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Nutritive Value of Heated Vegetable Oils

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Abstract

Peanut, sesame and coconut oils were heated at 270C for 8 hr, in an open iron pan. These fats were fed to albino rats at 15% level in otherwise adequate diets. All rats fed heated fats showed a growth depression. Livers of rats receiving heated oil were congested and showed extensive periportal fatty infiltration. Rats on heated peanut oil showed i) reduced B-vitamin storage in the liver, ii) increased glucose and cholesterol levels in the blood and iii) a disruption in the digestion and absorption of carbohydrates.

Introduction

HEATED OILS have been shown to be poorly absorbed (13), to produce cancerous tumors (11) and to cause symptoms resembling that due to vitamin E deficiency (9). Lower nutritive value is also believed to be due to the destruction of certain vitamins, especially vitamin A (6). Further, it has been shown that the oxidation products of fats produced inhibitory effect on certain enzyme systems (4,12).

Consumption of fried foods in India is probably higher than in any other country. The conditions of heating are known to vary widely. The oils are usually heated in open air pans to about 200–300C depending on the type of preparation and the heated oils are reused.

The present investigations were carried out to evaluate the effect of heat on some edible oils commonly used in the country.

Experimental and Results

Preparation of Heated Oils

Peanut, sesame and coconut oils were heated continuously at 270C for 8 hr in an open pan made of iron. The heated oils were stored in pyrex glass bottles at 0C.

Influence of Heating the Oil on Its Nutritive Value and Fat Deposition in the Liver

Thirty-six Wistar strain albino rats, five weeks old and weighing about 40–50 g from our laboratory stock colony were allotted to six groups in a randomized block design and were housed in individual cages. They were fed *ad lib.* on purified diets (fat, 15%; casein, 12%; sugar, 10%; salt mixture, 4%; vitaminized starch, 1% and corn starch, 58%) in which the fat was supplied by raw or heated peanut, sesame or coconut oils. At the end of six weeks feeding, the animals were killed by bleeding through the abdominal aorta. Livers, spleens, stomachs and kidneys were removed and weighed. The liver lipids were estimated by the method of Hawk (7). The results are presented in Table I.

The results show clearly that in all the three cases the heated oil has adversely affected the gain in weight. The feed efficiency ratio, calculated as increase in weight per gram of fat consumed, is significantly lower in groups receiving the heated oils. The livers of rats receiving the heated oil are significantly heavier than the controls. The lipid content of the livers is nearly twice that of the control groups. The livers showed signs of congestion and extensive periportal fatty infiltration. No significant changes were found in the weights of stomach, kidney and spleen of the two groups of animals.

TABLE I

Influence of Heating the Oil on Its Nutritive Value and Fat Deposition in the Liver

Dietary fat	Body weight		Mean gain per week (g)	Mean daily food intake (g)	Feed efficiency*	Liver weight		Liver fat %
	Initial g	Final (g)				(g)	g/100 g body weight	
Peanut oil:								
Raw	42.5	120.7	13.0 ± 0.5[b]	11.3	1.1	4.58	3.8 ± 0.3	3.9 ± 0.3
Heated	42.0	72.1	5.0 ± 0.9	7.1	0.67	4.11	5.7 ± 0.6	7.1 ± 0.4
Sesame oil:								
Raw	42.5	102.5	10.0 ± 0.6	9.5	1.0	4.30	4.2 ± 0.7	4.8 ± 0.3
Heated	42.4	68.3	4.3 ± 0.7	5.9	0.7	4.10	6.0 ± 0.5	7.8 ± 0.3
Coconut oil:								
Raw	43.1	109.2	11.0 ± 1.1	9.5	1.1	4.70	4.3 ± 0.3	4.5 ± 0.3
Heated	42.8	69.9	4.5 ± 0.7	6.1	0.7	4.05	5.8 ± 0.4	7.0 ± 0.3

* Increase in weight per gram of fat intake.
[b] Standard error of the mean.

TABLE II

Increase in the Liver Storage of B-Vitamins (μg/liver)

Vitamin	Diet	
	Raw peanut oil	Heated peanut oil
Thiamine	25.6 ± 2.3*	12.3 ± 1.6
Riboflavin	96.4 ± 8.6	67.4 ± 7.5
Niacin	652.0 ± 34.4	590.0 ± 34.6
Pantothenate	322.0 ± 21.6	175.0 ± 16.7
Pyridoxine	5.8 ± 0.3	2.5 ± 0.1

* Standard error of the mean.

they were sacrificed and the livers analyzed for the different B-vitamins. Thiamine, riboflavin and niacin were estimated according to the standard methods of the American Association of Vitamin Chemists (2). Pyridoxine and pantothenate were estimated microbiologically according to Barton-Wright (3).

It is evident from the results (Table II) that the liver storage of the different B-vitamins is adversely affected in the case of rats receiving the heated oil. This may be partly due to the lower food intake. It should be noted, however, that the different B-vitamins intake even by the rats receiving the heated oil diet is more than adequate.

Effect of Heated Oils on the Absorption of Carbohydrates

Two groups of thirty growing rats, weighing 120–130 g were fed purified diets containing 15% of raw or heated peanut oil for a period of 3 weeks. All the animals were fasted for a period of 24 hr. At this stage six animals in each group were sacrificed, and the total carbohydrate in the intestinal and stomach washings was determined. In the case of the fasted animals, the carbohydrate content in the intestinal and stomach washings was practically negligible. The remaining rats were fed orally a mixed diet at 1 g/100 g body weight of the rat, consisting of 120 mg casein, 150 mg oil, raw or heated, 690 mg of starch (includes 10 mg given as vitaminized starch) and 40 mg salt mixture. Six rats in each group were killed at intervals of 2, 3, 4 and 5 hr, and the intestinal and stomach washings were analyzed for starch and sugars according to the official methods of the Association of Agricultural Chemists (1). From the data, the percentage digestion and absorption of carbohydrates at different periods after feeding was calculated according to the following formulae:

$$\text{Digestion \%} = \frac{\text{Intake of starch} - \text{Amount of starch in the stomach and intestinal washings}}{\text{Intake of starch}} \times 100$$

$$\text{Absorption \%} = \frac{\text{Intake of starch} - (\text{Amount of starch in the stomach and intestinal washing} + \text{starch equivalent to the sugars present})}{\text{Intake of starch}} \times 100$$

The results given in Table III bring out clearly that digestion and absorption of carbohydrates are adversely affected by the presence of heated oil in the diet.

Influence of Heated Oils on the Blood Sugar and Cholesterol Levels

Two groups of six weanling rats weighing about 40–50 g were fed on purified diets containing 15% of raw or heated peanut oil for a period of 4 weeks. The animals were anaesthetized with ether and the blood was removed by heart puncture. Glucose and cholesterol in the whole blood were estimated by methods described by King and Wootton (10). The results are given in Table IV.

The results show that the presence of heated oil in the diet has been responsible for higher blood glucose and cholesterol levels.

TABLE III

Digestion and Absorption of Carbohydrates in Rats Receiving Raw or Heated Peanut Oil Diets (Values are mean of 6 male rats in each group)

Time in hours after feeding	Raw peanut oil diet		Heated peanut oil diet	
	Digestion %	Absorption %	Digestion %	Absorption %
2	11.0 ± 0.6	7.0 ± 0.3	9.0 ± 0.5	4.0 ± 0.2
3	34.0 ± 5.6	21.0 ± 4.4	22.0 ± 4.3	13.0 ± 1.2
4	62.0 ± 8.7	49.0 ± 7.6	39.0 ± 3.5	23.0 ± 2.0
5	78.0 ± 6.4	68.0 ± 5.6	68.0 ± 5.4	35.0 ± 3.5

Discussion

Present results indicate that heated oils have a growth-depressing action. The mechanism of this action is not clearly understood. Some workers (5) have attempted to correlate growth-depressing action of heated oils to polymer formation, but as no reliable method is available for determining the percentage of polymers in heated oils, this relationship is difficult to determine. Johnson et al. (8) observed that there was a rapid recovery of albino rats which had been changed from a thermally oxidized corn oil diet to a fresh corn oil diet, and this would seem to indicate that the thermally oxidized oil did not cause permanent metabolic damage. Some of our other investigations (in progress) have shown that the growth-depressing effect of heated oils could be counteracted by increased intake of sulfur amino acids. The diet used in the present study is essentially a low protein diet, and the effect of the sulfur amino acids may be due to the fact that these are the limiting amino acids in a purified 10% casein diet. The growth-depressing effect might also be, to a certain extent, due to the destruction of vitamins in the diet (6) or to a lowering in the activity of some enzymes (4,12).

The present studies have shown that there are large differences in the liver stores of B-complex vitamins of rats receiving raw and heated oil rations. These large differences cannot be entirely due to the destruction of the vitamins by the heated oil; faulty absorption of the vitamins is a point to be elucidated. The decreased digestion and absorption of carbohydrates in the case of rats receiving the heated oil may be due to lowered enzyme activity. The growth-depressing action of the heated oil may be due to the combined action of all the different factors and there is a necessity for further work to elucidate the correct mechanism.

Another significant observation, in the present study appears to be the effect of the heated oil in increasing the blood cholesterol of rats. The studies so far carried out on the effect of fats on cholesterol metabolism have been restricted to fats in raw form. Effect of heated fats on blood cholesterol merits a further study.

TABLE IV

Blood Glucose and Cholesterol Levels of Rats Receiving Raw or Heated Peanut Oil Diets

Constituent ml/100 ml blood	Raw peanut oil	Heated peanut oil
Glucose	105 ± 5.6*	131 ± 7.7
Cholesterol	76 ± 5.8	107 ± 3.8

* Standard error of the mean. All values are averages of 6 male rats in each group.

ACKNOWLEDGMENTS

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Comparitive Study of Monocarbonyl Compounds Formed During Deep Frying in Different Fats[1]

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Abstract

Fresh commercial corn oil, fresh commercial lard, and hydrogenated vegetable shortening were analyzed for carbonyl compounds before and after deep frying. The frying was carried out in an apparatus with a capacity for 2000 g of oil designed to quantitatively trap the volatile materials evolved during frying and which would ordinarily escape into the atmosphere. The trapped distillate was also subjected to carbonyl analysis.

Analysis of the fats and distillates showed a carbonyl pattern in essential agreement with the classical autoxidation mechanism for the different fats, i.e., the typical alkanals, alk-2-enals, and alk-2,4-dienals. The pattern correlated generally with the fatty acid composition of the fats. Comparison of the concentrations of the monocarbonyl compounds in the fats before and after frying, and in their distillates, indicated that the deodorization process which accompanies frying is effective in preventing the accumulation of the more volatile compounds formed. The less volatile products, mainly deca-2,4-dienal, were not efficiently removed. Accordingly, it was observed that the oils containing higher proportions of linoleic acid contained more residual monocarbonyl compounds after frying.

CONSIDERABLE INTEREST has been stimulated by the possibility of toxic chemical alterations in fats during cooking. Thermal oxidation of unsaturated fats at 200C for 24 hr has been reported to produce substances toxic to rats (1). The efficacy of extrapolating from extreme laboratory conditions of heating and oxidation to the milder conditions of practical cooking has been vigorously questioned by Melnick (2,3,4) and Kaunitz (5). With the exception of the surveys made in the potato chip industry by Melnick, (2,3,4), there have been no definitive chemical studies of actual frying operations. The present work was designed to study the production of carbonyl compounds during the frying of potatoes under controlled laboratory conditions which would simulate good cooking practice. Carbonyl compounds were chosen for study because of the availability of reliable analytical methods and the belief that as an important class of oxidation products, their study could provide information on the extent and type of oxidation. Knowledge of this type is necessary to help settle the controversy over the biological significance of heated fats.

Experimental

Potato Frying Operation. In order to quantitatively trap the vapor evolved during the frying process, the apparatus illustrated in Figure 1 was designed. Vessel No. 1 was a 3 liter resin kettle adapted to a still-head. The kettle was heated with an electric mantle connected to a variable transformer. The opening at B was vented to a nitrogen source. Instead of a thermometer at D, a stainless steel wire entering the apparatus around a rubber stopper was used to suspend the stainless steel basket containing the potatoes. This basket could be lowered and raised without removing the stopper. The distillate was collected in three low-temperature traps 2, 3, and 4. Trap No. 2 (2 liters) was mounted under a cold-water condenser, and was cooled by an ice-water bath. The major portion of the distillate was retained at this point. Traps 3 and 4 were mounted under cold-finger condensers charged with an ethanol-dry ice mixture. They were also cooled by ice-water baths. Trap No 5, containing 300 ml of 3 M phosphoric acid saturated with 2,4-dinitrophenylhydrazine, was used only when the system was under positive nitrogen pressure.

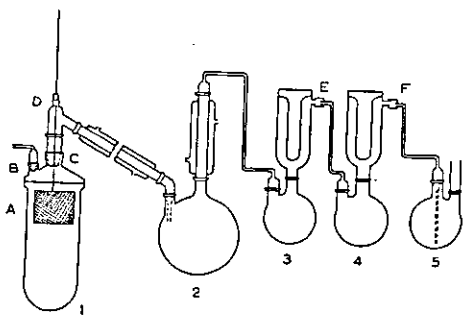

FIG. 1

The kettle was charged with 2000 g of fat which was then heated to 200C. The temperature was observed by the periodical insertion of a thermometer at B. Potatoes (300–400 g), sliced uniformly with a wire grid to dimensions of 14 × 14 × 50 mm, were placed in the stainless steel basket suspended over the hot oil by means of the stainless steel wire and a hook (Fig. 1). After the kettle-head had been bolted down and the ball-joint clamps secured, the basket was lowered into the fat without removal of the stopper at D. The potatoes were fried for 10 min. The basket was then raised above the oil, trap No. 5 was connected, and nitrogen pressure was applied at B to sweep the volatiles remaining in the head space into the traps. The flow was maintained at a rate of about 500 ml per min for a period of 10 min, during which the oil temperature, which had fallen to 175C during frying, returned to 200C. The nitrogen pressure was then discontinued and trap No. 5 was disconnected. Some of the data concerning the frying operation are presented in Table I.

Analysis of the Oils and Distillates. After the completion of the frying process, the oil was allowed to cool to room temperature overnight and then stored in the freezer for not more than 24 hr. The distillate fractions from the four traps were combined and thoroughly mixed with 400 ml of 8 M phosphoric acid saturated with 2,4-dinitrophenylhydrazine. After reacting overnight, this solution had lost its characteristic carbonyl odor and the oily material which had been floating on the surface had disappeared. The solution was then extracted with one liter of methylene chloride in 5 portions. The combined extracts were dried under reduced pressure in a rotary film evaporator and the residue was stored in the freezer.

The fatty acid compositions of the oils were determined before and after frying by the transesterification with HCl-methanol and gas-liquid chromatography. A column of 20% diethylene glycol succinate polyester on 60–80 mesh acid-washed celite was used in a conventional instrument equipped with an argon ionization detector containing a Sr^{90} source. The column was operated at 188C. Fatty acid analysis is presented in Table II.

The carbonyl compounds were analyzed before and after frying according to the method of Schwartz, Haller, and Keeney (6). Briefly, carbonyls were converted to 2,4-dinitrophenylhydrazones in a fat-hexane solution on a celite-phosphoric acid-column, followed by separation of the fat on a magnesia column. The

TABLE I

Data Pertaining to the Frying Operation[a]

	Corn oil	Lard	Hydrogenated vegetable oil
Weight of fat before frying	2000 g	2000 g	2000 g
Weight of fat after frying	1863 g	1835 g	1844 g
Volume of distillate	1575 ml	1600 ml	1730 ml
Color of oil after frying	Dark	Very dark	Light

[a] The amount of 3400 g raw potatoes was fried in 10 batches. Total heating time 240 min; frying time, 100 min.

TABLE II

Fatty Acid Composition of Fats Before and After Frying in Wt %

Fatty acid	Fat					
	Corn oil		Lard		Shortening	
	Fresh	Heated	Fresh	Heated	Fresh	Heated
12:0			Trace	Trace	3.65	3.45
14:0			1.24	1.32	1.43	1.52
16:0	14.1	13.6	30.1	29.5	21.4	22.3
16:1			1.74	2.04		
17:0			Trace	Trace		
18:0	2.00	2.20	16.3	15.7	10.6	10.3
18:1	28.6	30.6	42.0	42.3	49.3	47.8
18:2	55.0	53.7	8.56	8.18	13.7	14.7
18:3	Trace	Trace	Trace	Trace	Trace	Trace

TABLE III

Concentrations of Carbonyl Compounds Isolated from Fats Before and After Frying and from Their Distillates

Compound class, chain length	Micromoles of carbonyl compound per kg fat								
	Corn oil			Lard			Hydrogenated vegetable oil		
	Fresh	Heated	Distillate	Fresh	Heated	Distillate	Fresh	Heated	Distillate
Alkanones									
C_5	49	55	8.2	9.1	5.0	18			
C_6	0.74					1.3			25
C_8	1.4	1.2			0.44	2.0			tr
C_9	2.3	1.9			1.1	3.2			tr
C_7	1.4	1.4				2.9			tr
C_8	5.9	6.8				2.9			tr
C_9				0.14		2.9			tr
C_{10}				0.14		1.6			tr
C_{12}	0.46	0.57		0.20		1.3		1.5	tr
Alkanals									
C_5						50			
C_6			4.8			10			36
C_8			3.3			8.1			11
C_8			31						
C_9	2.5		28			86	1.5	1.9	28
C_9	3.8		41	9.3		49	2.8	6.6	25
C_7			6.6	13		61	0.64	2.5	25
C_9			1.7	4.2		22	0.48	5.4	6.8
C_9			6.6	5.8	1.9	16	0.64	2.9	4.9
C_{10}				2.6		32	tr	tr	9.1
C_{12}				5.7		4.9		3.9	2.4
Alk-2-enals				4.2		7.2			
C_5						1.6			
C_6						3.3		1.8	
C_8			5.1			5.4		1.9	6.2
C_7			38	4.0	1.8	58		7.9	26
C_8	1.3		12	2.4	1.4	28		8.0	20
C_9	1.4	0.79	4.3	3.9	2.0	19		2.0	5.4
C_{10}	0.72	1.3	11	5.2	8.6	53	tr	6.2	12
C_{11}		1.5	7.1	5.5	15	29		5.6	10
C_{12}		3.6		2.9	9.5	1.1			0.53
Alk-2,4-dienals									
C_5				2.2		10			8.2
C_8				0.88					
C_9			15	4.4		1.8	0.28		
C_{10}		160	70	15	28	87	1.4	9.0	44
C_{11}				13	23		1.1	11	
Total volatile mono-carbonyls	70.9	234	284	114	101	622	8.84	72.6	306

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palm and palm kernel oils are given in Table VI. The fatty acid composition of a hypothetical 4:1 mixture of these two oils (their approximate molar ratio in the total palm fruit [14]) was also calculated and recorded in Table VI.

The carbon number distribution of triglycerides for palm oil, palm kernel oil, and their 4:1 mixture was calculated from the data in Table VI using the random distribution hypothesis (7). The carbon number distribution of the 4:1 mixture was also calculated from the predicted triglyceride compositions of the original palm and palm kernel oils. Figure 6 compares the carbon number distribution of triglycerides in the randomized mixture and in the mixture of the two random oils. It is very clear that the two predicted triglyceride compositions are very different.

Discussion

The integration technique described above now makes it possible to accurately apply triglyceride distribution hypotheses to natural fats originating in tissues having regional differences in fatty acid composition. The accuracy of the integration method is limited only by the experimental accuracy with which such regional differences can be defined. Although only one-dimensional regional differences have been treated here, the technique is inherently applicable to three-dimensional differences. In the latter case, however, a more complicated mathematical treatment is required, so that simplification to a one-dimensional model is often desirable.

When the relative amounts but not the types of fatty acids vary with location, the triglyceride composition can be predicted from the average fatty acid composition without introducing appreciable error. Discrepancies between results from the integration technique and results based on average fatty acid composition are less than the experimental error in current triglyceride analysis procedures. Where different types of fatty acids exist in different regions, however, these differences must be taken into account to avoid large errors. These conclusions apply to both intra- and interseed differences in fatty acid composition.

The five fats and three distribution hypotheses examined here were chosen as typical examples, but they do not necessarily cover all possible cases. They are meant to serve only as a guide showing how regional differences can be handled when distribution hypotheses are tested against experimental results.

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SYMPOSIUM: THERMAL OXIDATION AND POLYMERIZATION IN FATS

conducted by The American Oil Chemists' Society at its 55th Annual Meeting, New Orleans, Louisiana April 19-22, 1964

MADHU R. SAHASRABUDHE, PRESIDING

H. P. DUPUY, PROGRAM CHAIRMAN

Introduction: Studies on Heated Fats

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DURING THE LAST TWO decades more than 100 publications have appeared in the literature on the chemical and nutritional aspects of heated fats. Perkins (1) reviewing the most pertinent literature up to 1960, concluded that sufficient data exist to justify the suspicion that the use of heated unsaturated oils may not be desirable from the nutritional standpoint. Seven papers presented at the symposium on Lipids and Their Oxidation (2) reported on the biological significance of auto-oxidized lipids.

Various symptoms of toxicity ranging from growth depression to death have been observed as a result of feeding oxidized fats to laboratory animals (1,3–6). Potentiation of known carcinogens fed to animals with heated oil fractions has also been reported (7,8). Some experimental evidence also exists to indicate that the edible fats in normal usage do not produce toxic products (9,10).

Oils and fats are normally processed at temperatures lower than those which will cause polymerization or any excessive degradation, but in frying where reuse of the oil occurs in commercial establishments a hazard might exist (11,12).

During frying, oil is heated to temperatures between 180C and 200C. Mechanisms involved during frying involve thermal oxidation and polymerization in presence of air. The three main groups of products formed are (a) hydroperoxides (b) secondary degradation products which include carbonyl compounds. Epoxy-, and hydroxy fatty acids and (c) polymers. Cyclized products may also be formed in the absence of air.

In strongly heated fats hydroperoxides do not accumulate and the toxicity is generally attributed to the degradation products and the polymeric material. Although some progress has been made on the study of separated fractions from heated fats, the evidence is not enough to arrive at any definite conclusions.

In a recent study on heated corn oil (13), the author fractionated corn oil heated at 200C into 8 fractions. The first four fractions, constituting about 62% of the original oil, were found to be triglycerides. The remaining 4 fractions constituted polymeric and degraded products with molecular weights ranging from 1320 to 4800. A number of hydroxyacids and short chain fatty acids were also identified in the polymeric fractions.

