The authors wish to acknowledge that such a heating bath was suggested by the late Alton E. Bailey, and his counsel was most helpful throughout the early part of the development. Comments and suggestions from members of the current AOM Stability Subcommittee of the American Oil Chemists' Society are likewise gratefully acknowledged.

Summary

A constant temperature bath of a type not heretofore reported has been developed. It eliminates the objectionable features of previous apparatus and provides precise control of the very critical temperature factor in AOM stability measurement. The apparatus is easily constructed from readily available materials; it operates reliably, safely and without attention for extended periods of time. Certain matters of technique are discussed, such as submersion of the sample below the top of the bath.

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Biological Effects of the Polymeric Residues Isolated from Autoxidized Fats[1,2]

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FOR A LONG TIME it has been recognized that highly autoxidized (rancid) fats are not the nutritional equivalents of fresh, wholesome fats, but the reason for the difference has not been explicitly stated. This loss has been variously attributed to peroxides or oxygenated products, but in our earlier studies (8) no signs of toxicity resulted from the feeding of adequate diets containing 5% of methyl monohydroxystearate, methyl 9,10-dihydroxystearate, methyl *cis*-epoxystearate, or a methyl oleate peroxide concentrate (85% peroxide). These results suggest that simple oxidized substances are not the principal agents responsible for the growth-depressing effects. It has been assumed that the peroxides destroy the vitamins in the fats, but this can be eliminated as a possible cause by separate administration of an adequate supply of vitamins.

The possibility that the nutritional effect is caused by the destruction of essential fatty acids is not tenable because, as we shall show later, the effect is extremely dramatic when the actual growth-depressing ingredients are fed at a high level to animals which have been receiving a nutritionally adequate diet and continue to receive essential fatty acids.

Information which may eventually be of value in clarifying our present-day ideas concerning autoxidized fats developed from two main sources. In studies of the chemistry of drying oils used for paints and varnishes it was slowly recognized that fats are capable of undergoing autoxidatively produced polymerization. At the same time knowledge was accumulating concerning the biological properties of heated fats (1). Both lines of investigation gradually led to the recognition of the importance of fat polymers and to the need for a distinction between polymerization brought about by heat alone and by autoxidation. It is not yet possible however to distinguish clearly between the bio-responses brought on by the two kinds of polymers because it is difficult to say whether the polymers are the only cause of these effects. Nutritional studies in which concentrates of autoxidatively produced polymers were used have not yet been reported. In any discussion of autoxidized fats however the effects caused by oxygen and by heat often overlap.

As an indication of the lack of agreement concerning the nutritional effects of polymers derived from fats, some workers (1a, 10, 11, 12) have stressed the undesirable nutritional properties of partly polymerized fats while others (3, 4, 13, 15) have deliberately polymerized certain oils to stabilize them for human consumption and reported no ill effects. These differences may result, in part, from the fact that the latter group examined partly polymerized fish oils and the others used fats of different origin.

Earlier (19) we had shown that molecular distillation was useful to isolate autoxidatively produced polymers from autoxidized methyl oleate. It was decided therefore to apply this technique to various autoxidized fats with the hope of removing unoxidized and oxidized monomeric triglycerides (molecular weight 1,000 or less) as the distillate fractions, leaving the autoxidatively produced polymers, if any, as the non-volatile residue. This separation technique was found to be satisfactory. It permitted the isolation of polymeric residues from drastically autoxidized lard, drastically autoxidized cottonseed oil, and a hydrogenated fat which had been used in commercial deep-fat frying.

Present Study

Lard aerated at 95°C. for about 200 hrs. and then molecularly distilled yielded an amber-colored polymeric residue which usually amounted to about 17% of the original fat. Cottonseed oil, similarly treated, yielded about 40% of the polymer. It should be noted

[1] This paper is XXI in the series "Reactions of Fatty Materials with Oxygen." Paper XX is reference 18.
[2] Presented at the Fall meeting of the American Oil Chemists' Society, Philadelphia, Pa., October 10–12, 1955.
[3] A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

that these yields are roughly proportional to the content of polyunsaturated acids in each of the fats. A hydrogenated shortening which had been used for 80 hrs. at 190°C. in the commercial manufacture of French-fried potatoes yielded 7% of polymeric residue.

Our nutritional studies in this field were carried out on albino rats from a colony which had been used for experiments of a similar type for 15 years. We have followed the procedures previously described for setting up well-matched groups and for paired and restricted feeding (6, 7). The basic diet consisted of 30% casein (G.B.I. vitamin-free test casein), 4% salts (U.S.P. No. 2), 2% cellulose (Alphacel), 10–20% fat, and 54–44% dextrose (Cerelose). To this were added liberal amounts of all known vitamins. When highly rancid fats were part of the diet, vitamin suspensions were fed by dropper. Our techniques permit the assumption that eventual differences among the groups of one experimental series are caused by the factors purposely varied. The conclusions arrived at were drawn only if the differences described were statistically significant. To be absolutely certain however, most of the experiments were repeated several times. This work has been carried out during the last three years, and about 3,000 rats have been used.

Results and Discussion

In Figure 1 are shown the results of experiments in which weanling rats were fed diets containing 10% of either fresh lard, the molecular-distillate fractions from autoxidized lard, or the autoxidatively produced polymeric residue. A weight deficit resulting from the feeding of any of the fractions was an expression of its growth-depressing characteristics.

As Figure 1 shows, the volatile fraction obtained by alembic distillation to degas the autoxidized fat was highly toxic when fed to two rats. Since it constituted only 1% of the total autoxidized fat, there was an insufficient quantity for the protracted feeding of additional animals. This fraction, known to consist largely of aldehydes, was not studied further.

Three fractions (M-I, M-II, M-III), obtained by molecular distillation up to a temperature of 280°C., produced no significant weight deficit. A fourth fraction (M-IV), obtained from 280–300°, exerted a mild growth-depressant effect. The non-volatile residue however was significantly active, as shown by the large weight deficit. These results have been confirmed several times.

When 10% of the polymer from autoxidized cottonseed oil was included in the diet, only about half of the rats were alive after four weeks; at the 20% level all of the rats died within one week. Initially, all rats developed diarrhea, a phenomenon also observed in rats fed unfractionated, autoxidized cottonseed oil (containing 40% polymer). When the diarrhea was at its height, all tissues around the anus were highly irritated. The penis was often swollen and deformed, which might have caused some deaths by interfering with urination. Histological examinations of most of the organs of six animals maintained on residues from autoxidized lard and from autoxidized cottonseed oil for four weeks showed no abnormalities except for differences in organ size, as discussed later in this paper. The rats merely showed signs of starvation.

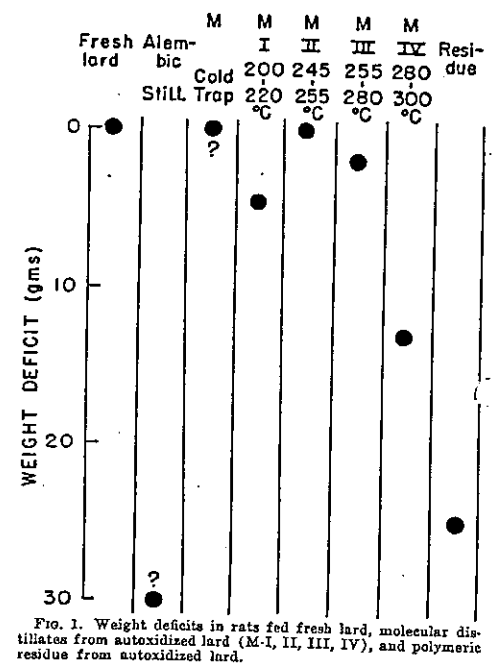

FIG. 1. Weight deficits in rats fed fresh lard, molecular distillates from autoxidized lard (M-I, II, III, IV), and polymeric residue from autoxidized lard.

