Changes in Levels of Liver Malondialdehyde, Liver and Serum Vitamin E, Serum GOT and GPT Activities, and Pathological Changes of Liver in the Case of Massive Liver Necrosis in Rats Fed a Low Casein, Vitamin E Deficient Diet

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In these present experiments, rats were fed a low casein, vitamin E and selenium deficient diet and were killed at various time intervals up to the occurrence of massive liver necrosis. Liver malondialdehyde, and liver and serum vitamin E levels were analyzed simultaneously with a pathological investigation of the features of the liver and measurement of serum GOT and GPT activities. These investigations were also performed on control rats given a high casein, vitamin E deficient diet or a low casein, vitamin E supplemented diet.

The results show that the liver malondialdehyde level was not increased concomitant with the decrease in liver and serum vitamin E level nor with the occurrence of massive liver necrosis. Liver cells of the rats fed on the low casein, vitamin E deficient diet showed swelling of the cytoplasm at the initial stage, and progressive centrilobular lipid deposition was observed by Sudan III stain.

It was already known that fatal liver lesion occurred when rats were fed diets deficient in sulfur containing amino acid, vitamin E and selenium.1~5 In these diets, cod liver oil or stripped lard was used as the lipid source. In the case of cod liver oil, large amounts of lipid peroxide would be formed as Tappel suggested, and this would be the principal reason for the occurrence of disease. However, if stripped lard was used, the situation would be different. Lard is low in polyunsaturated fatty acid, and it is considered unlikely that such an acceleration of lipid peroxidation would be the principal reason for the occurrence of liver disease.

To bring about this disease, known as massive liver necrosis, a low protein diet made from casein or torula yeast was used. Even by feeding animals a low protein diet containing other adequate nutrients, various unfavorable physiological effects are inflicted upon them. Damage to tissues and organs by protein deficiency or by feeding a low protein diet have been reported pathologically,7~10 and the liver is the organ most largely affected. Therefore, it is reasonable to suppose that such tissue and organ damage might have some relationship with the occurrence of massive liver necrosis when vitamin E deficiency is superimposed on such a low protein diet.

The present experiments consider these facts and attempt to clarify the mechanism for the occurrence of massive liver necrosis in rats fed a low protein, vitamin E deficient diet containing lard by investigating any abnormal changes in the liver which occurred before the appearance of obvious disease. Furthermore, the involvement of malondialdehyde and vitamin E levels in these changes was also investigated. Rats were fed the low casein, vitamin E deficient diet containing lard, and were killed at various time intervals up to the beginning of massive liver necrosis. The liver malondialdehyde and liver and serum vitamin E levels were then determined, and simultaneously, the pathological features of the liver were investigated. In addition, serum GOT and GPT activities were
measured because these enzymes have been adopted as a useful tool for the detection of hepatocellular damage.

Although there is some criticism in using malondialdehyde level as the extent of occurrence of lipid peroxidation, this has been widely adopted as a sensitive and convenient assay method for lipid peroxidation in animal tissues. It has been demonstrated by May et al.\textsuperscript{11} in an \textit{in vitro} experiment, that the relationship between the amount of malondialdehyde formed and the extent of the fatty acid oxidized is a stoichiometric one within certain limits, although it does not absolutely represent the fatty acid peroxides.

The results show that liver malondialdehyde level did not increase concomitant with the occurrence of fatal liver lesion nor with the decrease in liver and serum vitamin E levels in rats fed the low casein, vitamin E deficient diet containing lard. Other reasons for the occurrence of fatal liver disease in these rats are discussed.

MATERIALS AND METHODS

\textit{Animals and diets.} Male rats of the Wistar strain, weighing about 175g, were used. They were housed in individual cages in a temperature-controlled room at about 22°C.

The composition of diets used is shown in Table I. The low casein, vitamin E deficient diet contained 4\% vitamin free casein and 5\% vitamin E free lard. Two kinds of control diets were used. One, a high casein, vitamin E deficient diet, contained 25\% vitamin free casein and 5\% vitamin E free lard, and the other, a low casein, vitamin E supplemented diet, contained 4\% vitamin free casein and 5\% vitamin E free lard which was supplemented by 100 mg of \textit{\alpha}-tocopherol per kg of diet. Salt and vitamin mixtures were made according to Harper,\textsuperscript{12} and selenium was not added to all the diets.

