

TABLE IX
Effect of Soybean Hulls on Growth, Feed Efficiency,
and Fat Absorbability^a

Diet number and treatment ^c	Average weight 4 weeks		Feed/gain ^b		Fat absorbability	
	Exp.8	Exp.9	Exp.8	Exp.9	Exp.8	Exp.9
	<i>gm.</i>	<i>gm.</i>			<i>%</i>	<i>%</i>
I. Basal.....	591	598	1.58	1.82
II. Soybean oil.....	639	683	1.36	1.42	94.8
III. Soybean flakes.....	643	685	1.36	1.44	92.1	92.3
IV. Ground dehulled soybeans.....	643	667	1.47	1.58	72.9	67.3
V. Ground unextracted soybeans.....	614	658	1.57	1.71	70.4	60.0
VI. Soybean oil + hulls.....	662	663	1.34	1.49
VII. Soybean flakes + hulls.....	640	649	1.38	1.47
VIII. Ground dehulled soybeans + hulls.....	612	663	1.49	1.60	74.0	65.0
IX. Basal + hulls.....	569	627	1.62	1.75

^a Data represent averages of duplicate lots of chicks.

^b Experimental periods: Experiment 8, 0-4 weeks; Experiment 9, 1-4 weeks.

^c All soybean products were heated.

unrelated to its energy contribution, or to a factor present therein, the availability of which is directly proportional to the quantity of oil in the diet capable of being absorbed by the chick.

In studies employing the basal diet described in these experiments it was found that a dietary level of approximately 9% soybean oil with an absorbability of 95% is required to promote maximum growth stimulation in male chicks to four weeks of age. With an average absorbability of 69%, ground unextracted soybeans supply the equivalent of about 8.9% absorbable oil to the diet. Therefore, with this level of absorbable oil in the diet, it is possible that diets containing ground unextracted soybeans may be marginal in the growth-promoting property of soybean

oil. The possibility cannot be excluded however that the fraction of oil in ground unextracted soybeans, which is unavailable to chicks, contains a higher concentration of the growth-promoting property compared with the oil which is readily absorbed.

Acknowledgments

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Heated Fats. I. Studies of the Effects of Heating on the Chemical Nature of Cottonseed Oil

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When cottonseed oil was heated at 225°C. in the presence of air for long periods of time, nonurea adduct-forming monomers and dimers were formed which were toxic to rats. Analyses showed that the toxic fractions contained moderate amounts of carbonyl and hydroxyl and that they contained unsaturation difficult to remove by hydrogenation. Cyclic structures appeared to be present in the dimer fraction. The production of nonurea adducting monomers and dimers is associated with polymerization and other reactions of linoleic acid.

IN STUDIES from several laboratories evidence has been presented that oxidized and polymerized fats contain substances which are toxic to experimental animals. Crampton and coworkers (1-6) found that vegetable oils polymerized at 275°C. in an inert atmosphere caused growth depression in rats. The nonurea adduct-forming monomers from linseed oil were lethal to rats at 10% levels in the diet. It was suggested that these toxic monomers contain a cyclic

structure. Other studies (7-12) indicate that cyclic monomers are produced from polyunsaturated acids by way of Diels-Alder reactions.

Perkins and Kummerow (13) observed that thermal oxidation of corn oil at 200°C. produced polymeric substances containing high percentages of hydroxyl and carbonyl groups. The unsaturation present in the polymers was difficult to remove by hydrogenation. No monomers or polymers with cyclic structures were found in the thermally oxidized oil, and it was suggested that the free radical mechanism proposed by Sunderland (14), which results in polymerization through a single carbon-carbon bond, could account for the polymers derived from the thermally oxidized corn oil. A group of weanling rats were fed the non-distillable residue (dimers and high polymers) of the nonurea-forming fatty acids from corn oil thermally oxidized at 200°C. for 48 hrs.; all died within 7 days (15). The molecular distillate obtained from the non-

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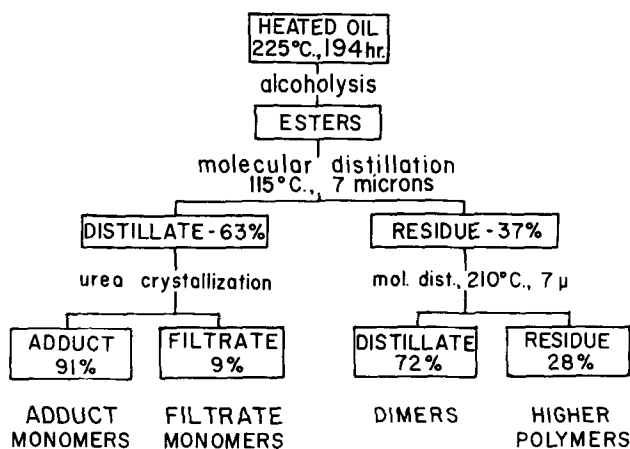