In our original feeding studies on unfractionated, autoxidized lard and autoxidized cottonseed oil, we assumed that the greater activity of autoxidized cottonseed oil was attributable to the presence of about 2½ times as much polymer as was present in autoxidized lard (8). Further studies however strongly suggest that the polymer from autoxidized cottonseed oil has a greater growth-depressant effect than that from autoxidized lard. This is shown graphically in Figure 2, in which the average growth of rats fed fresh lard or three polymeric residues at a 10% level are compared. In this and subsequent figures giving rat growth, we have followed the procedure of Zucker and Zucker (20) in plotting the logarithm of the rat's weight against the reciprocal value of the age. This results in a straight line for the growth of normal rats, as is shown in the graph for fresh lard. With the residue from autoxidized cottonseed oil the rats lost weight rapidly, and half of them died within four weeks. With the corresponding residue from autoxidized lard the rats were just able to maintain their weight and eventually grow a little, although a significant weight deficit compared to animals fed fresh lard was again noted. All the animals were alive after four weeks on the diet containing polymer from autoxidized lard.

Figure 2 also shows the growth of rats fed a polymer isolated from the hydrogenated shortening used in making French-fried potatoes. Compared to fresh lard, the growth of rats was retarded, but the effect was considerably less than that caused by the other two polymeric residues.

As noted earlier, inclusion in the diet of 20% of

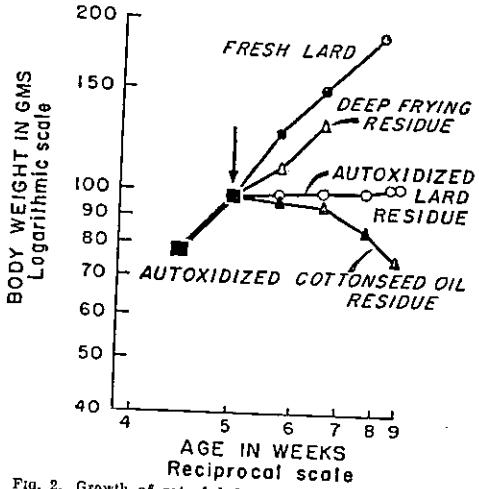

FIG. 2. Growth of rats fed fresh lard and polymeric residues from three autoxidized fats at a 10% level in the diet.

polymer from the autoxidized cottonseed oil led to the rapid death of all the rats. Addition of fresh fat to this diet however exerted a protective effect. A similar protective effect was observed when fresh fat was added to a diet containing autoxidized, unfractionated fat. Thus, with 15% of autoxidized cottonseed oil in the diet, about half of the rats died within three weeks, and all died within six weeks. When the diet also contained 15% of fresh cottonseed oil, all rats were alive after three months. The circumstance that the growth-depressant properties of one fat can be counteracted by the addition of a second fat to the diet has also been noted by Moore and co-workers (14).

Figure 3 compares the growth of rats fed 15% fresh cottonseed oil with those receiving 15% autoxidized cottonseed oil and those receiving 15% of autoxidized cottonsed oil plus 10% fresh cottonseed oil. Rats receiving the autoxidized oil plus fresh oil grew, although not as well as those receiving only fresh fat. Rats fed autoxidized cottonseed oil showed substantially no growth.

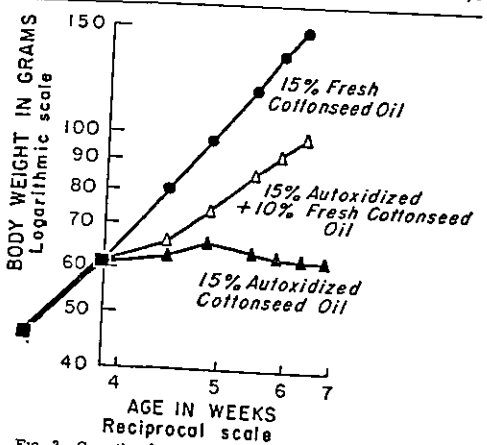

FIG. 3. Growth of rats fed 15% fresh cottonseed oil, 15% autoxidized cottonseed oil, 15% autoxidized cottonseed oil + 10% fresh cottonseed oil.

The results in Figure 3 are all the more remarkable because the food intake of the rats on autoxidized oil was roughly half that of normal rats whereas that of rats eating both fresh and autoxidized oils was nearly normal. Therefore the last-named group was consuming more autoxidized oil than the rats receiving only autoxidized oil. Nevertheless, for some unknown reason, the fresh oil protected the animals. Tocopherol in oral doses of 30 mg. per week exerted hardly any protective effect.

Noticeable changes were also observed in the organ-weight/body-weight relationship, which is shown in Figure 4. Normal organ-weight data were obtained by examining 130 male rats varying in body weight from 18 to 450 g. The limits of the spread in normal organ-weight formed parallel lines on a log-log scale. These data are in line with previous studies (17). For the sake of clarity the lines are given in Figure 4 instead of the individual points. The individual organ-weights of rats fed 15% autoxidized cottonseed oil are indicated by closed circles; those of rats fed 15% autoxidized plus 10% fresh cottonseed oil, by open circles.

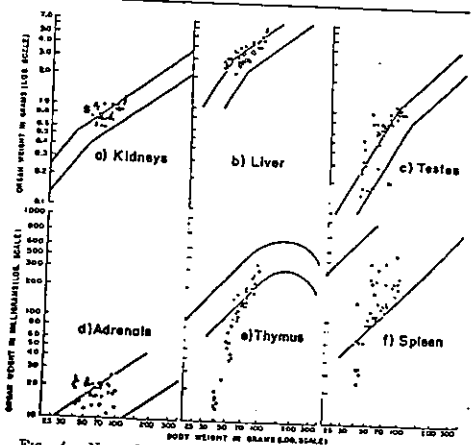

FIG. 4. Normal organ-weights (solid lines) and organ weights of rats fed 15% autoxidized cottonseed oil (closed circles), and 15% autoxidized cottonseed oil + 10% fresh cottonseed oil (open circles).

It is evident that nearly all kidneys, livers, and adrenals from animals on autoxidized oil were above the upper weight limit for normal organs while those from animals receiving autoxidized plus fresh oil were within normal limits. On the other hand, the lymphocytic organs, such as thymus and spleen, were reduced in size after the intake of autoxidized oil. This condition was also improved by fresh oil.

We have also been interested in investigating the caloric requirement for weight maintenance in rats

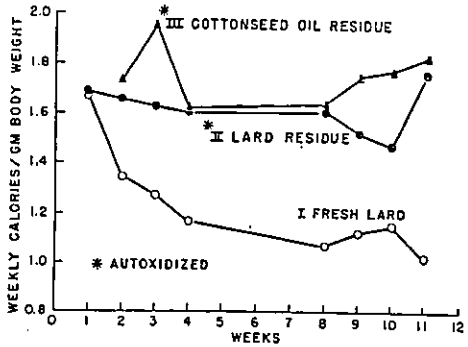

FIG. 5. Average caloric values for weight maintenance of rats fed 10% fresh lard (I), 10% autoxidized lard residue (II), or 10% autoxidized cottonseed oil residue (III).

under widely different experimental conditions. Figure 5 shows the caloric requirements of rats fed just enough food to maintain their weight. Under normal circumstances, continued growth requires an increasing caloric intake. The ordinate of Figure 5 indicates the weekly caloric requirement for the maintenance of one gram of body weight; the abscissa gives the time in weeks after the beginning of food restriction.

