Toxicity of Cyclic Compound of β-Eleostearic Acid*

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When rats were fed on diet containing about 20 per cent of thermally polymerized oil, intoxication of the rats was observed, and the symptom was particularly acute in case such a highly unsaturated oil as fish oil was used as raw material (1–3).

The cause of toxicity was ascertained to be the cyclic monomer formed by the thermal polymerization previously reported (4, 5). This report concerns the preparation of a cyclic compound of β-eleostearic acid and with its toxicity for rats.

EXPERIMENTAL AND RESULTS

Preparation of Pure β-Eleostearic Acid—Tung oil, indicated in Table I-i, was isomerized by the method of Hoffman et al. (6). About 500 g. of tung oil were mixed with 1 ml. of saturated potassium iodide solution, and the mixture was thoroughly stirred, kept air-tight and exposed to the sun-light to accelerate isomerization. Then the oil was saponified with alkali as usual and fatty acid was obtained. This was washed with distilled water, dissolved in ethanol and let stand overnight at −15°C. The precipitated β-eleostearic acid was filtered and dried in vacuum. The melting point of the acid was 70–71°C, and its infrared absorption spectrum was as shown in Fig. 1-a, exactly the same as that reported by Hoffman et al. (6).

Then ethyl ester of β-eleostearic acid was prepared as usual and distilled in vacuum. The fraction collected at 193–196°C showed the properties indicated in Table I-ii, which were almost comparable to the theoretical values. The infrared absorption spectrum of this ester was shown in Fig. 1-b.

TABLE I
Properties of Samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>n_D^20</th>
<th>Iodine Value (Wijs)</th>
<th>Saponification Value</th>
<th>Acid Value</th>
<th>Molecular Weight (Rast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>Tung oil</td>
<td>1.5063</td>
<td>173.7</td>
<td>194.2</td>
<td>5.4</td>
<td>—</td>
</tr>
<tr>
<td>ii</td>
<td>β-Eleostearic acid ethyl ester</td>
<td>1.4892</td>
<td>165.5 (166.0)</td>
<td>182.2 (183.0)</td>
<td>2.0</td>
<td>301 (306)</td>
</tr>
<tr>
<td>iii</td>
<td>β-Eleostearic acid ethyl ester-acrolein adduct compound</td>
<td>1.4847</td>
<td>139.8 (140.3)</td>
<td>153.6 (154.7)</td>
<td>1.6</td>
<td>367 (362)</td>
</tr>
</tbody>
</table>

( ) shows theoretical value.

* A brief account of this paper was reported at the annual meeting of the Japan Chemical Society held in Tokyo in April, 1958. The full text written in Japanese appeared in Nippon Kagaku Zasshi, 81, 467 (1960).

Preparation of Adduct Compound of β-Eleostearic Acid Ethyl Ester with Acrolein—The preparation was carried out according to the method of Miyoshi and Kurata (7) as shown in Table II.
TABLE II
Preparation of β-Eleostearic Acid Ethyl Ester-Acrolein Adduct Compound

<table>
<thead>
<tr>
<th>Tung Oil</th>
<th>Isomerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isomerized Tung Oil</td>
<td>Saponification, Refining</td>
</tr>
<tr>
<td>β-Eleostearic Acid (m.p. 70–71°C)</td>
<td>Esterification</td>
</tr>
<tr>
<td>β-Eleostearic Acid Ethyl Ester</td>
<td>Vacuum distillation (2mm Hg)</td>
</tr>
<tr>
<td>β-Eleostearic Acid Ethyl Ester (193–196°C)</td>
<td>Acrolein</td>
</tr>
</tbody>
</table>

Heating (in sealed tube) | 130–145°C 3.5 hrs. |
β-Eleostearic Acid Ethyl Ester-Acrolein Adduct Compound (100) | Vacuum distillation (2mm Hg) |

<table>
<thead>
<tr>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>~187.5°C</td>
<td>187.5–210°C (35.5)</td>
<td>210–218.5°C</td>
</tr>
<tr>
<td>Vacuum distillation (2mm Hg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>~187.5°C</td>
<td>187.5–203°C (19.1)</td>
<td></td>
</tr>
<tr>
<td>Vacuum distillation (1.8 mm Hg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>~187.5°C</td>
<td>187.5–192°C (7.6)</td>
<td>192–196°C</td>
</tr>
</tbody>
</table>

The β-eleostearic acid ethyl ester and acrolein were mixed in sealed tube in the ratio of 4:1 by weight, and heated at 130–145°C for 3.5 hours. The reaction product was distilled in vacuum. The distillation was repeated three times and the final distillate at 187.5–192°C/1.8 mm Hg was collected (yield 7.6 per cent).

