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 caloric 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 nonoxidized 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

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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^{40}	Polymeric material	
								Hexane insoluble	NDFA[a]
0	112.8	0.03	11.2	195.0	0.09	182	1.4628	1.4	0.75
22	109.1	0.08	7.8	196.3	0.10	269	1.4635	4.1	6.7
70	103.7	0.16	5.1	198.2	0.22	317	1.4652	8.0	14.8
123	97.6	0.27	—	200.3	—	563	1.4674	13.3	19.5
166	91.9	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
228	87.6	0.55	2.3	203.0	0.51	179	1.4701	22.3	33.9
262	84.9	0.60	1.7	204.4	0.50	195	1.4705	23.7 (25.4)[b]	32.8
334	82.2	0.93	2.1	205.3	0.65	213	1.4714	25.8	39.8
382	79.0	1.04	1.5	207.0	0.66	206	1.4727	30.0	45.6

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

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 nonvolatile 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.

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^{40}	Polymeric material	
								Hexane insoluble	NDFA[a]
0	110.6	0.02	2.1	195.0	0.17	190	1.4629	5.4	1.3
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	196.8	0.57	1165	1.4650	12.8	11.6
145	93.1	0.55	2.5	200.3	0.61	1324	1.4676	18.0	16.9
241	88.5	0.72	1.4	202.7	0.65	1251	1.4691	20.6	20.5
334	85.5	0.50	1.2	203.2	0.66	1427	1.4704	23.0	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^{60}	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	1.4648	8.7	5.4
46	101.1	0.23	7.1	198.3	0.34	202	1.4658	11.7	9.3
54	100.1	0.25	4.8	199.2	.38	191	1.4662	13.1	11.0
54 M[b]	99.0	—	7.0	—	.41	241	1.4662	13.0	11.5
54 MH[c]	99.8	—	5.0	—	.38	257	1.4662	13.3	12.9
62	98.7	0.31	3.3	190.5	.39	297	1.4668	16.9	13.5
62 M	98.3	0.31	7.3	190.6	.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^{60}	Polymeric material	
							Hexane insoluble	NDFA[a]
0	112.2	0.03	11.1	194.8	0.11	1.4627	2.1	2.3
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	1.4686	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.

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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^{60}	Polymeric material	
								Hexane insoluble	NDFA[a]
0	110.9	0.03	1.7	195.0	0.14	0	1.4612	4.0	2.3
31	104.9	0.22	2.7	196.0	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	2.1	200.3	0.49	1195	1.4653	12.5	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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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, Ota 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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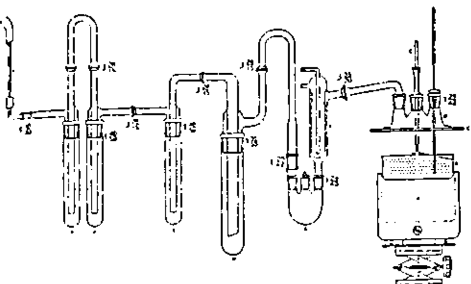

FIG. 1. Laboratory deep fat frying apparatus used for preliminary experiments.

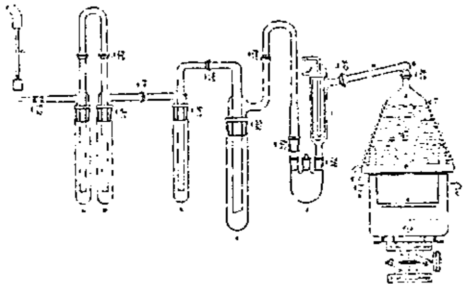

FIG. 2. Modified laboratory apparatus for deep fat frying under simulated restaurant conditions.

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-78100). 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 (D) 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	2.04	2.20
Iodine value (Wijs)	128	128	127	126	126	123	124	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	2.92	4.58	5.26	8.04	8.56	12.47
Viscosity (centistokes) 37.7C	39.7	40.0	40.3	43.2	42.3	44.9	42.9	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.

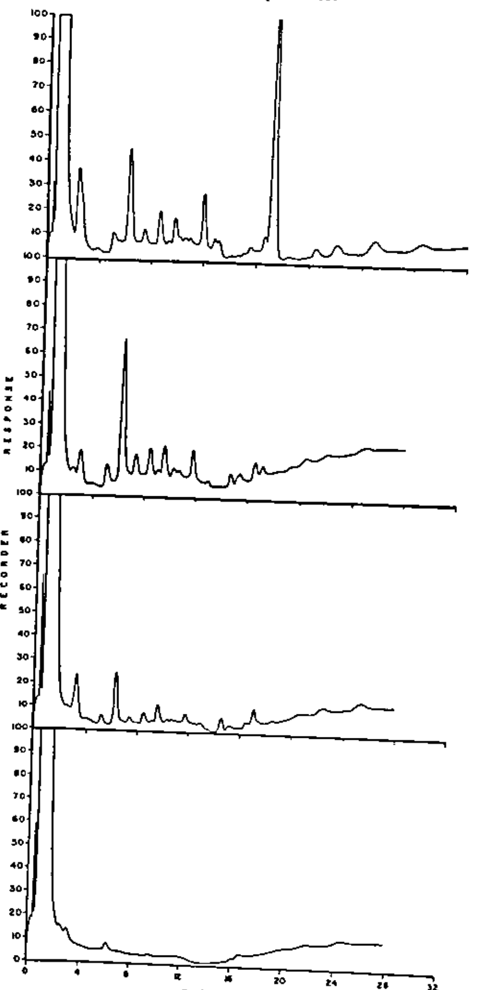

FIG. 3. Gas chromatogram of volatile acidic decomposition products collected during deep fat frying at 0–6 hr (bottom curve) 6–12 hr (lower center curve), 12–30 hr (upper center curve) and 60–90 hr (top curve).

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 wih 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 interreaction 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.

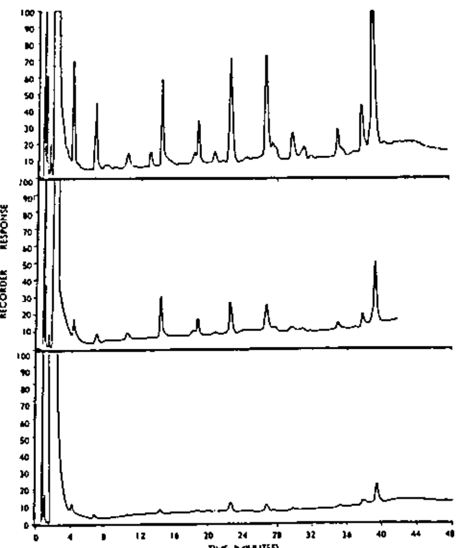

FIG. 4. Gas chromatogram of volatile nonacidic decomposition products collected during frying at 3–6 hr (bottom curve), 6–12 hr (center curve), and 12–30 hr (top curve).

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

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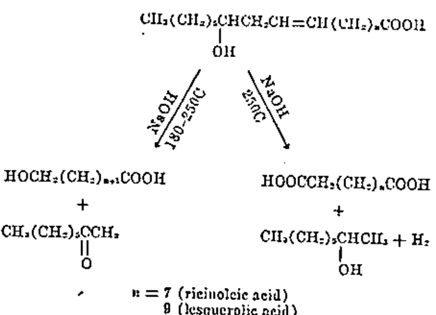
Abstract

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:



In this investigation the effects of reaction media, reaction times, and temperatures on the alkaline cleavage of ricinoleic 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.

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 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	2.3	37	23	40	....
2	0.10	0.85	0.5	0.38	184	6.6	67	19	....	14
3	0.40	3.40	2.0	1.50	183	11.5	11	80	....	9
4	0.10	0.22	13	0	201	2.1	22	20	58	....
5	0.20	1.70	1.5	0.13	192	7.0	53	34	13	....
6	0.10	0.85	0.5	0.82	191	24.6	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	183	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.

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TABLE II

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

[a] 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[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

[a] 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 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 (2X-crystallized, Mann), pepsin (2X-crystallized, Mann), α-chymotrypsin (3X-crystallized, Mann), ovalbumin (crystalline, Nutritional Biochemicals Corp.), and hemoglobin (2X-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^{-3} *M* ascorbate and 10^{-5} *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

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.

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 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.

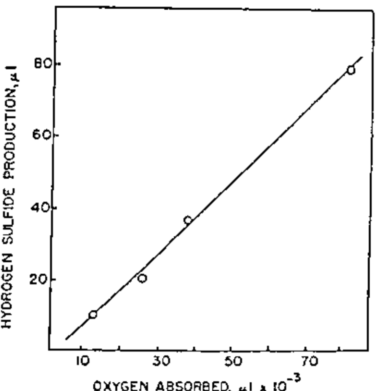


FIG. 1. Formation of hydrogen sulfide as a function of oxygen absorption in a cystein-arachidonate system.

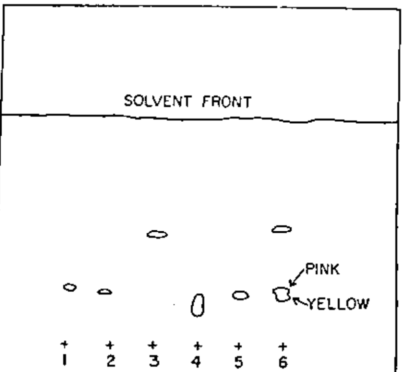


FIG. 2. Thin-layer chromatography of cysteine-arachidonate reaction products.

		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)	

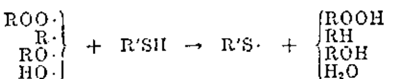
DISCUSSION

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.

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



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

followed by $R'S\cdot + R'S\cdot \rightarrow R'SSR'$. 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 d mercaptooctanoic 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-

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Chronic Toxicity of Methyl Linoleate Hydroperoxide for the Rabbit. (31459)

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Although the toxicity of oxidized fats for animals is well known(1-6), the explanation of their toxic effect is still unclear. There is evidence that the oral toxicity of air-oxidized oils is associated with their peroxide concentration; however, since peroxides apparently are destroyed in the intestine their toxicity presumably is exerted at this site(5,6). Whether fatty acid peroxides are formed in the tissues of animals in general, and of vitamin E deficient animals in particular, is a subject of current controversy. Clearly, if peroxides are formed *in vivo* they must be rapidly decomposed, as no appreciable accumulation occurs and their continued presence would have serious implications for the structural integrity of cell membranes and subcellular particles(7).