\textit{Plan of experiments.} The experiments were carried out in three phases as experiments 1, 2 and 3.

In experiment 1, all the rats were fed a low casein, vitamin E deficient diet. At the start of feeding with the experimental diet, another three rats purchased at the same time were killed and treated similarly to the test diet fed rats, the data from these rats being recorded as the 0 day control value. One quarter of the rats fed with the test diet were killed at the 68th day of feeding with the experimental diet and another quarter at the 91st day, the remaining animals being killed at the onset of obvious disease.

In experiment 2, the rats were divided into two groups, one group being fed a low casein, vitamin E deficient diet and the other a high casein, vitamin E deficient diet. In this experiment, as in experiment 1, another three rats were killed for the 0 day control value. One third of the rats fed with the low casein, vitamin E deficient diet were killed at the 62nd day of feeding with the experimental diet and another one third at the 100th day, the remaining animals being killed at the onset of obvious disease. All the rats fed with the high casein, vitamin E deficient diet were killed at the same time as the last low casein, vitamin E deficient

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>4% Casein – vitamin E (%)</th>
<th>25% Casein – vitamin E (%)</th>
<th>4% Casein + vitamin E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein*</td>
<td>4</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>\textit{\alpha}-Starch</td>
<td>85.6</td>
<td>64.6</td>
<td>85.6</td>
</tr>
<tr>
<td>Lard*</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Salt mixture\textsuperscript{d}</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mixture\textsuperscript{e}</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>\textit{\alpha}-Tocopherol in lard\textsuperscript{f}</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All the diets were further supplemented with 6000 IU of vitamin A and 600 IU of vitamin D/kg of diet.
\textsuperscript{b} Vitamin free casein was used.
\textsuperscript{c} Vitamin E free lard purchased from Oriental Kobo Kogyo Co. was used.
\textsuperscript{d} Made according of Harper.\textsuperscript{12} Selenium was not added.
\textsuperscript{e} Made according of Harper.\textsuperscript{12}
\textsuperscript{f} One gram of \textit{\alpha}-tocopherol was dissolved in 100 g of vitamin E free lard. Therefore, the 4\% casein, vitamin E supplemented diet contained 100 mg of \textit{\alpha}-tocopherol/kg of diet.
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diet fed rat (121st day of feeding with the experimental
diet).

In experiment 3, the rats were again divided into two
groups, one group being fed a low casein, vitamin E
supplemented diet and the other a low casein, vitamin E
deficient diet. All the rats were killed at 121st day of
feeding with the experimental diet.

All the rats were anesthetized with nembutal, blood
was collected by heart puncture, and the liver was perfused
with ice-cold 0.9% NaCl before excising. A portion of the liver
was fixed in 10% formalin and another portion in 2.5%
glutaraldehyde immediately after excising. The liver ma-
londialdehyde level and serum GOT and GPT activities
were determined for all the sacrificed rats. Liver and serum
vitamin E levels were determined in experiments 2 and 3.

Assay of liver malondialdehyde, liver and serum vitamin E
and serum GOT and GPT activities. Liver malondialdehyde
level was determined by using the method described by
Ohkawa et al.\textsuperscript{13} utilising thiobarbituric acid. Liver and
serum vitamin E levels were both determined fluorometri-
cally. That is, the liver vitamin E level was determined by
the method of Taylor et al.\textsuperscript{14} and serum vitamin E level by
the method of Abe and Katsui.\textsuperscript{15} Serum GOT and GPT
activities were determined by using the kit system of
Amco. Inc. which is based on the method of Reitman-
Frankel and adapted for a Unimeter 300.

Histological and electron microscopical procedures. For
histological observations, blocks of tissue from the liver
were fixed in 10% formalin buffered with sodium phos-
phate. Paraffin embedded sections were stained with
hematoxylin and eosin. To stain the lipid, frozen sec-
tions were made from the fixed tissues of each experi-
ment and stained with Sudan III.

