Rabbits were fed a vitamin E-deficient diet for 7 weeks. Control rabbits, paired for weight, were pair-fed an identical diet supplemented with vitamin E. After 3 weeks the experimental animals showed a rise in serum creatine kinase activity which was attributable to the muscle isoenzyme (MM). No rise in creatine kinase activity or appearance of MM was noted in the serum of the control rabbits. Total creatine kinase activity and MM activity were reduced in the gastrocnemii of the experimental animals while the activities of the brain (BB) and hybrid (MB) isoenzymes were increased. The specific activity of BB based on immunochemically determined BB protein was not different from normal in either the experimental or control group. Activation of pre-existing inactive BB is probably not the explanation for increased BB activity in the gastrocnemii of the experimental group.

Vitamin E deficiency produces a type of muscular dystrophy in rabbits (1). The etiology and pathogenesis of the dystrophy are unknown but the rise in serum creatine kinase activity appears to be a sensitive indicator of the onset of the disease (2). It is not known which isoenzymes of creatine kinase are responsible for the elevated serum creatine kinase activity.1 In the dystrophic rabbit muscle the activities of the brain isoenzyme (BB) and the hybrid isoenzyme (MB) are raised (3). This is reminiscent of the early fetal pattern in normal skeletal muscle before the muscle isoenzyme (MM) activity predominates (4). A similar resemblance to fetal muscle has also been made in denervated rabbit muscle (4) and in human muscular dystrophy (5).

The regulating mechanisms responsible for these changes in creatine kinase isoenzymes are unknown. In the preceding publication (6) we have demonstrated the presence of an inactive BB isoenzyme in normal rabbit and human skeletal muscle. We now report changes in BB activity in relation to BB protein as measured in dystrophic muscle of the vitamin E-deficient rabbit.

EXPERIMENTAL PROCEDURES

The following procedures were used as described previously: creatine kinase assay (7), cellulose acetate electrophoresis of creatine kinase isoenzymes (6), and immunochemical quantitation of BB protein (6).

**Vitamin E Deficiency in Rabbit** Initially, 14 female California white rabbits weighing 0.9 to 1.4 kg were paired by weight and five pairs were randomly selected. Each member of a pair was then randomly assigned to either the experimental or pair-fed control group. The remaining four animals served as a further control group.

Blood was obtained from the marginal ear veins of the animals. Serum was collected following centrifugation of the clotted blood at 2000 × g for 15 min. It was frozen and stored at -20° in 0.5-ml aliquots.

All animals were fed ad libitum for 8 days with normal rabbit chow. During this period their serum creatine kinase activity was measured on 3 different days. The serum isoenzyme pattern was also examined on these occasions. Daily body weights were recorded.

The vitamin E-deficient and control diets were prepared in pellet form to our specifications by Nutritional Biochemicals Corp. (Cleveland, Ohio). The composition of the experimental diet was as follows: casein (vitamin-free), 25%; sucrose, 47.5%; cellulose flour, 15%; cod liver oil, 3%; salt mixture (Rogers and Harper, 1965), 5%; vitamins A and D (E omitted) 4%; m-methionine, 0.5%.

Once the experiment had commenced, the control animals were pair-fed to the experimental ones. The four additional animals ate control diet ad libitum. Daily body weights and food consumption were measured for all animals. Every week serum creatine kinase activity was measured and examined qualitatively by cellulose acetate electrophoresis. The experimental period lasted 60 days at which time all animals were killed by an intravenous overdose of sodium pentobarbital.

**Tissue Extracts** — Gastrocnemius, heart, and brain were removed from the animals. These tissues were frozen and stored at -20°. After thawing they were trimmed free of fat and connective tissue. Homogenization was performed at 2% in 0.01 M Tris/acetate (pH 7.2) containing 0.014 M 2-mercaptoethanol. A Sorvall Omni-Mixer (Ivan Sorvall Inc., Newtown, Conn.) was used to homogenize muscle and a glass tube with motor-driven Teflon pestle was used for brain. All investigations were conducted on the tissue supernatant (10,000 × g for 30 min at 2°).

**Protein Assay** — The overnight solubilization of tissue homogenate protein in 0.5 N NaOH (10 volumes), as described by Lilienthal et al. (8), was performed prior to protein determination by an automated version of the procedure described by Lowry et al. (10).

**RESULTS**

Clinical and Pathological Aspects of Muscular Dystrophy in Rabbits Fed Diet Deficient in Vitamin E — When the rabbits

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first arrived in the laboratory, many of them had a high serum creatine kinase activity which fell sharply during the next few days. All this creatine kinase activity was attributable to the BB isoenzyme.

Before the experimental (vitamin E-deficient) and control diets were introduced on Day 9, all rabbits showed a brisk weight gain when maintained on normal rabbit chow ad libitum (Figs. 1 and 2). On Day 9, there was no significant difference according to the paired t test (p > 0.4) between the mean body weights of the experimental and control groups.

