growth of the seed do not represent a correct picture of the formation of fatty acids in the seeds. The rapid variation of the ratio of the weight of the kernel to the seed coat, as well as the fact that the seed coat does not contain appreciable amount of oil, will indicate that calculation of the amounts of individual fatty acids in 1 g of the seed does not give us a correct picture of the formation of fatty acids. The number of seeds that would produce one g of kernel differ at different stages of growth of the seeds. As each seed is a unit center of synthesis and storage of fatty acids, it is considered that calculations of the amounts of individual fatty acids in one seed, or a fixed number of seeds at each stage of growth, would give a better picture of biosynthesis of the fatty acids.

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Complex Formation Between Oxidized Lipids and Egg Albumin[1]

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Abstract

The nature of the reactions leading to the formation of complexes between oxidized lipids and proteins is little understood. Complexes were formed between thermally oxidized and thermally polymerized corn oils and egg albumin. The results indicated that the thermally oxidized oil was much more reactive than the thermally polymerized oil. The nature and extent of oxidative groups in the lipid were varied and reacted with egg albumin. The keto and epoxy groups seemed to have a pronounced influence on complex formation while the hydroxy and hydroperoxide groups were observed to be less reactive. Acetylation of egg albumin did not significantly decrease its complexing ability with thermally oxidized corn oil. Further confirmation that the reactive groups in the protein were not covalently tied up to the lipid was obtained by estimating these groups in the original protein and in the complexed protein. IR analyses of oxidized lipid-alumina, oxidized lipid-egg albumin and fatty acid-urea complexes indicated that the characteristic absorption was due to bonded O—H stretching vibration in the first two complexes and the bonded N—H stretching vibration in the two latter complexes.

Introduction

THE BROWNING of food materials is a familiar example of complex formation between proteins and reducing sugars (1,2). Complex formations between carbohydrates and lipids are generally not well known (3,4). The complex reactions that occur during the oxidation of fats in the presence of proteins have been considered to be responsible for the yellowing of bacon and rusting of fish, and oxidized fats have been implicated in the formation of brown pigment in the adipose tissue of vitamin E deficient rats and ceroid pigment in choline deficient rats (5,6,7). However very little work has been devoted to studying the chemical and physical properties of the oxidized lipid-protein complexes which may be formed *in vitro*.

The linkage between the lipid and the protein in the case of the complex conjugated proteins, known as lipoproteins, probably involves Vander Waals forces and weak electrostatic attractions (8,9,10). In most cases, these lipoprotein complexes are labile and the lipid can be separated by suitable extraction methods. On the other hand, oxidized lipid-protein complexes are not amenable to similar easy separations (11).

It has been recently observed that oxidized methyl linoleate interacts *in vitro* with serum low density lipoproteins while unoxidized methyl linoleate does not (12). Hartroft has observed the presence of ceroid pigments in the aortas of man exhibiting atheromatous changes, and he also observed the formation of ceroid-like pigments *in vitro* by reacting heparinized red cells with cod liver oil in the presence of air (13,14). The system used in these *in vitro* experiments has been complex and therefore very little information could be obtained regarding the type of interaction between the oxidized lipid and the protein.

In a previous study (11), a relatively simple system such as oxidized linoleic acid and egg albumin was studied and the results indicated absence of covalent bonding between the oxidized lipid and the protein. It was further suggested that hydrogen bonds were apparently responsible for complex formation. However no definite information could be obtained from that study as to the extent to which each of the oxidative groups in the lipid was involved in complex formation with the protein. Therefore, in the present investigation, an atempt was made to understand the nature of the linkage between the oxidized lipid and the protein by a) varying the nature and extent of the reactive groups in the lipid, b) blocking the reactive groups in the protein and estimating the total reactive groups in the original pro-

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tein and in the complexed protein, and c) bringing out points of similarity between oxidized lipid-egg albumin, oxidized lipid-alumina and fatty acid-urea complexes. The data obtained confirmed previous findings and further emphasized the role of keto and epoxy groups in complex formation.

