THE CREATINE CONTENT OF THE LIVER IN THE MUSCULAR DYSTROPHY OF VITAMIN E DEFICIENCY

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Creatinuria is one of the results of vitamin E deficiency. This was first demonstrated by Morgulis and Spencer (1) on rabbits. The effectiveness of α-tocopherol in reducing the excretion of creatine to normal levels was shown by Mackenzie and McCollum (2) and has been verified by many others. The creatine lost from muscle tissue during dystrophy (3) undoubtedly accounts for the creatinuria, and the administration of α-tocopherol prevents this loss (4). Biopsy experiments on dystrophic rabbits (5) indicated that the restoration of normal muscle creatine content, after the administration of tocopherol phosphate by vein, was delayed some hours as compared with relatively prompt reduction of the high oxygen consumption. This latter dropped in about 4 hours, whereas the already low creatine content was initially still further reduced. A suggested explanation for these changes was that the loss of creatine from muscle continued, while a previously accelerated process of synthesis had already been depressed.

There is no proof that the synthesis of creatine is accelerated in dystrophic animals, but the high rate of excretion would quickly exhaust the existing stores unless these were being replenished at a greater than normal rate.¹

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¹ No direct evidence is available on this question. From existing data, only an approximate calculation can be made. If the creatine content of normal rabbit muscle is 0.5 per cent, the total body creatine of an animal weighing 1 kilo would be 1000 × 0.4 × 5 mg., or 2000 mg. ((6) p. 494). The excess creatine excretion of a dystrophic rabbit ((2) p. 354) over a period of 27 days was approximately this same amount. If the dystrophic muscles still contained 1280 mg. (800 × 0.4 × 4 mg.) of creatine ((7) p. 304), this amount had to be manufactured during the 27 days, in addition to that which continued to be excreted as creatinine rather constantly throughout the period. If urinary creatinine has its origin in the phosphocreatine of muscle (6), the creatinine excretion of dystrophic animals should be reduced. Since it is not, the prior transformation of creatine to phosphocreatine may be lacking or incomplete in these animals. In dystrophic muscle the decline of phosphocreatine seems to be greater than that of creatine (8). Coupled phosphorylation of creatine has, in fact, been shown to be greatly diminished in homogenates of dystrophic muscles from guinea pigs and hamsters (9), but this could not be demon-
The liver is apparently the principal site of final creatine synthesis (10). If the production of creatine is accelerated in vitamin E deficiency, a higher concentration of it might be expected in the liver, even though this organ may not store it extensively. In any case, the active participation of the liver in the fluctuations of creatine can hardly be excluded.

It seemed important, therefore, to examine into the rôle of the liver in the alterations of creatine metabolism found in vitamin E deficiency. A beginning was made by determining the creatine (and creatinine) content of liver tissue from normal rabbits and rats and from these animals after various periods of maintenance on vitamin E-deficient diets. Delayed and immediate effects of the administration of α-tocopherol acetate or phosphate were also studied.

EXPERIMENTAL

The liver contains large amounts of chromogens other than creatinine which respond to the Jaffé test. Since absolute and not relative values for creatine were desired, the specific enzymatic method of Miller, Allinson, and Baker (11) was chosen, even though it is cumbersome and has been abandoned by some (12–14).

Lloyd's reagent was found to be unsatisfactory for the separation of creatinine from other chromogens found in autoclaved liver; it was non-specific and variable in its behavior. In addition to removing creatinine, it adsorbed other Jaffé-positive chromogens and additional colored compounds. Shaking 25 mg. of the reagent with 6 ml. samples of liver filtrate for 2 minutes removed as much as 40 per cent of the color. Incubating the filtrate with bacteria removed no color. Furthermore, the amount of chromogen removed by Lloyd's reagent was variable, from a fraction to several times the amount of creatinine removed from the same filtrate by bacterial digestion. After longer periods of shaking, some of the color and chromogen originally adsorbed by Lloyd's reagent returned to the solution. This is not a new observation.

The suspensions of Corynebacterium creatinovorans (NC)² were prepared³ and assayed according to the recommended procedure (11).

Minor changes were made in the protein precipitation preceding creatinine estimation, and in the order of color development. The proteins were precipitated by the acid cadmium reagent as used in the original method of Fujita and Iwatake (15). A simplification was introduced by

strated with rabbit muscle. A study of creatine-creatinine excretion as related to muscle creatine and phosphocreatine in dystrophic animals is greatly needed.

