The Proteins of the Dilution Precipitate Obtained from Salt Extracts of Pregnant and Non-Pregnant Uterus

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When finely divided uterus muscle is extracted with buffered 0.5M-potassium chloride (to which about 2.0 mM-ATP has been added) and when after some hours the extract is diluted with water to 0.04M, a protein precipitate (designated below 'the first dilution precipitate') is obtained, as with skeletal muscle. This was first observed for the uterus by Csapo (1948). For skeletal muscle this precipitate consists mainly of actomyosin and myosin, the proportions depending on the conditions of extraction. With uterus at the end of pregnancy also much actomyosin is found. Csapo (1948, 1949, 1950), using a viscometric method, calculated the actomyosin content from the fall in specific viscosity of uterus extracts on addition of ATP and the myosin content from the final specific viscosity. The viscosity change with ATP became progressively greater as pregnancy proceeded, and this has been confirmed by other workers [Schwalm, Cretius & Lange (1955) for human; Mirovich (1959) for rabbit; D. M. Needham (unpublished work) for rat]. Since the fall in viscosity with ATP addition has not been observed with any other system it may be taken as valid evidence for the presence of an actomyosin-like protein in uterus, increasing in amount during pregnancy. Early work on sedimentation and electrophoretic behaviour (Csapo, Erdös, Naeslund & Snellman, 1950; Naeslund, Snellman, Csapo & Erdös, 1949-1950; Naeslund & Snellman, 1951) also indicated the presence of actomyosin in pregnant uterus.

The use of the viscometric method to obtain quantitative results depended on two assumptions: that solutions of uterus actomyosin and myosin have similar specific viscosities to the skeletal-muscle proteins; and that no other proteins are present contributing significantly to the viscosity.

Recent work (Huys, 1960; Needham & Williams, 1963*b*) has shown the first assumption to be justified. Other properties have also been shown to be similar in proteins from the two sources. Thus Kominz & Saad (1956) found with human uterus at term, before and after treatment with ATP, sedimentation constants close to those of skeletal actomyosin and myosin respectively. Needham & Williams (1959) prepared electrophoretically homogeneous actomyosin from pregnant rabbit uterus

and found it to have similar mobility to the protein from skeletal muscle. Huys (1960) has confirmed this with cow uterus.

The second assumption, however, is, as we shall see, not well grounded, mainly because small and variable quantities of highly viscous salt-soluble collagen are extracted, particularly from non-pregnant uterus (Needham & Williams, 1963*a*), and also because a considerable quantity of protein of low viscosity is present. In the experiments described here, myosin was assessed by finding the fall in log relative viscosity with ATP after carefully controlled addition of actin, and subtracting from this the fall after ATP alone. It was ascertained (Needham & Williams, 1963*b*) that uterus myosin behaves normally with skeletal-muscle actin. Similar observations on the interaction of uterus myosin and skeletal-muscle actin have been made by Nagy (see Csapo, 1959).

It was then found in experiments to be described below on pig, rabbit and cow uterus that, even at term, the first dilution precipitates contain considerable amounts of unidentified protein; precipitates from non-pregnant uterus contain only some 25-30% of actomyosin plus myosin.

It has been observed by the method of Derrien (1947) with a variety of smooth muscles—from uterus (Snellman & Tenow, 1954), stomach (Oppel & Serebrenikova, 1959) and oesophagus (Tonomura & Sasaki, 1957)—that addition of ammonium sulphate to an extract (made with buffered 0.5M-potassium chloride), or to the solution of a dilution precipitate, leads at 16-20% saturation to precipitation of a considerable part of the protein. No precipitation in this zone takes place with skeletal-muscle preparations (Tonomura & Sasaki, 1957). It has been suggested by the various workers concerned that actin is responsible for this precipitation in smooth muscle since ammonium sulphate concentrations in the ranges 9-20%, 28-32% and 33-45% saturation are needed to precipitate actin, actomyosin and myosin respectively from solutions of the purified or partly purified proteins (Snellman & Tenow, 1954). No other evidence for this conclusion was brought forward, except in Oppel & Serebrenikova's experiments. These workers obtained from extract of dog-stomach muscle at 16-

