Relationship between vitamin E and erythrocyte hemolysis in rat fed on autoxidized methyl linoleate

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RELATIONSHIP BETWEEN VITAMIN E AND ERYTHROCYTE HEMOLYSIS IN RAT FED ON AUTOXIDIZED METHYL LINOLEATE

By

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Autoxidized oils including peroxide have been generally known to cause certain diseases in animals. It has been also known that the erythrocytes of vitamin E deficient animals easily hemolyze in the presence of hydrogen peroxide or diuric acid in vitro due to the peroxidation of lipids in their membranes. However, very few observations have been reported whether the erythrocytes of rats hemolyze or not when autoxidized oil is given orally.

In the present paper, the authors examined the relation between the H$_2$O$_2$-induced hemolysis of the erythrocytes and the vitamin E contents of the sera of the rats fed on autoxidized methyl linoleate (AOML) or a mixture of AOML and vitamin E. The diet shown in Table-2 was given to rats in each experimental group for 7 days, respectively.

The following results were obtained:
1. The hemolysis of the erythrocytes in the rats fed on methyl linoleate appeared more frequently than in the rats fed on the basal diet.
2. Higher hemolysis was observed in the erythrocytes of the rats fed on AOML than in those of the rats fed on methyl linoleate.
3. Any hemolysis of the erythrocytes was hardly observed in the rats fed on the additional vitamin E with the basal diet containing methyl linoleate (fresh or autoxidized).
4. The serum vitamin E content of the rat in the AOML group was the lowest of all the groups tested.

From these results, the authors presumed that the hemolysis of the erythrocytes of the rats fed on AOML might be caused by the following process: Some digestible and absorbable components from AOML decreased in vivo serum vitamin E content below a level susceptible of vitamin E deficiency.
1. INTRODUCTION

Highly unsaturated fatty acid, one of the components of the phospholipids in the erythrocytic membrane, is readily peroxidated to become hydroperoxides if there is a deficiency of antioxidants. Due to this change, such erythrocytic membrane becomes fragile, resulting in hemolysis in the presence of oxidizing agents such as hydrogen peroxide, dialuric acid, etc. Consequently, it has been noted that hemolysis of this type takes place frequently in animals deficient in vitamin E, a natural antioxidant\textsuperscript{1).} On the other hand, autoxidized oils containing hydroperoxide have been known to produce abnormal metabolism of various kinds when they are externally given to a variety of species\textsuperscript{2).} But the relationship between externally administered autoxidized oils and peroxidation in vivo which causes hemolysis is still unexplored. Moreover, there have been very few cases in which hemolysis is examined using the erythrocytes of animals fed on autoxidized oils.

The present report examines the $\text{H}_2\text{O}_2$-induced hemolysis of erythrocytes of rats fed on fresh or autoxidized methyl linoleate, and the effect of vitamin E in preventing such hemolysis, as well as the serum vitamin E content of such animals.

2. EXPERIMENTAL

2.1 Experimental Groups and Feeding Conditions

Rats of the Donryū strain (approximate body weight of 120 g in males and 110 g in females) were used as the experimental animals. Solid diet prepared by the Nippon Kurea Chemical Inc. was given from the time of weaning until the onset of the experimental feeding. The
duration of experimental feeding was 7 days, during which 10 g of the basal diet shown in Table 1 was given to each animal each day at a fixed time (10-11 a.m.). The type and the amount of added oils and the vitamins given to each experimental group are shown in Table 2.

### Table 1: Composition of basal diet (%).

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Starch</td>
<td>51.7</td>
</tr>
<tr>
<td>Sugar</td>
<td>25.8</td>
</tr>
<tr>
<td>McCollum's mixture</td>
<td>4.0</td>
</tr>
<tr>
<td>Vitamin-free casein</td>
<td>15.0</td>
</tr>
<tr>
<td>Vitamin mixture*</td>
<td>0.5</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*The diet (1 kg) contained the following vitamins: retinol 2,000 IU, thiamine 5 mg, riboflavin 8 mg, pyridoxine 5 mg, cyanocobalamin 0.03 mg, ascorbic acid 100 mg, cholecalciferol 200 IU, dl-α-tocopherol acetate 50 mg, nicotinic acid 40 mg, Ca-pantothenate 40 mg, p-aminobenzoic acid 100 mg, folic acid 2 mg, biotin 0.4 mg, menadione 5 mg, inositol 100 mg.