Rabbits eating either the experimental or control diets required about 10 days to "develop a taste" for their food. During this period, they all showed a mild to moderate weight loss (Figs. 1 and 2). As soon as their daily food consumption increased, they gained weight again, albeit at a slower rate (Figs. 1 and 2). According to the paired t test, there were no significant differences in either total final weight gain or average daily weight gain between the experimental rabbits and those eating control diet ad libitum.

After 3 to 5 weeks on the vitamin E-deficient diet, the rabbits in the experimental group showed a variable but sustained rise in serum creatine kinase activity (Table I and Fig. 1). This rise in creatine kinase activity was correlated in each animal with the appearance of the MM isoenzyme in the serum creatine kinase electrophoretogram (Table I and Fig. 3). MM isoenzyme never appeared in the serum of animals in either of the control groups. An isolated rise in serum creatine kinase activity (500 to 1000 units/liter) owing to the BB isoenzyme was seen in two of the pair-fed controls and in two of the rabbits eating control diet ad libitum in the remaining control animals, serum creatine kinase activity was consistently below 600 units/liter (Fig. 2). At the end of the study, serum creatine kinase was markedly elevated in all animals receiving the vitamin E-deficient diet (Table I) and in each case the elevation was attributable to the MM isoenzyme. Muscle weakness was inapparent.

Striking histopathological differences were noted between the gastrocnemii of a rabbit pair that was fed the vitamin E-

![Graph of body weight and serum creatine kinase (CK) activity for rabbit (No. 3, Table I) fed vitamin E-deficient diet ad libitum from Day 9 (arrow). Prior to Day 9, diet was normal rabbit chow. Note high serum creatine kinase activity upon arrival in laboratory. This was due to BB isoenzyme. Subsequent rise (Day 29) was owing to MM isoenzyme. Transient fall in body weight upon commencement of experimental diet was related to temporary decreased food intake.](http://www.jbc.org/)

![Graph of body weight and serum creatine kinase (CK) activity for rabbit pair-fed with control diet to rabbit discussed in Fig. 1. Prior to Day 9, diet was normal rabbit chow. Note similar transient fall in body weight upon commencement of pair-feeding (arrow). Serum creatine kinase activity (BB) was elevated on Day 1 but no further increases were noted in contrast to rabbit fed vitamin E-deficient diet.](http://www.jbc.org/)

![Cellulose acetate electrophoresis of creatine kinase isoenzymes in serum of rabbit (No. 2, Table I) fed vitamin E-deficient diet ad libitum over 7-week interval. MM isoenzyme appeared after 4 weeks on diet and was more apparent thereafter as serum creatine kinase activity increased. BB activity did not seem to change appreciably. Pink staining in MM region was noted on control strip (phosphorylcreatine omitted from enzyme stain) throughout and accounted for all apparent MM activity after 1, 2, and 3 weeks on the diet.](http://www.jbc.org/)
deficient and control diets for 6 weeks. A cross-section of muscle from the control animal (Fig. 4) shows normal myofibers and muscle architecture. By contrast (Fig. 5) dystrophic changes were seen in the muscle of a rabbit eating the vitamin E-deficient diet for the same period of time. The essential pathological features of the dystrophic muscle are variation in fiber size, central nuclei, fiber necrosis, and phagocytosis and increased connective tissue.

Creatine Kinase Isoenzymes in Tissues of Dystrophic Rabbits - The electrophoretic patterns of creatine kinase isoenzymes seen in the brain, heart, and gastrocnemius of a dystrophic rabbit and its pair-fed control counterpart are seen in Fig. 6. In dystrophic gastrocnemius there was a conspicuous increase in the activity of MB and BB. For brain and heart there were no significant differences in the specific activity of creatine kinase or any of its isoenzymes when the experimental rabbits were compared to the control group (Table II). However, several interesting results were apparent when this comparison was made for gastrocnemius (Table II). Creatine kinase specific activity was reduced in the dystrophic muscle. This was due to a reduction in the specific activity of MM as the specific activities of both MB and BB were increased (Table II). These changes were not related to a difference in

![Cellulose acetate electrophoresis of creatine kinase isoenzymes in tissues of vitamin E-deficient rabbit and its pair-fed control.](image)

**FIG. 6.** Cellulose acetate electrophoresis of creatine kinase isoenzymes in tissues of vitamin E-deficient rabbit and its pair-fed control. 1, brain, 3, heart, and 5, gastrocnemius of vitamin E-deficient animal; 2, brain, 4, heart, and 6, gastrocnemius of control. Note increased MB and BB activity in gastrocnemius of vitamin E-deficient animal. MM activity is also decreased in this tissue (see also Table II).

