e.g., alkylation versus cross-linking.

The data for two hemoglobin experiments are summarized in Table 2. On the basis of this data it would appear that cross-linking of hemoglobin with lipid carbonyls is accelerated under acid conditions and is suppressed in alkaline environment. In practice, however, the approach described above (in the gelatin-hematin studies) is more difficult to interpret than the pure gelatin-M.L. systems used in the past. In the case of the hemoglobin, for example, a physical change occurred in the molecule during the heating process which reduced digestibility of the protein by trypsin.

The pH dependency of the reaction is also apparent from the tables which indicate that the oxidation pathway may be different at high and at low pH values. The data indicate that with this protein aldehyde cross-linking is enhanced in acid environment. This is contrary to data normally found in the literature regarding aldehyde tannage.

TABLE 2

Liberation of α-Amino Nitrogen in Methyl Linoleate-Hemoglobin Foams After Autoxidation and Trypsin Hydrolysis

Reaction Time in Hours	Mg. of Liberated N_2/g. Protein							
	At pH 4.7				At pH 9.2			
	Control	Exptl.		%	Control	Exptl.		%
0	20.0	18.3	1.7	8.5	-	-	-	-
24	19.2	17.1	2.1	10.9	-	-	-	-
48	18.1	15.6	2.5	13.8	10.4	9.6	0.8	7.7
72	15.8	13.5	2.3	14.5	7.8	9.2	1.4	17.9
96	13.5	10.9	2.6	19.3	9.4	8.4	1.0	10.2
120	10.6	7.8	2.8	26.4	-	-	-	-

= Change in liberated N_2.

% = Reduction in hydrolysis rate.

In order to obtain a clearer insight into the types of interactions which appear to occur in the presence of pro-oxidants, an insulin-methyl linoleate-hematin model was studied chromatographically. Chromatograms of acid hydrolysates of the autoxidized mixtures failed to reveal any differences in amino acid composition in the samples containing autoxidized M.L. No evidence was obtained which indicated that either an alkylation reaction

(from an epoxide interaction) or a disulfide oxidation reaction had occurred. It is quite likely that insulin, because of its small size and amino acid composition, cannot serve as a useful model in pro-oxidant research.

B. Free Radical Studies Using Protein-Methyl Linoleate Models

The possible interaction of methyl linoleate radicals with proteins is most difficult to study because of the rapid and complex decomposition of such species. Although the radicals formed are short-lived it seemed quite likely that their influence on model proteins could be detected under suitable reaction conditions since the latter contain large numbers of labile protons. In the presence of oxygen lipid radicals are so extremely short-lived that for all practical purposes they exist as hydroperoxides, and in the linoleate molecule the latter decompose quickly to carbonyls. Based on previous experience, using chromatographic techniques in oxygen rich atmospheres, it seemed unlikely that either radicals or peroxides existed for a long enough time to interact with proteins. It was felt, however, that if ultraviolet light catalysis were used to generate linoleate radicals and if the model systems were held in vacuo to stabilize the radicals, an interaction might be demonstrated using paper chromatographic technique.

Unfortunately, the simple chromatographic approach yielded little in the way of reproducible data. In both control and experimental insulins, for example, the only detectable difference found was a conversion of cystine to cysteine in both samples. Oddly enough the data indicated that methyl linoleate protects disulfide groups during irradiation.*

Since the disulfide cross-links of insulin were reduced under the test conditions, an attempt was made to separate the resulting polypeptides using paper electrophoresis. This technique failed to separate insulin fragments, however, thereby suggesting that other reactions in addition to the disulfide reduction were simultaneously occurring. This suggestion was further strengthened when attempts to electrophoretically separate irradiated hemoglobin-M.L. systems also failed. Because neither the chromatographic nor the electrophoretic approaches proved useful in studying lipid free radical reactions it was decided to explore the possible adaptation of the Sanger technique to this problem.

Although the Sanger 1-fluoro 2,4-dinitrobenzene technique is generally used to mark N-terminal amino acids in protein structures, it was felt that it might also be useful for obtaining information on free radicals within the protein. It was reasoned that if radicals were formed in irradiated M.L., which is intimately associated with a protein such as insulin, the latter might provide protons to restabilize the lipid and in turn the protein would become a free radical

*This same observation was made in earlier work on irradiated aqueous solutions of insulin containing methyl linoleate where animal assay techniques for biological activity were used to determine the extent of lipid interactions with proteins.