### Table 2: Experimental groups and compositions of diets.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Group No.</th>
<th>Number of rat</th>
<th>Added oils &amp; vitamins (per 10 g of basal diet*)</th>
<th>Methyl linoleate</th>
<th>Autoxidized ML</th>
<th>dl-α-Tocopherol</th>
<th>dl-α-Tocopheryl acetate</th>
<th>dl-α-Tocopherol acetate</th>
<th>dl-α-Tocopherol acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>1, 2, 3, 9, 10</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4, 5, 6</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td></td>
<td></td>
<td>100 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>1</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>4</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>7</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>11</td>
<td>8</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>9</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
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<tr>
<td></td>
<td>13</td>
<td>10</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>11</td>
<td></td>
<td>50 mg**</td>
<td>240 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1 The daily dose of basal diet per rat is 10 g.
*2 Methyl linoleate.
*3 Autoxidized ML.
*4 dl-α-Tocopherol.
*5 dl-α-Tocopheryl acetate.
*6 POV = 3.778 meq/kg.
*7 POV = 3.660 meq/kg.
These oils and vitamins were mixed with the diet described above, and the rats had free access to it. Compared with the basal diet of Table 1, the added vitamins in Table 2 were 100 times the concentration for dl-α-tocopherol, the vitamin E, and 3 times the concentration for vitamin B₂ (riboflavin). These vitamins were added to the soybean oil in the basal diet. Added vitamin E was free-type dl-α-tocopherol for Group 2 and dl-α-tocopheryl acetate for all the other groups. The amount of added oils was 0.5 ml per animal. The present investigation consisted of four separate experiments (Experiments I-IV in Table 2) whose objectives were as follows: Experiment I examined the preventive effect of vitamin E against hemolysis seen in the basal diet group, and the difference between the effects of free-type and acetate-type vitamin E. Experiments II and III studied the changes in the rate of hemolysis when fresh or autoxidized methyl linoleate was added to the basal diet and the preventive effect of vitamin E in such conditions. Finally, Experiment IV investigated the relationship between the rate of hemolysis and the serum vitamin E content when diet containing fresh or autoxidized methyl linoleate was given.

2.2 Preparation of Added Oils

Fresh and autoxidized methyl linoleate were prepared in accordance with a previous publication. The purity of the fresh methyl linoleate was 90% and the peroxide value (POV) of autoxidized methyl linoleate was 3,778 meq/kg (a) in Experiments I-III, and 3,600 meq/kg (a) in Experiment IV. The iodine value (IV), saponification value (SV), acid value, average molecular weight (measured by the cryoscopic method), and conjugated diene acid content of the fresh and autoxidized methyl

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(a) In this method, the peroxide value eventually reaches approximately 5,000 meq/kg and then declines as decomposition of hydroperoxide increases.
linoleate used in Experiments I-III are shown in Table 3.

<table>
<thead>
<tr>
<th></th>
<th>POV (mg/Kg)</th>
<th>IV (Wt%)</th>
<th>SV (%)</th>
<th>AV</th>
<th>Average molecular weight*</th>
<th>Conjugated double bond (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh ML</td>
<td>0</td>
<td>146</td>
<td>168</td>
<td>0</td>
<td>290</td>
<td>0</td>
</tr>
<tr>
<td>Autoxidized ML</td>
<td>3.778</td>
<td>49</td>
<td>245</td>
<td>4.7</td>
<td>336</td>
<td>14</td>
</tr>
</tbody>
</table>

