based on the peptide-bond absorption, differential measurements being used in which the extinction at 225 $m\mu$ is subtracted from that at 215. The use of these wavelengths would not, however, have the effect of reducing the contribution of the aromatic amino acids, and cannot be applied to cases such as gramicidin, since this peptide actually has a greater absorption at 225 than at 215 $m\mu$.

From our results it seems that measurement in the region of 210 $m\mu$ can be used as a reliable, precise and quick method for the estimation of serum proteins. The use of measurements at this wavelength also appears to provide a fairly general method of protein estimation, and serious error is unlikely unless the protein contains exceptionally large amounts of aromatic amino acids, as with gramicidin or insulin.

A further advantage of this method lies in the great sensitivity. When no interfering substances were present it was possible to detect concentrations down to 2 $\mu g.$ of protein/ml., corresponding to an extinction of 0.04. Advantage was taken of this in experiments with anion-exchange cellulose for the column chromatography of serum proteins. Amounts of serum as low as 0.1 ml. were used satisfactorily and the quantitative interpretation of results was aided by the uniformity of specific absorption in the various fractions.

SUMMARY

1. Absorption measurements at 210 $m\mu$ can be usefully employed for protein estimation. At this wavelength the total proteins of 40 normal and pathological sera had a mean specific extinction coefficient $E_{1cm}^{0.1\%}$ of 20.5 ± 0.14 . Albumin, α -, β - and γ -globulin all had specific extinction $E_{1cm}^{0.1\%}$ near 20.

The method is directly applicable to all proteins studied except those containing unusually large amounts of aromatic amino acids.

2. A method for the estimation of total serum proteins and for albumin:globulin ratios based on the above is described. Bovine serum albumin had almost the same specific extinction coefficient as serum proteins and formed a useful reference standard.

3. The use of the extinction at 210 $m\mu$ for the detection of very small quantities of protein (2 $\mu g./ml.$) and its application to column chromatography are discussed. In this connexion it is necessary to avoid the use of buffers containing carboxylic acids.

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Some Properties of Uterus Actomyosin and Myofilaments

By D. M. NEEDHAM AND J. M. WILLIAMS
Department of Biochemistry, University of Cambridge

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Some properties of the actomyosin-like adenosine triphosphatase of the uterus have already been described (see Needham & Cawkwell, 1956). It was shown that this enzyme splits off only one phosphate group and has a pH-activity curve in 0.5M-potassium chloride resembling that of myosin from skeletal muscle. The activity, however, is much lower than that of skeletal-muscle actomyosin; rabbit- and pig-uterus actomyosin (with which

most of this work was done) also differ from skeletal muscle actomyosin (which is somewhat inhibited at higher concentrations of potassium chloride) in being less active in media containing 0.1M-potassium chloride than in those containing 0.5M-potassium chloride. Thus at 28°, with Ca^{2+} ion activation, the values for $\mu moles$ of inorganic phosphorus liberated/mg. of nitrogen/min. are about 0.2 and 0.6 in media containing 0.1 and

0.5M-potassium chloride respectively; the corresponding values for skeletal-muscle actomyosin are about 6.0 and 3.6.

In the presence of Mg^{2+} ions skeletal-muscle actomyosin has an activity of about 3 μ moles/mg. of nitrogen/min., under optimum conditions (calculated for 28° from the data at 20° of Hasselbach, 1952). Uterus actomyosin had negligible activity with Mg^{2+} ions under the same conditions and a variety of others tested.

In the present work the further purification of uterus actomyosin is described and the action upon it of crystalline trypsin under conditions closely resembling those used with skeletal-muscle myosin (Szent-Györgyi, 1953). In the latter case a decrease of 13% was found in total adenosine triphosphatase activity by Mihalyi & Szent-Györgyi (1953*b*), whereas Gergely, Gouvea & Karibian (1955) report an increase of about 25%. The myosin is split into two components, heavy and light meromyosin (H- and L-meromyosin), the former constituting a little more than 50% of the protein; it contains all the adenosine triphosphatase activity and power of complex formation with actin. Skeletal-muscle actomyosin is digested at the same rate as myosin; in this case L-meromyosin is released, but H-meromyosin is obtained in combination with actin (Szent-Györgyi, 1953; Mihalyi & Szent-Györgyi, 1953*b*).

With uterus actomyosin, there is a striking rise in total adenosine triphosphatase activity upon treatment with trypsin: 400–800% tested in 0.1M-potassium chloride and about 50% tested in 0.5M-potassium chloride; moreover, a fraction

could be obtained corresponding to the acto-H-meromyosin of skeletal muscle, with adenosine triphosphatase activity as high as that of the latter.

This high potential adenosine triphosphatase activity of the uterus protein led us to examine various possibilities of partial inactivation during preparation of the enzyme from the native smooth-muscle filaments.

Snellman & Tenow (1954) have claimed that the uterine contractile mechanism contains actotropomyosin instead of actomyosin, and suggest that the former consists of a complex of actin, a 'phosphate-absorbing protein' and nucleotropomyosin. In view of this we have estimated tropomyosin in pig uterus and also in the usual actomyosin precipitates.

A preliminary account of some of this work has been published (Needham & Cawkwell, 1958).

