8-1965

A Comparison of Tissue Lipids of Rats Fed Fresh, Commercially-Used, and Laboratory Heated Oil

Sarah Margaret Nutt
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Sarah Margaret Nutt entitled "A Comparison of Tissue Lipids of Rats Fed Fresh, Commercially-Used, and Laboratory Heated Oil." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

Ada Marie Campbell, Major Professor

We have read this dissertation and recommend its acceptance:

Mary Rose Gram, Bernadine Meyer, John T. Smith

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

July 30, 1965

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A COMPARISON OF TISSUE LIPIDS OF RATS FED FRESH, COMMERCIALLY-USED, AND LABORATORY-HEATED OIL

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Sarah Margaret Nutt

August 1965

ACKNOWLEDGMENT

The author wishes to express her gratitude to Dr. Ada Marie Campbell for her assistance in planning and carrying out this study, and for her invaluable guidance through the years of graduate study.

The author also wishes to thank Dr. Bernadine Meyer and Dr. Mary Rose Gram for their reviews and evaluations of this dissertation and for their suggestions.

The author is grateful to Dr. John T. Smith for his assistance in planning the study, for his criticisms and evaluations, and for his aid with photography. Thanks are also expressed to Miss Grace Button for the reproduction of the photograph of the figure, and to Miss Carolyn Nutt for proof-reading and typing of drafts of the report. Mrs. Pirkko Turkki and Miss Mary Ann Harvey are also acknowledged for their cooperation and assistance in the laboratory.

The author received a National Defense Education Act fellowship for the first three years of her graduate study. She is grateful to the United States government for the funds which made uninterrupted study possible.

The author further desires to express her appreciation to her parents for their encouragement, and to Lt. Randy L. Perry for his understanding and patience through the completion of this study.

S. M. N.

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CHAPTER I

INTRODUCTION

In the early 1930s reports of research with rats indicated that certain fat substances were essential for growth and general health of the rat. Later these substances were found to be essential fatty acids.

In the late 1940s reports concerning toxic factors in fats which had been heated and fed to rats began to appear. Later work related these toxic factors to oxidation and/or decomposition of the thermally-treated fats and oils. Although most of the fats used for study were subjected to more extensive damage than one would expect from ordinary frying procedures, the mere fact that so much fried food is consumed in this country makes the problem important.

Very few studies have been done with fats and oils which have been subjected to actual frying processes. The ones that have been reported indicate that when such oils are incorporated into the diet of a rat, mild growth depression and enlarged livers develop. Whether such symptoms might occur in humans is not known.

Frying procedures and the use and storage of fats vary greatly among the commercial establishments that prepare fried foods. The common practice is to fry food in oil at the temperature appropriate for the particular food, to strain the oil when sediment begins to be noticeable, to replenish the supply with fresh oil or fat when the volume is considerably decreased, and to discard the oil when the color becomes darkened and/or an off-flavor becomes apparent.

The length of time an oil is used depends on the type of commercial operation and the amount of frying that is done in one day's time. Some institutions discard the frying fat after one day's use; however, many find this procedure too expensive for the amount of food they fry. In these cases, the fat is exposed to air, light, and high temperatures for varying lengths of time.

Oil manufacturing companies have recognized this problem and have attempted to alleviate it. Various processes and additions of substances are used in an attempt to combat decomposition of oils and fats. One of the latest products is a soybean oil* which has been lightly hydrogenated and fractionated so that it remains an oil at room temperature, but becomes a solid at lower temperatures. It is advertised to be resistant to heat treatment, even under heavy and continuous frying use. This oil is of special interest in that it is soybean oil, which usually is not used for frying because of its susceptibility to flavor reversion (Swern, et al., 1964). It is used more commonly in margarines and in salad oils where heat treatment normally is not expected. The label on the can of the new type of soybean oil, however, states "for frying only."

This oil is used to a considerable extent in the commercial eating establishments in the Knoxville area and the users believe that they can use it almost indefinitely. The economic aspect of higher-stability oils is an important one; yet further study of such oils from the nutritional standpoint is desirable.

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*Crystal Oil, produced by the Hunt-Wesson Co., Fullerton, Calif.

Very few studies have been made of tissue lipids of rats after ingestion of heated fats. In the light of current concern over fat in the diet in relation to health, it seems important to investigate the lipid components of various tissues in animals which have been fed heated oils.

The purpose of this investigation was to study growth of rats fed diets containing a partially hydrogenated, fractionated soybean oil that was untreated, or thermally treated in the laboratory, or used extensively for frying. A further purpose was to compare the tissue lipids of the rats after the ingestion of such treated fats.

CHAPTER II

REVIEW OF LITERATURE

That fats are necessary in the diets of rats was first reported by Burr and Burr (1930). With fat-free diets their animals developed scaliness of tails, decline in growth, and kidney degeneration. The symptoms were cured when linoleic acid was added to the diet. Recovery was even more rapid when linoleic, linolenic, and arachidonic acids were included, indicating that these fatty acids could not be synthesized by the rat and were essential in the diet.

Although only minute amounts of these fatty acids were found to be necessary (Deuel, 1954; Mohrauer and Holman, 1963), there is some evidence that additional quantities of fat in the diet further promote growth of rats. Hoagland and Snider (1941) showed that on diets containing 5, 15, 30, or 54 per cent steam-rendered lard, rats grew most on regimens containing 30 per cent and least on the 5 per cent diet. Deuel *et al.* (1947) reported that weanling rats fed on diets containing 0 to 40 per cent of cottonseed oil or margarine fat showed the best growth when the fat constituted 20 to 40 per cent of the weight of the diet. In contrast to these reports, Hoagland *et al.* (1952) found no difference in the rate and efficiency of growth of young male rats fed diets containing 4, 9, or 15 per cent fat. Thomasson (1955) studied twenty different fats and oils at varying levels in the diet. The various oils and fats showed differences in growth-promoting effect,

but the feed efficiencies seemed to have a constant value and to be independent of the type of fat used.

In 1943 Burr and Barnes in reviewing studies on the need for fat in the diet were the first to suggest that fat must be fresh for best results in feeding experiments. They postulated that rancid fat is unpalatable, destructive to other vital nutrients, and possibly slightly toxic in itself.

In that same year, Morris et al. (1943) reported a definite toxic effect when heated lard was fed to rats. Growth depression, a paralysis similar to that occurring in vitamin E deficiency, and chronic gastric ulcers were observed when rats were fed a diet containing 50 per cent heated lard. But it was not until 1949 that much attention was called to oxidized fat in rat diets. At that time polymerized oils were being used in the food industry in an attempt to prevent flavor reversion in oils. Lassen et al. (1949) studied the digestibility of heated sardine oil because it was being used as a carrier for vitamins A and D in diets. They fed this heated oil at the 5 per cent level of the diet to adult rats and reported that digestibility of the oil decreased as the degree of polymerization increased. No toxic symptoms were reported with their adult rats.

Since that time most of the studies with heated fats and their possible toxicity have been conducted with young rats. Various methods have been used for thermal treatment in these studies and as a consequence, the reported results have been difficult to interpret and correlate (Perkins, 1960). Three main types of treated fats have been

used: 1) fats which have been treated thermally in the absence of oxygen, 2) fats which have been oxidized thermally in the presence of air, and 3) fats which either have been used for actual frying or have been treated by a process simulating frying.

Crampton et al. (1951c) first reported a study concerning the toxic effect of a fat which had been polymerized thermally in the absence of oxygen. When linseed oil, soybean oil, rapeseed oil, corn oil, peanut oil, and herring oil were heated at 275°C. in the absence of air and fed to rats for thirty-eight days, growth was not as great as was that of the controls fed fresh oil. Growth depression was greatest with the most unsaturated oil heated the longest time.

Later Crampton et al. (1951a) used only linseed oil for study because of its high percentage of unsaturated fatty acids. The heated oil was fed at the level of 20 per cent in the diet of weanling rats. The rats developed oily, matted coats and diarrhea. They failed to grow and soon died. To test the hypothesis that thermal treatment caused the oil to be more easily oxidized, feeding trials with rats were conducted to examine the effects of baking the diet containing heat-polymerized linseed oil and of vitamin E supplementation either directly to the diet or by dosage of the animals. Baking the diet prior to feeding produced no further deleterious effects and neither means of supplementation with vitamin E lessened the effects of feeding polymerized oil. The authors considered this to be evidence that the low nutritional value of thermally-polymerized linseed oil was not due to autoxidation but rather to some products produced in the oil during the heat treatment.

In an attempt to isolate the toxic product from thermally-polymerized fat, Crampton et al. (1951b) separated heated linseed oil into fractions: a monomeric ester fraction, a dimeric ester fraction, and several fractions containing monomeric and dimeric glycerides and intrapolymers. When these fractions were incorporated into the diets of weanling rats, the monomeric ester (distillable) fraction produced a growth deficiency, but no other toxic symptoms were observed. However, the undistillable dimeric fraction was lethal in approximately five days. It was concluded that the dimerization of an oil occurring during heat treatment removes essential fatty acids and also produces toxic radicals.

A further separation of polymerized linseed oil fractions was accomplished by Crampton et al. (1953). The separation consisted of distillation and urea adduct formation and resulted in detection of cyclized compounds. When the separated fractions were fed to weanling rats, the straight chain monomeric and dimeric esters were equally well metabolized, but the cyclized materials caused a reduction in digestibility and produced toxic effects.

In 1956 Crampton et al. compared the effects of feeding the esters of cyclized monomeric and cyclized polymeric acids from heat polymerization of linseed oil, soybean oil, and sunflower seed oil. This comparison was made because of the differences in the unsaturated fatty acid content of the three oils. The linseed oil that was used contained 47.4 per cent linolenic acid, 24.1 per cent linoleic acid, and 19.0 per cent oleic acid. The soybean oil contained 3.5 per cent

linolenic acid, 51.2 per cent linoleic acid, and 23.5 per cent oleic acid; and sunflower seed oil had no linolenic acid, 66.2 per cent linoleic acid, and 21.3 per cent oleic acid. The oils, therefore, differed chiefly in their contents of linolenic acid. The feeding trials with urea-adduct- and non-urea-adduct-forming fractions of the oils showed the linseed oil with its original high linolenic acid content to be most toxic, soybean with moderate linolenic acid content next, and sunflower seed oil least. However, the non-urea-adduct-forming portions of all three oils were toxic.

A later study of heat-polymerized menhaden oil by Common et al. (1957) gave results similar to those from the linseed oil studies. Since menhaden oil is highly unsaturated, the chief interest from this work was the additional evidence it provided for the association of toxicity of the non-urea-adduct-forming fraction of heat-polymerized oil with the presence of polyene acids in the original oils.

Alfin-Slater et al. (1959) studied the effects of thermal non-oxidative treatment of soybean oil, cottonseed oil, and lard on growth, reproduction, and longevity of rats which were fed diets containing 15 per cent of these fats. After ten weeks on the diets, the animals showed no differences in growth. At the end of four months, a slight depression in growth of the animals receiving the highly polymerized soybean oil was observed and the effect became more pronounced with time. When the rats from the groups fed polymerized oil were mated with rats from the stock colony, reproductive performance of the males was not affected by the polymerized oil; with the females there was a

decrease in the number of young born as well as a decrease in survival rate.

Addition of linoleic acid resulted in a slight improvement in reproductive performance, but the addition of twice the normal amount of alpha tocopherol yielded results comparable to those obtained with unheated oil. This seemed to indicate that highly polymerized soybean oil increased the requirement for vitamin E.

In the above study, slight increases in liver weights were noted in males fed the highly polymerized soybean oil, but not in the females. No increases in liver total lipids, liver cholesterol, plasma total lipids, or plasma cholesterol were noted in any of the rats.

Autoxidized fats and oils produced by heating in the presence of air have been shown to produce in rats an effect similar to that of fats thermally treated in the absence of oxygen. Kaunitz et al. (1955a) showed that when cottonseed oil that had been heated and aerated for 300 hours at 90°C. was incorporated into rat diets, the toxic effects were much greater than those of lard that had been treated in the same manner. With 10 per cent heated cottonseed oil in the diet, females did not become pregnant, growth was depressed, and kidney and liver weights in relation to body weight were increased. The effects could be counteracted by the addition of fresh cottonseed oil.