20% saturation a protein that would react with myosin to give markedly increased viscosity. No effect of ATP on this increased viscosity is mentioned. Oppel & Khlyustina (1960) further showed that the protein, after purification by repeated salting-out, resembled actin in its iso-ionic point and number of ionizable groups. They used values calculated from their electrometric titration curve to compare with values calculated from the amino acid analyses of Kominz, Hough, Symonds & Laki (1954). Ivanov & Mirovich (1959) were unable to demonstrate the presence of free actin when they added myosin solution to extracts of pregnant or non-pregnant uterine muscle; no increase in viscosity occurred. The presence of an inhibitor in uterine-muscle extract preventing the interaction of actin and myosin was ruled out by showing that skeletal actin and myosin would react in its presence. In a later paper Ivanov, Mirovich, Zhakhova & Tukachinsky (1962) could find evidence for no more than possible traces of free actin in uterus and stomach extracts.

The present work is concerned with the composition, studied by viscometric and other methods, of the first dilution precipitate itself and of various fractions obtained from it. The effect of various methods of extraction of the tissue upon the amount and composition of the dilution precipitate has also been studied, as well as the effect of repeated solution and reprecipitation upon the composition of the final precipitate. We were unable to obtain any evidence for the presence of actin in the precipitate at 20% saturation with ammonium sulphate.

Preliminary accounts of this work have been published (Needham, 1961, 1962).

EXPERIMENTAL

Materials

Preparation of the dilution precipitate. The tissue was cooled immediately upon removal from the animal. The extract was prepared in the cold room at about 4°, and the preparation during all stages was kept at this temperature.

The pieces of uterus were thoroughly scraped with a sharp scalpel to remove as much as possible of the endometrium; when possible, collagenous layers were stripped off from the outer surface, but this could not always be done. When stomach was used, the mucosa was removed by scraping. The tissue was then minced and ground in small quantities with Merck's washed quartz and 3 vol. of 0.5M-KCl containing 0.04M-potassium phosphate; 1.5 mM-ATP was also present, and the final pH of the solution was 7.1. After standing for about 5 hr. with frequent stirring, the suspension was centrifuged at 2000 g on the refrigerated MSE centrifuge with horizontal yoke. The supernatant was removed and the residue washed once with 1 vol. of the buffered KCl solution. Next day (when all ATP would have disappeared through adenosine-triphosphatase activity) the extract was centrifuged at 25 000 g for 60 min. in the

Spinco ultracentrifuge, model L, to sediment cell particles; then to the supernatant 14 vol. of water was added and the pH was adjusted to 6.5-6.7. After settling had taken place the supernatant was decanted and the precipitate was centrifuged down at 700 g. Its volume was judged by eye and solution was effected by adding solid KCl and 2M-potassium phosphate, pH 7.1, to bring to 0.5M-KCl and 0.04M-phosphate. Any adjustments necessary were made after the measurement of the actual volume. The solution was stored at 4°, containing about 10 mg. of protein/ml. Variations from this procedure are described where necessary.

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 actin. This was by the method of Straub (1943). The dried fibre was stored at 4°. The extract was made with water at 16° or in later experiments at 4° (see Drabikowski & Gergely, 1962). The precipitate obtained at pH 4.7 in the filtered extract was centrifuged down and dissolved by addition of the minimum amount of 5% (w/v) NaHCO₃. About 0.5 vol. of 0.5M-KCl-0.04M-potassium phosphate, pH 7.1, was added. The solution, which contained about 7 mg. of protein/ml., was stored at 4° and used within 3 days.

Preparation of myosin. This was prepared from skeletal muscle by the method of Szent-Györgyi (1951a). When storage was necessary the solution of the thrice-precipitated protein in 0.4M-KCl-0.07M-potassium phosphate, pH 6.8 (containing about 20 mg./ml.), was treated at about 4° with glycerol to 30% (v/v) and kept at -10°. It was used after removal of glycerol by dialysis against the buffered KCl solution.

Special chemicals. ATP was used as the sodium salt supplied by Sigma Chemical Co. It was neutralized with KOH to pH 6.9. Glyoxaline was supplied by L. Light and Co. Ltd. Ascorbic acid and cysteine hydrochloride were from Roche Products Ltd. Solutions of MgCl₂ and CaCl₂ were made from A.R. reagents and were standardized by titration with AgNO₃. Phosphate buffers were prepared from KH₂PO₄ (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 with the glass electrode.