For electron microscopy, small blocks of the liver tissue
were fixed by immersion in cacodylate buffered 2.5%
glutaraldehyde solution and embedded in Epon. Thin
sections were cut with glass knives on a Porter–Blum
ultramicrotome, stained with 1% uranylacetate and lead
using Sato's method, and photographed in a JEOL-100CX
electron microscope at 80 kV.

Statistical analysis. The significance of the difference
between two groups of values was determined by Student's
t test.

RESULTS

Mortality and macroscopical state of the liver

In experiment 1, apparent disease occurred
in all the rats fed on the 4% casein, vitamin E
deficient diet which were remaining after 91
days on the test diet. The beginning of the
disease occurred between the 113th and 132nd
day of feeding on the experimental diet and the
average time they lived on test diet was 122
days. The disease began so suddenly that the
rats were eating and behaving normally the
day before they fell sick. The disease began
with loss of normal alacrity and with almost
complete loss of appetite.

All of the livers from the rats fed on the 4% casein,
vitamin E deficient diet for 68 and 91
days appeared to be macroscopically normal.
On the contrary, all of the livers from the
diseased rats appeared to be abnormal, that is,
they were enlarged and shiny, the surface and
also the cut surface of which were mottled with
the dark reddish parts and rather yellowish
parts.

In experiment 2, apparent disease also oc-
curred in all the rats remaining after 100 days
on the test diet. This time, the beginning of the
disease occurred between the 111th and 121st
day, and average time they lived on the test
diet was 117 days. The macroscopic state of
the liver from the diseased rats was similar to
that of experiment 1 and the appearance of the
complete animal and the liver of all of rats fed
on the 25% casein, vitamin E deficient diet
seemed to be normal when they were killed.

In experiment 3, apparent disease did not
occur in all the rats during 121 days of feeding
on the experimental diet. The state of the
animal seemed to be normal when they were
killed. The livers from the 4% casein, vitamin
E deficient diet fed rats seemed to be somewhat
enlarged, but those from the vitamin E sup-
plemented group seemed to be normal.

Body and liver weights

In Table II, values for final body weight,
final liver weight and liver weight to body
weight ratio of all the sacrificed rats are shown.

In experiment 1, it can be seen from this
Table that liver weight increased somewhat in
the diseased rats, and the differences between
diseased, 68 and 91 day fed groups are all
statistically significant. Consequently, the liver
weight to body weight ratio was also higher in
the diseased rats, and the differences between
diseased, 68 and 91 day fed groups are also all
statistically significant.
Table II. Body and Liver Weights

<table>
<thead>
<tr>
<th>Dietary conditions</th>
<th>Final body weight (g)</th>
<th>Final liver weight (g)</th>
<th>Liver weight x 100 Body weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial* (3)*</td>
<td>Experiment 1</td>
<td>174 ± 7c</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>4% Casein, - vitamin E, 68 days (3)</td>
<td>216 ± 10</td>
<td>6.7 ± 0.2</td>
<td>3.09 ± 0.14</td>
</tr>
<tr>
<td>4% Casein, - vitamin E, 91 days (3)</td>
<td>219 ± 4</td>
<td>6.8 ± 0.2</td>
<td>3.11 ± 0.14</td>
</tr>
<tr>
<td>4% Casein, - vitamin E, diseased (6)</td>
<td>208 ± 17</td>
<td>7.7 ± 0.5d</td>
<td>3.71 ± 0.26e</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (3)</td>
<td>Experiment 2</td>
<td>173 ± 10</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>4% Casein, - vitamin E, 62 days (4)</td>
<td>195 ± 8</td>
<td>5.9 ± 0.6</td>
<td>3.02 ± 0.24</td>
</tr>
<tr>
<td>4% Casein, - vitamin E, 100 days (4)</td>
<td>198 ± 5</td>
<td>6.3 ± 0.6</td>
<td>3.16 ± 0.25</td>
</tr>
<tr>
<td>4% Casein, - vitamin E, diseased (4)</td>
<td>194 ± 3</td>
<td>7.2 ± 0.4d</td>
<td>3.74 ± 0.20g</td>
</tr>
<tr>
<td>25% Casein, - vitamin E, 121 days (5)</td>
<td>351 ± 13a</td>
<td>11.1 ± 0.6a</td>
<td>3.15 ± 0.10</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4% Casein, - vitamin E, 121 days (2)</td>
<td>228 ± 2</td>
<td>7.3 ± 0.2</td>
<td>3.19 ± 0.12</td>
</tr>
<tr>
<td>4% Casein, + vitamin E, 121 days (4)</td>
<td>222 ± 13</td>
<td>6.7 ± 0.4</td>
<td>3.00 ± 0.10</td>
</tr>
</tbody>
</table>

