THE METABOLISM OF CREATINE–1–C^{14} BY MICE WITH HEREDITARY MUSCULAR DYSTROPHY *

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Creatinuria, diminished urinary creatinine excretion and low concentrations of creatine in skeletal muscle are characteristic findings in progressive muscular dystrophy in man (1). These abnormalities of creatine metabolism are sometimes thought to be nonspecific changes secondary to a smaller muscle mass (2), but this has not been proven; and, because of the probable importance of phosphocreatine to energy metabolism in skeletal muscle (3), it is possible that an abnormality of creatine metabolism would contribute to the weakness if not to the actual damage of muscle fibers in muscular dystrophy. It is therefore important to establish the nature of the defect in creatine metabolism in muscular dystrophy.

Since the hereditary myopathy of mice described by Michelson, Russell and Harman (4) resembles progressive muscular dystrophy, an investigation of creatine metabolism in these mice was undertaken. It was found that the muscle creatine of the dystrophic mouse has a shortened turnover time [1] which suggests that the low concentration of creatine in the skeletal muscle in this condition is due to an impaired ability to retain creatine.

METHODS

Strain 129 dystrophic mice and their normal litter mates, approximately 8 weeks of age and of both sexes, were obtained from the Roscoe B. Jackson Memorial Laboratory. They were given a purified diet consisting of casein,[2] 30 g; sucrose, 59 g; hydrogenated vegetable fat (Crisco), 3 g; cod liver oil, 3 g; salt mixture (6), 5 g; choline chloride, 100 mg; DL-α-tocopheryl acetate, 15 mg; thiamine hydrochloride, 1 mg; riboflavin, 1 mg; nicotinamide, 4 mg; inositol, 20 mg; calcium pantothenate 2 mg; pyridoxine hydrochloride, 1 mg; biotin, 10 μg; 2-methyl-1,4-naphthoquinone, 25 μg; and vitamin B_{12}, 1 μg. Food and water were given *ad lib.* except during the collection of urine when food was withheld. Twenty-four-hour urine samples were collected under toluene by placing individual animals in small wire cages over glass funnels designed to minimize the mixing of urine and feces. Urine was not collected more often than at 2-day intervals. At least 3 urine collections were obtained from each animal, and creatine and creatinine were determined in appropriate aliquots of the urine by minor modifications of the Folin method (7).

After the animals had received the purified diet for approximately 1 month, each of 6 dystrophic mice and 6 of their normal litter mates was given an intraperitoneal injection of 1 μc (0.5 μc per μmole) of creatine-1-C^{14} per 25 g of body weight. Fifteen and 30 minutes after injection, 0.01-ml samples of blood were taken from a tail vein for C^{14} assay. Four animals from each group were killed 30 minutes after the injection, and 2 animals from each group were killed 38 days after the injection. One normal mouse (no. 6) was given an intraperitoneal injection of 1 μc of the creatine-1-C^{14} when it weighed 30 g, and it was not killed until 47 days after the injection. The concentration and specific activity of skeletal muscle creatine were determined by slight modifications of previously described methods (8, 9). Similar methods were used to measure heart creatine concentration and specific activity, but it was usually necessary to pool two hearts to obtain sufficient tissue for analysis. The major portion of the radioactive creatine was assumed to be intracellular. No corrections were made for the small amount of labeled creatine that may have been extracellular.

At intervals of 2 or more days, individual 24-hour urine collections were obtained from the mice that were kept 38 days or longer following creatine-1-C^{14} injection. Creatinine was separated from creatine by adjusting the 24-hour urine collection to pH 4 and mixing with Lloyd's reagent to adsorb creatinine. After washing the Lloyd's reagent three times with 5-ml portions of 0.01 N HCl,

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[1] The definition of this and other related terms may be found in the paper by Zilversmit (5).

[2] Vitamin-free casein purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

the creatinine was removed by washing twice with 3-ml portions of 0.5 N NaOH. The sodium hydroxide washings were acidified, and the specific activity of the creatinine was obtained by measuring its concentration and isolating creatinine zinc chloride after addition of carrier creatinine (9). This method eliminated over 99 per cent of creatine-1-C^{14} added to urine samples containing unlabeled creatine and creatinine.

