anti-oxidant for edible fats, increasing stability even in baked products (Mattil et al., '45; Higgins and Black, '45).

The question of vitamin E deficiency in heated oils also seemed worthy of consideration at this time, since polymerization at 275°C. would destroy any of this vitamin inherent in the original oil. The absence of vitamin E, a natural antioxidant, presumably permits a more rapid onset of autoxidation. In addition, a relation of vitamin E to growth, over and above its stabilization of linoleic acid, has been shown in insects by Fraenkel and Blewett ('46). In the feeding of heated lard to rats, Morris and his co-workers ('43) noted a depression of growth and also a paralysis simulating a vitamin E deficiency. Therefore it was possible that in our tests thermal destruction of vitamin E was responsible in part for the adverse effects we had observed to result from feeding heat-polymerized linseed oil.

EXPERIMENTAL

Feeding trials were conducted to examine the effects of baking, of controlled autoxidation, and of vitamin E supplementation of diets containing heat-polymerized linseed oil.

Oil preparation

Polymerization and acetone segregation were performed according to the method of Privett, McFarlane and Gass ('47). Preliminary trials in our laboratories showed no nutritional difference between whole heated oil and its acetone soluble fraction, beyond a decrease in intake of the diets containing the latter. This effect was thought to be due to residual traces of acetone (Langerman, '49). Therefore in the present test, in which acetone segregation of heated oil was involved, the control (unheated) oil was subjected to like treatment with acetone.

Where oils were artificially stabilized (test II), an antioxidant mixture of NDGA with citric acid was added as 0.05% of the oil. Peroxide values on these oils when freshly prepared

and after 28 days of storage at room temperature were estimated by the method of Skellon and Wills ('48). Peroxide determinations on chloroform extracts of the oil-containing diets were made at intervals throughout the 28-day feeding trial by a modification of the Skellon and Wills method (see appendix).

Biological procedure

The animals used in these trials were albino rats ranging in age from 21 to 28 days at the outset of the test period. They received, ad libitum, the diets described in table 1.

TABLE 1

Composition of diets

INGREDIENTS	DIET A (test II)	DIET B (tests I and III)
White flour (%)	47	54
Milk powder (%)	19	19
Casein (%)	11.5	11.5
Oil (%)	20	10
Bone meal (%)	2	2
Salt (%)	0.5	0.5
Yeast (%)	..	3
Vitamins		
Thiamine	10 p.p.m.	..
Niacin	230 p.p.m.	..
Riboflavin	5 p.p.m.	..

Diets minus vitamins or yeast were baked (except where specified in test I) for 20 minutes at 375°F. and the resulting biscuit granulated and air-dried for 24 hours. Vitamin supplements in diet A, or yeast in the case of diet B, were then added. Weekly doses of cod liver oil were administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

In addition to these vitamins, half of the animals in test III each week received orally alpha-tocopherol dissolved in corn oil. The doses, administered two days following the A and D supplementation, were of such amount as to supply 7 mg of vitamin E to each animal.

RESULTS

When the oils were stabilized, there was no appreciable accumulation of easily reducible peroxides. However, definite signs of progressive peroxidation were shown in the unstabilized oil over the 28-day trial. These facts are evident from the peroxide values given in table 2 and calculated at the beginning and the end of the test period on oil extracted from the feed and also on oil that was not combined with the feed.

It is true that peroxide values are not a full indication of rates of total oxygen absorption and that other diet components may have been attacked by peroxides as rapidly as

TABLE 2

Peroxide values

(Calculated as milligrams of peroxide oxygen per kilogram)

	OIL EXTRACTED FROM FEED		STORED OIL	
	Fresh	After 28 days	Fresh	After 28 days
Unheated oil	99	276	47	1,334
Unheated stabilized oil	26	55	49	172
Heated oil	116	333	0	270
Heated stabilized oil	25	38	0	51

these were formed. In such an event, no accumulation of easily reducible peroxides would have been noted. However, a steady increase in peroxide values in unstabilized oils throughout the test points to the likelihood that extensive peroxide decomposition had not yet begun in the stabilized samples.

The growth rates and feed intakes of the animals in test II, given in table 3, show that the stabilized oil was no better nutritionally than the unstabilized control oil. The equality of the growth responses of animals fed heated oils regardless of anti-oxidant treatment makes it unlikely that autoxidation is responsible for the low nutritive value of polymerized linseed oil.

This is further supported by the data from test I, where the growth responses of the animals show that baking did not influence the growth inhibition exerted by the heated oil. Exposure to oven temperatures would, if anything, encourage

TABLE 3

Effect of baking, of anti-oxidant stabilization, and of vitamin E supplementation on the nutritive value of unheated or heated whole linseed oil or on the acetone soluble fraction of the heated oil

TEST NO.	OIL USED	TREATMENT	OIL IN DIET	NO. ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAINS PER 1,000 CAL.[1]
			%		gm	gm	gm
I	Unheated	Diet baked	10	8	4.3	12.1	72
	Unheated	Diet unbaked	10	8	4.2	12.4	69
	Heated	Diet baked	10	8	3.1	10.6	60
	Heated	Diet unbaked	10	8	2.7	9.7	57
II	Unheated	nil	20	10	3.7	10.1	68
	Unheated	Oil stabilized[2]	20	10	3.8	9.6	73
	Heated (acetone soluble fraction)	nil	20	10	—0.1	6.0	..
	Heated (acetone soluble fraction)	Oil stabilized[2]	20	10	—0.1	5.8	..
III	Heated	nil	10	24	2.4	10.0	49
	Heated	Vitamin E supplementation to diet	10	24	2.3	9.8	48

[1] Gross calories (calculated from heats of combustion): 10% fat diet contained 4.9 Cal./gm; 20% fat diet contained 5.4 Cal./gm.

[2] Protected against autoxidation with NDGA and citric acid.

autoxidation. These results imply that the damaging factor in heated oil is not produced oxidatively.

Supplementation with vitamin E failed to alter the nutritive value of polymerized linseed oil (test III).

CONCLUSIONS

The results of these experiments show that peroxidation of polymerized linseed oil is not concerned in the development of any toxic factor, and also suggest that a lack of vitamin E is not involved in the growth inhibition resulting from the ingestion of heated oil.

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APPENDIX

Modification of the Skellon and Wills procedure for peroxide determinations on oil extracted from diets

A weighed sample of biscuit containing about 1 gm of the oil was shaken up with a 25-ml portion of chloroform and the filtrates were combined. The extract was evaporated to 10 ml by passing over it oxygen-free nitrogen *without heating*, and transferred to a 250-ml flask containing 2 gm sodium bicarbonate. A stopper was fitted, carrying a short piece of glass tubing capped by a rubber Bunsen valve. This served as a one-way pressure relief valve. Fifteen milliliters of glacial acetic acid were used to rinse the last traces of chloroform extract into the reaction flask, and the acid-bicarbonate reaction released CO_2 which swept air out of the flask through the relief valve. When foaming subsided, 1 ml of saturated fresh potassium iodide solution was added, and the tightly stoppered flask was left in the dark for one hour. Fifty milliliters of boiled, cooled water were then added, and the iodine titrated with 0.002 N thiosulfate.