Experimental

Thermally oxidized corn oil was prepared by blowing 300 ml air/min through a fritted glass stick into 2 kg corn oil heated to 200C. Heating was carried out in a 5-liter stainless steel beaker, using a hot plate and an electrical heating tape wrapped around the beaker. The temp was regulated by two separate powerstats. The oil was kept well agitated with a mechanical stirrer. Thermally oxidized corn oils A (TOCO A), B (TOCO B), C (TOCO C) were prepared by heating corn oil under these conditions for 20,36 and 60 hr, respectively. Thermally oxidized corn oil D (TOCO D), obtained from a commercial source, was also used in some of the experiments. This oil was prepared on a large scale by blowing 200 ml air/min/kg corn oil heated to 200C for 24 hr.

Thermally oxidized hydrogenated cottonseed oil (TOHCO) was also prepared according to the method used for TOCO A by heating hydrogenated cottonseed oil (iodine number 1.4) to 200C for 72 hr. The product on cooling was dissolved in seven volumes of acetone and stored at 0C for 72 hr. The acetone-soluble material was freed from actone under vacuum. It was freed from the last traces of acetone by repeated washing with water, dissolved in ethyl ether, washed again with water, dried over anhydrous sodium sulfate, and freed from solvent under vacuum.

Thermally polymerized corn oil was prepared under two different conditions:

1. *Under an Atmosphere of Nitrogen.* This product (TPCON 1) was obtained by heating corn oil contained in a 3-neck round bottom flask at 200C with a heating mantle for 48 hr with mechanical stirring under a blanket of H. P. tank nitrogen. Another sample (TPCON 2) was obtained in the same manner by heating corn oil at 300C for 96 hr.

2. *Under Vacuum.* This oil (TPCO) was prepared by heating corn oil contained in a round bottom flask under vacuum (200 micron pressure) at 400C with a heating mantle for 96 hr.

A highly polymeric fraction (TOCO BL) was obtained from TOCO B by removing unreacted triglyceride through enzymatic digestion, using commercial steapsin and phosphate buffer solution pH 8.0 (15).

Acetylated TOCO D was prepared by reacting 50 g TOCO D with 10 ml acetylchloride and 20 ml pyridine in an ether-toluene medium at room temp. The oxime was prepared by reacting 50 g TOCO D, dissolved in 100 ml pyridine, with hydroxylamine hydrochloride in 200 ml 95% ethanol at room temp. The epoxy derivatives were prepared according to the method of Findley (16) and the chlorohydroxy derivatives by reacting the epoxy substances with 0.2 N hydrochloric acid in ether medium. Treatment of linoleic acid with lipoxidase was accomplished by reacting 250 ml of a 5% solution of sodium linoleate with 10 mg lipoxidase (Mann Research Laboratories) in 0.1 M ammonia-ammonium chloride solution (pH 9.0) for 4 hr at 5C. The mixture was then acidified to pH 3.0 with dilute HCl, extracted with ether, washed and freed from solvent. One g of the linoleic acid hydroperoxide thus obtained was subsequently treated with 100 mg catalase (Nutrition Biochemicals Corporation) in a large excess of 0.1 M phosphate buffer pH 7.0 at 20C for 24 hr. After acidification to pH 3.0, the mixture was extracted with ether, washed and freed from solvent.

Formation of Lipid-Protein Complexes. A 1% solution of the protein was reacted with 30 g of the lipid at 60C for varying lengths of time. The denatured protein-lipid complex was obtained by filtration. It was freed from the unreacted lipid by exhaustive solvent extraction with acetone and ethyl ether—first by soaking in a large excess of acetone, followed by filtration and washing of the precipitate with acetone-ether (50-50 v/v) solution and finally by soxhlet extraction with acetone for 24 hr followed by ether for 24 hr. The lipid in the complex was estimated by alkali hydrolysis and gravimetric determination of the fatty acids as previously described (11). Suitable correction was made for the loss of glyceride structure due to the alkali treatment.

Lauroylation of Egg Albumin and the Complex. The protein or the complex was treated with a large excess of lauroyl chloride in an ether-pyridine medium for 72 hr at room temp (11). The number of acyl groups introduced was estimated by gravimetric determination of lauric acid after alkali hydrolysis of the acylated product and acidification.