In order to obtain adequate urine for the determination of the specific activity of both creatine and creatinine it was necessary to pool three 24-hour urine collections from each mouse. Three urine collections, obtained during the last week before the animals were killed, were pooled and aliquots were taken for creatine and creatinine determination and for isolation of creatinine. Another aliquot of the pooled urine samples was made approximately 1 N by adding concentrated HCl and heated in an autoclave at 118° C for 45 minutes. This procedure converted creatine to creatinine. The specific activity of the creatinine isolated from the autoclaved urine represented the average of the creatinine and creatine. Since the concentrations of creatine and creatinine and the specific activity of creatinine had been determined for the pooled urine samples, it was possible to calculate the specific activity of creatine.

All creatinine zinc chloride samples were counted in metal planchets with an ultra-thin window continuous gas-flow Geiger tube, and the observed counts were corrected to infinite thinness. The blood samples were mixed with 1 ml of a 1 : 1 mixture of acetone and water, dried in metal planchets and counted with an end-window Geiger tube. Therefore, the actual counts obtained with blood samples and creatinine zinc chloride samples are not comparable.

RESULTS

Both groups of animals continued to gain weight slowly on the purified diet, as is shown in Table I. The dystrophic mice weighed only about 65 per cent as much as their normal litter mates but, since all the animals gained weight throughout the experiment, it is apparent that involvement of the jaw muscles by the myopathy did not induce severe starvation.

TABLE I

Weight changes in normal and dystrophic mice

Age	Weight	
	Normal	Dystrophic
days	*g*	
67	21.0	15.0
77	23.7	15.4
85	24.6	15.3
97	24.9	15.9
104	25.3	16.4
135*	26.6	17.1

* Only 2 animals remained in each group at this time. The other weights are the averages of 4 to 6 animals.

The combined urinary excretion of creatine and creatinine (Table II) was similar in the two groups, although the dystrophic mice weighed less. The amount of creatinine excreted per gram of body weight was also similar in the two groups of animals, but the urinary creatine to creatinine ratio was higher in the dystrophic mice. The concentration of creatine was reduced in the skeletal muscle but not in cardiac muscle of the dystrophic mice. These observations agree in general with the report of Kandutsch and Russell (11) but differ somewhat with the findings of Perkoff and Tyler (12) who reported a very low urinary excretion of creatinine by the dystrophic mouse.

The specific activities of creatine of some of the tissues following creatine-1-C^{14} injection are given in Table III. The radioactivity present in the blood at 15 and 30 minutes following injection indicates that both groups of animals received similar doses of creatine-1-C^{14} and that the peak of

TABLE II

Effects of muscular dystrophy on creatine storage and excretion in mice

Measurement	Normal	Dystrophic	p*
Urinary creatine† + creatinine (μg/day)	599 [6]‡	649 [5]	>0.5
Urinary creatinine (μg/day/g body wt)	13.0[6]	14.1 [5]	>0.2
Urinary creatine/creatinine	1.15 [6]	2.26 [5]	<0.01
Skeletal muscle creatine (mg/g)	3.25 [7]	2.14 [6]	<0.001
Heart creatine (mg/g)	1.38 [7]	1.20 [6]	>.04

* Probability that the differences observed between normal and dystrophic mice are due to chance (10).
† Creatine concentrations are expressed as creatinine.
‡ Numbers in brackets indicate the number of animals used.

TABLE III

Incorporation of creatine-1-C^{14} into various tissues of the mouse

Measurement	Time after injection of creatine-C^{14}	Normal	Dystrophic	p*
Skeletal muscle creatine (cpm/μmole)	30 min	351 [4]	635 [4]	<0.001
Skeletal muscle creatine (cpm/μmole)	38 days	508 [2]	266 [2]	<0.05
Heart creatine (cpm/μmole)	30 min	2,710 [4]	3,380 [4]	>0.40
Heart creatine (cpm/μmole)	38 days	191 [2]	97 [2]	
Blood creatine (cpm/ml blood)	15 min	2,075 [4]	1,875 [4]	>0.50
Blood creatine (cpm/ml blood)	30 min	1,600 [4]	1,375 [4]	>0.50

* See footnotes (*‡) to Table II.

radioactivity in the blood had been reached earlier than 30 minutes after injection. The specific activity of skeletal muscle creatine in the dystrophic mice was higher than normal 30 minutes after injection and lower than normal 38 days after injection. This indicates that the turnover time of muscle creatine is shorter in the dystrophic mouse. In both groups of animals the specific activity of heart creatine 30 minutes after injection was about seven times that of skeletal muscle. The reduced turnover time of heart creatine was confirmed by finding specific activities lower than that of skeletal muscle 38 days after injection of labeled creatine.

