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

Summary

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

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

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

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

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

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

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

Present Study

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

[1] This paper is XXI in the series "Reactions of Fatty Materials with Oxygen." Paper XX is reference 18.

[2] Presented at the Fall meeting of the American Oil Chemists' Society, Philadelphia, Pa., October 10–12, 1955.

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

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

Results and Discussion

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

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

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

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

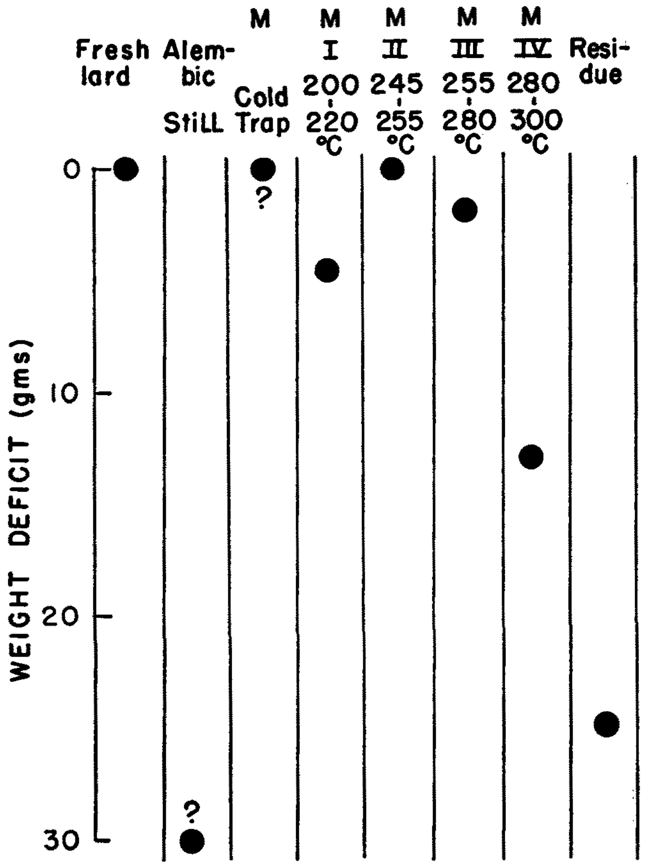

FIG. 1. Weight deficits in rats fed fresh lard, molecular distillates from autoxidized lard (M-I, II, III, IV), and polymeric residue from autoxidized lard.

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

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

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

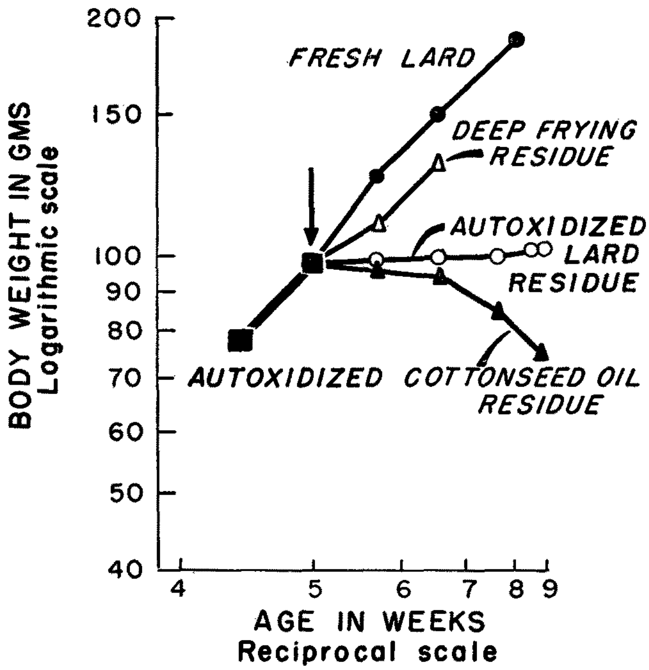

FIG. 2. Growth of rats fed fresh lard and polymeric residues from three autoxidized fats at a 10% level in the diet.

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

Figure 3 compares the growth of rats fed 15%

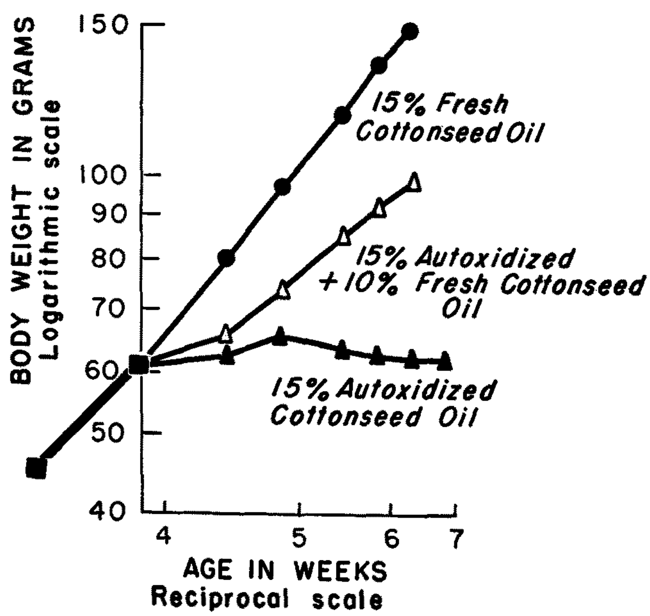

FIG. 3. Growth of rats fed 15% fresh cottonseed oil, 15% autoxidized cottonseed oil, 15% autoxidized cottonseed oil + 10% fresh cottonseed oil.

