Observations on the Actin Content of the Rabbit Myofibril

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1. On extraction of whole muscle by the procedure of Hasselbach & Schneider (1951), the amount of actin that passes into solution seems to account for little more than 10% of the protein content of the myofibrils. 2. Extraction of isolated myofibrils with suitable media that allow identification and estimation of dissolved proteins seems to give about the same yield of actin (10–13% of the total). 3. A comparatively large residue of myofibrillar components remains after extraction. The amount of actin present in the residue can be only hypothetical.

Actin has been generally assumed to account for 20–25% of the myofibrillar protein, after the work of Hasselbach & Schneider (1951). They reported that after the removal of myosin from minced muscle it is possible to bring into solution another protein fraction, accounting for 13–15% of total muscle protein, by homogenizing the residue in a solution of any ionic strength from 0 to above 0.6, at pH above 6.0. In their opinion that fraction is made up entirely of F-actin because it is soluble in water and shows ATP sensitivity (in the viscometer) only after the addition of myosin, and also because most of it (80–90%) is precipitated at low ionic strength after the addition of myosin. The present results are at variance with these findings and conclusions.

METHODS

Protein fractions. These were prepared according to the method of Hasselbach & Schneider (1951). Back muscles of the rabbit were minced and extracted five times at 2–3° with 0.47 M-KCl in 10 mM-potassium pyrophosphate–10 mM-potassium phosphate buffer, pH 6.2. The extracts were separated by centrifugation and the residue was homogenized for 2–3 min. in a Waring Blendor with 0.6 M-KCl. The residue was washed twice with the same solution.

Myofibrils. These were prepared as follows (Perry & Corsi, 1958): minced muscle was homogenized in 80 mM-KCl in 4 mM-EDTA–20 mM-borate buffer, pH 7; myofibrils were resuspended and washed with 0.1 M-KCl in 39 mM-borate buffer, pH 7 (about 31. for myofibrils from 100 g. of muscle).

L-myosin. This was prepared by the method described by Perry (1955). 'Natural' actomyosin was obtained by extracting the isolated myofibrils with Weber's solution (0.6 M-KCl–10 mM-Na₂CO₃–40 mM-NaHCO₃); actomyosin was precipitated by dialysis against 34 mM-KCl in 2.5 mM-potassium phosphate buffer, pH 7 and 1.0–0.4.

Electrophoretic separations. These were carried out with the Perkin-Elmer model 38 instrument in 0.26 M-KCl in 50 mM-potassium phosphate buffer, pH 6.9.

Chromatography. Conditions were very similar to those used by Perry & Zydowo (1959). DEAE-cellulose (medium grade) supplied by Sigma Chemical Co. (St Louis, Mo., U.S.A.) was suspended first in N-NaOH and then in the buffer in which the protein was dissolved. After being washed three times by centrifuging and resuspension with fresh buffer, the DEAE-cellulose was poured into a column (40 cm. × 1.6 cm. diam.) and washed overnight. Protein solutions were applied to the column in 0.1 M-KCl in 20 mM-tris-HCl buffer, pH 7.6. Elution was carried out at 2–3° under pressure of 60–80 cm. H₂O with a KCl gradient to 1.5 M-KCl. Chloride in the eluate was estimated by titration with Hg(NO₃)₂ according to the method of Schales & Schales (1941). Protein solutions before and after elution were concentrated by suspending them at 0° in a dialysis bag in front of a fan and by filtration in the LKB 6300A ultra-filter.

Viscometry. Viscosity estimations were carried out at 0° in 0.5 M-KCl in 40 mM-potassium phosphate buffer, pH 7.1. ATP sensitivity was measured by determining the fall in viscosity obtained when ATP was added to give a final concentration of 1 mM. Tropomyosin was estimated by measuring the fall in viscosity on the addition of KCl, according to the method of Perry (1953).

