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	Brood 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
			g.	g.	g.	g.
Fat-free basal	F	♂	−2	−2	4	5.25
	G	♂	−2	1	9	
	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.

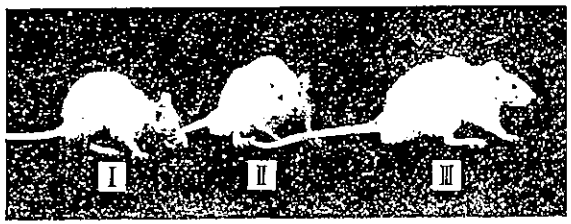

FIG. 2. Test rats, each fed with different type of ester: Oxidized ester of highly unsaturated acids for I, Ethyl ester of genuine highly unsaturated acids for II, Ethyl ester of oleic acid (control) for III.

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
		g.	g.	%	g.	%	
Fat-free basal	4	*	1.0412	2.31	*	*	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 oil, it is highly improbable that caloric losses need consideration in the evaluation of the experimental results.

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

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

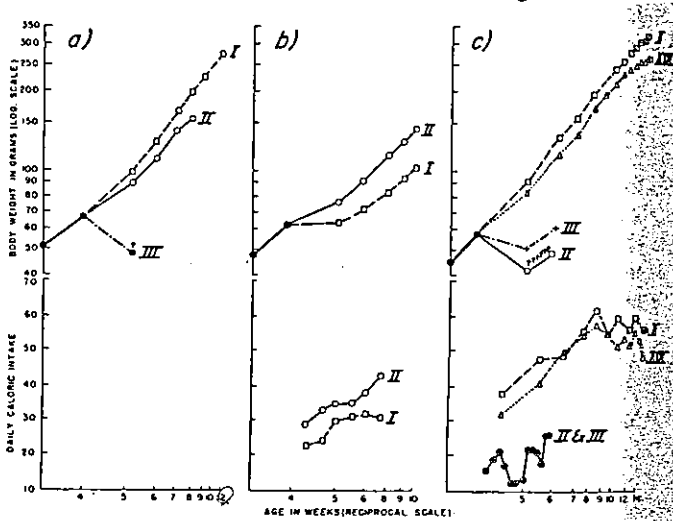

Fig. 1 Effect of the addition of fresh or "treated" cottonseed oil or both on growth and food consumption of albino rats fed diets containing 30 or 60% of casein. The "treated" cottonseed oil was aerated and heated to 95°C. for 50 to 300 hours.

(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.

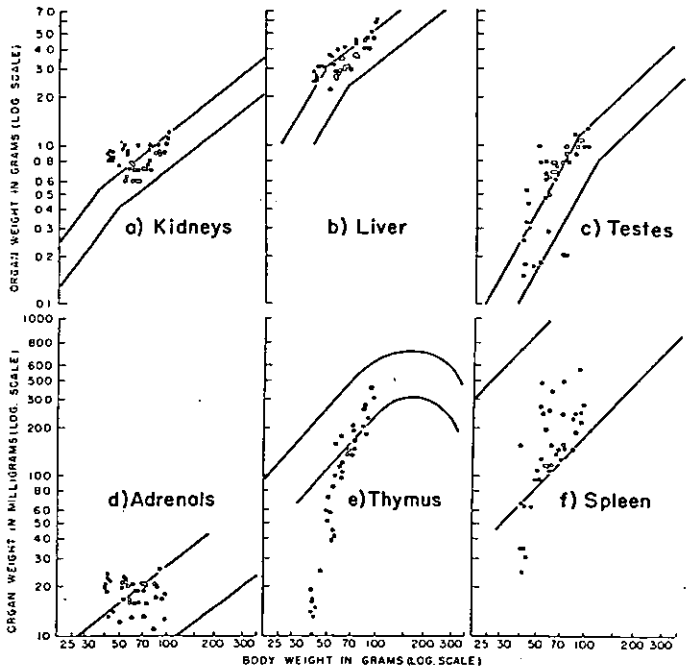

Fig. 2 Relation of organ weight to body weight in rats fed a purified diet containing 60% casein and refined cottonseed oil aerated and heated to 95°C. for 200 to 300 hours. The closed circles refer to animals freely eating a diet containing 15% of the treated oil; the open circles, to animals pair-fed 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.

The liver weights (fig. 2 b) showed similar, but somewhat less pronounced, changes. Most of the livers of animals on treated oil were well above those of normal rats; fresh oil prevented the changes. Organ and body weights of rats fed rancid lard have been reported by Greenberg and Frazer ('53). Their average results for kidneys and livers of animals fed rancid or fresh lard fall within the range for normal rats as given in figure 2a and b. It is true, however, that the liver and kidney weights for the rats fed rancid lard tend to be on the upper limit for normal rats while those of the animals fed fresh lard are lower. This is further evidence that heated and aerated lard is much less toxic than similarly treated cottonseed oil.

The scattering of the normal adrenal weights was much greater than that of the liver and kidneys (fig. 2 c). However, in 9 out of 16 cases, the adrenals of those fed treated oil exceeded the maximum weight of the normals while, in the group fed fresh as well as treated oil, this occurred only 4 times.

