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Proteins of the Uterine Contractile Mechanism

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Work on purified actomyosin from uterus muscle has already been described. Needham & Williams (1959) measured the adenosine triphosphatase activity of actomyosin from this source; it was electrophoretically homogeneous and showed mobility resembling that of skeletal-muscle actomyosin. The present enzymic work was done mainly on preparations after a larger number of precipitations at ionic strength 0.27–0.3, and values for adenosine-triphosphatase activity considerably higher than those previously reported were found. Huys (1960) has also studied the electrophoretic mobility, sedimentation rate and relative viscosity of purified uterus actomyosin, and found them to resemble the values for skeletal actomyosin.

We report in addition some experiments on the soluble form of actomyosin recently described by Laszt (1960) and Laszt & Hamoir (1961) in extracts of muscle from carotid artery, and by Huys (1961) in extracts of uterus muscle.

Myosin was first prepared from uterus by Nagy (see Csapo, 1959) by the potassium iodide method of Szent-Györgyi (1951*b*). He showed that it reacted with skeletal-muscle actin, giving a rise in viscosity which could be reversed by addition of ATP. We have now prepared myosin from uterus actomyosin by ultracentrifuging in the presence of ATP and Mg²⁺ ions (see Weber, 1956). Its adenosine-triphosphatase activity, actin-combining power and viscosity have been studied.

So far as we know, actin has not previously been prepared from smooth muscle by biochemical methods. However, Hanson & Lowy (1963), by homogenizing *Taenia coli* of the guinea pig, did obtain filaments which, examined in negatively stained preparations in the electron microscope,

were indistinguishable from filaments of F-actin preparations. In the present work we have made preparations consisting of actin to the extent of about 60%. The remainder was probably tropomyosin B.

Tropomyosin A or paramyosin, so important in the holding mechanism of some invertebrate smooth muscles (see, e.g., Hanson & Lowy, 1961; Rüegg, 1961), has not been observed in mammalian smooth muscle. We have used with uterus the various methods described for its preparation (Bailey, 1956, 1957; Laki, 1957*a*) from invertebrate muscle, but none could be obtained.

Some experiments are included on the possible function of tropomyosin B as a building-stone for actomyosin, but no evidence was found in favour of this.

EXPERIMENTAL

Preparation of actomyosin. The extract of uterus muscle was made as described by Needham & Cawkwell (1956) except that the tissue was first washed by grinding with 7 vol. of 16 mM-potassium phosphate, pH 7.0; after being centrifuged the tissue was well blotted before extraction with either 0.5M-KCl or M-KCl containing 1.5 mM-ATP. The number and nature of the precipitations by dilution are given in Table 1 for each extract.

For the labelling experiments the actomyosin was precipitated five times: twice at 1.0–0.5, three times at 1.0–3; on electrophoresis it showed one fairly symmetrical peak (Fig. 1*a*). The preparation was treated by the method of Mirsky & Pollister (1946) (see also Herrmann, Lerner & White, 1958) for removal of basic nucleoproteins. The method of Simkin & Work (1957) was used for removal of any amino acids adsorbed but not incorporated into the peptide chain. The method also serves to remove contaminating nucleic acids and lipids. The excess of unlabelled amino acids was added in the form of a 1% solution of

mixed amino acids in the same proportions as they occur in the radioactive algal hydrolysate used as the source of radioactive amino acids.

Water distilled from glass was used throughout, and for the preparations and solutions to be described. All values given for *g* are average values.

Preparation of myosin. For uterus myosin, a solution of purified uterus actomyosin, about 3 mg./ml. in 0.6M-KCl-0.04M-potassium phosphate containing 5 mM-ATP and 1 mM-MgCl₂, was centrifuged with cooling at 100000*g* for 3 hr. in the Spinco model L ultracentrifuge. At the end of the run the top part of the supernatant (about 33% of the volume) was removed. For myosin from skeletal muscle, the method of Szent-Györgyi (1951*a*) was used.

Preparation of actin. For preparation from skeletal muscle the method of Straub (1943) was used. One attempt with this method and uterus muscle led to extraction of nucleotropomyosin but little or no actin from the fibre. The method of Tsao & Bailey (1953) was used for uterus muscle.

Preparation of nucleotropomyosin B. This was done by two methods: (a) a modification of the method of Shêng & Tsao (1955), in which the minced tissue was first extracted with buffered KCl (pH 6.3) solution containing 0.01M-Na₄P₂O₇ to remove actomyosin, then, after straining, homogenized with M-KCl; (b) the method recommended by Tsao & Bailey (1953), in which the dried fibre obtained after butanol and acetone treatment is first extracted for actin, then, by means of M-KCl, for tropomyosin B. In both cases the isoelectric and ammonium sulphate precipitations (Bailey, 1948) were used. Crystallization was effected by prolonged dialysis against 0.1M-KCl or 1.6% (NH₄)₂SO₄, the pH being gradually reduced to about 5.0 (Bailey, 1948). For removal of nucleic acid the methods of Hamoir (1952) and of Sevag, Lackman & Smolens (1938) were used.

