ON THE NATURE OF THE NUTRITIONALLY DELETERIOUS CONSTITUENTS OF HEATED VEGETABLE OILS

A Thesis

by

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HISTORICAL INTRODUCTION

During World War II, the serious shortage of edible fats and oils in Canada stimulated investigations on the use of domestic vegetable oils for foods. Attention was concentrated on linseed oil, which could be produced in large quantities; but hydrogenated linseed shortenings developed serious flavor reversion. This made them totally unacceptable to the consumers.

Evidence was obtained in this Department that linolenic acid on hydrogenation forms precursors of reversion compounds. Investigation of means for the removal of linolenic acid from linseed or soybean oil triglycerides led to the use of thermal polymerization, followed by solvent segregation into an "edible" fraction and a drying fraction of superior film-forming properties.

The "edible"fraction yielded a hydrogenated shortening of acceptable stability and flavor. However, it showed markedly lowered nutrutuve value when included in the diet of rats at levels of 10% or over. The primary aim of the research reported herein has been to concentrate and identify the material responsible for the deleterious nature of heat-polymerized linseed oil. It is likely that similar products are responsible in heated soybean or other oils. Linseed oil was chosen for study because the damage occurs with short heating periods, and because most previous data were obtained with linseed oil.

REVIEW OF THE LITERATURE.

FLAVOR REVERSION:

Flavor reversion is defined by Bailey (1) as "The appearance of objectionable flavor in fats from less oxidation than is required to produce true rancidity". It is an ill-chosen term, since the characteristic taste and odor of a reverted fat seldom resemble those of the original crude fat, except in certain fish oils; neither do they resemble necessarily the taste and odor of the rancid fat.

The fats which show the most clearly defined reversion, as distinct from rancidity, are vegetable and fish oils containing linolenic acid. These revert with very slight oxidation, so that it is almost impossible to protect such oils after thorough deodorization. Those oils which revert seem to do so at least as easily after as before hydrogenation. Indeed, Armstrong and McFarlane (2) found that hydrogenated linseed oil reverted more rapidly than did the unhydrogenated oil.

The relation of linolenic acid content to reversion was emphasized by Armstrong (2), who added ethyl linolenate to a non-reverting shortening. He produced reversion whose extent varied with the linolenate content. Fishy flavors and odors, however, must be due to nitrogenous nonglyceride components of the oil (3), which appear to be bound by the highly unsaturated fatty acids. Phosphatides could be

responsible for the fishy odor of reverted soybean oil, since it is extremely difficult to remove them completely. The German processors refine soybean oil drastically in order to take out lecithin very thoroughly (4), in margarine manufacture.

Why hydrogenated linseed oil should revert even more than the refined oil was extremely puzzling, until ultraviolet absorption spectroscopy was applied to fat chemistry. Lemon (5) found that during hydrogenation of linseed oil, a considerable amount of isomeric dienoic acid was produced from linolenic acid; this isomeric acid could not be conjugated by isomerization with alkali. Lemon assumed that the middle, or 12:13 double bond of linolenic acid was hydrogenated to yield a 9:10, 15:16- "isolinoleic" acid. The disappearance of flavor reversion on further hydrogenation paralleled the disappearance of this acid. Various workers have prepared concentrates of isolinoleic acid, and have produced reversion in normally stable fats by addition of the isolinoleic acid in small amounts.

A number of recent processes have as their objective the elimination of reversion by specialized hydrogenation techniques. Presumably these techniques tend to inhibit the formation of isolinoleic acid in favor of normal linoleic acid (6, 7, 8).

If linolenic acid or its glycerides could be removed

from an oil, it should be possible to process non-reverting shortenings from the treated oil. Solvent crystallization of the less unsaturated glycerides or fatty acids at low temperatures (9) is a good, but elaborate and expensive technique. Selective liquid-liquid extraction is likewise applicable to both glycerides and fatty acids (10). Privett (11) developed a method comprising thermal polymerization of linseed oil, under conditions supposed to favor selective reaction of linolenic acid. The polymerized oil was then segregated with acetone, according to the method of Kolthoff (12); the soluble fraction yielded a non-reverting shortening on hydrogenation, while the polymeric insoluble fraction constituted a high-grade drying oil.

THERMAL POLYMERIZATION:

Modern views regarding the primary mechanism of thermal polymerization stem mainly from the theories of Kappelmeier (14) and of Scheiber (13, 15). Scheiber observed that the refractive indices of nonconjugated oils always increased during the early stages of heat bodying, and assumed that the double bonds in polyethenoid chains isomerized to conjugated structures before condensing into polymers. Kappelmeier suggested that a conjugated fatty chain added a second unsaturated chain by a Diels-Alder addition. Scheiber then included this concept in a scheme whereby the initial reaction during heat bodying was the

thermal shift of double bonds to conjugated positions; then the conjugated chains condensed with double bonds from other chains to form polymeric molecules. Much additional evidence for these views has been provided by ultraviolet absorption spectroscopy.

Brod, France and Evans (21) showed that 9:11 and 9:12 linoleates gave the same products on heat bodying. However, Radlove and Falkenburg (22) noted that previously conjugated linseed and soybean oils, when bodied to the same viscosities as the nonconjugated oils, had superior drying properties to the latter oils. This does not contradict the Scheiber-Kappelmeier hypothesis; rather, it indicates the existence of other, little-known mechanisms as well.

Studies on pure and mixed fatty monoesters have yielded the most useful information on polymerization kinetics and products. The work of Bradley and associates (18, 19, 20) has been of inestimable value in the elucidation of thermal polymerization phenomena. Their findings have been summarized admirably by Wheeler (23), and this summary is given here:

1. Dimers predominate in polymerized monoesters.
2. Conjugated esters dimerize much more rapidly than nonconjugated esters.
3. Conjugated and nonconjugated esters yield similar dimers.
4. Dienoic ester dimers are monocyclic, having a

cyclohexene ring.

5. Trienoic esters dimerize similarly, then close another ring to become bicyclic.

6. Side reactions (pyrolytic scission of double bonds, decarboxylation) form smaller fragments. The latter may react among themselves or with intact chains to give odd-sized "abnormal" polymers.

7. Monomeric derivatives of trienoic esters are found, with low iodine values and high densities, indicating intramolecular cyclization.

No one has succeeded in separating higher polymers than trimers from polymerized monoesters. Indeed, Marcusson (24) and Petit (25) emphasize that only dimeric fatty acids are recoverable from bodied linseed oil. The author has obtained like results (see Experimental section).

Whether dimers of trienoic acids are bicyclic, tricyclic, etc., is questionable. Ault et al. (26) argue for the tricyclic structure; but in all likelihood, several isomers are formed in the same system.

Side reactions are said to yield, according to Kass (27), aldehydes, free fatty acids, hydrocarbons, and (in the case of triglycerides) acrolein.

The cyclic monomers mentioned above were also reported by Steger and van Loon (28) in heated ethyl eleostearate, and were actually separated by Sunderland (32), who completely hydrogenated polymerized esters and removed the polymers and stearate. The amount of such material is appreciable in

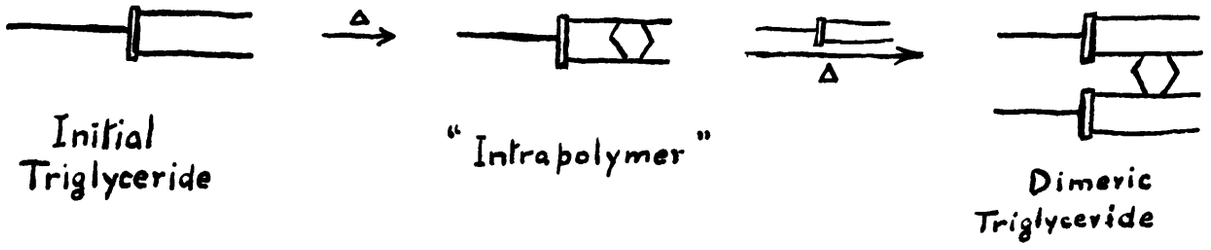
polymerized eleostearates; Kass (27) states it to be at least 10%.

While studies on fatty monoesters offer much useful information, triglycerides are far more complex systems, and the literature contains many conflicting claims. Bradley (16,17) extended the concept of functionality to drying oil polymerization. Unsaturated fatty acids which can add only one other molecule to form a dimer are monofunctional; those which can yield a trimer are bifunctional. Thus a system of bifunctional molecules can, theoretically, form a linear polymer chain. To form the insoluble, infusible cross-linked polymer structure, trifunctional molecules must participate. In triglyceride molecules, the effective functionality is the sum of the functionalities of the component fatty acids. Hence the common drying oils can be gelled completely by heat, while their monoesters merely thicken.

Champetier and Petit (29) polymerized ethyl esters of linseed oil, and compared the polymers with those prepared by ethanolysis of linseed stand oil. The products were very similar, suggesting that the reaction mechanisms operating in triglyceride oils are much the same as in their monoesters. However, the greater functionality of the oils gives rise to a much greater range of polymer sizes and species.

Adams and Powers (30) followed the course of thermal polymerization in linseed oil by changes in viscosities, cryoscopic mean molecular weights, densities, and iodine values. They found that iodine values decreased rapidly at

first, while viscosities and molecular weights did not increase rapidly until later stages of bodying. This was ascribed to intramolecular dimerization of fatty chains occurring in the early stages of heating, followed by later transesterification to polymeric glycerides, as shown:



This would explain the slow initial rise in viscosity and molecular weight, in spite of the rapid fall in iodine value. When the intrapolymers have accumulated sufficiently, transesterification begins to predominate, and polymeric glycerides are formed with a rapid rise in viscosity and molecular weight.

Bernstein (31) separated polymerized linseed and soybean oils into fractions of progressively greater molecular size, by successive extractions with an ascending series of n-aliphatic alcohols. He dismissed the idea of intrapolymers on purely theoretical grounds. The data presented were interpreted by a statistical theory of polymerization which ignores the Scheiber mechanism entirely. Since several of his assumptions lack supporting arguments, Bernstein's theory cannot be accepted nor rejected until the experimental background is more complete.

Thermal polymerization may be accelerated by various classes of catalysts. Some of these are rather specific for

certain stages of the process. For example, Raney nickels, metal carbonyls, and diphenyl disulfide (22) are efficient conjugation catalysts. Mercuric acetate, according to Goebel (34), is a very good addition catalyst. Certain metal oxides are catalysts for triglycerides, but not for monoesters; these may, in fact, behave as catalysts for transesterification of intrapolymers to polymeric glycerides. Waterman (33) claims that sulfur dioxide acts as a conjugation catalyst at moderate temperatures, and as an addition catalyst at higher temperatures. At very low temperatures, only tin or aluminum chlorides, and especially boron trifluoride are known polymerization catalysts (27).

Besides ordinary methods of heat bodying under an inert gas, in a vacuum, or with an inert gas blown into the oil, there are some special techniques. The use of sulfur dioxide to exclude oxygen and to catalyze the reaction simultaneously has been referred to (33). Goebel (34) bodied fatty acids under superheated steam, at 350 deg.C. and pressures of 5-30 atmospheres. He was able to get high yields of polymerized acids, high dimer/trimer ratios, and very little decarboxylation. An excellent survey of special bodying methods is that by Rheineck (35).