* Fed until sacrifice with a laboratory stock diet made by Oriental Kobo Kogyo Co. (MF).
* Numbers in parenthesis represent number of killed animals.
* Mean ± Standard deviation.
* Significantly different from groups fed on the 4% casein, vitamin E deficient diet for 68 and 91 days (in the case of 68 days, $p<0.01$, and in the case of 91 days, $p<0.025$).
* Significantly different from groups fed on the 4% casein, vitamin E deficient diet for 68 and 91 days (in both cases, $p<0.01$).
* Significantly different from groups fed on the 4% casein, vitamin E deficient diet for 62 and 100 days (in the case of 62 days, $p<0.025$, and in the case of 100 days, $p<0.05$).
* Significantly different from groups fed on the 4% casein, vitamin E deficient diet for 62 and 100 days (in the case of 62 days, $p<0.005$, and in the case of 100 days, $p<0.025$).
* Significantly different from all other groups (in all cases, $p<0.001$).

In experiment 2, the values of liver weight and liver weight to body weight ratio were also higher in the diseased rats, and the differences between diseased, 62 and 100 day fed groups are all statistically significant. The value of liver weight to body weight ratio of rats fed on the 25% casein, vitamin E deficient diet was almost the same as that of rats fed on the 4% casein, vitamin E deficient diet for both 62 and 100 days.

In experiment 3, the final body weights of the two groups were almost the same, that is, the final body weight of the vitamin E supplemented group was no greater than that of the vitamin E deficient group. The liver weight of the 4% casein, vitamin E deficient group was somewhat lower than that of the 4% casein, vitamin E deficient group, but the difference is not significant.

Liver malondialdehyde level.

The values of liver malondialdehyde level are shown in Table III.

In experiments 1 and 2, the values for the diseased group were almost the same as those of the other groups. This means that the liver malondialdehyde level was not increased concomitantly with the occurrence of fatal liver lesion. Moreover, no gradual increase in malondialdehyde level in the 4% casein, vitamin E deficient diet fed rats was observed, but rather, the values of the 91 and 100 day test diet fed groups were slightly lower than those of the 68 and 62 day fed groups. The values of liver malondialdehyde level of all the 4% casein, vitamin E deficient diet fed rats and 25%.
Table III. Liver Malondialdehyde Concentration

<table>
<thead>
<tr>
<th>Dietary conditions</th>
<th>Liver malondialdehyde concentration (nmol MDA(^a)/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Initial(^b)</td>
<td>157.7 ± 32.6(^c,d)</td>
</tr>
<tr>
<td>4% Casein, -vitamin E, 68 days</td>
<td>360.2 ± 34.7</td>
</tr>
<tr>
<td>4% Casein, -vitamin E, 91 days</td>
<td>303.2 ± 31.8</td>
</tr>
<tr>
<td>4% Casein, -vitamin E, diseased</td>
<td>393.6 ± 87.8</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>156.5 ± 9.6(^e)</td>
</tr>
<tr>
<td>4% Casein, -vitamin E, 62 days</td>
<td>338.4 ± 31.3</td>
</tr>
<tr>
<td>4% Casein, -vitamin E, 100 days</td>
<td>282.0 ± 21.1(^f)</td>
</tr>
<tr>
<td>4% Casein, -vitamin E, diseased</td>
<td>341.1 ± 76.1</td>
</tr>
<tr>
<td>25% Casein, -vitamin E, 121 days</td>
<td>325.3 ± 30.5</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
</tr>
<tr>
<td>4% Casein, -vitamin E, 121 days</td>
<td>303.8 ± 18.0(^g)</td>
</tr>
<tr>
<td>4% Casein, + vitamin E, 121 days</td>
<td>154.6 ± 10.5</td>
</tr>
</tbody>
</table>

\(^a\) Abbreviation of malondialdehyde.

