NAFD displayed a high proportion of difficultly conjugable diene unsaturation, as shown by the increase in absorption at 233 mμ on extending the isomerization time from 25 minutes to 6 hours. This increase could be explained by the presence of much *cis-trans* acid (Jackson, Paschke, Tolberg, Boyd and Wheeler, '52). The results with linseed oil suggest a much lower proportion of difficultly conjugable *cis-trans* isomer. In point of fact, oxidation with permanganate by Bertram's method showed that linseed NAFD contained less than 2% of saturated material, and the spectrometric data and iodine value suggest that linseed NAFD contained a high proportion of non-conjugable diene, and perhaps as much as 80%. But we are not yet in a position to state what feature of the chemical construction is responsible for the failure to form an urea adduct. It is, of course, possible that this feature may be related to the nutritional defectiveness of the fraction; at the same time it must be borne in mind that failure to form urea adducts may result from a variety of structural features.

SUMMARY

The non-adduct-forming fraction (NAFD) of the distillable esters from heated soybean oil was toxic, though to a lesser degree than that from the comparable fraction obtained from linseed oil. The NAFD from heated sunflower seed oil, however, was only slightly injurious to the rats.

The adduct-forming fractions from both the heated soybean oil and the heated sunflower seed oil were nutritionally harmless.

The chief chemical difference between the NAFD fractions from the three heated oils was in respect to their behaviour on alkali isomerization. The NAFD from heated linseed oil displayed relatively little increase of its absorbance at 233 mμ, whereas the results for the soybean and sunflower seed oils suggested the presence of high proportions of difficultly conjugable diene unsaturation.

These results suggest that the non-adduct-forming fraction of the distillable esters of heated linseed may contain a high proportion of non-conjugable diene cis-isomers, possibly of cyclic structure.

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The authors are indebted to the Canadian Committee on Edible Fats and Oils, National Research Council of Canada, Ottawa, for financial support and for frequent helpful discussions. They wish also to thank Dr. W. D. McFarlane and Dr. W. F. Parker, Canadian Breweries Limited, Toronto, Ont., for providing the sample of soybean oil.

APPENDIX

Preparation of ethyl esters of polymerized soybean, sunflower seed and linseed oils.

Raw solvent-extracted soybean oil (Victory Mills Limited, Toronto) was alkali-refined in batches of 1.5 kg with 3.6% 20° Baumé sodium hydroxide, washed and dried with sodium sulphate. The correct amount of alkali was calculated from the acid value and the tables given by Bailey ('51). Batches of 500 gm of the oil were then bleached with 2% Super Filtrol, filtered and polymerized at 275°C. for 20 hours under a stream of CO_2. From that point the preparation was as described by Crampton et al. ('55).

Raw sunflower seed oil (Co-operative Vegetable Oils Limited, Altona, Man.) was handled in the same fashion, except that a heating time of 26 hours was selected for the polymerization. Preliminary experiments showed that there was not any appreciable formation of NAFD fraction until this oil had been heated for 20 hours. The subsequent steps in the preparation of ester fractions were performed as for the linseed and soybean oils.

The yields and mean molecular weights of the various fractions are given above in table 2. The corresponding iodine numbers and refractive indices are reported in table 6.

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DEVELOPMENT OF FATTY LIVERS DURING LACTATION OF RATS FED AMINO ACID RATIONS[1]

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During earlier studies on the reproduction of rats fed protein-free amino acid rations, it was noted (Schultze, '55) that after completion of lactation the livers of rats were greatly enlarged, pale colored, mottled and friable. Since this condition was not observed in non-lactating animals of similar age fed the same rations, it appeared that fatty livers had been induced by the stress of lactation. This condition was investigated in conjunction with extended studies (Schultze, '56) of the adequacy of protein-free amino acid rations for reproduction and lactation. Moreover, since the literature does not appear to contain a record of previous systematic observations on this point, it was necessary to investigate the effect of pregnancy and lactation on the lipid content of livers of rats fed adequate rations composed largely of natural products. The results of this work are summarized in this paper.

EXPERIMENTAL

Rations. The rations contained the ingredients listed in table 1. Amino acid mixtures I and II which contained 10 and 16 amino acids respectively had the same composition as previously described (Schultze, '55) except that the isoleucine

[1] Paper no. 3513, Scientific Journal Series, Minnesota Agricultural Experiment Station.

TABLE V

A.O.C.S. Glycerine Analysis—International Collaborative Study—Summary of Means and Variances (% Glycerine)

	Mean		Components of Variance								
	Indicator	Electrometric	Within			Between			Total		
			Ind.	Elect.	Avg.	Ind.	Elect.	Avg.	Ind.	Elect.	Avg.
C. P. Glycerine											
A.O.C.S. method											
Acidified reagent	89.87	90.17	0.0176	0.0155	.0166	0.1633	0.1460	.1546	0.1809	0.1615	.1712
Neutral reagent	89.86	90.14	0.0039	0.0052	.0046	0.1373	0.1240	.1306	0.1412	0.1293	.1302
British method											
Acidified reagent	89.96	90.18	0.0048	0.0056	.0052	0.2080	0.1956	.2018	0.2128	0.2012	.2070
Neutral reagent	89.85	90.07	0.0079	0.0075	.0077	0.1872	0.2228	.2050	0.1951	0.2303	.2127
Acidified reagent (without N_2)	90.11	90.35	0.0841	0.0084	.0462	0.2622	0.4329	.3476	0.3463	0.4413	.3938
Made-Up Crude Glycerine											
A.O.C.S. method											
Acidified reagent	67.85	68.03	0.0038	0.0034	.0036	0.0862	0.0996	.0929	0.0900	0.1030	.0965
Neutral reagent	67.83	68.03	0.0126	0.0268	.0197	0.0587	0.0606	.0596	0.0713	0.0874	.0794
British method											
Acidified reagent	67.85	68.05	0.0033	0.0050	.0042	0.1218	0.1083	.1150	0.1241	0.1133	.1192
Neutral reagent	67.87	68.00	0.0025	0.0026	.0026	0.1066	0.1248	.1157	0.1091	0.1274	.1183
Acidified reagent (without N_2)	68.01	68.22	0.0030	0.0033	.0032	0.1144	0.2010	.1577	0.1174	0.2043	.1609

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Studies on the Nutritional and Physiological Effects of Thermally Oxidized Oils

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EDIBLE OILS which had been heated to 200°C. in the presence of air were shown to have less nutritive value than the comparative fresh oils (1). It has been shown that the polyunsaturated fatty acids in these oils were attacked and the viscosity and oxygen content of the oil were increased. Crampton *et al.* have suggested that polymeric or cyclic products were formed in heat-polymerized oils and that these products caused at least some of the results observed when diets containing the polymerized oils were fed to rats (2, 3). When oils were aerated at 90°–100°C., the nutritive value of the oils also decreased (4). Kaunitz *et al.* have suggested that this growth depression was related to polymeric products since the residue which remained after molecular distillation of these oils proved to be more growth-depressing than the whole oil (5).

The studies to date therefore suggest that polymeric products formed during oxidation of edible oils cause at least part of the growth depression. It was also implied that these polymers were formed from the polyunsaturated fatty acids. Several mechanisms have been suggested which would lead to polymeric or cyclic materials from unsaturated fatty acid esters. Sunderland has proposed a direct reaction between a double bond in one molecule and a methylene group of a second molecule to give a carbon-to-carbon linkage (6). Other workers have suggested that 1:4 addition reactions lead to the polymeric products (7). Paschke and Wheeler found that cyclic products were produced in polymerization of methyl eleostearate (8, 9, 10). Since it has been shown that linoleic acid can undergo these polymerizing reactions, an attempt was made in the present study to relate the linoleic acid content of a fat to thermal oxidative damage as measured by comparative growth-rates in rats.

The physiological effects of oxidized oils are not known, but studies have shown that organ-body weight-ratios are affected by these products (4, 11). Other workers have found some loss in the coefficient of digestibility, but most heated oils retain a high coefficient of digestibility (12). In the present work the rate of absorption and the *in vitro* rate of hydrolysis of thermally oxidized corn oil and the effect of thermally oxidized corn oil on the livers of rats fed diets containing 20% of the thermally oxidized corn oil were studied.

Experimental Procedures

The thermal oxidation of oils was carried out as in previous studies (1). Approximately one kilogram of the oil was thermally oxidized at 180°C. for 24 hrs. The air was bubbled through the hot oil at a rate of 200 ml. per hour except where lower rates are specifically mentioned. Linoleic acid was determined by the spectrophotometric method of Brice *et al.* (13).

The diet was similar to the one used in previous studies (1). It was composed of 21% casein, 44% cerelose, 5% Wesson-salt mixture, and 20% of either the fresh or the thermally oxidized oil (1). The water-soluble vitamins were added to the diet; the fat-soluble vitamins were dissolved in hydrogenated coconut oil and given every five days by dropper. In cases where very viscous oils were fed, only 10% could be added to the diet since the rats would not eat diets containing 20% of such oils. The urea separation of the thermally oxidized-oil fatty acids was carried out, using a modification of a procedure reported by Swern (14). A 100-g. sample of the oil was saponified in alcoholic potassium hydroxide, the solution was acidified, and the fatty acids were extracted with petroleum ether. The extracts were dried over sodium sulfate, and the solvent was removed at room temperature under vacuum. The fatty acids were then added to a mixture of urea and methanol in a ratio of 1:3:7, and the mixture was heated on a steam bath to dissolve all of the urea and fatty acids. The clear solution was cooled to 6°C., then filtered. The crystalline adducts were washed twice with small portions of cold ethyl ether. Additional urea was added to the filtrate to bring the ratio of fatty acids to urea to methanol to 1:5:10, and the above procedure was repeated. A third urea addition to a ratio of 1:5:10 was also made in order to remove as much of the unreacted material as possible. The urea adduct and nonurea adduct were then isolated by the normal procedures.

Results and Discussion

The greatest nutritional loss was observed in the sample containing the largest percentage of linoleic acid. Several oils were thermally oxidized under the standard conditions and then fed to male weanling-rats in the test diet (Table 1). The growth rate of the animal was expressed as the ratio of the average gain of six animals on the thermally oxidized oil to the average gain of six animals on the fresh oil. The test period was 10 days, and all animals were restricted to the same amount of diet.

TABLE I

Linoleic Acid Content of Oils and Growth Ratio of Diets Containing Thermally Oxidized Oils

Fat	Iodine value	Linoleic acid content, %	Growth[a] ratio
Corn oil	122	54.0	.30
Hydrogenated cottonseed oil	81	17.5	.61
Olive oil	77	4.8	.90
Oleo oil	47	2.8	.84

[a] Ratio of average gain of rats on diet containing thermally oxidized oil to gain of rats on diet containing fresh oil.

To ascertain the relationship of linoleic acid to total unsaturation, two sets of oils were prepared. The first consisted of three samples with similar iodine values but decreasing amounts of linoleic acid (Table II). These samples were thermally oxidized at 180°C. for 24 hrs., with 100 ml. of air per minute per kilo. The second set of oils consisted of two oils with iodine values of 102, the first with a linoleic acid content of 33%, and the second with a linoleic acid content of 20%. The first sample had a growth ratio of .75, and the second .81, both higher than those observed in samples with similar linoleic acid content from the first set of oils. The improvement in the nutritive value of the thermally oxidized oils of higher iodine values was surprising, but it is possible that the dilution of the linoleic double bonds by oleic-acid double bonds has actually lowered the polymerization of the linoleic acid. If one assumes that most of the less nutritive products are produced by the reactions of the double bonds of linoleic acid, the addition of double bonds which do not give rise to these less nutritive products when thermally oxidized could lead to a more stable product.

TABLE II

Effect of Linoleic Acid Content on Nutritional Value of Oils After Thermal Oxidation

Sample No.	Treatment	Iodine value	Linoleic acid, %	Average gain in 10 days, g.	Growth ratio
A	None	76	33.0	39.7 ± 1.8	
A	T.O. 24 hr.	48	10.5	26.2 ± 0.3	.62
B	None	72	21.4	41.0 ± 1.7	
B	T.O. 24 hr.	51	6.5	31.4 ± 1.6	.76
C	None	72	7.0	41.0 ± 1.6	
C	T.O. 24 hr.	68	3.1	36.5 ± 1.7	.88

The portion of the thermally oxidized oil which has the greatest growth-depressing action is that portion of the oil which does not form urea adducts (Table III). The corn oil for this test was thermally oxidized for 48 hrs. at 180°C. with 75 ml. of air per minute per kilo passing through it. The oils in all diets were fed at the 10% level in the basal diet since the nonurea adduct-forming fatty acids were too viscous to feed at the 20% level. The animals on the nonurea adduct-forming fraction would consume only five to six grams of diet a day after the first week. All animals were on equalized feeding, and the relatively low feed-intake is reflected by the small gain of animals even on the fresh corn oil diet. Comparison of the gains however shows that most of the growth-depressing material was concentrated in the nonurea adduct-forming materials. These products are probably similar to the polymeric residues which other workers have isolated

TABLE III

Growth of Rats on Diets Containing Urea Adduct-Forming or Nonurea Adduct-Forming Fractions of Thermally Oxidized Corn Oil

Oil and treatment	Iodine value	No. of rats	Initial weight, g.	Gain, g., 14 days
Corn oil, none	122	4	89.4	28.4 ± 1.1
Corn oil, thermally oxidized, 48 hr.	84	6	85.4	9.6 ± 1.8
Nonurea adducts from thermally oxidized corn oils, 48 hr.	75	6	89.5	−7.8 ± 2.4
Urea adduct from thermally oxidized corn oil, 48 hr.	92	6	87.2	25.0 ± 1.6

by molecular distillation and which have also shown marked growth-depressing action (5).

The relationship of the nonurea adduct-forming fatty acids to growth depression indicates a possible method of detecting nutritive changes in thermally oxidized oils (Table IV). Three of the changes which

TABLE IV

Comparison of Iodine Value, Viscosity, and Percentage of Nonurea Adduct of Thermally Oxidized Corn Oil to Growth of Rats Fed Diets Containing Thermally Oxidized Oil

Corn oil, treatment	Iodine value	Viscosity, poises, 25°C.	Percentage of nonurea adduct	Growth ratio
None	124	.65	6.0	1.00
8 hr. T.O.	115	.86	18.2	.89
16 hr. T.O.	108	1.25	29.3	.31
24 hr. T.O.	101	2.50	38.7	.24
48 hr. T.O.	92	7.55	47.0	.17

I… been observed in thermally oxidized oil—a de-…rease in iodine value, an increase in viscosity, and …n increase in percentage of nonurea adduct-forming material—offer possible methods for estimating the nutritive loss. The iodine value, while decreasing steadily, does not parallel the loss in nutritive value. The rather sudden decrease in nutritive value after eight hours is not reflected by any marked decrease in iodine value. The viscosity appears to offer a closer relationship to the growth ratio. It shows a rapid increase; however this increase is greater during the last 24 hrs. Decrease in growth ratio is greatest during the first 24 hrs. and is much less during the following 24 hrs.

The percentage of nonurea adduct-forming mate-…ial parallels the loss in nutritive value more closely …han either changes in iodine value and viscosity (Figure 1). The rapid increase in nonurea adduct-…orming material during the early stages of thermal …xidation and the subsequent slower formation dur-…ng the latter stages give a possible method for the …etermination of the nutritive loss during thermal …ation.

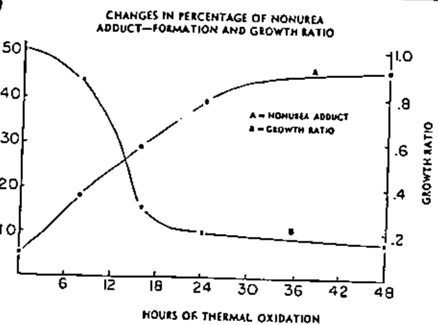

FIG. 1.