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

$$PV = \frac{V \times N \times 16 \times 10^3}{W \times P}$$

where,

V = Volume of thiosulfate used (ml)
N = Normality of thiosulfate
W = Sample weight (gm)
P = Per cent oil in sample.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT[1, 2]

III. THE SEGREGATION OF TOXIC AND NON-TOXIC MATERIAL FROM THE ESTERS OF HEAT-POLYMERIZED LINSEED OIL BY DISTILLATION AND BY UREA ADDUCT FORMATION

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ONE FIGURE

(Received for publication July 28, 1952)

INTRODUCTION

The deleterious effects of heating certain vegetable oils at polymerization temperatures on their nutritional value have been described in a previous paper (Crampton et al., '51c). There is some evidence (Crampton et al., '51a) that these deleterious effects are not a consequence of peroxidation. It has also been shown (Crampton et al., '51b) that the deleterious effects may be demonstrated by feeding the ethyl esters of heated linseed oil.

When the whole esters of heated linseed oil were separated by distillation at pressures of the order of 0.5 mm into a distillable fraction and a non-distillable fraction, then the non-distillable fraction was deleterious to experimental ani-

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada, Journal Series No. 304.

[2] Issued as paper 277, Canadian Committee on Food Preservation.

mals. Rats receiving this fraction at the level of 20% of the diet failed to grow; had oily, matted coats; excreted dark, sticky feces; and displayed a heavy mortality. Rats receiving the distillable fraction survived the experiment in good condition and excreted well-formed feces. However, they did not grow as well as rats receiving the whole esters of unheated linseed oil. In fact, their growth was no better than that of rats receiving the whole esters of heated oil. At this stage it seemed possible that the major defect of the heated oil arose from the presence of dimeric acid radicals. However, the distillable monomeric ester fraction was also nutritionally inferior to the esters of whole unheated linseed oil. This fact could not be related to differences in susceptibility to peroxidation, for the esters of the whole unheated oil were considerably more susceptible to peroxidation than the distillable esters of the heated oil (unpublished results).

It seemed possible, therefore, that the monomeric ester fraction might contain material other than straight chain esters, and that such material might play a part in determining the deleterious nature of the heated oils.

The formation of such materials by the exposure of the oils to polymerization temperatures carries implications of some importance so far as oils intended for food are concerned. Accordingly, the preliminary work was repeated and extended, and this paper deals with these later experiments.

EXPERIMENTAL

The nutritional test was performed in two replicates, each comprising observations on 60 animals. Six diets were tested. These diets differed only in (a) the nature of the oil component and (b) the proportion of white flour as adjusted to compensate for the use of 10 or 20% levels of oil.

The formula for the diets, in per cent, was as follows: white flour 53.65 or 43.65; skim milk powder 19; casein 11.5; oil preparation 10 or 20; brewers' dried yeast 3; feeding bone-meal 2; iodized salt 0.5; chromium sesquioxide[1] 0.25; and ferric citrate 0.1.

The oil preparations were certain fractions of the ethyl esters of heat-polymerized linseed oil (12 hours at 275°C. in a strong current of carbon dioxide). The preparation of these fractions is described in detail in the Appendix. They will be designated in this paper for convenience as follows:

1. *Straight chain monomers from whole oil:* that portion of the ethyl esters of heated linseed oil which formed urea adducts with ease.
2. *"Cyclized" monomers and dimers from whole oil:* that portion of the esters of heated oil which did *not* form urea adducts.
3. *Straight chain plus "cyclized" monomers from distillate:* that portion of the esters of heated linseed oil which was distillable at pressures around 0.5 mm.
4. *Straight chain monomers from distillate:* that portion of the distillable esters which formed urea adducts.
5. *"Cyclized" monomers from distillate:* that portion of the distillable esters which did *not* form urea adducts.
6. *Dimers by distillation:* the residue after removal of the monomeric esters by distillation.

The probable general chemical relationships of these 6 fractions are conveniently indicated by a flow sheet (fig. 1). It is to be understood that the designations of the fractions and the symbols used in the flow sheet do not imply that the nature of the fractions is established. While it is reasonably certain that fraction 6 consists mainly of esters of dimeric acids (see table 5) it cannot be considered as finally established whether these are linked by single carbon-to-carbon bonds or by a cyclohexene system. Again, whether the non-adduct-forming monomeric material of fractions 2, 3 and 5 is cyclized or branched is not established: the term "cyclized" must be read as denoting a mode of preparation and not as asserting the presence of a cyclic structure. The chemical characteristics of the fractions are reported in table 5: the

[1] For determination of digestibility.

mean molecular weights confirm the validity of the designations so far as the terms monomeric and dimeric are concerned, but do not provide evidence as to the presence or otherwise of cyclic structures. It may be recalled, however, that Paschke and Wheeler ('49) suggested the presence of cyclic monomers in heat-polymerized methyl linoleate.

The hydroxyl contents of the fractions were determined by the method of Ogg, Porter and Willits ('45). The data

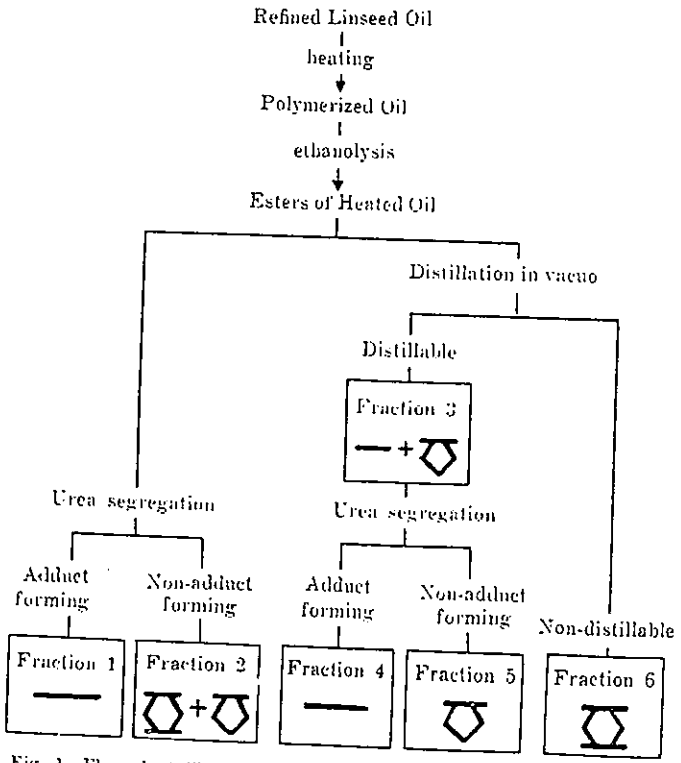

Fig. 1 Flow sheet illustrating preparation of fractions of esters of heated linseed oil used in feeding trials. (For details of preparation, see Appendix.)

are reported in table 5. So far as the monomeric fractions 1, 4 and 5 are concerned, the values correspond to not more than 8, 5 and 15%, respectively, of monohydroxy C18 acids. The available evidence therefore supports the view that hydroxy acids did not constitute a major proportion of the non-adduct-forming monomeric material.