When heated to high temperatures in the absence of air, fatty acids, particularly the poly unsaturated acids, can cyclize without increase in molecular weight to form 1,2 di-substituted cyclohexanes. Polymerization can also occur through a Diels-Alder reaction which also gives rise to a 1,2 di-substituted cyclohexane. No evidence is available to indicate the presence of such hydro-aromatic systems which would be formed if polymerization occurred by Diels-Alder reaction in edible oils.

In the presence of air, decomposition of initially formed hydroperoxides at temperatures below 100C gives rise to oxygen linked polymers. While at higher temperatures carbon linked polymers predominate.

Questions that still remain unanswered are: (i) whether or not thermal polymers are in fact formed in

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frying oils during normal processing. (ii) what particular fractions are toxic and (iii) what are the effects of long term feeding of oxidised fats.

The papers presented at this symposium are not intended to review all the work but to present further observations to elucidate the questions.

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Chromatographic Studies on Oxidative and Thermal Fatty Acid Dimers[1]

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Abstract

A chromatographic study was carried out to investigate the nature of polymeric products in edible oils. Dimers from low-temp oxidation of methyl linoleate were compared with thermal dimers from high-temp polymerization of conjugated methyl linoleate. The distilled dimers were subjected to liquid-partition chromatographic separations on silicic acid columns as methyl esters, as free acids, and as methyl esters prepared by saponification and reesterification. Chromatographically isolated dimer fractions were also rechromatographed before and after each treatment.

When thermal dimer esters are saponified and reesterified, chromatographic recoveries are quantitative, and the expected changes in polarity result; whereas, with oxidative dimer esters, gross changes in polarity occur. Chromatographic separations of dimer esters or their acids fractionate into distinct areas of increasing polarity.

Introduction

POLYMERIC MATERIALS in glyceride oils may result from thermal treatment, oxidation, or a combination of both. Some of the most effective catalysts that cause dimerization of fatty acids are those that generate free radicals. UV light, peroxides, anthraquinones and metals in the presence or absence of air induce polymerization. High temps and the absence of air are required to form thermal polymers. Although the composition and structure of the thermal and dehydro fatty acid polymers have been well characterized, the structure of polymers formed during active oxidation is unknown and their composition varies. Studies designed to characterize oxidative polymers have relied upon distillation, solvent fractionation, selective adsorption or chromatographic separations to isolate a homogeneous material suitable for analysis.

Polymer formation in edible fats concerns the oil processor, the food technologist, the nutritionist and the consumer because of the changes polymers induce in the properties and characteristics of the fat. In 1960 Perkins (19) reviewed the literature on the chemical and nutritional changes that occur in heated fats. Firestone (9) in 1963 reviewed the methods for the determination of polymers in fats and oils. A method for the determination of 0.01 to 1.0% thermal dimer was published by Rost (21,22); but he states that it is not suitable for the determination of oxidatively derived polymers in fats, and cautions that oxidation of the thermal polymer through exposure to air must be avoided. We have described a method of partition chromatography using silicic acid to separate either thermal or oxidative polymeric fatty acids from the unaltered natural acids (12). Structural differences between the thermal and oxidative type of polymers and the presence of polar groups in the oxidative-type polymer indicate that a difference in chromatographic polarity should exist. The ability to distinguish between thermal and oxidative polymers would contribute to a better understanding of the behavior of fats and oils in industrial and edible applications.

The present paper describes chromatographic studies designed to distinguish between thermal and oxidative dimers by the analysis of their methyl esters, the free dimeric acids obtained by saponification of the esters, and the esters obtained by reesterification of isolated dimeric acids.

Experimental

Materials. Methyl linoleate used for the preparation of the oxidative dimer was obtained by esterification of a linoleic acid cone obtained from safflower fatty acids through Podbielniak extraction with furfural and hexane (3). The fraction boiling at 147–150C at 0.5 mm was used, which by gas-liquid chromatography (GLC) showed a purity of 98.8% and the presence of 1.2% methyl oleate. The alkali-conjugated methyl linoleate was prepared from safflower fatty acids obtained through crystallization in hexane at −40C. Isomerization was conducted for 45 min at 190–200C in an ethylene glycol solution containing 15% potassium hydroxide. The acids were methylated in methanol and H_2SO_4 and purified by distillation at 144–150C 0.4 mm. The conjugated methyl linoleate esters have an absorptivity of 76.9 at 232 mμ, indicating a conjugation of 82%. Soybean methyl esters were prepared from refined soybean oil by transesterification with sodium methoxide. At the start of each experiment esters were freshly distilled.

Autoxidation. Methyl linoleate was oxidized without catalyst at 25C in a closed oxygen system at atmospheric pressure to peroxide levels of approx 500 meq/kg. The methyl ester hydroperoxides were coned by the countercurrent extraction procedure of Zilch et al. (27) employing the solvent system of 80% aqueous ethanol and petroleum ether. The recovered unoxidized esters were again subjected to oxidation and the hydroperoxides extracted. The combined methyl linoleate hydroperoxides had a peroxide level of 5,200 (theoretical value for the pure monohydroperoxide of methyl linoleate is 6126 meq).

Soybean methyl esters were oxidized at 6C by bubbling oxygen through a sintered-glass filter stick submerged in the sample. The esters were oxidized to a peroxide level of 1,400 in 144 hr. During the working day the esters were exposed to UV radiation from a 100-w mercury vapor lamp. Hydroperoxides were not extracted from the unoxidized soybean methyl esters, and the entire mixture was used for polymerization.

Polymerization. The purified methyl linoleate hydroperoxide esters were polymerized in 20-g batches in evacuated and sealed flasks made from 100 ml distilling bulbs. Flasks were completely immersed in a 200C oil bath and held for 20 min. A similar technique was used for the thermal polymerization of conjugated methyl linoleate except the time was extended to seven hr at 290C. Before sealing, the polymerization flasks were subjected to repeated thawing and freezing (under dry ice) to insure complete removal of any dissolved air or oxygen. The autoxidized soybean methyl esters were polymerized under nitrogen at atmospheric pressure for 30 min at 210C. Nitrogen was continuously passed through the esters during the heating and cooling cycle to blanket the samples from air and serve as a means of collecting volatiles for autoxidation studies.

Distillation. Esters from several polymerization flasks were combined and after thorough degassing were subjected to molecular distillation in the Asco "50" Roto film still. Monomers were distilled at 5–10 μ by the first pass at 140C, the dimers and trimers by the second and third passes at 200 and 250C, respectively.

Saponification and Reesterification. Polymers and subfractions were saponified according to AOCS method Cd-3-25 (1). Reesterification was carried out with dimethoxy propane (DMP) at room temp according to the method of Radin and Hajra (20). A few subfractions were also reesterified with diazomethane, and the results agreed with those obtained by the DMP procedure.

Chromatography. The liquid-partition benzene-methanol system previously described (11,12) was used for the various fractionations. All silicic acid chromatographic columns were prepared with an immobile solvent of 16% by wt methanol in benzene and an eluting solvent of 2% methanolic benzene. To remove the highly polar materials when esters are chromatographed, diethyl ether was added after 350 ml of mobile solvent had passed through the column. Recovery of the sample from the column was almost quantitative, except for the monomeric fraction obtained from the oxidative polymerizations.

Mol wt of the polymers were determined with a Mechrolab vapor pressure osmometer, Model 301. Temp depression readings were taken for several different polymer conen in benzene, and by extrapolating to a zero conen the number-average mol wt was obtained. Isolated *trans* values were determined in carbon disulfide solutions by IR absorption at 10.3 μ and expressed as elaidate. Hydroxyl contents were determined by absorption at 2.86 μ and reported as a percentage of the absorption shown by pure methyl ricinoleate (13). Hydroperoxide groups are completely destroyed during dimer preparation and therefore offer no interference in the spectral method.

Results

Oxidative and thermal dimers prepared from methyl linoleate were typical polymeric products described previously (10,12); and their preparation, distillation and characterization offered no particular problems. Table I shows the distillation yields of the various fractions and their isolated *trans* contents. The mol wt and hydroxyl contents of the respective fractions are reported in Tables II and III.

Oxidative Dimers. The hydroxyl content of the oxidative dimer shows a close relationship to the polarity of the various chromatographic fractions. The similarity in analysis of two oxidative dimers, prepared several months apart, is shown by runs A and B in Table II. Although not an exact duplication, the results do indicate that a fairly reproducible dimeric material can be prepared by rapid thermal decomposition of fatty acid hydroperoxides. Hydroxyl analyses are based on methyl ricinoleate and results of 123% indicate 1.23 times as many hydroxyls as the standard. The mol wt of the distilled polymers are in the expected ranges and show good agreement with the values previously reported in the literature (10,21). The mol wt of the four oxidative dimeric fractions obtained by liquid-partition chromatography are slightly lower than theoretical values, but clearly indicate the dimeric nature of all fractions. Mol wt of the saponified and reesterified dimer fractions are almost the expected theoretical values. Slight increases in mol wt would be expected in any lactones, epoxides or other cyclic groups, and any free acids were present in the polymers and therefore available for esterification. Reasons for the apparently large increase in

TABLE I

Distillation of Polymers

Fraction	Temp °C	Oxidative		Thermal	
		Yield %	Isolated *trans* %	Yield %	Isolated *trans* %
Monomer	140	17	15.5	39	14.9
Dimer	200	50	10.1	47	22.3
Trimer	250	20	18.2	...	...
Residue	<250	13	16.8	14	20.3

TABLE II

Hydroxyl Content of Fractions from Methyl Linoleate Oxidative Dimer

Chromatographic fraction	Hydroxyl %	
	Run A	Run B
Peak I	Trace	Trace
Peak II	27.1	18.5
Peak III	48.7	57.4
Peak IV (ether)	123.5*	123.0*
Original dimer	20.9	32.1

* Methyl ricinoleate = 100%.

TABLE III

Mol Wt of Polymers

Type	Distilled ester	Chromatographed ester		Distilled ester saponified and reesterified
Thermal				
Monomer	265	.....		.....
Dimer	590	.....		.....
Residue	840	.....		.....
Oxidative				
Monomer	275	I*	540	465
Dimer	560	II	570	600
Trimer	840	III	886	1085
Residue	1930	IV	540	1660

* Peak I, of chromatographed dimer.

[1] Presented at the AOCS Meeting, New Orleans, 1964. Honorable Mention, Bond Award Competition.
[2] A laboratory of the No. Utiliz. Res. & Dev. Div., ARS, USDA.

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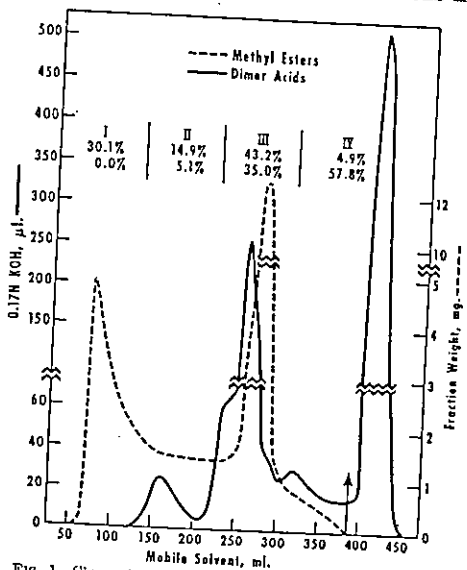

FIG. 1. Chromatograms of oxidative methyl linoleate dimers as esters and after saponification as free acids. Arrow indicates when diethyl ether added as eluting solvent.

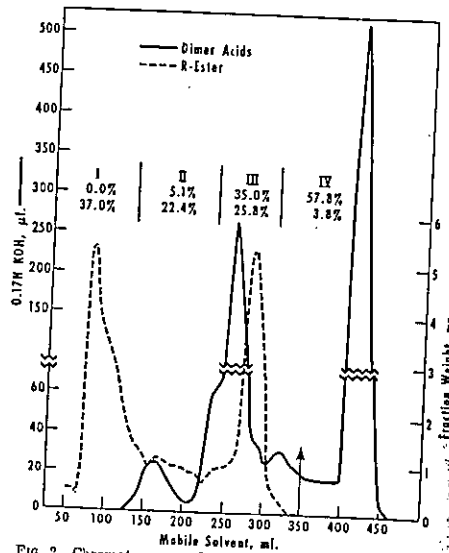

FIG. 2. Chromatograms of oxidative methyl linoleate dimers as free acids and after reesterification (R-ester).

mol wt of the trimer fraction are not known. Probably the increase in mol wt of monomeric fraction results from a loss of the short-chain components. Reduction in mol wt of residue material also probably results through the splitting of intramolecular esters of polymeric units.

Figures 1 and 2 show the separation obtained for the oxidative dimer when prepared, distilled and chromatographed 1) as the methyl esters, 2) after saponification of the methyl esters, and 3) after reesterification of the isolated acids. Four distinct chromatographic areas can be seen at different elution volumes: Peak I below 125 ml; peak II 125–220 ml; peak III 220–350 ml; and peak IV in the neighborhood of 400 ml. Oxidative esters show well-resolved peaks in areas I and III, and an appreciable amt of unresolved material in the peak II area. Dimeric acids show better resolution of fractions in peak areas II, III, and IV. The quantities of an oxidative dimer in each of the acid-chromatogram peaks bear no simple relationship to its distribution in the ester chromatogram. Reesterification of either the entire saponified oxidative dimer-acid mixture or the individual fractionated acids does not result in the identical products(s) from which the polymeric acids are obtained (Figs. 1 and 2, and Table IV). Reesterification of the oxidative dimer acids gives an approx chromatographic redistribution to the original ester. However, when isolated chromatographic fractions or subfractions of oxidative dimers are similarly treated by saponification and reesterification, the initial distribution of the fractions is not attained as shown in Table IV.

The monomeric fraction from distillation of the oxidative polymer is exceedingly complex and contains a wide distribution of polar materials. Repeated attempts to raise the column recovery failed; even washing with methanol followed by aqueous hydrochloric acid did not give complete recovery. Some loss, ca. 10–15%, is known to occur on drying the eluted materials to constant wt. GLC analysis of the monomer shows some 15 peaks eluted by temp programming to 240C. Characterizations of these components have not been undertaken.

Extended studies have not been made on the trimer and residue fractions. As shown in Table IV, trimeric material behaves chromatographically much like dimeric material. Increased amt of the highly polar fractions are found in the trimer and residue fractions of both oxidative and thermal dimers. Shorter chain secondary oxidation products and scission acids incorporated into the polymeric material could account for this increased chromatographic polarity.

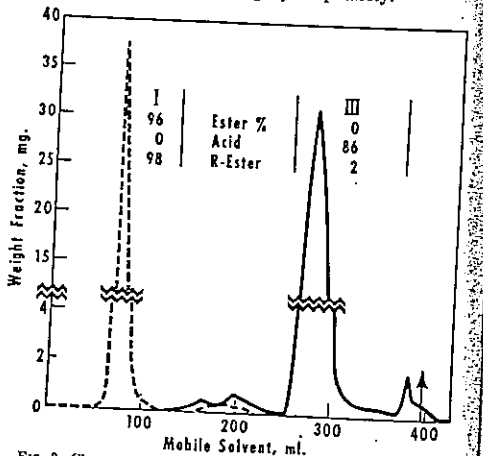

FIG. 3. Chromatograms of thermal methyl linoleate dimers as esters, as free acids after saponification, and as esters after reesterification (R-ester). The two ester curves coincide.

TABLE IV
Chromatographic Fractionation of Oxidative and Thermal Polymers

Chromatogram	Peak area I %	II %	III %	IV %	Column recovery %
Oxidative polymers					
Monomer					
Ester	20	25	14	3	62
Acid	.....	12	44	51	107
Reesterified	20	31	33	4	88
Dimer					
Acid	34	28	36	5	101
Ester	.....	4	42	50	96
Reesterified	31	28	34	5	99
Trimer					
Ester	24	27	44	6	101
Acid	.....	2	26	72	100
Reesterified	16	22	46	8	89
Residue					
Ester	(←26.2→)		59.1	12.2	97.0
Acid	Mostly insoluble in mobile solvent				
Reesterified	(←22.6→)		56.1	10.4	89.1
Thermal polymers					
Monomer					
Ester	100	2.1	0.9	2.1	105.1
Acid	.....	92.9	4.2	1.7	99.3
Reesterified	78.6	4.3	5.5	2.5	85.8
Dimer					
Ester	95.8	1.6	.....	1.4	99
Acid	.....	4.7	85.5	5.6	96
Reesterified	98.2	.....	1.7	1.0	100.9
Residue					
Ester	94.4	2.1	1.7	1.3	99.5
Acid	.....	1.4	62.3	29.4	92.1
Reesterified	84.0	(←16.4→)		1.5	101.9
Subfractions of oxidative dimer					
Dimer ester	29.3	13.8	39.3	4.3	86.6
Peak I	[29.3]				
Saponified	.....	8.0	47.4	40.0	95.4
Reesterified	67.2	15.6	17.1	1.6	97.0
Peak III			[39.3]		
Saponified	.....	.....	17.9	7.6 + 70.9	96.4
Reesterified	19.0	15.1	49.5	13.5	97.1
Subpeak III			[17.9]		
Reesterified	30.3	35.7	33.7	2.9	102.6
Subpeak IVa				[7.6]	
Reesterified	28.8	44.9	26.7	6.5	107.3
Subpeak IVb				[70.9]	
Reesterified	8.1	15.5	67.2	10.8	98.5
Subfractions of thermal dimer					
Dimer ester	95.8	1.6	.....	1.4	99.0
Peak I	[95.8]				
Saponified	.....	1.5	79.1	14.6	95.2
Reesterified	99.8	.....	.....	1.7	101.9
Dimer acid	.....	4.7	85.5	5.6	95.8
Peak III			[85.5]		
Reesterified	95.3	.....	.....	2.1	98.6
1:1 Mixture of oxidative and thermal polymers					
Dimer 1:1					
Esters	58.8	12.6	20.8	2.8	94.5
Acids	.....	4.8	58.4	31.5	94.8
Reesterified	61.6	9.9	21.0	4.2	96.8
Theoretical for 1:1 mixture					
Dimer 1:1					
Esters	62.6	7.8	19.7	2.8	.....
Acids	.....	5.0	60.2	31.7	.....
Reesterified	67.6	11.2	13.8	2.4	.....

Thermal Dimers. Dimers prepared by thermal polymerization of the conjugated esters and subjected to saponification and reesterification behave chromatographically in the expected fashion according to their polarities. Figure 3 shows that the nonpolar esters are exclusively in peak I with less than 2% in peak II.

These minor components in peak II are either monomeric free acids or half esters because they disappear on reesterification. Dimer esters can be freed of small amt of acid constituents by passage through ion-exchange resins. Upon saponification, the dimer acids move quantitatively to peak III with a small percentage which probably results from oxidation in the most polar area. Upon reesterification of the acids, the dimeric material again moves quantitatively (98.2%) back to peak I. Only one ester curve is shown in Figure 3 since the ester and reesterified curves coincide.

The monomeric fraction obtained through distillation of the thermal polymerization products contains unpolymerized acids and scission acids with less than 4.2% of any polymeric acid. Data presented in Table IV show 92.9% are nonpolymeric acids (peak II), and although the total recovery upon reesterification is not quantitative, there is no doubt about the chemical nature of these acids. GLC analysis of the reesterified monomeric fraction shows eight peaks of which linoleic acid and two unknown components constitute over 80% of the material.

Chromatographic analysis of the residue fraction indicates a high percentage of polymeric acids—62.3% in peak III, plus a large fraction (28.4%) of acids more polar than polymeric acids. This latter fraction probably results mostly from fission or cracking of the fatty acid chain, since all polymeric acids having a ratio of 1 carboxyl group/18 carbons always chromatograph in peak III. Trace oxidation occurring during preparation of esters or polymers would also contribute to this fraction.

Chromatographic Subfractionation. Subfraction data on oxidative dimers (Table IV) indicate that chromatographically separated fractions are not homogeneous and, upon saponification and reesterification, split into fractions of various polarities. The nonpolar peak I components of an oxidative dimer would be expected to behave chromatographically, and perhaps chemically, like a thermal or dehydro dimer. However, upon saponification of peak I (29.3% of the dimer) the polymeric acids were divided almost equally between peak areas III (47.4%) and IV (40.0%). Reesterification of the oxidative dimeric acids returns only 67.2% to the nonpolar peak I area, whereas for true thermal dimers reesterification returns 98+% to peak I.

When peak III, the major oxidative dimer ester component, is subfractionated after saponification (Fig. 4) two distinct acid components (7.6% + 70.9%)

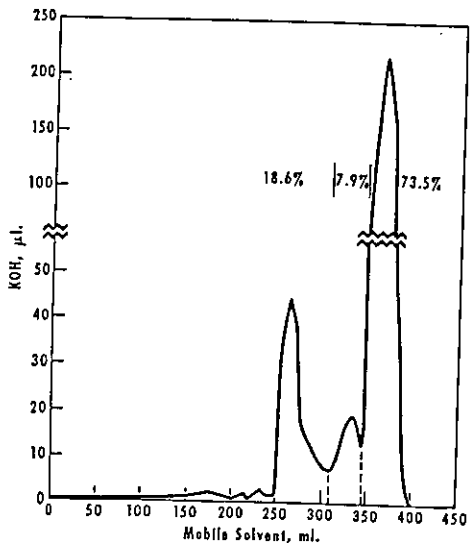

FIG. 4. Chromatogram of isolated peak III of oxidative methyl linoleate dimer after saponification and chromatographed as dimer acids.

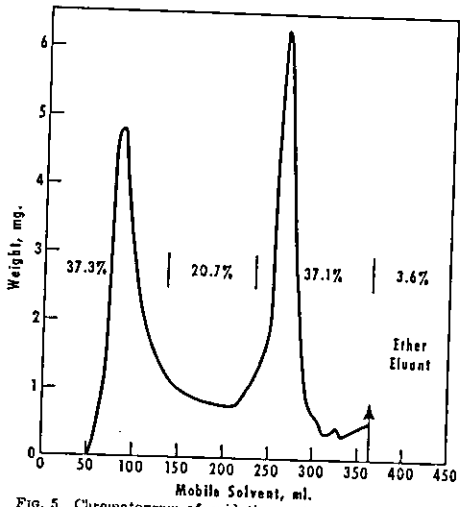

FIG. 5. Chromatogram of oxidative soybean oil methyl ester dimers.

occur in the peak IV area and a small amt (17.9%) remains in the peak III area. Thus 78.5% of the ester is composed of highly polar fatty acids. When these acids (all from peak III ester) are reesterified, a redistribution occurs to all four peak areas; i.e., 19.0, 15.1, 49.5 and 13.5%, respectively. In this subfraction of the oxidative dimer, only 49.5% returned to the original chromatographic area and polarity of the starting ester. The absence of any peak III in thermal esters does not allow a comparison, but because of the total absence of any thermal ester in this area, it is possible to distinguish between thermal and oxidative dimers by the ester concn found at peak III.

