3-Methylhistidine in Actin and Other Muscle Proteins

By P. JOHNSON, C. I. HARRIS and S. V. PERRY
Department of Biochemistry, University of Birmingham

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1. By the use of the extended elution system for basic amino acid analysis, 3-methylhistidine has been detected in hydrolysates of actin isolated from mammalian, fish and bird skeletal muscle. 2. Evidence is presented to indicate that 3-methylhistidine forms part of the primary structure and that in rabbit actin this residue is restricted to one peptide fraction obtained from the tryptic digest. 3. Rabbit skeletal-muscle actin has a 3-methylhistidine:histidine ratio 1:7·6, indicating a minimum molecular weight of 47 600. 4. Adult rabbit myosin contains approximately 2 3-methylhistidine residues/mol. These residues are localized in the heavy meromyosin part of the molecule, and are restricted to the major component obtained after succinylation.

Methylations of histidine in the 1- and 3-positions of the imidazole ring are well established pathways of histidine metabolism (Meister, 1965). Anserine (β-alanyl-1-methylhistidine) has long been recognized as a major peptide component of many muscles and, although 1-methylhistidine and its catabolic derivatives have been isolated from other tissues and from urine, anserine probably accounts for the bulk of the 1-methylhistidine in the animal body.

More recently, 3-methylhistidine has been reported to be present in urine as the free amino acid (Searle & Westall, 1951; Tallan, Stein & Moore, 1954). Its presence in some muscles has also been established, although there have been varying reports as to the precise form in which it occurs in this tissue. Tallan *et al.* (1954) reported the presence of a small amount of ninhydrin-reacting material that was eluted from Dowex 50 at a rate identical with that of 3-methylhistidine in the non-protein nitrogen fraction of cat gastrocnemius muscle. In whale muscle 3-methylhistidine is present as a peptide isomeric with anserine (Pocchiari, Tentori & Vivaldi, 1962; Cocks, Dennis & Nelson, 1964). None of these investigations has provided evidence that 3-methylhistidine occurs in peptide combination in protein, although Laki, Maruyama & Kominz (1962), in a footnote, comment on the presence of methylhistidine, without specifying the isomer, in actin hydrolysates, but imply that it is present as a contaminant.

The work reported in the present paper provides evidence for the presence of 3-methylhistidine as part of the primary structure of actin and of myosin. These findings have been reported briefly elsewhere (Johnson, Harris & Perry, 1967), and since this work was completed Asatoor & Armstrong (1967) have independently briefly reported the presence of 3-methylhistidine in actin hydrolysates.

METHODS

Protein preparations

Actin. Acetone-dried fibre was prepared from mixed adult rabbit skeletal muscle, and actin was extracted from it and further purified by the method of Carsten & Mommaerts (1963), with the modification that after the initial extraction the protein was isoelectrically precipitated with 0·01 m-sodium acetate buffer, pH 4·7. The isoelectric precipitate was redissolved in 2 mM-tris–HCl buffer, pH 7·6, containing ATP (0·2 mM) and ascorbic acid (0·2 mM), the pH readjusted to 7·6 with saturated NaHCO$_3$ solution, and the solution equilibrated overnight against 2 mM-tris–HCl, pH 7·6, containing ATP (0·2 mM) and ascorbic acid (0·2 mM) and then clarified by centrifugation for 1 hr. at 100 000g. The supernatant after centrifugation was then taken through two cycles of polymerization and depolymerization (Carsten & Mommaerts, 1963). Actins from human and fowl skeletal muscle were also prepared in this way.

Rabbit actin was extracted without the use of organic solvents as follows: (1) from natural actomyosin, prepared as described by Perry & Corsi (1958), by the method of Martonosi, Gouvea & Gergely (1960); (2) directly from myofibrils obtained by the routine method used in these Laboratories (Perry & Zydowo, 1959). Actin was isolated by precipitation with 0·8 m-phosphate buffer, pH 7·0, from the extract of myofibrils obtained with 5 mM-tris–HCl buffer, pH 8·6 (Perry & Corsi, 1958).

For the preparation of actin from foetal muscle, whole carcasses of 28-day-old rabbit foetuses were converted into acetone-dried fibre, as described by Carsten & Mommaerts (1963) for adult muscle, with the inclusion of three washes of the muscle residue with 0·1 m-KCl immediately after the myosin extraction, to remove the large quantities of

myoglobin and haemoglobin present. Isoelectrically precipitated foetal and some adult actin preparations after dialysis and clarification were chromatographed on Sephadex G-200 according to the method of Adelstein, Godfrey & Kielley (1963). The actin peak from the column was concentrated by vacuum dialysis.

For amino acid and peptide analysis, nucleotides were removed from native actin as described by Kasai, Nakano & Oosawa (1965). The nucleotide-free actin was analysed as such, or in some cases after carboxymethylation (Crestfield, Moore & Stein, 1963) or aminoethylation (Raftery & Cole, 1963) of the thiol groups.

Myosin. This protein was prepared by the routine method used in these Laboratories (Perry, 1955) and further purified by DEAE-cellulose chromatography (Perry, 1960). Only material from the main peak eluted at 0·3 M-KCl in tris–HCl buffer, pH 8·0, was used for analysis. Light meromyosin fraction I and heavy meromyosin were prepared by tryptic digestion of myosin according to the methods of Szent-Gyorgyi (1953) and Szent-Gyorgyi, Cohen & Philpott (1960), and subfragment I was obtained from heavy meromyosin as described by Jones & Perry (1966).

