CREATINE AND GLYCOCYAMINE METABOLISM IN RABBITS IN VITAMIN E DEFICIENCY*

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As is well known, many animals, when deprived of vitamin E (α-tocopherol), develop a paralysis of the voluntary muscles (1). The partly grown rabbit becomes completely helpless in 3 to 4 weeks. The progress of the disease is characterized by histological changes and alterations in chemical composition. Thus muscle creatine declines considerably (2), in rats even before any abnormality in structure is evident (3). There is a marked increase in urinary creatine (4), which may ultimately be as much as 30-fold the usual excretion (5). Since muscle loses little more than half its normal creatine content, the rate of creatine synthesis must be increased.

According to presently accepted views, the kidney is responsible for the initial synthesis of glycocyamine from glycine and arginine, and, in most species examined, the subsequent methylation of this compound is confined to the liver (6, 7). If the synthesis of creatine is accelerated, one might expect to find a higher content of creatine in the liver of dystrophic animals.

In preliminary experiments (8), this was found to be true. If glycocyamine is necessarily also synthesized in larger amounts, its concentration in the kidney might be elevated, together with a rise in the blood. If the rate of methylation in the liver were equally increased, the level of glycocyamine might remain unchanged. The source of the component units of creatine, especially in the later stages of dystrophy, is probably muscle protein; in the early stages, while the animals are still gaining weight, the origin of the extra creatine is not immediately obvious. When the animals can no longer eat, the creatinuria of dystrophy is compounded with that of inanition.

The excretion of creatinine in nutritional muscular dystrophy appears to be relatively unchanged (4). The view that urinary creatinine has its origin primarily in the creatine phosphate of muscle (9) cannot be reconciled with the observed diminution of creatine phosphate in dystrophic

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muscle (10) and with the conclusion that the phosphorylation of creatine is impaired (11).

In order further to elucidate some of these relationships, it seemed necessary to have information on the progressive changes in glycocyamine and creatine content of muscle, liver, kidney, blood, and urine during the development of the disease.

**EXPERIMENTAL**

Newly weaned albino rabbits weighing about 1 kilo were reared on the commonly used dystrophy-producing diet (12), to which were added 15 \( \gamma \) of vitamin B\(_{12} \) per kilo.\(^1\)

Two of each lot of six rabbits were used as controls and were given orally 15 mg. of \( \alpha \)-tocopherol acetate\(^2\) in olive oil every 4th day. They continued to thrive and gain weight after an initial loss accompanying the gradual transition from rabbit chow to the experimental diet. The animals which received no vitamin E also gained for 12 to 18 days, after which their weights declined sharply and continuously, with external evidence of advancing paralysis. Weight changes and total urinary excretion of creatine and glycocyamine were determined in 2 day periods.

At various intervals, animals were used for chemical and histological studies. After anesthesia by a non-lethal dose of nembutal (0.05 gm. per kilo. of body weight) injected into the marginal ear vein, the peritoneal cavity was opened and a sample of blood was obtained from the hepatic vein. Portions of liver, kidney cortex, and muscle (hind leg) were removed for analysis. Samples of muscle for microscopic study were fixed in Bouin's solution and stained with hematoxylin and eosin.\(^3\) Care was always taken to obtain the liver samples from the same lobe to minimize possible variations in chemical composition. The tissues were blotted dry on filter paper, weighed, and homogenized in a Potter-Elvehjem type homogenizer with 5 ml. of distilled water. After the tissues were thoroughly macerated, 5 ml. of 10 per cent trichloroacetic acid were added, and the solutions were centrifuged and decanted into 25 ml. volumetric flasks. The residues were again homogenized with water, trichloroacetic acid was added, and, after centrifuging, the supernatant fluids were decanted into the corresponding flasks. The contents of each flask were diluted to 25 ml. with distilled water. Creatine and glycocyamine were determined in aliquots of these solutions.

Because the Jaffe reaction is not specific for creatine, the direct method

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\(^{1}\) Kindly supplied by E. R. Squibb and Sons, New Brunswick, New Jersey.