The majority of the testicular weights of both groups of experimental animals were above those of the normal ones (fig. 2 d). In many instances, therefore, the testes did not lose weight, and some even increased in size despite the loss in body weight resulting from the feeding of the treated oil. This was not changed by the addition of fresh oil to the diet.

The shape of the thymus — body weight relationship in normal animals (fig. 2 e) expresses the involution of the thymus. The curve found in these studies is not appreciably different from that described by Stoerk ('46). Extremely small thymuses were found in the majority of animals fed treated oil. The thymuses of about half of the pair-fed controls receiving treated and fresh oils were also smaller than normal (presumably as an expression of semi-starvation) but were much larger than those fed only the treated oil.

The variation in splenic weights among the animals far exceeded the variations in any of the other organs. Yet, it seems evident that the spleens of about half of the animals fed only treated oil are below the lower limit for normal

spleens while those of their pair-fed controls are just within normal limits.

In further studies, an attempt was made to modify the toxic effect of the treated oil by feeding extra amounts of the fat-soluble vitamins and by the feeding of fresh oil by dropper rather than by inclusion in the diet.

When animals receiving 15% of the treated oil were given daily feedings by dropper of about 25 mg of synthetic DL-alpha-tocopherol acetate containing 125 μg of crystalline beta-carotene and 2.5 μg of vitamin D_2, their average weight after two weeks was 75 gm, while that of the rats receiving no supplement was 61, the difference being statistically significant. However, a group receiving 15% of the treated oil and 10% of fresh oil weighed 99 gm on the average at this time. Therefore, the mildy protective effect of tocopherol (and the other fat-soluble factors) does not explain the strongly protective action of fresh oil added to the diet.

Daily feeding by dropper of 0.5 to 0.8 gm of fresh, refined cottonseed oil exerted only a mild protective effect as to growth and survival time in rats fed 15% of the treated oil. After three weeks on the experimental diet, those receiving no supplement weighed, on the average, 60 gm and three out of 12 had died; those fed fresh oil in addition weighed, on the average, 69 gm and none had died. However, when a comparable amount of fresh oil was added to the diet, the average weight was well above 100 gm. We have so far no explanation for this difference.

DISCUSSION

In studies on heated and aerated lard (Kaunitz and Slanetz, '50), it was concluded that the amount of peroxides present is not related to the degree of toxicity. In the current studies, similar results were obtained. In the experiments shown in figure 1a, it was found that a sample of cottonseed oil, heated and aerated to a peroxide number of 191, brought about only very mild growth retardation. When heating and aeration were continued, the peroxide number dropped to 141, but the

sample had become very toxic. It would therefore seem that, if the toxic products are the result of oxidation, they may be breakdown products of peroxides; but it is quite possible that the toxic products are not at all, or only partly, related to oxidative changes. Crampton and his co-workers ('53), in their studies on heated linseed oil advanced this latter viewpoint. They made it seem probable that some products formed during thermal polymerization are toxic.

Concerning the antagonism between treated and fresh oils, we had first believed that an antimetabolite relationship might exist between the toxic products and fresh oil. However, in view of the fact that the protective effect of fresh oil is only mild when it is not added to the diet but is fed separately, the antimetabolite theory may not be a good explanation for the facts observed. The possibility must be considered that addition of fresh oil to heated oil may change the toxic properties of the latter, perhaps by influencing the state of polymerization.[5] But whatever explanation may eventually prove to be correct, it seems noteworthy that fresh oil may, under certain conditions, exert a life-saving effect which is not due to its caloric properties.

SUMMARY

1. The inclusion in a rat diet of 15 to 20% of refined cottonseed oil, aerated and heated to 95°C. for 200 to 300 hours, led to rapid loss of weight and death within three weeks.

2. The condition was accompanied by diarrhea and by the occurrence of large livers, kidneys, and adrenals and small spleens and thymuses. Histologically, the only change was an occasional intestinal edema.

3. Addition of fresh oil to the diet containing the heated and aerated oil protected the animals against the toxicity. Only growth retardation persisted. This protective effect could also be observed in paired-feeding experments in which the paired rats received the same number of calories and equal amounts of protein and of treated oil. When fresh cottonseed oil was fed by dropper instead of being included in the diet, its protective effect was only slight.

4. Extra feeding of 25 mg of DL-alpha-tocopherol acetate (and other fat-soluble factors) gave mild protection.

5. Peroxides are probably not responsible for the toxic effect of the heated and aerated cottonseed oil; polymerization may be a better explanation. No definite explanation for the antagonism of fresh to treated oil can be given; the effect is probably not due to an antimetabolite relationship but could be caused by a change in the state of polymerization.

[5] This possibility was discussed with a number of workers in the fields of fat and of polymer chemistry. While they agreed that no definite opinion could be given, some of the workers felt that at least changes in the kind of polymerizations could be effected by the addition of fresh to polymerized oil.

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In these two types of salicylate inhibition, they were thus proved to be different in their reaction natures. The relation between the logarithm of the velocity constants of inactivation reaction by higher salicylate concentrations and the reciprocal of absolute temperature are shown in Fig. 6. From the slope of this straight line, the Arrhenius energy was calculated to be 42000 cal. From these results obtained, it may be inferred that the inhibition of cholinesterase by sodium salicylate in its higher concentrations is brought about through the denaturation of enzyme protein or some similar reaction, as it was suggested also in the reaction by urethane.

