Fractional catabolic rates of myosin and actin estimated by urinary excretion of N^τ-methylhistidine: the effect of dietary protein level on catabolic rates under conditions of restricted food intake

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1. Critical studies on the distribution of N^τ-methylhistidine (3-methylhistidine; Me-His) among organs and tissues in adult rats are reported. Adult rats contained 46.5 ± 3.6 mg Me-His/kg body-weight. Almost 90 % of the Me-His in the body was recovered from skeletal muscle. These results support the hypothesis that fractional catabolic rates of myosin and actin in skeletal muscle can be estimated by measuring urinary excretion of Me-His.

2. Dietary protein level did not affect the total amount of Me-His in the body. However, urinary excretion of Me-His increased as dietary protein intake was increased.

3. From these results it was concluded that fractional catabolic rates of myosin and actin increase as dietary protein intake increases.

The turnover rate of skeletal muscle protein is a very important factor in the over-all regulation of the whole-body protein mass, because of its large mass (Yamaguchi & Kandatsu, 1967; Millward, 1970*a, b;* Young, 1970; Waterlow, 1975). Many studies have been reported on the catabolic rate of myofibrillar proteins in skeletal muscle. Many of them suggest that catabolic rates of muscle proteins are more rapid than was once thought. However, results are still surely equivocal, especially because of the high re-utilization of labelled amino acids in this tissue (Swick & Song, 1974; Hegsted, 1975; Waterlow, 1975).

Asatoor & Armstrong (1967) proposed that the catabolic rate of muscle protein could be measured by estimating the urinary excretion of N^τ-methylhistidine (3-methylhistidine; Me-His). As it was proved thereafter, certain histidines are methylated after the formation of the peptide chains of myosin and actin. This Me-His in muscle protein could not be re-utilized for protein synthesis after it is released by the breakdown of the proteins within the tissue (Young, Alexis, Baliga, Munro & Muecke, 1972). In experiments on rats and man, it was shown further that administered Me-His is not catabolized and is excreted quantitatively in urine (Young *et al.* 1972; Long, Haverberg, Young, Kinney, Munro & Geiger, 1975). These results confirmed the hypothesis proposed by Asatoor & Armstrong (1967). However, critical studies on the distribution of Me-His within the animal and human body have not been reported. This is another important point to prove the validity of the hypothesis.

Table 1. *Composition of the experimental diets given to rats (g/kg)*

Protein content ...	100	200	400	600
Ingredients				
Egg albumen*	100	200	400	600
Maize starch	800	700	500	300
Soya-bean oil	50	50	50	50
Salt mixture†	40	40	40	40
Vitamin mixture†	8·5	8·5	8·5	8·5
Choline chloride	1·5	1·5	1·5	1·5

* Supplied by Wako Pure Chemical Industries Co. Ltd, Osaka, Japan.
† Composition as described by Harper (1959).

Therefore, the first object of this paper was a study of Me-His distribution among organs and tissues in rats.

It is well known that the rates of synthesis and breakdown of the muscle proteins are very sensitive to protein deficiency and refeeding (Millward, 1970*b*; Waterlow, 1975). However, little work has been done concerning the changes in the turnover rate of the special proteins, myosin and actin, in skeletal muscle under various nutritional conditions, although nutritional effects on mixed muscle proteins have sometimes been reported (Waterlow & Stephen, 1968; Young, Stothers & Vilaire, 1971; Millward, Garlick, James, Nnanyelugo & Ryatt, 1973; Garlick, Millward, James & Waterlow, 1975; Millward, Garlick, Stewart, Nnanyelugo & Waterlow, 1975).

Several authors have measured urinary Me-His in order to estimate the catabolic rate of muscle proteins in various physiological conditions. For example, it was shown that the catabolic rate of muscle proteins decreases during starvation in men (Young, Haverberg, Bilmazes & Munro, 1973). The technique was also used to show the increased degradation rate of the contractile proteins in skeletal muscle during experimentally-induced sandfly fever in man (Wannemacher, Dinterman, Pekarek, Bartelloni & Beisel, 1975). In an earlier study (Nishizawa, Funabiki & Hareyama, 1975), we found that fractional catabolic rate of myosin and actin in skeletal muscle increases as the dietary casein intake increases. In the present paper, details of this experiment will be reported together with some similar results using egg albumen as the protein source.