Curve I, Figure 5, gives the average caloric values for weight maintenance of eight rats on a diet containing 10% fresh lard over a period of nearly three months. The rats were gradually able to get along on less and less food as the experiment progressed, a condition which parallels that in humans. The adaptation to reduced food intake did not occur when the rats were fed 10% of residue from autoxidized lard (Curve II) or residue from autoxidized cottonseed oil (Curve III). Eventually the caloric requirement of the rats fed autoxidized fat residues was more than 50% higher than that of rats given 10% of fresh lard. This tendency toward an increased caloric requirement was also observed when the rats were given diets containing 4 to 7% of the residue fraction.

The mechanism of the effect of polymeric residues on weight maintenance requirements is not clear. One possibility is that the increased caloric requirements are a reflection of feed losses due to diarrhea. The effect however was still found in animals fed 10% of the polymeric residues after the diarrhea had subsided as well as in those receiving 4 or 7% of the residue, which had never had diarrhea.

The fecal output of animals on polymeric residues was increased, perhaps partly because of reduced intestinal absorption, but it is doubtful that this explains the changes sufficiently. Quantitative analysis of the feces, now in progress, may be helpful.

Qualitatively, the changes produced by highly autoxidized (but not molecularly distilled) cottonseed oil are similar to those brought on by the polymeric residues isolated from either autoxidized cottonseed oil or autoxidized lard. There are several facts however which cannot as yet be explained by the assumption that the biological effects of autoxidized fats are produced by the polymeric residues. One of these is that, on a weight-for-weight basis, the growth-depressant effect of residue from the autoxidized cottonseed oil is only slightly greater than that of the undistilled autoxidized oil although the latter contains only 40% polymer. Also, when 15% of the autoxidized cottonseed oil, equivalent to only 6% of the polymeric residue, was included in a diet, all rats were dead at a time when those on a diet containing 10% polymeric residue were still alive. The fact that the activity of the autoxidized, but undistilled, fat is relatively more pronounced than that of the polymeric residue clearly demonstrates that the biological effects are not caused by substances formed in the process of molecular distillation.

Furthermore the volatile fractions obtained by molecular distillation of autoxidized fats, which had little or no growth-depressant properties when fed to rats with an otherwise normal diet, were hardly protective in diets also containing polymeric residues in contrast to fresh fats. A possible explanation for the absence of the protective effect in molecularly distilled fractions from autoxidized fats is their content of *trans* and/or conjugated unsaturated triglycerides. This point is now being investigated.

Some evidence is accumulating that the distribution of the rat's body fat changes under the influence of highly autoxidized fats or their polymeric fractions. Over-all, the emaciated animals lost a great deal of their depot fat, but the liver lipids seemed to increase (9). The composition of the liver lipids in rats after the intake of polymers obtained from autoxidized fat is presently being studied.

When highly autoxidized fats are consumed by rats fed diets deficient in some essential nutrient, such as protein or a vitamin, the deficiency state becomes evident more quickly than when the diet contains fresh fat. Thus it has been reported (11) that the acrodynia in pyridoxine-deficient animals is increased by autoxidized fats. It seems unlikely however that this effect is a specific one because the pyridoxal phosphate content of the liver of the animals fed polymeric residues in this study is normal. Deficiencies in vitamin K (12), vitamin A (16), and riboflavin (6) as well as the effect of cortisone (2) are intensified by autoxidized fat.

In studies with highly autoxidized lard only little effect on the growth of rats was noted when the diet contained 30% casein. With 5% casein animals fed the autoxidized fat lost weight and soon died whereas rats fed fresh fat were able to maintain their weight and eventually grow slowly (5). It may be of great biological importance that some products of autoxidation, which in the presence of an adequate diet produce scarcely any adverse biological effects, may damage the animal severely when fed in a marginal diet.

Finally studies were conducted to determine whether the intake of polymeric residues produced any permanent lesions in rats which had received these fats for several months and were then returned to a normal diet. Figure 6 shows the results of such a study. The broken line represents the growth of controls and the solid line that of the experimental animals. At the age of 33 days (Arrow 1) rats were placed on a diet containing 10% autoxidized cottonseed oil residue and maintained on such a diet for six weeks. As anticipated, no growth was observed during this period. Thereafter (Arrow 2) the rats were replaced on a normal diet. Realimentation occurred rapidly, and the rats finally reached the

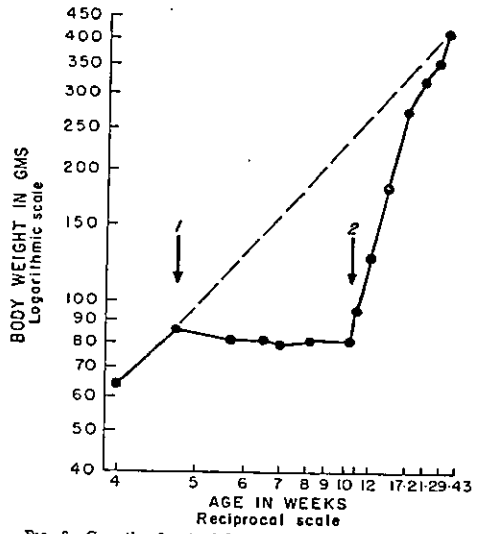

FIG. 6. Growth of rats fed a normal diet to arrow 1, 10% autoxidized cottonseed oil residue from arrow 1 to arrow 2, then returned to normal diet at arrow 2.

same weight as the controls. More than 50 rats which had previously received polymeric residues for several weeks to several months and had then been returned to normal diets have been studied for nearly one year, and in all cases immediate realimentation occurred without signs of subsequent injury from the intake of autoxidatively produced polymer.

Acknowledgments

We are indebted to Waldo C. Ault for his unceasing interest in this work. We also wish to thank Herbert Stoerk of the Merck Institute for Therapeutic Research, Rahway, N. J., for the histological examinations and George Boxer of the same Institute for pyridoxal phosphate determinations. Leo A. Pirk of Hoffman-La Roche Inc., Nutley, N. J., provided us with most of the synthetic vitamins used in the experiments. We are indebted to the Sterling-Winthrop Research Institute, Rensselaer, N. Y., for crystalline vitamin D_2, to the Barnett Laboratories, Long Beach, Calif., for crystalline beta-carotene, and to Merck and Company, Rahway, N. J., for vitamin B_{12}.

Summary

There is increasing evidence that the abnormal nutritional properties of highly autoxidized fats are related to the polymers which develop during autoxidation. Lard and cottonseed oil were aerated at 95°C. for 200 hrs. and molecularly distilled; and the residue fractions, non-volatile at 275 to 300°C., were studied.

Diets containing 20% of autoxidatively produced polymeric residue, fed to albino rats, led to diarrhea and rapid death, but when this residue was reduced to 10%, most of the animals were gradually able to tolerate it. At the 4 or 7% level it was well tolerated, but growth was reduced. There were no distinctive histological lesions, and withdrawal of the polymer permitted immediate realimentation without evidence of subsequent injuries.

The polymeric residue from autoxidized cottonseed oil exerted a greater growth-depressant effect than that from lard, and the latter, more than that from a hydrogenated vegetable oil used for deep-fat frying for 80 hrs. at 190°C. Addition of fresh fat to the polymeric residues decreased their growth-depressant effect.

When rats were fed a measured amount of diet sufficient to maintain their weight, the caloric requirement necessary for weight maintenance gradually decreased. When the dietary fat source consisted of polymeric residue to the extent of 4 to 10%, the caloric requirement for weight maintenance decreased relatively little, if at all. The polymeric residue from autoxidized lard was, in this respect, as effective as that from autoxidized cottonseed oil.

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The Chemistry of Polymerized Oils. VII. Cyclization of Methyl Beta-Eleostearate

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RECENTLY PASCHKE AND WHEELER (5) studied the cyclic monomer obtained on polymerization of a 10% solution of mixed methyl eleostearates in methyl laurate at 250°C. for 48 hrs.