AUTOXIDATION:

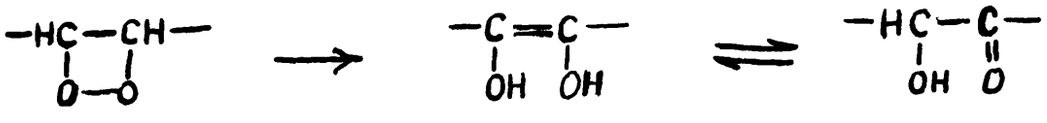
Two opposite classes of reactions occur when fatty molecules are oxidized by air. The first group results in scission of fatty chains, with production of oxygenated residues of low molecular weight and high volatility. The

other group of reactions gives rise to polymer formation via oxygen-containing linkages, whose nature is little known.

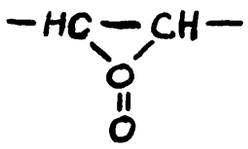
In the initial stages of autoxidation, oxygen is absorbed to form unstable peroxides. These are the reactive intermediates which initiate the decomposition and polymerization processes. There is usually a well-defined induction period, during which oxygen is absorbed but peroxides do not accumulate much. This is the period during which natural antioxidants in the fat reduce peroxides as rapidly as they are formed, inhibiting decomposition reactions. When the antioxidants are exhausted, peroxide concentration rises rapidly, and the peroxides then catalyze degradative or polymerization reaction chains.

The older concepts of peroxide structure will be mentioned only briefly, since they are mainly inferential and fail to give a satisfactory physical picture of chain initiation or propagation. The classical theory is that of -O-O- addition across a double bond (36):

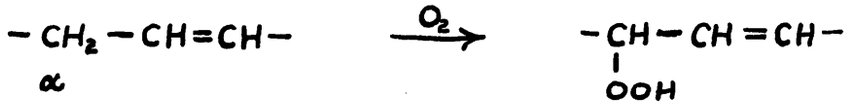
Fahrion (37) postulated that the <u>moloxide</u> so formed decomposed to a <u>ketol</u>:



Staudinger (38) thought that the Engler-Bach structure represented a second step, following the initial formation of

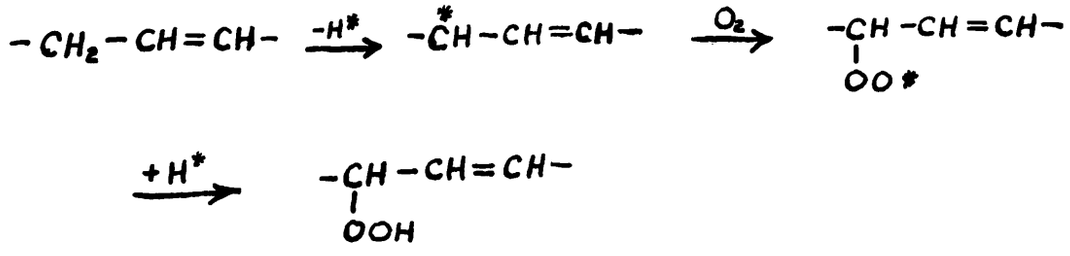


More recently, E. H. Farmer and his associates have proposed the hydroperoxide theory, and have actually separated fairly pure hydroperoxides (39-41) with the expected structures. It is well known that an ethylenic group confers high reactivity on an adjacent or α-methylene carbon atom. According to Farmer, a hydroperoxide is formed thus:



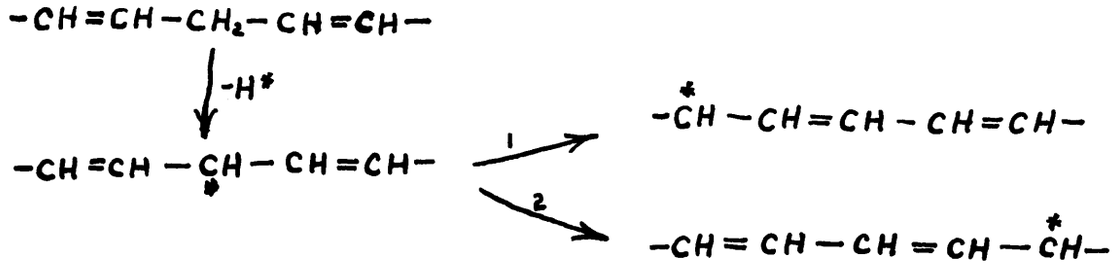
Farmer and Sutton (40) separated methyl hydroperoxido oleate by high-vacuum distillation of slightly oxidized methyl oleate. Swift et al (42) achieved a similar end by fractional crystallization from acetone. Both groups found the molecule to contain one double bond and one molecule of oxygen, as expected.

Sunlight, ultraviolet light, heat and catalysts promote hydroperoxide formation; iron salts, prolonged irradiation, and high temperatures promote decomposition. These facts suggest a free radical mechanism, which Farmer believes may follow this course:



Farmer pointed out further that a $-CH_2-$ group with

double bonds adjacent on each side is very sensitive to free radical formation, and hence to hydroperoxidation:



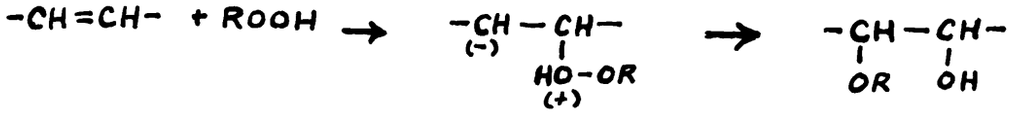
Thus we might expect increased conjugation in autoxidizing linoleates. This was verified by Lundberg and Chpault (43), who noted that diene absorpotion at 2325 Angstroms increased directly with peroxide value in autoxidizing methyl linoleate. These workers also found (44) that reduction of the peroxides by potassium iodide had no effect on diene conjugation.

Conjugated dienoic acids are attacked directly across both double bonds, without much rise in peroxide values, although total unsaturation decreases almost linearly with oxygen uptake (45). Gunstone and Hilditch (46) claim that oxygen attacks a double bond directly in normal linoleates as well as in the conjugated types; but conclusive evidence is lacking.

Saturated acids are rather inert, but have been shown to form some peroxides (47). These decomposed to ketones and alcohols, the ketones predominating. The situation with respect to autoxidation of trienoic acids is extremely complicated and vague; no simple conclusions are possible from the mass of experimental results obtained.

The reactions of hydroperoxides with other fatty

molecules and with antioxidants are very poorly understood. They appear to attack double bonds, and to give up the active O atom, with its high energy, to the double bond attacked. In polymerization, the simplest concept is that of direct attack by a hydroperoxide on a double bond (39):



If R above is also unsaturated, repeating additions are possible, with the formation of complex polymers. Bolland and Gee (48) presented another scheme based on -O-O- bridges. In oxidative drying of oils, new ester linkages also play an important part, as shown by Long and McCarter (49). They added free fatty acids to linseed oil, and observed falling acid values during drying.

Oxidative reactions are susceptible to acceleration or inhibition by the use of pro-oxidants or antioxidants, respectively. In drying oils, salts of cobalt, lead, iron and manganese are used as "driers", to spped the drying rate. These metals, and especially copper, are most undesirable contaminants in any processing or handling of edible fats, since they accelerate deterioration. Peroxides are, of course, pro-oxidants.

Antioxidants are present in most natural fats, and there are some very powerful synthetic types. It appears that an antioxidant functions by reducing peroxides as rapidly as they are formed, thus preventing the propagation of reaction chains. How they do this is not clear, though

Golumbic (50) has contributed much to our knowledge of the subject. When the active centres of the antioxidant have all been used up, peroxides can accumulate unhindered. This point is the end of the induction period, and the onset of organoleptic rancidity occurs about the time that peroxide values begin to increase rapidly. It follows, then, that antioxidants added after the end of the induction period are of no value in stabilizing a fat against rancidity; there are already sufficient peroxides to initiate more reaction chains than the antioxidant can possibly block.

Golumbic (51) has also essayed an explanation of synergism in antioxidants. He says that the synergist acts as a reservoir of hydrogen, which can regenerate the antioxidant reversibly at its own expense. The latter actually passes hydrogen on to the active peroxides, using up the active oxygen atoms. Only after the synergist is "spent" does the antioxidant begin to break down irreversibly.

A very detailed review, with an extensive bibliography, on the entire field of autoxidative reactions, is that of Swern, Scanlan and Knight (52).

TOXICITY OF HEATED OILS:

Most of the work on physiological effects of heated fats has been on fats heated in air, where oxidation plays a major role. Roffo (53) heated lard, beef and mutton tallows, and olive oil to 350 deg.C. for ½ hour; these heated fats were fed to rats with an equal amount of bread and milk.

Benign and malignant tumours developed in the stomachs and livers in 12 to 30 months. No one has been able to repeat these observations, although Morris (54) found that 50% of heated lard in the diet of rats would not maintain the body weight. Many of the animals also developed paralytic symptoms resembling the vitamin E deficiency described by Mackenzie, Mackenzie and McCollum (55).

In the case of linseed oil processed by the method of Privett (11), there is interference with normal growth when fed to rats at levels of as low as 8% in the diet (56). At higher levels, there is loss of weight, lack of thrift, diarrhoea, some anemia, and oily, staring fur. Many of the animals die in less than four weeks.

The possibility of linoleic acid deficiency has not been considered seriously, in spite of the rather similar symptoms; there is plenty of unpolymerized linoleic acid in the heated acetone-soluble oil, and the effects occur much more rapidly than those produced by Burr and Burr (57).

Gass (58) and Wills (59) have attempted to classify the toxic materials in heated linseed oil, preparing fractions which were fed to rats at the Nutrition Department of Macdonald College. Their more important findings are summarized briefly here, as they bear closely on subsequent studies reported herein:

1. Heated, acetone-soluble linseed oil is equally toxic whether or not it is hydrogenated. Thus poor removal of the nickel catalyst is not involved.

2. Soybean, corn, peanut and rapeseed oils can be made harmful to rats by heating for longer periods than linseed oil; the length of heating required seems to be connected with the unsaturation, i.e., with the relative rates of polymerization.
3. Crude or alkali-refined linseed oils yield heated fractions of identical toxicity. Ergosterol and lecithin, added to commercial shortening and heated at 275 deg.C. for 18 hours, did not render the samples any poorer than the pure shortening control.
4. Condensible volatile products of polymerization, made to volume with commercial shortening, had no deleterious effects.
5. Alcohol-soluble material removed from heated linseed oil by batch extraction was without any toxicity.
6. Acetone-soluble fractions from two successive liquid-liquid extractions of heated linseed and soybean oils were equally toxic in the respective oils.

Digestibilities of all the diets used did not vary significantly. No marked decrease in absorbability of heated oils was indicated, although the incidence of diarrhoea would suggest this.

It should be pointed out that nothing is known as to the degree to which polymerized triglycerides are susceptible to enzymatic lipolysis, either in vivo or in vitro. Similarly, nothing is known as to the extent, if at all, to which they

are absorbed or deposited in the body.

The lack of information on the intermediate metabolism of polymeric triglycerides, or even of dimeric fatty acids, is due in large measure to the difficulty of tracing such compounds in the tissues, organs and excreta. Progress is being made in the characterization of cyclic groupings in polymeric fatty acids via infrared spectroscopy. It may be that this technique will afford a means of estimating polymeric fatty acids or their metabolic residues in the body and excretory products.

METHODS

1. THERMAL POLYMERIZATION:

The apparatus is illustrated in Fig. 1. It comprises a 2-litre round-bottomed flask, with 24/40 Standard Taper side necks and a 45/50 Standard Taper centre neck. A gas bubbling tube, with a 24/40 inner glass joint, leads down close to the bottom of the flask, where several outlet holes ensure vigorous agitation of the charge by many small bubbles. The centre neck is closed by a 45/50 inner glass joint, drawn down to fit closely about a 360 deg.C. thermometer. The thermometer is suspended by a short sleeve of rubber tubing. The outlet neck is fitted with an inner glass joint, which is drawn down and curved over sharply to permit rapid drain-out of volatile products with a minimum of refluxing back into the flask.