…While many growth studies have been made with …rmally oxidized or oxidized oils, few studies on …cific physiological effects have been reported. It been shown that at least 70% of the polymeric due from cotton-seed oil oxidized at 100°C. is …rbed (15). Studies on heat-polymerized oils also have indicated only slight decreases in absorption. The thermally oxidized corn oil, which has been fed in this study, also shows little loss in the coefficient of absorption (Table V). While the six-hour rate of absorption of the thermally oxidized oil was significantly less than that of the fresh oil, the rate of absorption is the same at the end of 24 hrs. Since the initial rate of absorption might be decreased because of a slower rate of hydrolysis, *in vitro* hydrolysis of fresh and thermally oxidized corn oil by pancreatic lipase was carried out. A sample of the oil weighing 250 mg. was added to a flask containing 10 ml. of Sorensen buffer, pH 8.0; 3.0 ml. of an enzyme suspension containing 50 mg. lipase; and 1 ml. of 10% sodium taurocholate. About 5 ml. of small glass beads were added, and the flask was shaken on a mechanical shaker in a 37°C. constant-temperature room. After the digestion period was complete, 0.1 ml. of a 1% methanolic thymol blue solution was added, and the solution was titrated with 3*N* hydrochloric acid to a salmon-pink color. The acidified mixture was transferred to a separatory funnel and extracted with several portions of water-saturated ethyl ether. The ether extract was combined, washed free of HCl, and then filtered. The filter paper was washed with two 10-ml. portions of 95% ethanol, and the ether extract plus the ethanol wash was titrated with 0.02*N* alcoholic potassium hydroxide (Table VI). The rate of

TABLE V

Absorption of Corn Oil and Thermally Oxidized Corn Oil When Fed to Fasted Male Rats at a Level of 400 mg. per 100 $cm.^2$ Body Surface [a]

Fat fed	Absorption in mg./100 $cm.^2$/hr.[b]		
	6 hours	10 hours	24 hours[c]
Corn oil	50.1 ± 3.2(8)	33.0 ± 2.4(8)	22.2 ± 1.2(6)
Corn oil, T.O. 24 hr.[c]	36.4 ± 2.8(10)	28.3 ± 2.1(7)	21.1 ± 2.6(9)

[a] Weights of rats ranged from 145 to 190 g.
[b] ± Standard error of the mean.
[c] In the 24-hr. tests the corn oil was fed at a level of 600 mg./100 cm. body surface.

TABLE VI

Relationship Between Time and the Extent of the in Vitro Hydrolysis of Corn Oil and Thermally Oxidized Corn Oil with Pancreatic Lipase

Oil	Percentage of Hydrolysis					
	0.5	2.0	4.0	8.0	16.0	24.0 hrs.
	%	%	%	%	%	%
Corn oil, fresh	20.1±3.2	60.2±1.8	82.5±3.1	88.3±2.3	98.6±1.5	98.3±2.9
Corn oil, T.O. 8 hr.	21.3±4.0	....	....	87.2±4.2	....	....
Corn oil, T.O. 24 hr.	15.2±1.8	50.3±3.3	75.2±2.5	81.4±3.6	83.6±4.6	90.1±1.8

hydrolysis for the 24-hr. thermally oxidized oil was significantly less than that for the eight-hour thermally oxidized oil and the fresh corn oil. However, as the time of hydrolysis was increased, the difference between the percentage hydrolyzed for the fresh oil and the thermally oxidized oil decreased. While part of this decrease in hydrolysis could be caused by poorer surface-contact because of the solubility of the polymeric products, the other oxidation products found during thermal oxidation might be responsible for part of the decreased hydrolysis. Weinstein and Wynne have shown that carbonyl compounds can act as inhibitors of pancreatic lipase (16). Recent studies have shown that the carbonyl group is present in oils following thermal oxidation, therefore some decrease in rate of hydrolysis is possible (17).

The weight and composition of livers from rats fed diets containing thermally oxidized oil were also studied, and a significantly larger liver weight–body weight ratio was found in the animals fed the diets containing the thermally oxidized oil. One hundred and ten animals which had been fed the basal diet that contained 20% thermally oxidized corn oil for periods of two to four weeks gave an average liver weight–body weight ratio of .0466. Ninety-six animals fed diets which contained 20% fresh corn oil for similar lengths of time had an average liver–body weight ratio of .0352. No difference in lipide content or total solids was noted in the livers. The livers of animals which had been fed the thermally oxidized oil diet contained 3.95% lipide and had a total solids content of 32.23% while those fed the fresh oil diet contained 4.10% lipide and had a total solids content of 32.26%. The histopathological examination of the livers from animals fed the thermally oxidized oil diet indicated very little change or none.

This increase in liver–body weight ratio has been noted in animals fed oil oxidized at 100°C. and in animals fed heat-polymerized oil. No explanation has been given, but it would appear that some change must be taking place in normal metabolism which leads to the larger liver–body weight ratio. The increased ratio was found even in animals which were transferred to a grain basal diet after three weeks on a diet containing thermally oxidized oil. Twelve male rats were fed a diet containing 20% thermally oxidized oil for three weeks and then transferred to a grain diet and kept on it until they attained a body weight of 275–340 g. A second group of 12 animals were fed the basal diet containing 20% fresh corn oil for three weeks and then transferred to the grain diet and kept on it for the same period of time as the first group. The liver–body weight ratio of the first group on a thermally oxidized oil diet was .043 while that of the second group was .0301. Further studies are necessary in order to determine the cause of the increased liver–body weight ratio.

Summary

The present results indicated that the thermal oxidation products from the polyunsaturated fatty acids, primarily linoleic acid, are responsible for much of the loss of nutritional value in thermally oxidized edible oils. Oils which have a high linoleic acid content are more likely to undergo thermal oxidative damage than those with lower linoleic contents. Also the ratio of linoleic acid to total unsaturation has some effect on the nutritive stability of the oil when it has been thermally oxidized. An oil with a high iodine value but with a low linoleic acid value appears to be more stable to thermal oxidation than an oil with an iodine value one half as great but with most of the unsaturation in the oil caused by linoleic acid.

The products formed during thermal oxidation which cause the loss of nutritional value are those which do not form urea-inclusion compounds. They are probably polymeric in nature, but thermally oxidized oils also contain carboxylic acids and carbonyl groups which might cause some of the nutritional loss observed when thermally oxidized oils are fed.

The rate of *in vitro* hydrolysis of the thermally oxidized corn oil by pancreatic lipase, also the rate of absorption from the intestine of the male rats, were found to be decreased. However the percentage of absorption in 24 hrs. was the same with both fresh and thermally oxidized oil.

The liver–body weight ratio of rats fed a diet containing the thermally oxidized oil were found to be significantly larger than the liver–body weight ratio in animals fed diets containing fresh oil. However the livers of animals fed the thermally oxidized oil diets did not differ in lipide percentage or total solid content, and histopathological investigations did not show any abnormal conditions.

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The Pigments of Crude Cottonseed Oils. II. Nitrogen-Containing Pigments Derived from Gossypol

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THE GOSSYPOL in crude cottonseed oils of commercial origin exists mainly in a combined form (1), but oils containing uncombined or native gossypol can be obtained from cottonseed by mild extraction-procedures (3). Native gossypol in fresh oils undergoes rapid reaction with oil constituents to yield alkali-insoluble derivatives, but if the oils are treated immediately with p-aminobenzoic acid, an oil-insoluble Schiff base is formed instead. Crude oils treated in this manner may be stored at elevated temperatures for extended periods of time and still yield refined and bleached oils of low photometric color and normal stability (2). Apparently p-aminobenzoic acid can compete successfully for gossypol in some of the alkali-insoluble derivatives.

[1] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

marked loss of liver iron. The data suggest that the reduction of iron is responsible for the production of the anemic condition and presumably the depression of the activity of some iron-containing enzymes. A lowered liver copper may also occur and the data indicate that it may be the result of the reduced liver iron rather than an effect of the zinc. Copper probably acts in counteracting the anemia and reduced enzyme activity of zinc toxicity by further mobilizing the iron in the liver.

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Nutritional Properties of Fresh Fats Added to Diets Containing Autoxidized Cottonseed Oil[1]

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Diets containing highly autoxidized cottonseed oil lead to rapid weight loss when fed to rats and result in a high death rate. Addition of fresh cottonseed oil has been observed to exert a protective effect (Kaunitz et al., '55). In these studies, it was not clear whether this protective effect was a property of any fresh fat or whether various fats differ in this respect. This question invited further studies, particularly because it had meanwhile been observed that medium-chain and long-chain saturated triglycerides frequently differ in their nutritional effects (Kaunitz, et al., '58a, b; '59). Furthermore, such studies could be helped by the more detailed information which had been obtained as to the effect of such oxidized fatty materials on water intake and organ weights (Kaunitz, et al., '56, '59, '60).

MATERIALS AND METHODS

Refined cottonseed oil was aerated at 95°C for 300 hours. This oxidized cottonseed oil (OCSO) was included at levels of 10 and 15% in a purified diet shown in table 1. When desired, 20 or 15%, respectively, of a fresh fat was added to the diet at the expense of the carbohydrate. All diets were kept refrigerated.

The fresh fats used were commercially-available, refined cottonseed oil (CSO), refined corn oil, refined coconut oil, sweet butter, refined olive oil, and soybean oil and freshly rendered leaf lard, chicken fat, and perirenal beef tallow. In addition, medium-chain and long-chain saturated triglycerides (MCT and LCT) and ethyl esters of CSO were studied. The MCT was prepared from coconut oil by fractionation of the split fatty acids and reconstitution of the desired fraction (6 to 12 C) into triglycerides. The oil was clear, thin, odorless, with a melting point below 0°C and an iodine value of less than one. LCT was derived from coconut or other palm-kernel oils by hydrogenation of the fatty acids of 14 to 18°C and their reconstitution into triglycerides. This material had a melting point of about 40°C and an iodine value of 3 to 5. The ethyl esters of CSO were prepared by refluxing the oil with aqueous NaOH in alcohol, acidification and esterification with ethanol.[2]

The weanling rats used were males (except for one series) derived from a colony of the Sherman strain. When they were delivered to the laboratory at 24 days of age, they were placed on a diet similar to that in table 1 but containing lactalbumin instead of casein and 10% of fresh lard as fat. In about 5 days they were earmarked and weighed. After reweighing 4 or 5 days later, they were distributed into matching groups of 8 rats (7 in some series) so that average body weights were the same for the first weighing and again for the second weighing. The rats were placed in individual cages and supplied with non-dripping water bottles suitable for water consumption measurements. Vitamin supplements were fed by dropper to compensate for destruction of vitamins in the diets containing oxidized fats. The rats were weighed at least once weekly. After three weeks (4 in some instances), they were

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[2] Dr. Daniel Swern and Mr. H. B. Knight of the Eastern Regional Research Laboratories of the U. S. Department of Agriculture suggested the use of the esters and prepared them for us.

TABLE 1
Composition of purified diet containing oxidized cottonseed oil

Ingredient	Amount
	%
Oxidized cottonseed oil	10 or 15
Dextrose[1]	54 or 49[2]
Casein[3]	30
Cellulose[4]	2
Salt mixture (USP XIII)	3.5
Calcium carbonate	0.5
Vitamins and accessory factors[5,6]	

[1] Cerelose.
[2] When fresh fat was fed with the oxidized cottonseed oil, it was added at the expense of the carbohydrate.
[3] G.B.I. Vitamin-Free.
[4] Alphacel.
[5] For details, see J. Nutrition, *64*: 514, '58.
[6] We wish to thank Dr. Leo A. Pirk of Hoffman-La Roche, Inc., Nutley, New Jersey, for most of the synthetic vitamins.

anesthetized with chloroform, as much blood as possible was withdrawn from the heart and the organs were weighed. The ventricles, rather than the whole heart, were weighed because they can be separated with considerable accuracy.

Organ weight data are presented in figure 1 as a log-log plot of organ weight against body weight. This method was used because the ratio of the weight of an organ to the corresponding body weight is not linear but varies continuously, and a log-log plot gives a straight line distribution for most organs which may have one or more changes in slope with increasing body weight. The distribution has a uniform spread throughout its range. The normal organ weight distributions used for comparison in these experiments were derived from organ weight data collected for over 4 years from 427 male rats fed a diet similar to that given in table 1 but with fresh lard as fat source. The weights of an organ were grouped according to the corresponding body weights so that the body weight range for each subgroup was small. The organ weights of each subgroup were averaged and this average was plotted against the body weight representing the midpoint of the group range. A straight line resulted which had the same slope as the distribution. This line became the source of the "normal" weight of the organ for any given body weight. These lines are given in figure 1.

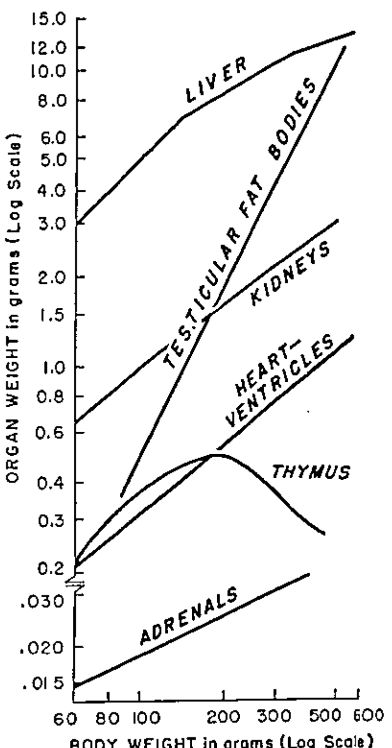

Fig. 1 Lines derived from plotting average organ weight-body weight relationships on a log-log scale.

The actual weight of an organ of an experimental animal was compared with the "normal" weight of the organ for the animal's body weight as derived from the appropriate line, and the difference between the two was expressed as a percentage of the normal weight. The individual percentages were used for statistical analyses. Although this method avoids some difficulties in comparing weights of animals of different body weights, it may have its limitations. One can hypothesize that a particular treatment could lead to the maximum enlargement of a certain organ for animals of a given age, which would make any differences in percentages of enlargement specious; the latter would

represent only differences in body weight rather than true differences in effect on the organ.

For statistical purposes, P values were calculated by t tests. A P value of 0.05 was considered as just significant. The ± values given are standard errors.[3]

EXPERIMENTS

Ten experimental series were carried out in which OCSO was fed alone and with various fresh fats. In order to facilitate the presentation of the data, all results involving any one fresh fat were combined and compared with the data from the corresponding groups fed OCSO alone.

In figure 2 are given the survival rates and the differences in body weight between corresponding series on OCSO alone and with an added fresh fat. When only 10% of OCSO was fed, most animals survived. With 15% of OCSO alone, a considerable number of animals died; with 15% of MCT, CSO and chicken fat, the survival rates were higher. The chi square for OCSO + MCT compared with OCSO alone was 5.8 and for chicken fat, 4.6.

When OCSO alone was fed, most rats lost weight. The groups fed 10% lost an average of 6 gm and those fed 15%, 16 gm (not significant). There was a decided difference in how the addition of fresh fats influenced the body weights. The use of 20% added to 10% of OCSO, MCT and CSO led to body weights which were 33 and 41 gm higher, respectively, than their controls ($P < 0.001$ for each). With 20% of lard and 20% of ethyl esters of CSO, the differences were less pronounced but still significant; with LCT, average body weights were lower than those with OCSO alone, but not significantly.