* Determined by the use of the Hitachi Molecular Weight Measuring Apparatus (Model 113)

2.3 Hemolysis Test

When the experimental feeding period was completed, the rat was deprived of food for one day and, under ether anesthesia, its abdominal cavity was exposed. Immediately afterwards, a blood sample was taken from the descending vena cava with a syringe treated with heparin. An appropriate amount of the blood sample was dropped into a sodium citrate--sodium chloride solution (5 g, 4.5 g/l) in order to prepare the sample for the hemolysis test. The hemolysis test followed the \( \text{H}_2\text{O}_2 \) method of Ikehata et al.\(^4\). It is believed that the rate of hemolysis measured by this method is easily affected by such conditions as the concentration of \( \text{H}_2\text{O}_2 \), external temperature, etc., and that it is consequently difficult to compare the absolute values of different experimental groups except for those whose measurements are taken at the same time\(^4\). Therefore, the present report deals only with comparisons among groups in the same experiment in order to make sure that such measurements have been taken under identical experimental conditions.
2.4 Measurement of Serum Vitamin E Content

The method of Abe and Katsui\(^5\) was followed.

3. RESULTS

The results of Experiments I-III and of Experiment IV are summarized in Figure 1 and Figure 2, respectively. In the present study, approximately equal numbers of female and male rats were used in each group. Since there was no tendency for differences with respect to sex, this variable is not considered further in this report.

3.1 Experiment I

As seen in Figure 1, the majority of rats in the basal diet group (Group 1) showed a high rate of hemolysis (10-40%). In the other two groups that were given vitamin E (Groups 2 and 3), very few rats showed a rate of hemolysis higher than 10%. Thus, adding a large dose of vitamin E to the diet proved effective in almost completely suppressing the hemolysis observed in the rats on the basal diet alone. This

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**Fig. 1** \( \text{H}_{2}\text{O}_{2}\)-induced hemolysis of erythrocytes in rats fed on different diets (Expt. I~III)
result is in agreement with a previous report\(^6\).

Free-type and acetate-type vitamin E did not differ in their preventive effect against hemolysis under the present experimental conditions.

### 3.2 Experiment II

The group administered methyl linoleate (Group 5) had a greater number of animals showing an extremely high rate of hemolysis (more than 30%) than the basal diet group (Group 4). In the group administered with methyl linoleate and a large dose of vitamin E (Group 6), however, hemolysis was almost completely suppressed in all the rats, replicating the results of a previous study\(^7\). The rate was below 10% under the present conditions.

### 3.3 Experiment III

There were a greater number of animals which showed an extremely high rate of hemolysis (more than 30%) in Group 8, administered with antoxidized methyl linoleate, than in Group 7, administered fresh methyl linoleate. In other words, the rate of erythrocytic hemolysis in rats, which increased with administration of fresh methyl linoleate, underwent a further increase when autoxidized methyl linoleate was given. In addition, a large dose of vitamin E was extremely effective again in preventing the hemolysis which was found to be enhanced by administration of autoxidized methyl linoleate.

### 3.4 Experiment IV

The relationship between serum vitamin E content and the rate of hemolysis was examined in this experiment.

As shown in Figure 2, the results differed from those of
Experiments I and II in that the animals in the basal diet group (Group 10) showed hardly any hemolysis, and that the increase in the rate of hemolysis in the group administered fresh methyl linoleate was only minimal. But the results were congruent with those of Experiments I-III with respect to the increase in the rate of hemolysis in the group administered autoxidized methyl linoleate and to the preventive effect of vitamin E against such hemolysis. As will be discussed later, vitamin E intake prior to the experimental feeding seems to have played some role in this discrepancy.

Serum vitamin E content in the basal diet group (Group 10) and the other four groups (Groups 11, 12, 13, and 14) are compared in Figure 2. It is clear that the serum vitamin E content of the group administered autoxidized methyl linoleate (Group 12) was generally lower than those of the other four groups. On the other hand, in the
groups administered with a large dose of vitamin E (Groups 13 and 14), most of the animals showed a vitamin E level higher than the maximum in Groups 10 and 11.

