Oxygen at moderate temperatures (60°C.) causes little or no decline in nutritive value until the lag phase is overcome; then available energy values go down, and liver sizes of test animals increase. The values in Table IV are for samples taken from cottonseed oil which has been stirred and blown with air.

TABLE IV
Influence of Oxidation Upon the Nutritive Value of Cottonseed Oil

Days of oxidation at 60°C.	I.P.V.	Available energy [a]	Liver size [b]
		%	% of body wt.
0	<1	100	4.0
15	310	100	3.9
19	1130	71	5.3
29	400	0	5.8

[a] Three rats per group, each rat getting 0.6 g. of test fat per day in addition to 5 g. of basal diet. At 1.2-g. test-fat levels rats receiving the fats oxidized for 19 days or more did very poorly and refused part of the diets.

TABLE V
Influence of Time Upon the Effect Which Heat Has Upon the Nutritive Value of Cottonseed Oil

Length of heating period [a]	Energy availability [b]	Liver size [b]
days	%	% of body wt.
0	100	5.0
1	95	5.7
2	95	6.1
3	93	6.4
4	72	6.9
5	75	7.1

[a] Heated in 3,000-g. quantities, not stirred, in household deep-fat fryer at 182°C.
[b] All animals fed 1.5-g. of test fat daily in addition to 5 g. of basal diet. Four rats per group.

Similarly heating fat in 3,000-g. quantities at 182°C. (360°F.) in a household deep-fat fryer causes gradual reduction of nutritive value (Table V). It should be noted that these are not applied conditions. No food was cooked, there was no addition of fresh fat, nor was there removal of volatile materials with steam. Even so, changes are slight during the early stages of heating.

THE INFLUENCE of varying the percentage of oil exposed to air at any given temperature is indicated in Table VI. In this case varying amounts of oil were heated in the same pan for the periods of time shown. In other tests constant amounts of oil were heated at different temperatures. It may be noted that a change in the amount of fat heated per unit of surface area has more influence than a change in temperature. The 50-g. quantities of oil rapidly became viscous, and the ones which had been heated for 6 hrs. had to be scraped from the pan with rubber spatulas. In general, samples which showed heat damage were quite viscous and certainly would not be suitable frying aids. Various fats respond differently although all tend to show the same changes.

TABLE VI
Influence of Surface Exposure Upon Changes in the Nutritive Values of Hot Cottonseed Oil

Heating period	Fat heated at 180°			Fat heated at 200°C.	Fat heated at 220°C.
Minutes	50 g. [b]	100 g. [b]	200 g. [b]	100 g.	100 g.
Energy Availabilities as Percentage of Control Oil [a]					
	%	%	%	%	%
0	100	100	100	100	100
30	102	106	108	102	112
120	98	98	94	94	90
360	50	80	98	77	69
Liver Weights as Percentage of Body Weights [a]					
0	4.4	4.4	4.4	4.4	4.4
30	4.6	4.4	4.0	4.2	4.5
120	5.4	5.3	4.5	5.0	5.4
360	6.8	6.3	5.4	6.2	6.7

[a] Samples fed at a level of 1.2 g. per animal, in addition to 5 g. of basal diet, to group of 4 animals on each test.
[b] The 50-, 100-, 200-g. quantities correspond to an oil depth of approximately 2, 4, and 8 mm., respectively, for oil heated in an 8½-in. diameter aluminum pan.

SINCE THE ORIGINAL OUTBREAK of poultry disease, various types of food and feed-grade fatty materials have been examined. The application of sensitive tests by Food and Drug Administration officials led to the detection, in some shipments of oleic acid, of traces of materials which gave chickens mild symptoms of hydropericardial disease. On the basis of these findings officials of the Food and Drug Administration are requiring all producers of oleic and stearic acids to chick-test products intended for food use. A detailed method for this purpose has been distributed (18). This involves measurements of the volume of pericardial fluid in chickens after they have been fed test materials at a 16% level for three weeks. Normally chickens have almost no fluid in the heart sac, and the presence of as little as 0.2 ml. is considered by Food and Drug officials as a positive symptom of the condition. Others who have used the method or variants of it do not agree that such a low volume necessarily indicates the presence of toxic materials.

Except for the work with fats producing chick edema, most of the reports of biological damage have resulted from the feeding of fat damaged by severe laboratory treatments. In order to obtain more data on cooking fats a series of samples was obtained from various commercial operations: potato chip or doughnut fryers, restaurants, grills, and so forth. These were obtained at the time of maximum heat treatment, often actually after the user had discarded the product as unsuitable for further cooking. When tested by our rat-feeding procedure, none of these showed marked changes from unheated fats, as has been reported elsewhere (19, 20).

In a study of oils from 89 potato chip manufacturers, Melnick (21) found insignificant changes in iodine values during processing and claimed on this basis that the products have not been changed in any significant amount. This same conclusion was reached a number of years ago by Deuel and his co-workers (22), who fed oils obtained from potato chip preparation to rats and were unable to detect changes caused by the processing.

Other studies in our own laboratories have led to substantially the same conclusion. In these studies two commercial types of 28-lb. deep-fat fryers were filled with refined cottonseed oil and heated to 182°C. (360°F.). Sliced potatoes to yield five pounds of potato chips were fried twice daily in one of the fryers. After the second frying each day sufficient fresh oil was added to restore the original volume. Except for sampling, oil in the other fryer was undisturbed until the end of the experiment. Samples of potato chips and of oil in each fryer were taken after each 24-hr. interval.

While the cooking of two 5-lb. quantities of potato chips twice daily did not provide the intensive usage experienced by fats in the vats of a grill or a commercial potato chipper, it did cause vigorous agitation and introduced food particles and steam. In addition, each day about 10% of heated fat was removed on the potato chips and had to be replaced. After only two days of use the oil foamed violently during cooking and could be kept in the kettle only by immersing small lots of sliced potatoes. Commercial usage would have been impossible. It is interesting to note that changes in biological quality appeared about the same time that foaming made use impractical.

TABLE VII
Potato Chip Frying

Treatment of cottonseed oil	Calorie Availability [a] %	Liver size		14-Day gains		Liver size	
		% of Body wt.	% of Control	Actual	Adjusted to aver. food intake	% of Body wt.	% of Control
1. 28 lb. CSO heated to 182°C.—5 lb. potato chip fried immediately	100	4.42	100	74.3	65.6	6.2	100
2. Heated oil at 182°C. for 24 hrs.—5 lb. chips fried	89	5.16	116	70.0	63.6	6.7	104
3. Heated oil at 182°C. for 48 hrs.—5 lb. chips fried [b]	89	5.58	128	63.3	64.3	6.5	102
4. Heated oil at 182°C. for 72 hrs.—5 lb. chips fried [b]	95	5.41	121	53.5	57.3	7.1	107
5. Heated oil at 182°C. for 96 hrs.—5 lb. chips fried [b]	91	6.23	138	54.5	59.7	7.2	108
6. Heated oil at 182°C. for 120 hrs.—5 lb. chips fried [b]	86	5.98	125	53.3	58.0	6.7	104
7. Heated oil at 182°C. for 120 hrs.—no frying	72	7.14	150				
8. Heated oil at 182°C. for 120 hrs.—then 5 lb. chips fried [b]	63	6.80	139	40.0	54.4	6.6	103
9. Commercial chips				80.0	72.1	6.1	99

[a] Based on an arbitrary value of 100 for the fresh, heated oil.
[b] Oil foamed violently when potato slices were placed in it.

As a source of energy Oil No. 7, which had been heated without use for cooking, was less effective than Oil No. 6, in which chips had been periodically cooked. Livers of rats fed Oil No. 7 also were heavier than livers of the No. 6 group. It must be remembered however that fresh make-up fat had been added periodically to No. 6 to keep the volume constant.

Chips produced during the experiment were mixed into diets in amount sufficient to supply 20% of fat. The rations provided generously for all nutrient needs of weanling rats. Previous 12-week studies had indicated that maximum effects on growth would occur in two weeks (19, 20). After that, rats fed severely heated and unheated fats grew at about the same rate. Hence the growth studies were restricted to a two-week period.

The gains of the groups fed fat heated for several days were smaller than when fresh fat was fed. However much of this decline in weight probably resulted from the decreased palatability of the diet since statistical adjustment of body weights to a common level of food intake evened out the gains. Certainly there is no evidence of decreased nutritive value on the basis of the adjusted weight gains or on liver sizes for animals fed products from the first three treatments. Livers of animals that were fed oil heated 48 hrs. were heavier than those of the first two groups, but at this time the oil was already foaming when used. Further evidence that the 48-hr. heated oil was beyond practical usage was provided by Schaal accelerated stability tests of the potato chips produced in it. These chips gave a stability of only two days as compared to 19 days for chips fried in fresh oil.

Studies of fats extracted from fried, broiled, or roasted meats have similarly shown no decrease in energy availability or in fractions which cause increase in liver weights (23).

SO FAR, most attention has been given to conditions which produce substances that cause diarrhea, retard growth, and alter organ size, and little mention has been made of the nature of the substances formed or their mode of action. Actually little is known in either of these areas. Almost all of the research workers who have produced damage in their samples have been able to show that the active substances are in the unsaponifiable material or in a fraction that is not adducted by urea. Attempts to obtain pure substances have been somewhat unsuccessful.

Kaunitz and others (24, 25) have reported that simple oxidation products, such as monohydroxy stearate, 9-10 dihydroxy stearate, *cis*-epoxy stearate, or oleate-peroxide, do not produce symptoms of tox-

icity and are at least not the principal toxic agents. Andrews *et al.* (12) however showed that t-butyl hydroperoxide depressed growth when fed. Polymeric residues obtained after molecular distillations were more toxic than distillable factions, leading to the conclusion by Kaunitz that polymers of some type were responsible for the physiological effects.

Crampton (7), considering the relative potencies of fractions separated from thermally-polymerized linseed oil by distillation and urea segregation, concluded that at least two factors were involved: acyl radicals whose esters could be easily distilled and which would not form urea adducts, and dimeric or polymeric factions which neither distilled nor formed adducts. The monomeric substances were digestible, *i.e.*, they disappeared from the gastrointestinal tract, and they depressed growth, but the polymeric materials were neither digestible nor toxic (26). Perkins and Kummerow (27, 28) also demonstrated that the urea nonadducts obtained from oxidized fats were the most potent factors.

We also have been able to concentrate the biologically effective materials in unsaponifiable or nonadductible fractions. In one series of studies the nonadduct-forming material (NAF) from cottonseed oil which had been heated to 360°F. for 120 hrs. was serially extracted three times with Skellysolve B (Fraction 1), three times with 30% ethyl ether in Skellysolve B (Fraction 2), three times with 60% ethyl ether in Skellysolve B (Fraction 3), and with pure ethyl ether (Fraction 4). Insofar as material was available, 0.3 g. of each of these fractions was fed with 5 g. of basal diet to rats to determine biological responses. The molecular weights of the fractions were estimated by the technique of Donnelly (29). Results of the study in Table VIII show the fraction soluble in pure Skellysolve B to be as active as an equivalent amount of the unfractionated NAF, and the fractions extracted with ethyl ether to be more potent. Not enough material was obtained for feeding after the extraction with 60% ether.