A charge of oil is heated by a hemispherical Glas-Col electric mantle, operating from a 7.5 amp. Powerstat variable transformer. This permits sensitive manual control of the oil temperature. The apparatus will handle about one kg. of oil without excessive splashing.

In carrying out a polymerization, a very slow flow of CO_2 was bubbled through the oil while the mantle was operated at the maximum safe voltage (about 80 volts). The oil reached 275 deg.C. in about 1½ hours. When the temperature was about 180 deg.C., the flow of CO_2 was increased somewhat, and at 275 deg. the flow was made rapid enough to keep the oil surging vigorously. This powerful stream of CO_2.

FIG. 1. POLYMERIZATION APPARATUS

A--Gas Bubbler

B--Outlet for Volatiles

C--2-l. 3-neck Flask

D--Thermometer

E--Glascol Mantle

F--Powerstat Transformer

G--Carbon Dioxide Cylinder

was found to yield products of remarkably low acid values and excellent color and blandness.

At the end of the desired polymerization time, the heating mantle was switched off and removed, to facilitate rapid cooling of the oil in the flask. The full flow of gas was maintained until the charge was down to 120 deg.C., and a slow flow was continued almost down to room temperature. Batches of polymerized oil were stored in full flasks (without headspace) closed by corks wrapped in tinfoil. These were kept in a refrigerator at 5 deg.C.

2. ACETONE SEGREGATION:

1 kg. of polymerized oil was weighed into a large flask, and 7 litres of acetone (Merck, U.S.P.) was added. The flask was warmed on the steam bath, with intermittent shaking, until the acetone began to boil. The emulsion was cooled and allowed to separate at 5 deg.C. overnight. The soluble upper layer was decanted, and the acetone was distilled off; the last traces were removed under the full vacuum of a Hyvac pump, being condensed in a dry ice trap.

3. PROPANOL SEGREGATION:

The directions of Bernstein (31) were followed. One kg. of oil and 4 litres of n-propanol (Brickman & Co.) were shaken in a nitrogen-flushed bottle for 20 minutes. The mixture was allowed to separate overnight at 5 deg.C., and the soluble upper layer was decanted. The solvent was removed

from both layers as above.

4. ALCOHOL WASHING OF OILS:

500 g. of oil was placed in a 2-litre 2-necked flask; one neck was fitted with a reflux condenser, while the other neck carried a vapor delivery tube almost to the bottom of the flask. Commercial absolute alcohol was boiled in a bulb, the vapor being blown into the oil and condensed. When a volume of alcohol equal to that ofoil had been blown in, the mixture was allowed to cool until two sharp layers formed. The upper alcohol layer was siphoned off. The procedure was repeated twice more. The alcohol - soluble material was combined, and the alcohol removed from both fractions by distillation, finishing under high vacuum on the steam bath.

5. ALCOHOLYSIS OF OILS:

Commercial absolute ethanol, or C.P. methanol, was made anhydrous by the magnesium method of Lund and Bjerrum. The alcohols were prepared freshly before use to prevent absorption of atmospheric moisture (60).

For each 100 g. of oil (free fatty acids having been removed by alcohol washing) there was used 30 g. of dry ethanol, or 22 g. of dry methanol. 0.5 g. of C.P. KOH per 100 g. oil was dissolved in the alcohol. The oil was held at about 60 deg. C. on the steam bath, and the KOH - alcohol was stirred in rapidly.

After about ½ a minute, the mixture darkened considerably if all reagents were dry and the oil contained less

than 0.3% F.F.A. The mixture was held at about 60 deg.C. for 2 hours, and a dark red, glycerol-rich layer settled out. This was drawn off in a separatory funnel and discarded. The esters were washed several times with hot water, until entirely free of cloudiness due to soaps. Using very hot water minimized emulsification.

The esters were transferred to a bulb and dried on the steam bath at about 1 mm. Hg. absolute. Dry esters were kept in a refrigerator, in filled flasks.

6. DISTILLATION OF ESTERS:

Distillations were carried out in the apparatus shown in Fig. 2. The distilling and receiving bulbs were of sizes suitable to the quantities of esters distilled. The distilling bulb was filled with clean glass wool to minimize foaming and bumping. No suitable manometer was available to measure the absolute pressure accurately, so distillation temperature limits were established on esters of alkali-refined linseed oil, and these were observed closely in all distillations of esters from polymerized oils.

The distilling adapter was wrapped with glass wool to prevent excessive refluxing and to ensure the most rapid possible removal of distillable esters. These usually distilled entirely between 155 and 175 deg.C. When the vapor temperature reached a maximum and began to fall, the heating bath was taken steadily up to 240 deg.C. If no further rise in vapor temperature occurred, the bath was removed and the

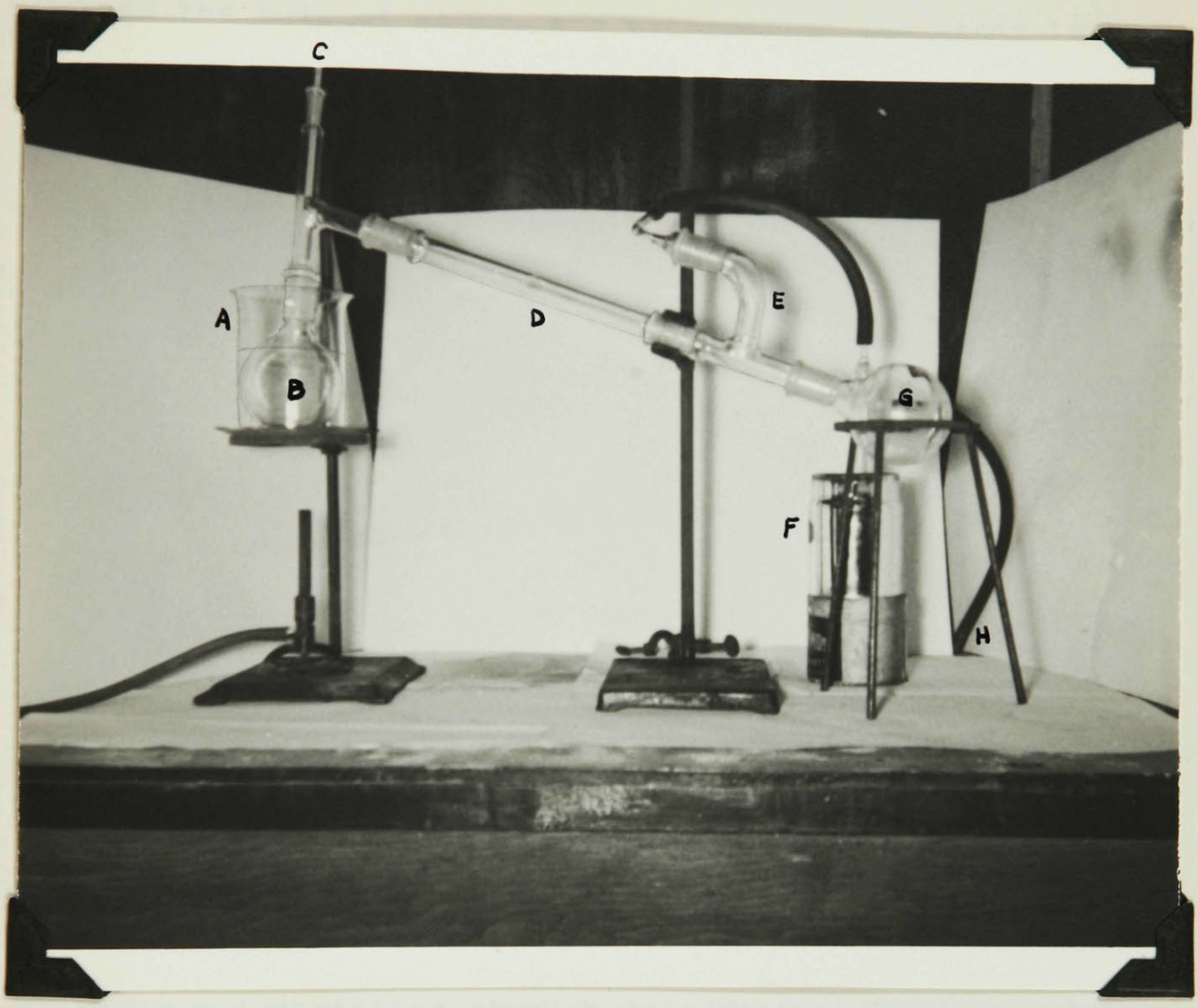


FIG. 2. VACUUM DISTILLATION APPARATUS

A--Glycerol Heating Bath

B--Distilling Bulb

C--Thermometer

D--Condenser

E--Vacuum Takeoff Adapter

F--Dry Ice Trap

G--Receiving Bulb

H--To Hyvac Pump

residue allowed to cool rapidly in the bulb, without breaking the vacuum.

Residues had to be recovered by washing out the glass wool in the bulb of ether, and evaporating the ether in a stream of CO_2. This ensured complete recovery of the residues, and accurate estimation of yields by collecting the ether washings in tared flasks.

7. MEAN MOLECULAR WEIGHTS:

Cyclohexane (B.D.H. Certified Chemical grade) was purified for cryoscopy as follows:

1 litre of cyclohexane and 120 c.c. of 20% fuming sulfuric acid were placed in a glass-stoppered flask, which was shaken mechanically for 12 hours. The acid layer was drawn off, and the cyclohexane was shaken for another 12 hours with 120 c.c. of fresh acid. This was again drawn off, and about 150 g. of granular barium hydroxide was added to the cyclohexane. After standing overnight, the cyclohexane was decanted and distilled through an efficient Stedman column. The product (yield about 650 g.) had the following constants:

M.P. = 6.4 deg.C. Ref. Index (25 deg.C.) = 1.4237.

Recently published values for very pure cyclohexane are (61):

M.P. = 6.547 deg.C. Ref. Index (25 deg.C.)= 1.42354.

Oil samples sufficient to give about 5% concentrations of solute were used. The solutions were cooled by a cold water bath, and were stirred with a stainless steel loop stirrer. A Beckmann thermometer enabled freezing points to

be estimated to within 0.002 deg.C. The cryoscopic constant was evaluated using resublimed naphthalene as the "known" solute. Observed molecular weights were corrected, after estimating free fatty acid values, by the method of Bernstein (31); this method assigns to the free fatty acids present a dimeric average molecular weight of 558. Duplicate samples checked to within about 1%.

8. IODINE VALUES:

In previous studies on heated oil fractions in this laboratory, the 2-hour Kaufmann (62) method was used, with the modification of using a 0.3 N reagent instead of 0.1N. This method was compared with the "Rapid Wijs" method of Hoffman and Green (63). It was found that the two methods gave closely agreeing values on several samples of unheated and heated oils, and also on monoesters. Cowan et al. (64) made an extensive study of several methods on dimeric esters, and concluded that no method gives true or highly reproducible iodine values. Since the results are of comparative value only, it was decided to adopt the Rapid Wijs method for its speed and convenience. The values so obtained were comparable with earlier results obtained by the Kaufman method.

9. ACID VALUES:

A sample of 5 - 10 g. of oil was weighed into a tared 250 ml. flask, and dissolved in 50 ml. of a mixture of 95% ethanol and toluene (50;50 by volume) which had been made

neutral to phenolphthalein just before use. 1 ml. of phenolphthalein indicator was added, the solution was shaken vigorously and titrated at room temperature with 0.06N KOH. Acid values were calculated as percent linoleic acid.