\(^{2}\) Kindly supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

\(^{3}\) The microscopic sections were prepared in the Department of Pathology, and Dr. E. D. Warner generously aided in assessing the apparent extent of tissue damage.
of Ennor and Stocken (13) was chosen. It depends on the condensation of diacetyl with substances containing the guanidine group to produce a pink color (Vosges-Proskauer reaction). The addition of \( \alpha \)-naphthol, as suggested by Barritt (14), intensifies the color. To remove the inhibitory effect of sulfhydryl compounds on color formation, \( p \)-chloromercuribenzoic acid was uniformly used and was particularly necessary in connection with the samples of liver. With its use, 96 to 100 per cent of creatine, added to tissue and urine samples as the internal standard, could be accounted for. Creatinine, allantoin, and glucose seemed to cause no interference in concentrations as high as 4 times those found physiologically. Arginine, guanidine, and glycocyamine produce about 10 per cent of the color produced by equivalent amounts of creatine. The interference by arginine was removed by preliminary treatment with arginase, and the amounts of glycocyamine were too small to require a correction. Since trichloroacetic acid develops a blue color with \( \alpha \)-naphthol in daylight, the procedure was carried out in a darkened room under diffuse light from a tungsten lamp. Trichloroacetic acid was also added to the standards.

Glycocyamine was determined by MacPherson's modification (15) of the Sakaguchi reaction, which involves the interaction of guanidine groups with \( \alpha \)-naphthol in alkaline solution to produce a compound with a red color when hypobromite is added. The color obeys the Beer-Lambert law over a limited range. By keeping the standards and unknowns within this range, reliable and reproducible results could be obtained. Creatine, nitroguanidine, and glycocyamidine do not respond to the test, and close agreement was uniformly obtained.

All colorimetric readings were made with the Coleman Universal spectrophotometer and were evaluated from standard curves checked at three points with each set of determinations. Creatine was read at 520 m\( \mu \), glycocyamine at 505 m\( \mu \).

**Creatine and Glycocyamine Excretion**—For purposes of correlation, three stages in the development of the dystrophic condition were defined as incipient, moderate, and severe. These are approximately stages I, II, and III as described by Mackenzie and McCollum (5). Rabbits in the incipient stage had been on the deficient diet for at least 15 days; the skeletal muscles show no gross abnormalities or histological changes. In the moderate stage of dystrophy, there are symptoms of paralysis and a noticeable slowness in regaining normal posture; histologically, there is an increased number of histiocytes, some degeneration of the sarcolemma, and some loss of cross-striations. Severely dystrophic rabbits are prostrate and show a severe loss of longitudinal and cross-striations and

4 A preparation of arginase was given us by Dr. Theodore Winnick of the Radiation Research Laboratory.
extensive areas of replacement by connective tissue, with many giant cells and polymorphonuclear leucocytes.

In the sixteen control animals, the average daily output of creatine varied greatly, from as little as 1 mg. to more than 100 mg. per 24 hours.8 Almost invariably, an increase accompanied a loss in body weight. Thus, in the first few days, during the transition from rabbit chow to the experimental diet when a loss of weight was usual, creatine excretion rose; as soon as the lost weight was regained, it dropped back to normal figures (2 to 5 mg. per 24 hours, occasionally 10 to 15 mg.). A few of the control animals unaccountably lost weight at the very end of the experiment and showed a creatinuria probably due to inanition, but records of food consumption were not made.

Glycocyamine excretion was slightly higher and never more than about twice that of creatine, and the fluctuations showed some parallelism. Perhaps, normally, more glycocyamine is produced than is needed for conversion into creatine and this excess is excreted. In normal human subjects (16), creatinemia (and creatinuria) increased the excretion of glycocyamine; this was due to reduced tubular reabsorption rather than to more rapid synthesis or methylation of glycocyamine, and there was no evidence that excess creatine retarded the methylation of glycocyamine (17).

The excretion of creatine by the twenty-four vitamin E-deficient animals showed some initial variability, but generally followed the pattern of the control animals until about the 10th day, when it mounted very rapidly by as much as 50 mg. on each succeeding day. The highest figure recorded for any animal was 310 mg. per day. The initial increase took place fully 1 week before the animals ceased to gain weight, and it preceded the beginning of the moderate stage of dystrophy by several days. All animals showed essentially the same degree of creatinuria, once the symptoms of dystrophy became apparent, and no correlation could be made between the stage of severity of the disease and the amount of creatine in the urine. As the creatine output began to increase, there was also a gradual rise in the amount of glycocyamine excreted. This increase was only about 3-fold of the normal in any stage of the disease; the highest figure recorded was 35 mg. per day.

