Factors Influencing the Formation of Complexes Between Oxidized Lipids and Proteins[1]

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Abstract

Complexes were formed between egg albumin and oxidized lipids, such as thermally oxidized corn oil and autoxidized linoleic acid, under a standardized set of conditions. No complex could be obtained under these conditions when lysine, glycine, bactopeptone, gelatin or sodium caseinate were substituted for egg albumin. Lactalbumin was equally as reactive, casein much less and fresh egg white superior to egg albumin in their complexing ability with oxidized corn oil. As the time of reaction was increased, the amount of the complex formed also increased. Optimum complex formation took place at a concentration of 1% protein, a pH of 7 and 60C. The ease of formation of these complexes *in vitro* indicated that similar complexing could readily take place *in vivo* under suitable pathological conditions.

Introduction

WE REPORTED earlier that oxidized linoleic acid could complex with egg albumin (1). Most other studies on oxidized lipid-protein complexes have been connected with degenerative or pathological changes in specific organs or tissues from animals or human beings (2,3,4,5). More recently, it has been shown that either oxidized fatty acids such as 12-oxo-*cis*-9-octadecenoic acid or methyl linoleate hydroperoxide could accelerate the development of encephalomalacia in chicks (6,7). Methyl linoleate hydroperoxide was also shown to interact with serum low density lipoproteins and it was suggested that this type of interaction may represent a significant step in the development of atherosclerosis (8). There is therefore need for more specific information regarding the type or types of interactions that take place between the oxidative groups in the lipid, and the reactive groups or sites in the protein or conjugated protein, as the case may be, and as well as the factors that bring about these interactions.

Very little information is available regarding the factors that influence the formation of oxidized lipid-protein complexes. Furthermore, the previous investigation was mainly restricted to oxidized linoleic acid and a single protein, viz. egg albumin. In the present investigation, complex formation was studied using oxidized and unoxidized oils, triglycerides, fatty acids, and several proteinaceous materials. An attempt was also made to show how factors such as pH, temperature, time of reaction, lipid, and protein concentration influence the formation of these complexes. From the data, it will be apparent that the optimum conditions are such that complexing could spontaneously take place *in vivo* under suitable conditions.

Experimental

Materials. Oleic acid (iodine number, observed 89.9, theory 89.9) was prepared by a slight modification of the fractional crystallization method described by Wheeler and Riemenschneider (9). Linoleic acid was prepared by a modification of the method described by Brown and Frankel (10). It had an iodine number of 180.1 (theory 181.1) and an acid number of 199.5 (theory 200.0).

Thermally oxidized corn oil was prepared by heating corn oil at 200C for 20–60 hr. Air was blown through the oil at the rate of 300 ml/ minute by means of a fritted glass stick. Thermally oxidized corn oil A (TOCO A) was prepared by heating corn oil under these conditions for 20 hr and thermally oxidized corn oil C (TOCO C) was prepared by heating corn oil under these conditions for 60 hr.

Formation of Lipid-Protein Complexes

Typical Procedure. Ten g of egg albumin was gradually dissolved in 1000 ml of distilled water, transferred to a three neck round bottom flask fitted with a mechanical stirrer and 30 g of the lipid material was added and emulsified into the protein solution by continued agitation. The flask was then placed in an oil bath and heated by means of a hot plate. A powerstat connected to the hot plate was so adjusted that the internal temperature of the lipid-protein emulsion was maintained constant at 60 ± 2C. The emulsion was kept vigorously stirred for varying lengths of time. In the standardized procedure, a 2 hr reaction was used. The terminal necks of the flask were left unstoppered in order to stimulate autoxidation of the lipid. At the end of a specified time, the denatured protein-lipid aggregate was separated by filtration. The precipitate was dispersed in 1000 ml of acetone and stored at room temperature for 24 hr with occasional stirring by means of a glass rod. The precipitate was separated by filtration, washed with acetone-ether (50:50 by vol) solution and extracted in a Soxhlet apparatus for 24 hr with acetone. This was followed by a 24 hr extraction with ethyl ether. In most cases, the second extraction with ether was found to be unnecessary. The extracted product, which has been designated as the lipid-protein complex was dried at 60C for 24 hr under vacuum and weighed.