The use of 15% each of OCSO and fresh fat, MCT, corn oil, olive oil, CSO, and soybean oil very significantly prevented body weight losses; the action of lard was somewhat less pronounced. With chicken fat, the difference in body weight over the OCSO controls was not quite on the borderline of significance. Butter, coconut oil, and the ethyl esters of CSO had no effect, and beef significantly aggravated the condition.

Water intake measurements were carried out in three series. For purposes of comparing the intakes of animals of widely

[3] Dr. John W. Fertig of the Department of Public Health and Administrative Medicine, Columbia University, kindly helped us with the statistical analyses.

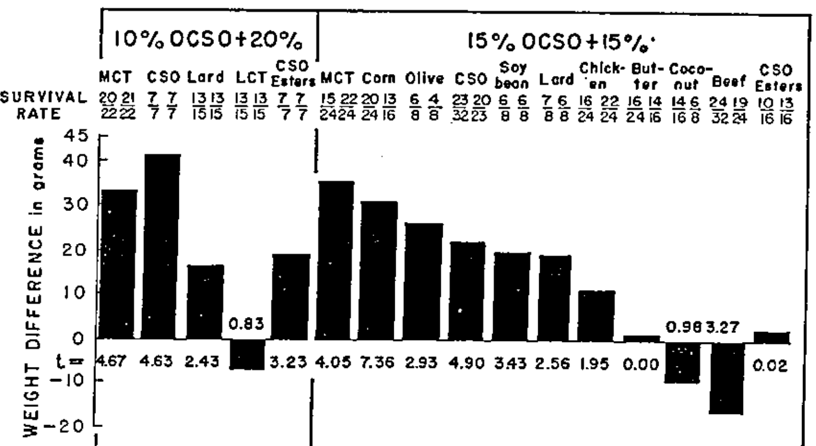

Fig. 2 Influence on body weight and survival rate of fresh fat added to a diet containing OCSO. Effects on body weight are expressed as weight difference between corresponding groups on OCSO alone and with a fresh fat. The t values refer to these differences. In each pair of survival rates, the first refers to that with OCSO alone.

differing body weights, intakes were expressed in terms of body surface. The body weights for each animal for the period of measurement were averaged and average surface calculated according to Lee's formula, $S = 12.54\ W^{2/3}$ where S is the surface in cm^2 and W is the weight in grams (Lee, '29). The average weekly water intake of each rat for the three-week period was divided by its S/100 to give $cm^3/100\ cm^2/week$. Five control groups fed only fresh fat (not otherwise used in these studies) had average intakes of 36 to 38 $cm^3/100\ cm^2/week$ for body weights of 100 to 428 gm. The groups fed OCSO alone had intakes of $66 \pm 8.6\ cm^3$ with 10% and, in the two series fed 15%, 85 ± 3.0 and $7.3\ cm^3$. This is in agreement with the finding that some fractions of such oxidized oils greatly increase water intake (Kaunitz et al., '59). Rats fed OCSO and 20% of MCT had an intake of $48 \pm 3.6\ cm^3$; with lard, it was $55 \pm 4.1\ cm^3$ and with LCT, $72 \pm 9.4\ cm^3$. The difference between the intakes of those fed OCSO alone and those fed OCSO with MCT were just significant; between those fed MCT and LCT, it was more pronounced. With 15% each of oxidized and fresh fats, MCT and other fats had no influence. Therefore, MCT was capable of reducing the high water intake associated with the intake of OCSO, but only when fed at rather high levels.

In figure 3 are presented the more pertinent data on the organ weight-body weight relationship. Average organ weight values and standard errors are also given. Kidney values show that the kidney was enlarged using both levels of OCSO alone; MCT and CSO led to significantly less enlargement when 20% was fed. With 20% of LCT, the percentage of deviation from normal was higher than with OCSO alone, but not significantly so. Lard and coconut oil had no effect even at the 20% level. With 15%, only MCT had a significant effect; beef fat aggravated the condition. CSO, corn oil, lard, chicken fat, coconut oil, butter, and the ethyl esters of CSO had no effect and are not included in the figure.

The enlargement of adrenals ran more or less parallel with that of the kidneys. OCSO alone led to adrenals with an average weight which was higher than their calculated weights at the start of the experiments as derived from the adrenal weight-body weight line. This increase occurred while the animals lost weight. With both levels of MCT, the percentage of deviation was less. With 20% of LCT, the adrenals were significantly heavier than in the corresponding groups on MCT although the body weight of the latter groups was so much higher.

Lard and coconut oil fed at the 20% level and CSO, chicken fat, lard, butter, beef fat, and the ethyl esters of CSO fed at the 15% level were studied; they had no influence and are not given in the tables.

The degree of liver enlargemnt was significantly reduced by 20% MCT and CSO but not by 20% of LCT. Also studied, but not included in the table, were lard at the 20% level and MCT, CSO, corn oil, chicken fat, lard, coconut oil, butter, beef fat, and ethyl esters of CSO, none of which had any effect.

Thymus weights were reduced more than 50% when OCSO alone was fed. MCT and fresh CSO reduced the losses significantly; ethyl esters of CSO did not. Also studied, but not included in the table, were lard on the 20% level and lard, butter, and beef fat on the 15% level. They had no effect.

Testicular fat bodies were weighed because it has been shown that the weight of these is proportional to the total neutral fat in the rat (Hausberger, '37; Stoerk and Porter, '50). With OCSO alone, the weight of this organ was consistently below normal. All fresh fats except beef fat and the ethyl esters of CSO increased the weight of the testicular fat body in relation to the body weight, i.e., increased the total neutral fat depots. MCT, although counteracting body weight losses at least as well as fresh CSO, led to smaller fat bodies than did CSO. This is in agreement with the observation that MCT does not easily induce deposition of neutral fat (Weitzel et al., '55; Kaunitz et al., '58b).

Changes in the heart ventricle weight-body weight relationship were not pronounced and are not included in the figure. However, one must take into consideration that the standard deviation of ventricle weights is the smallest one for any

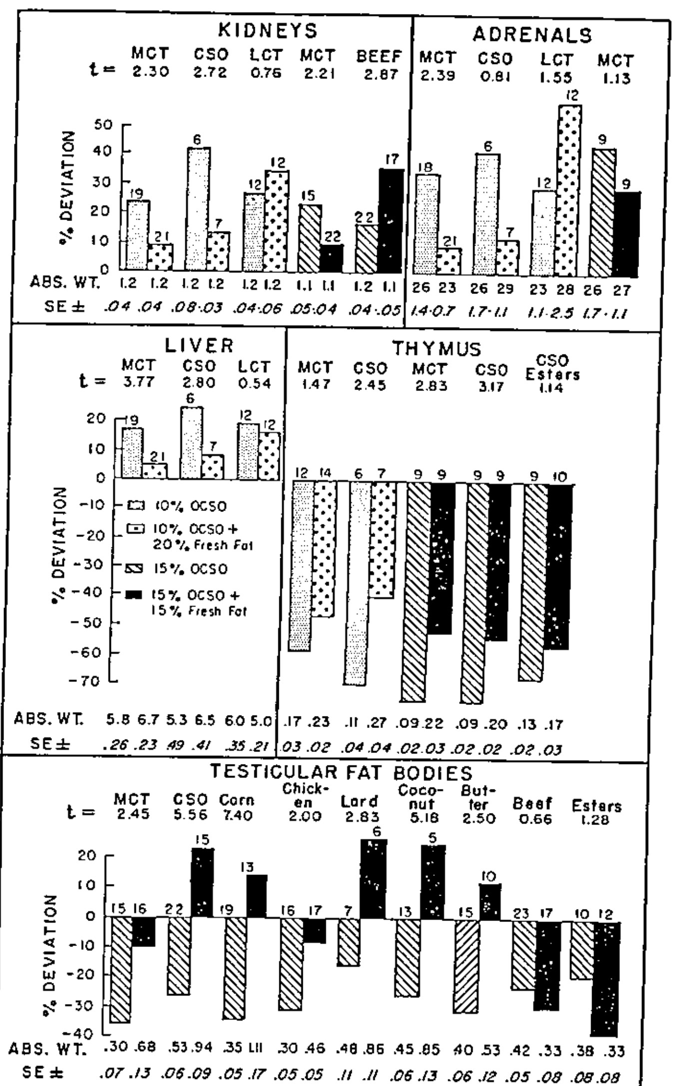

Fig. 3 Influence on organ weight-body weight relationship of adding fresh fat to a diet containing oxidized cottonseed oil (OCSO). Effects are expressed as percentages of deviation from normal. The t values refer to differences in percentage of deviation between corresponding groups fed OCSO alone and with a fresh fat. Numbers at the top of the columns are number of observations; below are given the average absolute organ weights in grams (milligrams for adrenal weights).

organ. It was noted in all series that OCSO alone induced ventricular weights approximately 5% smaller than expected from the standard line. This was also true when MCT, CSO, chicken fat, lard, coconut oil, and the ethyl esters of CSO were fed. With LCT and beef fat, the weights were about 2% above normal. When the data from corresponding series fed MCT and LCT were compared statistically, a P of 0.02 resulted; thus, ventricle weights were relatively higher when LCT was fed.

From some of the aforementioned observations, it was evident that the ethyl esters of CSO gave much less protection against weight losses and organ changes than did fresh CSO. In order to establish whether the glycerol moiety had any effect, one group was given 5% of glycerol with 10% of OCSO. Neither body weights nor organ weights were different from those of the group fed OCSO alone.

DISCUSSION

The various fats can be divided, with respect to their effect on body weights, into three groups: those strongly counteracting weight loss (MCT, CSO, corn oil, olive oil, and soybean oil), those having little or no influence (lard, coconut oil, butter, and chicken fat) and those aggravating the condition (LCT and beef fat).

Comparison of effects on organ weights of the fats strongly counteracting body weight losses shows that there are differences. Only MCT exerted a beneficial effect on kidneys and adrenals, and it also induced less neutral fat deposition than the other fats. Thus, a certain specificity in the action of these fats can be assumed.

If one attempts to relate any of the biological effects of these fresh fats to their physical properties, considerable correlation seems to exist between melting point and effect on body weight. Those with a low melting point (MCT, corn oil, CSO, soybean oil, and olive oil) gave the most protection against weight loss. Those with high melting points (beef fat and LCT) increased weight losses. Lard, chicken fat, butter, and coconut oil formed an intermediate group with respect to melting point and effect.

That the beneficial effect depends upon the presence of the fatty acids as triglycerides is suggested by the fact that the ethyl esters derived from CSO had little effect and neither did glycerol. Furthermore, although fats containing high percentages of linoleate in triglycerides (corn oil) had a beneficial effect, the addition of ethyl linoleate, in one experiment, to a diet containing OCSO led to the death of all 8 animals within 10 days. Those fed ethyl linoleate alone were normal.

The beneficial effect of certain oils is, in some ways, paradoxical. Previous studies (Kaunitz et al., '55) have shown that addition of fresh CSO to a diet containing OCSO led to increased food consumption and, therefore, to increased intake of the toxic material itself. Moreover, paired feeding experiments have shown that animals given fresh CSO in addition to OCSO did not show the same weight loss or organ enlargement; this is evidence that the findings are not the result of hunger, per se. Of some relevance may be the observation that polymerized fats decrease lipase activity in the feces (Peretti and Reale, '36) and reduce fat absorption[4] (Lassen et al., '49). If it is possible that the melting point of a fat influences both enzyme activity and food absorption, the difference between protective and non-protective fats may rest partly in their intestinal activity. However, this would not explain why fats with low melting points, which increase the absorption of oxidized material, have a beneficial effect. One may speculate that there exists competitive antagonism between oxidized and fresh fats after absorption.

The data may have some relation to the question of why fats have beneficial effects in some stress conditions. Feeding of OCSO subjects the animal to severe stress, and it may be of general interest that this stress is counteracted by liquid fats and aggravated by hard fats. The saturated but liquid fat, MCT, had effects at least as beneficial as the highly unsaturated oils.

[4] Saunders, D. H., H. B. Knight, D. Swern, H. Kaunitz, C. A. Slanetz and R. E. Johnson 1957 Composition of fecal lipids of rats fed diets containing polymers from autoxidized fats. Abstracts of the 48th Annual Meeting, Am. Oil Chemists' Society, no. 48.

SUMMARY

1. Purified diets containing 20 or 15% of medium-chain saturated triglycerides (MCT), refined cottonseed oil (CSO), corn oil, chicken fat, lard, coconut oil, butter, beef fat, long-chain saturated triglycerides (LCT) and ethyl esters derived from CSO in addition to 10 or 15%, respectively, of cottonseed oil aerated at 95°C for 300 hours (OCSO) were fed to weanling rats for three weeks.

2. Survival rate was significantly improved by MCT.

3. Body weight was considerably increased by MCT, CSO, corn oil, olive oil, and soybean oil; mildly improved by lard and chicken fat; not influenced by butter and coconut oil; and lessened by beef fat and LCT.

4. The elevated water intake of animals fed OCSO was reduced by MCT when 20% was added to 10% of OCSO.

5. Studies of organ weights in relation to body weight showed that the enlargement of kidneys and adrenals produced by OCSO was significantly reduced by MCT and fresh CSO, accentuated by beef fat and LCT and unaffected by other fats. Heart ventricle weights, in comparison with those of normal animals, were somewhat reduced with OCSO alone and with MCT and mildly increased with beef fat and LCT. (The difference between LCT and MCT was significant). The liver enlargement observed with OCSO was reduced by 20% of MCT or CSO. The reduction of testicular fat body weight associated with the feeding of OCSO was counteracted by most fresh fats.

6. The beneficial effect of the ethyl esters of CSO was slight compared with that of fresh CSO.

7. It is suggested that the beneficial effect of triglycerides on body weight can be correlated with their melting point.

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• *Technical*

Influence of Feeding Fractionated Esters of Autoxidized Lard and Cottonseed Oil on Growth, Thirst, Organ Weights, and Liver Lipids of Rats[1,2]

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THE NUTRITIONAL properties of autoxidatively and thermally polymerized fats have been given considerable attention. Such studies were usually undertaken with the objective of discovering what chemical changes or classes of compounds were associated with toxicity. The large number of products resulting from autoxidative or thermal polymerization of fats and oils made isolation of individual substances impracticable, but fractions have been isolated and used in feeding studies to assess their effect on the animal (1).

In our earlier work it was observed that feeding rats autoxidized fats and, in particular, the polymeric residues from autoxidized fats increased the caloric requirements for weight maintenance (2). This suggested studies of other "pharmacological" effects. It was thought that the substances responsible for these effects could be separated and/or concentrated by more detailed fractionation. Also, by utilizing other biological criteria, it was hoped that the pharmacological effects could be related to structural types. Therefore autoxidatively polymerized lard and cottonseed oil were fractionated by high-vacuum distillation and ...-complex-separation techniques. The fractions were fed to rats, which were observed for growth, fatalities, water intake, organ weights, liver lipids, and liver and serum cholesterol levels. Some of the nontoxic, as well as the toxic, fractions proved to be of biological interest.

Experimental Procedures

Commercial samples of winterized cottonseed oil hereafter CSO) and of prime steam lard (containing no antioxidant) were both oxidized with vigorous streams of oxygen at a temperature of 95–100°C. ... 210 hrs. The oxidations were conducted in 12-...ter flasks in batches of 8 to 10 kg.

A flowsheet summarizing the preparation of the fractions is given as Figure 1. Most of the autoxidized lard and autoxidized CSO were fractionated by first subjecting them to molecular distillation through a falling film, cyclic type of still. The distillate fractions were collected up to a temperature ... 275°C. at a pressure of 6 to 12 microns. The autoxidized lard yielded 54% distillate and 46% polymeric residue; the autoxidized CSO gave 42% distillate and 52% polymeric residue. The distillates and residues were saponified by refluxing with aqueous NaOH in alcohol and acidified; the resulting acids were esterified with ethanol.