When the polar acids, obtained by saponification of peak III oxidative dimer esters, are reesterified and chromatographed, fractions appear in each of the four peak areas. A greater concn of components in the nonpolar peaks results from saponification and reesterification of individual fractions of the lowest polarity. Thus the three acid subfractions (17.9, 7.6 and 70.9%) show components having peak I polarities of 30.3, 28.3 and 3.1%, respectively. Although the most polar acid subfraction (70.9%) originally came entirely from the peak III oxidative ester, upon reesterification it returned only 67.2% to the area of its original polarity. When the thermal dimeric acids in peaks III and IV are reesterified, they returned 95–99% to nonpolar peak I.

Separation of Mixed Oxidative and Thermal Dimers. Equal wt mixtures of the methyl linoleate thermal dimer and of the methyl linoleate oxidative dimer were chromatographed as esters and as free acids after saponification and after reesterification. These chromatographic results shown at the end of Table IV, agree closely to the expected fractionation as calculated from the chromatographic separations of the original dimers. As indicated previously, the true thermal dimer shows no ester components in peak III and the oxidative dimer will show approx 40% wt distribution in the peak III area. Since the 20.3% peak III ester fraction equals approx 40% of the oxidative dimer, the mixture contains 50% of that dimer. Agreement in known systems with the amt of added

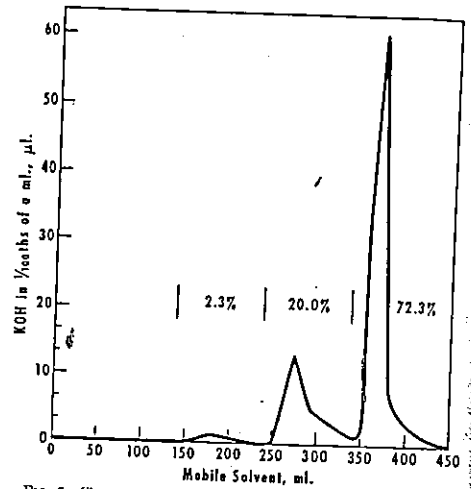

FIG. 6. Chromatogram of oxidative soybean oil methyl ester dimers after saponification.

thermal dimer indicates the possibility of developing a chromatographic method suitable for determining the respective amt of oxidative and thermal dimers in the presence of each other. It is believed that such a chromatographic method is feasible based on the polarity differences between the two.

Figures 5–7 show the behavior of oxidative dimers made from soybean methyl esters. Since linoleic makes up 52% of soybean oil, fair agreement would be expected with dimers made from pure linoleate esters. Good agreement exists between soybean ester dimers and methyl linoleate dimers on the chromatographic distribution of the components within the four polar fractions. Soybean dimeric acids show a considerably higher proportion (72.3%) of material in the highly polar peak IV area. This percentage might be expected because both the high levels of oxidation and the oxidation of linolenic acid lead to higher values in the extremely polar fraction (11). Changes of this type in the chromatographic distribution of polymer components indicate the need of detailed knowledge on the effect of conditions of oxidation and hydro-

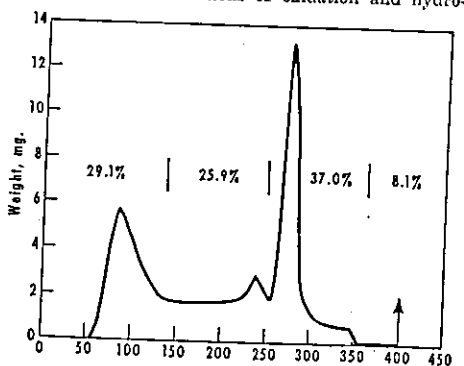

FIG. 7. Chromatogram of oxidative soybean methyl ester dimer after saponification and reesterification.

peroxide decompositions, as well as fat composition on the polarity of the oxidation products.

Discussion

Application of a chromatographic method to the determination of oxidative and thermal dimers has been discussed in previous publications (7,12). When acid dimers are chromatographed, the effects of the less polar carbonyl and hydroxyl groups are largely depressed. When chromatographed as acids, both oxidatively and thermally prepared fatty acid dimers are eluted from a chromatographic column in the same position. However, if these dimers are chromatographed as esters, the strong polar effect of the acid groups is depressed, and the polarity of the carbonyl and hydroxyl groups determines the position or elution-volume of the esters. The elution-volume of thermal dimers esters having no polar groups is the same as a normal or unoxidized fatty acid ester. Oxidatively prepared dimer esters have a greater polarity because of the presence of hydroxyl, carbonyl and other oxygen-containing groups. These polarity differences are the basis for determining oxidative dimers in the presence of thermal dimers. Published chromatograms (12) of thermal or oxidative dimers show four definite peak areas where fractions are eluted. The first elution area, or the nonpolar peak (I), contains the normal esters, thermal polymer esters, hydrocarbons and similar nonpolar materials. The second elution area (II), which is less defined, contains mildly polar materials, probably keto esters, epoxy esters, hydroxy fatty acid esters and similar products. In the peak III area, the concn of methanol in the eluant increases sharply, and the polar materials are coned into a narrow band and quickly eluated. This peak includes the dimer acids, hydroxy acids and hydroperoxidic acids, if any are present. The relationship of polarity to elution-volume of the various acid and ester dimeric materials is depicted in Table V.

Considerable confusion exists in the literature on the terminology and description of fat polymeric materials. Fixed definitions are not yet possible, but thermal dimers imply that polymerization has occurred in the absence of air. These thermal polymers will contain various cyclic Diels-Alder addition products, noncyclic dehydro polymers of various structures, and perhaps hybrid dimers in which one of the monomeric units has cyclized before dimerization occurs. Oxidative polymer implies that polymerization takes place in the presence of active oxygen, where probably the first reaction is the formation of monomeric hydroperoxides. When heat is applied during oxidation, polymerization reactions become exceedingly complex, not only through free radical reactions of the decomposing hydroperoxides, but by simultaneous formation of thermal dimers and through combinations of different active monomeric materials, many of which will contain oxygen. Polarity of the dimeric material, which largely depends on oxygen-containing groups, may in part be affected by the temp of polymerization and of hydroperoxide decomposition. Fedeli et al. (8) report that thermal polymerization of vegetable oils at temp up to 260C involves linoleic acid solely and that linolenic acid becomes involved only at temp above 280C. In studies on thermal scission of cod-liver oil peroxides, Aure et al. (2) report that at temp below 125C conjugation and polymerization are avoided. They also reported that unsaturated aldehydes begin to polymerize at 150C and that the rate increases rapidly at higher temp. Johnson et al. (16) found that methyl linoleate hydroperoxides decompose and form polymers, even when stored under nitrogen at 0C. Williamson (26) decomposed methyl linoleate hydroperoxide by continous heating at 100C for 23 hr. The polymer obtained after molecular distillation was free of acid groups but contained hydroxyl, carbonyl and epoxide groups; and he noted that chromatographic separation was impractical.

Through solvent fraction and distillation, the three types of polymer fractions obtained by Williamson were: 1) dimers carbon-to-carbon linked that contained no hydroxyl groups and retained a large proportion of the original fatty acid unsaturation; 2) dimers carbon-to-carbon linked that contained an appreciably hydroxyl content and had a relatively low degree of unsaturation; and 3) trimers containing a higher proportion of oxygen than the dimers and a high degree of unsaturation. Chang and Kummerow (5) oxidized ethyl linoleate at 30C and obtained a series of polymeric fractions of increasing mol wt by solvent fractionation. The unheated polymeric fractions were depolymerized by strong acids, and characterization of the split products indicated that the oxidative polymers were joined by carbon-to-oxygen bonds. Swern et al. (24) obtained 30–40% yield of polymers from 65C air oxidation of methyl oleate and concluded that the polymers obtained were oxygen-linked, probably as ethers since the polymers were not saponifiable to monomeric units.

Saponification of fats and fatty products is somewhat an arbitrary procedure, and for the more difficult saponifications, higher boiling solvents and longer times of saponification have been employed. The difficulty of saponifying paint films and drying oils is well-known (23). Steric hindrance within oxidative polymers may contribute to the difficulties of saponification. Gould (14) states that it is likely that acid-catalyzed hydrolysis, esterification and saponification are subject to virtually the same steric effects. Rates of esterification are known to be goverened primarily by the total number of substituents in the α and β positions (17). Since hydroperoxide decomposition is through a free radical mechanism, it is quite likely that some hydrogen abstraction may occur on the α and β carbons of the fatty acid chain, as well as in the allyl position of the double bonds. Dimers derived from hydroperoxides in this manner would be nonpolar and behave chromatographically like thermal dimers or the dehydro polymers.

Dehydro polymers, as discussed by Clingman and Sutton (6) and by Wheeler and coworkers (15,18), derived through free radical decompositoin of a peroxide, would be free of oxygen and similar to the oxidatively derived nonpolar dimers that occur in the peak I area. Fatty-acid hydroperoxides are effective free radical polymerization catalysts (25), and conditions for similar action are present during fat

TABLE V

Chromatographic Separations and Properties of Oxidative and Thermal Dimers

Area	Chromatographed as	
	Esters	Acids
Peak I	Unoxidized esters, thermal dimers, no hydroxyl groups	Nonacidic material
Peak II	Intermediate material, unresolved mixture with few hydroxyl groups	Unoxidized acids
Peak III	Oxidative dimers with hydroxyl groups	Oxidative and thermal dimer acids
Peak IV	Secondary oxidation products and highly polar products show very strong hydroxyl absorption	Secondary oxidation products and highly polar materials

autoxidation. Previous studies (10) on distilled oxidative dimers (not chromatographically fractionated) indicate diene conjugation as high as 23%, and with double bonds randomly distributed from the C-6 to C-10 carbon atom of the fatty-acid chains.

Chromatographic fractionation offers a method of characterization and analysis based on polarity of the various components in oxidative polymers. Although confirming our results with the chromatographic method, Bernard and Rost (4) question the nature of the dimeric material and maintain that in normally processed soybean oil, thermal polymers constitute less than 0.1%. Since Rost's method (21,22) determines thermal polymers only, it offers a means of checking the type of dimer found in the chromatographic peak I areas obtained from the oxidative dimers.

Distilled oxidative dimers do not give highly resolved chromatographic fractions, but show a large peak of ca. 30% of the same polarity as the thermal dimer, and have a major peak approx 50% in an area of much higher polarity. Saponification may not be complete, and reesterification of these two chromatographically isolated fractions shows that they are not composed of homogenous material because fractions of the various polarities are recovered. Internal ester linkages in oxidative dimer offer a partial explanation for polarity changes where hydrolysis would release hydroxyl groups to give a polar monomeric unit within the dimer structure. Polarity of a hydroxylated dimer would be different from the original dimer.

Many parameters which influence the conditions of oxidation and hydroperoxide decomposition must be investigated, and the various interactions evaluated before any chromatographic method of dimer analysis can be fully evaluated. So far results indicate that considerable chemical and physical information regarding the composition of oxidative dimers is available through a detailed analysis of the chromatographic fractions. Temp of oxidation and the environment of peroxide breakdown are extremely important, and these two factors probably contribute most to the diversity of results recorded in the literature. Many of the usual analytical techniques need critical evaluation in dimer analysis since basic distinction of dimer types (thermal, dehydro and oxidative) are made on unsaturation, type of unsaturation, mol wt, saponification value, functional group analysis and the presence of various cyclic and heterocyclic groups. No definition of oxidative polymers is possible until these materials are fractionated and the components chemically and physically characterized. Currently it might be advantageous to define, or at least partially describe, oxidative dimers in terms of polarity as determined by some chromatographic procedure.

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A Long-Term Nutritional Study with Fresh and Mildly Oxidized Vegetable and Animal Fats[1]

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Abstract

Fresh and oxidized cotton seed oil (CO) olive oil (OO), chicken fat (CF) and beef fat (BF) were fed to male weanling rats for 33 to 108 weeks. Groups fed oxidized fats except OO showed a higher death rate than those fed the corresponding fresh fats. Groups fed oxidized CO and BF had the highest death rate. Histological studies of animals dying from natural causes showed more pronounced cardiac lesions in the animals fed oxidized CO. Serum, liver and brain cholesterol levels were not influenced by oxidized fats. Fatty acid composition of depot fats and of heart and liver lipids did not show significant differences between groups fed fresh and the corresponding oxidized fats.

[1] Presented at the Spring Meeting of the AOCS 1964.

IN SPITE OF THE GREAT interest in the biological effects of natural fats which have been exposed to heat and/or air, there are comparatively few reports as to any pathological findings after long-term feeding of such fats. In acute experiments, McKay (1) induced the generalized Shwartzman reaction in pregnant rats by feeding them fractions of oxidized cod liver oil during the gestation period. These episodes of disseminated intravascular clotting, which are especially noticeable in lungs and kidneys, can be prevented by the feeding of tocopherol. Raulin (2) found that the degenerative heart lesions are more frequent in rats on a low tocopherol diet. Earlier short-term studies carried out in the authors' laboratory with highly polymerized fractions of autoxidized fats revealed no recognizable histological lesions except for some edema of the gut despite the fact that the animals had enlarged livers, kidneys, and adrenals before they died (3).

TABLE I

Death Rate at Different Ages of Male Rats Fed Various Fats

Age (wk)	Cottonseed oil		Olive oil	
	Fresh	Autoxidized	Fresh	Autoxidized
4–33	1/40	0/40	0/40	0/40
33–73	2/33	4/34	4/34	0/34
73–99	7/25	9/24	5/24	6/28
99–108	4/12	5/9	4/10	3/16
	Chicken fat		Beef fat	
4–99	2/20	6/20	2/20	4/20
99–108	6/12	3/9	2/12	4/12

In cooperation with the Human Nutrition Research Division of the U. S. Department of Agriculture, a long-term study of several food fats commonly used in the United States was undertaken. This report will give some of our findings with regard to cottonseed and olive oils and chicken and beef fats.

The fats were aerated for 40 hr at 60C at an airflow of 1–2 liters/min. Peroxide values for successive batches of rancid cottonseed oil were 122.6, 43.8 and 97.0 and for rancid olive oil, 12.7, 324.0 and 50.8. Although these values varied considerably, no attempt was made to keep the peroxide values at the same levels because conditions of oxidation had been predetermined and were kept as constant as possible. Variations in peroxide number may have been due to different amounts of antioxidants present in different batches of the oils. The fresh and oxidized oils were included at a level of 20% in a diet composed of 30% alcohol-washed casein, 44% dextrose, 3.5% USP XIII salt mixture, 0.5% calcium carbonate, 2% cellulose, and the following vitamin supplements (in mg/kg): (choline dihydrogen citrate 1000, inositol 1000, nicotinamide 100, p-aminobenzoic acid 300, thiamine · HCl 2, pyridoxine · HCl 4, riboflavin 4, Ca pantothenate 10, folic acid 2.5, biotin 0.025, ascorbic acid 25, vitamin K 10, vitamin B_{12} (0.1% trituration in mannose) 5, crystalline beta-carotene 5, alpha-tocopherol acetate 50, free alpha-tocopherol 10 and crystalline vitamin D_2 0.5). To insure an adequate vitamin intake despite the oxidized fat in the diet, each rat was given a weekly oral supplement of 3 drops of Vi Penta Multivitamin suspensions from Hoffman-LaRoche.

The studies were carried out on groups of weanling male rats of the Columbia-Sherman strain. The groups fed cottonseed and olive oils contained 40 rats each and those fed chicken and beef fats contained 20 rats each. All rats were observed for weight gain, food intake, life span and pathology at autopsy. From the groups fed the cottonseed and olive oils, six rats each were sacrificed at 33, 73, 99 and 108 weeks of age for histological examinations and for lipid analyses of serum and tissues. Groups of six rats each were sacrificed at 99 and 112 weeks of age from those fed the chicken and beef fats.

The rats were killed by drawing blood from the heart under chloroform anesthesia. Their organs were removed and immediately weighed; sections were fixed in 10% formalin for histological examination and the rest, as well as the serum, were quickly frozen and stored at −20C. Serum cholesterol was determined according to Bloor et al. (4), and tissue cholesterol, by the method of Sperry and Webb (5). Lipid was extracted from serum and tissues with chloroform:methanol according to Folch et al. (6). Fatty acid compositions were determined by gas-liquid chromatography of their methyl esters prepared by transesterification with 5% methanolic HCL in benzene (2:1). GLC was carried out on a Perkin-Elmer Model 154C vapor fractometer with a 2 meter column packed with diethylene glycol succinate on chromosorb W and with a hydrogen flame ionization detector. The carrier gas was helium at 20 psi; the operating temperature was 225C.

TABLE II

Average Body Weights and Food Intake of Rats on Different Fats at 96 Weeks

	Fresh oil		Autoxidised oil	
	Food intake, g	Body weight, g	Food intake, g	Body weight, g
Cottonseed oil	108.5 ± 5.10[a]	618 ± 25	108.8 ± 3.88	633 ± 23.9
Olive oil	94.1 ± 4.67[a]	588 ± 19.8	97.3 ± 3.32	565 ± 20.0
Chicken fat	86.8 ± 3.73	501 ± 16.3	85.0 ± 5.49	583 ± 21.5
Beef fat	123.0 ± 3.94	599 ± 19.4	113.8 ± 4.76	582 ± 23.3

[a] Standard error of the mean.
[b] P = <.05 *vs.* fresh cottonseed oil.

Table I gives the number of rats in each group dying between successive withdrawals of rats. The data shows that the rats fed oxidized cottonseed oil and oxidized chicken fat had a higher death rate than all other groups; the groups with the lowest rates were those fed fresh beef fat and oxidized olive oil. With the exception of the latter, the groups fed oxidized oils had higher death rates than did the corresponding groups fed the fresh oils.

Table II gives the body weight and food intake of all groups at two years of age. At one year, the group fed fresh cottonseed oil had the highest average weight. After two years, the average weights of the animals fed the cottonseed oils were still higher than those of the other groups but not significantly so. Monthly records of body weights and food intake for all groups were maintained through the study.

In the groups fed cottonseed oil, neither age nor the oxidation of the fat influenced the amount of food eaten, which was approximately 15 g per day. The rats fed the olive oils tended to eat somewhat less—particularly those fed the oxidized oil after about one year of age, when the second batch of oxidized olive oil with the higher peroxide number was being fed. However, they evidently adapted to the diet because their intake increased significantly although never to the levels of the rats fed cottonseed oil. The best survival rate was attained by this group fed an oxidized oil with a peroxide number of about 300 for over one year. This suggests that at least these peroxides taken orally were not toxic. This confirms previous studies (7). The food intakes of the groups fed chicken and beef fats for almost the first one and a half years did not differ from those of the rats fed vegetable oils. Later, however, the intakes of those fed chicken fat declined to about 12 g per day, whereas those fed beef fat ate approximately 17 g per day. Fecal fat analyses showed that the latter group excreted 200 mg fat per day whereas those fed chicken fat and the vegetable oils excreted 20

TABLE III

Serum, Liver and Brain Cholesterol Levels in Male Rats Fed Various Fats, for 108 Weeks

	Cholesterol, mg		
	Serum[a]	Liver	Brain
Fresh cottonseed oil	118 ± 0.9	209	3920
Oxidized cottonseed oil	118 ± 9.6	264	4320
Fresh olive oil	99 ± 8.0	562	3930
Oxidized olive oil	95 ± 9.0	607	4190
Fresh chicken fat	123 ± 11.3	527	3870
Oxidized chicken fat	138 ± 13.1	320	3780
Fresh beef fat	155 ± 16.0	336	3870
Oxidized beef fat	119 ± 8.8	384	4110

[a] The serum values are averages of six samples; the liver and brain values were derived from pooled samples. ± values are standard errors.

TABLE IV

Fatty Acid Compositions (%) of Tissue Lipid Extracts from Male Rats Fed Fresh and Oxidized Fats. Each Analysis was Carried Out on the Pooled Tissue from Six Rats.