10. PEROXIDE VALUES OF OILS:

The method of Skellon and Wills (65) was found to give values as reproducible as those claimed for any of the iodometric methods using an external source of inert gas. Its simplicity made it ideal for estimations on numerous samples at short intervals.

11. PEROXIDE VALUES ON EXTRACTS FROM DIETS:

A modification of the Skellon and Wills (65) procedure was used, as follows:

A weighed sample of biscuit, containing about 1g. of oil (the percent oil contained being known) was shaken up with 25 ml. of cold chloroform, and filtered with suction. The residue was extracted twice more with 25 ml. portions of chloroform, and the filtrates were combined. The extract was evaporated to 10 ml. by passing purified nitrogen without heating, and transferred to a 250 ml. flask containing 2g. sodium bicarbonate. A stopper was fitted, carrying a short piece of glass tubing capped by a rubber Bunsen valve. This served as a one-way pressure relief valve. 15 ml. of glacial acetic acid was used to rinse the last traces of chloroform extract into the reaction flask, and the acid-bicarbonate

reaction released CO_2 which swept air out of the flask through the relief valve. When foaming subsided, 1 ml. of saturated fresh potassium iodide solution was added, and the tightly stoppered flask was left in the dark for 1 hour. 50 ml. of boiled, cooled water was then added, and the iodine titrated with 0.002 N thiosulfate.

Peroxide values were calculated as milligrams peroxide oxygen per kilogram oil, thus:

$$P.V. = \frac{V \times N \times 16 \times 10^5}{W \times P}$$

where V = volume of thiosulfate used, ml.

N = normality of thiosulfate

W = sample weight, grams

P = percent oil in sample

12. FATTY ACID COMPOSITIONS:

Spectrophotometric analyses were kindly performed by members of Dr. N. H. Grace's staff in the Division of Applied Biology, National Research Council, Ottawa. The methods and calculations were those recommended by the Spectroscopy Committee, American Oil Chemists' Society (66).

13. REFRACTIVE INDICES, VISCOSITIES:

These were measured at 25 deg. C. A Zeiss immersion refractometer with auxiliary prism and metal cell was used, to permit measurements on a few drops of oil. Gardner - Holdt bubble viscometer tubes were used for viscosity measurements. The letters of the Gardner-Holdt scale were converted

to poises via the conversion table supplied with the tubes. This conversion to poises is permissible only when measurements are made at 25 deg.C.

14. FEEDING EXPERIMENTS:

These were performed by members of the Department of Nutrition, Macdonald College. White rats were used in all experiments, and males were used wherever possible. The duration of each trial was 28 days. The animals were given feed and water ad libitum, and records were kept of feed consumption and liveweight gains. The organs of animals which died during experiments were examined post mortem.

The original basal diets were:

White flour	47.0 or 57.0% (depending on oil level)
Milk powder	19.0
Casein	11.5
Oil	20.0 or 10.0
Bone Meal	2.0
Salt	0.5

The dry ingredients were blended, the oil worked in, and the mixture was baked 20 minutes at 375 deg. F. The cakes were granulated and air-dried.

Thiamin (10 p.p.m.), niacin (230 p.p.m.) and riboflavin (5 p.p.m.) were added and dispersed in the feeds, which were stored in capped jars in a dark, cool place. Vitamins A and D were administered orally once a week to provide 25 I.U. of vitamin A and 5 I.U. of vitamin D per day.

Later, the B vitamins were added in the form of 3% of yeast in the basal diet, replacing an equal amount of flour. This was considered a more complete source of vitamin B complex than the supplements.

EXPERIMENTAL

SECTION A. STUDIES ON PROPANOL SEGREGATION.

Objectives:

It has been suspected that the acetone-soluble fraction of heated linseed oil contained appreciable quantities of polymeric fatty acids. On the strength of Bernstein's (31) claim that n-propanol is a highly selective solvent for monomeric triglycerides, it was decided to refractionate acetone-soluble heated oil with propanol, in the hope of segregating polymeric material more completely.

Procedure:

Crude linseed oil was heated for 12 hours at 275 deg. C. The acetone-soluble fraction was prepared, and this was further segregated with propanol. The low yield of propanol-insoluble oil (about 10% of the original oil) necessitated the processing of about 23 kg. of crude oil to provide sufficient material for the feeding experiments.

Analytical data only are reported here (Table 1). Results of the feeding experiments have been reported elsewhere (59).

In a later feeding trial, it was possible to include groups receiving propanol-soluble and propanol-insoluble fractions from a direct propanol segregation of 12-hour heated linseed oil. These were compared with acetone-soluble heated oil and unheated alkali-refined oil. The oils were fed at 20% level in the diets. Analytical data and adjusted average

TABLE 1. Analytical Data for Acetone and Propanol Segregates from Heated Raw Linseed Oil.

Fraction	Yield Wt. %	Ref. Ind. 25°C.	Iodine Value	F.F.A. %	Mol. Wt. (Observed)	Mol. Wt. (Corrected)
1. Raw Oil	-	1.4777	180.4	1.17	845	850
2. Heated Oil	-	1.4827	124.8	3.58	1280	1330
3. Acetone-Sol. from #2	48	1.4776	123.0	4.18	790	805
4. Propanol-Sol. from #3	78	1.4785	117.7	5.59	775	810
5. Propanol-Insol. from #3	22	1.4833	113.5	1.73	1290	1320

TABLE 2. Analyses and Comparative Nutritive Values of Acetone and Propanol Segregates from Heated Alkali-Refined Linseed Oil.

Fraction	Yield Wt. %	Ref. Ind. 25°C.	Viscosity 25°C.,cp.	Iodine Value	Adjusted 28-Day Liveweight Gain, G.
1. Unheated, Reserved from Acetone	-	1.4789	50	183	96.1
2. Heated, Acetone-Soluble	45	1.4811	180	134	1.5
3. Heated, Propanol-Soluble	28	1.4791	127	135	13.6
4. Heated, Propanol-Insoluble	72	1.4851	890	121	4.2

NOTE: Oils fed at 20% level in diets of rats.

liveweight gains are listed in Table 2.

Discussion:

The data of Table 1 indicate that propanol-insoluble oil resegregated from acetone-soluble heated oil contains considerable polymeric material (probably not higher than dimeric glycerides). The acid values reached on heating were considered excessive, and no subsequent work was done with crude linseed oil. It is especially noteworthy that the refractive index, iodine value and mean molecular weight of the propanol-soluble oil all point to further polymerization during the propanol segregation.

The correction to the observed molecular weights assumed that free fatty acids present were entirely C_{18} monomers, but that they associated in cyclohexane as virtual dimers. This assumption has been shown to be approximately true for pure fatty acids, but there may have been dimeric fatty acids in the free fatty acid content of some samples, and the correction must be treated with reserve, except when acid values are very small.

Examination of Table 2 shows a markedly greater selectivity of propanol as against acetone in segregating monomeric glycerides. Liveweight gains, however, were so slightly different for all three heated oil fractions that propanol appeared not to have segregated the deleterious constituents to a useful degree. It was decided that solvent fractionation methods offered little hope of achieving quant-

itative separation of non-harmful material, and that such methods had best be abandoned or deferred until later.

SECTION B. THE RELATION OF AUTOXIDATION TO DECREASES IN NUTRITIONAL VALUE.

Objectives:

It has been suggested that the deleterious effects of heat-treated linseed oil as compared with the unheated oil may be related to a greater susceptibility to autoxidation. In view of this possibility, it was thought desirable to study the effects of controlling the extent of autoxidation during baking and storage of diets. If it could be shown that varying the autoxidation influenced the deleterious nature of heated oils at all favorably, there should be other means available to protect oils during processing.

Procedure:

A preliminary test was performed to ascertain the degree of protection which could be conferred on heated and unheated linseed oils by the use of an antioxidant. The antioxidant composition was a mixture of nordihydroguaiaretic acid (N.D.G.A.) and citric acid, each at 0.05% level in the oils.

An oven test was carried out at 98 deg.C. on stabilized and unstabilized samples of alkali-refined linseed oil, also on corresponding samples of acetone-soluble heated linseed oil. The oil samples were heated in shallow dishes, and were stirred vigorously just before withdrawing aliquots by pipette for peroxide value estimations.

The results are given in Tables 3 and 4, and are plotted in Fig.3. It is evident that the antioxidant afforded good

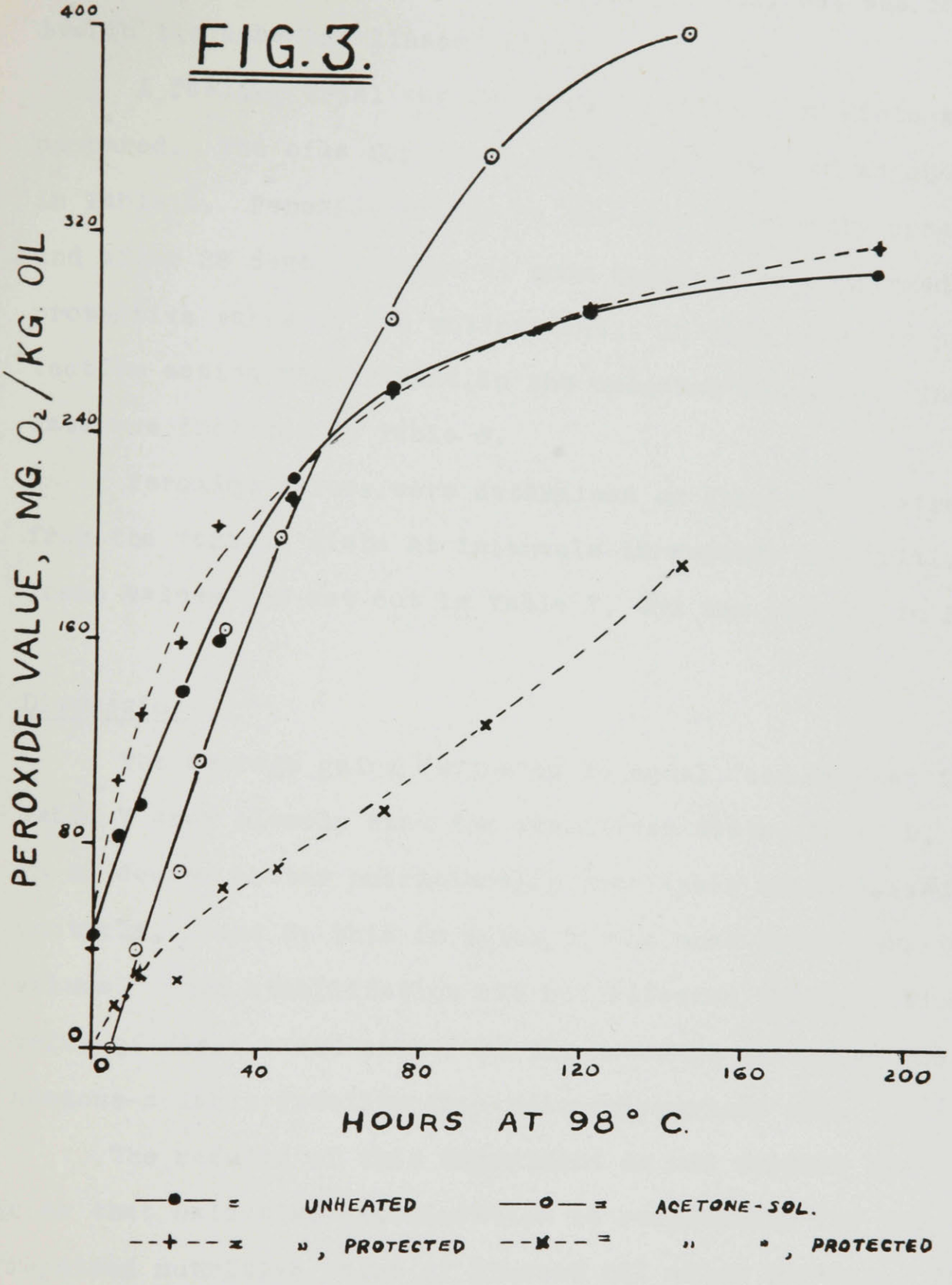
TABLE 3. Oven Test on Alkali-Refined Linseed Oil

Hours at 98°C.	Peroxide Values	
	Plain	Treated
0	44	40
1	53	51
2	57	60
6	83	106
12	96	132
22	140	158
30	160	204
48	224	215
72	261	258
120	292	293
192	309	318

TABLE 4. Oven Test on Acetone-Soluble Fraction from Heat-Polymerized Alkali-Refined Linseed Oil.