For the labelling experiments the tropomyosin had to be prepared from small amounts of uterus muscle. Since the small homogenizer available did not disintegrate the rather fibrous tissue very efficiently, a different method was used. The minced tissue was frozen in liquid N₂ and ground to a fine powder. The powder was allowed to thaw and stand at room temperature for 1 hr. so that the ATP might break down and the actomyosin be thus less readily extracted. The material was refrozen and freeze-dried overnight to

denature more actomyosin. It was then powdered again and extracted with M-KCl without treatment with organic solvents. The precipitation at the isoelectric point and with (NH₄)₂SO₄ followed, being carried through twice; the heating described by Bailey (1948) for denaturation of remaining contaminating proteins was also carried out twice. Such preparations contained about 0.08% of tryptophan; the yield was about 1 mg./g. wet wt. The method of Simkin & Work (1957) was used for removal of adsorbed radioactive amino acids and nucleic acid. Since experiments considered below in this paper indicate that nucleic acid is the impurity giving rise to the inhomogeneity in electrophoresis patterns (Fig. 1*b*) of tropomyosin B, and since the nucleic acid is removed by the later stages of the present procedure, it was considered that the purification described here was adequate.

Preparation of the residual and soluble protein fractions for the labelling experiments. The residual fraction was prepared from the material centrifuged down at low speed after the extraction of actomyosin. The residue was extracted six to ten times by being ground in a mortar with 0.1N-NaOH at 4° to extract all proteins except collagen and elastin (Lowry, Gilligan & Katersky, 1941). One of the extractions was overnight and the others were each for 30 min. The soluble fraction was prepared from the supernatant after the first precipitation of actomyosin by dialysis. The whole of the protein in this supernatant was precipitated by trichloroacetic acid at a final concentration of 5% (w/v). These fractions also were then treated by the method of Simkin & Work (1957).

Preparation of tropomyosin A. Both methods described by Bailey (1956, 1957) for extraction of tropomyosin A from lamellibranch muscle were used, with some modifications, in the attempt to prepare this protein from uterus. In the 'wet' method, in which the washed minced tissue is extracted directly with M-KCl without previous drying with organic solvents, much actomyosin is extracted and is precipitated by dilution to 0.25M. In some of our experiments this actomyosin-containing fraction was reprecipitated and freeze-dried to denature actomyosin, then re-extracted in case tropomyosin A had been carried down with the actomyosin. In some experiments the protein content of the M-KCl extract was increased before attempts at crystallization by alternate dialysis against 0.5M-KCl and pervaporation in front of a fan at 4°. The method that Laki (1957*a*) used with clam adductors was also tried.

Preparation of tonactomyosin. This was made from cow uterus (4 months pregnant) by the method of Huys (1961) with slight modifications. Muscle (15 g.) was thoroughly chopped and well ground with quartz and 1.5 vol. of cold extractant, 1 0.05 (0.016M-NaCl-0.0126M-potassium phosphate, pH 7.3). After 10 min. the suspension was centrifuged for 30 min. at 12500 rev./min. on the MSE angle-head centrifuge. The supernatant was left to stand for 3 hr. at room temperature, and during this time gel formation began. After a further 15 hr. at 4°, gel formation was very marked; the gel was centrifuged down in the MSE centrifuge at 5000 rev./min. and was washed twice with water.

Special chemicals. The ATP used was the crystalline sodium salt supplied by Sigma Chemical Co. It was neutralized by means of KOH solution to pH 6.9. Glyoxaline was supplied by L. Light and Co. Ltd. Solutions of MgCl₂ and CaCl₂ were made from A.R. reagents and were standardized by titration with AgNO₃. Phosphate buffers

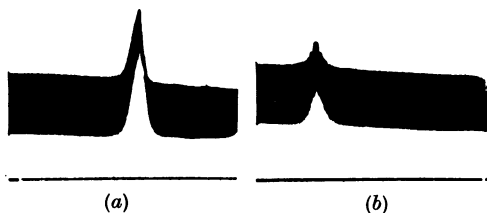


Fig. 1. (a) Electrophoresis pattern of uterus actomyosin used in the labelling experiments. Electrophoresis for 10.5 hr. at 1.79V/cm. Medium: 0.4M-KCl-16 mM-potassium phosphate, pH 6.5; concn. 4.5 mg./ml. Descending boundary. (b) Electrophoresis pattern of tropomyosin B from uterus, after removal of nucleic acid by the method of Sevag *et al.* (1938). Electrophoresis for 115 min. at 7.1V/cm. Medium: 0.05M-potassium phosphate, pH 6.5; concn. about 5 mg./ml. Ascending boundary.

were prepared from KH_2PO_4 (analytical reagent, Hopkin and Williams Ltd.), brought to the desired pH by means of KOH solution. All pH values for buffers or buffered solutions were measured by means of the glass electrode.

Ammonium sulphate was recrystallized from 50% (v/v) ethanol in the presence of EDTA (A.R. reagent) at pH 8.

The amino acid mixture used for injection consisted of uniformly ^{14}C -labelled L-amino acids prepared from an algal-protein hydrolysate and obtained from The Radiochemical Centre. The specific activity was $100\ \mu\text{c}/\text{mg}$.

The crystalline trypsin used was the salt-free product of the Novo Terapeutisk Laboratorium, Copenhagen. The trypsin inhibitor was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Injection of amino acids. The ^{14}C -labelled amino acids in 0.9% NaCl were injected intravenously into the ear vein of the rabbits. They were killed at different times after the injection, and the uteri were removed, cooled and trimmed as usual. After being minced and weighed, the tissue was divided into two approximately equal parts for the tropomyosin and actomyosin preparations.