TABLE VIII

Influence of Fractions of Heated Cottonseed Oil Upon the Growth and Liver Sizes of Rats

Diet	Gain	Liver size	Average molecular wt.
Basal	7	4.1	
Basal + 0.3 g. CSO	15	3.7	
Basal + 0.3 g. heated CSO[a]	13[b]	4.0[b]	
Basal + 0.3 g. NAF of heated CSO	11	5.0	
Basal + 0.3 g. NAF—Fraction 1	11	4.8	673
Basal + 0.3 g. NAF—Fraction 2	10	5.3	752
Basal + 0.3 g. NAF—Fraction 3	6	5.5	1320
NAF—Fraction 4	..	....	1420
NAF—Residue	..	....	very large

[a] 182°C. for 120 hrs.
[b] Interpolated from standard curve.

In another study cottonseed oil heated for 120 hrs. at 182°C. (360°F.) was treated with propanol. Two layers formed the upper or more soluble layer containing 86% of the original oil. When fed in the energy-restriction technique at a 1.5-g. level per day (in addition to 5 g. basal), the energy of the insoluble fraction proved to be only 17% available in contrast to 67% for the soluble fraction and 65% for the unfractionated heated oil. Livers from the corresponding animals averaged 5.5, 7.9, and 7.5% of the body weights. This suggests, as does Crampton's work, that a relatively indigestible but harmless fraction and a readily digestible but harmful fraction exist.

EFFORTS TO PURIFY these materials further have been frustrating, partially because of a lack of test methods applicable to small quantities. Even a 7-day rat test, using 0.3 g. per day for four rats requires 8.4 g. of product. Some urea nonadductable materials from heated fats have been separated into major fractions chromatographically, using silica gel and alumina preparations. There are some differences in the spectral characteristics of such fractions, but so far they have not been tested adequately on a biological basis to permit definition of the active fractions. The very limited work done indicates that several chromatographically distinct fractions have activity. This suggests that a family of compounds of similar physical properties and perhaps similar structures may be involved rather than one or two substances. Such a development would not be unexpected in view of the variety of structures available in unsaturated fatty acids, especially in partially hydrogenated fats where isomerization occurs.

It seems very probable that separations achieved by use of the various types of distillation, chromatography, solvent distributions, molecular sieves, etc., will soon result in much better understanding of the chemical nature of the substances responsible for the several biological effects that have been noted. This will permit more exact study of the amounts of these substances in experimental and food fats and will aid in evaluating the acceptability of heated food fats.

To date little is known of the biological causes of the effects observed. Under selected conditions it is possible to cause diarrhea, rough fur, decreased growth, and even death, but we do not know exactly why. There have been demonstrations that abused fats are less digestible than fresh fats (8, 20, 24) and that organs of animals fed the abused fats have altered sizes and enzyme activities (9, 12, 23, 24), that some fractions may accelerate the formation of certain types of abnormal tissues (1, 2, 3, 4, 5), and that oxidized fatty acids alter enzyme activity *in vitro* (30, 31), but the surface of this type of problem has scarcely been scratched. We can be certain that there will be much more work of this nature in the future.

We hope that, in interpreting the data, investigators will remember that alterations in rates of growth or organ size or in enzyme activity are not necessarily indicative of undesirable changes. They may be be beneficial. For example, moderate repeated exercise develops (enlarges) muscles although sustained violent exercise leads to sore, exhausted muscles. Furthermore many changes in quantity or quality of diet result in changes in organ size and composition. Before any substance can be considered harmful, the biological changes induced by its ingestion must be proved to be detrimental to temporary or long-term health. It must be borne in mind however that pathologists are suspicious of any change from the accepted normal.

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Newer Analytical Methods for the Fat and Oil Industry

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THE NEWER METHODS for the analysis of fats and oils fall for the most part into two groups, that is, those relating to physical characteristics and those used for the estimation of composition. This is altogether fitting because certainly the functional properties of fats and oils are intimately related to their composition and to their physical properties. Major advances in analysis in the last several years have occurred in the following specific areas: chromatography, dilatometry, nuclear magnetic resonance, urea fractionation, and spectroscopy—with a few miscellaneous methods in other areas.

Chromatography certainly heads the list of important advances in fat analysis. It has probably been the single most active area of exploration in the field in the last several years and still remains very active. The word chromatography which identifies this technique stems from the work of Tswett and probably is not a good choice since it does not denote the action or the process. However we do not need to be concerned here with nomenclature.

Chromatography does provide a means of obtaining quantitative separations of certain mixtures, and if you will permit a prediction, the applications will undoubtedly be greatly expanded in years to come.

Chromatography can for our purpose be very simply defined as a technique which utilizes such phenomena as surface adsorption, partition between solvents, and ion exchange to bring about separations of simple and complex mixtures into their various components. It is recognized that the definition may not be altogether adequate or complete but it will furnish a basis for our discussion.

The materials commonly involved in chromatography as applied to the analysis of fats include a) a solid support, such as diatomaceous earth, silicic acid, various chemical salts, paper, and other substances, b) a liquid stationary phase, usually adsorbed to the surface of the support, of varying composition depending upon the specific application, and c) a mobile phase which passes over or by the stationary phase. The mobile phase may be a liquid or a gas and in the case of the former may be designated as solvent, eluant, etc.

A good though elementary example of column adsorption chromatography is the A.O.C.S. method for the estimation of total neutral oil. Briefly this procedure involves pouring the sample, dissolved in a solvent, onto a column of aluminum oxide, and allowing the solution to percolate through the column. The eluate, *i.e.* the portion that passes through the column, is collected and the solvent is evaporated. The weighed residue represents neutral triglyceride.

The reason for being able to separate neutral oil from free fatty acids under the prescribed conditions for this method is that the less strongly held neutral triglycerides pass through the column with the solvent and the more polar, free fatty acids are adsorbed on the surface of the aluminum oxide and thus do not pass through the column.

In the case of partition chromatography separation is attained by distribution of the components of the mixture between the mobile and stationary phases based on partition coefficients.

Broadly speaking then, the fact that the different components of a mixture can be retained on or can be made to pass over or through a column at different rates by suitably adjusting the conditions and by properly selecting the solvents and other materials is the basis for the technique of chromatography. The separation may involve adsorption as in the procedure just mentioned, or partition between liquids as is applied to the fractionation of fatty acids. Ion exchange is not to my knowledge applied in many areas of fat analysis.

Separations employing column adsorption or partition chromatography have been successfully applied to the fractionation of fatty acids, to the determination of individual fatty acids such as butyric acid in butterfat and others, and to the determination of saturated fatty acids. It obviously is a good technique but when the mixture becomes complex the labor involved is not inconsiderable. Therefore in such instances paper chromatography and gas chromatography are more practical.

employed. A 100% difference in relative humidity was maintained across the films. The permeability constant, P, was calculated using the equation,

$$P = \frac{(w)(x)}{(A)(t)(p)}$$

where w is the weight of water vapor, in grams, diffusing through a film of thickness x, in centimeters, and area, A in square centimeters, during the time t, in seconds, when the vapor pressure difference p is measured in millimeters of mercury. The permeability constants obtained are recorded in Table I and those for the saturated acyl groups are shown graphically in Fig. 4.

Permeability decreased as the chain length of the fatty acid group increased. The presence of unreacted hydroxyl groups apparently had no significant effect on permeability, which is in agreement with data on glycerides obtained in our laboratory. Heretofore, no data on the permeability of amylose esters have been published. Using slightly different test conditions, Rankin *et al.* (12) obtained a permeability constant of 760×10^{-12} for a film of amylose. The permeability of amylose acetate is slightly higher than that reported for cellulose acetate (17). The lowest value obtained, 15×10^{-12} for the stearate, was slightly larger than that of polystyrene (1,5). The value for paraffin wax, probably the most impermeable of organic compounds, has been reported to be 0.17×10^{-12} (15).

When the amylose ester films were removed from the moisture cups after the measurements had been completed, it was observed that the more permeable films had become hazy.

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Report of the Uniform Methods Committee, 1961

The meeting of the Uniform Methods Committee was held at 2 p.m., October 30, 1961, during the Chicago fall meeting. K. E. Holt, R. J. Houle, R. A. Marmor, L. D. Metcalf, E. F. Sipos, E. M. Sallee, editor, and D. L. Henry were present. Visitors were: R. W. Bates, L. A. Baumann, R. C. Stillman, and M. E. Whitten.

Progress reports submitted by many of the Technical Committees were discussed with interest. These reports indicate that much good work is being done on needed methods. No recommendations were received to change existing methods or to adopt new ones, and no changes are recommended by the Uniform Methods Committee.

Respectfully submitted,

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Nutritive Value of Methyl Linoleate and Its Thermal Decomposition Products

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Methyl linoleate was heated for 10 hrs. at 300°C. in the absence of air and fractionated by alembic distillation and urea adduct-formation.

Intestinal absorptions of the urea adduct-forming monomeric nonadduct-forming monomeric, and dimeric fractions were determined. It was found that dimers were half as well absorbed as the monomers.

When fed to rats, dimers were better accepted and exhibited some toxicity symptoms different from the nonadduct-forming monomers. The dimers caused diarrhea, irritability, and loss of hair during the early period of administration. The nonadduct-forming monomers were lethal and produced an increase in liver weight. Both fractions depressed growth.

HEATING of unsaturated oils in the absence of air is known to result in the formation of a mixture of monomeric and polymeric substances, both linear and cyclic. For reviews on the nutritional significance of such compounds the reader is referred to the papers of Brown (1) and of Rice *et al.* (2).

Crampton and co-workers (3,9) have been able to demonstrate that, when linseed, soybean, and sunflower oils are heated at approximately 275°C. for 12 to 26 hrs. in a CO_2 atmosphere, a polymeric fraction, which is very slightly absorbed from the intestine of the rat, and a nonurea adduct-forming monomeric fraction, which is harmful to the same animal, are formed. Comparison of the fatty acid composition of these oils with their deleterious action indicates that linolenic acid is the main precursor. Nevertheless some toxicity has been found to develop in heated oils containing relatively high proportions of linoleic acid and no linolenic acid.

More unsaturated oils, such as fish oils, seem able to develop toxic effects and form poorly absorbed substances if heated and administered under similar conditions (10–15).

Methyl and ethyl linoleate seem also capable of forming cyclic and polymeric substances by heating, as has been suggested by Paschke *et al.* (16,17).

In order better to characterize the toxic derivatives and their biological properties, relatively pure methyl linoleate was prepared, heated in the absence of air, then fractionated. The fractions were tested for intestinal absorption and deleterious action in the rat.

Recently Kaunitz *et al.* (18) have made a similar study on thermal oxidation products of the same ester.

