• *Technical*

Influence of Feeding Fractionated Esters of Autoxidized Lard and Cottonseed Oil on Growth, Thirst, Organ Weights, and Liver Lipids of Rats[1,2]

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THE NUTRITIONAL properties of autoxidatively and thermally polymerized fats have been given considerable attention. Such studies were usually undertaken with the objective of discovering what chemical changes or classes of compounds were associated with toxicity. The large number of products resulting from autoxidative or thermal polymerization of fats and oils made isolation of individual substances impracticable, but fractions have been isolated and used in feeding studies to assess their effect on the animal (1).

In our earlier work it was observed that feeding fats autoxidized fats and, in particular, the polymeric residues from autoxidized fats increased the caloric requirements for weight maintenance (2). This suggested studies of other "pharmacological" effects. It was thought that the substances responsible for these effects could be separated and/or concentrated by more detailed fractionation. Also, by utilizing other biological criteria, it was hoped that the pharmacological effects could be related to structural types. Therefore autoxidatively polymerized lard and cottonseed oil were fractionated by high-vacuum distillation and urea-complex-separation techniques. The fractions were fed to rats, which were observed for growth, fatalities, water intake, organ weights, liver lipids, and liver and serum cholesterol levels. Some of the fractions, as well as the toxic fractions proved to be of biological interest.

Experimental Procedures

Commercial samples of winterized cottonseed oil (hereafter CSO) and of prime steam lard (containing no antioxidant) were both oxidized with vigorous streams of oxygen at a temperature of 95–100°C. for 210 hrs. The oxidations were conducted in 12-liter flasks in batches of 8 to 10 kg.

A flowsheet summarizing the preparation of the fractions is given as Figure 1. Most of the autoxidized lard and autoxidized CSO were fractionated by first subjecting them to molecular distillation through a falling film, cyclic type of still. The distillate fractions were collected up to a temperature of 275°C. at a pressure of 6 to 12 microns. The autoxidized lard yielded 54% distillate and 46% polymeric residue; the autoxidized CSO gave 42% distillate and 52% polymeric residue. The distillates and residues were saponified by refluxing with aqueous NaOH in alcohol and acidified; the resulting acids were esterified with ethanol.

The ethyl esters of the molecular distillate from autoxidized lard were fractionally distilled through a 2 × 20-in. Vigreux column to yield Distillate 1, MDDD (1%, 65–120°C./2 mm.); Distillate 2 (44%, 160–180°C./0.3 mm.); and a residue, MDDR (8%). (The yields of the various fractions are percentages of the autoxidized material originally charged to the molecular still.) Distillate 2 was separated into four fractions by urea complex formation and alembic distillations: Complex-Distillate, MDCD (33%), Complex-Residue, MDCR (1%), Noncomplex Filtrate-Distillate, MDFD (4%), and Noncomplex Filtrate-Residue, MDFR (2%). The alembic distillations in this report were conducted in a high-vacuum, short-path apparatus with an alembic type of distillate collector at pressures of less than 0.1 mm. of mercury.

The ethyl esters of the molecular distillation residue from autoxidized lard were molecularly distilled to give the following fractions: Distillate 1, MRMD1 (23%, 100–150°C.); Distillate 2, MRMD2 (9%, 150–225°C.); and a Residue, MRMR (14%). The pressure at the start of distillation was 20 microns. It decreased to 8 microns as the more volatile materials were removed.

The ethyl esters of the molecular distillate from autoxidized CSO were fractionated by urea-complex formation, followed by alembic distillation of the complex- and noncomplex-forming portions. The resulting fractions were: Complex-Distillate, MDCD (22%); Complex-Residue, MDCR (2%); Noncomplex Filtrate-Distillate, MDFD (7%); and Noncomplex Filtrate-Residue, MDFR, MDFR (7%).

The ethyl esters of the molecular distillation residue from autoxidized CSO were subjected to an alembic distillation to give a monomeric distillate, MRAD (16%). The large quantity of material remaining as the residue from this distillation was further separated by means of molecular distillation to give an apparently dimeric distillate, MRMD (11%), and a residue of higher polymeric materials, MRMR (23%).

The fractions were analyzed for the characteristics given in Table I (3). Molecular weights were deter-

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TABLE I

Chemical Properties of Fractions of Autoxidized Lard and Cottonseed Oil

Sample [a]	Acid No.	Sap. No.	Iodine No.	% Hydroxyl oxygen	% Carbonyl oxygen	Mol. wt.	Fatty acid chain-length
Lard fractions							
MDDD	32	219	38	0.4	2.2	.....	C_{18} and shorter
MDDR	22	193	44	1.1	0.9	350	C_{18}
MDCD	5	192	27	0	0.3	.....	C_{18}
MDCR	8	188	67	1.1	2.0	800	Dimer and trimer of C_{18}
MDFD	34	197	67	0.4	0.6	.....	C_{18}
MDFR	25	191	53	1.7	1.3	1100	Trimer of C_{18}
MRMD1	5	199	30	1.0	0.5	.....	C_{18}
MRMD2	15	223	48	2.8	1.3	1000	Trimer of C_{18}
MRMR	14	240	47	3.0	1.4	1500	Higher mol. wt.
CSO fractions							
MDCD	5	192	45	0.4	0.2	.....	C_{18}
MDCR	15	201	67	1.6	1.5	850	Dimer of C_{18}
MDFD	18	192	121	1.0	0.4	.....	C_{18}
MDFR	16	185	73	2.1	0.9	640	Dimer of C_{18}
MRAD	8	202	58	0.8	0.8	.....	C_{18}
MRMD	9	196	70	3.8	0.9	550	Dimer of C_{18}
MRMR	8	209	70	2.5	0.8	1500–2000	Higher mol. wt.

[a] As can be seen from the flowsheet, the first two letters of each fraction (MD and MR) indicate whether the fraction was obtained from the original molecular distillate or residue. The meaning of the last two letters can also be gained from the flowsheet, e.g., MRMR is the molecular residue of the original residue, and MRMD, its distillate.

mined by the comparative ebulliometric method, using two ebulliometers and a 10-junction differential iron-constantan thermopile (4).

All fractions were incorporated at a level of 8% in a purified rat diet containing 30% casein (G.B.I. Vitamin-Free Test Casein), 56% dextrose (Cerelose), 4% salts (U.S.P. XIII), 2% cellulose (Alphacel), and, per kilogram of diet, 1 g. of choline dihydrogen citrate, 1 g. of inositol, 300 mg. of p-aminobenzoic acid, 100 mg. of nicotinamide, 2 mg. of thiamine hydrochloride, 4 mg. of riboflavin, 4 mg. of pyridoxine, 10 mg. of calcium pantothenate, 2.5 mg. of folic acid, 5 micrograms of vitamin B_{12}, 25 micrograms of biotin, 10 mg. of synthetic vitamin K, 25 mg. of ascorbic acid, and 1 cc. of a linoleic acid suspension containing 5 mg. of *beta*-carotene, 50 mg. of *alpha*-tocopherol acetate, 10 mg. of free *alpha*-tocopherol, and 0.5 mg. of crystalline vitamin D_3. The control diet contained 8% lard. A fat level of 8% was chosen because preliminary studies had shown that ethyl esters of naturally-occurring fatty acids permitted the same growth as fresh lard when 8% was included in the diet.

The feeding experiments were carried out on albino rats from a homogeneous colony. From the time of their delivery to the laboratory until the start of the experiment when they were 35 days old, the weanling rats were given a lard diet similar to that described above but containing lactalbumin instead of casein. They were ear-marked and weighed at 31 days and reweighed at 35 days. At this time they were distributed into matching groups, the average weights of which were the same at 31 days and again at 35 days. The rats were kept in individual cages on shavings and were given water in nondripping bottles suitable for water-intake measurements. The animals were weighed twice weekly.

At the end of the experiment the animals were anesthetized with ether, and blood was drawn from the heart for cholesterol analyses, which were done on conveniently pooled samples according to Schoenheimer and Sperry (5).

Kidneys, testicular fat bodies, and livers were weighed, and the latter were immediately frozen on dry ice. All livers and sera of the groups studied were analyzed; two to four livers and the matching sera were pooled in corresponding samples. The liver sample was homogenized, and a sample of this was dried to constant weight at 100°C. A second sample was extracted with a 3:2 alcohol-ethyl ether mixture. An aliquot of the extract was dried to constant weight at 100°C. for the total lipid content. A second aliquot was analyzed for cholesterol according to the method of Sperry and Webb (6).

For the evaluation of the effects of the fractions on organs, the organ weight-body weight relationship was used. This relationship is not linear, but a log-log plot gives a straight-line distribution, the slope and spread of which are characteristic for each organ. Such distributions usually show one or more changes in slope with increasing body weight (7). To compare organ weights of groups having widely different average body weights, the organ weights of the control animals fed lard were used as the reference. These were plotted against the corresponding body weights on log-log paper, and the best straight line was drawn through them, with the established slope for the organ. This line became the source of "normal" organ weights for various body weights. The actual organ weights observed in the experimental groups were compared with the "normal" weights for the same body weights as derived from this line, and the differences between the two were expressed as percentages of the "normal" organ weights. Thus even the control organ weights sometimes showed slight deviations from the ideal, depending on how accurately the ideal line had been drawn. The slopes for livers and kidneys have been given in a previous report (2). The slope for the testicular fat bodies was 64°.

For the statistical analysis of the results, standard errors are given after average values, from which t values can be calculated and the P's read from a table because the number of observations is given with the data. A P of 0.05 was considered to be on the borderline of significance.

Results

In Tables II and III are summarized the data concerning survival rate, weight gain, water intake, and organ weights. The highest death rates were observed with the predominantly dimeric fractions, MRMD2 from lard and MRMD from CSO. With all other fractions the survival rates after three or four

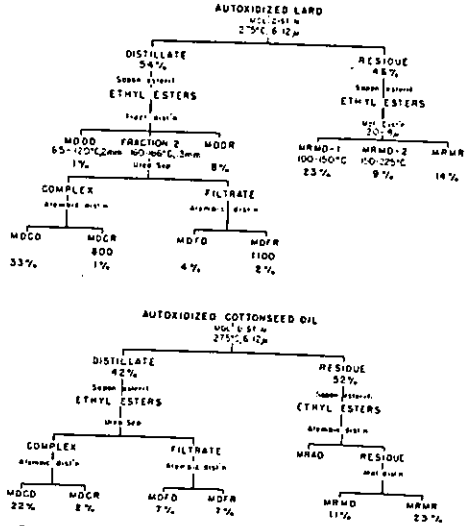

FIG. 1. Flowsheet for fractionation of autoxidized lard and cottonseed oil. The percentage yields are based on the amount originally fed into the molecular still. Molecular weights are given below some fractions.

weeks were not significantly different from those of the animals on lard.

In evaluating the effects of the fractions on body weight, an attempt was made to correlate them with the chemical characteristics of the fractions. If one relates the average body weights of the groups after three weeks (Tables II and III) to the percentage of hydroxyl oxygen in the respective fractions (Table I), a rough inverse relation is observed; the least oxidized fractions permitted the best growth and *vice versa*. However most of the fractions having higher OH-oxygen concentrations were dimeric or trimeric, and such fractions have previously been shown to be particularly toxic (8). Therefore the presence of dimers may account for the toxicity, especially since the higher polymers of both autoxidized CSO and lard depressed growth considerably less although they had high OH-oxygen concentrations. On the other hand, OH-oxygen concentrations would seem to have had some effect on body weight because the dimeric fraction from lard, MDCR, which contained less OH-oxygen than any other dimeric fraction, led to comparatively little depression of growth and the unpolymerized fraction, MDDR, with a high OH-oxygen content, markedly depressed growth.

For the comparison of the water intakes it was necessary to take into consideration the fact that the average body weights of the groups differed considerably and that water consumption of animals of different body weights could not be compared directly. Therefore each animal's surface was calculated by Lee's formula, $S = 12.44\ W^{2/3}$, where S is the surface in $cm.^2$ and W is the body weight in g. (9). The body weight of each rat was considered to be the average of its weights at the beginning and end of the experimental period. Each animal's water intake for this period was divided by the number of weeks in the period, and this average intake per week was divided by S/100 to give average intake per week per 100-$cm.^2$ surface. Five control groups (not otherwise used in these studies) had intakes of 36–38 cc./100 $cm.^2$/week for body weights of 100 to 428 g.

It can be seen from Tables II and III that some of the fractions increased the water intake significantly; none depressed it. On the average, fractions from the original residues (MR..) were more active than those from the original distillates (MD..), with P less than .01. In most instances, increased water intake was associated with enlarged kidneys, suggesting some renal damage. However the atoxic, unpolymerized fractions, MDCD, from autoxidized lard and CSO, which contained mainly C_{18} chains, brought about significantly increased water-intakes without significant renal enlargement. MRMD1 from lard and the comparable MRAD from CSO had only a mild influence on body and kidney weights but increased water intakes markedly (P less than .02 and .01, respectively).

If one examines the degree of kidney and liver enlargement of the experimental groups in relation to their average body weights, one sees the inverse relationship commonly noticed under various stress

TABLE II

Survival Rate, Body Weight, Water Intake, and Percentage of Kidney, Liver, and Fat Body Enlargement of Male Rats Fed Fractions of Autoxidized Lard for Three Weeks

Sample	Fatty acid chain-length	Survival rate	Av. body weight (g.)	Av. total water intake (cc./100 g. body wt.)	Av. wt. difference: Kidney	Liver (%)	Fat body
Lard	.....	12/13	196 ± 7.4[b]	193 ± 4.9	− 2 ±2.5	+ 2 ± 2.7	+ 2 ± 3.8
MDDD	C_{18} and shorter	4/4	137[b]	146[b]	− 1	−23	.....
MDDR	C_{18}	11/14	108 ± 16.8	212 ± 7.5	±3.4	± 2.6	
MDCD	C_{18}	13/14	188 ± 6.1	212 ±21.6	+10 ±3.9	−34 ± 3.7	−16 ± 5.9
MDCR	Dimer and trimer of C_{18}	4/4	138 ± 4.2	224 ± 9.6	− 3 ±2.2	− 3 ± 2.4	− 1 ± 4.7
MDFD	C_{18}	11/12	155 ± 9.8	.....	+12 ±7.3	+48 ±19.0	.....
MDFR	Trimer of C_{18}	4/4	133 ± 9.0	225 ±18.4	+20 ±3.5	+ 6 ± 3.4	+ 8 ± 6.5
MRMD1	C_{18}	13/14	175 ±13.0	125 ±10.3	+28 ±7.4	+31 ± 6.4	.....
MRMD2	Trimer of C_{18}	9/14	93 ± 8.9	231 ±11.4	+11 ±2.0	+10 ± 3.2	+13 ± 6.2
MRMR	Higher mol. wt.	12/13	137 ± 7.5 ± 7.7	297 ±21.8 ±12.6	−31 ±4.3 +14 ±2.3	+42 ± 4.7 +13 ± 2.2	− 3 ±24.6 0 ± 6.8

[a] Standard error. [b] After 2 weeks when lard animals had body wt. of 182 g. and water intake per 100 g. of 127 cc.

TABLE III

Survival Rate, Body Weight, Water Intake, and Percentage of Kidney, Liver, and Fat Body Enlargement of Male Rats Fed Fractions of Autoxidized Cottonseed Oil for Three Weeks

Sample	Fatty acid chain-length	Survival rate	Av. body weight (g.)	Av. total water intake (cc./100 g. body wt.)	Av. wt. difference Kidney	Liver (%)	Fat body
Lard	.....	14/16	190 ±6.6	203 ± 4.9	− 1 ±3.6	+ 1 ±3.6	+ 4 ±6.2
MDCD	C18	14/16	186 ±6.1	217 ± 6.6	+ 8 ±2.7	+ 2 ±1.6	− 6 ±2.9
MDCR	Dimer of C18	3/4	103 ±5.2	.....	+32 ±4.4	+19 ±7.4	.....
MDFD	C18	7/8	137 ±2.5	.....	+21 ±6.9	+16 ±5.1	.....
MDFR	Dimer of C18	0/8	104 ±1.9	.....	+ 7 ±4.2	+49 ±4.3	.....
MRAD	C18	16/16	179 ±3.6	268 ±15.2	+11 ±3.0	+ 6 ±2.4	−16 ±5.1
MRMD	Dimer of C18	12/16	85 ±3.1	228 ±22.1	+25 ±3.0	+45 ±3.5	.....
MRMR	Higher mol. wt.	15/16	126 ±3.3	274 ±17.0	+ 5 ±2.1	+10 ±2.7	−24 ±9.8

conditions. Those groups having the most depressed body weights in general had the largest kidneys and livers in relation to body weight. However certain fractions had more pronounced effects on one or both organs than could be ascribed unspecifically to various stresses, *i.e.*, the dimeric fraction from lard, MDCR, brought about severe enlargement of the liver whereas the kidneys of these animals were only slightly larger than those of other animals with the same body weight. Also noteworthy was the disproportionately small effect of the high polymer fraction from CSO, MRMR, which led to depressed growth but permitted normal kidneys and only slightly enlarged livers. Another fraction with relatively little effect on the kidney was the dimeric CSO fraction, MDFR, which led to almost normal kidneys but large livers.

Testicular fat body weights were studied because it has been shown that they are proportional to the total neutral fat in the body (10). However the relation of this fat to total body weight is somewhat variable, and the slope of the log-log plot is steep. Thus small differences are of dubious reliability. A study of the percentage of fat body enlargement in relation to body weight loss or OH-oxygen concentration of the particular fraction responsible for it failed to show any correlation.

However comparison of all available corresponding fractions from autoxidized lard and CSO, which were the two MDCD fractions, MRMD1 and MRAD, and the two MRMR fractions, shows that those fractions derived from CSO led to less fat deposition than the corresponding lard fractions although corresponding fractions depressed growth to almost the same degree. If one averages the fat body enlargement brought about by the three CSO fractions and compares the result with that from the lard fractions, the difference is significant (P less than .01). Examination of the chemical properties of these six fractions (Table I) reveals that the iodine numbers of the lard fractions range from 27 to 47 and those of the CSO fractions, from 45 to 70. However no conclusion is possible from these data.

In Table IV are summarized data from liver lipid and serum cholesterol studies. The average percentage of dry substance of the livers enlarged more than 30% was 31.4 ± .10, that of the smaller livers, 30.4 ± .18; the difference was significant (P less than .01). However the average total lipid content of the large livers was 20.8% ± 1.6 whereas that of the smaller was 23.5 ± .67. Therefore, if it is permissible to consider the carbohydrate content of the livers as negligible, one can conclude that enlargement was accompanied by accumulation of protein.

Liver and serum cholesterol levels varied with the degree of liver enlargement. The average liver cholesterol value for the three groups having the largest livers was 702 mg. % dry wt. ± 13; the average of the others was 788 ± 14, with P less than .01. Since a low liver cholesterol level, when accompanied by a low serum level, is a sign of depressed cholesterol synthesis (11), it seems likely that such was the case with the large livers. The low levels reflected damage to the livers induced by the toxic fractions. However the percentage of cholesterol in the total liver lipids was 3.6% in the large livers and 3.4% in the small livers. Therefore the difference in cholesterol levels was probably an expression of the lipid depletion of the damaged livers.

Similar lipid studies were carried out on rats fed fresh fats and autoxidized CSO. The diets contained 15% fat but were otherwise the same as those containing the ethyl esters. The fresh fats used were cottonseed oil, lard, a commercial margarine oil, corn oil, butter, and saturated medium chain triglycerides. The autoxidized CSO had been aerated at 90–95°C. for 300 hrs. The rats were placed on the diets at weaning and maintained for two to four months.

TABLE IV

Liver Lipid and Liver and Serum Cholesterol Levels of Male Rats Fed Fractions of Autoxidized Lard and Cottonseed Oil

Sample	Liver dry substance (%)	Total liver lipids (% dry wt.)	Liver cholesterol (mg. % dry wt.)	Serum cholesterol (mg. %)
Lard	29.9 ±2.5	21.3 ± .2	803 ± 5.5	68.5 ±2.8
Lard Fractions				
MDDR	31.2 ± .15	19.7 ±2.3	692 ±12.5	45.4 ±1.0
MDCD	29.8 ± .1	22.6 ±2.2	806 ±38.5	64.0 ±2.2
MDFD	34.1 ± .1	26.7 ±1.2	775 ±18.0	67.2 ±1.3
MRMD1	30.4 ±2.25	24.1 ± .6	737 ± 4.5	56.2 ±1.1
MRMD2	31.5	24.0	692	44.3
MRMR	29.9 ± .65	22.0 ± .4	805 ±50.0	53.8 ±5.4
CSO Fractions				
MDCD	30.0 ± .1	23.7 ± .4	795 ±17.0	74.2 ±1.2
MRAD	30.6 ± .05	24.3 ±2.2	766 ±16.0	64.0 ±2.2
MRMD	31.5 ± .1	14.7 ±2.2	717 ±32.0	35.7
MRMR	30.9 ± .5	21.3 ±1.0	757 ±10.0	48.5

In Table V are given the results of these studies. As with the toxic fractions of autoxidized CSO and lard, the livers of the animals fed the autoxidized CSO were relatively depleted in lipid, and their cholesterol was substantially lower than that of the animals on fresh fats. Also the cholesterol content of the total liver lipids in the animals on autoxidized CSO was significantly lower than that of the animals fed fresh fats. Inasmuch as it has been shown in earlier work (2) that the livers of animals on autoxidized CSO are damaged, these results would bear out the findings with the toxic fractions of autoxidized CSO and lard. In fact, the results with autoxidized CSO were more pronounced than were those with the fractions. This may have been caused by the higher level of autoxidized fat (15 instead of 8%) or by the fact that triglycerides rather than ethyl esters were fed.

