Manganese, Copper, Zinc, and Iron Concentrations and Subcellular Distribution in Two Types of Skeletal Muscle (43166)

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Abstract. **To clarify trace element distribution in red and white muscle, and to verify two populations of muscle mitochondria, the iron, zinc, copper, and manganese concentrations of whole muscle and their subcellular fractions were determined. The iron, zinc, copper, and manganese concentrations of red muscle were 1.83, 4.31, 2.05, and 1.67 times higher than those of white muscle, respectively. In skeletal muscle subcellular distribution of iron, zinc, and copper were entirely different and that of manganese was relatively similar as compared with those in liver reported previously. The pattern of mineral distribution in all fractions of red muscle was similar to that of white muscle, but their concentrations in some fractions were different between red and white muscle, e.g., iron, zinc, and manganese in supernatant fraction and copper in nuclear and microsomal fractions. The differences between subsarcolemmal and interfibrillar mitochondria were ascertained by the distribution of trace elements.**

[P.S.E.B.M. 1991, Vol 196]

Recently it has been assumed that some minerals in muscles are related to the pathogenesis of neuromuscular diseases like Duchenne muscular dystrophy (1–3). It is also known that some dietary mineral deficiencies lead to neuromuscular dysfunction (4–6), and muscle biopsies are being used for the diagnosis of mineral disorders (7, 8).

Muscle fiber type 1 and type 2 are different in physiologic and biochemical properties (9). Therefore, the type of fiber in a specimen should have special consideration in the physiologic and biochemical study of skeletal muscle. The study of mineral in muscle is no exception. Red muscle (predominant type 1) and white muscle (predominant type 2) have been known to have different sodium, potassium, calcium, and zinc concentrations (7, 10–12). However, there have been few reports on the contents of trace elements in red and white muscles, e.g., manganese, copper, and iron, which have been shown to be related to muscular function and muscular diseases (2, 3, 6). The objective of this study was to clarify the differences between iron, zinc, copper, and manganese concentrations in whole tissue and subcellular fractions between two muscle types.

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Received April 16, 1990. [P.S.E.B.M. 1991, Vol 196]
Accepted July 31, 1990.

0037-9727/91/1961-0083$2.00/0

Materials and Methods

Animals. Male Wistar rats (12 weeks old) were used. They were given a commercial diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and double-distilled water. Mineral concentrations in the diet were as follows: copper, 5.84 μg/g; zinc, 44.3 μg/g; manganese, 55.8 μg/g; iron, 105.3 μg/g.

Rats were exsanguinated under sodium pentobarbital anesthesia, and extensor digitorum longus (EDL), anterior tibialis, soleus, and gastrocnemius muscles were collected. The water contents of soleus and EDL muscles were the same (72–73%), and so we measured wet tissue. To measure mineral concentrations in whole muscle, soleus and EDL were used as red and white muscle, respectively. To prepare subcellular fractions, the soleus and red portion of gastrocnemius were used as red muscle, and EDL and the white portion of anterior tibialis were used as white muscle. Because the muscles of rats are very small, we prepared one sample from four rats for the subcellular experiment.

Reagents. Special grades of KCl, $MgCl_2$, sucrose, and Hepes and EGTA were purchased from Nacalai Tesque Inc. (Kyoto, Japan); trypsin (from bovine pan-

creas), trypsin inhibitor (from soybean), and bovine serum albumin were from Sigma Chemical Co.; and Percoll was from Pharmacia LKB Biotechnology Inc. (Tokyo, Japan). All other reagents were of the best grade available commercially.

Preparation of Subcellular Fractions. The procedure of preparations of subcellular fractions is shown in Figure 1. All procedures were performed at 0–4°C. Specimens were freed of extracellular fat and connective tissues and finely minced using stainless steel razors. The minced tissue was suspended in ice-cold isolation medium A (100 mg/ml). The medium A consisted of 120 m*M* KCl, 20 m*M* Hepes, 5 m*M* $MgCl_2$, and 1 m*M* EGTA, at pH 7.4, nearly similar to that described by Sherrat *et al.* (13). The tissue was homogenized with Potter-Elvehjem type homogenizer using two different Teflon pestles (clearances of 0.3 and 0.05 mm).