\(^b\) Fed until sacrifice with a laboratory stock diet made by Oriental Kobo Kogyo Co. (MF).

\(^c\) Mean ± Standard deviation.

\(^d\) Significantly different from all other test diet fed groups in experiment 1 (\(p < 0.005\) except 91 day fed group, in that case \(p < 0.01\)).

\(^e\) Significantly different from all other test diet fed groups in experiment 2 (\(p < 0.001\) except diseased group, in that case, \(p < 0.01\)).

\(^f\) Significantly different from groups fed on the 4% casein, vitamin E deficient diet for 62 days and 25% casein, vitamin E deficient diet for 121 days (in the former case, \(p < 0.025\), in the latter case, \(p < 0.05\)).

\(^g\) Significantly different from group fed on the 4% casein, vitamin E supplemented diet (\(p < 0.001\)).

Casein, vitamin E deficient diet fed rats increased about 2 fold compared with those of the initial control group.

In experiment 3, the value of liver malondialdehyde level of the 4% casein, vitamin E deficient diet fed group was about twice that of the 4% casein, vitamin E supplemented diet fed group, and the difference between the two groups is statistically highly significant.

Liver and serum vitamin E level

In Table IV, the values of liver and serum vitamin E level are shown.

In experiment 2, the values of both liver and serum vitamin E level decreased to about one third of that of the initial control group after 62 days feeding with the 4% casein, vitamin E deficient diet. Thereafter, a gradual decrease of both values occurred with the serum vitamin E level decreasing more rapidly. In particular, the value of serum vitamin E level for the diseased group decreased to about one half of that of the 100 day test diet fed group, indicating that the serum vitamin E decreased abruptly concomitant with the occurrence of massive liver necrosis. The value of serum vitamin E level of the 25% casein, vitamin E deficient diet fed group was a little higher than that of the 4% casein, vitamin E deficient diet fed groups, and the differences between these two groups were always significant. However, the value of liver vitamin E level for these two groups was almost the same, there being just a slight difference between this and the diseased group.

In experiment 3, the values of liver and serum vitamin E level of the 4% casein, vitamin E supplemented diet fed group were about five and three times those of the 4% casein, vitamin E deficient diet fed group respectively, the differences between two groups both being highly significant.
**Table IV. Liver and Serum Vitamin E Concentration**

<table>
<thead>
<tr>
<th>Dietary conditions</th>
<th>Liver vitamin E concentration (mg/100 g)</th>
<th>Serum vitamin E concentration (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>3.09 ± 0.29&quot;c</td>
<td>0.76 ± 0.06&quot;</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 68 days</td>
<td>1.07 ± 0.17</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 100 days</td>
<td>0.93 ± 0.11</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, diseased</td>
<td>0.80 ± 0.02&quot;d</td>
<td>0.09 ± 0.02&quot;</td>
</tr>
<tr>
<td>25% Casein, —vitamin E, 121 days</td>
<td>0.91 ± 0.05</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 121 days</td>
<td>0.68 ± 0.11&quot;f</td>
<td>0.17 ± 0.00&quot;</td>
</tr>
<tr>
<td>4% Casein, +vitamin E, 121 days</td>
<td>3.52 ± 0.39</td>
<td>0.53 ± 0.04</td>
</tr>
</tbody>
</table>

* Fed until sacrifice with a laboratory stock diet made by Oriental Kobo Kogyo Co. (MF).
* Mean ± Standard deviation.
" Significantly different from all other test diet fed groups in experiment 1 (in all cases, p < 0.001).
"d Significantly different from the group fed on the 4% casein, vitamin E deficient diet for 62 days (p < 0.025).
"e Significantly different from groups fed on the 4% casein, vitamin E deficient diet for 62 and 100 days and from the group fed on the 25% casein, vitamin E deficient diet (in all cases, p < 0.001).
"f Significantly different from the group fed on the 4% casein, vitamin E supplemented diet (p < 0.001).