Enzymic assays. Adenosine-triphosphatase activity was determined in 50 mM-tris-HCl buffer (pH 7.4)–0.2 M-KCl–5 mM-CaCl₂–4 mM-ATP. Incubations were carried out at 25° for 10 min. Aldolase activity was determined by the method described by Wu & Racker (1959).

RESULTS

Extraction of whole muscle by the procedure of Hasselbach & Schneider (1951). As much as 70% of the total nitrogen was extracted from minced muscle by a solution of potassium chloride in pyrophosphate buffer at pH 6.2. Fractionation of the extract by

precipitating the proteins with 10% (w/v) trichloroacetic acid or by precipitating myosin at low ionic strength (1.0 M) gave results similar to those described by Hasselbach & Schneider (1951). No actin could be detected by electrophoresis or viscometry.

After homogenizing the muscle residue with 0.6 M-potassium chloride a further 12–13% of total nitrogen passed into solution, in good agreement with the value reported by Hasselbach & Schneider (1951), but this extract did not appear to be made up only of actin. Although no fall in viscosity occurred on adding ATP, when first dialysed against 0.5 M-potassium chloride in 40 mM-phosphate buffer, pH 7.2, the addition of ATP to the extract caused a very appreciable fall. Adenosine-triphosphatase activity was high, amounting to almost 20% of the activity of the first extract on the basis of total nitrogen.

Only negligible amounts of material were precipitated at 1.03 (pH 6.5–6.8). By dialysing against 34 mM-potassium chloride in 2.5 mM-potassium phosphate buffer, pH 7 and 1.0 M, about 60% of the extracted protein was precipitated. Electrophoretic analysis of the precipitate redissolved by adding potassium chloride revealed one main peak, the mobility of which was about $4.2 \times 10^{-5} \text{ cm}^2/\text{v}/\text{sec}$. (4.0×10^{-5} in the ascending limb and 4.3×10^{-5} in the descending limb: values were the average of two independent determinations). Such values are different from those of 'natural' actomyosin under the same conditions of electrophoresis (about $3.3 \times 10^{-5} \text{ cm}^2/\text{v}/\text{sec}$). It was thought that the difference might be the consequence of different proportions of actin and myosin: since most myosin was removed with the first extract, it appeared that a smaller proportion of myosin should be obtained from the residue. In fact it was claimed by Hasselbach & Schneider (1951) that added myosin can be bound in the second extract. We attempted an approximate estimation of the amount of actin in the fraction precipitated at 1.0 M by mixing constant amounts of it with increasing amounts of pure myosin and measuring the variation of ATP sensitivity with different proportions of the components in the mixture. ATP sensitivity increased with increasing amounts of added myosin up to a maximum that appeared to indicate that the largest amount of myosin that could be bound was slightly less than double the amount of protein present in the fraction. Obviously the value that may be calculated for actin on the basis of this experiment depends on the combining ratio of myosin to actin, which is assumed. On the basis of the ratio 2.5:1 this would mean that actin represents about 75% of the fraction (a lower percentage is calculated if a higher ratio is assumed).

Fig. 1. Chromatography of the supernatant at low ionic strength of the extract in 0.6 M-KCl of whole muscle homogenized after the removal of myosin. Protein solution (27.5 ml.; $E_{280}^{1\text{cm}}$ 7.9) was applied in 0.1 M-KCl in 20 mM-tris buffer, pH 7.6. A gradient to 0.4 M-KCl in 20 mM-tris buffer was started at point A; a gradient to 2.0 M-KCl in 20 mM-tris buffer was started at point B. ●, $E_{280}^{1\text{cm}}$; □, concn. of KCl.

The supernatant at 1.0 M had only negligible adenosine-triphosphatase activity. Viscometric assays gave values of about 20% for tropomyosin content.