Measurement of radioactivity. The protein, suspended in several millilitres of ether, was poured rapidly on to aluminium planchets (area $8.2\ \text{cm}^2$) which had been coated with lacquer. The protein dried into a very even film and was counted in a gas-flow counter. The time for 1000 counts was recorded automatically and each planchet was counted at least three times. Each protein fraction was plated on to two planchets (if there was enough material), which were weighed before and after addition of the protein. After the counting the protein was washed and scraped off the planchets into Kjeldahl flasks for estimation of total nitrogen.

Electrophoresis. This was done in the Tiselius apparatus (Perkin-Elmer Corp., Conn., U.S.A.) at 4° .

Viscosity measurements. For most experiments two Ostwald viscometers were used. For 4 ml. of buffer, the flow-times were 29.6 and 28.7 sec. at 19.5° . For the measurements on actin a Tsuda viscometer was used with flow-time of 17.5 sec. for buffer, with pressure head of 15.5 cm. water at 20° .

Measurement of adenosine-triphosphate activity. This was done as described by Needham & Williams (1959), inorganic P being estimated by the method of Weil-Malherbe

& Green (1951). Except where otherwise stated, the activating ion was Ca^{2+} .

Trypsin treatment of myosin. This was carried out as described by Needham & Williams (1959) for actomyosin.

Estimation of protein nitrogen. Protein was precipitated by addition of trichloroacetic acid to 5% (w/v), or, with very dilute protein solutions, by addition of 40% trichloroacetic acid to give a final concentration of 10%. Nitrogen was estimated by the micro-Kjeldahl method, digestion being effected with the mixture used by Umbreit & Bond (1936) and NH_3 being estimated according to Ma & Zuazaga (1942).

Estimation of tryptophan. This was by the method of Spies & Chambers (1949).

RESULTS

Actomyosin. As shown in Table 1, an increased number of precipitations at $I\ 0.27$ or 0.3 gave actomyosin preparations with adenosine-triphosphatase activity some 50% higher than the highest reported by Needham & Williams (1959). The most active preparations had viscosity numbers before and after addition of ATP closely similar to those of skeletal-muscle actomyosin and myosin. The last experiment in Table 1 shows that actomyosin of high activity could be prepared from non-pregnant as well as pregnant uterus. A few tests were made with Mg^{2+} ion activation at low ionic strength (I about 0.025). The activity was never greater than $0.02\text{--}0.045\ \mu\text{mole}$ of phosphorus liberated/mg. of nitrogen/min.

In certain experiments on ammonium sulphate fractionation of dilution precipitates, the adenosine-triphosphatase activity as well as the actomyosin content (by fall in viscosity with ATP) and the myosin content (by actin-combining power) were estimated (see Needham & Williams, 1963a). In such experiments the adenosine-triphosphatase activity for the actomyosin agreed well with the

Table 1. Adenosine-triphosphatase activity of purified actomyosin from uterus

Experimental details are given in the text. Δ_{ATP} , Fall in $\log\ \eta_{\text{rel}}$. on addition of ATP. $Z = 2.3\ \log\ \eta_{\text{rel.}}/c$ (mg./ml.).

Expt.	Conditions of precipitation	P liberated ($\mu\text{moles}/\text{mg. of}$ N/min.)		Δ_{ATP} for 1 mg. of protein/ml.	Z	
		In 0.1 M-KCl	In 0.5 M-KCl		Before ATP	After ATP
Pregnant rabbits						
40	Three at $I\ 0.3$	0.57	1.28	0.096	0.376	0.168
39	One at $I\ 0.27$, one at $I\ 0.3$	0.49	1.04	0.080	0.341	0.166
35	One at $0.03\ \text{M}$, four at $I\ 0.27$	—	—	0.11	0.399	0.166
42	One at $0.04\ \text{M}$, three at $I\ 0.27$	—	1.1	—	—	—
Non-pregnant pig						
	Two at $I\ 0.27$, one at $I\ 0.3$	0.56	1.33	—	—	—

values given here. On the other hand, the myosin content indicated by the actin-combining power seemed to be correlated with very little, if any, adenosine-triphosphatase activity, although myosin preparations with activity not greatly below that of actomyosin can be obtained (see below). This is perhaps not surprising, in view of the observations of Tsao (1953) and Rüegg (1961) on myosin from skeletal muscle and from the smooth adductor of *Pecten* respectively: in both cases the adenosine-triphosphatase activity was very low after ammonium sulphate precipitation.

The values for adenosine triphosphatase also explain the very high activity (4.6μ moles of inorganic phosphorus liberated/mg. of nitrogen/min.) of the H-meromyosin formed from uterus actomyosin by trypsin treatment followed by ultracentrifuging in the presence of ATP (Needham & Williams, 1959). If the adenosine-triphosphatase activity of purified actomyosin in 0.1 M-potassium chloride is about 0.53μ mole of phosphorus liberated/mg. of nitrogen/min., a sixfold increase of this activity on trypsin treatment would give an activity of 3.2. If H-meromyosin makes up about 73% of the myosin molecules, as is suggested for skeletal-muscle myosin (see Szent-Györgyi, 1960), then a value for the H-meromyosin of about 4.4 would be expected.