This fraction was transparent, light yellowish, and contained no acrolein when tested by the reaction with sodium nitroprusside and piperidine (β). The properties of adduct compound were shown in Table I-iii, comparable to the theoretical value. Its infrared absorption spectrum was also shown in Fig. 1-c.

β-Eleostearic acid ethyl ester has three conjugated double bonds, whose configurations are all trans form. Cyclohexene ring as shown in the following equation might be formed by the addition of acrolein though Diels-Alder reaction.

When the infrared absorption spectrum
Toxicity of Cyclic Compound of \( \beta \)-Eleostearic Acid

\[-\text{CH}_2\text{CH} \cdot \text{CH} = \text{CH} \cdot \text{CH} = \text{CH} \cdot \text{CH} \cdot \text{CH} \cdot = \text{CH} - \rightarrow \text{CH}_2 \text{CH} = \text{CH} \cdot \text{CH} = \text{CH} \cdot \text{CH} = \text{CH} \cdot \text{CH} = \text{CH} \cdot \text{CH} \cdot \text{CHO} \]

of \( \beta \)-eleostearic acid ethyl ester-acrolein adduct compound was compared with that of the original \( \beta \)-eleostearic acid ethyl ester, the following difference was observed:

The strong absorption of 10.1 \( \mu \) (990 cm\(^{-1}\)) due to the existence of conjugated trans-trans double bond, which was found in \( \beta \)-eleostearic acid ethyl ester, almost disappeared as a result of the addition of acrolein, and 10.3 \( \mu \) (970 cm\(^{-1}\)) absorption, due to trans double bond appeared on the other hand. 15.2 \( \mu \) (660 cm\(^{-1}\)) absorption was not found in the spectrum of the original ester. This absorption band was also ascertained by MacDonald (9) in the monomeric, non-urea-adduct-forming material from the ethanolysis of heated linseed oil. Cyclohexene has a band 670 cm\(^{-1}\) (14.97 \( \mu \)) (9).

Bands at 663 and 671 cm\(^{-1}\) (15.1 and 14.9 \( \mu \)) are characteristic of \( \Delta^2 \)- and \( \Delta^3 \)-steroids, respectively (10). Cis \( \alpha \)-ionone (11) also absorbs in this region. This absorption may be attributable to the cyclohexene ring. Another absorption, due to the aldehyde radical, was observed at 3.7 \( \mu \) (2700 cm\(^{-1}\)).

These differences seem to be caused by the fact that the addition of acrolein to \( \beta \)-eleostearic acid ethyl ester as pictured in the above formula.

From the properties and infrared absorption spectrum, the product obtained might be the adduct compound of one molecule of acrolein with one molecule of \( \beta \)-eleostearic acid ethyl ester.

\textbf{Preparation of Intra-Molecular Cyclic Compound}

\textbf{TABLE III}

\begin{center}
\textit{Separation of Cyclic Monomer from Thermally Treated Product of \( \beta \)-Eleostearic Acid Ethyl Ester}
\vspace{0.5em}
\hspace{2em} \( \beta \)-Eleostearic Acid Ethyl Ester (100) \\
\hspace{2em} Thermally Treated Product from Heating (in sealed tube) 180-185\(^\circ\)C, 5 hrs. \\
\hspace{2em} \( \beta \)-Eleostearic Acid Ethyl Ester (99) \\
\hspace{2em} Vacuum distillation (3 mm Hg) \\
\vspace{0.5em}
\hspace{2em} Loss \\
\hspace{2em} Distillate \\
\hspace{2em} Residue \\
\hspace{2em} 161.5-200\(^\circ\)C (75.5) \\
\hspace{2em} (4.8) \\
\hspace{2em} Urea-adduct forming method \\
\hspace{2em} Stand overnight (2.5\(^\circ\)C) \\
\hspace{2em} Urea (5 volumes) \\
\hspace{2em} Ethanol (8 volumes) \\
\hspace{2em} 50-55\(^\circ\)C \\
\vspace{0.5em}
\hspace{2em} Filtrate I \\
\hspace{2em} (Not forming urea-adduct) \\
\hspace{2em} Concentration, Stand overnight (2.5\(^\circ\)C) \\
\hspace{2em} Urea-Forming Adduct \\
\hspace{2em} Washing with ethanol \\
\hspace{2em} Hot water \\
\hspace{2em} Straight Chain Ethyl Ester (60.1) \\
\vspace{0.5em}
\hspace{2em} Crystal of Urea \\
\hspace{2em} Separated \\
\hspace{2em} Filtrate II \\
\hspace{2em} Concentration \\
\hspace{2em} Stand 48 hrs. (3-4\(^\circ\)C) \\
\hspace{2em} Crystal of Urea Separated \\
\hspace{2em} Filtrate III \\
\hspace{2em} Ethanol removed \\
\hspace{2em} Reddish Brown Ester (8.4) \\
\vspace{0.5em}
\hspace{2em} Vacuum distillation (2 mm Hg) \\
\hspace{2em} Fraction I \\
\hspace{2em} 109-168\(^\circ\)C \\
\hspace{2em} (2.0) \\
\hspace{2em} Fraction II \\
\hspace{2em} 168-174.5\(^\circ\)C \\
\hspace{2em} (4.0) \\
\hspace{2em} Residue \\
\hspace{2em} (1.1) \\
\hspace{2em} Loss \\
\hspace{2em} (1.3) \\
\vspace{0.5em}
\end{center}
of β-Eleostearic Acid Ethyl Ester—The process of preparation was shown in Table III. After heating 230 g. of β-eleostearic acid ethyl ester in a sealed tube at 180–185°C for 5 hours, 228 g. of the contents of the tube were distilled in vacuum. The distillate at 161.5–206°C/3 mm Hg was collected. The yield was 174 g. (75.5 per cent).