Fig. 1. Fractionation of ethyl esters from cottonseed oil heated at 225°C.

adducting acids showed no toxicity. All monomeric material, both urea-adducting and nonadducting, was shown to be normal fatty acid by spectrophotometric methods.

Kaunitz and coworkers (16,17) observed that the polymeric residues from fats oxidized at 95–100°C. and the dimeric and higher polymer ethyl esters derived from these fats severely depressed the growth of rats. No signs of toxicity were found from the feeding of adequate diets containing 5% of methyl monohydroxystearate, methyl 9,10-dihydroxystearate, methyl *cis*-epoxystearate, or a methyl oleate hydroperoxide concentrate containing 85% of the peroxide, suggesting that simple oxidized materials prepared from long-chain fatty acids are not the principal agents responsible for the growth-depressing effects that were observed.

Experiments in this laboratory have confirmed the production of substances toxic to rats from overheated cottonseed oil. Both monomeric and dimeric fractions have been recovered from the heated oil which are toxic to rats. The monomeric fractions showed a greater degree of toxicity than the dimeric material.

Experimental

Cottonseed oil was heated at $205^{\circ} \pm 2^{\circ}\text{C}$. in 3,500-g. batches in a covered 6-qt. capacity aluminum-well household deep-fat fryer. (Temperatures varying between 150° and 210°C. are used in various commercial deep-fat drying operations.) The warm-up time was about 30 min. The oil was heated 7 to 8 hrs. each day for 40 days, stirring several seconds every 2 hrs. during the heating period. The oil was kept covered when it was not heated. Periodically 200-g. samples were withdrawn for examination and stored under nitrogen at -15°C . Methyl esters were prepared by saponification with potassium hydroxide in 95% ethanol, ether extraction of unsaponifiables, and esterification of the acidified soaps with methanol containing 1% sulfuric acid. The fatty acid esters were subjected to urea fractionation (4) and micromolecular distillation (18). The monomers and polymers were analyzed by the spectrophotometric method for polyunsaturated acids (19) although this method is not strictly applicable to compounds other than normal unsaturated fatty acids and esters.

Additional batches of heated oil were prepared for rat-feeding studies by heating 30 lbs. of U.S.P. cot-

tonseed oil at $225^{\circ}\text{C.} \pm 3^{\circ}\text{C}$. in an uncovered 25-qt. capacity aluminum pot. (The higher temperature was chosen to accelerate chemical changes occurring in the heated oil.) The oil was heated continuously for 170–195 hrs. to a refractive index (Abbe, 30°C.) of 1.4786 ± 0.0002 since a maximum amount of nonurea adduct-forming monomers was found in the previous oil heated at 205°C. when this refractive index had been reached. A stainless steel immersion heater² was used, and the oil was stirred continuously at 100–150 r.p.m. with a 2-in. stainless steel turbine propeller and shaft. The heated oil was transferred to large Pyrex flasks, cooled 10 min. in a cold-water bath under nitrogen, and transferred while warm to pint-size amber-colored bottles and stored under nitrogen in a freezer. Ethyl esters were prepared from the heated cottonseed oil by alcoholysis (4). The esters were distilled in a 2-in. "Rota Film" molecular still.³ The distilled monomers were fractionated with urea (4). The fractionation steps are outlined in Figure 1. The distilled monomers and the urea filtrate monomers (nonurea adduct-forming monomers) were analyzed by gas chromatography, using a Perkin-Elmer Vapor Fractometer Model 154C with a hot wire temperature of 325°C., bridge current of 250 ma., and oven temperature of 200°C. The inlet helium flow was 42 cc./min. (measured by flow meter), and the outlet helium flow was 55.6 cc./min. (measured by soap bubble burette). A 6-ft. column of $\frac{1}{4}$ -in. o.d., $\frac{3}{16}$ in. i.d. aluminum tubing was prepared, using a packing consisting of 30% (w/w) diethylene glycol-glutarate polyester on a support of 80–100 mesh Johns-Manville Chromosorb W. The polyester was prepared by the method of James (20). The urea filtrate monomers were hydrogenated in a low-pressure, Parr reaction apparatus,⁴ using 1 part of 10% palladium on charcoal catalyst to 10 parts of sample dissolved in 10 volumes of 95% ethanol and a pressure of 50 p.s.i. of hydrogen at room temperature for 40 min. Under these conditions cottonseed fatty acids are hydrogenated to a Wijs iodine value of 0.