In an effort to determine what factor in oxidized fat was responsible for the toxic effect, Kaunitz et al. (1955b) and Kaunitz et al. (1956) fractionated oxidized cottonseed oil by alembic and molecular distillation. The oil had been aerated at 100°C. for 200

hours. The fractions were added to rat diets at 10 per cent and 20 per cent levels. The volatiles obtained by alembic distillation were highly toxic. Molecular distilled fractions were nontoxic, but the residues were highly toxic and seemed to contain mostly polymers. The authors postulated that the amount of such polymers formed was due to the amount of unsaturated fatty acids present in the original oil. By feeding monohydroxy stearate, methyl dihydroxysterate, or methyl oleate peroxide concentrate at the 5 per cent level in the diet and observing no deleterious effect, they were able to rule out the possibility that simple oxidized substances were responsible for the toxic effects.

When the enlarged livers from rats fed the polymeric fraction from the autoxidized cottonseed oil at the 8 per cent level in the diet were analyzed for fat content, no difference was found between these livers and those of rats fed fresh lard at the 8 per cent level (Kaunitz et al., 1959). The authors concluded that the enlargement of the livers was due to accumulation of protein. The concentrations of lipids in the livers resulting from feeding 15 per cent autoxidized cottonseed oil were different from the concentrations found after feeding the fractions at 8 per cent. When autoxidized cottonseed oil, fresh cottonseed oil, lard, corn oil, butter, and saturated medium-chain triglycerides were fed at the 15 per cent level and the liver lipids of the rats were compared, total liver lipids were lowest in the rats fed autoxidized cottonseed oil. Cholesterol content of liver and serum was also lowest in the animals receiving the autoxidized cottonseed oil.

Liver weights and percentage fat in the livers were increased significantly and feed efficiencies were decreased significantly in rats fed diets containing 15 per cent thermally-oxidized groundnut, sesame, and coconut oil (Raju and Rajagopalan, 1955). When the level of fat in the diet was increased to 30 per cent, the rats died within a week. This study was done in India and the heating conditions simulated those most commonly used for cooking in that country.

Friedman et al. (1961) heated cottonseed oil in an open vessel at 225°C. for 190 hours with continuous stirring at 100 r.p.m. Linoleic acid in the oil decreased from 49.4 per cent to 9.0 per cent. Polymerization occurred; monomers decreased from 100.0 per cent to 58.2 per cent, dimers were formed to the percentage of 29.8 per cent, and trimers and higher polymers were formed to the extent of 12.0 per cent. The fresh oil and the heated oil were fed to rats at 10 per cent and 20 per cent levels in the diet. Growth depression, lowered feed efficiency, and increased liver weights were observed in the rats fed the heated oil at both levels. Although data on serum lipids were too limited for statistical analysis, heating had no effect on total serum lipids at the 10 per cent level; at the 20 per cent level in the diet, the heated fats appeared to raise the total serum lipid level in females but had no effect on serum lipids of males. Serum lipids were lower at the 20 per cent level than at the 10 per cent level with both heated and unheated oil. The authors considered their limited data to indicate that an alteration in lipid metabolism occurred when heated fat was fed. When non-urea-adduct-forming monomer and dimer fractions were separated from

this same thermally treated oil and fed to weanling rats, a severe toxicity was observed (Firestone et al., 1961).

Corn oil has been studied widely. Johnson et al. (1956) compared the nutritive values of thermally-oxidized corn oil, margarine base stock (a hydrogenated vegetable oil), and butter oil. The oils were heated with stirring for twenty-four hours at 200°C. Air was bubbled through the oil at 100 ml. per minute. With all oils a decrease in iodine value, an increase in acid value and peroxide value, and a development of a reddish color and painty odor were observed. These oils were fed to weanling rats along with corresponding fresh oils by the paired feeding technique. Growth was depressed greatly when oxidized corn oil was fed and some depression was noted with the margarine stock base. No depression was observed with butter oil. Again the authors thought that the depression was due to the unsaturated fatty acids present in the original oil. The possibility that a vitamin deficiency might be responsible for the depression in growth was eliminated because of the rapidity of the onset of the symptoms. Although a possibility exists that inhibition of an enzyme is involved, the rapid recovery obtained when the animals were changed to fresh corn oil would indicate that there was no destruction of enzyme-producing sites.

Johnson et al. (1957) attempted to relate linoleic acid content of a fat to thermal oxidative damage as measured by comparative growth rates in rats. Corn oil, hydrogenated cottonseed oil, olive oil, and oleo oil were heated at 180°C. for twenty-four hours with air bubbled

through at a controlled rate. The linoleic acid content was determined by alkali isomerization and spectrophotometry. The fat was introduced into a rat diet at the level of 20 per cent and fed for only ten days. Growth rate comparisons showed that the greatest nutritional loss was in the corn oil, which originally had the highest amount of linoleic acid. As original linoleic acid content and iodine value decreased, growth rates of the rats fed the heated oils increased. A further experiment was conducted to differentiate between the effects of linoleic acid level and those of total unsaturation of the oil. Three samples were prepared so that they had an iodine value of seventy-two and varying amounts of linoleic acid. The three samples then were thermally oxidized as described above. In this trial the decrease in growth corresponded directly to the original linoleic acid content. When the iodine value was set at 102 instead of seventy-two before thermal oxidation for a similar test, the decreased growth with increased original level of linoleic acid was slight. The fats of the higher iodine number had higher nutritive values than those of the lower iodine number at all three original levels of linoleic acid. The authors could not explain this effect, but they suggested that the dilution of the linoleic acid double bonds by oleic acid double bonds might have decreased the polymerization of the linoleic acid.

Perkins and Kummerow (1959b) fed a non-distillable residue from non-urea-adduct-forming fatty acids from corn oil which had been heated for forty-eight hours at 200°C. When this fraction, representing approximately 30 per cent of the original oil, was fed at the 10 per

cent level in the diet of weanling rats, the rats died within seven days. Dilution with an equal amount of fatty acids from fresh oil assured survival for twenty-one days but only partially counteracted the growth-depressing effect.

In a later study Perkins et al. (1961a) were concerned with the effects of dilution of fatty acids from oxidized corn oil with fatty acids from fresh corn oil on absorption, liver weights, and carcass and fecal fat composition. Again they used a 10 per cent level of fat in the diet of weanling rats for a fifty-nine-day feeding period. Both with oxidized corn oil and with fatty acids from the oxidized oil, weight gain and digestibility were lower than with fresh oil. Dilution of the fatty acids from oxidized oil with 90 per cent fatty acids from fresh corn oil counteracted the effects. Fatty acid analyses by gas-liquid chromatography showed a decrease in linoleic acid content from 54.5 per cent to 14.6 per cent after oxidation. Hydroxy acids increased from zero to 36.6 per cent. The carcass fatty acids reflected the fatty acid pattern of the diet; i.e., those fed fresh oil had 36.10 per cent linoleic acid, those fed oxidized oil had only 15.20 per cent linoleic acid, and those fed fatty acids from oxidized oil had 23.70 per cent linoleic acid. The fact that 6.98 per cent hydroxy acids were found in the carcass fat of those fed oxidized corn oil is noteworthy.

The deposition of hydroxy acids in carcass lipids was not found as a result of feeding milk fat which had been heated for twenty-four hours at 200°C. with air bubbled through it (Bhalerao et al., 1961). These workers also used gas-liquid chromatography to analyze fatty acids

of rat carcass and liver after the feeding of oxidized milk fat, fresh milk fat, corn oil, and lard. No statistical difference was observed in the fatty acid composition of the carcasses of rats fed fresh and oxidized milk fat, though the oxidized milk fat did seem to increase the fatty acid tentatively identified as a C_{18} branched acid. The most dramatic effect of the kind of fat was observed in the carcass lipid concentration of linoleic acid which varied from 2 per cent in the rats fed milk fat to 39 per cent in those fed corn oil. Liver lipids from the rats fed milk fat also contained 13 per cent arachidonate as compared with 10 per cent for the liver lipids of those fed corn oil.

The absorption of fresh and thermally-oxidized corn oil, coconut oil, milk fat, and olive oil was studied in lymph-cannulated rats by Bhalerao et al. (1963). The various fats were given to the animals in 1-ml. portions by stomach tube and the lymph was collected over a period of twenty-four hours. Approximately 10 per cent less of thermally-oxidized fat than fresh fat was absorbed. The difference in absorption was greater for the highly unsaturated oils, corn oil, and olive oil, than for the other oils. There was practically no difference in the percentage of saturated fatty acids absorbed from heated and fresh fats. It was interesting that in the corn oil studied the linoleic acid was reduced only from 60 per cent to 54 per cent during oxidation.

The problem of toxic effects of oxidized fats and oils was evaluated in a different manner by Andrews et al. (1956) and Andrews et al. (1960). These workers assumed that the products of fat oxidation

initiated by whole body irradiation are essentially the same as the products initiated by *in vitro* autoxidation. They oxidized soybean oil in a 60°C. steam bath until the peroxide number reached 1200. Three dilutions of this oil were made with fresh oil to produce oil samples having peroxide values of 1200, 800, 400, and 100. When the samples were fed to rats at the 20 per cent level in the diet, rate of growth varied with peroxide number. All the rats fed the diet containing oil with a peroxide number of 1200 died within three weeks. Growth depression was observed with the diets containing oils with peroxide values of 800 and 400. Those fed oils with peroxide values of 100 grew well. At the age of eight weeks these rats and a control group fed fresh oil were subjected to whole body irradiation. During the next thirty days, twice as many of the rats fed the fat with the peroxide number of 100 died as of those fed the control diet, indicating that the consumption of a fat with a moderate high peroxide number caused a slower than normal recovery from the stress of irradiation.

Poling *et al.* (1962) investigated the influences of temperature, heating time, and aeration upon the nutritive value of fats. Cottonseed oil aerated at 60°C. for sixteen days or exposed in thin layers to air at 180° to 220°C. caused growth depression and enlarged livers when fed to rats for only ten days. Heating the fat in deep layers prior to feeding caused less depression of growth than did heating in thin layers, indicating that exposure to oxygen accelerated nutritional impairment. They also compared the effects of heating corn oil, cottonseed oil, lard, hydrogenated vegetable oil, and tallow in different size

portions. Samples of the fat were heated for six hours at 200°C. with gentle stirring in 3-kg., 400-g., and 200-g. portions. No differences were observed with the different fats fed, but a highly significant difference was observed in growth rates with the quantity of fat heated. The smaller quantities heated produced greater growth depression when fed to weanling rats than did larger quantities heated and fed to the rats at the same level in the diet. The authors concluded that the changes induced by thermal oxidation of fats are proportional to the severity of the conditions imposed and that more extensive treatment than is usually encountered in processing or cooking is necessary to produce physiologically-detectable damage to a fat.

Various researchers (Melnick, 1957a, 1957b; Rice et al., 1957; Poling et al., 1960; Keane et al., 1959; Perkins, 1960) support the conclusion that ordinary frying procedures do not produce harmful products; however, very few studies on the nutritional value of fats which actually have been used for frying have been conducted. Bennion and Hanning (1956) showed that the type of food fried affected the decomposition rate of the frying medium. They observed more pronounced increases in free fatty acids and decreases in smoke point in the fat when fritters were fried than when potatoes were fried. Peroxide values were lower in the fat used for frying fritters than for potatoes and iodine values and saponification values were about the same. The color and general appearance of the fritter frying medium was much more affected than was the potato frying medium.

The method of caring for the fat between frying periods has been investigated by Rust and Harrison (1960). They observed that dilution with 20 per cent fresh fat had a greater effect in preventing deterioration, as estimated by palatability scores and acid values, than did the addition of only the amount of fat that was lost during an eight-hour frying period. Filtering and refrigeration between fryings also were shown to prolong the life of a fat.

Melnick (1957a) collected samples of oil used for potato chip frying for four days from eighty-nine potato chip producers throughout the country. These fats were found to have decreased only 1 per cent in iodine value from that of the fresh fat. Melnick concluded from these data that no polymers had been formed and that such fats were not damaged to such an extent that they would be harmful. He did not do any feeding trials to support his hypothesis.

