The Metabolism of Anserine and Carnosine in Normal and Vitamin E-deficient Rabbits*  

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Among the alterations observed in the nitrogenous constituents of skeletal muscle of severely vitamin E-deficient rabbits is a decrease in the concentrations of carnosine and anserine (1, 2). The experiments of Razina (3) on the synthesis of carnosine in hepatectomized and nephrectomized rats implicate skeletal muscle as a major site of their synthesis. This site is also indicated by studies on the synthesis in vitro of anserine and carnosine from the constituent amino acids by carnosine synthetase from chick pectoral muscle (4, 5). However, it is of interest that the rat may not utilize the same pathway for the formation of anserine (6, 7) and the liver is not completely excluded as a possible site of synthesis (8).

An increased urinary amino acid excretion is also a prominent feature of a vitamin E deficiency in rabbits (9) and in human progressive muscular dystrophy (10, 11). Excretion of L-methionylhistidine has been observed in these conditions (12, 13) and Fink et al. (13) have observed that L-methylhistidinuria generally precedes the appearance of creatininuria, thus making it one of the earliest detectable symptoms of nutritional muscular dystrophy.

These observations suggested the presence of an abnormality in the metabolism of anserine and carnosine as a result of, or concomitant with, the appearance of muscular dystrophy and formed the basis for the experiments reported in this paper. Concentrations of L-methylhistidine excreted in the urine and the levels of anserine and carnosine in muscle were determined by a column chromatographic procedure at various stages in the development of a nutritional muscular dystrophy in rabbits. In addition, the utilization in vivo of L-methionine-methyl-C\textsuperscript{14} and DL-histidine-2-C\textsuperscript{14} for the synthesis of anserine and carnosine was determined as measured by their incorporation into the peptides after varying periods of time.

**EXPERIMENTAL**

**General Experimental Procedure**—Rabbits of the New Zealand strain weighing 600 to 1200 g were maintained on a purified diet (14) with or without oral supplements, given three times a week, of 1 mg of \( \alpha \)-tocopherol per kg of body weight. The animals were weighed and urine samples were collected every other day. At times indicated by the creatine to creatinine ratios and alterations in muscle tonus, the rabbits were given an intraperitoneal injection of \( \text{L-methionine-methyl-C}^{14} \) or \( \text{DL-histidine-2-C}^{14} \) dissolved in 2 ml of 0.9% sodium chloride solution at levels of 4 to 5 \( \mu \)curies per 10 \( \mu \)moles per kg of body weight. After periods of from 5 to 48 hours the animals were killed by dislocation of the cervical vertebrae and the leg muscles were excised and frozen immediately in a Dry-Ice-acetone mixture. Samples of 20 g of skeletal muscle were freed of gross fat and connective tissue and the imidazole constituents were isolated by a procedure described previously (7).

**Chromatographic Isolation Procedures**—The isolation of \( \beta \)-alanine, histidine, and the methylhistidines is based on the ion exchange carrier displacement chromatographic method described by Buchanan (15). Dowex 50-X4(200-400 mesh) was generated in the \( \text{H}^{+} \) form (16) and then washed repeatedly with 0.2\% 2,6-lutidine or \( \alpha \)-picoline until it was saturated. It was ready for use after washing with water until the eluate was neutral. The fraction containing the imidazole constituents was applied to a Dowex 50-2,6-lutidine column, 0.9 X 9 cm, and the column was then eluted with 0.1 \( \mu \)M 2,6-lutidine. This separated histidine and L-methylhistidine from the peptides. The peptide fraction was concentrated to dryness under reduced pressure in a rotary evaporator and hydrolyzed in 6 \( \text{N} \) HCl in a sealed tube at 110\(^\circ\)C for 5 to 16 hours. After removing HCl by repeated concentration in a stream of air, the hydrolysate was chromatographed on a column of Dowex 50-\( \alpha \)-picoline, 0.8 X 9 cm, and the column was eluted with 0.1 \( \mu \)M \( \alpha \)-picoline. This column also separated 3-methylhistidine as well as \( \beta \)-alanine, histidine, and 1-methylhistidine. After concentration to dryness followed by dilution to a given volume with water, the compounds were assayed by reaction with ninhydrin (18) with the appropriate amino acids as standards. Results are expressed in terms of millimoles per 100 g of tissue or per g of noncollagenous nitrogen and the concentrations of histidine and 1-methylhistidine are representative of carnosine and anserine respectively.

