*Biochemical Studies on Oils and Fats. X. Toxicity of Thermally Denatured Ethyl Linolenate**

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In previous papers the author has reported that thermally denatured fish oil (cuttle fish oil)[1,2], rape-seed oil[3], and the ethyl esters of highly unsaturated fatty acids[4] show toxicity due to monomeric cyclization products formed as a result of heating.

For the purpose of elucidating the properties and structure of the cyclic products, experiments were conducted to investigate whether or not ethyl linolenate shows a similar toxicity when thermally denatured. The present paper describes the results of the experiments.

Experimental and Results

Separation of Linolenic Acid from Linseed Oil.—Refined linseed oil, whose properties are shown in Table I, was saponified by the ordinary method, and the separated fatty acids were dissolved in ether. Bromine was dropped into the ether solution with cooling. The precipitated bromide was collected by filtration, washed with cold ether, and then dried. For purification, the bromide was dissolved in a large quantity of benzene, and the solution was boiled on a hot water bath. The hexabromide of linolenic acid was then obtained as white precipitate on cooling. The precipitate was collected, washed with cold ether and dried. The hexabromide thus obtained was treated with zinc, ethanol and hydrochloric acid to effect debromination and esterification simultaneously. The ester mixture was distilled in vacuo (1.5 mmHg), and the fraction boiling at 167~170°C was used in the experiment. The properties of this fraction are shown in Table I.

Thermal Denaturation of Ethyl Linolenate and Separation of Cyclic Monomer.—Thermal denaturation of ethyl linolenate was carried out by heating it for 40 hr. at 250°C in a carbon dioxide stream. During this process the iodine value decreased from 242.5 to 157.5, as is shown in Fig. 1. Straight chain ester and cyclic ester were separated from the denatured ethyl linolenate by the urea adduct method[1]. The yields of the straight chain ester and the cyclic ester were 21.2 and 63.3% respectively. By vacuum distillation, the latter was separated into distillate (153~175°C/1.8~2.0 mmHg) and residue. The distillate is a transparent light yellow, and its yield was 24.7% of the total cyclic ester. The residue is somewhat viscous, transparent, and reddish brown, and its yield was 70.8%.

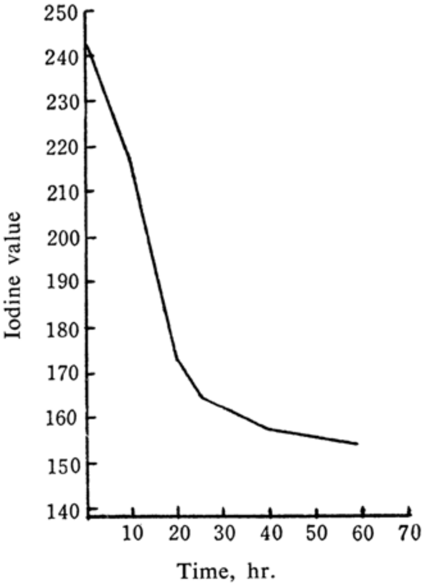

Fig. 1. Change in iodine value of ethyl linolenate due to thermal denaturation.

TABLE I. PROPERTIES OF LINSEED OIL AND ETHYL LINOLENATE

	n_D^{20}	Iodine value (Wijs)	Sap. value	Acid value	Molecular weight (Rast)
Linseed oil	1.4833	191.8	186.9	0	—
Ethyl linolenate	1.4688	242.5	183.1	1.0	302

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* IX: N. Matsuo, *J. Biochemistry*, **49**, 635 (1961).

1) N. Matsuo, *J. Biochemistry (Japan)*, **29**, 885 (1958).

2) N. Matsuo, *Food and Nutrition (Japan)*, **10**, 255 (1958).

3) N. Matsuo, ibid., **12**, 118 (1958).

4) N. Matsuo, *J. Japan Oil Chemists' Soc.*, **9**, 37 (1960).

Animal Experiments with Various Esters.—An animal experiment was conducted in which rats, each weighing about 60 g., were fed on the basal diet containing 20% of a sample ester, as shown in Table II.