Tropomyosin B was prepared by the method of Bailey (1948) as modified by Bailey (1951), and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954) and used at the precrystallization stage of method B.

Tryptic digestion of proteins

Actin. Carboxymethylated actin suspended in water (10 mg./ml.) was digested with trypsin at 25° for 8 hr. (1 mg. of trypsin/60 mg. of actin at the start and two further additions of similar amounts of enzyme at 2 hr. intervals). The pH of the digestion was maintained at 8·0 by addition of N-NH₃ with continuous monitoring in the pH-stat. The digest was deproteinized by lowering the pH to 6·5 with an equal volume of 5% (v/v) pyridine–0·2% (v/v) acetic acid buffer, pH 6·5, and centrifuged, and the supernatant solution was freeze-dried and stored.

Myosin. The acetone-dried protein, prepared as described by Jones & Perry (1966), was suspended in water (50 mg./ml.) at 37°, the pH was adjusted to 8·5 and maintained at this value on the pH-stat, and the protein was digested with 1 mg. of trypsin/100 mg. of myosin. After 4 hr. an equal amount of trypsin was again added and the digestion continued for a total of 8 hr. The pH was adjusted with N-HCl to 3·0 and the insoluble material removed by centrifugation. Peptides were fractionated on Dowex 1 (X 2; 200–400 mesh) by the method of Wittmann & Braunitzer (1959) as modified by Landon (1964).

Separation and determination of peptides and amino acids

Paper electrophoresis was carried out on Whatman 3MM paper on a horizontal cooled plate at pH 1·8 [2·2% (v/v) formic acid–8·7% (v/v) acetic acid] and pH 6·5 [5% (v/v) pyridine–0·2% (v/v) acetic acid] with a potential gradient of 100 v/in. Electrophoreses were also conducted in glass tanks with buffer-equilibrated toluene as coolant (Michl, 1951; Ryle, Sanger, Smith & Kitai, 1955) at pH 3·5 [2·5% (v/v) acetic acid–0·25% (v/v) pyridine], pH 6·5 and pH 9·0 [1% (w/v) (NH₄)₂CO₃] with a potential gradient of 75 v/in.

For peptide 'mapping', about 1 mg. of material was subjected to electrophoresis at pH 6·5 followed by descend-ing chromatography in pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.) (Baglioni, 1961).

Larger amounts of 3-methylhistidine were isolated from gram quantities of isoelectrically precipitated actin dissolved in water with the aid of a little saturated NaHCO₃ solution, and precipitated with 0·5vol. of 15% (w/v) trichloroacetic acid. The washed precipitate was then suspended in 6 N-HCl at a concentration of about 20 mg./ml. After refluxing at 110° for 24 hr. the HCl was removed on a rotatory evaporator. Preliminary separation of the combined histidine and 3-methylhistidine fraction was achieved by paper electrophoresis and the 3-methyl-histidine was finally separated on a 45 cm. × 1 cm. column of Dowex 50 (X 8; 200–400 mesh) equilibrated with sodium citrate buffer (0·38 M with respect to Na⁺), pH 4·26. The column was run at 30° at a flow rate of 40 ml./hr. and 5 ml. fractions were collected. Samples were assayed by the ninhydrin method (Hirs, Moore & Stein, 1959) and the peaks were pooled and desalted as described by Tallan et al. (1954).

The imidazole amino acids were chromatographed on Whatman 3MM paper in the following solvents: (I) pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.); (II) butan-1-ol–acetic acid–butyl acetate–water (75:15:3:25, by vol.); (III) phenol–water–ammonia (Smith, 1958); (IV) phenol–water–HCl (Cocks et al. 1964).

Preparative chromatography of tryptic peptides from carboxymethylated actin. A 190 cm. × 2·8 cm. column of Dowex 1 (X 2; 200–400 mesh; acetate form) was equilibrated with N-ethylmorpholine (0·2 M)–acetic acid buffer, pH 7·5. The N-ethylmorpholine used was freshly redistilled before use and the buffers and the Dowex column were shielded from daylight by aluminium foil.

About 600 mg. of peptide material was applied to the column in 15 ml. of N-ethylmorpholine (0·2 M)–acetic acid buffer, pH 8·0. Then 350 ml. of N-ethylmorpholine (0·2 M)–acetic acid, pH 7·5, was applied and the fractionation continued by using the linear gradient systems indicated in (a)–(e). The volumes and solution composition in each of the two mixing chambers are listed in each case: (a) 700 ml. of N-ethylmorpholine (0·2 M)–acetic acid, pH 7·5, and 700 ml. of pyridine (0·2 M)–acetic acid, pH 5·3; (b) 700 ml. of pyridine (0·2 M)–acetic acid, pH 5·3, and 700 ml. of pyridine (0·2 M)–acetic acid, pH 3·85; (c) 700 ml. of pyridine (0·2 M)–acetic acid, pH 3·85, and 700 ml. of 0·2 N-acetic acid; (d) 700 ml. of 0·2 N-acetic acid and 700 ml. of N-acetic acid; (e) 700 ml. of N-acetic acid and 700 ml. of 50% (v/v) acetic acid.