Molecular weights were determined by the cryoscopic method of Gay (21). Viscosities were obtained with a ball and cup absolute microviscometer (22). Epoxide oxygen was determined by the method of Swern and coworkers (23). Dielectric constants were obtained by using an impedance bridge and a cell consisting of two coaxial brass cylinders separated with a Teflon collar. The method for the determination of dielectric constant is discussed by Hazlehurst (24). Wijs iodine values, peroxide values, and free fatty acids were determined according to the official A.O.C.S. methods (19). Carbonyl oxygen was determined by the method of Knight and Swern (25). Hydroxyl contents were determined by using acetic anhydride in pyridine as the acetylating reagent (26).

Samples of cottonseed oil heated at 225°C. for 194 hrs. were fed to rats at levels of 10–20% in a sucrose-casein diet for a period of 6 months. Control animals were fed unheated cottonseed oil under the same conditions. In addition, the fractions obtained by molecular distillation and urea fractionation of the ethyl esters were fed to rats according to a short-term restricted-feeding test similar to one developed by Rice (27). The toxicity of individual fractions was also

² "Lo Lag," 1,000 watts, American Instrument Company, Silver Spring, Md.

³ A. F. Smith Company, Rochester, N.Y.

⁴ Parr Instrument Company, Moline, Ill.

TABLE I
 Cottonseed Oil Heated in Deep-Fat Fryer at 205°C.

Sample	1	2	3	4	5	6	7	8
Heating time in hrs.	0	24	56	96	123	198	243	307
Molecular weight	850	895	956	1100	1140	1446	1515	1790
Viscosity (poises) at 25°C.	0.4	0.8	1.2	1.7	2.8	9.5	27	110
Dielectric constant at 28°C.	3.18	3.27	3.37	3.52	3.60	3.84	3.93	4.07
Refractive index at 30°C.	1.4687	1.4694	1.4708	1.4726	1.4742	1.4770	1.4788	1.4812
Peroxide value (m.e.q./kg.)	0.8	4.5	1.6	1.6	1.6	1.4	1.2	1.5
Iodine value, 30 min. Wijs.	110.3	105.1	99.09	91.99	88.67	78.90	74.14	68.43
% Free fatty acids, as oleic.	0.09	0.39	0.65	1.3	1.8	2.0	2.1	2.0
% Conjugated diene	0.25	0.77	0.92	1.19	1.20	1.06	1.02
% Conjugated triene	0.06	0.16	0.20	0.16	0.18	0.19	0.16
% Conjugated tetraene	0.00	0.02	0.02	0.02	0.02	0.03	0.01

observed by feeding orally 0.4-ml. portions on each of two successive days to weanling rats weighing between 30-40 g. The animals were observed for a period of 7 days.

Results

Chemical Changes in Oil Heated at 205°C. Heating cottonseed oil at 205°C., as described, resulted in a decrease in the content of linoleic acid whereas the amounts of oleic and saturated acids were essentially unchanged (Figure 2). These results seem to indicate

low values of peroxide and epoxide found. Infrared examination indicated a steadily increasing hydroxyl and carbonyl content as the oil is heated. Increasing absorption at 10.36 microns indicated a progressively increasing content of isolated *trans*-unsaturation with heating time, presumably associated with the changes occurring as a result of linoleate oxidation, isomerization, and polymerization. The concentration of conjugated diene increased to a maximum and then decreased while conjugated triene and tetraene increased initially, then remained at stationary levels.

The concentration of urea filtrate monomers increased to a maximum of 7% at 250 hrs. of heating, then slowly decreased (Figure 2). The dimer content increased rapidly during the first 50 hrs. of heating to a value of 7% whereas the trimer content increased slowly during this period to 1.5%. Between 100 and 250 hrs. the dimers and trimers increased at the same steady rate. After 250 hrs. the dimer content levelled off at about 19% while the increase in trimers continued at the same rate. Linoleic acid decreased steadily for the first 200 hrs. of heating and at a decreasing rate thereafter. The low initial rate of trimer formation and the steady increase in trimer content after 250 hrs. suggest that trimers were formed from the dimers by additional polymerization. The dimers and higher polymers were not depolymerized with hydrochloric acid according to the method of Chang and Kummerow (28), indicating that the dimers and trimers were formed through carbon-to-carbon bonds. The urea filtrate monomers, isolated from the oil heated 243 hrs. at 205°C., consisted largely of monoene material (Table II). The urea filtrate monomers contained approximately 18% diene, largely nonconjugated.