The muscular dystrophy which results from a deficiency of vit E may be attributable to peroxidation of lipids in the cellular and subcellular membranes which proceeds in the absence of this vitamin(8). If this explanation is correct, chronic parenteral administration of small amounts of preformed hydroperoxides might be expected to enhance the appearance of the disease by increasing the rate of vit E consumption in the tissues for decomposition of the peroxide or for destruction of additional free radicals whose formation may be catalyzed in its presence. This hypothesis was tested in the present study by determining the effects on the occurrence of vit E deficiency of administering small doses of purified methyl linoleate hydroperoxide to rabbits over protracted periods of time.

Experimental. New Zealand White weanling rabbits weighing 1100-1600 g were housed individually in metal cages and maintained on a vit E deficient diet for 10 days to deplete their tocopherol reserves. The composition of the diet was as follows(%): Labco casein 20.0, glucose (Cerelose) 20.4, starch 40.0, distilled lard 7.0, salts 4164(9) 4.0, cellulose 5.0, cod liver oil 3.0, choline chloride 0.1 and vit premix(9) 0.5. Methyl linoleate hydroperoxide (MLHP), prepared fresh according to a method described previously(10), was suspended in isotonic saline with the aid of 2% Tween 80. An emulsion of pure methyl linoleate was prepared in the same way.

In Experiment I, 2 groups of 6 vitamin E-depleted rabbits each were injected daily over a period of 10-14 days, one group with 1 ml of an emulsion containing 50 mg of MLHP and the other with the same quantity of emulsified methyl linoleate. The dose was administered intravenously through an ear vein. During the course of injections observations were made on the excretion of creatine and creatinine in the urine(11), body weight changes and development of muscular incoordination. At the end, the animals were examined for gross lesions of the internal organs and the livers were preserved in 10% formalin for histopathological examination.

Experiment II was designed to determine the influence of tocopherol or selenium administration on the chronic toxicity of MLHP. Thirty-two animals depleted of vit E reserves as described above were divided into 5 groups. Group 1 was maintained as an untreated control, Group 2 received daily intravenous injections of 50 mg of methyl linoleate in emulsion and the remaining animals were given a similar daily dose of MLHP. Group 5 received, in addition, a daily oral supplement of 100 mg of d-α-tocopheryl acetate prior to the injection, and Group 6 was given a supplement of 1 μg of selenium per g of diet in the form of sodium selenite. All the animals were killed after 10-14 days and histological examinations made made of their livers.

Experiment III was carried out to determine whether the incipient signs of vit E deficiency could be induced in rabbits depleted of their tocopherol stores by continuous intravenous infusion of MLHP. As peroxides are known to be rapidly metabolized *in vivo*, continuous administration of these compounds is probably necessary to maintain a circulating titer. After being maintained on a vit E deficient diet for 12-14 days, weanling animals were shaved in the neck region and fine polyethylene tubing was introduced 2-3 inches into the jugular vein through an 18-gauge heparinized hypodermic needle. The rabbits subsequently were kept in a restraining cage and given free access to food and water. Fresh emulsions of MLHP (peroxide value 4800-5400 meq per kg) were prepared in isotonic saline containing 100 mg% Tween 80 and 50 mg% animal lecithin. The hydroperoxide was dispersed in this medium at a concentration of 2 mg per ml by shaking gently and then bubbling nitrogen through the mass vigorously for 5 minutes. Emulsions thus prepared were stable for the duration of the infusion. As intravenous administration of fat emulsions is known to result in some physiological reactions which may be confused with those observed in vit E deficiency(12), a group of control rabbits was infused with a preparation containing methyl linoleate in place of MLHP. The emulsions were administered by means of a continuous infusion pump at a rate of 0.103 ml (206 μg MLHP) per minute for a period of 26-30 hours. The parameters chosen as criteria of incipient vit E deficiency were red blood cell hemolysis and creatinuria. Samples of blood and urine were taken at the beginning and end of the infusions (and, where possible, during the experiment) for analysis by published methods(11,13).

Results. Experiment I. The rabbits which received daily MLHP injections underwent a sharp increase in the ratio of creatine to creatinine in the urine during a 10-14 day period (Table I) and exhibited either a general loss of muscle tone or gross symptoms of incoordination which were evident in the righting reaction. In the methyl linoleate-treated group, no gross signs of dystrophy were detected and most of the creatine:creatinine ratios remained within the normal range. Two animals in the control group exhibited a mild creatinuria as might be expected of rabbits maintained on the deficient diet for 3 weeks. On the other hand, 5 of the 6 MLHP-injected rabbits experienced pronounced creatinuria as indicated by values in excess of 1.00. The range of ratios for the 12 animals at the start of the injection period was 0.03-0.33. Whereas the linoleate-treated group gained some weight during the experiment, the weights of most of the hydroperoxide-treated animals remained about constant or declined.

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TABLE I. Effect of Intravenous Administration of Methyl Linoleate or MLHP on Urinary Creatine Excretion and Occurrence of Gross Symptoms of Muscular Dystrophy in Rabbits.

Rabbit No.	Methyl linoleate group*: Body wt Initial	Body wt Final	Urinary creatine/creatinine Initial	Urinary creatine/creatinine Final	Righting reaction	MLHP group*: Body wt Initial	Body wt Final	Urinary creatine/creatinine Initial	Urinary creatine/creatinine Final	Righting reaction
1	1100	—	.16	.04	—	1350	—	.10	.65	—
2	1570	1900	.10	.54	—	1550	1440	.03	2.92	+
3	1470	1510	.12	.73	—	1380	1080	.10	1.38	+
4	1095	1190	.33	.20	—	1090	1110	.17	1.73	—
5	1490	1570	.11	.12	—	1400	1380	.09	1.81	+
6	1320	1390	.16	.08	—	1470	1580	.10	1.08	—

* 50 mg per day injected intravenously for 10-14 days. Peroxide value of the MLHP preparation was 5000 meq/kg.

Gross examination of the livers of the MLHP-injected rabbits revealed the presence of white mottled areas on the surface of the lobes. Histopathological examination showed that these areas were marked by diffuse centrolobular fatty changes of varying degree with scattered foci of necrosis and calcification. Giant cell formation and bile duct proliferation also were observed in some livers. These lesions were not observed in any of the animals which received methyl linoleate.

Experiment II. Administration of large amounts of α-tocopherol orally prevented the occurrence of creatinuria and other gross signs of toxicity in rabbits injected with small daily doses of MLHP, but did not entirely prevent the liver lesions (Table II). Selenium had no influence on the incidence of hepatic lesions or on the increased creatine excretion. Interestingly, one case of hepatic lesions was observed in the methyl linoleate group, suggesting that peroxidation of this compound occurred in the vit E-depleted liver of this animal.

Experiment III. Table III shows that the continuous infusion of either methyl linoleate or MLHP for 26-30 hours was associated with an increase in erythrocyte hemolysis and creatine excretion. However, the hemolysis values for the MLHP-treated rabbits were consistently greater than those for the control group, and in 3 animals the creatine:creatinine ratios reached exceptionally high levels. The results suggest that the hydroperoxide induced a degeneration of red blood cell membranes and initiated additional destructive processes affecting other cells. These effects appear to be more extensive than can be attributed to the non-specific effect of infusing lipid emulsions.

Discussion. The rate of MLHP administration used in these studies (about 12 μM per 100 g body weight per day) was well below the LD_{50} intraperitoneal dose of 150-170 μM per 100 g reported for rats(6) and a comparable figure reported for mice(4). The toxicity of linoleate hydroperoxides is much greater by the intraperitoneal route than by

TABLE II. Influence of Vit E or Selenium on Toxic Effects of Intravenously Administered MLHP.

Treatment*	Oral supplement†	No. of rabbits: In group	With liver lesions	With incoordination	Urinary creatine/creatinine: Avg	Range
None	none	3	0	0	.15	.10- .20
Methyl linoleate	"	8	1	2	.14	.08- .30
MLHP	"	6	5	3	.69	.13-1.81
"	vit E	7	3	1	.12	.04- .20
"	Se	8	8	1	.64	.23-1.67

* 50 mg per day injected intravenously for 10-14 days. Peroxide value of the MLHP preparation was 5800 meq/kg.
† 100 mg d-α-tocopheryl acetate per day; 1 ppm Se as sodium selenite.

TABLE III. Effect of Continuous Infusion of Methyl Linoleate or MLHP on Erythrocyte Hemolysis and Urinary Creatine Excretion in Rabbits.

Rabbit No.	Methyl linoleate group: Infusion time, hr	% RBC hemolysis	Urinary creatine/creatinine	MLHP* group: Infusion time, hr	% RBC hemolysis	Urinary creatine/creatinine
1	0	0	.11	0	0	.13
	29	31	.34	30	49	.59
2	0	0	.01	0	0	.32
	30	9	.73	30	100	3.12
3	0	0	.31	0	0	.08
	30	32	.37	30	49	12.90
4	0	0	.004	0	0	.20
	26	21	.38	30	100	3.80

* Peroxide values of the MLHP preparations ranged from 4800 to 5400 meq/kg. Infusion rate was 206 μg/min.

the oral route(3,4,6), and at acute doses seems to be unaffected by tocopherol administration (6). The ameliorating effect of vit E on peroxide-induced creatinuria observed in the present study indicates that if the amounts of hydroperoxide administered are small the vitamin is capable of partially destroying them *in vivo*, probably by reduction to the corresponding hydroxy acid. Considering the large amounts of vit E employed in this experiment, however, and the incomplete protection obtained, it is not surprising that at much higher rates of hydroperoxide administration no protective effect was discernible(6).

The results of these experiments indicate that the chronic administration of small doses of hydroperoxide leads to a more rapid destruction of tocopherol in the tissues and an accelerated appearance of deficiency symptoms (creatinuria, erythrocyte hemolysis, muscular dystrophy). It has been reported by several workers that the concentration of vit E in the tissues of exhaustively depleted animals declined to undetectable levels. Under these conditions the formation of hydroperoxides *in vivo* may be presumed to increase, and there is considerable evidence that the damaging effect of these compounds on subcellular particles, sulfhydryl enzymes and other proteins(14) may account for the biochemical and histological changes observed in vit E deficiency. It has been reported(15) that cerebellar disorders characteristic of tocopherol deficiency can be induced in chicks by intravenous injection of 10 mg of methyl linoleate hydroperoxide.