The distillate was dissolved in 1.5 liters of ethanol and was gradually added with 870 g. of urea at 50–55°C. After thoroughly stirring for 30 minutes, the mixture was let stand overnight. The precipitation of urea adduct was collected and washed with 500 ml. of ethanol (washings were added to the filtrate).

By treating the precipitate with a large quantity of hot water, straight chain ester was separated from the precipitate. Its yield was 60.1 per cent for the original β-eleostearic acid ethyl ester.

The mixture of filtrate and washings was concentrated to about 800 ml. and kept overnight at 2.5°C. The crystal of urea was removed and the filtrate II was again concentrated to 500 ml. and kept at 3–4°C for 48 hours. In this way, filtrate III was obtained free from any precipitates. When ethanol was evaporated out of this filtrate, a residue of reddish brown colour was obtained. Its yield was 8.4 per cent.

Then the residue was distilled in vacuum (2 mm Hg) and the distillate at 168–174.5°C was collected. The yield of the fraction was 4.0 per cent.

It was transparent, light yellow liquid with properties as shown in Table IV, and its main component might be a cyclic monomer of β-eleostearic acid ethyl ester. Its infrared absorption spectrum was shown in Fig. 2.

![FIG. 2. Infrared absorption spectrum of cyclic monomer of β-eleostearic acid ethyl ester.](image)

When the spectrum in Fig. 2 was compared with the spectrum of the original β-eleostearic acid ethyl ester, the following difference was observed:

The strong 10.1 μ (990 cm⁻¹) absorption in the spectrum of β-eleostearic acid ethyl ester, which was due to the conjugated trans-trans double bond, almost disappeared, and new absorption due to cis double bond appeared at 14.4 μ (690 cm⁻¹). This seems to be caused by the formation of double bond in a cis ring, replacing conjugated trans-trans double bond, as a result of cyclization of β-eleostearic acid ethyl ester caused by heating. New weak absorption at 15.2 μ seems to be caused by the cyclohexene ring.

From the above mentioned findings on molecular weight, infrared absorption spectrum and non adduct forming character with urea, it is known that the main component of the distillate at 168–174.5°C is a cyclic monomer produced by intra-molecular cyclization of β-eleostearic acid ethyl ester.

**Toxicity of Cyclic Compound**—When rats, each weighing 60 to 70 g., were fed on basal

<table>
<thead>
<tr>
<th>Sample</th>
<th>$n^{20}_D$</th>
<th>Iodine Value (Wijs)</th>
<th>Saponification Value</th>
<th>Acid Value</th>
<th>Molecular Weight (Rast)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic Monomer of β-Eleostearic Acid Ethyl Ester</td>
<td>1.4781</td>
<td>115.3</td>
<td>164.4</td>
<td>4.0</td>
<td>300 (306)</td>
</tr>
</tbody>
</table>

( ) shows theoretical value.

TABLE IV

Properties of Cyclic Monomer Separated from Thermally Treated Product of β-Eleostearic Acid Ethyl Ester
Toxicity of Cyclic Compound of ƒÀ-Eleostearic Acid

A diet (Table V), containing 10 per cent of the ƒÀ-eleostearic acid ethyl ester, the rats grew normally.