TABLE V

Liver Lipid and Liver and Serum Cholesterol Levels of Male Rats Fed Fresh Fats or Autoxidized Cottonseed Oil

Sample	No. of expts.	Total liver lipid (% dry wt.)	Cholesterol level (% liver lipid)	Cholesterol level (mg. % dry substance)
Fresh fats	12	24.6±.91	4.1±.15	999±24
Autoxidized cottonseed oil	8	21.6±.58	3.6±.08	768±14

Discussion

The results of this attempt at screening the many substances occurring in autoxidized lard and CSO indicate that some of the materials produced were toxic to rats; the survival rate declined and growth was depressed. Certain types of polymers (particularly dimers, as noted before [8]) and higher levels of oxidation (as indicated by high OH-oxygen content) were associated with toxicity. However, if one considers that the toxic fractions were prepared from materials autoxidized far beyond that occurring when fats are used for human consumption, extrapolation of these findings to the action of commercially-used fats seems unwarranted.

Probably more significant than the expected toxicity of some of the fractions is the fact that some of the atoxic fractions had characteristic effects which may deserve pharmacological study. For instance, the atoxic fractions MDCD from autoxidized lard and CSO, which contained mainly straight C_{18} chains, increased fluid intake significantly. Also of interest may be the fractions tending to depress neutral fat deposition and some of the fractions increasing liver and kidney weight.

The depression of serum and liver cholesterol by the toxic fractions may have some relevance to the current interest in the relation of fats to serum cholesterol. It suggests that the mere depression of serum cholesterol by a fat does not necessarily imply an advantage to the animal.

Summary

Lard and cottonseed oil which had been autoxidized at about 100°C. for 210 hrs. were fractionated by a technique involving molecular distillation, conversion to ethyl esters, urea-complex formation, and redistillation. The ethyl esters were then fed to rats for three weeks at a level of 8% in a purified diet. Growth, water intake, organ weights, total liver lipids, and serum and liver cholesterol levels were determined. Groups fed 8% lard served as controls.

Growth was severely depressed by the residue fractions of the urea-complex- and noncomplex-forming portions of the original molecular distillates. Of the three fractions from the original molecular distillation residues, the dimeric and polymeric fractions were the most active. The relative liver and kidney weights were usually increased by feeding the growth-depressing fractions. However there were a number of exceptions indicating more specific effects from some of the fractions. Water intakes were lower with the fractions derived from the original molecular distillates than with those from the original molecular distillation residues. Testicular fat body weights suggested that feeding of autoxidized CSO fractions led to less neutral fat deposition than feeding of corresponding autoxidized lard fractions. Dry weight of the enlarged livers was higher, and the total lipid lower than of the control livers. Total liver cholesterol was higher in animals with smaller livers, but there was no difference in the cholesterol content of the total liver lipids. Serum cholesterol levels were lower in animals with large livers.

Further study of those fractions having pharmacological properties is suggested.

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REFERENCES

1. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Knight, H. B., and Swern, Daniel, "Metabolism," in press.
2. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Knight, H. B., Saunders, D. H., and Swern, Daniel, J. Am. Oil Chemists' Soc., *33*, 630 (1956).
3. Knight, H. B., and Swern, Daniel, J. Am. Oil Chemists' Soc., *26*, 366 (1949).
4. Swietoslawski, "Ebulliometric Measurements," New York, Reinhold, 1945.
5. Schoenheimer, R., and Sperry, W. M., J. Biol. Chem., *106*, 745 (1934).
6. Sperry, W. M., and Webb, M., J. Biol. Chem., *187*, 97 (1950).
7. Stoerk, H. C., and Zucker, T. F., Proc. Soc. Exper. Biol. Med., *61*, 297 (1946).
8. Crampton, E. W., Common, R. H., Pritchard, E. T., and Farmer, F. A., J. Nutrition, *60*, 13 (1956).
9. Lee, M. O., Am. J. Physiology, *89*, 24 (1929).
10. Stoerk, H. C., and Porter, C. C., Proc. Soc. Exper. Biol. Med., *74*, 65 (1950).
11. Cook, R. P., "Cholesterol," 1st ed., p. 152, New York, Academic Press, 1958.

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all physical symptoms of molybdenosis. Sodium and ammonium sulfate had similar effects, although the ammonium salt exhibited toxic properties. The sulfate salts practically eliminated the mortality produced by sodium molybdate, but failed to prevent the additional mortality characteristic of ammonium molybdate. Addition of graded levels of sulfate to a high-molybdenum diet failed to prevent greatly increased storage of molybdenum in the tibiae of chicks, although growth improved as sulfate concentration was increased.

Methionine failed to affect the growth depression caused by a high level of molybdenum.

LITERATURE CITED

Arrington, L. R., and G. K. Davis 1953 Molybdenum toxicity in the rabbit. J. Nutrition, *51:* 295.

Arthur, D., I. Motzok and H. D. Branion 1958 Interaction of dietary copper and molybdenum in rations fed to poultry.. Poultry Sci., *37:* 1181.

Dick, A. T. 1952 The effect of diet and of molybdenum on copper metabolism in sheep. Australian Vet. J., *28:* 30.

——— 1953a The effect of inorganic sulphate on the excretion of molybdenum in the sheep. Ibid., *29:* 18.

——— 1953b The control of copper storage in the liver by inorganic sulphate and molybdenum. Ibid., *29:* 233.

Ferguson, W. S., A. H. Lewis and S. J. Watson 1938 Action of molybdenum in nutrition of milking cattle. Nature, *141:* 553.

——— 1943 The teart pastures of Somerset. I. The cause and cure of teartness. J. Agr. Sci., *33:* 44.

Gray, L. F., and L. J. Daniel 1954 Some effects of excess molybdenum on the nutrition of the rat. J. Nutrition, *53:* 43.

Kratzer, F. H. 1952 Effect of dietary molybdenum upon chicks and poults. Proc. Soc. Exp. Biol. Med., *80:* 483.

Marmoy, F. B. 1939 The determination of molybdenum in plant materials. J. Soc. Chem. Ind., *58:* 275.

Motzok, I., D. Arthur and H. D. Branion 1957 Feeding of molybdenum to poultry. Poultry Sci., *36:* 1144.

Reid, B. L., A. A. Kurnick, R. L. Svacha and J. R. Couch 1956 The effect of molybdenum on chick and poult growth. Proc. Soc. Exp. Biol. Med., *93:* 245.

Toxicity of Air-Oxidized Soybean Oil[1,2]

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Although it has long been recognized that overheated or oxidized fat causes toxic manifestations in the rat, neither the exact nature of materials causing these symptoms nor the specific mechanisms by which they are caused have been understood with any certainty. Work prior to 1940 established that highly oxidized fat accelerates the destruction of a large variety of essential nutrients (Burr and Barnes, '43). Subsequent work, however, has indicated that certain oxidized oils contain materials which are toxic to the rat even though apparently adequate steps are taken to protect the easily-oxidizable foodstuffs in the diet.

The identity of the toxic materials in oxidized or overheated fat has been investigated by two routes, differing in the manner by which the oxidized fat was obtained. Several groups of investigators, including Crampton et al. ('51a, '51b, '51c, '53, '56), Common et al. ('57), Granados et al. ('49), and Raju and Rajagopalan ('55) have changed the chemical nature of oils primarily by means of heat treatment, often with complete exclusion of air. Other groups of investigators (Matsuo, '54; Kaneda et al., '55; Kaunitz et al., '55; Andrews et al., '56) oxidized various oils by aeration with little or no elevation in temperature during the oxidation process. Grossly, the results obtained are similar when either type of oxidized oil is fed, namely, rough fur and unkempt appearance and decreased weight gain often followed by death. When, however, those groups feeding heat-treated oil measured the peroxide content of their product, the peroxide concentration in the toxic oil was found to be very low compared with values obtained with aerated oil. On the other hand, in those cases in which oils were oxidized by aeration and some attempt was made to measure the toxicity due to polymers, the polymer content appeared completely innocuous, the toxicity apparently correlating best with peroxide concentration.

In the experiments to be reported in this paper, an attempt was made to reconcile these apparently contradictory results and, further, to identify the toxic mechanism or mechanisms which so frequently lead to death in the rat. There are apparently few or no definitive data on the latter point altbough it would seem to be of considerable importance in many areas of the world in which cooking customs involve intermittent high-temperature and open air heating of highly unsaturated oils (Raju and Rajagopalan, '55). Consequently, in a preliminary experiment, various levels of peroxidized fat were fed to determine the levels of toxicity and toxic symptoms. Second, the peroxidized oil was fractionated in such a way as to separate peroxide and polymeric material and the fractions were fed. Third, the absorption of peroxidized fat was studied, and fourth, an attempt was made to ascertain the site of the damage.

METHODS AND MATERIALS

Growth experiments. Unless otherwise noted, weanling rats[4] were fed diets containing air-oxidized soybean oil. The animals were individually housed in sus-

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pended cages and, where pertinent, individual diet consumption records were kept. Each rat was weighed twice weekly.

Two diets were utilized in the growth experiments. The percentage composition of diet A was as follows: casein, 25; sucrose, 46; salt mixture, 4; brewers' yeast, 5; fat, 20. The composition of diet B is presented in table 1, as well as the composition of the vitamin and salt mixtures.

Semiweekly administration to each rat of 175 U.S.P. units of vitamin A, 35 units of vitamin D and 1.75 mg of α-tocopherol acetate dissolved in 0.05 ml of sesame oil was utilized to satisfy the fat-soluble vitamin requirement. Initially, this supplement was administered by intramuscular injection in order to avoid the possibility of oxidation by peroxide in the gut. Subsequent experimentation, however, established that similar growth could be obtained in rats given this supplement orally at noon on a semiweekly basis (most of the diet was consumed during the night), and this method of supplementation was used in later experiments.

Preparation of oxidized soybean oil. The raw oil was oxidized by aeration in a water bath at 60°C for approximately one week with the addition of 2 mg/kg each of $CuCl_2$ and $FeCl_3$. Toward the end of the oxidation period the peroxide number (PN), milliequivalents of peroxide oxygen per kilogram of oil, was determined at intervals according to the method of Polister and Mead ('53) so that oxidation might be halted at maximum peroxide concentration inasmuch as the PN decreases rapidly after the maximum is reached. The oxidized oil was then stored at −20°C under nitrogen.

Fractionation of oxidized soybean oil. Two kilograms of raw oil were oxidized in the usual manner and one-half of the oxidized oil was stored at −20°C (fraction C). The remaining oil was dissolved in 1.7 l of petroleum ether (b.p. 30 to 60°) saturated with methanol and was then extracted with 4 l of methanol saturated with petroleum ether. The oil contained in the petroleum ether layer was separated on a silicic acid column into two fractions. This fractionation was accomplished by adding 25-gm quantities of oil in petroleum ether to 8 by 14-cm silicic acid columns and eluting with 500-ml portions of 5% ethyl ether in petroleum ether. Each fraction was collected separately and evaporated to dryness under nitrogen in a warm-water bath. All fractions weighing more than 1 gm were tested for peroxide, as previously described, and those fractions having a peroxide number of less than 20 were pooled. The resulting colorless oil (fraction A) had a peroxide value of 13.2 and contained little or no polymeric material as shown by complete distillation of a methylated sample. After removal of the low-peroxide material from the columns, the remaining oil was eluted with three- to 4-column volumes of methanol. The methanol was evaporated under reduced pressure at a temperature under 40°C. The last traces of solvent were removed under high vacuum at room temperature. The combined sample from all

TABLE 1

Composition of diet B

Dietary component main mixture		Salt mixture		Vitamin mixture	
		Salt	Amount	Vitamin	Amount
	gm/100 gm		gm		mg/gm
Oxidized oil	15.0	$CaCO_3$	300.0	Choline	120,000
Casein	18.0	$CaHPO_4$	75.0	Thiamine	200
Sucrose	60.8	K_2HPO_4	322.5	Riboflavin	200
Glycerol	1.0	NaCl	167.4	Niacin	400
l-Cystine	0.2	$MgSO_4$	49.8	Calcium pantothenate	800
Salt mixture	4.0	$MnSO_4 \cdot 2H_2O$	0.5	Folic acid	8
Vitamin mixture	1.0	Zn acetate	0.402	Biotin	4
		Fe citrate	27.4	Pyridoxine	100
		KI	0.8	Vitamin B_{12}	0.8
		$CuSO_4 \cdot 5H_2O$	0.3	*i*-Inositol	4.0
				Sucrose	273,500

of the columns was an amber, moderately viscous oil with a peroxide number of 927 (fraction B). The original methanol fraction was extracted with petroleum ether at 0°C for 6 hours in a liquid-liquid extractor. The sample, in methanol, was stored under nitrogen at −20°C until shortly before use. The methanol was then removed under reduced pressure in an ice bath with a slow stream of nitrogen. High vacuum was utilized to remove the last traces of the solvent. The sample (fraction D) was colorless, extremely viscous and had a peroxide number of 3,185. Peroxide numbers and spectral properties of the fractions are listed in table 2.

Absorption studies. The animals used in these investigations were mature rats[4] which were subjected to thoracic duct cannulation according to the technique of Bollman et al. ('48). Preliminary investigation revealed that pre-operative administration of 2 ml of oxidized soybean oil (PN = 1200) was frequently fatal to the subject. A similar amount of peanut oil was employed, therefore, in the pre-operative procedure. Lymph was then collected for a 20- to 24-hour period at room temperature and was stored at −20°C. These samples served as the source of control lymph fat. After the initial period of lymph collection, a dose of air-oxidized soybean oil was administered to the cannulated rat. Lymph collection was then continued for periods extending up to 12 hours. These samples were also stored at −20°C. Similar volumes of both control and experimental samples were then extracted with a 3:1 mixture of alcohol and ether, the protein removed by centrifugation, and the alcohol-ether layer evaporated in a tared flask under nitrogen on a warm-water bath, for determination of peroxide number. Other lymph fat samples were examined for conjugated diene by dissolving the fat isolated from 2 ml of lymph in 50 ml of ethanol and examining solution at 232 μ with a Carey recording spectrophotometer.

TABLE 2

Peroxide number and conjugated diene concentration in the various fractions of oxidized soybean oil

Fraction	Peroxide number	Meq. conj. diene/kg oil[1]
A	13	0
B	927	1190
C (unseparated)	1156	780
D	3185	1602

[1] The fact that the peroxide numbers and conjugated diene concentrations do not change in parallel fashion probably indicates that the unsaturated centers of many molecules have suffered further attack after the initial peroxide formation.

Xanthine oxidase assay. The intestinal xanthine oxidase of rats was assayed as follows: the animals were decapitated and the upper third of the small intestine excised; intestinal contents were flushed out with approximately 10 ml of saline and 0.5 gm of the upper end of the washed intestinal section placed on dry ice until homogenized; homogenization was conducted at 5°C in 10 ml of an 0.015 M sodium pyrophosphate buffer contained in an all glass homogenizer. The general method of enzyme assay was that of Dhungat and Sreenivasan ('54). Milk xanthine oxidase was purified according to the method of Ball ('39).

RESULTS

Rat growth experiments. In the initial growth study, oxidized oil (1200 PN) was diluted with fresh oil to give peroxide numbers of 800, 400 and 100. Four variations of diet A were prepared: three with each of the diluted oils and the 4th with the original 1200 PN oil. A 5th diet containing fresh oil served as the control. Extraction of the fat in these diets showed that the peroxide-number did not change on mixing, after three weeks' storage at −20°C, or on standing at room temperature for three days.

After consuming the control diet for 6 days, 50 weanling rats were divided into 5 groups of 10 each, equally divided between males and females. One of these groups was assigned to each of the diets previously described, and the animals were weighed individually twice a week for the ensuing 70 days. The average growth curves of the female rats in each group are presented in figure 1. The diets containing 20% of fat with peroxide numbers

[4] Sprague-Dawley strain.

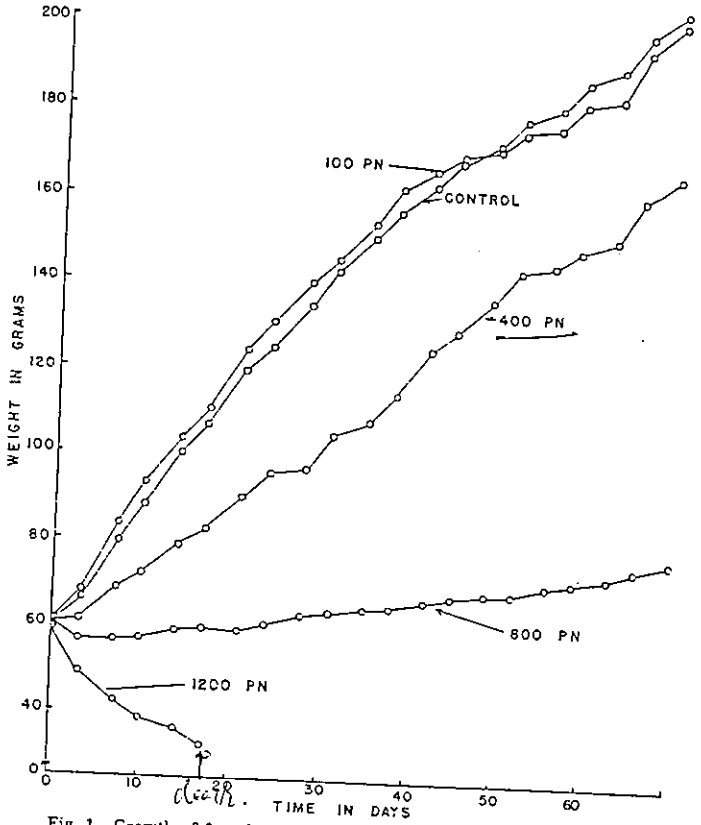

Fig. 1 Growth of female rats receiving various levels of oxidized fat.

of 400 to 1200 caused a reduction in growth rate directly related to the peroxide number. Consumption of the 1200 PN diet caused death within approximately three weeks. The only other symptoms observed in the animals fed this diet were weight loss and severe diarrhea. Animals receiving the 800 PN diet developed moderate to severe diarrhea, but this symptom had subsided somewhat by the 8th week. Similar effects were observed in the growth of male rats fed these diets.

The data obtained in this experiment indicate that the diet containing 100 PN oil has no effect on growth. It seemed conceivable, however, that under conditions of stress a difference between the control and 100 PN diets might become apparent. Accordingly, an experiment was conducted in which 25 rats, 11 males and 14 females, consumed the 100 PN diet for 8 weeks and then were subjected to a whole-body X-irradiation dose of 500 r.[3] A similar group of animals served as concomitant controls. In both the control and 100 PN diets the concentration of brewers' yeast was increased from 5 to 7.5%

[3] Irradiation was carried out using a Picker 250 KV Industrial X-Ray apparatus, with the following factors: 15 ma, 0.28 Cu parabolic, 0.21 Cu inherant and 1 Al; field size 47 cm², FOD 55 cm.

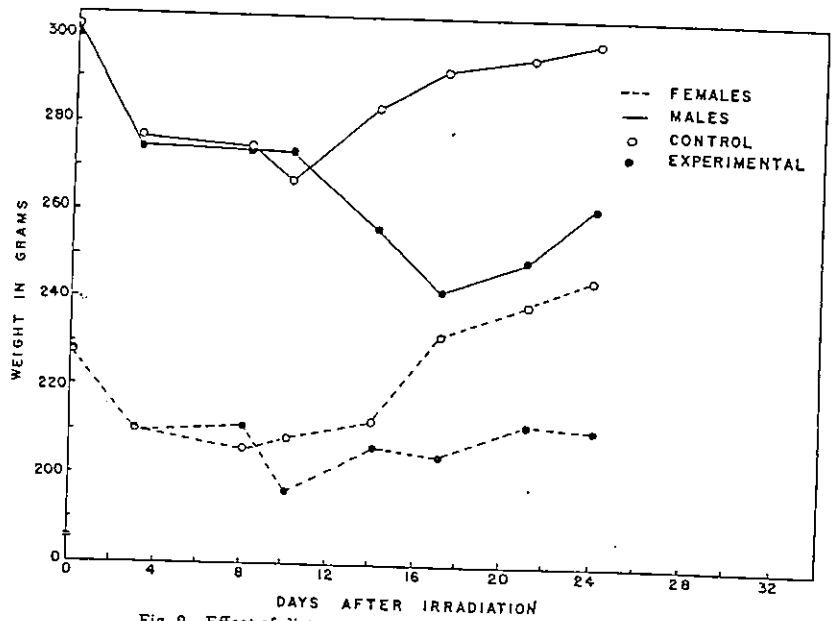

Fig. 2 Effect of diet on recovery from whole body irradiation.

at the expense of sucrose. Figure 2 shows the average group weight changes over the 27-day period following irradiation. The results obtained in this experiment indicate that consumption of the 100 PN diet caused a slower than normal recovery from the stress of irradiation.

In an attempt to compare the effect of the lipid peroxide with that of a simple water-soluble peroxide, and to eliminate diet flavor as a factor, *t*-butyl hydroperoxide, synthesized according to the method of Milas and Surgenor, ('46) was used in both feeding and injection studies. In preliminary injection experiments, 8 mg of this compound in a non-physiological solution was found to be immediately fatal when intravenously administered to rats weighing approximately 400 gm. Intravenous injection of a physiological solution of 1 mg of *t*-butyl hydroperoxide every three or 4 days over a two-week period caused a weight loss and some loss of hair. A growth study was conducted with 4 weanling rats fed control diet A by substituting a solution of 4×10^{-5} M *t*-butyl hydroperoxide for the drinking water. Figure 3 illustrates the average growth of these animals compared with three rats consuming the same diet and drinking tap water. This figure also illustrates the marked loss of weight which occurs when a high-PN diet is substituted for the control diet.