Acetylation of Egg Albumin. The procedure employed was the method described by Fraenkel-Conrat et al. (17).

Alumina-Lipid Adsorption Complexes. These complexes were formed by mixing 100 g activated alumina with 25 g of the lipid for 10 min using a spatula (11). The unreacted lipid was removed by exhaustive washing with ethyl ether followed by soxhlet extraction with ethyl ether for 24 hr and finally by soxhlet extraction with 1, 4 dioxane for 24 hr.

Thermal Differential Analyses. The equipment for these analyses consisted of an electrically heated tubular furnace, a nickel block 1 in. x 1 in. x ⅜ in containing four holes ¼ in. deep by 3/16 in. diam, an amplifier and an Esterline graphic ammeter. Two of the holes in the nickel block were connected by a differential thermocouple consisting of platinum-platinum-rhodium junctions. The sample was placed in one of these holes and the other hole was filled with alumina. The differential thermocouple was connected to the graphic ammeter through the amplifier and a sensitivity control. The third hole was also filled with alumina and a thermocouple placed in it was connected to a pyrometer which registered the temperature of the block. The fourth hole was left empty.

As soon as the holes were filled, the nickel block was placed in the furnace and the current turned on. The rate of heating was controlled by means of a powerstat and was kept constant at about 18C/min. The difference in the temp of the hole containing the sample and the reference hole containing alumina was registered automatically on a constantly moving chart. In order to obtain suitable thermograms with lipid-protein complexes, a 2% sample on alumina (2 parts sample mixed with 98 parts alumina) was employed.

Two techniques were used to obtain the IR spectra, the *smear* technique and the potassium bromide disc method (18,19). The iodine and acid numbers were determined by the official AOCS methods (20). The peroxide numbers were determined according to the method of Wheeler (21), the carbonyl values were determined by the method described by Lappin and

TABLE I

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

Lipid	Conditions of polymerization	Code	Reaction time hr	Complex [b] initial protein %	Lipid [c] complex %
Corn oil	20 hr 200C in air	TOCO A	48	86.1	7.1
Corn oil	36 hr 200C in air	TOCO B	48	89.1	17.4
Corn oil	60 hr 200C in air	TOCO C	48	123.0	40.7
Fatty acids from TOCO B	36 hr 200C in air [d]	....	48	75.2	3.7
Linoleic acid	unoxidized initially	....	31	89.9	6.0
Non-lipase hydrolysate from TOCO B	36 hr 200C in air [d]	TOCO BL	48	120.0	28.6
Corn oil	48 hr 200C under nitrogen	TPCON 1	48	76.2	0.4
Corn oil	96 hr 300C under nitrogen	TPCON 2	48	88.2	16.0
Corn oil	96 hr 400C under vacuum	TPCO	2	90.0	9.2
Hydrogenated cottonseed oil	72 hr 200C in air	TOHCO	16	82.7	6.6

[a] Ten g egg albumin, 30 g lipid, 1000 ml distilled water at 60C.
[b] Indicates yield of the complex expressed as per cent of the wt of the initial dry protein.
[c] Indicates the percentage of the extractable material obtained from the complex on hydrolysis after correcting for the extractable material in the native protein.
[d] These conditions refer to TOCO B and not to the material derived from it.

Clark (22), the hydroxyl value by the method of Smith and Bryant (23) and the epoxy value by the method of Swern et al. (24).

Results

The results confirm a previous observation that only oxidized or polymerized fatty acids are capable of complexing with egg albumin (11). However, the relative reactivities of thermally oxidized and thermally polymerized glycerides have not been previously studied.