EXPERIMENTAL

Materials

Preparation of the purified actomyosin. The early stages were similar to those described by Needham & Cawkwell (1956), except that when small quantities of tissue were used (20–30 g.) it was disintegrated at 4° by thorough grinding with acid-washed sand or quartz, the extracting fluid being added gradually. This had the advantage over the use of the Waring Blendor that frothing was avoided. After two precipitations at 0.04M-KCl, three further precipitations were carried out; the final concentration of KCl in each case was adjusted to 0.25M, and the pH to 6.85 with the use of 0.66M-glyoxaline-HCl buffer.

Cell-particle suspension. This was made as described by Needham & Cawkwell (1956). The residues obtained after centrifuging the 0.5M-KCl extract at 100 000 g always

Fig. 1. Separation of myofilament-containing fractions from uterus homogenates. The tissue was homogenized in 0.025M-KCl–0.039M-borate. Collagenase when used was at a concentration of 0.18 mg./ml. of homogenate; it was added after the first centrifuging and was allowed to act at 4° for 4–20 hr.

showed a transparent, jelly-like uppermost layer, probably consisting of a little deposited actomyosin; to free the particles from this, the suspension made as described in 0.5 M-KCl containing 0.05 M-potassium phosphate (pH 7.5) was allowed to stand for a few hours and then centrifuged again and the sediment resuspended. Before use in the tests for adenosine triphosphatase activity it was dialysed for some hours against 0.5 M-KCl.

Preparation of myofilament-containing fractions. Homogenization of the minced tissue in 0.039 M-borate buffer (pH 7.0) containing 0.025 M-KCl was carried out at 4° in a Waring Blendor for large quantities of pig uterus, or in a small plastic blender (about 40 ml. capacity) for rabbit uterus. The latter had the advantage that frothing could be almost completely avoided; the tissue used with it was first passed through a Latapie mincer. The medium used was that found by Perry & Grey (1956) to give least leaching out of myofibrillar protein in skeletal muscle. Several trials led to the adoption of the procedure shown in Fig. 1. All values given for *g* are average values.

The collagenase treatment mentioned in Fig. 1 consisted in adding collagenase (0.18 mg./ml.) to the homogenate and keeping it for different periods (4–20 hr.) at 4°.

Enzymes. The crystalline trypsin used was the salt-free product of the Novo Terapeutisk Laboratorium, Copenhagen. The trypsin inhibitor (made from soya bean and crystallized from ethanol) was from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

The collagenase used was ABC Form II, from the Agricultural Biologicals Corp., Lynbrook, N.Y., U.S.A. It is described as containing only traces of other proteinase.

Special chemicals. The adenosine triphosphate (ATP) used was the sodium salt supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A.

Glutathione was obtained from the National Biochemical Corp., Cleveland, Ohio, U.S.A. Solutions were made up and neutralized immediately before the experiment.

2:3-Dimercaptopropanol (BAL) was the gift of Sir Rudolph Peters. It was obtained as a saturated solution in water by allowing the oil to stand covered by a few millilitres of water for some days with occasional shaking. The solution was standardized by titration against 0.01 N-iodine and neutralized just before use.

Diisopropyl phosphorofluoridate (DFP) was the gift of Dr B. C. Saunders to Dr M. Dixon. It was used as a solution (0.1 M) in propan-2-ol.

Buffers. Phosphate buffers were prepared from KH_2PO_4 (analytical reagent, Hopkin and Williams Ltd.) brought to the desired pH by means of KOH solution. Glyoxaline was supplied by L. Light and Co. Ltd.; if not completely colourless it was recrystallized first from benzene and then from ethanol. Buffer solutions were made by addition of the required amounts of N-HCl. Borate buffers about pH 7.0 were made from appropriate mixtures of 0.05 M-sodium tetraborate and 0.2 M-boric acid (Palitzsch, 1915; Britton, 1955); for higher pH values mixtures of 0.2 N-NaOH and 0.2 M-boric acid were used (Britton, 1955). All pH values given for buffers or buffered solutions were measured by means of the glass electrode.

Methods

Estimation of tropomyosin. The assay method of Bailey (1948) was carried out on pig myometrium for the quanti-

tative separation of tropomyosin. Tropomyosin was also estimated in an actomyosin preparation from pig myometrium. The preparation was freeze-dried overnight, then ground with 96% (v/v) ethanol and centrifuged. This was repeated with ethanol and then with ether. The residue was air-dried, then extracted at room temperature overnight with 8 vol. of M-KCl. Extraction with this salt solution was repeated twice more and the assay method of Bailey was continued from this point.

Trypsin treatment of actomyosin. This was done on the lines described by Szent-Györgyi (1953) for skeletal-muscle myosin. Actomyosin was used in the present work because experiments attempting to prepare free myosin from the uterus with Guba-Straub solution (see Szent-Györgyi, 1951) were unsuccessful. A solution of uterus actomyosin (containing about 10 mg. of protein/ml.) in 0.6 M-KCl was used. For every 10 ml., 1 ml. of 0.1 M-borate buffer (pH 8.8) and 0.5 mg. of crystalline trypsin were added. The digestion was carried on for 30 min. at 23°, then stopped by addition of 0.5 mg. of trypsin inhibitor dissolved in a small volume of 0.1 M-sodium acetate. In the first experiments the reaction was ended by adding 0.2 ml. of 0.1 M-DFP.