The homogenate was centrifuged at 600*g* for 10 min to get supernatant (S) 1 and pellet (P) 1. P1 was treated with trypsin as described by Fischer *et al.* (14), homogenized with a Teflon pestle of 0.05 mm clearance, and centrifuged at 600*g* for 15 min to obtain S2 and P2. To make nuclear fraction, P2 was washed twice with medium A, followed by suspension in a small volume of medium B, consisting of 250 m*M* sucrose, 2 m*M* Hepes, and 0.1 m*M* EGTA, at pH 7.4.

S1 and S2 were centrifuged at 16,000*g* for 10 min to obtain S3 and P3, and S4 and P4, respectively. P4, which was treated with trypsin, was purified by density gradient centrifugation with Percoll as described by Mickelson *et al.* (15), washed twice with medium A, and suspended in a small volume of medium B to make interfibrillar mitochondrial fraction. P3, which was not treated with trypsin, was treated as P4 to make subsarcolemmal mitochondrial fraction.

S3 and S4 were centrifuged at 100,000*g* for 60 min to get S5 and P5, and S6 and P6, respectively. S5 was used as supernatant fraction. S6 was discarded. P5 and P6 were combined, washed twice with medium A, and suspended in a small volume of medium B to make microsomal fraction.

Protein was determined according to the method of Peterson (16).

Assessment of Purity of Fractions. To assess the contamination of fractions like nuclear fraction by mitochondria and microsome, marker enzymes were assayed. Succinate dehydrogenase as a marker enzyme of mitochondria was assayed as described by Singer (17), and NADPH cytochrome P-450 reductase as a marker enzyme of microsome was assayed as described by Masters *et al.* (18). The results are shown in Table I, which indicated that the contaminations were less than 10%.

Analysis. To prevent trace element contamination, all possible precautions were taken. All laboratory ware used in this study, including Potter-Elvehjem homogenizer, centrifugation tube, and tips, were soaked in 10% nitric acid solution for several hours followed by several rinsings with deionized double-distilled water.

Samples were wet-ashed with nitric acid. Zinc and iron were determined with an inductively coupled plasma atomic emission spectrometer (ICP-1000II; Shimadzu Co., Ltd., Kyoto, Japan). Copper and Manganese concentrations were determined with a flameless atomic absorption spectrophotometer with a graphite furnace (AA-646; Shimadzu Co. Ltd.) by standard addition technique.

Statistics. The data were expressed as the mean ± SE and analyzed statistically by paired *t* test. A value of $P < 0.05$ was considered significant.

Results

The mineral concentrations in white and red muscles are shown in Table II. The manganese, copper, zinc, and iron concentrations in red muscle were significantly higher as compared with those in white muscle.

Subcellular distribution of iron is shown in Table III. Iron concentrations of the fractions were in the following descending order: mitochondria > supernatant > microsome > nucleus. Iron concentrations of supernatant and microsomal and nuclear fractions in red muscle were significantly high compared with those in white muscle. The iron concentration of interfibrillar mitochondrial fraction in red muscle was significantly low as compared with that in white muscle. In red muscle the iron concentration of subsarcolemmal mitochondrial fraction was significantly low compared with that of interfibrillar mitochondrial fraction.

Subcellular distribution of zinc is shown in Table IV, which was in the following descending order: supernatant ≫ microsome ≈ mitochondria > nucleus. Zinc concentrations of all fractions except for interfibrillar mitochondrial fraction in red muscle were significantly high compared with those in white muscle. The zinc concentration of interfibrillar mitochondrial fraction in red muscle was significantly low compared with that in white muscle. In white and red muscles, zinc concentrations of subsarcolemmal mitochondrial fractions were significantly different from those of interfibrillar mitochondrial fractions.

Subcellular distribution of copper is shown in Table V. Copper concentrations of fractions were in the following descending order: mitochondria > microsome > nucleus ≈ supernatant. Copper concentrations of microsomal and nuclear fractions in red muscle were significantly high compared with those in white muscle.

Subcellular distribution of manganese is shown in Table VI, which was in the following descending order: mitochondria > supernatant > nucleus > microsome. Mn concentrations of all fractions except for interfi-