Following our earlier work on the thermal dimer of methyl β-eleostearate (1), we examined the cyclic monomer. From our previous investigations we had concluded that it was important in thermal polymerization studies to keep the reaction conditions as mild as possible, in order to minimize secondary changes. For this reason the cyclic monomer was separated from material which had only been heated at 180° for 4½ hrs.

Experimental

Preparation of Methyl β-Eleostearate Cyclic Monomer. Methyl β-eleostearate (m.p. 12.4 to 13.6°), $E^{1\%}_{1cm.} = 1{,}850$ in ethanol at 268 mμ, was prepared by esterification from the acid, m.p. 71°, with *p*-toluenesulfonic acid as catalyst.[1] Some 115 g. of this ester were heated in a sealed evacuated ampoule for 4½ hrs. at 180°. Monomeric material (69 g.) was separated by repeated passages through a falling-film molecular still at 105°. A solution of 66.2 g. of this distillate in 50 ml. of methanol was added at 40° to a solution of 330 g. of urea in 1,500 ml. of methanol. After 24 hrs. at room temperature the precipitate (which on decomposition with water gave 37.0 g. of oil) was filtered off, and the filtrate was concentrated to 800 ml. After two days the precipitate (which on decomposition with water gave 10.5 g. of oil) was removed, and the filtrate was concentrated to 500 ml. This solution was left at 5° over-night, and the precipitate was filtered off. On treatment with water the latter afforded 0.55 g. of oil. The filtrate, after removal of urea and methanol, gave 11.2 g. of ester. On distillation in a small falling-film molecular still at 105°, this material yielded 10.0 g. of cyclic monomer, or 9% based on the original eleostearate. Its ultraviolet spectrum, which is shown in Figure 1(a), is very different from that recorded by Paschke and Wheeler (5). It had the following properties: Number of double bonds (Adams' catalyst in ethanol) = 2.00 (calc. 2.00); C = 77.42% (calc. 78.03%); H = 11.32% (calc. 11.04%).

Conversion of the Cyclic Monomer to Orthophthalic Acid. Four grams (0.0134 mole) of this cyclic monomer were refluxed with 2.45 g. of N-bromosuccinimide (0.0134 mole) in 30 ml. of carbon tetrachloride for 3 hrs. The solution was cooled, and the solid succinimide (1.27 g.) was filtered off. The filtrate was evaporated under vacuum, and the residue was heated with 5 ml. of redistilled diethylaniline at 130° under nitrogen for 1 hr. to dehydrobrominate the diene to the benzene derivative. When cooled, the mixture set semi-solid. Water and hexane were added, and the

[1]Ester prepared from the acid by treatment with diazomethane in ether had $E^{1\%}_{1cm.} = 1{,}980$, m.p. 14.0–15.1°. Pure ester should have $E^{1\%}_{1cm.} = 2{,}060$ based on $E^{1\%}_{1cm.} = 2{,}161$ for the acid (4).

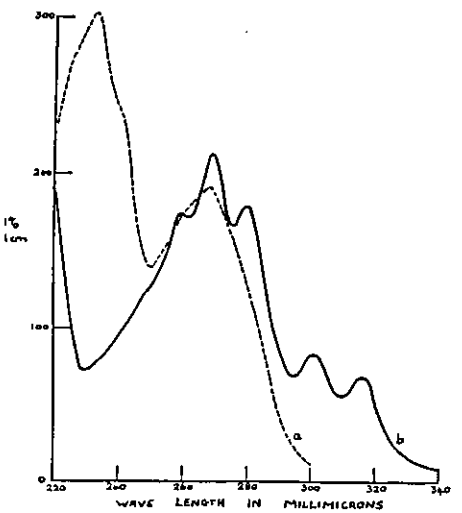

FIG. 1. Ultraviolet absorption spectra. a) Cyclic monomer. b) Material obtained by substitutive bromination and dehydrobromination of cyclic monomer.

mixture was shaken. The hexane layer was separated and washed three times with dilute HCl, then four times with water. The hexane solution was dried over Na_2SO_4, and the solvent was removed to give 4.26 g. of dark oil, which was molecularly distilled at 105° to afford 2.58 g. of a straw-yellow distillate. The ultraviolet absorption curve of this material is shown in Figure 1(b).

A mixture of 0.67 g. (0.0023 mole) of the straw-yellow distillate, 0.82 g. (0.0046 mole) of N-bromosuccinimide and 12 ml. of carbon tetrachloride, was refluxed for 12 hrs. Solvent was removed under vacuum, and the residue was extracted several times with boiling hexane. After evaporation of the hexane 1.07 g. of brominated ester was obtained. This was refluxed with 2.5 g. of Na_2CO_3 in 50 ml. of water for 6 hrs. (*cf.* [5]). The solution was acidified with diluted H_2SO_4, and extracted continuously with ether for ½ hr. The ethereal extract was washed several times with water and evaporated to dryness under vacuum to give 0.72 g. of resinous material. This was dissolved in 100 ml. of acetone (previously treated with $KMnO_4$ and dried over K_2CO_3) and 2.0 g. of $KMnO_4$ added in portions, with shaking, over several hours. Finally the solution was refluxed until the permanganate color had disappeared. The acetone was removed in vacuum, and the residue was extracted three times with 150 ml. of boiling water

STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF MENHADEN OIL FROM HEAT TREATMENT[1]

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INTRODUCTION

Crampton et al. ('56) have compared the nutritive values of various fractions of the ethyl esters prepared from heat-polymerized linseed, soybean and sunflower seed oils. Particular attention was paid to the fraction of the distillable ethyl esters that failed to form urea adducts (the NAFD fraction). The NAFD from linseed oil was highly injurious to the well-being of young growing rats, despite the fact that the material was a bland, neutral oil. The corresponding fraction from soybean oil was injurious, though less so than linseed NAFD. The NAFD from sunflower seed oil was much less injurious than that from the other two oils. The adduct-forming fractions of the distillable esters (AFD) from all three oils were nutritionally innocuous.

Crampton et al. ('53) and Wells and Common ('53) have considered the possibility that failure of the NAFD fraction to form urea adducts might be due to formation of cyclic monomeric acids during the thermal polymerization of the triglyceride. Since that time considerable evidence has been forthcoming to demonstrate that cyclized monomeric acids are formed during heating of methyl eleostearate (Paschke and Wheeler,

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada, Journal Series no. 398.

'55) and of linseed oil (Macdonald, '56). We ourselves have applied the procedure described by Paschke and Wheeler ('55) to the NAFD fraction from linseed oil and have secured confirmatory evidence, from infrared spectrophotometry, of the presence of aromatic material in the product. Our use of the term "cyclic monomers" in our previous papers (Crampton et al., '53, '55) is justified, therefore, to the extent that this fraction has been shown to contain some cyclized material, though it is probably not composed entirely of "cyclic monomers." *Cis-trans* isomerizations may account, in part at least, for failure of NAFD fractions to form urea adducts; and comparable evidence for the presence or absence of cyclic monomeric acids in the NAFD fractions of soybean and sunflower seed oils is not yet available.

The results of comparison of NAFD fractions from linseed, soybean and sunflower seed oils, however, suggested that it could be of interest to conduct similar studies on an oil containing acids of higher unsaturation. Menhaden oil was chosen for this purpose. The results are presented in the present paper.