**Table V. Serum GOT and GPT Activities**

<table>
<thead>
<tr>
<th>Dietary conditions</th>
<th>GOT Activity (Karmen units)</th>
<th>GPT Activity (Karmen units)</th>
<th>GOT/GPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>144 ± 45&quot;b</td>
<td>47 ± 15</td>
<td>3.05 ± 0.22</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 68 days</td>
<td>137 ± 24</td>
<td>67 ± 11</td>
<td>2.08 ± 0.49</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 91 days</td>
<td>157 ± 3</td>
<td>64 ± 5</td>
<td>2.45 ± 0.18</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, diseased</td>
<td>480 ± 26&quot;c</td>
<td>505 ± 53&quot;c</td>
<td>0.96 ± 0.09c</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>134 ± 16</td>
<td>64 ± 14</td>
<td>2.13 ± 0.23</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 62 days</td>
<td>157 ± 18</td>
<td>70 ± 9</td>
<td>2.25 ± 0.13</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 100 days</td>
<td>206 ± 31&quot;d</td>
<td>67 ± 5</td>
<td>3.08 ± 0.47e</td>
</tr>
<tr>
<td>4% Casein, —vitamin E, diseased</td>
<td>543 ± 38&quot;f</td>
<td>590 ± 12&quot;f</td>
<td>0.92 ± 0.05f</td>
</tr>
<tr>
<td>25% Casein, —vitamin E, 121 days</td>
<td>216 ± 64</td>
<td>77 ± 15</td>
<td>2.77 ± 0.46</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4% Casein, —vitamin E, 121 days</td>
<td>183 ± 11&quot;g</td>
<td>82 ± 3&quot;h</td>
<td>2.23 ± 0.21</td>
</tr>
<tr>
<td>4% Casein, +vitamin E, 121 days</td>
<td>100 ± 7</td>
<td>47 ± 14</td>
<td>2.23 ± 0.54</td>
</tr>
</tbody>
</table>

* Fed until sacrifice with a laboratory stock diet made by Oriental Kobo Kogyo Co. (MF).
* Mean ± Standard deviation.
*c Significantly different from all other groups (in all cases, p < 0.001).
"d Significantly different from the initial control group and from the group fed on the 4% casein, vitamin E deficient diet for 62 days (p < 0.025, in the former case, p < 0.05).
"e Significantly different from the initial control group and from the group fed on the 4% casein, vitamin E deficient diet for 62 days (in both cases, p < 0.025).
"f Significantly different from all other groups (in all cases, p < 0.001).
"g Significantly different from the group fed on the 4% casein, vitamin E supplemented diet (p < 0.001).
Serum GOT and GPT activities

In Table V, the values of serum GOT and GPT activities are shown.

In experiments 1 and 2, the serum GPT activity of rats fed on the 4% casein, vitamin E deficient diet was constant until the 91st or 100th day, increasing abruptly at the onset of disease. For GOT activity, this abrupt increase was also observed in rats fed with the 4% casein, vitamin E deficient diet at the onset of disease, but contrary to the case of GPT, a gradual increase was seen even before the onset of disease in experiment 2. Consequently, the ratio of GOT activity divided by GPT activity increased gradually until the 100th day, and then decreased abruptly from the onset of disease. For all values, the differences between the diseased and all other groups are all statistically highly significant.

In experiment 3, both the GOT and GPT activities were higher in the 4% casein, vitamin E deficient diet fed group than those of the 4% casein, vitamin E supplemented diet fed group, the differences between the two groups being statistically significant.

Histological and electron microscopical observation

In experiments 1 and 2, the liver tissues of the 0 day control animals and 25% casein, vitamin E deficient diet fed group were quite similar. The hepatic cell cord structure was well preserved and each hepatic parenchymal cell showed a compact cytoplasm in which granular structures were evenly distributed (Fig. 1).

The liver of rats killed on the 62nd day on the 4% casein, vitamin E deficient diet showed slight to moderate swelling of the parenchymal cells and the hepatic cell cord structure became indistinct. In the cytoplasm, some vacuolar pale eosinophilic areas appeared to displace the granular structures. When fixation of the...
Fig. 7. Electron Micrograph of the Yellow Part of the Diseased Rat Liver.
The cytoplasm contains numerous lipid droplets mixed with mitochondria, lysosome, endoplasmic reticulum etc. The droplets are relatively small in size and have a boundary membrane. Uranylacetate and lead stain. × 5940 (Exp. 2).