Ammonium sulphate fractionation. This was done according to Szent-Györgyi (1953), with slight modifications. The cooled digest was dialysed overnight at 4° against 10 vol. of 6.6 mM-potassium phosphate buffer (pH 7.0) and any precipitate was centrifuged off. The solution was brought to 0.4 satn. with $(\text{NH}_4)_2\text{SO}_4$ by adding the solid; the pH was maintained about 7.0 (as shown by tests with bromothymol blue on drops of the diluted solution) by addition of dilute ammonia. The precipitate (fraction 2) was removed by centrifuging. The content of $(\text{NH}_4)_2\text{SO}_4$ was raised to 0.55 satn. with ammonia addition if necessary, and this precipitate (fraction 3) was removed by centrifuging. Fractions 1–3 were each dissolved in a small volume of water and these solutions, as well as the supernatant (fraction 4) were dialysed against water until free from ammonia.

Measurement of adenosine triphosphatase activity. The reaction mixture (final volume 2 ml.) contained (unless otherwise stated): 2 mM-ATP; 0.066 M-glyoxaline buffer (pH 7.0); 0.01 M- CaCl_2 . The concentration of KCl is given for the different experiments. The reaction was carried on for 3 min. at 28°. It was started by addition of enzyme, care being taken that the amounts used fell within the range of proportionality with the rate of reaction. The reaction was stopped by addition of 1 ml. of trichloroacetic acid (15%, w/v).

Measurement of viscosity. An Ostwald viscometer, needing 3.5 ml., was used; its flow time was 26.5 sec. for buffer at 22°.

Methods of estimation. Inorganic P was measured by the method of Fiske & Subbarow (1925) or by the method of Weil-Malherbe & Green (1951) if the quantities were below 20 µg. Protein N was estimated by the method of Chibnall, Rees & Williams (1943), followed by distillation in the apparatus of Markham (1942). For estimation of soluble N after trypsin treatment, the digest was brought to 2.5% (w/v) with trichloroacetic acid and kept at 4° overnight before the precipitated protein was centrifuged off (see Mihalyi, 1953).

Electrophoresis. This was done in the Tiselius apparatus (Perkin-Elmer Corp., Conn., U.S.A.; model 38) at 4°. The actomyosin solutions used contained 0.5 mg. of N/ml. in

0.4M-KCl to which potassium phosphate (0.016M; pH 6.75) had been added. They were dialysed overnight against this same buffered salt solution.

Sedimentation. For sedimentation of acto-H-meromyosin and of actin the Spinco ultracentrifuge model L was used, with cooling.

For the examination of the homogeneity of acto-H-meromyosin the model E analytical Spinco ultracentrifuge was used at 270 000 *g* and temp. 5–7°; the protein (1.1 mg. of N/ml.) was dissolved in 0.6M-KCl containing 0.016M-potassium phosphate (pH 7.0).

RESULTS

Tropomyosin in actomyosin preparations. The amount of tropomyosin present in an actomyosin preparation was estimated according to Bailey (1948). The thrice-precipitated actomyosin was made from 150 g. of non-pregnant pig uterus. After the heating procedure of the final stage of the assay, 16.5 mg. of protein was found in the supernatant. This amounts to about 2.5% of the total protein of the actomyosin preparation. Another portion of the actomyosin preparation (also from 150 g. of uterus) was subjected to a leaching-out process, similar to that used by Perry (1953) with skeletal myofibrils. The actomyosin was kept for 12 days at 4° suspended in 0.039M-borate buffer (pH 7.1) in the presence of a trace of toluene. At the end of this time the suspension was centrifuged at 4° at 10 000 *g* and the supernatant was used for stages I–III of Bailey's (1948) method of tropomyosin preparation. Protein was found to be 23 mg. (corresponding to about 3.5% of the total protein). In each case the protein prepared showed the characteristic sharp fall in relative viscosity on addition of salt. Thus the first preparation, a solution containing 1.27 mg. of protein/ml. of 0.02M-potassium phosphate buffer (pH 6.3), had a relative viscosity of 1.51, which fell to 1.14 on addition of KCl to 0.05M.

Tropomyosin was also estimated in the whole myometrium from pregnant pig and compared

with the actomyosin content. The procedure described by Needham & Cawkwell (1957) was used for the latter. The values found were 2.76 mg. of actomyosin N/g. wet wt. and 0.28 mg. of tropomyosin N. For bovine uterus Sheng & Tsao (1955) found about 0.25 mg. of tropomyosin N/g. wet wt., about 10% of the structural proteins. The corresponding values for skeletal muscle are 10 mg. (Hasselbach & Schneider, 1950–51) and 0.8 mg. (Bailey, 1948).

Purification of uterus actomyosin. Repeated precipitation of the actomyosin by dilution to 0.25M-KCl at pH 6.85 (see under Materials) resulted in the isolation of a protein which gave a single peak on electrophoresis. For the descending boundary $\mu = 3.31\text{--}3.36 \times 10^{-5}$ cm.²/v/sec. After two precipitations at 0.04M-KCl, three such precipitations were adequate in Expt. 1 (Table 1); in another experiment (Expt. 3, Table 1) in which a single precipitation at 0.25M-KCl was carried out, electrophoresis showed a second small peak behind the main peak. In both cases the adenosine triphosphatase activity had been significantly raised by the purification. These experiments were done on rabbit myometrium.