The fatty degeneration of the liver found in this study does not occur in vit E deficient rabbits and has not been reported in other animals following short-term hydroperoxide administration. The lesion is distinct from the dietary necrotic liver degeneration described by Schwartz(16), as indicated by the histopathology and the failure of selenium to exert a protective effect. Intravenous administration of preformed hydroperoxides may lead to rapid localized liver damage before these compounds are reduced by tissue antioxidants. Holman and Greenberg(1) have observed that a hydroxy acid with a conjugated diene system (the likely reduction product of a linoleate hydroperoxide) is much less toxic for rats than the corresponding hydroperoxide.

Summary. Small quantities of purified methyl linoleate hydroperoxide (MLHP) were administered by daily intravenous injection (50 mg/day) or by continuous intravenous infusion (206 μg/min) to rabbits depleted of their vitamin E stores. After 10-14 days the injected animals exhibited fatty degeneration and necrosis of the liver, creatinuria and an increased incidence of muscular incoordination. The creatinuria was prevented by large oral doses of vitamin E (100 mg/day) but sodium selenite had no effect on the creatinuria or the incidence of liver lesions. Infusion of MLHP for 26-30 hr led to an increased fragility of the red blood cells and a marked creatinuria. These results indicate that chronic administration of small quantities of MLHP leads to a more rapid de-

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struction of vitamin E in the tissues and an accelerated appearance of deficiency symptoms.

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Thyroxine Augmentation of Growth Hormone-Induced Endochondral Osteogenesis.* (31460)

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The actions of thyroxine in enhancing body growth, skeletal and somatic, are among the most sensitive of this hormone's effects(1), but remain incompletely studied and understood. Thyroxine participates both in (*A*) incremental growth, and in (*B*) differentiation.

A, 1. It has long been known that thyroxine augments the action of pituitary growth hormone, in the absence of the pituitary and/or the thyroid glands, as well as in intact animals (2). Whether this augmentation is true synergism is unresolved, but the demonstration by Geschwind and Li(3) that a dose of thyroxine so minute as to have no discernible effect alone could yet increase the sensitivity of the "tibia line" assay for growth hormone, appears strong evidence for synergism.

A, 2. Alone, thyroxine induces a discernible, but slight and unsustained, elongation of bones in hypophysectomized rats, a transient effect which is thus independent of pituitary growth hormone(4).

B. In differentiation, thyroxine can induce the appearance of new epiphyseal ossification centers and subsequently their fusion to the diaphysis through resorption of the epiphyseal cartilage plate. This action occurs in the absence of the pituitary(4) or the thyroid(5,6), or both(7). It is apparently effected through continued erosion of cartilage in the absence of an equivalent rate of chondrogenesis; "chondroclasts" have appeared at the marrow-cartilage junction during thyroxine administration(8).

In most circumstances, the principles underlying these actions are not known; in one circumstance (growth in thyroxine-treated thyroidectomized rats) it has been well-documented that repair of the pituitary and restoration of endogenous growth hormone secretion is a substantial part of the action(9,10, 11). To complicate the situation, distinctions have not always been well drawn between the immediate effects of hormone administration (*i.e.*, observation after a few days or even weeks) and the effects seen following nearly a year of sustained treatment. Nor does it follow that the widening of an epiphyseal cartilage plate (such as is used as an indicator of growth in the assay of growth-promoting substances) is always associated with active bony elongation.†

The present inquiries arose, in part, from the observation that it proved impossible to stimulate skeletal growth to the point of gigantism in thyroidectomized rats by long-continued administration of high doses of growth hormone, although it was possible to achieve this effect in intact or hypophysectomized rats(12). The growth hormone stimulated osteogenesis, as such, as judged by widening and thickening of bones, but not the elongation of bones which depends on chondrogenesis at epiphyseal cartilage plates. Only when traces of thyroxine were added to the treatment was full endochondral osteogenesis resumed. As a possible explanation, it appeared that growth hormone alone might support osteogenesis, but that thyroxine might be necessary for growth hormone to stimulate sustained chondrogenesis; conceivably, the needs for thyroid hormone were so low that they might be met by endogenous activity even in hypophysectomized rats. (Taurog *et al*(13) have shown continued, albeit low-level thyroxine formation in rats 8-9 weeks after hypophysectomy.)

The present study is intended to search for the lower limit of this potential thyroxine requirement for endochondral ossification. It was decided to use, as the basic test situation, the standard tibia line assay procedure, for this test depends upon evocation of chondrogenesis in the epiphyseal cartilage plate. The conditions for performing the test have been defined carefully by Geschwind and Li(3), who also describe the earlier contribution of Marx, Simpson, and Greenspan in the test's development. In brief, female rats are hypophysectomized when 26-28 days of age, and after a 12-day post-operative interval are screened for good condition and presumptive evidence of completeness of the operation. The hormonal material under test is injected daily for a 4-day period; 24 hours after the last injection the animals are autopsied and the tibias dissected free, split, and "stained" with silver nitrate solution to mark calcified regions (von Kossa reaction). The width of the uncalcified portion of the proximal epiphyseal cartilage plate is measured in a compound microscope with a micrometer eyepiece. Typically the width in untreated controls is of the order of 150 μ. If, following treatment, the width does not achieve at least 200 μ, the effect is considered to be in the "non-specific" range(3). With a potent growth hormone a linear dose-response increase is found over a wide range of dosages. Dose is always expressed as total administered during the 4-day test.

Experiment 1. A standard dose of growth hormone was sought to form the base of comparison for all further tests. The preparation of growth hormone employed was highly purified. To give still further assurance of minimal contamination with thyrotropic hormone —TSH—(which might stimulate the thyroid, if present, or even aberrant thyroid follicles such as have been seen in the thymus(15)), the hormone was treated with periodate(16) to inactivate any residual glycoprotein moieties.‡ Table I shows the epiphyseal plate widths encountered in the various experimental groups. At 40 μg total dose this preparation gave a response in the "non-specific" range; 50 μg gave a well-defined response, almost 50% above the control level. The dose chosen for further testing was 60 μg, since this gave an increase which was invariably significantly above the non-specific range.

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† Of several situations which support this remark, one may mention an experiment in which bone length was greater in a thyroxine-growth hormone-treated group than with growth hormone alone, but the epiphyseal cartilage plates were narrower in the doubly-treated group(14).

‡ We acknowledge gratefully a generous supply of such growth hormone from Dr. Lawson L. Rosenberg.

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Long-term Rat Feeding Study with Used Frying Fats

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ABSTRACT A study was made to learn whether fats which had been exposed to the heat and aeration of actual frying differ significantly from fresh fats in their nutritional properties. Partially hydrogenated soybean oils, cottonseed oil, and lard were used for frying under practical restaurant-type frying conditions until they became unfit for further use owing to excessive foaming during frying. The used fats were fed to groups of 50 male and 50 female rats as 15% of the diet for 2 years. The used fats were slightly less absorbable than unheated control fats, and gave correspondingly slower growth rates. Other than this there were no differences in clinical, metabolic, or pathological criteria to suggest that the used fats adversely affected the rats consuming them. Mortality among the heated-fat groups was no higher than among the control groups. Distillable non-urea-adductable fractions concentrated from the used fats proved somewhat toxic when large doses of them were administered by stomach tube to weanling rats. The results indicate that, although heating of fats under actual frying conditions does cause the formation of substances which can be shown to be toxic, the level of such substances and the degree of their toxicity are so low as to have no practical dietary significance.

Fatty materials undergo chemical reactions when they are strongly heated in air. Evidence has been offered that some of the reaction products which form under certain heating conditions are toxic when fed to rats (1, 2). It seemed important to learn whether fats which had been exposed to the heat and aeration of actual frying differ significantly from fresh fats in their nutritional properties.

Of the publications reporting toxicity in heated fats, some (3–5) have dealt with fats which had been heated under extreme conditions of temperature, or aeration, or both. Others (6, 7) report the concentration of small quantities of toxic materials from large volumes of heated fat. And in still others it appears that manifestations ascribed to fat toxicity should have been ascribed to oxidative deterioration of improperly stored mixed diets (8–11). The significance of these results as they relate to human nutrition is not clear.

Work has been reported in which fats that actually had been used for restaurant and commercial frying were evaluated nutritionally by short-term feeding studies and were found to be unimpaired in value (12, 13). The long-term feeding studies which have been reported used fats that had been oxidized to high peroxide value at low temperature (14), or polymerized at high temperature in the absence of air (15), or bubbled with air during heating (16). In each case no ill effects were observed in the animals that consumed these fats, but, since the conditions used for preparing the fats differed considerably from practical frying conditions, these experimental results also are not directly applicable to practical human nutrition.

In a recently published study, Lanteaume et al. (17) fed rats with diets containing 15% of heated fats. The fats were grapeseed oil which had been used 60 times for frying potatoes without replenishment of fat lost by absorption in the potatoes, or grapeseed oil which had been heated 2 days or 4 days in an open beaker at 200°. The feeding study started with rats 7 months of age and lasted for one year. Unheated grapeseed oil was slightly more absorbable than the fat used for frying and gave slightly greater weight gains. The artificially heated fats were slightly less absorbable, and gave slightly lower weight gains. Otherwise no significant differences were observed among the groups according to the several clinical, biochemical, and histological observations made.

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We wished to conduct an experiment whose results, more nearly than any of those cited above, would demonstrate whether fats repeatedly used in actual frying practice have any meaningful effects in normal diets. For this experiment we studied 7 fats. Two of these were unheated controls. Four had been used for frying until they reached the end of their frying usefulness as evidenced by excessive foaming tendency. One was used for frying until it had attained a steady state of foaming tendency. The distillable non-urea-adducting fractions of these fats were isolated and bioassayed, and the fats themselves were fed to rats over a 2-year period.

EXPERIMENTAL

Fats. The fresh and used fats studied in this experiment are described in table 1. The table also shows the code letter assigned to each fat, which will be used to identify it in the other tables. All the fats had been prepared by normal factory or pilot plant processes. The cottonseed oil and soybean oils had been refined, bleached, and deodorized. Analyses of the fats are reported in table 2.