Table II. Composition of diet (%)

Starch (rice powder):	65
Casein (ether extracted):	9
McCollum salt mixture:	3
Yeast	3
Liver oil:	One drop a day
Ester	20

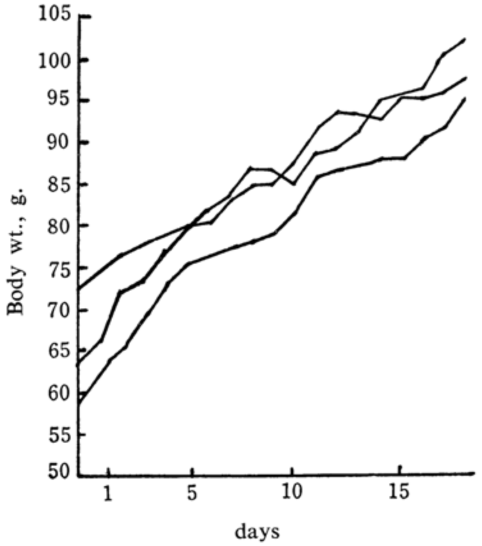

Fig. 2. Growth of rats fed on basal diet containing 20% ethyl linolenate.

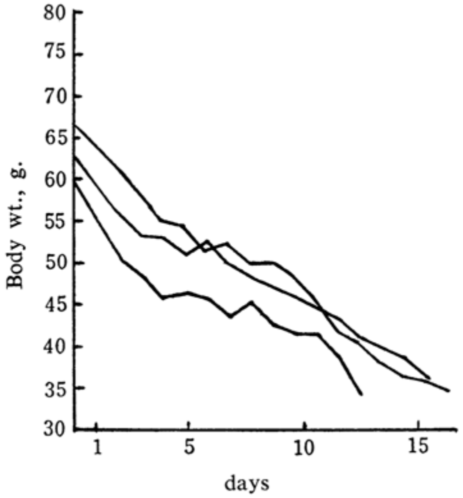

Fig. 3. Growth of rats fed on basal diet containing 20% denatured ethyl linolenate. (All rats died at the ends of the curves.)

Ethyl Linolenate. — The rats fed on a diet containing ethyl linolenate showed a healthy growth, as is seen in Fig. 2.

Denatured Ethyl Linolenate. — As shown in Fig. 3, these rats gradually became thinner and died in 15 days or so.

Distilled Cyclic Ester and Residual Cyclic Ester. — When rats were fed on a basal diet containing 20% distilled cyclic ester, extremely acute toxicity was shown, and all rats died in three or four days. However, no particular change was noticed in the appearance of the rats. In the case of feeding on a basal diet containing 20% residual cyclic ester, toxicity was considerably weaker, and there were some increases in the weight of the rats, although they excreted soft feces (Fig. 4).

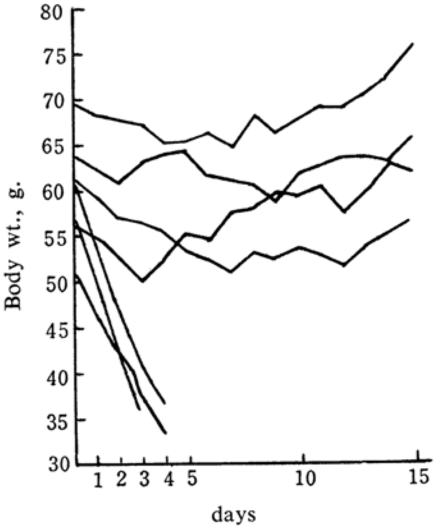

Fig. 4. Growth of rats fed on basal diet containing 20% of distilled cyclic ester (A) and residual cyclic ester (B).
(A: all rats died at the ends of the curves.)

From these observations, it was apparent that distilled cyclic ester was severely toxic.

Properties of Various Esters.—The properties of these esters are shown in Table III.

From the iodine value 242.5 of ethyl linolenate (theoretical value: 247.4), it is assumed that the linolenate contained about 6% ethyl linoleate if the contaminant was only ethyl linoleate. The saponification value of this ethyl linolenate was 183.1 (theoretical value: 183.0) and its molecular weight 302 (theoretical value: 306).

The molecular weight of the distilled cyclic ester was 293. From its boiling point, the main component of this cyclic ester is considered a cyclic monomer. Since its iodine value was 159.4, it seems that the original

TABLE III. PROPERTIES OF ESTERS

	n_D^{20}	Iodine value (Wijs)	Sap. value	Acid value	Molecular weight (rast)
Ethyl linolenate	1.4688	242.5	183.1	1.0	302
Denatured ethyl linolenate	1.4838	157.5	170.1	6.5	
Straight chain ester	1.4692	201.5	177.4	0.4	
Cyclic ester	1.4891	142.5	168.6	7.3	
Distilled ester	1.4751	159.4	171.0	7.4	293
Residual ester	1.4948	135.8	168.5	8.2	

linolenate with three double bonds lost one on cyclization. The iodine value of ethyl linoleate, with two double bonds, is 166.0.