Effects of trypsin-treatment on uterus actomyosin. Table 2 shows some of these experiments. In Expt. 3 the adenosine triphosphatase activity was increased by 60% (tested in 0.5M-KCl) and by nearly 500% (tested in 0.1M-KCl). As a result of these changes the adenosine triphosphatase activity was now about 40% lower in the higher KCl concentration, as is found with skeletal-muscle actomyosin. That the enhanced ATP breakdown was due to true adenosine triphosphatase activity was shown by ascertaining that the reaction was complete when only one phosphate group was split off.

Fig. 2 shows the rapid fall in viscosity consequent on trypsin-treatment and the course of the formation of acid-soluble N during the trypsin-digestion. These curves may be compared with the rather

Table 1. *Effect of purification on the adenosine triphosphatase activity of actomyosin preparations*

Conditions of the activity tests were as described in the text.

Expt. no.	Procedure	Inorganic P liberated (μ moles/mg. of N/min.)	
		With 0.1M-KCl	With 0.5M-KCl
1	Actomyosin made by two precipitations at 0.04M-KCl	0.22	0.6
	With three further precipitations at 0.25M-KCl	0.24	0.84
2	Actomyosin made by two precipitations at 0.04M-KCl	0.08	0.59
	With one further precipitation at 0.25M-KCl	0.12	0.74

Table 2. *Effect of treatment with trypsin and subsequent ammonium sulphate fractionation on actomyosin preparations*

In each case the uterus actomyosin had been precipitated three times at 0.04M-KCl. The conditions of digestion and fractionation and the measurement of adenosine triphosphatase activity were as described in the text.

Expt. no.	Actomyosin preparation	Protein N (mg.)	Inorganic P liberated (μ moles/mg. of N/min.)	
			With 0.1M-KCl	With 0.5M-KCl
3	From pig uterus (not pregnant)			
	Digest before trypsin-treatment	30	0.3	0.74
	Digest after trypsin-treatment	—	1.7	1.2
	Fraction 1	2.61	0.15	0.1
	Fraction 2	7.7	4.5	2.0
	Fraction 3	3.7	No activity	—
4	From pregnant rabbit uterus			
	Digest before trypsin-treatment	48	0.21	0.52
	Digest after trypsin-treatment	—	1.5	0.89
	Fraction 1	2.7	0.23	0.15
	Fraction 2	17.3	2.2	1.6
	Fraction 3	4.8	No activity	—
5	From pregnant pig uterus			
	Digest before trypsin-treatment	51	0.12	0.34
	Digest after trypsin-treatment	—	1.08	0.91
	Fraction 2	14.3	2.6	2.1
6	From pregnant rabbit uterus			
	Digest before trypsin-treatment	44.4	0.15	0.42
	Digest after trypsin-treatment	—	0.79	0.59
	Fraction 1	2.52	1.54	0.99
	Fraction 2	12.5	3.27	1.9
	Fraction 3	3.6	No activity	—
	Fraction 4	3.2	No activity	—
	From rabbit skeletal muscle; once pptd. at 0.04M-KCl, once at 0.3M-KCl			
	Digest before trypsin-treatment	47.4	4.5	1.23
	Digest after trypsin-treatment	—	5.3	1.4
	Fraction 1	3.0	2.0	1.2
	Fraction 2	11.8	8.2	4.1
	Fraction 3	11.3	11.6	6.8
	Fraction 4	—	No activity	—

similar ones for skeletal-muscle myosin, given by Gergely (1953) for viscosity changes, and by Mihalyi & Szent-Györgyi (1953b) and Gergely *et al.* (1955) for acid-soluble N.

Attempts were then made, by application of the $(\text{NH}_4)_2\text{SO}_4$ fractionation method of Szent-Györgyi (1953), to see whether a protein fraction corresponding to the acto-H-meromyosin from skeletal-muscle myosin, had been formed. As for skeletal-muscle protein this method has only been described for separation of the products of myosin digestion; in Expt. 6 (Table 2) a digestion of skeletal-muscle actomyosin was included.

A study was made of the time course of the increase in adenosine triphosphatase activity during the treatment with trypsin. Small samples were removed at different time intervals, treated at once with trypsin inhibitor and tested for adenosine triphosphatase activity. The results are shown in Fig. 3.

This fractionation with actomyosin as the sub-

strate for digestion did not give the clear-cut results obtainable with skeletal-muscle myosin. From the adenosine triphosphatase results it appears that acto-H-meromyosin contaminated fraction 1 and could spread over fractions 2 and 3. Also recovery of the protein was incomplete, since solution of the fractions and complete clearing of the supernatants on centrifuging were often very slow. About 66% of the original protein was usually accounted for; more than 80% of the adenosine triphosphatase activity could be recovered in the fractions.

Another method for the separation of the acto-H-meromyosin from the uterus was therefore tried, based on a procedure described by Mihalyi & Szent-Györgyi (1953b) for separating acto-H-meromyosin after actin had been added to digested myosin.