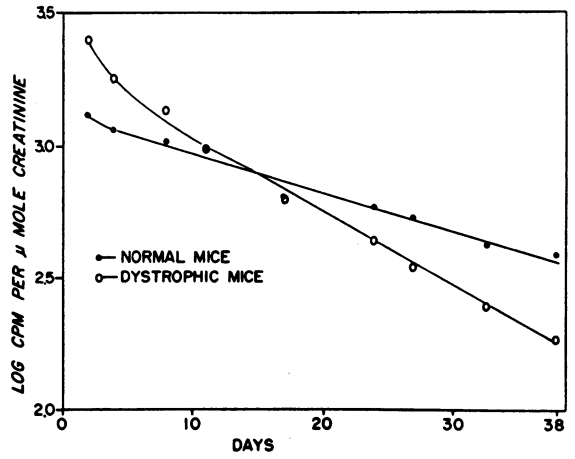

FIG. 1. RADIOACTIVITY OF URINARY CREATININE. Creatinine was separated from creatine by using Lloyd's reagent. The creatinine was then isolated for counting as creatinine zinc chloride by a carrier procedure. There were two female mice in each group.

The logarithm of the specific activity of urinary creatinine is plotted against time in Figure 1. It is apparent that the specific activity of the urinary creatinine is diminishing much more rapidly in the dystrophic mice than in their normal litter mates. In both groups of animals the curve is not a straight line until about 10 days following the injection of labeled creatine. If the straight portion of the curve is used to estimate the half-life of body creatine, half-lives of 20 and 11 days are obtained for the normal and dystrophic mice, respectively.

In Table IV the specific activity of creatinine in the urine collected on the day the animals were killed is compared with the specific activity of the

TABLE IV

Muscle creatine and urinary creatinine specific activities *

Animal	Condition	Muscle creatine	Urinary creatinine†
2‡	Normal	546	377
5	Normal	469	409
6§	Normal	497	314
2	Dystrophic	241	174
5	Dystrophic	290	195

* Counts per minute per μmole of creatine.
† The urine was collected during the last 24 hours before the animals were killed.
‡ Animals having the same number are litter mates.
§ This mouse was killed 47 days following injection of creatine-C^{14}; the other animals were killed 38 days after injection.

TABLE V

Urinary creatine and creatinine specific activity *

Animal	Condition	Creatine	Creatinine
2	Normal	296	415
5	Normal	355	421
6	Normal	238	378
2	Dystrophic	95	249
5	Dystrophic	122	252

* Counts per minute per μmole of creatine. Data were obtained from urine collected during the week before the mice were killed. No. 6 was killed 47 days after injection of creatine-C^{14}. The other animals were killed 38 days after injection.

skeletal muscle creatine from the same animal. The urinary creatinine in every case has a considerably lower specific activity than has the skeletal muscle creatine. It is not likely that this is due to the transformation of relatively large quantities of unlabeled creatine to creatinine while the urine was being collected, since the urinary creatine had an appreciable specific activity (Table V) a short time before the animals were killed.

DISCUSSION

Since most of the creatine of the body is located within skeletal muscle and since there is no evidence for enzymatic formation of creatinine (13, 14), it is commonly assumed that urinary creatinine has its origin largely in skeletal muscle. However, some of the data from the present experiment indicate that this is not a valid assumption for the mouse. When the logarithm of urinary creatinine specific activity is plotted against time, a straight line is not obtained until about 10 days following the injection of labeled creatine. This suggests that during the earlier time intervals there are two or more sources of radioactive urinary creatinine. If the curved portions of the graphs in Figure 1 are due to the formation of creatinine in sites other than skeletal muscle, then these sources of creatinine have a relatively short turnover time. It would, therefore, be expected that urinary creatinine, isolated a short time after administering labeled creatine, would contain a higher concentration of the label than would total body creatine (largely skeletal muscle creatine). This phenomenon has been observed in rats (15).

Further evidence against the unique origin of creatinine from skeletal muscle creatine is provided by the finding of a low urinary creatinine specific activity in comparison with that of skeletal muscle creatine (Table IV). The percentage of urinary creatinine that has its origin in skeletal muscle can be estimated by dividing urinary creatinine specific activity by skeletal muscle creatine specific activity and multiplying by 100. The calculation is based on the assumption that skeletal muscle creatine is the only body pool of creatine that is highly labeled 38 days following a single injection of creatine-1-C^{14}. The results of this calculation are recorded in Table VI. If the creatine outside the skeletal muscle were still significantly labeled at the time these specimens were obtained, then the numbers shown in the table would be too large. Thus it is evident that 25 per cent or more of the urinary creatinine of these mice did not come from skeletal muscle. This large source of creatinine might account for the excretion of normal amounts of urinary creatinine (based on body weight) by the dystrophic mice even though they exhibited low concentrations of creatine in skeletal muscle (Table II).