Experimental

Preparation of the Fractions. Methyl linoleate was prepared from the methyl esters of commercial sunflower seed oil [2] by the urea-adduct procedure (19). The esters were heated at 300°C. for 10 hrs. in the presence of CO_2 in an apparatus similar to that described by Bradley and Johnston (20). The heated product was fractionated by alembic distillation under reduced pressure into a distillable monomeric fraction and a residual polymeric fraction. The latter, as may be seen from the data in Table I, is essentially dimeric.

[1] With the technical assistance of Oscar Giacomantone and Perla Mordujovich.
[2] Generously supplied by Alba, S.A., Argentina.

TABLE I
Typical Properties of Tested Products

Fraction	Iodine number [a]		Molecular weight [b]	
	Expti.	Theor.	Expti.	Theor.
Methyl linoleate	154.5	172.4	287 [c]	294.4
Heated methyl linoleate [d]	103.3	....	....	....
Distillable monomers [d]	112.1	....	290.5	294.4
Adduct-forming monomers [d]	114.9	172.4	....	294.4
Nonadduct-forming monomers [d]	95.2	85.6	....	294.4
Polymers [d]	92.2	D = 85.6 [e] T = 57.5	640	D = 588 T = 882

[a] Hanus, 30 min.
[b] Cryoscopy in benzene.
[c] From saponification value.
[d] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[e] D = dimers; T = trimers.

The monomeric fraction was then separated by means of the procedure of Wells and Common (8) into urea adduct-forming monomers and nonurea adduct-forming monomers. Some of the chemical characteristics of the prepared methyl linoleate and its products of heating are shown in Table I. Iodine number was determined by the Hanus (30 min.) procedure. Mean molecular weights were obtained by means of cryoscopy in benzene.

Determination of the Absorption Coefficient. The technique described by Irwin *et al.* (21) and by Deuel *et al.* (22) for the determination of intestinal absorption was followed with minor modifications. Adult female albino rats belonging to the strain of the Institute and weighing between 165 and 300 g. (average 233 g.) were fasted for 48 hrs. They were fed by stomach tube approximately 300 mg. of accurately weighted ester per dm^2 of body surface. After 4 hrs. the rats were sacrificed with ether, and the gastrointestinal tract was flushed with 150-ml. portions each of physiological saline, petroleum ether, and saline in that order. The aqueous-ethereal extract was acidified, and the fat in the ether phase was determined gravimetrically. For control the fat was extracted from the intestinal contents of similarly conditioned animals not given the test material. The absorption coefficient was calculated from the difference between the fat content of the control and test animals. The method was tested by giving separately adduct-forming monomers and dimers to rats in identical fasting conditions and by immediately washing the digestive tract. The absorption coefficients were expressed as mg. of fat absorbed per dm^2 of body surface per hour. The percentage of fat recovered was also determined. The data from all animals which exhibited diarrhea were discarded (Table II).

Test of Biological Action. Adult male albino rats weighing between 144 and 233 g. (average 186 g.) were distributed in groups of 10 animals each. One group was made up of only six rats because of the small amount of nonadduct-forming monomers available. During periods of 25 to 36 days the group 1 animals were fed a fat-free diet and the other groups a diet containing 10% by weight of the following lipids: group 2, methyl linoleate; group 3, adduct-forming monomers; group 4, dimers; group 5, non-

TABLE II

Absorption Coefficients of Thermal Decomposition Products of Methyl Linoleate

Fraction	No. of expts.	Avg. weight of rats	Absorp. period	Absorp. coeffic.[a]	Ester absorbed
		(g.)	(hrs.)	(mg./dm²/h)	(%)
Adduct-forming monomers	12	204	4	43.3 ± 5.5[c]	55.6
	3	266	0		89.5
Nonadduct-forming monomers	6	203	4	45.8 ± 9.6	62.4
Dimers	7	232	4	26.5 ± 5.0	31.7
	9	246	0		76.4
				Average recovery	
None	10	241	0	26.4 ± 18.1 mg./rat	

[a] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[b] Determined by the procedure of Irwin *et al.* (21) and Deuel *et al.* (22).
[c] Standard deviation.

TABLE III

Composition of the Diets

Component	Percentage in the diet	
	Fat-free	With fat
Sucrose	72	62
Casein	20	20
Ester or oil tested	0	10
Yeast (dry)	4	4
Salts (McCollum-Davis)	4	4
Supplement:		
vitamin A, 200 i.u./100 g.		
vitamin D, 20 i.u./100 g.		

adduct-forming monomers; group 6, corn oil (Mazola); group 7, dimers plus corn oil (1 + 1). The composition of the diets is presented in Table III. Rations were prepared daily in order to prevent alteration of the fat and were placed in the cages in metal boxes with lids. A circular hole in the lid allowed easy access to feed and prevented losses. Feed was given *ad libitum* to all groups except group 6, which received 5 g. of feed per day per rat. The animals were weighed daily. Groups 5 and 6 were kept in individual cages because otherwise the weaker or dead animals were eaten by the others. It was also necessary to isolate the rats of group 4 because their diarrheic feces acquired a varnish consistency which kept the animals stuck to each other and to the floor. At the end of the experimental period the rats were sacrificed with gas, and their livers were weighed and histologically studied (Table V).

Results and Discussion

Degree of Absorption. The amount of fat found in the intestine of fasted rats (Table II), averaging 26.4 mg. per rat, agrees with previously reported values (22). The recovery of adduct forming monomers (about 90%) indicates the acceptable capability of the procedure for washing the digestive tract. Published recoveries obtained with different oils and procedures are of the same order of magnitude (22). On the other hand, the amount of fat obtained from the intestine by washing immediately after giving dimers was lower than after other fractions even though the dimers are less well absorbed than monomers, as shown by the data from the 4-hr. absorption period. No correction for this anomaly was applied. The recorded values show no differences in the absorption coefficients of the adduct-forming and nonadduct-forming monomeric substances tested but do show differences between the monomers and dimers.

Biological Action. The growth curve (Fig. 1) of the group fed adduct-forming monomers [consisting, as found by Paschke *et al.* (16,17) of a main portion of normal methyl linoleate and about 14% of its conjugated isomer] shows no striking difference as compared with the controls fed methyl linoleate (group 2) or the fat-free diet (group 1). The growth curves of groups 4 and 5, which received dimers and nonadduct-forming monomers, respectively, are remarkably abnormal. After only two days on experiment the average weight of group 4 was less than that at the beginning, even with good acceptance of feed. At the third day the animals had marked diarrhea, accompanied by colorless fluid feces at the moment of excretion, which became darker, semisolid, and sticky in the contact with the air and acquired a varnish aspect. After five days the rats showed pronounced irritability, being almost constantly in a fighting position, one in front of the other. Hair became yellow and absent in small but increasing areas. Both diarrhea and the loss of weight and hair continued until around the 15th day, after which slight improvement in the general aspect was noted with diminishing diarrhea and loss of hair, and an increase in weight. The growth curve of this group during the first two weeks coincides remarkably with that of group 6, which was restrictively fed. After two weeks the curves separate; the weight of group 4 increases slightly and that of group 6 decreases steadily. Feed consumption of group 4 (Table IV) averaged 10.0 g./day/rat during the first two weeks and 14.6 g./day/rat for the whole experimental period, indicating that loss of weight cannot be explained as resulting from the rejection of feed by the animals. The results could be due to an adverse effect of the dimers on the utilization of the other nutrients, as suggested by Raulin and co-workers (23). As can be seen in Table V, rats receiving the dimer diet for 25 days showed a smaller ratio of body to liver weight than animals fed identical fat for 36 days. The values in the latter test were at the level of the controls. The number of dead animals in

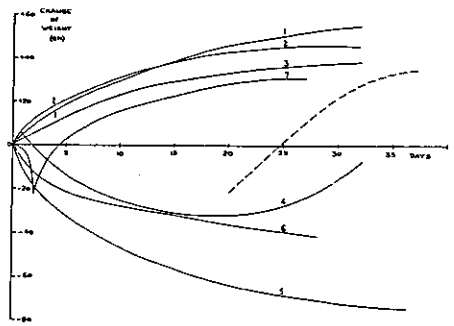

FIG. 1. Change, with time, of the weights of rats fed methyl linoleate and its thermal decomposition products.

Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.

Curve No. 1 corresponds to a group fed a fat-free diet. The other curves correspond to groups fed diets containing 10% by weight of the following lipids: No. 2, methyl linoleate; No. 3, adduct-forming monomers; No. 4, dimers; No. 5, nonadduct-forming monomers; No. 6, corn oil; No. 7, dimers plus corn oil (1 + 1). Feeding was *ad libitum* in all groups except in group 6, which received only 5 g. of feed per day per rat. The dotted line represents the change of weight of a group fed dimers (group 4) until the 20th day, and corn oil afterwards.

TABLE IV

Feed Consumption

Group No.	Fat in the diet	Duration of the experiment	Consumption
		(days)	(g./day/rat)
1	None	25	15.8
2	Methyl linoleate	25	19.0
3	Adduct-forming monomers[a]	32	11.7
4	Dimers[a]	36	14.6
5	Nonadduct-forming monomers[a]	36	6.2
6	Corn oil	25	5.0[b]
7	Dimers + corn oil (1 + 1)	25	12.8

[a] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[b] Restricted.

group 4 was one to ten, similar to the ratio of some of the control groups accordingly considered normal.

In a separate experiment, rats given a diet of 10% dimers for 20 days were then fed the same proportions of corn oil. After 12 days improvement in the general condition was observed, including an increase of weight to normal levels (Fig. 1), growth of hair, and disappearance of diarrhea. The growth curve corresponding to group 7, which was fed a mixture of equal parts of dimers and corn oil, runs slightly under the control curves, but the difference seems to be insignificant. The aspect of the animals was completely normal, indicating either a dilution of dimers to nontoxic levels or a protection by the nonheated oil, as previously pointed out by others (24).

The above evidences suggest that the dimeric fraction, although absorbed in relatively small degree, has a weak toxic effect to which the rat appears to develop a tolerance. The toxic effect also disappears as soon as other fat is substituted for dimers in the diet or fresh nontoxic fat is added to the diet.

The group fed nonadduct-forming monomers (group 5) showed rather different characteristics. Loss of weight was much more marked than in groups fed dimers and corn oil. Hair became yellowish after five days but did not fall out. At the end of the 32-day period the only surviving rat looked meager and inactive. There was one death on the 14th day, two on the 15th, and one each on the 18th and 23rd days. The daily average feed consumption of 6.2 g./day/rat was small as compared with that of 11.7 to 15.8 g./day/rat of the control groups (Table IV). However it was larger than the 5 g./day/rat of the corn oil-fed group. The loss of weight was also much more remarkable than in this last group, thus indicating a deleterious effect. The ratio of body weight to liver weight (Table VI) was notably diminished in the only animal of this group that was autopsied.

It is thus evident that nonadduct-forming monomers from methyl linoleate possess a lethal effect although to a lesser degree than similar fractions from heated linolenic acid-rich oils (6). This weaker toxic activity, as well as the very small amounts of this fraction formed on heating linoleic acid-rich oils, may explain the lack of harmful effects found when sunflower seed and soybean oils were tested by Crampton.

