2. Chumakov, M. P., J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 7, 125 (1963).
3. Simpson, D. I. H., Knight, E. M., Courtois, G., Williams, M. C., Weinbren, M. P., and Kibukamusoke, J. W., E. African Med. J. 44, 87 (1967).
4. Woodall, J. P., Williams, M. C., and Simpson, D. I. H., E. African Med. J. 44, 93 (1967).
5. Wisseman, C. L., Jr., personal communication, 1963.
6. Taylor, R. M., ed., U. S. Public Health Serv. Publ. 1760, (1967).
7. Clarke, D. H. and Casals, J., Am. J. Trop. Med. Hyg. 7, 561 (1958).
8. Casals, J., Japan. J. Med. Sci. Biol. 20 (Suppl.), 119 (1967).
9. Haldane, J. B. S., Nature 187, 879 (1960).
10. Causey, O. R., personal communication, 1967.
11. Woodall, J. P., Williams, M. C., Simpson, D. I. H., Ardoin, P., Lule, M., and West, R., E. African Virus Res. Inst. Rept. No. 14 (1963-64), 34 (1965).

Received Dec. 16, 1968. P.S.E.B.M., 1969, Vol. 131.

Creatine Entry into Skeletal Muscle of Normal and of Dystrophic Mice* (33848)

COY D. FITCH[1] AND M. RAHMANIAN

Departments of Medicine and Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas 72201

Since there is no evidence for creatine synthesis in skeletal muscle or for creatine degradation other than by slow, nonenzymatic formation of creatinine, one of three hypotheses should explain the low creatine content of skeletal muscle of dystrophic mice (1–3). Entry of creatine into muscle might be inhibited, loss of creatine from muscle might be accelerated, or the two defects might coexist. Relevant to choosing the correct hypothesis are the following two observations from studies of creatine kinetics *in vivo*: Dystrophic mice have higher than normal specific activity of muscle creatine 30 min after creatine-^{14}C injection, and they have a shortened turnover time of body creatine (1). These observations are consistent with the hypothesis of accelerated loss of creatine as the cause of the low creatine content of dystrophic muscle.

Additional evidence is needed, however, because interpretation of data from studies *in vivo* requires a large number of assumptions (4), despite the apparent simplicity of creatine metabolism. Therefore, the present studies of creatine entry into isolated extensor digitorum longus muscles were undertaken. In agreement with the interpretation of creatine kinetics *in vivo*, we found greater than normal entry of creatine into dystropic muscles. In addition, we found the creatine entry process in mouse muscle to differ from that of rat muscle (5, 6) by having a major nonsaturable component.

Materials and Methods. One- to 2-month-old, dystrophic mice of Bar Harbor Strain 129 and matching normal mice of the same strain, sex, and age were obtained from the Roscoe B. Jackson Memorial Laboratory. Normal and dystrophic mice were always treated similarly and studied concurrently. Some of the normal mice were heterozygous for the dystrophic trait, but they were indistinguishable from homozygous normal mice in these studies. After being fed a complete, purified diet (1) for a minimum of 1 week, the mice were decapitated, and their extensor digitorum longus muscles were removed and placed in vessels containing media for incubation. Approximately 3 min were required to obtain each muscle.

To study entry, 0.1–0.2 μCi of creatine-1-^{14}C (sp act, 2.62 or 5.08 mCi/mmole)[2] per

* Paper no. 4 in a series: Creatine Metabolism in Skeletal Muscle; paper no. 3 is Ref. (6). Supported in part by Research Grant AM-03615 from the National Institutes of Health, USPHS.

[1] Present address: Division of Biochemistry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C. 20012.

[2] Radioactive compounds were purchased from New England Nuclear Corporation.

TABLE I. Some Characteristics of the Mouse Extensor Digitorum Longus.

Measurement	Normal mice	Dystrophic mice
Wt[a] (mg)	6.5 ± 1.2 (56)[b]	3.4 ± 1.0 (56)
Total water[a] (% of wet wt)	78.0 ± 1.0 (21)	77.4 ± 1.7 (21)
Inulin space[a] (% of wet wt)	26.6 ± 4.3 (50)	40.7 ± 5.6 (44)
Creatine[c] (mg/g of wet wt)	5.06, 3.76	3.14, 2.74
Noncollagen protein[c] (% of wet wt)	25.4, 26.6	23.0, 24.4

[a] After 60-min incubation at 37° in 2 ml of Krebs–Ringer bicarbonate solution (pH 7.4) under an atmosphere of O_2:CO_2, 95:5. The average weights of the mice were 25 g (normal) and 15 g (dystrophic).

[b] Mean ± SD with number of determinations in parentheses.