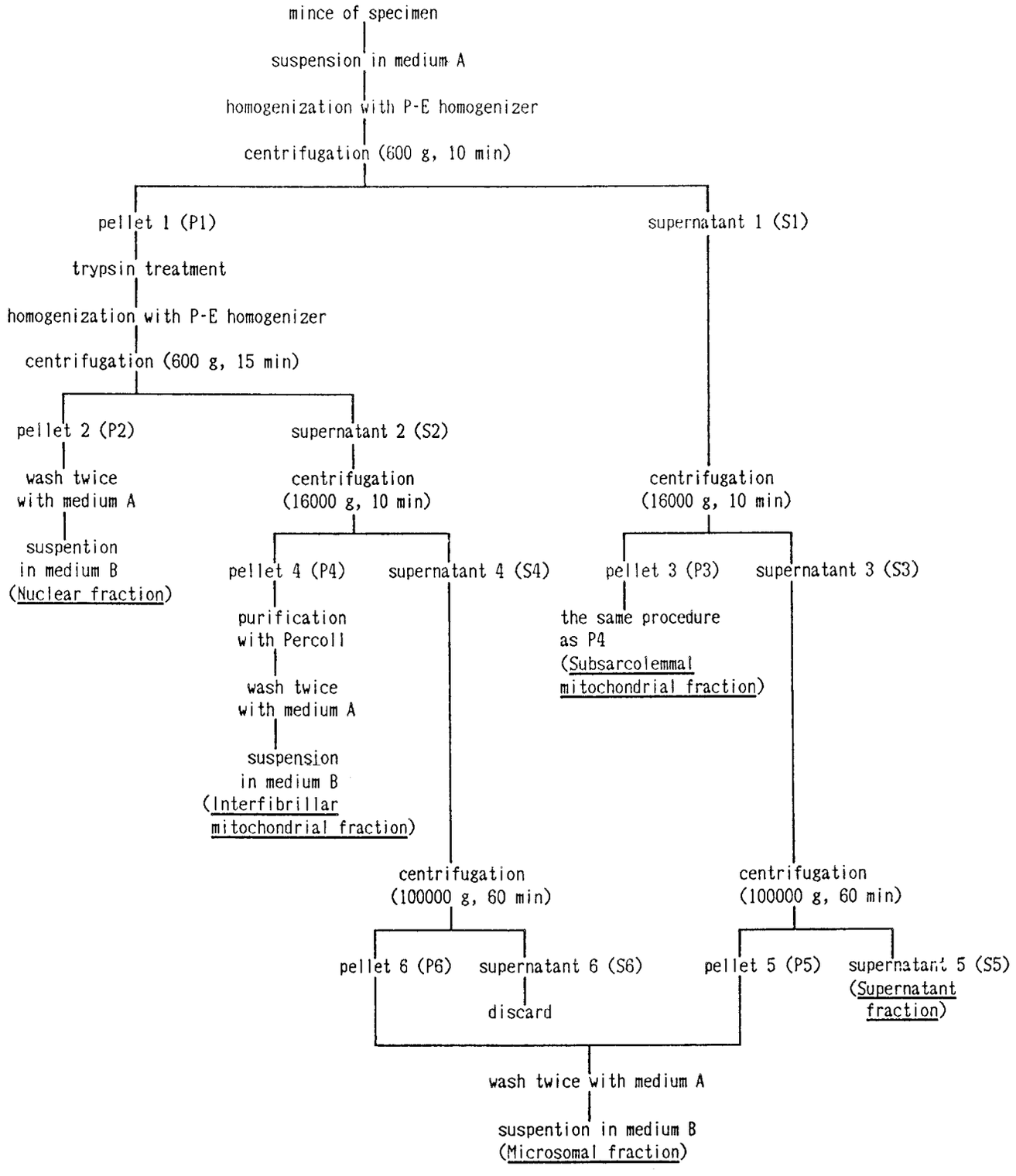

Figure 1. Procedure for preparation of skeletal muscle subcellular fractions.

brillar mitochondrial fraction in red muscle were significantly high compared with those in white muscle. In white and red muscle, manganese concentrations of subsarcolemmal mitochondrial fractions were significantly low compared with those of interfibrillar mitochondrial fractions.

Discussion

As far as we know, this is the first report on the distributions of iron, copper, and manganese in subcellular fractions of skeletal muscle. Subcellular distributions of iron and copper in the muscle are entirely different from those in the liver reported by Thiers and

Table I. Activities of Marker Enzymes in Subcellular Fractions[a]

	nmol/min/mg protein				
	Nucleus	Mitochondria		Microsome	Supernatant
		Subsarcolemmal	Interfibrillar		
Succinate dehydrogenase[b] (mitochondrial marker enzyme)	4.37 ± 0.16	299 ± 16	357 ± 5	29.6 ± 0.4	8.12 ± 0.26
NADPH cytochrome P-450 reductase[b] (microsomal marker enzyme)	0.077 ± 0.003	1.16 ± 0.11	1.16 ± 0.18	18.6 ± 1.4	0.027 ± 0.002

[a] Values are mean ± SE (n = 4).
[b] The activity of succinate dehydrogenase was assayed as a mitochondrial marker enzyme to assess mitochondrial contamination, and the activity of NADPH cytochrome P-450 reductase was assayed as a microsomal marker enzyme to assess microsomal contamination.