TABLE V

Ratio of Body to Liver Weight[a]

Group No.	No. of rats	Fat in the diet	Avg. body weight	Avg. liver weight	Body weight/ liver weight
			(g.)	(g.)	(g./g.)
1	5	None	243	10.2	23.8
2	5	Methyl linoleate	260	12.4	21.0
3	5	Adduct-forming monomers[b]	233	10.2	22.8
4[c]	2	Dimers[b]	202	12.7	15.9
4[d]	7	Dimers[b]	171	7.8	21.9
5	1	Nonadduct-forming monomers[b]	108	9.4	11.5

[a] Liver histology was normal in all animals.
[b] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[c] Sacrificed after 25 days of feeding.
[d] Sacrificed after 36 days of feeding.

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Toxicity of Fatty Acid Ester Hydroperoxides. (28809)

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The acute toxicity of fatty acid hydroperoxides is well recognized(1-3). Such toxicity is demonstrable by feeding tests; by injection or oral dosage(4,5); and even by inunction on the skin of rats(6). The mechanism for this toxicity is unknown but it is a reasonable hypothesis that hydroperoxides may be a source of damaging free radicals (7-9). The experiments to be described were done in an attempt to determine whether large doses of tocopherol or ethoxyquin might modify the lethality of hydroperoxides.

Experimental. Peroxide concentrates were prepared by aerating samples of crude methyl linoleate at room temperature (22-24°C). Methyl esters of safflower seed oil fatty acids, furnished by the Pacific Vegetable Oil Co., contained approximately 73% methyl linoleate (by gas chromatography). No antioxidants had been added during its preparation. One sample was aerated at 40-50°C for one day and thereafter at room temperature. On days 3, 4, 5 and 6 the peroxide values were 1990, 2560, 2570, and 2600, respectively (in milliequivalents per kg fat).

Peroxide values were determined by a modification of AOCS standard procedure Cd 8-53 as follows: 20-30 mg samples, 3 ml of chloroform-glacial acetic acid (3:1), 50 ml flask, 1 ml of saturated potassium iodide, 2 minutes reaction at room temperature under nitrogen atmosphere, 10 ml water, titration with 0.01 N $Na_2S_2O_3$.

Hydroperoxide concentrates were prepared by a modification of the procedures described by Banks(10). The reaction mixture was separated between petroleum ether and 85% aqueous methanol. The 85% aqueous methanol-soluble fraction was then recovered after dilution with water, followed by extraction with petroleum ether. Such preparations had peroxide values varying from 3800 to 4700 (theoretical for methyl linoleate hydroperoxide 6135). They were stable for months at —18°C. Preparations made from samples which had been aerated longer than necessary to reach maximum peroxide values contained a fraction characterized by its insolubility in petroleum ether. Peroxide values of these fractions varied from 3300 to 4700. A determination of average molecular weight on one preparation (by osmometry) gave 510. The petroleum-ether-soluble peroxide concentrate had a molecular weight by this method, 340; calculated for methyl linoleate hydroperoxide, 326. The petroleum-ether-insoluble fraction thus appeared to be in part a dimer. It was found to be at least as toxic as the soluble fraction but has not yet been studied in more detail.

Toxicity of these preparations in rats was measured after intraperitoneal injection (Table I). Particularly viscous preparations were diluted with crude methyl linoleate which, by itself, was innocuous by the same route. Rats were kept on a normal stock diet. Death usually occurred within 24 hours but occasionally rats died after 2 or 3 days. Those that survived longer showed no obvious long-term ill-effects. One rat was injected on several occasions with close to lethal doses without evidence of accumulated damage. The only gross symptom in all rats that died was massive ascites. These observations are in accord with those of Horgan *et al*(4) with mice and of Vishida and Kummurow(11) with methyl linoleate hydroperoxide injected into rats.

Intraperitoneal injection of tocopherol suspended in crude methyl linoleate into numerous rats caused no death or obvious discomfort. Results obtained with rats first injected with tocopherol and 24 hours later with hydroperoxide preparations are shown in Table I. If there was a protective effect, it was very slight. Preliminary experiments had shown that simultaneous injections of tocopherol and hydroperoxide also did not change the level of the LD_{50} dose. Similarly in a limited series the antioxidant ethoxyquin appeared not to effect the lethality of the hydroperoxide.

TABLE I. Effect of Tocopherol and Ethoxyquin on Intraperitoneal Toxicity of Hydroperoxide.

Pretreatment*†					
None		Tocopherol		Ethoxyquin	
Hydroperoxide, μM/100 g	Lethality	Hydroperoxide, μM/100 g	Lethality	Hydroperoxide, μM/100 g	Lethality
43–56	0/3	—	—	—	—
61–74	2/2	—	—	—	—
82–109	0/3	93–95	1/2	—	—
113–118	1/2	—	—	—	—
149–150	2/6	150	0/4	150	2/4
155–170	8/8	170	4/5	—	—
180	7/8	180	5/8	185	1/1
185	25/25	188–238	0/4	196	0/1
—	—	240	10/10	238	1/1

* Rats (300-350 g) were injected intraperitoneally with an amount of α-tocopherol or ethoxyquin equivalent to the dose of hydroperoxide injected 24 hr later.

† Rats kept on a stock diet manufactured by Diablo Laboratories in Berkeley, Calif., which contained 4.8% fat, supplemented with Vit. E.

Discussion. The LD_{50} of methyl linoleate hydroperoxide was 150-170 μM per 100 g body weight, whether or not tocopherol in equimolar amounts had been presented to the animal. Horgan *et al* reported that the LD_{50} of the peroxides of autoxidized methyl linoleate was 45-60 μM per mouse. In contrast, the LD_{50} of autoxidized linoleic acid was 6-8 μM peroxide per mouse (about 25 μM *per* 100 g). Holman and Greenberg(5) found that ethyl linoleate hydroperoxide had a LD_{50} of 12 mg (35 μM) in mice. The hydroperoxide of methyl oleate acid was more toxic, LD_{50}, 6 mg (18 μM per mouse).

Introduction of the peroxide by stomach tube killed three rats at the level of 1600 μM per g. Lower levels caused drastic loss of fluid from the intestinal tract but no deaths. In the experiments of Holman and Greenberg (5) mice were not killed by single 200 mg doses of either ethyl linoleate hydroperoxide (588 μM) or methyl oleate hydroperoxide (610 μM) nor by 75 mg doses daily for two weeks of either. These combined observations indicate that hydroperoxides do not easily pass through the intestinal wall (cf. Mead, 12), and also that they are highly irritating. In a separate experiment carried out with the help of F. DeEds and J. O. Thomas at the Western Regional Research Laboratory, USDA, Albany, California, hydroperoxide concentrate (5-10 μM) was injected into the lumen of a tied-off segment of a rat small intestine. In comparison with a control segment the injected segment more than doubled in weight in one hour, mostly due to an increase in mucus excretion into the intestine, clear indication of intense irritation (cf. 13).

TABLE II. Toxicity of Methyl Linoleate Hydroperoxide Concentrate when Administered by Stomach Tube.

Hydroperoxide, μM/100 g	Symptoms*	Loss in wt, %*
400	None	5, 5, 7
800	Diarrhœa	5, 7, 11
1600	Dead	11, 16, 17

* Original weights, 300-350 g. Weighed again at 18 hr. There were no subsequent deaths. Surviving animals recovered and gained weight.

These combined observations suggest that hydroperoxides kill by attack on some vital tissue not yet identified. It is possible that sulfhydryl enzymes(14) or cytochromes(8) are the vulnerable tissue components. Whatever the mechanism, neither tocopherol nor ethoxyquin are effective in reducing the dose required to kill, either when made available simultaneously or 24 hours previous to the administration of the toxic dose.

The lack of effectiveness of tocopherol is parallel to the observed relative ineffectiveness of tocopherol in combating radiation

toxicity which, it has been hypothesized, may be mediated through the formation of hydroperoxides(4).

Summary. Intraperitoneal injection of concentrates of methyl linoleate hydroperoxide into adult rats was lethal at a level of about 150 μM per 100 g. Previous injection of equal amounts of tocopherol or ethoxyquin did not change the LD_{50} level. Sixteen hundred but not 800 μM per 100 g were lethal when administered by stomach tube.

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Inactivation of Streptococcal Bacteriophage by Sulfhydryl Reagents.* (28810)

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Work reported previously indicated that the host range of beta-hemolytic streptococcal bacteriophage is a group-specific phenomenon. Thus, the Group A phage lyses Group A streptococci but not Group C and similarly, Group C phage lyses Group C streptococci but not Group A. Such group specificity of these phages suggests that the cell wall carbohydrate, a significant constituent shared by all streptococcal strains of the same serologic group, may serve as a receptor substance for attachment of the phage to the organism(1). Additional support for this view has been obtained by experiments which demonstrated the inactivation of Group C phage but not Group A phage with the purified cell wall carbohydrate of Group C streptococci. It is clear, however, that factors other than the nature of the specific attachment substance of the bacterial cell wall control the adsorption and penetration of the virus into the host cell. For example, recent studies by Kosloff have emphasized the role of sulfhydryl groups in T2 bacteriophage invasion of *E. coli*(2).

In related experiments with animal viruses, Choppin and Philipson reported inactivation of enteroviruses by treatment with mercaptide forming reagents such as p-chloromercuribenzoate; mercuric ion and Thimerosal; an inactivation which was reversed with subsequent treatment by the thiol compound, reduced glutathione(3). In the experiments reported here, inactivation of streptococcal phage with the mercaptide forming reagents was also partially reversed by reduced glutathione.

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Materials and methods. Group A phage lysates were made by lysis of streptococcal Group A strain T25/41 with A25 phage stock and similarly Group C phage lysates were prepared by the lysis of Group C strain 26RP66 with C1 phage stock. The soft agar layer technique was employed to determine the number of plaque forming phage particles (4). The special media employed as the nutrient source for the agar plates has been described(1). Streptococcal strains T25/41 and 26RP66 were used as indicator strains in the soft agar layer for phages A25 and C1 respectively.

The various phage inactivation experiments were carried out as follows. Two-tenths ml of an appropriate concentration of sulfhydryl reagent was added to 1.8 ml of phage lysate which had been diluted in dialysate broth to a concentration of approximately 10^7 plaque forming units per ml. The mixture was incubated at 24°C and 0.1 ml samples, taken at intervals were diluted immediately into 9.9 ml of Todd-Hewitt broth to halt inactivation. Additional dilutions made from this diluted sample were plated to determine the titer of plaque forming units. Controls were performed in a manner similar to the experimental system and final results were calculated as percent of control values. The dialysate broth employed in the inactivation step has been described(1).

Stock solutions of p-hydroxymercuribenzoate (PHMB)§ were prepared in glycylglycine buffer at pH 8.6. The reagent remained in solution upon subsequent dilution into dialysate broth at pH 7.5. Aqueous solutions of Thimerosal, $HgCl_2$, $ZnCl_2$ and $CdCl_2$, were diluted to appropriate concentration with the dialysate broth.