Fractionation experiments. In an attempt to settle the question of whether the toxic principle in oxidized oils is peroxidic or polymeric in nature, the fractions obtained from air-oxidized soybean oil were fed to weanling rats. It was assumed that fraction A contained most of the non-hydroxylated or peroxidized glycerides, while fraction D consisted primarily of glycerides containing fatty acid peroxides. Fraction B was assumed to be a mixture of the two materials. Since incorporation of a highly oxidized oil, namely, fraction D, in the diet makes the food unpalatable, the rats were fed a fat-free diet similar to diet A and the appropriate oil was force-fed. Diet A was modified by increasing the brewers' yeast from 5 to 7.5% and adding

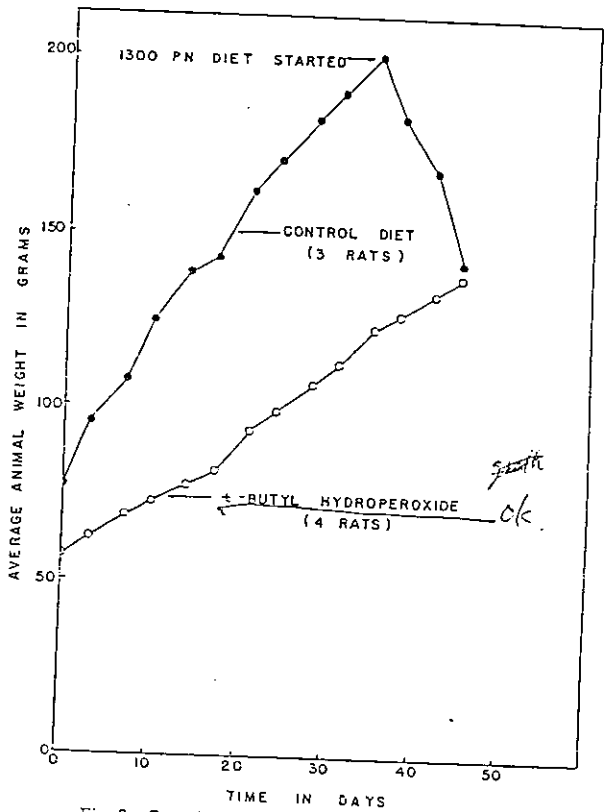

Fig. 3 Growth of rats under several dietary regimes.

2% of guar gum,[6] both at the expense of sucrose. Preliminary considerations indicated that each rat should receive an amount of oil equivalent to 38% of the total amount of calories consumed during the previous 24 hours. Ten grams of the dry diet were weighed into tared glass feed cups for each rat. Two milliliters of water were then mixed with the diet in each cup to make a thick paste which soon hardened and which the animals could eat with a minimum of spillage. An extra cup of diet was prepared each day and used to correct food consumption figures for water evaporation. Fifteen male and 5 female weanling rats ate the fat-free diet for an initial 8-day period. During this period some diarrhea was observed but weight gains were satisfactory and the animals appeared otherwise healthy. At the end of the 8-day period, the male rats were divided into three groups (A, B and C) of 5 each with the female rats comprising a 4th group (D). These group designations correspond to the fraction designations of the oxidized oil. All animals received orally twice the previously described volume of fat-soluble vitamin mix once a week about three hours before any fat was force-fed. If the amount of oil to be administered was more than 0.5 ml, it was given in two equal doses at noon and 4:00

[6] Donated by the Stein-Hall Co., New York.

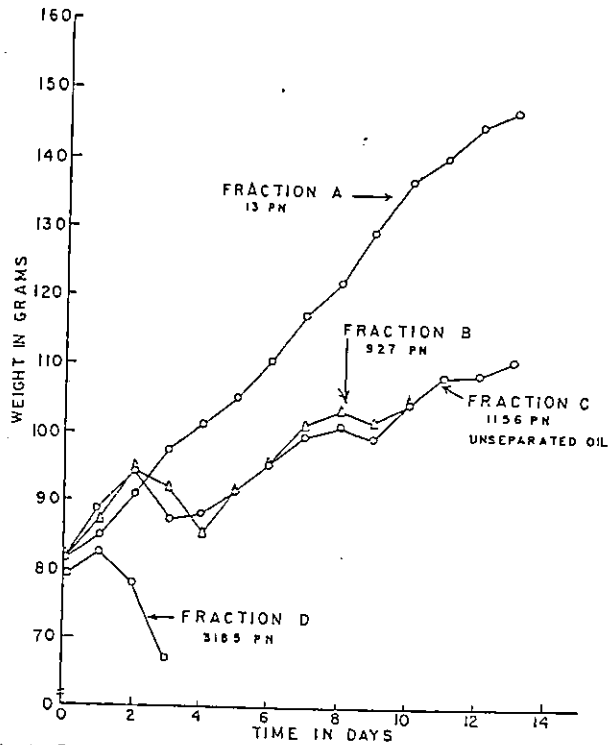

Fig. 4 Growth of rats receiving various fractions of oxidized soybean oil.

P.M. The rats were weighed daily during the experimental period. The average weight gains for the 4 diet groups are presented in figure 4.

The rats in group A appeared healthy and ate well in contrast to those in groups B, C and D. Those in groups B and C were indistinguishable in appearance but rats in group D began losing weight after the first day of fat administration. On the third day of the experiment two of the rats in group D were found dead and, since the remaining three appeared moribund, they were killed for pathological examination.

Histopathological examination[7] of these three animals revealed no obvious anatomical basis for their condition. The livers of these rats showed slight enlargement of the nuclei of the parenchymal cells and prominent nucleoli. Moreover, the small intestines of these animals had cytoplasmic vacuoles distending the mucosal walls. Examination of all other organs generally gave negative findings.

Absorption experiments. In order to ascertain the actual site of action of the toxic principle in oxidized fat, it was deemed of primary importance to determine whether any of the peroxide-containing material could be absorbed through the intestinal wall. For this study, rats with thoracic duct cannulae were given small doses of oxidized soybean oil, and the lymph was collected for periods varying between two and 24 hours. After isolation, the lymph fat was analyzed for peroxide and conjugated

[7] Histopathology performed under the direction of Dr. D. Moyer of the Dept. of Pathology, UCLA School of Medicine.

TABLE 3

Peroxide numbers of lymph fat obtained from rats fed either 0.5 to 1.0 ml of air-oxidized soybean oil (PN = 1200) or 2 ml of peanut oil

Rat no.	Oil fed	Lymph extracted	Weight of extracted fat	Peroxide no. of fat
		ml	*mg*	
A	Peanut	4.0	213.7	0.9
	Oxidized soybean	4.0	242.9	32.7
B[1]	Peanut	4.0	188.5	13.1
	Oxidized soybean	4.0	135.0	13.9
C[1]	Peanut	3.0	71.1	4.2
	Oxidized soybean	3.0	161.5	4.3

[1] Rats B and C are presented as typical of the 25 rats in which no significant change in the PN of the lymph fat was found.

diene. The results of the peroxide determinations are shown in table 3. The results of the experiment with rat A led to the conclusion that fatty acid peroxides may be absorbed as such. However, in subsequent experiments involving 25 cannulated rats it has been impossible to detect a significant rise in the peroxide number of lymph fat as the result of feeding oxidized soybean oil with a PN of 1200. The results obtained with rat A were, therefore, assumed to be either an artifact or an exceptional case of absorption.

Ultraviolet examination of a 1:50 dilution in ethanol of lymph fat from cannulated rats yielded the results typical of those presented in table 4. It can be seen

Table 4

Optical densities at 232 mμ observed in lymph fat from rats fed oxidized soybean oil

Rat no.[1]	Amt. ox. oil fed	Length of collection time	232 mμ O.D. at[2]
	ml	*hours*	
9	0.5–1.0	4.5	0.075
	0.5–1.9	4.0	0.024
6	3.0	21.5	0.030
		5.0	0.130
		3.5	0.021
		16.0	0.058
		8.0	0.008
		16.0	0.000

[1] These results are presented as typical of several experiments performed.

[2] The optical density at 232 mμ (the maximal for conjugated diene) is a measure of the absorption of the fatty acid chain containing the unsaturated center prior to feeding the oxidized oil. The lymph fat had no significant absorption at this point under these conditions of dilution.

that there was an increase in light absorption at 232 mμ (the maximum for conjugated diene) in the lymph of those rats which had been fed the oxidized oil. In the case of rat 6, 3 ml of oxidized oil (PN = 1100) were fed by stomach tube before the cannulation. Collection was begun immediately after the operation (two hours after feeding) and at no time did this animal receive further administrations of oxidized oil. It therefore appears that even though the peroxide itself may not appear in the lymph, its reduction products may be absorbed. The results of the absorption experiments suggest that the fatty acid hydroperoxides are destroyed during or before the absorption process and further, that the toxic effect probably takes place primarily in the intestinal cells.

Intestinal xanthine oxidase assay. Since the absorption studies had led to the hypothesis that the primary site of toxicity was the intestine, and the pathology of these animals was generally negative, an investigation of the effect of oxidized fat on intestinal xanthine oxidase as a representative intestinal enzyme was undertaken. This enzyme was selected because it is fairly well characterized and is sensitive to lack of a dietary component (McQuarrie and Venosa, '45).

Twelve mature male rats were fed a control diet similar to diet B except that unoxidized soybean oil was substituted for the oxidized oil and the level was increased from 15 to 20% at the expense of sucrose. This control diet was continued for 10 days, at the end of which period the animals were paired on the basis of weight.

TABLE 5

Inhibition of intestinal xanthine oxidase by the ingestion of oxidized fat

Days on experiment	Diet	Feed ingested	Enzyme activity[1]	Intestinal protein[2]	Animal weight	
					Initial	Final
		gm/day	*μl O_2*	*%*	*gm*	
0	Control	0	23.5	73.9	226.5	
	Control				212.5	
2	Oxidized	2.4	8.0	62.4	211.0	207.0
	Control	2.4	28.5	59.4	201.5	—
5	Oxidized	3.0	1.5	64.5	193.0	178.5
	Control	3.0	4.5	63.6	194.0	180.0
7	Oxidized	3.7	0.5	68.7	212.0	189.0
	Control	3.7	10.0	62.1	204.0	194.0
9	Oxidized	4.9	1.0	66.9	203.0	177.5
	Control	4.9	13.0	63.6	204.0	209.0
12	Oxidized	5.9	0.0	62.1	200.5	188.0
	Control	5.9	25.0	56.7	197.0	203.5

[1] Corrected for endogenous activity.

[2] Determined by method of Lowry et al. ('51).

One pair was killed at this time for enzyme assay. One animal in each of the 5 remaining pairs was randomly assigned to an experimental diet containing 20% of air-oxidized soybean oil (PN = 1200). The control diet was pair-fed for the duration of the experiment. Pairs of rats were killed at intervals of 2, 5, 7, 9 and 12 days after initiation of the experiment and the intestinal xanthine oxidase of each animal assayed. The results of these assays are presented in table 5.

The observed inhibition of intestinal xanthine oxidase appears to be specific since it cannot be due to either lower protein intake or intestinal edema. One possible explanation for this enzyme inhibition is oxidation of sulfhydryl groups as suggested by Potter and DuBois ('43) in their study of succinic dehydrogenase. For an investigation of this possibility a group of 12 weanling female rats were paired and subjected to the same dietary regimen as in the previous experiment. Pairs of rats were killed each day for 6 days and the levels of SH in each intestine determined. The intestinal samples were prepared as described above and the method of Grunert and Phillips ('51) was used to determine the levels of SH, reported as glutathione. The results obtained in this experiment are given in table 6.

Apparently, the inhibition of intestinal xanthine oxidase is not directly connected with oxidation of sulfhydryl groups. In order to investigate further the mechanism of this enzyme inhibition, milk xanthine oxidase was purified by the method of Ball ('39) and *t*-butyl hydroperoxide was used as the inhibitory compound. The assay

TABLE 6

Levels of intestinal glutathione in weanling female rats consuming either oxidized or unoxidized soybean oil

Rat no.	Oil	No. of days on experiment	Intestinal glutathione level
			μg/mg fresh tissue
7	Control	1	0.38
8	Oxidized		0.57
9	Control	2	0.51
10	Oxidized		0.75
23	Control	3	0.27
24	Oxidized		0.21
11	Control	4	0.24
12	Oxidized		0.37
19	Control	5	0.19
20	Oxidized		0.22
25	Control	6	0.27
26	Oxidized		0.43

TABLE 7

Warburg assay of the inhibition of milk xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[4]
Buffer[1]	Enzyme	t-Butyl hydroperoxide 1.1×10^{-4} M	FAD[2,3] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.3				1.7	0.0
1.0	0.3			1.0	0.7	80.3
1.0	0.3	0.1		1.0	0.6	73.9
1.0	0.3	0.1	0.1	1.0	0.5	90.6

[1] 0.039 M K–$NaPO_4$, pH 7.54.
[2] Flavin adenine dinucleotide.
[3] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.
[4] Average of duplicate flasks.

TABLE 8

Warburg assay of the inhibition of rat intestinal xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[1]
Buffer[2]	Enzyme[3]	t-Butyl hydroperoxide 1.1×10^{-4} M	FAD[4,5] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.5				1.5	43.6
1.0	0.5			1.0	0.5	69.1
1.0	0.5	0.1		1.0	0.4	42.4
1.0	0.5	0.1	0.1	1.0	0.3	71.7

[1] Average of duplicate flasks.
[2] 0.039 M K–$NaPO_4$, pH 7.54.
[3] A 5% intestinal homogenate.
[4] Flavin adenine dinucleotide.
[5] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.

technique was essentially the same as that previously described except that a 0.039 M phosphate buffer with equal concentrations of sodium and potassium at pH 7.5 gave higher oxygen uptake and was, therefore, substituted for the pyrophosphate buffer. In view of the work of Bernheim, et al. ('52) and Ottolenghi et al. ('55) the effect of flavin adenine dinucleotide (FAD) on the inhibited enzyme was investigated. The data obtained in this experiment are reported in table 7.

The results of this experiment indicate that *t*-butyl hydroperoxide is capable of inhibiting milk xanthine oxidase and further, that this inhibition may be reversed by FAD. The high value for the flask containing FAD probably means that the enzyme preparation is somewhat deficient in FAD. A similar assay using a 5% intestinal homogenate from a stock rat as the enzyme source yielded the results presented in table 8.

This experiment corroborates the similarity of the inhibitory effects of the hydroperoxide and the ability of FAD to reverse these effects although apparently not as completely as with the purer preparation.

DISCUSSION

The investigations described above support earlier work in establishing that the toxicity ascribed to air-oxidized oils correlates well with the fatty acid peroxide content of these materials. The feeding experiment in which *t*-butyl hydroperoxide was utilized substantiates, at least in some degree, the toxicity of fatty acid peroxides, although this substantiation must be qualified because of differences in structure and probable mode of absorption. Nevertheless, from the data obtained in this experiment it can be calculated that the consumption of peroxide in the form of *t*-butyl hydroperoxide corresponds to a diet containing 20% of oil with a PN of approximately 600. It is interesting to note that the average growth curve of these animals falls between those for rats receiving diets with oils of 400 and 800 PN. The fractionation of air-oxidized soybean oil showed predominantly polymers of a polar nature, probably polyperoxides, and little or no polymers of the type found in heat-oxidized oil. This study also illustrates the complexity of the reactions occurring in the autoxidation of an oil, since if a simple generation of peroxide groups were the only reaction occurring, equivalence between peroxide oxygen and conjugated diene should be observed. Such is not the case, however (table 2). Although the work reported here demonstrates the toxicity of oxidized oil to be due entirely to peroxide formation, we have not investigated the toxic principle of anaerobically heated oils. Thus, it may be suggested that two separate classes of toxic compounds are possible in autoxidized oil—fatty acid peroxides when the fat is oxidized in air at only slightly elevated temperatures for 1.5 weeks or less and fatty acid polymers when the oil is heated to temperatures in the neighborhood of 250°C with the exclusion of oxygen for periods of one to two days. The conditions of autoxidation for a maximum yield of one class or the other will vary, however, with different oils. In our laboratory we have observed that extended aeration of soybean oil results in an oil of lower than expected peroxide content, and that the application of high temperatures at any point during the autoxidation process will have the same effect. It is probable that either of these modifications tends to decrease the peroxide content of the oil by formation of degradation products and polymers.

The primary site of toxicity, as demonstrated in the cannulated rats, appears to be the intestine. The gross observations and the limited pathology data available appear to support this conclusion. Even the irradiation experiment lends some indirect support to this hypothesis since it is known that the intestine is one of the most sensitive organs to whole-body irradiation, and extended recovery periods were observed in those animals receiving a dietary fat containing peroxide.

The mechanism or mechanisms by which fatty acid peroxides cause the observed toxic effects still need clarification and amplification. In the absence of observable gross changes in any of the organs examined, it seems likely that the injury is of a more subtle nature. The inhibition of intestinal xanthine oxidase, however, points to the possibility that choline oxidase and amine oxidase may be inhibited in a similar manner and that succinoxidase and cytochrome oxidase may also be inhibited but in a manner as yet unknown (Bernheim et al., '52; Ottolenghi et al., '55).

SUMMARY

The toxicity of air-oxidized soybean oil to weanling rats was investigated in several aspects.

Growth studies demonstrated that the concentration of the toxic principle corresponded closely to the peroxide concentration of the oil. Moreover, separation of the oxidized oil into high-, medium- and low-peroxide fractions revealed that toxicity again followed peroxide concentration.

Histopathological examination of rats fed oxidized soybean oil gave generally negative findings but indicated that the intestine might be involved. Moreover, absorption of fatty acid peroxides in mature rats, studied by means of thoracic duct cannulation, indicated that although the reduced products of the peroxides were absorbed, the peroxides themselves were destroyed in the intestine and probably had their action at that site. Recovery from the effects of whole-body irradiation, a condition also affecting the intestine, was delayed by diets containing fat with peroxide numbers as low as 100.

Inhibition of intestinal xanthine oxidase by air-oxidized soybean oil and its reversal by exogenous flavin adenine dinucleotide suggest that the specific toxicity of the lipid peroxides may be at the level of the intestinal enzymes.

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LITERATURE CITED

Andrews, J. S., J. F. Mead and W. H. Griffith 1956 Toxicity of lipid peroxides in the rat. Federation Proc., *15:* 918.

Ball, E. G. 1939 Xanthine oxidase; purification and properties. J. Biol. Chem., *128:* 51.

Bernheim, F., K. M. Wilbur and C. B. Kenaston 1952 The effect of oxidized fatty acids on the activity of certain oxidative enzymes. Arch. Biochem. Biophys., *38:* 177.

Bollman, J. L., J. C. Cain and J. H. Grindlay 1948 Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat. J. Lab. Clin. Med., *33:* 1949.

Burr, G. O., and R. H. Barnes 1943 Non-caloric functions of dietary fats. Physiol. Rev., *23:* 256.

Common, R. H., E. W. Crampton, F. Farmer and A. S. W. DeFreitas 1957 Studies to determine the nature of the damage to the nutritive value of menhaden oil from heat treatment. J. Nutrition, *62:* 341.

Crampton, E. W., F. A. Farmer and F. M. Berryhill 1951a The effect of heat treatment on the nutritional value of some vegetable oils. Ibid., *43:* 431.

Crampton, E. W., R. H. Common, F. A. Farmer, F. M. Berryhill and L. Wiseblatt 1951b Studies to determine the nature of the damage to the nutritive value of some vegetable oils from heat treatment. I. The relation of autoxidation to decrease in the nutritional value of heated linseed oil. Ibid., *43:* 533.

——— 1951c Studies to determine the nature of the damage to the nutritive value of some vegetable oils from heat treatment. II. Investigation of the nutritiousness of the products of thermal polymerization of linseed oil. Ibid., *44:* 177.

Crampton, E. W., R. H. Common, F. A. Farmer, A. F. Wells and D. Crawford 1953 Studies to determine the nature of the damage to nutritive value of some vegetable oils from heat treatment. III. The segregation of toxic and non-toxic material from the esters of heat-polymerized linseed oil by distillation and by urea adduct formation. Ibid., *49:* 333.

Crampton, E. W., R. H. Common, E. T. Pritchard and F. A. Farmer 1956 Studies to determine the nature of the damage to the nutritive value of some vegetable oils from heat treatment. IV. Ethyl esters of heat-polymerized linseed, soybean and sunflower seed oils. Ibid., *60:* 13.

Dhungat, S. B., and A. Sreenivasan 1954 The use of pyrophosphate buffer for the manometric assay of xanthine oxidase. J. Biol. Chem., *208:* 845.

Granados, H., E. Aaes-Jorgensen and H. Dam 1949 Influence of certain nutrients on changes in adipose and dental tissues of vitamin E-deficient rats. Brit. J. Nutrition, *3:* 320.

Grunert, R. R., and P. H. Phillips 1951 A modification of the nitroprusside method of analysis for glutathione. Arch. Biochem., *30:* 217.

Kaneda, T., H. Sakai and S. Ishii 1955 Nutritive value or toxicity of highly unsaturated fatty acids. J. Biochem. (Japan), *42:* 561.

Kaunitz, H., C. A. Slanetz and R. E. Johnson 1955 Antagonism of fresh fat to the toxicity of heated and aerated cottonseed oil. J. Nutrition, *55:* 577.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.

Matsuo, N. 1954 Studies on the toxicity of fish oil. J. Biochem. (Japan), *41:* 647.

McQuarrie, E. B., and A. T. Venosa 1945 The effect of dietary protein intake on the xanthine oxidase activity of rat liver. Science, *101:* 493.

Milas, N. A., and D. M. Surgenor 1946 Studies in organic peroxides. VIII. *t*-Butyl hydroperoxide and di-*t*-butyl peroxide. J. Am. Chem. Soc., *68:* 205.

Ottolenghi, A., F. Bernheim and K. M. Wilbur 1955 The inhibtion of certain mitochondrial enzymes by fatty acids oxidized by ultraviolet light or ascorbic acid. Arch. Biochem. Biophys., *56:* 157.

Polister, B. H., and J. F. Mead 1953 Effect of certain vitamins and antioxidants on irradiation-induced autoxidation of methyl linoleate. J. Agr. Food Chem., *2:* 199.

Potter, V. R., and K. P. DuBois 1943 Studies on the mechanism of hydrogen transport in animal tissues. VI. Inhibitor studies with succinic dehydrogenase. J. Gen. Physiol., *26:* 391.

Raju, N. V., and R. Rajagopalan 1955 Nutritive value of heated vegetable oils. Nature, *176:* 513.

The Growth, Breeding and Longevity of Rats Fed Irradiated or Non-Irradiated Pork[1,2]

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The age-old problem of food preservation has received a new impetus with the use of ionizing radiation. Although many problems exist, the practical use of this method has a promising future.