The buffer flow rate was maintained at 60 ml./hr. by means of a Milton–Roy Mini-Pump, and 12 ml. fractions were collected.

Elution of peptides was followed by ninhydrin colour estimations after alkaline hydrolysis of 0·2 ml. samples of the fractions, by the method of Hirs et al. (1959). Appropriate fractions were pooled and then concentrated by rotatory evaporation.

DNP derivatives. The DNP derivatives of the imidazole amino acids were prepared as described by Biserte, Holleman, Holleman-Dehove & Sautiere (1959) and chromatographed in the toluene solvent of Biserte & Osteux (1951). Thin-layer chromatography was carried out with 0·3mm.-thick layers of silica gel G (E. Merck A.-G., Darmstadt, Germany) on glass using the solvent I system of Brenner, Niederwieser & Pataki (1964).

Phenylthiohydantoin derivatives. The phenylthiohydantoin derivatives of amino acids were synthesized by the micro method of Sjöquist (1960) and chromatographed in the heptane–butan-1-ol–75% formic acid (40:30:9, by vol.) system (Sjöquist, 1960). For thin-layer chromatography, the heptane solvent for water-soluble phenylthiohydantoin amino acid derivatives (Jeppsson & Sjöquist, 1967) was used.

Enzymic assays. Histidine decarboxylase activity was followed by the method of Gale (1957) and histidase activity by that of Tabor & Mehler (1955).

Amino acid analysis. Amino acid analyses were carried out on the Technicon and Beckman model 120B automatic instruments. For the Technicon system the standard 20hr. run on grade B resin was used. Most of the analyses were carried out on the Beckman instrument by the system of Spackman, Stein & Moore (1958) as modified for accelerated analysis by Spackman (1963). The columns used were as follows: (1) the normal basic amino acid analysis column (0·6cm. × 15cm.); (2) the expanded basic amino acid column (0·9cm. × 15cm.), as used for aminoethylcysteine estimations (Raftery & Cole, 1963); (3) the physiological fluids column (0·9cm. × 20cm.). Elution was carried out at 52·5° with sodium citrate buffer (0·35M with respect to Na+), pH5·28, for (1) and (2), and at 30° with sodium citrate buffer (0·38M with respect to Na+), pH4·26, for (3). Technicon grade B resin was used in (1) and Beckman 15A resin in (2) and (3). In all the Beckman systems, the buffer pumps were set to deliver 40ml./hr. and the ninhydrin pump was set at half this rate.

Estimation of 3-methylhistidine. Samples of actin, in the buffer used for precipitation by polymerization and depolymerization, and samples of acetone-dried myosin were hydrolysed at 110° in 6N-HCl at a concentration of about 3mg./ml. in an evacuated sealed tube, as recommended by Moore & Stein (1963). After hydrolysis, the HCl was rapidly removed in a rotatory evaporator and the hydrolysate, equivalent to 8–10mg. of actin or 10–12mg. of myosin, was applied to the physiological fluids column. Under these conditions, histidine and 3-methylhistidine were eluted as separate peaks and the amounts could be accurately estimated.

Materials

Trypsin was twice-recrystallized freeze-dried material, purchased from Seravac Laboratories, Maidenhead, Berks.; ATP and tris were supplied by C. F. Boehringer und Soehne, Mannheim, Germany; 3-methylhistidine was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and Mann Research Laboratories Ltd., New York, N.Y., U.S.A. 1-Methylhistidine was also supplied by Koch–Light Laboratories Ltd.

RESULTS

During routine amino acid analysis of actin preparations by the standard Beckman system for protein hydrolysates, it was noted that the histidine peak did not return to the base line before the ammonia peak began to be eluted. When hydrolysates were analysed by using the expanded system for the basic amino acids (Raftery & Cole, 1963) a new peak not corresponding to any of the normal amino acid components of protein was eluted after the histidine. With the physiological fluids system this peak was resolved (Fig. 1) in a position corresponding to that reported for 3-methylhistidine (Spackman, Stein & Moore, 1958). Application of known samples of 3-methylhistidine to the analytical system confirmed that this amino acid was eluted in a position identical with that occupied by the new compound (Fig. 1), which was therefore provisionally identified as 3-methylhistidine.

In view of previous reports (Tallan *et al.* 1954; Pocchiari *et al.* 1962; Cocks *et al.* 1964) of the occurrence of 3-methylhistidine in the non-protein fraction of cat and whale muscle, the actin preparations were subjected to procedures that would be expected to remove peptide contaminants. The suspected 3-methylhistidine was not removed from actin by three washings of isoelectrically precipitated F-actin with 5% (w/v) trichloroacetic acid or by treatment of aqueous solutions of G-actin, pH7·0, with Dowex 50 (X 2) and Dowex 1 (X 2). The 3-methylhistidine peak was also present in actin that had been fully reduced by mercaptoethanol in the presence of 8M-urea (Crestfield *et al.* 1963) before the protein thiol groups had been blocked by iodoacetate or ethyleneimine.