The ethyl esters of the molecular distillate from autoxidized lard were fractionally distilled through a 2 × 20-in. Vigreux column to yield Distillate 1, MDDD (1%, 65–120°C./2 mm.); Distillate 2 (44%, 160–180°C./0.3 mm.); and a residue, MDDR (8%). (The yields of the various fractions are percentages of the autoxidized material originally charged to the molecular still.) Distillate 2 was separated into four fractions by urea complex formation and alembic distillations: Complex-Distillate, MDCD (33%), Complex-Residue, MDCR (1%), Noncomplex Filtrate-Distillate, MDFD (4%), and Noncomplex Filtrate-Residue, MDFR (2%). The alembic distillations in this report were conducted in a high-vacuum, short-path apparatus with an alembic type of distillate collector at pressures of less than 0.1 mm. of mercury.

The ethyl esters of the molecular distillation residue from autoxidized lard were molecularly distilled to give the following fractions: Distillate 1, MRMD1 (23%, 100–150°C.); Distillate 2, MRMD2 (9%, 150–225°C.); and a Residue, MRMR (14%). The pressure at the start of distillation was 20 microns. It decreased to 8 microns as the more volatile materials were removed.

The ethyl esters of the molecular distillate from autoxidized CSO were fractionated by urea-complex formation, followed by alembic distillation of the complex- and noncomplex-forming portions. The resulting fractions were: Complex-Distillate, MDCD (22%); Complex-Residue, MDCR (2%); Noncomplex Filtrate-Distillate, MDFD (7%); and Noncomplex Filtrate-Residue, MDFR, MDFR (7%).

The ethyl esters of the molecular distillation residue from autoxidized CSO were subjected to an alembic distillation to give a monomeric distillate, MRAD (16%). The large quantity of material remaining as the residue from this distillation was further separated by means of molecular distillation to give an apparently dimeric distillate, MRMD (11%), and a residue of higher polymeric materials, MRMR (23%).

The fractions were analyzed for the characteristics given in Table I (3). Molecular weights were deter-

[1] Carried out with the aid of a grant from the United States Public ...alth Service.
[2] Presented at the 30th fall meeting, American Oil Chemists' Society, ...tember 28–30, 1956, Chicago, Ill.
[3] A Laboratory of the Eastern Utilization Research and Development ...ision, Agricultural Research Service, United States Department of ...riculture.

TABLE I

Chemical Properties of Fractions of Autoxidized Lard and Cottonseed Oil

Sample [a]	Acid No.	Sap. No.	Iodine No.	% Hydroxyl oxygen	% Carbonyl oxygen	Mol. wt.	Fatty acid chain-length
Lard fractions							
MDDD	12	249	28	0.4	2.2	......	C_{16} and shorter
MDDR	22	193	44	1.1	0.9	350	C_{18}
MDCD	5	192	27	0	0.3	......	C_{18}
MDCR	8	188	67	1.1	2.0	800	Dimer and trimer of C_{18}
MDFD	34	197	67	0.4	0.6	......	C_{18}
MDFR	25	191	53	1.7	1.3	1100	Trimer of C_{18}
MRMD1	5	199	30	1.0	0.5	......	C_{18}
MRMD2	15	223	48	2.8	1.3	1000	Trimer of C_{18}
MRMR	14	230	47	2.0	1.4	1500	Higher mol. wt.
CSO fractions							
MDCD	5	192	45	0.4	0.2	......	C_{18}
MDCR	15	201	67	1.6	1.5	650	Dimer of C_{18}
MDFD	18	192	121	1.0	0.4	......	C_{18}
MDFR	16	185	73	2.1	0.9	640	Dimer of C_{18}
MRAD	8	202	58	0.8	0.3	......	C_{18}
MRMD	9	196	70	3.8	0.9	550	Dimer of C_{18}
MRMR	8	209	70	2.5	0.8	1500–2000	Higher mol. wt.

[a] As can be seen from the flowsheet, the first two letters of each fraction (MD and MR) indicate whether the fraction was obtained from the original molecular distillate or residue. The meaning of the last two letters can also be gained from the flowsheet, e.g., MRMR is the molecular residue of the original residue, and MRMD, its distillate.

mined by the comparative ebulliometric method, using two ebulliometers and a 10-junction differential iron-constantan thermopile (4).

All fractions were incorporated at a level of 8% in a purified rat diet containing 30% casein (G.B.I. Vitamin-Free Test Casein), 56% dextrose (Cerelose), 4% salts (U.S.P. XIII), 2% cellulose (Alphacel), and, per kilogram of diet, 1 g. of choline dihydrogen citrate, 1 g. of inositol, 300 mg. of p-aminobenzoic acid, 100 mg. of nicotinamide, 2 mg. of thiamine hydrochloride, 4 mg. of riboflavin, 4 mg. of pyridoxine, 10 mg. of calcium pantothenate, 2.5 mg. of folic acid, 5 micrograms of vitamin B_{12}, 25 micrograms of biotin, 10 mg. of synthetic vitamin K, 25 mg. of ascorbic acid, and 1 cc. of a linoleic acid suspension containing 5 mg. of *beta*-carotene, 50 mg. of *alpha*-tocopherol acetate, 10 mg. of free *alpha*-tocopherol, and 0.5 mg. of crystalline vitamin D_2. The control diet contained 8% lard. A fat level of 8% was chosen because preliminary studies had shown that ethyl esters of naturally-occurring fatty acids permitted the same growth as fresh lard when 8% was included in the diet.

The feeding experiments were carried out on albino rats from a homogeneous colony. From the time of their delivery to the laboratory until the start of the experiment when they were 35 days old, the weanling rats were given a lard diet similar to that described above but containing lactalbumin instead of casein. They were ear-marked and weighed at 31 days and reweighed at 35 days. At this time they were distributed into matching groups, the average weights of which were the same at 31 days and again at 35 days. The rats were kept in individual cages on shavings and were given water in nondripping bottles suitable for water-intake measurements. The animals were weighed twice weekly.

At the end of the experiment the animals were anesthetized with ether, and blood was drawn from the heart for cholesterol analyses, which were done on conveniently pooled samples according to Schoenheimer and Sperry (5).

Kidneys, testicular fat bodies, and livers were weighed, and the latter were immediately frozen on dry ice. All livers and sera of the groups studied were analyzed; two to four livers and the matching sera were pooled in corresponding samples. The liver sample was homogenized, and a sample of this was dried to constant weight at 100°C. A second sample was extracted with a 3:2 alcohol-ethyl ether mixture. An aliquot of the extract was dried to constant weight at 100°C. for the total lipid content. A second aliquot was analyzed for cholesterol according to the method of Sperry and Webb (6).

For the evaluation of the effects of the fractions on organs, the organ weight-body weight relationship was used. This relationship is not linear, but a log-log plot gives a straight-line distribution, the slope and spread of which are characteristic for each organ. Such distributions usually show one or more changes in slope with increasing body weight (7). To compare organ weights of groups having widely different average body weights, the organ weights of the control animals fed lard were used as the reference. These were plotted against the corresponding body weights on log-log paper, and the best straight line was drawn through them, with the established slope for the organ. This line became the source of "normal" organ weights for various body weights. The actual organ weights observed in the experimental groups were compared with the "normal" weights for the same body weights as derived from this line, and the differences between the two were expressed as percentages of the "normal" organ weights. Thus even the control organ weights sometimes showed slight deviations from the ideal, depending on how accurately the ideal line had been drawn. The slopes for livers and kidneys have been given in a previous report (2). The slope for the testicular fat bodies was 64°.

For the statistical analysis of the results, standard errors are given after average values, from which t values can be calculated and the P's read from a table because the number of observations is given with the data. A P of 0.05 was considered to be on the borderline of significance.

Results

In Tables II and III are summarized the data concerning survival rate, weight gain, water intake, and organ weights. The highest death rates were observed with the predominantly dimeric fractions, MRMD2 from lard and MRMD from CSO. With all other fractions the survival rates after three or four

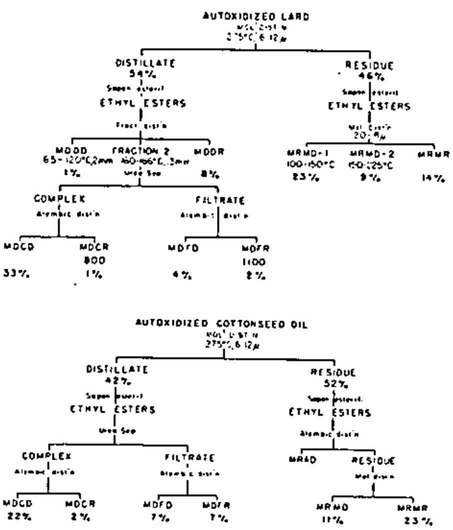

FIG. 1. Flowsheet for fractionation of autoxidized lard and cottonseed oil. The percentage yields are based on the amount originally fed into the molecular still. Molecular weights are given below some fractions.

weeks were not significantly different from those of the animals on lard.

In evaluating the effects of the fractions on body weight, an attempt was made to correlate them with the chemical characteristics of the fractions. If one relates the average body weights of the groups after three weeks (Tables II and III) to the percentage of hydroxyl oxygen in the respective fractions (Table I), a rough inverse relation is observed; the least oxidized fractions permitted the best growth and *vice versa*. However most of the fractions having higher OH-oxygen concentrations were dimeric or trimeric, and such fractions have previously been shown to be particularly toxic (8). Therefore the presence of dimers may account for the toxicity, especially since the higher polymers of both autoxidized CSO and lard depressed growth considerably less although they had high OH-oxygen concentrations. On the other hand, OH-oxygen concentrations would seem to have had some effect on body weight because the dimeric fraction from lard, MDCR, which contained less OH-oxygen than any other dimeric fraction, led to comparatively little depression of growth and the unpolymerized fraction, MDDR, with a high OH-oxygen content, markedly depressed growth.

For the comparison of the water intakes it was necessary to take into consideration the fact that the average body weights of the groups differed considerably and that water consumption of animals of different body weights could not be compared directly. Therefore each animal's surface was calculated by Lee's formula, $S = 12.44\ W^{2/3}$, where S is the surface in cm.² and W is the body weight in g. (9). The body weight of each rat was considered to be the average of its weights at the beginning and end of the experimental period. Each animal's water intake for this period was divided by the number of weeks in the period, and this average intake per week was divided by S/100 to give average intake per week per 100-cm.² surface. Five control groups (not otherwise used in these studies) had intakes of 36–38 cc./100 cm.²/week for body weights of 100 to 428 g.

It can be seen from Tables II and III that some of the fractions increased the water intake significantly; none depressed it. On the average, fractions from the original residues (MR..) were more active than those from the original distillates (MD..), with P less than .01. In most instances, increased water intake was associated with enlarged kidneys, suggesting some renal damage. However the atoxic, unpolymerized fractions, MDCD, from autoxidized lard and CSO, which contained mainly C_{18} chains, brought about significantly increased water-intakes without significant renal enlargement. MRMD1 from lard and the comparable MRAD from CSO had only a mild influence on body and kidney weights but increased water intakes markedly (P less than .02 and .01, respectively).

If one examines the degree of kidney and liver enlargement of the experimental groups in relation to their average body weights, one sees the inverse relationship commonly noticed under various stress

TABLE II

Survival Rate, Body Weight, Water Intake, and Percentage of Kidney, Liver, and Fat Body Enlargement of Male Rats Fed Fractions of Autoxidized Lard for Three Weeks

Sample	Fatty acid chain-length	Survival rate	Av. body weight (g.)	Av. total water intake (cc./100 g. body wt.)	Av. wt. difference: Kidney	Av. wt. difference: Liver (%)	Av. wt. difference: Fat body
Lard	....	12/13	196 ± 7.4[a]	193 ± 4.9	− 2 ±2.5	+ 2 ± 2.7	+ 2 ± 3.8
MDDD	C_{16} and shorter	4/4	137[b] ±16.8	148[b] ± 7.5	− 1 ±3.4	−23 ± 2.6	....
MDDR	C_{18}	11/14	108 ± 6.1	212 ±21.6	+18 ±3.9	+34 ± 3.7	−16 ± 5.9
MDCD	C_{18}	13/14	188 ± 4.2	224 ± 9.6	− 3 ±2.2	− 3 ± 2.4	− 1 ± 4.7
MDCR	Dimer and trimer of C_{18}	4/4	138[b] ± 9.3	....	+12 ±7.7	+48 ±19.0	....
MDFD	C_{18}	11/12	155 ± 9.0	225 ±18.4	+20 ±3.5	+ 6 ± 3.4	+ 8 ± 6.5
MDFR	Trimer of C_{18}	4/4	133 ±13.0	125 ±10.3	+28 ±7.4	+31 ± 6.4	....
MRMD1	C_{18}	13/14	175 ± 6.9	231 ±11.4	+11 ±2.0	+10 ± 3.2	+13 ± 6.2
MRMD2	Trimer of C_{18}	9/14	93 ± 7.5	297 ±21.8	+31 ±4.3	+42 ± 4.7	− 3 ±14.6
MRMR	Higher mol. wt.	12/13	137 ± 7.7	281 ±12.6	+14 ±2.3	+13 ± 2.2	0 ± 6.8

[a] Standard error. [b] After 2 weeks when lard animals had body wt. of 162 g. and water intake per 100 g. of 127 cc.

TABLE III

Survival Rate, Body Weight, Water Intake, and Percentage of Kidney, Liver, and Fat Body Enlargement of Male Rats Fed Fractions of Autoxidized Cottonseed Oil for Three Weeks

Sample	Fatty acid chain-length	Survival rate	Av. body weight (g.)	Av. total water intake (cc./100 g. body wt.)	Av. wt. difference: Kidney	Av. wt. difference: Liver (%)	Av. wt. difference: Fat body
Lard	....	14/16	190 ±6.6	201 ± 4.9	− 1 ±3.6	+ 1 ±3.6	+ 4 ±6.2
MDCD	C_{18}	14/16	186 ±6.1	217 ± 6.6	+ 8 ±2.7	+ 2 ±1.6	− 6 ±2.9
MDCR	Dimer of C_{18}	3/4	103 ±5.2	....	+32 ±4.4	+19 ±7.4	....
MDFD	C_{18}	7/8	137 ±2.5	....	+21 ±6.9	+16 ±5.1	....
MDFR	Dimer of C_{18}	6/8	104 ±1.9	....	+ 7 ±4.2	+49 ±4.3	....
MRAD	C_{18}	16/16	179 ±3.6	268 ±15.2	+11 ±3.0	+ 8 ±2.4	−18 ±5.1
MRMD	Dimer of C_{18}	12/16	85 ±3.1	228 ±22.1	+25 ±3.0	+45 ±3.5	....
MRMR	Higher mol. wt.	15/16	126 ±3.3	274 ±17.0	+ 5 ±2.1	+10 ±2.7	−24 ±9.6

conditions. Those groups having the most depressed body weights in general had the largest kidneys and livers in relation to body weight. However certain fractions had more pronounced effects on one or both organs than could be ascribed unspecifically to various stresses, *i.e.*, the dimeric fraction from lard, MDCR, brought about severe enlargement of the liver whereas the kidneys of these animals were only slightly larger than those of other animals with the same body weight. Also noteworthy was the disproportionately small effect of the high polymer fraction from CSO, MRMR, which led to depressed growth but permitted normal kidneys and only slightly enlarged livers. Another fraction with relatively little effect on the kidney was the dimeric CSO fraction, MDFR, which led to almost normal kidneys but large livers.