The research of Poling and coworkers ('55) failed to demonstrate any "unwholesomeness" in diets of rats fed irradiated beef, although some loss of vitamin E was noted. Cathode or beta rays were used in the irradiation process. A similar study was reported by Bubl and Butts ('56) using mixed organ meats subjected to gamma irradiation. No toxic effects were noted in breeding performance or longevity of the rats. The nutritional value of irradiated synthetic diets was studied by Richardson and Brock ('58). Reproduction and longevity were measured. They concluded that there was a slight difference in favor of the non-irradiated diet, but the difference was so small that they did not consider it to be of any practical importance. In a short-term experiment Read and coworkers ('58) reported no toxicity in 14 irradiated foods when fed to rats for 8 to 12 weeks at 35% of the dry weight of the diet.

The work reported here is part of the contractual program of the Office of the Surgeon General, Department of the Army, to determine the wholesomeness of irradiated foods, based on the procedure recommended by Lehman and Laug ('54). This broad research program was established to test a wide spectrum of representative foods from which extrapolation to most others could occur. Pork was one of these foods as it represented a fresh meat of high lipid and high moisture content.

The long-term feeding experiments were designed to obtain data concerning food consumption, growth, reproduction, lactation, size and viability of young and longevity. Four direct-line generations of rats were used in obtaining the data reported here.

EXPERIMENTAL

Both the irradiated and non-irradiated pork used in these experiments was supplied by the Quartermaster Food and Container Institute, Chicago. The cuts used were boned loin or shoulder which had been minced in a mechanical grinder, then passed through a fine-plate sausage grinder. The mass was then thoroughly mixed, usually in quantities of 1000 pounds or greater. The mixture was then packed into no. 2 (307 by 405) "C" enamel cans, sealed under vacuum and sharp frozen. The cans of pork serving as the control (non-irradiated) were shipped to this station frozen and stored at −10°F. until needed.

Two levels of irradiation were used: 2.79 megarad (3 megarep.) and 5.58 megarad (6 megarep.) to prepare the experimental meat. These levels were obtained in the canal of the Materials Testing Reactor, Phillips Petroleum Company, Idaho Falls, Idaho, using spent fuel rods giving mixed gamma radiation. Upon completion of irradiation, the cans of pork mixture were shipped to this station at ambient temperature. They were stored at room temperature, 78°F. (range 72 to 84°F.), for from three to 8 months from the date of irradiation prior to mixing in the diet.

Both the irradiated and control pork were cooked prior to mixing in the ration.

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In planning diets for clinical studies, the guiding principles have been: replacement of a portion of the more saturated fats with polyunsaturated vegetable oils with a simultaneous reduction in total fat intake, and, if necessary, reduction in total caloric intake (7,11,12,18,25,26,32,37,39,40,45,46). In other words there has been a substitution of one type of fat for another, rather than addition of a new fat and, hence, no drastic change in normal dietary habits. Whether or not this modification will have a desirable effect on the development of coronary heart disease is a matter that can be determined only after many years of study of such diets. Several large studies of this type are currently in progress and, although no definite conclusions are yet possible, the trend seems to be toward a lower incidence of new heart cases in the vegetable oil diet group.

Regardless of what these studies eventually prove, it seems obvious that the tendency in the United States is going to be toward a less rich diet, if only to control obesity. Fats are the most concentrated sources of calories and are added in large quantities during food preparation. It is reasonable to believe that the use of tablespreads, shortenings, and the like could be cut appreciably without nutritional loss and, in fact, with a possible benefit. The present American diet containing 40% of calories from fat is unnecessarily rich and the tendency at present would seem to be toward recommending a fat intake of 25 to 30% of the total calories.

Members of the fats and oils industry know, of course, that there are important differences in shortening value, stability, and so forth, between the ordinary animal fats, the hydrogenated oils, and the natural edible oils. Recently it has become obvious there are also important nutritional differences which can be related to fatty acid composition.

The evidence now indicates that the time is approaching when diet planning will include a balancing of the fatty acids just as we already balance amino acids, vitamins, and minerals to ensure that intakes of all essential ones are adequate. It seems probable that the present American diet has too low a ratio of linoleic to saturated fatty acids and would be improved by a cut in total fat calories with an increased use of the unhydrogenated vegetable oils in place of a portion of the more saturated solid fats.

In particular we are becoming increasingly aware of the unique value of linoleic and other polyunsaturated fatty acids. There now seems little doubt that these are very important constituents of the fats, far too valuable to be destroyed by such processes as hydrogenation. Recognizing the requests of nutritionists for higher linoleate-content shortenings and margarines, many companies in the industry have been working on the development of such products and several are already in the markets.

Because the immediately obvious merits of such new products are related to the cholesterol problem, a recent statement of the Food and Drug Administration (14) deserves comment. This states in part: "The role of cholesterol in heart and artery diseases has not been established. A causal relationship between blood cholesterol levels and these diseases has not been proved. The advisability of making extensive changes in the nature of the dietary fat intake of the people of this country has not been demonstrated."

This is a laudably conservative version of the same conclusions reached in the present discussion. Unfortunately this ruling has been interpreted in many quarters as a) discrediting all the evidence that vegetable oils do differ from animal fats in effects on serum cholesterol levels and b) discounting indications from clinical and epidemiological studies that coronary-prone individuals may be benefited by diets lower in saturated fats. Such an interpretation is not in accord with the facts. Many clinicians are convinced that the evidence is impressive enough to warrant large-scale testing and that, meanwhile, the prudent coronary-prone individual is well advised to make some changes in his diet with respect to fats. The interpretation put on the FDA statement has to some extent tended to hamper development of new high-linoleate food products just at the time when these are most needed for clinical study.

Of course if foods are to have a higher linoleic to saturated fatty acid (L/S) ratio there will be shelf-life problems. The linoleate-rich oils naturally oxidize or become rancid more rapidly than do the more saturated, linoleate-poor fats. It is well known that from a nutritional point of view rancid fats have several undesirable properties. Thus, if a higher L/S ratio is desirable, new ways may have to be found to stabilize foods containing these fats, perhaps with new antioxidants or new packaging materials. As you well know, this is going to be a complex problem because the edible oils vary widely in stability. For example refined soybean oil reverts easily whereas refined corn and cottonseed oils are quite stable, presumably because of their high tocopherol contents.

Summary

So far as the industry is concerned, the evidence indicating a relationship of dietary fat to heart disease presents some interesting challenges. Undoubtedly it portends a change in the fat consumption pattern toward a lower *per capita* use coupled with a shift from solid fats toward a higher proportion of edible oils. Most important of all, however, is the growing recognition that fats and oils are nutritionally valuable foods, intimately related to health and well-being, and should by no means be regarded merely as a source of calories.

Although there are innumerable factors involved in the etiology of heart disease, dietary fat is an important one and fortunately is one that can be modified in whatever way proves desirable. Because the more saturated types of fats lead to higher serum cholesterol levels than do the polyunsaturated oils, and because cholesterol is somehow involved in the course of atherosclerotic heart disease, clinical tests are now in progress to determine whether prolonged use of a diet rich in these oils will lead to fewer heart attacks than does the usual American diet rich in saturated fats. So far, data are encouraging enough to merit recommendation of the modification in dietary fat to the coronary-prone individual and to justify development of new high-linoleate fat products by the industry.

REFERENCES

1. Ahrens, E. H. Jr., Am. J. Med. *22*, 928–953 (1957).
2. Ahrens, E. H. Jr., Hirsch, J., Insull, W. Jr., Tsaltas, T. T., Blomstrand, R., and Peterson, M. L., J. Am. Med. Assoc. *164*, 1905–1911 (1957).
3. Anderson, J. T., Keys, A., and Grande, F., J. Nutrition *63*, 421–444 (1957).
4. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A., Can. J. Biochem. Physiol. *34*, 441–455 (1956).
5. Beveridge, J. M. R., Connell, W. F., Mayer, G. A., and Haust, H. L., J. Nutrition, *71*, 61–65 (1960).
6. Beveridge, J. M. R., Connell, W. F., Mayer, G. A., Haust, H. L., and White, M., Can. J. Biochem. Physiol. *36*, 895–911 (1958).
7. Brock, J. F., Postgrad. Med. J. *35*, 216–217 (1959).
8. Brock, J. F., and Gordon, H., Postgrad. Med. J. *35*, 223–232 (1959).
9. Bronte-Stewart, B., Brit. Med. Bull. *14*, 243–252 (1958).
10. Bronte-Stewart, B., Postgrad. Med. J. *35*, 198–202, 232 (1959).
11. Brown, H. B., and Page, I. H., J. Am. Med. Assoc. *165*, 1989–1995 (1958).
12. Brown, H. B., and Page, I. H., J. Am. Med. Assoc. *173*, 248–252 (1960).
13. Dewber, T. R., Moore, F. E., and Mann, G. V., Am. J. Public Health *47*, Suppl. 4, 4–24 (1957).
14. Federal Register *24*, 9990 (Dec. 10, 1959); Food and Drugs 21.1.A.3.41.
15. Gertler, M. M., Woodbury, M. A., Gottsch, L. G., White, P. D., and Rusk, H. A., J. Am. Med. Assoc. *170*, 149–152 (1959).
16. Gordon, H., Lewis, B., Eales, L., and Brock, J. F., Lancet, 1957-II, 1299–1308.
17. Gore, I., Hirst, A. E., and Koseki, Y., Am. J. Clin. Nutrition *7*, 50–54 (1959).
18. Grayhiel, A., J. Am. Med. Assoc. *172*, 2057–2060 (1960).
19. Hartroft, W. S., O'Neal, R. M., and Thomas, W. A., Federation Proc. *15*, Suppl. 3, 36–46 (1956).
20. Hashim, S. A., Clancy, R. E., Hegsted, D. M., and Stare, F. J., Am. J. Clin. Nutrition *7*, 30–34 (1959).
21. Haust, H. L., and Beveridge, J. M. R., Arch. Biochem. Biophys. *78*, 367–375 (1958).
22. Hellman, L., Rosenfeld, R. S., Insull, W. Jr., and Ahrens, E. H. Jr., Circulation *16*, 497 (1957).
23. Holman, R. L., McGill, H. C. Jr., Strong, J. P., and Geer, J. C., Am. J. Pathol. *34*, 209–235 (1958).
24. Jolliffe, N., and Archer, M., J. Chronic Diseases *9*, 636–652 (1959).
25. Jolliffe, N., Rinzler, S. H., and Archer, M., Am. J. Clin. Nutrition *7*, 451–462 (1959).
26. Katz, L. N., Stamler, J., and Pick, R., "Nutrition and Atherosclerosis," Lea and Febiger, Philadelphia, 1958.
27. Keys, A., J. Am. Med. Assoc. *164*, 1912–1919 (1957).
28. Keys, A., J. Chronic Diseases *6*, 552–559 (1957).
29. Keys, A., Anderson, J. T., and Grande, F., Circulation *16*, 480 (1957).
30. *Ibid.* *19*, 201–214 (1959).
31. Kinsell, L. W., J. Am. Med. Assoc. *170*, 2201–2202 (1959).
32. Kinsell, L. W., Michaels, G. D., Walker, G., Wheeler, P., Splitter, S., and Flynn, P., Diabetes *8*, 176–188 (1959).
33. Lawry, E. Y., Mann, G. V., Peterson, A., Wysocki, A. P., O'Connell, R., and Stare, F. J., Am. J. Med. *22*, 605–623 (1957).
34. Lewis, B., Lancet 1958-I, 1090–1092.
35. Malmros, H., and Wigand, G., Lancet 1957-II, 1-8.
36. McCann, M. B., Trulson, M. F., Waddell, W. R., Dalrymple, W., and Stare, F. J., Am. J. Clin. Nutrition *7*, 35–42 (1959).
37. Olson, F. E., Splitter, S., Balch, H., Flynn, P. F., and Kinsell, L. W., Am. J. Clin. Nutrition *6*, 632–634 (1958).
38. Olson, R. E., Am. J. Public Health *49*, 1120–1128 (1959).
39. Pilkington, T. R. E., Stafford, J. L., Hankin, V. S., Simmonds, P. M., and Koerselman, H. B., Brit. Med. J. 1960, 23–26.
40. Rathmann, D. M., "Unsaturated Fats and Serum Cholesterol," Corn Products Refining Co., New York, 1958.
41. Scarborough, W. R., Smith, E. W., and Baker, B. M., Am. Heart J. *59*, 19–35 (1960).
42. Sinclair, H. M., Lancet 1959-I, 252–254, 474–475; Internatl. J. Geriatrics *1*, 33–40 (1959).
43. Stamler, J., Postgrad. Med. *25*, 685–701 (1959).
44. Stare, F. J., Food Technol. *12*, 33–36 (1959).
45. Stare, F. J., Von Hallie, T. B., McCann, M. B., and Portman, O. W., J. Am. Med. Assoc. *164*, 1920–1925 (1957).
46. Stefanik, P. A., and Trulson, M. F., Postgrad. Med. *26*, 533–536 (1959).
47. Thomas, W. A., Hartroft, W. S., and O'Neal, R. M., A.M.A. Arch. Pathol. *69*, 104–109 (1960).
48. U.S.D.A., Agr. Research Service, Publ. ARS 62-8, "Food Consumption and Dietary Levels of Households in the United States," Aug. 1957.
49. Yerushalmy, J., and Hilleboe, H. E., N. Y. State J. Med. *57*, 2343–2354 (1957).
50. Yudkin, J., Lancet 1957-II, 155–162.

A Nutritive Evaluation of Over-Heated Fats

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According to the tests used, harmful substances do not occur in fried foods or in fats used in preparing foods. It is possible to obtain biologically undesirable materials by excessively heating and/or oxidizing fats in the laboratory, but the conditions required for the production of such materials differ greatly from those used in practical cooking or processing of foods. There appears to be no reason to believe that fats are nutritionally damaged when handled by normally-accepted good practice in present-day food preparation.

FATS ARE IMPORTANT and essential in the diet. They are not something we can take or leave alone; they provide energy, supply essential fatty acids, carry fat-soluble vitamins, improve flavors, modify textures, and add satiety values to meals.

Much of their utility depends upon their stability to heat. In frying operations they prevent sticking and transfer heat from hot surfaces to food. The stability of fats at high temperatures (up to 200°C. in some frying operations) invites repeated or continuous use, and questions have been raised concerning the nutritive value and wholesomeness of fats after long usage. Some data in the scientific literature show that undesirable changes occur in fats if they are heated in the laboratory to high temperatures for long periods or if they are subjected to severe oxidizing conditions. Other reports indicate that fats which have been used for prolonged commercial or home cooking retain their nutritive value and remain wholesome.

There are two major reasons why food technologists handling fats should be familiar with this subject. The nutritive values of fats at all stages of processing and use should be known, and there are significant and frequently adverse public relation aspects which must be handled. Publicity problems usually arise from misinterpretations or from unjustified extrapolations of laboratory findings. Even though the implied effects may not be true, headlines such as "The Carcinogenic Action of Heated Fats and Lipoids" (1) cannot be considered in the best interests of the fat industry. The facts must be known in order to understand the problems and to combat misleading reports or inferences.

It is not our intention to review this subject exhaustively. Instead we plan to consider published and unpublished research findings which indicate typical changes that can occur in food fats during laboratory treatments or cooking procedures and to contrast the findings with those obtained when food fats are tested. Noncritical review of the scientific literature relating to heated fats can lead to very erroneous conclusions since reports show that it is possible to mistreat fats experimentally with sufficient heat and/or oxygen to cause, when the abused fat is fed to test animals, retarded growth, poor feed efficiencies, rough, greasy matted coats, diarrhea, starvation, enlarged livers and kidneys, abnormal fat depots, impaired enzymatic functions, abnormal water metabolism, papillomas and other growth formations, and shortened life spans. In extreme cases animals may die in a few days after severely abused fats have been fed. There is thus no question whatsoever that fat can be damaged by purposeful abuse. The critical question is: "are fats damaged during processing or cooking operations?" To answer that question we must examine some of the conditions which produce the effects listed above. They may have been

deliberately designed to produce measurable damage rather than to simulate cooking procedures.

THERE IS NO EVIDENCE that food fats have carcinogenic properties unless they have been abused far beyond normal conditions. Nevertheless inferences that food fats may be involved in the disease are too frequent. One of the early suggestions that heated fats might cause cancer was made by A. H. Roffo (2), who claimed that sunflower and olive oils oxidized by heating to 250–350°C. (482–662°F.) had carcinogenic potencies when fed to rats. This should be recognized as very drastic treatment. Lane, Blickenstaff, and Ivy (3) however did not get tumors when lard "browned" at 350°C. (662°F.) for 30 min. was fed to rats from the colony of Dr. Roffo, but there were increased incidences of papillomas of the forestomach and ulcers in the glandular stomach. Peacock and Eck made similar claims for the feeding or injection of cottonseed oil heated to 350°C. (662°F.) for 4 hrs. (4, 5). He was unable to find any known carcinogen in the fat. Two mice out of 300 showed tumors after 15 months on the diets. Other investigators have had varying degrees of success in efforts to demonstrate carcinogenic properties in heated fats.

While there have been demonstrations that painting overheated fat repeatedly upon the skin, injecting it into the skin or muscle, or feeding it can result in conditions suggestive of the formation of cancer, it is perhaps significant that A. A. Newman, after going to considerable length to imply that epoxidized and heated fats are a nutritional hazard (6), states: ". . . the introduction of autoxidized and thermally-affected fats into test animals can produce pathological lesions ranging, according to conditions, from benign papillomas in the forestomach to malign neoplastic growth in the glandular region. While it must be emphasized that none of the latter type of lesions so far produced have satisfied the rigorous conditions of true carcinogenicity, in several instances the difference was not wide from a practical viewpoint." It might be added that the fats tested were not at all representative of cooking fats.

Fats which have been heated to unrealistically high temperatures do not necessarily indicate intensification of cooking conditions. They may have undergone chemical changes quite different from those occurring under cooking conditions. Nevertheless inferences drawn from reports of exaggerated conditions can result in scare propaganda that is of no value to anyone. More research is needed to clarify the situation completely, and this research needs to be done with food sources of fat as well as with unrealistically-abused fats.

Feeding studies have been conducted by many different laboratories in studies of "abused" fats. No effort will be made to review all of the relevant reports. Only a few will be selected to illustrate the nature of the studies and typical findings. The treatments fall into three general categories: heating in the absence of air, heating in the presence of air, and oxidation at low temperatures.

ONE OF THE FIRST groups to study the biological effects of polymerization was that of E. W. Crampton and his co-workers (7). They blew carbon dioxide through various oils heated to 275°C. (527°F.) in all glass equipment for various intervals of time. Table I, adapted from their Table 2 (7), illustrates several things. The inclusion of severely heat-treated oils in rat diets at levels of 10 or 20% reduced rates of gain and decreased caloric efficiencies. The amounts of damage were proportional to the length of heat treatments. Different types of oils differ in response. Linseed, the most unsaturated, shows the most damage.

These workers observed that appetite was depressed when thermally-polymerized oils were fed and that the feces of the animals were dark and sticky. Hair coats were also oily and matted whereas the controls were sleek and clean.

TABLE I
Effects of Heat Treatment of Oils[a]

Test oil used	Duration of heating	Aver. daily gain	Aver. daily feed intake	Gain per 1,000 cal.
	hours	g.		
Linseed	0	3.9		
	2	3.8	9.6	91
	4	3.4[b]	9.6	91
	8	2.5[b]	9.5	80
Corn	0	3.6	8.1	69
	15	2.8	10.4	78
	30	2.3[b]	8.7	71
Soybean	0	4.6	8.0	64
	3	4.0	10.2	90
	9	2.9[b]	9.4	65
			7.7	76

[a] Adapted from the data of Crampton, Farmer, and Berryhill (7). Ten or 12 animals were used per group, and fats were fed at 10% of the diet except for 20% of soybean oil.
[b] Significantly lower than controls.

In contrast to these findings Lassen, Baron, and Dunn (8) report that adult rats fed edible, polymerized sardine oils at a level of 5% in the diet were healthy after short experiments and showed no significant changes in urine analyses. They demonstrated however that sardine oil which was 30% polymerized was only 85% digestible in comparison with the 98% digestible found for unpolymerized oils.

O. C. Johnson and his co-workers at the University of Illinois added the stress of oxidation to heat treatment by blowing 100 milliliters per minute of air through 1,500 g. of various oils heated to 200 ± 10°C. (392°F.) in stainless steel beakers for various periods up to 24 hrs. (9). This is a very rigorous treatment and results in increased acid values, increases in viscosity and color, and decreased iodine values. Rats fed thermally-oxidized butter oil gained as well as controls fed fresh butter oil; but rats fed thermally-oxidized corn oil gained poorly in comparison to their controls on fresh corn oil. Final weights after nine weeks on corn oil tests were 124 and 332 g., respectively. That these responses were not caused by differences in food intake was demonstrated by a paired feeding experiment, in which the thermally-oxidized corn oil gave significantly less growth (1% level) than the fresh oil. Margarine stock heated and oxidized under the same conditions depressed growth slightly when pair-fed in comparison with fresh oil.

Temperatures as high as 200°C. (392°F.) cause rapid destruction of fatty peroxides, and the course of polymerization at such severe conditions may be very different from what it is at lower temperatures. In this connection it is of importance to note that when Kaunitz and Slanetz (10) fed 15% cottonseed oil, which had been aerated at 95°C. (203°F.) for 200 hrs. (iodine No. 141), immediate diarrhea and weight loss occurred and about half of the animals died within three weeks. At a level of 10% peroxidized fat only a few rats died, and the others seemed to adjust to the diet, overcoming the diarrhea and gaining slightly in weight. The addition of fresh oil along with the heated oil seemed partially to overcome the effects of the peroxidized oil. The only symptom was slower gains. The addition of Vitamins E, A, and D did not result in a corresponding improvement.