Ribonuclease was from bovine pancreas, five-times recrystallized, salt- and protease-free. It was supplied by Sigma Chemical Co.

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

Lithium chloride (Hopkin and Williams Ltd. dry powder) was recrystallized from hot water in the presence of EDTA. A 5M solution was prepared by use of a specific-gravity bottle and data from the Landolt-Bornstein *Physikalisch-chemische Tabellen*.

Methods

Ammonium sulphate fractionation. This was carried out at about 4° with protein solutions containing 8-10 mg./ml. in 0.5M-KCl buffered with 0.04M-potassium phosphate, pH 7.0. Where LiCl was used, an equal volume of the 5M solution was added drop by drop to the protein solution before the solution of (NH₄)₂SO₄. The solution of (NH₄)₂SO₄ was adjusted if necessary to pH 7 just before use by means of aq. NH₃ soln. (sp.gr. 0.88) and added gradually, with stirring, to the nominal saturation percentages given, with-

out regard to any effect upon the saturation value of the LiCl also present. After addition of $(\text{NH}_4)_2\text{SO}_4$ the mixture stood for 1 hr., being allowed to warm up to about 12° before centrifuging for 30 min. at this temperature at 8000 rev./min. in the refrigerated MSE centrifuge with conical head. Each fraction was dissolved in the buffered 0.5M-KCl, pH 7.0, and dialysed against this solution until NH_3 -free. Where immediate solution proved difficult, the suspension was dialysed. Fractions were precipitated as follows: F1 at about 20% saturation, F2 at 26–32% or 26–45% saturation, and F3 at 32–45% saturation. Details for each experiment are given in Tables 4 and 5.

Salting-out curves. These were obtained by the method of Derrien (1947) as described by Azzone & Aloisi (1958), except that readings on the Beckman spectrophotometer were made at room temperature.

Use of diethylaminoethylcellulose for DNA removal. The DEAE-cellulose (Whatman powder, DE 50 manufactured by W. and R. Balston) was treated as described by Perry & Zydowo (1959) for regeneration and then washed four times with 100 vol. of 0.35M-KCl–0.02M-potassium phosphate, pH 7.2, and suspended in the same medium (1 g./10 ml.). This salt concentration was chosen as the highest at which nucleic acid was likely to be retained. The protein solutions in the same medium were treated with the DEAE-cellulose by adding the suspension of the latter in the proportion of 1 g. of the solid to 2–5 mg. of protein N. After 1 hr. at 4° with gentle agitation the DEAE-cellulose was centrifuged off and the protein was reprecipitated.

Sedimentation. The Spinco model E analytical ultracentrifuge was used with cooling.

Electrophoresis. This was done in the Tiselius apparatus (Perkin-Elmer Corp., Conn., U.S.A.; model 38) at 4°.

Starch-gel electrophoresis. This was carried out at pH 8.7 in tris-citrate buffer, with borate buffer in the electrode vessels (Poulik, 1957).

Measurement of viscosity. Two Ostwald viscometers were used. For 4 ml. of buffer the flow-times were 28.4 and 29.3 sec. at 20°. Since the concentration of myosin or actomyosin is proportional to the logarithm of the relative viscosity (Portzehl, Schramm & Weber, 1950) all results are expressed as $\log \eta_{\text{rel.}}$ or by means of the viscosity number, Z ($2.3 \times \log \eta_{\text{rel.}}/c$, where c is concentration of protein in mg./ml.; Weber & Portzehl, 1952).

Measurement of adenosine-triphosphatase activity. This was done as described by Needham & Williams (1959), inorganic P being estimated by the method of Weil-Malherbe & Green (1951). When cysteine was added (the hydrochloride being neutralized just before use) it was at 15 mM concentration.

Methods of estimation. Total N was estimated by the micro-Kjeldahl method. Digestion was effected with the mixture used by Umbreit & Bond (1936) and NH_3 was estimated according to Ma & Zuazaga (1942). Protein N was precipitated by addition of trichloroacetic acid to 5% (w/v); with very dilute protein solutions (such as samples of viscometer contents, with only about 1 mg. of protein/ml.) addition of 40% (w/v) trichloroacetic acid to give a final concentration of 10% was used.