Careful examination of the elution diagrams obtained by amino acid analysis of many actin samples failed to show the presence of β-alanine, indicating that the 3-methylhistidine was not present in the actin samples as an isomer of the dipeptide anserine, such as has been reported to be present in whale muscle (Pocchiari *et al.* 1962).

The possibility that one of the histidine residues of actin was fortuitously methylated during the preparation of the protein was also considered. Although rather unlikely, such a reaction could conceivably occur as a result of the extensive use of acetone in the preparation of the dehydrated muscle fibre from which the actin was extracted. Such an explanation for the presence of 3-methylhistidine was excluded by the analysis of actin prepared without the use of organic solvents, as indicated in the Methods section. These preparations contained 3-methylhistidine, although the 3-methylhistidine:histidine ratio in the phosphate-precipitated fraction from myofibril extracts was lower than that in the normal actin preparations. It was clear, however, that these preparations contained other proteins in addition to actin.

Confirmatory identification of 3-methylhistidine. The suspected 3-methylhistidine was isolated from acid hydrolysates of actin by paper electrophoresis at pH6·5, elution of the 3-methylhistidine band and repeating the electrophoresis at pH6·5 and elution until only one ninhydrin-staining band was obtained on electrophoresis at pH3·5, 6·5 and 9·0.
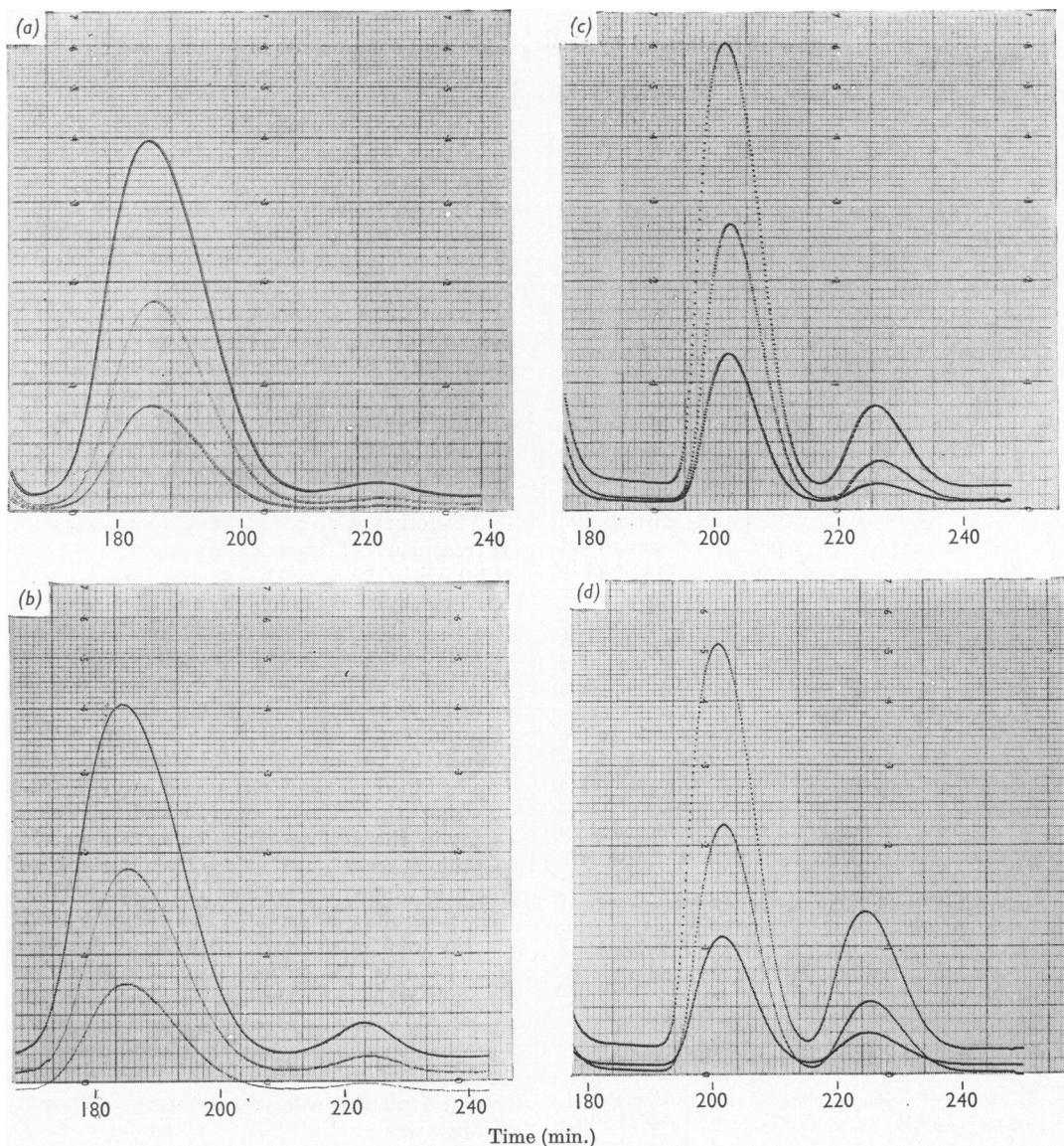
Fig. 1. Histidine and 3-methylhistidine analyses of hydrolysates of actin and myosin. The Figure shows elution patterns, from the basic amino acid column of the Beckman physiological fluids system, of hydrolysates of the following proteins: (a) 12 mg. of myosin; (b) 12 mg. of myosin+0·05 μmole of 3-methylhistidine; (c) 10 mg. of actin; (d) 10 mg. of actin+0·2 μmole of 3-methylhistidine.