**TABLE II**

Enzymatic specific activities of creatine kinase and its isoenzymes in extracts of pair-fed control and dystrophic rabbit tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total creatine kinase</th>
<th>MM Specific activities</th>
<th>MR Specific activities</th>
<th>BR Specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dystrophic</td>
<td>Control</td>
<td>Dystrophic</td>
</tr>
<tr>
<td>Heart</td>
<td>13,800 (1,400)</td>
<td>13,400 (1,400)</td>
<td>12,400 (1,100)</td>
<td>11,700 (1,100)</td>
</tr>
<tr>
<td></td>
<td>p &gt;0.6</td>
<td></td>
<td>p &gt;0.2</td>
<td>&gt;0.6</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>43,800 (3,200)</td>
<td>32,700 (3,200)</td>
<td>43,600 (3,200)</td>
<td>31,800 (4,100)</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Brain</td>
<td>4,800 (190)</td>
<td>5,000 (190)</td>
<td>0 (190)</td>
<td>0 (190)</td>
</tr>
<tr>
<td>p</td>
<td>&gt;0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Values are means ± standard errors for five pairs of rabbits. Activities are expressed per g of protein in the tissue homogenate.
b Probability, according to the paired t test, that the observed differences are due to random variation. Because variances for control and dystrophic animals with respect to MB in gastrocnemius were significantly different (p < 0.05), paired t test was performed on a logarithmic transformation of these data only.
active and inactive brain creatine kinase in dystrophic muscle

Creatine kinase isoenzyme activity was assayed as units per g of protein in the tissue homogenate. The noncollagenous protein content (6) of control and dystrophic muscles did not differ, thus variations of the activity of the creatine kinase isoenzymes were not simply due to changes in the amount of protein in the muscle. No changes in the specific activities of the creatine kinase isoenzymes were apparent in the heart or brain of the dystrophic rabbits. The disease seemed to be selective for skeletal muscle.

We noted a 3-fold increase of total immunoreactive BB in the dystrophic gastrocnemius. Both active and inactive BB appeared to be increased as the specific enzymatic activity of BB based on immunoreactive BB was not different from that found in normal rabbits (6). Thus, it is unlikely that increased BB activity was related to activation of pre-existing inactive BB protein. If M subunits combined with inactive or active B subunits, then when levels of M are decreased due to leakage there would be less of the hybrid isoenzyme and inactive, as well as active BB would increase. However, while we found these increases in BB protein, the levels of MB also increased. We therefore believe the change in BB protein is a primary one. It may not be the defect in muscular dystrophy but it is worthy of further study since it might lead to a better understanding of the underlying disease process.

Acknowledgment—We gratefully acknowledge the technical assistance of Felicity Howard.

Table III

<table>
<thead>
<tr>
<th>A</th>
<th>BB isoenzyme of creatine kinase in pair-fed control and dystrophic rabbit gastrocnemius</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
</tr>
<tr>
<td>Control</td>
<td>Dystrophic</td>
</tr>
<tr>
<td></td>
<td>units/g</td>
</tr>
<tr>
<td>x</td>
<td>0</td>
</tr>
<tr>
<td>(88)</td>
<td>(0.1)</td>
</tr>
</tbody>
</table>

* Values are means ± standard errors for five pairs of rabbits. Activities are expressed per g of protein in the tissue homogenate.

REFERENCES

Discussion
The dystrophic rabbits showed a rise in serum creatine kinase activity which was entirely attributable to the MM isoenzyme. Elevated serum creatine kinase activity is widely recognized as a manifestation of various human muscular dystrophies and it is the MM isoenzyme which is generally increased (5). Other muscle enzymes frequently show high activities in the serum (e.g. fructose-1,6-diphosphate aldolase and glutamic oxalacetic transaminase) (11). The mechanism of enzyemia is unknown but a leakage of muscle enzymes in muscular dystrophy has been postulated (12, 13), perhaps on the basis of decreased ATP (14). MM specific activity was about 25% reduced in the gastrocnemius of our dystrophic rabbits. The resulting effects on muscle cell energy metabolism could ultimately be responsible for muscle cell necrosis which is a histopathological hallmark of muscular dystrophy.

MB and BB specific activities were increased significantly in the dystrophic gastrocnemius. This resemblance to the fetal isoenzyme pattern is not unique for creatine kinase. Dreyfus et al. (15) reported similar findings for isoenzymes of lactate dehydrogenase in pathological muscle.

MB and BB protein, measured immunohistochemically, was increased 3-fold in dystrophic gastrocnemius (Table III). When BB activity was expressed as units per antigen unit, it was essentially no different from the values reported previously (6) for normal rabbit heart and gastrocnemius.

Acknowledgment—We gratefully acknowledge the technical assistance of Felicity Howard.

p<0.05

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Brain isoenzyme of creatine kinase. III. Active and inactive forms in dystrophic muscle of vitamin E-deficient rabbit.
J B Armstrong, J A Lowden and A L Sherwin


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