METHODS

Twenty-nine male Wistar rats weighing 60–99 g were given a diet with 200 g casein/kg *ad lib.* for 30 d. After this period, mean body-weight of rats was 212 g. The animals were then randomly divided into four groups and given the experimental diets, shown in Table 1, for 20 d. Food intake was restricted to 12 g/d per rat, in order to minimize the effect of body-weight gain on the urinary excretion of Me-His. This procedure made kinetic treatment of the results easy. The animals were individually housed in stainless-steel cages in an air-conditioned room maintained at $22 \pm 2°$. The diets were almost isoenergetic and did not contain Me-His.

Collection and preparation of urine samples for Me-His determination

On the last day of feeding, 24 h urine samples were collected in flasks containing 2 ml 4 M-hydrochloric acid, and stored at $-20°$ until they were analysed. Portions

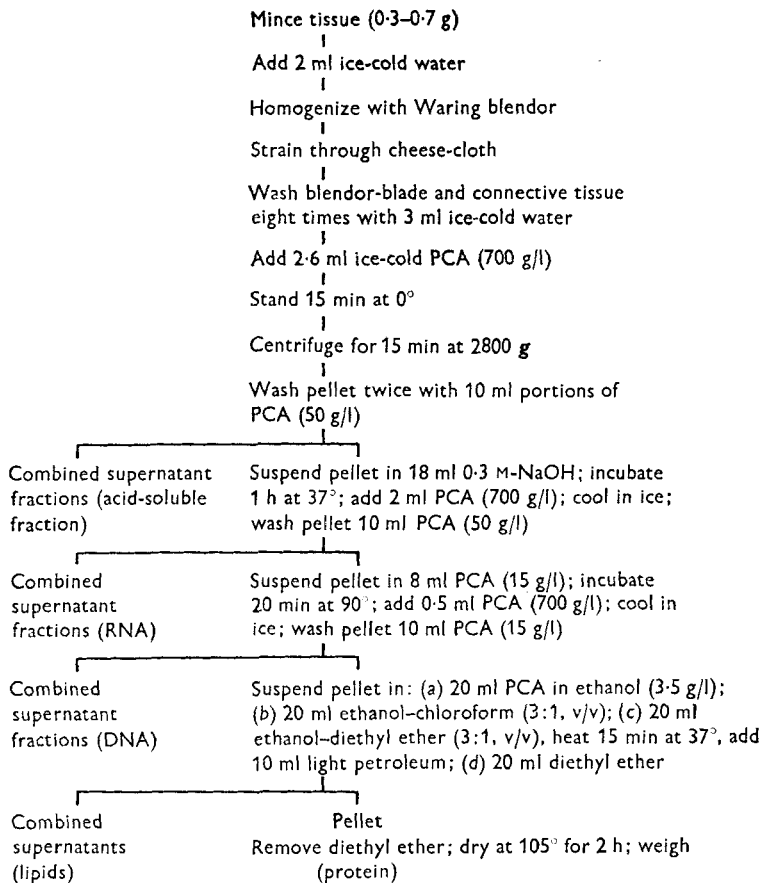Mince tissue (0·3–0·7 g)
|
Add 2 ml ice-cold water
|
Homogenize with Waring blendor
|
Strain through cheese-cloth
|
Wash blendor-blade and connective tissue
eight times with 3 ml ice-cold water
|
Add 2·6 ml ice-cold PCA (700 g/l)
|
Stand 15 min at 0°
|
Centrifuge for 15 min at 2800 **g**
|
Wash pellet twice with 10 ml portions of
PCA (50 g/l)

Combined supernatant   Suspend pellet in 18 ml 0·3 M-NaOH; incubate
fractions (acid-soluble   1 h at 37°; add 2 ml PCA (700 g/l); cool in ice;
fraction)   wash pellet 10 ml PCA (50 g/l)

Combined   Suspend pellet in 8 ml PCA (15 g/l); incubate
supernatant   20 min at 90°; add 0·5 ml PCA (700 g/l); cool in
fractions (RNA)   ice; wash pellet 10 ml PCA (15 g/l)

Combined   Suspend pellet in: (a) 20 ml PCA in ethanol (3·5 g/l);
supernatant   (b) 20 ml ethanol–chloroform (3:1, v/v); (c) 20 ml
fractions (DNA)   ethanol–diethyl ether (3:1, v/v), heat 15 min at 37°, add
                              10 ml light petroleum; (d) 20 ml diethyl ether

Combined   Pellet
supernatants   Remove diethyl ether; dry at 105° for 2 h; weigh
(lipids)   (protein)

Fig. 1. Diagrammatic representation of procedure for separation of mixed tissue proteins.
PCA, perchloric acid.

of 0·5 ml were mixed with 0·5 ml concentrated HCl and hydrolysed at 110° for 24 h to convert N-acetyl-Me-His to Me-His (Young *et al.* 1972). Following hydrolysis, HCl was removed by rotary evaporation under reduced pressure. The hydrolysates were dissolved in water and evaporated again in the presence of a small amount of sodium hydroxide in order to facilitate removal of ammonia. Just before applying them to the amino acid analyser, the samples were acidified with 2 M-HCl, evaporated to dryness and dissolved in 4 ml 0·01 M-HCl.