	C_{16}	C_{18}	$C_{18:1}$	C_{20}	$C_{20:1}$	$C_{20:2}$	$C_{20:4}$
				Depot fat			
Cottonseed oil							
Fresh							
33 weeks	0.9	20.2	2.2	1.4	22.8	52.5	.....
73 weeks	1.2	18.8	3.1	2.5	26.6	47.6	.....
99 weeks	1.3	17.2	1.6	1.6	24.2	54.1	.....
108 weeks	0.3	16.0	0.6	2.3	30.6	49.3	.....
Oxidized							
33 weeks	0.8	21.6	2.9	2.0	23.7	49.0	.....
73 weeks	0.9	19.0	1.7	2.0	28.6	47.7	.....
99 weeks	1.1	16.2	1.6	2.2	25.7	53.2	.....
108 weeks	....	14.3	0.4	0.7	28.5	56.0	.....
Olive oil							
Fresh							
33 weeks	0.5	11.2	2.6	1.4	78.5	5.9	.....
73 weeks	0.9	17.6	3.7	3.2	70.0	11.2	.....
99 weeks	....	12.4	2.7	1.5	74.6	8.8	.....
108 weeks	....	8.5	1.6	1.2	83.4	5.2	.....
Oxidized							
33 weeks	0.3	11.2	2.0	1.0	79.2	5.4	.....
73 weeks	0.4	16.4	3.7	1.7	66.8	11.0	.....
99 weeks	0.5	11.0	2.7	1.5	72.6	11.7	.....
108 weeks	....	10.2	1.8	2.1	82.8	3.1	.....
Chicken fat							
Fresh							
112 weeks	....	15.2	3.4	1.3	64.7	15.3	.....
Oxidized							
112 weeks	....	14.5	4.0	0.5	69.5	11.3	.....
Beef fat							
Fresh							
112 weeks	0.5	17.1	3.9	3.2	73.6	1.5	.....
Oxidized							
112 weeks	0.4	17.3	3.2	2.9	74.8	1.3	.....
				Liver			
Cottonseed oil							
Fresh							
33 weeks	0.8	25.3	1.2	10.4	13.2	34.1	14.3
73 weeks	0.4	21.1	1.1	11.9	12.7	32.1	17.2
99 weeks	0.2	21.1	0.3	12.7	10.4	33.6	22.0
108 weeks	....	21.5		20.1	15.9	19.3	22.0
Oxidized							
33 weeks	0.1	24.1	1.3	11.7	13.4	34.0	14.5
73 weeks	0.3	20.4	....	12.3	15.9	30.1	18.7
99 weeks	....	22.2	0.2	12.8	11.3	31.4	22.8
108 weeks	....	21.0		23.0	6.0	21.2	26.0
Olive oil							
Fresh							
33 weeks	0.6	17.0	2.9	6.0	61.6	5.5	5.1
73 weeks	0.4	19.8	1.7	7.1	47.9	13.4	9.0
99 weeks	0.3	18.2	1.1	8.3	43.7	13.1	15.3
108 weeks	....	19.7	0.6	7.9	55.1	3.8	11.4
Oxidized							
33 weeks	0.2	16.0	3.1	7.5	61.2	5.8	6.9
73 weeks	0.8	19.7	1.1	10.9	46.5	9.6	11.5
99 weeks	0.3	16.3	1.9	13.6	44.9	8.7	16.2
108 weeks	....	22.7		8.7	51.3	4.6	10.7
Chicken fat							
Fresh							
112 weeks	....	22.7	1.2	15.1	29.6	13.5	17.9
Oxidized							
112 weeks	....	23.7	1.5	14.9	27.1	12.6	20.2
Beef fat							
Fresh							
112 weeks	0.1	21.7	0.5	20.2	35.6	2.7	19.2
Oxidized							
112 weeks	....	19.6	1.5	22.6	40.0	2.6	14.1
				Heart			
Cottonseed oil							
Fresh							
33 weeks	1.2	17.5	2.6	23.3	10.6	30.2	12.4
73 weeks	0.6	11.0	0.7	16.9	13.3	23.4	22.6
99 weeks	....	12.4	0.1	23.1	7.9	25.9	30.7
108 weeks	....	15.3		28.8	9.2	21.9	24.7
Oxidized							
33 weeks	1.6	24.9	3.9	17.5	24.9	22.5	4.3
73 weeks	....	20.4	..	20.5	12.7	28.7	10.2
99 weeks	....	13.4	0.1	22.9	10.1	23.9	29.7
108 weeks	....	13.5		30.5	6.8	23.9	24.9
Olive oil							
Fresh							
33 weeks	0.4	19.4	3.4	16.3	35.3	16.2	7.9
73 weeks	0.5	11.3	1.0	18.0	26.3	13.9	26.4
99 weeks	....	13.7	0.4	21.2	16.0	18.4	31.2
108 weeks	....	12.2	....	29.1	39.9	6.8	10.3
Oxidized							
33 weeks	0.8	14.4	1.1	24.2	35.9	9.8	14.8
73 weeks	0.1	11.1	0.9	16.8	32.8	14.3	21.8
99 weeks	....	11.7	0.2	21.2	24.8	9.9	33.3
108 weeks	....	11.5		26.7	23.8	13.1	24.1
Chicken fat							
Fresh							
112 weeks	....	12.9	0.3	25.9	15.2	17.1	28.3
Oxidized							
112 weeks	....	11.5	0.1	30.3	12.1	16.5	29.2
Beef fat							
Fresh							
112 weeks	....	13.1	0.1	30.4	18.7	6.0	31.7
Oxidized							
112 weeks		16.9	0.3	30.9	20.3	6.9	23.8

to 70 mg per day. This difference in fat excretion does not explain that the groups gained weight at similar rates despite wide variations in their food intakes. The result is in line with observations that weight maintenance can be affected by adaptation to widely varying food intakes (8).

Table III gives serum, liver and brain cholesterol values for all groups. The serum levels of the animals fed olive and cottonseed oils appeared to increase slightly with age, but were not affected by the kind of oil fed. Serum levels of the rats fed chicken and beef fats were somewhat higher but oxidation of the fat did not seem to have any effect.

The liver cholesterol values of the animals fed olive oil were higher than those of the other groups. Although strict statistical evaluation of the data is hardly possible, the consistency of the differences between the animals fed olive oils and those fed other fats suggest that the feeding of olive oils was associated with high liver cholesterol levels. When the animals were 108 weeks old, liver cholesterol values of those fed the cottonseed oils were probably lower than those of any other group.

The cholesterol values of the brain increased with age. The average values were 1.43% at 33 weeks, 1.83% at 73 weeks, 3.03% at 99 weeks and 3.96% at 108 weeks. Feeding of various fats was without affect. Cholesterol analyses of kidney and heart lipids did not reveal any differences.

Thus, the various fats seemed o exert only a mild influence on cholesterol levels and what differences there were did not appear to be correlated to survival rate.

Analyses of fatty acid were carried out on all lipid extracts which had been analyzed for cholesterol. For brevity, only the results obtained for depot fat, liver and heart are given in Table IV. Depot fat analyses revealed no differences other than those reflecting the composition of the dietary fats. Neither age nor feeding of oxidized fat brought about definite changes. Liver lipids, although influenced by the dietary fat, had their own characteristics. Again, no influence of age or oxidation of the dietary fat could be established. The heart lipids seemed to be somewhat less influenced by the composition of the dietary fat. Here, too, age and oxidation of the dietary fat had no detectable effect. Whether or not the characteristic lipid composition of various organs is related to special functions remains to be seen. However, it may be pointed out that all organs of the animals fed the beef fats had a low linoleate content, and that, in the case of those eating fresh beef fat the low linoleate content was associated with a low mortality rate.

In our earlier feeding studies with more highly polymerized fats, we had seen marked enlargement of liver, kidneys and adrenals. In the present studies, liver weights were not significantly influenced by the oxidized fats. The hearts of the two oldest groups fed cottonseed oil were relatively heavier than those of the other groups, especially those fed beef and chicken fats. Two of the four animals fed oxidized cottonseed oil and sacrificed at 108 weeks had particularly heavy hearts. The kidney weights were greater than those of any other group.

Table V summarizes the main histological findings in the animals dying from natural causes. In general, they died from the usual causes of death in older rats: lung infections, cardiorenal diseases and malignant tumors, and there did not appear to be

TABLE V

Incidence of Deaths from Various Causes Among Male Rats Fed the Different Fats. Diagnosis was Based on Histologic Evidence

	No. of deaths	Inflammatory lung disease	Cardiorenal insufficiency	Cancers*	Undetermined
Cottonseed oil					
Fresh	15	6	2	0	5
Autoxidized	18	6	3	3	6
Olive oil					
Fresh	16	4	3	4	5
Autoxidized	13	3	4	3	3
Chicken fat					
Fresh	6	0	2	2	2
Autoxidized	11	3	5	3	0
Beef fat					
Fresh	5	0	1	1	3
Autoxidized	8	2	1	1	4

* Metastasizing tumors of the pituitary and of the adrenal have been excluded.

definite differences between the groups. One possible exception may have been a higher incidence of malignant tumors in the groups fed olive oil and chicken fat. A chi-square analysis gave a P of about 5%, which is just enough to invite further studies. Chicken fat contained 70 μg per 100 g of stilbesterol whereas the other fats contained about one-third this amount. Among the 91 rats sacrificed at about two years of age, the difference did not exist; eight malignant tumors distributed over all the groups were observed. It is known that old rats develop unspecific cardiac and renal lesions. In the heart, muscle fibers were damaged by accumulations of round cells and the appearance of fibrous tissue. The greatest difference was found between the groups fed fresh and oxidized cottonseed oils. In the latter there were significantly more scarring and round cell infiltration, that is, muscle damage before the laying down of connective tissue. The group fed this diet and sacrificed after two years had by far the largest hearts. In other groups there seemed to be more severe lesions in the groups fed oxidized chicken and beef fat than in their controls, but not significantly so.

The kidneys were graded with regard to hyaline casts and accumulation of round cells. Among those dying spontaneously, the number of lesions was higher in the animals fed oxidized fats, with the exception of oxidized olive oil. In the animals that had been sacrificed, there was not much difference between those on fresh and oxidized fats, but there was a highly significant difference between the relatively few animals with lesions among those fed the olive oils and the greater frequency in the other groups.

It is evident that even the relatively mild damage to the fats led to significant biological changes. Oxidized fats reduced the survival rate except for the curious case of oxidized olive oil. The feeding of oxidized cottonseed oil was associated with the occurrence of more marked heart and kidney lesions.

It is evident that the biological activities of the various fats could hardly be correlated with their chemical properties, which makes it more necessary to carry out long-term nutritional studies.

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Nutritive Value of Heated Vegetable Oils

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Abstract

Peanut, sesame and coconut oils were heated at 270C for 8 hr, in an open iron pan. These fats were fed to albino rats at 15% level in otherwise adequate diets. All rats fed heated fats showed a growth depression. Livers of rats receiving heated oil were congested and showed extensive periportal fatty infiltration. Rats on heated peanut oil showed i) reduced B-vitamin storage in the liver, ii) increased glucose and cholesterol levels in the blood and iii) a disruption in the digestion and absorption of carbohydrates.

Introduction

HEATED OILS have been shown to be poorly absorbed (13), to produce cancerous tumors (11) and to cause symptoms resembling that due to vitamin E deficiency (9). Lower nutritive value is also believed to be due to the destruction of certain vitamins, especially vitamin A (6). Further, it has been shown that the oxidation products of fats produced inhibitory effect on certain enzyme systems (4,12).

Consumption of fried foods in India is probably higher than in any other country. The conditions of heating are known to vary widely. The oils are usually heated in open air pans to about 200–300C depending on the type of preparation and the heated oils are reused.

The present investigations were carried out to evaluate the effect of heat on some edible oils commonly used in the country.

Experimental and Results

Preparation of Heated Oils

Peanut, sesame and coconut oils were heated continuously at 270C for 8 hr in an open pan made of iron. The heated oils were stored in pyrex glass bottles at 0C.

Influence of Heating the Oil on Its Nutritive Value and Fat Deposition in the Liver

Thirty-six Wistar strain albino rats, five weeks old and weighing about 40–50 g from our laboratory stock colony were allotted to six groups in a randomized block design and were housed in individual cages. They were fed *ad lib.* on purified diets (fat, 15%; casein, 12%; sugar, 10%; salt mixture, 4%; vitaminized starch, 1% and corn starch, 58%) in which the fat was supplied by raw or heated peanut, sesame or coconut oils. At the end of six weeks feeding, the animals were killed by bleeding through the abdominal aorta. Livers, spleens, stomachs and kidneys were removed and weighed. The liver lipids were estimated by the method of Hawk (7). The results are presented in Table I.

The results show clearly that in all the three cases the heated oil has adversely affected the gain in weight. The feed efficiency ratio, calculated as increase in weight per gram of fat consumed, is significantly lower in groups receiving the heated oils. The livers of rats receiving the heated oil are significantly heavier than the controls. The lipid content of the livers is nearly twice that of the control groups. The livers showed signs of congestion and extensive periportal fatty infiltration. No significant changes were found in the weights of stomach, kidney and spleen of the two groups of animals.

Influence of Heated Oils on the Levels of B-Vitamins in the Livers

Eighteen male weanling albino rats, about 4 weeks old, were distributed into three groups in a random block design and were depleted of their B-complex stores by feeding a deficient diet for a period of two weeks. One group was sacrificed at this stage for determining the basal stores of B-vitamins in the liver which were as follows: thiamine, 11.7 μg; riboflavin, 44.8 μg; niacin, 285 μg; pantothenate, 185 μg and pyridoxine, 12.5 μg. The two other groups were fed on a purified diet containing 15% of raw or heated peanut oil for a period of two weeks, after which

TABLE I

Influence of Heating the Oil on Its Nutritive Value and Fat Deposition in the Liver

Dietary fat	Body weight: Initial (g)	Body weight: Final (g)	Mean gain per week (g)	Mean daily food intake (g)	Feed efficiency[a]	Liver weight (g)	Liver weight: g/100 g body weight	Liver fat %
Peanut oil:								
Raw	42.5	120.7	13.0 ± 0.5[b]	11.3	1.1	4.58	3.8 ± 0.3	3.9 ± 0.3
Heated	42.0	72.1	5.0 ± 0.9	7.1	0.67	4.11	5.7 ± 0.6	7.1 ± 0.4
Sesame oil:								
Raw	42.5	102.5	10.0 ± 0.6	9.5	1.0	4.30	4.2 ± 0.7	4.8 ± 0.3
Heated	42.4	68.8	4.3 ± 0.7	5.0	0.7	4.10	6.0 ± 0.5	7.8 ± 0.3
Coconut oil:								
Raw	43.1	109.2	11.0 ± 1.1	9.5	1.1	4.70	4.3 ± 0.3	4.5 ± 0.3
Heated	42.8	69.9	4.5 ± 0.7	6.1	0.7	4.05	5.8 ± 0.4	7.0 ± 0.3

[a] Increase in weight per gram of fat intake.
[b] Standard error of the mean.

TABLE II

Increase in the Liver Storage of B-Vitamins (µg/liver)

Vitamin	Diet: Raw peanut oil	Diet: Heated peanut oil
Thiamine	25.6 ± 2.3[a]	12.3 ± 1.6
Riboflavin	96.4 ± 8.6	67.4 ± 7.5
Niacin	652.0 ± 35.4	590.0 ± 34.6
Pantothenate	322.0 ± 21.6	175.0 ± 18.7
Pyridoxine	5.8 ± 0.3	2.5 ± 0.1

[a] Standard error of the mean.

they were sacrificed and the livers analyzed for the different B-vitamins. Thiamine, riboflavin and niacin were estimated according to the standard methods of the American Association of Vitamin Chemists (2). Pyridoxine and pantothenate were estimated microbiologically according to Barton-Wright (3).

It is evident from the results (Table II) that the liver storage of the different B-vitamins is adversely affected in the case of rats receiving the heated oil. This may be partly due to the lower food intake. It should be noted, however, that the different B-vitamins intake even by the rats receiving the heated oil diet is more than adequate.

Effect of Heated Oils on the Absorption of Carbohydrates

Two groups of thirty growing rats, weighing 120–130 g were fed purified diets containing 15% of raw or heated peanut oil for a period of 3 weeks. All the animals were fasted for a period of 24 hr. At this stage six animals in each group were sacrificed, and the total carbohydrate in the intestinal and stomach washings was determined. In the case of the fasted animals, the carbohydrate content in the intestinal and stomach washings was practically negligible. The remaining rats were fed orally a mixed diet at 1 g/100 g body weight of the rat, consisting of 120 mg casein, 150 mg oil, raw or heated, 690 mg of starch (includes 10 mg given as vitaminized starch) and 40 mg salt mixture. Six rats in each group were killed at intervals of 2, 3, 4 and 5 hr, and the intestinal and stomach washings were analyzed for starch and sugars according to the official methods of the Association of Agricultural Chemists (1). From the data, the percentage digestion and absorption of carbohydrates at different periods after feeding was calculated according to the following formulae:

$$\text{Digestion \%} = \frac{\text{Intake of starch} - \text{Amount of starch in the stomach and intestinal washings}}{\text{Intake of starch}} \times 100$$

$$\text{Absorption \%} = \frac{\text{Intake of starch} - (\text{Amount of starch in the stomach and intestinal washing} + \text{starch equivalent to the sugars present})}{\text{Intake of starch}} \times 100$$

The results given in Table III bring out clearly that digestion and absorption of carbohydrates are adversely affected by the presence of heated oil in the diet.

Influence of Heated Oils on the Blood Sugar and Cholesterol Levels

Two groups of six weanling rats weighing about 40–50 g were fed on purified diets containing 15% of raw or heated peanut oil for a period of 4 weeks. The animals were anaesthetized with ether and the blood was removed by heart puncture. Glucose and cholesterol in the whole blood were estimated by methods described by King and Wootton (10). The results are given in Table IV.

The results show that the presence of heated oil

TABLE III

Digestion and Absorption of Carbohydrates in Rats Receiving Raw or Heated Peanut Oil Diets (Values are mean of 6 male rats in each group)

Time in hours after feeding	Raw peanut oil diet: Digestion %	Raw peanut oil diet: Absorption %	Heated peanut oil diet: Digestion %	Heated peanut oil diet: Absorption %
2	11.0 ± 0.6	7.0 ± 0.3	9.0 ± 0.5	4.0 ± 0.2
3	34.0 ± 5.6	21.0 ± 4.4	22.0 ± 4.8	13.0 ± 1.2
4	62.0 ± 8.7	49.0 ± 7.6	39.0 ± 3.5	23.0 ± 2.6
5	78.0 ± 6.4	68.0 ± 5.6	68.0 ± 5.4	35.0 ± 3.5

in the diet has been responsible for higher blood glucose and cholesterol levels.

Discussion

Present results indicate that heated oils have a growth-depressing action. The mechanism of this action is not clearly understood. Some workers (5) have attempted to correlate growth-depressing action of heated oils to polymer formation, but as no reliable method is available for determining the percentage of polymers in heated oils, this relationship is difficult to determine. Johnson et al. (8) observed that there was a rapid recovery of albino rats which had been changed from a thermally oxidized corn oil diet to a fresh corn oil diet, and this would seem to indicate that the thermally oxidized oil did not cause permanent metabolic damage. Some of our other investigations (in progress) have shown that the growth-depressing effect of heated oils could be counteracted by increased intake of sulfur amino acids. The diet used in the present study is essentially a low protein diet, and the effect of the sulfur amino acids may be due to the fact that these are the limiting amino acids in a purified 10% casein diet. The growth-depressing effect might also be, to a certain extent, due to the destruction of vitamins in the diet (6) or to a lowering in the activity of some enzymes (4,12).

The present studies have shown that there are large differences in the liver stores of B-complex vitamins of rats receiving raw and heated oil rations. These large differences cannot be entirely due to the destruction of the vitamins by the heated oil; faulty absorption of the vitamins is a point to be elucidated. The decreased digestion and absorption of carbohydrates in the case of rats receiving the heated oil may be due to lowered enzyme activity. The growth-depressing action of the heated oil may be due to the combined action of all the different factors and there is a necessity for further work to elucidate the correct mechanism.

Another significant observation, in the present study appears to be the effect of the heated oil in increasing the blood cholesterol of rats. The studies so far carried out on the effect of fats on cholesterol metabolism have been restricted to fats in raw form. Effect of heated fats on blood cholesterol merits a further study.

TABLE IV

Blood Glucose and Cholesterol Levels of Rats Receiving Raw or Heated Peanut Oil Diets

Constituent mg/100 ml blood	Raw peanut oil	Heated peanut oil
Glucose	105 ± 5.6[a]	131 ± 7.7
Cholesterol	78 ± 5.8	107 ± 3.8

[a] Standard error of the mean. All values are averages of 6 male rats in each group.

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Comparitive Study of Monocarbonyl Compounds Formed During Deep Frying in Different Fats[1]

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Abstract

Fresh commercial corn oil, fresh commercial lard, and hydrogenated vegetable shortening were analyzed for carbonyl compounds before and after deep frying. The frying was carried out in an apparatus with a capacity for 2000 g of oil designed to quantitatively trap the volatile materials evolved during frying and which would ordinarily escape into the atmosphere. The trapped distillate was also subjected to carbonyl analysis.

Analysis of the fats and distillates showed a carbonyl pattern in essential agreement with the classical autoxidation mechanism for the different fats, i.e., the typical alkanals, alk-2-enals, and alk-2,4-dienals. The pattern correlated generally with the fatty acid composition of the fats. Comparison of the concentrations of the monocarbonyl compounds in the fats before and after frying, and in their distillates, indicated that the deodorization process which accompanies frying is effective in preventing the accumulation of the more volatile compounds formed. The less volatile products, mainly deca-2,4-dienal, were not efficiently removed. Accordingly, it was observed that the oils containing higher proportions of linoleic acid contained more residual monocarbonyl compounds after frying.

CONSIDERABLE INTEREST has been stimulated by the possibility of toxic chemical alterations in fats during cooking. Thermal oxidation of unsaturated fats at 200C for 24 hr has been reported to produce substances toxic to rats (1). The efficacy of extrapolating from extreme laboratory conditions of heating and oxidation to the milder conditions of practical cooking has been vigorously questioned by Melnick (2,3,4) and Kaunitz (5). With the exception of the surveys made in the potato chip industry by Melnick, (2,3,4), there have been no definitive chemical studies of actual frying operations. The present work was designed to study the production of carbonyl compounds during the frying of potatoes under controlled laboratory conditions which would simulate good cooking practice. Carbonyl compounds were chosen for study because of the availability of reliable analytical methods and the belief that as an important class of oxidation products, their study could provide information on the extent and type of oxidation. Knowledge of this type is necessary to help settle the controversy over the biological significance of heated fats.

Experimental

Potato Frying Operation. In order to quantitatively trap the vapor evolved during the frying process, the apparatus illustrated in Figure 1 was designed. Vessel No. 1 was a 3 liter resin kettle adapted to a still-head. The kettle was heated with an electric mantle connected to a variable transformer. The opening at B was vented to a nitrogen source. Instead of a thermometer at D, a stainless steel wire entering the apparatus around a rubber stopper was used to suspend the stainless steel basket containing the potatoes. This basket could be lowered and raised without removing the stopper. The distillate was collected in three low-temperature traps 2, 3, and 4. Trap No. 2 (2 liters) was mounted under a cold-water condenser, and was cooled by an ice-water bath. The major portion of the distillate was retained at this point. Traps 3 and 4 were mounted under cold-finger condensers charged with an ethanol-dry ice mixture. They were also cooled by ice-water baths. Trap No 5, containing 300 ml of 3 M phosphoric acid saturated with 2,4-dinitrophenylhydrazine, was used only when the system was under positive nitrogen pressure.

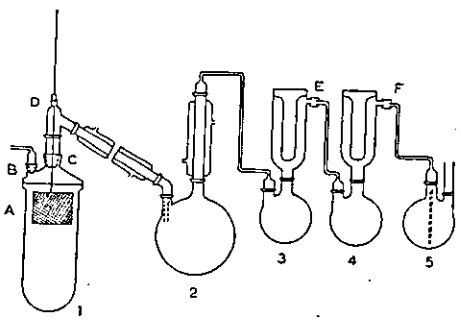

FIG. 1

The kettle was charged with 2000 g of fat which was then heated to 200C. The temperature was observed by the periodical insertion of a thermometer at B. Potatoes (300–400 g), sliced uniformly with a wire grid to dimensions of 14 × 14 × 50 mm, were placed in the stainless steel basket suspended over the hot oil by means of the stainless steel wire and a hook (Fig. 1). After the kettle-head had been bolted down and the ball-joint clamps secured, the basket was lowered into the fat without removal of the stopper at D. The potatoes were fried for 10 min. The basket was then raised above the oil, trap No. 5 was connected, and nitrogen pressure was applied at B to sweep the volatiles remaining in the head space into the traps. The flow was maintained at a rate of about 500 ml per min for a period of 10 min, during which the oil temperature, which had fallen to 175C during frying, returned to 200C. The nitrogen pressure was then discontinued and trap No. 5 was disconnected. Some of the data concerning the frying operation are presented in Table I.