Testicular fat body weights were studied because it has been shown that they are proportional to the total neutral fat in the body (10). However the relation of this fat to total body weight is somewhat variable, and the slope of the log-log plot is steep. Thus small differences are of dubious reliability. A study of the percentage of fat body enlargement in relation to body weight loss or OH-oxygen concentration of the particular fraction responsible for it failed to show any correlation.

However comparison of all available corresponding fractions from autoxidized lard and CSO, which were the two MDCD fractions, MRMD1 and MRAD, and the two MRMR fractions, shows that those fractions derived from CSO led to less fat deposition than the corresponding lard fractions although corresponding fractions depressed growth to almost the same degree. If one averages the fat body enlargement brought about by the three CSO fractions and compares the result with that from the lard fractions, the difference is significant (P less than .01). Examination of the chemical properties of these six fractions (Table I) reveals that the iodine numbers of the lard fractions range from 27 to 47 and those of the CSO fractions, from 45 to 70. However no conclusion is possible from these data.

In Table IV are summarized data from liver lipid and serum cholesterol studies. The average percentage of dry substance of the livers enlarged more than 30% was 31.4 ± .10, that of the smaller livers, 30.4 ± .18; the difference was significant (P less than .01). However the average total lipid content of the large livers was 20.8% ± 1.6 whereas that of the smaller was 23.5 ± .67. Therefore, if it is permissible to consider the carbohydrate content of the livers as negligible, one can conclude that enlargement was accompanied by accumulation of protein.

Liver and serum cholesterol levels varied with the degree of liver enlargement. The average liver cholesterol value for the three groups having the largest livers was 702 mg. % dry wt. ± 13; the average of the others was 788 ± 14, with P less than .01. Since a low liver cholesterol level, when accompanied by a low serum level, is a sign of depressed cholesterol synthesis (11), it seems likely that such was the case with the large livers. The low levels reflected damage to the livers induced by the toxic fractions. However the percentage of cholesterol in the total liver lipids was 3.6% in the large livers and 3.4% in the small livers. Therefore the difference in cholesterol levels was probably an expression of the lipid depletion of the damaged livers.

Similar lipid studies were carried out on rats fed fresh fats and autoxidized CSO. The diets contained 15% fat but were otherwise the same as those containing the ethyl esters. The fresh fats used were cottonseed oil, lard, a commercial margarine oil, corn oil, butter, and saturated medium chain triglycerides. The autoxidized CSO had been aerated at 90–95°C. for 300 hrs. The rats were placed on the diets at weaning and maintained for two to four months.

TABLE IV

Liver Lipid and Liver and Serum Cholesterol Levels of Male Rats Fed Fractions of Autoxidized Lard and Cottonseed Oil

Sample	Liver dry substance (%)	Total liver lipids (% dry wt.)	Liver cholesterol (mg. % dry wt.)	Serum cholesterol (mg. %)
Lard	29.9 ±2.5	21.3 ± .2	808 ± 5.5	68.5 ±2.3
Lard Fractions				
MDDR	31.2 ± .15	19.7 ±2.3	692 ±12.5	45.4 ±1.0
MDCD	29.8 ± .1	22.6 ±2.2	896 ±38.5	64.0 ±2.2
MDFD	31.1 ± .1	26.7 ±1.2	775 ±78.0	67.2 ±1.2
MRMD1	30.4 ±2.25	24.1 ± .6	737 ± 4.5	56.2 ±1.1
MRMD2	31.5	24.0	692	44.3
MRMR	29.9 ± .65	22.0 ± .4	805 ±50.0	53.8 ±5.4
CSO Fractions				
MDCD	30.0 ± .1	23.7 ± .4	795 ±17.0	74.2 ±1.2
MRAD	30.6 ± .05	24.3 ±2.2	766 ±36.0	64.0 ±2.2
MRMD	31.5 ± .1	18.7 ±2.2	717 ±32.0	35.7
MRMR	30.9 ± .5	21.3 ±1.0	757 ±10.0	48.5

In Table V are given the results of these studies. As with the toxic fractions of autoxidized CSO and lard, the livers of the animals fed the autoxidized CSO were relatively depleted in lipid, and their cholesterol was substantially lower than that of the animals on fresh fats. Also the cholesterol content of the total liver lipids in the animals on autoxidized CSO was significantly lower than that of the animals fed fresh fats. Inasmuch as it has been shown in earlier work (2) that the livers of animals on autoxidized CSO are damaged, these results would bear out the findings with the toxic fractions of autoxidized CSO and lard. In fact, the results with autoxidized CSO were more pronounced than were those with the fractions. This may have been caused by the higher level of autoxidized fat (15 instead of 8%) or by the fact that triglycerides rather than ethyl esters were fed.

TABLE V

Liver Lipid and Liver and Serum Cholesterol Levels of Male Rats Fed Fresh Fats or Autoxidized Cottonseed Oil

Sample	No. of expts.	Total liver lipid (% dry wt.)	Cholesterol level (% liver lipid)	Cholesterol level (mg. % dry substance)
Fresh fats	12	24.8±.91	4.1±.15	999±24
Autoxidized cottonseed oil	6	21.6±.58	3.6±.08	768±14

Discussion

The results of this attempt at screening the many substances occurring in autoxidized lard and CSO indicate that some of the materials produced were toxic to rats; the survival rate declined and growth was depressed. Certain types of polymers (particularly dimers, as noted before [8]) and higher levels of oxidation (as indicated by high OH-oxygen content) were associated with toxicity. However, if one considers that the toxic fractions were prepared from materials autoxidized far beyond that occurring when fats are used for human consumption, extrapolation of these findings to the action of commercially-used fats seems unwarranted.

Probably more significant than the expected toxicity of some of the fractions is the fact that some of the atoxic fractions had characteristic effects which may deserve pharmacological study. For instance, the atoxic fractions MDCD from autoxidized lard and CSO, which contained mainly straight C_{18} chains, increased fluid intake significantly. Also of interest may be the fractions tending to depress neutral fat deposition and some of the fractions increasing liver and kidney weight.

The depression of serum and liver cholesterol by the toxic fractions may have some relevance to the current interest in the relation of fats to serum cholesterol. It suggests that the mere depression of serum cholesterol by a fat does not necessarily imply an advantage to the animal.

Summary

Lard and cottonseed oil which had been autoxidized at about 100°C. for 210 hrs. were fractionated by a technique involving molecular distillation, conversion to ethyl esters, urea-complex formation, and redistillation. The ethyl esters were then fed to rats for three weeks at a level of 8% in a purified diet. Growth, water intake, organ weights, total liver lipids, and serum and liver cholesterol levels were determined. Groups fed 8% lard served as controls.

Growth was severely depressed by the residue fractions of the urea-complex- and noncomplex-forming portions of the original molecular distillates. Of the three fractions from the original molecular distillation residues, the dimeric and polymeric fractions were the most active. The relative liver and kidney weights were usually increased by feeding the growth-depressing fractions. However there were a number of exceptions indicating more specific effects from some of the fractions. Water intakes were lower with the fractions derived from the original molecular distillates than with those from the original molecular distillation residues. Testicular fat body weights suggested that feeding of autoxidized CSO fractions led to less neutral fat deposition than feeding of corresponding autoxidized lard fractions. Dry weight of the enlarged livers was higher, and the total lipid lower than of the control livers. Total liver cholesterol was higher in animals with smaller livers, but there was no difference in the cholesterol content of the total liver lipids. Serum cholesterol levels were lower in animals with large livers.

Further study of those fractions having pharmacological properties is suggested.

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all physical symptoms of molybdenosis. Sodium and ammonium sulfate had similar effects, although the ammonium salt exhibited toxic properties. The sulfate salts practically eliminated the mortality produced by sodium molybdate, but failed to prevent the additional mortality characteristic of ammonium molybdate. Addition of graded levels of sulfate to a high-molybdenum diet failed to prevent greatly increased storage of molybdenum in the tibiae of chicks, although growth improved as sulfate concentration was increased.

Methionine failed to affect the growth depression caused by a high level of molybdenum.

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Toxicity of Air-Oxidized Soybean Oil[1,2]

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Although it has long been recognized that overheated or oxidized fat causes toxic manifestations in the rat, neither the exact nature of materials causing these symptoms nor the specific mechanisms by which they are caused have been understood with any certainty. Work prior to 1940 established that highly oxidized fat accelerates the destruction of a large variety of essential nutrients (Burr and Barnes, '43). Subsequent work, however, has indicated that certain oxidized oils contain materials which are toxic to the rat even though apparently adequate steps are taken to protect the easily-oxidizable foodstuffs in the diet.

The identity of the toxic materials in oxidized or overheated fat has been investigated by two routes, differing in the manner by which the oxidized fat was obtained. Several groups of investigators, including Crampton et al. ('51a, '51b, '51c, '53, '56), Common et al. ('57), Granados et al. ('49), and Raju and Rajagopalan ('55) have changed the chemical nature of oils primarily by means of heat treatment, often with complete exclusion of air. Other groups of investigators (Matsuo, '54; Kaneda et al., '55; Kaunitz et al., '55; Andrews et al., '56) oxidized various oils by aeration with little or no elevation in temperature during the oxidation process. Grossly, the results obtained are similar when either type of oxidized oil is fed, namely, rough fur and unkempt appearance and decreased weight gain often followed by death. When, however, those groups feeding heat-treated oil measured the peroxide content of their product, the peroxide concentration in the toxic oil was found to be very low compared with values obtained with aerated oil. On the other hand, in those cases in which oils were oxidized by aeration and some attempt was made to measure the toxicity due to polymers, the polymer content appeared completely innocuous, the toxicity apparently correlating best with peroxide concentration.

In the experiments to be reported in this paper, an attempt was made to reconcile these apparently contradictory results and, further, to identify the toxic mechanism or mechanisms which so frequently lead to death in the rat. There are apparently few or no definitive data on the latter point although it would seem to be of considerable importance in many areas of the world in which cooking customs involve intermittent high-temperature and open air heating of highly unsaturated oils (Raju and Rajagopalan, '55). Consequently, in a preliminary experiment, various levels of peroxidized fat were fed to determine the levels of toxicity and toxic symptoms. Second, the peroxidized oil was fractionated in such a way as to separate peroxide and polymeric material and the fractions were fed. Third, the absorption of peroxidized fat was studied, and fourth, an attempt was made to ascertain the site of the damage.

METHODS AND MATERIALS

Growth experiments. Unless otherwise noted, weanling rats[4] were fed diets containing air-oxidized soybean oil. The animals were individually housed in sus-

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pended cages and, where pertinent, individual diet consumption records were kept. Each rat was weighed twice weekly.

Two diets were utilized in the growth experiments. The percentage composition of diet A was as follows: casein, 25; sucrose, 46; salt mixture, 4; brewers' yeast, 5; fat, 20. The composition of diet B is presented in table 1, as well as the composition of the vitamin and salt mixtures.

Semiweekly administration to each rat of 175 U.S.P. units of vitamin A, 35 units of vitamin D and 1.75 mg of α-tocopherol acetate dissolved in 0.05 ml of sesame oil was utilized to satisfy the fat-soluble vitamin requirement. Initially, this supplement was administered by intramuscular injection in order to avoid the possibility of oxidation by peroxide in the gut. Subsequent experimentation, however, established that similar growth could be obtained in rats given this supplement orally at noon on a semiweekly basis (most of the diet was consumed during the night), and this method of supplementation was used in later experiments.

Preparation of oxidized soybean oil. The raw oil was oxidized by aeration in a water bath at 60°C for approximately one week with the addition of 2 mg/kg each of $CuCl_2$ and $FeCl_3$. Toward the end of the oxidation period the peroxide number (PN), milliequivalents of peroxide oxygen per kilogram of oil, was determined at intervals according to the method of Polister and Mead ('53) so that oxidation might be halted at maximum peroxide concentration inasmuch as the PN increases rapidly after the maximum is reached. The oxidized oil was then stored at −20°C under nitrogen.

Fractionation of oxidized soybean oil. Two kilograms of raw oil were oxidized in the usual manner and one-half of the oxidized oil was stored at −20°C (fraction C). The remaining oil was dissolved in 1.7 l of petroleum ether (b.p. 30 to 60°) saturated with methanol and was then extracted with 4 l of methanol saturated with petroleum ether. The oil contained in the petroleum ether layer was separated on a silicic acid column into two fractions. This fractionation was accomplished by adding 25-gm quantities of oil in petroleum ether to 8 by 14-cm silicic acid columns and eluting with 500-ml portions of 5% ethyl ether in petroleum ether. Each fraction was collected separately and evaporated to dryness under nitrogen in a warm-water bath. All fractions weighing more than 1 gm were tested for peroxide, as previously described, and those fractions having a peroxide number of less than 20 were pooled. The resulting colorless oil (fraction A) had a peroxide value of 13.2 and contained little or no polymeric material as shown by complete distillation of a methylated sample. After removal of the low-peroxide material from the columns, the remaining oil was eluted with three- to 4-column volumes of methanol. The methanol was evaporated under reduced pressure at a temperature under 40°C. The last traces of solvent were removed under high vacuum at room temperature. The combined sample from all

TABLE 1
Composition of diet B

Dietary component main mixture		Salt mixture		Vitamin mixture	
		Salt	Amount	Vitamin	Amount
	gm/100 gm		gm		mg/gm
Oxidized oil	15.0	$CaCO_3$	300.0	Choline	120,000
Casein	18.0	$CaHPO_4$	75.0	Thiamine	200
Sucrose	60.8	K_2HPO_4	322.5	Riboflavin	200
Glycerol	1.0	NaCl	167.4	Niacin	400
l-Cystine	0.2	$MgSO_4$	49.8	Calcium pantothenate	800
Salt mixture	4.0	$MnSO_4 \cdot 2H_2O$	0.5	Folic acid	8
Vitamin mixture	1.0	Zn acetate	0.402	Biotin	4
		Fe citrate	27.4	Pyridoxine	100
		KI	0.8	Vitamin B_{12}	0.8
		$CuSO_4 \cdot 5H_2O$	0.3	i-Inositol	4.0
				Sucrose	273,500

of the columns was an amber, moderately viscous oil with a peroxide number of 927 (fraction B). The original methanol fraction was extracted with petroleum ether at 0°C for 6 hours in a liquid-liquid extractor. The sample, in methanol, was stored under nitrogen at −20°C until shortly before use. The methanol was then removed under reduced pressure in an ice bath with a slow stream of nitrogen. High vacuum was utilized to remove the last traces of the solvent. The sample (fraction D) was colorless, extremely viscous and had a peroxide number of 3,185. Peroxide numbers and spectral properties of the fractions are listed in table 2.

Absorption studies. The animals used in these investigations were mature rats[4] which were subjected to thoracic duct cannulation according to the technique of Bollman et al. ('48). Preliminary investigation revealed that pre-operative administration of 2 ml of oxidized soybean oil (PN = 1200) was frequently fatal to the subject. A similar amount of peanut oil was employed, therefore, in the pre-operative procedure. Lymph was then collected for a 20- to 24-hour period at room temperature and was stored at −20°C. These samples served as the source of control lymph fat. After the initial period of lymph collection, a dose of air-oxidized soybean oil was administered to the cannulated rat. Lymph collection was then continued for periods extending up to 12 hours. These samples were also stored at −20°C. Similar volumes of both control and experimental samples were then extracted with a 3:1 mixture of alcohol and ether, the protein removed by centrifugation, and the alcohol-ether layer evaporated in a tared flask under nitrogen on a warm-water bath, for determination of peroxide number. Other lymph fat samples were examined for conjugated diene by dissolving the fat isolated from 2 ml of lymph in 50 ml of ethanol and examining solution at 232 μ with a Carey recording spectrophotometer.

