TABLE V

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

	Mean		Components of Variance								
			Within			Between			Total		
	Indicator	Electrometric	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	0.1292	.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

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.

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.

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.

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.	58	3.1	36.5 ± 1.7	.88

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.

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

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

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

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	.85	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

have been observed in thermally oxidized oil—a decrease in iodine value, an increase in viscosity, and an 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 material parallels the loss in nutritive value more closely than either changes in iodine value and viscosity (Figure 1). The rapid increase in nonurea adduct-forming material during the early stages of thermal oxidation and the subsequent slower formation during the latter stages give a possible method for the determination of the nutritive loss during thermal oxidation.

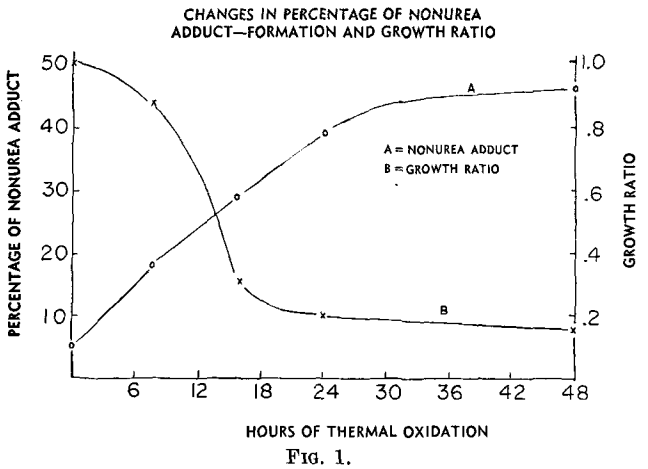

FIG. 1.

While many growth studies have been made with thermally oxidized or oxidized oils, few studies on specific physiological effects have been reported. It has been shown that at least 70% of the polymeric residue from cotton-seed oil oxidized at 100°C. is absorbed (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.[b] Body Surface [a]

Fat fed	Absorption in mg./100 cm.[b]/hr.[b]		
	6 hours	10 hours	24 hours[a]
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.

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