Chemical Changes in Oil Heated at 225°C. The results of heating one batch of cottonseed oil at 225°C. are summarized in Table III. Increases in molecular weight and viscosity and decreases in iodine number are again indicative of progressive polymerization. Low levels of peroxides and epoxides, unstable at high temperature, were again observed. Carbonyl ox-

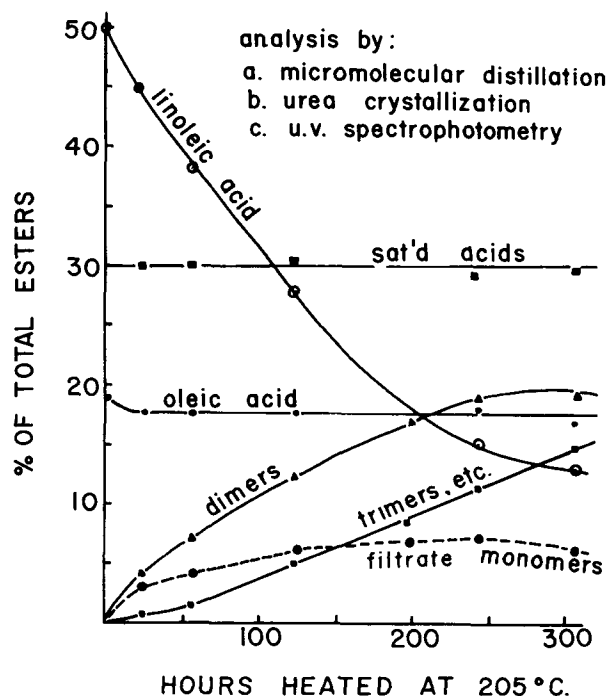


FIG. 2. Composition of methyl esters from cottonseed oil heated at 205°C.

that the changes in chemical composition of the heated oil are largely related to polymerization and other reactions involving linoleic acid. When cottonseed oil was heated for 300 hrs. at 205°C. in the absence of air, the molecular weight and iodine value did not change, indicating that at this temperature oxygen is necessary for the oil to undergo the changes observed in the presence of air. The progressive decrease in iodine number and increase in molecular weight, refractive index, and viscosity during heating in the presence of air are indicative of continuous polymerization of the oil (Table I). A linear increase in dielectric constant with time of heating points to the continuous incorporation of oxygen into the oil. The oxygen uptake is not accounted for by the uniformly

TABLE II
 Analysis of Methyl Ester Fractions from Oil Heated 243 hrs. at 205°C. by U.V. Spectrophotometry

	Unheated Oil	Heated Oil Fractions		
	Methyl esters	Urea adduct monomer	Urea filtrate monomer	Dimer
	%	%	%	%
Conjugated diene	0.36	1.06	4	7
Conjugated triene	0.07	0.19	0	0
Conjugated tetraene	0.00	0.03	0	0
Monene	19.3	28.9	81	70
Nonconjugated diene	50.0	24.2	14	0.7
Nonconjugated triene	0.0	0.1	0.0	0.0
Saturated acids (by difference)	30.0	45.5
Iodine number	110	73	105	75

TABLE III
 Cottonseed Oil Heated in Uncovered Aluminum Pot at 225°C.

Sample	1	2	3	4	5	6	7	8
Heating time in hrs.	0	24	48	72	96	144	168	194
Molecular weight	850	936	1020	1080	1155	1350	1430	1510
Viscosity (poises) at 25°C.	0.6	1.0	1.4	2.1	3.2	8.6	12.5	18.1
Dielectric constant at 28°C.	3.18	3.27	3.37	3.47	3.57	3.70	3.82
Refractive index at 30°C.	1.4685	1.4697	1.4710	1.4724	1.4738	1.4762	1.4774	1.4786
Iodine value, 30 min. Wijs	109.8	104.3	97.87	91.45	86.82	79.07	76.05	73.38
Peroxide value (m.e.g./kg.)	2.5	3.0	2.5	1.5	0.5	0.0	0.0	0.0
% Free fatty acids, as oleic	0.09	0.20	0.39	0.50	0.60	0.75	0.85	0.80
% Epoxide oxygen	0.000	0.020	0.028	0.045	0.036	0.035	0.025
% Carbonyl oxygen	0.02	0.17	0.15	0.13	0.10
% Hydroxyl oxygen	0.14	0.23	0.29	0.40	0.43	0.47