Frying procedure. The used fats were prepared by heating them in 60-kg (fat capacity) gas-fired kettles. The fats were heated at 182° each weekday from 8 AM to 4 PM. At 9 AM and at 2 PM in each kettle 3.95 kg each of frozen potatoes, breaded scallops, and onion rings, in that order, were fried for 5 minutes, 2.5 minutes, and 3 minutes, respectively. These foods were obtained through normal commercial food channels. At 4 PM daily 2.47 kg of fat were added to each kettle to replace the fat that had been absorbed by the fried foods. The kettles were allowed to cool overnight and on weekends. During the frying of the onion rings, the height of foam was measured above the previously measured level of still fat in the kettle. When the foam height exceeded 75 mm that fat was removed from the kettles and protected against oxidative changes which might have occurred during storage by the addition of 39 ppm butylated hydroxytoluene plus 31 ppm butylated hydroxyanisole. The fats were sealed in 453-g cans under nitrogen before storage in a frozen-food warehouse. Fat D (soybean oil, IV 108, with silicone) never attained the 75-mm foam height end point owing to the stabilizing effect of the silicone. Frying in that fat was discontinued after it became apparent that a steady state of foam height had been reached due to fat turnover.

Total duration of the heating ranged from just over a week to over 5 weeks. Table 2 shows the number of hours each of the fats was heated and the analytical values after heating. Samples of used fats were withdrawn from storage and analyzed at intervals up to 2 years; the zero- and 2-year analyses are also shown in the table.

Feeding study. The fats were incorporated at a level of 15% into the semipurified diet shown in table 3. The diets were prepared fresh each week and kept refrigerated until dispensed into the feeding cups. Feeding was carried out 3 times per week, and any feed remaining in the cups was discarded, so that the longest period that any of the feed was unrefrigerated after mixing was 3 days. Previous experiments (unpublished) had shown that the results obtained with such a system of diet handling were indistinguishable from results obtained with daily mixing of diets, whereas weekly feeding had led to relatively poor growth with diets containing used fats.

Four hundred each of male and female rats[3] were received as weanlings. After 3 days the extremely light and heavy animals were discarded. The remainder were sorted into 7 groups of 50 males and 50 females, balanced on the basis of weight and litter. The rats were kept individually

TABLE 1

Fats studied

Code	Description
A	Soybean oil, fresh (not used for frying)
B	Soybean oil, hydrogenated to IV 108, fresh
C	Soybean oil, hydrogenated to IV 108, used for frying
D	Soybean oil, hydrogenated to IV 108, with 1.6 ppm methyl silicone added, used for frying
E	Soybean oil, hydrogenated to IV 70, used for frying
F	Cottonseed oil, used for frying
G	Lard, used for frying

[3] Simonsen Standard Laboratory, Sprague-Dawley-originated, Caesarean-derived rats from Simonsen Laboratories, White Bear Lake, Minnesota 55110.

TABLE 2

Analyses of fats

Fat	Frying time	Storage time	Lovibond color [1]	Free fatty acid [1]	Iodine value [1]	Peroxide value	n_D^{60}	Polar fraction [2]	Conjugated diene [3]	Conjugated triene [3]	Fatty acid composition by gas-liquid chromatography						
											14:0	16:0	16:1	18:0	18:1	18:2	18:3
	hours	years		%				%	%	%	%	%	%	%	%	%	%
A	0	0	10/1.0	0.04	129.0	1.6			50.6	7.3	0	11	0	5	27	51	6
	0	2	12/1.2	0.03	130.0	0.5	1.4603	1.4	51.2	7.7	0	11	0	4	26.5	52	6
B	0	0	3/0.3	0.02	108.2	1.2	1.4578		33.5	3.0	tr	11	0	5	45	35	4
	0	2	4/0.4	0.02	108.5	0.1	1.4577	1.3	33.2	3.1	0	11.5	0	6	46	34.5	2
C	0	0	3/0.3	0.02	108.2	1.2	1.4578		33.5	3.0	0	12	0	6	50	31	1
	60	0	300/80	0.65	100.5	3.8	1.4597	13.6	28.1	2.3	0	13	0	6.5	50	30	0.3
	60	2	300/80	0.76	101.0	3.5	1.4597	14.6	28.1	2.3							
D	0	0	3/0.3	0.02	108.5		1.4578				tr	11	0	5	46	35	3
	216	0	3500/430	8.10	101.5	0	1.4589	30.2	33.6	3.0	0	11	0	6	50	32	1
	216	2	1800/400	8.20	101.5	0.7	1.4588	32.5	28.5	2.7	0	12	0	6.5	49.5	30.5	1.5
E	0	0	3/0.2	0.03	69.7	0	1.4533		5.1	0.5	0	15	0	12	66	7	0
	84	0	300/60	1.30	63.5	5.5	1.4552	20.1	3.2	0.08	tr	16	0	14	65	5	tr
	84	2	300/80	1.30	66.4	1.9	1.4551	19.0	2.6	0.1	0	16	0	14.5	65.5	4.0	0
F	0	0	8/0.8	0.03	109.0	0.6	1.4581		53.8	0.44	1	27	0	2	17	53	0
	49	0	200/50	0.45	102.5	4.6	1.4601	14.2	44.9	0.33	1	31	0	2	17	48	0
	49	2	200/60	0.48	102.0	4.4	1.4598	15.2	46.5	0.6	1	30	0	2.5	17	49.5	0
G	0	0	1/0.1	0.05	64.4	0	1.4527		13.1	0.98	1	24	0	13	46	10	3
	116	0	600/110	2.30	55.6	3.0	1.4550	23.4	7.5	0.42	1	30	0	15	46	6	0
	116	2	500/140	2.40	55.7	1.3	1.4550	25.5	7.5	0.40	2	31	1.5	15	44.5	5.5	0

[1] AOCS method (24)
[2] Measured by adsorption chromatography on silicic acid containing 4% methanol; eluted with methanol:benzene 1:49. Method of E. N. Frankel (unpublished).
[3] AOCS method (24) involving alkali isomerization and UV absorption, as linoleate and linolenate.

TABLE 3
Composition of diet

	%
Casein [1]	5.0
Non-fat dry milk [2]	21.0
Ground whole wheat	43.0
Dried egg white [3]	3.0
Dried liver, defatted [4]	3.0
Vitamin mixture in sucrose [5]	3.0
L-Lysine·HCl	0.5
Calcium phosphate, dibasic [6]	1.0
Salt mixture [7]	3.5
Vitamin mixture in soybean oil [8]	2.0
Experimental fat	15.0
Kilocalories/g	4.4

[1] Labco, Vitamin-Free, Whitson Products Division of the Borden Company, New York.
[2] Land O'Lakes Creameries, Minneapolis.
[3] Nutritional Biochemicals Corporation, Cleveland; biotin was added at a level of 1 mg/100 g of albumen.
[4] Wilson Laboratories, Inc., Chicago.
[5] Furnished the following in mg/kg of diet: menadione, 1.8; thiamine, 2.4; riboflavin, 3.0; niacin, 12.0; folic acid, 0.15; Ca pantothenate, 12.0; pyridoxine, 2.4; inositol, 12,000; p-aminobenzoic acid, 60.0; biotin, 0.015; cyanocobalamin, 90.0; ascorbic acid, 60.0; and choline chloride, 1800.
[6] Added to maintain calculated Ca and P at 2% and Ca:P ratio at 1.1:1.0.
[7] Phillips and Hart (25) but with the addition of 0.005% cobalt chloride; obtained from Nutritional Biochemicals Corporation, Cleveland.
[8] Soybean oil furnished essential fatty acids, and the mixture furnished the following IU per kg of diet: 12,000 vitamin A; 3,200 vitamin D_2; and 100 vitamin E as d-α-tocopheryl acetate.

in galvanized steel cages, 26 × 30 × 17 cm, at 23 ± 1° and 50 ± 5% relative humidity. They received 12 hours of light per day uniformly. The individuals within the groups were so distributed as to avoid bias caused by position within the racks or the room. Feed and water were given ad libitum. Feed consumption records were maintained for each animal. Each week the animals were weighed and carefully examined for abnormalities. As they aged, sick ones were separated from the rest, and their values were removed from the subsequent growth and feed data, but were included in the longevity and pathology data.

Metabolic studies. For the determination of fat absorbability, and coefficient of absorbability, feces were collected from 10 randomly selected rats per sex per dietary group for two weeks at 2, 12, and 21 months, by means of wire-screen collectors fastened below the regular cages. The feces were dried in vacuo at 80°, cleaned, and pulverized. Total fatty acids were determined by saponification with alcoholic KOH, acidulation, dilution with water, extraction with petroleum ether, drying and evaporating the extract, and weighing the dried residue. Fecal nitrogen was determined by the Kjeldahl method. For the calculations, endogenous fat was assumed to be 0.03 g/day for males and 0.02 g/day for females; these values were based on our experience with similar animals fed purified rations.

Urine was collected from 10 randomly selected animals per sex per group at 2, 12, and 21 months. The rats were given water but not feed during the 12-hour collection period, which followed a 12-hour fast. Rats were kept in metabolism cages for the collection. The urine was collected under toluene; each sample was diluted to 25 ml and analyzed for nitrogen (Kjeldahl), and for sugar, albumin, bilirubin, and ketone bodies by means of test tablets and strips.[4]

Clinical studies and pathology. From each group and sex there were chosen at random 5 rats at 12 months and 10 rats at 24 months. Blood samples were obtained from these animals by caudal artery puncture for blood cell counts, hematocrit, hemoglobin, and blood glucose analyses. The same animals were anesthetized with ethyl ether, and 5-ml samples of blood were drawn from the heart for plasma cholesterol (18) and plasma phospholipid determinations (19). The animals were then put to death by excess of the ether; the heart, liver, and kidneys were removed and weighed for the calculation of organ-to-body weight ratios. The heart, liver, and kidneys were assayed for sodium, potassium, and calcium by flame photometry at 12 months and by atomic absorption spectroscopy at 24 months. Liver cholesterol and phospholipid values were determined. Peritoneal fats were converted to methyl esters, and their fatty acid compositions were determined by gas-liquid chromatography. Thymus, heart, lung, liver, stomach, pancreas, spleen, adrenal, kidney, mesenteric lymph nodes, ileum, gonads, and any apparent neoplasms were removed for histological examination. Slides were prepared and studied within our laboratories, and were also examined by a consulting veterinary pathologist. Animals that died or were killed because moribund were autopsied, and if putrescence had not started the same tissues were removed for similar histology.

[4] Ames Company, Inc., Elkhart, Indiana.

Statistical treatment. Where applicable the analysis of variance was used to test for significance. The final growth and feed consumption data were tested by the use of harmonic means because of the extreme disproportionalities produced in the data by intermediate deaths (20). Minimum significant differences were computed by the method of Tukey as described by Scheffe (21).