Preparation of mixed tissue proteins

Six rats given the diet with 200 g protein/kg were lightly anaesthetized with diethyl ether and killed by exsanguination. Liver, kidneys, spleen, heart, stomach, intestinal tract, lungs, testicles and brain were immediately removed, weighed and stored at −20° until they were analysed. After removal of the viscera, the skin was flayed and skeletal muscles were dissected out carefully with a dissecting knife and

scissors in a cold room (4°). Skin and skeletal muscles were also weighed and stored at $-20°$. Tissue proteins were prepared by partial modification of the method of Shibko, Koivistoinen, Tratnyek, Newhall & Friedman (1967) (Fig. 1). Approximately 10 mg tissue proteins was hydrolysed in 2 ml 6 M-HCl in sealed evacuated tubes at 110° for 24 h. The hydrolysates were dried under reduced pressure and dissolved in 5 ml 0·01 M-HCl. The samples were stored at $-20°$ until they were analysed.

Preparation of whole-animal homogenate

The total amount of Me-His in the body was estimated by grinding a whole frozen body. Rats were killed by diethyl ether anaesthesia and frozen at $-20°$. Frozen animals were cut into several pieces using a chopper, and then ground four to five times using a meat grinder. The ground samples were dried at 60° under an air-stream and ground further using a mill. Approximately 1 g of each of the samples was hydrolysed under reflux with 80 ml 6 M-HCl at 110° for 24 h. The hydrolysates were cooled in ice-cold water and filtered through a sintered-glass filter in order to eliminate the black precipitate and solidified fat. The filtrates were dried three times and dissolved in 250 ml water. These samples were also kept refrigerated at $-20°$ until they were analysed.

Measurement of Me-His

Me-His in the hydrolysates was analysed using an amino acid analyser (JEOL 5AH; Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan) operated at high sensitivity. The methods were presented in a previous paper (Nishizawa et al. 1975). Me-His in the urine hydrolysates was analysed by the method described previously. However, the pH of the buffer was changed to 4·55, because, when buffer at pH 4·26 was used, an unknown substance appeared as a 'shoulder' of the Me-His peak. By increasing the pH of the elution buffer, both histidine and Me-His could be chromatographed as separate single peaks. Under these conditions, recovery of Me-His added to the urine samples was 98·9–100·0 % (mean 99·6 %, n 3).

Carcass analysis

Nitrogen content in whole-animal homogenates was estimated by the semi-micro-Kjeldahl method (Association of Official Analytical Chemists, 1975). Body lipid was extracted with diethyl ether and weighed (Association of Official Analytical Chemists, 1975).

Measurement of urinary N and creatinine

N in the urine samples was estimated by the semi-micro-Kjeldahl method. Urinary creatinine was determined by the alkaline picrate method (Grafnetter, Janošová & Červinková, 1967).

Statistical analysis

The procedure described by Snedecor & Cochran (1967) was used for the analysis of variance.
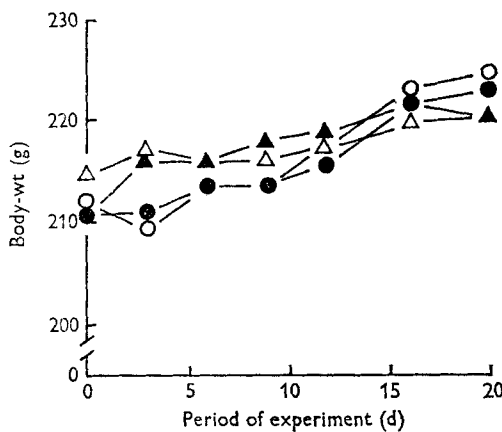
Fig. 2. Changes in body-weight (g) in rats given diets with various levels of protein (g/kg); (○), 100; (●), 200; (△), 400; (▲), 600; for details of diets, see Table 1. Points represent mean values for groups of rats containing five, twelve, six and five animals which were given 100, 200, 400 and 600 g protein/kg diet respectively.