[c] Measured in fresh, unincubated tissue; the individual values were obtained from separate pools of extensor digitorum longus muscles.

milliter of incubation medium served as tracer, and the movement of radioactivity from medium into muscle was measured by previously described methods (5, 6). Incubation conditions are given in the legends to Fig. 1 and 2. A one-hr incubation period was used routinely after experiments with shorter periods of incubation showed that a 1-hour period was suitable for measurement of initial rates over the range of creatine concentrations under consideration.

To determine some of the characteristics of the mouse extensor digitorum longus muscle, creatine concentrations were measured by the method of Tanzer and Gilvarg (7) as modified by Bernt *et al.* (8); noncollagen protein was dissolved in dilute NaOH according to the method of Lilienthal *et al.* (9) and measured by the method of Lowry *et al.* (10); and total water was measured by drying the muscles to constant weight in an oven at 105°. Inulin space was measured by incubating the muscles in media containing inulin-carboxyl-^{14}C (sp act, 2.68–2.84 mCi/g) or inulin methyl-^{3}H (sp act, 231 mCi/g) at concentrations ranging from 2 to 8 mg/100 ml. In many instances, inulin space and creatine entry were measured simultaneously by using both creatine-^{14}C and inulin-^{3}H in the incubation medium.

Results and Discussion. Some characteristics of the extensor digitorum longus muscles are given in Table I. Since the experimental design required intact muscles from mice matched for age rather than size, the dystrophic muscles were about one-half as large as normal. Weights are given in Table I. Also given in Table I are creatine and noncollagen protein concentrations obtained from pools of 5 or 6 extensor digitorum longus muscles which individually would have been too small for the analyses. In agreement with the usual results from skeletal muscle (1–3), the creatine content of normal extensor digitorum longus muscles was high and the creatine content of dystrophic extensor digitorum longus muscles was relatively low, using either wet weight of muscle or noncollagen protein as a reference measurement (Table I). For comparison, creatine and noncollagen protein were measured in individual samples of skeletal muscle from the thighs of the same mice, yielding the following results: creatine, 3.94 ± 0.48 mg/g of wet weight (mean ± SD) for 11 normal mice and 3.18 ± 0.75 for 11 dystrophic mice; and noncollagen protein, 19.3 ± 3.8% of wet weight for 11 normal mice and 19.8 ± 4.3 for 11 dystrophic mice. The difference in creatine concentrations is significant with a p value of less than 0.01 (11).

Two other characteristics, of importance for a study of creatine entry, are the total amounts of water in the muscles and the distribution of this water between extracellular and intracellular spaces. As the data in Table I show, there was no significant difference in the total water contents of normal and dystrophic muscle, although the dystrophic muscles had much more water accessible to inulin. A time-course study of inulin deposition, depicted in Fig. 1, reveals an ap-

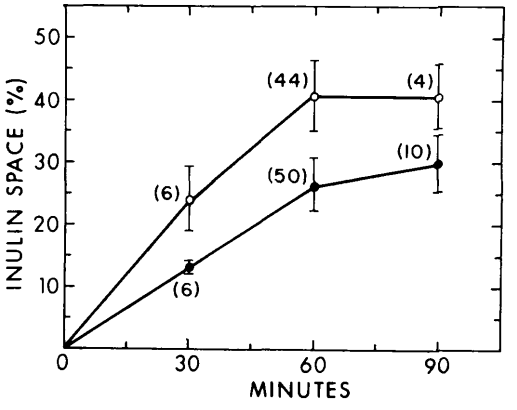

FIG. 1. Time-course of inulin deposition in extensor digitorum longus muscles: individual muscles were incubated for the specified lengths of time at 37° under an atmosphere of O_2:CO_2, 95:5, in 2 ml of Krebs–Ringer bicarbonate solution (pH 7.4) containing either ^{14}C- or ^{3}H-labeled inulin. In many instances, the incubation medium also contained one of the concentrations of creatine shown in Fig. 2. The means ± SD are shown; the number of determinations at each time interval is given in parentheses; (●), normal muscles; (○), dystrophic muscles.

proach to steady-state by both groups of muscles during incubation periods of 1 or 1.5 hr. To what extent the approach to a steady-state represents the filling of potential but unfilled spaces *in vivo* and whether or not inulin space is identical to extracellular fluid volume in either group of muscles remains unknown. Nevertheless, calculation of the amount of creatine entering intracellular space requires an estimate of extracellular space and the inulin space is used for this purpose.

In Fig. 2, the initial rates of creatine entry into normal and dystrophic muscles are plotted as functions of external creatine concentration. Both curves exhibit a major linear (nonsaturable) component; in experiments not shown, linearity extended to 20 m*M* external creatine concentration. The curves in Fig. 2 also suggest that a saturable component of entry may be operative at physiologic serum creatine concentrations, 0.02–0.05 m*M* (3), but this possibility is difficult to evaluate in the presence of such a large nonsaturable component of entry. These observations on creatine transport into mouse muscle stand in contrast to observations from earlier studies of the rat; a saturable process is responsible for most of creatine entry into rat skeletal muscle *in vitro* (5, 6). Perhaps a similar difference in creatine transport by the renal tubules of rats and mice is responsible for the greater creatinuria of adult mice (12).