At pH 6·5, 3-methylhistidine moved more slowly than histidine and 1-methylhistidine and could be readily isolated from the latter two amino acids (Fig. 2). In this way about 3–4 mg. of 3-methyl-histidine could be isolated from 2 g. of actin.

On being stained with ninhydrin, the suspected 3-methylhistidine gave the blue–grey colour that is characteristic of all the imidazole amino acids, but like authentic 3-methylhistidine did not give the Pauly reaction (Smith, 1958). A positive reaction to the iodine test for imidazole compounds (Smith, 1958) was obtained. The suspected and known amino acids both gave the green-spot test for 3-methylhistidine (Dent, 1948; Searle &

Westall, 1951), although in our hands a positive reaction to this test was also obtained with the other imidazole amino acids.

On electrophoresis of the suspected 3-methylhistidine at pH 1·8, 3·5, 6·5 and 9·0, its mobility was indistinguishable from that of authentic 3-methylhistidine. When 3-methylhistidine was mixed with the material isolated from actin, one ninhydrin-staining band was obtained at all pH values.

Identity with 3-methylhistidine was further confirmed by chromatography in the four solvent systems listed in Table 1. In all cases the R_F values of the suspected and authentic 3-methylhistidine were not significantly different, nor was separation obtained on co-chromatography.

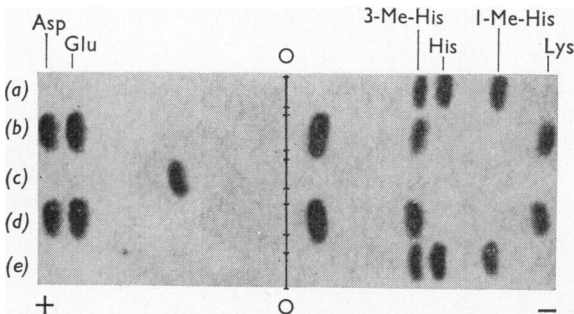The identity of the suspected 3-methylhistidine

Fig. 2. Paper electrophoresis of a 3-methylhistidine-containing peptide fraction isolated from a tryptic digest of actin. The peptide was separated by Dowex 1 (X 2) chromatography (fraction XIII) followed by pH 9·0 electrophoresis. This fraction cannot be resolved by electrophoresis but is partially resolved into two components by chromatography (see the text). The electrophoretic run was at pH 6·5 for 90 min. at 1500 v. O, Origin. (a) Known samples of histidine, 1-methylhistidine and 3-methylhistidine; (b) 3-methylhistidine-containing peptide after acid hydrolysis; (c) 3-methylhistidine-containing peptide; (d) 3-methylhistidine-containing peptide to which 0·05 μ-mole of 3-methylhistidine was added before hydrolysis; (e) as (a).

was further confirmed by comparing the DNP and phenylthiohydantoin derivatives of the material isolated from actin and of known 3-methylhistidine. In each case the derivatives obtained from the known and the suspected 3-methylhistidine appeared to be identical, for they moved with similar R_F values and did not separate on co-chromatography. Blocking of the imino group of the imidazole ring was indicated by the fact that, with both the suspected and the known 3-methylhistidine, only one DNP derivative was obtained at all relative concentrations of fluorodinitrobenzene (Fig. 3). With histidine, either the α-mono-DNP or the bis-DNP derivative was obtained, depending on the fluorodinitrobenzene:histidine ratio.

Samples of histidine decarboxylase and histidase, which control experiments showed to be active against histidine, had no action on a sample of known 3-methylhistidine, on that isolated from actin, or on 1-methylhistidine.

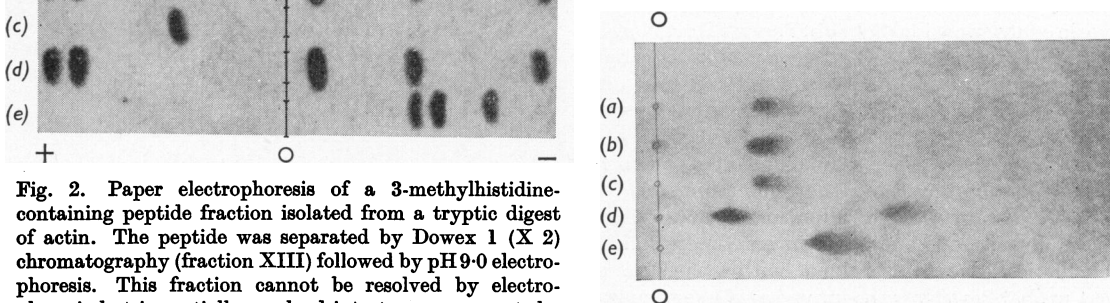*3-Methylhistidine content of actin.* Actin preparations from rabbit muscle were purified as

Fig. 3. Chromatography of the DNP derivative of 3-methylhistidine isolated from actin. The solvent system was described in the Methods section. Of each substance 0·05 μmole was applied. O, Origin. (a) DNP derivative of 3-methylhistidine isolated from actin; (b) DNP derivatives of known 3-methylhistidine and 3-methylhistidine isolated from actin; (c) DNP-3-methylhistidine; (d) α-DNP-histidine and bis-DNP-histidine; (e) 2,4-dinitrophenol.