SUMMARY

1. Sodium salicylate inhibits the cholinesterase in two different ways as the incubated concentration of the former differs.

2. It was indicated that the reversible inhibition, which is observed in the lower concentration range of salicylate, is non competitive. The inhibition seems to be brought about by combination of two molecules of salicylate to one enzyme molecule. The binding of one molecule of substrate to one enzyme molecule may result a hindrance of the binding of salicylate molecule with the latter.

3. In the irreversible inhibition by sodium salicylate which occurs in the higher concentration of the latter, the inactivation reaction of enzyme proceeds according to the first order kinetics, and is independent of substrate concentrations. The Arrhenius energy of this reaction was calculated to be 42,000 cal.

The present investigation was supported by Prof. K. Kaziro, to whom the author's deep gratitude is due.

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STUDIES ON THE TOXICITY OF FISH OIL

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It was first depicted by Sahashi (*1*) and Somekawa (*2*) that some sorts of fish oil including whale oil are toxic to rat. But it remains undecided whether they are equally toxic to man or not. Since in Japan, where resource of fat is extremely short, the use of fish oils as nutrient is highly desirable, the wide experiments on man were carried out in many clinical as well as biochemical laboratories. The result of such cooperative works revealed that fish oil is not so toxic to man in dosage of 50–100 gms. per day. But quite recently Kaneda and Ishii (*3*) made an interesting observation that fish oil per se is not so toxic even to rat, but when it is once oxidised simply by being exposed to air, it turns out to be toxic.

In this case it is well assumed that unsaturated fatty acid in fish oil is the substance easily oxidised and at the same time responsible for toxic effect. Using the unsaturated fatty acids obtained from cuttle fish oil, the author carried out the experiment and fully confirmed the results obtained by Kaneda and Ishii (*3*). Further, some chemical nature of the toxic substance was studied, the details of which are partly reported here.

EXPERIMENTAL

Preparations of Unsaturated Fatty Acids

The raw cuttle fish oil produced in Hokkaido was used. Its properties are shown in Table I.

TABLE I

The Properties of Raw Cuttle Fish Oil

Density (15°)	Iodine value (Wijis)	Acid value	Sap. value	Unsap. matter (%)
0.9305	182.5	2.03	189.3	2.19

The unsaturated fatty acids were separated by the soda-acetone process (4). Namely, raw cuttle fish oil was saponified by ethyl alcoholic solution of NaOH, mixed with acetone, left over night at 0° and filtered. From the filtrate, which contained acetone soluble sodium-salts of highly unsaturated acids, acetone was distilled off, and its residue was decomposed by dil. HCl.

The free acids, dissolved in petroleum-ether (b. p. below 65°) were washed several times with water, and dehydrated by anhydrous sodium-sulphate and then petroleum-ether was distilled off, giving the highly unsaturated acids as residue.

Since the sample obtained as above, used to contain somewhat of unsaponificable matter which was existing in raw oil, so it was desirable to remove it as much as we could.

Next, the sample was dissolved in twice the volume of absolute ethyl alcohol containing 2.5 per cent HCl, and heated on the water-bath for about three hours. By this process ethyl esters of the acids were prepared. Then, the ethyl esters were fractionated under a vacuum of 2 mm. Hg at various temperatures. The 205~215° fraction was chiefly used for the author's experiment. This fraction has light yellow colour and no odour. Since these esters are liable to be rapidly oxidised when they are in contact with air, special precaution was paid throughout the preparation.

The container of the esters was always filled with CO_2 gas and was kept in dry-ice during the experiments. An aliquote portion of the sample thus prepared were put into a petri dish and borught into contact with air at the room temperature. As the oxidation proceeded the esters developed light yellow colour. At a certain point of oxidation the reaction was stopped and the ester was kept again in dry-ice avoiding further change.

Ethyl ester of oleic acid was used as control. Some chemical properties of the esters and of their oxidised compounds are indicated in Table II together with those of oleic acid ester for comparison.

TABLE II

Analysis of the Samples Used in the Experiments

	d_4^{15}	n_D^{20}	Iodine value	Sap. value	Unsap. matter (%)
Ethyl ethylesters of highly unsaturated fatty acids	—	1.4823	358.70	163.90	Trace
Oxidised esters of highly unsaturated fatty acids	0.9568	—	264.82	201.49	Trace
Ethyl ester of oleic acid	0.8822	—	82.96	187.69	0

RESULTS

Effect on the Growth of the Rat—The rats of nearly same size and body weight (about 60 g.) were divided into three groups, each comprising 4 rats. To the first group the feed was given containing 5 per cent of the unsaturated fatty acid esters in the basal diet, to the second group that of the oxidised esters and to the third that of oleic acid ester, which considered as control. The composition of the basal diet is given in Table III.

TABLE III

Ingredients of Basal Diet

Starch (rice powder)	80%	Yeast	3%
Casein (ether extracted)	9%	Liver oil	1 drop/day
McCollum salt mixture	3%		

Total amount of diet per day is 10 g. (from 1st day to 5th day), 12.5 g. (from 6th day to 10th day), 15 g. (from 11th day to the 30th day).