Isolation and bioassay of distillable non-urea-adducting fractions (DNUA). The DNUA's were isolated by a modification of the method of Crampton (22). A 1-kg portion of each fat was saponified with aqueous-alcoholic KOH. The soaps were acidulated, and the free fatty acids were extracted. The acids were converted to ethyl esters by twice refluxing for 30 minutes with 2 volumes of ethanol containing 0.5% H_2SO_4. The esters were distilled under high vacuum at pot temperatures up to 240°. Each distillate was mixed with 4 times its weight of powdered urea and 4 times its volume of ethanol. The slurry was held at 55° for 30 minutes before cooling overnight to room temperature. After filtration the filter cake was washed with urea-saturated alcohol. The combined alcohol solutions were mixed with water and extracted with hexane, then ether. The extracts were concentrated, dried, and evaporated under reduced pressure, leaving behind the DNUA. Yields of DNUA are shown in table 4.

For bioassay 0.5 ml of a DNUA was administered daily for 3 consecutive days to each of 5 weanling male rats (7). Administration was by stomach tube. The rats were given a commercial pelleted ration and water ad libitum during the experiment. Survivors were killed on the tenth day, at which time body and thymus weights were determined. As controls, similar groups of rats were not given any DNUA, or else were given the DNUA from olive oil which had been heated 50 hours at 182°, the latter having previously been shown in our laboratory to be toxic under these conditions. Results of the bioassay are shown in table 4.

RESULTS

Fats. Analytical values on the fresh fats (table 2) showed them to be typical of their respective kinds. The analytically measurable changes which took place in the fats during frying were generally similar to the changes observed by many other

TABLE 4
Acute toxicity test

Sample characteristics [1]	Level of DNUA in fat	Avg body wt gain [2]		Thymus wt [3]	Diarrhea incidence	Mortality
		3 days	10 days			
	% by volume	g	g	mg	%	%
DNUA of Fat A	2.0	17.2	69.4	425	0	0
DNUA of Fat B	1.5	19.6	71.2	446	0	0
DNUA of Fat C	2.1	0.3	54.7	359 (81)	0	40
DNUA of Fat D	2.0	9.2	59.6	391	0	0
DNUA of Fat E	2.0	−10.4	24.0	167 (32)	100	20
DNUA of Fat F	2.3	7.8	55.6	328	0	0
DNUA of Fat G	1.7	9.8	57.3	409 (88)	0	20
Positive control [4]	2.4	−10.0	39.3	142 (84)	40	40
Negative control, pellets only	—	21.6	66.6	445	0	0

[1] There were 5 weanling male rats/group, and each rat was given 0.5 ml of lipid/day for 3 consecutive days. DNUA = distillable non-urea-adductable fraction.
[2] These results are for surviving animals only.
[3] Weights shown are averages for animals living at 10 days. Values in parentheses are for dead rats; normal rats of the same age as the dead ones have thymus weights averaging 210 mg.
[4] DNUA of olive oil heated 50 hours at 182°

TABLE 5

Cumulative growth, feed consumption, and feed efficiency [1]

Fat	Avg wt gain 2 months	Avg wt gain 12 months	Avg wt gain 21 months	Avg feed consumption 2 months	Avg feed consumption 12 months	Avg feed consumption 21 months	Feed efficiency [2] 2 months	Feed efficiency [2] 12 months	Feed efficiency [2] 21 months	Coefficient of absorbability [3] 2 months	Coefficient of absorbability [3] 12 months	Coefficient of absorbability [3] 21 months
	g	g	g	g	g	g	%	%	%	%	%	%
						Male						
A	365 ^{a}	727 ^{a}	783 ^{a}	974 ^{a}	6619 ^{ab}	11870 ^{a}	37.4 ^{a}	11.0 ^{a}	6.6 ^{a}	96.2 ^{a}	96.5 ^{a}	97.0 ^{a}
B	358 ^{ab}	725 ^{ab}	763 ^{a}	963 ^{a}	6511 ^{a}	11747 ^{ab}	37.2 ^{a}	11.1 ^{a}	6.5 ^{a}	95.5 ^{a}	96.8 ^{a}	96.6 ^{a}
C	339 ^{bc}	677 ^{b}	764 ^{a}	951 ^{a}	6435 ^{a}	11674 ^{ab}	35.7 ^{b}	10.5 ^{abc}	6.5 ^{a}	90.3 ^{b}	92.4 ^{b}	94.2 ^{b}
D	344 ^{abc}	704 ^{ab}	774 ^{a}	965 ^{a}	6604 ^{ab}	11848 ^{ab}	35.6 ^{b}	10.6 ^{ab}	6.5 ^{a}	91.5 ^{b}	92.7 ^{b}	92.2 ^{b}
E	340 ^{bc}	703 ^{ab}	747 ^{a}	1001 ^{a}	6929 ^{b}	12292 ^{ab}	34.0 ^{b}	10.1 ^{bc}	6.0 ^{b}	82.2 ^{c}	88.2 ^{c}	86.9 ^{c}
F	339 ^{bc}	682 ^{ab}	727 ^{a}	962 ^{a}	6506 ^{a}	11861 ^{ab}	35.3 ^{b}	10.5 ^{abc}	6.1 ^{ab}	87.9 ^{d}	90.0 ^{d}	89.8 ^{d}
G	335 ^{c}	679 ^{ab}	737 ^{a}	976 ^{a}	6855 ^{b}	12380 ^{b}	34.7 ^{b}	9.9 ^{c}	6.0 ^{b}	87.8 ^{d}	89.4 ^{cd}	89.3 ^{d}
						Female						
A	200 ^{de}	405 ^{c}	525 ^{b}	732 ^{b}	4897 ^{c}	9409 ^{c}	27.5 ^{d}	8.2 ^{a}	5.6 ^{a}	96.8 ^{e}	97.5 ^{e}	97.6 ^{e}
B	204 ^{d}	392 ^{c}	518 ^{b}	726 ^{b}	4825 ^{c}	9238 ^{c}	28.0 ^{d}	8.1 ^{ab}	5.6 ^{a}	96.4 ^{e}	96.8 ^{e}	96.4 ^{e}
C	196 ^{de}	375 ^{c}	466 ^{b}	731 ^{b}	4901 ^{c}	9164 ^{c}	26.9 ^{de}	7.6 ^{bc}	5.0 ^{ab}	92.1 ^{fg}	92.7 ^{fg}	92.5 ^{fg}
D	201 ^{de}	377 ^{c}	463 ^{b}	744 ^{b}	4974 ^{c}	9323 ^{c}	27.1 ^{d}	7.6 ^{bc}	4.9 ^{ab}	92.7 ^{f}	94.0 ^{f}	93.6 ^{f}
E	189 ^{e}	362 ^{c}	446 ^{b}	746 ^{b}	5018 ^{c}	9262 ^{c}	25.5 ^{f}	7.1 ^{c}	4.8 ^{b}	87.6 ^{h}	89.6 ^{h}	89.6 ^{h}
F	196 ^{de}	374 ^{c}	486 ^{b}	741 ^{b}	4913 ^{c}	9145 ^{c}	26.4 ^{ef}	7.6 ^{bc}	5.3 ^{ab}	91.1 ^{gi}	91.8 ^{g}	92.0 ^{g}
G	196 ^{de}	372 ^{c}	454 ^{b}	749 ^{b}	5131 ^{c}	9441 ^{c}	26.1 ^{ef}	7.2 ^{c}	4.8 ^{b}	89.8 ^{i}	91.9 ^{g}	90.3 ^{h}

[1] Any 2 values within a column which are followed by the same letter are not significantly different ($P \geqq 0.05$) from each other.
[2] Feed efficiency = weight gain × 100/feed consumption.
[3] For experimental fat level only. Allowance was made for fat-soluble vitamin mixture and metabolic fat.

workers in the past. There were small but irregular increases in the free fatty acid levels, peroxide values, and refractive indices, and small decreases in the iodine values. There were small decreases in the relative levels of polyunsaturated fatty acids, presumably as a result of oxidation and polymerization. The content of polar material in each fat increased markedly during frying. The ultraviolet, gas chromatographic, and urea adduction results show that the majority of the fatty acid chains were unaffected by the heating procedures. The properties of the fats did not change appreciably during storage for 2 years, showing that the storage conditions were adequate for preservation of the fats in their originally prepared states.

Two-year feeding study. Table 5 shows mean growth and feed consumption data for 2, 12, and 21 months. Values for intermediate times were generally similar to the ones shown, and are omitted for brevity. The 24-month growth data are omitted since deaths among the senescent animals caused extreme variations within the groups. During the early, rapid growth stage the two fresh fats produced generally more rapid growth than the others, although Fat D (soybean oil, IV 108, with silicone) was nearly equivalent. The growth differences were more pronounced among the male than the female rats. The differences in body weights which arose during the early growth period tended to persist throughout the experiment, although they became relatively smaller with time, and statistically insignificant with increasing variance among the individuals. Part of the reason for the diminution of weight differences with time was that the heavier groups, namely those receiving Fats A (fresh soybean oil), B (fresh soybean oil, IV 108), and D (used soybean oil, IV 108, with silicone), had slightly higher mortality rates than the others, and these deaths occurred mostly among the more obese individuals. Deaths of the more obese animals in the heavier groups lowered the mean weights of those groups.

The differences in growth could not be attributed to differences in feed consumption. Instead, rapid growth appears to have been associated with high feed efficiency, and is accounted for by the values given for coefficient of absorbability (table 5). Two factors appear to have influenced absorbability: heating, and degree of unsaturation. The unheated fats were most absorbable, and also, on account of the experimental design, the most unsaturated. Among the heated fats, the 2 samples of lightly hydrogenated soybean oil (C and D) were more absorbable than the lard or cottonseed oil, while Fat E, the soybean oil which had been hydrogenated to IV 70 before frying, showed the lowest values for coefficient of absorbability. This same relationship of absorbabilities prevailed in both sexes at all ages. The lower absorbability of the heated fats is probably due to their content of unabsorbable (22) polymeric materials, formed during heating.