A preparation was made from pregnant pig uterus (150 g.) by the procedure for obtaining actomyosin electrophoretically homogeneous. A

Fig. 2. Effect of trypsin-treatment on the relative viscosity of pig-uterus actomyosin, and on formation of acid-soluble N from rabbit-uterus actomyosin. Viscosity measurements were made at 22° in an Ostwald viscometer. For protein precipitation 2.5% of trichloroacetic acid was added and the mixtures were kept 15 hr. at 4° before centrifuging. ●, Viscosity change; ○, acid-soluble N as percentage of the original protein N.

Fig. 3. Effect of trypsin-treatment on the adenosine triphosphatase activity of pig-uterus actomyosin, tested in 0.1M-KCl (●) and in 0.5M-KCl (○). The digests were prepared as described in the text, and the trypsin activity was stopped in each sample by addition of trypsin inhibitor.

solution (23 ml. containing 7.5 mg. of protein/ml. in 0.6M-KCl) was treated as usual with trypsin. After addition of trypsin inhibitor the solution was diluted with 0.6M-KCl to a content of 3.7 mg. of protein/ml. and centrifuged for 2 hr. at 100 000 g. The semi-transparent pellets deposited contained 49% of the original protein. In the supernatant 45% was recovered.

The pellets were dissolved in 0.5M-KCl buffered with 0.01M-potassium phosphate (pH 7.0). A portion was used for the formation of H-meromyosin by dissociating the acto-H-meromyosin. A solution containing 10 mg. of protein/ml., 3.3 mM-ATP and 5 mM-MgCl₂ was centrifuged for 2 hr. at 100 000 g. A small pellet, presumably of actin and undissociated acto-H-meromyosin, was deposited. The second supernatant (H-meromyosin) as well as the first supernatant and the solution of the first pellets (acto-H-meromyosin) were tested for adenosine triphosphatase activity (Table 3).

In the analytical ultracentrifuge the acto-H-meromyosin showed one main component, *S*₂₀ about 73 (compare Mihalyi & Szent-Györgyi, 1953b). No protein resembling L-meromyosin was precipitated from the first supernatant on prolonged dialysis against 0.04 M-KCl containing 3 mM-phosphate, pH 7.0 (compare Velick, 1956).

The effect of the same treatment with trypsin on the adenosine triphosphatase activity of the particulate fraction from the myometrium was also tested. There was a marked clearing of the suspension and about 20% of the protein N became acid-soluble, but the adenosine triphosphatase activity did not increase by more than 10%.

Possible causes of the low adenosine triphosphatase activity of purified uterus actomyosin. Since the results of trypsin-treatment show that the uterus actomyosin has potentially a high adenosine triphosphatase activity, an investigation was made of possible causes of partial inactivation during the isolation and purification of the protein, and of possible reasons for the specially low activity in 0.1M-KCl.

Table 3. Adenosine triphosphatase activity of the products of trypsin-digestion of uterus actomyosin

Conditions of trypsin-digestion and measurement of adenosine triphosphatase activity were as described in the text.

	Inorganic P liberated (μmoles/mg. of N/min.)
Digest centrifuged (2 hr. at 100 000 g)	
Supernatant	0.38
Pellet	5.5
Solution of pellet in 0.5M-KCl (containing 0.01M-potassium phosphate, pH 7, 3.3 mM-ATP and 5 mM-MgCl ₂) centrifuged (2 hr. at 100 000 g)	
Supernatant	4.6

The effect of -SH reagents was tried, but glutathione (0.01M) and BAL (5 mM–75 μ M) were without effect. Besides its effect in restoration of -SH groups, BAL has marked capacity for combination with heavy metals (Webb & van Heyningen, 1947). With the idea of removing these possible inhibitory factors, glycine (0.04M) was also used but the enzyme activity was unchanged. The glyoxaline buffer used in the routine tests probably also protected the enzyme against effects of heavy metals.

The possibility that combination with actin might be masking active groups was tested by using much higher ATP concentration (8 mM) in the hope of increasing dissociation of the actomyosin. Little effect was shown when the test was made in 0.5M-KCl; in 0.1M-KCl there was even less activity with the high substrate concentration.

The suggestion was made that one effect of trypsin might be to remove an inhibitory substance bound to the actomyosin. This was investigated in the following way. Trypsin-treatment was carried out as usual and, after dilution to 4 mg. of protein/ml., the digest was centrifuged for 2 hr. at 100 000 g. The pellet of acto-H-meromyosin was dissolved in 0.6M-KCl and centrifuged again, to remove any remaining trypsin. The second pellet was dissolved in 0.6M-KCl. In order to find whether any diminution of the high adenosine triphosphatase activity occurred upon mixing with untreated actomyosin, a solution in 0.5M-KCl containing 0.12 mg. of acto-H-meromyosin N/ml. and 1.4 mg. of actomyosin N/ml. was left at 4° for 12 hr. The adenosine triphosphatase activity of the diluted mixture was compared with the sum of the activities of the actomyosin and acto-H-meromyosin (Table 4). It is seen that there is no diminution in activity but an increase, which could be perhaps accounted for by adsorption of a trace of trypsin. The activation is slight; thus if it concerns only the actomyosin activity it can be calculated that the P liberated has risen from 0.12 to 0.15 μ mole/mg. of N/min. after standing with the acto-H-meromyosin. This experiment gives no evidence for the presence of an inhibitory factor, but the latter is not ruled out since it might not affect the acto-H-meromyosin under the conditions used.