In another report Melnick (1957b) reviewed the literature and suggested that if any harmful degradation were taking place in the oils used to fry potato chips, a flavor deterioration would occur along with this breakdown. In 1958 Melnick et al. compared unused oils and oils used for frying potato chips as to degree of unsaturation, melting point, setting point, fatty acid composition, free fatty acids, concentration of fatty acid isomers, solids content index, and crystallizing properties. No significant differences were found and they again concluded that oils which had been used for frying potato chips contained no polymers and were not harmful.

Keane et al. (1959) compared the growth of rats fed unheated hydrogenated cottonseed oil, hydrogenated cottonseed oil which had been heated in the laboratory, and hydrogenated cottonseed oil which had been used for several days for frying food. The oils were incorporated into the diets at the level of 20 per cent. Slight growth depression and slightly enlarged livers were observed with the feeding of the laboratory-heated fat, but none were observed with the commercially-used fat. The authors did not indicate how their laboratory-heated fat was treated. Analysis of fatty acids of the oils showed that the heated oils were more unsaturated than the unheated oils. Since it might be expected that a hydrogenated oil would be less susceptible to damage during heating, they repeated the experiment with corn oil and again found no harmful effects.

Slightly increased feed efficiencies also were reported by Deuel et al. (1951) when rat growth on a diet containing heated margarine fat was compared with growth on a diet containing unheated margarine fat. The diet used in the study contained only 11 per cent fat and the fat was heated for eight hours at 250°C.

Further evidence that fats used in commercial frying procedures are not damaged enough to be detrimental in diets was presented by Poling et al. (1960). Samples of fats and oils used for commercial frying procedures for varying lengths of time and under various conditions were collected for thirty-four establishments. The fats were incorporated into rat diets at the level of 20 per cent for a seven-day feeding period. No significant differences were found in the

nutritive value of the fats. Slight growth depression and slightly enlarged livers were noted when the most unsaturated fats were fed, but these were not significant. The writers suggested that the exposure of the fat during frying to the varying quantities of water, to foods of different types, to agitation, and to supplementation with fresh fat may either increase or decrease the nutritive value of the fat used for frying.

Witting et al. (1957) found definitely poorer growth of rats fed commercially used corn oil at the 10 per cent level in the diet than of those fed fresh corn oil. The same effect was observed with lard but not with hydrogenated shortening.

It is difficult to draw valid conclusions from the studies concerning the nutritive value of fats which have been used for frying because of the use of different oils, different conditions of heating, different levels of oil in the diets, and different levels of other components in the diets. Other studies have been carried out in attempts to determine whether compounds that might have been produced in such heated fats are absorbed and metabolized in the rat and to investigate other factors that might be involved. In 1939 Longenecker observed that the fatty acid pattern in adipose tissue resembled that of the fat or oil being fed, especially if long-chain unsaturates were present in the diet. Linoleic acid was present in appreciable quantities in the adipose tissue of rats fed corn oil, but not in that of rats fed less unsaturated fats.

In 1944 Harris et al. investigated the use of hydroxy fatty acids in the rat because they were found to be present in both animal and vegetable fat. Control animals were fed a diet containing 25 per cent hydrogenated vegetable shortening and the experimental diets had 2.5 per cent of the hydrogenated fat replaced with mono-, di-, or trihydroxy stearic acid. The animals fed the mono- and dihydroxy acids grew better than the controls and had better feed efficiencies. Those fed trihydroxy acids had a better feed efficiency than the controls, but total weight was less. The authors felt that the difference was due to the difference in the melting points of the fats.

Perkins and Kummerow (1959a) found rather high amounts of hydroxy acids in certain fractions isolated from oxidized corn oil and suggested that these high amounts might be responsible for the growth depression and toxicity observed when heated fats were fed. No growth depression was noted when they studied the incorporation of hydroxy acids by feeding them in the form of triricinolein and ricinoleic acid (Perkins et al., 1961b). Carcass and liver fatty acid analysis of rats fed these fats and others as controls indicated that dietary hydroxy acids were deposited. They also were able to show that both saturated and unsaturated hydroxy fatty acids were converted to monoenoic acids in the rats. A larger amount of oleic and hexedecenoic acid seemed to be deposited and a preferential excretion of stearic and linoleic acids seemed to occur in rats fed sources of hydroxy fatty acids in comparison to those fed a source of linoleic acid. In a later study Perkins (1964) studied the carcass glyceride structures

after the ingestion of this type of fat. He concluded that the hydroxy acids are metabolized much the same as normal triglycerides and exert no particular influence on the structure of the glycerides in the rat.

Kaunitz and Johnson (1964) fed purified diets containing 5 per cent dihydroxy stearic acid. Absorption studies showed that at least 80 per cent of the ingested dihydroxy stearic acid was absorbed. Growth was depressed in the animals for the first four weeks and followed by rapid weight gain. The authors felt that this was an adaptation to the type of dietary fat. However, they were unable to detect the presence of hydroxy acids in serum, kidney, liver, and epididymal fat.

Deuel et al. (1951) studied the effects of feeding isopropyl and stearyl citrates on the growth of rats because citrates were sometimes used in fats as anti-flavor-reversion agents. They found such fatty acids to be nontoxic and to promote normal growth.

As a result of hydrogenation of vegetable oils, positional and stereoisomers of unsaturated fatty acids are formed in appreciable quantities (Swern, et al., 1964). Melnick and Deuel (1954) tested such isomers for nutritional value in rats and found that they were not antimetabolites and could be used by the animal. Later Johnston et al. (1958) fed diets containing trans isomers and found that they were deposited in the adipose and liver tissues of rats. The percentage of these trans acids in the tissues remained at the same concentration after about a month if feed intake was unaltered. The remainder appeared to be metabolized. When trans isomers were removed from the diet, depletion of the trans fatty acids from the liver was slower than

from the carcass fat, possibly due to a metabolic pool in which the adipose fatty acids are mobilized first.

Holman (1951) fed isomers of linoleic and linolenic acids to animals which had been on fat-free diets for ten to twelve months. The fatty acid deficiency symptoms which had developed could be relieved by the administration of linoleic acid, but the administration of the trans form of either linoleic or linolenic acid had no curative effect on the deficiency symptoms. It would seem, therefore, that trans acids cannot be used by the rat to meet the essential fatty acid requirement. Holman and Aaes-Jorgensen (1956) found that with a diet used for fatty acid depletion male rats developed severe testicular degeneration. Administration of trans fatty acid isomers to these animals failed to cure the symptoms. Growth of the rats after the administration of these fatty acids was about half that obtained when an equal amount of linseed oil or linolenate was administered in the same manner.

Hopkins et al. (1957) were able to show that eicosenoate and erucate were utilized by the rat, were deposited in the carcass lipids, and produced no serious nutritional or metabolic disturbance. These acids are found in some vegetable oils in minute quantities.

The effects of feeding fatty acids of different chain lengths to rats on the fatty acid pattern in the carcass were studied by Kaunitz et al. (1961). They were able to show that the feeding of fat-free diets or diets containing medium-chain or long-chain fatty acids resulted in differences in the concentrations of the various acids

deposited, especially oleate. With the addition of 2 per cent linoleate to either of the three types of diets, even more dramatic differences in deposited fatty acids were noted. The addition of linoleate to fat-free diets resulted in a deposition of 21 per cent linoleate. The addition to diets containing medium-chain fatty acids resulted in a deposition of 11 per cent linoleate and substantial amounts of C_8, C_{10}, and C_{12} acids. With long-chain fatty acids the addition of linoleate resulted in deposition of only 6 per cent linoleate, along with increased amounts of C_{12} and C_{14} acids. The authors concluded that linoleate in the diet regulates the type of fat deposited.

Kirschner and Harris (1961) also studied chain length in relation to metabolism. They compared animals receiving C^{14}-labeled butyric, caprylic, lauric, and palmitic acid by stomach tube. Activity measurements were made on collected samples of breath, urine, and feces. Activity measurements of fecal samples indicated that palmitic was absorbed less than the shorter chains. The rapid expiration of labeled CO_2 with the short-chain fatty acids indicated that they are metabolized rapidly and do not enter fat depots.

Babayan *et al.* (1964) studied polyglycerol esters prepared with fatty acids from cottonseed and peanut oils. Polyglycerol esters were utilized by weanling rats just as well as lard fed at the same level. These polymers were not toxic.

Methyl linoleate was heated in the absence of air for ten hours at 300°C. to determine whether this treatment would produce symptoms observable with heated fats when fed to rats (Bottino, 1961). The

heated lineolate was fractionated by distillation and urea adduct formation. When the fractions were fed to rats, the non-urea-adduct-forming monomers produced increased liver weights and proved lethal in a short time. The observable symptoms in the rat were much the same as those observed when polymerized oils were fed.

CHAPTER III

PROCEDURE

As stated in the introduction, soybean oil generally has not been used as a frying medium because of its tendency toward flavor reversion (Swern, et al., 1964). However, a product now available and made especially for frying is prepared from soybean oil that has been lightly hydrogenated and fractionated.* It contains antioxidants, butylated hydroxyanisole and butylated hydroxytoluene. Methyl silicones have been added as antifoaming agents. The product is a liquid at 60°F., but it becomes a solid when the temperature is lowered. It is being used extensively as a frying medium for varying but extended lengths of time. Since reports have indicated that the feeding of diets containing thermally-oxidized soybean oil is detrimental to the health of a rat (Alfin-Slater et al., 1959; Andrews et al., 1960; Andrews et al., 1956; Crampton et al., 1951b; Crampton et al., 1956; and Reporter and Harris, 1961), it was decided to incorporate the processed soybean oil into an animal study concerning the effects of feeding heated oil on tissue lipid concentrations.

The study was designed to test the nutritive value of such an oil when it was fresh, when it had been thermally oxidized in the laboratory, and when it had been used as a frying medium by a local cafeteria.. Analyses of the tissue lipids isolated from rats which had been

*Crystal Oil--Hunt-Wesson Co., Fullerton, California.

fed the oils also were included in the plan. The phospholipids were emphasized because they have received relatively little attention in the studies in which heated oils have been fed to rats.

I. OIL SAMPLES

Three samples of Crystal Oil were compared. Sample I was untreated. Sample II was obtained from a local cafeteria where it had been used for thirty-nine days. At the establishment where the oil was obtained, two deep-fat fryers were used simultaneously. Each fryer had a capacity of five gallons. The manager stated that approximately one gallon of oil was added daily to replace that which was lost during frying. The oil was filtered twice daily, once after lunch and once after dinner, and returned to the cleaned fryer until time for the next frying. The average temperature of the oil during frying periods was 163°C. The fryer was turned on each morning at 6:30 A. M. and turned off at approximately 8:30 A. M. Doughnuts were fried during this period of heating. The oil was heated again from 10 A. M. until 2 P. M. and from 4 P. M. until 10 P. M. During these periods fish, potatoes, fritters, and eggplant were fried. The average heating time each day was 12 hrs.

Sample III was taken from the same lot of oil as was sample I, but it was subjected to heat treatment in the laboratory. One gallon of oil was heated in an electric deep-fat fryer of the type designed for home use. Heating took place on four consecutive days, 12 hrs. each day, for a total of 48 hrs. at 200°C. $\pm$ 5°.

The three samples were poured into small containers, flushed with nitrogen, covered tightly, and frozen until time of use.

II. ANIMALS AND DIETS

Albino rats of the Wistar strain from the University of Tennessee colony were used. Weanling rats twenty-one days of age with an average weight of 34 g. were put on test diets. Six rats, two females and four males, were assigned to each experimental group. Each animal in a group had a littermate of the same sex in each of the other groups. The animals were housed in individual wire cages. Distilled water was given ad libitum and fresh feed was supplied each morning.

The composition of the experimental diets is given in Table I. Diets Ia, IIa and III were fed ad libitum. Since it was expected that the animals would eat less of the laboratory-heated fat, animals in groups I and II were restricted to the amount of feed eaten by their littermates in group III. Whenever the animals in groups I and II ate less than did those in group III, the remaining feed was weighed and an equal amount of fresh feed was added to the next day's allotment.

The diets were mixed weekly and stored in dark colored, tightly-closed jars in the refrigerator. The oil to be included in the diet mixture was kept in the freezer until the night before the diet was to be mixed. At that time it was put into the refrigerator.