EXPERIMENTAL

The sample of commercial alkali-refined menhaden oil used in this work had the following characteristics: sp.gr. (25°C.), 0.9251; refractive index (25°C.), 1.4784; iodine no. (Benham and Klee, '50), 180; saponification no. 188; mean molecular weight (cryoscopic), 839; unsaponifiable, 1.04%. This oil was heated in batches of 500 gm for 15 hours at a temperature of 275°C. while passing a strong current of CO_2. The time of heating was selected on the basis of the results of preliminary kinetic studies. The heated oil was converted to the ethyl esters by transesterification in the presence of hydrochloric acid gas as catalyst (see appendix). The use of acid catalysts was preferred because of the sensitivity of the polyene acids of menhaden oil to alkali isomerization. Fractionation of the esters by urea adduct formation and distillation were performed as described by Crampton et al. ('53). In addition,

TABLE 1

Yields and characteristics of fractions of ethyl esters of menhaden oil used in feeding trial

FRACTION[1]	YIELD AS % OF TOTAL ESTERS OF HEATED OIL		REFRACTIVE INDEX 25°C.	IODINE NO.[2]	FREE FATTY ACIDS	SAP. NO.[3]	PEROXIDE VALUE[4]	
	Linseed[2] (12 hr. at 275°C.)	Menhaden (15 hr. at 275°C.)			%		(a)	(b)
							mg/meq. O_2/kg oil	
AF	46 (293)[5]	45 (299)	1.4483	86	0.73	183	276	6712
NAF	54 (472)	45 (497)	1.4964	151	1.33	151	0	1047
DE	60 (294)	60 (296)	1.4517	84	1.15	166	0	861
AFD	40 (203)	34 (290)	1.4420	45	0.41	189	360	1782
NAFD	11 (300)	20 (310)	1.4650	143	1.92	169	0	1215
NDE	40 (550)	..	.....	...	...	...	...	....
WE	..	100 (314)	1.4598	156	1.32	182	0	1100

[1] AF = Adduct-forming fraction of total esters.
NAF = Non-adduct-forming fraction of total esters.
DE = "Distillable" esters.
AFD = Adduct-forming fraction of distillable esters.
NAFD = Non-adduct-forming fraction of distillable esters.
NDE = Non-distillable esters.
WE = Whole esters of unheated oil.
[2] Data for linseed oil quoted from Crampton et al. ('53).
[3] Iodine numbers determined by the method of Benham and Klee ('50).
[4] Saponification numbers determined by the method of the A.O.A.C. ('55).
[5] Peroxide values determined by the method of Skellon and Wills ('48).
(a) Value of ester fraction before its inclusion in the diet.
(b) Value determined on oil fraction extracted from the diets by cold chloroform at the conclusion of the 28-day feeding trial.
[6] Figures within parentheses are cryoscopic mean molecular weights determined in cyclohexane.

ethyl esters of the whole unheated oil were prepared by acid-catalyzed transesterification. The yields and characteristics of the various fractions are presented in table 1. It will be noted that the yield of the NAFD ester fraction was 20%, based on weight of whole esters.

The basal diet for the nutritional experiment was similar to that used in our previous experiments, except that wheat flour was replaced by ground whole wheat. The percentage compositions of the diets were as follows: — 10% level diet: whole wheat 54; dried skimmilk 19; casein 12; ester fraction 10; dried yeast 3; dicalcic phosphate 1; iodized salt 0.5; ferrous sulphate 0.1; vitamin A and D supplement 0.15; chromic oxide 0.25. Twenty per cent level diet: as above, but with 44% ground wheat and 20% ester fraction.

The experimental animals comprised 12 groups each of 10 rats. Six of these groups received each a different ester fraction at a level of 10% of the diet and the other 6 groups received the same ester fractions at a level of 20%. Ester fraction 6 (see table 1) was not included in the feeding experiment. Its place was taken by the esters of whole unheated menhaden oil which were also fed both at the 10% and the 20% level. The various diets were offered ad libitum.

The results for survival, food intake, liveweight gain, digestibility of the ether extract and gain per 1000 digested calories are presented in table 2. Tables 1 and 2 include comparable data for similar fractions from heated linseed oil. The latter data have been cited from Crampton et al. ('53).

The results presented in table 2 may be summarized in the general statement that the various fractions from heated menhaden oil had about the same effects on the well-being of the rats as had similar fractions from linseed oil in previous comparable experiments (Crampton et al., '53). A slight apparent superiority of the NAFD from menhaden oil was not sufficiently marked to warrant any assertion as to its superiority over the NAFD from linseed oil. The menhaden NAFD,

TABLE 2

Effects of esters of heated menhaden oil on survival and live weight gains of rats and the digestibility of ether extracts of the diets

FRACTION	RATS SURVIVING 28-DAY TEST		AV. LIVE WEIGHT GAINS PER 28-DAY PERIOD		AV. FOOD INTAKE PER RAT PER 28-DAY PERIOD		APPARENT DIGESTIBILITY OF ETHER EXTRACT		AV. LIGHT WEIGHT GAIN PER 1000 CAL. DIGESTED	
	20%[1]	10%	20%	10%	20%	10%	20%	10%	20%	10%
	%	%	gm	gm	gm	gm	%	%	gm	gm
AF	100	100	118	113	233	286	94	87	105	95
	(100)[2]	(100)	(101)	(107)	(266)	(300)	(96)	(95)	(61)	(61)
NAF	0	90	...	3	...	132	...	73	...	37
	(20)	(100)	(-32)	(30)	(141)	(205)	(76)	(77)	(9)	(50)
DE	80	100	2	46	95	152	95	88	43	67
	(90)	(100)	(4)	(66)	(118)	(228)	(97)	(92)	(43)	(63)
AFD	100	100	111	122	233	261	94	91	92	103
	(100)	(100)	(57)	(108)	(193)	(290)	(96)	(96)	(54)	(62)
NAFD	10	60	-50	-13	81	96	85	87	...	...
	(0)	(0)	..	..	..	..	..	(93)	..	..
WE	100	100	61	93	165	233	93	94	73	87
	...	...	..	..	..	..	..	..	..	..
LSD			18	18	30	30			18	18
(P = 0.05)			(23)	(23)	(36)	(36)			(16)	(16)

[1] The ester fractions were fed at 20 and 10% of the diet.

[2] Figures within parentheses related to comparable data for linseed oil cited from Crampton et al. ('53).

241

however, was definitely and markedly inferior nutritionally to the NAFD from soybean or sunflower seed oil.

The chief interest of the present work lies in the additional evidence that it provides for the association of toxicity of the NAFD fraction of heat-polymerized oils with the presence of polyene acids in the original oil.

SUMMARY

The non-adduct-forming fraction (NAFD) of the distillable esters from heated menhaden oil was toxic to rats to a degree comparable with the toxicity of the similar fraction from heated linseed oil.

The adduct-forming fraction (AFD) of the distillable esters from the heated oil was nutritionally innocuous.

The results provide some additional evidence for an association between the toxicity of the NAFD fraction and the presence of polyene acids in the original oil.

ACKNOWLEDGMENTS

The authors are indebted to the Canadian Committee on Edible Fats and Oils, National Research Council of Canada, for financial support and for advice. They are specially indebted to Dr. C. Y. Hopkins for details of the acid-catalyzed interesterifications, and to Professor Alfred Taurins, McGill University, for determining the infrared spectrograms and advising on their interpretation. They wish also to thank Canada Packers Limited, Montreal, for donating samples of menhaden oil used in this study.

APPENDIX

A. Preparation of ethyl esters. One kilogram absolute ethanol was weighed into a tared 2-liter round bottomed flask with ground glass joint. Dry hydrochloric acid gas was passed in until the weight had increased by 20 gm.

The oil (500 gm) was then added and the mixture was refluxed for 24 hours. The mixture was then allowed to cool, finally in a large separatory funnel. The upper layer was separated and distilled from a steam bath until the volume was reduced by one half. The concentrate was poured into 4 volumes of water, and to the mixture the lower layer from the separation was added.

The esters were extracted from the aqueous mixture with peroxide-free ethyl ether. The ether was removed under reduced pressure using a stream of N_2 instead of air. The esters thus prepared were stored under CO_2 in tightly stoppered flasks in the freezer until required.