Hours at 98°C.	Peroxide Values	
	Plain	Treated
0	0	0
2	0	0
4	0	0
5	0	13
6	9	18
9	28	23
11	38	29
22	69	26
26	113	31
32	165	61
46	201	70
71	287	93
96	350	127
144	402	191

FIG. 3.


PEROXIDE VALUE, MG. O_2 / KG. OIL
400
320
240
160
80
0
0
40
80
120
160
200
HOURS AT 98° C.
—●— = UNHEATED
—○— = ACETONE-SOL.
--+-- = " , PROTECTED
--×-- = " " , PROTECTED

protection to the acetone-soluble heated oil, but was ineffective in the unheated linseed oil.

A feeding trial was designed in which five diets were compared. The oils for these diets were prepared as shown in Table 5. Peroxide values on the oils as freshly prepared and after 28 days' storage at room temperature confirmed the protective value of the antioxidant. In this case the protective action was evident in the unheated oil also. These data are included in Table 6.

Peroxide values were determined on chloroform extracts from the various diets at intervals throughout the trial. These values are set out in Table 7, and are plotted in Fig.4.

Discussion:

The average gains (adjusted to equal feed intake) in Table 7 show clearly that the stabilized diets, 2 and 5, were in no degree better nutritionally than their unstabilized controls, 1 and 3; this in spite of the much lower peroxide values. Thus stabilization has not affected the nutritional value of diets based either on the unheated oil or on the acetone-soluble fraction from heat-polymerized oil.

The results of this experiment do not support the view that oxidative deterioration is responsible for the impaired nutritive value of linseed oil after heating. It is true that peroxide values are not a full indication of rates of total oxygen absorption, and that other dietary components may have been attacked by peroxides as rapidly as these were

TABLE 5. Preparation of Oils for Experiment on Autoxidation.

Alkali-refined linseed oil.	Dissolve in 7 vols. acetone; Remove acetone.	- Diet 1 (20% oil)
	Dissolve in 7 vols. acetone; Add antioxidant; Remove acetone; Add antioxidant.	- Diet 2 (20% oil)
Heated alkali-refined linseed oil.	Segregate with 7 vols. acetone; Remove acetone from soluble fraction.	- Diet 3 (20% oil)
		- Diet 4 (10% oil)
	Segregate with 7 vols. acetone; Add antioxidant to sol. fraction; Remove acetone; Add antioxidant.	- Diet 5 (20% oil)

NOTES: (a) Each addition of antioxidant consisted of 0.025% NDGA and 0.025% citric acid dissolved directly in the hot oil or solution.

(b) Oils stored under same conditions as the diets used in the trials.

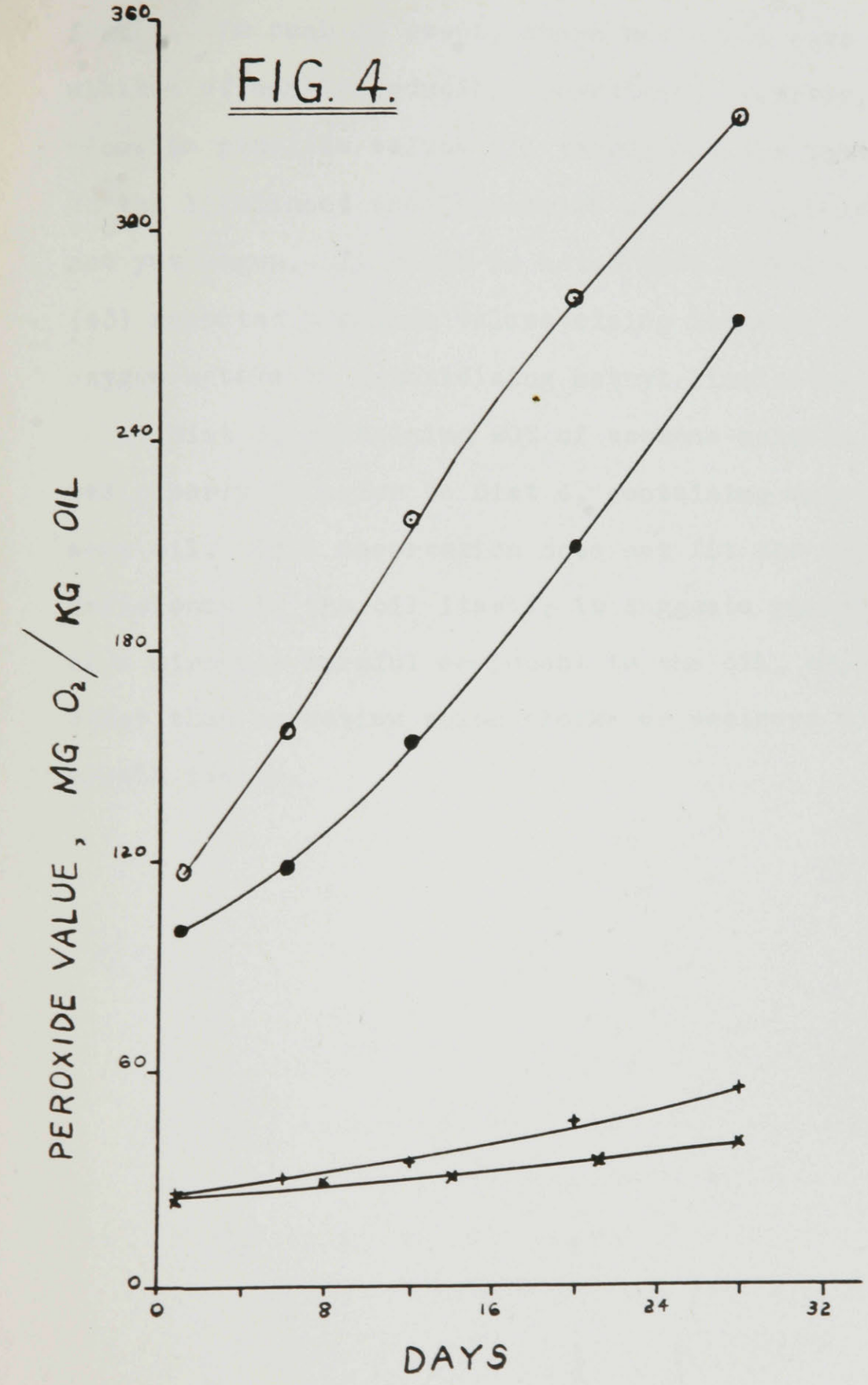
TABLE 6. Analytical Data for Oils Used in Experiment on Autoxidation.

Oil Fraction	Viscosity 25°C. cp.	Ref. Index 25°C.	Iodine Value	Peroxide Value as mg. Peroxide Oxygen per Kg.	
				Fresh	After 28 days
1. Unheated, no Antioxidant	50	1.4789	183	47	1334
2. Unheated, Antioxidant	50	1.4777	183	49	172
3 & 4. Heated Acetone-Sol., no Antioxidant	180	1.4811	134	0	270
5. Heated Acetone-Sol., Anti-oxidant	173	1.4805	134	0	51

TABLE 7. Peroxide Values of Oils Extracted from Feeds and Average Adjusted Liveweight Gains.

Days since baking = days of feeding trial	DIET 1 Unheated 20% Oil	2 Unheated Stabilized 20% Oil	3 Heated Acetone Sol. 20% Oil	4 Heated Acetone Sol. 10% Oil	5 Heated Acetone Sol. Stabilized 20% Oil
1	99	26	116	62	25
6	117	30	157	71	-
8	-	-	-	-	30
12	153	33	218	-	-
14	-	-	-	101	30
20	210	45	281	-	-
21	-	-	-	107	34
28	276	55	333	119	38
Average adjusted liveweight gains for 28 day period	96.1	100.6	1.5	60.9	2.0

FIG. 4.


360
300
240
180
120
60
0
PEROXIDE VALUE, MG. O_2 / KG. OIL
0
8
16
24
32
DAYS
= (UNHEATED) DIET 1
= DIET 2
= (ACETONE-SOL.) DIET 3
= DIET 5

formed. In such an event, there would not have been an accumulation of easily reducible peroxides. However, the steady rises in peroxide values all throughout the test period point to the likelihood that extensive peroxide decomposition had not yet begun. It might be noted that Lundberg and Chipault (43) reported peroxide values rising linearly with total oxygen uptake in autoxidizing methyl linoleate.

Diet 3, containing 20% of acetone-soluble heated oil, was clearly inferior to Diet 4, containing only 10% of the same oil. This observation does not fit the view of a simple deficiency in the oil itself; it suggests rather the presence of a directly harmful component in the oil, or of some agent other than oxidation which blocks or destroys a necessary growth factor.

SECTION C. PRELIMINARY STUDIES ON METHYL ESTERS OF ACETONE-SOLUBLE SEGREGATE FROM POLYMERIZED LINSEED OIL.

Objectives:

High-vacuum distillation is a recognized technique for separation of monomeric from polymeric esters in mixtures of partially polymerized monoesters (19,25,26,67). In such separations it is important to ascertain whether the charge undergoes polymerization or decomposition in the flask during distillation. Norris and associates (68) distilled methyl linolenate in a column at 1 mm. pressure, refluxing the ester for about 3½ hours at a temperature at least 20 deg.C. higher than was used in this laboratory. They checked on decomposition spectroscopically, and found an insignificant amount. Hence it seemed safe to attempt simple distillation of mixed esters at about 0.2-0.3 mm. pressure.

Procedure:

The following samples of esters were prepared:

1. Ethyl esters of acetone-soluble oil from heated, alkali-refined linseed oil, by direct alcoholysis.
2. Methyl esters of acetone-soluble oil from heated, alkali-refined linseed oil, by direct alcoholysis.
3. Methyl esters of acetone-soluble oil from heated, crude linseed oil, by saponification, hydrolysis, and esterification of fatty acids with methanol saturated with dry HCl gas.
4. Methyl esters of acetone-soluble oil from heated, alkali-

refined linseed oil, by the procedure used for Sample 3.

This scheme permitted comparison of esters prepared by the two different methods, and also of esters derived from curde and alkali-refined linseed oils. The analytical data on these sample and their fractions are given in Table 8.

Discussion:

The first point to be noted in connection with the distillations is that the analytical constants of the "recombined samples" agree very closely with those of the original samples. Since there was very little net loss of material, this recombination should have yielded essentially the original ester mixture in each instance. The refractive indices and iodine values show that this is substantially what did happen. It is evident, therefore, that no serious degree of thermal alteration occurred during the distillations.