Weights of the rats, weights of feed given, and weights of feed remaining in the feed cups were recorded daily. Animal weights and feed consumed (feed given - feed remaining in the cup) were used to

TABLE I

EXPERIMENTAL DIETS

Substance (g./100 g.)	Groups I, Ia	Groups II, IIa	Groups III
Vitamin Free Casein[a]	18.0	18.0	18.0
Cornstarch	60.8	60.8	60.8
Salt Mixture[b]	4.0	4.0	4.0
Vitamin Mixture[c]	2.2	2.2	2.2
Untreated Crystal Oil	15.0	----	----
Commercially-Used Crystal Oil	----	15.0	----
Laboratory-Treated Crystal Oil	----	----	15.0

[a]Nutritional Biochemicals Corp., Cleveland, Ohio.

[b]Nutritional Biochemicals Corp., Cleveland, Ohio, according to Hubbell, Mendel and Wakeman (1937).

[c]Vitamin Diet Fortification Mixture, Nutritional Biochemical Corp., Cleveland, Ohio. g./kg. of vitamin mixture: Vitamin A concentrate (200,000 units/g.), 4.5; Vitamin D concentrate (400,000 units/g.), 0.25; Alpha tocopherol, 5.0; Ascorbic acid, 45.0; Inositol, 5.0; Choline chloride, 75.0; Riboflavin, 1.0; Menadione, 2.25; P-aminobenzoic acid, 5.0; Calcium pantothenate, 3.0; Niacin, 4.5; Pyridoxine hydrochloride, 1.0; Thiamine hydrochloride, 1.0. Mg./kg. of vitamin mixture: Biotin, 20; Folic acid, 90; Vitamin B_{12}, 1.35; triturated in dextrose to a final weight of one kg.

calculate weekly feed efficiency

$$\frac{\text{weight gain, g.}}{\text{feed consumed, g.}}$$

At the end of forty-two days, the animals were sacrificed by decapitation after a blow on the head. Individual blood samples were collected in 50-ml. conical centrifuge tubes, allowed to clot for 15 min. and centrifuged at more than 750X gravity for 10 min. The serum was drawn from the top with a pipette and the blood was recentrifuged for 5 min. The remainder of the serum was taken from the top and added to the first collection. The tubes containing the collected serum were flushed with nitrogen, stoppered tightly, and stored at -20°C.

Immediately after decapitation of the rats, the livers were removed and dropped into weighed jars, covered quickly, and weighed. After flushing with nitrogen and covering tightly, the individual livers were stored at -20°C.

The visible adipose tissues from the peritoneal cavity also were removed, placed in small jars, flushed with nitrogen, covered, and frozen. The remainder of each rat carcass was wrapped with aluminum foil and frozen.

Further analyses on the rat tissue lipids were carried out only with the littermates which had been pair fed.

III. OIL ANALYSES

Viscosity measurements. Viscosity measurements of the three oils were made in triplicate by means of the Brookfield Synchro Lectric Viscometer, Model LVF. Samples of the oils were allowed to come to room

temperature and poured into glass jars which contained approximately 250 ml. The jar with the oil was set into sand in a large beaker and heated in the oven until the temperature of the oil reached 45°C. Readings were taken immediately with the no. 1 spindle on speeds six, twelve, and thirty. Each reading was converted to centipoises by multiplying by a factor specific for the speed and spindle used. The three values for each oil sample were averaged.

Hydroxy acid analyses. Hydroxy acid content of the oils was obtained by a modification of the method of Smith and Shriner (1956). Duplicate 8-10 g. samples of each oil were placed in weighed Kjeldahl flasks and weighed to the nearest tenth of a milligram. Five milliliters of acetic anhydride-pyridine* (200:800, v:v) and 5 ml. of pyridine* were pipetted into each flask. Any of the sample adhering to the side of the flask was washed down with the reagent. The flasks were placed on a steam bath for 30 min. After cooling to room temperature, 5 ml. of carbon dioxide-free water were added and the flasks were returned to the steam bath for 5 min. Again the contents of the flasks were cooled to room temperature and transferred quantitatively to 250-ml. Erlenmeyer flasks with the use of two 10-ml. washings of carbon dioxide-free water and 25 ml. of butyl alcohol. The excess acid was titrated with 1.084 N. NaOH to the phenolphthalein end point. A reagent blank was carried with each set of samples. Per cent hydroxy acid was calculated by the following formula:

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*Pyridine was distilled prior to use.

$$\text{Per cent hydroxyl} = \frac{\text{N. of NaOH} \times (B - S) \times 17}{1000 \times \text{g. sample}} \times 100$$

where:

B = ml. of sodium hydroxide required by blank

S = ml. of sodium hydroxide required by sample.

Fatty acid analyses. Fatty acid analyses were carried out on the oil samples. No attempt was made to separate phospholipid fractions from the oils because of the precautions taken by the oil manufacturers to eliminate them (Taylor, 1949; Beal et al., 1956). In preparation for gas-liquid chromatography (G. L. C.) of fatty acids, the acids were converted to their methyl esters by an interesterification procedure. Approximately 40 mg. of each oil sample were placed in a 15-ml. conical centrifuge tube. Five milliliters of 5 per cent sulfuric acid in methanol (v:v) were added and the samples were refluxed for one hour in a water bath held at approximately 70°C. At the end of the reflux period, samples were chilled and any visible fat remaining in each tube was removed. Six milliliters of petroleum ether and 2 ml. of distilled water were added to each tube. The contents then were poured into a 30-ml. separatory funnel and the bottom layer, containing sulfuric acid and water, was allowed to drain out. Washing with water was repeated twice. The petroleum ether layer, containing the methyl esters, was poured into a clean centrifuge tube and 0.5 g. of sodium sulfate and 0.5 g. of silicic acid were added to remove water

and other contaminants. The mixture was covered and allowed to stand at least one hour, after which the extract was decanted into another centrifuge tube. If the extract was not used immediately, the tube was flushed with nitrogen, stoppered, and stored in the freezer.

Just prior to G. L. C. analysis, the solvent was evaporated under a stream of nitrogen, leaving the methyl esters in the tube. The methyl esters then were diluted with hexane* and injected into a Barber-Colman model 61C Gas-Liquid Chromatograph. The chromatograph was equipped with an argon ionization detector with radium source and an argon gas supply. A seven-foot packed column with a polar liquid phase was used.** The following operating conditions were maintained:

Column Temperature	175°C.
Cell, Split, and Flash Heater Temperatures	220°C.
Cell Voltage	900
Gas Pressure	22 p.s.i.
Sensitivity Setting	10

Duplicate chromatograms were obtained for each sample. The major components were identified by comparisons of retention times with those of a standard fatty acid methyl ester mixture. The concentrations of fatty acids present were found by multiplying the height of each peak by the width at half-height. Percentage of each fatty acid present was calculated by the following formula:

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*Matheson Coleman and Bell, Chromatoquality.

**Thirteen per cent ethylene glycol succinate polyester by weight on 80-100 mesh Gas-Chrom P.

$$\text{Per cent F. A. in sample} = 100 \times \frac{\text{"area" of peak}}{\text{total "area" under all peaks in the chromatogram}} .$$

IV. SERUM ANALYSES

Extraction of lipids. Within seven days after the rats were killed, extraction of the serum lipids was accomplished by a modification of the method of Bloor (1928). Jesting and Bang (1963) found Bloor's method to give as complete extraction as any of the three commonly used methods of lipid extraction that they compared.

Serum was allowed to thaw 15 min. at room temperature. The volume of as much serum as could be drawn into a graduated pipette was measured. The serum was added dropwise to 100 ml. of boiling 95 per cent ethanol-ether mixture (3:1, v:v). The mixture was reheated in boiling water to the boiling point and filtered through Whatman no. 1 filter paper. Three 25-ml. portions of the ethanol-ether mixture were used to rinse the flask and filter paper. The filtrate was collected in a standard taper, round bottom flask and evaporated to dryness on a flash evaporator. The lipid in the flask was transferred quantitatively with chloroform into a 15-ml. conical centrifuge tube. A stream of nitrogen was allowed to flow into the tube, after which the tube was stoppered tightly and stored at -20°C. for later analysis.

For determination of serum lipid concentration, the serum lipid was transferred quantitatively to a weighed 2-ml. volumetric flask and evaporated to dryness in a vacuum desiccator. The volumetric flask

with the sample was weighed and the concentration of lipid in serum was calculated.

The lipid in the volumetric flask was diluted to volume with chloroform in preparation for separation of neutral lipids and phospholipids by thin layer chromatography.

Thin layer chromatography. The thin layer chromatography (T. L. C.) procedure to separate the neutral lipids and the classes of phospholipids was a modification of the method of Wood and Kinsell (1963). Twenty grams of Silica Gel G* were mixed with 38 ml. of distilled water and applied to four 100 x 200 mm. glass plates in layers approximately 500 μ thick with the use of a Camag applicator. Prior to application of the silica gel, the plates had been washed with sulfuric-nitric acid mixture (1:1, v:v) and rinsed with distilled water to prevent contamination of the sample. The coated plates were dried in a 250°F. oven for one hour and stored in a desiccator until time for use, usually not more than one day.

The serum lipid samples were diluted with chloroform to contain 10 to 15 μg. lipid per μl. solution and applied to the bottom of the T. L. C. plate with a microsyringe in 5-μl. portions in order to keep spot size small. Four spots were applied to each plate. Application was repeated until each spot contained approximately 750 μg. lipid. Preliminary work with serum lipids indicated that this amount of sample would yield phospholipid classes having phosphorus concentrations

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*E. Merck, Ag., Darmstadt, Germany.

appropriate for the determination that was to follow. A standard mixture of phospholipid containing a lecithin, a cephalin, and sphingomyelin dissolved in chloroform was applied to each plate as a fifth spot for identification purposes. For development, the plate was placed in an enclosed chamber with troughs at the bottom containing a solvent mixture of chloroform:methanol:water (80:35:5, v:v:v).* The solvent mixture had been placed in the chamber one hour before the plate to allow saturation of the chamber to take place. Filter papers were put on each side of the chamber and wet with solvent. The length of saturation time was critical in obtaining reproducible chromatograms. Development was carried out for one hour. The plates were removed, air dried for one minute, sprayed with 0.2 per cent ninhydrin in acetone** and heated in a 250°F. oven for one minute. The ninhydrin reaction with amino groups produced a blue color, and made possible the identification of cephalins and other amino compounds. Further spraying with an 0.004 per cent aqueous solution of Fluorescein*** made the other lipid-containing spots visible. Separation of lipid classes is shown in Figure 1. The plates were viewed under ultraviolet light and marked with a sharp spatula. Each marked spot and a corresponding

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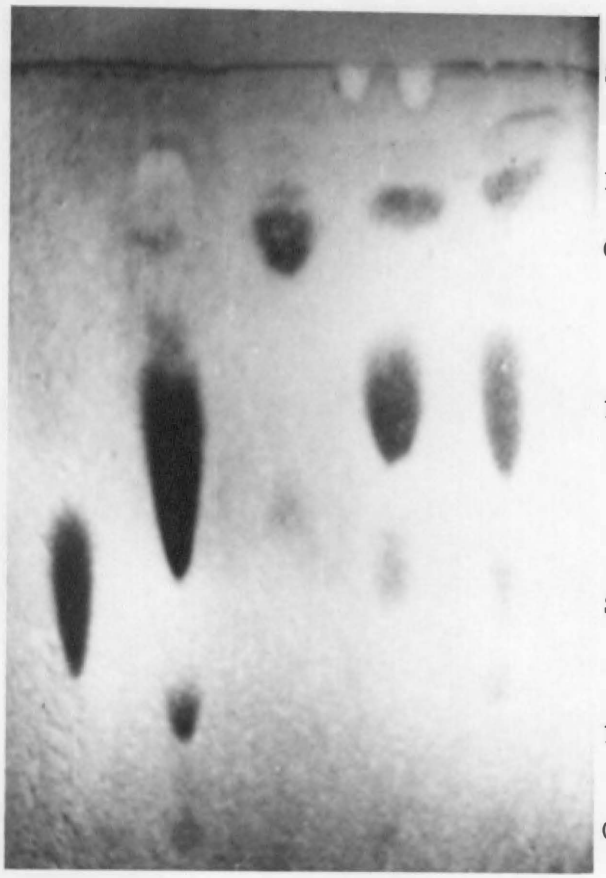
*It was sometimes necessary to reduce the water to 4 ml. on humid days.