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

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

Noticeable changes were also observed in the organ-weight/body-weight relationship, which is shown in Figure 4. Normal organ-weight data were obtained

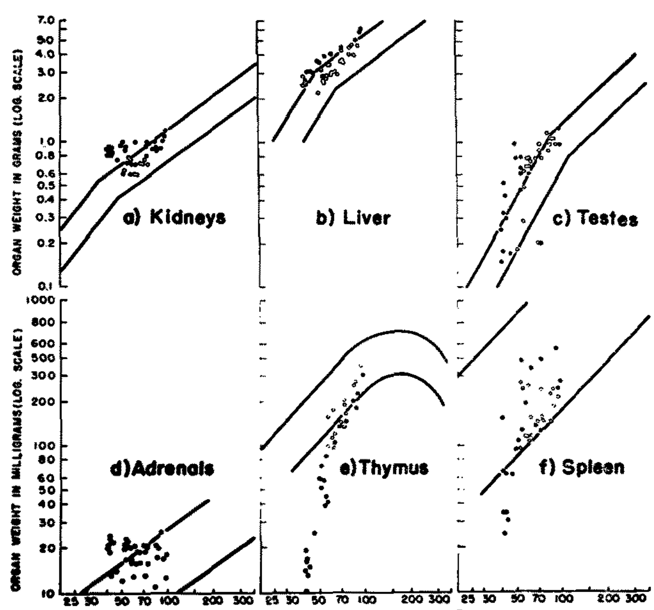

FIG. 4. Normal organ-weights (solid lines) and organ weights of rats fed 15% autoxidized cottonseed oil (closed circles), and 15% autoxidized cottonseed oil + 10% fresh cottonseed oil (open circles).

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

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

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

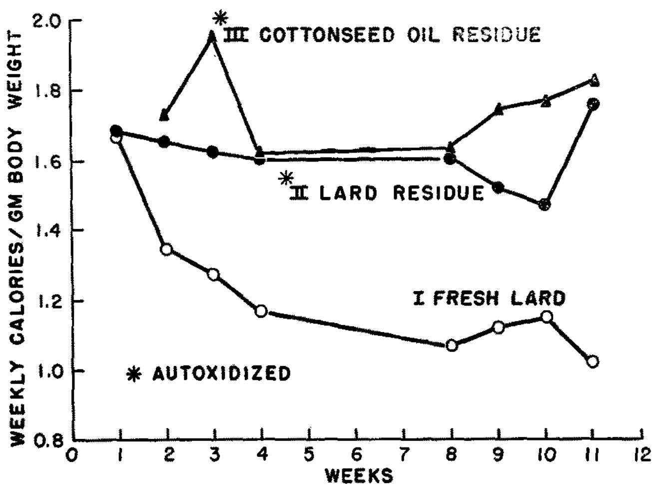

FIG. 5. Average caloric values for weight maintenance of rats fed 10% fresh lard (I), 10% autoxidized lard residue (II), or 10% autoxidized cottonseed oil residue (III).

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

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

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

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

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

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

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

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

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

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

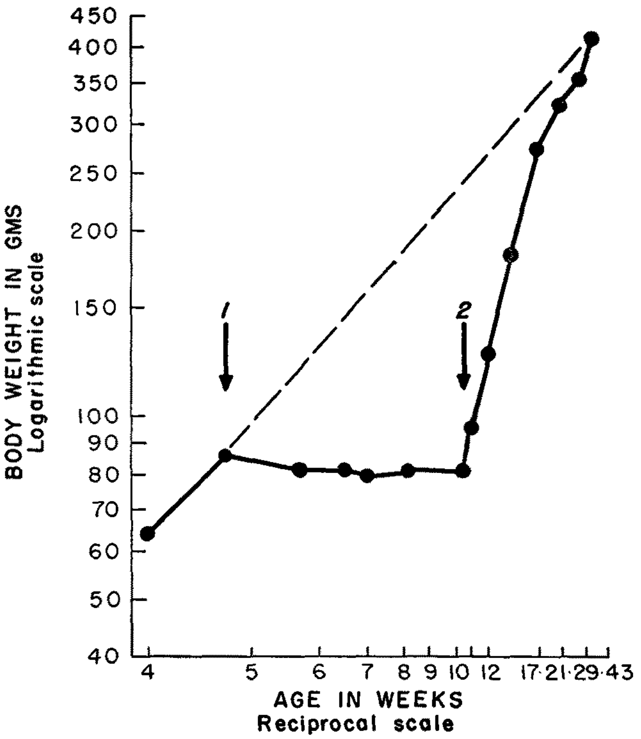

FIG. 6. Growth of rats fed a normal diet to arrow 1, 10% autoxidized cottonseed oil residue from arrow 1 to arrow 2, then returned to normal diet at arrow 2.

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

Acknowledgments

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Summary

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

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

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

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

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