The experimental results are recorded in Table IV and also illustrated in the accompanying Fig. 1 and photographs (Figs. 2, 3, and 4). It is apparent that the rats fed on the unsaturated fatty acid ester containing diet showed the same satisfactory growth as those on the oleic acid ester diet. But it is quite remarkable that all rats that fed on the oxidised ester diet not only showed retarded growth but died within few days after the beginning of the experiment. It was also noticed in this group that white hair turned into brown.

TABLE IV

Feeding Records

Kind of ester fed	Sex	Increase of body weight (g.) 10th day	average	20th day	average	30th day	average
Ethyl ester of oleic acid.	♀	+11.0	+13.1	+34.0	+43.3	+ 61.0	+82.1
	♂	+14.0		+51.2		+113.5	
	♀	+15.0		+40.5		+ 70.8	
	♀	+12.5		+47.5		+ 83.0	
Ethyl ester of unsaturated fatty acid.	♂	+ 7.7	+ 6.3	+41.0	+38.1	+ 85.0	+75.8
	♀	+ 7.0		+38.5		+ 77.0	
	♂	+ 7.0		+44.5		+ 75.0	
	♀	+ 3.2		+28.5		+ 66.0	
Oxidised form of the above ester.	♀	−3.8 (died on 6th day)					
	♀	−5.0 (died on 7th day)					
	♀	−5.0 (died on 8th day)					
	♂	−8.5 (died on 3rd day)					

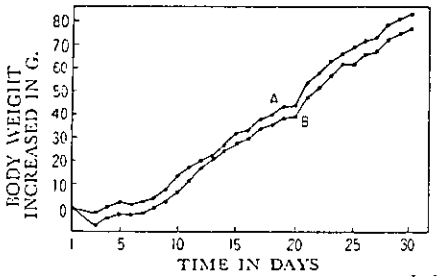

FIG. 1. The curve of average body weight increased. A (Control): average body weight increment of the rats fed on the diet containing 5 per cent of the ethyl ester of oleic acid. B: that of rats fed on the diet containing 5 per cent of the ethyl ester of highly unsaturated fatty acids.

FIG. 2. The rat fed on the diet containing 5 per cent of the ethyl ester of oleic acid. (Control) On the 27th day after the commencement of the experiment.

FIG. 3. The rat fed on the diet containing 5 per cent of the ethyl ester of highly unsaturated acid. On the 27th day after the commencement of the experiment.

Effect of More Mild and Chronic Intoxication by the Oxidised Ester—For this purpose the grown-up rats were fed on the diet containing 5 per cent of the oxidised ester not daily but at some intervals.

One of the typical examples is illustrated in Fig. V with regard to the change of body weight.

As shown in Fig. 5, the retarted growth of the animal continued during the feeding of the toxic diet, but somewhat recovering was observed when that feeding was stopped. It ended, however, in fatal at the end of 23th day after the commencement

FIG. 4. The rat fed on the diet containing 5 per cent of the oxidised ethyl ester of highly unsaturated acid. On the 4th day after the commencement of the experiment.

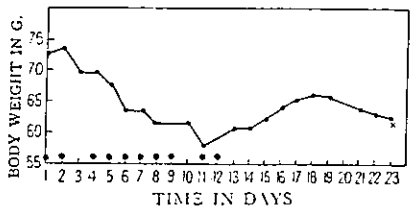

FIG. 5. The curve of body weight changed.
The mark • shows the day on which the oxidised ester diet was given.

of the experiment. The appearance of the animal was quite particular. The hair of the face was diplated off, the mouth and rear legs were swollen, the remaining hair changed into brown, and hemorrhages were seen here and there in the skin. (see Figs. 6 and 7.)

DISCUSSION

The above results substantially proved the finding first made by Kaneda and Ishii that the highly unsaturated acid from fish oil is not toxic but rather its oxidised form is toxic. As it is usually assumed that unsaturated fatty acid absorbs oxygen forming peroxide at double bonds in the molecule, so it might be plausible that the toxicity of the oxidised acid is caused by peroxide. In this regard the next paper will give full details. At any rate it is promising that fish oil can be utilised

Fig. 6

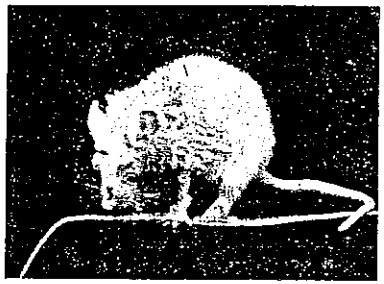

FIG. 7

as human nutrient when it is prepared and preserved avoiding spontaneous oxidation. Or it may be more safe to hydrogenate fish oil to certain extent to get rid of double bonds.

Besides such important problems of fish oil as fat resources in nutrition, the essential feature of toxic effect of the oxidised unsaturated fatty acid from fish oil is interesting to investigate. In this line the work of Kaunitz and Johnson (*5*) is noteworthy. They observed that rat fell into flavin-shortage when rancid lard was given. But it seems to the author that the toxicity is too acute to ascribe it solely to riboflavin shortage.

SUMMARY

1. When the diet containing 5 per cent ethyl esters of highly unsaturated fatty acids obtained from cuttle fish oil was given to rat, it showed quite the same good nutritive effect as the diet containing oleic acid ester.

2. To the contrary the oxidised form of the above unsaturated fatty acid esters showed an extremely toxic effect to rat.

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reginous substance.