**Mann Research Laboratories, Inc., New York.

***Matheson Coleman and Bell.



Samples from 1-5 are: 1) sphingomyelin (brain); 2) lecithin (dipalmitoyl, synthetic); 3) cephalin (dipalmitoyl, synthetic); 4) beef muscle lipid; 5) rat liver lipid.

FIGURE 1

SEPARATION OF LIPID CLASSES BY THIN LAYER CHROMATOGRAPHY

blank spot of the same area and approximate position on the plate were scraped into acid-washed micro-Kjeldahl flasks for phosphorus determinations.

Phosphorus determinations. In order to determine the amount of total phospholipid and of each phospholipid class, spots from the plates and also a total lipid sample were analyzed for phosphorus by modifications of the methods of Marinetti (1962) and Bartlett (1959).

Two glass beads and 1.2 ml. of 70 per cent perchloric acid were added to the flask containing the sample. Digestion was carried out on a micro-Kjeldahl digestion rack for one hour. After cooling to room temperature, 7 ml. of distilled water and 1.5 ml. of 2.5 per cent ammonium molybdate solution were added. The contents of the flask were mixed thoroughly* and then 0.2 ml. of Fiske-Subbarow reagent** were added. The mixture was heated in a boiling water bath for 7 min. for color development. The flasks were removed from the water and cooled for 10 min. at room temperature. The contents were poured into 15-ml. conical centrifuge tubes and centrifuged for 10 min. at approximately 1000X gravity. The solution was decanted from the silica gel into a cuvette. Optical density was read in a Bausch and Lomb Spectronic

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*Failure to mix completely at this point caused the production of a bluish-black color.

**Reagent was prepared by adding 0.5 g. of 1-amino-2-naphthol 4-sulfonic acid (Matheson Coleman and Bell) to 200 ml. of freshly prepared 15 per cent sodium bisulfite (anhydrous) and stirring. After the addition of 1.0 g. of anhydrous sodium sulfite, the solution was filtered and stored in a dark bottle. Fresh solutions were made weekly.

20 spectrophotometer at a wave length of 650 mμ. A reagent blank was carried with each set of samples.

Prior to the use of the above method, a standard phosphorus curve was prepared from varying concentrations of standard potassium phosphate (monobasic). The relationship was linear between 1 μg. and 6 μg. of phosphorus.

The reading for the blank spot corresponding to the sample being read was subtracted from the sample optical density reading. This optical density then was read from the standard phosphorus curve to give the amount of phosphorus present. The phosphorus content was multiplied by the factor twenty-five to give a value for phospholipid. The calculated amounts of phospholipid per spot were added to give total phospholipid content. Total phospholipid also was estimated from the total lipid phosphorus determinations. Percentage of each class was calculated by dividing the amount of class phosphorus by the total amount of phosphorus and multiplying by 100.

Fatty acid analyses. Methyl ester preparation and G. L. C. of the total serum lipids were accomplished in the same manner as for the oil samples except that smaller amounts of serum lipids were available for analyses.

V. LIVER ANALYSES

Extraction of lipids. The liver lipids were extracted by a modification of the method of Ostrander and Dugan (1961). Each frozen liver was allowed to thaw at room temperature and then transferred

quantitatively into a Waring Blendor jar with one Kim-Wipe and 75 ml. of absolute methanol. A few crystals of hydroquinone and a small piece of dry ice were added to prevent oxidation. The contents of the jar were blended for 5 min. with the speed controlled by means of an Adjust-A-Volt regulator set at thirty. Twenty milliliters of chloroform were added and the mixture was blended again for 5 min. at the same setting. An additional 20-ml. portion of chloroform was added and the mixture was blended for 30 sec. at a setting of thirty-five. Thirty milliliters of distilled water containing 0.5 g. zinc acetate were blended with the sample for 10 sec. with the voltage regulator set at thirty-five.

The extraction mixture was poured into a no. 2A Buchner funnel and filtered through Whatman no. 1 filter paper with suction. A blanket of carbon dioxide over the filtering flask was supplied through a tube and an inverted funnel attached to an overhead flask containing dry ice and water. The residue, with filter paper, was transferred to a blendor jar. The funnel was wiped with one Kim-Wipe and the residue, filter paper, and Kim-Wipe were reblended with 45 ml. of chloroform for 2½ min. The mixture was transferred to the funnel and refiltered with the use of 50 ml. of chloroform for washing.

The filtrate was transferred to a 250-ml. graduated cylinder with a small amount of chloroform. The volume of the chloroform layer was recorded and the contents were poured into a 250-ml. separatory funnel. The bottom layer was collected into a 500-ml. evaporating flask. Two 3-ml. portions were taken and put into weighed 25-ml.

beakers. Most of the solvent was allowed to evaporate from the samples at room temperature under the hood. The samples were dried further to constant weight in a vacuum desiccator. Per cent lipid in the livers was calculated from the average weight of the two samples. The solvent in the remaining lipid-solvent mixture was evaporated on a flash evaporator. The extracted lipids were transferred to a 10-ml. volumetric flask with chloroform and stored under nitrogen at -20°C.

Thin layer chromatography. Portions of the liver lipid extracts were subjected to T. L. C. for phosphorus determinations of the phospholipid classes in the same manner as were the serum lipids. In addition another T. L. C. separation was made in preparation for G. L. C. of total phospholipids and neutral lipids from a pooled liver lipid sample. In the latter separation the thickness of the plates was increased to 1000 μ. and the amount of lipid applied was increased to 1500 μg. per spot. It was necessary to make two plates to provide enough of each fraction for G. L. C. After the separation, the neutral lipids were scraped into a test tube for methylation and the phosphorus-containing spots were scraped into another.

Phosphorus determinations. The phosphorus content of the liver phospholipid classes was analyzed by the same procedure as was used for the serum samples. Calculations of the per cent of total phospholipid and percentages of phospholipid classes were made. Phospholipid concentrations also were calculated on the basis of lipid per whole liver.

Fatty acid analyses. Methylation and G. L. C. were accomplished on total liver lipids, phospholipids, and neutral lipids by the same procedure as described for the oil samples.

VI. MUSCLE ANALYSES

Just prior to lipid extraction the frozen carcasses were thawed at room temperature for one hour. Then the muscle tissue was dissected from the entire carcass and weighed. The muscle lipids were extracted and analyzed for phospholipids and fatty acids in the same way as were the liver lipids.

VII. ADIPOSE TISSUE ANALYSES

The adipose tissue was thawed, weighed, and extracted by the modification of the Ostrander and Dugan (1961) method described above. Separation of phospholipids was not attempted because of the minute amounts present. Methylation and G. L. C. were accomplished on the total lipids by the same procedures as described for the oil samples.

VIII. STATISTICAL ANALYSES

The experimental design was a complete randomized block, and the data were subjected to analysis of variance. Orthogonal comparisons were used for the feed efficiency means and the liver weight means. All other means were compared by application of Duncan's multiple range test (Steel and Torrie, 1960). Significance of difference between means was tested at the 5 per cent level.

CHAPTER IV

RESULTS AND DISCUSSION

I. OILS

The values for fatty acids, hydroxy acids, and viscosities of the oils are presented in Table II. The chief component of the unheated oil was oleic acid, 69.7 per cent. Linoleic acid constituted 22.7 per cent of the oil and palmitic 7.6 per cent. Crampton et al. (1956) reported unhydrogenated soybean oil to contain only about 25.0 per cent oleic acid with approximately 51.0 per cent linoleic, 8.5 per cent linolenic, 2.4 per cent arachidonic acid, and 13.0 per cent total saturates. The light hydrogenation and fractionation in the production of Crystal Oil apparently result in a considerable decrease in polyunsaturation, a roughly corresponding increase in monounsaturation, and some decrease in concentration of saturates.

The fatty acid composition of the commercially-used oil appears to have been affected more by treatment than that of the laboratory-heated oil. The commercially-used oil had larger amounts of myristate, palmitate, and stearate than did the fresh oil, totaling 24.3 per cent as compared with 7.6 per cent; the laboratory-heated oil differed little from fresh oil in its content of saturates. Total unsaturates constituted only 75.7 per cent of the commercially-used oil as compared to 92.4 per cent of the fresh oil; both monoenoic and dienoic fatty acids apparently were decreased with use.

TABLE II

FATTY ACID,[a] HYDROXY ACID,[b] AND VISCOSITY[c] VALUES OF FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[d]

	Oils		
	I	II	III
Fatty Acids:			
C_{14}	0.0	1.6	0.2
C_{16}	7.6	21.0	7.4
$C_{16:1}$	tr	tr	0.2
C_{18}	tr	1.7	0.9
$C_{18:1}$	69.7	61.3	75.8
$C_{18:2}$	22.7	14.4	15.5
Total Saturates	7.6	24.3	8.5
Total Monoenes	69.7	61.3	76.0
Total Unsaturates	92.4	75.7	91.5
Hydroxy Acids	0.0	0.1	0.1
Viscosity (centipoises)	28.6	36.3	38.0

[a]Per cent of total fatty acids. Each value is an average from duplicate chromatograms.

[b]Per cent of total lipid. Each value is an average from duplicate determinations.

[c]Average of three readings taken at 45°C. at three different spindle speeds.

[d]I, II, and III, respectively.

The laboratory-heated oil and the fresh oil were similar in concentrations of total unsaturates, but they differed in relative proportions of monoenoic and dienoic fatty acids. The primary effect of laboratory heating apparently was some decrease in linoleate with a corresponding increase in oleate.

Hydroxy acids were not detectable in the fresh oil and not more than 0.1 per cent was found in the other two samples. Viscosity values were greater for samples II and III than for I. Since increased viscosity generally is associated with increased polymerization (Swern et al, 1964), it appears that the two heated samples were polymerized to about the same extent.

The colors of the oils also were different. The fresh oil was light yellow. The laboratory-heated oil was a dark reddish yellow and the commercially-used oil was a dark brown. No odor was apparent with fresh oil or laboratory-heated oil, but the commercially-used oil had a definite, though non-rancid, odor.

II. ANIMAL GROWTH

Weight gains and feed efficiencies of the rats are shown in Tables III and IV, respectively. Orthogonal comparisons indicated that the slight differences were due to an interaction of the effects of the dietary oil and of restricted feeding. The effect of dietary oil on feed efficiency depended on whether animal feed was restricted or fed ad libitum. The feed efficiency was affected by the oil with feeding at a controlled level (groups I, II, and III) but not with ad libitum feeding (groups Ia, IIa, and III). No significant differences between groups I and II were observed. Keane et al. (1959) found

TABLE III

WEIGHT GAINS[a] OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

Litter	Groups				
	I	Ia	II	IIa	III
1 ♀	120	145	119	145	122
2 ♂	200	211	232	223	234
3 ♀	128	138	127	142	130
4 ♂	190	186	185	222	194
5 ♂	218	221	220	216	231
6 ♂	194	207	196	225	193
Mean	175	185	180	196	184

[a]Weight gain in grams.

[b]Groups I and Ia, II and IIa, and III, respectively. Groups Ia, IIa, and III were fed ad libitum. Groups I and II were restricted to the amount of feed eaten by group III.

TABLE IV

FEED EFFICIENCIES[a] OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

Litter	Groups				
	I	Ia	II	IIa	III
1 ♀	.32	.38	.30	.36	.33
2 ♂	.39	.44	.42	.44	.44
3 ♀	.32	.34	.32	.36	.35
4 ♂	.40	.42	.44	.44	.42
5 ♂	.43	.42	.41	.42	.46
6 ♂	.41	.43	.43	.42	.46
Mean	.38	.41	.39	.41	.41

[a]Feed efficiency = $\frac{\text{weight gain in grams}}{\text{feed intake in grams}}$.

[b]Groups I and Ia, II and IIa, and III, respectively. Groups I and II were restricted to the amount of feed eaten by group III. Groups Ia, IIa, and III were fed ad libitum.

slightly better feed efficiencies with commercially-used cottonseed oil than with fresh oil and a slight depression with laboratory-heated oil. Deuel et al. (1951) found slightly better feed efficiencies with laboratory-heated margarine fat than with fresh margarine fat. Alfin-Slater et al. (1959) found no difference in growth with laboratory-heated soybean oil and fresh oil until after ten weeks on the diet and then there were only slight depressions.