Whereas TPCON 1 reacted with egg albumin to the extent of only 0.4%, TOCO B complexed with the protein to the extent of 17.4% (Table I). As the time of thermal oxidation of corn oil was increased, the reactivities of the thermally oxidized oils were also found to increase progressively. Linoleic acid and thermally oxidized corn oil fatty acids (TOCO B) reacted with egg albumin to a considerably lesser extent than TOCO B. Corn oil, which was thermally polymerized under nitrogen at 300C for 96 hr, had a much darker color and higher viscosity when compared with corn oil polymerized under vacuum at 400C for 96 hr. The former complexed with egg albumin to the extent of 16% in 48 hr while the latter reacted with egg albumin to the extent of 9.2% in 2 hr. Thermally oxidized hydrogenated cottonseed oil was also observed to react with egg albumin. Apart from giving the yield of the complex, the data presented in column 5, Table I, could be used to evaluate the relative amounts of the protein tied up by the lipid and expressed as per cent original dry

TABLE II

The Chemical and Physical Properties of Some of the Lipid Materials Used for Complex Formation

Lipid material	Viscosity poises 25C	Iodine number	Peroxide number meq/kg	Carbonyl value meq/g
TOCO A	2.1	107.4	4.7	0.32
TOCO B	3.5	99.9	20.7	0.44
TOCO BL	15.4	92.9	12.5	0.41
TOCO C	22.7	80.6	3.7	0.50
Unreacted linoleic acid [a]	......	93.7	215.8	1.43
TPCO	6.3	97.5	1.7	0.11
TOHCO	27.0	17.3	3.1	1.30

[a] Isolated from the corresponding experiment mentioned in Table I.

TABLE III

The Influence of the Reactive Groups in the Lipid on Complex Formation [a]

Lipid	Treatment	Epoxy value meq/g	Hydroxyl value meq/g	Carbonyl value meq/g	Complex initial protein %	Lipid complex %
TOCO D	None	0.3	0.9	0.5	95	20.0
TOCO D	Hydroxylamine, hydrochloride	....	....	....	35	3.5
TOCO D	Sodium bisulfite	....	....	....	93	19.0
TOCO D	Chlorohydroxylated	0.0	1.2	0.5	96	14.0
TOCO D	Acetylated	....	....	....	60	10.5
Castor oil	None	....	....	....	40	1.0
Castor oil	Epoxidized	....	....	....	75	4.6
Castor oil	Chlorohydroxylated	....	....	....	45	1.2
Linoleic acid	None	....	....	....	30	0.7
Linoleic acid	Lipoxidase (peroxide value 2300)	0.0	0.2	0.3	72	2.1
Linoleic acid	Lipoxidase followed by catalase (peroxide value 550)	0.1	0.8	1.0	81	5.1

[a] Complexes were formed in a 250 ml Erlenmeyer flask by shaking the oxidized lipid-protein emulsion at 37C for 12 hr.

protein. This amount was greatest in the case of TOCO BL and linoleic acid. Although in two instances (TOCO C and TOCO BL) the wt of the complex far exceeded the wt of the original protein, the amount of protein in these complexes worked out to be 73% and 86% respectively of the original wt of the protein.

In an attempt to understand the nature of the linkage between the protein and the oxidized or polymerized lipid in these complexes, the chemical and physical properties of the oxidative and thermal polymers of corn oil were studied (Table II). In the case of the thermally oxidized oils, a decrease in the iodine number was attended by an increase in the amount of the complex and complexed lipid (Table I and II). However, a similar trend was not observed in the case of peroxide numbers. The carbonyl values increased only slightly as the duration of the thermal oxidation of the corn oil was increased.

The unreacted TOCO isolated from these experiments showed a slight increase in the peroxide number and a small decrease in the carbonyl value which might indicate that very little further oxidation of these oils had occurred. The unreacted TPCO exhibited a small increase in both the peroxide and carbonyl value.

The only difference that could be observed in the IR spectra of TOCO and TPCO over that of fresh corn oil was the pronounced band at 10.36 μ indicative of *trans* configuration. The material isolated from the complexes by alkali hydrolysis exhibited all the characteristic absorption band of fatty acids; the C—H stretching near 3.4 μ; the O—H . . . O association band at 3.7 μ; the C=O stretching at 5.8 μ; the C—H deformation around 7.0 μ, the broad band at 10.6 μ, due to the vibrations of the O—H group in and out of the plane of the COOH group and the CH_2 wagging mode near 13.7 μ.