Results. The inactivation of Groups A and C phage with p-hydroxymercuribenzoate, (which will hereafter be referred to as PHMB), is shown in Fig. 1. Rapid inactivation of the Group C phage Cl occurred with a concentration of 1.0×10^{-3} M PHMB; the Group A phage A25, on the other hand, was consistently less sensitive to the action of this reagent. At a concentration of 1×10^{-3} M PHMB 99% of the Cl phage was inactivated in 10 minutes whereas only 50% of the A25 phage was inactivated.

§ Nutritional Biochemicals Co.

Fig. 2 illustrates the inactivation of phage by Hg^{++} and demonstrates the fact that phage is less sensitive to PHMB than to this ion. For instance, at a concentration of 3.6×10^{-4} M Hg^{++}, Cl phage inactivation is comparable to that achieved with 1×10^{-3} M PHMB as depicted in Fig. 1. This difference in the effectiveness of the inactivation by these 2 substances was a consistent finding in several experiments over a range of inactivator concentrations. In general a concentration of PHMB 3 to 5 times that of $HgCl_2$ gave comparable inactivation. 1×10^{-3} M Thimerosal, another organic mercurial, also inactivates the phage, but is somewhat less effective than PHMB at the same concentration.

Because mercuric ion is bivalent and in Group IIb of the periodic table the inactivation of phage by the other elements of this group is of interest. Fig. 3 illustrates a comparison of the inactivation of Group C phage by zinc ion, cadmium ion, and mercuric ion. At a concentration of 1×10^{-3} M zinc or cadmium ion, phage is inactivated more slowly than by 3.6×10^{-4} M mercuric ion. Thus the ions of the elements of Group IIb inactivate the Cl phage, but Hg^{++} is more effective in this respect.

In view of the recent finding of Choppin and Philipson that the inactivation of enterovirus infectivity with mercaptide forming reagents is reversed with subsequent treatment by reduced glutathione(3), it was of interest to determine the effect of similar treatment on bacteriophage previously inactivated with PHMB or mercuric ion. In the following experiments Cl bacteriophage inactivated by PHMB or mercuric ion was subsequently treated with reduced glutathione. Cl phage was treated with either 5×10^{-4} M PHMB or 3.3×10^{-5} M mercuric ion. After 5 min-

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

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

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

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

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

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Abstract

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

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

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

TABLE I

Death Rate at Different Ages of Male Rats Fed Various Fats

Age (wk)	Cottonseed oil		Olive oil	
	Fresh	Autoxidized	Fresh	Autoxidized
4-33	1/40	0/40	0/40	0/40
33-73	2/33	4/34	4/34	0/34
73-99	7/25	9/24	8/24	6/28
99-108	4/12	5/9	4/10	3/16
	Chicken fat		Beef fat	
4-99	2/20	5/20	2/20	4/20
99-108	6/12	5/9	2/12	4/12

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

The fats were aerated for 40 hr at 60C at an airflow of 1–2 liters/min. Peroxide values for successive batches of rancid cottonseed oil were 122.6, 43.8 and 97.0 and for rancid olive oil, 12.7, 324.0 and 20.8. Although these values varied considerably, no attempt was made to keep the peroxide values at the same levels because conditions of oxidation had been predetermined and were kept as constant as possible. Variations in peroxide number may have been due to different amounts of antioxidants present in different batches of the oils. The fresh and oxidized oils were included at a level of 20% in a diet composed of 30% alcohol-washed casein, 44% dextrose, 3.5% USP XIII salt mixture, 0.5% calcium carbonate, 2% cellulose, and the following vitamin supplements (in mg/kg): (choline dihydrogen citrate 1000, inositol 1000, nicotinamide 100, p-aminobenzoic acid 300, thiamine · HCl 2, pyridoxine · HCl 4, riboflavin 4, Ca pantothenate 10, folic acid 2.5, biotin 0.025, ascorbic acid 25, vitamin K 10, vitamin B_{12} (0.1% trituration in mannose) 5, crystalline beta-carotene 5, alpha-tocopherol acetate 50, free alpha-tocopherol 10 and crystalline vitamin D_2 0.5). To insure an adequate vitamin intake despite the oxidized fat in the diet, each rat was given a weekly oral supplement of 3 drops of Vi Penta Multivitamin suspensions from Hoffman-LaRoche.

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

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

TABLE II

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

	Fresh oil		Autoxidized oil	
	Food intake, g	Body weight, g	Food intake, g	Body weight, g
Cottonseed oil	108.5 ± 5.19[a]	613 ± 25	106.8 ± 3.88	633 ± 23.9
Olive oil	94.1 ± 4.67[b]	588 ± 19.8	97.3 ± 3.32	565 ± 20.0
Chicken fat	86.5 ± 3.73	591 ± 16.3	85.0 ± 5.49	583 ± 21.5
Beef fat	123.6 ± 3.94	599 ± 19.4	113.8 ± 4.76	582 ± 23.3

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

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

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

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

TABLE III

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

	Cholesterol, mg		
	Serum[a]	Liver	Brain
Fresh cottonseed oil	118 ± 9.9	209	3920
Oxidized cottonseed oil	116 ± 9.6	264	3930
Fresh olive oil	99 ± 8.9	562	4190
Oxidized olive oil	95 ± 9.0	607	4190
Fresh chicken fat	123 ± 11.3	527	3870
Oxidized chicken fat	138 ± 13.1	320	3780
Fresh beef fat	155 ± 16.9	346	3670
Oxidized beef fat	119 ± 3.8	384	4110

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

TABLE IV

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

	C14	C16	C16:1	C18	C18:1	C18:2	C20:4
				Depot fat			
Cottonseed oil							
Fresh							
33 weeks	0.9	20.2	2.2	1.4	22.8	52.5	.....
73 weeks	1.3	18.8	3.1	2.5	26.6	47.8	.....
99 weeks	1.3	17.2	1.6	1.6	24.2	54.1	.....
108 weeks	0.3	16.9	0.6	2.3	30.6	49.3	.....
Oxidized							
33 weeks	0.8	21.6	2.9	2.0	23.7	49.0	.....
73 weeks	0.9	19.0	1.7	2.0	28.6	47.7	.....
99 weeks	1.1	16.2	1.6	2.2	25.7	53.2	.....
108 weeks	...	14.3	0.4	0.7	28.5	56.0	.....
Olive oil							
Fresh							
33 weeks	0.5	11.2	2.6	1.4	74.5	5.9	.....
73 weeks	0.3	13.6	3.7	1.2	70.0	11.2	.....
99 weeks	...	12.4	2.7	1.5	74.6	4.8	.....
108 weeks	....	8.5	1.6	1.2	83.4	5.2	.....
Oxidized							
33 weeks	0.3	11.2	2.9	1.0	79.2	5.4	.....
73 weeks	0.4	16.4	3.7	1.7	66.8	11.0	.....
99 weeks	0.5	11.0	2.7	1.5	72.6	11.7	.....
108 weeks	....	10.2	1.8	2.1	82.8	3.1	.....
Chicken fat							
Fresh							
112 weeks	....	15.2	3.4	1.3	64.7	15.3	.....
Oxidized							
112 weeks	...	14.5	4.0	0.5	69.5	11.3	.....
Beef fat							
Fresh							
112 weeks	0.5	17.1	3.9	3.2	73.8	1.5	.....
Oxidized							
112 weeks	0.4	17.3	3.3	2.9	74.8	1.3	.....
				Liver			
Cottonseed oil							
Fresh							
33 weeks	0.8	25.3	1.2	10.4	13.2	34.1	14.3
73 weeks	0.4	23.1	1.1	11.9	12.7	32.1	17.2
99 weeks	0.2	21.1	...	12.7	10.4	33.6	22.0
108 weeks	....	21.5	0.3	20.1	15.9	19.3	22.9
Oxidized							
33 weeks	0.1	24.1	1.3	11.7	13.4	34.0	14.5
73 weeks	0.3	19.4	....	12.3	15.9	39.1	14.7
99 weeks	...	22.3	...	12.8	11.3	31.4	22.3
108 weeks	....	21.0	0.2	23.6	6.0	21.2	28.0
Olive oil							
Fresh							
33 weeks	0.6	17.9	2.9	6.0	61.6	5.5	5.1
73 weeks	0.4	19.8	1.7	7.1	47.9	13.4	9.0
99 weeks	0.3	18.2	1.1	8.3	43.7	13.1	15.3
108 weeks	...	19.7	0.6	7.9	55.1	3.8	11.4
Oxidized							
33 weeks	0.2	16.0	2.1	7.5	61.2	5.8	6.9
73 weeks	0.2	19.7	1.1	10.9	46.5	8.6	11.5
99 weeks	0.3	16.3	...	13.6	44.9	8.7	16.2
108 weeks	...	22.7	1.9	6.7	51.3	4.6	10.7
Chicken fat							
Fresh							
112 weeks	....	22.7	1.2	15.1	29.6	13.5	17.9
Oxidized							
112 weeks	....	23.7	1.5	14.9	27.1	12.6	20.2
Beef fat							
Fresh							
112 weeks	0.1	21.7	0.5	20.2	35.6	2.7	19.2
Oxidized							
112 weeks	....	19.9	1.5	22.6	40.0	2.6	14.1
				Heart			
Cottonseed oil							
Fresh							
33 weeks	1.2	17.5	3.6	23.3	10.6	30.2	12.4
73 weeks	0.6	11.9	0.7	16.9	13.2	23.4	22.6
99 weeks	....	12.4	...	23.1	7.9	25.9	30.7
108 weeks	....	15.3	0.1	28.8	9.2	21.9	24.7
Oxidized							
33 weeks	1.6	24.0	3.9	17.5	24.9	22.5	4.3
73 weeks	....	10.4	...	20.5	12.7	28.7	19.2
99 weeks	....	13.4	...	22.9	10.1	23.9	29.7
108 weeks	....	13.5	0.1	30.5	6.8	23.9	24.9
Olive oil							
Fresh							
33 weeks	0.4	10.4	3.4	16.3	35.9	16.2	7.9
73 weeks	0.5	11.3	1.0	18.0	26.3	11.9	26.4
99 weeks	....	13.7	0.4	21.2	16.0	19.4	31.2
108 weeks	....	12.2	....	29.1	39.9	6.8	10.1
Oxidized							
33 weeks	0.8	14.4	1.1	24.2	35.0	9.8	14.8
73 weeks	0.1	11.1	0.9	16.6	32.8	14.3	21.8
99 weeks	....	11.7	...	21.2	24.8	9.0	33.3
108 weeks	....	11.5	0.2	26.7	23.8	13.1	24.1
Chicken fat							
Fresh							
112 weeks	...	12.9	0.3	25.9	15.2	17.1	28.3
Oxidized							
112 weeks	....	11.5	0.1	30.3	12.1	16.5	29.2
Beef fat							
Fresh							
112 weeks	....	13.1	0.1	30.4	18.7	6.0	31.7
Oxidized							
112 weeks		16.9	0.3	30.9	20.3	6.9	23.8

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

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

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

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

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

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

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

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

TABLE V

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

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

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

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

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

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

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

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Nutritive Value of Heated Vegetable Oils

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Abstract

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

Introduction

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

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

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

Experimental and Results

Preparation of Heated Oils

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

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

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

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

TABLE I

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

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

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

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

TABLE II

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

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

[a] Standard error of the mean.