Such a free radical within the protein could possibly stabilize via a cross-linking mechanism, through destruction (alteration) of one or more amino acid residues, or it might possibly remain as a free radical in an evacuated system.

If amino acid destruction were to occur, new terminal amino groups would be generated which would react with the FDNB reagent. Since the fluorine group on FDNB is quite labile, it is also possible that a protein free radical might use it to gain its electronic equilibrium. In the event that either of the above possibilities should occur, the FDNB technique would show differences between samples containing M. L. from those which do not. Cross-linked protein (resulting from free radical formation) could not be studied by this technique, however.

Paper chromatographs of the irradiated insulin-M. L. models after the FDNB reaction and acid hydrolysis did not yield any reproducible data. In a few experiments insulin-M. L. samples differed from the irradiated insulin controls, i.e., as many as three amino acids were missing on the chromatograms. We were unable, however, to duplicate the experiments in this series and were forced to conclude that ultraviolet catalysis is not specific enough to yield useful information unless a means is found to successfully separate the various fractions of the reaction mixture.

It is quite likely that any interaction occurring between a protein and a lipid free radical would be nonspecific and incomplete when the ultraviolet technique is employed. If an interaction occurs between the radical and a few amino acid residues, the chromatographic FDNB method employed here would not reveal the occurrence of an interaction unless a complete quantitative amino acid analysis were made, and even then it is doubtful that it would shed light on the type of interaction which takes place.

Polymers, such as proteins, may be cross-linked through a radiation induced free radical mechanism, and it is possible that this type of interaction may occur under the conditions of the ultraviolet experiments. The problem of analysis becomes one of separating native, acylated, and cross-linked species.

The separation of native from cross-linked proteins forms the basis for another program in this laboratory, and the methods which were developed there have been applied to the present problem.

The method in question stems from the observations of J. J. Katz* who demonstrated the solubility of proteins in anhydrous hydrogen fluoride and showed that this is a true solvent action, not decomposition of any kind. Hydrolysis, of course, could not occur in the complete absence of water, and the true solvent nature was confirmed by the fact that enzymes dissolved in hydrogen fluoride upon precipitation retained their activity.

*Katz, J. J., Arch. Biochem. Biophys. 51, 293-305 (1954).

Katz also pointed out that, in some instances, cross-linked proteins were not soluble in anhydrous hydrogen fluoride in contradistinction to noncross-linked proteins.

The solubility data for eight proteins, both native and cross-linked, are summarized in Table 3. All of the compounds in the native (noncross-linked) state dissolved quite rapidly in anhydrous hydrogen fluoride at -78°C, as was expected in view of Katz's findings. None of the formaldehyde or p-benzoquinone cross-linked samples was soluble in HF although the tubes were slowly warmed for two hours to 25°C. The much milder cross-linking agent, epichlorohydrin, gave, in some instances, reaction products soluble in HF as shown below.

TABLE 3

Solubility of Native and Cross-Linked Proteins in Anhydrous Hydrogen Fluoride at 25°C*

Purified Protein	Solubility in Anhydrous Hydrogen Fluoride[b.]			
	Control[a.]	Form-aldehyde	Epichloro-hydrin	p-Benzo-quinone
Lysozyme	+	-	-	-
Edestin	+	-	-	-
Trypsin	+	-	-	-
Pepsin	+	-	±	-
Bovine Albumin	+	-	-	-
Protamine Sulfate	+	0	+	-
b-Lactoglobulin (bovine)	+	-	-	-
Ovalbumin	+	-	-	-

a. All samples dissolved quickly at -78°C.

b. Solubility of the cross-linked preparations was read after 2 hrs. at approximately 25°C.

(+) indicates that sample is soluble; (-) that it is insoluble; (±) that it is partly dissolved; (0) that the sample could not be isolated by acetone precipitation, and it was therefore not tested for solubility in HF.

The solubility data for six proteins which have been used as models in methyl linoleate oxidation studies are summarized in Table 4.

*For procedural details see Bjorksten, J., et al Finska Kemists Medd. 71, 69-76 (1962).