Variations in the Procedure. In some instances, buffer, salt or acetone-water solutions were used instead of distilled water. The protein was also mixed in the dry state with the lipid at different temperatures and for varying lengths of time. In one instance, the egg albumin which had been denatured by lauric acid was used both in the dry as well as in the wet state. The egg albumin denatured by lauric acid was also used before and after removal of the lauric acid by Soxhlet extraction.

Estimation of Lipid in the Complexes

Two to three g of the complex was hydrolyzed on a steam bath for 10 hr in a 10% solution of aqueous KOH. The resulting solution was acidified with HCl and extracted with ethyl ether. The residue obtained upon evaporation of the ether extract was taken as indicative of the percentage of lipid complexed with the protein. Whenever glycerides were used for com-

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TABLE I

The Effect of the Nature of the Lipid on Complex Formation [a]

Expt. No.	Lipid material	Reaction time, hr	Product[b]/ initial protein, %	Lipid[c]/ product, %
1	Trilaurin	60	70.0	0.0
2	Lauric acid	60	77.0	0.1
3	Oleic acid	32	66.0	0.4
4	Cottonseed oil	24	73.0	1.3
5	Corn oil	2	34.4	0.5
6	Corn oil	24	74.5	3.3
7	Linoleic acid	2	69.0	0.3
8	Linoleic acid	28	90.0	7.7
9	TOCO A[d]	2	70.1	4.4

[a] Using 10 g of egg albumin, 30 g of the lipid, 1000 ml of distilled water at 60C.

[b] Product/initial protein means yield of the product expressed as per cent of the initial dry protein. The initial protein contained 2.5% moisture as determined by drying at 110C in a vacuum oven to constant weight.

[c] Lipid/product means the percentage of extractable material obtained from the product on hydrolysis after correcting for the extractable material in the native protein.

[d] Corn oil thermally oxidized at 200C for 20 hr in air.

plex formation, this amount was multiplied by a factor of 1.05 to account for the loss of glyceride structure by saponification. Egg albumin under similar conditions gave rise to 0.8% of extractable material.

Results

The results indicated that lipid-protein complexes were not formed from lipid and protein unless the fat or fatty acid was oxidized or polymerized. Egg albumin is easily denatured and forms denatured aggregates, particularly in the presence of any foreign material like lipid. Therefore, the term complex could be misleading, especially when it is applied to the denatured protein. The criteria for denaturation of the protein in these experiments was based mainly on solubility. However, lipid polymers seemed to possess an ability to combine with denatured protein aggregates under suitable conditions.

Saturated glycerides and fatty acids for example, did not react wtih egg albumin to any appreciable extent, while a monounsaturated fatty acid like oleic acid complexed with the protein to the extent of 0.4% (Table I). On the other hand, a polyunsaturated acid like linoleic acid complexed to the extent of 7.7% in 28 hr. However, it reacted with egg albumin only to the extent of 0.3% in 2 hr. Corn oil and cottonseed oil produced about the same amount of denaturation, although the percentage of complexed lipid was greater with corn oil.

In order to study the effect of complex formation in a homogeneous media, the sodium soap of the fatty acids from thermally oxidized corn oil was used, but no complex was formed. A similar result was observed when egg albumin was reacted with cottonseed oil in an acetone-water medium. Under the standard condition, TOCO A reacted with egg albumin to the extent of 4.4% in 2 hr as compared with 0.5% in the case of fresh corn oil. The lipid material obtained upon alkali hydrolysis of the egg albumin-TOCO complex was subjected to various physical and chemical analyses. The acid and iodine numbers (11) of this material were 237 and 59, respectively. The corresponding figures for TOCO A fatty acids obtained by direct alkali hydrolysis of TOCO A were 208 and 112, respectively. The infrared spectrum of the complexed lipid resembled the spectra of fatty acids. A close agreement was noted between the observed values for nitrogen of the lipid-protein complexes and the values calculated on the basis of the lipid content of the complexes.