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245

greater part of his professional life, he advised on national medical problems, wrote for medical journals, and spoke to medical audiences. As he took part in the development of nutrition he became convinced, and so urged, that it be looked upon as an exceedingly important factor in preventive medicine. He was frequently consulted by the practitioner regarding clinical problems; the discussion usually revolved about normal physiology and chemistry upon the basis of which guidance to the solution of the problem was usually given.

During his lifetime many honors came to Dr. Mendel. He was proud to have been a charter member of the Yale Chapter of Sigma Xi. Later, honorary degrees were conferred by the University of Michigan, Rutgers University, and Western Reserve University. He was long a member of the National Academy of Sciences and of the American Philosophical Society. In 1929 he was elected to membership in the Societe de Biologie in Paris and became a member of the American Academy of Arts and Sciences a year later. His academic service was recognized by a gold medal given by the American Institute of Chemists and a year before his death, the Chemists' Club of New York conferred upon him the Conne Medal for outstanding chemical service to medicine. When he was 60, his friends, students, and professional associates presented him with his portrait and for the same occasion, there was published an Anniversary Number of the *Yale Journal of Biology and Medicine* containing articles by some of his former pupils. While he valued these various honors, he prized most of all the successes of his many former students.

In Dr. Mendel's death on December 9, 1935, at the age of 63, there passed not only one of the pioneers in the science of nutrition, but also a gentle friend to many whose lives were enriched by contact with him.

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246

STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT[1]

IV. ETHYL ESTERS OF HEAT-POLYMERIZED LINSEED, SOYBEAN AND SUNFLOWER SEED OILS

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INTRODUCTION

The literature on the effects of polymerization temperature on the nutritive value of edible oils has been reviewed up until 1952 by Crampton et al. ('53). Since then Frahm et al. ('53) have reported deleterious effects of heat-polymerized whale oil when it is fed to mice. Raju and Ragagopalan ('55) have reported the results of feeding rats with diets containing 15% of peanut or sesame or cocoanut oil which had been heated at 270°C. in open pans in contact with air. The effects included depression of live weight gain, decrease in food efficiency and increases in liver weight as percentage of body weight and in percentage of liver fat. However, although the temperature used by Raju and Ragagopalan suggests that there was polymerization, the experiments of these workers are not comparable with those carried out in our laboratories where the oils were heated in a current of CO_2.. Kaunitz et al. ('55) have found that cottonseed oil heated and aerated at 90 to

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Province of Quebec, Canada. Journal Series no. 394.

95°C. for periods of up to 300 hours became injurious to rats. Incorporation of fresh oil gave a degree of protection against some of the deleterious effects; peroxides were considered not likely to be responsible for the ill effects. The conditions of heating differed greatly from those used in our laboratories.

In a previous paper (Crampton et al., '53) we have reported a study of the nutritional properties of certain fractions prepared from the ethyl esters of heat-polymerized linseed oil by distillation and urea adduct formation. The preparation and the designations of the different fractions are described in this previous paper, as are also the diets and plan of the feeding trials. For convenience of reference the flow sheet for fractionation is reproduced in figure 1.

It should be pointed out here that polymerization of triglyceride oils is now known definitely to include formation of significant amounts of trimeric, and even of some higher polymeric acyl radicals, as well as of dimeric acyl radicals (Paschke and Wheeler, '54). In the present paper, therefore, fraction 6 is designated "polymer" rather than "dimer."

From this earlier work it seemed reasonable to suppose that heated linseed oil was nutritionally injurious, firstly, because of the presence of polymerized material that is poorly absorbed, if at all, and secondly, because of the presence of monomeric acyl radicals incapable of forming urea adducts by reason of some structural feature, possibly a cyclization. In this connection it is noteworthy that Paschke and Wheeler ('55) have now demonstrated the formation of a cyclic monomer during heat polymerization of methyl eleostearate and have shown that this cyclic monomer is mainly an ortho-disubstituted cyclohexadiene.

The question that next presented itself was the degree to which formation of non-adduct-forming monomeric material could be related to the fatty acid composition of the original oil itself. Accordingly, in 1953-'54 further rat feeding trials were carried out involving 24 lots of 10 animals each in order to study the nutritional value of similar fractions prepared from soybean oil and sunflower seed oil. Soybean oil was

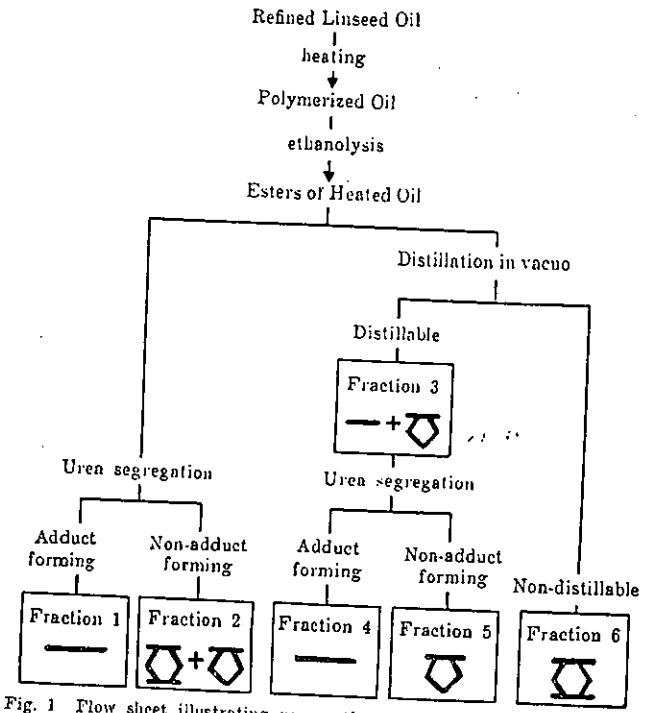

Fig. 1 Flow sheet illustrating preparation of fractions of esters of heated linseed oil used in feeding trials.

chosen because it is a food oil that contains some linolenic acid. Sunflower seed oil was chosen because of its high linoleic acid content and negligible linolenic acid content. Typical data for the fatty acid composition of the three oils are summarized in table 1.

Preliminary experiments with soybean and sunflower seed oils showed that non-adduct-forming distillable ester (NAFD) fractions could be obtained from both oils using the same polymerization temperature (275°C.) and heating in a current of CO_2. It was necessary to heat these oils for longer times

TABLE 1

Fatty acid composition by weight of linseed, soybean and sunflower seed oils

	SATURATED			UNSATURATED		
	C_{16}	C_{18}	C_{20}	Oleic	Linoleic	Linolenic
	%	%	%	%	%	%
Linseed N. American (I No. 179.8)[1]	6.3	2.5	0.5	19.0	24.1	47.4
Soybean (I No. 132.5)[2]	10.6	2.4	2.4	23.5	51.2	8.5
Sunflower seed[3]	6.4	1.3	4.0	21.3	66.2	..

[1] Rose and Jamieson ('41), cited by Bailey ('52).
[2] Hilditch, Meara and Holmberg ('47), cited by Bailey ('52).
[3] Barker, Crossley and Hilditch ('50), cited by Bailey ('52).