Further heating of a cottonseed oil with peroxide value of 191 caused a reduction in peroxide value to 141 but an increase in severity of symptoms when fed, leading Kaunitz and co-workers to conclude that the amount of peroxide present was not related to the degree of toxicity. In addition to the growth-depressing effects Kaunitz has reported that the feeding of heated fats causes enlarged kidneys, livers, and adrenals, also small spleens and thymus glands. Water intake is also greatly increased (11).

Recently Andrews and co-workers (12) have published data to indicate that growth depression in rats fed oxidized soybean oil is proportional to the extent of oxidation from peroxide numbers of 100 to 1,200. Using cuprie and ferric ions as catalysts, they oxidized soybean oil by aeration at 60°C. (140°F.) to peroxide numbers as high as 1,200. When fed as 20% of the diet, 1,200 peroxide number fat caused immediate, severe diarrhea and sustained losses in weight, also fatalities of all animals in three weeks. Dilution of this product with fresh soybean oil to give mixtures with peroxide numbers of 800 and 400 lessened the severity of the symptoms and prevented the fatalities.

THESE STUDIES and a number of others proved that fat can be damaged, but the conditions needed for damage were much different from home or commercial cooking operations. The need for severe laboratory treatment of highly unsaturated oils to get the marked changes suggested that sensitive methods might be essential if one were to study practical operating conditions. Furthermore rapid methods for measuring changes in nutritive value and wholesomeness are essential if many fats are to be examined. The tests commonly used required 8 to 12 weeks of test feeding of experimental animals.

With this in mind we undertook the development of methods which would quickly detect changes of fat quality. Perhaps the most successful of these has been a restricted-feeding technique, which permits exact comparison of control and experimental animals (13). This grew out of the postulate that fatty substances which had been damaged might not be available for energy. If this were true, under proper conditions the rate of gain of animals fed abused fats should be proportional to the amount of undamaged fat remaining. In practice weanling rats are fed 5-g. quantities of a basal ration containing only enough energy-containing substances to permit slight growth but formulated to supply an excess of the daily needs of all essential nutrients. Additions of energy-containing materials to such a diet permits growth in proportion to the amount of energy added. This is true whether the extra food is carbohydrate, fat, or protein in nature.

Table II illustrates the application of such a technique to laboratory-heated and/or oxidized fats. These are typical values, and it may be seen that the available energy is markedly reduced by severe heating or oxidation. A 7-day period is adequate for such determinations. The results are quite uniform and reproducible, permitting the use of small groups of animals. In addition to demonstrating reductions in energy value, animals fed fats which have been excessively heated or oxidized experimentally showed the organ enlargements that others have reported in longer feeding-studies. The increase in liver weight occurs very rapidly, being easily detectable in three days and maximal in five to seven days. These livers are not fatty; by analysis and by gross and limited microscopic inspection the tissue is normal.

TABLE II
Energy Values of Abused Fats

Substance added	Aver. gain in 7 days[c]	Available energy[d]	Liver size
	g.	%	% body wgt.
None	5.0		
Fresh cottonseed oil	27.0	100	4.0
Severely heated CSO[a]	21.0	72	5.4
Severely oxidized CSO[b]	4.7	0	7.0

[a] 120 hrs. at 162°C. in household cooker.
[b] Air blown through oil held at 60°C. for 10 days.
[c] 2.5 g. of fat fed each day in addition to basal.
[d] In term of % of theoretical, based on fresh CSO as 100%.

This technique has been applied in a number of studies of factors which might damage fat. These will be reported elsewhere in more detail, but an outline of the findings will indicate the magnitude of the changes which may be expected with common food fats when heated or oxidized.

That these changes (Table II) are typical is shown by the decreases in available energy values listed in Table III for several samples of salad oil and shortenings. Values for any one sample are quite reproducible, but different lots of a single type of oil vary in response to heat as the two corn oils indicate.

Hence the values in Table III cannot be taken as an indication of the relative stabilities of the fats. Some of these tests were made before liver weights were routinely checked, but liver weights taken in other tests indicate increased size when various types of fats are excessively heated during experiments.

TABLE III
Available Energy of Various Fats After Heating at 182°C. for 120 Hours[a]

Type of oil heated	Available energy in heated fat as % of energy available from unheated product[b]
Cottonseed salad oil, Sample 1	72
Cottonseed salad oil, Sample 2	68
Peanut salad oil	67
Corn salad oil, Sample 1	86
Corn salad oil, Sample 2	75
Hydrogenated meat and vegetable fat shortening	81
Hydrogenated vegetable oil shortening	70
Hydrogenated vegetable oil shortening	84
Meat fat and vegetable fat shortening	76
Lard	66[c]

[a] Approximately 3,000 g. quantities of fat were heated for 120 hrs. at 182°C. in a household deep-fat fryer. Four animals were fed at a level of 1.5 g. per day in addition to 5 g. of basal diet.
[b] These values are for samples of commercial products and do not necessarily reflect the relative merits of the type of product because there is variability from one supplier to another, as the two corn oil samples indicate.
[c] 168 hrs. of heating rather than 120.

Oxygen at moderate temperatures (60°C.) causes little or no decline in nutritive value until the lag phase is overcome; then available energy values go down, and liver sizes of test animals increase. The values in Table IV are for samples taken from cottonseed oil which has been stirred and blown with air.

TABLE IV

Influence of Oxidation Upon the Nutritive Value of Cottonseed Oil

Days of oxidation at 60°C.	I.P.V.	Available energy[a]	Liver size[a]
		%	% of body wt.
0	<1	100	4.0
15	310	100	3.9
18	1130	71	5.3
28	400	0	5.8

[a] Three rats per group, each rat getting 0.6 g. of test fat per day in addition to 5 g. of basal diet. At 1.2-g. test-fat levels rats receiving the fats oxidized for 19 days or more did very poorly and refused part of the diets.

TABLE V

Influence of Time Upon the Effect Which Heat Has Upon the Nutritive Value of Cottonseed Oil

Length of heating period[a]	Energy availability[b]	Liver size[b]
days	%	% of body wt.
0	100	5.0
1	95	5.7
2	95	6.1
3	93	6.4
4	72	6.9
5	75	7.1

[a] Heated in 3,000-g. quantities, not stirred, in household deep-fat fryer at 182°C.
[b] All animals fed 1.5-g. of test fat daily in addition to 5 g. of basal diet. Four rats per group.

Similarly heating fat in 3,000-g. quantities at 182°C. (360°F.) in a household deep-fat fryer causes gradual reduction of nutritive value (Table V). It should be noted that these are not applied conditions. No food was cooked, there was no addition of fresh fat, nor was there removal of volatile materials with steam. Even so, changes are slight during the early stages of heating.

THE INFLUENCE of varying the percentage of oil exposed to air at any given temperature is indicated in Table VI. In this case varying amounts of oil were heated in the same pan for the periods of time shown. In other tests constant amounts of oil were heated at different temperatures. It may be noted that a change in the amount of fat heated per unit of surface area has more influence than a change in temperature. The 50-g. quantities of oil rapidly became viscous, and the ones which had been heated for 6 hrs. had to be scraped from the pan with rubber spatulas. In general, samples which showed heat damage were quite viscous and certainly would not be suitable frying aids. Various fats respond differently although all tend to show the same changes.

TABLE VI

Influence of Surface Exposure Upon Changes in the Nutritive Values of Hot Cottonseed Oil

Heating period	Fat heated at 180°			Fat heated at 200°C.	Fat heated at 220°C.
Minutes	50 g.[b]	100 g.[b]	200 g.[b]	100 g.	100 g.
	Energy Availabilities as Percentage of Control Oil[a]				
	%	%	%	%	%
0	100	100	100	100	100
30	102	106	108	102	112
120	98	90	94	94	90
360	50	80	98	77	69
	Liver Weights as Percentage of Body Weights[a]				
0	4.4	4.4	4.4	4.4	4.4
30	4.6	4.4	4.0	4.2	4.5
120	5.4	5.3	4.5	5.0	5.4
360	6.8	6.3	5.4	6.2	6.7

[a] Samples fed at a level of 1.2 g. per animal, in addition to 5 g. of basal diet, to group of 4 animals on each test.
[b] The 50-, 100-, 200-g. quantities correspond to an oil depth of approximately 2, 4, and 6 mm., respectively, for oil heated in an 8½-in. diameter aluminum pan.

In the present tests none of the fats changed rapidly enough to give cause for concern. The treatments in which changes were detectable were more severe than conditions encountered in reasonable home or commercial cooking. In fact, undesirable increases in viscosity, color, and flavor precede the detectable biological effects even in those relatively sensitive tests where the treated fat is the sole additive to the diet. Hence, while these data suggest that slight changes may occur during normal usage, such changes will be minimal and by no means as severe as those reported in the scientific literature on abused fats.

A different type of response to a substance in fat was noted in 1957 when a heavy incidence of an edematous condition in the broiler type of chickens appeared in flocks fed specific lots of fat, which were later shown to include residues from fat-processing operations (14). When the contaminated fats were fed, fluid accumulated in the heart sac and/or in the abdominal cavity, sometimes in spectacular quantities. Severely afflicted birds developed distended abdomens, resulting in the designation "water belly" in trade areas. Pathological examination also revealed gross liver and kidney damage. Extensive studies by the regulatory officials and in the laboratories of many feed manufacturers and feed-ingredient suppliers proved that feed-grade fats were harmless if they did not include a particular type of residue from one type of fat processing.

The toxic material could be concentrated in the unsaponifiable fraction of fats, and extensive studies of the chemical and physical properties of concentrates have been made (14, 15, 16, 17). So far, these have not led to identification of the toxic material or to rapid tests for it although Harman *et al.* (17) have recently reported crystallization of about a milligram of material which they believe to be the toxic substance. This crystalline material is reported to be effective at a level of 0.1 mg. per kg. of feed or at a concentration of one-tenth part per million in the diet. There are no indications that this material is related in any way to the factors which are produced when food fats are heated.

SINCE THE ORIGINAL OUTBREAK of poultry disease, various types of food and feed-grade fatty materials have been examined. The application of sensitive tests by Food and Drug Administration officials led to the detection, in some shipments of oleic acid, of traces of materials which gave chickens mild symptoms of hydropericardial disease. On the basis of these findings officials of the Food and Drug Administration are requiring all producers of oleic and stearic acids to chick-test products intended for food use. A detailed method for this purpose has been distributed (18). This involves measurements of the volume of pericardial fluid in chickens after they have been fed test materials at a 16% level for three weeks. Normally chickens have almost no fluid in the heart sac, and the presence of as little as 0.2 ml. is considered by Food and Drug officials as a positive symptom of the condition. Others who have used the method or variants of it do not agree that such a low volume necessarily indicates the presence of toxic materials.

TABLE VII

Potato Chip Frying

Treatment of cottonseed oil	Calorie Availability[a] %	Liver size		14-Day gains		Liver size	
		% of Body wt.	% of Control	Actual	Adjusted to aver. food intake	% of Body wt.	% of Control
1. 28 lb. CSO heated to 182°C.—5 lb. potato chip fried immediately	100	4.42	100	74.3	65.6	6.2	100
2. Heated oil at 182°C. for 24 hrs.—5 lb. chips fried	89	5.16	116	70.0	63.6	6.7	104
3. Heated oil at 182°C. for 48 hrs.—5 lb. chips fried[b]	89	5.58	128	63.3	64.3	6.5	102
4. Heated oil at 182°C. for 72 hrs.—5 lb. chips fried[b]	95	5.41	121	53.5	57.8	7.1	107
5. Heated oil at 182°C. for 96 hrs.—5 lb. chips fried[b]	93	6.23	138	54.5	59.7	7.2	108
6. Heated oil at 182°C. for 120 hrs.—5 lb. chips fried[b]	86	5.58	125	53.3	58.0	6.7	104
7. Heated oil at 182°C. for 120 hrs.—no frying	72	7.14	159	...	...	...	...
8. Heated oil at 182°C. for 120 hrs.—then 5 lb. chips fried[b]	63	6.80	139	40.0	54.4	6.6	103
9. Commercial chips	...	...	...	80.0	72.1	6.1	99

[a] Based on an arbitrary value of 100 for the fresh, heated oil.
[b] Oil foamed violently when potato slices were placed in it.

In a study of oils from 89 potato chip manufacturers, Melnick (21) found insignificant changes in iodine values during processing and claimed on this basis that the products have not been changed in any significant amount. This same conclusion was reached a number of years ago by Deuel and his co-workers (22), who fed oils obtained from potato chip preparation to rats and were unable to detect changes caused by the processing.

Other studies in our own laboratories have led to substantially the same conclusion. In these studies two commercial types of 28-lb. deep-fat fryers were filled with refined cottonseed oil and heated to 182°C. (360°F.). Sliced potatoes to yield five pounds of potato chips were fried twice daily in one of the fryers. After the second frying each day sufficient fresh oil was added to restore the original volume. Except for sampling, oil in the other fryer was undisturbed until the end of the experiment. Samples of potato chips and of oil in each fryer were taken after each 24-hr. interval.

While the cooking of two 5-lb. quantities of potato chips twice daily did not provide the intensive usage experienced by fats in the vats of a grill or a commercial potato chipper, it did cause vigorous agitation and introduced food particles and steam. In addition, each day about 10% of heated fat was removed on the potato chips and had to be replaced. After only two days of use the oil foamed violently during cooking and could be kept in the kettle only by immersing small lots of sliced potatoes. Commercial usage would have been impossible. It is interesting to note that changes in biological quality appeared about the same time that foaming made use impractical.

As a source of energy Oil No. 7, which had been heated without use for cooking, was less effective than Oil No. 6, in which chips had been periodically cooked. Livers of rats fed Oil No. 7 also were heavier than livers of the No. 6 group. It must be remembered however that fresh make-up fat had been added periodically to No. 6 to keep the volume constant.

Chips produced during the experiment were mixed into diets in amount sufficient to supply 20% of fat. The rations provided generously for all nutrient needs of weanling rats. Previous 12-week studies had indicated that maximum effects on growth would occur in two weeks (19, 20). After that, rats fed severely heated and unheated fats grew at about the same rate. Hence the growth studies were restricted to a two-week period.

The gains of the groups fed fat heated for several days were smaller than when fresh fat was fed. However much of this decline in weight probably resulted from the decreased palatability of the diet since statistical adjustment of body weights to a common level of food intake evened out the gains. Certainly there is no evidence of decreased nutritive value on the basis of the adjusted weight gains or on liver sizes for animals fed products from the first three treatments. Livers of animals that were fed oil heated 48 hrs. were heavier than those of the first two groups, but at this time the oil was already foaming when used. Further evidence that the 48-hr. heated oil was beyond practical usage was provided by Schaal accelerated stability tests of the potato chips produced in it. These chips gave a stability of only two days as compared to 19 days for chips fried in fresh oil.

Studies of fats extracted from fried, broiled, or roasted meats have similarly shown no decrease in energy availability or in fractions which cause increase in liver weights (23).

SO FAR, most attention has been given to conditions which produce substances that cause diarrhea, retard growth, and alter organ size, and little mention has been made of the nature of the substances formed or their mode of action. Actually little is known in either of these areas. Almost all of the research workers who have produced damage in their samples have been able to show that the active substances are in the unsaponifiable material or in a fraction that is not adducted by urea. Attempts to obtain pure substances have been somewhat unsuccessful.

Kaunitz and others (24, 25) have reported that simple oxidation products, such as monohydroxy stearate, 9-10 dihydroxy stearate, *cis*-epoxy stearate, or oleate peroxide, do not produce symptoms of tox-

icity and are at least not the principal toxic agents. Andrews *et al.* (12) however showed that t-butyl hydroperoxide depressed growth when fed. Polymeric residues obtained after molecular distillations were more toxic than distillable factions, leading to the conclusion by Kaunitz that polymers of some type were responsible for the physiological effects.

Crampton (7), considering the relative potencies of fractions separated from thermally-polymerized linseed oil by distillation and urea segregation, concluded that at least two factors were involved: acyl radicals whose esters could be easily distilled and which would not form urea adducts, and dimeric or polymeric factions which neither distilled nor formed adducts. The monomeric substances were digestible, *i.e.*, they disappeared from the gastrointestinal tract, and they depressed growth, but the polymeric materials were neither digestible nor toxic (26). Perkins and Kummerow (27, 28) also demonstrated that the urea nonadducts obtained from oxidized fats were the most potent factors.

We also have been able to concentrate the biologically effective materials in unsaponifiable or nonadductible fractions. In one series of studies the nonadduct-forming material (NAF) from cottonseed oil which had been heated to 360°F. for 120 hrs. was serially extracted three times with Skellysolve B (Fraction 1), three times with 30% ethyl ether in Skellysolve B (Fraction 2), three times with 60% ethyl ether in Skellysolve B (Fraction 3), and with pure ethyl ether (Fraction 4). Insofar as material was available, 0.3 g. of each of these fractions was fed with 5 g. of basal diet to rats to determine biological responses. The molecular weights of the fractions were estimated by the technique of Donnelly (29). Results of the study in Table VIII show the fraction soluble in pure Skellysolve B to be as active as an equivalent amount of the unfractionated NAF, and the fractions extracted with ethyl ether to be more potent. Not enough material was obtained for feeding after the extraction with 60% ether.

TABLE VIII

Influence of Fractions of Heated Cottonseed Oil Upon the Growth and Liver Sizes of Rats

Diet	Gain	Liver size	Average molecular wt.
Basal	7	4.1	
Basal + 0.3 g. CSO	15	3.7	
Basal + 0.3 g. heated CSO[a]	13[b]	4.0[b]	
Basal + 0.3 g. NAF of heated CSO	11	5.0	
Basal + 0.3 g. NAF—Fraction 1	11	4.8	673
Basal + 0.3 g. NAF—Fraction 2	10	5.3	552
Basal + 0.3 g. NAF—Fraction 3	6	5.5	1320
NAF—Fraction 4	..	....	1320
NAF—Residue	..	....	very large

[a] 182°C. for 120 hrs.
[b] Interpolated from standard curve.

In another study cottonseed oil heated for 120 hrs. at 182°C. (360°F.) was treated with propanol. Two layers formed the upper or more soluble layer containing 86% of the original oil. When fed in the energy-restriction technique at a 1.5-g. level per day (in addition to 5 g. basal), the energy of the insoluble fraction proved to be only 17% available in contrast to 67% for the soluble fraction and 65% for the unfractionated heated oil. Livers from the corresponding animals averaged 5.5, 7.9, and 7.5% of the body weights. This suggests, as does Crampton's work, that a relatively indigestible but harmless fraction and a readily digestible but harmful fraction exist.

EFFORTS TO PURIFY these materials further have been frustrating, partially because of a lack of test methods applicable to small quantities. Even a 7-day rat test, using 0.3 g. per day for four rats requires 8.4 g. of product. Some urea nonadductable materials from heated fats have been separated into major fractions chromatographically, using silica gel and alumina preparations. There are some differences in the spectral characteristics of such fractions, but so far they have not been tested adequately on a biological basis to permit definition of the active fractions. The very limited work done indicates that several chromatographically distinct fractions have activity. This suggests that a family of compounds of similar physical properties and perhaps similar structures may be involved rather than one or two substances. Such a development would not be unexpected in view of the variety of structures available in unsaturated fatty acids, especially in partially hydrogenated fats where isomerization occurs.

It seems very probable that separations achieved by use of the various types of distillation, chromatography, solvent distributions, molecular sieves, etc., will soon result in much better understanding of the chemical nature of the substances responsible for the several biological effects that have been noted. This will permit more exact study of the amounts of these substances in experimental and food fats and will aid in evaluating the acceptability of heated food fats.

To date little is known of the biological causes of the effects observed. Under selected conditions it is possible to cause diarrhea, rough fur, decreased growth, and even death, but we do not know exactly why. There have been demonstrations that abused fats are less digestible than fresh fats (8, 20, 24) and that organs of animals fed the abused fats have altered sizes and enzyme activities (9, 12, 23, 24), that some fractions may accelerate the formation of certain types of abnormal tissues (1, 2, 3, 4, 5), and that oxidized fatty acids alter enzyme activity *in vitro* (30, 31), but the surface of this type of problem has scarcely been scratched. We can be certain that there will be much more work of this nature in the future.

We hope that, in interpreting the data, investigators will remember that alterations in rates of growth or organ size or in enzyme activity are not necessarily indicative of undesirable changes. They may be be beneficial. For example, moderate repeated exercise develops (enlarges) muscles although sustained violent exercise leads to sore, exhausted muscles. Furthermore many changes in quantity or quality of diet result in changes in organ size and composition. Before any substance can be considered harmful, the biological changes induced by its ingestion must be proved to be detrimental to temporary or long-term health. It must be borne in mind however that pathologists are suspicious of any change from the accepted normal.

REFERENCES

1. Peacock, P. R., Brit. Med. J., *1*, 364–367 (1947).
2. Roffo, A. H., Bol. Inst. Med. Exp., *21*, 1–134 (1944).
3. Lane, A., Blickenstaff, D., and Ivy, A. C., Cancer, *3*, 1044–1051 (1950).
4. Peacock, P. R., and Eck, S., Acta. Unio. Intern. Contra. Cancrum, *7*, 612–615 (1951).
5. Peacock, P. R., Brit. J. Nutrition, *2*, 201–204 (1948).