The feeding trials lasted 28 days. Live weight changes and feed consumption were recorded. The apparent digestibilities were determined by the Cr_2O_3 index technique (Schürch et al., '50). Digestible calories were calculated from the gross calorific values of the diets, the feed intake and the simultaneously determined digestibility coefficients. Live

TABLE 1

Comparison of the effect of 10% versus 20% of fractions of the esters of heat-polymerized vegetable oil in the diet of rats (60 rats at each level)

OIL IN DIET	RATS SURVIVING 28 DAYS OF TEST	AVERAGE GAIN IN 28 DAYS	AVERAGE FEED IN 28 DAYS	DIGESTIBILITY OF FEED	GAIN ADJUSTED TO EQUAL INTAKE OF DIGEST. CALORIES
%	%	gm	gm	%	gm
10	83	73	262	91	67
20	63	27	177	83	52
Least sig. diff. (P = 0.05)	..	11	17	..	8

weight gains were adjusted by simple regression to equal intake of digestible calories to provide a measure of the relative efficiency of the diets. Least significant differences between means, where shown, were calculated from the expression

$$\text{L.S.D.} = \sqrt{n} \times \sqrt{2} \times t_{\overline{n-4}} \, s$$

In the preliminary tests, the use of diets containing 20% of certain ester fractions similar to those used in the present experiments had resulted in heavy mortality. Consequently, half of the rats in each of the lots in the tests now reported received a diet containing 10% oil. The differences between the responses of the rats receiving these two levels of oil are summarized in table 1.

Efficacy of protection of the fractions and diets against autoxidation

Since peroxidized fats and fatty acid esters may be toxic to rats, it is necessary to ensure that the results of feeding such materials are not vitiated by the effects of peroxidation. It has been shown that the deleterious effects of heated linseed oil are not related to the degree of susceptibility of the heated oils or of the diets containing the oils to peroxidation (Crampton et al., '51a). In the present experiments the fractions used in the diets were prepared immediately

TABLE 2

Peroxide values of oils extracted from the mixed feeds at two weeks and 4 weeks after preparation. Feeds stored in refrigerator

FRACTION CONTAINED IN THE DIET	PEROXIDE VALUE, MG PEROXIDE OXYGEN PER KG OIL	
	After 2 weeks	After 4 weeks
Fraction 1	44	98
Fraction 2	38	88
Fraction 3	42	90
Fraction 4	46	102
Fraction 5	38	92
Fraction 6	52	75

before feeding, and were protected by addition of an antioxidant and storage in the refrigerator in the dark. The stability of the oils in the diets was tested at the end of the second week of the second replicate and again at the end of the 4th week. Peroxide values of the lipid components of the feed were determined by the method of Skellon and Wills ('48) as described by Crampton et al. ('51a). Peroxidation was relatively slight in all fractions (table 2). From unpublished observations it is believed that peroxidation would not have begun to interfere with the nutritional well-being of the rats until the peroxide value of the oil extracted from the feed attained values of the order of 1,000 mg per kilogram of oil.

It may be concluded, therefore, that the results of the present feeding experiments were due to the effects of constituents other than products of peroxidation.

RESULTS

With diets containing 20% oil

The results of feeding these 6 oil fractions in diets in which they constituted 20% by weight are summarized in table 3. The first salient feature of the results relates to the *mortality*. It is clear that the presence of the "cyclized" monomers in the diet was associated with toxicity, though the mode of action of this material remains obscure. Rats consuming fraction 5, which contained the highest concentration of this material, survived only 5 to 10 days in spite of a greatly reduced feed intake (and hence low intake of toxic material).

A comparison of the results for diet 2, containing a mixture of approximately three parts dimeric esters to one part "cyclized" monomer, with the results for diet 6, shows clearly the much more harmful nature of the monomeric material. The deaths in group 6 were associated with partial starvation and excessive diarrhoea, whereas the animals of group 2 displayed neither diarrhoea, digestive disturbances, nor overt clinical symptoms suggestive of the cause of death.

The animals receiving fractions consisting mainly of straight chain monomers — i.e., groups 1 and 4 — all survived the test and remained in good health. Their gains were comparable to those secured in previous tests with esters of whole unheated linseed oil.

The *gains* of the rats receiving the various fractions also reflected the toxic effects of the "cyclized" monomeric material and the adverse effects of the dimeric esters. The dimeric ester fraction was associated with low digestibility, and this was doubtless the cause of the diarrhetic condition of the rats receiving this material.

The *relative efficiencies* of the diets *per unit of digestible calories eaten* showed that the straight chain monomeric

TABLE 3

The effect of feeding fractions of polymerized linseed oil at 10% level in rat diets (10 rats per lot)

OIL FRACTION FED	PRINCIPAL OIL CONSTITUENTS	NO. OF ANIMALS SURVIVING TEST	AVERAGE GAIN	AVERAGE FEED INTAKE (dry matter)	DIG. OF DRY MATTER	DIG. OF FAT FRACTION	MEAN GAINS ADJUSTED TO EQUAL INTAKE OF DIG. CAL.
			gm	*gm*	%	%	*gm*
1	Straight chain monomers of whole oil	10	101	266	93	94	72
2	"Cyclized" monomers plus dimers of whole oil	2	−32	141	81	77	4
3	Straight chain plus "cyclized" monomers of distillate	9	4	118	94	97	52
4	Straight chain monomers of distillate	10	57	193	94	95	63
5	"Cyclized" monomers of distillate	0	..	..	..	..	
6	Dimers	7	13	168	55	29	71

esters and the dimeric esters were equally well "metabolized," whereas the presence of "cyclized" monomeric material resulted in a sharp reduction in the utilization of digestible calories.

The *low feed intake* by group 5 made it possible that this might have caused the death of some of the rats receiving the "cyclized" monomers. This point was examined by feeding a supplementary group of 5 rats the basal oil-free diet ad libitum. They were dosed daily by dropper with a quantity of "cyclized" monomer equal to 10% by weight of their measured voluntary intake of the fat-free mixture. The intake of the basal diet under these conditions was low, but it was adequate for maintenance and in one case permitted some gain. Nonetheless, all of these rats died within 16 days, thus confirming that the mortality in group 5 could not be explained on the grounds of low food intake.

It will be noted from the schematic outline (fig. 1) of the preparation of the fractions that fractions 1 and 4 ought to consist mainly of the straight chain adduct-forming material. However, fraction 1 had a lower iodine number and a lower refractive index than fraction 4 (table 5). Moreover, the yield of fraction 1 represented 46%, whereas that of fraction 4 was 49%, of the total esters. These differences in yield and in the characteristics of presumptive straight chain monomeric material according to the route by which it has been prepared, suggest that in the case of fraction 4 the adduct-forming fraction had probably carried with it by entrainment a small proportion of material incapable of itself of forming an adduct: i.e., of "cyclized" monomer.

Fraction 1 was also superior to fraction 4 in nutritional value. This circumstance reinforces the view that fraction 4 contained a small proportion of the non-adduct-forming material ("cyclized" monomer), and that the "cyclized" monomeric ester is either of itself the deleterious component or that the deleterious material accompanies this ester fraction.

TABLE 1

The effect of feeding fractions of polymerized linseed oil at 10% level in rat diets (10 rats per lot)

LOT NUMBER	DIETARY FAT CONSISTED ENTIRELY OF	NO. OF ANIMALS SURVIVING YEAR	AVERAGE GAIN	AVERAGE FEED INTAKE (CONSUMED)	NO. OF DAYS ON DIET	DOSE OF FAT PER RATION	MEAN GAINS ADJUSTED TO EQUAL INTAKE OF 100 G. FAT
			gm.	gm.		g.	gm.
1	Straight chain monomers of whole oil	10	207	809	96	16	72
2	"Cyclized" monomers plus dimers of whole oil	10	89	505	28	32	88
3	Straight chain plus "cyclized" monomers of distillate	10	99	858	96	96	66
4	Straight chain monomers of distillate	10	188	699	96	96	72
5	"Cyclized" monomers of distillate	0	..	..	16	96	..
6	Dimers	10	77	925	28	69	64
Least significant difference ($P = 0.05$)		..	55	96	..	..	16

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With diets containing 10% fat

The results of feeding the diets in which the fat levels were reduced to 10% are shown in table 4. They were in all respects what would have been predicted from the results of feeding the 20% fat levels. The toxic effect of the "cyclized" monomers had been lessened, though it was still clearly in evidence. Gains, feed intake, digestibility and efficiency of utilization from the straight chain monomers were practically identical as between rats in lot 1 with 20% fat and those of lots 1 and 5 with 10% fat. Even at this lower fat level none of the rats receiving the "cyclized" monomeric material (lot 5) survived 28 days: where this "cyclized" monomeric material constituted only 2.5% of the diet intake (lots 2 and 3), the rats were able to complete the test, but with significantly smaller gains.