Nucleic acid was estimated by a modification of the method of Ogur & Rosen (1950). To 2 ml. of protein solution (containing about 3 mg./ml.) at 0°, perchloric acid was added to give a final concentration 0.2M. After 30 min. at 4° the precipitate was centrifuged down and washed by re-

suspension and sedimentation with cold 0.2N- HClO_4 . The residue was then treated successively with cold 70% (v/v) ethanol, ethanol and ether (twice), being resuspended and centrifuged each time. The insoluble material was treated with 2 ml. of 0.5N- HClO_4 for 20 min. at 70°; this hot extraction was repeated after centrifuging down the precipitate. The clear supernatants were combined and used for the determination of total nucleic acid P by the method of Allen (1940) and of DNA by the method of Burton (1955), calibrated on a sample of DNA of known P content (kindly supplied by Dr A. A. Newton).

RESULTS

Myosin and actomyosin content of the first dilution precipitate

Solutions of dilution precipitates in 0.5M-potassium chloride–0.04M-potassium phosphate, pH 7.1, were used; they were diluted to contain 1–2 mg. of protein/ml. and centrifuged at about 800 g after dilution. For assessment of actomyosin, measurements of relative viscosity were made before and after addition of ATP and Mg^{2+} ions, each to 2.5 mM. For assessment of actin-combining power, the actin solution made as described was used, successive additions of 0.1 or 0.05 ml. being made as needed. Magnesium ions (to 2.5 mM) were added before the actin. When the rise in viscosity corresponded to that expected from the actin addition alone, or was clearly less than that to be expected from complete interaction of myosin with the addition, ATP was added.

The data of Szent-Györgyi (1951*b*) showed that, when a solution of myosin containing 2.2 mg./ml. was treated with a sufficiency of actin, a fall in $\log \eta_{\text{rel.}}$ of 0.28 took place on addition of ATP. The fall obtained with ATP was proportional to the actin added up to the point of myosin saturation. With skeletal-muscle myosin we found, at a myosin concentration of 1–2.5 mg./ml., a fall of about 0.14 in $\log \eta_{\text{rel.}}$ calculated for 1 mg./ml. This agrees satisfactorily with the results of Szent-Györgyi. At very low myosin concentrations, however, there was considerable variation in the fall calculated for 1 mg./ml. and a definite tendency for the fall to become much larger (some 50% larger when the measurements were done at 0.2 mg./ml.). These effects may be due to anomalous behaviour of actin present in excess (see also Szent-Györgyi, 1951*b*). In the experiments described here the increased fall in $\log \eta_{\text{rel.}}$ with ATP after addition of actin was usually only about 0.01/mg. of protein in the dilution precipitate or fraction derived from it (Tables 1–5). The value of 0.14 for the fall with 1 mg. of myosin/ml. would imply only about 0.07 mg. of myosin/mg. of protein used; the actual amount is probably less. It should be mentioned that a case was noticed in which a preparation of

actin from fibre stored at 4° for some months gave a much greater fall, and on further storage this change was progressive. The actin preparations should therefore be frequently checked.

Experiments made with purified myosin from uterus (Needham & Williams, 1963*b*) have so far given with ATP a fall in viscosity after actin treatment only some 80 % of that given in the same conditions by skeletal myosin. This is probably due to the presence of impurities, or some denaturation of the myosin.

Since one part of myosin combines, in skeletal-muscle actomyosin, with one-quarter to one-fifth of its weight of actin (Szent-Györgyi, 1951*b*) dissociation of about 1.25 mg. of actomyosin would give the fall in $\log \eta_{rel.}$ of 0.14. It is assumed in this work that the same actin:myosin ratio holds for uterus actomyosin. A fall of 0.1–0.11 for 1 mg./ml. was indeed found on addition of ATP to solutions of purified actomyosin made from uterus (Needham & Williams, 1963*b*).

The following conclusions can be drawn from Table 1. For the non-pregnant pig uterus the first dilution precipitate contained about 22 % of myosin as actomyosin, and not more than 27 % of free plus combined myosin. For pregnant rabbit the value for myosin as actomyosin was about 44 %, the value including the free myosin being scarcely any higher. A very unusual case was encountered in rabbit 14, where the precipitate had the properties of relatively pure actomyosin plus a little myosin. The cow uterus in early pregnancy resembled the non-pregnant pig uterus.