RESULTS

Changes in body-weight during the experimental period are presented in Fig. 2. Mean gain of body-weight was 0·47 g/d.

Me-His contents of various tissue proteins

Table 2 shows tissue or organ weights and their protein contents, together with Me-His contents in their proteins. Skeletal muscle and skin comprised 40 and 26·5 % total body-weight respectively. These results are consistent with others (Miller, 1969; Munro, 1969; Yamaguchi & Kandatsu, 1973). Distribution of Me-His among tissues and organs has been calculated, taking the total amount of body Me-His as 100 %.

Me-His content of skeletal muscle protein was 520 μg/g, the highest value in the tissues. Proteins in stomach and intestines contained 260 and 210 μg Me-His/g respectively. Me-His contents of spleen and heart proteins were almost the same and were 170 and 150 μg/g respectively. The other tissue proteins showed far lower content of Me-His than those. The high concentration of Me-His in the spleen may be due to leucocytes and platelets in this tissue. These cells have been shown to contain a large amount of Me-His (Nishizawa, Funabiki & Hareyama, unpublished results). This fact may also explain the relatively high concentration of Me-His in lung. Whole blood was also analysed for Me-His but we failed to detect a significant amount, presumably because leucocytes and platelets make an insignificant contribution to total blood protein. The results shown in Table 2 are compatible with those reported by Haverberg, Omstedt, Munro & Young (1975), although our results are slightly lower in general.

Since the whole-body concentration of Me-His in rats given the diet with 200 g protein/kg was 46·5 mg/kg body-weight (Table 3), skeletal muscle contains 75 % of

Table 2. *N^τ-methylhistidine (3-methylhistidine; Me-His) content in mixed proteins of tissues and organs of the rats*

(Mean values with their standard errors for six rats weighing 203–249 g)

	Tissue				Me-His content in:				
	Fresh weight (g/kg body-wt)		Protein concentration (mg/g fresh tissue)		Mixed proteins (μg/g protein)		Total organ* (mg/g fresh tissue per kg body-wt)		Tissue Me-His relative to total body Me-His† (%)
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Brain	7·36	0·28	95·8	1·7	19	4	0·013	0·003	0·02
Testicle	7·86	0·58	74·1	1·0	23	5	0·013	0·004	0·02
Spleen	1·60	0·07	129	3	167	9	0·035	0·003	0·07
Kidney	6·29	0·14	167	2	39	5	0·041	0·007	0·08
Liver	27·0	1·3	196	7	9	2	0·052	0·011	0·11
Lung	5·37	0·26	115	5	116	7	0·077	0·004	0·16
Heart	3·84	0·06	154	2	152	5	0·090	0·003	0·19
Stomach	4·83	0·15	76·2	2·8	261	16	0·096	0·008	0·20
Intestine	23·4	1·8	82·6	3·5	211	16	0·402	0·030	0·86
Skin and hair	265	10					3·93	0·54	8·45
Skeletal muscle	400	9	164	5	519	10	34·9	1·2	75·1
Others‡	141	7							
Total	893						39·6		
Total body Me-His§							46·5		
Recovery‖ (%)							85·1		

* (g Fresh tissue/kg body-wt) × (protein concentration ÷ 1000) × (μg Me-His/g protein ÷ 1000).
† Tissue Me-His (mg/g fresh tissue per kg body-wt) ÷ total body Me-His (mg/kg body-wt).
‡ Bones, adipose tissue and tail.
§ For details, see Table 3.
‖ Total organ Me-His ÷ total body Me-His.

Table 3. *N^τ-methylhistidine (3-methylhistidine; Me-His), nitrogen and lipid in whole bodies of rats given diets with different levels of protein*

(Mean values with their standard errors)

Dietary protein level* (g/kg)	No. of rats	N (g/kg body-wt)		Lipid (g/kg body-wt)		Me-His (mg/kg body-wt)	
		Mean	SE	Mean	SE	Mean	SE
100	6	30·8	0·8	174	11	48·1	2·2
200	6	29·3	1·2	196	34	46·5	3·6
400	6	31·6	0·6	140	16	47·5	2·2
600	5	30·6	0·5	162	18	48·1	1·6

Differences between the groups were not statistically significant.
* For details of diets, see Table 1.

the total Me-His in the body. Skin and hair contributed 8·4% to the total body Me-His and for the gastrointestinal tract 1%. Other tissues contributed less than 1% of the total body Me-His.