TABLE 4

Solubility of Proteins in Anhydrous Hydrofluoric Acid After Interreaction With Autoxidizing Methyl Linoleate

Test Protein	Molecular Weight	pI	HF Solubility		
			Zero Time	10 Minutes	120 Minutes
Hemoglobin	68,000	6.9	+	+	+
Hemoglobin + M.L.			-	-	-
Gelatin	95,000	4.6	-	+	+
Gelatin + M.L.			-	-	-(gel)
Trypsin	23,000	10.4	+	+	+
Trypsin + M.L.			-	-(gel)	-(gel)
Ovomucoid*	27,000	3.9	+	+	+
Ovomucoid + M.L.			+	+	+
Ovalbumin*	45,000	4.5	gel	gel	gel
Ovalbumin + M.L.			gel	gel	gel
Insulin	12,000	(5.3)**	+	+	+
Insulin + M.L.**			-	±	±

* These proteins contain appreciable amounts of carbohydrate.

** The insulin used here was dried from acid solution; therefore the isoelectric point does not apply.

As shown in Table 4, hemoglobin and gelatin are firmly cross-linked by autoxidizing methyl linoleate while trypsin and insulin are at least partially cross-linked. Ovomucoid and ovalbumin, on the other hand, are apparently not cross-linked. Since both of the latter compounds are glycoproteins, i.e., contain substantial amounts of carbohydrate, and since carbohydrates are rapidly depolymerized to monomeric sugar derivatives in HF, it is possible that this phenomenon alters their solubility properties.

The solubility for insulin in HF after irradiation in vacuo is given in Table 5.

TABLE 5

Solubility of Insulin in Anhydrous Hydrofluoric Acid After Ultraviolet Irradiation in Vacuo

	Solubility in HF	
	Zero Time	120 Minutes
Insulin	+	+
Insulin + M.L.	+	+
DNP-Insulin (Control)*	-	±
DNP-Insulin**	+	+
DNP-Insulin + M.L.**	-	±

*This sample was not irradiated.
**The DNP derivatives were synthesized after completion of the irradiated step.

NOTE: The characteristic yellow colored DNP-insulin is colorless in HF solution.

Since all of the samples tested were found to be predominantly soluble in HF, it is doubtful that ultraviolet irradiation-induced free radical cross-linkage occurs in insulin. However, because of the data shown in Table 4 (where insulin is found to be only weakly cross-linked) and since the disulfide bonds are reduced as a result of irradiation, this protein is obviously a poor model for free radical study. It would appear that hemoglobin or gelatin would be more satisfactory models.

Based on the data given in Tables 4 and 5, it was decided to explore in greater detail the interaction between gelatin and autoxidizing methyl linoleate. In a preliminary experiment the effect of pH and HF solubility was determined. The data, shown below in Table 6, show clearly that in the presence of oxygen cross-linking occurs most completely at or around neutrality. At pH 4 carbonyl cross-linkage is greatly diminished. The data suggest a simple method for minimizing one result of rancidity, namely, reduced nutritional efficiency which involves interaction of carbonyl with the ϵ-amino group of lysine.

TABLE 6

Effect of pH on the Gelatin-Methyl Linoleate Cross-Linking Reaction as Shown by the HF Solubility Test*

Reaction Time (days)	HF Solubility at Various pH Levels											
	pH 4.0				pH 7.0				pH 9.0			
	0	10"	60"	120"	0	10"	60"	120"	0	10"	60"	120"
1	±	±	+	+	-	-	-	-	-	+	+	+
2	±	+	+	+	-	-	-	-	-	-	±	+
3	+	+	+	+	-	-	-	-	-	-	±	±
4	±	±	+	+	-	-	-	-	0**	0	0	0
5	-	-	-	-	-	-	-	-	-	-	+	+
6	±	+	+	+	-	-	-	-	0	0	0	0
7	+	+	+	+	-	-	-	-	0	0	0	0
8	+	+	+	+	-	-	±	±	-	-	-	-

*Gelatin control films for each reaction period and pH were all soluble in HF.