**TABLE V**

*Composition of Basal Diet (%)*

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch (Rice Powder)</td>
<td>75</td>
</tr>
<tr>
<td>Casein (Ether Extracted)</td>
<td>9</td>
</tr>
<tr>
<td>McCollum Salt Mixture</td>
<td>3</td>
</tr>
<tr>
<td>Yeast</td>
<td>3</td>
</tr>
<tr>
<td>Liver Oil</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>10</td>
</tr>
</tbody>
</table>

(i) **Toxicity of Eleostearic Acid Ethyl Ester-Acrolein Adduct Compound for Rats**—Rats, each weighing about 60g., were fed on basal diet (Table V), containing 10 per cent of the adduct compound of ƒÀ-eleostearic acid ethyl ester with acrolein.

In this experiment, as shown in the growth curves given in Fig. 3, all rats died in 4 to 6 days after showing an abrupt decrease in body weight.

(ii) **Toxicity of Cyclic Monomer Obtained from Thermally Treated Product of Eleostearic Acid Ethyl Ester**—Rats, weighing about 60g. each, were fed on basal diet containing 10 per cent of cyclic monomer separated from thermally treated product of ƒÀ-eleostearic acid ethyl ester as mentioned above. In this experiment, as illustrated in the growth curves given in Fig. 4, all rats died in 7 days or less after showing a remarkable loss of weight.

**DISCUSSION**

Thermally polymerized oil, obtained by heating cuttlefish oil in a carbon dioxide stream or in the air at 250°C for 10 hours (no catalyst), has toxicity even though it contains an extremely small quantity of peroxide. When rats, each weighing 50 to 70g., were fed on basal diet containing 20 per cent of this thermally polymerized oil, they all died within 15 days. As a result of ethanoly-
ysis of the thermally polymerized oil and subsequent separation into straight chain and cyclic compounds by the urea adduct, it was found that the toxicity was almost attributable to the cyclic constituent (1, 2).

When refined rape-seed oil was used as a sample, it was also observed that cyclic product was responsible for toxicity in the case of heat treatment of the oil at 250°C for 50 hours (3). Furthermore, when cyclic ethyl ester, separated from the thermal polymer of highly unsaturated fatty acid ethyl ester and ethyl linolenate (4, 5) was distilled into distillable ester and non-distillable ester, it was found that the distillable ester shows particularly acute toxicity and that the non-distillable ester has only weak toxic effect. Judging from the boiling point of the distillable ester obtained when ethyl linolenate was used as a sample, and also comparing the molecular weight and other properties of the distillable fraction with those of the original ester, it was confirmed that the main origin of toxicity was due to the cyclic monomer. According to infrared absorption spectrum, it was also found that this cyclic monomer contained a cyclohexene structure in the molecule.

Several studies (12, 13) were reported concerning the cyclization of β-eleostearic acid because it has three double bonds in a trans form, which was suitable for such study. Therefore, research on cyclization was conducted, using β-eleostearic acid, and toxicity for rats were examined with its cyclic product.

The mixture of β-eleostearic acid ethyl ester and acrolein, even by heating in a sealed tube, formed a cyclohexene ring by the addition reaction. Thermally treated product was thrice distilled in vacuum, and distillate at 187.5-192°C/1.8 mm Hg. was obtained. Judging from its molecular weight and infrared absorption spectrum, the main component of this fraction has probably a cyclic structure.

Cyclic monomer was obtained by heating β-eleostearic acid ethyl ester under such moderate conditions as at 180-185°C for 5 hours, minimizing the formation of dimer or any products other than monomer. Although its yield is small, judging from its molecular weight and infrared absorption spectrum, it is evidently a monomer containing intra-molecular cyclic constituent.

In animal experiments, in which rats were fed on basal diet containing each one of these products, the rats unexceptionally died in 7 days or less. This fact gave further endorsement to the previous reports that the main origin of toxicity is the cyclic monomer.

**SUMMARY**

1. An adduct compound of β-eleostearic acid ethyl ester and acrolein having cyclohexene ring in its molecule was obtained. This showed clear toxicity when rats were fed on basal diet containing 10 per cent of it.

2. An intramolecular cyclic compound obtained by heating β-eleostearic acid ethyl ester were found also quite toxic to rats.

3. The cyclic constituents in the above substances were confirmed by infrared absorption spectrum and other several properties.

The author expresses his heartfelt thanks to Dr. K. Kodama, President of Tokushima University, for his continual guidance given in the course of this experiment. Deep appreciation is expressed to Dr. S. Tanaka of Tokyo University's Over-all Research Laboratory for his assistance in the infrared spectrum research. Thanks are also due to Mr. K. Kuwamoto for his cooperation in the experiment.

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(3) Matsuo, N., *J. Soc. Food and Nutrition (Japan)*, 12, 118 (1959)
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