When various amounts of dimethyl 5-hydroxykynurenamine hydrochloride were injected intravenously to rabbits, the result as indicated in the following table was obtained.

TABLE I

The compound tested	Blood pressure lowering activity	Duration of the activity
	γg./kg.	*mm. Hg*	
Dimethyl 5-hydroxy-kynurenamine			
	50	14	18 *sec.*
	100	20-22	
	200	32	1 *min.* 40 *sec.*
	500	40	3 *min.* 50 *sec.*
Dimethyl kynurenamine			
	100	15	
	200	28	
	500	30	

Judging from this result, it seems that the action of dimethyl 5-hydroxykynurenamine is stronger than that of dimethyl kynurenamine.

By interrupting the incubation of 5-hydroxykynurenine with liver homogenate at the early stage and fractionating the cleavage products by paper chromatography we obtained a substance, which was judged to be 5-hydroxykynurenamine from its properties. The diluted solution of this substance showed also the blood pressure lowering action.

This work was aided by a grant from the scientific research fund of the Ministry of Education of Japan. We wish to express our thanks to the Takeda Research Laboratory for performing elementary analysis.

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NUTRITIVE VALUE OR TOXICITY OF HIGHLY UNSATURATED FATTY ACIDS. II

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(Received for publication, June 5, 1955)

In the previous paper (*1*), the authors have established that highly unsaturated fatty acids are not harmful at all when the fatty acids are thoroughly refined so as not to autoxidize themselves. Moreover, it has been found that the widely-believed toxic effects of highly unsaturated acids are primarily caused, not by the unsaturation of fatty acids, but by the autoxidized products which are yielded from highly unsaturated acids.

Since the authors presumed that this conclusion might apply not only to the highly unsaturated acids but to all of the members of natural unsaturated fatty acids, the same course of feeding experiments was carried out, using ethyl esters of liquid acids of linseed oil.

The results of experiment were entirely identical as expected. Based upon these results, it is considered that all the unsaturated acids, which are not easily autoxidized, become harmful once they autoxidize themselves. It was, therefore, tried to examine whether the autoxidized products show toxic effects, by the way of separating such products from other non-toxic ones.

In this case, it was presumed that all the autoxidized products of the unsaturated fatty acids, such as peroxides, conjugated acids, polymerized products, aldehydes, ketones, *etc.* might not be equally toxic but only one or some specific products might be highly toxic and the rest not being toxic or at the least toxic.

In order to verify this presumption, each product was separated and each of them was subjected to the animal test.

When autoxidized slightly, a greater part of unsaturated acids tend to remain for a while with the original structure, showing the most harmful results as stated in our previous report (*1*).

As regard to the method of concentrating the autoxidized products from autoxidized unsaturated acids, several papers have been reported. Privett *et al.* (*2*) separated autoxidized products, using Skellysolve F

and ethyl alcohol, the Changs (*3*) did it, using Skellysolve F and diethyl ether. Zilch (*4*), Fugger (*5*), Cannon (*6*), *etc.* separated by countercurrent extraction procedure and Atherton, Hilditch (*7*) did it by chromatographic method using silica gel. Furthermore, in these several years, Catravas (*8*), Coleman (*9*), Swern (*10*) have reported the method of fractionation by urea complexes.

Therefore, these methods were adopted to separate the autoxidized products. Firstly, adopting the Privett's method (*2*), petroleum ether and 85 per cent ethyl alcohol were used for separation. Most of the autoxidized products solved into alcohol and some remained in the part of the petroleum ether.

The both fractions were harmful for the rats.

The experiment means that, autoxidized products were condensed in this method, but the method was not effective to separate the wholly unharmful fraction out of the harmful one as desired by the authors.

The Atherton and Hilditch's chromatographic method (*7*) of using silica gel showed some toxic substances remaining in the fraction without being absorbed by the silica gel, and did not satisfy our object primarily. However, when chromatographic method is applied, using alminium oxide, the fraction not absorved to alminium oxide is totally harmless. But it is difficult to elute fully the absorbed products from the alminium oxide, endangering the materials to be autoxidized further in the course of experiments.

Having tried the separation by urea complexes, 75 per cent of ethyl esters of autoxidized linseed oil produced urea adduct, showing harmless, but the fraction which did not produce urea adduct was quite toxic.

The fraction which produced urea adduct contains unchanged fatty acids of linseed oil, slightly polymerized products, aldehydes, *etc.* and a very little of autoxidized products. On the other hand, the fraction which did not produce urea adduct contains a little aldehyde, showing a high peroxide value and also contains some conjugated fatty acids which were produced by autoxidation.

As a result of the above, aldehyde does not have any conspicuous toxic effect but the actual harmful products in autoxidized fatty acids are some products with branched-chain fatty acids, lactone, polymerized products, conjugated acids and peroxide.

When unsaturated fatty acids are polymerized, nature of the products varies according to the temperature. At the lower temperature

peroxide value goes higher, while at the higher temperature it does not go up, as well verified by Atherton and Hilditch as did by the authors.