Xanthine oxidase assay. The intestinal xanthine oxidase of rats was assayed as follows: the animals were decapitated and the upper third of the small intestine excised; intestinal contents were flushed out with approximately 10 ml of saline and 0.5 gm of the upper end of the washed intestinal section placed on dry ice until homogenized; homogenization was conducted at 5°C in 10 ml of an 0.015 M sodium pyrophosphate buffer contained in an all glass homogenizer. The general method of enzyme assay was that of Dhungat and Sreenivasan ('54). Milk xanthine oxidase was purified according to the method of Ball ('39).

TABLE 2
Peroxide number and conjugated diene concentration in the various fractions of oxidized soybean oil

Fraction	Peroxide number	Meq. conj. diene/kg oil[1]
A	13	0
B	927	1190
C (unseparated)	1156	780
D	3185	1602

[1] The fact that the peroxide numbers and conjugated diene concentrations do not change in parallel fashion probably indicates that the unsaturated centers of many molecules have suffered further attack after the initial peroxide formation.

RESULTS

Rat growth experiments. In the initial growth study, oxidized oil (1200 PN) was diluted with fresh oil to give peroxide numbers of 800, 400 and 100. Four variations of diet A were prepared: three with each of the diluted oils and the 4th with the original 1200 PN oil. A 5th diet containing fresh oil served as the control. Extraction of the fat in these diets showed that the peroxide-number did not change on mixing, after three weeks' storage at −20°C, or on standing at room temperature for three days.

After consuming the control diet for 6 days, 50 weanling rats were divided into 5 groups of 10 each, equally divided between males and females. One of these groups was assigned to each of the diets previously described, and the animals were weighed individually twice a week for the ensuing 70 days. The average growth curves of the female rats in each group are presented in figure 1. The diets containing 20% of fat with peroxide numbers

[4] Sprague-Dawley strain.

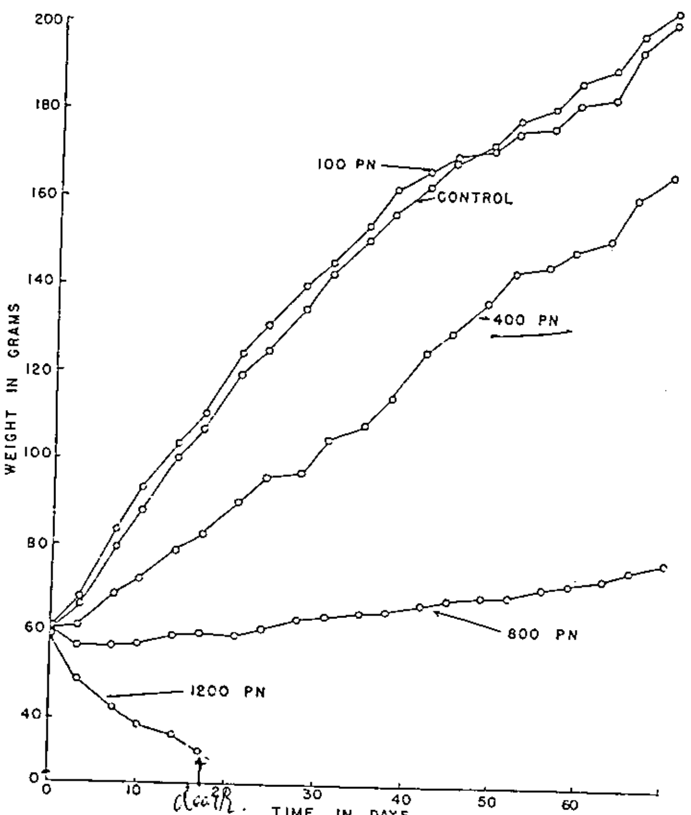

Fig. 1 Growth of female rats receiving various levels of oxidized fat.

of 400 to 1200 caused a reduction in growth rate directly related to the peroxide number. Consumption of the 1200 PN diet caused death within approximately three weeks. The only other symptoms observed in the animals fed this diet were weight loss and severe diarrhea. Animals receiving the 800 PN diet developed moderate to severe diarrhea, but this symptom had subsided somewhat by the 8th week. Similar effects were observed in the growth of male rats fed these diets.

The data obtained in this experiment indicate that the diet containing 100 PN oil has no effect on growth. It seemed conceivable, however, that under conditions of stress a difference between the control and 100 PN diets might become apparent. Accordingly, an experiment was conducted in which 25 rats, 11 males and 14 females, consumed the 100 PN diet for 8 weeks and then were subjected to a whole-body X-irradiation dose of 500 r.[3] A similar group of animals served as concomitant controls. In both the control and 100 PN diets the concentration of brewers' yeast was increased from 5 to 7.5%

[3] Irradiation was carried out using a Picker 250 KV Industrial X-Ray apparatus, with the following factors: 15 ma, 0.28 Cu parabolic, 0.21 Cu inherant and 1 Al; field size 47 cm², FOD 55 cm.

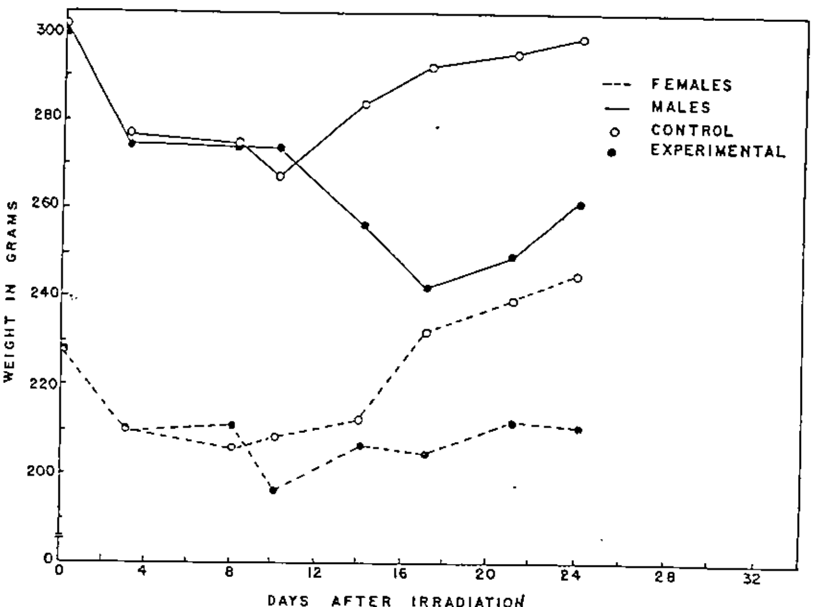

Fig. 2 Effect of diet on recovery from whole body irradiation.

at the expense of sucrose. Figure 2 shows the average group weight changes over the 27-day period following irradiation. The results obtained in this experiment indicate that consumption of the 100 PN diet caused a slower than normal recovery from the stress of irradiation.

In an attempt to compare the effect of the lipid peroxide with that of a simple water-soluble peroxide, and to eliminate diet flavor as a factor, t-butyl hydroperoxide, synthesized according to the method of Milas and Surgenor, ('46) was used in both feeding and injection studies. In preliminary injection experiments, 8 mg of this compound in a non-physiological solution was found to be immediately fatal when intravenously administered to rats weighing approximately 400 gm. Intravenous injection of a physiological solution of 1 mg of *t*-butyl hydroperoxide every three or 4 days over a two-week period caused a weight loss and some loss of hair. A growth study was conducted with 4 weanling rats fed control diet A by substituting a solution of 4×10^{-2} M *t*-butyl hydroperoxide for the drinking water. Figure 3 illustrates the average growth of these animals compared with three rats consuming the same diet and drinking tap water. This figure also illustrates the marked loss of weight which occurs when a high-PN diet is substituted for the control diet.

Fractionation experiments. In an attempt to settle the question of whether the toxic principle in oxidized oils is peroxidic or polymeric in nature, the fractions obtained from air-oxidized soybean oil were fed to weanling rats. It was assumed that fraction A contained most of the non-hydroxylated or peroxidized glycerides, while fraction D consisted primarily of glycerides containing fatty acid peroxides. Fraction B was assumed to be a mixture of the two materials. Since incorporation of a highly oxidized oil, namely, fraction D, in the diet makes the food unpalatable, the rats were fed a fat-free diet similar to diet A and the appropriate oil was force-fed. Diet A was modified by increasing the brewers' yeast from 5 to 7.5% and adding

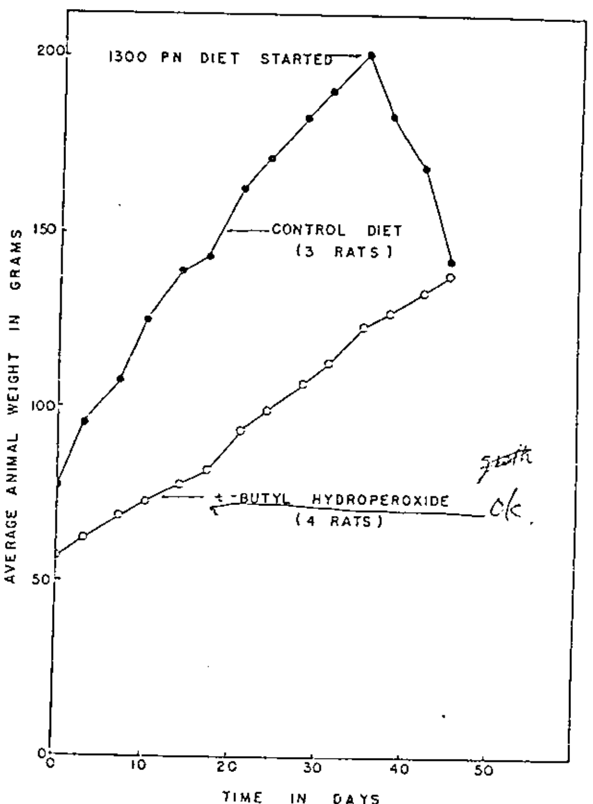

Fig. 3 Growth of rats under several dietary regimes.

2% of guar gum,[6] both at the expense of sucrose. Preliminary considerations indicated that each rat should receive an amount of oil equivalent to 38% of the total amount of calories consumed during the previous 24 hours. Ten grams of the dry diet were weighed into tared glass feed cups for each rat. Two milliliters of water were then mixed with the diet in each cup to make a thick paste which soon hardened and which the animals could eat with a minimum of spillage. An extra cup of diet was prepared each day and used to correct food consumption figures for water evaporation. Fifteen male and 5 female weanling rats ate the fat-free diet for an initial 8-day period. During this period some diarrhea was observed but weight gains were satisfactory and the animals appeared otherwise healthy. At the end of the 8-day period, the male rats were divided into three groups (A, B and C) of 5 each with the female rats comprising a 4th group (D). These group designations correspond to the fraction designations of the oxidized oil. All animals received orally twice the previously described volume of fat-soluble vitamin mix once a week about three hours before any fat was force-fed. If the amount of oil to be administered was more than 0.5 ml, it was given in two equal doses at noon and 4:00

[6] Donated by the Stein-Hall Co., New York.

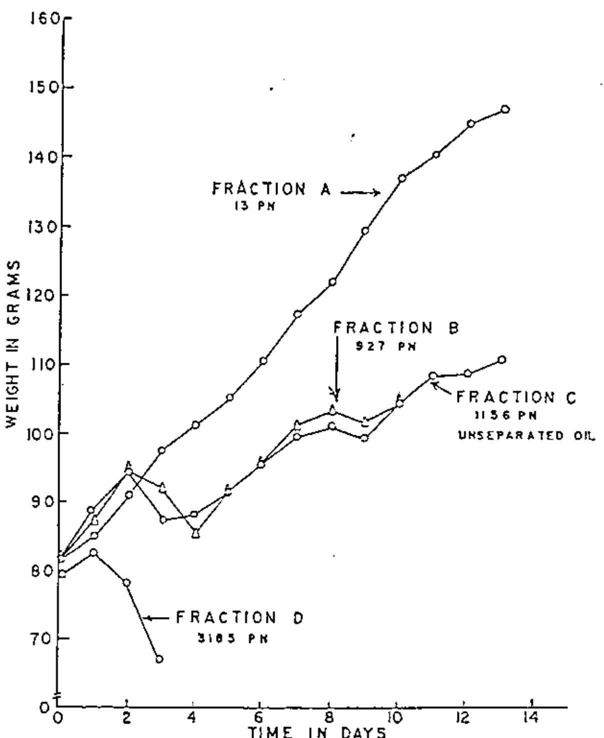

Fig. 4 Growth of rats receiving various fractions of oxidized soybean oil.

P.M. The rats were weighed daily during the experimental period. The average weight gains for the 4 diet groups are presented in figure 4.

The rats in group A appeared healthy and ate well in contrast to those in groups B, C and D. Those in groups B and C were indistinguishable in appearance but rats in group D began losing weight after the first day of fat administration. On the third day of the experiment two of the rats in group D were found dead and, since the remaining three appeared moribund, they were killed for pathological examination.

Histopathological examination[7] of these three animals revealed no obvious anatomical basis for their condition. The livers of these rats showed slight enlargement of the nuclei of the parenchymal cells and prominent nucleoli. Moreover, the small intestines of these animals had cytoplasmic vacuoles distending the mucosal walls. Examination of all other organs generally gave negative findings.

Absorption experiments. In order to ascertain the actual site of action of the toxic principle in oxidized fat, it was deemed of primary importance to determine whether any of the peroxide-containing material could be absorbed through the intestinal wall. For this study, rats with thoracic duct cannulae were given small doses of oxidized soybean oil, and the lymph was collected for periods varying between two and 24 hours. After isolation, the lymph fat was analyzed for peroxide and conjugated

[7] Histopathology performed under the direction of Dr. D. Moyer of the Dept. of Pathology, UCLA School of Medicine.

TABLE 3

Peroxide numbers of lymph fat obtained from rats fed either 0.5 to 1.0 ml of air-oxidized soybean oil (PN = 1200) or 2 ml of peanut oil

Rat no.	Oil fed	Lymph extracted	Weight of extracted fat	Peroxide no. of fat
		ml	*mg*	
A	Peanut	4.0	213.7	0.9
	Oxidized soybean	4.0	242.9	32.7
B[1]	Peanut	4.0	188.5	13.1
	Oxidized soybean	4.0	135.0	13.9
C[1]	Peanut	3.0	71.1	4.2
	Oxidized soybean	3.0	161.5	4.3

[1] Rats B and C are presented as typical of the 25 rats in which no significant change in the PN of the lymph fat was found.

diene. The results of the peroxide determinations are shown in table 3. The results of the experiment with rat A led to the conclusion that fatty acid peroxides may be absorbed as such. However, in subsequent experiments involving 25 cannulated rats it has been impossible to detect a significant rise in the peroxide number of lymph fat as the result of feeding oxidized soybean oil with a PN of 1200. The results obtained with rat A were, therefore, assumed to be either an artifact or an exceptional case of absorption.

Ultraviolet examination of a 1:50 dilution in ethanol of lymph fat from cannulated rats yielded the results typical of those presented in table 4. It can be seen that there was an increase in light absorption at 232 mμ (the maximum for conjugated diene) in the lymph of those rats which had been fed the oxidized oil. In the case of rat 6, 3 ml of oxidized oil (PN = 1100) were fed by stomach tube before the cannulation. Collection was begun immediately after the operation (two hours after feeding) and at no time did this animal receive further administrations of oxidized oil. It therefore appears that even though the peroxide itself may not appear in the lymph, its reduction products may be absorbed. The results of the absorption experiments suggest that the fatty acid hydroperoxides are destroyed during or before the absorption process and further, that the toxic effect probably takes place primarily in the intestinal cells.