For isolation of 1- and 3-methylhistidines from urine, the urine samples were adjusted to pH 3 and aerated to remove carboxylic acids and dissolved in 2 ml of 0.9% sodium chloride solution at levels of 4 to 5 \( \mu \)curies per 10 \( \mu \)moles per kg of body weight. After periods of from 5 to 48 hours the animals were killed by dislocation of the cervical vertebrae and the leg muscles were excised and frozen immediately in a Dry-Ice-acetone mixture. Samples of 20 g of skeletal muscle were freed of gross fat and connective tissue and the imidazole constituents were isolated by a procedure described previously (7).

* A preliminary report of this investigation was presented before the Forty-ninth Annual Meeting of the American Society of Biological Chemists at Philadelphia, Pennsylvania, April 14 to 18, 1958. This investigation was supported by a grant from the Muscular Dystrophy Associations of America, Inc.
bon dioxide, and then they were treated similarly to the muscle extracts except for elimination of acid hydrolysis.

All of the compounds were checked for purity by paper chromatography on Whatman No. 3 paper in phenol-0.1 m phosphate buffer, pH 6.6, 7:1 and in ethanol-ether-phosphate buffer, pH 6.6, 15:12:4 (6). The identity of representative samples of urinary 1-methylhistidine was checked by comparison of their infrared absorption spectrum with that of an authentic sample.

Noncollagenous nitrogen was determined by the method of Lilienthal et al. (19). Creatine and creatinine were determined by the method of Lambert (20). Methionine was assayed by the procedure described by Williams et al. (21) and degraded by the Baernstein degradation (22) as used by Berg (23). Carnosinase activity was determined by procedures described by Hanson and Smith (24).

Radioactive samples were diluted to a given volume and 1- to 2-ml aliquots were plated and dried on aluminum planchets and counted as infinitely thin samples to ±2% accuracy in a gas flow Geiger counter with a Micromil window.

RESULTS

Chromatographic Separation of β-Alanine, Histidine, 1-Methylhistidine, and 3-Methylhistidine—These constituents may be separated on either Dowex 50-2,6-lutidine or Dowex 50-α-picoline columns. The α-picoline columns have proved to be more useful since complete resolution is effected on a comparatively small column and the 2,6-lutidine columns have been used particularly in preliminary separation of the free amino acids from the peptides. A typical separation of a mixture of 15 μmoles samples of histidine, 1-methylhistidine, carnosine, and anserine on a Dowex 50-2,6-lutidine column, 0.8 × 15 cm, with 0.1 M 2,6-lutidine as eluting agent gave a breakthrough of histidine after 13 ml, appearance of 1-methylhistidine after collecting 17 ml of eluate, and breakthrough of a mixture of carnosine and anserine after collecting 41 ml.

Fig. 1 shows a separation of 15-μmole samples of β-alanine, 1-methylhistidine, 3-methylhistidine, and histidine on a Dowex 50-α-picoline column, 0.8 × 9 cm. β-Alanine appears with the solvent front followed by 3-methylhistidine, then histidine, which is separated completely from 1-methylhistidine. Recoveries varied between 93 and 100% in repeated isolations.

Excretion of Urinary 1-Methylhistidine—The chromatographic procedures were first applied to a study of the excretion of the methylhistidines in the rabbit in varying stages of vitamin E deficiency. Fig. 2 shows the results of measurement of 1-methylhistidine excretion from zero to 35 days by rabbits on a deficient diet. Levels of 1-methylhistidine are expressed as μmoles excreted in a 24-hour period per kg of body weight and these are compared with the changes in the creatine to creatinine ratio. Normal rabbits excreted 3.7 to 9.5 μmoles of 1-methylhistidine in a 24-hour period. Tallan (17) has also reported a low level excretion of this compound by normal rabbits. However, Datta et al. (25) failed to detect it and Fink et al. (13) have recently reported that only 2 out of 22 normal rabbits showed a detectable irregular 1-methylhistidine excretion.