Table 1. R_F *values of the imidazole amino acids and of 3-methylhistidine isolated from actin*

The imidazole amino acids were chromatographed in various solvents; for details of the solvent systems see the Methods section.

Solvent... ... Amino acid	R_F values			
	I	II	III	IV
3-Methylhistidine from actin	0·11	0·19	0·93	0·52
3-Methylhistidine	0·11	0·20	0·93	0·52
Histidine	0·10	0·17	0·74	0·23
1-Methylhistidine	0·09	0·15	0·92	0·44

Table 2. 3-*Methylhistidine content of actin from skeletal muscle of various species*

Duplicate analyses were carried out on each preparation with the exception of the trout and foetal rabbit actins. For analysis methods, see the text. The amount of 3-methylhistidine in rabbit actin is given as mean ± S.D.

Species	No. of preps.	3-Methylhistidine: histidine ratio	Amount of 3-methyl-histidine (moles/10^5 g. of actin)
Rabbit	5	1:7·6	2·10 ± 0·04‡
Human	1	1:8·62	
Chicken	1	1:7·46	
Trout	1	1:10·1	
Rabbit foetus (28-day-old)	{ 1	1:12·2*	
	1	1:11·4†	

* Purified by polymerization and depolymerization.
† Purified by Sephadex G-200 chromatography.
‡ Based on a value of 15·9 moles of histidine/10^5 g. of actin.

indicated in the Methods section, particular care being taken to minimize tropomyosin contamination by extraction at low temperatures (Drabikowski & Gergely, 1964), and repeated cycles of polymerization and depolymerization. Tests on the Ca^{2+}-activated ATPase of desensitized actomyosin gave no indication of significant tropomyosin contamination (Schaub, Perry & Hartshorne, 1967). Such preparations had a consistent 3-methylhistidine content and analyses suggested that one out of every nine histidine residues in actin was methylated (Table 2). Actin isolated from the skeletal muscle of other animals also contained 3-methylhistidine, implying that this amino acid is probably a component of all actins obtained from skeletal muscle. The slightly lower 3-methylhistidine:histidine ratio obtained with the actin of trout probably reflects the impurity of the preparation rather than a significant species difference. Lower ratios were also obtained for the 3-methylhistidine content of rabbit foetal actin, but there are special problems in purifying actin from this tissue and the preparations used showed heterogeneity on chromatography on Sephadex G-200. It is possible that this is the explanation of the lower 3-methylhistidine:histidine ratio, but further work is necessary to decide finally whether actin from skeletal muscle of the rabbit foetus has a significantly lower content of 3-methylhistidine than its adult counterpart.

3-*Methylhistidine-containing peptide.* On exhaustive tryptic digestion of carboxymethylated actin prepared as described in the Methods section, a peptide mixture representing about 75% of the total actin N was obtained. When treated with fresh trypsin, the trypsin-resistant core slowly broke down with further liberation of peptides, all of which corresponded to those present in the original digest. By 'fingerprinting' techniques about 26
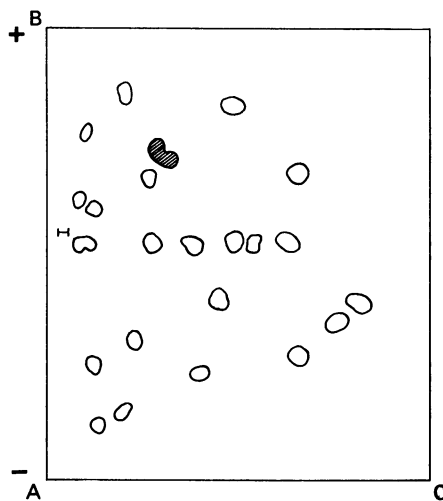

Fig. 4. Peptide 'map' of a tryptic digest of carboxymethylated actin. Actin digest (1 mg.) was applied and electrophoresis at pH 6·5 in the direction AB and chromatography in pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.) in the direction AC were carried out. The shaded area indicates the position of the peptide containing 3-methylhistidine.

ninhydrin-staining spots could be recognized (Fig. 4). On ion-exchange chromatography on Dowex 1 (X 2) the tryptic digest of carboxymethylated actin could be separated into 18 fractions (Fig. 5), in only one of which (fraction XIII) could detectable amounts of 3-methylhistidine be identified after acid hydrolysis by analysis on the Technicon system and paper electrophoresis at pH 6·5. On paper electrophoresis at pH 9·0 fraction XIII separated into two peptide bands. Only the faster of the two bands obtained on electrophoresis at

pH 9·0 produced 3-methylhistidine on acid hydrolysis (Fig. 2). Although the 3-methylhistidine-containing peptide band appeared homogeneous on electrophoresis at pH 3·5, 6·5 and 9·0, it could be resolved into two closely moving bands by chromatography in pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.) (Baglioni, 1961). These two components correspond to the ninhydrin-staining spots in the area shaded on the 'fingerprint' of tryptic digests of actin illustrated in Fig. 4. The two bands resolved by chromatography were not well separated, but preliminary attempts were made to elute them separately and determine their amino acid content. It was clear that although the
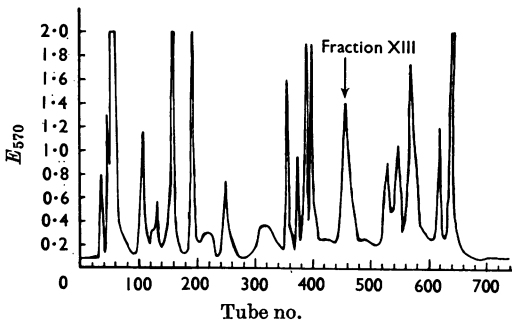