Analysis of the Oils and Distillates. After the completion of the frying process, the oil was allowed to cool to room temperature overnight and then stored in the freezer for not more than 24 hr. The distillate fractions from the four traps were combined and thoroughly mixed with 400 ml of 8 M phosphoric acid saturated with 2,4-dinitrophenylhydrazine. After reacting overnight, this solution had lost its characteristic carbonyl odor and the oily material which had been floating on the surface had disappeared. The solution was then extracted with one liter of methylene chloride in 5 portions. The combined extracts were dried under reduced pressure in a rotary film evaporator and the residue was stored in the freezer.

The fatty acid compositions of the oils were determined before and after frying by the transesterification with HCl-methanol and gas-liquid chromatography. A column of 20% diethylene glycol succinate polyester on 60–80 mesh acid-washed celite was used in a conventional instrument equipped with an argon ionization detector containing a Sr^{90} source. The column was operated at 188C. Fatty acid analysis is presented in Table II.

The carbonyl compounds were analyzed before and after frying according to the method of Schwartz, Haller, and Keeney (6). Briefly, carbonyls were converted to 2,4-dinitrophenylhydrazones in a fat-hexane solution on a celite-phosphoric acid-column, followed by separation of the fat on a magnesia column. The

TABLE I

Data Pertaining to the Frying Operation[a]

	Corn oil	Lard	Hydrogenated vegetable oil
Weight of fat before frying	2000 g	2000 g	2000 g
Weight of fat after frying	1803 g	1835 g	1844 g
Volume of distillate	1575 ml	1600 ml	1730 ml
Color of oil after frying	Dark	Very dark	Light

[a] The amount of 8400 g raw potatoes was fried in 10 batches. Total heating time 240 min; frying time, 100 min.

TABLE II

Fatty Acid Composition of Fats Before and After Frying in Wt %

Fatty acid	Fat					
	Corn oil		Lard		Shortening	
	Fresh	Heated	Fresh	Heated	Fresh	Heated
12:0			Trace	Trace	3.65	3.45
14:0			1.24	1.32	1.42	1.52
16:0	14.1	13.6	30.1	29.5	21.4	22.8
16:1			1.74	2.04		
17:0			Trace	Trace		
18:0	2.00	2.30	16.3	16.7	10.6	10.8
18:1	28.6	30.8	42.0	42.8	40.8	47.8
18:2	55.0	53.7	8.56	8.18	13.7	14.7
18:3	Trace	Trace	Trace	Trace	Trace	Trace

TABLE III

Concentrations of Carbonyl Compounds Isolated from Fats Before and After Frying and from Their Distillates

Compound class, chain length	Micromoles of carbonyl compound per kg fat								
	Corn oil			Lard			Hydrogenated vegetable oil		
	Fresh	Heated	Distillate	Fresh	Heated	Distillate	Fresh	Heated	Distillate
Alkanones									
C_5	49	55	8.2	9.1	5.0	18			
C_6	0.74					1.3			25
C_7	1.4	1.2			0.44	2.0			tr
C_8	2.3	1.9			1.1	3.2			tr
C_9	1.4	1.4				2.9			tr
C_{10}	5.9	6.8		0.14		2.9			tr
C_{11}				0.14		1.6			tr
C_{12}				0.20		1.3			tr
C_{13}	0.46	0.57					1.5		tr
Alkanals									
C_5						50			
C_6			4.8			10			36
C_7			3.3			8.1			11
C_8			21						
C_9	2.5		28	0.3		36	1.5	1.9	28
C_{10}	3.8		41	13		49	2.8	6.6	25
C_{11}			6.6	4.2		61	0.64	2.5	25
C_{12}			1.7	5.8	1.9	22	0.48	5.4	8.8
C_{13}			6.6	2.6		16	0.64	2.9	4.9
C_{14}				5.7		32	tr	tr	9.1
C_{15}				4.2		4.9		3.9	2.4
Alk-2-enals						7.2			
C_5						1.6			
C_6			5.1			3.3		1.3	
C_7			38			5.4		1.9	6.2
C_8	1.3		12	4.0	1.8	53		7.9	26
C_9	1.4	0.79	4.3	2.4	1.4	28		3.0	20
C_{10}	0.72	1.3	11	3.9	2.0	19		2.0	5.4
C_{11}		1.5	7.1	5.2	8.6	53	tr	6.2	12
C_{12}		3.6		5.5	18	29		5.6	10
Alk-2,4-dienals				2.9	9.5	1.1			0.53
C_5				2.2		10			
C_6				0.88					8.2
C_7			15	4.4		1.8	0.28		
C_8		160	70	15	28	87	1.4	9.0	44
C_9				13	23		1.1	11	
Total volatile monocarbonyls	70.9	234	294	114	101	622	8.84	72.6	306

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monocarbonyl and carbonyl ester derivatives were then separated by chromatography on hydrated alumina. The carbonyl ester derivatives were not studied in detail. Separation of the monocarbonyl derivatives into classes of methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals was carried out on magnesia columns (7) followed by resolution of the classes on a hexane-acetonitrile column (8). This column was monitored by a Canalco ultraviolet flow analyzer at 350 mμ to yield recorded chromatograms which were correlated with collected eluate fractions and which could reliably record less than 0.01 μmole of derivative. The concentrations of the compounds isolated were determined spectrophotometrically and are presented in Table III. Identifications were made on the basis of partition chromatographic peak volumes, chromatographic mobilities, and ultraviolet spectra.

The distillate derivative residues were handled in a similar manner to the fats with the addition of two more steps. Excess reagent was removed from this residue by ion-exchange on Dowex-50 (9). Monocarbonyl and dicarbonyl derivatives were then separated on magnesia which had been deactivated with heat (10). This separation was necessary before class separation of the monocarbonyls. The concentrations of the compounds isolated from the distillates are shown in Table III.

Discussion

General Appearance of the Oils. The degree of darkening and the odor after frying was in proportion to the refinement of the oils. Before frying, all of the oils were fairly light in color and bland in odor. Lard was the darkest with its typical odor and the hydrogenated vegetable shortening was the lightest. Corn oil was darker after frying and gave a strong odor of deca-2,4-dienal. Hydrogenated vegetable oil developed very little odor. These general observations are consistent with the quantitative and qualitative monocarbonyl patterns shown in Table III.

Ketone Distribution. The demonstration of a homologous series of methyl ketones produced in the lard during frying represents a break with the tradition associating these compounds exclusively with heated milk fat. Evidence of this series in trace quantities was also obtained from the hydrogenated vegetable oil distillate and the unheated corn oil. Mechanisms for the production of ketones by thermal oxidation have been proposed by Crossley (11) and, for their production by low temperature oxidation, by Bell (12). However, the present data are insufficient to permit discussion of the relative merits of these. Crossley also proposed vinyl ketones as intermediates. The apparent absence of these, as well as alpha-beta unsaturated ketones in general, was studied carefully. Derivatives of synthetic alpha-beta unsaturated ketones were subjected to chromatographic and spectral analysis and were found to conform to behavior which should have allowed their observation if they were present.

Aldehyde Distribution. The aldehyde patterns of all three fats are in general agreement with the observations on autoxidized fats made by Gaddis (13). This, along with the absence of unsaturated ketones and the small quantities of ketones in general, indicates that autoxidation is the principal degradative process occurring during frying. A supporting observation shows the apparent absence of acrolein from the fats and distillates. Acrolein is a typical product of the thermal degradation of fats.

Significance of Steam Evolution. A comparison of the total monocarbonyl content of each of the oils before and after frying and of their distillates shows that steam generation during frying is quite effective in removing some of the compounds. The residual compounds were identified as C_{11} and C_{12} enals and the C_{10} and C_{11} dienals which arise primarily from the degradation of hydroperoxides originating from linoleate. Corn oil, with 55% linoleic acid, contained 2 to 3 times as much residual monocarbonyls after frying than the other fats with only 8 to 14% linoleic acid. Sixty percent of this material was deca-2,4-dienal. It is apparent that the residual monocarbonyl content of the fats after frying depends largely upon the linoleate content of the fats.

Degree of Oxidation. An important point to be made from this work is that deep fat frying does not result in alarming degradation of fats. The total volatile monocarbonyl compound production ranged from 380 to 733 μmoles per kilogram for the three fats. There was no significant change in the fatty acid composition of the fats as a result of frying. It is doubtful that most frying operations, either commercial or in the home, would be more drastic than those used here.

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The Reaction of an Autoxidized Lipid with Proteins

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Abstract

Evidence is presented which indicates that an interaction occurs between proteins and an autoxidizing unsaturated lipid. Using a model system approach, it has been established that two purified proteins (gelatin and insulin) are chemically modified in the presence of an autoxidizing lipid, methyl linoleate.

The insulin-methyl linoleate interaction has been studied chromatographically after acid and alkaline hydrolysis, and also by using the Sanger end group analysis method. The data indicate that lipid intermediates react with the ε-amino group of lysine, and also with phenylalanine and glycine, the N-terminal amino groups of insulin.

Hydrogen fluoride solubility and enzyme hydrolysis determinations indicate that the autoxidation products of methyl linoleate interact with protein to produce new chemical entities through cross-linking.

Introduction

AN EVER INCREASING number and variety of dehydrated foods are being developed for both human and animal consumption. Many of these contain lipids which become oxidized upon removal of water from the finished product (1).

The autoxidation of unsaturated lipids produces several active chemical intermediates: free radicals, hydroperoxides, oxiranes, and carbonyl compounds (2). These highly reactive intermediates, in turn, are capable of entering into reactions with one or more of the functional groups within protein molecules (3). As a consequence, one would expect a variety of complex interactions to occur between proteins and the degradation products of autoxidizing lipids under suitable reaction conditions, and in most instances the effect on food items could be undesirable.

The present study was designed to determine whether autoxidizing lipids do form addition products with proteins in the absence of water. A simplified model system approach has been employed with both gelatin and insulin serving as receptors for such intermediates. The assumption was made that the most likely interaction between the model protein and the lipid would involve either an alkylation or a cross-linkage of free amino groups; our approach was so tailored.

When protein alteration occurs in the presence of autoxidizing lipid a change in hydrolysis rate with proteolytic enzymes may be used as an index of such reactions. For this reason a trypsin assay technique was adapted to the problem.

Because the enzyme approach does not yield exact information concerning either the chemical nature of the interaction or the sites of reaction within the protein, two specific techniques were employed.

In order to identify the sites of lipid interactions with protein amino groups, the 1-fluoro-2,4-dinitrobenzene (FDNB) reagent of Sanger (4) can be used indirectly. Native insulin, for example, contains two N-terminal amino groups (phenylalanine and glycine) and one free ε-amino group (lysine) which react with FDNB. If, however, any of these amino groups are involved in an interaction with lipid intermediates, subsequent reaction with FDNB will not yield the usual yellow colored dinitrophenyl derivative. Therefore, by indirection, the sites of lipid-protein interactions for amino groups can be determined.

Solubility of the reacted proteins was also determined in anhydrous hydrogen fluoride (HF). This test has been used to differentiate cross-linked proteins from their denatured or alkylated counterparts because the former are insoluble while the latter are soluble in this reagent (5).

Materials and Methods

Materials

Pigskin gelatin (312 Bloom) obtained from the Grayslake Gelatin Co., Grayslake, Ill., was used in the preparation of model foams. Commercially available crystalline zinc insulin in acid solution (500 units/ml) obtained from Eli Lilly & Co., Indianapolis, Ind., was used for end group marking studies.

Reagents used in the hydrolysis and chromatographic phases of this study were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Amino acid controls and 1-fluoro-2,4-dinitrobenzene were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, while hydrogen fluoride (HF) was obtained from Matheson Co., Joliet, Ill. Methyl linoleate (ML) having an iodine value of 172 was obtained from Hormel Institute, Austin, Minn. This high purity lipid was used throughout as a model lipid.

Methods

Gelatin-ML Foam Preparation. Five-gram samples of gelatin were dissolved in 100 ml. of distilled water at 45–50C; 1.0 g of ML was added and the resulting reaction mixture was whipped to a stiff foam in a Sunbeam mixer. The foams were freeze-dried and the dehydrated materials were autoxidized in either air or nitrogen for five days at 50C. After autoxidation the foams were shredded in a Waring blender with ethanol-benzene azeotrope. The resulting slurries were poured into thimbles and Soxhlet extracted with the azeotrope for 16 hr to remove residual lipid. As a final step in preparation for enzymatic hydrolysis, the defatted foams were further dried overnight in vacuum desiccators.

In one series of tests, standard gelatin foams were prepared as detailed above, except that 1.4 g of sodium bisulfite were dissolved in gelatin-ML solution to act as an aldehyde trap.

Trypsin Assay of Gelatin-ML Foams. Samples of defatted foams (0.5 g) were mixed with 59 ml of Sprensen'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 reaction mixture precipitated immediately upon mixing with 20 ml of 20% trichloroacetic acid (TCA). The second flask was shaken for 20 hr at 38C and then precipitated with 20 ml of 20% TCA. Both samples were filtered

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TABLE I

Liberation of Alpha-Amino Nitrogen in Gelatin-ML Foams After Autoxidation and Trypsin Hydrolysis

Gelatin foam	g ML/5 g gelatin	Mg of N_2 lib./g gelatin (ave.*)	% Reduction by hydrolysis
Control	0.	28.8	0
Experimental (in air)	1.0	22.4	−22.2
Experimental (in N_2)	1.0	28.0	− 2.8
Experimental (with $NaHSO_3$)	1.0	28.1	− 2.1

* Significant at the 5% level based on an average of six analyses.

and washed with 5% TCA into 100 ml volumetric flasks The filtrates were used for α-amino nitrogen determinations with the standard Van Slyke apparatus.

Preparation of Insulin-ML Samples. Three-milliliter aliquots (estimated to contain 60 mg of insulin) of Lilly U-500 zinc insulin solution were placed in interjoint boiling flasks. The amount of 50 μl of ML was added to the flasks designated as experimentals, while nothing was added to the controls. Both control and experimental samples were shell-frozen and freeze-dried. The dried samples were oxygenated, stoppered, and placed in an oven at 50C. At the end of five days the samples were removed from the oven and both control and autoxidized insulins were ether extracted before further treatment with chemical agents.

Hydrolysis of Insulin. Control and experimental insulins, after ether extraction, were hydrolyzed with 10 ml of either 6N HCl or 14% $Ba(OH)_2$ by refluxing for 20 hr. The hydrolysates were then prepared for paper chromatographic analysis according to the method of Block et al. (6).

Paper Chromatographic Technique. Control and experimental hydrolysates were carefully spotted on Whatman No. 1 paper and dried. The paper was treated with ammonia vapors and placed in the Chromatocab. Butanol:water:acetic acid (250:250:60) was used as the developing solvent (6). Color development was accomplished by spraying with ninhydrin (0.2%) in acetone, and duplicate chromatograms were dipped in 0.2% isatin in acetone containing 4% acetic acid.

N-Terminal Amino Group Determinations. Control and lipid oxidized insulins were reacted with FDNB to tag the free N-terminal and ε-amino groups of the protein according to the method of Sanger (4). The resulting dinitrophenyl (DNP)-insulins were hydrolyzed with 6N HCl for 6 hr and then extracted with ether to separate amino acids and DNP derivatives. Both the ether and water phases were evaporated in vacuo and prepared for chromatographic analysis. The water phase was developed in butanol:water:acetic acid as described above and the ether-soluble derivatives were developed in benzene: 1% aqueous acetic acid (1:1) (Ref. 7).

Hydrogen Fluoride Solubility Test. The technique of Katz (8) as modified by Bjorksten et al. (5) was used as a qualitative test for cross-linking of both insulin and gelatin after reaction with autoxidizing ML as described earlier.

Discussion and Results

Gelatin-ML Reactions

The interreaction of autoxidized lipid with proteins can be demonstrated by the liberation of nitrogen through an enzyme approach. In a protein, such as gelatin, the hydrolysis rate with trypsin is fairly constant. An alteration of the protein, whether it be in the form of alkylation, denaturation, or cross-linkage, will alter the rate of enzyme action. If, after incubation with autoxidizing ML, a measurable change in hydrolysis rate (either higher or lower) is found, it may be taken as an indication of protein-lipid interaction. In general, alkylated and denatured proteins are more easily attacked by proteolytic enzymes than their native counterparts (9). Cross-linked proteins, on the other hand, resist hydrolysis by proteolytic enzymes (10).

The results in Table I show the influence of the autoxidizing ML on the digestibility of gelatin foams with trypsin.

It may be noted that gelatin-ML foams after 5 days' incubation in air at 50C become considerably resistant to hydrolysis by trypsin. The data also indicate that the interaction requires oxygen, since little inhibition of hydrolysis was noted in foams incubated under nitrogen. The addition of $NaHSO_3$ to model foams effectively inhibits the interaction between gelatin and autoxidizing ML. Since $NaHSO_3$ forms an addition product with aldehydes it is quite likely that lipid-derived carbonyls are preferentially complexed with this salt and the protein is thus unaltered.

Insulin-ML Reactions

In preliminary studies of the insulin interreaction with autoxidizing ML, a biological assay method was used to follow the course of the reaction. It was observed in previous work that insulin loses biological activity and becomes quite insoluble in acids as autoxidation proceeds (11). However, when insolubilized material was hydrolyzed in 6N HCl and compared chromatographically with control insulin, the amino acid compositions were found to be identical. Chromatographic comparisons of control and experimental (insolubilized) alkaline hydrolysates, on the other hand, revealed that appreciable amounts of lysine are lost as a result of autoxidation. Since amino acid-aldehyde complexes are acid-unstable but base-stable under reflux, and amino acid-epoxy complexes are acid-stable but base-unstable, the results of the hydrolysis studies suggested that lipid-derived aldehydes were reacting with the protein.

Hydrolyses, either acid or basic, are rather severe methods and as such may break down many of the amino acid-aldehyde complexes. Because of this it was decided to attack the problem indirectly by using the FDNB technique.

In the Sanger technique the free amino and hydroxyl groups of the protein are reacted with FDNB. The resulting bright yellow N-dinitrophenyl (DNP)-amino acid derivatives are relatively stable in strong acids, are ether-soluble with the exception of DNP-lysine, and are easily identified chromatographically after acid hydrolysis. The reaction is shown below:

$$R\text{–}NH_2 + FDNB \longrightarrow RNH(DNP) \xrightarrow[\text{Reflux}]{HCl}$$

DNP–amino acids + amino acids

If pure insulin is reacted with FDNB, acid hydrolyzed, then ether extracted to remove DNP derivatives, and finally paper chromatographed, no spot is found for lysine in the aqueous phase, although a bright yellow spot for ε-DNP-lysine is seen. Likewise, appreciably less phenylalanine and glycine are found than in pure insulin hydrolysates. On the other hand, insulin which has been in intimate contact with the autoxidizing ML prior to FDNB treatment will not react with the reagent since the reaction sites have already reacted with the oxidation products. Subsequent hydrolysis in strong mineral acid then will liberate free amino acids as follows:

$$\text{Protein–NH–CRHOH} + FDNB \xrightarrow[\text{(amino acids)}]{HCl} R\text{–}NH_2 +$$

dinitrophenol + RCHO

Therefore, by indirection, under relatively mild conditions the sites of ML interaction within the protein molecule may be determined by comparing the missing amino acids on the control samples with those present in the autoxidized material.

Chromatographic separation of the amino acids in the aqueous phase of acid hydrolyzed DNP-insulins clearly indicated that lysine was primarily involved in the insulin-ML interaction. Lysine was clearly evident in the experimental sample but not in the control. It was also noted that in the experimental sample no water-soluble DNP derivative was found, while in the controls a very sharp bright yellow spot was found at Rf = 0.70. This spot was cut from the chromatogram, hydrolyzed with $Ba(OH)_2$ to regenerate the parent amino acid, and was identified chromatographically as lysine. ε-DNP-lysine was then synthesized and its Rf value was determined in the partitioning solvents mentioned above. The synthetic derivative gave a bright yellow spot Rf = 0.70, thus confirming the involvement of the ε-amino group of lysine in lipid-protein interactions.

Chromatographic separations of the ether extracts of DNP-insulin hydrolysates were also studied. These derivatives were spotted on paper and then chromatographed with benzene:1% acetic acid solution. This solvent separates DNP-glycine (Rf = 0.07) from DNP-phenylalanine (Rf = 0.70). Both DNP-amino acid derivatives were detected in the insulin controls, but not in the experimentals (lipid oxidized), thereby indicating that N-terminal amino acids were also involved in the protein-lipid interaction.

Since the reactions of lipid intermediates with proteins may be pH-dependent, it was necessary to conduct the end group analyses in both acid (pH2–3) and alkali (pH~9). Although it was expected that such widely diverse conditions would considerably affect the N-terminal lipid-protein interaction, the results obtained under alkaline conditions were quite similar to those obtained from acid-solubilized insulin.

In addition to free amino groups, insulin contains an appreciable number of carboxyl, phenolic, guanido, imidazole, amido, hydroxyl and disulfide groups, any one of which could potentially interreact with products of autoxidized lipid. Chromatograms of insulin hydrolysates, however, did not indicate that any stable derivatives involving these groups had resulted during autoxidation. Although time did not permit a complete examination of all reactive groups, attempts were made to obtain data on the possible involvement of the carboxyl and guanidine groups of insulin since known methods may be applied to such study.

The Bradbury modification (12) of the Akabori hydrazinolysis method (13) was employed to determine whether or not the C-terminal residues of acid or alkaline insulins react with ML intermediates. Hydrazinolysates of both acid and alkaline insulin samples were found to contain the C-terminal amino acids in free form when chromatographed on paper. This indicated that the C-terminal groups are not important sites in the insulin-ML interaction under the autoxidizing conditions employed.

The qualitative Sakaguchi reaction (14) used as a measure of guanidine interaction did not indicate a chemical alteration of this group; results from autoxidation for both acid- and alkaline-derived insulins gave positive reactions for unaltered guanidine groups. The results, therefore, suggest that the guanidine group of arginine is not involved in the insulin-ML interaction.

Because of the rather restricted reaction conditions used in the insulin studies, an apparent absence of reaction with active hydrogen groups, aside from that of the free amino groups, is not surprising. Aldehyde reactions with hydroxyl, thiol and other hydrogen groups, for example, would normally be studied in aqueous solution rather than in anhydrous systems such as those used here (15). Quite likely the presence of water and prooxidants would influence the types of interaction within a protein molecule.