With the creatine to creatinine ratio as an indicator of the onset and increasing severity of the experimental dystrophy (26), it is seen that there is a slight increase in methylhistidine excretion by the 7th day which is within the limits of a high normal excretion. However, the creatine to creatinine ratio is unchanged. At 25 days, both methylhistidine excretion and the creatine to creatinine ratio are elevated and by the 30th day, they are distinctly indicative of muscular dystrophy. At 35 days when the rabbits have reached the terminal stage of an acute vitamin E deficiency and the creatine to creatinine ratio is 4.2, the excretion of 1-methylhistidine reaches a value of 38 μmoles.
per 24-hour urine sample. In two animals, values of 60 to 62 μmoles were seen. These findings agree qualitatively with those reported by Fink et al. (13), but our animals excreted smaller amounts than reported in their investigation. No definite conclusion may be drawn from this study as to whether the increased creatine to creatinine ratio occurs subsequent to the increase in 1-methylhistidine excretion as has been reported by Fink et al. (13). No 3-methylhistidine was detected in any of the urine samples.

Concentration of Peptides in Muscle in Vitamin E Deficiency—Several workers (1, 2) have observed that severe vitamin E deprivation in rabbits leads to decreased levels of muscle anserine and carnosine. Talman (2) reported a decrease in the late stages of deficiency, but he did not observe a decrease in the combined carnosine-anserine peak obtained in chromatographic analysis of muscle from a rabbit in the early stages of dystrophy. Table I shows the results of two experiments in which the anserine and carnosine concentrations were measured in rabbits maintained on a chow diet, on a purified diet supplemented with α-tocopherol, and on a purified diet deficient in vitamin E. Concentrations were determined on rabbits in which the creatine to creatinine ratio was elevated to 1 to 2 with no other symptoms (Stage I), and on rabbits in which gross symptoms of muscular dystrophy were apparent (Stage III) (26). Results are expressed in terms of millimoles of peptide per 100 g of wet weight of muscle and as millimoles per g of noncollagenous nitrogen. The latter unit is necessary since dystrophic muscle undergoes varying degrees of replacement by collagen and lipids (27). It should be noted that the noncollagenous nitrogen determinations in Experiment 1 were done on untreated tissue, whereas the tissue in Experiment 2 was first freed of lipids with Bloor’s reagent. It is apparent in Experiment 1 that animals in deficiency Stage III have a reduced concentration of anserine and carnosine, expressed as millimoles per g of noncollagenous nitrogen, amounting to a 70% and a 64% reduction in anserine compared to the chow-fed rabbits and the supplemented rabbits, respectively. A similar percentage decrease in carnosine is observed. Anserine levels show a decrease in deficiency Stage III in Experiment 2, but the depression is less marked when expressed in terms of noncollagenous nitrogen, perhaps due to the preliminary lipid extraction of the tissue. Thus, anserine decreases 54.7% in terms of millimoles per 100 g of tissue, but only 33% when calculated as millimoles per g of noncollagenous nitrogen. Rabbits, killed when the creatine to creatinine ratios were 1.6 to 2.6, show only a 12.3% decrease in anserine. However, carnosine is very significantly decreased. These changes were studied in a small group of animals and more analyses are desirable to establish the point at which a measurable decrease in peptide levels is first observed. This is of particular interest in attempting to relate the urinary excretion of 1-methylhistidine to the breakdown of muscle anserine and to evaluate its importance in terms of the nutritional muscular dystrophy.