Adenosine triphosphatase activity of native myofilaments. It is possible that the procedure of solution of the actomyosin and reprecipitation might themselves affect the original adenosine triphosphatase activity; such changes have been reported by Perry (1951) for skeletal myofibrils. Efforts were therefore made to get preparations of native myofilaments free from cell particles and collagen fibres. This aim has not yet been satisfactorily achieved, but since some conclusions can be drawn from the experiments they will be described here.

Table 4. *Investigation of the possible effect of actomyosin on the adenosine triphosphatase activity of acto-H-meromyosin*

Conditions of the activity tests were as described in the text.

	Inorganic P liberated in 5 min. at 28° (μ g.)	
	With 0.1M-KCl	With 0.5M-KCl
Actomyosin N (0.07 mg.)	1.2	5.0
Acto-H-meromyosin N (0.019 mg.)	6.2	5.9
Actomyosin (0.07 mg.) plus 0.0067 mg. of acto-H-meromyosin		
N:		
Observed	4.0	9.1
Calculated for the mixture	3.4	7.1

When the collagenase treatment had been given in the preparation of the homogenate (see Methods section and Fig. 1), after the second homogenization and centrifuging the main residue R was covered by a fine sludge which could easily be poured or pipetted off separately (R_1). R_3 was obtained in the same way after the third homogenization and centrifuging. When no collagenase treatment was given, this layer was absent and only R_2 and R_4 were obtained. The yields were very poor and variable: only about 50 mg. of protein N/100 g. wet wt. for R_1 plus R_3 , and much less for R_2 plus R_4 . Treated with van Gieson's stain and examined microscopically, all four fractions showed much muscle and little connective tissue. No unbroken cells and usually no nuclei, or very few, were seen. Under the phase-contrast microscope at magnification $\times 850$, masses of fine fibrils, rather more than 50 m μ in diameter, were seen lying side by side or fanning out. Since the individual filaments in the uterus-muscle cell examined with the electron microscope were found to be only about 50 Å in width (Shoenberg, 1958) the fibrils seen here must consist of artificially aggregated filaments.

Representative analyses of these filaments are given in Table 5. The protein going into solution upon treatment with 0.5M-KCl (containing 0.05M-potassium phosphate, pH 7, and 1.5 mM-ATP) was taken as actomyosin, the assumption being made that soluble protein from the cell particles would already have been extensively extracted in the initial procedures with solutions of low ionic strength (compare Hillarp & Nilson, 1954). The undissolved residue was treated with 0.1N-NaOH at 4° for 20 hr.; the protein soluble in NaOH was taken as particle protein, that insoluble as collagen.

Table 5 shows that preparations could be obtained consisting of muscle probably to the extent of 60–70 %; the collagen content was always low

Table 5. *Nitrogen distribution in the fractions obtained by centrifuging uterus homogenates*Preparation of the fractions R₁, R₂ and R₄ and the method of analysis are described in the text.

Expt. no.	Fraction used	Conditions	Total N	Actomyosin N	Particle N	Collagen N
F3	R ₄	No collagenase treatment. The 0.5M-KCl soln. was centrifuged (1500 g for 20 min.) to obtain the particles	6.86	4.9	1.68	0.19
F6	R ₁ + R ₂	Collagenase treatment for 20 hr. The 0.5M-KCl soln. was centrifuged (1500 g) to obtain the particles The 0.5M-KCl ('actomyosin') solution was centrifuged again (15000 g for 40 min.)	7.0	4.5	0.6	0.08
			—	3.6	0.63	—

Table 6. *Assessment of the adenosine triphosphatase activity of the native myofilaments of the uterus*

Preparation of the fractions R₁-R₄ and the method of subdividing these into actomyosin solution and particles + collagen are described in the text. Adenosine triphosphatase activity is expressed as μ moles of inorganic P liberated/mg. of N/min. estimated in 0.1M-KCl. In Expts. F2, F6 and F10, pregnant rabbit uterus was used; in F3 and F9, pregnant pig uterus was used. In Expts. F2 and F3, the actomyosin solution was centrifuged for 20 min. at 1500 g to get the particles + collagen. In Expts. F6, F9 and F10, centrifuging at this stage was for 60 min. at 15 000 g.

Expt. no.	Fraction used	Actomyosin (%)	Particles + collagen (%)	Adenosine triphosphatase activity of R fractions	Adenosine triphosphatase activity of particles + collagen	Particle contribution to adenosine triphosphatase activity in 1 mg. of R-fraction N	Myofilament contribution to adenosine triphosphatase activity in 1 mg. of R-fraction N	Adenosine triphosphatase activity of myofilaments
F2*	R ₂ + R ₄	56	44	0.96	1.6	0.69	0.27	0.5
F3*	R ₄	72	28	0.6	0.8	0.22	0.38	0.52
F6†	R ₁ + R ₂	70	30	2.0	2.2	0.66	1.34	1.9
F9*	R ₂ + R ₄	38	62	0.7	0.71	0.45	0.35	0.6
F10†	R ₁ + R ₂	63	37	0.55	0.53	0.2	0.35	0.6
	R ₂ + R ₄	45	55	1.2	1.7	0.94	0.26	0.6

* No collagenase treatment.