The rat extracts showed rather anomalous behaviour, since the protein in the first dilution precipitate was always much greater in amount per g. wet wt. than with the other animals tested. The

actomyosin content of the precipitates was, however, particularly low, so that the amount of actomyosin per g. wet wt. would not differ much from that found with the other animals.

Table 2 shows the results of variation in pH of the extracting fluid, and also of preliminary freezing of the tissue. In some cases the minced tissue at 4°, spread out in a covered mortar, was placed at –15°. After 4 hr. it was ground (without previous thawing) with quartz and the extractant fluid at 4°. In other cases the cooled tissue was frozen in liquid nitrogen and pounded to a powder before adding the extractant. No obvious effects were found. For large quantities of tissue the freezing method is inconvenient; higher pH values did not give significantly greater amounts of extracted protein, so values of 7.0–7.2 were used as a routine.

Some experiments illustrating the effect of a second and third precipitation at 0.04*M*-potassium chloride are shown in Table 3. There was little effect on the composition of the precipitate, though a tendency for slightly increased actomyosin and myosin content in the second and third precipitates as compared with the first is seen; 80–85 % of the actomyosin was recovered at each precipitation.

As might be expected from the high nucleic acid content of the tissue, the dilution precipitate from uterus contained nucleic acid, the DNA content being particularly high in the precipitates from non-pregnant animals. Results for the RNA phosphorus and DNA phosphorus for a non-pregnant rabbit were 29.2 and 13.8 $\mu\text{g.}/\text{mg.}$ of protein nitrogen; for a pregnant rabbit (23 days) 25.14 and 4.86. Similar values have been reported by Mirovich (1959). The nucleic acids present might affect the composition of the dilution precipitate by carrying down extraneous protein, and might, if

Table 1. *Values of $\log \eta_{rel.}$ of solutions of dilution precipitate, with and without actin addition, and the effect of addition of adenosine triphosphate*

All values for $\log \eta_{rel.}$ are calculated for 1 mg. of protein/ml. For conditions see text.

	Protein N in dilution precipitate (mg./g. wet wt.)	$\log \eta_{rel.}$		Δ_{ATP}	$\log \eta_{rel.}$ With actin		$\Delta_{ATP, actin}$
		Before ATP	After ATP		Before ATP	After ATP	
Non-pregnant pig							
11	1.66	0.076	0.043	0.033	0.107	0.068	0.039
12	1.0	0.060	0.035	0.025	0.090	0.056	0.034
13	0.5	0.072	0.046	0.026	0.10	0.064	0.036
17	0.78	0.091	0.056	0.035	0.124	0.083	0.041
18	1.43	0.051	0.033	0.018	0.070	0.050	0.020
Non-pregnant rabbit							
30	1.75	0.070	0.046	0.024	0.109	0.082	0.027
24	1.70	0.085	0.057	0.028	0.116	0.085	0.031
Pregnant rabbit							
29 days: 43	1.97	0.082	0.035	0.047	0.086	0.041	0.045
25 days: 14	0.9	0.21	0.08	0.13	0.230	0.088	0.142
Pregnant rat							
Near term: 68	2.6	0.056	0.039	0.017	0.087	0.065	0.022
Near term: 27	3.74	0.043	0.029	0.014	—	—	—

Table 2. *Effects of various methods of extraction on the amount and composition of the first dilution precipitate*For further details see text. All values for $\log \eta_{rel.}$ are calculated for 1 mg./ml.

Experimental conditions	Protein N in dilution ppt. (mg./g. wet wt.)	Fall in log $\eta_{rel.}$	
		With ATP	With ATP after actin
Rabbit 43: pregnant, 29 days			
Extracted with 0.5M-KCl-0.04M-potassium phosphate- 14 mM-ATP; pH 7.0	1.97	0.046	0.046
Extracted with 0.5M-KCl-0.02M-NaHCO ₃ -14 mM-ATP; pH 8.0	1.36	0.053	0.053
Fig 18: non-pregnant			
Each sample extracted with 0.5M-KCl-0.04M-potassium phosphate-14 mM-ATP at 4°; pH 7	0.86	—	—
after 4 hr. at -15°; pH 7	1.43	0.018	0.020
after 4 hr. at -15°; pH 7.4	1.43	0.020	0.020
Cow: pregnant, 4 months (frozen in liquid N ₂)			
Extracted for 18 hr. with 0.5M-KCl-0.04M-potassium phosphate-14 mM-ATP; pH 7.0	1.66	0.022	0.052
Extracted for 18 hr. with Weber solution*	1.44	0.023	0.032