CONCLUSIONS

1. Heat polymerization of alkali-refined linseed oil results in the formation of monomeric acyl radicals whose esters can be distilled at low pressures but which do not form urea adducts. These esters are highly digestible when eaten but are not utilized for growth. The esters contain a small proportion of hydroxyl groups, but are apparently mainly esters of "cyclized" or branched structure.

2. When a fraction consisting essentially of these esters of "cyclized" monomeric acids comprises as much as 10% of the diet, it renders the diet "toxic." The harmful effect is measurable when this fraction comprises 2.5% of the diet. The effect is ascribed tentatively to the "cyclized" monomeric esters as such, rather than to the small proportion of hydroxy acid present in this and other fractions.

3. Dimeric acyl radicals are also formed on heating linseed oil at polymerization temperatures. A fraction consisting mainly of the esters of the dimeric acids is non-toxic, but this material is largely unabsorbed from the gastrointestinal tract. Its presence results in excreta of the nature and consistency of a mixture of soft feces and fresh varnish.

4. Esters of straight chain monomeric acids (i.e., capable of forming urea adducts) are apparently as nutritious as are esters prepared directly from normal unheated triglyceride oils which, in turn, are but slightly inferior to the triglycerides of normal food.

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APPENDIX

Preparation of ethyl esters of polymerized linseed oil

Commercial alkali-refined linseed oil (Canada Linseed Oil Mills, Montreal, P.Q.) was heated in a stream of CO_2 for 12 hours at 275°C. The oil was heated in batches of about 1,200 gm at a time, and the CO_2 was passed at a rate sufficient to keep the oil surging fairly freely. The resultant polymerized oil was of a clear light straw color.

The polymerized oil was allowed to cool while maintaining the current of CO_2. The polymerized oil was then shaken up with one-third of its volume of absolute ethanol to remove any fatty acids present. The washings were discarded.

The washed polymerized oil was used for the preparation of the ethyl esters by alcoholysis, using caustic soda at the rate of 0.5% by weight of the oil as catalyst (Feuge and Gros, '49). The esters were washed with large volumes of hot distilled water in a large separatory funnel. The total yield of mixed esters reckoned on the weight of oil taken was approximately 90% (varying from 88 to 92 with different batches).

Fractionation of the ethyl esters for the feeding trials

A portion of the total ethyl esters was subjected to vacuum distillation as described by Crampton et al. ('51b). The non-distillable fraction, comprising the ethyl esters of dimeric fatty acids, constituted fraction 6. The distillable portion, comprising the ethyl esters of monomeric acids, constituted fraction 3.

Immediately after distillation Tenox II * (Tennessee Eastman Corp.) was added to the portions of fractions 3 and 6 reserved for feeding at the rate of 0.1%. The protected ester fractions were at once removed to the cold room (5°C.), and held there until incorporated in the diets.

To another portion of fraction 3, Tenox II was added at the rate of 0.025%. This portion was subjected to fractionation by urea adduct formation (Schlenk, '49). The following technique was used.

Five hundred grams of the esters were dissolved in 2 l absolute commercial ethanol in a 4 l beaker. Solid urea was added slowly with stirring, while maintaining the mixture at 50°C. In all 2 kg urea were added, since approximately 3 gm urea are required for each gram of C18 ester for adduct formation. The mixture was maintained at 50°C. for about 30 minutes and then allowed to stand at room temperature overnight. Next morning the adduct and excess urea were filtered off and washed on the Buchner funnel with absolute ethanol saturated with urea. The adduct was decomposed by being thrown into warm water. Immediately the oily layer separated, Tenox II was added at the rate of approximately 0.25% of the adduct-forming material. The oily material was washed with water and dried under high vacuum with shaking. This material constituted fraction 4, and presumably consisted mainly of straight chain monomeric esters (see table 5). The non-adduct-forming material in the supernatant and ethanolic washings was separated by the addition of copious amounts of water. No Tenox

* Tenox II is a preparation containing 20% butylated hydroxyanisole, 4% citric acid, 6% propyl gallate and 70% propylene glycol.

II was added, since presumably that present before adduct formation would tend to be concentrated in this fraction, which constituted fraction 5. The addition of small quantities of sodium chloride facilitated the separation of both fractions 4 and 5 from the aqueous phase. Fraction 5 was dried in the same fashion as was fraction 4. Both fractions were now further protected against autoxidation by the further addition of 0.1% Tenox II and stored under the same conditions as fractions 3 and 6.

Another portion of the total esters was separated into adduct-forming (fraction 1) and non-adduct-forming fractions (fraction 2) by the technique described above, except that the total weights of the esters and ethanol were correspondingly reduced, since the total esters of heated oil contained a much smaller proportion of straight chain monomeric material than did the distillable esters (fraction 3). The same precautions were taken against peroxidation as when preparing fractions 4 and 5.

The analytical characteristics of the fractions are presented in table 5.

TABLE 5

Yields and characteristics of fractions of ethyl esters used in the feeding trials

FRACTION NUMBER	YIELD AS % TOTAL ESTERS OF HEATED LINSEED OIL	IODINE NO.[1]	REFRACTIVE INDEX n_D^{25}	MEAN MOLECULAR WEIGHT	HYDROXYL[2] VALUE AS PER CENT (— OH)
1 Adduct-forming fraction of total esters	46	118.2	1.45345	293	0.23
2 Non-adduct-forming fraction of total esters	54	162.7	1.47561	472	0.16
3 Distillable esters	60	130.1	1.45684	294	0.22
4 Adduct-forming fraction of distillable esters	49	124.8	1.45494	293	0.11
5 Non-adduct-forming fraction of distillable esters	11	170.7	1.46986	300	0.39
6 Non-distillable esters	40	159.9	1.48017	550	0.64

[1] Iodine numbers by the method of Benham and Klee ('50), using one-hour reaction time (Klee and Benham, '50).

[2] Hydroxyl values by the method of Ogg, Porter and Willits ('45).

EFFECTS OF PROLONGED INGESTION OF XYLOSE ON RATS

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Xylose is a pentose sugar which might conceivably be incorporated into foods for human consumption. In terms of sweetness it has a value of 40 when compared to sucrose at 100. Monogastric animals are unable to utilize this 5-carbon sugar (Pflüger, '05; Miller and Lewis, '32; Anderson, '50), which suggests that it might be used as a dietary sweetening agent by individuals who desire to reduce body weight. Xylose might also serve as a sweetening agent for diabetics, either in addition to or in lieu of saccharin. The tendency of this sugar to cause diarrhea in laboratory animals suggests its possible use in laxative preparations. In the event a use is found for xylose, it could be produced in quantity from cottonseed hulls and corn cobs, thanks to the methods of production developed by Dunning and Lathrop ('45).