Solubility Studies Using Anhydrous Hydrogen Fluoride

The results in both the trypsin assay and insulin studies suggested that lipid-derived aldehydes covalently cross-link gelatin under the test conditions. In cross-linking reactions the molecular weight of the protein greatly increases and the resulting polymer is generally quite insoluble in the usual protein solvents. Because of this insolubility it is not possible to use classical methods to determine increases in molecular weight as a proof that cross-linkage has occurred. It has been shown, however, that anhydrous hydrogen fluoride dissolves a large number of native and acylated proteins while their firmly cross-linked counterparts do not dissolve (5). By indirection, therefore, insolubility of a protein in HF can be used as a test for covalent cross-linkage.

Both insulin and gelatin were found to be readily soluble in the solvent, while after reaction with autoxidizing ML they were quite insoluble. On the basis of the trypsin hydrolysis and HF solubility tests it was concluded that, in the autoxidation of ML, reactive intermediates arise which insolubilize proteins via a cross-linking reaction.

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D. K. Behrens of Eli Lilly & Co. provided us with crystalline zinc insulin.

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Heated Fats. IV. Chemical Changes in Fats Subjected to Deep Fat Frying Processes: Cottonseed Oil

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Abstract

The effects of deep fat frying processes on the formation of polymeric and oxidized materials were determined under practical conditions. Experiments which determined the effect of continuous heating, turnover rate, intermittent heating, steam, and deep frying were carried out. The amounts of polymeric material and other parameters of fat deterioration were determined. The results obtained indicated that the amount of polymeric material formed increased regularly as heating time increased. A high overall turnover of used oil may not increase the useful life of a frying fat when the specific daily turnover of fat is small. Intermittent heating of a fat alternated with cooling cycles increased the deterioration of cottonseed oil. The act of frying a food product (potatoes) or simple addition of water to hot fat exerts a strong deteriorative effect on heated cottonseed oil.

Introduction

GREAT MASSES OF EVIDENCE have accumulated which indicate that the heating of edible fats and oils at high temperatures results in the formation of nutritionally harmful substances. This material has been recently reviewed (1–4). It is also apparent that much of the published data cannot be directly applied to the problem concerning the effect of heat on fats under practical conditions. Much of this work has been carried out on carefully controlled and laboratory treated samples. The conditions employed are usually more severe than those encountered in practice. Several publications (5–9) have appeared which represent attempts to report work of a more practical nature in an approach to the heated fat problem. Rice (10), Poling (11,12) and Keane (13) have reported that fats obtained from restaurants and those prepared during frying experiments are not significantly damaged by heating during normal use unless they are abused. When abused fats were fed to young weanling rats using a calorie energy restriction feeding technique developed by Rice et al. (14), harmful effects were observed. Poling (11) has reported the effects of various treatments upon the nutritional characteristics of edible fats using this method.

It has been generally accepted that growth depressing materials and other nutritionally harmful materials are present in the oxidized or polymeric portions of heated fats. The deterioration of an oil during frying has been followed by conventional means, usually by measurement of unsaturation and free fatty acid content. Little interest has been shown in the actual amounts of polymeric or polar materials produced in oils heated under practical deep frying conditions, as a true parameter of the deterioration of an oil.

The series of experiments reported in this paper represent part of our general program to determine the chemical changes which take place during deep fat frying processes. The effects of several variables on the stability of cottonseed oil toward polymer formation and general breakdown were investigated. The nutritional value of the resulting heated fats was also determined and will be reported elsewhere.

Methods

A representative oil, cottonseed oil, was used in the present study. The cottonseed oil employed was winterized and commercially available. (Armour Star salad oil; analysis by GLC indicated the following fatty acid composition (%): 14:0, 1.07; 16:0, 26.63; 16:1, 0.82; 18:0, 2.33; 18:1, 20.91; 18:2, 48.25.) All experiments were carried out using the same lot of sample.

A standard method of heating was used in all of the experiments to be described unless otherwise specified. Eleven liters of fat were heated with a minimum amount of stirring to maintain even heat distribution in a commercial type electrically heated, 15 lb, stainless steel deep fat fryer, with a surface area of 182 sq in. In order to prevent localized overheating, the oil temperature was raised to 175C in a stepwise manner; this required about 30 min for final temperature equilibration. Samples of the oil were taken at various time intervals, blanketed with nitrogen gas and stored in the cold (−20C) until required.

Determinations of the saponification number (15), iodine value (15), peroxide value (15), percentage of free fatty acids (15), percentage of hydroxyls (16), and carbonyl values (17) were carried out on all samples.

In order to determine the concentration of oxidized, polymerized materials in the samples of heated fat obtained during the experiments, three different methods were used. These methods were: *a*) distillation of the fatty acids of the hydrolyzed heated fat samples at reduced pressure (less than 0.5 mm Hg in order to separate unreacted, monomeric and higher polymeric materials); *b*) a batchwise countercurrent distribution process similar to that reported by Zilch, Dutton and Cowan (18) employing redistilled skellysolve F and ethanol (80%) for separating oxidized from non-oxidized products based on polarity differences; and *c*) determination of the percentage of non-urea adduct forming fatty acids in the heated fats according to the method of Johnson et al. (19). The urea complex method was used initially and then dropped in favor of the previously discussed methods.

Experimental Results

Experiment 1

The more highly unsaturated fats, cottonseed oil, for example, are widely used in deep fat frying. It is therefore of interest to investigate the effects of heating on the deterioration of cottonseed oil. A commercial deep fat fryer was employed as previously illustrated. Cottonseed oil was heated continuously for a total of 382 hours; potatoes fried in this oil at the termination of the experiment were acceptable when sampled fresh and hot; after cooling, however, they became soggy and were not acceptable. Samples of the oil were removed at intervals for analysis. The data obtained are shown in Table I. Iodine values decreased to about 80 during heating with the formation of a dark colored viscous oil. The free fatty acid content increased to about 1%. While the hydroxyl group content increased to 0.66%, the carbonyl value increased markedly indicating the formation of non-volatile carbonyl compounds. Increasing concentrations of polymeric material developed during heating; from an initial value of 1.4% to 30.0% as determined by the solvent partition method and from 0.75% to a maximum of 45.6% as determined by the distillation method. In one case, the percentage of nonurea adduct forming acids was determined. The value obtained (35.4%) was in agreement with that obtained by the distillation method.

TABLE I

Effect of Continuous Heating on Cottonseed Oil

Hours heating	Iodine value	Free fatty acid (%)	Peroxide value (mEq/kg)	Saponification value	Hydroxyl (%)	Carbonyl value (mEq/kg)	Index of refraction n_D^{25}	Polymeric material	
								Hexane insoluble	NDFA[a]
0	112.8	0.03	11.2	195.0	0.09	182	1.4628	1.4	0.75
23	109.1	0.08	7.8	196.5	0.19	269	1.4625	4.1	6.7
70	103.7	0.16	5.1	198.2	0.22	317	1.4652	6.0	14.8
123	97.6	0.27	—	200.3	—	563	1.4674	13.8	19.5
166	91.0	0.38	3.1	201.0	0.43	239	1.4681	16.7	24.6
190	88.1	0.44	2.1	201.5	0.48	191	—	20.0	26.0
225	87.6	0.55	2.3	203.0	0.51	179	1.4701	22.3	33.0
262	84.9	0.60	1.7	204.4	0.50	195	1.4705	23.7 (35.4)[b]	32.8
334	83.2	0.83	2.1	205.3	0.65	213	1.4714	25.8	39.8
382	79.0	1.04	1.5	207.9	0.66	206	1.4727	30.0	45.6

[a] NDFA, Nondistillable fatty acids.
[b] NUAFA, Nonurea adduct forming fatty acids.

Experiment 2

The rate of exchange of fresh oil with used oil or the turnover rate may be an important factor in determining the nutritional quality of a used oil. The following experiment was designed to determine the effect of a small turnover rate on the stability of cottonseed oil. Cottonseed oil was heated continuously for 334 hr as in experiment 1, but when samples were removed for analysis (250 cc) an identical amount of fresh oil was added to the fryer. The result was a small but constant turnover of fat (2.5% per day) and a large total turnover during the experimental period (45%). Marked effects on the stability of the oil were observed. The data obtained are shown in Table II. The iodine values of the oil at the termination of the experiment remained about equal to those obtained when the oil was continuously heated without the addition of fresh oil, as did the other constants which were determined. The percentage of hexane insoluble material was about the same as that obtained for continuously heated oil, but the amount of nondistillable acids obtained was quite different. Much less nondistillable material appeared to be formed when small amounts of fresh oil were added constantly to the oil during the heating period. The carbonyl value of the replenished oil also increased tremendously and may be due to the formation of carbonyl compounds by decomposition of precursors during storage. While a high total turnover of fat may suggest that the original fat has been diluted and thereby protected from deterioration, such an assumption may be misleading since a high total turnover does not correctly express the length of time that some part of the fat has been heated. If the turnover rate of an oil per day is fairly small, the addition of fresh oil has little effect on the oil's stability.

Experiment 3

In many instances where deep fat frying is employed, the oil utilized is not heated continuously; the effect of intermittent heating on the stability of cottonseed oil was therefore investigated. Cottonseed oil was heated for a period of 7–8 hr during single time intervals; allowed to cool to room temperature and to remain thus for 12 to 16 hr. The heating cycle was then repeated and alternated with the cooling cycle. The oil was subjected to a total of 62 hr of actual heating; but the experiment was continued for a total of 223 hr to achieve this. Samples were taken at three intervals during each cycle: (*a*) when the sample had been heating for several hours prior to cooling, (*b*) after standing at room temperature for 12 to 16 hr, prior to reheating, and (*c*) one-half hour after reaching the final frying temperature. The data obtained are shown in Table III. When heated cottonseed oil is allowed to cool at room temperature, an expected buildup of fatty acyl peroxides takes place. When the oil is again heated, these peroxides decompose and cause increased damage to the oil as shown by the hydroxyl value and carbonyl value which increase during this period. Other constants showed predictable increases or decreases. When compared to an oil heated continuously, this oil, heated for only 62 hr (223 hr elapsed time) contained as much hexane insoluble or polar material as was present in cottonseed oil which had been heated continuously for 166 hours.

Experiment 4

In order to simulate the effect of frying a wet prod-

TABLE II

Effect of Turnover Rate on Cottonseed Oil

Hours heating	Iodine value	Free fatty acid (%)	Peroxide value (mEq/kg)	Saponification value	Hydroxyl (%)	Carbonyl value (mEq/kg)	Index of refraction n_D^{25}	Polymeric material	
								Hexane insoluble	NDFA[a]
0	110.6	0.02	2.1	195.0	0.17	190	1.4629	5.4	1.8
25	104.6	0.14	2.6	197.0	0.40	561	1.4641	9.7	8.9
73	98.1	0.30	2.7	198.8	0.57	1185	1.4650	12.8	11.6
145	92.1	0.55	2.5	200.2	0.61	3324	1.4676	18.0	16.9
241	88.5	0.72	1.4	202.7	0.65	1251	1.4691	20.6	20.5
274	85.5	0.80	1.2	203.2	0.66	1427	1.4704	25.0	21.9

[a] NFDA, Nondistillable fatty acids.

TABLE III

Effect of Intermittent Heating on Cottonseed Oil

Hours heating	Iodine value	Free fatty acid (%)	Peroxide value (mEq/kg)	Saponification value	Hydroxyl (%)	Carbonyl value (mEq/kg)	Index of refraction $n_D^{25°}$	Polymeric material	
								Hexane insoluble	NDFA[d]
0	110.5	0.01	2.4	195.2	.14	35.3	1.4630	2.4	1.4
30 E[a]	104.8	0.14	5.6	197.4	.27	135	1.4646	8.7	5.4
46	101.1	0.23	7.1	198.3	0.34	202	1.4656	11.7	9.3
54	100.1	0.25	4.8	199.2	.38	191	1.4652	13.1	11.0
54 M[b]	99.9	—	7.0	—	.41	241	1.4662	13.0	11.5
54 MH[c]	99.8	—	5.0	—	.38	267	1.4662	13.9	11.5
62 M	98.7	0.31	3.3	199.5	.39	297	1.4668	16.0	13.5
	98.3	0.31	7.3	199.6	.41	307	1.4668	15.6	13.8

[a] E, sampled before heat off.
[b] M, sampled when cold before heating.
[c] MH, sampled when temp. of 175C is reached.
[d] NDFA, Nondistillable fatty acids.

uct such as fresh potatoes without the added variable of the food product itself, water was discharged into the oil in the form of small droplets at an average rate of 28.8 cc per hour for 188 hr. A total of 54.14 liters of water was added to 9 liters of oil during the total heating period. The data obtained are shown in Table IV. The addition of water to the oil resulted in a decreased iodine value, increased the free fatty acid content by a factor of seven, doubled the hydroxyl content and increased the polymer content by almost 30% when compared to cottonseed oil heated continuously for the same amount of time. It is apparent that water (steam) exerts a considerable influence on the stability of a fat. The formation of free fatty acids indicates an increased degree of hydrolytic cleavage. The decrease in unsaturation and an increase in the percentage of hydroxylated compounds and polymeric material indicates that the presence of water (as such or from a food product) strongly accelerates the deterioration of a frying fat.

Experiment 5

In this experiment the effects of deep frying potatoes in continuously heated cottonseed oil was investigated. During a period of 100 hr, approximately 43 lb of cut potatoes were fried in 1 lb batches at regular intervals (1 batch/1 hour) during 8 hr time periods. Fresh unheated oil (approximately 200 cc) was added each day to replace that absorbed by the fried product. After frying had been carried out for approximately 30 hr, foaming of the oil when the potatoes were introduced became a serious problem, and the frying time was increased from 6 to 10 min in order to obtain a good quality product. The potatoes which were used were commercially prepared, precut, French fries. They were washed, and drained for 2 hr prior to frying and had been sodium sulfite treated (before being washed with water) for increased storage life. The data obtained are given in Table V.

A comparison of these data with that obtained from the heating of cottonseed oil alone for identical time periods indicated that the process of frying contributed substantially to the deterioration of an oil; a decrease in iodine value; and increase in free fatty acid values, hydroxyl value and polymeric materials was observed. The deteriorative effect of frying may result from a combination of at least four effects: 1) the effect caused by the composition of the food product; 2) the extra agitation and subsequent increased incorporation of air into the hot fat resulting from the introduction of the food product; 3) the effect of that portion of water displaced from the food product into the hot oil as steam; and 4) the amount of oil absorbed by the food product would ultimately reflect itself in the useful life of an oil. It is difficult to separate the effects of water in such a system but it would seem that its presence would be one of the predominant factors involved in fat deterioration. When the data resulting from this experiment was compared with that obtained when water alone was added to heated cottonseed oil, it was evident that frying drained potatoes in cottonseed oil resulted in less overall damage to the oil. It is apparent that the larger amounts of water added to the heated oil in the previous experiment caused a considerable increase in both the rate and extent of deterioration of cottonseed oil.

It has been reported previously (20) that foaming becomes a serious problem during deep fat frying. During the course of this study it was observed that a fine dense foam formed in cottonseed oil used to fry potatoes when the oil contained about 8% of polymeric materials. The polymer content of cottonseed oil which had been heated in the presence of water was adjusted to 8% by the addition of fresh oil. Potatoes were fried in the resulting oil. No fine dense foam was formed. When the same procedure was applied to an oil which had been used to fry potatoes, the foam was produced. Some foam was observed when this method was applied to an oil which had been heated continuously. An increase in frying temperature from 175C to 195C had no effect on foam formation. These results indicate that the formation of foam during frying may be dependent upon the types of oxidized and polymeric products present in a heated oil.

Discussion

The effects of practical frying conditions upon the deterioration of an oil are many and varied. When cottonseed oil is heated (Table I), the iodine value decreases as expected; the free fatty acid content, saponification values, and hydroxyl values increase as

TABLE IV

Effect of Water on Cottonseed Oil

Hours heating	Iodine value	Free fatty acid (%)	Peroxide value (mEq/kg)	Saponification value	Hydroxyl (%)	Index of refraction $n_D^{25°}$	Polymeric material	
							Hexane insoluble	NDFA[a]
0	113.2	0.03	11.1	194.8	0.11	1.4627	2.1	2.8
44	104.0	0.19	4.2	197.7	0.35	1.4653	7.5	12.7
123	91.8	1.48	2.6	203.7	0.68	1.4696	18.7	28.6
164	87.7	1.70	2.1	205.7	0.76	1.4701	21.9	32.0
188	84.4	3.12	3.0	207.1	0.89	1.4709	26.0	38.0

[a] NDFA, Nondistillable fatty acids.

TABLE V

Effect of Frying Potatoes in Cottonseed Oil

Hours heating	Iodine value	Free fatty acid (%)	Peroxide value (mEq/kg)	Saponification value	Hydroxyl (%)	Carbonyl value (mEq/kg)	Index of refraction $n_D^{25°}$	Polymeric material	
								Hexane insoluble	NDFA[a]
0	110.9	0.03	1.7	195.0	0.14	0	1.4612	4.0	2.8
31	104.0	0.22	2.7	196.9	0.36	184	1.4630	7.0	5.0
70	100.4	0.55	2.4	199.7	0.48	1187	1.4641	11.5	8.4
100	98.1	0.87	3.1	200.3	0.49	1195	1.4653	13.5	10.7

[a] NDFA, Nondistillable fatty acids.

well. The data obtained in the present study indicates that an appreciable amount of carbonyl containing material is also formed. The amount of polymeric material formed in such an oil increased regularly as it was heated for longer time periods. The amount of polar or highly oxidized material increased more rapidly than did the amount of high molecular weight nonpolar material as evidenced by larger percentages of material insoluble in hexane.

The useful life of an oil employed in deep fat frying may be strongly dependent upon the turnover rate, or the rate of addition of fresh oil to the used oil. Melnick et al. (6,21) have indicated that fats used in large volume potato chip fryers are not appreciably damaged. It has also been argued that fats resulting from small deep frying operations, where fat turnover is very small, may be considerably damaged. The results obtained from the present experiments confirm the suggestion of these authors. The rate of turnover is one of the factors which determine the degree to which a heated fat has been abused. The overall turnover rate of a fat may be quite large, but even though such a high overall turnover rate may indicate great dilution of the fat, it does not indicate the true length of time that a fat may have been heated. The detailed study of Poling et al. (11) concerning the effects of aeration on the nutritive value of fats has shown that the degree of exposure of a fat to oxygen is proportional to its nutritional effect. It is reasonable to expect that a fat which is continuously heated in the presence of air may actually be harmed to a lesser extent than an oil which has been heated intermittently. Such intermittent heating may be frequently employed by smaller establishments in attempts to prolong the useful life of a frying fat. It is possible that such intermittent heating, cooling, and heating cycles would encourage the destruction of the oil through an increased build-up of peroxides and carbonyl compounds which may then be destroyed by subsequent heating. Our results indicate that an oil which had been heated for 62 hr intermittently (or a total of 223 hr total time elapsed) contained 16.9% oxidized material; an oil heated continuously for 166 hr contained 16.7% oxidized material. The constants such as iodine value, percentage of free fatty acids, index of refraction, etc., remained comparable. These preliminary results indicate that such an oil had a shorter useful life when compared to an oil heated continuously as measured by the time required to reach a given polymer content as determined by the amount of hexane insoluble fatty acids present in the heated fat.

It would be desirable to know the effects of water upon deep frying and oil stability, since most products which may be cooked by deep frying contain an appreciable water content. The presence of steam in a deep fryer may aid in protecting an oil because of a deodorization effect causing the removal of some free fatty acids and decomposition products. Our results (Table IV) indicate that steam exerts a strong deteriorative effect on cottonseed oil. Reaction of hot cottonseed oil in a deep fryer with water injected into the oil quickly results in an oil of very inferior quality when compared to continuously heated oil. The addition of water to the hot fat caused greatly increased free fatty acid content, presumably due to hydrolytic cleavage of the triglycerides. A sizable increase in the amount of polymeric material was also observed when compared to the amounts formed during continuous heating. For example, after 23 hr heating time, the amount of high molecular weight and polar material were 18.7 and 28.6% compared to 13.3 and 14.5% for continuously heated oil.

The act of frying a food product, French fries, in the present study contributed substantially to the breakdown of cottonseed oil, but it appeared to be less damaging than the addition of water to hot fat. Although the amount of water added to the fat was probably the determining factor in the effect of water on a hot fat, the greater surface area of moisture contained upon a food product and that trapped within the product may be of greater significance than the actual percentage of water added to fat during frying. It is of interest to note that even though potatoes fried in such oil are completely acceptable, the fat remaining in the fryer after only 31 hr of use contained about 7% of oxidized high molecular weight material, and foamed extensively during the frying process. The study of Robinson et al. (20) indicated a correlation between the time that an oil has been used for frying and the frying temperature with foaming tendencies. This was also correlated with the development of viscosity in a fat and the formation of free fatty acids. These authors found that the addition of 25% of highly polymerized blown cottonseed oil to a sample of shortening caused it to foam immediately while the addition of 2.5% of the same blown oil cause foaming within 24 hr. Our results confirm this and indicate that foaming is related to the formation of oxidation products of high molecular weight.

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were converted to acetate esters for increased resolution. The analyses were made using a Pye-Argon chromatograph, equipped with a radium-D detector. The 4 ft by ¼ in. O.D. glass column was packed with 14% EGSS-X organosilicon polyester on Gas Chrom P. Good separation of the saturated cyclic alcohol acetates from straight-chain alcohol acetates was obtained at 175C and 35 cc per minute argon flow rate. GLC analysis showed the conversion of ester or acid to saturated alcohols to be almost quantitative with the formation of less than 2% hydrocarbons, which were easily separated by distillation. Gas chromatograms of saturated cyclic alcohol acetates are shown in Fig. 1.

Saturated C_{18}-cyclic alcohol monomers (containing 40% cyclic, 53% stearyl, and 7% palmityl) and saturated C_{20}-cyclic alcohol monomers (containing 50.7% cyclic, 36.8% stearyl, and 12.5% palmityl) were evaluated in cosmetic formulations. In several different emulsion systems—hand creams, hand and body lotions, and an antiperspirant compound—saturated cyclic alcohol monomers imparted a better feel and esthetic appearance than could be obtained with either cetyl or stearyl alcohol. In deodorant antiperspirant formulations containing aluminum salts, the saturated cyclic alcohols had much greater emolliency and reduced the tackiness inherent with the use of aluminum salts. When present in an aerosol medium, the hydrogenated cyclic alcohols had good lubricating properties for the valves. When incorporated in cosmetic formulations, little difference between C_{18}- and C_{20}-cyclic alcohols was noticed. In almost every product where saturated cyclic alcohols were used, the viscosity of the solution was reduced and the normal "body" of the finished formula was less than when cetyl and stearyl alcohols were used. These disadvantages were overcome by increasing the amount of saturated cyclic alcohol used between 50% and 100%. Hand and body lotions prepared with saturated cyclic alcohols were stable at 45C.