Although Tappel (12) has indicated that amino acids and partial hydrolysates of proteins also form complexes with oxidized lipids, the present study indicated that amino acids like glycine and lysine, and proteinaceous materials such as bactopeptone, gelatin and sodium caseinate did not form stable complexes with TOCO A under the experimental conditions described above (Table II). On the other hand, egg albumin, whole egg white, lactalbumin, and casein did form complexes with TOCO A.

The various conditions that influenced the formation of these complexes were studied using egg albumin and TOCO A. Reproducible results could be obtained if the method described under "Experimental" was strictly adhered to. As the time of reaction was increased the amount of the complex and the percentage of the lipid in the complex increased in a linear fashion (Table III). The amount of lipid in the complex increased as the volume of water used in the experiment was increased to an optimum level of 1000 ml and decreased thereafter. A similar trend in the weight of the complex was however not observed in this case. The extent of denaturation of the protein seemed to be directly proportional to the amount of lipid used in the experiment (Table IV). The results indicated that 60C was the optimum temperature for complex formation (Table V). Considerable difficulty in filtration was experienced with egg albumin complexed at 30 and 90C, but not in that complexed at 60C.

The pH of the reacting solution was found to have a pronounced influence on complex formation (Table VI). As the pH was varied on either side of the isoelectric pH of the protein, the amount of denaturation decreased. However, the percentage of lipid in the complex increased as the pH was increased to a value of 7.0. It will be noted that the reaction with distilled water produced more complex than any of the buffered solutions with the exception of pH 5.0, which gave rise to more denaturation but less combined lipid. A pH of 7.0 appeared to be the optimum for complex formation.

A 2% solution of sodium chloride increased the denaturation of the protein and produced a slight increase in the amount of the complexed lipid as compared with a distilled water medium under the same

TABLE II

The Effect of the Nature of the Proteinaceous Material Upon Complex Formation [a]

Material	Medium	Reaction, hr	Product/ initial protein, %	Lipid/ product, %
Glycine	Water	24	0	.....
Lysine	Water	24	0	.....
Bactopeptone	Water	24	0	.....
Gelatin	Water	48	0	.....
Sodium caseinate	Water	24	0	.....
Fresh egg white	Water	20	41.4	7.3
Egg albumin powder	Water	20	81.4	4.7
Lactalbumin	Water	2	85.2	3.8
Casein	Dry state	4	.....	1.8

[a] Using 10 g of the proteinaceous material and 30 g of TOCO A at 60C.

TABLE III

The Effect of Time of Reaction on Complex Formation [a]

Time, hr	Product/ Initial protein, %	Lipid/ product, %
2	70.1	4.4
16	78.6	5.0
24	81.4	5.0
48	86.1	7.1
68	91.7	9.2

[a] Using 10 g egg albumin, 30 g TOCO A, 1000 ml distilled water at 60C.

conditions. Although a 1% lysine solution increased the denaturation of the protein, very little difference could be observed in the percentage of the complexed lipid in the product.

In view of the fact that denaturation of the protein seemed to be linked with the reaction between the oxidized lipid and the protein, experiments were devised to study this relationship. As mentioned previously, oleic acid did not complex with egg albumin to any appreciable extent, however, it was capable of denaturing protein. When only 3 g of TOCO C was used for forming the complex under the standard conditions, 63.4% of the product and 2.6% of the complexed lipid was obtained. However, when 3 g of TOCO C was mixed with 27 g of oleic acid, the corresponding figures were 75.4% and 6.1%, respectively.

When the protein was mixed in the dry state with TOCO A, no reaction could be observed in 2 hr (Table VII). Prolonged mixing seemed to enhance the combining capacity of the protein. Egg albumin which had been denatured by lauric acid seemed to react under these conditions somewhat more than the native protein. The same denatured protein would not react with TOCO A under the standardized conditions in which water was present. However, the denatured protein was found to react with TOCO A under the latter conditions if it was not freed from the lauric acid which had been used as a denaturing agent.