6. Newman, A. A., Food Manufacture, October, 423–425 (1958).
7. Crampton, E. W., Farmer, F. A., Berryhill, F. M., J. Nutrition, *45*, 431–440 (1951).
8. Lassen, F., Baron, E. K., and Dunn, H. J., Arch. Biochem., *23*, 1–7 (1949).
9. Johnson, O. C., Sakuragi, T., and Kummerow, F. A., J. Am. Oil Chemists' Soc., *33*, 433–435 (1956).
10. Kaunitz, Hans, and Slanetz, C. A., Proc. Soc. Exptl. Biol. Med., *75*, 322–326 (1950).
11. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Babayan, U. K., and Barsky, G., J. Nutrition, *64*, 523–524 (1958).
12. Andrews, J. S., Griffith, W. H., Mead, J. F., and Stein, R. A., J. Nutrition, *70*, 199–210 (1960).
13. Rice, E. E., Warner, W. D., Mone, P. E., and Poling, C. E., J. Nutrition, *61*, 253–266 (1957).
14. Brew, W. E., Dore, J. B., Benedict, J. H., Potter, G. C., and Sipos, E., J. Assoc. Offic. Agric. Chem., *42*, 120–128 (1959).
15. Friedman, L., Firestone, D., Horwitz, W., Banes, P., Austead, M., and Shire, G. J., Assoc. Offic. Agric. Chem., *42*, 129–140 (1959).
16. Woolum, J. C., Alexander, J. C., J. Assoc. Offic. Agric. Chem., *43*, 141–148 (1959).
17. Harman, R. E., Davis, G. E., Ott, W. H., Brink, N. G., and Kuehl, F. A. Jr., J. Am. Chemists' Soc., *52*, 2078–2079 (1960).
18. Private Communication, Leo Friedman, Division of Nutrition, Bureau of Biological and Physiol. Sciences, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington 25, D.C.
19. Rice, E. E., Mone, P. E., and Poling, C. E., Fed. Proc., *16*, 398 (1957).
20. Poling, C. E., Warner, W. D., Mone, P. E., and Rice, E. E., in press, J. Nutrition.
21. Melnick, D., J. Am. Oil Chemists' Soc., *34*, 351–356 (1957).
22. Deuel, H. J. Jr., Greenberg, J. M., Colbert, C. E., Baker, R., and Fisher, H. F., Food Research, *16*, 258–280 (1951).
23. Warner, W. D., Poling, C. E., and Rice, E. E., unpublished data.
24. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Knight, H. B., Saunders, D. H., and Swern, Daniel, J. Am. Oil Chemists' Soc., *33*, 630–634 (1956).
25. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Knight, H. B., Saunders, D. H., and Swern, Daniel, Fed. Proc., *14*, 408 (1955).
26. Crampton, E. W., Common, R. H., Farmer, F. A., Wells, A. F., and Crawford, D., J. Nutrition, *40*, 333–348 (1953).
27. Perkins, E. G., and Kummerow, F. A., J. Am. Oil Chemists' Soc., *36*, 371–375 (1959).
28. Perkins, E. G., and Kummerow, F. A., J. Nutrition, *68*, 101–108 (1959).
29. Donnelly, T. H., Abstracts, 136th Meeting, Am. Chem. Soc., 14 T, September, 1959.
30. Bernheim, F., Wilbur, K. M., and Kenaston, C. B., Arch. Biochem. and Biophys., *35*, 177–184 (1951).
31. Olinlevathi, A., Bernheim, F., and Wilbur, K. M., Arch. Biochem. and Biophys., *56*, 157–164 (1955).

Newer Analytical Methods for the Fat and Oil Industry

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THE NEWER METHODS for the analysis of fats and oils fall for the most part into two groups, that is, those relating to physical characteristics and those used for the estimation of composition. This is altogether fitting because certainly the functional properties of fats and oils are intimately related to their composition and to their physical properties. Major advances in analysis in the last several years have occurred in the following specific areas: chromatography, dilatometry, nuclear magnetic resonance, urea fractionation, and spectroscopy—with a few miscellaneous methods in other areas.

Chromatography certainly heads the list of important advances in fat analysis. It has probably been the single most active area of exploration in the field in the last several years and still remains very active. The word chromatography which identifies this technique stems from the work of Tswett and probably is not a good choice since it does not denote the action or the process. However we do not need to be concerned here with nomenclature.

Chromatography does provide a means of obtaining quantitative separations of certain mixtures, and if you will permit a prediction, the applications will undoubtedly be greatly expanded in years to come.

Chromatography can for our purpose be very simply defined as a technique which utilizes such phenomena as surface adsorption, partition between solvents, and ion exchange to bring about separations of simple and complex mixtures into their various components. It is recognized that the definition may not be altogether adequate or complete but it will furnish a basis for our discussion.

The materials commonly involved in chromatography as applied to the analysis of fats include a) a solid support, such as diatomaceous earth, silicic acid, various chemical salts, paper, and other substances, b) a liquid stationary phase, usually adsorbed to the surface of the support, of varying composition depending upon the specific application, and c) a mobile phase which passes over or by the stationary phase. The mobile phase may be a liquid or a gas and in the case of the former may be designated as solvent, eluant, etc.

A good though elementary example of column adsorption chromatography is the A.O.C.S. method for the estimation of total neutral oil. Briefly this procedure involves pouring the sample, dissolved in a solvent, onto a column of aluminum oxide, and allowing the solution to percolate through the column. The eluate, *i.e.* the portion that passes through the column, is collected and the solvent is evaporated. The weighed residue represents neutral triglyceride.

The reason for being able to separate neutral oil from free fatty acids under the prescribed conditions for this method is that the less strongly held neutral triglycerides pass through the column with the solvent and the more polar, free fatty acids are adsorbed on the surface of the aluminum oxide and thus do not pass through the column.

In the case of partition chromatography separation is attained by distribution of the components of the mixture between the mobile and stationary phases based on partition coefficients.

Broadly speaking then, the fact that the different components of a mixture can be retained on or can be made to pass over or through a column at different rates by suitably adjusting the conditions and by properly selecting the solvents and other materials is the basis for the technique of chromatography. The separation may involve adsorption as in the procedure just mentioned, or partition between liquids as is applied to the fractionation of fatty acids. Ion exchange is not to my knowledge applied in many areas of fat analysis.

Separations employing column adsorption or partition chromatography have been successfully applied to the fractionation of fatty acids, to the determination of individual fatty acids such as butyric acid in butterfat and others, and to the determination of saturated fatty acids. It obviously is a good technique but when the mixture becomes complex the labor involved is not inconsiderable. Therefore in such instances paper chromatography and gas chromatography are more practical.

employed. A 100% difference in relative humidity was maintained across the films. The permeability constant, P, was calculated using the equation,

$$P = \frac{(w)(x)}{(A)(t)(p)}$$

where w is the weight of water vapor, in grams, diffusing through a film of thickness x, in centimeters, and area, A in square centimeters, during the time t, in seconds, when the vapor pressure difference p is measured in millimeters of mercury. The permeability constants obtained are recorded in Table I and those for the saturated acyl groups are shown graphically in Fig. 4.

Permeability decreased as the chain length of the fatty acid group increased. The presence of unreacted hydroxyl groups apparently had no significant effect on permeability, which is in agreement with data on glycerides obtained in our laboratory. Heretofore, no data on the permeability of amylose esters have been published. Using slightly different test conditions, Rankin *et al.* (12) obtained a permeability constant of 760×10^{-12} for a film of amylose. The permeability of amylose acetate is slightly higher than that reported for cellulose acetate (17). The lowest value obtained, 15×10^{-12} for the stearate, was slightly larger than that of polystyrene (1,5). The value for paraffin wax, probably the most impermeable of organic compounds, has been reported to be 0.17×10^{-12} (15).

When the amylose ester films were removed from the moisture cups after the measurements had been completed, it was observed that the more permeable films had become hazy.

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REFERENCES

1. American Society for Testing Materials, ASTM Designation (a) E 28-51T, (b) D 882-56T, Philadelphia, The Society.
2. Bua, W. C., Muelgaert, J., and Hiemstra, F. (to Cooperatieve Verkoop- en Productievereniging van Aardappelmeel en Derivaten "Avebe" G.A.), U.S. Pats. 2,829,987–2,829,990 (April 8, 1958).
3. Fischer, K., Angew. Chem., *43*, 394–396 (1935).
4. Gensing, L. B., and Mallatt, R. C., Ind. Eng. Chem., Anal. Ed., *13*, 370–374 (1941).
5. Lovegren, N. V., Guice, W. A., and Feuge, R. O., J. Am. Oil Chemists' Soc., *35*, 327–331 (1958).
6. Malm, C. J., Mench, J. W., Kendall, D. L., and Hiatt, G. D., Ind. Eng. Chem., *43*, 684–688 (1951).
7. Malm, C. J., Mench, J. W., Kendall, D. L., and Hiatt, G. D., *Ibid.*, *43*, 688–691 (1951).
8. Matchett, J. R., and Lavine, J., Ind. Eng. Chem., Anal. Ed., *13*, 98–99 (1941).
9. Meyer, K. H., Bernfeld, P., and Hohenemser, W., Helv. Chim. Acta, *23*, 885–890 (1940).
10. Mikus, F. F., Hixon, R. M., and Rundle, R. E., J. Am. Chem. Soc., *68*, 1115–1123 (1946).
11. Mullen II, J. W., and Pacsu, E., Ind. Eng. Chem., *34*, 1209–1217 (1942).
12. Rankin, J. C., Wolff, I. A., Davis, H. A., and Riat, C. E., Chem. & Eng. Data Ser., *3*, 120–123 (1958).
13. Senti, F. R., and Dimler, R. J., Food Technol., *13*, 663–667 (1959).
14. Sheppard, S. E., and Newsome, P. T., J. Phys. Chem., *39*, 143–152 (1935).
15. Taylor, R. L., Herrmann, D. B., and Kemp, A. R., Ind. Eng. Chem., *35*, 1255–1263 (1936).
16. Wagner, R. H., and Russell, J., Anal. Chem., *20*, 151–155 (1948).
17. Whistler, R. L., and Hilbert, G. E., Ind. Eng. Chem., *36*, 796–798 (1944).
18. Whistler, R. L., Advances in Carbohydrate Chemistry, *1*, 279–307 (1945).
19. Wolff, I. A., Olds, D. W., and Hilbert, G. E., J. Am. Chem. Soc., *73*, 346–349 (1951).
20. Wolff, I. A., Olds, D. W., and Hilbert, G. E., Ind. Eng. Chem., *43*, 911–914 (1951).
21. Youngs, C. G., Epp, A., Craig, B. M., and Sallans, H. R., J. Am. Oil Chemists' Soc., *34*, 107–108 (1957).

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Report of the Uniform Methods Committee, 1961

The meeting of the Uniform Methods Committee was held at 2 p.m., October 30, 1961, during the Chicago fall meeting. K. E. Holt, R. J. Houle, R. A. Marmor, L. D. Metcalf, E. F. Sipos, E. M. Sallee, editor, and D. L. Henry were present. Visitors were: R. W. Bates, L. A. Baumann, R. C. Stillman, and M. E. Whitten.

Progress reports submitted by many of the Technical Committees were discussed with interest. These reports indicate that much good work is being done on needed methods. No recommendations were received to change existing methods or to adopt new ones, and no changes are recommended by the Uniform Methods Committee.

Respectfully submitted,

J. J. GANUCHEAU	T. D. METCALF
K. E. HOLT	E. F. SIPOS
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R. A. MARMOR	E. M. SALLEE

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Nutritive Value of Methyl Linoleate and Its Thermal Decomposition Products

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Methyl linoleate was heated for 10 hrs. at 300°C. in the absence of air and fractionated by alembic distillation and urea adduct-formation.

Intestinal absorptions of the urea adduct-forming monomeric nonadduct-forming monomeric, and dimeric fractions were determined. It was found that dimers were half as well absorbed as the monomers.

When fed to rats, dimers were better accepted and exhibited some toxicity symptoms different from the nonadduct-forming monomers. The dimers caused diarrhea, irritability, and loss of hair during the early period of administration. The nonadduct-forming monomers were lethal and produced an increase in liver weight. Both fractions depressed growth.

HEATING of unsaturated oils in the absence of air is known to result in the formation of a mixture of monomeric and polymeric substances, both linear and cyclic. For reviews on the nutritional significance of such compounds the reader is referred to the papers of Brown (1) and of Rice *et al.* (2).

Crampton and co-workers (3,9) have been able to demonstrate that, when linseed, soybean, and sunflower oils are heated at approximately 275°C. for 12 to 26 hrs. in a CO_2 atmosphere, a polymeric fraction, which is very slightly absorbed from the intestine of the rat, and a nonurea adduct-forming monomeric fraction, which is harmful to the same animal, are formed. Comparison of the fatty acid composition of these oils with their deleterious action indicates that linolenic acid is the main precursor. Nevertheless some toxicity has been found to develop in heated oils containing relatively high proportions of linoleic acid and no linolenic acid.

More unsaturated oils, such as fish oils, seem able to develop toxic effects and form poorly absorbed substances if heated and administered under similar conditions (10–15).

Methyl and ethyl linoleate seem also capable of forming cyclic and polymeric substances by heating, as has been suggested by Paschke *et al.* (16,17).

In order better to characterize the toxic derivatives and their biological properties, relatively pure methyl linoleate was prepared, heated in the absence of air, then fractionated. The fractions were tested for intestinal absorption and deleterious action in the rat.

Recently Kaunitz *et al.* (18) have made a similar study on thermal oxidation products of the same ester.

Experimental

Preparation of the Fractions. Methyl linoleate was prepared from the methyl esters of commercial sunflower seed oil[2] by the urea-adduct procedure (19). The esters were heated at 300°C. for 10 hrs. in the presence of CO_2 in an apparatus similar to that described by Bradley and Johnston (20). The heated product was fractionated by alembic distillation under reduced pressure into a distillable monomeric fraction and a residual polymeric fraction. The latter, as may be seen from the data in Table I, is essentially dimeric.

[1] With the technical assistance of Oscar Giacomantone and Perla Merdujovich.
[2] Generously supplied by Alba, S.A., Argentina.

TABLE I
Typical Properties of Tested Products

Fraction	Iodine number [a]		Molecular weight [b]	
	Expti.	Theor.	Expti.	Theor.
Methyl linoleate	154.6	172.4	287 [c]	294.4
Heated methyl linoleate [d]	103.8	....	....	....
Distillable monomers [e]	111.1	....	290.5	294.4
Adduct-forming monomers [e]	114.9	172.4	....	294.4
Nonadduct-forming monomers [e]	95.2	85.6	....	294.4
Polymers [e]	92.2	D = 85.6 [f] T = 57.5	640	D = 588 T = 882

[a] Hanus, 30 min.
[b] Cryoscopy in benzene.
[c] From saponification value.
[d] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[e] D = dimers; T = trimers.

The monomeric fraction was then separated by means of the procedure of Wells and Common (8) into urea adduct-forming monomers and nonurea adduct-forming monomers. Some of the chemical characteristics of the prepared methyl linoleate and its products of heating are shown in Table I. Iodine number was determined by the Hanus (30 min.) procedure. Mean molecular weights were obtained by means of cryoscopy in benzene.

Determination of the Absorption Coefficient. The technique described by Irwin *et al.* (21) and by Deuel *et al.* (22) for the determination of intestinal absorption was followed with minor modifications. Adult female albino rats belonging to the strain of the Institute and weighing between 165 and 300 g. (average 233 g.) were fasted for 48 hrs. They were fed by stomach tube approximately 300 mg. of accurately weighted ester per dm^2 of body surface. After 4 hrs. the rats were sacrificed with ether, and the gastrointestinal tract was flushed with 150-ml. portions each of physiological saline, petroleum ether, and saline in that order. The aqueous-ethereal extract was acidified, and the fat in the ether phase was determined gravimetrically. For control the fat was extracted from the intestinal contents of similarly conditioned animals not given the test material. The absorption coefficient was calculated from the difference between the fat content of the control and test animals. The method was tested by giving separately adduct-forming monomers and dimers to rats in identical fasting conditions and by immediately washing the digestive tract. The absorption coefficients were expressed as mg. of fat absorbed per dm^2 of body surface per hour. The percentage of fat recovered was also determined. The data from all animals which exhibited diarrhea were discarded (Table II).

Test of Biological Action. Adult male albino rats weighing between 144 and 233 g. (average 186 g.) were distributed in groups of 10 animals each. One group was made up of only six rats because of the small amount of nonadduct-forming monomers available. During periods of 25 to 36 days the group 1 animals were fed a fat-free diet and the other groups a diet containing 10% by weight of the following lipids: group 2, methyl linoleate; group 3, adduct-forming monomers; group 4, dimers; group 5, non-

TABLE II

Absorption Coefficients of Thermal Decomposition Products of Methyl Linoleate

Fraction	No. of expts.	Avg. weight of rats	Absorp. period	Absorp. coeffic.[b]	Ester absorbed
		(g.)	(hrs.)	(mg./dm²/h)	(%)
Adduct-forming monomers	12	294	4	43.3 ± 5.5[c]	55.8
	3	266	0		80.5
Nonadduct-forming monomers	6	262	4	45.8 ± 9.6	62.4
Dimers	7	252	4	25.5 ± 5.0	31.7
	9	246	0		76.4
None	10	241	0	Average recovery 26.4 ± 18.1 mg./rat	

[a] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[b] Determined by the procedure of Irwin *et al.* (21) and Deuel *et al.* (22).
[c] Standard deviation.

adduct-forming monomers; group 6, corn oil (Mazola); group 7, dimers plus corn oil (1 + 1). The composition of the diets is presented in Table III. Rations were prepared daily in order to prevent alteration of the fat and were placed in the cages in metal boxes with lids. A circular hole in the lid allowed easy access to feed and prevented losses. Feed was given *ad libitum* to all groups except group 6, which received 5 g. of feed per day per rat. The animals were weighed daily. Groups 5 and 6 were kept in individual cages because otherwise the weaker or dead animals were eaten by the others. It was also necessary to isolate the rats of group 4 because their diarrheic feces acquired a varnish consistency which kept the animals stuck to each other and to the floor. At the end of the experimental period the rats were sacrificed with gas, and their livers were weighed and histologically studied (Table V).

Results and Discussion

Degree of Absorption. The amount of fat found in the intestine of fasted rats (Table II), averaging 26.4 mg. per rat, agrees with previously reported values (22). The recovery of adduct forming monomers (about 90%) indicates the acceptable capability of the procedure for washing the digestive tract. Published recoveries obtained with different oils and procedures are of the same order of magnitude (22). On the other hand, the amount of fat obtained from the intestine by washing immediately after giving dimers was lower than after other fractions even though the dimers are less well absorbed than monomers, as shown by the data from the 4-hr. absorption period. No correction for this anomaly was applied. The recorded values show no differences in the absorption coefficients of the adduct-forming and nonadduct-forming monomeric substances tested but do show differences between the monomers and dimers.

Biological Action. The growth curve (Fig. 1) of the group fed adduct-forming monomers [consisting, as found by Paschke *et al.* (16,17) of a main portion of normal methyl linoleate and about 14% of its conjugated isomer] shows no striking difference as compared with the controls fed methyl linoleate (group 2) or the fat-free diet (group 1). The growth curves of groups 4 and 5, which received dimers and nonadduct-forming monomers, respectively, are remarkably abnormal. After only two days on experiment the average weight of group 4 was less than that at the beginning, even with good acceptance of feed. At the third day the animals had marked diarrhea, accompanied by colorless fluid feces at the moment of

TABLE III

Composition of the Diets

Component	Percentage in the diet	
	Fat-free	With fat
Sucrose	72	62
Casein	20	20
Ester or oil tested	0	10
Yeast (dry)	4	4
Salts (McCollum-Davis)	4	4
Supplement: vitamin A, 200 i.u./100 g. vitamin D, 20 i.u./100 g.		

excretion, which became darker, semisolid, and sticky in the contact with the air and acquired a varnish aspect. After five days the rats showed pronounced irritability, being almost constantly in a fighting position, one in front of the other. Hair became yellow and absent in small but increasing areas. Both diarrhea and the loss of weight and hair continued until around the 15th day, after which slight improvement in the general aspect was noted with diminishing diarrhea and loss of hair, and an increase in weight. The growth curve of this group during the first two weeks coincides remarkably with that of group 6, which was restrictively fed. After two weeks the curves separate; the weight of group 4 increases slightly and that of group 6 decreases steadily. Feed consumption of group 4 (Table IV) averaged 10.0 g./day/rat during the first two weeks and 14.6 g./day/rat for the whole experimental period, indicating that loss of weight cannot be explained as resulting from the rejection of feed by the animals. The results could be due to an adverse effect of the dimers on the utilization of the other nutrients, as suggested by Raulin and co-workers (23). As can be seen in Table V, rats receiving the dimer diet for 25 days showed a smaller ratio of body to liver weight than animals fed identical fat for 36 days. The values in the latter test were at the level of the controls. The number of dead animals in

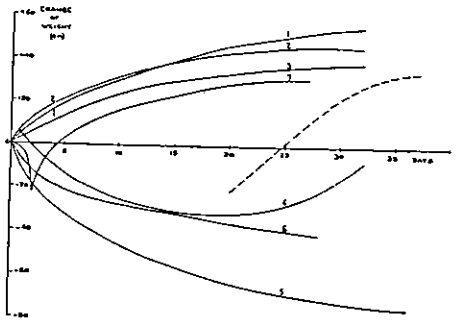

FIG. 1. Change, with time, of the weights of rats fed methyl linoleate and its thermal decomposition products.

Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.

Curve No. 1 corresponds to a group fed a fat-free diet. The other curves correspond to groups fed diets containing 10% by weight of the following lipids: No. 2, methyl linoleate; No. 3, adduct-forming monomers; No. 4, dimers; No. 5, nonadduct-forming monomers; No. 6, corn oil; No. 7, dimers plus corn oil (1 + 1). Feeding was *ad libitum* in all groups except in group 6, which received only 5 g. of feed per day per rat. The dotted line represents the change of weight of a group fed dimers (group 4) until the 20th day, and corn oil afterwards.

TABLE IV

Feed Consumption

Group No.	Fat in the diet	Duration of the experiment	Consumption
		(days)	(g./day/rat)
1	None	25	15.8
2	Methyl linoleate	25	13.0
3	Adduct-forming monomers[a]	32	11.7
4	Dimers[a]	36	14.6
5	Nonadduct-forming monomers[a]	36	6.2
6	Corn oil	25	5.0[b]
7	Dimers + corn oil (1 + 1)	25	12.8

[a] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[b] Restricted.

group 4 was one to ten, similar to the ratio of some of the control groups accordingly considered normal.

In a separate experiment, rats given a diet of 10% dimers for 20 days were then fed the same proportions of corn oil. After 12 days improvement in the general condition was observed, including an increase of weight to normal levels (Fig. 1), growth of hair, and disappearance of diarrhea. The growth curve corresponding to group 7, which was fed a mixture of equal parts of dimers and corn oil, runs slightly under the control curves, but the difference seems to be insignificant. The aspect of the animals was completely normal, indicating either a dilution of dimers to nontoxic levels or a protection by the nonheated oil, as previously pointed out by others (24).

