• *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 rats 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 nontoxic, 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-

[1] Carried out with the aid of a grant from the United States Public Health Service.

[2] Presented at the 30th fall meeting, American Oil Chemists' Society, September 28–30, 1956, Chicago, Ill.

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TABLE I

Chemical Properties of Fractions of Autoxidized Lard and Cottonseed Oil

Sample [a]	Lard fractions						Fatty acid chain-length
	Acid No.	Sap. No.	Iodine No.	% Hydroxyl oxygen	% Carbonyl oxygen	Mol. wt.	
MDDD	12	249	28	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	230	47	2.0	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	650	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	0.3	.....	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.

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_2. 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

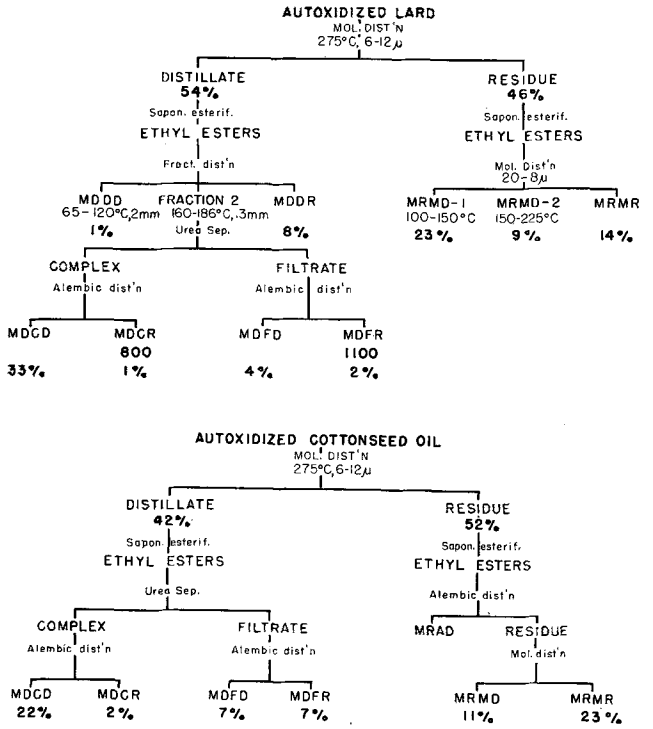

FIG. 1. Flowsheet for fractionation of autoxidized lard and cottonseed oil. The percentage yields are based on the amount originally fed into the molecular still. Molecular weights are given below some fractions.

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, $S = 12.44\ W^{3/5}$, 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[a]	193 ± 4.9	− 2 ±2.5	+ 2 ± 2.7	+ 2 ± 3.8
MDDD	C_{18} and shorter	4/4	137[b] ±16.8	146[b] ± 7.5	− 1 ±8.4	−23 ± 2.6	.....
MDDR	C_{18}	11/14	108 ± 6.1	212 ±21.6	+18 ±3.9	+34 ± 3.7	−16 ± 5.9
MDCD	C_{18}	13/14	188 ± 4.2	224 ± 9.6	− 3 ±2.2	− 3 ± 2.4	− 1 ± 4.7
MDCR	Dimer and trimer of C_{18}	4/4	138[b] ± 9.8	.....	+12 ±7.3	+48 ±19.0	.....
MDFD	C_{18}	11/12	155 ± 9.0	225 ±18.4	+20 ±3.5	+ 6 ± 3.4	+ 8 ± 6.5
MDFR	Trimer of C_{18}	4/4	133 ±13.0	125 ±10.3	+28 ±7.4	+31 ± 6.4	.....
MRMD1	C_{18}	13/14	175 ± 6.9	231 ±11.4	+11 ±2.0	+10 ± 3.2	+13 ± 6.2
MRMD2	Trimer of C_{18}	9/14	93 ± 7.5	297 ±21.8	+31 ±4.3	+42 ± 4.7	− 3 ±14.6
MRMR	Higher mol. wt.	12/13	137 ± 7.7	281 ±12.6	+14 ±2.3	+13 ± 2.2	0 ± 8.8

[a] Standard error. [b] After 2 weeks when lard animals had body wt. of 162 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	201 ± 4.9	− 1 ±3.6	+ 1 ±3.6	+ 4 ±6.2
MDCD	C_{18}	14/16	186 ±6.1	217 ± 8.6	+ 8 ±2.7	+ 2 ±1.6	− 6 ±2.9
MDCR	Dimer of C_{18}	3/4	103 ±5.2	.....	+32 ±4.4	+19 ±7.4	.....
MDFD	C_{18}	7/8	137 ±2.5	.....	+21 ±6.9	+16 ±5.1	.....
MDFR	Dimer of C_{18}	6/8	104 ±1.9	.....	+ 7 ±4.2	+49 ±4.3	.....
MRAD	C_{18}	16/16	179 ±3.6	268 ±15.2	+11 ±3.0	+ 8 ±2.4	−18 ±5.1
MRMD	Dimer of C_{18}	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 ±9.6

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	808 ± 5.5	68.5 ±2.3
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	896 ±38.5	64.0 ±2.2
MDFD	31.1 ± .1	26.7 ±1.2	775 ±18.0	67.2 ±1.2
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 ±36.0	64.0 ±2.2
MRMD	31.5 ± .1	18.7 ±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	Cholesterol level	Cholesterol level
		(% dry wt.)	*(% liver lipid)*	*(mg. % dry substance)*
Fresh fats	12	24.8±.91	4.1±.15	999±24
Autoxidized cottonseed oil	6	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.

Acknowledgments

Waldo Ault of the Eastern Regional Research Laboratory has contributed greatly to this work by his advice, encouragement, and criticism. We are indebted to Leo A. Pirk of Hoffmann-La Roche Inc., Nutley, N. J., for most of the vitamins used in these studies and to M. L. Tainter of the Sterling-Winthrop Research Institute, Rensselaer, N. Y., for crystalline vitamin D_2.

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[Received February 27, 1959]