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

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

Effect of Heated Oils on the Absorption of Carbohydrates

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

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

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

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

Influence of Heated Oils on the Blood Sugar and Cholesterol Levels

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

The results show that the presence of heated oil

TABLE III

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

Time in hours after feeding	Raw peanut oil diet: Digestion %	Raw peanut oil diet: Absorption %	Heated peanut oil diet: Digestion %	Heated peanut oil diet: Absorption %
2	11.0 ± 0.6	7.0 ± 0.3	9.0 ± 0.5	4.0 ± 0.2
3	34.0 ± 5.6	21.0 ± 4.4	22.0 ± 4.3	13.0 ± 1.2
4	62.0 ± 8.7	49.0 ± 7.6	39.0 ± 3.5	23.0 ± 2.6
5	78.0 ± 6.4	68.0 ± 5.6	58.0 ± 5.4	35.0 ± 3.5

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

Discussion

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

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

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

TABLE IV

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

Constituent ml/100 ml blood	Raw peanut oil	Heated peanut oil
Glucose	105 ± 5.6[a]	131 ± 7.7
Cholesterol	76 ± 6.6	107 ± 3.8

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

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

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Abstract

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

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

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

Experimental

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

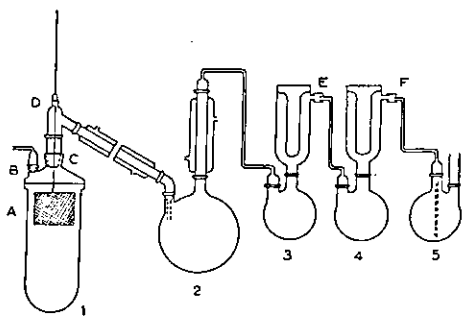

FIG. 1

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

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

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

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

TABLE I

Data Pertaining to the Frying Operation[a]

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

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

TABLE II

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

Fatty acid	Fat					
	Corn oil		Lard		Shortening	
	Fresh	Heated	Fresh	Heated	Fresh	Heated
12:0			Trace	Trace	3.65	3.45
14:0			1.24	1.32	1.42	1.52
16:0	14.1	13.6	30.1	29.5	21.4	22.3
16:1			1.74	2.04		
17:0			Trace	Trace		
18:0	2.00	2.30	16.3	16.7	10.6	10.3
18:1	28.6	30.8	42.0	42.3	49.3	47.8
18:2	55.0	53.7	8.56	8.18	13.7	14.7
18:3	Trace	Trace	Trace	Trace	Trace	Trace

TABLE III

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

Compound class, chain length	Micromoles of carbonyl compound per kg fat								
	Corn oil			Lard			Hydrogenated vegetable oil		
	Fresh	Heated	Distillate	Fresh	Heated	Distillate	Fresh	Heated	Distillate
Alkanones									
C_6	49	55	8.2	9.1	5.0	18			25
C_7	0.74					1.3			tr
C_8	1.4	1.2			0.44	2.0			tr
C_9	2.3	1.9			1.1	3.2			tr
C_{10}	1.4	1.4				2.9			tr
C_{11}	5.9	6.8		0.14		2.9			tr
C_{12}				0.14		1.6			tr
C_{13}				0.20		1.3		1.5	tr
C_{11}	0.46	0.57							
Alkanals									
C_5						50			36
C_6			4.8			10			11
C_8			3.3			6.1			
C_7			31			36	1.5	1.9	26
C_8	2.5		28	9.3		49	2.8	6.8	35
C_9	3.8		41	13		61	0.64	2.5	25
C_{10}			6.6	4.2		22	0.48	5.4	8.8
C_{11}			1.7	5.8	1.9	16	0.64	2.0	4.9
C_9			5.6	2.6		32	tr	tr	9.3
C_{12}				5.7		4.9		3.9	2.4
C_{13}				4.2		7.2			
Alk-2-enals									
C_5						1.6			
C_6						3.3		1.3	
C_8			5.1			5.4		1.0	6.2
C_9			38	4.0	1.8	53		7.9	26
C_{10}	1.3		12	2.4	1.4	28		3.0	20
C_8	1.4	0.79	4.3	3.9	2.0	19		2.0	5.4
C_{12}	0.72	1.8	11	5.2	8.6	53	tr	6.2	12
C_{11}		1.5	7.1	5.5	18	29		5.6	10
C_{13}		3.6		2.9	9.5	1.1			0.53
Alk-2,4-dienals									
C_7				2.2		10			8.2
C_8				0.88					
C_9			15	4.4		1.6	0.28		
C_{10}		160	70	15	28	87	1.4	9.0	44
C_{11}				13	23		1.1	11	
Total volatile monocarbonyls	70.9	234	294	114	101	622	8.84	72.6	306

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

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

Discussion

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

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

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

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SYMPOSIUM: THERMAL OXIDATION AND POLYMERIZATION IN FATS

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

MADHU R. SAHASRABUDHE, PRESIDING
H. P. DUPUY, PROGRAM CHAIRMAN

Introduction: Studies on Heated Fats

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

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

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

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

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

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

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

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

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

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

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

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

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Abstract

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

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

Introduction

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

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

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

Experimental

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

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

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

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

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

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

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

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

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

TABLE I

Distillation of Polymers

Fraction	Temp °C	Oxidative		Thermal	
		Yield %	Isolated *trans* %	Yield %	Isolated *trans* %
Monomer	140	17	15.5	39	14.9
Dimer	200	50	19.1	47	22.3
Trimer	250	20	18.2	....	....
Residue	<250	13	16.6	14	20.3

TABLE II

Hydroxyl Content of Fractions from Methyl Linoleate Oxidative Dimer

Chromatographic fraction	Hydroxyl %	
	Run A	Run B
Peak I	Trace	Trace
Peak II	27.1	18.5
Peak III	48.7	57.4
Peak IV (ether)	123.5[a]	123.0[a]
Original dimer	29.9	32.1

[a] Methyl ricinoleate = 100%.

Results

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

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

TABLE III

Mol Wt of Polymers

Type	Distilled ester	Chromatographed ester		Distilled ester saponified and reesterified
Thermal				
Monomer	265	.....		.....
Dimer	590	.....		.....
Residue	840	.....		.....
Oxidative				
Monomer	275	I[a]	546	465
Dimer	560	II	570	600
Trimer	840	III	586	1085
Residue	1930	IV	540	1660

[a] Peak I, of chromatographed dimer.

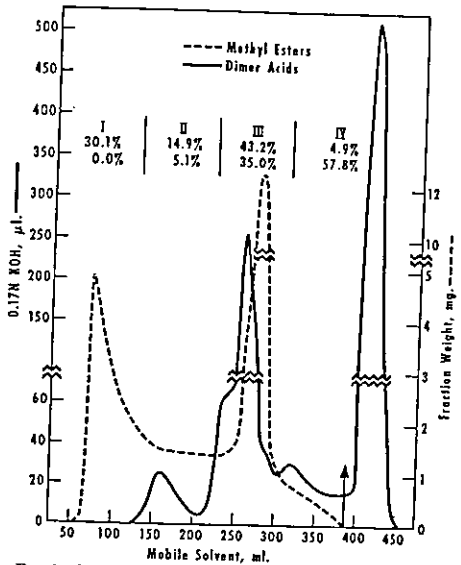

FIG. 1. Chromatograms of oxidative methyl linolenate dimers as esters and after saponification as free acids. Arrow indicates when diethyl ether added as eluting solvent.

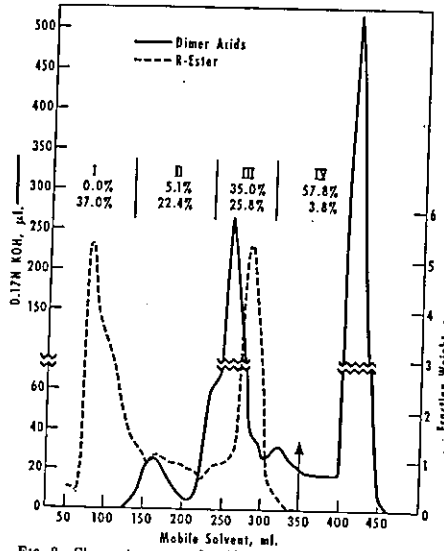

FIG. 2. Chromatograms of oxidative methyl linoleate dimers as free acids and after reesterification (R-ester).

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

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

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

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

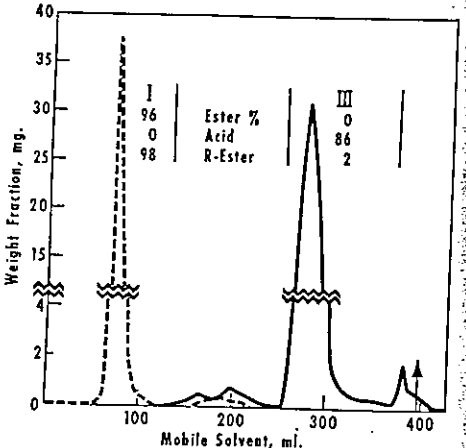

FIG. 3. Chromatograms of thermal methyl linoleate dimers as esters, as free acids after saponification, and as esters after reesterification (R-ester). The two ester curves coincide.

TABLE IV
Chromatographic Fractionation of Oxidative and Thermal Polymers

Chromatogram	Peak area I %	II %	III %	IV %	Column recovery %
Oxidative polymers					
Monomer					
Ester	20	25	14	3	62
Acid	.....	12	44	51	107
Reesterified	20	31	33	4	88
Dimer					
Acid	34	26	36	5	101
Ester	.....	4	42	50	96
Reesterified	31	29	34	5	99
Trimer					
Ester	24	27	44	6	101
Acid	.....	2	26	72	100
Reesterified	15	22	46	6	89
Residue					
Ester	(←26.1→)		59.1	12.2	97.9
Acid	Mostly insoluble in mobile solvent				
Reesterified	(←22.6→)		56.1	10.4	89.1
Thermal polymers					
Monomer					
Ester	100	2.1	0.0	2.1	105.1
Acid	.....	92.9	4.2	1.7	99.3
Reesterified	73.6	4.3	5.5	2.5	85.8
Dimer					
Ester	95.6	1.8	.....	1.4	99
Acid	.....	4.7	85.5	5.6	96
Reesterified	98.2	.....	1.7	1.0	100.9
Residue					
Ester	94.4	2.1	1.7	1.3	99.5
Acid	.....	1.4	62.3	28.4	92.1
Reesterified	84.0	(←16.4→)		1.5	101.9
Subfractions of oxidative dimer					
Dimer mtar	29.3	13.8	30.3	4.3	86.6
Peak I	[29.3]				
Saponified	.....	8.0	47.4	40.0	95.4
Reesterified	67.2	15.6	17.1	1.6	97.0
Peak III			[30.3]		
Saponified	.....	.....	17.9	7.6 + 70.9	96.4
Reesterified	19.0	15.1	49.5	13.5	97.1
Subpeak III			[17.9]		
Reesterified	30.3	35.7	33.7	2.9	102.6
Subpeak IVa				[7.6]	
Reesterified	28.3	44.9	26.7	6.5	107.3
Subpeak IVb				[70.9]	
Reesterified	3.1	15.5	67.2	10.8	96.5
Subfractions of thermal dimer					
Dimer ester	95.8	1.8	.....	1.4	99.0
Peak I	[95.8]				
Saponified	.....	1.5	79.1	14.6	95.2
Reesterified	99.3	..	..	1.7	101.0
Dimer acid	.....	4.7	85.5	5.6	95.8
Peak III			[85.5]		
Reesterified	95.3	.....	.....	2.1	98.6
1:1 Mixture of oxidative and thermal polymers					
Dimer 1:1					
Esters	58.6	12.8	20.3	2.8	94.5
Acids	.....	4.8	58.4	31.5	94.8
Reesterified	61.8	9.9	21.0	4.2	96.8
Theoretical for 1:1 mixture					
Dimer 1:1					
Esters	62.6	7.8	19.7	2.8	.....
Acids	.....	5.0	60.2	31.7	.....
Reesterified	67.6	11.2	13.8	2.4	.....