The assumption that only skeletal muscle creatine is highly labeled 1 month after a single injection of creatine-1-C^{14}, permits the estimation of the percentage of urinary creatine which is derived from skeletal muscle creatine. The urinary creatine specific activity divided by the muscle creatine specific activity and multiplied by 100 would give the percentage of the urinary creatine that came from skeletal muscle. Table VI records the results of this calculation. The values obtained are slightly high because the urine collections were obtained a few days before the animals were killed. Nevertheless, it is evident that only about 50 per cent of the urinary creatine represents

TABLE VI

Contribution of skeletal muscle to urinary creatine and creatinine

Animal	Condition	Urinary creatinine derived from muscle creatine*	Urinary creatine derived from muscle creatine*
		%	%
2	Normal	69.0	54.2
5	Normal	87.0	75.7
6	Normal	63.2	47.9
2	Dystrophic	72.2	39.4
5	Dystrophic	67.2	45.4

* See text for method of calculation.

TABLE VII

Daily loss of skeletal muscle creatine as urinary creatine and creatinine

Animal	Condition	Lost as urinary creatine*	Lost as urinary creatinine*
		%	%
2	Normal	0.43	0.51
5	Normal	0.73	1.14
6	Normal	0.53	0.85
2	Dystrophic	1.67	2.00
5	Dystrophic	2.44	2.05

* See text for method of calculation.

newly synthesized creatine and that the relative amount of newly synthesized creatine which is excreted in the urine is similar in the two groups of animals.

The percentage of skeletal muscle creatine excreted daily as urinary creatine or creatinine can be estimated by dividing the amount of urinary creatine or creatinine that has its origin in skeletal muscle (based on the percentages previously calculated) by the estimated amount of skeletal muscle creatine and multiplying by 100. The amount of skeletal muscle was estimated to be 40 per cent of the total body weight, and this was multiplied by the concentration of skeletal muscle creatine for a particular animal in order to arrive at the total amount of skeletal muscle creatine for that animal. The errors of this estimation would tend to minimize the differences between the dystrophic and normal mice. Even so, it is apparent from Table VII that the relative daily loss of skeletal muscle creatine as urinary creatine or creatinine is twice as great in the dystrophic mice as in their normal litter mates. This reflects the greatly reduced turnover time of skeletal muscle creatine in the dystrophic mice.

A reduced turnover time of skeletal muscle creatine in the dystrophic mice means that the molecules of creatine reside for a shorter than normal period of time in the muscle. There are two possible explanations for this finding; either there is defective metabolism of creatine by skeletal muscle, or the creatine within the muscle must be in rapid equilibrium with a relatively larger extramuscular pool of creatine. Which of these explanations is chosen depends in part upon the nature of the mechanisms that maintain muscular creatine levels.

The mechanisms responsible for maintaining the high muscle creatine concentrations are not known, but active transport of creatine across the cell membrane and intracellular trapping of creatine are two possible explanations. If the latter possibility were the primary mechanism, a rapid exchange of creatine across cell membranes would not be expected. On the other hand, if an active transport system maintained the high concentrations of creatine within the cell it would be possible for labeled intracellular creatine to exchange rapidly with nonlabeled extracellular creatine. If a rapid exchange normally occurs, the difference in pool size between normal and dystrophic animals might be enough to account for the differences in turnover times of skeletal muscle creatine. However, this would not explain the low concentration of creatine in the skeletal muscle of the dystrophic mice, which is present regardless of whether the concentration is based on wet weight of tissue, fat-free dry weight of tissue (11), or noncollagen nitrogen (16).

A decrease in the intracellular concentration of creatine must be the result of a higher rate of loss of creatine than its rate of entry. If the rate of entry of creatine into skeletal muscle is normal or increased and the rate of loss is abnormally high, it would suggest that a mechanism which normally prevents the loss of creatine from the cell is impaired by the dystrophic process. If the rate of entry of creatine into the skeletal muscle is lower than normal and its rate of loss normal or decreased, it would suggest that a mechanism for the transport of creatine into the cell is not functioning properly. If the rate of entry of creatine into the muscle were low and the rate of exit high, it would suggest that both the above mechanisms for maintaining muscle creatine concentrations are operative but functioning abnormally.