III. SERUM LIPIDS

Serum lipid concentrations are given in Table V. No significant difference was found in the serum lipids from groups I and II, but group III serum had significantly less lipid than did that of the other two groups. Although Friedman et al. (1961) did not have enough data for statistical analysis, their limited data indicated that rat serum lipids were lower when either fresh or heated cottonseed oil was fed at the 20 per cent level in the diet than when they were fed at the 10 per cent level. At the higher level of oil in the diet serum lipids were higher for females fed the heated oil than for those fed the fresh oil, but a difference was not observed with males.

Group III serum lipids also had a significantly higher percentage of phospholipids than did those of the other groups (Table VI). When phospholipid concentrations are expressed as milligrams per milliliter of serum, the mean values are 1.47, 1.50, and 1.49 for groups I, II, and III, respectively, indicating that the differences in total lipid content shown in Table V (page 49) were due to the non-phosphorus-containing lipids. Whether serum cholesterol levels were lower in the animals fed laboratory-heated oil than in the other groups is not known, as serum cholesterol determinations were not carried out.

TABLE V

TOTAL LIPID EXTRACTED FROM SERUM OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[a]

Litter	Groups I	II	III
	mg. lipid/ml. serum		
1 ♀	20.3	16.9	4.9
2 ♂	14.7	11.5	4.2
3 ♀	16.6	9.1	7.7
4 ♂	7.4	11.5	2.6
5 ♂	5.7	4.8	5.1
6 ♂	3.4	5.5	4.6
Mean[b]	11.3	9.9	4.9

[a]Groups I, II, and III, respectively.

[b]Means not joined by a straight line are significantly different.

TABLE VI

PHOSPHOLIPIDS IN SERUM FROM RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[a]

Litter	Groups I	II	III
	Per Cent of Total Serum Lipid		
1 ♀	10.3	13.3	36.9
2 ♂	13.6	17.0	33.4
3 ♀	10.4	14.2	26.8
4 ♂	13.0	13.1	30.6
5 ♂	23.4	20.8	29.4
6 ♂	22.4	19.0	30.3
Mean[b]	15.5	16.2	31.2

[a]Groups I, II, and III, respectively.

[b]Means not joined by a straight line are significantly different.

Recovery of phospholipids from the T. L. C. plates averaged 93 per cent. The per cent of each fraction was calculated from the total of all phosphorus-containing fractions. The phosphorus-containing compounds at the solvent front were not identified. Perhaps this fraction contained cardiolipins. Skipsky et al. (1964) identified small amounts of cardiolipins at the solvent front of their chromatograms of rat liver lipids. Wood and Kinsell (1963) reported a phosphorus-containing fraction at the solvent front of their serum lipid chromatograms, but they did not identify it. It is also possible that some glycerylphosphorylcholine was present. Since there were rather high amounts of lysolecithins in the serum lipids, it would seem feasible that further degradation produced glycerylphosphorylcholine.

No significant differences in the amounts of phospholipid fractions in the serum of the three groups were found except in the content of sphingomyelin (Table VII). Per cent of sphingomyelin was significantly lower in the serum of animals fed laboratory-heated oil than in that of the other groups.

It should be noted that no cephalins were found in the serum. Wood and Kinsell (1963) used T. L. C. for qualitative identifications of phospholipid fractions of different species. Although they did not study rat serum, they found cephalins in human plasma and hog serum, but none in sheep or steer serum. As in the present study, lecithins, sphingomyelin, and lysolecithins, and small amounts of phosphorus-containing compounds at the solvent front were found in all of the samples they studied.

TABLE VII

PHOSPHOLIPID FRACTIONS[a] FROM SERUM LIPIDS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Lecithin Groups			Lysolecithin Groups			Sphingomyelin Groups			Phospholipid at S. F.[c] Groups		
Litter	I	II	III	I	II	III	I	II	III	I	II	III
1 ♀	48.3	41.2	50.2	7.3	24.3	21.8	29.9	19.7	10.2	14.4	14.8	17.9
2 ♂	40.3	44.8	46.2	28.8	23.0	21.5	24.5	23.0	7.0	6.4	9.2	25.4
3 ♀	60.3	46.3	56.0	11.1	19.5	21.7	22.5	13.7	15.1	5.4	20.5	7.2
4 ♂	79.8	40.9	65.0	8.3	22.1	22.5	11.9	11.0	7.3	0.0	26.0	5.2
5 ♂	37.5	56.5	55.0	32.2	35.9	29.9	5.4	6.1	8.4	24.9	1.6	6.7
6 ♂	57.7	61.0	61.8	24.4	25.5	16.3	13.7	13.5	13.3	4.2	0.0	8.7
Mean[d]	54.1	48.5	55.7	18.7	25.1	22.3	18.0	14.5	10.2	9.2	12.0	11.8

[a]Expressed as per cent of total phospholipid.

[b]Groups I, II, and III, respectively.

[c]S. F. = Solvent Front.

[d]Means not joined by a straight line are significantly different.

Vogel et al. (1962) used silicic acid-impregnated paper chromatography to study human serum lipids. They found 66 per cent lecithin, 21 per cent sphingomyelin, 9 per cent lysolecithin, and 3 per cent cephalin. These values are of similar magnitude to those obtained in the present study for rat serum with the exception of cephalin. Doizaki and Zieve (1963) used T. L. C. for human serum studies and found results comparable to those of Vogel et al.

Fatty acid content of the serum lipids of the three groups is given in Table VIII. The only significant differences were with myristic and linolenic acids. Myristic acid was lower in group III than in group I and linolenic acid was greater in group III than in either group I or II. Group differences in total polyenes, total monoenes, and total saturates were not significant.

IV. LIVER LIPIDS

Orthogonal comparisons of liver weight data showed that the increases in liver weights were due to the level of feed intake and not to the kind of oil incorporated into the diets (Table IX). With no restriction on feed intake (groups Ia, IIa, and III) liver weight increased with body weight. When feed was restricted (groups I and II) liver weight did not increase proportionally with body weight. All values for liver weight as per cent of body weight are in agreement with values for rats fed unheated groundnut oil, sesame seed oil, and coconut oil (Raju and Rajagopalan, 1955); fresh corn oil, hydrogenated castor oil acids, and commercial shortening (Perkins et al.,

TABLE VIII

FATTY ACID CONCENTRATIONS[a] IN SERUM LIPIDS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Groups		
	I	II	III
Fatty Acids:			
C_{12}	0.3	0.3	0.1
C_{14}	1.8	1.2	0.7
C_{16}	28.5	31.1	19.8
$C_{16:1}$	0.2	0.5	0.5
C_{18}	8.4	8.5	8.3
$C_{18:1}$	31.3	28.7	33.9
$C_{18:2}$	19.4	15.3	19.5
$C_{18:3}$	3.2	4.9	7.8
$C_{20:4}$	7.0	7.8	9.5
Total Saturates	38.8	41.1	28.9
Total Monoenes	31.5	29.3	34.4
Total Polyenes	29.6	28.0	36.7

[a]Per cent of total fatty acids. Each value is an average of the values for six animals in each group. Means not joined by a straight line are significantly different.

[b]Groups I, II, and III, respectively.

TABLE IX

LIVER WEIGHTS[a] OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

Litter	Groups				
	I	Ia	II	IIa	III
1 ♀	2.93	3.90	2.80	4.07	3.49
2 ♂	3.87	3.50	3.82	3.88	3.73
3 ♀	3.19	4.04	2.91	3.56	3.56
4 ♂	3.82	3.78	3.33	3.86	3.87
5 ♂	3.60	3.71	3.66	3.83	3.80
6 ♂	3.81	4.09	3.89	3.91	4.39
Mean	3.54	3.84	3.40	3.85	3.81

[a]Expressed as per cent of body weight.

[b]Groups I and Ia, II and IIa, and III, respectively. Groups I and II were restricted to the same amount of feed eaten by group III. Groups Ia, IIa, and III were fed ad libitum.

1961b); fresh cottonseed oil (Poling et al., 1962); and fresh milk fat and fresh lard (Bhalerao et al., 1961).

No significant difference was found in total lipid content of the livers when expressed either as per cent of liver tissue or as milligrams of lipid per liver (Table X). The small differences in milligrams lipid per liver reflect the differences in feed efficiency. Group III grew more on a given amount of feed and thus had larger livers with more lipid than the other groups. The data on a percentage basis agree with the findings of Johnson et al. (1957) in their study of heated corn oil fed to rats. However, all of the per cent lipid values are higher than those of approximately 4 per cent reported for animals fed fresh oils (Raju and Rajagopalan, 1955 and Johnson et al., 1957). Griffin and Wade in 1939 studied "normal" rats. From a series of studies they concluded that above 4.01 per cent lipid in the liver should be considered an abnormally high amount. In view of these studies, the liver lipid contents reported here might be considered high. However, Channon and Wilkinson (1936) used a 40 per cent fat diet for rats and found lipids to constitute approximately 6 per cent of liver weight when beef drippings, palm oil, coconut oil, and olive oil were fed. With butter and cod liver oil diets, the livers contained approximately 3.5 per cent lipid. Fruton and Simmonds (1960) reported 5 per cent lipid in the livers as normal.

Phospholipids as per cent of total lipid also were not significantly different between groups (Table XI). When calculated as milligrams of phospholipid per total liver, there was a significantly

TABLE X

TOTAL LIPIDS EXTRACTED FROM LIVERS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[a]

Litter	Per Cent of Liver Tissue (Wet Weight Basis) Groups I	II	III	Mg. Lipid/Liver (Wet Weight Basis) Groups I	II	III
1 ♀	7.39	6.87	5.93	333.2	298.4	327.6
2 ♂	4.53	4.90	5.16	412.2	501.6	519.1
3 ♀	6.87	7.60	6.83	346.5	346.5	383.6
4 ♂	4.59	5.38	4.75	385.4	392.1	418.0
5 ♂	4.62	6.18	5.39	419.7	572.0	541.2
6 ♂	4.79	4.48	4.95	420.3	401.9	497.7
Mean[b]	5.46	5.90	5.50	386.2	418.8	447.8

[a]Groups I, II, and III, respectively.

[b]Means joined by a straight line are not significantly different.

TABLE XI

PHOSPHOLIPIDS FROM LIVERS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[a]

Litter	Per Cent of Liver Lipid (Wet Weight Basis) Groups			Mg. Phospholipid/Liver (Wet Weight Basis) Groups		
	I	II	III	I	II	III
1 ♀	35.06	30.94	46.41	117	112	153
2 ♂	44.12	25.26	29.51	181	127	153
3 ♀	39.25	40.91	46.48	138	142	178
4 ♂	42.66	35.82	50.85	164	140	213
5 ♂	44.78	51.61	58.27	188	295	316
6 ♂	45.65	42.25	43.30	192	170	215
Mean[b]	41.92	37.80	45.80	163	164	204

[a]Groups I, II, and III, respectively.

[b]Means not joined by a straight line are significantly different.

greater amount in the group III livers than in the other two groups. This was probably only a reflection of the increased size of the livers since phospholipids are considered to be a part of the cell structure. The concentrations of liver lecithin, cephalin, sphingomyelin, and lysolecithin are given in Table XII. There is very little difference in the per cent of any class of phospholipids between groups except for lysolecithin. None of this compound could be detected in the livers of animals from groups I and II, but group III had an average of 6.8 per cent of total phospholipid as lysolecithin. Although it has been found that lysolecithin may be formed in lipid preparations during storage or during some laboratory procedures (Vogel and Zieve, 1962), group III samples were stored and analyzed in the same manner as were the other samples. There is no reason to believe that the higher content of lysolecithin in this group of samples was a result of laboratory treatment.

Analysis of variance of the liver fatty acids showed significantly larger amounts of saturates and smaller amounts of monoenes in group II than in groups I and III (Table XIII). The higher saturation in group II was reflected in palmitic acid content. Total polyenes were not affected significantly by the diet. There was, however, somewhat less linoleic acid in the liver lipids of groups II and III than in those of group I. No linolenate was found in any group. This agrees with previous findings that linolenate concentration is very small in the livers unless the acid is fed (Mohrhauer and Holman, 1963).