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

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

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

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

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

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

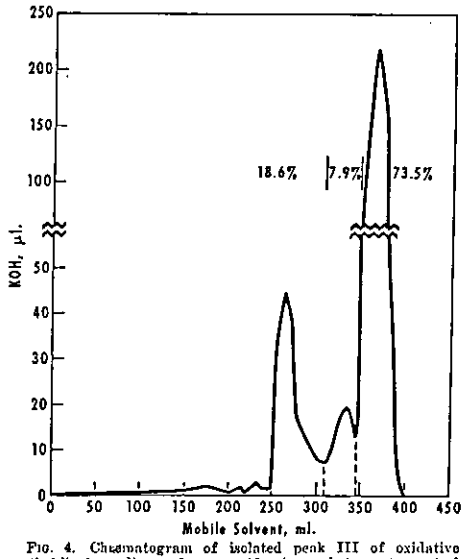

FIG. 4. Chromatogram of isolated peak III of oxidative methyl linolcate dimer after saponification and chromatographed as dimer acids.

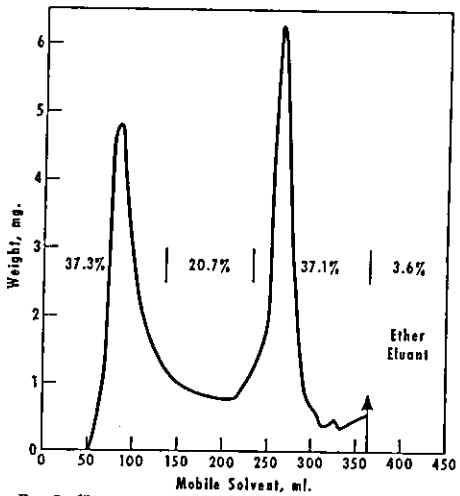

FIG. 5. Chromatogram of oxidative soybean oil methyl ester dimers.

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

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

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

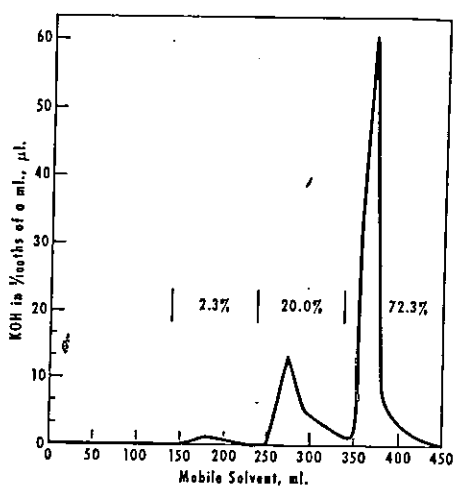

FIG. 6. Chromatogram of oxidative soybean oil methyl ester dimers after saponification.

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

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

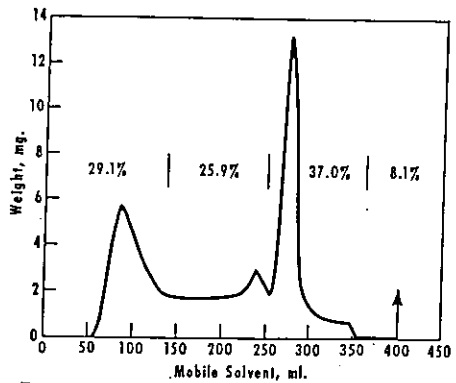

FIG. 7. Chromatogram of oxidative soybean methyl ester dimer after saponification and reesterification.

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

Discussion

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

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

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

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

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

TABLE V

Chromatographic Separations and Properties of Oxidative and Thermal Dimers

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

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

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

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

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

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

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Abstract

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

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

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

TABLE I

Death Rate at Different Ages of Male Rats Fed Various Fats

Age (wk)	Cottonseed oil		Olive oil	
	Fresh	Autoxidized	Fresh	Autoxidized
4–33	1/40	0/40	0/40	0/40
33–73	2/33	4/34	4/34	0/34
73–99	7/25	9/24	8/24	6/28
99–108	4/12	5/9	4/10	3/16
	Chicken fat		Beef fat	
4–99	2/20	5/20	2/20	4/20
99–108	6/12	5/9	2/12	4/12

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

The fats were aerated for 40 hr at 60C at an airflow of 1–2 liters/min. Peroxide values for successive batches of rancid cottonseed oil were 122.6, 43.8 and 97.0 and for rancid olive oil, 12.7, 324.0 and 20.8. Although these values varied considerably, no attempt was made to keep the peroxide values at the same levels because conditions of oxidation had been predetermined and were kept as constant as possible. Variations in peroxide number may have been due to different amounts of antioxidants present in different batches of the oils. The fresh and oxidized oils were included at a level of 20% in a diet composed of 30% alcohol-washed casein, 44% dextrose, 3.5% USP XIII salt mixture, 0.5% calcium carbonate, 2% cellulose, and the following vitamin supplements (in mg/kg): (choline dihydrogen citrate 1000, inositol 1000, nicotinamide 100, p-aminobenzoic acid 300, thiamine · HCl 2, pyridoxine · HCl 4, riboflavin 4, Ca pantothenate 10, folic acid 2.5, biotin 0.025, ascorbic acid 25, vitamin K 10, vitamin B_{12} (0.1% trituration in mannose) 5, crystalline betacarotene 5, alpha-tocopherol acetate 50, free alpha-tocopherol 10 and crystalline vitamin D_2 0.5). To insure an adequate vitamin intake despite the oxidized fat in the diet, each rat was given a weekly oral supplement of 3 drops of Vi Penta Multivitamin suspensions from Hoffman-LaRoche.

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

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

TABLE II

Average Body Weights and Food Intake of Rats on Different Fats at 95 Weeks

	Fresh oil		Autoxidized oil	
	Food intake, g	Body weight, g	Food intake, g	Body weight, g
Cottonseed oil	108.5 ± 5.10[a]	613 ± 25	108.8 ± 3.88	633 ± 23.9
Olive oil	94.1 ± 4.67[b]	588 ± 19.8	97.3 ± 3.33	585 ± 20.0
Chicken fat	86.5 ± 3.73	591 ± 16.3	85.0 ± 5.49	563 ± 21.5
Beef fat	123.6 ± 3.94	599 ± 19.4	113.8 ± 4.76	582 ± 23.3

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

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

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

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

TABLE III

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

	Cholesterol, mg		
	Serum[a]	Liver	Brain
Fresh cottonseed oil	118 ± 9.9	209	3920
Oxidized cottonseed oil	118 ± 9.6	264	4220
Fresh olive oil	99 ± 9.9	562	3930
Oxidized olive oil	95 ± 9.0	607	4190
Fresh chicken fat	123 ± 11.3	527	3870
Oxidized chicken fat	138 ± 13.1	320	3780
Fresh beef fat	155 ± 16.0	336	3670
Oxidized beef fat	119 ± 8.8	384	4130

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

TABLE IV

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

	C_{14}	C_{16}	C_{16}:1	C_{18}	C_{18}:1	C_{18}:2	C_{20}:4
Depot fat							
Cottonseed oil							
Fresh							
33 weeks	0.9	20.2	2.2	1.4	22.8	52.5	.....
73 weeks	1.2	18.8	3.1	2.5	26.6	47.6	.....
99 weeks	1.3	17.2	1.6	1.6	24.2	54.1	.....
108 weeks	0.3	16.9	0.6	2.3	30.6	49.3	.....
Oxidized							
33 weeks	0.8	21.6	2.9	2.0	23.7	49.0	.....
73 weeks	0.9	19.0	1.7	2.0	28.6	47.7	.....
99 weeks	1.1	16.2	1.6	2.2	25.7	53.2	.....
108 weeks	....	14.3	0.4	0.7	28.5	56.0	.....
Olive oil							
Fresh							
33 weeks	0.5	11.2	2.6	1.4	76.5	5.9	.....
73 weeks	0.3	13.6	3.7	1.2	70.0	11.2	.....
99 weeks	....	12.4	2.7	1.5	74.6	8.8	.....
108 weeks	....	8.5	1.6	1.2	83.4	5.2	.....
Oxidized							
33 weeks	0.3	11.2	2.9	1.0	79.2	5.4	.....
73 weeks	0.4	16.4	3.7	1.7	66.8	11.0	.....
99 weeks	0.5	11.0	2.7	1.5	72.6	11.7	.....
108 weeks	....	10.2	1.8	2.1	82.8	3.1	.....
Chicken fat							
Fresh							
112 weeks	....	15.2	3.4	1.3	64.7	15.3	.....
Oxidized							
112 weeks	....	14.5	4.0	0.5	69.5	11.3	.....
Beef fat							
Fresh							
112 weeks	0.5	17.1	3.0	3.2	73.8	1.5	.....
Oxidized							
112 weeks	0.4	17.3	3.3	2.9	74.8	1.3	.....
Liver							
Cottonseed oil							
Fresh							
33 weeks	0.8	25.3	1.2	10.4	13.2	34.1	14.3
73 weeks	0.4	21.1	1.1	11.9	12.7	32.1	17.2
99 weeks	0.2	21.1		12.7	10.4	33.6	22.0
108 weeks	....	21.5	0.3	20.1	15.9	19.3	22.9
Oxidized							
33 weeks	0.1	24.1	1.3	11.7	13.4	34.0	14.5
73 weeks	0.3	19.4	....	12.8	15.9	29.1	18.7
99 weeks	....	22.2	....	12.8	11.3	31.4	22.3
108 weeks	....	21.0	0.2	23.6	6.0	21.2	28.0
Olive oil							
Fresh							
33 weeks	0.6	17.9	2.9	6.0	61.6	5.5	5.1
73 weeks	0.4	19.8	1.7	7.1	47.9	13.4	9.0
99 weeks	0.3	18.2	1.1	8.3	43.7	13.1	15.3
108 weeks	....	19.7	0.6	7.9	55.1	8.8	11.4
Oxidized							
33 weeks	0.2	16.9	2.1	7.5	61.2	5.8	6.9
73 weeks	0.3	19.7	1.1	10.0	46.5	8.6	11.5
99 weeks	0.3	16.3		13.6	44.9	8.7	16.2
108 weeks	....	22.7	1.9	8.7	51.3	4.6	10.7
Chicken fat							
Fresh							
112 weeks	....	22.7	1.2	15.1	29.6	13.5	17.9
Oxidized							
112 weeks	....	23.7	1.5	14.9	27.1	12.6	20.2
Beef fat							
Fresh							
112 weeks	0.1	21.7	0.5	20.2	35.6	2.7	19.2
Oxidized							
112 weeks	....	19.0	1.5	22.6	40.0	2.6	14.1
Heart							
Cottonseed oil							
Fresh							
33 weeks	1.2	17.5	2.6	23.3	10.6	30.2	12.4
73 weeks	0.6	11.9	0.7	16.9	13.2	21.4	22.6
99 weeks	....	12.4		23.1	7.9	25.9	30.7
108 weeks	....	15.3	0.1	28.8	9.2	21.9	24.7
Oxidized							
33 weeks	1.6	24.9	3.9	17.5	24.9	22.5	4.3
73 weeks	....	19.4	....	20.5	12.7	28.7	19.2
99 weeks	....	13.4	....	22.9	10.1	23.9	29.7
108 weeks	....	13.5	0.1	30.5	6.8	23.9	24.9
Olive oil							
Fresh							
33 weeks	0.4	19.4	3.4	16.3	35.3	16.2	7.9
73 weeks	0.5	11.3	1.0	18.0	26.2	13.9	26.4
99 weeks	....	13.7	0.4	21.2	16.0	19.4	31.2
108 weeks	....	12.2	....	29.1	30.9	6.8	10.1
Oxidized							
33 weeks	0.8	14.4	1.1	24.2	35.0	9.8	14.8
73 weeks	0.1	11.1	0.9	16.8	32.8	14.3	21.8
99 weeks	....	11.7		21.2	24.8	9.9	33.3
108 weeks	....	11.5	0.2	26.7	23.8	13.1	24.1
Chicken fat							
Fresh							
112 weeks	....	12.9	0.3	25.9	15.2	17.1	28.3
Oxidized							
112 weeks	....	11.5	0.1	30.3	12.1	16.5	29.2
Beef fat							
Fresh							
112 weeks	....	13.1	0.1	30.4	18.7	6.0	31.7
Oxidized							
112 weeks		16.9	0.3	30.9	20.3	6.9	23.8