Table 4

Optical densities at 232 mμ observed in lymph fat from rats fed oxidized soybean oil

Rat no.[1]	Amt. ox. oil fed	Length of collection time	232 mμ O.D. at[2]
	ml	*hours*	
9	0.5–1.0	4.5	0.075
	0.5–1.9	4.0	0.024
6	3.0	21.5	0.030
		5.0	0.130
		3.5	0.021
		16.0	0.058
		8.0	0.008
		16.0	0.000

[1] These results are presented as typical of several experiments performed.

[2] The optical density at 232 mμ (the maximal for conjugated diene) is a measure of the absorption of the fatty acid chain containing the unsaturated center prior to feeding the oxidized oil. The lymph fat had no significant absorption at this point under these conditions of dilution.

Intestinal xanthine oxidase assay. Since the absorption studies had led to the hypothesis that the primary site of toxicity was the intestine, and the pathology of these animals was generally negative, an investigation of the effect of oxidized fat on intestinal xanthine oxidase as a representative intestinal enzyme was undertaken. This enzyme was selected because it is fairly well characterized and is sensitive to lack of a dietary component (McQuarrie and Venosa, '45).

Twelve mature male rats were fed a control diet similar to diet B except that unoxidized soybean oil was substituted for the oxidized oil and the level was increased from 15 to 20% at the expense of sucrose. This control diet was continued for 10 days, at the end of which period the animals were paired on the basis of weight.

TABLE 5

Inhibition of intestinal xanthine oxidase by the ingestion of oxidized fat

Days on experiment	Diet	Feed ingested	Enzyme activity[1]	Intestinal protein[2]	Animal weight Initial	Animal weight Final
		gm/day	*μl O_2*	*%*	*gm*	
0	Control	0	23.5	73.9	226.5	
	Control				212.5	
2	Oxidized	2.4	8.0	62.4	211.0	207.0
	Control	2.4	28.5	59.4	201.5	—
5	Oxidized	3.0	1.5	64.5	193.0	178.5
	Control	3.0	4.5	63.6	194.0	180.0
7	Oxidized	3.7	0.5	68.7	212.0	189.0
	Control	3.7	10.0	62.1	204.0	194.0
9	Oxidized	4.9	1.0	66.9	203.0	177.5
	Control	4.9	13.0	63.6	204.0	209.0
12	Oxidized	5.9	0.0	62.1	200.5	188.0
	Control	5.9	25.0	56.7	197.0	203.5

[1] Corrected for endogenous activity.

[2] Determined by method of Lowry et al. ('51).

One pair was killed at this time for enzyme assay. One animal in each of the 5 remaining pairs was randomly assigned to an experimental diet containing 20% of air-oxidized soybean oil (PN = 1200). The control diet was pair-fed for the duration of the experiment. Pairs of rats were killed at intervals of 2, 5, 7, 9 and 12 days after initiation of the experiment and the intestinal xanthine oxidase of each animal assayed. The results of these assays are presented in table 5.

The observed inhibition of intestinal xanthine oxidase appears to be specific since it cannot be due to either lower protein intake or intestinal edema. One possible explanation for this enzyme inhibition is oxidation of sulfhydryl groups as suggested by Potter and DuBois ('43) in their study of succinic dehydrogenase. For an investigation of this possibility a group of 12 weanling female rats were paired and subjected to the same dietary regimen as in the previous experiment. Pairs of rats were killed each day for 6 days and the levels of SH in each intestine determined. The intestinal samples were prepared as described above and the method of Grunert and Phillips ('51) was used to determine the levels of SH, reported as glutathione. The results obtained in this experiment are given in table 6.

Apparently, the inhibition of intestinal xanthine oxidase is not directly connected with oxidation of sulfhydryl groups. In order to investigate further the mechanism of this enzyme inhibition, milk xanthine oxidase was purified by the method of Ball ('39) and *t*-butyl hydroperoxide was used as the inhibitory compound. The assay

TABLE 6

Levels of intestinal glutathione in weanling female rats consuming either oxidized or unoxidized soybean oil

Rat no.	Oil	No. of days on experiment	Intestinal glutathione level
			μg/mg fresh tissue
7	Control	1	0.38
8	Oxidized		0.57
9	Control	2	0.51
10	Oxidized		0.75
23	Control	3	0.27
24	Oxidized		0.21
11	Control	4	0.24
12	Oxidized		0.37
19	Control	5	0.19
20	Oxidized		0.22
25	Control	6	0.27
26	Oxidized		0.43

TABLE 7

Warburg assay of the inhibition of milk xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[4]
Buffer[1]	Enzyme	t-Butyl hydroperoxide 1.1×10^{-4} M	FAD[2,3] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.3				1.7	0.0
1.0	0.3			1.0	0.7	80.3
1.0	0.3	0.1		1.0	0.6	73.9
1.0	0.3	0.1	0.1	1.0	0.5	90.6

[1] 0.039 M K–$NaPO_4$, pH 7.54.
[2] Flavin adenine dinucleotide.
[3] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.
[4] Average of duplicate flasks.

TABLE 8

Warburg assay of the inhibition of rat intestinal xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[1]
Buffer[2]	Enzyme[3]	t-Butyl hydroperoxide 1.1×10^{-4} M	FAD[4,5] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.5				1.5	43.6
1.0	0.5			1.0	0.5	89.1
1.0	0.5	0.1		1.0	0.4	42.4
1.0	0.5	0.1	0.1	1.0	0.3	71.7

[1] Average of duplicate flasks.
[2] 0.039 M K–$NaPO_4$, pH 7.54.
[3] A 5% intestinal homogenate.
[4] Flavin adenine dinucleotide.
[5] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.

technique was essentially the same as that previously described except that a 0.039 M phosphate buffer with equal concentrations of sodium and potassium at pH 7.5 gave higher oxygen uptake and was, therefore, substituted for the pyrophosphate buffer. In view of the work of Bernheim, et al. ('52) and Ottolenghi et al. ('55) the effect of flavin adenine dinucleotide (FAD) on the inhibited enzyme was investigated. The data obtained in this experiment are reported in table 7.

The results of this experiment indicate that *t*-butyl hydroperoxide is capable of inhibiting milk xanthine oxidase and further, that this inhibition may be reversed by FAD. The high value for the flask containing FAD probably means that the enzyme preparation is somewhat deficient in FAD. A similar assay using a 5% intestinal homogenate from a stock rat as the enzyme source yielded the results presented in table 8.

This experiment corroborates the similarity of the inhibitory effects of the hydroperoxide and the ability of FAD to reverse these effects although apparently not as completely as with the purer preparation.

DISCUSSION

The investigations described above support earlier work in establishing that the toxicity ascribed to air-oxidized oils correlates well with the fatty acid peroxide content of these materials. The feeding experiment in which *t*-butyl hydroperoxide was utilized substantiates, at least in some degree, the toxicity of fatty acid peroxides, although this substantiation must be qualified because of differences in structure

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and probable mode of absorption. Nevertheless, from the data obtained in this experiment it can be calculated that the consumption of peroxide in the form of *t*-butyl hydroperoxide corresponds to a diet containing 20% of oil with a PN of approximately 600. It is interesting to note that the average growth curve of these animals falls between those for rats receiving diets with oils of 400 and 800 PN. The fractionation of air-oxidized soybean oil showed predominantly polymers of a polar nature, probably polyperoxides, and little or no polymers of the type found in heat-oxidized oil. This study also illustrates the complexity of the reactions occurring in the autoxidation of an oil, since if a simple generation of peroxide groups were the only reaction occurring, equivalence between peroxide oxygen and conjugated diene should be observed. Such is not the case, however (table 2). Although the work reported here demonstrates the toxicity of oxidized oil to be due entirely to peroxide formation, we have not investigated the toxic principle of anaerobically heated oils. Thus, it may be suggested that two separate classes of toxic compounds are possible in autoxidized oil—fatty acid peroxides when the fat is oxidized in air at only slightly elevated temperatures for 1.5 weeks or less and fatty acid polymers when the oil is heated to temperatures in the neighborhood of 250°C with the exclusion of oxygen for periods of one to two days. The conditions of autoxidation for a maximum yield of one class or the other will vary, however, with different oils. In our laboratory we have observed that extended aeration of soybean oil results in an oil of lower than expected peroxide content, and that the application of high temperatures at any point during the autoxidation process will have the same effect. It is probable that either of these modifications tends to decrease the peroxide content of the oil by formation of degradation products and polymers.

The primary site of toxicity, as demonstrated in the cannulated rats, appears to be the intestine. The gross observations and the limited pathology data available appear to support this conclusion. Even the irradiation experiment lends some indirect support to this hypothesis since it is known that the intestine is one of the most sensitive organs to whole-body irradiation, and extended recovery periods were observed in those animals receiving a dietary fat containing peroxide.

The mechanism or mechanisms by which fatty acid peroxides cause the observed toxic effects still need clarification and amplification. In the absence of observable gross changes in any of the organs examined, it seems likely that the injury is of a more subtle nature. The inhibition of intestinal xanthine oxidase, however, points to the possibility that choline oxidase and amine oxidase may be inhibited in a similar manner and that succinoxidase and cytochrome oxidase may also be inhibited but in a manner as yet unknown (Bernheim et al., '52; Ottolenghi et al., '55).

SUMMARY

The toxicity of air-oxidized soybean oil to weanling rats was investigated in several aspects.

Growth studies demonstrated that the concentration of the toxic principle corresponded closely to the peroxide concentration of the oil. Moreover, separation of the oxidized oil into high-, medium- and low-peroxide fractions revealed that toxicity again followed peroxide concentration.

Histopathological examination of rats fed oxidized soybean oil gave generally negative findings but indicated that the intestine might be involved. Moreover, absorption of fatty acid peroxides in mature rats, studied by means of thoracic duct cannulation, indicated that although the reduced products of the peroxides were absorbed, the peroxides themselves were destroyed in the intestine and probably had their action at that site. Recovery from the effects of whole-body irradiation, a condition also affecting the intestine, was delayed by diets containing fat with peroxide numbers as low as 100.

Inhibition of intestinal xanthine oxidase by air-oxidized soybean oil and its reversal by exogenous flavin adenine dinucleotide suggest that the specific toxicity of the lipid peroxides may be at the level of the intestinal enzymes.

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The Growth, Breeding and Longevity of Rats Fed Irradiated or Non-Irradiated Pork[1,2]

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The age-old problem of food preservation has received a new impetus with the use of ionizing radiation. Although many problems exist, the practical use of this method has a promising future.

The research of Poling and coworkers ('55) failed to demonstrate any "unwholesomeness" in diets of rats fed irradiated beef, although some loss of vitamin E was noted. Cathode or beta rays were used in the irradiation process. A similar study was reported by Bubl and Butts ('56) using mixed organ meats subjected to gamma irradiation. No toxic effects were noted in breeding performance or longevity of the rats. The nutritional value of irradiated synthetic diets was studied by Richardson and Brock ('58). Reproduction and longevity were measured. They concluded that there was a slight difference in favor of the non-irradiated diet, but the difference was so small that they did not consider it to be of any practical importance. In a short-term experiment Read and coworkers ('58) reported no toxicity in 14 irradiated foods when fed to rats for 8 to 12 weeks at 35% of the dry weight of the diet.

The work reported here is part of the contractual program of the Office of the Surgeon General, Department of the Army, to determine the wholesomeness of irradiated foods, based on the procedure recommended by Lehman and Laug ('54). This broad research program was established to test a wide spectrum of representative foods from which extrapolation to most others could occur. Pork was one of these foods as it represented a fresh meat of high lipid and high moisture content.

The long-term feeding experiments were designed to obtain data concerning food consumption, growth, reproduction, lactation, size and viability of young and longevity. Four direct-line generations of rats were used in obtaining the data reported here.

EXPERIMENTAL

Both the irradiated and non-irradiated pork used in these experiments was supplied by the Quartermaster Food and Container Institute, Chicago. The cuts used were boned loin or shoulder which had been minced in a mechanical grinder, then passed through a fine-plate sausage grinder. The mass was then thoroughly mixed, usually in quantities of 1000 pounds or greater. The mixture was then packed into no. 2 (307 by 405) "C" enamel cans, sealed under vacuum and sharp frozen. The cans of pork serving as the control (non-irradiated) were shipped to this station frozen and stored at −10°F. until needed.

Two levels of irradiation were used: 2.79 megarad (3 megarep.) and 5.58 megarad (6 megarep.) to prepare the experimental meat. These levels were obtained in the canal of the Materials Testing Reactor, Phillips Petroleum Company, Idaho Falls, Idaho, using spent fuel rods giving mixed gamma radiation. Upon completion of irradiation, the cans of pork mixture were shipped to this station at ambient temperature. They were stored at room temperature, 78°F. (range 72 to 84°F.), for from three to 8 months from the date of irradiation prior to mixing in the diet.

Both the irradiated and control pork were cooked prior to mixing in the ration.

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[2] Technical paper no. 1215, Oregon Agricultural Experiment Station.

In planning diets for clinical studies, the guiding principles have been: replacement of a portion of the more saturated fats with polyunsaturated vegetable oils with a simultaneous reduction in total fat intake, and, if necessary, reduction in total caloric intake (7,11,12,18,25,26,32,37,39,40,45,46). In other words there has been a substitution of one type of fat for another, rather than addition of a new fat and, hence, no drastic change in normal dietary habits. Whether or not this modification will have a desirable effect on the development of coronary heart disease is a matter that can be determined only after many years of study of such diets. Several large studies of this type are currently in progress and, although no definite conclusions are yet possible, the trend seems to be toward a lower incidence of new heart cases in the vegetable oil diet group.

Regardless of what these studies eventually prove, it seems obvious that the tendency in the United States is going to be toward a less rich diet, if only to control obesity. Fats are the most concentrated sources of calories and are added in large quantities during food preparation. It is reasonable to believe that the use of tablespreads, shortenings, and the like could be cut appreciably without nutritional loss and, in fact, with a possible benefit. The present American diet containing 40% of calories from fat is unnecessarily rich and the tendency at present would seem to be toward recommending a fat intake of 25 to 30% of the total calories.

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Members of the fats and oils industry know, of course, that there are important differences in shortening value, stability, and so forth, between the ordinary animal fats, the hydrogenated oils, and the natural edible oils. Recently it has become obvious there are also important nutritional differences which can be related to fatty acid composition.

The evidence now indicates that the time is approaching when diet planning will include a balancing of the fatty acids just as we already balance amino acids, vitamins, and minerals to ensure that intakes of all essential ones are adequate. It seems probable that the present American diet has too low a ratio of linoleic to saturated fatty acids and would be improved by a cut in total fat calories with an increased use of the unhydrogenated vegetable oils in place of a portion of the more saturated solid fats.

In particular we are becoming increasingly aware of the unique value of linoleic and other polyunsaturated fatty acids. There now seems little doubt that these are very important constituents of the fats, far too valuable to be destroyed by such processes as hydrogenation. Recognizing the requests of nutritionists for higher linoleate-content shortenings and margarines, many companies in the industry have been working on the development of such products and several are already in the markets.

Because the immediately obvious merits of such new products are related to the cholesterol problem, a recent statement of the Food and Drug Administration (14) deserves comment. This states in part: "The role of cholesterol in heart and artery diseases has not been established. A causal relationship between blood cholesterol levels and these diseases has not been proved. The advisability of making extensive changes in the nature of the dietary fat intake of the people of this country has not been demonstrated."