The possible inclusion of xylose in foods immediately raises the question as to whether any health hazard would be involved. The literature concerning the utilization of this substance by man is neither very extensive nor conclusive, but some experimental work has been done using laboratory animals. Darby and Day ('39) reported on the cataractogenic action of xylose in rats. These same workers (Darby and Day, '40) and others (Anderson, '50; Blatherwick et al., '36) observed diarrhea and abdominal distension when rats received this sugar orally. Blatherwick and co-workers ('36) fed

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NUTRITIVE VALUE OR TOXICITY OF HIGHLY UNSATURATED FATTY ACIDS. I

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According to the result from the experiments on the nutritive value of fish oils conducted by Ozaki (*1*) and Yoshida (*2*), highly unsaturated fatty acids in fish oils give toxic effects on the growth of rats. So far, this has been generally conceded, and the existence of highly unsaturated fatty acids in fish oils has been thought to account for the inferiority of fish oils to other edible oils from nutritive point of view. If this is actually the case, it might be surmised that eating of sardines, for example, in a large amount at a time or successively at short intervals would bring about harmful effects on our health.

It is a well known fact that highly unsaturated acids are rapidly oxidized when exposed to air. For this reason, a special attention must be paid when we carry out a feeding experiment by administrating highly unsaturated acids to test animals, and even if the experiment is started with genuine highly unsaturated acids, there will be a danger of supplying more or less autoxidized acids to the animals.

As has been reported in the previous paper (*3*), we examined the nutritive values of ethyl ester of highly unsaturated acids with iodine value of 365 and their partially reduced products. During the assay period the rats receiving ethyl ester of these acids showed a symptom of losing hairs around their mouths and legs. The ethyl ester used for the previous test was preserved in a brown bottle, and every time the bottle was opened, CO_2 gas was introduced, so that the acid might be kept from oxidation. In the later course of the experiment, however, we found that it was impossible to prevent the acid from deterioration by such a treatment as described above. Therefore, there was a possibility that we were measuring the nutritive value of the acid which was not so genuine as we thought to be, but which had become slightly oxidized. On the other hand, it was revealed by a successive experiment carried out by us that the partially reduced products of highly unsaturated acids, though they had fairly high iodine values, were almost as nutritious as oleic acid.

It has been known that natural unsaturated acids are to be easily oxidized when the number of the double bond increases, and the oxidation starts from the most unstable carbon existing in the molecules of unsaturated acids. On the other hand, oleic acid may be regarded as one of the unsaturated acids of stable structure. These facts may suggest that the partially reduced products of highly unsaturated acids have become as nearly stable as oleic acid, dispite their fairly high iodine values, when unstable factors have been removed from highly unsaturated acids by the reduction. And again because of the improved stability they became less oxidizable, and therefore, more nutritious than before.

The assumption has been further advanced that natural unsaturated acids, except one which has the conjugated double bond, would not always become less nutritious, as it has been generally believed, by the increase of double bond. In other words, unsaturated acids with the same number of carbon, even if they vary in degree of unsaturation, are almost equal in their nutritive values; and the long accepted differences among the nutritive values of unsaturated acids should certainly be attributable mainly to the degrees of the oxidation which is caused by the unstability of unsaturated acids. According to our opinion therefore, highly unsaturated acids such as contained in fresh sardines, without being oxidized, are not only harmless but also very nutritious for animals. Highly unsaturated acids which showed retarding effects in previous studies must have been those which became autoxidized to some extent.

In confirmation of this hypothesis, we extracted a sample of highly unsaturated acids as genuinely as possible from sardine oil and administered it to the rats, while oleic acid and palmitic acid were used as the control. Just as we expected, the rats did not show any distaste in taking the diet prepared with the unoxidized highly unsaturated acid, and they showed, from the nutritive point of view, almost as good results as those fed with oleic acid. They had a better result than those fed with palmitic acid. Whereas, the other groups of the animals which had received the diet containing highly unsaturated acids obtained from the same source, but oxidized by atmospheric oxygen, all perished after losing their weight and hairs. A slight deficiency in the nutritive value of the test acid in comparison to that of oleic acid might be explained by a supposition that the test acid was likely autoxidized a little in spite of our effort to keep it unchanged.

During the experiment the amount of yeast fed to the rats was made minimum so as to avoid the effect of vitamin B group upon them. The

idea was to re-examine the view that the toxic effect of highly unsaturated acids on animals can be antidoted by giving a large quantity of riboflavin. However, the administration of that few amount of vitamin B did not seem to have ill effect on the animals fed with highly unsaturated acids which were not much oxidized. The fact led us to the belief that it might not be the acids themselves that riboflavin counteracts against, but the oxidized products which turned out of highly unsaturated acid. However, further research should be made in this respect.

FIG. 1. A test rat fed with autoxidized ester of highly unsaturated fatty acid. Note depilated condition around the face which occurred in practically all of the test animals fed with the ester.

EXPERIMENTAL

A. Preparation of Fatty Acids—Bromination method usually applied to separate highly unsaturated acids was not feasible for the present purpose, because it was thought that bromination of polyethenoid fatty acid would result in forming two or more isomeric bromo derivatives. The authors, therefore, carried out the separation by low temperature crystallization method, of which procedures are as follows:

Extract oil from fresh sardine by boiling to saponify it with alcoholic sodium hydroxide solution, and acidify by adding HCl. The fatty acids thus obtained are washed with hot water, then dehydrated. After adding acetone ten times as much as the quantity of the mixed fatty acids, keep them standing in a cold storage over night to filtrate solid acids crystallized from the solution and remove unsaponified matters with ether. Apply sodium salt-acetone method twice to the above mixed acids to obtain the crude product of highly unsaturated acids, which will be changed into ethyl ester in the

presence of HCl-catalyst, and subjected to the vacuum distillation (2 mm, 190–200°). The sample, when deodorized by blowing H_2 gas into it at 80–100°, for 30 minutes, will bear a slightly yellowish tint. It is recommended to store the sample in a thermos with crushed dry ice so as to prevent it from autoxidation.

The sample of oxidized acids was prepared by leaving a 4 mm-layer of genuine highly unsaturated acids in an open basin at room temperature. One of the controls, ethyl ester of oleic acid was prepared from mixed fatty acids of camellia-oil by lead salt-alcohol method, and the other, ethyl ester of palmitic acid, by vacuum distillation of the commercial product.

B. Feeding Procedures—Weanling male and female rats obtained from our stock colony were depleted by feeding them with 9.5 g. of the fat-deficient diet per day per rat. When the weight remained constant over two weeks, they were separated into different groups, each consisting of the same sex of rats in an approximately similar number and weight. The supplements of unsaturated acids were daily administered 0.5 g. per rat over a 30-day period to observe growth and other physiological conditions of the animals.

Daily doses of vitamin B contained in beer yeast of the basal diet were 14.2 γ of thiamin and 11.4 γ of riboflavin per rat. Ingredients of the basal diet are given in Table I, and properties of the supplemented unsaturated acids in Table II.