The viscosity numbers (Z) given in Table 1 are to be compared with the values given by Portzehl, Schramm & Weber (1950) for skeletal muscle (Z for actomyosin, 0.34–0.38 under the conditions of I and pH used above; Z for myosin, 0.17–0.2); and with values given by Huys (1960) for actomyosin from cow uterus, 0.37 and 0.18 before and after ATP.

Tonoactomyosin. Huys (1961) has examined the tonoactomyosin of uterus muscle from the cow. This form of actomyosin could be extracted from the muscle by means of solutions of low ionic strength (I about 0.05). For its solubility in such solutions after extraction and precipitation the presence of a small amount of ATP (0.15%) was necessary. The results of electrophoresis, sedimentation and viscosity experiments led to the conclusion that the tonoactomyosin consisted of an aggregated form of actomyosin. Its state was affected by addition of ATP but the effects were different from those seen with the usual actomyosin. At higher ionic strength it behaved electrophoretically and in the ultracentrifuge like typical actomyosin, before and after addition of ATP.

We prepared tonoactomyosin as described under Experimental. The gel was treated with the solution (I 0.05) to which 0.15% of ATP had been added. The ionic strength was raised to 0.075 by adding solid sodium chloride and rapid solution took place. A small residue was removed by centri-

fuging and 10 ml., containing 6.7 mg. of protein/ml., was obtained. The relative viscosity with 5.7 mg./ml. was 1.60, and this fell to 1.44 on addition of ATP to 9 mM. This relative viscosity, as well as the fall with ATP, is very low compared with that to be expected with a comparable amount of typical actomyosin. Solid sodium chloride and 0.08 ml. of 2 M-phosphate buffer were now added to the viscometer contents, bringing the ionic strength to 0.45. The relative viscosity rose to 1.77, and was unchanged on further addition of ATP.

The rest of the solution was also treated in the same way and left at 4° overnight. The relative viscosity was now 3.31 and this fell on addition of ATP (to 2.5 mM) to 1.66. It thus appears that the rise in ionic strength has led to formation of a much more viscous substance, much more sensitive to ATP. Although the measurements were made only after some hours, there is no reason to suppose that the change requires the lapse of time. If, as seems likely from Huys' measurements of electrophoretic mobility and sedimentation coefficients, this substance is normal actomyosin, the amount present would be 3 mg./ml. This is calculated on the basis of a fall on addition of ATP of 0.1 in $\log \eta_{rel.}$ for 1 mg. of actomyosin/ml. (Needham & Williams, 1963a). Since the solution contained 5.7 mg. of protein/ml. this would mean that it contained 53% of actomyosin. The yield of tonoactomyosin from 15 g. of muscle is thus about 36 mg.

A parallel experiment was made on muscle from the same uterus, treated with the usual extracting solution (buffered 0.5 M-potassium chloride containing ATP) and the first dilution precipitate was prepared as usual. This amounted to 8.7 mg./g. wet wt., of which 25% was actomyosin. The yield is thus about 33 mg. of actomyosin for 15 g. of muscle. These results are not highly quantitative but suggest that the greater part of the actomyosin of the uterus may be in the form of tonoactomyosin.

Myosin. The results obtained with four different myosin preparations from pregnant-rabbit uterus are shown in Table 2. The yield was very small—less than 1 mg./g. wet wt.

In only two cases was the actin-combining power high enough to indicate that nearly 90% of the protein in the upper supernatant was myosin. Great variability in adenosine-triphosphatase activity was found, but it seems that purified myosin can have activity (μ moles of phosphorus liberated/mg. of nitrogen/min.) at least up to about 0.7 in 0.5 M-potassium chloride, and about half this in 0.1 M-potassium chloride. If it is assumed that only the protein capable of combining with actin has adenosine-triphosphatase activity then the activity of this myosin might be as high as 0.9. That skeletal-muscle myosin preparations can be obtained with unchanged actin-combining power but little or no

Table 2. *Adenosine-triphosphatase activity of uterus myosin*

Experimental details are given in the text. Correction for adenosine-triphosphatase activity of actomyosin is based on the following values for μ moles of P liberated/mg. of N/min.: in 0.1 M-KCl, 0.6; in 0.5 M-KCl, 1.3; after trypsin treatment, in 0.1 M-KCl, 3.6; in 0.5 M-KCl, 2.6. It is taken that a fall in $\log \eta_{rel.}$ of 0.1 with ATP alone indicates the presence of 1 mg. of actomyosin/ml.

Expt.	Approx. percentage of myosin by actin-combining power	Δ_{ATP} before actin addition	P liberated (μ moles/mg. of N/min.)		Z
			In 0.1 M-KCl	In 0.5 M-KCl	
39	85	0	0.4	0.4	0.198
42	50	0	0.14	0.14	—
46	50	0.006	0.26	0.52	0.192
		Corrected for actomyosin	0.23	0.46	—
		After trypsin treatment	1.02	0.85	—
		Corrected for actomyosin	0.84	0.72	—
47	85	0.012	0.37	0.81	0.125
		Corrected for actomyosin	0.29	0.64	—

adenosine-triphosphatase activity has been observed by Baranyi & Baranyi (1959).