With highly unsaturated acids polymerized at the higher temperature and decreased iodine value, no toxic effect appeared at the animal test and the rats grew up well. This means the polymerized products of highly unsaturated acids without oxidized product are harmless, and some substances which contain conjugated linkage often turns out to be toxic as has been known well for a long time. The unsaturated fatty acids which autoxidized at the lower temperature, according to our experiment, contains about 6 per cent of conjugated acids, equivalent to 0.03 g. of daily feed for rats. The test of feeding experimentally about 5 times of conjugated acids of tung oil show harmless result.

According to the results gained, conjugated fatty acids are not considered to be the prime cause of producing toxic effect in the autoxidized unsaturated acids.

From the above results, the most harmful fraction contained in autoxidized unsaturated acids must be branched-chain fatty acids, lactone or peroxide.

Judging from these results, the authors assumed that the toxic effects should certainly be attributable mainly to the formation of preoxide-structure.

Supposing the main toxicity is to be attributed to peroxide, autoxidized product, which were freed from peroxide, must be quite harmless or slightly toxic. Then, using the same method as applied to the Lea's peroxide determination method (*11*), potassium iodide and acetic acid were added to the autoxidized liquid esters of linseed oil and freed from peroxide structure, and then checked how harmful the feed was. We found out, as expected, the toxicity was quite weak and the rats did not die (rats had slightly loose bowels).

The same experiment was applied to the autoxidized highly unsaturated fatty acids and the result was also harmless.

In other words, it was confirmed from the above result that the most harmful factor among the autoxidized fatty acids was peroxide structure.

The lethal dose for mice of the peroxide in autoxidized fatty acids was confirmed by the fact that about 278 mg. of total peroxide oxygen per kg. of mice caused the death of the half of the mice tested.

Now, the remaining question is that by what process and mechanism

the peroxide in autoxidized acids gives toxicity to the rats.

As the means of inquiring into this question, the authors asked Mr. Saito and Mr. Ushiki of Tokyo University, Medical Department to make sample tips of the internal organs of the rats which were fed with autoxidized acids and about to die. They have found that the tubuli uriniferi of kidney was extended at the border of medulla and cortical layers, and the cell-infiltration was observed at the mucous membrane of small intestine.

Moreover, the mitochondria were isolated from the rats liver with 0.25 *M* saccharose solution, and when one drop of autoxidized highly unsaturated acids added to half of this solution, the number of mitochondria decreased quickly. On the other hand, when the peroxide-free acids were added to another half, the mitochondria did not decrease. And we observed that the peroxide was found from fats of liver and muscle of rats when autoxidized unsaturated acids were fed.

Having concluded from the above results obtained, peroxide in the autoxidized fatty acids might directly affect the digestive organs of rats, or indirectly might be absorved, through the rats digestive organs, into the rats organs and give toxic effects to them by the peroxide itself or its secondary disintergrated product.

EXPERIMENTAL

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 their body weight remained almost constant, they were separated into different groups.

TABLE I

Composition of Diets

Ingredients	Amount (%)	Ingredients	Amount (mg./100 g. diets)
Polished rice powder	83	Vitamin A	80 (U.S.P.U.)
Casein (ether extracted)	10	Tocopherol	3
McCollum salts mixture	3	Thiamine	0.125
Sample lipides	2-5	Rivoflavin	0.1
		Pyridoxine	0.1
		Pantothenic acid	1
		Niacin	1.5
		Choline	100
		Inositol	200
		p-Aminobenzoic acid	0.5
		Vitamin B_{12}	0.0015

0.2-0.5 g. of sample lipides were daily administered per rat over a 30-day period to observe growth and other physiological conditions of these animals.

Experiment I

Preparation of Ethyl Ester of Liquid Acids of Linseed Oil—The mixed fatty acids of linseed oil were dissolved in aceton, fractionally crystallized in cold storage and separated the solid acids portion. The aceton of filtrate was distilled under vacuum. The obtained liquid acids were changed into ethyl ester in the presence of HCl-catalyst, and subjected to the vacuum distillation. Then the sample was purified by chromatography of aluminium oxide.

The sample of autoxidized acids was prepared by leaving a 4 mm. layer of genuine liquid acids of linseed oil in an open basin at 30°, 50 hours, and 100 g. of this autoxidizes liquid ester was added to a hot solution of 700 g. urea in 1000 ml. of methanol. A precipitate was formed immediately, but the mixture was heated to effect complete solution, and then it was allowed to cool to the room temperature. The well-defined needles of urea complexes were filtered with suction (Fr. 1), and the filtrate was then dissolved to 85 per cent methanol solution and separated the petroleum ether soluble fraction (Fr. 2). Moreover, the petroleum insoluble fraction was extracted with ether (Fr. 3).

TABLE II

Diagram showing Scope of Fractionation of Autoxidized Liquid Acids of Linseed Oil by Urea Adduct Formation