The distillations showed that methyl esters prepared by direct methanolysis are practically identical with those prepared from the fatty acids. Methanolysis is certainly to be preferred, since the technique is simpler, more rapid and economical, and does not expose the oils to strongly alkaline conditions at high temperatures.

The methyl esters derived from crude linseed oil were not greatly different than those derived from alkali-refined oil. Approximately 17-18% of polymeric material was present in each sample of methyl esters, and hence in each batch of acetone-soluble heated oil. The mean molecular weights of

TABLE 8. Properties of Methyl Esters and Fractions Obtained in Distillations.

Sample	Fraction	Percent yield	Molecular Weight	Ref. Ind. at 25°C.	Iodine Value
1	Charge	-	370	1.4617	130.5
	Distillate	-			
	Residue	-	(Sample was lost in unsuccessful attempt at distillation)		
	Recombined Sample*	-			
2	Charge	-	334	1.4648	128.0
	Distillate	82.0	296	1.4600	137.2
	Residue	17.5	587	1.4896	105.5
	Recombined Sample*	-	-	1.4647	127.6
3	Charge	-	333	1.4639	128.4
	Distillate	80.7	292	1.4574	136.4
	Residue	18.6	578	1.4887	105.0
	Recombined Sample*	-	-	1.4643	129.0
4	Charge	-	336	1.4647	129.0
	Distillate	81.9	295	1.4600	135.6
	Residue	17.6	598	1.4904	102.8
	Recombined Sample*	-	-	1.4647	128.4

* Distillate and residue blended in the exact ratio of their yields, to approximate original sample.

the distillates and residues suggested strongly that only monomers and dimers, respectively, were present. This is in agreement with the observations of Marcusson (24) and Petit (25). The percentages of polymers, assuming they are dimers only, can be calculated from the mean molecular weights of the original samples. The calculated percentages agree quite closely with the actual yields of residues, lending support to the view that monomers and dimers only were present.

With the above information suggesting the absence of decomposition during distillation, it was considered justified to attempt larger scale distillations with a view to using distilled ester fractions for feeding experiments.

SECTION D. EXPERIMENT ON NUTRITIVE VALUES OF ETHYL ESTER FRACTIONS FROM HEATED AND UNHEATED LINSEED OIL.

Objectives:

The studies reported in Section C verified that the acetone-soluble fraction of heated linseed oil contained a substantial percentage of polymeric (mainly dimeric) fatty acids. It will be shown in Section F that some of this material was in the form of "intrapolymers", which presumably can not be separated from normal monomeric glycerides by common solvents. It was felt that, if dimeric fatty acids were the chief source of toxicity in heated oils, vacuum distillation of the monoesters would afford the best way of removing the offending polymeric material quantitatively from a mixture containing it. Thus the distillable monomeric esters should support better growth than the mixed esters, while the residual dimers should be extremely toxic.

Procedure:

The preparations used in this feeding experiment were as follows (all esters made by ethanolysis):

1. Whole alkali-refined linseed oil. This fraction served as a control for the whole ethyl esters.
2. Ethyl esters of whole linseed oil.
3. Ethyl esters of acetone-soluble oil from heated linseed oil. (12 hours at 275 deg.C.) The acetone-soluble oil was alcohol-washed prior to ethanolysis.
4. Ethyl esters of whole linseed oil containing 10% of esterified alcohol washings from No. 3. The alchohol

washings were esterified with ethanol, using 3% of H_2SO_4 as catalyst.

5. Distillable ethyl esters of alcohol-washed acetone-soluble oil.
6. Undistillable ethyl esters from No. 5.

These six fractions were fed as 20% of the diet to six groups of rats for 28 days. Results of the feeding test are summarized in Table 9.

Discussion:

The rats receiving ethyl esters of whole linseed oil grew significantly less than those receiving linseed oil itself. However, their growths and appearance were satisfactory in every way. The ethyl esters of acetone-soluble heated oil (Diet 3) were distinctly poorer nutritionally than the whole linseed esters.

The main objective of the experiment was, of course, a comparison of the mixed, distillable and undistillable esters of acetone-soluble heated oil. The undistillable esters, as expected, were clearly the worst fraction, causing the deaths of all animals in an average of 9 days. However, the distillable fraction was not better than the mixed esters, except for the excellent appearance of the animals. It has been suggested that the distillable esters merely lacked an essential nutrient factor, or interfered with such a factor; while the undistillable esters contained an actively toxic principle in high concentration. This question is receiving

TABLE 9. Results of Feeding Trial with Ethyl Ester Fractions from Heated Linseed Oil.

Group	Average Daily Gain, Grams	Average Daily Feed, Grams	Gain per 100 Grams Feed, Grams	Remarks
1	3.4	9.7	35.2	Excellent appearance and thrift
2	2.3	7.5	30.2	" " " "
3	0.83	5.8	14.3	Small, good appearance. Some diarrhoea, fur slightly shabby
4	-	2.0	All animals dead at 11 days - not starvation	
5	0.67	4.5	14.9	Small, but active and thrifty. Fur sleek, and no diarrhoea
6	-	3.5	None of animals survived test	Oily, matted fur; feces black and sticky. Eyes kept closed, huddled attitudes.

NOTES: (a) Above figures averaged over 28 days.

(b) Differences necessary for significance: Gain = 0.4 g./day
Feed = 1.0 g./day.

further attention in this laboratory.

A surprising result was that obtained with Diet 4. It seemed that alcohol washing had extracted some deleterious material from the acetone-soluble oil. However, it was also possible that the H_2SO_4 catalyst used in esterifying the extract had somehow produced harmful material. These questions have been considered in another experiment, described in the next section. The possibility of starvation due to the very low feed intake (2 grams per day) was ruled out through an auxiliary experiment. In this test, 21-day old rats were fed 2 grams per day of a diet containing 20% of unheated linseed oil. All survived the 28-day test period satisfactorily.

SECTION E. FURTHER INVESTIGATIONS ON ALCOHOL-EXTRACTABLE MATERIALS FROM HEATED OILS.

Objectives:

As already pointed out, the following experiment was designed to elucidate the role of alcohol washings from heated oil in the serious toxicity of Diet 4 in the previous experiment. Since whole linseed esters were tolerated well, it appeared that the 2% of esterified alcoholic extract in Diet 4 must contain or give rise to exceptionally toxic material.

Procedure:

Groups were included to permit studies of the influence of alcohol washing on whole heated linseed oil, as well as on the acetone-soluble fraction. Other groups included the alcoholic extracts as the sole lipid material in otherwise fat-free diets; while one group received whole linseed ethyl esters plus 10% of alcohol washings from acetone-soluble heated oil. In each diet, the amount of alcoholic extract included was that obtained from as much oil as itself would constitute 10% of the diet. The alcoholic extracts all averaged close to 10% by weight of the respective washed oils. The preparations tested, and results of the test, are listed in Table 10.

Discussion:

The most salient observations emerging from this feeding trial are that neither whole linseed oil, whole

TABLE 10. Results of Feeding Trial with Alcohol-Washed Oils and Alcoholic Extracts Thereof.

Percent in Diet	Oil Treatment	Average Daily Gain, Grams	Average Daily Feed, Grams	Gain per 1000 cal., Grams
10	1. Linseed oil (no treatment)	4.2	10.6	102
9 + 1	2. Linseed oil + alcoholic extract from #4	3.8	10.3	95
10	3. Whole heated oil - as prepared	2.2	8.2	69
10	4. Whole heated oil - alcohol washed	2.3	7.9	75
10	5. Acetone-soluble heated oil	2.0	7.5	68
10	6. Acetone-soluble heated oil - alcohol washed	1.9	6.9	64
9 + 1	7. Ethyl esters of linseed oil + alcoholic extract from #6	0.8	7.2	28
0	8. Fat free diet	3.8	11.8	95
1	9. Fat free diet + alc. extract of heated oil	4.1	12.3	95
1	10. Fat free diet + esterified alc. extract of heated oil	4.0	12.9	89

heated oil, nor the acetone-soluble fraction is changed in nutritive value by alcohol washing. The alcoholic extracts were innocuous either alone or added to whole linseed oil; but when added to linseed ethyl esters, the extract from acetone-soluble oil proved harmful. Recalling that Diet 4 in the previous trial contained esterified alcohol washings, it appears that the toxicity was not produced during the esterification.

One is forced, therefore, to assume that the ill effects of the alcoholic extract occurred only through interaction with ethyl esters; an interaction which did not take place with triglycerides. This interaction may have been physiological, or it may have occurred during baking of the diets. Although the possible relationship of this type of toxicity to that found in alcohol-washed heated oils is unknown, the latter case remains of primary importance.

SECTION F. SOME OBSERVATIONS ON THE KINETICS AND SELECTIVITY OF THERMAL POLYMERIZATION.

Objectives:

Privett and associates (69) have laid particular emphasis on the selective nature of thermal polymerization at moderate temperatures, with regard to the fatty acids involved. For linseed oil, at temperatures up to 275 deg.C., it is claimed that linolenic acid reacts almost exclusively until it reaches a very low concentration. Only then does linoleic acid begin to react rapidly; while oleic acid is used up very slowly, at late stages of polymerization.

While these workers showed good evidence for the preferential reaction of the most highly unsaturated fatty acids, it would be instructive to study the actual rates of disappearance of the individual fatty acids in an oil undergoing thermal "bodying". This is the major aim of the experiments reported here.

Another question meriting investigation is that of the existence of intrapolymers, produced by fusion of two fatty acid chains in the same triglyceride molecule. This subject has received little more than speculative attention in the literature to date. Positive evidence for intrapolymers might explain, in terms of the presumed toxicity of dimeric fatty acids, why solvent segregation methods have consistently failed to yield non-injurious fractions from heated linseed oil.

In Bernstein's (31) studies on solvent fractionation

of polymerized oils, he found that a series of fractions from one batch of heated oil exhibited a linear relation between (log viscosity) and (molecular weight)$^{\frac{1}{2}}$. It was proposed to test this relationship on a series of linseed stand oils bodied for various periods.

Procedure:

Samples from one lot of alkali-refined linseed oil were heat-polymerized at 275 deg.C. for 5, 9, 13 and 17 hours. Portions of the stand oils were segregated with acetone, and the soluble segregates were saved for study. Iodine values, refractive indices, acid values and mean molecular weights (in cyclohexane) were determined on the whole and acetone-soluble oils. Viscosities of the whole oils were measured. These data are compiled in Table 11. Molecular weights were corrected for free fatty acids.

All the stand oils, their acetone-soluble segregates, and a sample of the unheated oil, were converted to methyl esters and vacuum-distilled; refractive indices and molecular weights of the distillates and residues are listed in Table 14. Iodine values were determined on the monomers, and these were analyzed spectroscopically for fatty acid compositions (66).

Knowing the yields of dimeric esters, the fatty acid compositions of the total esters, and hence of the stand oils, were calculated. Table 12 contains these results in terms of linolenic, linoleic, oleic, saturated and dimeric acids. The data are plotted against iodine values of the

TABLE 11. Properties of Linseed Stand Oils and their Acetone-Soluble Segregates after Various Times of Polymerization.

	Whole Linseed Stand Oil					Acetone-Soluble Oil			
Hours Heated	0	5	9	13	17	5	9	13	17
Iodine Value	182	140	130	121	112	147	138	130	122
Refractive Index-25°C.	1.4788	1.4840	1.4852	1.4869	1.4886	1.4810	1.4818	1.4812	1.4799
Acid Value (% F. F. A.)	0.17	0.83	0.88	0.65	0.91	0.64	0.56	0.65	0.61
Molecular Weight	873	1140	1300	1490	1840	1010	1030	1060	970
Viscosity 25°C.-poise	.45	272	470	1030	2700				

TABLE 12. Fatty Acid Analyses of Monomeric Methyl Esters and Total Methyl Esters after Various Times of Polymerization.