† Collagenase treatment for 20 hr.

‡ Collagenase treatment for 4 hr.

Table 7. *Effect of collagenase treatment on the adenosine triphosphatase activity of actomyosin gels from the uterus*

Adenosine triphosphatase activity was measured as described in the text.

Expt. no.	Conditions	Inorganic P liberated (μ mole/mg. of N/min.)	
		In 0.1 M-KCl	In 0.5 M-KCl
1	In 0.04 M-borate buffer, pH 7.0;		
	12 hr. at 4°		
	Control	0.098	0.49
	With collagenase 12 hr.	0.74	0.89
2	With collagenase 18 hr.	0.69	0.74
		0.86	0.94
	In 0.04 M-borate buffer, pH 7.25;		
	15 hr. at 4°		
	Control	0.36	0.86
	With collagenase	1.00	1.29

but in some cases the particle content was higher than is shown here. With the very finely divided R_2 and R_4 it was assumed that all the particles which had come down originally with the fibrils would come down at this centrifugal force (1500 g) from the KCl solution. With R_1 and R_3 , less finely divided, it was found that greater centrifugal force was needed to remove all the particles, presumably because some lighter ones had been carried down entangled in the interstices of the fibrils. This would not be surprising when one remembers the extremely intimate relations of the mitochondria and cytoplasmic reticulum with the myofilaments, as seen in electron micrographs of the uterus-muscle cell (Shoenberg, 1958). In later experiments the KCl solution was always centrifuged at 15 000 g to obtain the particle-collagen fraction.

A summary of the results on the adenosine triphosphatase activity of the R_1 - R_4 fractions and the particle-collagen fractions is given in Table 6. It can be seen that the calculated adenosine triphosphatase activity of the myofibrillar fractions amounted to about 0.6 μ mole of P/mg. of N/min. when tested in 0.1 M-KCl, an activity about equal to that of purified actomyosin preparations tested in 0.5 M-KCl. It thus seems that the very low adenosine triphosphatase activity of purified preparations tested in 0.1 M-KCl may be caused by the repeated solution and reprecipitation of the protein. The adenosine triphosphatase activity of the particles was always found to be higher in the rabbit than in the pig uterus, as observed previously by Needham & Cawkwell (1956); the very low value found in Expt. F10 (Table 6) for the particle-containing fraction from R_1 plus R_3 is probably to be explained by the very high nucleic acid content noticed in this particular case. Nuclei had been in fact observed in the microscopic examination of R_1 .

The high value for myofibrillar adenosine triphosphatase in Expt. F6 (with collagenase treat-

ment) must be mentioned. This observation led to the examination of the effect of collagenase treatment on purified actomyosin gels. Thrice-precipitated actomyosin was used as a suspension in 0.04 M-borate buffer and left at 4° for different lengths of time, with collagenase (0.18 mg./ml.). The gels were then centrifuged, washed to remove collagenase, dissolved in 0.5 M-KCl and tested for adenosine triphosphatase activity. Table 7 shows the results: an increase in activity, particularly in the activity measured at low ionic strength. In another experiment the collagenase treatment was done at room temperature and the resulting viscosity change was followed. This was completed in 45 min. and was rather less than the viscosity change found with trypsin. During this time no change was found in adenosine triphosphatase activity. As a result of this observation, in Expt. F10 (Table 6) collagenase treatment was carried out at 4° for only 4 hr., with the aim of getting some effect on the collagen without effect on the myofibrillar adenosine triphosphatase. This seems to have been accomplished, since the adenosine triphosphatase activity resembled that in experiments without collagenase, and a good yield of R_1 and R_3 was obtained.

The adenosine triphosphatase activity of the actomyosin solutions made by extracting fractions R_1 - R_4 with 0.5 M-KCl was tested in some experiments. It was always lower than the calculated myofibrillar adenosine triphosphatase; the difference between activities in 0.1 M-KCl and 0.5 M-KCl was often much less than with purified actomyosin.

DISCUSSION

Our results do not confirm the finding of Snellman & Tenow (1954) that by dilution of uterus-muscle extract (made with 0.5 M-potassium chloride containing 0.1 M-potassium phosphate, pH 7.0) to 0.1 M-potassium chloride, a protein complex is

obtained containing roughly 40% of actin and 20–25% of tropomyosin. As has been described here, not more than 3.5% of tropomyosin could be found in the dilution precipitates even without extensive purification. The precipitates of Snellman & Tenow (1954) also contained nucleic acid and they suggest that the tropomyosin was present as nucleotropomyosin. It is true that nucleotropomyosin can be obtained from uterus muscle (Sheng & Tsao, 1955, from the cow; D. M. Needham & J. M. Williams, unpublished work, from the pig and rabbit, by the method of Sheng & Tsao). However, it is likely (compare Hamoir, 1952) that the nucleotropomyosin (if not an artifact but actually occurring as such in the tissue) is dissociated on the treatment with organic solvents involved in Bailey's method for tropomyosin isolation. So both nucleotropomyosin and tropomyosin would be estimated in the actomyosin precipitates by the methods used here. Sheng & Tsao (1955) found that the yield of tropomyosin itself was the same, whether the muscle was treated by Bailey's method (giving free tropomyosin) or by their own method, giving mainly nucleotropomyosin. Perry & Corsi (1958) found that by a still milder method of extraction of myofibrils a higher amount of tropomyosin could be obtained (10–12% of the total N of the myofibrils). However, it seems that even if up to half the tropomyosin were destroyed or left unextracted in the method of Bailey or of Sheng & Tsao, not more than about 7% of our dilution precipitates could have consisted of tropomyosin. Kominz, Saad, Gladner & Laki (1957) remark that tropomyosin impurity in myosin B (myosin plus actomyosin) preparations from human uterus becomes more significant if ATP is omitted from the extraction medium.