Fig. 5. Chromatography of the tryptic peptides of a tryptic digest of carboxymethylated actin. Peptide material (500 mg.) was applied to a Dowex 1 (X 2) column, which was developed as described in the Methods section. The ordinate represents ninhydrin colour after alkaline hydrolysis. Fraction XIII was the only one to contain significant amounts of 3-methylhistidine, as revealed by amino acid analysis and by paper electrophoresis at pH 6·5 after acid hydrolysis.

separation was not complete the slower peptide fraction contained twice as much 3-methylhistidine as the faster. The fraction containing most of the 3-methylhistidine also contained aspartic acid, threonine, glutamic acid, proline, glycine, isoleucine, lysine and tryptophan.

3-Methylhistidine content of myosin and other muscle proteins. Tropomyosin B and creatine phosphokinase, both isolated from rabbit skeletal muscle, possessed no detectable 3-methylhistidine, but significant amounts of ninhydrin-positive material were detected in the same position as 3-methylhistidine when acid hydrolysates of normal myosin preparations were analysed by the Beckman physiological fluids system (Fig. 1). The 3-methylhistidine:histidine ratio was much lower than in actin, and satisfactory analyses for the latter amino acid could only be obtained by applying larger amounts (10–12 mg.) of myosin hydrolysate to the amino acid analyser column.

The average value for myosin was 1·8–1·9 residues/mol. of mol.wt. 525 000 for the standard myosin preparations (Table 3). This value was not changed significantly if the protein was further purified by DEAE-cellulose chromatography (Perry, 1960) and subsequent fractionation by the lithium chloride–ammonium sulphate method (Luchi, Kritcher & Conn, 1965). If myosin was succinylated by the procedure of Oppenheimer, Barany, Hamoir & Fenton (1966), 3-methylhistidine was detected only in the major fraction precipitated by 55%-saturated ammonium sulphate. As with actin, the 3-methylhistidine was not removed from myosin by treatment with 5% (w/v) trichloroacetic acid, nor could β-alanine be detected in the acid hydrolysates of the myosin samples. Preliminary study indicates that the

Table 3. *3-Methylhistidine content of adult rabbit myosin and its fragments*

For the methods of analysis, see the text. The amount of 3-methylhistidine/mole of protein was calculated on the basis of the quoted histidine content and the 3-methylhistidine:histidine ratio determined. Amounts of 3-methylhistidine are given as mean ± S.D.

Protein	No. of preps.	No. of estimations	Amount of 3-methyl-histidine (moles/mole of protein)	Mol.wt. (assumed)	Amount of histidine (moles/10^5 g. of protein)	Amount of histidine (moles/mole of protein)
Myosin	7	11	1·9 ± 0·2	525 000*	14·4§	75·6
Myosin (purified on DEAE-cellulose)	2	4	1·8 ± 0·2	525 000*	14·4§	75·6
Heavy meromyosin	5	7	1·9 ± 0·2	350 000*	14·0†	49·0
Subfragment I	3	5	1·0 ± 0·1	129 000‡	15·0‡	19·3
Light meromyosin fraction I	2	3	Absent	151 000*	21·0†	31·7

* Mueller (1964).
† Lowey & Cohen (1962).
‡ Jones & Perry (1966).
§ Trayer (1966).

3-methylhistidine is localized in at least one peptide fraction isolated by Dowex 1 (X 2) chromatography of the tryptic digest of myosin.

When myosin was converted into the meromyosins by controlled tryptic digestion all the 3-methylhistidine was found in the heavy meromyosin. None could be detected in light meromyosin fraction I. On further digestion to subfragment I the 3-methylhistidine:histidine ratio increased further, but whereas heavy meromyosin contained about 2 residues of 3-methylhistidine/mol., this value had fallen to 1·0 in subfragment I (Table 3).