	Monomeric Methyl Esters					Total Methyl Esters				
Hours Heated	0	5	9	13	17	0	5	9	13	17
Iodine Value	182	143	138	128	117	182	140	130	121	112
% Linolenic	47.7	28.2	23.4	20.4	11.4	47.7	21.4	16.2	11.7	5.7
% Linoleic	12.7	12.8	12.4	5.2	4.8	12.7	9.7	8.6	3.0	2.4
% Oleic	31.1	-	-	-	-	31.1	36.2	35.9	34.2	33.0
% Saturated	8.5	-	-	-	-	8.5	8.7	8.7	8.6	8.6
% Dimeric	-	-	-	-	-	0	24.0	30.6	42.5	50.3

FIG. 5.

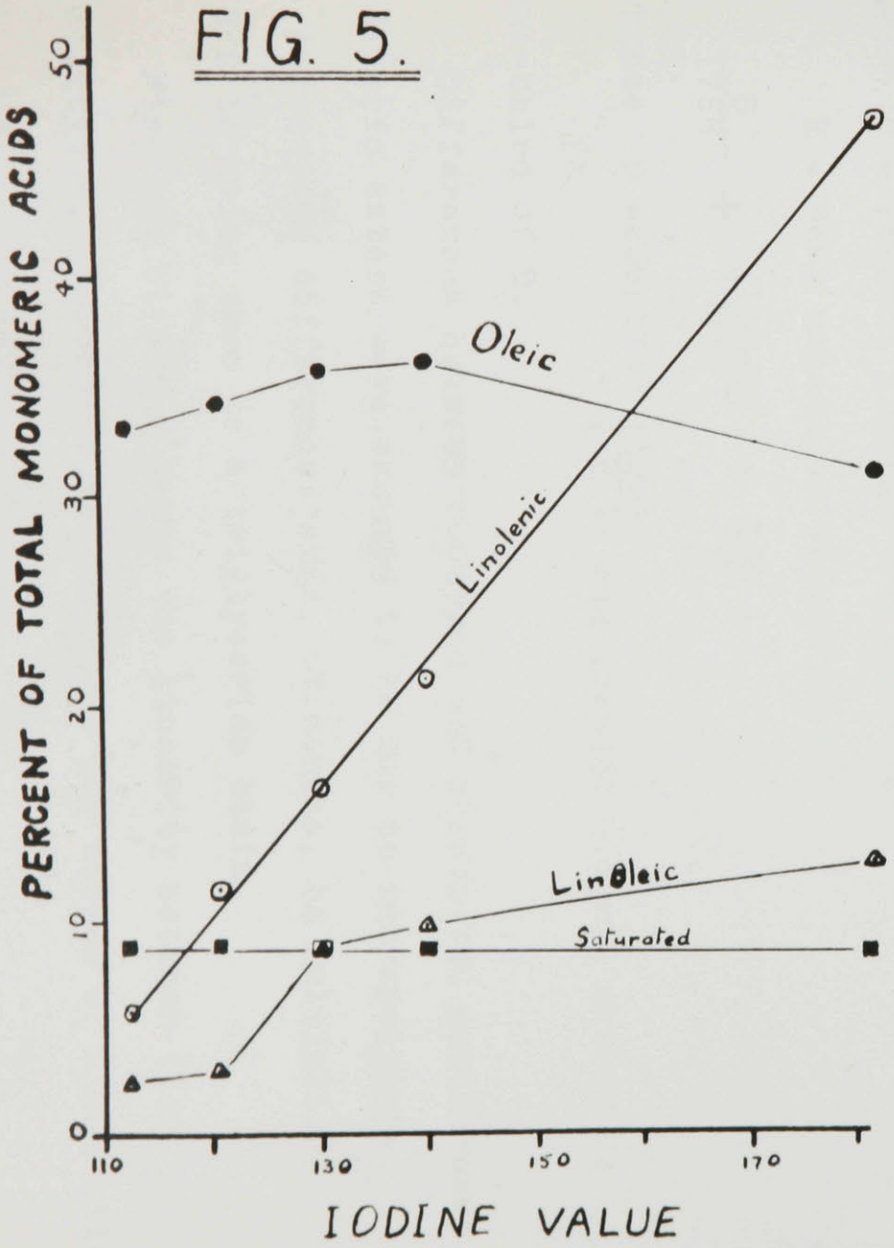
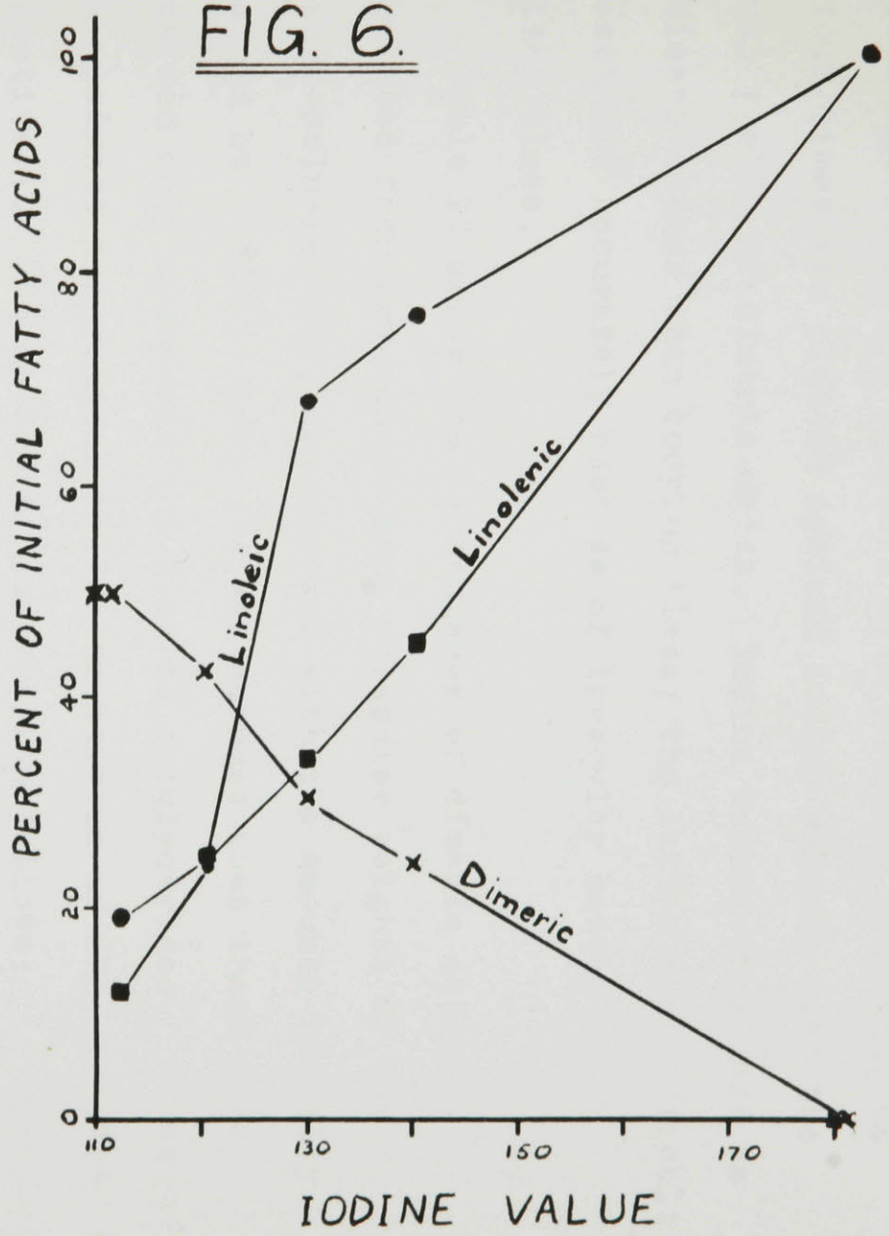

PERCENT OF TOTAL MONOMERIC ACIDS
0
10
20
30
40
50
Oleic
Linolenic
Linoleic
Saturated
110
130
150
170
IODINE VALUE

FIG. 6.


PERCENT OF INITIAL FATTY ACIDS
0
20
40
60
80
100
Linoleic
Linolenic
Dimeric
110
130
150
170
IODINE VALUE

stand oils in Figure 5. In Figure 6, percentages of the initial linolenic and linoleic acid contents remaining at various times are plotted against iodine values; also the actual yields of dimeric acids. Iodine values were used as abscissae rather than bodying times; the latter are difficult to estimate accurately because of irregular heatup and cooling times.

Table 13 shows the percentages of dimeric esters calculated from cryoscopic mean molecular weights of the acetone-soluble oils, as compared with the amounts actually obtained by distillation. It was assumed that these oils contained only monomeric and dimeric triglycerides. These were assigned theoretical molecular weights of 878 and 1756 respectively. The method of calculation follows:

Let D = Percent dimeric triglycerides

M = Mean molecular weight of oil

$$\frac{D}{1756} + \frac{(100-D)}{878} = \frac{100}{M};$$

whence $D = 200 - 1756 \left(\frac{100}{M}\right)$; and percent dimeric esters is one-third of D.

Differences between observed and calculated percentages of dimeric esters were assumed to be due to intrapolymers. The percentage differences must, of course, be multiplied by 3/2 to bring them to a triglyceride basis.

Finally, Figure 8 tests the linearity between (log viscosity) and(molecular weight)$^{\frac{1}{2}}$ for the series of stand oils.

TABLE 13. Calculation of Intrapolymer Content in Acetone-Soluble Segregates of Linseed Stand Oils.

Hours Heated	5	9	13	17
Molecular Wt.	1010	1030	1060	970
Calc. % Dimeric Triglycerides	26.1	29.6	34.4	19.0
Calc. % Dimeric Methyl Esters	8.7	9.9	11.5	6.3
Observed % Dimeric Methyl Esters	21.7	23.1	30.7	14.8
Difference, as "Intrapolymers"	13.0	13.2	19.2	8.5