Four fractions, which were numbered in order of elution, were separated by chromatography on DEAE-cellulose. Two fractions, I and IV, could be separated as sharp peaks (Fig. 1). Fraction I, representing about 30% of the total eluted material, was not retained when applied in 0.1 M-potassium chloride in 20 mM-tris buffer, pH 7.6. Fraction IV, representing about 24% of the total, was eluted between 0.4 M- and 1.2 M-potassium chloride in 20 mM-tris buffer, pH 7.6 (maximum at about 0.6 equiv. of Cl⁻/l.). Between 0.1 M- and 0.4 M-potassium chloride in tris buffer, pH 7.6, no separation was obtained even by using a slow increase in concentration of the eluting solution, although one peak generally appeared with a maximum corresponding to 0.3 equiv. of Cl⁻/l. Only by stepwise increase of the concentration of potassium chloride in the buffer to 0.22 M (Perry & Zydowo, 1959) and subsequently to 0.3 M could two fractions (II and III) be separated, representing about 27 and 20% of the total respectively. Most of fraction I was coloured. Aldolase activity was present in appreciable amounts. Dialysis of fraction II against water brought out of solution about 70% of the protein content: this globulin could be redissolved readily by the addition of potassium chloride. Fraction III contained tropomyosin; in fact it was the only one in which tropomyosin could be detected. Viscometric assays

gave values of almost 50% for tropomyosin content. Fraction IV appeared to contain nucleic acid. In most of it the E_{280}/E_{260} ratio was less than 1 and a positive test for pentose was obtained with the orcinol reaction (Mejbaum, 1939; Schneider, 1945).

In two experiments the muscle residue, after extraction of myosin, was homogenized with a buffer of low ionic strength ($I0.04$) at pH7. We were unable to confirm the observation by Hasselbach & Schneider (1951) that under such conditions the yield is the same as with 0.6M-potassium chloride. In our hands the yield was very poor, representing less than 2% of the total nitrogen of the muscle.

Extraction of isolated myofibrils. Extracting isolated myofibrils with a buffer of low ionic strength at pH above 7 appeared to be useful as an alternative approach to the estimation of actin in muscle. Under these conditions a depolymerized form of actin passes into solution along with tropomyosin and a small amount of a third component, which moves more slowly in the electric field and is probably sarcoplasmic in origin (Perry & Corsi, 1958; Perry & Zydowo, 1959).

Myofibrils were mixed with 6–9 vol. of 5 mM-tris buffer, pH8, and dialysed against 150–200 vol. of the same buffer, usually for 3–6 days. The extract was separated by centrifugation and it was assumed that the percentage of protein extracted was $100 \times$ concentration of nitrogen in the supernatant divided by the concentration of nitrogen in the whole mixture. In some experiments the residue was washed repeatedly with the tris buffer and the total nitrogen content of the assembled supernatants was compared with the total nitrogen content of the original sample of myofibrils used for extraction. The amount of protein extracted after 3–6 days never exceeded 25% of the total myofibrillar content and in most cases it was definitely less, namely about 20% (Table 1). These values are lower than some reported by Perry & Corsi (1958), but that may be due to the different methods used for the preparation of myofibrils. By washing the myofibrils with

larger volumes of buffer the sarcoplasmic component could have been removed and thus not detected by electrophoresis. It appeared that the admixture of the sarcoplasmic component in myofibrils prepared by the method used by Perry & Corsi (1958) may cause an overestimation of the extracted myofibrillar protein as high as 13%, and electrophoresis showed that the larger yield of poorly washed myofibrils was well accounted for by the amount of the sarcoplasmic component that was present in the extract. The more thorough washing of the myofibrils caused no appreciable loss of actin or tropomyosin.

Estimation of actin and tropomyosin was carried out by several methods: actin was selectively precipitated by the addition of potassium phosphate buffer, pH7.2, to give a final concentration of 0.75M (Perry & Corsi, 1958); the fall in viscosity on the addition of potassium chloride, which is a peculiarity of tropomyosin, was determined according to the method of Perry (1953); the areas of the peaks on the electrophoresis diagrams were also measured. Values for tropomyosin estimated by the viscometric method were slightly below 50% in most cases (the average of six estimations was 48%); values obtained by the other methods were in fair agreement.

