3-METHYLHISTIDINE, A COMPONENT OF ACTIN

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Tallan, Stein, and Moore (1954) identified 3-methylhistidine (3-MeHis) as a component of human urine, and commented that the source of this material was far from clear. The results of analyses made in this laboratory on samples of blood and urine collected under fasting conditions indicated that urinary 3-MeHis has an endogenous origin. Because of its possible physiological significance attempts were made to locate its source. Evidence will be presented here that 3-MeHis is a component of actin, an important functional protein of muscle.

Materials and Methods. L -Histidine-^{14}C (u.l.)(231 mC/mMole) and L -methionine-methyl-^{14}C (12.5 mC/mMole) were obtained from New England Nuclear Corporation. Amino acid analyses were made with a Spinco Model 120 amino acid analyzer using the procedure of Spackman, Stein, and Moore (1958) and with another analyzer using a system similar to that of Hamilton (1963). The basic ampholytes in urine, deproteinized filtrates of tissue homogenates, and protein hydrolysates were separated and concentrated with the aid of ion exchange resins to prepare them for paper electrophoresis. Paper electrophoresis was carried out with a Gilson Medical Electronics Model D high voltage electrophoresis apparatus. Samples were applied to a 35 x 57 cm. sheet of Whatman 3 MM paper and electrophoresis was effected at 2500 V for 90 min. in pyridine-acetic acid buffer, pH 6.1, using Varsol as coolant. For the experiments with ^{14}C-histidine, radioactive zones were located by autoradiography; when methionine-methyl-^{14}C was used, the 1- and 3-MeHis zones were located with ninhydrin. Appropriate areas were cut out, eluted,

and the concentrated eluates were transferred onto planchets as thin films. Radioactivity was measured with a Nuclear Chicago thin-window gas-flow counter.

Animals were killed, tissues were separated immediately, and filtrates were prepared by homogenizing 1 gm. of wet tissue with 5 ml. of cold 3% sulfosalicylic acid and separating the supernatant fluid by centrifugation. Aliquots were applied directly to the columns of the amino acid analyzers. For preparation of tissue proteins, the residue after removal of the supernatant fluids was again homogenized with 10 ml of 3% sulfosalicylic acid, the supernatant fluid was discarded, the residue was then washed with 10 ml of water and finally dried *in vacuo* over H_2SO_4. Protein hydrolysates were prepared by refluxing 5 - 15 mg. of protein dissolved in 50 ml of 6 N HCl for 22 hours and 72 hours at 135° according to the procedure of Hirs (1956).

Results and Discussion. It has been reported that labeled 3-MeHis was excreted by rats which were given an injection of α-^{14}C-DL-histidine (Wolf, *et al.*, (1956)). The endogenous formation of 3-MeHis was confirmed by experiments in which labeled histidine or methionine were administered to rats intraperitoneally, urine was collected for 2 consecutive 24 hr. periods while they were maintained fasting, and the specific activities of the 1- and 3-MeHis in the urine were measured. The results showed that histidine and the methyl group of methionine serve as precursors for 3-MeHis, and that urinary 1- and 3-MeHis come from pools with markedly different turnover rates.

A survey of several tissues of the rabbit was then made. The amounts of the histidines were determined in protein-free filtrates of homogenates, before and after hydrolysis, and in hydrolysates of the crude tissue proteins. Striated muscle proved to have the highest 3-MeHis content of all the tissues; 98% of the amino acid in the tissue was present in the sulfosalicylic acid precipitate. Striated muscle proteins from some other animals were also found to contain 3-MeHis (Table I).

Table I

3-Methylhistidine content of crude muscle protein from different species. (μmoles/gm. dried precipitate)

	Rabbit (Thigh)	Cow (Thigh)	Chicken (Breast)	Rat (Thigh)	Human (Calf)
1-Methylhistidine	ca..5	< .1	2.16*	< .1	< .1
3-Methylhistidine	2.72	2.00	2.34	2.85	1.76
Histidine	92.	139.	155.	136.	100.
% 3-MeHis residues in muscle protein	.0438	.0302	.0353	.0430	.0266
% Actin in muscle protein $\frac{60,000}{151}$ x % 3 MeHis	17.4	12.1	14.1	17.1	9.9

* This sample contained 2.5 μmoles of β-alanine/gm. β-Alanine could not be detected in any of the other hydrolysates.

These results showed that 3-MeHis is present in the protein of muscle tissue. Purification of myosin, actin, and tropomyosin was undertaken to determine if 3-MeHis might be a unique component of one of them. Myosin was prepared from rabbit muscle mince as described by Perry (1955) and was further purified as described by Tsao (1953). Actin was prepared by the procedure of Carsten and Mommaerts (1963). Tropomyosin was prepared by the method of Bailey (1948). 3-MeHis could not be detected in the tropomyosin hydrolysate. A small amount (0.29 moles/10^5 g. protein) was present in the hydrolysate of a crude myosin preparation, and much less (0.16 moles/10^5 g. protein) in myosin which had been further purified. Further work will be required to establish whether more highly purified myosin does contain 3-MeHis. All hydrolysates of actin preparations contained 3-MeHis in the proportion of 1 mole per 8 to 10 moles of histidine. This corresponds to somewhat more than 1 gm. mole per 60,000 gm. protein. The results of the analyses of actin are given in Table II, along with results taken from the literature for comparison. It may be noted that Kominz, et al. (1962) reported the presence of about 1 gm. mole of an uncommon amino acid per 60,000 gm. of