The mechanism of the trypsin effect in raising the adenosine triphosphatase activity of uterus actomyosin remains obscure. The fall in viscosity roughly parallels the increase in adenosine triphosphatase activity in 0.5M-potassium chloride. With skeletal-muscle myosin Mihalyi & Szent-Györgyi (1953*a*) found the viscosity fall to parallel the appearance of the H- and L-meromyosin assessed from sedimentation patterns. From Fig. 3 it is seen that the increase in activity measurable in 0.5M-potassium chloride is complete in about 10 min., whereas the increase measurable in 0.1M-potassium chloride needs about 30 min. This suggests that, besides splitting the protein into the meromyosins, trypsin may also affect the adenosine triphosphatase activity of the acto-H-meromyosin formed.

The effects of collagenase are difficult to interpret, since the preparation is described as containing traces of proteinase and peptidase; it is thus possible that the effects on actomyosin are due, in part at least, to an unspecific proteinase.

Chymotrypsin (Gergely *et al.* 1955; Middlebrook, 1958) and subtilisin (Middlebrook, 1958) both act upon myosin to give very similar products to those given by trypsin, and Middlebrook suggests that the reaction is non-hydrolytic. The effect of short collagenase treatment in causing fall in viscosity in uterus actomyosin without increase in adenosine triphosphatase activity differed from that of trypsin, with which fall in viscosity and increase in adenosine triphosphatase activity both started immediately. Collagenase treatment had no effect on Ca^{2+} ion-activated activity of adenosine triphosphatase in skeletal myofibrils (Perry, 1951).

The acto-H-meromyosin prepared from uterus is similar to that from skeletal muscle in sedimentation rate and solubility. Its adenosine triphosphatase activity is of the same order, somewhat less in Expt. 6 (Table 2), but in Table 3 higher than that given by Mihalyi & Szent-Györgyi (1953*b*) in their description of acto-H-meromyosin made from skeletal-muscle actomyosin by a similar sedimentation procedure.

So far no preparation of L-meromyosin has been obtained from uterus actomyosin. Further work is needed here.

The evidence from this work suggests that the myofilaments of pregnant-uterus muscle have indeed a low adenosine triphosphatase activity (about 10% of that of skeletal myofibrils); this value agrees with that found in 0.5M-potassium chloride solutions for actomyosin preparations from the uterus. Since the myofilament preparations showed similar activities in 0.5 and 0.1M-potassium chloride, it seems that the very low activity of uterus actomyosin preparations tested in 0.1M-potassium chloride (that is, in the gel form) may be an artifact.

The low adenosine triphosphatase activity of the myofilament preparations investigated at various stages of pregnancy is puzzling when one considers the task of uterus muscle at parturition. It may be of interest to consider the possible significance of the finding that from uterus actomyosin acto-H-meromyosin can be obtained, in amount comparable with that obtained from skeletal-muscle actomyosin and of similar activity. Whether an unmasking of this potential adenosine triphosphatase activity occurs *in vivo* at the end of pregnancy is not yet known. If so, some unmasking agent which did not, like trypsin, simultaneously disrupt myosin would presumably be at work.

SUMMARY

1. Actomyosin electrophoretically homogeneous has been prepared from pregnant rabbit uterus.
2. Short treatment of uterus actomyosin with trypsin led to increased adenosine triphosphatase

activity of the digest: 400–800 % when tested in 0.1M-potassium chloride, about 50 % when tested in 0.5M-potassium chloride. By ultracentrifuging, a product could be separated from the digest comprising half the total protein and containing the whole adenosine triphosphatase activity. This product, in its high adenosine triphosphatase activity (about 5 μ moles of phosphorus liberated/mg. of nitrogen/min.), sedimentation rate and solubility resembled the acto-H-meromyosin obtained by similar treatment of skeletal-muscle actomyosin.

3. No evidence could be obtained that the very low adenosine triphosphatase activity (especially when tested in 0.1M-potassium chloride) of purified actomyosin from uterus was due to oxidation of –SH groups or to inhibition by heavy metals.

4. Preparations containing myofilaments were made from the uterus by homogenization in a medium of low ionic strength followed by differential centrifuging. The adenosine triphosphatase activity of these myofilaments (about 0.5 μ mole of phosphorus liberated/mg. of nitrogen/min.) was the same tested in 0.1 and 0.5M-potassium chloride and resembled that of purified preparations tested in 0.5M-potassium chloride.

5. Treatment of uterus homogenates with a collagenase preparation made it possible to obtain better yields of the myofilament-containing preparation; these showed enhanced adenosine triphosphatase activity, probably due to a direct effect of the collagenase preparation on the myofilaments.

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