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R. L. Reichert conducted hydrogenations and Shulton, Inc. made valuable suggestions and evaluated the saturated cyclic alcohols in cosmetic formulations.

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Chemical Reactions Involved in the Deep Fat Frying of Foods. I. A Laboratory Apparatus for Frying Under Simulated Restaurant Conditions[1]

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Abstract

A laboratory apparatus has been designed which can be used to quantitatively collect the volatile decomposition products produced during deep fat frying under simulated restaurant conditions. In order to study the chemical reactions of frying fat without any inter-reaction with the food fried, moist cotton balls were fried in corn oil.

The oil used for frying was shown to differ considerably from oil which was continuously heated. The latter had a darker color and higher viscosity. It foamed significantly while the oil used for frying did not. Furthermore, the continuously heated oil had a much lower free fatty acid content than did the oil used for frying under simulated restaurant conditions.

The volatile decomposition products collected during frying of cotton balls in corn oil were separated into acidic and nonacidic compounds. Each group exhibited a definite gas chromatographic pattern after only a short period of frying. Part of the degradation products, particularly those of higher boiling points, were found to remain in the frying oil.

Introduction

THE NUTRITIVE VALUE of frying fats has been a controversial issue. Results of investigations from laboratories using commercial frying fats indicate that they have no deleterious effect in human nutrition (1–3). On the other hand laboratory treated fats have generally resulted in gross symptoms of nutritional disorders (4–7). However, the conditions under which the fats were treated for these experiments were quite different from those used in commercial deep fat frying or in restaurants.

The chemical reactions which take place during deep fat frying are far from completely understood. Perkins and Kummerow (8) heated corn oil at 200C for 48 hr while aerating it with a stream of oxygen. The polymerized oil was saponified and the fatty acids fractionated by forming urea adduct followed by molecular distillation. Some physical and chemical constants of the fatty acid fraction were determined. The volatile decomposition products formed by bubbling air through synthetic triglycerides were analysed by Endres et al. by GLC retention times (9). Recently, Ots et al. collected the volatile decomposition products by blowing air through preheated soybean oil at 250C. They also characterized the gas chromatographic fractions by retention times (10).

The present paper describes an apparatus used for chemical characterization of the volatile and nonvolatile decomposition products produced during deep fat frying under simulated commercial conditions.

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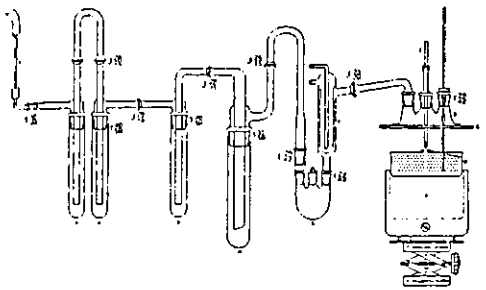

FIG. 1. Laboratory deep fat frying apparatus used for preliminary experiments.

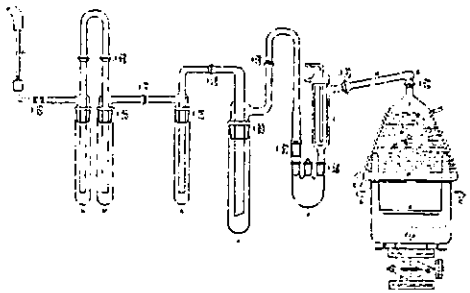

FIG. 2. Modified laboratory apparatus for deep fat frying under simulated restaurant conditions.

Experimental

Laboratory Deep Fat Frying Apparatus

The apparatus used in preliminary experiments for the quantitative collection of the volatile decomposition products under simulated restaurant conditions is shown in Figure 1. A Sunbeam household deep fat fryer (A), 8¼ in I.D. 9½ in O.D. containing 2300 ml of oil at 185C was used. The fryer was made to fit snugly a flat flange type resin reaction flask top (D), 5½ in. I.D. through an aluminum ring (C). The ring had an O.D. of 10½ in. and an I.D. of 6 in. A groove was engraved in the bottom side of the aluminum ring to fit the edge of the deep fat fryer. The center neck of (D) was fitted with a ground glass stirrer shaft (E) in a ground glass bearing (Kontes Glass Co. No. K-781000). The end of the stirrer shaft was attached to an aluminum basket (B) perforated with holes of ¼ in. diameter. The basket could therefore be lifted or lowered without leakage of gases.

For frying, the basket (B) was lifted out of the oil by the use of the shaft (E). The fryer was lowered with the aid of the laboratory jack. Ten moist cotton balls were then placed in the basket for frying. The cotton balls were previously washed thoroughly first with ethanol and then with redistilled hexane. Each of the cotton balls contained 75% of water and weighed 2 g. The laboratory jack was then used to raise the fryer to fit the resin reaction flask top snugly. A vacuum pump connected to the end of the flowmeter (M) was turned on to draw a current of air through the top of the fryer and then the train of traps at a rate of 7.2 l/min as indicated by the flowmeter (L). The basket was now dipped into the oil by lowering the shaft (E) and the cotton balls began to fry.

The volatile decomposition products and steam thus produced were drawn by the current of air flowing through the apparatus into the water cooled condenser (F). The condensate was collected in flask (G). The more volatile decomposition products were then condensed in the train of traps (H through K) which were cooled with dry ice. The frying was carried out once every 30 min. After each 6 hr of frying, the oil was allowed to cool to room temperature and the collected volatile decomposition products were washed out with ethyl ether.

Modified Deep Fat Frying Apparatus

The apparatus for the quantitative collection of volatile decomposition products used in the present investigation is shown in Figure 2. It was an improved and modified version of the apparatus used for preliminary experiments. The aluminum frying basket (A) was held in position by clamping at (B) and (C). The top of the Sunbeam deep fat fryer was fitted with an Alembic type cone (F) made of stainless steel. The cone was 10 in. high and had a top diameter of 5 in.; bottom diameter, 11 in. It was cooled with running water through aluminum coils wrapped around the outside of the cone. A glass connector (H) with an Alembic shaped head (G) was used to join the cone to the condenser (I).

For frying, the deep fat fryer containing 2300 ml of corn oil maintained at 185C was lowered until the aluminum basket was out of the oil. Ten moist cotton balls, prepared in the same manner as those used in the preliminary experiments, were placed in the aluminum basket. A vacuum pump connected to the end of the flowmeter (P) was turned on to draw a current of air through the top of the fryer and then through the train of traps at a rate of 7.2 liter/min as indicated by the flowmeter (O). The fryer was then raised until the cotton balls were immersed in the oil and fried.

The volatile decomposition products and steam thus produced were drawn by the current of air flowing through the apparatus into the stainless steel cone and then the condenser and the train of traps (K through N). The condensate collected on the inside of the cone could not drip back into the fryer because it was trapped by the Alembic edge (D). Excessive amount of volatile decomposition products and steam condensed in the cone would flow out from the exit

TABLE I

Physical and Chemical Changes of Corn Oil During Deep Fat Frying and During Continuous Heating

	Oil used for frying (hr)							Oil continuously heated
	0	3	6	12	30	60	90	90 hr
Free fatty acid (% oleic acid)	0.12	0.13	0.13	0.17	0.30	0.88	1.37	0.32
Peroxide number (meq/kg)	1.34	1.53	1.63	2.75	1.92	2.41	2.94	2.20
Iodine value (Wijs)	128	128	127	126	126	123	121	122
Refractive index (40C)	1.4675	1.4675	1.4680	1.4681	1.4681	1.4681	1.4681	1.4681
Color (photometric)	2.86	3.26	3.92	4.58	5.26	6.04	6.56	12.47
Viscosity (centistokes) 37.7C	39.7	40.0	40.3	43.2	42.3	44.9	48.9	50.4
Foaming (ml)	None	None	None	None	None	None	None	200

(E) and could be collected with a suitable container. Those which were not condensed in the cone were collected in the flask (J) and traps (K through N). Those condensed in the head of the connector tube (H) also could not drip back into the fryer because of the Alembic head (G).

Ten moist cotton balls containing approximately 16 g of water were fried every 30 min. Thirteen frying operations were done each day in 6 hr. After each 12 hr of frying, 800 ml of fresh corn oil was added into the fryer to replenish the oil absorbed by the cotton balls. After each 6 hr of frying, the oil was allowed to cool to room temperature. The apparatus was disassembled and all the condensates were washed out with distilled water and ethyl ether.

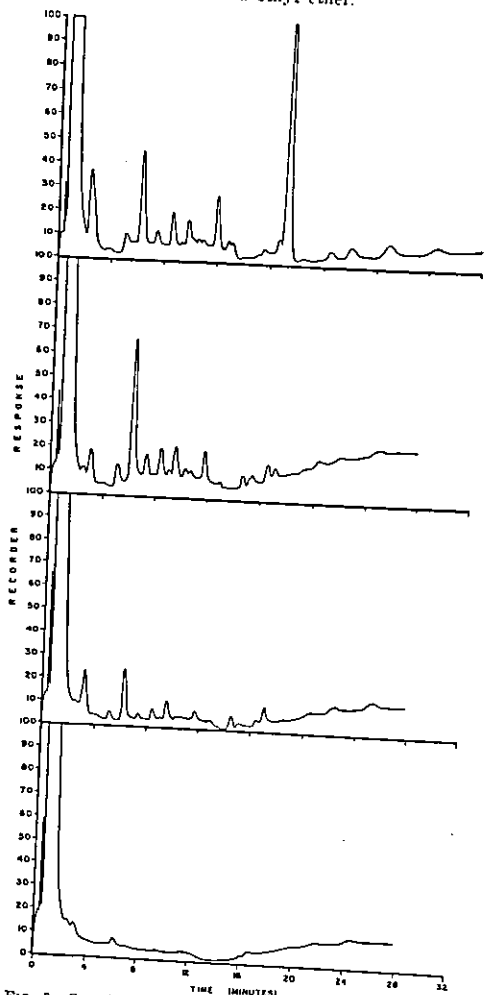

FIG. 3. Gas chromatogram of volatile acidic decomposition products collected during deep fat frying at 0–6 hr (bottom curve) 6–12 hr (lower center curve), 12–30 hr (upper center curve) and 60–90 hr (top curve).

Continuous Heating of Corn Oil

The modified deep fat frying apparatus was used. Corn oil, 2300 ml was placed in the Sunbeam deep fat frier and was continuously heated at 185C for 90 hr.

Extraction and Preliminary Fractionation of the Volatile Decomposition Products

The combined condensates and washings from the deep fat frying of cotton balls were extracted with ethyl ether and the extract concentrated according to the method of Chang (11). In order to remove the oil in the condensate due to entrainment during frying, the concentrated ether extract was then molecularly distilled in a bell-shaped flask with a flat bottom fitted with an Alembic distilling head. The distillations were done at 150C for 6 hr under a vacuum of 1–2 μ, while the sample was agitated with a magnetic stirrer.

The distillate was fractionated into acidic compounds and nonacidic compounds by extraction with 10% aqueous sodium carbonate solution.

Isolation of Volatile Decomposition Products Remaining in the Frying Oil

At the end of the experiment, the volatile decomposition products remaining in the corn oil were isolated by vacuum steam distillation. The distillate collected from distillation at 150C under 5 μ for 2 hr was extracted with ethyl ether and the ether extract treated in the same manner as described previously.

Gas Chromatography

The nonacidic volatile decomposition products were fractionated with a Beckman GC-2A gas chromatograph with an 8 ft column (¼ in. diameter) packed with 15% Ucon Polar on 80/100 Mesh Chromosorb W. The temperature was nonlinearly programmed from 50–200C in 40 min with a Beckman Thermotrac.

The acidic volatile decomposition products in ethyl ether solution were directly converted into their methyl esters by the use of diazomethane (12). The esters were fractionated with a Wilkens A-90-P gas chromatograph with a 6 ft column (¼ in. diameter) packed with 20% stabilized DEGS on 70/80 mesh Anakrom with temperature nonlinearly programmed from 60–200C in 18 min.

Analytical Methods

Free Fatty Acids, Photometric Color, Iodine Value, and Peroxide Number were determined according to the Official Methods of the American Oil Chemists' Society. Colors were measured with 1 cm rectangular cells instead of 2.18 cm diameter cylindrical cuvettes. Viscosity was determined at 37.7C with a Cannon-Fenske Viscometer (No. 200, Cannon Instrument Co.). Foaming was determined in a 250 ml high form graduated Griffin Beaker containing 160 ml of oil at 185C. Six square inches of Whatman No. 1 filter paper were moistened with 0.7 g of distilled water. The papers were strung on a Nichrome wire and inserted to the 50 ml mark in the center of the beaker. The highest volume to which the oil foamed was noted.

Results and Discussion

Apparatus for Laboratory Deep Fat Frying

The apparatus shown in Figure 1 could be used for deep fat frying in the laboratory. To avoid any interaction of the frying oil and the food fried, inert moist cotton balls were used for the frying. However, after some preliminary experiments, it was observed that considerable amount of volatile decomposition products of higher boiling point and some entrained oil were condensed on the resin reaction flask top. Since these condensates occasionally dripped back into the frying oil, they might undergo further decomposition to produce additional volatile decomposition products as artifacts. Furthermore, the resin reaction flask top was at an elevated temperature during the experiments. The condensate spread on the top as a film undoubtedly underwent further decomposition to produce volatile products which might not be the same as those produced from the oil during frying. Although this apparatus was abandoned after the exploratory experiments, it is described to avoid repetition by other researchers.

The modified apparatus shown in Figure 2 was found satisfactory for the quantitative collection of the volatile decomposition products produced during deep fat frying under simulated restaurant conditions.

Comparison of Frying Oil and Continuously Heated Oil

The appearance of the oil which was used for frying cotton balls in corn oil at 185C for 90 hr was quite different from the oil continuously heated for the same length of time at the same temperature. Some of the physical and chemical changes of corn oil when used for deep fat frying under simulated restaurant conditions are shown in Table I. These changes are also compared with those of corn oil which was continuously heated without frying and without replenishment with fresh oil every 12 hr.

The continuously heated oil had a darker color and higher viscosity. It foamed significantly while the oil used for frying did not. Furthermore, the continuously heated oil had a much lower free fatty acid content than the oil used for frying. Since free fatty acids remaining in the heated oil may further participate in chemical reactions, the chemical structures of the final decomposition products might also be different in oils heated continuously and in oils used for frying. Recently, Kritchevsky (13) reported that rabbits fed with cholesterol suspended in corn oil had higher atheromata when free fatty acid content was increased to 0.5%. The present data, therefore, seem to indicate a possibility that the frying oil may have effects upon the metabolic pattern of lipids which are different from those of the continuously heated oil.

Volatile Decomposition Products Remaining in the Oil

When corn oil was continuously heated, there was a significant amount of volatile decomposition products remaining in the oil. They could be isolated from the oil by vacuum steam distillation. According to gas chromatographic analyses, these compounds, both acidic and nonacidic, were not qualitatively different from those which were evaporated during the heating period. When the oil was used for frying, there was still a significant amount of volatile decomposition products, both acidic and nonacidic, remaining in the oil. However, they consisted predominantly of compounds with higher boiling points. It is therefore concluded that the effect of the volatile decomposition products upon human nutrition should be studied because these compounds, at least those with higher boiling points, will partially remain in the frying oil and are thus consumed with the fried foods.

Volatile Decomposition Products Collected During Various Intervals of Frying

The methyl esters of the volatile acidic decomposition products collected during 0–6, 6–12, 12–30, 30–60, and 60–90 hr of frying were gas-chromatographed under identical conditions (Fig. 3). The quantity of volatile acids produced during the first 6 hr of frying was small and the number of peaks few. After 12 hr of frying, the number of peaks increased to 23. When the concentration of the gas chromatographic sample was increased, a total of 27 peaks could be counted. Further frying to 30 and even 90 hr did not increase the number of peaks. However, in the early stage of frying, acids of lower boiling points were more predominant. They were probably produced by breakdown of carbon chains. In the later stage, when the free fatty acid content was increased, acids of higher boiling points were more predominant. They were probably produced by hydrolysis of triglycerides. Gas chromatograms therefore show that during the initial stage of frying more acidic volatile decomposition products are produced by chain breaking through autoxidation. But during the later stage of frying, more acidic decomposition products are produced by hydrolysis of the ester linkages of triglycerides.

The volatile nonacidic decomposition products collected during 0–3, 3–6, 6–12, 12–30, 30–60, and 60–90 hr were also gas-chromatographed under identical conditions (Fig. 4). The number of peaks increased from 6 hr of frying to 30 hr of frying. All the peaks observed in 6 and 12 hr of frying were also observed in 30 hr of frying. No new peaks were observed in the volatile decomposition products when the oil was further used for 60 and 90 hr. This seems to indicate that the decomposition pattern of the frying oil remained essentially the same after the oil was used for frying for 30 hr when the oil could be considered as good and reuseable by commercial standards, until the oil was used for 90 hr when the oil would be considered as bad enough to be discarded.

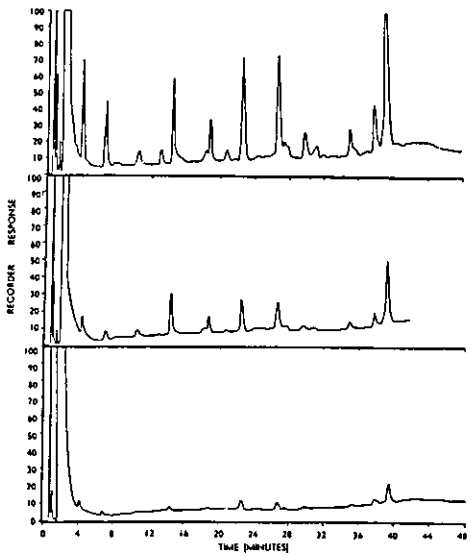

FIG. 4. Gas chromatogram of volatile nonacidic decomposition products collected during frying at 3–6 hr (bottom curve), 6–12 hr (center curve), and 12–30 hr (top curve).

ACKNOWLEDGMENTS

Mrs. Agnes S. Gibbons conducted the frying experiments and Mr. R. L. Hoffman constructed the apparatus of preliminary experiment. Supported by a PHS research grant HE-07610 from the National Heart Institute, Public Health Service.

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Alkaline Cleavage of Hydroxy Unsaturated Fatty Acids. I. Ricinoleic Acid and Lesquerolic Acid

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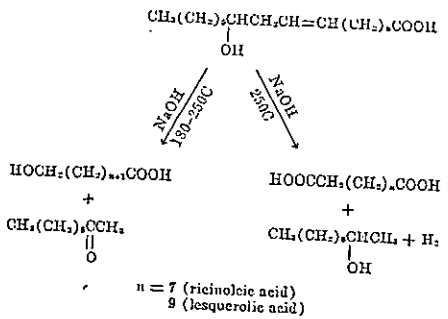
Abstract

The effects of temperature and media on the fusion of ricinoleic and lesquerolic acid derivatives with concentrated aqueous alkali were examined. Improved yields of ω-hydroxy acids were obtained by use of excess 2-octanol. The effect of excess 2-octanol is discussed in relation to a recently proposed reaction mechanism.

Introduction

HIGH TEMPERATURE (above 250C) alkaline fusion of derivatives of ricinoleic acid, 12-hydroxy-*cis*-9-octadecenoic acid, is a commercial method for manufacturing sebacic acid in high yield (1–9). Lower temperature (180–200C) alkaline fusion produces 10-hydroxydecanoic acid (1,5,9–12), but the yield is smaller than for sebacic acid. Analogous compounds are produced from lesquerolic acid, 14-hydroxy-*cis*-11-eicosenoic acid, which is the major fatty acid component of some *Lesquerella* seed oils (13,14). The high temperature alkaline fusion gives dodecanedioic acid (15). The low temperature reaction, which has not been reported previously, yields 12-hydroxydodecanoic acid. The following equations summarize the reactions:



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In this investigation the effects of reaction media, reaction times, and temperatures on the alkaline cleavage of ricinolec acid and lesquerolic acid were examined. A procedure for enhancing yields of ω-hydroxy acids is discussed in relation to possible reaction mechanisms (1,5,16,17).

Experimental

Materials

Methyl Ricinoleate. This ester was prepared by alcoholysis of castor oil followed by fractional distillation of the mixed methyl esters under reduced pressure to give a product of > 98% purity (18).

Methyl Lesquerolate. Lesquerella fendleri oil (13, 19) was alcoholized with methanolic sodium methoxide, and methyl lesquerolate (~95% purity) was isolated by fractional distillation at 190–195C and 100–275 μ Hg.

2-Octanol. Eastman Kodak 66 (ketone-free) 2-octanol was used as obtained.

Analytical Techniques

Programmed-temperature gas-liquid chromatographic analyses were run on a model 720 F and M Gas Chromatograph. The column was a 3-ft, 0.25-in. stainless steel tube packed with 10% ECNSS-S (Applied Science Laboratories, Inc., State College, Penn.) on 100–120 mesh Gas Chrom P.

Procedures for Alkaline Fusion of Hydroxy Unsaturated Acids

A. Preparation of Sebacic Acid by High Temperature Alkaline Cleavage of Ricinoleic Acid. A one-liter nickel resin kettle and top were fitted with a sealed steel stirrer, heated dropping funnel, and a condenser for collection of volatile by-products. The reaction vessel was charged with 17.8 g of sodium hydroxide, 2.5 g of water, and 3.9 g of lead oxide as catalyst (8), and brought to 250 C with an electrically heated silicone oil bath. Methyl ricinoleate (78 g) was saponified with 20 g of sodium hydroxide in 12 ml of water. The soap was dissolved in 138 ml of hot water, and was added dropwise to the stirred hot caustic. Sodium ricinoleate was sufficiently soluble in hot water to permit uniform dropwise addition as a homogeneous aqueous solution. The reaction mixture was then stirred at 250C for 2 hr and the volatile components collected. The mixture remaining in the kettle was cooled to about 90C and dissolved in 90 ml hot water. After filtration of the hot caustic solution, an aliquot of the filtrate was withdrawn for analysis. The aliquot was acidified to pH 1 with 50% aqueous sulfuric acid and extracted with ether. The ether solution was dried with sodium sulfate, and the ether was removed on a rotary evaporator to yield sebacic acid. This was converted to esters in refluxing excess methanol with 0.5% concentrated sulfuric acid as catalyst. The resultant dimethyl sebacate was examined by gas-liquid chromatography (GLC). The GLC pattern showed only one large peak, and no methyl 10-hydroxydecanoate was present. The remaining caustic solution was acidified, and 35.2 g (70%) of >95% pure sebacic acid was obtained; mp 127–131C.