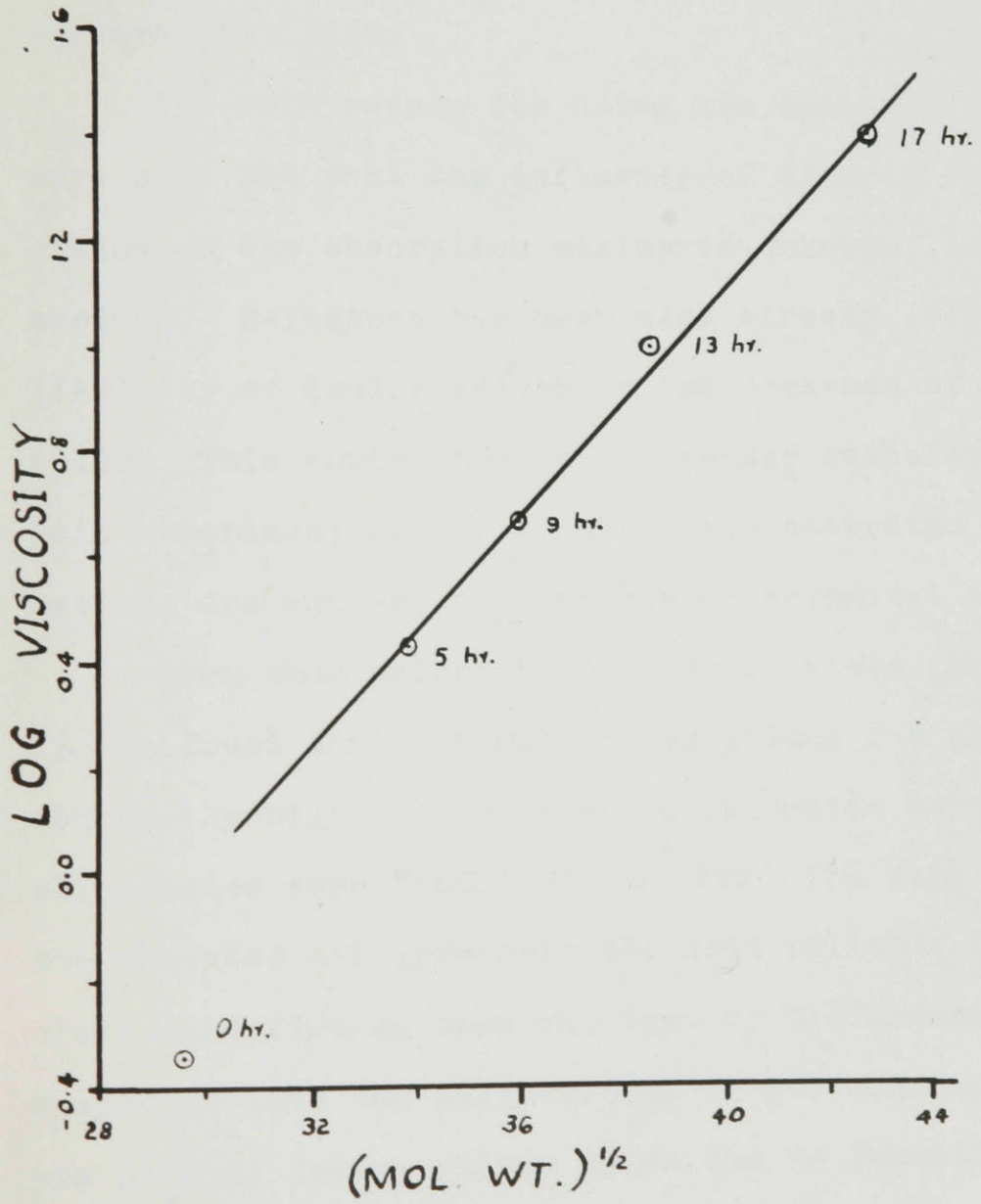
TABLE 14. Molecular Weights and Refractive Indices of Monomeric and Dimeric Esters from Linseed Stand Oils.

Hours Heated	Refractive Indices 25°C.		Molecular Weights	
	Monomers	Dimers	Monomers	Dimers
0	1.4620	-	294	-
5	1.4601	1.4860	291	599
9	1.4596	1.4831	288	588
13	1.4588	1.4813	290	595
17	1.4582	1.4797	293	584

IODINE VALUE ●
100
120
140
160
180
200
REFRACTIVE INDEX ⊙
1.4780
1.4800
1.4820
1.4840
1.4860
1.4880
PERCENT DIMERIC ESTERS ×
0
10
20
30
40
50
800
1000
1200
1400
1600
1800
2000
MOLECULAR WEIGHT

FIG. 7.

FIG. 8.


LOG VISCOSITY
1.6
1.2
0.8
0.4
0.0
-0.4
28
32
36
40
44
(MOL WT.)^1/2
0 hr.
5 hr.
9 hr.
13 hr.
17 hr.

Discussion:

Referring first to Table 14, it is clear that these distillations, like those reported in Section C, yielded substantially pure monomeric and dimeric ester fractions. Therefore the expedient of calculating fatty acid compositions of the total esters from those of the distillates appears justified.

The main reason for doing the analyses on the monomers only was that the influence of dimeric fatty acid chains on the absorption maxima is unknown, and may be serious. Reference has been made already (64) to the unreliability of iodine values in the presence of dimeric molecules. This would immediately render calculations of oleic acid worthless; as it is, oleic and saturated acids estimations are subject to numerous experimental errors.

Even when using the monomeric esters for analysis, it was found that the calculated values for oleic acid were abnormally high. Therefore the saturated acid values for all samples were "smoothed" to about the same value as for the unheated oil (probably the most reliable figure), and oleic acid figures were obtained by difference. It is suspected that the difficulties in calculating oleic acid via partial iodine values arose due to formation of cyclic monomers from linolenic acid. Such products, with two double bonds, would account for the high iodine values, which in turn gave rise to the abnormal oleic acid calculations.

Figures 5 and 6 show clearly that linolenic acid reacts much more rapidly than linoleic acid at 275 deg. C.,

and also that oleic acid plays a very minor part in polymerization during the period studied, at least. The linearity between gross decrease in unsaturation (i.e., in iodine value) and linolenic acid disappearance, helps to emphasize the dominant role of linolenic acid, or selectivity of polymerization.

It is notable that at about 130 iodine value, linoleic acid suddenly begins to react much more rapidly than before, while linolenic acid continues about as before. The combined effect is an increase in the rate of accumulation of dimers (Fig.6). This corresponds almost exactly to the beginning of "Stage 2" in Privett's stepwise reaction mechanism (69). It is unfortunate that the present experiments did not cover longer bodying times, so that the subsequent steps might have been observed also.

In Table 13, evidence is presented for the existence of intrapolymers. This evidence is predicated on an assumption that no glycerides higher than dimeric in size are extractable by acetone. The assumption is probably fairly sound; in any case, it would require a substantial concentration of trimeric glycerides to account for the total dimeric acids removed.

If dimeric fatty acids are indeed responsible for the nutritive defects of heated linseed oil, it is understandable how intrapolymers could defeat all efforts to segregate out non-injurious fractions by means of solvents. Presumably, other solvents besides acetone are unable to differentiate

between intrapolymers and monomeric glycerides.

Figure 7 shows the variations of iodine value, refractive index, and percent dimeric esters with mean molecular weight of the stand oils. One observation of special interest is that the refractive index and percent dimeric ester curves are nearly identical. By proper choice and placement of the scales, they could be superimposed. Thus a plot of dimeric esters vs. refractive index should be a straight line, as has been verified with the present data.

Hence, if it were desired to polymerize a batch of linseed oil to show specified dimeric acid content, a sample could be bodied for some convenient time. The refractive index and dimeric ester content of this bodies sample would supply one point on the dimeric ester vs. refractive index plot; the refractive index of the unheated oil (which has zero dimeric esters) would supply the other. On drawing a straight line through the points, the refractive index corresponding to the desired dimer level could be picked off. Then it would be simple to polymerize the main batch with regular withdrawals of samples for refractive index measurements, until the desired refractive index was reached. Such a procedure would eliminate the tedious determination of dimer content on a whole series of samples. It would, however, be feasible only for a single lot of oil; each new lot would require a new plot to be made.

Figure 8 demonstrates the straight-line relation between (log viscosity) and(molecular weight)$^{\frac{1}{2}}$ for the series

of linseed stand oils bodied from one lot. The unbodied oil, however, lies far off this line. A possible explanation is that the triglycerides in the original oil had a particular fatty acid distribution, which was changed by interesterification to a completely random distribution during bodying.

GENERAL DISCUSSION

It has been shown that solvent segregation techniques offer little hope of concentrating the harmful constituents in heated linseed oil. An explanation has been suggested in terms of intrapolymers, assuming that most solvents cannot discriminate between these and normal triglycerides. This explanation, of course, presupposes the toxicity of dimeric fatty acids and their esters.

In Section D, it was shown that dimeric ethyl esters, or some other material of similar molecular size and low volatility, were extremely toxic to rats. The value of this experiment was somewhat reduced by the failure of the rats to grow on the monomeric esters. Since this feeding trial was performed, another experiment has shown that baking seriously reduces the nutritive value of diets containing ethyl esters (70). Diets which were not baked were not nearly so toxic; the effects of baking on diets containing triglycerides were insignificant.

Thus the experiments in Section D are open to re-investigation using unbaked diets, and possibly with the inclusion of suitable antioxidants. Although antioxidants appeared without value in triglyceride oils (Section B), the low stability of ethyl esters appears to offer scope for their use. Perhaps monomeric esters adequately protected against oxidation may prove to be as nutritious as esters of unheated linseed oil.

Another distinct possibility, which has been ignored

thus far, is that the "monomeric" esters actually include some cyclic monomers formed from trienoic acids. The toxicity of the natural cyclic fatty acids is well-known. The thermally-created cyclic fatty acids may be toxic in a roughly analogous manner.

Concentration and removal of such cyclic monomers poses a difficult problem. An approach now under study in this laboratory is the formation of crystalline adducts between urea and straight-chain aliphatic compounds (71). By successive crystallizations of the normal monomers in distillable ethyl esters, it may be possible to remove everything but clyclic material, which will not form solid urea adducts.

If it can be shown that the injurious nature of a heated oil is a function of the amount of dimeric fatty acids present, these will be incriminated beyond question. A project soon to be carried out in this laboratory consists of preparing linseed and soybean stand oils of equal dimeric acid contents. If these show approximately equal depression of growth rates, the role of dimeric fatty esters will be established convincingly.

Once the nature of the toxicity is established finally, what will be the future of thermal treatment as a means of reducing the tendency of certain oils to revert in flavor? Certainly heat treatment is simpler, cheaper and more attractive commercially than most other conceivable treatments. Intensive studies on catalytic or other "special" methods of

polymerization may lead to reactions which are both highly selective, and yield large polymer molecules with but traces of intrapolymers. Under such conditions, it would be practical to use simple solvent segregation for the preparation of edible and drying fractions.

The significance of thermal damage to edible oils extends beyond the preparation of non-reverting shortenings. Present-day oil and fat technology is moving constantly towards higher processing temperatures, striving to step up plant capacities and efficiency. It is a matter of more than academic importance to assess the possible damage that may result from high-temperature processing.

SUMMARY

1. Heat-treated vegetable oils exhibit growth impairment and other toxic effects in the diets of rats. The extent of these effects is related to the unsaturation of the oil and the amount of heat treatment.

2. Segregation of heat-treated linseed oil with acetone or n-propanol yields fractions of identical nutritive value to the whole unsegregated oil.

3. The toxicity of heated linseed oil appears unrelated to oxidative changes during baking or storage of the diets. Diets containing oils stabilized with N.D.G.A. and citric acid were identical in value to diets containing unstabilized oils at the same level.

4. The fatty acids of heated linseed oil appear to contain monomers and dimers only. These are separable by vacuum distillation of their methyl or ethyl esters, into nearly pure monomers and dimers.

5. Dimeric ethyl esters from heated linseed oil are extremely toxic to rats. The monomers produce no pathological or clinical symptoms, but fail to support growth at 20% level.

6. Alcohol-extractable material from heated linseed oil interacts in some obscure way with linseed esters (but not with triglycerides) to produce serious toxic symptoms.

7. When linseed oil is heat-bodied at 275 deg.C., under CO_2, linolenic acid reacts in a highly selective manner until its concentration is greatly reduced. Then linoleic acid

is consumed more readily, in what appears to be a second stage in polymerization.

8. Evidence is presented for the accumulation of intrapolymers in linseed oil during the early stage of heat bodying. It is suggested that the inability of solvents to discriminate between intrapolymers and true monomeric glycerides may account for failure to segregate fractions of different toxicities.

9. During the heat bodying of linseed oil, its refractive index rises linearly with the accumulation of dimeric fatty radicals. A simple method is proposed for preparing stand oils of known dimeric acid content by refractive index measurements.

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