The above evidences suggest that the dimeric fraction, although absorbed in relatively small degree, has a weak toxic effect to which the rat appears to develop a tolerance. The toxic effect also disappears as soon as other fat is substituted for dimers in the diet or fresh nontoxic fat is added to the diet.

The group fed nonadduct-forming monomers (group 5) showed rather different characteristics. Loss of weight was much more marked than in groups fed dimers and corn oil. Hair became yellowish after five days but did not fall out. At the end of the 32-day period the only surviving rat looked meager and inactive. There was one death on the 14th day, two on the 15th, and one each on the 18th and 23rd days. The daily average feed consumption of 6.2 g./day/rat was small as compared with that of 11.7 to 15.8 g./day/rat of the control groups (Table IV). However it was larger than the 5 g./day/rat of the corn oil-fed group. The loss of weight was also much more remarkable than in this last group, thus indicating a deleterious effect. The ratio of body weight to liver weight (Table VI) was notably diminished in the only animal of this group that was autopsied.

It is thus evident that nonadduct-forming monomers from methyl linoleate possess a lethal effect although to a lesser degree than similar fractions from heated linolenic acid-rich oils (6). This weaker toxic activity, as well as the very small amounts of this

TABLE V

Ratio of Body to Liver Weight[a]

Group No.	No. of rats	Fat in the diet	Avg. body weight	Avg. liver weight	Body weight/ liver weight
			(g.)	(g.)	(g./g.)
1	5	None	243	10.2	23.8
2	5	Methyl linoleate	260	12.4	21.0
3	5	Adduct-forming monomers[b]	233	10.2	22.8
4[c]	2	Dimers[b]	202	12.7	15.9
4[d]	7	Dimers[b]	171	7.8	21.9
5	1	Nonadduct-forming monomers[b]	108	9.4	11.5

[a] Liver histology was normal in all animals.
[b] Methyl linoleate was heated at 300°C. for 10 hrs. in the presence of CO_2 and fractionated by distillation into distillable monomers, and polymers. The distillable monomers were later separated into urea adduct-forming monomers and nonadduct-forming monomers.
[c] Sacrificed after 25 days of feeding.
[d] Sacrificed after 36 days of feeding.

fraction formed on heating linoleic acid-rich oils, may explain the lack of harmful effects found when sunflower seed and soybean oils were tested by Crampton.

Acknowledgment

The author wishes to express his appreciation to Raymond Reiser for reading the paper, to V. Laghens for performing the histological analyses, and to the members of the Departamento de Química Tecnológica, Facultad de Química y Farmacia, Universidad de La Plata, La Plata, Argentina, for their generous cooperation.

REFERENCES

1. Brown, J. B., Nutrition Rev., *17*, 321 (1959).
2. Rice, E. E., Peling, C. F., Hume, P. E., and Warner, W. D., J. Am. Oil Chemists' Soc., *37*, 607 (1960).
3. Crampton, E. W., Farmer, F. A., and Berryhill, F. M., J. Nutrition, *43*, 431 (1951).
4. Crampton, E. W., Common, R. H., Farmer, F. A., Berryhill, F. M., and Wiseblatt, L., J. Nutrition, *43*, 523 (1951).
5. Crampton, E. W., Common, R. H., Farmer, F. A., Berryhill, F. M., and Wiseblatt, L., J. Nutrition, *44*, 177 (1953).
6. Crampton, E. W., Common, R. H., Farmer, F. A., Wells, A. F., and Crawford, D., J. Nutrition, *49*, 333 (1953).
7. Wiseblatt, L., Wells, A. F., and Common, R. H., J. Sci. Food Agr., *4*, 227 (1953).
8. Wells, A. F., and Common, R. H., J. Sci. Food Agr., *4*, 233 (1953).
9. Crampton, E. W., Common, R. H., Pritchard, E. T., and Farmer, F. A., J. Nutrition, *60*, 13 (1956).
10. Lossen, S., Bacon, E. K., and Dunn, H. J., Arch. Biochem., *23*, 1, (1949).
11. Frahm, H., Lembke, A., and von Rappard, G., Kiel Milchwirtschaft-Forschber., *5*, 443 (1953).
12. Kaneda, T., Saka, H., and Ishii, S., J. Biochem., *42*, 561 (1955).
13. Witting, L. A., "The Nutritional Value of Polymerized Fats," thesis, University of Illinois, 1956.
14. Common, R. H., Crampton, E. W., Farmer, F. A., and de Freitas, A., J. Nutrition, *63*, 341 (1957).
15. Matsuo, N., Seikagaku, *29*, 855 (1957-58); C. A., *53*, 11551 (1959).
16. Paschke, R. F., and Wheeler, D. H., J. Am. Oil Chemists' Soc., *26*, 278 (1949).
17. Paschke, R. F., Jackson, J. E., and Wheeler, D. H., Ind. Eng. Chem., Anal. Ed., *24*, 1113 (1952).
18. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Knight, H. B., and Swern, Daniel, Metabolism, *9*, 53 (1960).
19. Westerfeld, W. W., ed., Biochem. Prep., *4*, 86 (1955).
20. Bradley, T. F., and Johnston, W. B., Ind. Eng. Chem., Anal Ed., *32*, 802 (1940).
21. Irwin, M. H., Steenbock, H., and Templin, V. M., J. Nutrition, *12*, 55 (1936).
22. Deuel, H. J. Jr., Hallman, L., and Leonard, A., J. Nutrition, *20*, 215 (1940).
23. Raulin, J., Richir, C., Escribano, L., and Jacquot, R., C.R., *248*, 1229 (1959).
24. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., and Babayan, V. K., J. Nutrition, *70*, 521 (1960).

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Toxicity of Fatty Acid Ester Hydroperoxides. (28809)

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The acute toxicity of fatty acid hydroperoxides is well recognized(1-3). Such toxicity is demonstrable by feeding tests; by injection or oral dosage(4,5); and even by inunction on the skin of rats(6). The mechanism for this toxicity is unknown but it is a reasonable hypothesis that hydroperoxides may be a source of damaging free radicals (7-9). The experiments to be described were done in an attempt to determine whether large doses of tocopherol or ethoxyquin might modify the lethality of hydroperoxides.

Experimental. Peroxide concentrates were prepared by aerating samples of crude methyl linoleate at room temperature (22-24°C). Methyl esters of safflower seed oil fatty acids, furnished by the Pacific Vegetable Oil Co., contained approximately 73% methyl linoleate (by gas chromatography). No antioxidants had been added during its preparation. One sample was aerated at 40-50°C for one day and thereafter at room temperature. On days 3, 4, 5 and 6 the peroxide values were 1990, 2560, 2570, and 2600, respectively (in milliequivalents per kg fat).

Peroxide values were determined by a modification of AOCS standard procedure Cd 8-53 as follows: 20-30 mg samples, 3 ml of chloroform-glacial acetic acid (3:1), 50 ml flask, 1 ml of saturated potassium iodide, 2 minutes reaction at room temperature under nitrogen atmosphere, 10 ml water, titration with 0.01 N $Na_2S_2O_3$.

Hydroperoxide concentrates were prepared by a modification of the procedures described by Banks(10). The reaction mixture was separated between petroleum ether and 85% aqueous methanol. The 85% aqueous methanol-soluble fraction was then recovered after dilution with water, followed by extraction with petroleum ether. Such preparations had peroxide values varying from 3800 to 4700 (theoretical for methyl linoleate hydroperoxide 6135). They were stable for months at —18°C. Preparations made from samples which had been aerated longer than necessary to reach maximum peroxide values contained a fraction characterized by its insolubility in petroleum ether. Peroxide values of these fractions varied from 3300 to 4700. A determination of average molecular weight on one preparation (by osmometry) gave 510. The petroleum-ether-soluble peroxide concentrate had a molecular weight by this method, 340; calculated for methyl linoleate hydroperoxide, 326. The petroleum-ether-insoluble fraction thus appeared to be in part a dimer. It was found to be at least as toxic as the soluble fraction but has not yet been studied in more detail.

Toxicity of these preparations in rats was measured after intraperitoneal injection (Table I). Particularly viscous preparations were diluted with crude methyl linoleate which, by itself, was innocuous by the same route. Rats were kept on a normal stock diet. Death usually occurred within 24 hours but occasionally rats died after 2 or 3 days. Those that survived longer showed no obvious long-term ill-effects. One rat was injected on several occasions with close to lethal doses without evidence of accumulated damage. The only gross symptom in all rats that died was massive ascites. These observations are in accord with those of Horgan *et al*(4) with mice and of Vishida and Kummurow(11) with methyl linoleate hydroperoxide injected into rats.

Intraperitoneal injection of tocopherol suspended in crude methyl linoleate into numerous rats caused no death or obvious discomfort. Results obtained with rats first injected with tocopherol and 24 hours later with hydroperoxide preparations are shown in Table I. If there was a protective effect, it was very slight. Preliminary experiments had shown that simultaneous injections of tocopherol and hydroperoxide also did not change the level of the LD_{50} dose. Similarly in a limited series the antioxidant ethoxyquin appeared not to effect the lethality of the hydroperoxide.

TABLE I. Effect of Tocopherol and Ethoxyquin on Intraperitoneal Toxicity of Hydroperoxide.

Pretreatment*†					
None		Tocopherol		Ethoxyquin	
Hydroperoxide, μM/100 g	Lethality	Hydroperoxide, μM/100 g	Lethality	Hydroperoxide, μM/100 g	Lethality
43–56	0/3	—	—	—	—
61–74	2/2	—	—	—	—
82–109	0/3	93–95	1/2	—	—
113–118	1/2	—	—	—	—
149–150	2/6	150	0/4	150	2/4
155–170	8/8	170	4/5	—	—
180	7/8	180	5/8	185	1/1
185	25/25	188–238	0/4	196	0/1
—	—	240	10/10	238	1/1

* Rats (300-350 g) were injected intraperitoneally with an amount of α-tocopherol or ethoxyquin equivalent to the dose of hydroperoxide injected 24 hr later.
† Rats kept on a stock diet manufactured by Diablo Laboratories in Berkeley, Calif., which contained 4.8% fat, supplemented with Vit. E.

Discussion. The LD_{50} of methyl linoleate hydroperoxide was 150-170 μM per 100 g body weight, whether or not tocopherol in equimolar amounts had been presented to the animal. Horgan *et al* reported that the LD_{50} of the peroxides of autoxidized methyl linoleate was 45-60 μM per mouse. In contrast, the LD_{50} of autoxidized linoleic acid was 6-8 μM peroxide per mouse (about 25 μM *per* 100 g). Holman and Greenberg(5) found that ethyl linoleate hydroperoxide had a LD_{50} of 12 mg (35 μM) in mice. The hydroperoxide of methyl oleate acid was more toxic, LD_{50}, 6 mg (18 μM per mouse).

Introduction of the peroxide by stomach tube killed three rats at the level of 1600 μM per g. Lower levels caused drastic loss of fluid from the intestinal tract but no deaths. In the experiments of Holman and Greenberg (5) mice were not killed by single 200 mg doses of either ethyl linoleate hydroperoxide (588 μM) or methyl oleate hydroperoxide (610 μM) nor by 75 mg doses daily for two weeks of either. These combined observations indicate that hydroperoxides do not easily pass through the intestinal wall (cf. Mead, 12), and also that they are highly irritating. In a separate experiment carried out with the help of F. DeEds and J. O. Thomas at the Western Regional Research Laboratory, USDA, Albany, California, hydroperoxide concentrate (5-10 μM) was injected into the lumen of a tied-off segment of a rat small intestine. In comparison with a control segment the injected segment more than doubled in weight in one hour, mostly due to an increase in mucus excretion into the intestine, clear indication of intense irritation (cf. 13).

TABLE II. Toxicity of Methyl Linoleate Hydroperoxide Concentrate when Administered by Stomach Tube.

Hydroperoxide, μM/100 g	Symptoms*	Loss in wt, %*
400	None	5, 5, 7
800	Diarrhœa	5, 7, 11
1600	Dead	11, 16, 17

* Original weights, 300-350 g. Weighed again at 18 hr. There were no subsequent deaths. Surviving animals recovered and gained weight.

These combined observations suggest that hydroperoxides kill by attack on some vital tissue not yet identified. It is possible that sulfhydryl enzymes(14) or cytochromes(8) are the vulnerable tissue components. Whatever the mechanism, neither tocopherol nor ethoxyquin are effective in reducing the dose required to kill, either when made available simultaneously or 24 hours previous to the administration of the toxic dose.

The lack of effectiveness of tocopherol is parallel to the observed relative ineffectiveness of tocopherol in combating radiation

toxicity which, it has been hypothesized, may be mediated through the formation of hydroperoxides(4).

Summary. Intraperitoneal injection of concentrates of methyl linoleate hydroperoxide into adult rats was lethal at a level of about 150 μM per 100 g. Previous injection of equal amounts of tocopherol or ethoxyquin did not change the LD_{50} level. Sixteen hundred but not 800 μM per 100 g were lethal when administered by stomach tube.

1. Matsuo, N., in *Lipids and their Oxidation*, Schultz, H. W., Day, E. A., Sinnhuber, R. O., Avi Publishing Co., 1962, 321.
2. Kaneda, T., Sakurai, H., Ishii, S., *Bull. Jap. Soc. Sci. Fish.*, 1954, v20, 658.
3. Andrews, J. S., Griffith, W. H., Mead, J. F., Stein, R. A., *J. Nutrition*, 1960, v70, 199.
4. Horgan, V. J., Philpot, J. S. L., Porter, B. W., Roodyn, D. B., *Biochem. J.*, 1957, v67, 551.
5. Holman, R. T., Greenberg, S. I., *J. Am. Oil Chem. Soc.*, 1958, v35, 707.
6. Matsuo, N., *J. Japan. Biochem. Soc.*, 1957, v29, 769.
7. Uri, N., in *Oxidation and Antioxidants*, Lundberg, W. O., Interscience Publishers, 1961, 89.
8. Tappel, A. L., *Vitamins and Hormones*, 1962, v20, 493.
9. Mesrobian, R. B., Tobolsky, A. V., in *Oxidation and Antioxidants*, Lundberg, W. O., Interscience Publishers, 1961, 110.
10. Banks, A., Fazakerlay, S., Keay, J. N., Smith, J. G. M., *J. Sci. Food Agr.*, 1961, v10, 724.
11. Nishida, T., Tsuchiyama, H., Inone, M., Kummerow, F. A., Proc. Soc. Exp. Biol. and Med., 1960, v105, 308; Nishida, T., Kummerow, F. A., *J. Lipid Research*, 1960, v1, 450.
12. Mead, J. F., in *Lipids and Their Oxidation* (see Ref. 1), 360.
13. DeEds, F., Report of Sixth Annual Dry Bean Conference, Western Regional Research Laboratory, USDA, Albany 10, Calif., 1963, 32.
14. Wills, E. D., *Biochem. Pharm.*, 1961, v7, 7.

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Inactivation of Streptococcal Bacteriophage by Sulfhydryl Reagents.* (28810)

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Work reported previously indicated that the host range of beta-hemolytic streptococcal bacteriophage is a group-specific phenomenon. Thus, the Group A phage lyses Group A streptococci but not Group C and similarly, Group C phage lyses Group C streptococci but not Group A. Such group specificity of these phages suggests that the cell wall carbohydrate, a significant constituent shared by all streptococcal strains of the same serologic group, may serve as a receptor substance for attachment of the phage to the organism(1). Additional support for this view has been obtained by experiments which demonstrated the inactivation of Group C phage but not Group A phage with the purified cell wall carbohydrate of Group C streptococci. It is clear, however, that factors other than the nature of the specific attachment substance of the bacterial cell wall control the adsorption and penetration of the virus into the host cell. For example, recent studies by Kosloff have emphasized the role of sulfhydryl groups in T2 bacteriophage invasion of *E. coli*(2).

In related experiments with animal viruses, Choppin and Philipson reported inactivation of enteroviruses by treatment with mercaptide forming reagents such as p-chloromercuribenzoate; mercuric ion and Thimerosal; an inactivation which was reversed with subsequent treatment by the thiol compound, reduced glutathione(3). In the experiments reported here, inactivation of streptococcal phage with the mercaptide forming reagents was also partially reversed by reduced glutathione.

Materials and methods. Group A phage lysates were made by lysis of streptococcal Group A strain T25/41 with A25 phage stock and similarly Group C phage lysates were prepared by the lysis of Group C strain 26RP66 with C1 phage stock. The soft agar layer technique was employed to determine the number of plaque forming phage particles (4). The special media employed as the nutrient source for the agar plates has been described(1). Streptococcal strains T25/41 and 26RP66 were used as indicator strains in the soft agar layer for phages A25 and C1 respectively.

The various phage inactivation experiments were carried out as follows. Two-tenths ml of an appropriate concentration of sulfhydryl reagent was added to 1.8 ml of phage lysate which had been diluted in dialysate broth to a concentration of approximately 10^7 plaque forming units per ml. The mixture was incubated at 24°C and 0.1 ml samples, taken at intervals were diluted immediately into 9.9 ml of Todd-Hewitt broth to halt inactivation. Additional dilutions made from this diluted sample were plated to determine the titer of plaque forming units. Controls were performed in a manner similar to the experimental system and final results were calculated as percent of control values. The dialysate broth employed in the inactivation step has been described(1).

Stock solutions of p-hydroxymercuribenzoate (PHMB)§ were prepared in glycylglycine buffer at pH 8.6. The reagent remained in solution upon subsequent dilution into dialysate broth at pH 7.5. Aqueous solutions of Thimerosal, $HgCl_2$, $ZnCl_2$ and $CdCl_2$, were diluted to appropriate concentration with the dialysate broth.

Results. The inactivation of Groups A and C phage with p-hydroxymercuribenzoate, (which will hereafter be referred to as PHMB), is shown in Fig. 1. Rapid inactivation of the Group C phage C1 occurred with a concentration of 1.0×10^{-3} M PHMB; the Group A phage A25, on the other hand, was consistently less sensitive to the action of this reagent. At a concentration of 1×10^{-3} M PHMB 99% of the C1 phage was inactivated in 10 minutes whereas only 50% of the A25 phage was inactivated.

Fig. 2 illustrates the inactivation of phage by Hg^{++} and demonstrates the fact that phage is less sensitive to PHMB than to this ion. For instance, at a concentration of 3.6×10^{-4} M Hg^{++}, C1 phage inactivation is comparable to that achieved with 1×10^{-3} M PHMB as depicted in Fig. 1. This difference in the effectiveness of the inactivation by these 2 substances was a consistent finding in several experiments over a range of inactivator concentrations. In general a concentration of PHMB 3 to 5 times that of $HgCl_2$ gave comparable inactivation. 1×10^{-3} M Thimerosal, another organic mercurial, also inactivates the phage, but is somewhat less effective than PHMB at the same concentration.

Because mercuric ion is bivalent and in Group IIb of the periodic table the inactivation of phage by the other elements of this group is of interest. Fig. 3 illustrates a comparison of the inactivation of Group C phage by zinc ion, cadmium ion, and mercuric ion. At a concentration of 1×10^{-3} M zinc or cadmium ion, phage is inactivated more slowly than by 3.6×10^{-4} M mercuric ion. Thus the ions of the elements of Group IIb inactivate the C1 phage, but Hg^{++} is more effective in this respect.

In view of the recent finding of Choppin and Philipson that the inactivation of enterovirus infectivity with mercaptide forming reagents is reversed with subsequent treatment by reduced glutathione(3), it was of interest to determine the effect of similar treatment on bacteriophage previously inactivated with PHMB or mercuric ion. In the following experiments C1 bacteriophage inactivated by PHMB or mercuric ion was subsequently treated with reduced glutathione. C1 phage was treated with either 5×10^{-4} M PHMB or 3.3×10^{-5} M mercuric ion. After 5 min-

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autoxidation. Previous studies (10) on distilled oxidative dimers (not chromatographically fractionated) indicate diene conjugation as high as 23%, and with double bonds randomly distributed from the C-6 to C-10 carbon atom of the fatty-acid chains.

Chromatographic fractionation offers a method of characterization and analysis based on polarity of the various components in oxidative polymers. Although confirming our results with the chromatographic method, Bernard and Rost (4) question the nature of the dimeric material and maintain that in normally processed soybean oil, thermal polymers constitute less than 0.1%. Since Rost's method (21,22) determines thermal polymers only, it offers a means of checking the type of dimer found in the chromatographic peak I areas obtained from the oxidative dimers.

Distilled oxidative dimers do not give highly resolved chromatographic fractions, but show a large peak of ca. 30% of the same polarity as the thermal dimer, and have a major peak approx 50% in an area of much higher polarity. Saponification may not be complete, and reesterification of these two chromatographically isolated fractions shows that they are not composed of homogenous material because fractions of the various polarities are recovered. Internal ester linkages in oxidative dimer offer a partial explanation for polarity changes where hydrolysis would release hydroxyl groups to give a polar monomeric unit within the dimer structure. Polarity of a hydroxylated dimer would be different from the original dimer.

Many parameters which influence the conditions of oxidation and hydroperoxide decomposition must be investigated, and the various interactions evaluated before any chromatographic method of dimer analysis can be fully evaluated. So far results indicate that considerable chemical and physical information regarding the composition of oxidative dimers is available through a detailed analysis of the chromatographic fractions. Temp of oxidation and the environment of peroxide breakdown are extremely important, and these two factors probably contribute most to the diversity of results recorded in the literature. Many of the usual analytical techniques need critical evaluation in dimer analysis since basic distinction of dimer types (thermal, dehydro and oxidative) are made on unsaturation, type of unsaturation, mol wt, saponification value, functional group analysis and the presence of various cyclic and heterocyclic groups. No definition of oxidative polymers is possible until these materials are fractionated and the components chemically and physically characterized. Currently it might be advantageous to define, or at least partially describe, oxidative dimers in terms of polarity as determined by some chromatographic procedure.