B. Preparation of Dodecanedioic Acid by High Temperature Alkaline Cleavage of Lesquerolic Acid. The equipment and procedures used were identical to those described above except that the less soluble sodium lesquerolate was added in ethanol, or the more soluble potassium lesquerolate was added in water. The yield of crude dodecanedioic acid obtained was 62%; mp 110–122C. One crystallization from ethyl acetate (14:1) gave a 48% yield of dodecanedioic acid; mp 125–128C. A portion of the crude free acid was converted to dimethyl dodecanedioate, and examination by GLC indicated that no methyl 12-hydroxydodecanoate was present in the sample.

C. Preparation of 10-Hydroxydecanoic Acid by Low Temperature Alkaline Cleavage of Ricinoleic Acid. In one experiment without an organic diluent (run 1), the same apparatus and procedures described above were used except a lower temperature was maintained. The quantities of reactants, reaction conditions, and analytical results are shown in Table I.

In all other runs, the apparatus was modified by replacing the take-off arrangement with a reflux condenser. A mixture of concentrated aqueous alkali and an organic diluent such as 2-octanone or 2-octanol was stirred and heated to the desired temperature in the reaction pot. Methyl ricinoleate was added dropwise and the reaction mixture was stirred for the desired time. The mixture was cooled to 90C, water was added, and an aliquot was withdrawn from the vigorously stirred system for analysis. For isolation of the 10-hydroxydecanoic acid in large quantity, the mixture was acidified to pH 1, and the lower aqueous layer was separated from the supernatant organic layer. The hot organic solution was washed with hot water, dried with sodium sulfate, and filtered. Commercial pentane was added, and a copious crop of 10-hydroxydecanoic acid crystals formed. The mixture was stored at —25C overnight, and the crystals were filtered, washed with commercial pentane, and dried. The ω-hydroxy acid was further purified by recrystallization from benzene after decolorization with charcoal. See runs 2 and 3 for quantities of reactants, reaction conditions, and analytical results.

D. Preparation of 12-Hydroxydodecanoic Acid by Low Temperature Alkaline Cleavage of Lesquerolic Acid. In one experiment, the same apparatus and procedure described in (B) were used. The quantities of reactants, reaction conditions, and analytical results are shown in run 4. In all other runs the reaction was performed under reflux and worked up as described in (C). Methyl lesquerolate was added dropwise to a reaction system containing one of the following diluents: p-cresol, glycerol, or 2-octanol. The conditions and results are presented in the table.

TABLE I

Alkaline Fusion of Hydroxy Acids

Run No.	Starting materials: Fatty acid,[a] moles	NaOH, moles	H_2O, moles	Diluent,[b] moles	Temp, C	Time, hrs	Percent composition of crude reaction mixture[c]: Dibasic acid	ω-Hydroxy acid	Starting acid	Unknowns
1	0.10	0.22	15	0	202	3.3	37	29	40	....
2	0.10	0.85	0.5	0.38	184	5.8	67	19	....	14
3	0.40	3.40	2.0	1.50	183	11.5	11	60	....	0
4	0.10	0.22	13	0	201	2.1	22	20	58	....
5	0.20	1.70	1.5	0.13	192	7.9	53	34	12	....
6	0.10	0.85	0.5	0.82	191	24.8	0	0	98	2
7	0.10	0.85	0.5	0.19	187	10.5	21	63	11	5
8	0.10	0.85	0.5	0.38	183	12.7	14	66	13	7
9	0.10	0.85	0.5	0.57	186	22.5	23	64	5	8
10	0.10	1.70	0.5	0.38	185	12.6	12	66	9	13
11[d]	0.30	2.55	1.5	1.14	185	12.4	19	63	7	11

[a] Run 1: added as aqueous sodium ricinoleate; runs 2 and 3: added as methyl ricinoleate; run 4: added as aqueous potassium lesquerolate; runs 5 to 11: added as methyl lesquerolate.
[b] Run 2: 2-octanone; run 3: 2-octanol; run 5: p-cresol; run 6: glycerol; runs 7 to 11: 2-octanol.
[c] Analyzed as methyl esters by GLC. Not adjusted for nonvolatile products. Compositions reported on the basis of uncorrected areas of peaks.
[d] Blanketed with nitrogen.

Discussion

The nearly exclusive formation of dicarboxylic acids from the alkaline fusion of some hydroxy unsaturated acid derivatives at above 250C has been amply substantiated by numerous earlier investigators (1–9,15), and further confirmed by the results presented here. Manufacturing plants claim greater than 80% yields of sebacic acid from castor oil (3,6–8). Only a 21% yield of dodecanedioic acid from lesquerolic acid was previously reported (15), but by following procedure B, a 48% yield is obtained.

At lower temperature, the yields of dicarboxylic acids decrease and some ω-hydroxy acids form. We find, however, that the dicarboxylic-acid-to-ω-hydroxy-acid ratio favors the former acid even at temperatures as low as 183C (cf. runs 1,2,4, and 5). Hence, lowering the reaction temperature is not adequate to obtain high yields of ω-hydroxy acids. Furthermore, it has been shown that below 180C no appreciable cleavage occurs (5).

In a recent series of investigations, Weedon and co-workers (16,17,20–22) have extensively examined the reactions of fatty acid derivatives with concentrated alkali. The first step in their proposed mechanism for the alkaline cleavage of hydroxy acids is dehydrogenation to give a keto acid. In the case of unsaturated hydroxy acids, e.g., ricinoleic acid or lesquerolic acid, the dehydrogenation process which

TABLE II

RELEASE OF AMINO ACIDS FROM PEPTIDE FOUR BY CARBOXYPEPTIDASES B AND A*

Amino acid	Liberated, absorbed (μmoles)	Not absorbed, hydrolyzed acid (μmoles)	Total (μmoles)
Arginine	0.29	0	0.29
Isoleucine	0.26	Trace	0.26
Glutamine (as glutamic acid)	0.15	0.15	0.30
Serine (2)	0	0.54	0.54

* Partition of amino acids on Dowex 50 after hydrolysis with carboxypeptidases B and A. Average of values obtained in two experiments.

TABLE III

AMINO ACIDS LIBERATED FROM PEPTIDE FOUR BY CARBOXYPEPTIDASE A*

Amino acid	μmoles liberated after short-term incubation	μmoles liberated after long-term incubation
Serine	Trace	Trace
Glutamine (as glutamic acid)	Trace	0.16
Isoleucine	0.15	0.32

* Peptide Four was first incubated with carboxypeptidase B and the reaction run to completion. Carboxypeptidase A was then allowed to react with each of two aliquots of the peptide solution for different lengths of time. Details are as in text.

without being hydrolysed, and no free amino acids were detected. The failure of this peptide to react with ninhydrin reagent suggested that it was either very large or that its N-terminal amino acid was substituted. The reaction of Peptide 4 with hydrazine yielded a mixture of amino acid hydrazides and acetyl hydrazide which was identified by each of the two chromatographic systems (6).

Sequence of Peptide Four. Carboxypeptidase B is known to split arginine from a C-terminal position in peptides. The remaining amino acids may then be removed sequentially by carboxypeptidase A; in rate studies the residues most proximal to the C-terminus of the peptide should accumulate most rapidly during incubation with the enzyme. The liberated amino acids and any unreacted peptide should be absorbed on Dowex 50: the N-terminal acetylated amino acid and acetylated peptide fragments should pass through the resin. Figure 4 outlines the procedure and anticipated results.

Table II shows the results of duplicate long-term incubations with the carboxypeptidases. Serylserine is the acetylated or terminal peptide residue since no free serine was found. These data suggest that the peptide sequence is *N*-acetylseryl·seryl·glutaminyl·isoleucyl·arginine. This sequence is confirmed by a comparison of the amounts of amino acids liberated during the short- and long-term incubations of Peptide 4 with carboxypeptidase A (Table III). Arginine was not measured in this experiment. Since only isoleucine was found after short contact with the enzyme it must be adjacent to arginine in the peptide. The longer incubation produced more isoleucine, some glutamine and a trace of serine. Hence glutamine is adjacent to isoleucine and the second seryl residue must occupy the position between isoleucine and acetylserine. Therefore, the sequence of Peptide 4 is *N*-acetylseryl·seryl·glutaminyl·isoleucyl·arginine.

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Damage to Proteins, Enzymes, and Amino Acids by Peroxidizing Lipids

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Transient free-radicals are produced in peroxidizing lipid-protein reaction systems. The pattern of damage to proteins, induced by these radicals, is similar to that observed in the case of radiation damage; proteins and enzymes lose solubility and constituent amino acids are destroyed. Lipid peroxidation damage appears to be about one-tenth as effective as radiation damage. Amino acid destruction was measured in lipid-peroxidation damaged γ-globulin, catalase, serum albumin, hemoglobin, and and ovalbumin. Among the most labile amino acids are methionine, histidine, cystine, and lysine. Major products of lipid peroxidation-cysteine interaction are hydrogen sulfide and cystine.

Transient free-radicals, generated in peroxidizing unsaturated lipid-protein mixtures, participate in the chain of reactions leading to considerable damage. Chemical evidence for damage to cytochrome c has been reported (1). A recent paper describes the mechanism for lipid peroxidation induced free-radical polymerization of proteins and enzymes (2); it was also shown that soluble polymeric materials are produced in an irradiated cytochrome c solution.

This paper reports evidence for the radiomimetric effects of lipid peroxidation intermediates to proteins and amino acids.

MATERIALS AND METHODS

Experiments conditions of the reaction systems have been given (2); ethyl arachidonate was used unless otherwise indicated. Proteins and enzymes used in experiments on yield of insoluble protein were: trypsin (2×-crystallized, Mann), pepsin (2×-crystallized, Mann), α-chymotrypsin (3×-crystallized, Mann), ovalbumin (crystalline, Nutritional Biochemicals Corp.), and hemoglobin (2×-crystallized, Sigma). In order to initiate and maintain peroxidation in reactions containing nonmetalloproteins, the buffer employed was 0.05 *M*, pH 7.0 phosphate containing 10^{-5} *M* ascorbate and 10^{-4} *M* copper sulfate. Control reactions were run in lipid-free systems at 37°.

In studies of lipid peroxidation damage to constituent amino acids, the proteins and enzymes were: γ-globulin (Bovine, Calbiochem), catalase (Crude, Sigma), bovine serum albumin (Crystalline, Pentex, Inc.), hemoglobin and ovalbumin of the purity and source given above. Lipid peroxidation was allowed to proceed until sufficient protein was insolubilized; insoluble proteins were extracted and stored as previously described (2). Amino acid analyses of the acid hydrolyzed insoluble protein were made either with a Beckman or Technicon automatic amino acid analyzer.

In studies of peroxidation damage to cysteine, a reaction system composed of 1 gm of L-cysteine, 1 gm of ethyl arachidonate, and 8 ml of buffer was employed. Hydrogen sulfide was measured by the method of Marback and Doty (3). Nonvolatile cysteine degradation products were characterized by thin-layer chromatography using silica gel G plates and a migrating solvent composed of phenol–acetic acid–water (70 wt.:10 vol:20 vol). Spots were detected with the polychromatic copper-ninhydrin spray devised by Moffat and Lytle (4).

RESULTS

Expression of protein insolubilized per mole of peroxy radical in Table I is appro-

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priate because it allows a comparison with protein damage by ionizing radiation where product formation per free radical is given as ionic yield. Peroxy radicals are approximately equal to oxygen reacted in this well known lipid peroxidation mechanism involving free-radical intermediates.

Table II gives the percentage loss for each amino acid in reacted protein or enzyme. Extent of oxidation was 2.5, 1.6, 1.9, 1.9, and 1.6 moles of oxygen per mole of lipid for γ-globulin, catalase, bovine serum albumin, hemoglobin, and ovalbumin reaction mixtures, respectively. Lysine, histidine, tyrosine, methionine, and cystine, in descending order, are the most labile amino acids in γ-globulin; in catalase, lysine, serine, valine, methionine, and histidine are the most labile amino acids. Glycine, cystine, histidine, alanine, and valine are labile in bovine serum albumin while tyrosine, methionine, lysine, and histidine are labile in hemoglobin. Finally, methionine, histidine, threonine, proline, and glycine are the most labile amino acids in ovalbumin.

Peroxidizing arachidonate-cysteine interaction leads to sulfhydryl cleavage with the production of hydrogen sulfide. The data of Fig. 1 show that H_2S production increased in a linear fashion with extent of lipid peroxidation. Of the nonvolatile products of lipid-cysteine interaction, only cystine and a trace of alanine could be detected on thin-layer chromatograms (Fig. 2). Both cystine and alanine would be expected to react further to yield both ninhydrin-positive and ninhydrin-negative products and could account for the low yield of alanine at the termination of the experiment. However, no intermediate oxidation products of cysteine or cystine, based on spot color, could be identified. No insolubilized material was produced even under prolonged exposure of reaction mixture to high oxygen tension; tests for sulfate and free sulfur were negative.

TABLE I

PROTEIN INSOLUBILIZED BY PEROXIDIZING LIPID

Protein	Moles O_2/mole lipid	Yield (moles protein insolubilized/mole peroxy radical)
Trypsin	0.28	0.012
Pepsin	0.15	0.017
Ovalbumin	0.29	0.0087
α-Chymotrypsin	0.22	0.0025
Cytochrome c	0.25[a]	0.018
Hemoglobin	1.03[a]	0.0021

[a] Lipid consisted of a mixed ethyl ester containing 73% docosahexaenoate and 17% eicosapentaenoate.

TABLE II

LIPID PEROXIDATION DAMAGE TO AMINO ACIDS OF PROTEINS

Amino acid	% Amino acid loss				
	γ-Globulin	Catalase	Bovine serum albumin	Ovalbumin	Hemoglobin
Lysine	58.8	42.4	40.8	21.6	58.6
Histidine	51.8	18.2	54.1	38.3	57.6
Arginine	26.5	8.2	11.5	Slight damage	24.1
Aspartic acid	11.3	0	39.8	0	7.2
Threonine	14.8	10.9	46.2	28.0	37.7
Serine	24.4	22.4	43.2	25.0	43.0
Glutamic acid	24.1	—[a]	41.0	25.0	39.0
Proline	16.0	—[a]	Slight damage	27.9	38.8
Glycine	21.2	12.0	82.5	27.6	53.8
Alanine	18.8	10.3	50.0	24.5	33.7
½-Cystine	32.8	—[a, b]	64.0	—[a]	—[a]
Valine	21.0	21.4	47.7	25.8	31.3
Methionine	38.3	20.3	47.5	80.3	58.5
Isoleucine	20.0	12.3	42.8	21.4	—[a, b]
Leucine	22.2	2.6	34.4	5.4	32.8
Tyrosine	50.7	10.5	45.2	8.5	91.0
Phenylalanine	32.0	6.8	44.1	20.9	36.4

[a] Amino acid analyses were not suitable for an accurate measurement of loss.
[b] These amino acids are only present in small amounts in the protein.

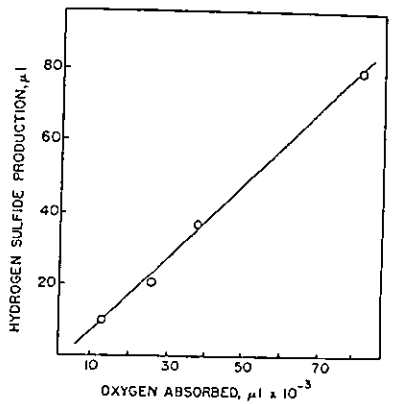

FIG. 1. Formation of hydrogen sulfide as a function of oxygen absorption in a cystein-arachidonate system.

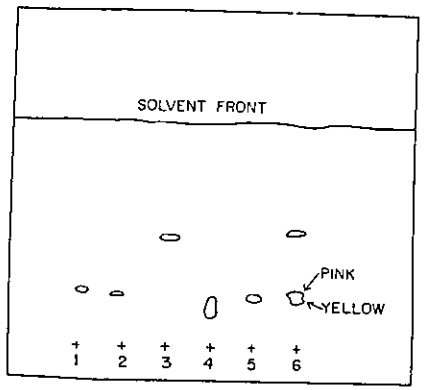

FIG. 2. Thin-layer chromatography of cysteine-arachidonate reaction products.

		R_f	Color
1	Cysteine standard	0.28	Pink
2	Cystine standard	0.27	Yellow
3	Alanine standard	0.50	Pink
4	L-Cysteic acid standard	0.22	Blue
5	L-Cysteine sulfinic acid standard	0.27	Blue
6	Aqueous cysteine-arachidonate reaction products	0.29	(Pink and yellow), 0.57 (pink)

DISCUSSION

Comparison of lipid peroxidation damage with that of ionizing radiation is appropriate because both reactions involve free-radical intermediates and because analogous information is available from studies of radiation damage to proteins and cysteine. Results of lipid peroxidation damage in Table I can be compared to radiation damage to proteins. Yield values of protein damaged per ion pair are 0.05, 0.03, 0.10, and 0.48 for invertase, catalase, cytochrome c, and ribonuclease, respectively (5). Another yield value is 0.20 for trypsin (6). Lipid peroxidation damage is less than radiation damage, an inequality caused by the biphasic lipid reaction system; many of the peroxy free-radicals are prevented from reacting with protein. Since both radiation and lipid peroxidation give rise to free-radical intermediates, it does not seem surprising that both types of damage should show similarities; Haissinsky has indicated that both ionizing radiation and lipid peroxidation may have a similar mechanism of damage at the molecular level (7).

The relationship between radiation and peroxidation damage is also able to explain the observation that the over-all pattern of amino acid damage in proteins is similar in both processes. In irradiated ovalbumin, histidine, cystine, methionine, phenylalanine, and threonine suffer greatest damage (8). Irradiation studies with hemoglobin show the most radiolabile amino acids to be methionine, histidine, threonine, tyrosine, and phenylalanine (8). Likewise, cystine, methionine, phenylalanine, histidine, and tyrosine are the most radiolabile amino acids of catalase (9).

Irradiated proteins often show an increased alanine content with increase in dose; however, the content of this amino acid drops at still higher dosage and remaining amino acids suffer increased destruction. Studies of lipid peroxidation damage to proteins have shown that there are no increases in alanine. Furthermore, α-amino-*n*-butyric acid, produced by cleavage of the terminal CH_3S-group from methionine, has not been detected. Evidently, the magnitude of peroxidation damage was sufficient to destroy these primary damage products.

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Hydrogen abstraction reactions, initiated by a variety of free-radical intermediates would account for disulfide production:

$$\left.\begin{matrix}\mathrm{ROO}\cdot\\ \mathrm{R}\cdot\\ \mathrm{RO}\cdot\\ \mathrm{HO}\cdot\end{matrix}\right\} + \mathrm{R'SH} \rightarrow \mathrm{R'S}\cdot + \left\{\begin{matrix}\mathrm{ROOH}\\ \mathrm{RH}\\ \mathrm{ROH}\\ \mathrm{H_2O}\end{matrix}\right.$$

followed by $\mathrm{R'S}\cdot + \mathrm{R'S}\cdot \rightarrow \mathrm{R'SSR'}$. Free-radical cleavage of the R—SH bond would lead ultimately to H_2S production. The radiolability of sulfur amino acids is well documented (10, 11).

The lability of histidine is of interest. Data of Table II show this amino acid to be labile in most of the examples given. Histidine acts prooxidatively to initiate a rapid uptake of oxygen in arachidonate emulsions with the formation of soluble polymeric products which are Folin reagent-positive (2). Recently, Saunders and Hampson have shown that the prooxidative effect of histidine in methyl linolenate emulsions appears to be associated with both the imidazole and amino groups (12). In the presence of activators, the histidine complement of β-casein is photolabile (13).

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Glycine Reduction to Acetate and Ammonia: Identification of Ferredoxin and Another Low Molecular Weight Acidic Protein as Components of the Reductase System

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Protein A, one of the catalytic components of the glycine reductase system of *Clostridium sticklandii* and related amino acid-fermenting clostridia, was partially purified and characterized as an acidic low molecular weight protein apparently possessing one or more sulfhydryl groups essential for activity.

Certain crude extracts unable to reduce glycine unless supplemented with protein A can be shown to require this protein irrespective of the electron donor system employed, i.e., with reduced methyl viologen, a DPNH-generating system or with dimercaptans such as 1,4-dimercaptothreitol. In contrast, ferredoxin and another unidentified factor(s) also removed by treatment of extracts with DEAE-cellulose are required for glycine reduction in the DPNH-linked system but are bypassed when dimercaptans serve as electron donors.

Glycine reduction to acetate and ammonia is catalyzed by a number of anaerobic amino acid-fermenting bacteria capable of carrying out Stickland type fermentations. Soluble enzyme preparations of these bacteria can utilize as electron donors certain dimercaptans structurally related to dimercaptooctanoic acid, DPNH, or reduced methyl viologen for the reductive deamination of glycine. Irrespective of the electron donor employed, concommitant with glycine reduction is an esterification of orthophosphate and the formation of ATP (1, 2). One enzyme, common to all of these systems, is a low molecular weight acidic protein (protein A) that is readily separated from crude extracts by treatment with DEAE-cellulose. The present communication documents the requirement for this component and describes a procedure for its purification together with some of its properties. It is also shown that treatment with DEAE-cellulose resolves the crude extracts for additional acidic protein components that are needed when DPNH serves as the electron donor for glycine reduction. One of these components can be replaced with ferredoxin. In contrast, with $R(SH)_2$ as electron donor, the addition of highly purified protein A preparations alone is sufficient to restore to full activity DEAE-cellulose-treated extracts.

MATERIALS AND METHODS

Materials

The following materials were purchased from commercial sources: 2X-crystallized horse liver alcohol dehydrogenase, Worthington Biochemical Corp.; methyl viologen and potassium pyruvate, Mann Research Laboratories; carbamylphosphate and 1,4-dimercaptothreitol, California Biochemical Corp.; Sephadex spherical gels from Pharmacia Fine Chemicals, Inc., and polyacrylamide spherical gels from Bio-Rad Laboratories. Other chemicals were purchased or prepared as described previously (2).

Ferredoxin isolated from *Clostridium pasteurianum* was a generous gift from J. E. Carnahan, duPont de Nemours and Co.; intrinsic factor, from L. Ellenbogen of Lederle Laboratories; and liver glucose dehydrogenase, from E. R. Stadt-