Creatine and Glycocyamine in Tissues—As seen in Table I, the creatine concentration in the liver and blood of the control animals was about...
double that of glycocyamine. In the kidney, the amount of glycocyamine was greater than that of creatine. In muscle the creatine content was many times as high as that of glycocyamine.

In the incipient stage, the creatine content of liver and kidney increased markedly. This increase occurred simultaneously with the rise in urinary excretion. Muscle creatine declined amazingly. More than half of the ultimate loss of creatine was sustained in this incipient stage when there

### Table I

**Average Creatine and Glycocyamine Concentration in Tissues of Normal and Experimental Animals**

<table>
<thead>
<tr>
<th>Dystrophy</th>
<th>Class</th>
<th>Creatine</th>
<th>Glycocyamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of animals</td>
<td>Average content per 100 gm. tissue</td>
</tr>
<tr>
<td>Control</td>
<td>Liver</td>
<td>16</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>10</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>8</td>
<td>652.0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>10</td>
<td>2.7</td>
</tr>
<tr>
<td>Incipient</td>
<td>Liver</td>
<td>7</td>
<td>61.1</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>3</td>
<td>65.9</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>3</td>
<td>444.0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>3</td>
<td>11.8</td>
</tr>
<tr>
<td>Moderate</td>
<td>Liver</td>
<td>10</td>
<td>47.9</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>7</td>
<td>45.9</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>4</td>
<td>489.0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>6</td>
<td>12.6</td>
</tr>
<tr>
<td>Severe</td>
<td>Liver</td>
<td>7</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>5</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>5</td>
<td>277.0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>5</td>
<td>6.3</td>
</tr>
</tbody>
</table>

was not histological evidence of altered structure, and while the animals were still gaining weight. The increased concentration of creatine in the blood would be expected as a matter of transport.

Muscle creatine in the moderate stage differed little from that in the incipient stage. In the severe stage, it declined to less than half the figure found in the control animals. Liver, kidney, and blood creatine values remained high throughout, although there was a slight decrease from the elevated values in the incipient stage. When the data from all the experimental animals were grouped together and examined statistically, the *t* value for differences between creatine content of control and of experimental tissues was highly significant at the 0.5 per cent level.
In all three stages, the glycocyamine content of liver, blood, and muscle differed little from that of the controls. At the 1 per cent level the figures had no statistical significance. In the incipient stage, kidney glycocyamine declined slightly and remained at this lower level, but this also was not significant.

DISCUSSION

It is surprising that the most extensive loss of creatine by muscle tissue of dystrophic rabbits occurs before any structural alterations are evident. The marked increase (5-fold) in liver creatine may be interpreted as indicating an increased rate of synthesis. Because of the creatinuria, the increase (3-fold) in creatine content of the kidney cortex might be an artifact, but a simple calculation shows that the amount of urine in glomeruli and tubules at any one time is not sufficient to account for this increase. Perhaps there is some storage in the kidney cells, as Borsook and Dubnoff (7) have suggested, and the same may be true of the liver. Both organs might temporarily store some of the creatine being released from muscle. The possibility that the kidney cells of the rabbit may also be able to methylate glycocyamine, in dystrophy if not normally, deserves further study by the more specific methods now available.

Since glycocyamine remains fairly constant (except for a slight decrease in the kidney) in the face of large variations in the levels of tissue creatine, there may be a series of balanced reactions, such that the amount of glycocyamine formed is always adequate for the needed production of creatine, but is never in excess. If the source of the glycocyamine is arginine and glycine, a study of the changes in these two amino acids and in methionine during the development of dystrophy might be promising.

These considerations leave the problem of creatinuria in dystrophy unsolved and provide no immediate explanation for the loss of creatine from muscle tissue in the absence of vitamin E.

SUMMARY

A study has been made of the excretion of creatine and glycocyamine and of their concentration in liver, kidney, muscle, and blood during the progressive development of the paralysis produced in young rabbits on a diet deficient in vitamin E.

Creatinuria and the loss of creatine from muscle tissue precede the external signs of paralysis and any observable histological changes. Most of the ultimate loss of creatine occurs before evidences of tissue damage appear.

Simultaneously, the amounts of creatine in liver, kidney, and blood increase; the liver contains 5 times that of control animals on the same diet.
plus tocopherol; kidney and blood, 4 times. Glycocyamine undergoes remarkably small alterations; its synthesis, if confined to the kidney, appears to be governed by the rate at which creatine is being made in the liver.

From these observations several suggestions emerge for further study of the problem of creatinuria in muscular dystrophy.

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