DISCUSSION

The natural occurrence in proteins of amino acids other than the commonly occurring 20 is remarkably rare despite the fact that the total number of amino acids that have been isolated from biological material is now quite large. Derivatives formed by the esterification of the side-chain hydroxyl groups or by the acetylation of the N-termini, which do not survive acid hydrolysis, are fairly common as protein constituents, but the only substituted amino acids surviving acid hydrolysis that have so far been reported to be present in peptide combination in proteins are the hydroxyl derivative of proline and the hydroxyl and N-methyl derivatives of lysine. Whereas the hydroxyl derivatives appear to be restricted to collagen, there is evidence suggesting that ϵ-N-methyl-lysine may be more widespread. In the flagellar protein of *Salmonella typhimurium* a high proportion of the lysine is methylated (Ambler & Rees, 1959), and ϵ-N-methyl-lysine has also been reported to be present in hydrolysates of histones from various sources (Murray & Luck, 1962; Murray, 1964). The present findings and the independent work of Asatoor & Armstrong (1967) are the first reports of the occurrence of a methylated histidine derivative as a component of a protein. It is not yet possible to say how widely the amino acid is distributed in other proteins, for in the elution system commonly used for amino acid analytical procedures 3-methylhistidine might escape detection as it is not clearly separated from histidine. Even if there are appreciable amounts of 3-methylhistidine relative to histidine, the presence of the former amino acid is revealed only by the failure of the elution trace to return to the base line between the histidine and ammonia peaks in the Beckman system for analysis of the basic amino acids. Thus, if it is present, unless special precautions are taken to extend the elution pattern of the basic amino acids, 3-methylhistidine would probably be evaluated with the histidine.

The data obtained with actin from different species suggest that 3-methylhistidine is a normal component of this protein. In actin from rabbit skeletal muscle, which has been most carefully purified and extensively studied, the 3-methylhistidine:histidine ratio 1:7·6 would imply a minimum molecular weight of 47 600. Assuming that 3-methylhistidine is present in all actin molecules of a given sample the results indicate that there is probably a total of 9 histidine residues/actin molecule, one of which is methylated. If this is the case, and taking the histidine content of actin as 15·9 residues/mol. of mol.wt. 100 000 (obtained from aminoethylated actin, which does not include 3-methylhistidine, analysed by the extended basic amino analysis system), the minimum molecular weight for actin would be 50 000. This value should be compared with the recently published values obtained by the standard physicochemical procedures (Ulbrecht, Grubhofer, Jaisle & Walter, 1960; Adelstein *et al.* 1963; Lewis, Maruyama, Carroll, Kominz & Laki, 1963; Mihashi, 1964; Krans, Van Eijk & Westenbrink, 1965). Although these range from 47 000 to 62 000, most values lie close to 60 000.

It is significant that on complete tryptic digestion of actin only about 26 peptides can be recognized, i.e. about half the number expected from the known lysine and arginine content and a molecular weight of 60 000. Taken together, the 3-methylhistidine content and the tryptic peptide data suggest that actin may be built up of two very similar sub-units, only one of which contains 3-methylhistidine.

The fact that the significant methylation of imidazole amino acids in rabbit, as exemplified by anserine formation, is much more active in post-natal life (Severin, 1955; Kendrick-Jones, 1966) poses the question whether a mechanism exists for the methylation of the histidine of actin in foetal muscle. The data obtained indicate that actin from the 28-day-old rabbit foetus contains an appreciable amount of 3-methylhistidine, although a somewhat smaller amount than actin from the adult. It is probable, however, that the lower values reflect the purity of the preparation rather than evidence of a significant difference between the 3-methylhistidine content of the adult and foetal forms of this protein. In any case it can be concluded that a mechanism exists in foetal muscle for the methylation of a histidine residue in actin.

The component present in myosin hydrolysates that appears to be identical with 3-methylhistidine has not been so intensively studied as that obtained from actin. Experience with the latter protein strongly suggests that 3-methylhistidine is also present in myosin, although in a much smaller amount than is in actin. Nevertheless, despite the fact that the value is low, it is somewhat higher than that briefly reported by Asatoor & Armstrong (1967), and is not significantly decreased by various purification procedures. Myosin is a notoriously

difficult protein to free from contaminating proteins, but it seems unlikely that the 3-methylhistidine is present as a contaminating protein impurity rather than as part of the myosin molecule. It is very unlikely, if the impurity were actin, that it would survive the purification procedures and the tryptic digestion used for the preparation of heavy meromyosin. Indeed, if actin contamination were to explain the 3-methylhistidine content of heavy meromyosin, it would have to be present as 30% of the preparation. This is a completely unrealistic value, for there is no evidence that the heavy meromyosin preparations contain actin at all.

The results for adult-rabbit myosin indicate there are approximately two 3-methylhistidine residues/mol., these being localized in the high-molecular-weight component obtained by ammonium sulphate fractionation of the succinylated protein. As this fraction represents the bulk of the myosin molecule the evidence suggests that the myosin molecule contains two major similar sub-units. The 3-methylhistidine residues appear to be localized either close to or in the head of the myosin molecule, judging from their distribution among the main tryptic fragments obtained from myosin. Subfragment I contains slightly more than half the number of 3-methylhistidine residues/mol. of myosin or heavy meromyosin, but until the distribution of the 3-methylhistidine in various fractions produced during subfragment I formation from heavy meromyosin has been studied it would be premature to conclude from this datum that 2mol. of subfragment I are produced/mol. of heavy meromyosin (Jones & Perry, 1966; Mueller, 1965).

Speculation on the significance of the methylation of histidine residues in actin and myosin must wait until more information is available about the distribution of 3-methylhistidine in other proteins. Nevertheless, if this substitution is shown to be restricted to actin and myosin, in which proteins the process is highly specific and limited to 1 and 2 residues/mol. respectively, it would not be unreasonable to assume that it is related to the unique biological properties of these myofibrillar proteins.

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