TABLE XII

PHOSPHOLIPID FRACTIONS[a] FROM LIVERS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

Litter	Lecithin Groups			Cephalin Groups			Sphingomyelin Groups			Lysolecithin Groups		
	I	II	III	I	II	III	I	II	III	I	II	III
1 ♀	48.8	44.0	48.0	47.9	41.2	31.5	8.3	14.8	13.1	0.0	0.0	7.5
2 ♂	49.5	57.1	47.2	31.6	35.5	30.6	18.9	7.4	17.4	0.0	0.0	4.8
3 ♀	64.7	61.0	55.6	27.4	32.3	26.8	7.9	6.7	10.3	0.0	0.0	7.3
4 ♂	65.3	61.3	59.3	27.1	28.5	20.8	7.6	10.2	13.0	0.0	0.0	6.9
5 ♂	74.8	59.9	56.6	18.5	27.8	28.8	6.8	12.2	7.2	0.0	0.0	7.4
6 ♂	57.9	61.8	56.2	28.1	31.4	24.9	14.0	6.8	11.9	0.0	0.0	7.0
Mean[c]	59.0	57.5	53.8	30.1	32.8	27.2	10.6	9.7	12.1	0.0	0.0	6.8

[a]Expressed as per cent of total phospholipid.

[b]Groups I, II, and III, respectively.

[c]Means joined by a straight line are not significantly different.

TABLE XIII

FATTY ACID CONCENTRATIONS[a] IN LIVER LIPIDS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Groups		
	I	II	III
Fatty Acids:			
C_{12}	tr	tr	0.1
C_{14}	0.4	0.4	0.3
C_{16}	19.3	28.7	21.5
$C_{16:1}$	0.2	0.5	0.3
C_{18}	10.8	11.1	10.1
$C_{18:1}$	42.9	37.2	45.1
$C_{18:2}$	16.1	12.2	14.9
$C_{20:4}$	10.4	9.9	7.7
Total Saturates	30.4	40.2	32.0
Total Monoenes	43.1	37.7	45.4
Total Polyenes	26.5	22.1	22.6

[a]Per cent of total fatty acids. Each value is an average of the values for six animals in each group. Means not joined by a straight line are significantly different.

[b]Groups I, II, and III, respectively.

Fatty acid concentrations in the livers varied between groups in the same direction as did those of the dietary oils although fatty acids of the livers were much more saturated than were those of the oils. Diet I, with its higher level of linoleic acid (Table II, page 44), did not produce a significantly higher level of arachidonic acid than did the other two diets. Mohrhauer and Holman (1963) in their study with different levels of ethyl linoleate (.009, 1.26, and 4.875 per cent of calories) showed that an increase of the acid in the diet produced a higher amount of arachidonic acid in the livers. They were using linoleate alone, however, and had no interactions of other acids.

Bhalerao et al. (1961) found 13 per cent arachidonic acid in the liver lipids of rats fed a diet containing milk fat with a linoleic acid content of only 1.2 per cent. With a diet containing corn oil, 54 per cent linoleic acid, the rat liver lipids contained only 10 per cent arachidonic acid. With this exception their fatty acid values for liver lipids reflected the fatty acid values of their diet.

The data for the fatty acids in the phospholipids and neutral lipids of the three groups of livers are presented in Table XIV. Although no statistical analyses of the pooled samples were possible, it may be seen that the phospholipids contained all of the arachidonic acid. Total saturates also were greater in the phospholipids than in the neutral lipids and total monoenes were lower. The relatively high concentration of palmitic acid observed in the liver total lipids of group II is seen here in both the phospholipids and the neutral lipids. The difference between groups in linoleic acid concentration is more pronounced in the neutral lipids than in the phospholipids.

TABLE XIV

FATTY ACIDS[a] IN NEUTRAL LIPIDS AND PHOSPHOLIPIDS FROM POOLED LIVERS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Phospholipids Groups			Neutral Lipids Groups		
	I	II	III	I	II	III
Fatty Acids:						
C_{12}	0.0	0.0	0.0	0.3	0.0	0.1
C_{14}	0.4	tr	0.6	0.1	0.5	0.6
C_{16}	19.5	28.8	24.5	21.1	39.5	21.6
$C_{16:1}$	0.2	0.1	0.4	0.9	1.8	1.7
C_{18}	19.9	23.6	12.4	2.5	1.0	tr
$C_{18:1}$	30.8	21.5	29.8	50.4	47.4	52.6
$C_{18:2}$	16.5	12.3	19.5	24.8	9.9	23.4
$C_{20:4}$	12.8	13.8	12.9	0.0	0.0	0.0
Total Saturates	39.7	52.3	37.5	24.0	41.0	22.3
Total Monoenes	31.0	21.6	30.2	51.2	49.2	54.4
Total Polyenes	29.3	26.1	32.4	24.8	9.9	23.4

[a]Per cent of total fatty acids. Each value is the average from duplicate determinations.

[b]Groups I, II, and III, respectively.

V. MUSCLE LIPIDS

On the wet weight basis, differences in percentage lipid in the muscle tissues of the three groups of animals were not significant (Table XV). Values ranged from 3 to 9 per cent and varied more within groups than between groups. No difference in phospholipid content, as per cent of total lipid, was found in the muscles (Table XVI). Percentages of phospholipid fractions also were not different among the three groups (Table XVII). Approximately 60 per cent of the muscle phospholipid was lecithin, 32 per cent was cephalin, and 8 per cent was sphingomyelin. These percentages are very similar to those for beef muscle (Turkki, 1965).

Fatty acid content of the muscles was little affected except for palmitoleic acid and linoleic acid (Table XVIII). Group I had less palmitoleic acid than did groups II and III. Group II had less linoleic acid than the other groups. Neither total saturates nor monoenes were very different in the three groups. No arachidonate was detected in the total lipid samples. When Perkins et al. (1961a) compared carcass fatty acids from rats fed corn oil and heated corn oil, they found decreases in linoleate and increases in oleate in the rats fed heated corn oil.

The six muscle lipid samples from each group were pooled and fatty acids were determined in the phospholipids and neutral lipids. No statistical analyses were possible. The percentages are given in Table XIX. In the phospholipid fraction an unidentified compound

TABLE XV

TOTAL LIPID EXTRACTED FROM MUSCLE TISSUE OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[a]

Litter	Groups I	Groups II	Groups III
	Per Cent Lipid (Wet Weight Basis)		
1 ♀	7.1	9.1	6.2
2 ♂	4.4	4.7	5.1
3 ♀	5.8	6.4	5.2
4 ♂	3.2	4.0	4.2
5 ♂	4.4	4.0	5.4
6 ♂	4.0	4.3	4.0
Mean[b]	4.8	5.4	5.0

[a]Groups I, II, and III, respectively.

[b]Means joined by a straight line are not significantly different.

TABLE XVI

PHOSPHOLIPIDS IN MUSCLE LIPID FROM RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[a]

Litter	Groups I	II	III
	Per Cent of Total Muscle Lipid		
1 ♀	7.9	5.8	9.0
2 ♂	8.4	6.1	7.2
3 ♀	7.7	9.1	9.3
4 ♂	8.9	7.9	8.2
5 ♂	9.6	10.6	7.9
6 ♂	9.9	7.9	7.8
Mean[b]	8.7	7.9	8.2

[a]Groups I, II, and III, respectively.

[b]Means joined by a straight line are not significantly different.

TABLE XVII

PHOSPHOLIPID FRACTIONS[a] FROM MUSCLE TISSUE LIPIDS OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Lecithin			Cephalin			Sphingomyelin		
	Groups			Groups			Groups		
Litter	I	II	III	I	II	III	I	II	III
1 ♀	63.2	63.5	64.0	27.6	31.7	31.3	9.2	4.8	4.7
2 ♂	51.3	56.5	63.4	40.4	32.3	31.7	8.2	11.2	4.9
3 ♀	60.3	64.4	64.0	25.7	29.0	26.9	14.1	6.6	9.1
4 ♂	59.5	63.0	60.2	32.2	29.9	34.0	8.4	7.2	5.9
5 ♂	61.5	60.6	62.5	33.9	33.0	32.2	4.6	6.4	5.3
6 ♂	64.6	64.2	65.1	30.8	29.4	31.0	4.6	15.0	3.9
Mean[c]	60.1	62.0	63.2	31.8	30.9	31.2	8.2	8.5	5.6

[a]Expressed as per cent of total phospholipid.

[b]Groups I, II, and III, respectively.

[c]Means joined by a straight line are not significantly different.

TABLE XVIII

FATTY ACIDS[a] IN MUSCLE LIPIDS FROM RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Groups		
	I	II	III
Fatty Acids:			
C_{12}	0.2	0.2	0.3
C_{14}	1.3	1.8	1.8
C_{16}	26.7	29.4	23.3
$C_{16:1}$	1.9	3.7	2.9
C_{18}	2.1	2.5	1.9
$C_{18:1}$	53.3	52.2	53.2
$C_{18:2}$	14.6	10.2	16.6
Total Saturates	30.2	34.0	27.3
Total Monoenes	55.2	55.9	56.2

[a]Per cent of total fatty acids. Each value is an average of the values for six animals in each group. Means not joined by a straight line are significantly different.

[b]Groups I, II, and III, respectively.

TABLE XIX

FATTY ACIDS[a] IN NEUTRAL LIPIDS AND PHOSPHOLIPIDS FROM POOLED MUSCLES OF RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Phospholipids Groups			Neutral Lipids Groups		
	I	II	III	I	II	III
Fatty Acids:						
C_{12}	tr	0.0	0.0	0.2	0.9	0.4
C_{14}	0.5	1.6	0.5	1.2	2.8	2.7
$C_?$	0.5	2.5	1.2	0.0	0.0	0.0
C_{16}	42.4	39.0	33.8	18.3	29.8	22.5
$C_{16:1}$	0.4	0.6	0.5	2.0	5.2	4.3
C_{18}	8.1	15.3	9.4	0.3	0.0	0.0
$C_{18:1}$	14.8	14.5	18.8	55.0	42.1	54.5
$C_{18:2}$	24.3	18.5	26.7	23.1	19.3	15.6
$C_{20:4}$	9.2	8.0	9.0	0.0	0.0	0.0
Total Saturates	50.8	55.9	43.7	20.0	33.5	25.6
Total Monoenes	15.2	15.1	19.4	57.0	47.3	58.8
Total Polyenes	33.5	26.5	35.7	23.1	19.3	15.6

[a]Per cent of total fatty acids. Each value is an average from duplicate determinations.

[b]Groups I, II, and III, respectively.

appeared just before palmitic acid on the chromatogram. It did not appear in the neutral lipid or the total lipid chromatograms. This compound has been observed previously in the phospholipid fractions of beef muscle lipid in this laboratory (Cook, 1963; Nutt, 1963; Taylor, 1964; and Campbell and Turkki, 1965). Stearate was essentially lacking in the muscle lipid. All of the laurate was in the neutral lipids. Arachidonate, which was present in the total lipids in such small quantities that it was not detected, was measurable in the phospholipids.

The phospholipids were more polyunsaturated than the neutral lipids. They also contained more saturates. The neutral lipids were very much higher in monoenes than were the phospholipids. Sinclair (1932) when feeding fat-free diets and cod liver oil diets observed that the animals fed cod liver oil had the higher levels of unsaturated acids in muscle phospholipids. Although no statistical analyses were possible with the pooled samples, phospholipids of groups I and III appeared to be more unsaturated than those of group II.

VI. ADIPOSE TISSUE LIPIDS

Adipose fatty acid data are presented in Table XX. Group II adipose tissue was consistently different from that of the other two groups; the acids varied in much the same direction as they did in the dietary oils (Table II, page 44).

Lauric acid was present in small amounts in all three groups even though no laurate was detected in the oils. Myristic acid also

TABLE XX

FATTY ACIDS[a] IN ADIPOSE TISSUE FROM RATS FED FRESH OIL, COMMERCIALLY-USED OIL, AND LABORATORY-HEATED OIL[b]

	Groups		
	I	II	III
Fatty Acids:			
C_{12}	0.2	0.2	0.2
C_{14}	0.9	2.1	1.1
$C_{14:1}$?	0.0	0.3	0.0
C_{16}	14.4	26.7	16.0
$C_{16:1}$	1.2	3.9	1.7
C_{18}	0.0	0.3	0.0
$C_{18:1}$	68.8	57.1	68.2
$C_{18:2}$	14.6	9.3	12.8
Total Saturates	15.5	29.4	17.3
Total Monoenes	70.0	61.3	69.3

[a]Per cent of total fatty acids. Each value is an average of the values for six animals in each group. Means not joined by a straight line are significantly different.