Incorporation of Labeled Precursors into Anserine and Carnosine—It is obviously not possible to ascertain the contribution of increased catabolic or decreased anabolic activity or both to the observed decrease in peptide concentrations simply from measurement of alteration in amounts of the peptides. As a first approach to this question, it was of interest to determine whether normal and dystrophic rabbit muscles were able to catalyze hydrolysis of the peptides. Carnosinase and anserinase activities are widely distributed in a number of tissues, including kidney, liver and spleen, but little or no activity has been reported in normal skeletal muscle in the rat (24) or rabbit (28). However, an increase in the activities of dipeptidases catalyzing the hydrolysis of glycyl-L-leucine and glycylglycine has been reported in extracts of dystrophic muscle of vitamin E-deficient rabbits (29). Muscle extracts were prepared from the skeletal muscle of normal and dystrophic rabbits and their ability to catalyze the hydrolysis of carnosine and anserine was investigated by the assay described by Hanson and Smith (24). Several concentrations of muscle extract were used and incubations were conducted over periods of from 1 to 17 hours, but results were consistently negative. Thus, in agreement with other workers, muscle appears to be devoid of carnosinase activity. Therefore, it must be concluded that hydrolysis of anserine, as evidenced by the increased 1-methylhistidine excretion, occurs external to the muscle. This aspect will be discussed later.

Several workers (20, 31) have demonstrated the incorporation of labeled β-alanine, histidine, 1-methylhistidine, and methionine into carnosine and anserine in normal animals and synthesis in vivo from β-alanine and the imidazole precursors has been obtained in chick pectoral muscle (4, 5). The latter findings and the studies of Rasima (3) in hepatocarcinized and nephrectomized rats show that muscle is able to effect synthesis of the peptides. Experiments by Winnick et al. (32) and this laboratory2 have demonstrated methyl transfer from 1-adenosylmethionine to form anserine by chick muscle extracts. Thus, it appears that the muscle itself has a significant role in the synthetic metabolism of the peptides. In the present study in vivo, the incorporation of labeled α-histidine and 1-methionine into the peptides has been used in an effort to evaluate the ability of dystrophic muscle to synthesize carnosine from L-histidine and β-alanine and to carry out transfer of the methyl group of methionine to the methylhistidine moiety of anserine.

Table II shows two experiments in which the incorporation of α-histidine-2-C4 has been determined after 5.5, 24, and 48 hours. Results are expressed as c.p.m. per 100 μmoles of peptide. Experiment 1 was limited to rabbits in Stage IIII deficiency whereas Experiment 2 included animals in both Stages I and III. α-Histidine-2-C4, measured after 24 hours, was incorporated only slightly into anserine as compared to its utilization for synthesis of carnosine in both normal and deficient animals. It was incorporated into carnosine in deficient muscle as efficiently or better than in normal muscle. In contrast, as shown in Table III, Experiment 1, incorporation of L-methionine-methyl-C4 into anserine, measured after 5 hours, was considerably better in the normal than in the deficient animals. In Experiment 2, this observation was confirmed after 5 and 16 hours, and extended to include animals in Stage I deficiency. In this experiment, a markedly decreased incorporation of C4 was observed even at an early stage of the deficiency.

In examining these results, at least three factors are of immediate importance. First, the problem of the site of synthesis must be considered. Evidence supporting the muscle as a major synthetic site has already been mentioned. Second, changes in the pool size of the precursors and in the total concentration of the product being measured as a result of the vitamin E deficiency must be evaluated. Third, it must be assumed that the precursors have been incorporated and mixed with the endogenous compounds in a homogeneous manner. Consideration of