TOCO BL, which is of a more polymeric nature than TOCO B, reacted with egg albumin to a greater extent than TOCO B, but to a considerably lesser extent than TOCO C. The solubility of these lipids decreased with increasing polymerization; however, they were all soluble in ethyl ether. Therefore, the increased amount of complexed lipid observed with

TABLE IV
Estimation of Lauroyl Groups Introduced into the Protein and the Complex

Protein	State of protein	Acylated product		Mole acyl group 100 g protein
		Total lipid %	Polymeric lipid %	
Egg albumin	native	0.8	0	0
Egg albumin	denatured by oleic acid	27.6	0	0.186
Egg albumin-TOCO complex	denatured	27.3	8.7	0.123
Egg albumin-TOCO complex	denatured	51.1	38.6	0.120
Egg albumin-linoleic acid complex	denatured	33.5	8.0	0.183

increasing polymerization of the reacting lipid was not due to incomplete extraction of the unreacted lipid. The lipid-protein complexes were extremely stable, the lipid being unextractable with polar and nonpolar solvents. For instance, 10 g of an egg albumin-TOCO complex containing 13% TOCO was extracted continuously in a soxhlet apparatus with ethyl ether for 10 days and only 6 mg of extractable material was obtained. Various other solvents, like benzene, skellysolve F, methanol, ethanol, dioxane and chloroform were used, and in only one instance, i.e., dioxane about 2% of extractable material was obtained.

Whereas TOCO D complexed with egg albumin to the extent of 20%, the oxime of TOCO D reacted only to the extent of 3.5% (Table III). However the bisulfite addition product of TOCO D did not significantly affect the complexing ability of the oxidized lipid. TOCO D, which was chlorohydroxylated reacted to a lesser extent than TOCO D but to a greater extent than the oxime of TOCO D. The castor oil experiments also indicated that the epoxy groups do contribute towards complex formation. The complex formed with oxidized linoleic acid with a peroxide value as high as 2300 contained only 2.1% complexed lipid. Although the peroxide value decreased from 2300 to 550 after catalase treatment, an increase rather than a decrease in complex formation was observed. Acetylation of TOCO D brought about a pronounced decrease in complex formation, which could be attributed to a decrease in the hydrophilic character of the lipid.

In order to determine whether any of the groups in the protein of the complexes were tied up, an attempt was made to estimate the reactive hydrogen groups in the protein and in the protein of the complexes. These included the amino, sulfhydryl and the hydroxyl groups but not the carboxyl groups. The lack of reaction observed between lauroyl chloride and native egg albumin indicated that the reactive groups became available only on denaturation of the protein (Table IV). The linoleic acid-egg albumin complex had almost the same number of reactive groups as the protein. The number of reactive groups in the egg albumin-TOCO complexes seemed to be less than in the protein.

Egg albumin after acetylation gave a negative ninhydrin test and a zero formal value. It combined with TOCO C almost to the same extent as egg albumin (Table V), which established that the amino groups in the protein molecule were not involved in the lipid-protein complex reaction. The addition of p-chloromercuribenzoate to the reaction mixture of TOCO D and egg albumin did not affect complex formation which indicated that the sulfhydryl groups in the protein were not involved in the reaction. All

TABLE V
The Effect of Acetylation of the Protein on Complex Formation

Protein	Lipid	Reaction conditions			Complex initial protein %	Lipid complex %
		Water ml	Temp C	Time hr		
Egg albumin 10 g	TOCO C 30 g	1000	60	2	90.2	18.5
Acetylated egg albumin 10 g	TOCO C 30 g	1000	60	2	92.3	17.0

the egg albumin-TOCO complexes gave a positive ninhydrin, Millon's and biuret tests with the single exception of the egg albumin-TOCO BL complex which gave a negative ninhydrin test. The acylated proteins and complexes gave negative ninhydrin and Millon's reactions but positive biuret tests.