The above results can be summarized as follows: In the group administered autoxidized methyl linoleate, most rats showed hemolysis, and at the same time serum vitamin E content was the lowest. The groups administered with vitamin E, in which hemolysis was completely prevented, showed very high vitamin E content.

4. DISCUSSION

It was found that the rate of hemolysis in rats administered fresh methyl linoleate added to the basal diet was somewhat higher than that in rats fed on the basal diet. It has been known that the dietary vitamin E requirement increases when unsaturated fatty acids such as linoleate are given to animals\(^1\). Harris and Embree\(^8\) have proposed that the ratio of the amount of vitamin E in mg to the amount of highly unsaturated fatty acids in grams (E/PUFA) be used as a criterion for the determination of the vitamin E requirement. Fukuba\(^9\) noted that, although there was hardly any hemolysis when the E/PUFA value was 0.8, the rate of hemolysis increased when the E/PUFA value declined to 0.6. This E/PUFA value roughly calculated for the diet with the fresh methyl linoleate used in the present investigation was higher than 0.8. In other words, hemolysis was found in some animals although the concentration of vitamin E was within the range considered sufficient for prevention of hemolysis according to Fukuba. Furthermore, the basal diet groups in Experiment I (Group 1) and Experiment II (Group 4) contained animals that showed a high rate of
hemolysis although the E/PUFA value of the basal diet was higher of course than that of the diet with methyl linoleate added. One possible reason for this is that the rats might have been deficient in vitamin E due to the lower E/PUFA value of the commercial diet used prior to the experiment. That is, it is conceivable that the vitamin E content of the commercial diet used for the pre-experimental feeding significantly affected the rate of hemolysis.

Rats administered autoxidized methyl linoleate showed a clearly higher rate of hemolysis than rats on the basal diet alone, and rats administered with fresh methyl linoleate. Yoshioka and Kaneda\textsuperscript{10} reported that low molecular hydroperoxi-arunen\textae鲁, among various products of the autoxidation of oils and fats, produced especially severe hemolysis. Hydroperoxi-arunen\textae鲁 is a low molecular secondary oxide produced at the most advanced stage of autoxidization of oils and fats. The autoxidized methyl linoleate used in the present study, however, was at a rather early stage of oxidization and its molecular weight was quite similar to that of fresh methyl linoleate (Table 3). Therefore, it seems justifiable to infer that hemolysis in the present investigation was due to a substance other than hydroperoxi-arunen\textae鲁. This inference is also supported by the fact that the peroxide value of lipids in the tissue increased following administration of hydroperoxi-arunen\textae鲁\textsuperscript{10}, whereas the value did not increase following the administration of autoxidized methyl linoleate roughly similar to that used in the present study\textsuperscript{11}. On the other hand, the serum vitamin E content of the rats administered autoxide was near or below 0.5 mg/ml.

\* Translator's note: as transliterated. (hydroperoxialkenal?)
Fujii et al\textsuperscript{12} reported that the rate of hemolysis increased rapidly as the serum vitamin E content dropped below 0.5 mg/ml. Thus it is reasonable to conclude that the serum vitamin E content of the rats administered with autoxide had declined to the level at which erythrocytes were highly susceptible to hemolysis. In relation to this, a previous report\textsuperscript{13} that even some products of autoxidization which do not have hydroperoxi radicals possessed strong toxicity suggests the following. It was not hydroperoxide, believed not to be digestible or absorbable\textsuperscript{14,15}, that directly peroxidated the lipids of the erythrocytic membrane. Instead, products of autoxidization other than hydroperoxide, upon absorption into the body, lowered the vitamin E concentration in the blood resulting in conditions conducive to peroxidization.

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*Translator's Footnote: There are alternate ways to pronounce and spell this first name.