TABLE 2

Yields of fractions of ethyl esters used in feeding trials

FRACTION		YIELD AS PER CENT OF TOTAL ETHYL ESTERS OF HEATED OIL		
		Linseed[1] (12 hr. at 275°C.)	Soybean (24 hr. at 275°C.)	Sunflower seed (26 hr. at 275°C.)
1. Adduct-forming fraction of total esters	——	46 (293)[2]	63 (296)	65 (..)
2. Non-adduct-forming fraction of total esters	◇ + ◇	54 (472)	37 (415)	35 (..)
3. "Distillable" esters	—— + ◇	60 (294)	74 (296)	75 (303)
4. Adduct-forming fraction of distillable esters	——	49 (293)	64 (294)	65 (295)
5. Non-adduct-forming fraction of distillable esters	◇	11 (300)	10 (294)	10 (296)
6. Non-distillable esters	◇	40 (550)	26 (415)	25 (613)

[1] Data for linseed oil quoted from Crampton et al. ('53).
[2] Figures in parentheses are cryoscopic mean molecular weights.

than linseed oil in order to attain reasonable yields of this fraction. The times used for bulk preparation of fractions are given in table 2, together with approximate yields of the various fractions. The data for linseed oil are quoted from Crampton et al. ('53).

RESULTS

Tables 3 to 7 summarize the results of the feeding experiments with soybean oil and sunflower seed oil, the data for linseed oil being cited from Crampton et al. ('53). In the discussion that follows, the data for linseed oil are considered along with those for the two other oils; the latter sets of data are here reported for the first time.

Survival of rats. The percentages of the rats surviving the 28-day feeding are shown in table 3.

TABLE 3

Comparison of the nutritional effects of ethyl esters of linseed, soybean and sunflower seed oils

(Ten rats per lot)
Percentage of rats surviving 28-day test

DIET	ESTER FRACTION	LINSEED		SOYBEAN		SUNFLOWER SEED	
		20%	10%	20%	10%	20%	10%
1	——	100	100	100	100	100	100
2	◇ + ◇	20	100	0[1]	100	50[2]	50[2]
3	—— + ◇	90	100	100	100	100	100
4	——	100	100	100	100	100	100
5	◇	0	0	100	80[3]	100	100
6	◇	70	100	0[1]	10[4]	100	100

[1] All animals removed after 10 days because of diarrhea and extreme viscosity of feces.
[2] Five animals removed because of diarrhea.
[3] Remaining rats in poor condition.
[4] Some diarrhea.

The only deaths recorded were in lots where the diets contained either non-adduct-forming monomers or dimeric or higher polymers, with the former displaying the greater toxicity. Also, in every lot receiving esters of polymeric acids there was diarrhea and the feces were varnish-like. This material was so sticky that at morning inspection the feet and tails were often found to be inseparable without washing. In some cases the animals were stuck to the wire floor of their cages. The diets, however, did not appear to be toxic in the usual sense of the term.

The ester fraction which consisted entirely of urea adduct-forming monomers had no harmful effect on survival. The one death in lot 3 may have been due to the "cyclic" monomers of linseed oil also present, since all rats died in lot 5 even where the diet contained but 10% of such material.

Gain of rats. The gain figures shown in table 4 are not directly comparable as between oils. For each ester source, however, figures are strictly comparable as between diets and between levels.

TABLE 4

Live weight gains — 28 days

DIET	ESTER FRACTION	LINSEED		SOYBEAN		SUNFLOWER SEED	
		20%	10%	20%	10%	20%	10%
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
1	——	101	107	93	99	143	150
2	⟨◇⟩ + ◇	32	39	..	63	107	150
3	—— + ◇	4	66	83	101	162	162
4	——	57	108	91	99	151	159
5	⟨◇⟩	..	..	16	36	97	142
6	⟨◇⟩	13	77	..	69	90	143
Least significant difference (P = 0.05)		23	23	10	10	20	20

The results leave little doubt that esters of both "cyclic" monomeric and polymeric acids from the heat polymerization of these three oils are undesirable components of rat diets. However, there would seem to be a difference in the degree of toxicity of the non-adduct-forming distillable fractions, insofar as that from sunflower seed oil was less damaging than those derived from flaxseed or soybean oil. These oils differ chiefly in their contents of the trienoic linolenic acid and there is a temptation to ascribe to this fatty acid the origin of the toxic "cyclic" monomers. Such an ascription requires an assumption that some non-adduct-forming material arises on heat polymerization from some fatty acid other than linolenic and perhaps also that this is a less toxic material than that formed from trienoic fatty acids.

Food intake. Fractions 1 and 4 were equally readily eaten, except at the 20% level of the linseed oil fractions. The polymer fraction 6 was practically equally acceptable, except at the 20% level of the linseed oil fraction; this is remarkable in view of the unattractive varnish-like nature of the materials in question. The "cyclic" monomer fraction (fraction 5) was less readily eaten than the other fractions; and here it is to be noted that these fractions were either colorless or very pale, bland oils. Fractions 2 and 3, containing some "cyclic" monomer, were as readily accepted as fractions 1 and 6, in the case of the soybean and sunflower seed oil fractions, but less readily in the case of the linseed fraction. On the whole, these results suggest that the only fraction that was definitely poorly acceptable was fraction 5.

Digestibility of the oils. There was reasonably clear evidence that the non-adduct-forming polymers were poorly digestible. This was predictable from the abnormal feces. It is probable that low digestibility was a major causal factor in the slower gain of the animals fed this oil fraction. The digestibility of other fractions was above 90%.

Efficiency of utilization of dietary calories (gain per 1000 digested calories). The data suggest that, in general, the rats used that portion of the calories which they absorbed about

equally well excepting for the "cyclic polymers." In the case of the linseed and soybean oils, the digested polymeric esters were in some cases as efficient as the straight-chain materials, but with sunflower seed oil this fraction was as unsatisfactory as the cyclic monomers (table 5).

TABLE 5

Gains per 1000 digested calories

DIET	ESTER FRACTION	LINSEED		SOYBEAN		SUNFLOWER SEED	
		20%	10%	20%	10%	20%	10%
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
1	——	61	61	99	99	82	89
2	⬡ + ⬡	9	50	..	88	69	101
3	—— + ⬡	43	63	92	104	95	90
4	——	54	62	99	106	92	87
5	⬡	..	..	85	85	77	93
6	⬡	61	56	..	88	65	84
Least significant difference (P = 0.05)		16	16	10	10	14	14

DISCUSSION

Certain characteristics of the non-adduct-forming distillable ester fractions from the three oils are presented in table 6. The order of decreasing injuriousness was also the order of decreasing iodine value and refractive index, while the cryoscopic mean molecular weights were, for practical purposes, the same and corresponded to a preponderance of C_{18} acids. The most marked difference between the three NAFD fractions was in respect to their behaviour on alkali isomerization (fig. 2). Linseed NAFD displayed a relatively low absorption at 233 mμ, while both soybean NAFD and sunflower seed

TABLE 6

Iodine values and refractive indices (n^{25}_d) of fractions of ethyl esters of linseed, soybean and sunflower seed oils

FRACTION		LINSEED	SOYBEAN	SUNFLOWER SEED
1. Adduct-forming fraction of total esters	Iodine no.	118.2	99	..
	n^{25}_d	1.45345	1.45302	..
2. Non-adduct-forming fraction of total esters	Iodine no.	162.7	125	..
	n^{25}_d	1.47561	1.46998	..
3. "Distillable" esters	Iodine no.	130.1	106	110
	n^{25}_d	1.45684	..	..
4. Adduct-forming fraction of "distillable" esters	Iodine no.	124.8	92	107
	n^{25}_d	1.45494	1.44935	1.45254
5. Non-adduct-forming fraction of "distillable" esters	Iodine no.	170.7	143	130
	n^{25}_d	1.46986	1.47001	1.45671
6. "Non-distillable" esters	Iodine no.	159.9	114	106
	n^{25}_d	1.48017	..	1.47655

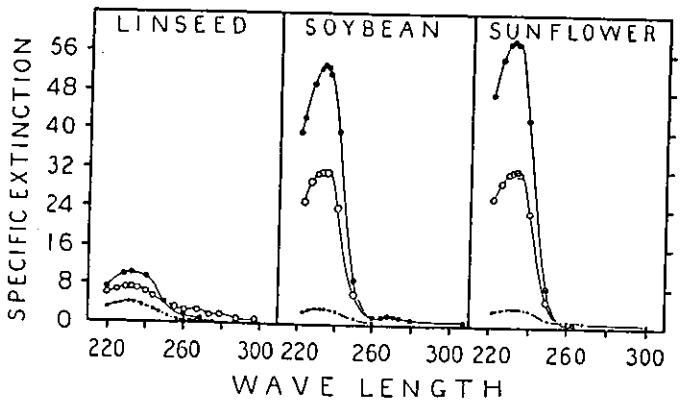

Fig. 2 Ultraviolet absorption spectra of non-adduct-forming distillable fractions of the esters of heat polymerized linseed, soybean and sunflower seed oils.