2 Unpublished experiments.
the pool size of the precursors presents difficulties, but some information may be derived from measurement of the concentrations and specific activities of the precursors in muscle. With this information and a knowledge of the total concentration and specific activity of the product, an approximation of the magnitude of synthesis of the peptides may be possible. Normal and deficient muscle (127 g) were obtained from rabbits given injections of L-methionine-methyl-C14 and killed after 5 hours and the concentration and specific activity of L-methionine and anserine were determined. Methionine was obtained in a mixture of neutral amino acids by taking the fraction eluted with 0.1 M pyridine from a Dowex 50-H+ column (7) and rechromatographing it on a Dowex 2-OH- column (33). Creatine was washed off with water and the neutral amino acids were obtained by elution with 0.1 N acetic acid. The eluate was concentrated and methionine was determined on an aliquot (21). The remainder of this fraction was reacted with hydroiodic acid and the methyl group of methionine was obtained as tetramethylammonium iodide (22). Anserine was isolated as described in the "Methods." Normal muscle contained 1.19 mg per 100 g of muscle with a specific activity of 24,721 c.p.m. per 100 μmoles. Deficient muscle had 5.6 mg of methionine with a specific activity of 7,760 c.p.m. per 100 μmoles, or 31.4% of the activity of normal muscle methionine. Thus, in agreement with results of other workers (2), methionine was elevated in the deficient muscle. Anserine (280 μmoles per g of noncollagenous nitrogen) with a specific activity of 516 c.p.m. per 100 μmoles was isolated from normal muscle. Deficient muscle had 5.6 mg of methionine-methyl-Cl4 and that the specific activity of activated methionine.


discussion
In recent years, several workers have described chromatographic methods which will separate carnosine and anserine, histidine and 1-methylhistidine (7, 17, 34, 35). In a few cases, 3-methylhistidine was also separated (17, 35). The procedure described here offers certain advantages over the earlier peptide isolations in cases where there is no need to distinguish between the β-alanine moieties of carnosine and anserine. Less time is needed for a separation since milligram quantities can be isolated on a small column with relatively small elution volumes and the procedure eliminates need for desalting the eluate since

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stage of deficiency</th>
<th>No. of rabbits</th>
<th>Anserine</th>
<th>Carnosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control on chow diet</td>
<td>3</td>
<td>0.8920 ± 0.012</td>
<td>0.4700 ± 0.022</td>
<td>0.1660 ± 0.022</td>
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<tr>
<td>Control</td>
<td>7</td>
<td>0.7430 ± 0.015</td>
<td>0.4110 ± 0.017</td>
<td>0.1490 ± 0.004</td>
</tr>
<tr>
<td>Stage III</td>
<td>6</td>
<td>0.2500 ± 0.018</td>
<td>0.1290 ± 0.004</td>
<td>0.0500 ± 0.004</td>
</tr>
<tr>
<td>2 Control</td>
<td>7</td>
<td>0.8000 ± 0.010</td>
<td>0.2770 ± 0.001</td>
<td>0.0670 ± 0.001</td>
</tr>
<tr>
<td>Stage I</td>
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<td>0.4620 ± 0.004</td>
<td>0.1060 ± 0.002</td>
<td>0.0220 ± 0.002</td>
</tr>
<tr>
<td>Stage III</td>
<td>3</td>
<td>0.3600 ± 0.002</td>
<td>0.0850 ± 0.002</td>
<td>0.0420 ± 0.002</td>
</tr>
</tbody>
</table>

* Stage I, creatine to creatinine ratio = 1.6-2.6; Stage III, moderate to severe paralysis (26).
† Calculations in Experiment 2 based on noncollagenous nitrogen determinations on lipid extracted tissue.
‡ Standard error of the mean.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Stage of deficiency</th>
<th>No. of animals</th>
<th>Anserine</th>
<th>Carnosine</th>
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<tbody>
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<td>1</td>
<td>III</td>
<td>6</td>
<td>0.1290 ± 0.004</td>
<td>0.0520 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>III</td>
<td>3</td>
<td>0.0850 ± 0.002</td>
<td>0.0420 ± 0.002</td>
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</table>

* Specific activity of DL-histidine-2-Cl4 is 1.0 μc per 10 μmoles; administered 5 μc per kg of body weight.
† Range of values.

<table>
<thead>
<tr>
<th>Experiment No.</th>
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<th>No. of animals</th>
<th>Time of experiment</th>
<th>Control</th>
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<td>5</td>
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<td>406 (370-443)</td>
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<tr>
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<td>4</td>
<td>24</td>
<td>1113 (629-1604)</td>
<td>419 (114-700)</td>
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<tr>
<td>4</td>
<td>III</td>
<td>5</td>
<td>701 (302-1040)</td>
<td>67 (17-116)</td>
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</tr>
<tr>
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<td>III</td>
<td>16</td>
<td>478</td>
<td>32</td>
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</tbody>
</table>

* Specific activity of L-methionine-methyl-C14 is 4.0 μc per 10 μmoles; administered 4.0 μc per kg of body weight.
† Range of values.
the eluate may be removed by evaporation. This is important when radioactivity measurements involve solid counting techniques. No 3-methylhistidine was found in rabbit urine by this procedure, but the compound has been obtained from cats, in agreement with Tallan (24) and in small amounts from rats (36).