² Obtained from the American Type Culture Collection.
³ The help of Dr. J. R. Porter and the Department of Bacteriology in culturing the bacteria is gratefully acknowledged.
adding at one time all the sodium hydroxide required to precipitate the cadmium. This had no effect upon the completeness of protein precipitation or on the final creatinine values. In the modified procedure, 8 ml. of acid cadmium reagent were added to the tissue extract, followed by 1.5 ml. of 1.1 N sodium hydroxide. The mixture was allowed to stand 5 minutes before filtering; the filtrate was used directly for color development or incubation. With large samples of liver, the glycogen extracted gave cloudy solutions which were not clarified by the cadmium treatment. A Klett-Summerson photoelectric colorimeter was used with filter No. 54.

Since in liver tissue the true creatine and creatinine usually constitute only 5 to 25 per cent of the total chromogen, the difference between the chromogen content before and after bacterial digestion is very small.

With liver filtrates the intensity of the color with picrate was found to increase for at least 45 minutes, whereas with pure solutions of creatinine maximum color development is attained in 12 minutes. In order to have color development simultaneous on each sample of original and incubated filtrates and on a blank, the addition of alkaline picrate was properly timed for reading after exactly 12 minutes. Notwithstanding these precautions, an occasional liver filtrate contained more chromogen after digestion than before, possibly as a result of bacterial synthesis. When this happened, the results were discarded.

Rabbits weighing 800 to 1000 gm. were maintained on the same vitamin E-deficient diet as was used heretofore (7), except that the carbohydrate was sometimes glucose only. After about 1 month on this diet, or less, when an animal was unable to rise after being placed on its side, it was killed by stunning; the liver and gastrocnemii, or portions of them, were weighed wet, minced, and analyzed for creatine and creatinine. The average weights of liver used for single creatine and creatinine estimations were about 2 and 8 gm. respectively, of muscle tissue about 0.5 and 2 gm. respectively. Control rabbits were fed the same vitamin E-deficient diet, with a supplement of 10 to 15 mg. per week of dl-α-tocopherol acetate given by mouth in olive oil.

The results of these analyses are given in Table I. In agreement with the observations of many workers not using the specific enzymatic method, the muscle of vitamin E-deficient animals contained much less creatine than is normally present. The amount of true creatine in the liver of normal rabbits is in the range of that reported for rats and dogs (16). No prior figures seem to be available for the rabbit. Liver tissue from vitamin E-deficient rabbits showed an appreciable accumulation of creatine, 2 to 6 times the amount in the livers of control animals. This increase was

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4 dl-α-Tocopherol acetate and α-tocopherol phosphate disodium salt were generously supplied by Hoffman-La Roche, Inc.
not in total chromogen but only in creatine. The characterization of the degree of severity of dystrophy is rather uncertain at best; this lack of any adequate criteria probably accounts for the great variability in the liver creatine figures. The creatinine content of both liver and muscle was in reasonable agreement with the figures for rats (16), and was so small that its determination was not continued.

A more direct method of studying the effect of vitamin E on creatine production in the liver was obviously desirable and biopsy experiments were therefore undertaken. Vitamin E-deficient rabbits were used for biopsy experiments when dystrophy was well developed. Control animals received the same diet plus 5 mg. of dl-α-tocopherol acetate in olive oil daily.

**Table I**

**True Creatine and Creatinine in Muscle and Liver of Rabbits**

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Creatine (mg./100 gm.)</th>
<th>Creatinine (mg./100 gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle</td>
<td>Liver</td>
</tr>
<tr>
<td>Vitamin E-deficient</td>
<td>245</td>
<td>42.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>107</td>
<td>11.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>292</td>
<td>37.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>128</td>
<td>34.3</td>
</tr>
<tr>
<td>&quot;</td>
<td>148</td>
<td>46.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>139</td>
<td>15.0</td>
</tr>
<tr>
<td>Control</td>
<td>426</td>
<td>10.3</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average of duplicates from later biopsy experiments.

Anesthesia for the duration of the operation was provided by divided doses of sodium amytal given intraperitoneally. Amytal appeared to have no effect on liver creatine. Severe hemorrhage was avoided by removing liver samples with electrosurgery, and by the use of thrombin solution. After removal of an initial sample through a mid-line incision, 10 to 25 mg. of disodium-α-tocopherol phosphate in distilled water were injected into the femoral vein. Further liver samples were removed at intervals of 3 to 4 hours. The incision was kept closed and the animal was covered and kept warm. Each liver specimen of 0.5 to 2 gm. was immediately minced, divided into two portions, weighed, and autoclaved with acid for creatine analysis by the enzymatic method. Large stock animals were easily main-

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5 Apparatus kindly loaned by Dr. W. R. Ingram, Department of Anatomy.