•—•—• unisomerized.
○—○—○ alkali-isomerized for 25 minutes.
●—●—● alkali-isomerized for 6 hours.

250

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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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								
			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	.2079
Neutral reagent	89.85	90.07	0.0079	0.0075	.0077	0.1872	0.2228	.2050	0.1951	0.2303	.2127
Acidified reagent (without Na_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	0.0605	.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 Na_2)	68.01	68.32	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.	46	10.5	28.2 ± 0.3	.62
B	None	72	21.4	41.0 ± 1.7	
B	T.O. 24 hr.	51	6.5	31.4 ± 1.8	.76
C	None	72	7.0	41.0 ± 1.6	
C	T.O. 24 hr.	56	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	9.5 ± 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

[illegible] 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 first 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.

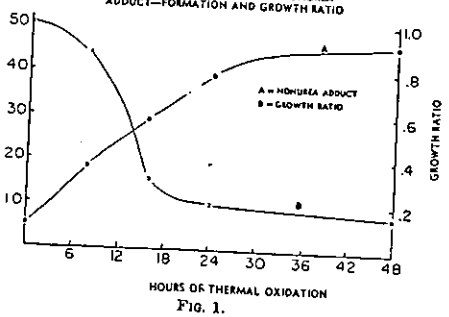

FIG. 1.

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 [illegible] from cotton-seed oil oxidized at 100°C. is reported (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)	22.3 ± 1.2(6)
Corn oil, T.O. 24 hr. [c]	36.4 ± 2.9(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 500 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	50.2±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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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.

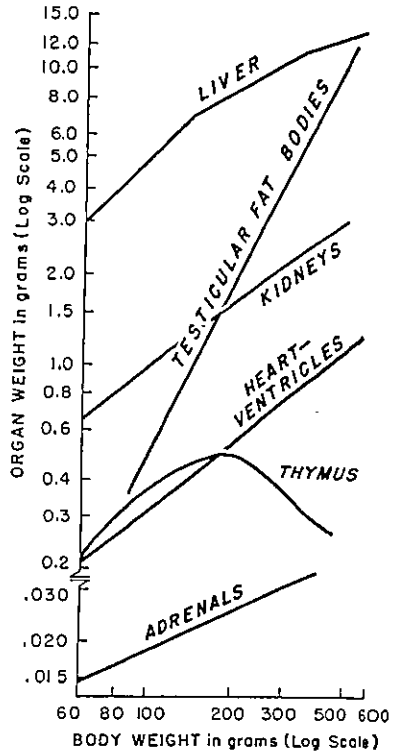

Fig. 1 Lines derived from plotting average organ weight-body weight relationships on a log-log scale.

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.

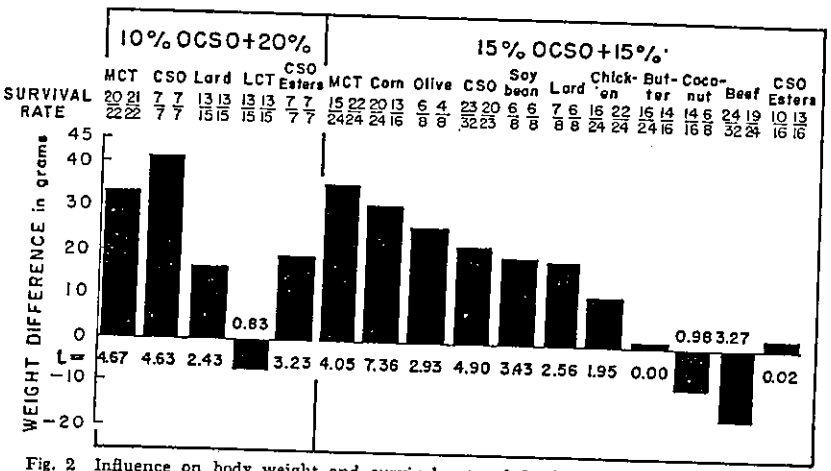

Fig. 2 Influence on body weight and survival rate of fresh fat added to a diet containing OCSO. Effects on body weight are expressed as weight difference between corresponding groups on OCSO alone and with a fresh fat. The t values refer to these differences. In each pair of survival rates, the first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

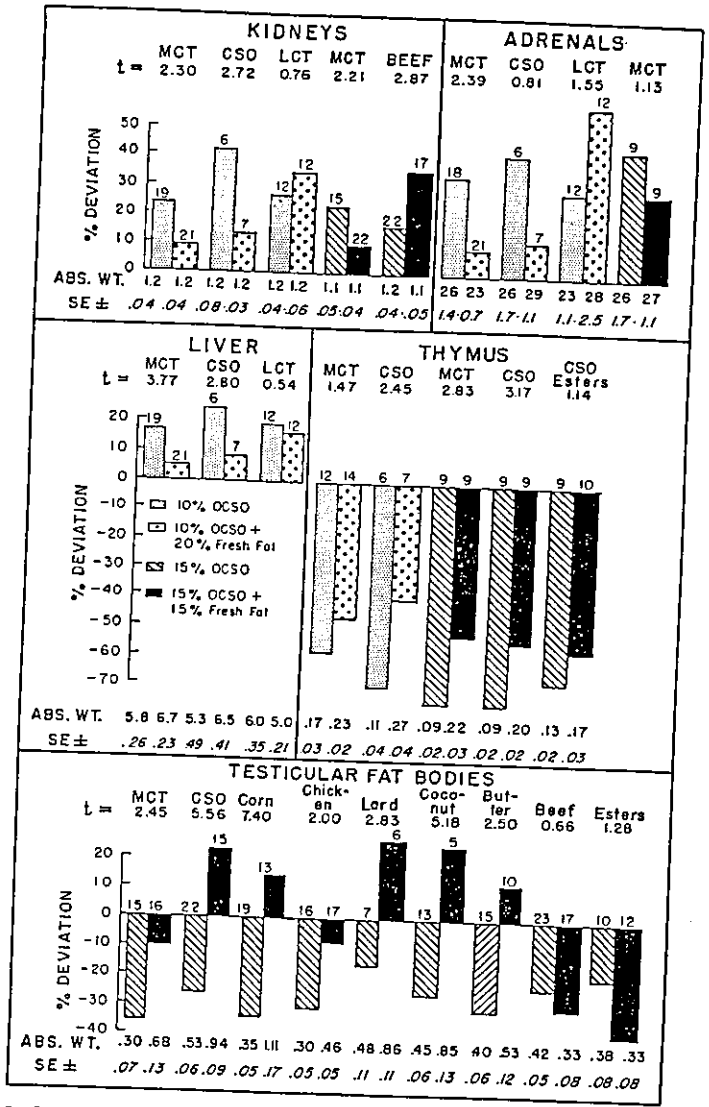

Fig. 3 Influence on organ weight-body weight relationship of adding fresh fat to a diet containing oxidized cottonseed oil (OCSO). Effects are expressed as percentages of deviation from normal. The t values refer to differences in percentage of deviation between corresponding groups fed OCSO alone and 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

[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.

at least as beneficial as the highly unsaturated oils.

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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