Tissues was inadequate, the cytoplasm of these cells appeared clearer or foamy (Fig. 2).

The livers of rats killed on the 100th day of the test diet showed basically the same appearance as those of the 62nd day killed animals, but swelling of the hepatic cells was more prominent (Fig. 3).

The livers of diseased rats fed on the 4% casein, vitamin E deficient diet revealed extensive loss of liver cells. Red and yellow parts were mixed in the liver from diseased rats, as previously described. The red part of these organs showed massive confluent necrosis and loss of the parenchymal cells extending from the centrilobular areas toward the peripheral areas. Massive hemorrhaging and some neutrophil infiltration were seen in the affected areas. A small number of living cells were preserved in insular fashion only in the perportal areas (Fig. 4). In the yellow part occasional necrosis was seen around the central veins, but this was less severe than that in the red part. In the non-necrotized areas many liver cells showed degenerative changes. On the other hand, regenerative activity manifested by parenchymal cell mitosis was quite prominent.

In experiment 1, the histological features of the liver were quite similar to those of experiment 2. In experiment 3, the livers of the two groups showed almost the same appearance and resembled those of rats killed on the 62nd or 100th day of the test diet in experiment 2, that is, the influence of vitamin E addition was not observed histologically. No necrosis of the parenchymal cells was found.

Sudan III stained sections of 0 day control animals showed a small amount of fat mainly at the peripheral zone of the liver lobules, and the central zone was not stained (Fig. 5A). Most of these tiny fat droplets were located in the peripheral part of the liver cells adjacent to
the Disse's space (Fig. 5B).

The livers of rats killed on the 100th day of feeding on the 4% casein, vitamin E deficient diet showed accumulation of a large amount of sudanophilic droplets diffused throughout the lobule (Fig. 6A). These droplets were mainly located in the hepatic cell cytoplasm and their size was relatively small (Fig. 6B).

The liver of rats killed on the 62nd day showed fat droplets similar to the 100th day rats, but these were seen mainly in the central zone of the lobules.

Sudan III stained sections of the yellow part of the diseased rat liver showed a quite similar appearance to those of 100th day animals, but in those of the red part, the sudanophilic materials had disappeared.

The electron micrograph shown in Fig. 7 represents the yellow part of the diseased rat liver. The liver cell contained many vacuolar structures in the cytoplasm and the size of these measured about 2 μm or less which was comparable with that of mitochondria. Occasionally they had a boundary membrane or fragments of this membrane within the droplets.

**DISCUSSION**

From the results of experiments 1 and 2, an increase of liver malondialdehyde level was not observed when massive liver necrosis occurred in the 4% casein, vitamin E deficient diet fed rats. Moreover, in experiment 2, the liver malondialdehyde level of the 4% casein, vitamin E deficient diet fed rats did not increase compared with that of the 25% casein, vitamin E deficient diet fed rats which never developed fatal liver disease.

Meanwhile, the liver and serum vitamin E level decreased gradually in the 4% casein, vitamin E deficient diet fed rats. Particularly, serum vitamin E level decreased markedly at the onset of massive liver necrosis. This could mean that at the beginning of this disease, certain processes requiring consumption of large amounts of vitamin E were taking place.

These results for the present experiments show that the liver malondialdehyde level did not increase despite the gradual decrease in liver and serum vitamin E. And, from these results, as a reason for the occurrence of massive liver necrosis in rats fed the 4% casein, vitamin E deficient diet containing lard, an acceleration of the peroxidation of liver lipids accompanying an increase of malondialdehyde level could not be considered. Bunyan et al.17) by titration of the tissue lipid peroxide iodometrically in rats developing liver necrosis due to a deficiency of selenium and vitamin E, also found no relationship between tissue lipid peroxide and liver necrosis. Moreover, from the fact that a small amount of vitamin E still remained in the liver and serum even when massive liver necrosis occurred, a decrease of vitamin E level could not be the primary reason for the occurrence of this disease.