Table II. Mineral Concentrations in White and Red Muscle[a]

	μg/g of wet tissue		Red/white ratio
	White muscle[b]	Red muscle[b]	
Iron	13.63 ± 0.38	24.88 ± 0.68[c]	1.83 ± 0.08
Zinc	13.62 ± 0.52	58.51 ± 1.43[c]	4.31 ± 0.09
Copper	1.180 ± 0.035	2.407 ± 0.036[c]	2.05 ± 0.08
Manganese	0.145 ± 0.001	0.242 ± 0.003[c]	1.67 ± 0.03

[a] Values are mean ± SE (n = 6).
[b] EDL and soleus muscles were used as white and red muscle, respectively.
[c] Significant difference ($P < 0.05$) from white muscle.

Table III. Subcellular Distribution of Iron in Red and White Muscle[a]

	μg/g protein	
	White muscle[b]	Red muscle[b]
Nucleus	56 ± 4	89 ± 10[c]
Subsarcolemmal mitochondria	317 ± 8[d]	334 ± 7
Interfibrillar mitochondria	456 ± 41	334 ± 8[c]
Microsome	96 ± 5	164 ± 2[c]
Supernatant	131 ± 10	302 ± 21[c]

[a] Values are mean ± SE (n = 5); each sample was prepared by tissues from four rats.
[b] The soleus and red portion of gastrocnemius as red muscle and EDL and white portion of anterior tibialis as white muscle were used.
[c] Significant differences ($P < 0.05$) from red muscle.
[d] Significant differences ($P < 0.05$) from interfibrillar mitochondria.

Table IV. Subcellular Distribution of Zinc in Red and White Muscle[a]

	μg/g protein	
	White muscle[b]	Red muscle[b]
Nucleus	8.8 ± 0.4	17.8 ± 0.1[c]
Subsarcolemmal mitochondria	40.2 ± 0.8[d]	54.0 ± 3.3[c, d]
Interfibrillar mitochondria	56.7 ± 4.3	37.8 ± 0.9[c]
Microsome	37.8 ± 1.5	69.5 ± 1.5[c]
Supernatant	169 ± 5	516 ± 13[c]

[a] Values are mean ± SE, n = 5; each sample was prepared by tissues from four rats.
[b] The soleus and red portion of gastrocnemius as red muscle and EDL and white portion of anterior tibialis as white muscle were used.
[c] Significant differences ($P < 0.05$) from red muscle.
[d] Significant differences ($P < 0.05$) from interfibrillar mitochondria.

Table V. Subcellular Distribution of Copper in Red and White Muscle[a]

	μg/g protein	
	White muscle[b]	Red muscle[b]
Nucleus	5.2 ± 0.4	14.1 ± 1.6[c]
Subsarcolemmal mitochondria	68.1 ± 1.3	67.5 ± 1.7
Interfibrillar mitochondria	67.2 ± 1.2	67.7 ± 1.8
Microsome	24.2 ± 0.8	41.6 ± 0.3[c]
Supernatant	8.2 ± 0.8	8.3 ± 0.8

[a] Values are mean ± SE (n = 5); each sample was prepared by tissues from four rats.
[b] The soleus and red portion of gastrocnemius as red muscle and EDL and white portion of anterior tibialis as white muscle were used.
[c] Significant differences ($P < 0.05$) from red muscle.

Vallee (19). In the muscle, iron concentrations of microsomal and supernatant fractions and copper concentration of supernatant fractions, especially the last one, were lower than that of the liver. The pattern of subcellular distribution of manganese in the muscle is relatively similar to that in the liver and kidney, reported by Sakurai *et al.* (20), but manganese concentrations of nuclear and supernatant fractions of the muscle are low and high, respectively, as compared with those of the liver and kidney.