Table II

Amino Acid Composition of Rabbit Actin
(Residues amino acid/60,000 gm. protein (16.1% N))

Amino Acid	This Lab	Carsten (1963)	Kominz, et al. (1962)	Amino Acid	This Lab	Carsten (1963)	Kominz, et al. (1962)
Asp	49	47	47	Met	22	22	21
Thr	36	38	35	Ile	40	37	28
Ser	33	32	31	Leu	38	35	34
Pro	26	25	27	Tyr	22	22	21
Glu	57	55	55	Phe	17	16	16
Gly	38	37	37	Lys	28	25	25
Ala	42	41	39	His	11	10	10
Val	28	25	22	Arg	26	24	26
				3-MeHis	1.4	-	(.9)

rabbit actin and 2 gm. mole per 60,000 gm. of Phormia actin. They assumed that it was 1-MeHis derived from anserine, present as a contaminant of the proteins. Neither 1-MeHis nor β -alanine has ever been definitely detected in any of our hydrolysates of purified proteins, and even in hydrolysates of crude muscle protein there is much more 3-methyl- than 1-methyl-histidine.

The identity of the 3-MeHis in the crude protein precipitates and in purified actin was established by the identical behavior of authentic and natural materials in the two different ion exchange chromatography systems and in the electrophoretic system. In addition, 3-MeHis from hydrolyzed rabbit actin was separated on the 50 cm. column of the Spinco analyzer and subjected to partition chromatography. Upon co-chromatography, authentic and natural 3-MeHis did not separate on paper with pyridine-acetone-3 N NH_4OH (50:30:25)(R_F, 0.33) or upon thin-layer chromatography on silica-gel G with chloroform - methanol - 17% ammonia (40:40:20)(R_F, 0.58). Authentic and natural compounds showed the same characteristic colors with ninhydrin

on paper (gray) and on silica-gel G (reddish-purple).

If there is one residue of 3-MeHis per molecule of actin (m.w. 60,000) the amount actually found in crude muscle protein (Table I) agrees well with what would be expected if all of it were in actin, since muscle protein contains 13 - 15% actin (Bailey, 1954). With the use of a rough approximation of the total muscle protein content of animals and the estimate that muscle actin (rat) has a half-life of 67 days (Velick, 1956), it can be calculated that about 16 mg. of 3-MeHis formed by the breakdown of muscle actin should be excreted daily by a 70 kg. man. Actually, about 40 - 50 mg. is excreted daily. The discrepancy might occur because of a different turnover rate of actin in man than in the rat, or, more probably, because actin occurs in contractile protein in other cells and tissues in addition to striated and smooth muscle, and has a more rapid turnover rate in those sites.

Whether 3-MeHis is formed from histidine and then incorporated into actin or histidine is methylated after it is incorporated into a peptide chain remains to be established. Cowgill and Freeburg (1957) could not detect any radioactivity in the carcass proteins of animals that had been given 3-^{14}C-methyl-histidine, and there is not an increased excretion of 3-MeHis after the ingestion of an oral dose of histidine (Block, et al., (1964)). It seems likely that the 3-MeHis in actin is formed by methylation of protein-bound histidine. Evidence consistent with this idea was gained by the following experiment. L-Histidine-^{14}C (u.l.)(50 μC, 0.0315 mg in 3 ml of saline solution) was given intraperitoneally, in 3 doses at 2 hr. intervals, to a 1.9 kg. rabbit. After 6 days the animal was killed and actin was isolated from its skeletal muscle and hydrolyzed. The specific activity of the histidine in the hydrolysate was 123.4 cpm/μ mole and of the 3-MeHis was 123.6 cpm/μmole. If the 3-MeHis had been formed in another organ, such as the liver, and transported to the muscle it would be unlikely that the 3-MeHis incorporated into actin would have the same specific activity as the histidine.

The occurrence of an uncommon amino acid as a component of a major functional protein is unusual. In order to establish conclusively that the 3-MeHis residue is a component of the polypeptide chain of actin it will be necessary to demonstrate it is a component of a peptide obtained by degrading actin. However, when purified actin was subjected to treatment at room temperature with 3% sulfosalicylic acid or 5% trichloroacetic acid no ninhydrin-positive material was present in the supernatant fluid, and the amino acid composition of the precipitated protein was essentially unaltered. In addition, filtration of solutions of actin through a column of Sephadex G-25 or carboxymethylation in 8 M urea followed by Sephadex filtration did not alter the 3-MeHis content. Any of these procedures might be expected to dissociate a loosely associated or physically bound smaller molecule. Thus, the evidence presented here supports the idea that 3-MeHis is a component of actin.

This unusual structural component might be involved in some manner in the function of actin as a part of the contractile element of muscle. Bailey (1954) has commented upon the possibility that the mechanisms underlying muscle contraction may be applicable in all biological systems involving contractility. If this amino acid is found to occur only in contractile proteins, its measurement in hydrolysates of tissue preparations might afford an estimate of their content of contractile protein. Measurement of the rate of 3-MeHis excretion by persons receiving a well-defined dietary regimen could provide an estimate of the turn-over rate of actin so that the effect of exertion, immobilization, and other environmental and physiological factors might be determined. Examination of 3-MeHis excretion and its content in muscle tissue of patients suffering from muscular diseases is an obviously important area for exploration.

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