Creatine entry was greater into dystrophic than into normal extensor digitorum longus muscles at every external creatine concentration, even after correcting for the greater inulin space of dystrophic muscles (Fig. 2). Some or all of this difference may be attributable to the greater surface to volume ratio of the smaller dystrophic muscles. But irrespective of its cause, the greater entry of creatine into dystrophic muscle *in vitro* is inconsistent with the hypothesis of inhibited creatine entry *in vivo*. On the other hand, greater than normal entry of creatine is entirely consistent with accelerated loss of creatine as the cause of the low creatine content of skeletal muscle of dystrophic mice.

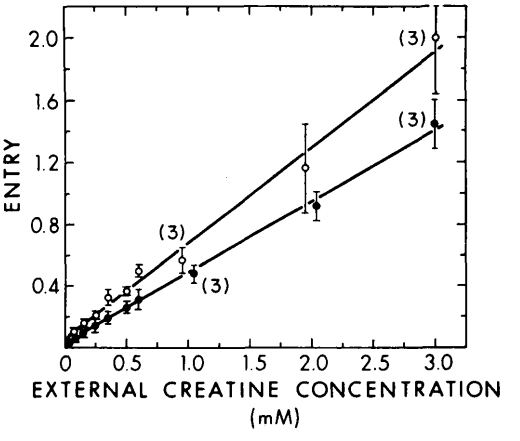

FIG. 2. Creatine entry into normal and dystrophic skeletal muscle: individual extensor digitorum longus muscles were incubated for 1 hr at 37° under an atmosphere of O_2:CO_2, 95:5, in 2 ml of Krebs–Ringer bicarbonate solution (pH 7.4) containing inulin-^{3}H and creatine-^{14}C as tracers. Individual inulin space values were used to calculate creatine entry. The units of entry are mmoles per liter of intracellular water per hour. Four control, (●), and 4 dystrophic, (○), muscles were studied at each concentration of creatine except as noted in parentheses; the means ± SD are shown.

Summary. Creatine entry into isolated extensor digitorum longus muscles of normal and of dystrophic mice was studied with the aid of creatine-^{14}C as a tracer. The creatine entry process in mouse muscle was found to differ from that of rat muscle by having a major nonsaturable component.

At all external creatine concentrations, entry of creatine into dystrophic muscles exceeded entry into normal muscles, even after correcting for a greatly expanded inulin space in dystrophic muscles. This finding is inconsistent with the hypothesis of inhibited creatine entry *in vivo*, leaving accelerated loss of creatine to explain the low creatine content of skeletal muscle of dystrophic mice.

We are indebted to Mr. Samuel C. Dillard for technical assistance.

1. Fitch, C. D., Oates, J. D., and Dinning, J. S., J. Clin. Invest. **40**, 850 (1961).

2. Kandutsch, A. A. and Russell, A. E., Am. J. Physiol. **194**, 553 (1958).

3. Banker, B. Q. and Denny-Brown, D., J. Neuropathol. Exptl. Neurol. **18**, 517 (1959).

4. Fitch, C. D., Lucy, D. D., Bornhofen, J. H., and Dalrymple, G. V., Neurology **18**, 32 (1968).

5. Fitch, C. D. and Shields, R. P., J. Biol. Chem. **241**, 3611 (1966).

6. Fitch, C. D., Shields, R. P., Payne, W. F., and Dacus, J. M., J. Biol. Chem. **243**, 2024 (1968).

7. Tanzer, M. L. and Gilvarg, C., J. Biol. Chem. **234**, 3201 (1959).

8. Bernt, E., Bergmeyer, H. U., and Möllering, H., *in* "Methods of Enzymatic Analysis" (H. U. Bergmeyer, ed.), p. 407 Academic Press, New York (1963).

9. Lilienthal, J. L., Jr., Zierler, K. L., Folk, B. P., Buka, R., and Riley, M. J., J. Biol. Chem. **182**, 501 (1950).

10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

11. Snedecor, G. W., "Statistical Methods Applied to Experiments in Agriculture and Biology," 4th ed., p. 75, Iowa State Univ. Press, Ames, Iowa (1948).

12. McGeer, E. G., McGeer, P. L., Miller, J. R., Derry, D., and Nichol, C., Can. J. Biochem. Physiol. **40**, 13 (1962).

Received Dec. 18, 1969. P.S.E.B.M., 1969, Vol. 131.