The formation of strongly bound oxidized lipid-alumina complexes have been reported (11). The lipid-alumina and the lipid-protein complexes resembled one another in their ease of formation. Since alumina complexed only with oxidized lipids, it was apparent that oxidized lipids possessed certain functional groups that were not present in fresh lipids. Thermal differential analyses have been used extensively as a very useful tool for the analyses of minerals and recently have been considered as a possible technique for the structural differentiation of complex molecules such as corn amylose, corn amylopectin, glycogen and cellulose (25). The exothermic and endothermic peaks are related to the thermal oxidation and decomposition of the samples. None of the lipid-protein complexes prepared in the present study gave any characteristic mp; however they all decomposed at temp over 230C. The results of a thermal differential analysis study with lipid-protein and lipid-alumina show in Figures 1–5. The pronounced peak observed in the thermogram of egg albumin-TOCO complex at 360C (block temp) was not present in the thermogram of the denatured egg albumin free from lipid. The thermogram of a sample of alumina-TOCO complex gave a broad peak around 360C. This peak was absent in the thermogram of a sample of palmitoyl egg albumin. However, the same peak was also observed in the thermogram of a sample of lauroylated egg albumin-TOCO complex thereby serving as confirmatory evidence for the existence of the peak near 360C. A mixture of alumina and tristearin gave two peaks, one near 300C; other ca. 400C. Palmitic acid gave rise to a single peak in the neighbourhood of 390C.

Very little information could be obtained from X-ray diffraction studies because all the lipid-protein complexes were amorphous in nature. The powder patterns of these complexes and the denatured protein were all alike. The X-ray diffraction patterns of alumina and alumina-lipid complexes were found to be identical. The free O—H and the free N—H stretching vibrations are around 2.8 μ and 2.9 μ, respectively. The bonded O—H and the bonded N—H stretching vibrations are shifted towards higher wave lengths (26). The IR spectrum of a sample of urea-oleic acid (27) exhibited all the absorption bands of oleic acid plus two bands around 3.0 μ and 3.15 μ. The IR spectrum of a sample of a TOCO-alumina complex run against alumina as potassium bromide discs exhibited the C—H stretching vibration at 3.3 μ but not the C=O stretching vibration. Also, it exhibited broad absorption bands around 2.9 μ and 3.1 μ. The absorption bands observed around 3.1 μ in these complexes were probably due to N—H . . . O

THERMOGRAMS OBTAINED AT ROOM TEMPERATURE TO 600C

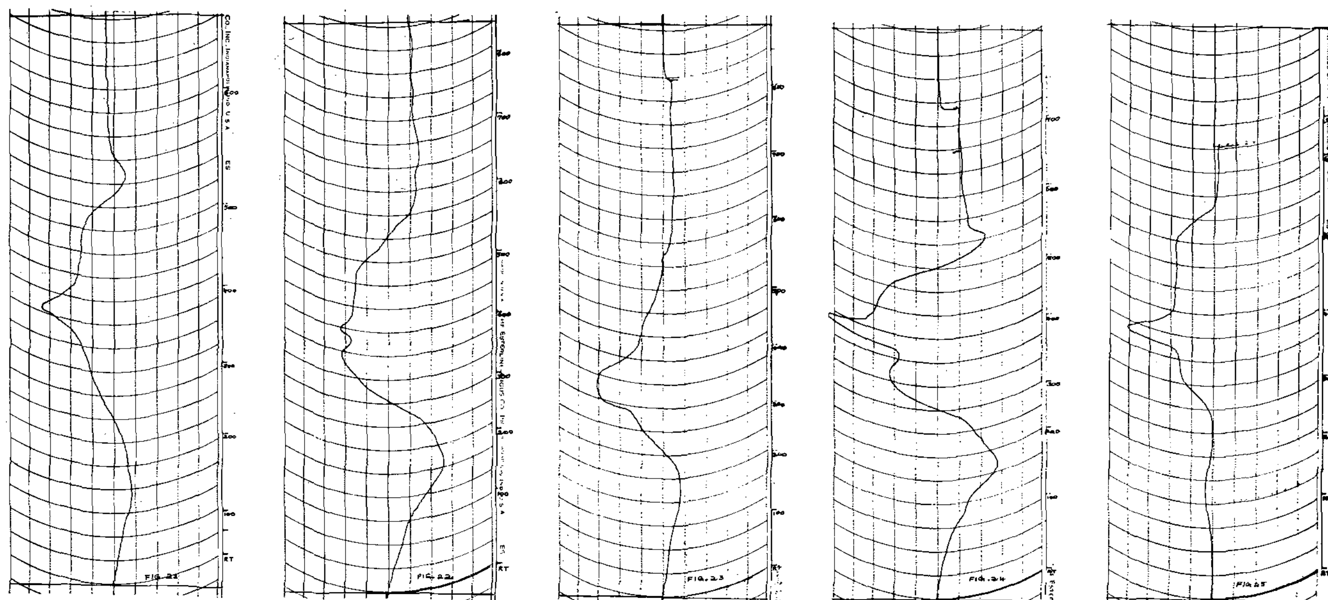

FIG. 1. FIG. 2. FIG. 3. FIG. 4. FIG. 5.