Actin. By the method of Tsao & Bailey (1953), from 10 g. of dried fibre (equivalent to about 100 g. of pregnant-pig uterus) 8 ml. of extract containing 10 mg. of protein/ml. was obtained. A portion of this solution, after addition of glycine to 0.1 M, was brought to pH 2.35 by very gradual addition of *n*-hydrochloric acid. After dialysis against 0.1 M-glycine at pH 2.35, electrophoretic mobilities were determined, in a final protein concentration of 6.6 mg./ml. Two peaks were observed, the slower, rather larger one having a mobility -5.61×10^{-5} cm.²/v/sec. ascending, -5.01×10^{-5} descending. This peak corresponds to actin and the faster probably to tropomyosin B (see Tsao & Bailey, 1953). From the electrophoresis pattern (Fig. 2) it would appear that about 40–50 mg. of actin was obtained/100 g. wet wt.

The myosin-combining power was studied with another portion of the extract. The myosin (from skeletal muscle) and the actin-containing protein were mixed to give a solution containing 1.5 of the former mg./ml., and 0.5 mg. of the latter/ml. On addition of ATP there was a fall in $\log \eta_{rel.}$ of 0.09, indicating the formation of 0.9 mg. of actomyosin, which would be expected to contain 0.2–0.3 mg. of actin. The fall in $\log \eta_{rel.}$ is about half that (0.174) obtained by Tsao & Bailey (1953) in a similar experiment with actin purified from skeletal muscle and almost homogeneous electrophoretically.

The question of the presence of free actin in extracts of smooth muscle (Oppel & Serebrenikova, 1959; Oppel & Khlyustina, 1960) has been studied further. In earlier work (Needham & Williams, 1963a) we could find no free actin in the fraction obtained from solutions of the dilution precipitate at 20% ammonium sulphate saturation. Ivanov, Mirovich, Zhakova & Tukachinsky (1962) could



Fig. 2. Electrophoresis pattern of actin preparation from uterus muscle. Electrophoresis for 100 min. and 140 min. at 5.4 v/cm. Medium: 0.1 M-KCl–0.1 M-glycine, 67 mM-HCl, pH 2.35; concn. 6.6 mg./ml. Ascending boundary.

find only traces of free actin in uterus and stomach extracts. It seemed possible that actin, although not existing in the uncombined state in salt extracts, might yet be in such a state of combination as to be set free on treatment with ammonium sulphate. Some experiments, closely following the procedure of Oppel & Serebrenikova (1959), were therefore made to determine whether free actin could be precipitated from the original

extract by 20% ammonium sulphate saturation. After solution of the precipitate formed and removal of the ammonium sulphate by dialysis, the fraction (F1) was tested with skeletal-muscle myosin, and the results are shown in Table 3. The myosin was free from actomyosin, but F1 did contain a little, as is shown by the fall in viscosity on addition of ATP. The viscosity of the mixture of myosin and F1 was slightly higher than was to be expected from the sum of the viscosities observed on the two proteins independently. That this increase was due to actomyosin formation is made very probable by its reversal on addition of ATP. If the actomyosin formed contained about 25% of actin, the amount of actin present (0.15 mg./ml.) would be less than 5% of the protein in F1. An attempt was made to purify the actin by repeating twice the precipitation with ammonium sulphate but again the actin seemed to account for only about 5% of the protein of F1.

Tropomyosin B. Crystalline preparations were made by the two methods mentioned under Experimental. Both contained much nucleic acid. With the product of the method of Tsao & Bailey (1953), involving drying of the tissue with organic solvents, the ratio extinction at 260 $m\mu$: extinction at 280 $m\mu$ was 1.18; with the method of Shêng & Tsao (1955) the nucleic acid content was higher and the ratio was 1.36. As in the experiments of Shêng, Tsao & Pêng (1956), the nucleotropomyosin on electrophoresis appeared inhomogeneous: one large peak beginning to divide into two and one small peak were seen. After treatment of the preparations by the method of Hamoir (1952) or of Sevag *et al.* (1938) for removal of nucleic acid, although removal was not complete the tropomyosin now gave in each case a single peak, the peak after treatment of the material by the second method being the more symmetrical (Fig. 1*b*). The mobilities of the latter were -6.65×10^{-5} cm.²/v/sec. ascending and -6.76×10^{-5} descending. These

values are similar to those of Kominz, Saad & Laki (1957) for *Venus*-adductor tropomyosin, and of Hamoir (1951) for carp-muscle tropomyosin: -6.7×10^{-5} and -7.1×10^{-5} cm.²/v/sec. at pH 7.1 and 7.4, *I* 0.15–0.2. The yield we obtained of such preparations was about 1 mg./g. wet wt. and the tryptophan content was about 0.07% (about 0.25 mole/mole of tropomyosin).

Some viscosity measurements were done on a crystalline preparation made by the method of Tsao & Bailey (1953). A solution in water containing about 3 mg./g. wet wt. gave a relative viscosity of about 50, and this fell to 1.35 in the presence of 0.6 M-potassium chloride.