TABLE I

Ingredients of Diets

	Basal diet	Test diet
Polished rice powder	79%	79%
Casein (Ether extracted)	10	10
Dried beer yeast	3	3
McCollum salts mixture	3	3
Ethyl ester of fatty acid	0	5
	95	100
Liver oil of tuna (1% ethanol soln.)	1 drop/day	

TABLE II

Analysis of the Samples Used in the Experiments

	Iodine value	Sap. value	Unsap. matter (%)
Ethyl ester of highly unsaturated acid	335.92	173.36	Trace
Oxidized ester of the above acid	161.08	——	Trace

C. Feeding Results—

(1) The results of the feeding experiment on genuine highly unsaturated acids and their oxidized product are summarized in Table III.

TABLE III

Increase of body Weight when the Samples Added

(Period: From September 9 to October 10, 1952)

Type of fat fed	Brood series	Sex	Weight gained in 32 days	Average gain in weight
Fat-free basal	Z	♂	8 g.	7 g.
	D	♂	6	
Ethyl ester of highly unsaturated acid	Z	♂	11	13.4
	A	♂	11	
	B	♂	12	
	D	♂	20	
	E	♂	13	
Oxidized ester of the above acid	Z	♂	−30 (died on 9th day)	
	A	♂	−53 (died on 11th day)	
	B	♂	−46 (died on 11th day)	
	D	♂	−47 (died on 23rd day)	
	E	♂	−42 (died on 11th day)	

Of the rats those which received unoxidized highly unsaturated acids exhibited no dislike in taking the supplemented diet. The others fed with the oxidized acids in the assay period manifested remarkable decrease in weight and the depilation. Most of them could not live longer than two weeks after the administration.

(2) In confirmation of these results, the assay was repeated over a 3-week period, using oleic acid and palmitic acid as the control. Properties of the supplements used and the data on the weight gained in the second assay are given in Tables IV and V, respectively.

TABLE IV

Analysis of the Samples Used in the Experiments

	N_D^{20}	Iodine value	Sap. value	Unsap. matter (%)
Ethyl ester of oleic acid (control)	1.4440	82.70	184.25	0
Ethyl ester of palmitic acid (control)	1.4348	0	196.20	0
Ethyl ester of highly unsaturated acid	1.4798	328.34	173.06	Trace
(Original free fatty acid)		(353.87)		
Slightly oxidized ester of the above acid	1.4800	312.40	——	Trace
Oxidized ester of the above acid	1.4854	285.74	171.20	Trace

TABLE V

Feeding Records

(Period: From December 9 to 31, 1952)

Type of fat fed	Breed series	Sex	Weight gained in 23 days: At the end of 1st week	At the end of 2nd week	Last day	Average gain in weight
Fat-free basal	F	♂	−2 g.	−2 g.	4 g.	g.
	G	♂	−2	1	9	5.25
	H	♂	−3	−2	5	
	H	♂	−7	−5	3	
Ethyl ester of oleic acid (control)	F	♂	3	14	23	21.5
	G	♀	3	9	22	
	H	♀	2	7	19	
	H	♂	0	9	22	
Ethyl ester of palmitic acid (control)	F	♂	2	5	15	13
	G	♀	0	4	12	
	G	♂	−1	4	13	
	H	♀	0	4	12	
Ethyl ester of highly unsaturated acid	F	♀	2	12	22	19.25
	G	♀	0	10	20	
	G	♂	1	5	18	
	H	♀	−4	5	17	
Slightly oxidized ester of the above acid	F	♀	0	7	18	15.25
	G	♀	−3	0	12	
	G	♂	1	9	16	
	H	♀	−3	5	15	
Oxidized ester of the above acid	F	♀	−6 (died on 4th day)			
	G	♀	−7 (died on 7th day)			
	G	♂	−6 (died on 6th day)			
	H	♀	−8 (died on 7th day)			

As it is obvious from Table V, the nutritive value of highly unsaturated acids was better than that of palmitic acid used as control, and not very much lower than that of oleic acid. In other words, the administration of unoxidized unsaturated acids was found to have no depressing effect on the growth of rats. However, the autoxidized acid (iodine value 285) obtained from the test acid had such a seriously toxic effect on the rats that they all lost their lives in the first week of the assay period.

In addition, the coefficient of digestibility of each supplement has been determined by chemical analysis of the fecal matter. For this purpose, the feces of each group were collected daily for two weeks of the test period and stored in ethyl alcohol. In Table 6 are presented the data obtained from the fecal fat analized by an ordinary method.

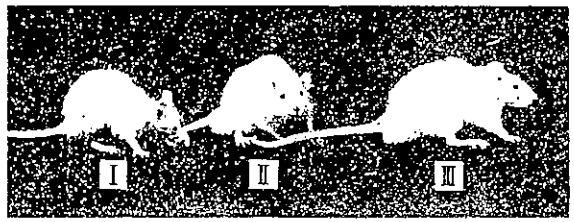

FIG. 2. Test rats, each fed with different type of ester: Oxidized ester of highly unsaturated acids for I, Ethyl ester of genuine highly unsaturated acids for II, Ethyl ester of oleic acid (control) for III.

TABLE VI

Analytical Results of Feces Excreted in Two Weeks

Type of fat fed	No. of rats	Total fat consumed	Total fat recovered from feces	Crude fat in feces	Unabsorbed fat from feces	Coefficient of digestibility	Iodine value of fecal fat
Fat-free basal	4	*g.	1.0412g.	2.31 %	*g.	*%	59.81
Et-ester of oleic acid (control)	4	28	1.9950	4.52	0.9538	96.59	74.92
Et-ester of highly unsaturated acid	4	28	2.6335	6.79	1.5923	94.31	160.75
Slightly oxidized ester of the above acid	4	28	4.1735	9.01	3.1323	88.81	74.92

* Not applicable.

a. The correction for metabolic fat was made by subtracting 1.04 g., the metabolic fat recovered in fat-free group, from each of the other values.

b. Coefficient of digestibility as used here was defined as that fraction of total ingested fat which was retained.

In respect to the coefficient of digestibility, genuine highly unsaturated acids were nearly the same as oleic acid. However, the iodine value of fecal fat of the former acid was higher than that of the latter.

Immediately after the experiment, the rats were put to death to examine thiamin and riboflavin contained in their livers.

It can be seen in Table VII that no special differences were obvious in the vitamin contents recovered from each group of the rats fed with different fatty acids.

TABLE VII

Vitamin B Contents in the Livers after Feeding Experiments

Type of fat fed	Vitamin B found in liver (γ/100 g.)	
	Thiamin	Riboflavin
Ethyl ester of oleic acid (control)	171	614
Ethyl ester of genuine highly unsaturated acid	160	634

DISCUSSION

Based on the above results, we recognized that highly unsaturated acids, when not oxidized, have a sufficiently high nutritive value for the growth of rats, and that it is not necessary to supplement the diet with a large quantity of yeast or riboflavin as it was believed to be. However, oxidized fatty acids are definitely inferior to genuine highly unsaturated acids in the nutritive value so that the administration of the former causes depilation to the test animals and even the death in the end. These evidences led us to a skepticism that most of the previous reports on the nutritive value of highly unsaturated acids might have been based on the acids which had been more or less oxidized.

It should be noted in this connection that the information obtained so far is not valid in explaining why oxidized highly unsaturated acids show an extremely retarding effect on the growth of animals. What we know at the present time is that hydroxy acids such as ricinoleic acid and dioxystearic acid are not nutritious. There are, however, only few reports on the oxidation of highly unsaturated acids which are supposed to be helpful in clarifying the results attained by our assay. The important among them are those which deal with rancid lard.