FIG. 1. Egg albumin denatured by lauric acid. Exothermic peak at 400C.
FIG. 2. Egg albumin-TOCO complex. Exothermic peaks at 360C and 400C.
FIG. 3. Alumina-TOCO complex. A broad exothermic peak around 360C.
FIG. 4. Lauroylated egg albumin-TOCO complex. A broad exothermic peak around 360C and a sharp exothermic peak at 400C.
FIG. 5. Palmitoyl egg albumin. Exothermic peak at 390C.

and O—H . . . O stretching vibrations respectively. The IR spectra of TOCO-egg albumin complexes containing more than 14% TOCO exhibited the various absorption bands of the lipid plus two broad bands around 3.0 μ and 3.2 μ. Deuteration studies indicated a decrease in the intensity of the 3.2 μ band and the appearance of two bands at 4.0 μ and 4.15 μ. Hence the 3.2 μ band was probably due to unresolved O—H . . . O and N—H . . . O stretching vibrations. It is, however, difficult to state that the 3.2 μ band observed in the case of the TOCO-egg albumin complexes was due to hydrogen bonding between the oxidized lipid and the protein, because hydrogen bonds existed in the protein itself.

Discussion

The results obtained with corn oil, which was thermally oxidized for 36 hr at 200C and corn oil which was thermally polymerized under nitrogen atmosphere for 48 hr at 200C, clearly demonstrated that oxidative groupings were responsible for complex formation. Corn oils (TPCON 2 and TPCO) which were thermally polymerized at higher temp for longer periods of time, both under nitrogen and vacuum, reacted with egg albumin to a much greater extent than TPCON 1 and this was probably due to the formation of new oxygen groupings in these polymers. Evidence for the presence of carbonyl and hydroperoxide groups in TPCO was obtained by direct chemical determination both before and after reaction with egg albumin. Furthermore, although TPCO was prepared by polymerization at a much higher temp and for a longer time than TOCO C, it complexed with egg albumin to a considerably lesser extent than TOCO C and this indicated that the thermally oxidized oil was much more reactive than the thermally polymerized oil (Table I,V). The results obtained with TOCO B fatty acids and linoleic acid indicated that the loss or absence of the glyceride structure caused a considerable decrease in the complexing ability of the lipid. It would appear from the results obtained with TOHCO that almost completely saturated glycerides could also form oxidative products which were capable of complexing with proteins. In the case of the thermally oxidized corn oils, the viscosities may be considered to reflect the molecular complexity of the polymers. On this basis, it was evident from the results obtained with TOCO A, TOCO B, TOCO BL and TOCO C that the mol wt of the polymers was apparently related to the extent to which a lipid-protein complex could be obtained.

An earlier study in which various aldehydes were reacted with egg albumin seemed to indicate that the lipid-protein complex reaction did not involve an aldehyde-amine condensation (11). The results obtained by reacting the bisulfite addition product of TOCO D with egg albumin indicated that the aldehyde groups did not contribute significantly towards complex formation. On the other hand, when the aldehyde and ketone groups in TOCO D were masked by formation of the oxime, a pronounced decrease both in the amount of the complex and complexed lipid was observed. From these two observations, apparently ketone groups in oxidized lipids play a major role in complex formation. The results obtained with TOCO D and TOCO D, which was chlorohydroxylated, indicated that the epoxide groups also contribute toward complex formation although much less than the ketone groups in the lipid. The linoleic acid experiments demonstrated that the peroxide groups per se were not responsible for complex formation. Oxidized linoleic acid having a peroxide value as high as 2300 reacted with egg albumin to the extent of only 2.1%. The hydroperoxide decomposed by catalase treatment gave rise to more carbonyl and hydroxyl groups which seemed to have enhanced the complex formation. The results obtained with castor oil indicated that the hydroxy groups were not sufficient to bind the lipid to the protein; apparently a combination of epoxy or keto and the hydroxy

groups seemed to be necessary. Preliminary experiments conducted with long chain keto and hydroxy acids have indicated that the keto acids complex readily with egg albumin while the hydroxy acids tend to be less reactive.