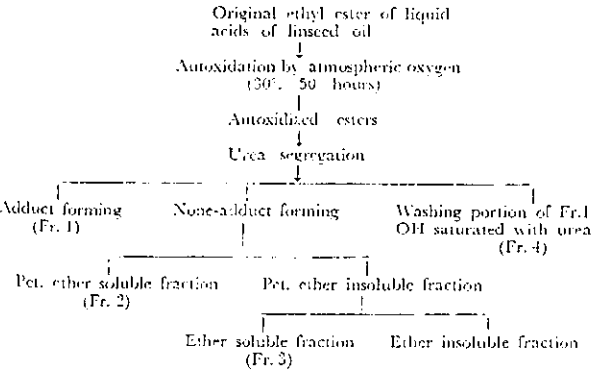

TABLE III

Analysis of the Samples Used in the Experiments

Sample ester	Yield (%)	Acid value	Sap. value	Iodine value	Peroxide value (S.C./kg.)	Kreis test	Conjugated acid (%) Diene	Triene	Tetraene
Original ethyl ester of liquid acids of linseed oil		0	176.01	168.28	0	—	4.97	0	—
Autoxidized esters of above acids		6.4	184.85	137.45	409	⊕	6.07	0.03	0
Fr. 1	74.9	3.5	177.30	143.41	33	⊕	1.01	0	—
Fr. 2	6.1	14.8	213.20	116.43	1216	⊕	30.15	0	0
Fr. 3	11.9	3.8	242.97	77.53	1585	⊖	17.63	0.69	—
Fr. 4	3.7		223.43	106.91	1203	—			—
Total	96.6								

TABLE IV

Feeding Records of Autoxidized Esters of Linseed Oil

(Period: From Mar. 16 to April 5, 1954)

Type of fat fed	Brood series	Sex	Body weight (g.) Initial	Last day	State of mortality
Autoxidized esters of linseed oil, 0.5 g.	C	♀	49	40	Died on 4th day
	C	♀	41	30	" 3rd "
	A	♀	39	44	" 3rd "
	A	♂	38	37	" 7th "
Fr. 1, 0.4 g.	C	♀	46	56	Survived over 20 days
	C	♀	48	64	" " "
	A	♂	51	79	" " "
Fr. 2, 0.2 g.	C	♀	46	46	Died on 2nd day
	C	♀	39	39	" " "
	A	♂	53	53	" " "
Fr. 3, 0.2 g	C	♀	48	48	Died on 2nd day
	C	♀	46	46	" " "
	A	♀	48	43	" 6th "
	A	♂	52	52	" 2nd "

From these results, it was made clear that the original autoxidized ester and Fr. 2 or Fr. 3 which did not form urea adduct showed the toxic effects upon the growth of rats, and they all died in 7 days. However, Fr. 1 (consisting mainly of un-autoxidized acids) did not give any retarding action to the rats.

Experiment II

Polymerized Ester of Highly Unsaturated Fatty Acids—Genuine ethyl ester of highly unsaturated acids are polymerized in the presence of Japanese acid clay at 120°, 6 hours in carbone dioxide. The properties of the samples were as follows:

TABLE V

Analysis of the Samples Used in the Experiments

	N_D^{15}	Iodine value	Sap. value	Peroxide value (m.m./kg.)
Ethyl ester of oleic acid (control)	1.4440	82.70	184.25	
Original ester of highly unsaturated fatty acids	1.4846	348.79	158.14	11
Polymerized ester of above acids	1.4854	310.62	156.23	62

TABLE VI

Feeding Record of Polymerized Ester of Highly Unsaturated Acids

(Period: April 16 to May 1, 1954)

Type of fat fed	Sex	Body weight gained in 15 days	Mean weight gained
None (Fat-free basal)	♀	8 g.	8 g.
	♀	8	
Ethyl ester of oleic acid (Control), 0.5 g.	♀	16	13
	♀	10	
Original ester of highly unsaturated acid, 0.5 g.	♀	19	17.5
	♂	17	
	♀	16	
	♀	18	
Polymerized ester of above acids, 0.5 g.	♀	20	18.7
	♀	19	
	♀	19	

Polymerized highly unsaturated acids show the nutritive effects to the rats and no retarding effects are noticed.

Experiment III

Nutritive Value of Peroxide-free Products of Autoxidized Unsaturated Fatty Acids—Autoxidized esters of unsaturated fatty acids are weighed into test tube, and the same weight of powdered potassium iodide is added. After adding glacial acetic acid and chloroform (2:1 by volume) 20 times as much as the quantity of the sample ester, carbon dioxide passed into the air space above the liquid to displace most of air, and the tube is heated. The peroxide-free products thus obtained are washed with sodium thiosulfate solution and water, then chloroform is distillated away *in vacuo*.

TABLE VII

Properties of Peroxide-free Products from Autoxidized Liquid Acids of Linseed Oil or Autoxidized highly Unsaturated Fatty Acids

	Iodine value	Sap. value	Peroxide value (S.C./kg.)
Original autoxidized liquid ester of linseed oil	137.45	184.85	415
Peroxide-free products of above acids	136.28		45
Original autoxidized ester of highly unsaturated acids	291.00	179.87	485
Peroxide-free products of above acids	301.04	170.64	33

TABLE VIII

Feeding Records of Peroxide-free Products of Autoxidized Linseed Ester

(Period: Mar. 26 to April 15, 1954)

Type of fat fed	Sex	Body weight		State of mortality
		Initial	Last day	
Autoxidized liquid ester of linseed oil, 0.5 g.	♀	50 g.	50 g.	Died on 2nd day
	♂	61	61	" 3rd "
	♀	89	94	" 11th "
Peroxide-free products of above acids, 0.5 g.	♂	53	55	Survived over 3 weeks
	♀	50	50	" "
	♀	91	100	" "

TABLE IX

Feeding Records of Peroxide-free Products of Autoxidized Highly Unsaturated Fatty Acids

(Period: May 24 to June 11, 1954)

Type of fat fed	Sex	Body weight		State of mortality
		Initial	Last days	
Non (Fat-free basal)	♂	76 g.	92 g.	Survived over 18 days
	♀	67	77	" "
Autoxidized ester of highly unsaturated acids 0.5 g.	♂	72	72	Died on 7th day
	♀	73	70	" 4th "
	♀	71	61	" 3rd "
Peroxide-free products of above acids 0.5 g.	♂	77	96	Survived over 18 days
	♀	71	84	" "
	♂	75	84	" "

As it is obvious from Tables VIII and IX, peroxide-free products from autoxidized unsaturated fatty acids become non-toxic, and showed nutritive effects upon the growth of rats.