According to Rusoffs and Hanson (*4*), the administration of rancid lard causes the biotin deficiency to rats, as the results that the biotin composition in the intestines of the rats is disturbed by the oxidized products. Kaunitz and his co-workers (*5*) pointed out that the diet containing rancid lard tends to cause the riboflavin deficiency to animals and the increase in requirements of various essential factors. Those facts seem to suggest something which may throw light on the results of our assay.

It has also been broght to our attention that the polymerized fish oil, depending on the treatments given, sometimes turns out highly

nutritious, and indeed better than the original sample, and that there are considerable differences in the nutritive value between autoxidized products of highly unsaturated acids and the oxidized acid produced in the course of polymerization. Further studies will be made in respect to these differences.

SUMMARY

It has been widely accepted that the nutritive value of unsaturated fatty acids is depleted when the number of double bond in the acids increases. In order to prove whether this is true or not, we have carried out the following experiments, using the diet consisting of highly unsaturated fatty acids.

1. When the diet, containing 5 per cent of ethyl ester of highly unsaturated fatty acids kept from the oxidation as much as possible, was administered to the test rats together with a small amount of yeast (3 per cent), the normal growth of the rats was attained without showing retarding effects on their health. The nutritive value of the test ester was not much lower than that of oleic acid used for the comparison.

2. The rats, which were fed with the acids obtained from the same source and autoxidized, exhibited a retarding effect upon their growth with the decrease in weight and the depilation around their mouths and legs. They all died in the later course of the test.

On the basis of these assays, we have come to the conclusion that highly unsaturated fatty acids contained in fresh fish will not be harmful at all for the health of animals.

We hereby highly appreciate the helpful advices given by Dr. Jun'ichi Ozaki, and the constant encouragement by Dr. Hideo Higashi. We are also much indebted to the unselfish devotion of Misses Hisae Sakurai and Kimie Arai in the feeding of animals.

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ANTAGONISM OF FRESH FAT TO THE TOXICITY OF HEATED AND AERATED COTTONSEED OIL[1]

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TWO FIGURES

It has previously been shown (Kaunitz and Slanetz, '50; Kaunitz, '53) that the inclusion in a complete rat diet of lard heated and aerated for 300 hours at 90°C. produces only mild toxicity symptoms. In the current paper, it will be shown that similarly treated cottonseed oil is severely toxic and that this effect can be counteracted by fresh oil.

PROCEDURE

The studies were carried out on albino rats from a homogeneous colony and were begun when the rats were 4 weeks old. The maintenance diet of the colony and the procedures used to obtain comparable groups have been previously described (Kaunitz et al., '54).

Samples of a commercially available refined cottonseed oil[2] were aerated and heated in a water bath at 90 to 95°C. for 50 to 300 hours. A clear oil always resulted. The peroxide numbers were repeatedly determined by the Stansby procedure.

The desired amounts of the treated or fresh oil or both, alcohol-washed casein and cerelose comprised 93.5% of the

[1] Aided by a grant from the Schenley Laboratories Inc., New York, N. Y.
[2] Wesson Oil.

purified diet employed. To these constituents were added 4% salt mixture (U.S.P. no. 2), 0.5% calcium carbonate, and 2% calcium carbonate, and 2% celluration and, per kilogram, 1 gm of inositol, 1 gm of choline, 300 mg of para-aminobenzoic acid, 100 mg of nicotinic acid, and 10 mg of vitamin K. Other food factors were supplied by feeding 4 times weekly two drops of a watery suspension containing, per milliliter, 4 mg of thiamine, 8 mg of riboflavin, 8 mg of pyridoxine, 20 mg of calcium pantothenate, 5 mg of folic acid, 0.05 mg of biotin, 10 μg of vitamin B_{12}, and 50 mg of ascorbic acid. The fat-soluble factors were administered in a linoleic acid suspension containing, per milliliter, 50 mg of alpha-tocopherol acetate, 10 mg of free alpha-tocopherol, 0.5 mg of vitamin D_2, and 5 mg of crystalline beta-carotene.[3]

In the course of the experiments, it became necessary to pair-feed rats on diets containing the treated oil with matching groups receiving fresh oil in addition. The compositions of the rations containing fresh oil were adjusted so that isocaloric amounts of the diets to be compared would contain identical amounts of protein and of treated oil. For instance, when 15% treated oil and 30% protein were used, 1 gm of the diet was equivalent to 4.49 calories. A diet containing 30% fat was equivalent to 5.27 calories per gram. Therefore, an amount of the high-fat diet equal to 85.2% of a given amount of the low-fat diet had to be fed. In order to make the protein and treated oil intakes of the animals on the two diets equal, $\frac{100}{85.2} \times 30\%$ protein and $\frac{100}{85.2} \times 15\%$ treated oil were used in the high-fat diet which thus contained 35.2% protein, 17.6% treated and 12.4% fresh oils. This procedure is acceptable only if it can be assumed that there was no significant difference in the calories lost in the feces of the paired groups. Despite the diarrhea observed in animals eating the treated

[3] Dr. Leo Pirk of Hoffmann-La Roche, Inc., Nutley, New Jersey, generously supplied us with most of the synthetic vitamins used. Vitamin D_2 was supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y., and the crystalline beta-carotene, by the Barnett Laboratories, Long Beach, California.

oil, it is highly improbable that caloric losses need consideration in the evaluation of the experimental results.

The rats were kept in single unit cages with wire bottoms and removable pans to facilitate the determination of food consumption. Body weights were recorded by plotting the logarithm of the weight against the reciprocal value of the age (Zucker and Zucker, '42).

The organ weight — body weight relationship has been presented as a log — log plot. To obtain data on normal rats, the organs of 130 male rats varying in body weight from 18 to 450 gm were examined. These animals had been on a complete diet containing 10% lard and 30% casein and were considered normal because animals on this diet grew normally (according to Zucker and Zucker, '42) and males remained fertile during this period. On the log — log plot, the upper and lower limits of the spread formed parallel lines. For brevity and clarity, only these lines and not the individual points for the normal rats are given below for the comparison with the experimental animals.

RESULTS

When rats were fed a diet containing 30% casein and 15% of cottonseed oil which had been aerated and heated to 95°C. for 200 hours, their daily caloric intake was less than half of that of rats receiving fresh fat or no fat at all. They began losing weight at once (fig. 1a, curve III). Three weeks after having been placed on the diets containing 15% of the treated material, 12 to 75% (on the average, roughly half) of 4 groups had died (table 1). When only 10% of the treated oil was used, few rats had died among 6 groups three weeks after the experiment started. They were somehow able partly to adjust to the treated oil in that they gradually lost their diarrhea (see below) and even increased in weight slightly (fig. 1b). The circumstance that the weight increase though present, was very small even in the absence of the diarrhea suggests that the weight reduction was not due primarily to diarrhea.