Detailed results of the many clinical analyses carried out — fecal nitrogen, urine volume, pH, nitrogen, and protein, blood counts, hemoglobin, hematocrit, blood glucose, plasma cholesterol and phospholipid, organ-to-body weight ratios, liver lipid, phospholipid and cholesterol, liver and kidney calcium, sodium, and potassium — are not shown in the tables. The values were generally within normal ranges and were not remarkable. Many of the values show differences between the sexes and at different ages, but there were few statistically significant ($P = 0.05$) differences assignable to dietary groups within the age-sex groupings. All of the statistically significant differences which were seen are summarized in table 6. Most of these differences appear to be of a random nature, showing no pattern which can be correlated with fresh vs. used fats. An exception to this is the liver cholesterol-to-phospholipid ratio at 24 months; the relatively high values observed in the male groups receiving unheated fats might be related to the relatively high incidence of obesity in those groups. The biological significance of differences in mineral content of the various organs is questionable, since these values showed wide variations, both within and among the various groups. Analyses of peritoneal fats showed their fatty acid compositions to be generally parallel to the fatty acid compositions of the respective dietary fats.

Longevity and pathology. Table 7 shows the percentage of animals surviving at

TABLE 6

Statistically significant differences in metabolic and clinical values

Analysis	Sex	Age	Direction of difference
		months	
Fecal nitrogen	F	21	B > A, D, G
Urine volume	F	12	E > A, C, D, F
Heart wt/body wt	M	12	C > E
Liver wt/body wt	F	12	E > A, B, D
Liver wt/body wt	F	24	F > A
Liver cholesterol	M	24	A > B, C, D, E, F, G
Liver cholesterol/phospholipid	M	24	A, B > C, D, E, F, G
Kidney calcium	F	12	F > D
Liver potassium	F	24	A, B, C > D, E, F, G
Kidney potassium	F	12	G > C
Kidney potassium	F	24	A, C > E
Kidney sodium	F	24	A > B, D, F, G

TABLE 7

Percentage of animals surviving at various ages

Diet group	Males, age in months				Females, age in months				Total, both sexes
	12	18	21	24	12	18	21	24	
A	96	84	80	62	92	80	60	33	48
B	98	87	73	53	100	91	80	58	56
C	96	96	87	67	98	91	80	60	63
D	88	73	66	53	98	89	80	58	55
E	100	87	82	69	96	89	80	67	68
F	100	96	82	64	100	93	87	56	60
G	98	93	91	62	98	96	93	84	73
Avg	96.5	88	80	61	97	90	80	59	60

several ages. Sixty percent of the animals survived for 2 years. There was only a slight difference between the sexes in most groups. Survival rates were slightly lower for animals receiving the two unheated fats and Fat D (soybean oil, IV 108, with silicone, used) than for animals receiving the other heated fats. This difference parallels and is attributed to the larger mean weights, the higher incidence of obesity, and the better utilization of fat in those same groups. The table shows what appears to have been a high incidence of early mortality in the males receiving Fat D (soybean oil, IV 108, with silicone, used). The value shown resulted from 6 deaths within the first year; two were from unknown causes, one from a tumor of the prostate, two from pneumonia, one from a bladder infection. The pneumonia and bladder infection are not uncommon among such animals as these in our laboratory, and we see no reason to suppose that the distribution of mortality was not random.

Table 8 shows the incidence of tumors, respiratory disease, nephritis, and liver pathology observed grossly in all rats. The females developed more tumors than the males, owing to their susceptibility to mammary carcinoma. The higher incidence of tumors in the females contributed to their having mortality rates equal to the males. No diet-related differences in tumor incidence appeared in the males, but the females receiving the two fresh fats had a higher incidence of tumors than those receiving used fats. It has been reported (23) that a direct relationship exists between caloric intake and tumor risk in the rat, and, as pointed out above, the two fresh fats furnished more absorbable energy than the used fats.

The histological findings on the 10 rats killed from each group are summarized in table 9. In both sexes the incidence of

TABLE 8

Percentage incidence of various pathological conditions [1] (all animals, all ages)

Fat	A		B		C		D		E		F		G	
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Tumors [2]	20.0	55.6	22.2	53.3	17.8	37.8	22.2	28.9	22.2	33.3	26.7	28.9	13.3	31.1
Respiratory disease [3]	22.2	11.1	15.6	6.7	22.2	13.3	20.0	11.1	11.1	8.9	2.2	6.7	11.1	6.7
Nephritis	30.0	8.0	24.0	14.0	20.0	12.0	22.0	6.0	30.0	12.0	18.0	12.0	30.0	18.0
Liver pathology	24.0	34.0	48.0	38.0	6.0	28.0	18.0	24.0	28.0	30.0	12.0	26.0	22.0	30.0

[1] Grossly observable tumors and disease symptoms only.
[2] Male tumor incidence, 20.6%; female tumor incidence, 38.4%.
[3] Severe respiratory disease with clinical symptoms; male respiratory disease incidence, 14.9%; female respiratory disease incidence, 9.2%.

fatty infiltration of the liver was relatively high in animals receiving the unheated fats and quite low in animals receiving Fat E (soybean oil, IV 70, used) or Fat G (lard, used). Thus it appears that this condition also may be related to the intake of absorbable energy, but not specifically to the ingestion of fresh or used fats. None of the other frequently seen pathology appears to have been related to dietary differences. Various other pathological conditions were observed only infrequently, and could not be related to dietary differences.

Acute toxicity test. Crampton et al. (22) showed that the toxic substances formed by heating linolenate under carbon dioxide could be concentrated into the DNUA fraction. The distillable urea-adducting fraction contained esters of unchanged fatty acids, while the non-distillable fraction contained polymers which, although not nutritious, were non-toxic by reason of their non-absorbability. For assaying the presence of toxic substances in the DNUA fractions of heated fats, we have used a method (7) which involves forced feeding of weanling rats with large quantities (ca. 30 g/kg) of the DNUA over a 48-hour period. Our experiences with this procedure show that its results parallel those observed by Crampton, but it is quicker and uses much less material. Table 4 shows that, as expected, DNUA fractions from the fresh fats did not harm the rats receiving them, while those from the heated fats were all harmful in one or more respects, causing diminished weight gains, premature involution of the thymus, diarrhea, or death. The number of animals used was too small to permit quantitative comparisons among the various used fats; it is clear that all of them contained low levels of substances toxic to the weanling rat.

DISCUSSION

The aim of this experiment was to feed high levels of fats which had been heated under conditions as severe as any ever likely to be encountered as constituents of fried foods in the normal American diet. Therefore we chose what we believed to be the worst possible conditions for preparing the fats, short of conditions which

TABLE 9

Percentage incidence of pathology in two-year-old rats (histological examination of ten rats/group, per sex)

Fat	A		B		C		D		E		F		G	
Sex	M	F	M	F	M[1]	F	M	F	M	F	M[1]	F	M	F
Fatty liver	40	50	30	20	22	10	20	20	0	—	11	10	10	—
Chronic pyelonephritis	50	50	80	50	77	20	80	30	60	10	55	50	40	50
Tubule mineralization in kidney	—	40	—	20	—	40	—	60	—	50	—	30	—	30
Adrenal telangiectasis	—	30	30	70	22	70	—	30	10	20	—	40	20	40
Alveolar foam cells	20	10	20	—	—	—	20	10	20	30	66	20	10	20
Tumors: Mammary	—	60	—	70	—	40	—	20	—	30	—	30	—	20
Others	20	—	10	20	33	0	20	20	10	—	22	—	30	10

[1] Only 9 slides/group were examined by the consulting pathologist.

would have been wholly unrealistic in relation to culinary practice.

Of the ways in which fats are used in cooking, three involve relatively severe heat treatment. We may distinguish these as 1) pan frying, 2) continuous frying, and 3) intermittent deep-fat frying. In pan-frying or sautéing, fats are heated strongly, but only for short periods of time. Such fats are not ordinarily saved and re-used, and hence there is little opportunity for any transformation products which might form to build up to substantial levels.

In many commercial frying operations, such as doughnut or potato chip manufacturing, where frying is continuous, fat is constantly absorbed by the food and carried out, to be replaced by fresh fat. The turnover time is short enough so that there is little opportunity for significant changes in fat composition to occur; the levels of color, flavor, foaming tendency, and free fatty acids are not objectionable, so it is seldom or never necessary to discard the fat and replace it with new.[5] It seems clear that if one is to look for nutritionally undesirable changes in used frying fat, they should be sought not here, but rather in the low fat-turnover operations of small volume, batch-frying establishments.

Some restaurants frequently keep their fat hot for many hours a day, fry in it occasionally, allow it to cool overnight, and reheat it next day. While this pattern of use continues, the polymer content and viscosity of the fat increase. Eventually it either foams over the sides of the kettle or becomes unacceptably dark in color; then it is discarded and replaced. (In many restaurants, of course, the frying volume is great enough so that there is a rapid turnover of fat, and here the situation is not unlike that which exists in the continuous frying operations discussed above.) In our opinion, those fats used for frying until they threaten to foam out of the kettle are the most severely heated fats likely to be consumed in the normal human diet, and it was such fats that we chose for our feeding study. Along with them we fed a fat (D) whose frying life had been greatly extended through the use of silicone, and which had been used for frying until its content of transformation products (as indicated by foam height) appeared to have reached a steady state through fat turnover, even though the quantity of food being fried in it was relatively small.

Most previous workers have studied fats which had been heated at higher temperatures or with more vigorous aeration than would be found in actual frying practice. Attempts to apply conclusions based on such work to human nutrition seem unwarranted, owing to the likelihood that such artificially abused fats may differ from practically used fats in the kind, as well as the levels, of transformation products which they contain.

Some factor of exaggeration is necessary and appropriate in feeding studies for detecting mild toxicity or low levels of toxic substances. Such exaggeration was achieved in the acute bioassay stage of our work by concentrating the altered fatty acids from used fats through urea adduction. The DNUA fractions of the used fats elicited manifestations of acute toxicity, and even caused death in some cases, when 1.5-ml doses of them were given to weanling rats over a 48-hour period. It is clear from this that toxic substances did form in our fats during the heating program, although at quite low levels. To administer in whole fat the same quantity of these toxic substances as was administered in the DNUA concentrate would have required dosing each rat with more than his own body weight of used fat during a 48-hour period.

Chronic toxicity of the DNUA's and of other used fat components were evaluated in the long-term stage of our experiment. In the 2-year study, a considerable degree of exaggeration compared with human diets was maintained (a) by using the fats up to the practical end-point of their usefulness for frying, rather than to some intermediate point corresponding to the average of the fats being used in actual food preparation, (b) by feeding them at the high level of 15% of the diet, and (c) by feeding them steadily throughout the lifetime of the animals. Even with these exaggerations, the experiment produced no evidence that used frying fats adversely affected the health or longevity of the animals consuming them.