Experiments on labelling of actomyosin and tropomyosin B. The resemblances in properties and amino acid composition between myosin and tropomyosin B led Bailey (1948) to suggest that the tropomyosin might be one of the units of which myosin is composed. Since that time this idea has received much attention but no firm conclusion has yet been reached. The evidence in favour of the view comes mainly from considerations of amino acid composition of myosin, actin and tropomyosin, and also of fragments of the myosin and meromyosin molecules prepared by different methods (see e.g. Laki, 1957*b*; Kominz, Saad, Gladner & Laki, 1957; Szent-Györgyi & Cohen, 1957; Kominz, Carroll, Smith & Mitchell, 1959). The experiments of Velick (1956) on labelling of skeletal-muscle proteins with radioactive amino acids did not settle this question. It seemed therefore worth while to try the injection of labelled amino acids into pregnant animals at a time when actomyosin synthesis was known to be occurring in the uterus, to determine the relative rates at which the actomyosin and tropomyosin were labelled. Actomyosin and not myosin was used owing to the very small yields of purified myosin obtainable from uterus.

Table 3. *Demonstration of traces of free actin in the precipitate formed at 20% ammonium sulphate saturation of stomach-muscle extracts*

Experimental details are given in the text.

	log $\eta_{rel.}$	log $\eta_{rel.}$ after ATP	Δ_{ATP}
1 Myosin, 5 mg./ml. F1 from pig stomach muscle, 3 mg./ml.	0.4113 } 0.5131	0.4017 } 0.4849	-0.0282
Mixture	0.5467	0.4847	-0.0620
Difference between sum of separate viscosities and viscosity of mixture	+0.0326	—	—
Difference between Δ_{ATP} values for sum and for mixture	—	—	-0.0338
2 Myosin, 4 mg./ml. F1 from dog stomach muscle, 3.9 mg./ml.	0.3743 } 0.5164	0.3715 } 0.4569	-0.0595
Mixture	0.5781	0.4382	-0.1399
Difference between sum of separate viscosities and viscosity of mixture	+0.0617	—	—
Difference between Δ_{ATP} values for sum and for mixture	—	—	-0.0804

Table 4 gives the results obtained in three experiments on rabbits. The time of pregnancy was chosen as the latest at which one could be certain that the weight of the uterus is still increasing and that the actomyosin content per g. wet wt. is still rising. Reynolds (1949) has found that the growth rate falls off after the twentieth day; the results recorded by Csapo (1953) show the most rapid increase in actomyosin content continuing until about the twenty-fifth day, but the results of Mirovich (1959) indicate that after the twentieth day the actomyosin content/g. wet wt. may be about constant.

The present results show that tropomyosin B and actomyosin incorporated labelled amino acids at the same rate in the pregnant-rabbit uterus. There is no indication that tropomyosin B is a precursor of myosin, since if it were, it would be expected to incorporate amino acids more rapidly and to reach a higher maximum activity. Even if tropomyosin had been more rapidly labelled than actomyosin, this would not have proved its precursor relationship, since actomyosin and not myosin was used. A delay in the labelling of actomyosin might be expected while the sub-units actin and myosin were being synthesized, but none was observed. Since actomyosin incorporates amino acids as rapidly as tropomyosin B it is unlikely that myosin would be found to incorporate them more slowly. The residual protein is more slowly labelled and the soluble protein fraction more rapidly labelled than these two proteins. Herrmann *et al.* (1958), studying the incorporation of [¹⁴C]glycine into embryonic and newly hatched chicks, found the maximum labelling of actomyosin within 1 hr., whereas the collagen fraction had not begun incorporation by

this time. They suggested that the lag was due to the presence of soluble procollagen acting as a precursor. In the present experiments the collagen fraction showed labelling within 20 min of injection. It has been shown (Needham & Williams, 1963*b*) that pregnant-rabbit uterus contains salt-soluble collagen; presumably here the transformation into insoluble collagen is proceeding more rapidly than in the embryo muscle

Tropomyosin A. Three experiments on pregnant- and three on non-pregnant pig-uteri were done, but no indication of the presence of tropomyosin A was found.

DISCUSSION

The evidence from our own work and that of other authors already cited shows that the actomyosin and myosin of vertebrate smooth muscle are very similar in their physicochemical properties to these proteins obtained from skeletal muscle. Cohen, Lowey & Kucera (1961) have also recently found that L-meromyosin can be obtained by controlled trypsin treatment of uterus myosin and has the same sedimentation coefficient as the protein from skeletal muscle. Needham & Williams (1959) had earlier shown that acto-H-meromyosin from uterus muscle behaves in the ultracentrifuge in the same way as the skeletal-muscle protein.

There are, however, two important respects in which the proteins from the two sources can differ. One is the much lower adenosine-triphosphatase activity of uterus myosin and actomyosin (although uterus H-meromyosin approaches skeletal H-meromyosin in activity; Needham & Williams, 1959). The other is the ability of uterus actomyosin to exist in an aggregated form, soluble at low ionic strength in the presence of ATP.