Cassens *et al.* (12) have previously reported zinc concentrations in whole muscles and their subcellular fractions in pigs, which differ from our observations. In their reports, zinc concentrations of subcellular fractions decreased in the following order: nuclear > supernatant ≫ mitochondria ≈ microsome. They used 0.25 M sucrose as medium for preparation of subcellular fraction; skeletal muscle, when homogenized in nonelectrolyte medium, often assumes a gelatinous consistency, which makes it difficult to obtain sufficient disintegration of the myofibrils (21). Thus, there is a possibility that the nuclear fraction prepared by Cassens

Table VI. Subcellular Distribution of Manganese in Red and White Muscle[a]

	μg/g protein	
	White muscle[b]	Red muscle[b]
Nucleus	0.642 ± 0.020	0.926 ± 0.028[c]
Subsarcolemmal mitochondria	2.35 ± 0.03[d]	3.18 ± 0.03[c, d]
Interfibrillar mitochondria	4.36 ± 0.10	4.34 ± 0.03
Microsome	0.392 ± 0.027	0.542 ± 0.014[c]
Supernatant	1.44 ± 0.04	2.27 ± 0.136[c]

[a] Values are mean ± SE (n = 5); each sample was prepared by tissues from four rats.
[b] The soleus and red portion of gastrocnemius as red muscle and EDL and white portion of anterior tibialis as white muscle were used.
[c] Significant differences ($P < 0.05$) from red muscle.
[d] Significant differences ($P < 0.05$) from interfibrillar mitochondria.

et al. (12) was contaminated by other fractions considerably. Our observations of subcellular distribution of zinc in the muscle is entirely different from that in the liver reported by Theirs and Vallee (19), e.g., zinc concentration of supernatant fraction of the muscle was very high as compared with that of the liver.

Our results showed that the difference in iron concentration in whole muscle depended mainly on that in supernatant fraction, which contains myoglobin, the iron-binding protein. High iron concentrations in the supernatant fraction of red muscle may be due to its high concentration of myoglobin (22).

The difference in zinc concentration between red and white muscles is mainly due to that of supernatant fraction. The zinc-binding proteins, such as malate dehydrogenase, lactate dehydrogenase, and neutral protease, and free zinc ion might be responsible for high zinc concentrations in the supernatant fraction.

The differences in copper distribution between red and white muscles were found in nuclear and microsomal fractions, which might contribute to the differences in copper concentrations in whole muscles. Red muscle is rich in mitochondria containing high concentrations of copper (22), which will also contribute to the differences in whole muscles. The copper-binding proteins, such as cytochrome oxidase, might be related to the high copper concentration of mitochondrial fractions.

Since the original observation of Hulsmann *et al.* (23), the existence of two populations of muscle mitochondria, namely, subsarcolemmal and interfibrillar mitochondria, has been controversial. Joffe *et al.* (24) mentioned a 1.5 times higher glutamate-dependent O_2 consumption by interfibrillar mitochondria than subsarcolemmal mitochondria. Muller (25) and Krieger *et al.* (26) reported that there are morphologic and biochemical differences between the two populations in relation to exercise. However, Matlib *et al.* (27) assumed that the differences between the two populations proposed by Hulsmann *et al.* (23) can be explained by artifacts of isolation and assay procedures that result in the loss of cytochrome *c* from mitochondria in ionic media. Also, Fischer *et al.* (14) failed to detect any evidence for the existence of two populations biochemically. In our results, zinc and manganese concentrations in red and white muscles and iron concentration in white muscle were different between two types of mitochondria, and thus support their existence.

It has been reported that interconversion of muscle fiber into one another takes place rapidly by various factors such as exercise, immobilization, denervation, hormone, and so on (28, 29). Considering that the manganese, copper, zinc, and iron concentrations and their subcellular distribution are different in two types of muscle, rapid conversion of muscle fibers will cause the change of such trace element concentrations and their subcellular distribution in the muscle. It is also known that the rates of mobility of elements across the cell membrane are not the same (30). Therefore, there is concern that the rapid conversion of muscle types can bring about the imbalance of elements in the muscle, which in turn will have an effect on the biologic condition of the muscle.

Rapid muscular atrophy is caused by certain conditions, such as immobilization and unweighting. In the course of rapid atrophy, the difference of mobility of elements is also expected to induce the imbalance of elements in the muscle. Furthermore, larger imbalance of elements will be induced in red muscle than in white muscle, because the former is rich in trace elements. This speculation is supported by the fact that the atrophy has proceeded more rapidly in the red muscle than in the white muscle (31).

We are grateful to Dr. M. Ujihara for the technical advice and Dr. S. M. Ahmed for assistance in the preparation of the manuscript and very kind encouragement (both from our laboratory).

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