The largest amount of actin that could be extracted from isolated myofibrils accounted for about 13% of the total myofibrillar protein. Estimations were usually restricted to extracts less than 4 days old, because after a more prolonged treatment actin and tropomyosin may be too altered for accurate estimation. Some extracts obtained after many days in tris buffer showed a fairly large peak, between the usual ones, which did not correspond to actin or tropomyosin. In any case, extraction appeared already complete after 3 days.

Analysis of the unextracted residues. After removal of actin an insoluble 'stroma' remained, accounting for about 15% of total nitrogen (and consisting almost entirely of connective-tissue fibres), as found by Hasselbach & Schneider (1951). Extraction of collagen and determination of hydroxyproline by the method of Neuman & Logan (1950) showed that collagen accounted for only 10–11% of the dry residue. Admixture of elastin and mucoproteins and other non-muscular components is not likely to increase that value appreciably (Meyer, Hoffman & Linker, 1957; Bowes, Elliott & Moss, 1957).

This result appears to agree with that obtained with isolated myofibrils. By extracting from them myosin, actin and tropomyosin, first at high ionic strength (30 mM-magnesium chloride–0.2 M-sodium pyrophosphate buffer, pH7) and subsequently at low ionic strength (5 mM-tris buffer, pH8) we were always left with an insoluble residue accounting for

Table 1. *Total nitrogen extracted from isolated myofibrils by tris buffer of low ionic strength*

Prep. no.	Extraction time (days)	N extracted (% of total N)
104	5	18
105	6	20
106	3	18.3
107	6	24.5
109	4	20.5
115	5	22.3
117	3	21
118	3	21.5
128	4	25

about 20% of the original nitrogen content. More efficient extracting media (e.g. potassium iodide) did not appear to provide solutions of proteins suitable for analysis and identification according to conventional criteria.

DISCUSSION

The results appear to indicate that actin extracted from minced muscle by the procedure of Hasselbach & Schneider (1951) cannot appreciably exceed 10% of the protein content of the myofibrils. At least one-half of the fraction considered to be actin by Hasselbach & Schneider (1951) consists of several components. One is myosin; the others seem to be the same as those of the so-called 'extra protein' fraction, which is extracted along with myosin from isolated myofibrils by solutions of high ionic strength containing ATP or pyrophosphate and is soluble at $I0.04$. In our hands chromatography of the protein material that was not precipitated at $I0.04$ gave results similar to those obtained by Perry & Zydowo (1959) with 'extra protein'. Fraction I was shown by them to be sarcoplasmic in origin: the red colour is probably accounted for by haemoglobin or myoglobin (Hartshorne & Perry, 1962). The other fractions seem to consist of myofibrillar proteins. However, according to Perry & Zydowo (1959), actin is not likely to be present in extra protein.

Extracting isolated myofibrils with buffer of low ionic strength does not appear to give a much better yield of actin.

Our results are at variance with the conclusion of Hasselbach & Schneider (1951) that the 'insoluble stroma' of muscle consists mainly of connective-tissue fibres. It appears that most of it is not collagen. The amount of the residue of whole muscle is about the same as the residue of isolated myofibrils after extraction under suitable conditions. It corresponds roughly to the value reported by Scopes (1964) for the insoluble protein after

maximum extraction at high ionic strength ($I1.0$) of myofibrils from rigor muscle. It seems unlikely that much of it is collagen. It may be suggested that the already known myofibrillar proteins are present in the residue, possibly in some denaturated form. In fact it is not unlikely that myosin and actin are present, because extracted myofibrils may still contract, though very slowly, on the addition of ATP (Perry & Corsi, 1958). But as long as the undissolved actin cannot be estimated and new methods are not found for dissolving the myofibrils completely and allowing a satisfactory analysis of the solution, no thorough estimation is possible of the actin content of the myofibrils. At present the usually accepted values do not seem to be supported by any direct experimental evidence.

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