Tonoactomyosin has so far only been prepared from the smooth muscle of artery and uterus; that it is more widely distributed in vertebrate smooth muscle has been suggested by Laszt & Hamoir (1961): e.g. in the primitive striated fibres of amphibian or of insect larvae. It might indeed be mentioned that in unpublished experiments we have observed very marked gel formation in aqueous extracts of frog muscle. Hamoir & Laszt (1962) prepared tonomyosin by ammonium sulphate precipitation in the presence of ATP from carotid tonomyosin dissolved at *I* 0.35. Its properties differed in several respects from those of rabbit myosin. Gaspard-Gotfroid (1962) found the adenosine triphosphatase activity of this tonomyosin to be only 6–10% of that recorded for rabbit myosin under similar conditions of assay. It seems, however, possible that some decrease in activity had been caused by the ammonium sulphate treatment. The adenosine-triphosphatase activity of the uterus myosin prepared in the present work was, in

Table 4. Incorporation of ¹⁴C-labelled amino acids into uterus proteins of the pregnant rabbit

Experimental details are given in the text. Each figure represents the results from counting one planchet.

	Counts/min./mg.		
	Expt. 3	Expt. 4	Expt. 5
Period of pregnancy (days)	21	18	18
Amount of ¹⁴ C-labelled amino acids injected (μC)	25	25	35
Time after injection when animals killed (min.)	60	30	20
Tropomyosin B	20.3	11.2	11.1
Actomyosin	19.5	9.1	11.1
		10.3	10.8
Soluble proteins	—	13.4	19.1
		12.0	15.0
		12.2	
Residue	—	3.32	7.6

0.5M-potassium chloride, about 12% of that of rabbit skeletal-muscle myosin.

The actin prepared from uterus muscle behaved in a quantitatively normal way with skeletal-muscle myosin and ATP. Huys (1960, 1961), however, brings forward evidence of differences in behaviour in the ultracentrifuge.

The question of the tropomyosin B content in relation to the actomyosin content in the two different types of muscle is of some interest. In skeletal muscle, Perry & Corsi (1958) determined the tropomyosin B content of washed myofibrils by viscosity measurements on extracts made by long leaching of the myofibrils. They found tropomyosin to make up 10–12% of the total nitrogen of the myofibril, a value corresponding to more than twice that given by Bailey (1948) for tropomyosin extracted and purified from whole muscle. For uterus, the amount of purified tropomyosin B obtained in the present work, and also by Shêng & Tsao (1955), was less than from skeletal muscle: only about 1.5 mg./g. wet wt., to be compared with Bailey's figure of 4.5 mg./g. wet wt.; as with skeletal muscle, this amount certainly represents only part of the tropomyosin present. Since the skeletal myofibril contains about 65% of actomyosin (Perry & Corsi, 1958) the ratio of tropomyosin B to actomyosin would be about 12:65. Uterus, even when pregnant, contains only about 6 mg. of actomyosin/g. wet wt. (Needham & Williams, 1963a); the ratio here would then be at least 1.5:6, probably considerably higher.

Jaisle (1960) has given much higher values for the tropomyosin B content of human uterus: about 8 mg./g. wet wt. in the corpus of pregnant uteri, about 10 mg. for non-pregnant. In Jaisle's method, the homogenized tissue was thoroughly washed with 0.1M-potassium chloride and then the residue was extracted several times with acetone and ether. The air-dried powder was first extracted with water to remove actin and other substances, then with buffered M-potassium chloride. The protein nitrogen of this solution was taken as the measure of tropomyosin B content without further purification. This solution was made salt-free by dialysis and the change in viscosity on addition of salt was followed. However, the fall in Z recorded (about 0.04) for the change from 0.1 to 0.3M-potassium chloride is only one-third of that (about 0.12) which can be calculated from the data of Tsao, Bailey & Adair (1951). It seems that, though tropomyosin B is certainly present, it is unlikely to account for a great part of the protein prepared by this method.

The mechanism of contraction of vertebrate smooth muscle is still obscure. The contraction cycle is characterized by its slowness, especially in the relaxation phase, and by consequent low energy

expenditure in maintenance. Bozler (1948) has emphasized that basically the contractile mechanism need not differ from that responsible for phasic contractions. However, reasons for the slowness of relaxation should be considered. Bozler (1953) brings forward evidence that resistance of a viscous type has to be met, and that this may be intramolecular in nature caused by weak bonds formed after shortening and slowly broken during extension. Johnson (1962), also discussing these questions, concludes that the slow contractions of vertebrate smooth muscle, often called tonic contractions, are basically tetanic, but that additional mechanisms may be involved. In the various suggestions that tropomyosin B (Shêng & Tsao, 1955) or the proteins of the T fraction (Ivanov *et al.* 1962), one component of which is tropomyosin B, are particularly concerned with tonus of vertebrate smooth muscle the authors probably had in mind such additional mechanisms.

Gansler (1960, 1961), in electron-microscope studies, has considered synaeresis, dependent on changes in state of actomyosin, as the main factor in contraction of vertebrate smooth muscle; Laszt & Hamoir (1961), dealing particularly with muscle of arterial walls, suggest that this tonus is mediated by increase in rigidity of a tonactomyosin network.

The filaments seen in the electron microscope are all alike and of diameter (50–80 Å) similar to that of actin filaments in skeletal muscle (Gansler, 1956; Shoenberg, 1958). That they consist of actin is made likely by the X-ray-diffraction work of G. F. Elliott (personal communication) on *Taenia coli* of the guinea pig, and by the preparation of actin filaments from the same material by Hanson & Lowy (1963). Elliott also finds lack of clear indications of an α -pattern, and non-appearance at low angles of myosin reflexions; these observations indicate that the myosin present is mainly in random arrangement. Choi (1962), on the other hand, has seen in the electron microscope with gizzard muscle (which is not, however, a typical smooth muscle) filaments of greater diameter, which he takes to be myosin.