xygen increased to a maximum, then decreased slowly. The content of hydroxyl oxygen increased steadily throughout the period of heating. Molecular distillation of the fatty acid ethyl esters from the heated oil yielded 57% urea adduct-forming monomers and 6% urea filtrate monomers in the distillate, and 27% dimers and 10% trimers and higher polymers in the residue (Figure 1). The composition of monomers in the unheated and heated oils, determined by ultraviolet spectrophotometry and gas chromatography, is summarized in Table IV. The content of oxygen-con-

 TABLE IV
 Monomers in Cottonseed Oil Heated 194 hrs. at 225°C.

Component	In unheated oil	In heated oil
	%	%
Conjugated diene	0.38	1.07
Conjugated triene	0.12	0.24
Conjugated tetraene	0.003	0.01
Cis	24.4	24.6
Cis	1.4	2.1
Cis monoene	18.6	16.4
Cis diene	54.4	17.3
Trace components	1.2	2.6

taining functional groups in the isolated monomers and polymers is shown in Table V. The percentage of hydroxyl and carbonyl-containing molecules in each of the ethyl ester fractions was calculated, assuming that each molecule contained no more than one carbonyl and one hydroxyl group and that the molecular weights of the monomers, dimers, and higher polymers were 310, 620, and 930, respectively. The urea filtrate monomers and dimers contained 0.41 and 0.47% carbonyl oxygen, respectively, or approximately 10–20% of carbonyl compounds. The fractions contained 1.20 and 0.61% hydroxyl oxygen, respectively, or approximately 20–25% hydroxyl compounds. Low levels (2%) of oxygenated fatty acids were found in the urea-adduct fraction. Gas chromatograms of the total monomers, urea filtrate monomers, and hydrogenated urea filtrate monomers were obtained (Figure 3). The bulk of the urea filtrate monomers formed a number of unresolved bands with retention times in the C₁₈ region. However, upon hydrogenation, these bands were split into two series of bands, one in the C₁₆ and the other in the C₁₈ region. By comparison of the retention times of the unresolved bands obtained from the hydrogenated and unhydrogenated urea filtrate monomers, it was estimated that about 57% of "abnormal" fatty acids were present in the urea filtrate monomers. "Abnormal" fatty acids are defined as those components not having retention volumes corresponding to normal fatty acids by gas chromatography.

Samples of urea filtrate monomers and dimers were treated according to the aromatization procedure of

Scholfield and Cowan (12). The products were examined by ultraviolet spectrophotometry for the presence of aromatic structures. None could be found in the case of the filtrate monomer. Absorption maxima in the 250–260 and 270–280 m μ regions in the case of the oxidized brominated-dehydrobrominated dimers suggest the presence of cyclic structures.

Nutritive Value of Oil Heated at 225°C. A sample of cottonseed oil heated at 225°C. for 170–190 hrs., as described, when fed to rats in a 6-month study or in 10- to 12-day "calorie assays," was shown to have decreased nutritive value for growth and food efficiency and to produce enlarged livers in the test animals. The isolated ethyl esters of the dimers and nonurea-adducting monomers were toxic to weanling rats in short-term feeding tests; the dimers were less toxic than the nonlinear monomers. When 0.4 ml. of urea filtrate monomers and dimers were force-fed to weanling rats, death occurred within 3 days. Unsaturation present in these fractions was only partially removed by hydrogenation (Table VI). The iodine number of the dimer fraction decreased only from 54 to 34 after hydrogenation. There was no loss in toxicity after hydrogenation of the urea filtrate monomers and dimers. Hydrogenation did not destroy the carbonyl oxygen present in the fractions. Nutritional studies of the heated oil and fractions isolated from it are presented in detail elsewhere (29).