In experiment 2, in the 4% casein, vitamin E deficient diet fed rats, serum GOT activity increased gradually but GPT activity was constant until the beginning of massive liver necrosis. GOT distributes widely in various organs such as the heart and skeletal muscle as well as in the liver, whereas GPT exists mainly in the liver. Therefore, the fact that GOT activity gradually increased but GPT activity was constant means that organs other than the liver would be damaged preceding the occurrence of massive liver necrosis.

From histological observations, the hepatic cells of rats fed with the low casein, vitamin E deficient diet showed swelling of the cytoplasm as an initial change. This was found in all animals fed on the test diet for 62 to 121 days as long as they behaved healthily. The cytoplasmic pale areas are thought to be attributed to the presence of lipid-containing droplets. From the facts that these droplets showed a boundary membrane and their size was relatively small, these droplets would be different from the true lipid inclusion commonly seen in the cytoplasm. The exact nature of these droplets is not known at present, and some organelles such as mitochondria, lysosome or endoplasmic reticulum might have some relationship to their pathogenesis.
From a clinical point of view, massive necrosis of the liver is seen most often in severe viral hepatitis and in toxic or drug-induced hepatitis. In an other case, with venous congestion centrlobular confluent necrosis, hemorrhaging and dilatation of the sinusoids are well documented pathological features. In the present experiments, there was little inflammatory reaction throughout the liver sections examined. Although the cardiovascular system was not studied in the present experiments, the centrlobular occurrence of confluent necrosis is suggestive of venous congestion due to cardiac failure rather than viral hepatitis. Also, the swollen state of the hepatic cells or an accumulation of lipid droplets which started from the central zone might be more susceptible to the relatively anoxic condition of this area.

If damage to the heart muscle occur primarily, the resulting cardiovascular disease might be an additional reason for the sudden occurrence of massive liver necrosis. There are some reports concerning the clinical or pathological evidence of myocardial disease on experimental animals by vitamin E deficiency.\(^1\) Also, it was reported by Wilson \textit{et al}.\(^1\) that large amounts of dietary vitamin E inhibited atherogenesis by preventing hypercholesterolemia in rabbits fed the low cholesterol atherogenic diet.

In the present experiments, the fact that lard was used as the only dietary lipid source would be of primary concern for the occurrence of massive liver necrosis. Two reasons for this hypothesis can be considered. Firstly, linoleic acid intake was low in addition to low protein intake and vitamin E deficiency. Up to the present, the reasons for the occurrence of massive liver necrosis were considered in relation to deficiencies of sulfur containing amino acid, vitamin E and selenium. However, linoleic acid might have some protective action for the occurrence of the disease, and when lard was used, it was induced without much acceleration of lipid peroxidation. As another reason, when lard was used, the intake of a considerable amount of saturated fatty acid might have had some influence on the occurrence of the disease, because saturated fatty acid intake accelerates heart failure.

Moreover, the occurrence of cardiac malfunction by malnutrition has been reported,\(^2\) and it has also been reported by Kyger \textit{et al}.\(^2\) that in rats, protein deficiency results in decreased myocardial contractility. Therefore, in the present experiments, a low protein and low linoleic acid intake would together accelerate cardiac malfunction leading further to the occurrence of massive liver necrosis.

However, in experiment 3, an apparent decrease in liver malondialdehyde level was observed in rats fed with the 4\% casein, vitamin E supplemented diet. This could mean that large amounts of vitamin E would prevent the formation of malondialdehyde even when lard was used as the only dietary lipid source. In experiment 3, although massive liver necrosis did not occur with the low casein, vitamin E deficient diet fed rats during the 121 day feeding period, considering the fact that an increase in serum GOT and GPT activities was observed, some hepatic damage would be occurring in these rats when they were sacrificed. The reason why in experiment 3, apparent massive liver necrosis did not occur in these rats is not clear. Although vitamin free casein purchased from Oriental Kobe Kogyo Co. was used throughout the experiments, the selenium content in it might not be identical for different materials, and such a factor might be the reason.

REFERENCES

7) M. G. Deo, S. K. Sood and V. Ramalingaswami, \textit{Arch. Pathol.}, 80, 14 (1965).