Discussion

Previous investigators did not directly estimate the amount of lipid in the complex. For instance, Casselman reacted unsaturated lipids with tissue preparations and merely indicated whether a complex was formed or not (13). Tappel estimated the amount of lipid in the complex from an elemental analysis (12). The close agreement obtained between the observed and calculated values for nitrogen in the complexes prepared in the present study indicated that the lipid was quantitatively isolated by hydrolysis.

The small amounts of extractable material (Table I) obtained upon hydrolysis of the denatured protein treated with lauric acid, trilaurin, oleic acid, corn oil (Expt. No. 5) and fresh linoleic acid (Expt. No. 7) are indicative of the efficiency of the extractive system used. Since saturated or monounsaturated fatty acids and glycerides did not complex with egg albumin to any appreciable extent, these lipid materials did not possess the necessary structure of functional groups that were apparently present in oxidized corn oil and oxidized linoleic acid (1).

The present data also indicated that complex formation was not solely restricted to egg albumin. TOCO A complexed with lactalbumin to about the same extent as with egg albumin. On the other hand, casein was observed to be less reactive than egg albumin. The increased complexing observed with fresh egg white seemed to indicate that the commercial egg albumin powder used in this study was partially denatured. As neither gelatin nor sodium caseinate formed any complex, this suggested that a certain specificity existed in order that a protein may react with a lipid polymer. From the present data, it is not possible to suggest what this specificity is. However, it was apparent that complexing was associated with the denaturing property of the protein. The reason for the increased complex formation with time was probably due to the increase in denaturation of the protein with time, thereby presenting more surface for the lipid to complex. A similar reasoning could be applied to the increase in complex formation observed by increasing the amount of lipid used in the experiment. The drop in the percentage of complexed lipid using a reaction temperature of 90C was apparently due to the fact that a certain physical state of the protein was required for a reaction of this type.

Under the present experimental conditions, all lipids, saturated or unsaturated, fresh or oxidized, seemed to denature protein to some extent by occupying specific spots in the protein molecule. A suitable solvent should displace almost all of these lipids from their positions, but a large part of the oxidative and thermal polymers probably stay combined with the protein. If this conjecture is correct, then thermally oxidized corn oil should displace lauric acid from its position in the protein molecule by a similar process. This seemed to have happened when the denatured protein was used before removal of lauric acid. After removal of lauric acid the denatured protein did not complex with TOCO A under the standardized conditions, which indicated that a simultaneous denaturation and lipid-protein reaction had to occur so as to bring about complex formation. This was also confirmed by the experiment in which a noncomplexing fatty acid such as oleic acid was used in conjunction with a small amount of TOCO A and resulted in enhanced denaturation of the protein as well as an increase in the amount of complexed lipid.

Tappel prepared oxidized lipid-protein complexes by reacting unsaturated fats and fatty acids with proteins in the presence of a hematin catalyst (12). In agreement with the observations made by Tappel, the present study indicated that oxidized lipid-protein complexes could be readily formed *in vitro*. While Tappel has stressed the need for a hematin catalyst in the lipid protein reaction, the present study clearly demonstrated that hematin was not necessary for complex formation.

Preliminary work conducted has confirmed the ear-

TABLE IV

The Effect of the Amount of Lipid on Complex Formation [a]

TOCO A wt, g	Product/initial protein, %	Lipid/product, %
5	38.6	1.9
10	45.0	2.0
18	56.1	2.2
30	70.3	4.4

[a] Using 10 g egg albumin, 1000 ml distilled water at 60C in 2 hr.

TABLE V

The Effect of Temperature on Complex Formation [a]

Temperature, C	Product/initial protein, %	Lipid/product, %
30	43.5	2.6
60	70.1	4.4
90	59.0	1.1

[a] Using 10 g egg albumin, 30 g TOCO A, 1000 ml distilled water and 2 hr reaction.

TABLE VI

The Effect of pH on Complex Formation [a]

pH	Product/initial protein, %	Lipid/product, %
1.2	66.8	1.1
5.0	75.0	2.6
7.0	52.5	4.3
9.4	59.0	3.6

[a] Using 10 g egg albumin, 30 g TOCO A, 1000 ml buffer solution at 60C for 2 hr.