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

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

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

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

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

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

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

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

TABLE V

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

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

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

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

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

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

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

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Nutritive Value of Heated Vegetable Oils

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Abstract

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

Introduction

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

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

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

Experimental and Results

Preparation of Heated Oils

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

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

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

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

TABLE I

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

Dietary fat	Body weight: Initial (g)	Body weight: Final (g)	Mean gain per week (g)	Mean daily food intake (g)	Feed efficiency[a]	Liver weight (g)	Liver weight: g/100 g body weight	Liver fat %
Peanut oil:								
Raw	42.5	120.7	13.0 ± 0.5[b]	11.3	1.1	4.58	3.8 ± 0.3	3.9 ± 0.8
Heated	42.0	72.1	5.0 ± 0.9	7.1	0.67	4.11	5.7 ± 0.6	7.1 ± 0.4
Sesame oil:								
Raw	42.5	102.5	10.0 ± 0.8	9.5	1.0	4.30	4.2 ± 0.7	4.8 ± 0.3
Heated	42.4	68.3	4.3 ± 0.7	5.9	0.7	4.20	6.0 ± 0.5	7.8 ± 0.3
Coconut oil:								
Raw	43.1	109.2	11.0 ± 1.1	9.5	1.1	4.70	4.3 ± 0.3	4.5 ± 0.3
Heated	42.8	69.9	4.5 ± 0.7	6.1	0.7	4.05	5.8 ± 0.4	7.0 ± 0.3

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

TABLE II

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

Vitamin	Diet: Raw peanut oil	Diet: Heated peanut oil
Thiamine	25.6 ± 2.3[a]	12.3 ± 1.6
Riboflavin	90.4 ± 8.6	67.4 ± 7.5
Niacin	652.0 ± 35.4	590.0 ± 34.6
Pantothenate	322.0 ± 21.6	175.0 ± 18.7
Pyridoxine	5.8 ± 0.3	2.5 ± 0.1

[a] Standard error of the mean.

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

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

Effect of Heated Oils on the Absorption of Carbohydrates

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

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

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

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

Influence of Heated Oils on the Blood Sugar and Cholesterol Levels

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

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

TABLE III

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

Time in hours after feeding	Raw peanut oil diet: Digestion %	Raw peanut oil diet: Absorption %	Heated peanut oil diet: Digestion %	Heated peanut oil diet: Absorption %
2	11.0 ± 0.6	7.0 ± 0.3	9.0 ± 0.5	4.0 ± 0.2
3	34.0 ± 5.6	31.0 ± 4.4	23.0 ± 4.3	13.0 ± 1.2
4	62.0 ± 8.7	49.0 ± 7.6	39.0 ± 3.5	23.0 ± 2.6
5	78.0 ± 6.4	68.0 ± 5.6	58.0 ± 5.4	35.0 ± 3.5

Discussion

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

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

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

TABLE IV

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

Constituent ml/100 ml blood	Raw peanut oil	Heated peanut oil
Glucose	105 ± 5.6[a]	131 ± 7.7
Cholesterol	76 ± 6.8	107 ± 3.8

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

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

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Abstract

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

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

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

Experimental

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

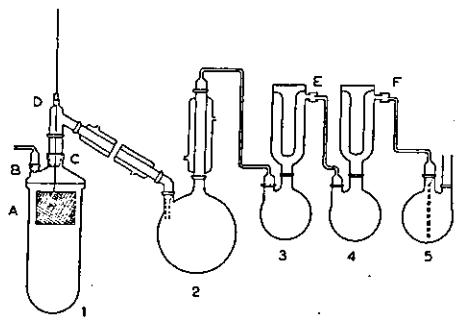

FIG. 1

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

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

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

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

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TABLE I

Data Pertaining to the Frying Operation[a]

	Corn oil	Lard	Hydrogenated vegetable oil
Weight of fat before frying	2000 g	2000 g	2000 g
Weight of fat after frying	1863 g	1836 g	1844 g
Volume of distillate	1575 ml	1600 ml	1730 ml
Color of oil after frying	Dark	Very dark	Light

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

TABLE II

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

Fatty acid	Fat					
	Corn oil		Lard		Shortening	
	Fresh	Heated	Fresh	Heated	Fresh	Heated
12:0			Trace	Trace	3.65	3.45
14:0			1.24	1.32	1.42	1.52
16:0	14.1	13.6	30.1	29.5	21.4	22.3
16:1			1.74	2.04		
17:0			Trace	Trace		
18:0	2.00	2.30	16.3	16.7	10.6	10.3
18:1	28.8	30.8	42.0	42.3	49.3	47.8
18:2	55.0	53.7	8.56	8.16	13.7	14.7
18:3	Trace	Trace	Trace	Trace	Trace	Trace

TABLE III

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

Compound class, chain length	Micromoles of carbonyl compound per kg fat								
	Corn oil			Lard			Hydrogenated vegetable oil		
	Fresh	Heated	Distillate	Fresh	Heated	Distillate	Fresh	Heated	Distillate
Alkanones									
C_6	49	55	8.2	9.1	5.0	18			25
C_7	0.74					1.3			tr
C_8	1.4	1.2			0.44	2.0			tr
C_9	2.3	1.9			1.1	3.2			tr
C_{10}	1.4	1.4				2.9			tr
C_{11}	5.9	6.8		0.14		2.9			tr
C_{12}				0.14		1.6			tr
C_{13}				0.20		1.3		1.5	tr
C_{14}	0.46	0.57							
Alkanals									
C_5						50			36
C_6			4.8			10			11
C_7			3.3			8.1			
C_8			31			35	1.5	1.9	26
C_9	2.5		28	9.3		49	2.8	6.6	25
C_{10}	3.8		41	13		61	0.64	2.5	25
C_{11}			6.6	4.2		22	0.48	5.4	8.8
C_{12}			1.7	5.8	1.9	16	0.64	2.9	4.9
C_{13}			6.6	2.6		32	tr	tr	9.1
C_{14}				5.7		4.9		3.9	2.4
C_{15}				4.2		7.2			
Alk-2-enals									
C_5						1.6			
C_6						5.3		1.3	
C_7			5.1			5.4		1.9	6.2
C_8			38	4.0	1.6	53		7.9	26
C_9	1.3		12	2.4	1.4	28		3.0	26
C_{10}	1.4	0.79	4.3	3.9	2.0	19		2.0	5.4
C_{11}	0.72	1.3	11	5.2	6.6	53	tr	6.2	12
C_{12}		1.5	7.1	5.5	18	29		5.6	10
C_{13}		3.6		2.9	9.5	1.1			0.53
Alk-2,4-dienals									
C_5				2.2		10			8.2
C_6				0.88					
C_7			15	4.4		1.8	0.28		
C_8		160	70	15	28	87	1.4	9.0	44
C_9				13	23		1.1	11	
Total volatile mono-carbonyls	70.9	234	294	114	101	622	8.84	72.6	306

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

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

Discussion

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

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

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

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

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

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The Reaction of an Autoxidized Lipid with Proteins

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Abstract

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

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Materials

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

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

Methods

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

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

Trypsin Assay of Gelatin-ML Foams. Samples of defatted foams (0.5 g) were mixed with 59 ml of Sprensen's buffer (pH 7.7) containing 10 mg of Difco trypsin (1:250) in 125 ml. Erlenmeyer flasks. Each sample was set up in duplicate with one reaction mixture precipitated immediately upon mixing with 20 ml of 20% trichloroacetic acid (TCA). The second flask was shaken for 20 hr at 38C and then precipitated with 20 ml of 20% TCA. Both samples were filtered

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TABLE I

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

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

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

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

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

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

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

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

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

Discussion and Results

Gelatin-ML Reactions

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

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

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

Insulin-ML Reactions

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

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

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

$$\text{R–NH}_2 + \text{FDNB} \longrightarrow \text{RNH(DNP)} \xrightarrow[\text{Reflux}]{\text{HCl}}$$

$$\text{DNP–amino acids} + \text{amino acids}$$

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

$$\text{Protein–NH–CRHOH} + \text{FDNB} \xrightarrow[\text{(amino acids)}]{\text{HCl}} \text{R–NH}_2 +$$

$$\text{dinitrophenol} + \text{RCHO}$$

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

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

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

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

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

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

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

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

Solubility Studies Using Anhydrous Hydrogen Fluoride

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

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

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

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