Moreover, linoleic acid was isolated from mixed fatty acids of soybean oil by urea complexes. (The yield of linoleic acid was 87.5 per cent.) Then we carried out the same experiment on the autoxidized liquid acids of linseed oil or highly unsaturated acids, and the same result was obtained.

From these results it has come to the conclusion that the most toxic structure in autoxidized fatty acids is peroxide.

Experiment IV

Lethal Dose of Peroxide Oxygen in Autoxidized Unsaturated fatty Acids to Mice—In order to survey the degree of the toxicity of peroxide produced in unsaturated fatty acids, 0.15–0.5 g. of autoxidized highly unsaturated acids (peroxide value 448 S.C./kg.) was given to mice and estimated the lethal dose of peroxide oxygen against mice.

TABLE X

Lethal Dose of Peroxide Oxygen in Autoxidized Highly Unsaturated Acids Against Mice

Total peroxide oxygen in samples fed to mice *mg.*	1.79	3.58	5.017	7.16
Mortality	0/12	2/12	6/12	10/12

From these results, the lethal dose was not estimated accurately, however, if calculated with 5.017 mg. peroxide oxygen, the LD_{50} is 278 mg. of total peroxide oxygen per kg. of mice.

Experiment V

Peroxide Content of Fats Separated from Liver and Muscle of Rats which Were Fed with Autoxidized Unsaturated Fatty Acids—In order to investigate the origin of toxicity of autoxidized unsaturated acids, the authors determined the peroxide contents of fats of liver and muscle of rats when the autoxidized acids were fed.

The rats which received 0.5 g. of autoxidized fatty acids per day per rat were killed when the retarding action was noticed. The liver and muscle of rats were separated from rats, treated with Na_2SO_4 and the fats were extracted with diethyl ether.

TABLE XI

Peroxide Value of Fats Separated from Liver and Muscle of Rats which Were Fed with Autoxidized Unsaturated Fatty Acids

Type of sample oil	Peroxide value of sample oil (S.C./kg.)	Peroxide value (S.C./kg.)	
		Liver oil	Muscle oil
None (Fat-free basal)			3
Ethyl ester of oleic acid (control)	0		2
Original ester of highly unsaturated acids	40		14
Autoxidized products of above acids	1405		37
Slightly autoxidized ester of liquid acids of linseed oil	409	20	
Autoxidized ester of liquid acids of linseed oil	3170	89	

No peroxide was found from tissues of the controlled rats which received unautoxidized oleic acid, however, rats fed with autoxidized unsaturated fatty acids contained a large quantity of peroxide in liver and muscle.

DISCUSSION

The survey of the above results leads us to assume that both autoxidized highly unsaturated fatty acids and autoxidized unsaturated fatty acids are all harmful for the rats, and we confirmed that the toxicity is actually caused by the peroxide product.

According to the Duboulozes (*12*), the half of the peroxide was found in the digestive tract of the rats 1.5 hours after the feeding was given to the rats through their mouths.

The authors as well confirmed that considerable volume of peroxide was in the muscle of the rats after autoxidized acids were fed to them.

Based upon these results, it might possibly be considered that the toxicity caused on the rats could be sorted into the direct action which the peroxide gives to the rat's digestive tract or their other internal systems and the indirect one which the absorbed peroxide itself or its secondary disintegrates give toxic effects to tissues.

In other words, just as reported in the previous paper that the rats which were contacted with the autoxidized acids exhibited a depilation around their mouths and legs and also some inflammation, the autoxidized acids are considered to affect the rat's internal systems, attesting it by the fact that the small intestines were injured. Autoxidized acids accumulated in the rat's liver and muscle, which as previously verified, tend to decrease mitochondria in the livers, are assumed to work upon various enzymes, resulting the decrease of enzyme function. However, further research should be made in this respect.

SUMMARY

In the previous paper it has been established that the nutritive value of highly unsaturated fatty acids is not much lower than that of oleic acid, and that generally-believed toxicity of highly unsaturated acids is not produced by the acids themselves but actually by the formation of autoxidized matters.

The observation led us to an assumption that natural unsaturated fatty acids, even if different in the autoxidation degree, would always become less nutritious by some degree of autoxidation.

The assumption was confirmed by the toxic effect on rats of the autoxidized product which was prepared from the ethyl ester of liquid acids of linseed oil, as the rats all died in a few days after the feeding.

On the basis of these findings, our efforts have been extended to throw light on the nature of the above-mentioned toxic product. The method used for and the result obtained from the present test are as follows:

1. The autoxidized products were prepared by leaving the original