TABLE VII

The Effect of the State of the Protein and Dry Mixing Under Various Conditions on Complex Formation

Protein	Reaction conditions				Extractable[a] material in product, %
	Water ml, protein g	Temp, C	Time, hr	Lipid	
10 g native egg albumin	0[b]	200	0.5	100 g TOCO A	0
10 g native egg albumin	0[b]	60	2.0	100 g TOCO A	0
10 g native egg albumin	0[b]	60	33.0	100 g TOCO A	4.8
10 g egg albumin denatured by lauric acid[c]	0[b]	60	33.0	100 g TOCO A	6.5
10 g egg albumin denatured by lauric acid[c]	100	60	33.0	30 g TOCO A	0
10 g egg albumin denatured by lauric acid[d]	100	60	24.0	30 g TOCO A	2.6

[a] After alkali hydrolysis and acidification.
[b] Indicates mixing in the dry state.
[c] After removal of lauric acid by Soxhlet extraction.
[d] Before removal of lauric acid.

lier observations of Casselman (13) and Hartroft (2) that the rat red blood cell proteins also complex with oxidized lipids under the present experimental conditions. The ease with which these complexes are formed suggested that lipid-protein complexes may also form *in vivo* under certain pathological conditions. The complexing of oxidized lipid with protein may be responsible for the formation of the brown pigments which have been noted in the uterus of vitamin E deficient rats (5) and for the accumulation of ceroid pigment in the liver of choline deficient rats (3). The observation that complexing can take place at 30C and that optimum complexing was noted at a pH of 7.0 would indicate that *in vivo* lipid-protein complexing could be involved in degenerative or pathological changes in specific organs or tissues (3,4,5,6,7,14).

While it is true that the oxidized lipid-protein complexes are different from the naturally occurring lipoproteins, it seems possible that the interaction between oxidized lipids and the low density lipoprotein of blood serum (8) may be similar to the interaction between the oxidized lipids and other proteins used in this study.

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The Acetylenic Acid in *Comandra pallida* and *Osyris alba* Seed Oils

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Abstract

Gas-liquid chromatographic (GLC) analyses are reported for fatty acid methyl esters from seed oils of two previously unreported species of Santalaceae, *Comandra pallida* A. DC. and *Osyris alba* L. The major component in each (43 and 57%, respectively) is an enynoic acid, probably *trans*-11-octadecen-9-ynoic (ximenynic) acid which has been found in seed oils of other members of this family. Equivalent chain lengths by GLC analysis and infrared and ultraviolet spectra agree with those obtained by our analyses of *Ximenia americana* L., in which ximenynic acid is known to occur. The spectral data also agree with those in literature reports on ximenynic acid. The positions of unsaturation have, however, not been rigorously established for the two species newly reported.

Introduction

POLYUNSATURATED acetylenic oils have been found in plant seeds from only two families, Olacaceae and Santalaceae. Ligthelm and Schwartz (9) proposed four possible structures for an unknown acetylenic acid in seed oil of *Ximenia caffra*, and proposed it be named ximenynic acid. Ligthelm et al. later characterized this acid as *trans*-11-octadecen-9-ynoic acid (10). Ximenynic acid was synthesized by Grigor et al. (2). Simultaneously Gunstone and McGee (3) characterized "santalbic" acid and found it had the same structure as ximenynic acid.

Ximenynic acid has now been reported in seed oil from two species of *Ximenia* (8) in the family Olacaceae and from four species of *Santalum* (3,4), two of *Exocarpus* (5), and one of *Leptomeria* (6) in the Santalaceae.

This paper reports the presence of apparent ximenynic acid in seed oils from two additional genera of Santalaceae. An analysis of seed oil from *Ximenia americana* L., in which ximenynic acid is known to occur (8), is also included.

Procedure

Materials and Methods

Oils were extracted with petroleum ether (30–60C) from the ground seed plus pericarp of *Comandra pallida* A. DC. and of *Osyris alba* L., and from the ground seed of *Ximenia americana* L.

Esters were prepared from the oils of *C. pallida* and *O. alba* by methanolysis with sodium methoxide

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