Table 4. *Effects of dietary protein level on urinary N^τ-methylhistidine (3-methylhistidine; Me-His), nitrogen and creatinine excretion (mg/kg body-weight per d) in adult rats with restricted food intake*

(Mean values with their standard errors)

Dietary protein level* (g/kg)	No. of rats	N		Creatinine		Me-His	
		Mean	SE	Mean	SE	Mean	SE
100	5	392	24	27·7	2·1	1·17	0·08[a]
200	12	905	56	27·8	1·1	1·24	0·04[a]
400	6	1900	160	33·0	1·4	1·45	0·06[b]
600	5	3630	130	31·3	2·5	1·47	0·07[b]

For creatinine, differences between the groups were not statistically significant.

[a,b] Values without common superscripts were statistically significantly different: 200 g protein/kg diet *v.* 400 and 600 g protein/kg diet, $P < 0.025$; 100 g protein/kg diet *v.* 400 and 600 g protein/kg diet, $P < 0.01$.

* For details of diets, see Table 1.

Relationship between whole-body Me-His content and dietary protein intake

Whole-body content of Me-His, N and lipid were not affected by dietary protein intake, when total food intake was restricted to the maintenance requirement (Table 3). These results indicate that Me-His content of the body protein itself is not affected by dietary protein level.

Effects of dietary protein levels on urinary excretion of N, creatinine and Me-His

N, creatinine and Me-His excretion in urine are given in Table 4. Higher protein intake was accompanied by a progressive increase in urinary N output. Creatinine output was not altered with the dietary changes. However, higher protein intake brought about increased urinary excretion of Me-His. When the protein level in the diet was increased from 200 to 400 g/kg and from 200 to 600 g/kg, urinary excretion of Me-His increased 17 % ($P < 0.025$) and 18% ($P < 0.025$) respectively. Urinary excretion of Me-His in rats given diets with 400 or 600 g protein/kg was significantly higher than in those given a diet with 100 g protein/kg ($P < 0.01$). However, no significant increase in Me-His excretion was found when the dietary protein level was increased from 100 to 200 g/kg or from 400 to 600 g/kg. These results, considered with the total Me-His content of the body (Table 3), show that fractional catabolic rates of myosin and actin tend to increase as dietary protein level increases under conditions of restricted food intake.

DISCUSSION

The sum of the Me-His recovered in the various tissues and organs analysed accounts for approximately 85% of the total Me-His in the body. The unrecovered 15% of Me-His would probably be found in the small amount of muscles which could not be removed from the bones. Taking into account these unremoved skeletal muscles, more than 90% of Me-His in the body would be found in skeletal muscle

$(75+15\%)$. These results confirm the assumption that catabolic rates of myosin and actin are measured by determining urinary excretion of Me-His. However, approximately 10% of the total Me-His occurs in the skin and the gastrointestinal tract. If the proteins in these tissues turn over rapidly, we have to take into account the amount of urinary Me-His contributed by these tissues. If it is assumed that these contributions are negligible, it can be concluded that the dietary protein level affects the rate of catabolism of myosin and actin in skeletal muscle. Especially when dietary protein level is increased from 200 to 400 g/kg, catabolic rate of myosin and actin increases about 17%. (The present studies were done on animals with a restricted food intake; the effect of energy intake under various protein levels on Me-His excretion (Nishizawa, Shimbo, Noguchi, Hareyama & Funabiki, unpublished results) will be published elsewhere.) Several authors have reported degradation of myosin and actin in a random manner (Funabiki & Kandatsu, 1968; Goldberg, 1969; Millward, 1970a; Rabinowitz, 1973). Funabiki & Cassens (1972) also showed that these two proteins have similar half-lives. Therefore, we can calculate the half-life of myosin and actin from the urinary excretion of Me-His by the following equation:

$$\text{half-life (d)} = 0.693 \left(\frac{\text{total body Me-His} \times 0.90}{\text{daily urinary excretion of Me-His}} \right).$$

From this equation, the half-life of myosin and actin of young adult rats given a diet with 200 g egg albumen/kg was calculated to be 23 d.

In the present paper, the method to calculate fractional catabolic rates of myosin and actin by urinary excretion of Me-His has been evaluated critically. This method seems to be useful for the purpose. However, we still need to know exactly how much urinary Me-His is contributed by other tissues than skeletal muscle. In particular, the contribution of skin and gastrointestinal tract should not be neglected.

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