The uptake of creatine-1-C^{14}, relative to the amount of creatine in skeletal muscle, is increased in the dystrophic mice (Table III), and it may be calculated that the total amount of creatine-1-C^{14} that enters each gram of skeletal muscle is similar in the two groups of animals. Coupling this observation with the reduced turnover time of skeletal muscle creatine and the low concentration of creatine in the muscle of the dystrophic mice leaves little doubt that there is a defect in a mechanism which normally prevents the loss of creatine from the cell. The excess loss of creatine could result

from increased conversion of creatine to creatinine, from leakage of creatine out of the muscle, or from a combination of both possibilities. Because of the limitations of the present experiment it is not possible to exactly define the defect, but it is clear that the metabolism of creatine by skeletal muscle is abnormal in mice with hereditary muscular dystrophy. The possibility thus arises that the muscular weakness or wasting of these mice may be related to the abnormal metabolism of creatine.

A similar defect in the muscular metabolism of creatine occurs in vitamin E-deficient animals (9, 17) which also exhibit muscular weakness and wasting, but the primary change in creatine metabolism in progressive muscular dystrophy in man is thought to be a decreased total uptake of creatine because of the smaller muscle mass (18, 19). Since the latter might be interpreted as indicating normal metabolism of creatine by the surviving skeletal muscle, it should be pointed out that some of the observations in patients with progressive muscular dystrophy suggest that the changes in creatine metabolism are similar to the changes found in dystrophic mice.

The experiments with humans which are most comparable with those reported here were performed by Roche (18) and Benedict (19) and their co-workers. These investigators administered N^{15}-labeled glycine to normal subjects and to patients with progressive muscular dystrophy. Urinary creatinine was subsequently isolated from both groups and urinary creatine was isolated from the dystrophic patients. Two of the findings are similar to those reported here. The N^{15} concentration of the urinary creatinine was always higher in the dystrophic than in the normal subjects, and in all of the dystrophic patients the N^{15} content of urinary creatinine decreased during the 2-week observation period. By contrast, the N^{15} concentration of urinary creatinine from the normal subjects did not change during the observation period. Because of these findings it was suggested that the fraction of muscle creatine converted to creatinine was increased in progressive muscular dystrophy (19).

It is well established for the rat that urinary creatinine is derived from body creatine (20). If this is also true for man, a decrease in the N^{15} concentration of urinary creatinine would indicate a dilution of body creatine with unlabeled creatine. However, Roche and Benedict and their associates (18, 19) found that the N^{15} concentration of urinary creatine was much higher than that of urinary creatinine even when the N^{15} concentration of urinary creatinine was decreasing. These two observations appear to be contradictory, unless it is assumed that the source of urinary creatine is different from the source maintaining tissue concentrations. Based on current knowledge of creatine metabolism, such a circumstance appears highly unlikely. Since glycine enters into so many metabolic pathways, more easily interpretable data might be obtained by using labeled creatine to investigate the metabolism of creatine by skeletal muscle in progressive muscular dystrophy. Until other evidence is available, it should not be assumed that the muscular metabolism of creatine is normal in patients with progressive muscular dystrophy.

SUMMARY

The urinary excretion of creatine and creatinine by mice with hereditary muscular dystrophy and by their normal litter mates was measured while the animals were receiving a purified diet. Normal and dystrophic mice were then given a single intraperitoneal injection of creatine-1-C^{14} and killed either 30 minutes or several days after the injection. The concentration and specific activity of the creatine of heart and skeletal muscle were measured in all the animals, and urinary creatine and creatinine specific activities were determined for the animals that were allowed to live for 38 days or longer after the injection of labeled creatine.

The combined excretion of urinary creatine and creatinine was similar in the two groups of animals, but the ratio of urinary creatine to creatinine was increased in the dystrophic mice. The urinary excretion of creatinine expressed as a function of body weight was not decreased in the dystrophic mice. The concentration of creatine was reduced in skeletal muscle but not in cardiac muscle of the dystrophic mice.

The turnover time of both the total body creatine and skeletal muscle creatine was reduced in the dystrophic mice. These findings indicate that the low concentration of creatine in the skeletal muscle of the dystrophic mice results from an impaired ability to retain creatine.

The turnover time of heart creatine was considerably less than that of skeletal muscle in both groups of animals, but no differences between the two groups were found.

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