The literature reports that artifically abused fats contained substances which are toxic to laboratory animals. From such reports it has been inferred that it may be harmful for humans to consume used frying fats. We have found that actual used frying fats contain only very small quantities of substances which are toxic when administered in large doses to weanling rats, and that the fats themselves produce no appreciable ill effects on animals consuming them.

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Protein Quality of a Soybean Protein Textured Food in Experimental Animals and Children [1,2]

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ABSTRACT Studies were carried out in experimental animals and children to evaluate the protein quality of a textured food simulating ground beef, and made from isolated soybean protein with added egg albumin and wheat gluten. On the basis of a PER value of 2.50 for casein, the PER of the textured food and of natural dehydrated beef was 2.30 and 2.34, respectively. Highest weight gain was obtained with diets containing 16.7 and 16.3% protein, from the soybean protein textured food and casein, respectively. NPU values were 62.6 for casein and 59.1% for the soybean protein textured food. Heating of the soybean protein textured food increased weight gain but not the PER. Heating of the protein isolate and of the fiber made from it improved both. Apparently this treatment caused the elimination of adverse physiological factors inherent in soybean, or the removal of substances in the product derived from the preparation process. At the 10% protein level, supplementation with lysine and methionine added together, but not alone, improved protein quality. Growth and nitrogen balance studies with dogs indicated that the soybean protein textured food had essentially the same protein quality as that of dehydrated beef. True protein digestibility and biological value were 92.3 and 65.3%, respectively, for the soybean protein textured food, and 87.0 and 67.4% for the dehydrated beef. The results in children show that, at a protein intake level of 2 g/kg/day, no difference in quality was evident between skim milk and the soybean protein textured food. Nitrogen equilibrium was obtained when the children received approximately 138 mg of nitrogen from the soybean protein textured food, as compared with 97 mg from milk. The true protein digestibility and biological value was 92.3 and 65.3%, respectively. It was concluded that the protein quality of the soybean protein textured food was about 80% of that from milk. It was readily accepted by the children and free of adverse physiological effects.

Highly purified proteins are being isolated now from oil-free, food-grade protein concentrates such as soybean, cottonseed and sesame. Among these, protein isolates prepared from oil-free soybean flakes have received the greatest attention. The protein content of the isolate runs as high as 95%, and the products are bland in taste and have none of the flavors normally associated with the flours and other similar products (1–3).

The protein isolates are available in monofilament, granular or powder forms, which make them suitable for a wide range of functional uses, such as whipping, emulsifying, gelling, stabilizing, thickening and moisture-binding. Thus, the number of food products which can be made from them is practically unlimited.[3,4]

The essential amino acid pattern present in such isolates is, in the majority of cases, essentially the same as that in the material from which it was prepared. However, the process of isolation, eliminating certain protein fractions, as well as the use of variable temperatures, treatment with chemicals and pH changes, may alter the nutritive value of such products. Information on the nutritive value of the protein of these isolates is not very extensive, and

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Purification of Cyclic Fatty Acid Esters: a GC-MS Study[1]

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ABSTRACT

Gas chromatographic analysis of cyclic monomeric concentrates and fractions from argentation chromatography on packed columns containing SE-30, OV-25 and Apiezon L stationary phases yielded incompletely separated peaks representing the various isomers present in the mixture. Somewhat better separation was achieved using a 6 ft x 1/8 in. column packed with 15% EGS on Chromosorb W. This column, when coupled to a mass spectrometer, yielded information concerning the composition of each of the isomeric components. Comparable results were obtained using a 50 ft x 0.02 in. S.C.O.T. column with DEGS stationary phase and a 150 ft x 0.01 in. capillary column coated with Apiezon L. While argentation thin layer chromatography proved useful, an argentation column method using silicic acid coated with 10% $AgNO_3$ proved more efficient for larger scale preparations. Elution of the column with 2% diethyl ether in petroleum ether yielded material essentially free of conjugated linolenate. A comparison of the behavior upon argentation thin layer chromatography of conjugated methyl linolenate, methyl linoleate and cyclic monomer esters indicated that these esters migrated to the same relative position as methyl oleate.

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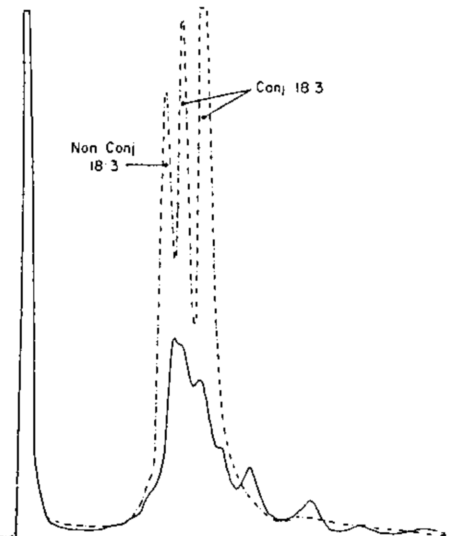

FIG. 1. Gas liquid chromatogram of cyclic monomer reaction product methyl esters with both conjugated and nonconjugated methyl linolenates (18:3) (6 ft x 1/8 in. S.S., with 15% EGS, Chromosorb W [AW] 60-80 mesh).

INTRODUCTION

The cyclization of linolenic and linoleic acids has been studied extensively. Schofield and Cowan demonstrated that linolenic acid could be converted to a cyclized structure by heating in the presence of solvent and alkali (1). Subsequent papers have concerned themselves with methods for improving the preparation and yields of saturated cyclized product (2), its separation from straight chain fatty acids (3), determination by gas liquid chromatography (GLC) (4), and a structural study of these products as mixtures of the corresponding saturated and aromatic isomers (5). Extensive earlier structural work has been reviewed by Friedrich (5), including work on cyclic products from eleostearate.

Much of this interest in cyclic fatty acids stems from their potential as industrial chemicals (6). However of equal if not greater importance is the fact that cyclic monomers of fatty acids (primarily linoleic acid) have been found in fats heated under relatively mild conditions. For instance, cyclic monomers have been found as a component of heated cottonseed oil (7) and heated linoleic acid (8,9). They have been isolated from soybean oil (10) and corn oil (11) heated in deep fryers. In addition, they are formed during the thermal oxidation in the laboratory of pure triglycerides containing oleic (12) and linoleic (13) acids. Furthermore cyclic monomers have been isolated as by-products from the hydrogenation of fats and oils (14,15). The field of cyclic compounds in fats and oils has recently been reviewed comprehensively by Artman (16).

As part of our program to determine the metabolic properties of heated fat components, a mixture of cyclic fatty acids was prepared from pure linolenic acid. These experiments were to precede our objective of preparing a purified ^{14}C-labeled cyclic monomer for use in metabolic studies. It was therefore important to remove as much starting material as possible from the product, since the

FIG. 2. Gas liquid chromatogram of hydrogenated cyclic monomer reaction product methyl esters (6 ft x 1/8 in. S.S., with 15% EGS, Chromosorb W [AW] 60-80 mesh).

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presence of such material, especially in the labeled conjugated form, would yield ambiguous results. The present paper is concerned with the preparation and partial characterization of a purified monomer fraction.

EXPERIMENTAL PROCEDURES

Pure linolenic acid was obtained from the Nu Chek Prep Co. and was analyzed as >99% by both GLC and thin layer chromatography (TLC). Pure aromatized cyclic monomer was obtained through J.C. Cowan, Northern Regional Research Lab., Peoria, Ill.

Methyl esters were prepared by reaction with diazomethane in diethyl ether followed by evaporation of solvent (17) and by reaction with methanol containing 2% H_2SO_4 (17) followed by the usual workup (17).

Samples were hydrogenated at atmospheric pressure with stirring by dissolving the sample (~5 mg) in ethyl acetate (10 ml) with 1-2 mg PtO_2 in an all glass system (18).

The cyclic monomers of pure linolenic acid were prepared according to a modification of the method of Schofield and Cowan (1) as follows: A typical preparation of the cyclic monomer of linolenic acid utilized 10 g pure trienoic acid dissolved in 100 g technical grade diethylene glycol and placed in a 250 ml three-necked pyrex flask. To this mixture was added 80-100% M excess of sodium hydroxide pellets (Reagent) and the flask and contents heated under an atmosphere of nitrogen gas for 1 hr at 240-245 C. During this time the level of solvent was kept constant by addition of diethylene glycol. The mixture was cooled, adjusted to pH 2 with 10% aqueous H_2SO_4 and the fatty acids extracted with petroleum ether. The organic extract was washed, dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was stored at 0 under nitrogen.

TLC was carried out according to Stahl (19). Glass plates (20 x 20 cm) were coated with a slurry of Silica Gel G (EM) containing 12% $AgNO_3$ in NH_4OH in layers 1 mm thick. Samples (80-100 mg) were applied as a narrow streak at 2 cm from the bottom of a plate. The plate was placed in a developing tank and developed twice with a solvent composed of petroleum ether-diethyl ether-glacial acetic acid 95:5:1. After drying, the plates were sprayed lightly with 1% of 2,7'-dichlorofluorescein in ethanol and viewed under UV light. The separated bands were removed by scraping into petroleum ether. The slurry was filtered and the fraction recovered by evaporation under N_2 to remove solvent. Analytical TLC was carried out under identical conditions as for preparative chromatography but employed a thinner silica gel layer (750 μ).

Purification of the crude cyclic monomer methyl ester was also carried out using column chromatography as follows: Mallinckrodt silicic acid (100-200 mesh) was washed with 50% aqueous HCl followed by distilled water rinses until succeeding rinses were Cl^- ion free. The washed silicic acid was heated in an oven at 150 C until dry. Silver nitrate (12.5% wt) was dissolved in distilled water and 100 g of the washed silicic acid added to form a slurry. This slurry was redried at 150 C and packed as a slurry in petroleum ether into a 2 cm diameter column. The average size column employed was 40 cm x 2 cm and contained ca. 80 g of packing. Samples of crude cyclic monomer methyl ester (2 g) were applied to the column and eluted with varying percentages of diethyl ether in petroleum ether. The elution was monitored by TLC and hydrogenation followed by GLC of the products.