Infrared Absorption Spectra of Ethyl Linolenate and Distilled Cyclic Ester.—The infrared absorption spectra of ethyl linolenate and distilled cyclic ester are shown in Fig. 5, I and II, respectively, measured with the Baird B type. The absorption band at about 14 μ for ethyl linolenate is considerably intense and is attributable to cis double bonds.

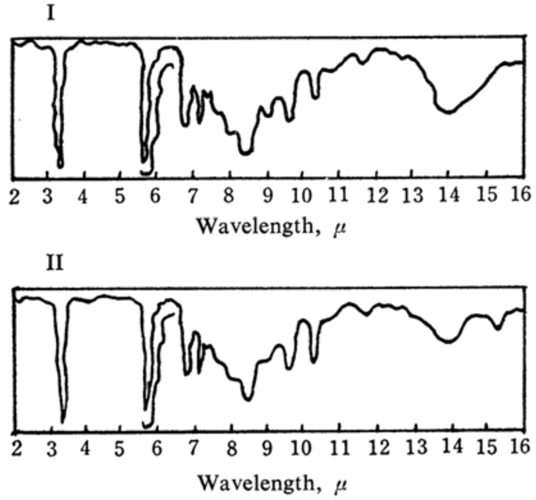

Fig. 5. Infrared absorption spectrum of ethyl linolenate (I) and distilled cyclic ester (II).

Linolenic acid has cis double bonds naturally. When it is separated by the bromination method, however, isomerization takes place to some extent, and an absorption band of 10.3 μ (970 cm^{-1}) attributable to trans double bonds appears. When the absorption band near 6 μ was examined with an increased concentration of the sample, a small absorption was noticed. This absorption is also considered to be attributable to cis double bonds.

When the infrared absorption spectrum (Fig. 5–II) of the distilled cyclic ester is compared with that (Fig. 5–I) of ethyl linolenate, it is found that the absorption attributed to cis double bonds is considerably weaker. This seems to be due to the disappearance of some double bonds. A new absorption band at 15.2 μ (660 cm^{-1}) was observed. Cyclohexene shows a band at 670 cm^{-1} (14.97 μ)[5]. Bands at 663 and 671 cm^{-1} (15.1 and 14.9 μ) are characteristic of Δ^2- and Δ^3-steroids respectively[6], *cis-α*-Ionone[7] also absorbs in this region. So this absorption band is considered to be attributable to the formation of a cyclohexene ring as a result of cyclization. This absorption band was also ascertained by MacDonald[5] in the monomeric, non-urea-adduct-forming material resulting from the ethanolysis of heated linseed oil.

Absorption due to trans double bonds became somewhat greater, but no significant change was observed. Examination of the absorption near 6 μ observed with the higher concentration of the sample showed the existence of two small bands. It seems that the new absorption band at about 6.1 μ (1650 cm^{-1}) originates from the cyclic compound.

Discussion

Acute toxicity is shown by the distilled cyclic ester separated from the denatured product obtained by heating ethyl linolenate for 40 hr. at 250°C. From its molecular weight and infrared absorption spectrum, this distilled ester is considered to be a cyclic monomer with a cyclohexene ring.

Paschke, Wheeler[8] and Rivett[9] have reported the possibility of the formation of intermolecular cyclic compounds. Apparent toxicity was shown also in an experiment feeding animals on a certain synthesized compound containing a cyclohexene ring and one double bond outside the ring[10,11].

Cyclic compounds showed toxicity also in an experiment[4] on highly unsaturated fatty acid

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5) J. A. MacDonald, *J. Am. Oil Chemists' Soc.* **33**, 394 (1956).

6) R. N. Jones and F. Herling, *J. Org. Chem.*, **19**, 1252 (1954).

7) G. Buchi and N. C. Yang, *Helv. Chim. Acta*, **38**, 1338 (1955).

8) R. F. Paschke and D. H. Wheeler, *J. Am. Oil Chemists' Soc.*, **32**, 473 (1955).

9) D. E. A. Rivett, ibid., **33**, 635 (1956).

10) N. Matsuo, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **81**, 469 (1960).

11) N. Matsuo, *Food and Nutrition* (*Japan*), **12**, 86 (1959).

from fish oil; a monomeric cyclization product, separated by vacuum distillation, was the main origin of the toxicity.

From the result of the experiment on ethyl linolenate, it can be concluded that the origin of toxicity is a monomeric cyclization product, and that the main component of this product is a compound having a cyclohexene ring.

Summary

When ethyl linolenate is thermally denatured at 250°C for 40 hr. in a carbon dioxide stream, the product is toxic to rats. The origin of the toxicity of thermally denatured ethyl linolenate is a monomeric cyclization product, which is mainly a compound having a cyclohexene ring.

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