[b]Groups I, II, and III, respectively.

was deposited in each group, but group II had significantly more than did groups I and III. The data confirm the statement of Deuel (1955) that myristate ordinarily constitutes only 1 per cent or less of the fatty acids in rat adipose tissue unless it is fed in the diet. Eckstein (1929) was able to show that relatively large amounts of myristate were laid down in the tissues when the acid was fed. Channon et al. (1937) found similar results when they fed coconut oil to rats. Since there was a larger amount of myristic acid present in oil II, the increase in myristic acid in the adipose tissue of the animals fed the oil is not unusual.

In the group II adipose samples a peak appeared on the chromatogram where tetradecenoic acid would be expected to appear. This peak was not seen in the samples from either group I or III. Lovern (1940) reported that the administration of ethyl myristate to eels was followed by an increased tetradecenoic acid content in the eel fat, but that no increase in myristic acid occurred. From his study it would seem possible that the compound reported here was tetradecenoic acid resulting from an increased intake of myristic acid.

Lovern also found an increase in palmitic acid and palmitoleic acid when ethyl palmitate was fed. Since there was a much larger amount of palmitic acid in oil II of this study, the larger amounts of palmitic acid and palmitoleic acid in the adipose tissue of group II appear to confirm Lovern's findings with ethyl palmitate.

Stearic acid was deposited only to a small extent and only in group II animals. Stearic acid also was in greater concentration in oil II than in the other two oils.

Of all the fatty acids found in adipose tissue of rats, oleic acid repeatedly has been found to be in highest concentration (Longenecker, 1939; Banks et al., 1933; Kaunitz et al., 1961; Spadola and Ellis, 1936; and Deuel, 1955). The concentration of this fatty acid in tissue lipids is less influenced by diet than any other unsaturated acid because it may be deposited directly from the diet or it may be synthesized or both (Deuel, 1955). Since the oleic acid in group II adipose fatty acids was significantly less than in groups I and III, and since it also was in smaller concentrations in oil II, it would appear that the diet did influence its deposition in this case. It is noteworthy, however, that oil III had considerably more oleate than did oil I and the deposition of the acid in these two groups of animals was not different.

Kaunitz et al. (1961) were able to show that the addition of as little as 2 per cent linoleate to purified rat diets containing 20 per cent of a mixture of long-chain saturated triglycerides (5 per cent C_{10}, 45 per cent C_{12}, 25 per cent C_{14}, 8 per cent C_{16}, and 16 per cent C_{18}) produced deposition in adipose tissue of 6 per cent linoleate. Linoleate in the present study constituted approximately 15 per cent of oils II and III and approximately 22 per cent of oil I; however, linoleate in the adipose tissue of the animals fed oil III was not significantly different from that of those fed oil I. Group II adipose lipid was significantly lower in linoleate than that of group I.

Spadola and Ellis (1936) fed cottonseed oil before and after hydrogenation to rats at the 8 per cent level in the diet and found

that the amount of linoleic acid deposited was proportional to the amount of the acid in the oil. They also reported that the ingestion of varying amounts of unsaturated acid affected the saturated depot fatty acids less than it did the unsaturated acids. Total saturates in the present study, however, were as much higher in group II, as compared to groups I and III, as total unsaturates were lower.

Longenecker (1939) studied the effects of ingestion of corn oil on the adipose tissue fatty acids of rats and found that the rats deposited fatty acids with concentrations almost identical to those of the corn oil fed. Although the lipids of the adipose tissue lipids were not as unsaturated as were the dietary oils in the present study, the fatty acid data do support Longenecker's conclusion that the concentrations of depot fatty acids are regulated by dietary fatty acid concentrations.

VII. SUMMARIZATION OF EFFECTS

Since differences among groups with respect to feed efficiencies and growth were slight, it seems apparent that the diets did not produce a toxic effect in the rats. Also no difference in liver weights as per cent of total body weight could be attributed to the feeding of heated oils.

The Crystal Oil apparently was not damaged enough by heating to produce detrimental effects on the health of the rats, even though the commercially-used oil had been used for thirty-nine days. Perhaps the daily addition of fresh oil, the agitation of the oil, and the

presence of food during frying processes counteracted damaging effects of heat treatment. Apparently the 48-hr. laboratory heating period was not sufficiently long to produce toxic polymers. Previous studies have shown that fats with high concentrations of saturated fatty acids were not as susceptible to heat damage, when nutritional value was estimated by animal feeding trials, as were more unsaturated oils (Johnson et al., 1956; Kaunitz et al., 1955b; Keane et al., 1959). In the present study, the light hydrogenation seemed to result in a higher proportion of oleate to polyunsaturated acids in the Crystal Oil than in the original oil. When Johnson et al. (1957) studied the effects of linoleate concentrations and of total unsaturation of oils on rat growth, they suggested that a dilution of linoleic acid double bonds by oleic acid double bonds might decrease the polymerization that occurred during heating. This suggestion might hold true for the present study and account for the fact that no growth depression was observed when the heated oils were fed; however, it does not account for the high feed efficiency when laboratory-heated oil was fed. Also, viscosity measurements did not indicate that less polymerization had occurred in the laboratory-heated oil than in the commercially-used oil.

Tissue lipid concentrations, as a result of feeding the heated oils, appeared to be affected by the manner in which the oil was heated. The fatty acid concentrations in the tissues were affected by the commercially-used oil more than by the laboratory-heated oil; the percentage total lipids, percentage phospholipid, and percentages of

phospholipid fractions were affected more by laboratory-heated oil than by commercially-used oil. To a certain extent the fatty acid proportions of the tissue lipids reflected the fatty acid proportions of the commercially-used oil. Although the values were significantly different only in liver and adipose tissue, in all tissues the lipids of the animals fed commercially-used oil were more saturated than were those of the animals fed the other oils. Also monoenes tended to be lower in the tissue lipids of the animals fed commercially-used oil than in those of the animals fed fresh or laboratory-heated oil.

Liver and adipose tissue fatty acid concentrations appeared to have been more affected by commercially-used oil than were those of serum and muscle tissue. Deuel (1955) states that blood and liver are most susceptible to alterations resulting from the fat consumed and that storage fats are more slowly altered by dietary means. The animals of the present study had been fed the experimental diets for six weeks, possibly long enough to adjust to the different dietary oils. Whether a greater difference in serum lipids could have been shown if samples of blood had been obtained at different stages of the rats' growth is not known.

The feeding of laboratory-heated oil appeared to affect total serum lipids, per cent phospholipids in serum lipids, per cent sphingomyelin in serum phospholipids, and per cents of myristate and linolenate in the serum fatty acids. The differences observed in per cent lipid in the serum were apparently due to relatively low levels of non-phosphorus-containing lipids in the rats fed laboratory-heated

oil. Sphingomyelin concentration in the phospholipids were significantly lower in the serum of the animals fed laboratory-heated oil than in that of the other animals and lysolecithin concentration was significantly higher. None of these effects, apparently brought about by the feeding of laboratory-heated oil, appear to be related to fatty acid content, hydroxy acid content, or viscosity of the oil since the values for each were similar to those for one or both of the other oils (Table II, page 44).

Although liver lipid concentrations and per cent phospholipid in the liver did not appear to be affected by feeding laboratory-heated oil, lysolecithin was detected only in the animals fed this oil. The effect could not be related to any known factor in the oil. Muscle and adipose tissue lipids did not appear to be affected appreciably by the feeding of laboratory-heated oil.

It cannot be said whether growth depression could have been shown in the animals fed the heated oils if they had been kept on the diets longer or whether the observed effects in the lipids are indicative of damage or alterations. However, it may be said that feeding heated oils to rats, even when no visible effects were apparent, definitely produced different concentrations of the various lipid components of the rat tissues, indicating that perhaps lipid metabolism is altered.

The findings of this study suggest that further work should be done with tissue lipids of rats which have been fed heated fats. Since results of studies concerning the feeding of polyunsaturated oils to

rats in other laboratories have been contradictory and since no toxicity was observed in this study with a lightly hydrogenated oil, it would seem desirable to compare unhydrogenated soybean oil with Crystal Oil under controlled conditions, i.e., with the same heating temperature and time and at the same level in the diet. It might be possible then to correlate unsaturation, heating time, and/or level in the diet to the tissue lipid levels. This might be done with either laboratory-heated or commercially-used oil with samples taken at different times during its use. It also would be desirable to have enough animals in the study to analyze tissue lipids at different stages of the growth period. If this were done, some indication of increases or decreases in serum and liver lipid levels in the individual animal could be obtained. Another phase of such investigations should include analyses of cholesterol levels in the serum and livers after the feeding of heated oils.

CHAPTER V

SUMMARY

In order to investigate the effects of the ingestion of heated oils on tissue lipids, weanling Wistar rats were fed fresh oil, commercially-used oil, and laboratory-heated oil for six weeks. The oil used was a new product, a partially fractionated, lightly hydrogenated soybean oil produced specifically for frying.

Oil samples were analyzed for fatty acid content, hydroxy acid content, and viscosity. Feed efficiency data were obtained weekly. After the rats were killed, livers were weighed and calculations of liver weight as per cent of total body weight were made. Analyses for total lipid, total phospholipids, percentages of phospholipid fractions, and percentages of fatty acids were carried out on animal serum, liver, muscle, and adipose tissue lipids.

The Crystal Oil contained primarily oleic acid, linoleic acid, and palmitic acid. Commercially-used oil had more myristic, palmitic, and stearic acids than did the fresh oil. Total unsaturates constituted only 75.7 per cent of the oil as compared to 92.4 per cent for the fresh oil. Laboratory-heated oil was similar to fresh oil in fatty acid composition, although there was some decrease in linoleate and increase in oleate with the laboratory heating. Viscosity was greater for both commercially-used and laboratory-heated oil than for the fresh oil. Very small amounts of hydroxy acids were detected in the heated oils but not in the fresh oil.

Feed efficiencies were found to be slightly higher for the rats fed laboratory-heated oil than for the other groups. No growth depression or apparent toxicity was noted as a result of feeding heated oil. Liver weights also were not affected.

Serum lipid concentrations were lower for animals fed laboratory-heated oil than for those fed the other oils. Phospholipids as per cent of total lipids were highest in this group, but calculation of milligrams phospholipid per milliliter of serum indicated that the differences actually were in the non-phosphorus-containing lipids. Phospholipid fractions of serum lipids were not significantly different between groups except for sphingomyelin, which was lower in the animals fed laboratory-heated oil than in the other animals. Higher amounts of linolenate and lower amounts of myristate were found in the serum lipids of rats fed laboratory-heated oil; the other fatty acid contents were not significantly different from those of the other groups.

No significant difference between groups was found in the total lipid concentrations or phospholipid concentrations. Lysolecithin was detected in the liver lipids of the animals fed the laboratory-heated oil, but not in the other groups. Fatty acids of the liver lipids varied between groups in the same direction as did the dietary oils, but the liver fatty acids were more saturated. Arachidonate and total saturates were in higher concentrations in the liver phospholipids of all groups than in the neutral lipids, and monoenes were lower.

In the muscle lipids, per cent lipid, per cent total phospholipid, and per cents of phospholipid fractions were not different

between groups. Neither total saturated nor unsaturated fatty acid concentrations were very different in the muscle lipids. Animals fed laboratory-heated oil and those fed commercially-used oil had higher palmitate content in the muscle lipids than did those fed fresh oil. Animals fed commercially-used oil had less linoleate than the other two groups.

Striking differences were observed in the fatty acid concentrations of the adipose tissues. The animals fed commercially-used oil deposited more myristate, palmitate, palmitoleate, and stearate, and less oleate and linoleate than did the other groups. The fatty acid concentrations of adipose tissues varied in the same direction as did the fatty acid content of the dietary oils.

The observed effects seem to indicate that even when no visible harmful effects are noted in animals fed heated fats, some alteration in lipid metabolism may occur, the alteration depending upon the conditions of heat treatment.

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