This is a laudably conservative version of the same conclusions reached in the present discussion. Unfortunately this ruling has been interpreted in many quarters as a) discrediting all the evidence that vegetable oils do differ from animal fats in effects on serum cholesterol levels and b) discounting indications from clinical and epidemiological studies that coronary-prone individuals may be benefited by diets lower in saturated fats. Such an interpretation is not in accord with the facts. Many clinicians are convinced that the evidence is impressive enough to warrant large-scale testing and that, meanwhile, the prudent coronary-prone individual is well advised to make some changes in his diet with respect to fats. The interpretation put on the FDA statement has to some extent tended to hamper development of new high-linoleate food products just at the time when these are most needed for clinical study.

Of course if foods are to have a higher linoleic to saturated fatty acid (L/S) ratio there will be shelf-life problems. The linoleate-rich oils naturally oxidize or become rancid more rapidly than do the more saturated, linoleate-poor fats. It is well known that from a nutritional point of view rancid fats have several undesirable properties. Thus, if a higher L/S ratio is desirable, new ways may have to be found to stabilize foods containing these fats, perhaps with new antioxidants or new packaging materials. As you well know, this is going to be a complex problem because the edible oils vary widely in stability. For example refined soybean oil reverts easily whereas refined corn and cottonseed oils are quite stable, presumably because of their high tocopherol contents.

Summary

So far as the industry is concerned, the evidence indicating a relationship of dietary fat to heart disease presents some interesting challenges. Undoubtedly it portends a change in the fat consumption pattern toward a lower *per capita* use coupled with a shift from solid fats toward a higher proportion of edible oils. Most important of all, however, is the growing recognition that fats and oils are nutritionally valuable foods, intimately related to health and well-being, and should by no means be regarded merely as a source of calories.

Although there are innumerable factors involved in the etiology of heart disease, dietary fat is an important one and fortunately is one that can be modified in whatever way proves desirable. Because the more saturated types of fats lead to higher serum cholesterol levels than do the polyunsaturated oils, and because cholesterol is somehow involved in the course of atherosclerotic heart disease, clinical tests are now in progress to determine whether prolonged use of a diet rich in these oils will lead to fewer heart attacks than does the usual American diet rich in saturated fats. So far, data are encouraging enough to merit recommendation of the modification in dietary fat to the coronary-prone individual and to justify development of new high-linoleate fat products by the industry.

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A Nutritive Evaluation of Over-Heated Fats

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According to the tests used, harmful substances do not occur in fried foods or in fats used in preparing foods. It is possible to obtain biologically undesirable materials by excessively heating and/or oxidizing fats in the laboratory, but the conditions required for the production of such materials differ greatly from those used in practical cooking or processing of foods. There appears to be no reason to believe that fats are nutritionally damaged when handled by normally-accepted good practice in present-day food preparation.

FATS ARE IMPORTANT and essential in the diet. They are not something we can take or leave alone; they provide energy, supply essential fatty acids, carry fat-soluble vitamins, improve flavors, modify textures, and add satiety values to meals.

Much of their utility depends upon their stability to heat. In frying operations they prevent sticking and transfer heat from hot surfaces to food. The stability of fats at high temperatures (up to 200°C. in some frying operations) invites repeated or continuous use, and questions have been raised concerning the nutritive value and wholesomeness of fats after long usage. Some data in the scientific literature show that undesirable changes occur in fats if they are heated in the laboratory to high temperatures for long periods or if they are subjected to severe oxidizing conditions. Other reports indicate that fats which have been used for prolonged commercial or home cooking retain their nutritive value and remain wholesome.

There are two major reasons why food technologists handling fats should be familiar with this subject. The nutritive values of fats at all stages of processing and use should be known, and there are significant and frequently adverse public relation aspects which must be handled. Publicity problems usually arise from misinterpretations or from unjustified extrapolations of laboratory findings. Even though the implied effects may not be true, headlines such as "The Carcinogenic Action of Heated Fats and Lipoids" (1) cannot be considered in the best interests of the fat industry. The facts must be known in order to understand the problems and to combat misleading reports or inferences.

It is not our intention to review this subject exhaustively. Instead we plan to consider published and unpublished research findings which indicate typical changes that can occur in food fats during laboratory treatments or cooking procedures and to contrast the findings with those obtained when food fats are tested. Noncritical review of the scientific literature relating to heated fats can lead to very erroneous conclusions since reports show that it is possible to mistreat fats experimentally with sufficient heat and/or oxygen to cause, when the abused fat is fed to test animals, retarded growth, poor feed efficiencies, rough, greasy matted coats, diarrhea, starvation, enlarged livers and kidneys, abnormal fat depots, impaired enzymatic functions, abnormal water metabolism, papillomas and other growth formations, and shortened life spans. In extreme cases animals may die in a few days after severely abused fats have been fed. There is thus no question whatsoever that fat can be damaged by purposeful abuse. The critical question is: "are fats damaged during processing or cooking operations?" To answer that question we must examine some of the conditions which produce the effects listed above. They may have been

deliberately designed to produce measurable damage rather than to simulate cooking procedures.

THERE IS NO EVIDENCE that food fats have carcinogenic properties unless they have been abused far beyond normal conditions. Nevertheless inferences that food fats may be involved in the disease are too frequent. One of the early suggestions that heated fats might cause cancer was made by A. H. Roffo (2), who claimed that sunflower and olive oils oxidized by heating to 250–350°C. (482–662°F.) had carcinogenic potencies when fed to rats. This should be recognized as very drastic treatment. Lane, Blickenstaff, and Ivy (3) however did not get tumors when lard "browned" at 350°C. (662°F.) for 30 min. was fed to rats from the colony of Dr. Roffo, but there were increased incidences of papillomas of the forestomach and ulcers in the glandular stomach. Peacock and Eck made similar claims for the feeding or injection of cottonseed oil heated to 350°C. (662°F.) for 4 hrs. (4, 5). He was unable to find any known carcinogen in the fat. Two mice out of 300 showed tumors after 15 months on the diets. Other investigators have had varying degrees of success in efforts to demonstrate carcinogenic properties in heated fats.

While there have been demonstrations that painting overheated fat repeatedly upon the skin, injecting it into the skin or muscle, or feeding it can result in conditions suggestive of the formation of cancer, it is perhaps significant that A. A. Newman, after going to considerable length to imply that epoxidized and heated fats are a nutritional hazard (6), states: ". . . the introduction of autoxidized and thermally-affected fats into test animals can produce pathological lesions ranging, according to conditions, from benign papillomas in the forestomach to malign neoplastic growth in the glandular region. While it must be emphasized that none of the latter type of lesions so far produced have satisfied the rigorous conditions of true carcinogenicity, in several instances the difference was not wide from a practical viewpoint." It might be added that the fats tested were not at all representative of cooking fats.

Fats which have been heated to unrealistically high temperatures do not necessarily indicate intensification of cooking conditions. They may have undergone chemical changes quite different from those occurring under cooking conditions. Nevertheless inferences drawn from reports of exaggerated conditions can result in scare propaganda that is of no value to anyone. More research is needed to clarify the situation completely, and this research needs to be done with food sources of fat as well as with unrealistically-abused fats.

Feeding studies have been conducted by many different laboratories in studies of "abused" fats. No effort will be made to review all of the relevant reports. Only a few will be selected to illustrate the nature of the studies and typical findings. The treatments fall into three general categories: heating in the absence of air, heating in the presence of air, and oxidation at low temperatures.

ONE OF THE FIRST groups to study the biological effects of polymerization was that of E. W. Crampton and his co-workers (7). They blew carbon dioxide through various oils heated to 275°C. (527°F.) in all glass equipment for various intervals of time. Table I, adapted from their Table 2 (7), illustrates several things. The inclusion of severely heat-treated oils in rat diets at levels of 10 or 20% reduced rates of gain and decreased caloric efficiencies. The amounts of damage were proportional to the length of heat treatments. Different types of oils differ in response. Linseed, the most unsaturated, shows the most damage.

These workers observed that appetite was depressed when thermally-polymerized oils were fed and that the feces of the animals were dark and sticky. Hair coats were also oily and matted whereas the controls were sleek and clean.

TABLE I
Effects of Heat Treatment of Oils[a]

Test oil used	Duration of heating	Aver. daily gain	Aver. daily feed intake	Gain per 1,000 cal.
	hours	*g.*		
Linseed	0	3.9	9.6	91
	2	3.8	9.6	91
	4	3.4[b]	9.5	80
	8	2.5[b]	8.1	69
Corn	0	3.6	10.4	78
	15	2.8	8.7	71
	30	2.3[b]	8.0	64
Soybean	0	4.6	10.2	90
	3	4.0	9.4	85
	9	2.9[b]	7.7	76

[a] Adapted from the data of Crampton, Farmer, and Berryhill (7). Ten or 12 animals were used per group, and fats were fed at 10% of the diet except for 20% of soybean oil.
[b] Significantly lower than controls.

In contrast to these findings Lassen, Baron, and Dunn (8) report that adult rats fed edible, polymerized sardine oils at a level of 5% in the diet were healthy after short experiments and showed no significant changes in urine analyses. They demonstrated however that sardine oil which was 30% polymerized was only 85% digestible in comparison with the 98% digestible found for unpolymerized oils.

O. C. Johnson and his co-workers at the University of Illinois added the stress of oxidation to heat treatment by blowing 100 milliliters per minute of air through 1,500 g. of various oils heated to 200 ± 10°C. (392°F.) in stainless steel beakers for various periods up to 24 hrs. (9). This is a very rigorous treatment and results in increased acid values, increases in viscosity and color, and decreased iodine values. Rats fed thermally-oxidized butter oil gained as well as controls fed fresh butter oil; but rats fed thermally-oxidized corn oil gained poorly in comparison to their controls on fresh corn oil. Final weights after nine weeks on corn oil tests were 124 and 332 g., respectively. That these responses were not caused by differences in food intake was demonstrated by a paired feeding experiment, in which the thermally-oxidized corn oil gave significantly less growth (1% level) than the fresh oil. Margarine stock heated and oxidized under the same conditions depressed growth slightly when pair-fed in comparison with fresh oil.

Temperatures as high as 200°C. (392°F.) cause rapid destruction of fatty peroxides, and the course of polymerization at such severe conditions may be very different from what it is at lower temperatures. In this connection it is of importance to note that when Kaunitz and Slanetz (10) fed 15% cottonseed oil, which had been aerated at 95°C. (203°F.) for 200 hrs. (iodine No. 141), immediate diarrhea and weight loss occurred and about half of the animals died within three weeks. At a level of 10% peroxidized fat only a few rats died, and the others seemed to adjust to the diet, overcoming the diarrhea and gaining slightly in weight. The addition of fresh oil along with the heated oil seemed partially to overcome the effects of the peroxidized oil. The only symptom was slower gains. The addition of Vitamins E, A, and D did not result in a corresponding improvement.

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Further heating of a cottonseed oil with peroxide value of 191 caused a reduction in peroxide value to 141 but an increase in severity of symptoms when fed, leading Kaunitz and co-workers to conclude that the amount of peroxide present was not related to the degree of toxicity. In addition to the growth-depressing effects Kaunitz has reported that the feeding of heated fats causes enlarged kidneys, livers, and adrenals, also small spleens and thymus glands. Water intake is also greatly increased (11).

Recently Andrews and co-workers (12) have published data to indicate that growth depression in rats fed oxidized soybean oil is proportional to the extent of oxidation from peroxide numbers of 100 to 1,200. Using cupric and ferric ions as catalysts, they oxidized soybean oil by aeration at 60°C. (140°F.) to peroxide numbers as high as 1,200. When fed as 20% of the diet, 1,200 peroxide number fat caused immediate, severe diarrhea and sustained losses in weight, also fatalities of all animals in three weeks. Dilution of this product with fresh soybean oil to give mixtures with peroxide numbers of 800 and 400 lessened the severity of the symptoms and prevented the fatalities.

THESE STUDIES and a number of others proved that fat can be damaged, but the conditions needed for damage were much different from home or commercial cooking operations. The need for severe laboratory treatment of highly unsaturated oils to get the marked changes suggested that sensitive methods might be essential if one were to study practical operating conditions. Furthermore rapid methods for measuring changes in nutritive value and wholesomeness are essential if many fats are to be examined. The tests commonly used required 8 to 12 weeks of test feeding of experimental animals.

With this in mind we undertook the development of methods which would quickly detect changes of fat quality. Perhaps the most successful of these has been a restricted-feeding technique, which permits exact comparison of control and experimental animals (13). This grew out of the postulate that fatty substances which had been damaged might not be available for energy. If this were true, under proper conditions the rate of gain of animals fed abused fats should be proportional to the amount of undamaged fat remaining. In practice weanling rats are fed 5-g. quantities of a basal ration containing only enough energy-containing substances to permit slight growth but formulated to supply an excess of the daily needs of all essential nutrients. Additions of energy-containing materials to such a diet permits growth in proportion to the amount of energy added. This is true whether the extra food is carbohydrate, fat, or protein in nature.

Table II illustrates the application of such a technique to laboratory-heated and/or oxidized fats. These are typical values, and it may be seen that the available energy is markedly reduced by severe heating or oxidation. A 7-day period is adequate for such determinations. The results are quite uniform and reproducible, permitting the use of small groups of animals. In addition to demonstrating reductions in energy value, animals fed fats which have been excessively heated or oxidized experimentally showed the organ enlargements that others have reported in longer feeding-studies. The increase in liver weight occurs very rapidly, being easily detectable in three days and maximal in five to seven days. These livers are not fatty; by analysis and by gross and limited microscopic inspection the tissue is normal.

TABLE II
Energy Values of Abused Fats

Substance added	Aver. gain in 7 days[c]	Available energy[d]	Liver sizes
	g.	%	*% body wgt.*
None	5.0	....	....
Fresh cottonseed oil	27.0	100	4.0
Severely heated CSO[a]	21.0	72	5.4
Severely oxidized CSO[b]	4.7	0	7.0

[a] 120 hrs. at 182°C. in household cooker.
[b] Air blown through oil held at 60°C. for 10 days.
[c] 1.2 g. of fat fed each day in addition to basal.
[d] In term of % of theoretical, based on fresh CSO as 100%.

This technique has been applied in a number of studies of factors which might damage fat. These will be reported elsewhere in more detail, but an outline of the findings will indicate the magnitude of the changes which may be expected with common food fats when heated or oxidized.

That these changes (Table II) are typical is shown by the decreases in available energy values listed in Table III for several samples of salad oil and shortenings. Values for any one sample are quite reproducible, but different lots of a single type of oil vary in response to heat as the two corn oils indicate.

Hence the values in Table III cannot be taken as an indication of the relative stabilities of the fats. Some of these tests were made before liver weights were routinely checked, but liver weights taken in other tests indicate increased size when various types of fats are excessively heated during experiments.

TABLE III
Available Energy of Various Fats After Heating at 182°C. for 120 Hours[a]

Type of oil heated	Available energy in heated fat as % of energy available from unheated product[b]
Cottonseed salad oil, Sample 1	72
Cottonseed salad oil, Sample 2	68
Peanut salad oil	67
Corn salad oil, Sample 1	86
Corn salad oil, Sample 2	75
Hydrogenated meat and vegetable fat shortening	81
Hydrogenated vegetable oil shortening	70
Hydrogenated vegetable oil shortening	64
Meat fat and vegetable fat shortening	76
Lard	66[c]

[a] Approximately 8,000-g. quantities of fat were heated for 120 hrs. at 182°C. in a household deep-fat fryer. Four animals were fed at a level of 1.5 g. per day in addition to 5 g. of basal diet.
[b] These values are for samples of commercial products and do not necessarily reflect the relative merits of the type of product because there is variability from one supplier to another, as the two corn oil samples indicate.
[c] 168 hrs. of heating rather than 120.