Discussion

When unsaturated fatty acids are heated at temperatures above 250°C. in the absence of air, cyclic monomers and polymers are formed by Diels-Alder reactions (7–12). High temperatures are generally required for these reactions. For example, no changes were observed by us in cottonseed oil heated at 205°C. *in vacuo* for a prolonged period of time. However Rivett (10) has prepared cyclic monomers and polymers from methyl eleostearate by heating *in vacuo* at 180°C. It was suggested that the primary cyclic monomer was a 1,2-dialkyl-3,5-cyclohexadiene. On the other hand, Perkins and Kummerow (13) pre-

 TABLE V
 Content of Oxygen Functional Groups in Isolated Ethyl Ester Fractions

	Oxirane oxygen	Peroxide oxygen	Carbonyl oxygen	Carbonyl-containing molecules	Hydroxyl oxygen	Hydroxyl-containing molecules
	%	%	%	%	%	%
Urea adduct monomers	0.00	0.00	0.10	2	0.08	2
Urea filtrate monomers	0.00	0.00	0.41	8	1.20	23
Dimers	0.00	0.00	0.47	18	0.61	24
Trimers and higher polymers	0.02	0.00	0.38	21	0.95	55

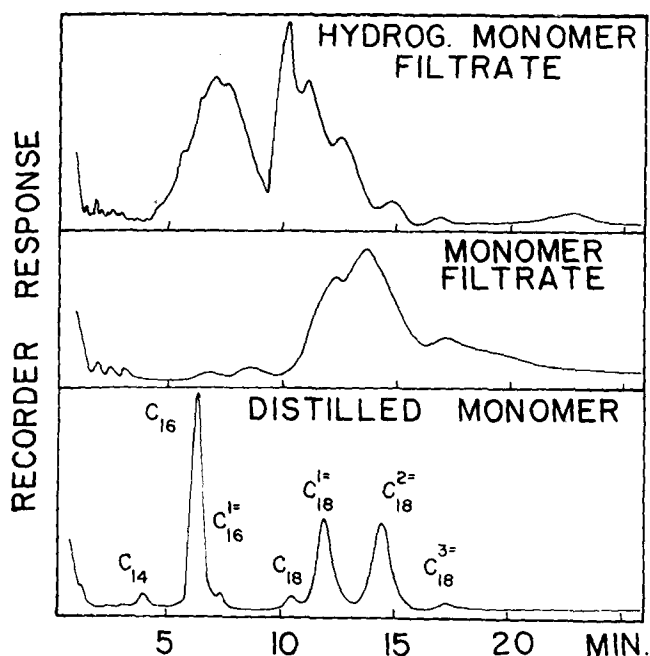


Fig. 3. Gas chromatograms of ethyl ester monomer, monomer urea filtrate, and hydrogenated monomer urea filtrate from cottonseed oil heated 194 hrs. at 225°C.

sented evidence which suggested that thermal oxidation of unsaturated acids produced polymers joined through a single carbon-carbon bond and containing large amounts of hydroxyl and carbonyl groups. Dimers with two or more hydroxyl groups in the molecules were proposed. These polymers were produced by bubbling air through corn oil at 200°C.

TABLE VI
Determination of Toxicity by Oral Feeding of Weanling Rats

	Urea adducts	Urea filtrate monomers		Dimers, 165°C., 7μ	
		Unhydrogenated	Hydrogenated	Unhydrogenated	Hydrogenated
Iodine number.....	65	73	23	54	34
Carbonyl oxygen, %.....	0.10	0.41	0.30	0.21	0.21
Hydroxyl oxygen, %.....	0.08	1.20	0.72	0.62	0.72
Toxicity ^a		^b	^b	^b	^b

^a 0.4 ml. fed by stomach tube to weanling rats (30-40 g.).

^b Indicates death within 3 days after dose.

Paschke and Wheeler (8) have suggested that linoleate, polymerized in the presence of oxygen, could form cyclic monomers and polymers. Privett and Lundberg (30) reported the formation of triene conjugation in the alkali isomerization of oxidized linoleate. Heating cottonseed oil, as described in the present study, results in a low peroxide level during the heating together with the appearance of increased amounts of conjugated diene, triene, and tetraene, available for further reaction including cyclization.

Attempts to aromatize the urea filtrate monomers according to the procedure of Scholfield and Cowan (12) were not successful. However cyclic structures that do not easily aromatize under the conditions used may nevertheless be present. Bromination-dehydrobromination and oxidation of the dimer fraction yielded products which showed aromatic character when examined by ultraviolet spectrophotometry. It seems more likely that, under the conditions used, noncyclic polymers similar to those suggested by Perkin and Kummerow would be largely produced. The lower concentration of oxygen available during the heating accounts for the moderate amounts of carbonyl and hydroxyl found and may also account for the simultaneous production of cyclic structures.

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