With 20% of the treated material, all rats died within three weeks. Similar results have been obtained with linseed oil heated to 275°C. in the absence of oxygen (Crampton et al., '51). The rats had the appearance of starving animals with

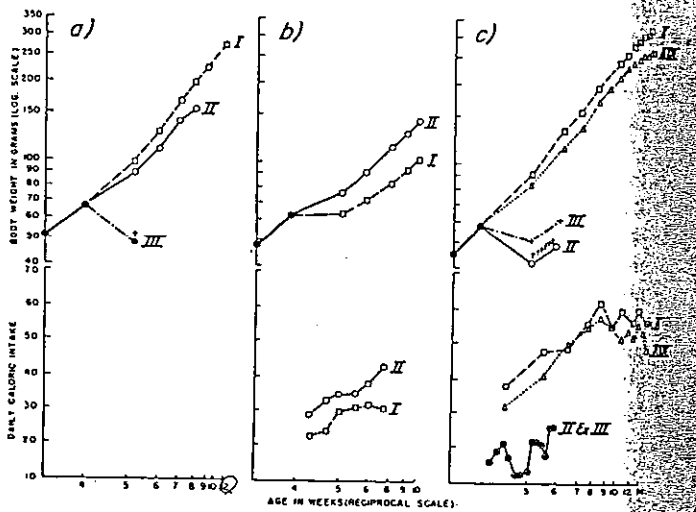

Fig. 1 Effect of the addition of fresh or "treated" cottonseed oil or both on growth and food consumption of albino rats fed diets containing 30 or 60% of casein. The "treated" cottonseed oil was aerated and heated to 95°C. for 50 to 300 hours.

(a) averages of 8 males fed 30% casein and
- I 20% fresh oil
- II 20% mildly treated oil (peroxide number: 191)
- III 20% more strongly treated oil (peroxide number: 141)

(b) averages of 12 females fed 60% casein and
- I 10% treated oil
- II 10% treated and 20% fresh oils

(c) averages of 16 males fed 30% casein and
- I 10% oil
- II 15% treated cottonseed oil
- III 17.6% treated and 12.4% fresh oil with 35.2% casein (pair-fed with II)
- IV fat-free with 2% linoleic acid

dirty, and sometimes sparse, fur. They had severe diarrhea, the feces containing appreciable amounts of mucus and being lighter in color than those of the controls. Histological examinations of nearly all organs[4] of 6 rats in an advanced stage of the disease failed to reveal any abnormalities except mild edema of the intestinal mucous membrane in some instances.

TABLE 1

Effect of the addition of fresh or "treated" cottonseed oil or both on growth and food consumption of albino rats fed diets containing different amounts of casein. The "treated" oil was aerated and heated to 95°C. for 50 to 300 hours.

	TREATED OIL ONLY			TREATED OR FRESH OIL OR BOTH			
NO. AND SEX	% treated oil in diet	% casein in diet	% dead after 3 weeks on diet	% treated oil in diet	% fresh oil in diet	% casein in diet	% dead after 3 weeks on diet
7 ♂[1]	10	5	29	...	10	5	0
7 ♂[1]	10	30	0	...	10	30	0
16 ♀	10	30	0	10	15	30	0
8 ♂[1]	10	49.5	12	12.8	17.2	63.5	0
12 ♀	10	60	0	10	20	60	0
7 ♂	10	74	43	...	10	74	0
16 ♂[1]	15	30	38	17.6	12.4	35.2	0
				17.6	12.4	35.2	0
16 ♂[1]	15	30	63	15.9	4.1	31.7	0
				17.6	12.4	35.2	0
8 ♂[1]	15	54	12	15	15	63.5	0
8 ♂[1]	15	54	75	17.6	12.4	63.5	25
8 ♂	20	5	100				
8 ♂	20	30	100				
8 ♂	20	30	100				

[1] Pair-fed with animals on the same line.

When fresh oil was added to the diet containing the treated oil, the effects of the latter could be nullified to a large extent. Male rats receiving 15% of the treated and 15% of the fresh oils and permitted to eat without restriction were alive three months after the experiment had started and showed no

[4] We are greatly indebted to Dr. Herbert Stoerk of the Merck Institute for Therapeutic Research, Rahway, New Jersey, for the histological studies reported in this paper.

signs of the disease except that their body weights were well below those of the rats receiving only fresh fat. This result was similar to those obtained with heated and aerated lard in which decreased growth was the only sign of abnormality. When female rats receiving 10% of the treated cottonseed oil were mated after several weeks on the diet and after they had been without diarrhea for several weeks, they either did not become pregnant or resorbed the fetuses. With fresh cottonseed oil in addition to the treated material, nearly normal litters were born and reared. The beneficial effects of adding fresh oil to diets containing treated material were the more remarkable because the higher food intake of the animals receiving supplements of fresh fat would result at the same time in an increase in the intake of heated and aerated oil (fig. 1 b). Paired feeding experiments demonstrated that the very low caloric intake of the freely-eating group fed treated oil was not responsible for most of the toxic symptoms observed. The animals receiving isocaloric amounts of the diet containing fresh as well as treated oil grew better and lived longer than the animals with which they were paired (fig. 1 c); this was ascertained in three separate experiments.

Determination of the hemoglobin content and counts of the red and white blood cells were made on pair-fed animals receiving 5 to 74% protein and 15% treated oil with and without fresh oil. There were no essential differences among the groups as to hemoglobin and red blood cells. Among 15 animals receiving 30% casein and 15% treated oil, 7 had white counts of over 9000 per cubic millimeter, while none of their pair-fed controls receiving fresh oil had counts this high. Despite great individual variations, there was a similar trend among the animals receiving 5 and 74% casein.

Organ weight studies are shown in figure 2. The curve of the normal kidney (fig. 2 a) revealed a break at 40 to 50 gm. body weight, which corroborates the findings of previous workers (Stoerk and Zucker, '46). It is evident that nearly all kidneys from animals on treated oil are considerably outside the upper limit for normal kidneys while the weights

of those receiving fresh and treated oil are within normal limits. In fact, the kidney weights of the animals fed only the treated oil were heavier than those of normal rats weighing 60 gm, which was the average weight of the groups just before the administration of the treated oil. Thus, while the body weight declined, the kidneys increased in size. Simultaneous administration of fresh oil kept the relation of kidney to body weight within normal limits.

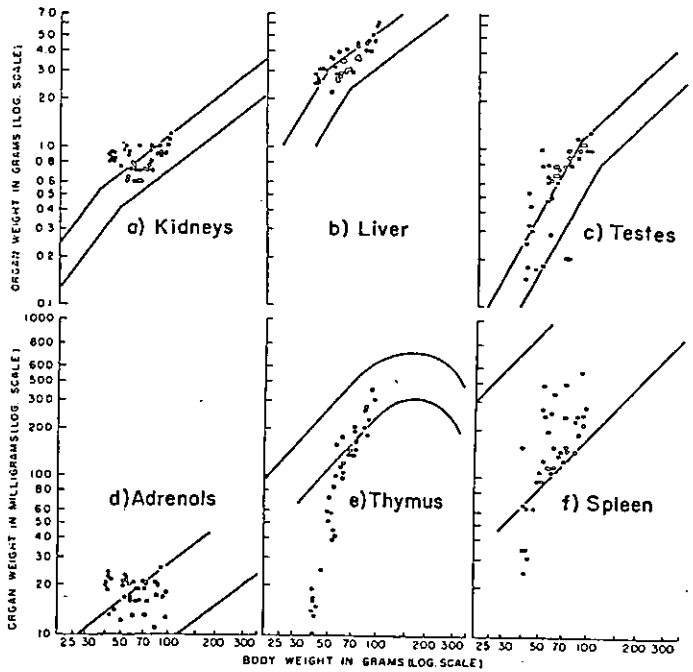

Fig. 2 Relation of organ weight to body weight in rats fed a purified diet containing 60% casein and refined cottonseed oil aerated and heated to 95°C. for 200 to 300 hours. The closed circles refer to animals freely eating a diet containing 15% of the treated oil; the open circles, to animals pair-fed with the latter and receiving 17.6% treated and 12.4% fresh oils. The parallel lines indicate the limits of variation in the organs from 130 normal animals.