The reduction in the number of reactive groups observed in the egg albumin-TOCO complexes, as compared with the uncombined protein, seemed to be the result of a steric hindrance rather than a decrease brought about by a chemical combination between the lipid and the protein, because the egg albumin-linoleic acid complex containing 8% lipid had about 32 more reactive groups than the egg albumin-TOCO complex containing 8.7% lipid (assuming a mol wt of 50,000 for egg albumin). If a chemical combination was involved, the complex containing 38.6% lipid would have considerably less available reactive groups than the complex containing only 8.7% lipid. Glavind and Pedersen (28) have suggested that the hydroperoxide groups in oxidized lipids could react with the carboxyl groups of adjacent peptide chains in protein molecules forming ester linkages. A comparison of the peroxide values of thermally oxidized oils and that of autoxidized linoleic acid revealed that the hydroperoxide groups in the lipid molecule were not directly responsible for the complex formation.

It has been suggested that thermograms may be used not only as a "fingerprint device" for the identification and characterization of organic compounds, but also to interpret molecular composition (29). Distinct differences have been observed in the thermograms of α and β glucose, which indicated considerable stereo-specificity of the method (25). Furthermore, the differences observed in the thermograms of the various polymers of glucose have been attributed to the differing degree of 1,4 and 1,6 linkages in those polymers. Since both alumina and egg albumin formed complexes with oxidized lipids rather than fresh lipids and since a physical mixture of alumina and a nonreactive lipid like tristearin or palmitic acid did not give rise to an exotherm near 360C, it can be stated that the bonding that existed in the two type of complexes was similar in nature. It should be pointed out that palmitoyl egg albumin did not give rise to this exotherm which suggested that a covalently bound lipid does not produce this type of thermal reaction.

All the data presented suggested that linkage between the lipid and the protein was more of a physical than of a chemical nature. Various kinds of fairly strong physical forces binding one molecule to another are known to exist (30). The combination between starch and fatty acids forming a starch-lipid complex is one example (4). The exact nature of the bonding of the fatty acid to the starch is not known. Another interesting example is the urea complexes of fatty acids. Approximately 14 molecules of urea wind around a long-chain fatty acid or hydrocarbon giving rise to crystalline compounds, the hydrogen bonding between adjacent urea molecules largely accounting for the stability of the complex (31). The fatty acids in these complexes cannot be extracted by ether, benzene or similar solvents but can be easily obtained by the addition of water which destroys the crystalline nature of these compounds. Palmer (32) has proposed a structure for the egg albumin-detergent complex which consisted of a polar protein monolayer with the detergent molecules adsorbed on the side only and with their long axes perpendicular to the protein layer.

In the case of the lipid-protein complex, it is not possible to express the adsorption of the lipid on the protein merely on the basis of either polar or nonpolar Vander Waals forces or the regular electrostatic forces for two reasons: 1) the lipid-protein complexes are extremely stable; and 2) the lipid does not carry any ionic charges. However, it is possible that the lipid is linked to the protein by means of weak electrostatic attractions such as hydrogen bonding. The hydrogen bonding may exist between the carbonyl oxygen in the lipid and the hydrogen in the amide groups of the protein and also between the carbonyl oxygen in the protein and the hydrogen of the hydroxy or the hydroperoxide groups in the oxidized lipid. Although bondings of this kind are individually weak (5–8 kcal/mole) the cumulative effect of all these bonds may be quite large (33). As suggested earlier, a combination of epoxy or keto and hydroxy groups in the lipid seemed to be necessary for complex formation. If only weak electrostatic attractions existed in these complexes, it could explain the relative ease with which they were formed.

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