6 Generously supplied by Dr. H. P. Smith, Department of Pathology.
tained in excellent condition under anesthesia for 12 hours, during which as many as nine samples of liver could be removed. Dystrophic rabbits, however, lived no longer than 4 hours after the injection of tocopherol phosphate. Their smaller size and weakened condition were probably responsible; a contributory factor may have been too large doses of tocopherol phosphate.

Table II shows the effect of the intravenous administration of \( \alpha \)-tocopherol phosphate upon liver creatine. The changes were not significant, except in one animal, whose high value of 65 mg. per cent had dropped to about 40 mg. per cent 3 hours after the injection of 10 mg. of the phosphate.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Tocopherol phosphate injected</th>
<th>Initial creatine</th>
<th>Creatine after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>Vitamin E deficient</td>
<td>25</td>
<td>15.4</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td>18.9</td>
<td>28.8</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>31.5</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td>20.9</td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>41.4</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>34.1</td>
<td>32.9</td>
<td>37.7</td>
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<tr>
<td></td>
<td>10</td>
<td>65.5</td>
<td>36.6</td>
</tr>
<tr>
<td></td>
<td>64.0</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>43.5</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>3.5</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6.2</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

In another, a large dose (25 mg.) of tocopherol phosphate was followed by an increased creatine level during the first 2 hours, followed by a decline toward the initial value. In this case the initial creatine level was low, perhaps implying a less severe dystrophic condition. Unequal distribution of creatine between the different lobes is another possible variable which could not be controlled. Apparently any effects of tocopherol injection cannot be expected in less than 3 or 4 hours, and unless the deficient animals can be made to survive for a longer time, the approach by the technique of biopsy is not promising. In control animals, the effect of the injections was indifferent and not significant.

With some hesitation, attempts were made to demonstrate the effects of \( \alpha \)-tocopherol acetate on the creatine content of the livers from vitamin
E-deficient rats. Some of the animals had been on the deficient diet for more than a year and were almost completely paralyzed. Other younger animals showed only slight disability in the use of the hind legs, and still others showed few if any external symptoms. Doses of \( \alpha \)-tocopherol acetate, 10 mg. per day, in divided doses, for 3 days preceding liver analysis, produced no apparent change in their condition, as was to be expected, and no significant change in liver creatine. The figures for control animals were similar to those found for control rabbits, 3 to 7 mg. per 100 gm. of tissue. The highest value in the deficient rats was 9.8 mg. per 100 gm.

DISCUSSION

The negative results with rats are not surprising in view of the dissimilarities in the responses of rabbits and rats to vitamin E deficiency. For muscle function, the need of the maturing rabbit for tocopherol is critical; that of the rat is not, once the nurslng stage has been passed. Differences in cellular metabolic processes must be the determinants in these variations. The guinea pig is subject to dystrophy, like the rabbit, and its liver homogenates methylate guanidoacetic acid at double the rate found for rat liver slices (14).

The high content of creatine in the livers of vitamin E-deficient rabbits is in keeping with the notion that the rapid turnover of creatine during dystrophy requires its synthesis at an abnormally rapid rate. Perhaps the administration of tocopherol phosphate slows up the accelerated process of creatine synthesis sooner than it restores the capacity of the muscles to conserve creatine by phosphorylation. This order of events would account for the preliminary decrease in the already diminished muscle creatine when \( \alpha \)-tocopherol is given to dystrophic rabbits (5).

A satisfactory solution of the problem will require a study of creatine synthesis in liver slices from dystrophic animals, and a complete balance sheet of liver, muscle, blood, and urinary creatine. Such studies will be greatly facilitated by less circumstantial and more precise methods of determining creatine in tissues as complex as liver, and in the blood.

SUMMARY

Creatine and creatinine were determined in the liver and skeletal muscles of vitamin E-deficient rabbits by the enzymatic method.

The concentration of creatine in the liver was much higher in dystrophic than in control animals; in both cases the creatinine content was small and without significant differences.

In biopsy experiments, the level of liver creatine of vitamin E-deficient rabbits was not lowered within 4 hours after injection of \( \alpha \)-tocopherol phosphate.
The injection of tocopherol phosphate had no influence on the liver creatine of control animals. The possibility is discussed briefly that lack of vitamin E in the rabbit involves an increased rate of synthesis of creatine by the liver and a decrease in the rate of its phosphorylation in muscle.

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