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REFERENCES

1. AOCS Official and Tentative Methods, 2nd ed. rev., Chicago, Ill. (1962).
2. Aure, L., K. M. Asthausen and E. Bjorsvik, Rappt. Nord. Fellsarskningssymp. 3, 119–23 (1961); C.A. 59, 15506 (1963).
3. Beal, R. E., and O. L. Brekke, JAOCS 36, 397–400 (1959).
4. Bernard, A. H., and H. E. Rost, Ibid. 39, 479 (1962).
5. Chang, S. S., and F. A. Kummerow, Ibid. 30, 403–407 (1953).
6. Clingman, A. L., and D. A. Sutton, Ibid. 30, 53–56 (1953).
7. Evans, C. D., Proc. Flavor Chemistry Symposium," Campbell Soup Co., 1961, pp. 123–146.
8. Fedeli, E., A. Valentini and G. Jacini, Fette, Seifen, Anstrichmittel 65, 402–410 (1963); C.A. 59, 5973 (1963).
9. Firestone, D., and JAOCS 40, 247–255 (1963).
10. Frankel, E. N., C. D. Evans and J. C. Cowan, Ibid. 37, 418–424 (1960).
11. Frankel, E. N., C. D. Evans, D. G. McConnell, E. Selke and H. J. Dutton, J. Org. Chem. 26, 4563–4569 (1961).
12. Frankel, E. N., C. D. Evans, Helen A. Moser, D. G. McConnell and J. C. Cowan, JAOCS 38, 130–134 (1961).
13. Frankel, E. N., D. G. McConnell and C. D. Evans, Ibid. 39, 297 (1962).
14. Gould, E. S., "Mechanism and Structure in Organic Chemistry," Henry Holt & Co., New York, 1959.
15. Harrison, S. A., and D. H. Wheeler, J. Am. Chem. Soc. 76, 2379–2382 (1954).
16. Johnston, A. E., K. T. Zilch, E. Selke and H. J. Dutton, JAOCS 38, 367–371 (1961).
17. Newman, M. S., J. Am. Chem. Soc. 72, 4783–4786 (1950).
18. Paschke, R. F., L. E. Peterson, S. A. Harrison and D. H. Wheeler, JAOCS 41, 58–60 (1964).
19. Perkins, E. G., Food Technol. 14, 508–514 (1960).
20. Radin, N. S., and A. K. Hajra, J. Lipid Res. 1, 250–251 (1960).
21. Rost, H. E., Fette, Seifen, Anstrichmittel 64, 427–433 (1962).
22. Rost, H. E., Ibid. 65, 463–466 (1963).
23. Shaw, J. N., and M. W. Formo, JAOCS 31, 448–451 (1954).
24. Swern, D., H. B. Knight, J. T. Scanlan and W. C. Ault, J. Am. Chem. Soc. 67, 1132–1135 (1945).
25. Swern, D., J. E. Coleman, H. B. Knight, K. T. Zilch, H. J. Dutton, J. C. Cowan and J. M. Gyenge, J. Polymer Sci. 17(5), 487–490 (1953).
26. Williamson, L., J. Appl. Chem. 3, 301–307 (1955).
27. Zilch, K. T., H. J. Dutton and J. C. Cowan, JAOCS 29, 244–246 (1952).

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A Long-Term Nutritional Study with Fresh and Mildly Oxidized Vegetable and Animal Fats[1]

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Abstract

Fresh and oxidized cotton seed oil (CO) olive oil (OO), chicken fat (CF) and beef fat (BF) were fed to male weanling rats for 33 to 108 weeks. Groups fed oxidized fats except OO showed a higher death rate than those fed the corresponding fresh fats. Groups fed oxidized CO and BF had the highest death rate. Histological studies of animals dying from natural causes showed more pronounced cardiac lesions in the animals fed oxidized CO. Serum, liver and brain cholesterol levels were not influenced by oxidized fats. Fatty acid composition of depot fats and of heart and liver lipids did not show significant differences between groups fed fresh and the corresponding oxidized fats.

[1] Presented at the Spring Meeting of the AOCS 1964.

IN SPITE OF THE GREAT interest in the biological effects of natural fats which have been exposed to heat and/or air, there are comparatively few reports as to any pathological findings after long-term feeding of such fats. In acute experiments, McKay (1) induced the generalized Shwartzman reaction in pregnant rats by feeding them fractions of oxidized cod liver oil during the gestation period. These episodes of disseminated intravascular clotting, which are especially noticeable in lungs and kidneys, can be prevented by the feeding of tocopherol. Raulin (2) found that the degenerative heart lesions are more frequent in rats on a low tocopherol diet. Earlier short-term studies carried out in the authors' laboratory with highly polymerized fractions of autoxidized fats revealed no recognizable histological lesions except for some edema of the gut despite the fact that the animals had enlarged livers, kidneys, and adrenals before they died (3).

TABLE I

Death Rate at Different Ages of Male Rats Fed Various Fats

Age (wk)	Cottonseed oil		Olive oil	
	Fresh	Autoxidized	Fresh	Autoxidized
4–33	1/40	0/40	0/40	0/40
33–73	2/33	4/34	4/34	0/34
73–99	7/25	9/24	8/24	6/28
99–108	4/12	5/9	4/10	3/16
	Chicken fat		Beef fat	
4–99	2/20	5/20	2/20	4/20
99–108	6/12	5/9	2/12	4/12

In cooperation with the Human Nutrition Research Division of the U. S. Department of Agriculture, a long-term study of several food fats commonly used in the United States was undertaken. This report will give some of our findings with regard to cottonseed and olive oils and chicken and beef fats.

The fats were aerated for 40 hr at 60C at an airflow of 1–2 liters/min. Peroxide values for successive batches of rancid cottonseed oil were 122.6, 43.8 and 97.0 and for rancid olive oil, 12.7, 324.0 and 20.8. Although these values varied considerably, no attempt was made to keep the peroxide values at the same levels because conditions of oxidation had been predetermined and were kept as constant as possible. Variations in peroxide number may have been due to different amounts of antioxidants present in different batches of the oils. The fresh and oxidized oils were included at a level of 20% in a diet composed of 30% alcohol-washed casein, 44% dextrose, 3.5% USP XIII salt mixture, 0.5% calcium carbonate, 2% cellulose, and the following vitamin supplements (in mg/kg): (choline dihydrogen citrate 1000, inositol 1000, nicotinamide 100, p-aminobenzoic acid 300, thiamine · HCl 2, pyridoxine · HCl 4, riboflavin 4, Ca pantothenate 10, folic acid 2.5, biotin 0.025, ascorbic acid 25, vitamin K 10, vitamin B_{12} (0.1% trituration in mannose) 5, crystalline beta-carotene 5, alpha-tocopherol acetate 50, free alpha-tocopherol 10 and crystalline vitamin D_3 0.5). To insure an adequate vitamin intake despite the oxidized fat in the diet, each rat was given a weekly oral supplement of 3 drops of Vi Penta Multivitamin suspensions from Hoffman-LaRoche.

The studies were carried out on groups of weanling male rats of the Columbia-Sherman strain. The groups fed cottonseed and olive oils contained 40 rats each and those fed chicken and beef fats contained 20 rats each. All rats were observed for weight gain, food intake, life span and pathology at autopsy. From the groups fed the cottonseed and olive oils, six rats each were sacrificed at 33, 73, 99 and 108 weeks of age for histological examinations and for lipid analyses of serum and tissues. Groups of six rats each were sacrificed at 99 and 112 weeks of age from those fed the chicken and beef fats.

The rats were killed by drawing blood from the heart under chloroform anesthesia. Their organs were removed and immediately weighed; sections were fixed in 10% formalin for histological examination and the rest, as well as the serum, were quickly frozen and stored at −20C. Serum cholesterol was determined according to Bloor et al. (4), and tissue cholesterol, by the method of Sperry and Webb (5). Lipid was extracted from serum and tissues with chloroform:methanol according to Folch et al. (6). Fatty acid compositions were determined by gas-liquid chromatography of their methyl esters prepared by transesterification with 5% methanolic HCL in benzene (2:1). GLC was carried out on a Perkin-Elmer Model 154C vapor fractometer with a 2 meter column packed with diethylene glycol succinate on chromosorb W and with a hydrogen flame ionization detector. The carrier gas was helium at 20 psi; the operating temperature was 225C.

TABLE II

Average Body Weights and Food Intake of Rats on Different Fats at 96 Weeks

	Fresh oil		Autoxidized oil	
	Food intake, g	Body weight, g	Food intake, g	Body weight, g
Cottonseed oil	108.5 ± 5.10[a]	613 ± 25	108.6 ± 3.68	633 ± 23.9
Olive oil	94.1 ± 4.67[a]	588 ± 19.8	97.3 ± 3.32	565 ± 20.0
Chicken fat	86.5 ± 3.73	591 ± 16.3	85.0 ± 3.49	583 ± 21.5
Beef fat	123.6 ± 3.94	589 ± 19.4	119.8 ± 4.76	582 ± 23.3

[a] Standard error of the mean.
[b] $P = <.05$ vs. fresh cottonseed oil.

Table I gives the number of rats in each group dying between successive withdrawals of rats. The data shows that the rats fed oxidized cottonseed oil and oxidized chicken fat had a higher death rate than all other groups; the groups with the lowest rates were those fed fresh beef fat and oxidized olive oil. With the exception of the latter, the groups fed oxidized oils had higher death rates than did the corresponding groups fed the fresh oils.

Table II gives the body weight and food intake of all groups at two years of age. At one year, the group fed fresh cottonseed oil had the highest average weight. After two years, the average weights of the animals fed the cottonseed oils were still higher than those of the other groups but not significantly so. Monthly records of body weights and food intake for all groups were maintained through the study.

In the groups fed cottonseed oil, neither age nor the oxidation of the fat influenced the amount of food eaten, which was approximately 15 g per day. The rats fed the olive oils tended to eat somewhat less—particularly those fed the oxidized oil after about one year of age, when the second batch of oxidized olive oil with the higher peroxide number was being fed. However, they evidently adapted to the diet because their intake increased significantly although never to the levels of the rats fed cottonseed oil. The best survival rate was attained by this group fed an oxidized oil with a peroxide number of about 300 for over one year. This suggests that at least these peroxides taken orally were not toxic. This confirms previous studies (7). The food intakes of the groups fed chicken and beef fats for almost the first one and a half years did not differ from those of the rats fed vegetable oils. Later, however, the intakes of those fed chicken fat declined to about 12 g per day, whereas those fed beef fat ate approximately 17 g per day. Fecal fat analyses showed that the latter group excreted 200 mg fat per day whereas those fed chicken fat and the vegetable oils excreted 20

TABLE III

Serum, Liver and Brain Cholesterol Levels in Male Rats Fed Various Fats, for 108 Weeks

	Cholesterol, mg		
	Serum[a]	Liver	Brain
Fresh cottonseed oil	118 ± 9.9	209	3920
Oxidized cottonseed oil	118 ± 9.6	264	4320
Fresh olive oil	99 ± 9.9	562	3930
Oxidized olive oil	95 ± 9.0	507	4190
Fresh chicken fat	123 ± 11.3	527	3670
Oxidized chicken fat	138 ± 13.1	320	3780
Fresh beef fat	155 ± 16.9	536	3670
Oxidized beef fat	119 ± 3.8	384	4110

[a] The serum values are averages of six samples; the liver and brain values were derived from pooled samples. ± values are standard errors.

TABLE IV

Fatty Acid Compositions (%) of Tissue Lipid Extracts from Male Rats Fed Fresh and Oxidized Fats. Each Analysis was Carried Out on the Pooled Tissue from Six Rats.

	C16	C18	C18:1	C16	C18:1	C18:2	C20:4
				Depot fat			
Cottonseed oil							
Fresh							
33 weeks	0.9	20.2	2.2	1.4	22.8	52.5	.....
73 weeks	1.2	18.8	3.1	2.5	26.6	47.6	.....
99 weeks	1.3	17.2	1.6	1.6	24.2	54.1	.....
108 weeks	0.3	16.9	0.6	2.3	30.6	49.3	.....
Oxidized							
33 weeks	0.8	21.6	2.9				
73 weeks	0.9	19.0	1.7	2.0	33.7	48.0	.....
99 weeks	1.1	16.2	1.6	2.0	28.6	47.7	.....
108 weeks	....	14.3	0.4	2.2	25.7	53.2	.....
				0.7	28.5	56.0	.....
Olive oil							
Fresh							
33 weeks	0.5	11.2	2.6	1.4	78.5		
73 weeks	0.3	13.6	3.7	1.2	70.0	5.9	.....
99 weeks	....	12.4	2.7	1.5	74.6	11.2	.....
108 weeks	....	8.5	1.6	1.2	83.4	8.8	.....
Oxidized						5.2	.....
33 weeks	0.3	11.2					
73 weeks	0.4	16.3	2.9	1.0	79.2		
99 weeks	0.5	11.0	3.7	1.7	66.8	5.4	.....
108 weeks	....	10.2	2.7	1.5	75.6	11.0	.....
			1.6	2.1	82.8	11.7	.....
Chicken fat						3.9	.....
Fresh							
112 weeks	....	15.2	3.4	1.3	64.7	15.3	.....
Oxidized							
112 weeks	...	14.5	4.0	0.5	69.5	11.3	.....
Beef fat							
Fresh							
112 weeks	0.5	17.1	3.0	3.2	73.8	1.5	.....
Oxidized							
112 weeks	0.4	17.3	3.3	2.9	74.8	1.3	.....
				Liver			
Cottonseed oil							
Fresh							
33 weeks	0.6	25.3	1.2	10.4	13.2	34.1	14.3
73 weeks	0.4	22.1	1.1	11.9	12.7	33.1	17.2
99 weeks	0.3	21.1		12.7	10.4	33.6	22.0
108 weeks	....	21.5	0.3	20.1	15.9	19.3	22.9
Oxidized							
33 weeks	0.1	24.1	1.3	11.7	13.4	34.0	14.5
73 weeks	0.3	19.4	....	12.3	15.9	29.1	18.7
99 weeks	....	22.2	....	12.8	11.3	31.4	22.3
108 weeks	....	21.0	0.2	23.6	6.0	21.2	26.0
Olive oil							
Fresh							
33 weeks	0.6	17.0	2.8	6.0	61.6	5.5	5.1
73 weeks	0.4	19.8	1.7	7.1	47.9	13.4	9.0
99 weeks	0.3	18.2	1.1	8.3	43.7	13.1	15.3
108 weeks	....	19.7	0.6	7.9	55.1	3.8	11.4
Oxidized							
33 weeks	0.2	16.0	2.1	7.5	61.2	5.8	6.9
73 weeks	0.3	19.7	1.1	10.9	46.5	8.6	11.5
99 weeks	0.3	16.3		13.0	44.9	8.7	16.2
108 weeks	....	22.7	1.9	8.7	51.3	4.6	10.7
Chicken fat							
Fresh							
112 weeks	....	22.7	1.3	15.1	29.6	13.5	17.9
Oxidized							
112 weeks	....	23.7	1.5	14.9	27.1	12.6	20.2
Beef fat							
Fresh							
112 weeks	0.1	21.7	0.5	20.2	35.6	2.7	19.2
Oxidized							
112 weeks	....	19.0	1.5	22.6	40.0	2.6	14.1
				Heart			
Cottonseed oil							
Fresh							
33 weeks	1.2	17.5	2.6	23.3	10.6	30.2	12.4
73 weeks	0.6	11.9	0.7	16.9	13.2	23.4	22.6
99 weeks	....	12.4		23.1	7.9	25.9	30.7
108 weeks	....	15.3	0.1	26.8	9.2	21.9	24.7
Oxidized							
33 weeks	1.8	24.9	8.9	17.5	24.9	22.5	4.3
73 weeks	....	10.4	....	20.5	12.7	24.7	19.2
99 weeks	....	13.4	....	22.9	10.1	23.8	29.7
108 weeks	....	13.5	0.1	30.5	6.8	23.9	24.9
Olive oil							
Fresh							
33 weeks	0.4	19.4	3.4	16.3	35.3	16.2	7.9
73 weeks	0.5	11.3	1.0	16.0	26.3	13.9	26.4
99 weeks	....	13.7	0.4	21.2	16.0	19.4	31.2
108 weeks	....	12.2	....	29.1	30.9	6.8	10.1
Oxidized							
33 weeks	0.8	14.4	1.1	24.2	35.0	9.8	14.8
73 weeks	0.1	11.1	0.9	16.6	32.8	14.3	21.8
99 weeks	....	11.7		21.2	24.8	9.0	33.3
108 weeks	....	11.5	0.2	26.7	23.8	13.1	24.1
Chicken fat							
Fresh							
112 weeks	....	12.9	0.3	25.9	15.2	17.1	28.3
Oxidized							
112 weeks	....	11.5	0.1	30.3	12.1	16.5	29.3
Beef fat							
Fresh							
112 weeks	....	13.1	0.1	30.4	18.7	6.0	31.7
Oxidized							
112 weeks		16.9	0.3	30.9	20.3	6.9	23.8

to 70 mg per day. This difference in fat excretion does not explain that the groups gained weight at similar rates despite wide variations in their food intakes. The result is in line with observations that weight maintenance can be affected by adaptation to widely varying food intakes (8).

Table III gives serum, liver and brain cholesterol values for all groups. The serum levels of the animals fed olive and cottonseed oils appeared to increase slightly with age, but were not affected by the kind of oil fed. Serum levels of the rats fed chicken and beef fats were somewhat higher but oxidation of the fat did not seem to have any effect.

The liver cholesterol values of the animals fed olive oil were higher than those of the other groups. Although strict statistical evaluation of the data is hardly possible, the consistency of the differences between the animals fed olive oils and those fed other fats suggest that the feeding of olive oils was associated with high liver cholesterol levels. When the animals were 108 weeks old, liver cholesterol values of those fed the cottonseed oils were probably lower than those of any other group.

The cholesterol values of the brain increased with age. The average values were 1.43% at 33 weeks, 1.83% at 73 weeks, 3.03% at 99 weeks and 3.96% at 108 weeks. Feeding of various fats was without affect. Cholesterol analyses of kidney and heart lipids did not reveal any differences.

Thus, the various fats seemed o exert only a mild influence on cholesterol levels and what differences there were did not appear to be correlated to survival rate.

Analyses of fatty acid were carried out on all lipid extracts which had been analyzed for cholesterol. For brevity, only the results obtained for depot fat, liver and heart are given in Table IV. Depot fat analyses revealed no differences other than those reflecting the composition of the dietary fats. Neither age nor feeding of oxidized fat brought about definite changes. Liver lipids, although influenced by the dietary fat, had their own characteristics. Again, no influence of age or oxidation of the dietary fat could be established. The heart lipids seemed to be somewhat less influenced by the composition of the dietary fat. Here, too, age and oxidation of the dietary fat had no detectable effect. Whether or not the characteristic lipid composition of various organs is related to special functions remains to be seen. However, it may be pointed out that all organs of the animals fed the beef fats had a low linoleate content, and that, in the case of those eating fresh beef fat the low linoleate content was associated with a low mortality rate.

In our earlier feeding studies with more highly polymerized fats, we had seen marked enlargement of liver, kidneys and adrenals. In the present studies, liver weights were not significantly influenced by the oxidized fats. The hearts of the two oldest groups fed cottonseed oil were relatively heavier than those of the other groups, especially those fed beef and chicken fats. Two of the four animals fed oxidized cottonseed oil and sacrificed at 108 weeks had particularly heavy hearts. The kidney weights were greater than those of any other group.

Table V summarizes the main histological findings in the animals dying from natural causes. In general, they died from the usual causes of death in older rats: lung infections, cardiorenal diseases and malignant tumors, and there did not appear to be

TABLE V

Incidence of Deaths from Various Causes Among Male Rats Fed the Different Fats. Diagnosis was Based on Histologic Evidence

	No. of deaths	Inflammatory lung disease	Cardiorenal insufficiency	Cancers*	Undetermined
Cottonseed oil					
Fresh	15	8	2	0	5
Autoxidized	18	6	3	3	6
Olive oil					
Fresh	16	4	3	4	5
Autoxidized	13	3	4	3	3
Chicken fat					
Fresh	6	0	2	2	2
Autoxidized	11	3	6	3	0
Beef fat					
Fresh	5	0	1	1	3
Autoxidized	8	2	1	1	4

* Metastasizing tumors of the pituitary and of the adrenal have been excluded.

definite differences between the groups. One possible exception may have been a higher incidence of malignant tumors in the groups fed olive oil and chicken fat. A chi-square analysis gave a P of about 5%, which is just enough to invite further studies. Chicken fat contained 70 μg per 100 g of stilbesterol whereas the other fats contained about one-third this amount. Among the 91 rats sacrificed at about two years of age, the difference did not exist; eight malignant tumors distributed over all the groups were observed. It is known that old rats develop unspecific cardiac and renal lesions. In the heart, muscle fibers were damaged by accumulations of round cells and the appearance of fibrous tissue. The greatest difference was found between the groups fed fresh and oxidized cottonseed oils. In the latter there were significantly more scarring and round cell infiltration, that is, muscle damage before the laying down of connective tissue. The group fed this diet and sacrificed after two years had by far the largest hearts. In other groups there seemed to be more severe lesions in the groups fed oxidized chicken and beef fat than in their controls, but not significantly so.

The kidneys were graded with regard to hyaline casts and accumulation of round cells. Among those dying spontaneously, the number of lesions was higher in the animals fed oxidized fats, with the exception of oxidized olive oil. In the animals that had been sacrificed, there was not much difference between those on fresh and oxidized fats, but there was a highly significant difference between the relatively few animals with lesions among those fed the olive oils and the greater frequency in the other groups.

It is evident that even the relatively mild damage to the fats led to significant biological changes. Oxidized fats reduced the survival rate except for the curious case of oxidized olive oil. The feeding of oxidized cottonseed oil was associated with the occurrence of more marked heart and kidney lesions.

It is evident that the biological activities of the various fats could hardly be correlated with their chemical properties, which makes it more necessary to carry out long-term nutritional studies.

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