The results of Shoenberg (1962) on *Taenia coli* of the guinea pig indicate that during contraction the fine filaments do not shorten or crumple but move past each other. This suggests that a sliding mechanism in some form may be involved in vertebrate smooth muscle.

SUMMARY

1. Further purification of uterus actomyosin gave preparations with adenosine-triphosphatase activity as high as 1.3μ moles of inorganic phosphorus liberated/mg. of nitrogen/min.

2. The presence of actomyosin soluble at low ionic strength in the presence of ATP (tonoactomyosin) in cow uterus was confirmed.

3. Myosin was prepared from purified uterus actomyosin, and found to have adenosine-triphosphatase activity at least 0.7 μ mole of inorganic phosphorus liberated/mg. of nitrogen/min. Its actin-combining power and viscosity were studied.

4. Actin was isolated from uterus muscle in the form of a preparation containing about 60% of actin and 40% of tropomyosin B. Evidence was obtained for the presence of small amounts of free (or loosely combined) actin in uterus extracts.

5. Labelling experiments with injection of 14 C-labelled amino acids into rabbits at the eighteenth to twentieth day of pregnancy gave no indication that uterus tropomyosin B was labelled before uterus actomyosin.

6. No evidence for the presence of tropomyosin A in the uterus could be found.

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The Action of Enzymes on Human α -Lipoprotein

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Two types of structure have been proposed for human α -lipoprotein. Gurd (1960) and Cook & Martin (1962) suggested that the protein maintained its integrity as a protein, the lipid being bound to its surface. On the other hand, Vandenheuvel (1962) concluded that the lipid could be sandwiched between sheets of peptide.

Investigations on whole plasma and partially purified preparations have indicated that both peptidases and phospholipases could attack α -lipoprotein (Canal & Girard, 1962; Condrea, de Vries & Mager, 1962). Using purified horse α -lipoprotein, Krumwiede (1958) studied the effects of these enzymes on the extraction of lipids from the complex. To obtain information on the lipoprotein structure, we have made a more detailed study of the effects of enzymic digestion. A preliminary account of this work has been published (Ashworth & Green, 1963*a*).

EXPERIMENTAL

Materials

Crystalline trypsin was obtained from British Drug Houses Ltd., Poole, Dorset, and crystalline α -chymotrypsin from L. Light and Co. Ltd., Colnbrook, Bucks. *Naja naja* venom was obtained from the London Zoo through the courtesy of Dr H. G. Vevers and was used without purification as a source of phospholipase A (EC 3.1.1.4). Phospholipase D (EC 3.1.4.4) was either from British Drug Houses Ltd. or prepared from Savoy cabbage ('stage 3 precipitate') as described by Davidson & Long (1958). The commercial preparation was used as a 0.1% solution. The 'stage 3 precipitate' was dissolved in water to give a solution containing 160 milliunits/ml. The enzyme units used are as recommended by the Commission on Enzymes of the International Union of Biochemistry (1961).

Human α -lipoprotein was prepared as described by Ashworth & Green (1963*b*), and purified by flotation in the

ultracentrifuge at a density of 1.21 (Green, Oncley & Karnovsky, 1960). For experiments with trypsin and chymotrypsin, it was dissolved in 0.16 M-phosphate buffer ($\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$), pH 7.65. For all other experiments, it was left in the NaCl-KBr solution used in its preparation.

Alumina (grade O) was from Peter Spence and Co., Widnes, Lancs., and silicic acid (analytical reagent; 100 mesh) from Mallinckrodt Chemical Works. Ether was dried over sodium and distilled before use.

Methods

Paper electrophoresis. This was performed as described by Green (1962).

Extraction and titration of fatty acids. These were carried out by the procedure of Davis (1947).

Determination of choline. This was carried out by the method of Shapiro (1953).

Extraction of total lipids. This was performed as described by Ashworth & Green (1963*b*).

Other analyses. All other analyses were performed as described by Ashworth & Green (1963*b*).

Ether extraction. The aqueous solution was added to twice its own volume of diethyl ether in a 15 ml. glass-stoppered tube and left at room temperature with intermittent shaking for 20 min., centrifuged to separate the phases and the ether phase removed with a capillary pipette. This procedure was carried out three times, the extracts being combined for analysis.

Separation of esterified and unesterified sterols. Chromatography on columns of silicic acid or alumina was used. Silicic acid columns consisted of a mixture of 3 g. of silicic acid and 2 g. of Celite, and alumina columns of 5 g. of alumina (partially deactivated to Brockmann grade 3). In each case, columns 1 cm. in diameter were prepared from suspensions of the adsorbent in light petroleum (b.p. 40–60°). The lipid was applied to the column in ether-light petroleum (1:24, v/v) and sterol esters were eluted with 100 ml. of this mixture. Unesterified sterols were eluted with 100 ml. of diethyl ether.

Enzyme studies. Trypsin and chymotrypsin reaction mixtures contained 12.5–20 mg. of α -lipoprotein and