Gas chromatography was accomplished using several columns. For ordinary analysis a 6 ft x 1/8 in. S.S. column packed with 15% EGS coated on 60-80 mesh Chromosorb W (Supelco, Inc., Bellefonte, Pa.) was employed. Other

FIG. 3. Argentation TLC of cyclic, conjugated and nonconjugated fatty acid methyl esters: 1. methyl stearate, 2. methyl oleate, 3. methyl linoleate, 4. methyl linolenate, 5. conjugated methyl linoleate isomer mixture, 6. conjugated methyl linolenate isomer mixture, 7. methyl esters of aromatized cyclic monomer from linolenate, 8. methyl esters of cyclic monomer reaction product (dimeric and polymeric materials removed).

SE-30 on 60-80 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), a 6 ft x 1/8 in. S.S. column packed with 3% OV-25 on 60-80 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), a 50 ft x 0.02 in. S.C.O.T. column with DEGS stationary phase (Perkin-Elmer Corp., Norwalk, Conn.), and a 150 ft x 0.01 in. capillary column coated with Apiezon L (Perkin-Elmer Corp.). These columns were utilized at a column temperature of 170-185 C, at optimum flow rates for each column, and coupled with a flame ionization detector. The instruments employed were a Beckman GC-5 and a Varian Aerograph A-60A.

Mass spectra were determined with a Perkin-Elmer Hitachi RMU6E double focusing mass spectrometer adjusted to a resolution of 1000 and coupled with a gas chromatographic inlet system. The helium separator was maintained at 250 C, as was the ion source and short heated transfer line from the GLC. The ionizing current was 55 μA and the voltage set at 70 volts. Spectra were recorded every 3 sec to m/e = 600 during the elution of a GLC peak (1-10 μg material) as determined by both the FID detector of the gas chromatograph (10% split) and the continuous record produced by the total ion monitor. The mass spectra so obtained were stored on magnetic tape for further processing. High resolution element maps were obtained on selected samples at a resolution of 10,000 with a Varian Mat SM-1B instrument. The Varian Aerograph series 1200 Gas Chromatograph coupled to the mass spectrometer employed a 6 ft x 1/8 in. S.S. column packed with 15% EGS coated on 60-80 mesh acid washed Chromosorb W. A helium flow rate of 20-22 ml/min was employed. Approximately 90% of the column effluent was diverted to the mass spectrometer. Elution of individual components was accomplished by isothermal operation of the column at 175 C. The mass spectra collected (30-40 per sample) were processed using an on-line data system. The background contribution of the EGS column and that due to peak overlap were subtracted to yield spectra representative of the eluted components. The spectra were also averaged for changing ion current to obtain a representative spectrum for any one component.

RESULTS AND DISCUSSION

The formation of cyclic monomers of fatty acids in oils, which have been heated under both simulated and actual deep fat frying conditions, represents a potential public

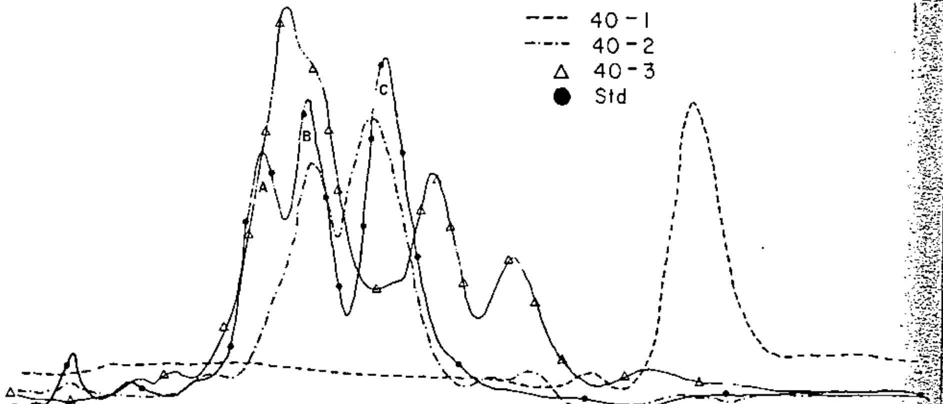

FIG. 4. Gas liquid chromatogram of fractions obtained by argentation thin layer chromatography of cyclic fatty acid methyl esters. Composition of standard: A. nonconjugated methyl linolenate, B. and C. conjugated isomers of linolenate (6 ft x 1/8 in. S.S., with 15% EGS on 60-80 mesh Chromosorb W [AW]).

cyclic monomer occurs in heated cottonseed oil as well as soybean oil heated in a deep fat fryer (10). Furthermore it appears that cyclic monomer may be the one component of heated oils with the most toxic potential that occurs in the largest quantities (7). For this reason it was essential to study the purification of cyclic monomer to remove as many other side reaction products as possible, with a view toward synthesis of approximately labeled compounds for future metabolic and toxicological studies.

The method of producing the cyclic monomer mixture used in the present study was a somewhat modified version of that reported previously (1). The formation of some small amounts of dimeric products appears to be a byproduct of the reaction at the high temperature and is probably due to a Diels Alder condensation between two molecules of linolenic acid. When the mechanism of cyclic acid formation (5) is considered, the side product of the reaction is, in addition to a small amount of dimeric material, primarily conjugated linolenic acid which is not readily separable from the reaction product.

Examination of the reaction products from a typical cyclization reaction using an ethylene glycol succinate (EGS) column (Fig. 1) indicated an ill-defined mixture of isomers similar to those reported for the cyclic monomer from linseed oil. There is considerable overlap indicated with the conjugated isomer mixture from methyl linolenate. The amount of conjugated linolenate isomers in the reaction mixture may be determined by hydrogenation and subsequent rechromatography of the hydrogenated crude reaction product (Fig. 2). The chromatogram of this sample indicated the presence of a small amount of methyl stearate (7-9%) and the presence of a component which did not change in retention time upon hydrogenation, probably due to an aromatic isomer.

Although the effect of conjugation on the Rf values of compounds separated via argentation TLC has not been fully investigated, it has been shown that the effect of silver complexing increased as the distance between double bonds increased (20). A comparison of the effects of argentation on the separation of conjugated and nonconjugated fatty acid methyl esters and cyclic monomer which demonstrates this effect is shown in Figure 3. The influence of increasing the number of double bonds in decreasing the Rf value in the series Rf 18:0>18:1>18:2>18:3, where the double bonds are all of the *cis* configuration, is readily apparent. However it is interesting to note that the complex mixture of geometrical and positional isomers representing conjugated 18:2 and 18:3 methyl esters exhibits the same Rf value as methyl oleate. The two lower spots in the conjugated 18:3 lane may be due to mixtures containing lower percentages of *trans* double bonds; all three components have identical mass spectra. The aromatized cyclic monomer and the present reaction product behave in the same fashion. The reaction product, methyl ester, separated into three poorly resolved bands upon preparative argentation TLC. Each of the fractions were collected and the organic material extracted and analyzed by GLC on an EGS column coupled directly to the mass spectrometer. The mass spectra of each peak was recorded. The gas chromatogram of each fraction is outlined in Figure 4, and the origin of the fractions is indicated on the photograph of the TLC plate (Fig. 3, lane 8). Fraction 1, the topmost spot, was composed of one major component, which did not change retention time upon hydrogenation and represented ca. 10% of the total cyclic material. The average mass spectrum of this peak indicated that it was a mixture of isomeric aromatic cyclic monomers with a molecular weight of 290 as the methyl ester (Fig. 5).

An examination of the mass spectrum indicates the presence of an intense molecular ion as well as ions at m/e 258 and 259, representing loss of methanol from the methyl ester. Cleavage of the alkylcarbonyl side chain leads to the formation of a series of ions at m/e 105 (C_8H_9, 105.0693, calculated 105.0704), 119 (C_9H_{11}, 119.0857, calculated 119.0861), and 133 ($C_{10}H_{13}$, 133.1015, calculated 133.1017) with the general structure [benzenium ring]$^+$–$(CH_2)_XCH_3$. The ions at *m/e* 119 and 133 contained a small percentage of ions (<2%) with the structure C_8H_7O (m/e 119.0490, calculated 119.0497) and C_9H_9O (m/e 133.0650, calculated 133.0653) due to a ketene ion. In addition, an intense ion at m/e 197 corresponding to $C_{15}H_7$ (m/e 197.1325, calculated 197.1330) may have originated from a corresponding ketene ion by loss of water and a proton. Less intense ions at m/e 215, $C_{15}H_{19}O$ (m/e 215.1436, calculated 215.1436) and m/e 229, $C_{16}H_{21}O_1$ (m/e 229.1592, calculated 229.1593) seem to be ketene type ions that have lost one proton. Ion structures that have been written in monosubstituted forms may also be written in the disubstituted form, as may be obtained by partial cleavage of one substituent. A fairly intense ion is present at m/e = 91 representing the tropilium ion.

The mass spectra of this fraction are quite similar to those published by Zeman et al. (21) for pure synthetic aromatic cyclic monomer, and by Scharmann et al. (8) for aromatic acids produced from linolenate and linoleate with the general structure: [cyclohexadiene ring with] $(CH_2)_YCO_2CH_3$ [and] $(CH_2)_XCH_3$ $X + Y = 10$

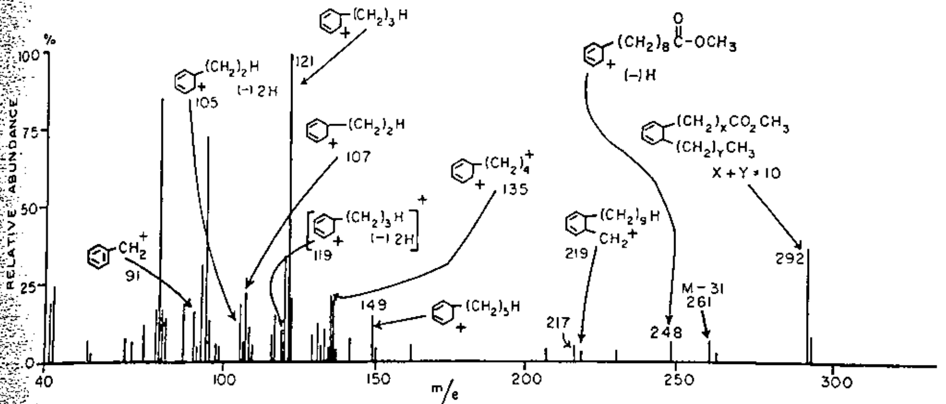

FIG. 6. Mass spectrum (70 eV) of isomeric cyclic monomer methyl esters (fraction 40-2).