The data described in this paper and the work of others cited above permit preliminary description of a pattern of the metabolism of the peptide during development of a nutritional muscular dystrophy. Increased excretion of 1-methylhistidine, presumably arising largely from hydrolysis of muscle anserine (12) becomes clearly apparent in our studies between the 16th and 25th day, coincident with a slight elevation in the creatine to creatinine ratio and well before the appearance of other symptoms of muscular dystrophy. A slight increase may occur at the 7th day but its significance has not been determined. Fink et al. (13) reported excretion of 1-methylhistidine beginning after about one week on the same diet used in our studies (14) and before the appearance of creatinuria. Further, we have observed consistently a normal low methylhistidinuria, indicating certain qualitative differences between our findings and Fink et al. (13).

There is no evidence either in the studies of Hanson and Smith (24) and Garkavi (28) or in this investigation to suggest that the peptides are enzymatically hydrolyzed in normal muscle or in dystrophic muscle, although increased proteolytic activity (29, 37) and phosphorylative activity (38) have been reported in dystrophic muscle. The changes which occur in a vitamin E deficiency include alteration in permeability properties of the cell membranes (39). Further, dystrophic changes from other causes may effect cell permeability as indicated by the finding of Zierler that aldolase leaks from the muscle cells of mice with hereditary muscular dystrophy (40). Loss of vitamin E or other changes resulting in alteration of the essential structural composition of the muscle could reduce the effectiveness of an active carrier system (41) in the mediated transport and cellular concentration of the amino acid constituents, and as a consequence, the peptides may be lost from the muscle cell and subsequently hydrolyzed in other tissues; e.g., the kidney. A methylhistidinuria would then occur as a result of the hydrolysis of anserine. Alternatively, the observations might be explained in terms of a release of these peptides from a bound form which is not normally permeable to the cell membrane. There is no evidence at this time to support this view. Failure to detect anserine or 1-methylhistidine in the peripheral circulation in this investigation is not unexpected with the assay procedures used since 1-methylhistidine has a low renal threshold (42) and would be removed rapidly from the circulation, presumably without further significant metabolic alteration (6). The incorporation of labeled methionine into anserine is significantly depressed between the 25th and 30th day and the available evidence is consistent with a depressed anserine synthesis. Thus, the decreased anserine levels may be a result of an increased leakage from muscle and a depressed synthesis of peptide by the muscle. A similar pattern is probable for carnosine although our data only suggest this.

The significance of these findings in the sequence of events leading to physical symptoms of muscular dystrophy requires elucidation of the metabolic role of the peptides in the normal functioning of muscle (43-49).

SUMMARY

A chromatographic procedure is described for the separation of β-alanine, histidine, 1-methylhistidine, and 3-methylhistidine.

The pattern of 1-methylhistidine excretion and the levels of carnosine and anserine in skeletal muscle have been examined during a developing vitamin E deficiency in rabbits. In agreement with other studies, 1-methylhistidine excretion increases markedly with increasing severity of the deficiency. Carnosine and anserine decrease to between 38 and 64% of the levels seen in normal animals.

Histidine-2-C14 was incorporated only slightly into anserine and was well utilized for carnosine synthesis by both normal and vitamin E-deficient animals. L-Methionine-methyl-C14 was incorporated into anserine of normal muscle 2- to 10-fold better than into deficient muscle. The available evidence is consistent with the view that vitamin E-induced muscular dystrophy results in a decreased synthesis of anserine in the muscle and its hydrolysis to β-alanine and 1-methylhistidine subsequent to its increased loss from the muscle.

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