**Zero (0) indicates that tests were not run on days indicated.

The data in Table 7 are an extension of the above experiment to determine the influence of both prolonged drying in vacuo and ultraviolet irradiation on model systems. Surprisingly, the data indicate quite clearly that gelatin at pH 7 can be dried to a point where it becomes partially insoluble in HF. A possible explanation is that bound water is removed and replaced by coordinate covalent cross-links. Another possible explanation is that drying brings the protein molecules closer to each other thereby favoring cross-linkage. Methyl linoleate appears to render the protein even more insoluble, as does ultraviolet light.

TABLE 7

Effect of Prolonged Drying In Vacuo and Ultraviolet Irradiation on Gelatin-Methyl Linoleate Systems at Various pH Levels

Gelatin Films In Vacuo 6 Days	HF Solubility at Various pH Levels											
	pH 4.0				pH 7.0				pH 9.0			
	0	10"	60"	120"	0	10"	60"	120"	0	10"	60"	120"
Control	+	+	+	+	±	±	±	±	±	±	+	+
M.L.	±	±g	±g	±g	-g	-g	-g	-g	±	±	±	±
Control + uv	+	+	+	+	-g	-g	-g	-g	±	±g	±g	±g
M.L. + uv	±g	±g	±g	+	-g	-g	-g	-g	-	±g	±g	±g

Abbreviations: M.L. = methyl linoleate
uv = ultraviolet
g = abbreviation used to indicate gelation of the protein

The data also show that lipid free radical studies on gelatin would be more successful at pH 4, since the controls are soluble but the experimental models are at least partially insoluble thereby indicating some type of lipid-protein interaction catalyzed by ultraviolet irradiation. Since it is doubtful that such reactions would go to completion, future work on lipid free radicals should be done at this pH with HF used as an extracting solvent to purify cross-linked proteins. The purified cross-linked material could then be subjected to hydrolysis and analysis with a better chance for success in determining the nature of the interaction.

C. Antioxidant Study

The hydrogen fluoride solubility technique also shows promise in screening potential lipid antioxidants. For example, in one study now concluded, four simple sugars were added at a low level (5 mg. /50 mg. gelatin) to standard autoxidizing methyl linoleate-gelatin models. Sugars were used for two reasons: (1) their chemical relationship to water and alcohol which are reported to inhibit autoxidation, and (2) their presence in ovalbumin and ovomucoid which are resistant to cross-linkage by autoxidizing lipid (Table 4).

Solubility tests of the oxidized model systems, which are summarized in Table 8, indicate that mannose is quite effective in blocking cross-linkage in gelatin; glucose and galactose are partially effective, while fructose has little inhibitory power.

TABLE 8

Inhibitory Effect of Sugars on the Cross-Linking Reaction of Gelatin-Methyl Linoleate Mixtures in Oxygen as Shown by the HF Solubility Test

Test Sugar*	HF Solubility of Test Mixture	
	0	120 Minutes
D(+) Mannose	+	+
D(-) Glucose	-	+
D(+) Galactose	±	± (gel)
D(-) Fructose	-	±

*Mixtures of all four sugars with gelatin were soluble in HF, while oxidizing mixtures of methyl linoleate and gelatin are always insoluble.

Time did not permit a more thorough study of sugar antioxidants, so at this time their mode of action cannot be discussed.

D. Lipid Hydroperoxide Studies

In this work it was assumed that if lipid hydroperoxides react with proteins the sulfur groups would most probably be the site of an interaction Accordingly, glutathione (glutamyl cysteinyl glycine) and insulin were selected as model compounds. Partially oxidized olive oil (peroxide value in excess of 3000) was used as a source of hydroperoxides and the reactions were run under nitrogen to avoid decomposition of the lipid. Both models were reacted for a prolonged period of time (one year) before being hydrolyzed and analyzed paper chromatographically.

Careful inspection of the chromatograms failed to reveal any evidence which would indicate that either cysteine or cystine had been oxidized by the hydroperoxide. It was concluded therefore that this lipid intermediate has little influence on proteins. Peracids, which would have a much higher oxidative potential were not investigated.

SUMMARY

A simple and direct method to determine cross-linking of proteins has been developed. This method, which is based on solubility of proteins in anhydrous hydrogen fluoride, shows clearly that proteins are cross-linked by both aldehyde and free radical intermediates in autoxidizing methyl linoleate. On the basis of this technique, four simple sugars have been screened for antioxidant potential. The data indicate that mannose is a very effective inhibitor of lipid-protein interactions.