* Weber solution: 0.6M-KCl-0.04M-NaHCO₃-0.01M-Na₂CO₃ (Weber & Meyer, 1933).Table 3. *Effect of repeated precipitation in 0.04M-potassium chloride solution on the behaviour of the dilution precipitate*Extraction in each case was according to the procedure described under Methods. All values for $\log \eta_{rel.}$ are calculated for 1 mg./ml.

Experimental material	Fall in $\log \eta_{rel.}$	
	With ATP	With ATP after actin
Fig 12: non-pregnant		
First dilution precipitate	0.025	0.034
Third dilution precipitate	0.021	0.030
Fig 20: non-pregnant		
First dilution precipitate	0.020	0.032
Second dilution precipitate	0.027	0.040
Dog stomach		
First dilution precipitate	0.048	0.054
Second dilution precipitate	0.052	0.065
Third dilution precipitate	0.056	0.065
Rabbit 43: pregnant, 29 days		
First dilution precipitate	0.046	0.045
Second dilution precipitate	0.060	0.064
Third dilution precipitate	0.061	0.064

combined with actomyosin or myosin in the precipitate, affect their behaviour with ATP or actin. It was therefore desirable to try the effect of removing the nucleic acids. This was readily done for RNA by treatment at 4° with 12 μ g. of ribonuclease/ml. in a solution containing about 4 mg. of the dilution precipitate/ml. of 0.5M-potassium chloride-0.04M-potassium phosphate, pH 7.0. The RNA content fell by about 90%. Removal of DNA was less straightforward. Treatment with deoxyribonuclease had to be on the gel form, since this enzyme is inhibited by 0.5M-potassium chloride.

Even with continuous gentle agitation for 24 hr. at 4° little effect was seen. Treatment of a solution of the precipitate in buffered 0.35M-potassium chloride, pH 7.2 or 7.6, with DEAE-cellulose (after treatment with ribonuclease) removed the whole of the DNA, but there was always some protein loss, which might be 20% greater than that due in the control to reprecipitation alone. The results of Perry (1960) make it unlikely that myosin would be retained under these conditions, but there are no data for actomyosin.

A test was therefore made with the first dilution precipitate of pig 13, non-pregnant, described in Table 1; after removal of 90% of the RNA, treatment with DEAE-cellulose and precipitation, the falls in $\log \eta_{rel.}$ were, with ATP before and after actin, 0.031 and 0.058 respectively. That there is no apparent effect on the actomyosin content as a result of nucleic acid removal, and an apparent rise in myosin content, was confirmed in another experiment on pig 20, non-pregnant. The Δ_{ATP} values measured on the first dilution precipitate before and after removal of 90% of the RNA were respectively 0.026, 0.027, 0.025 and after actin 0.030, 0.040 and 0.058. Whether the apparent rise in myosin content is significant is a question that needs further study.

Ammonium sulphate fractionation by the Derrien method

A few experiments were done by this method on solutions of the dilution precipitate from uteri of pregnant and non-pregnant rabbits. Fig. 1 shows the marked difference in the curves obtained in the two states for $-dS/dC$ plotted against C (S , extinc-

Fig. 1. Salting-out curve of the dilution precipitate. For details see text. O, From non-pregnant rabbit uterus; ●, from pregnant rabbit uterus (30 days).

tion; C , percentage saturation with ammonium sulphate). The interpretation of such curves is difficult for two reasons. The solutions of the dilution precipitates were opalescent, especially those from non-pregnant uteri, and the fall in extinction at $280\text{ m}\mu$ might be due to removal of lipid material as well as protein. After about 20% saturation with ammonium sulphate this opalescence had largely disappeared, as was observed also by Oppel & Serebrenikova (1961). Secondly, irregularities were often found on the salting-out curves due to re-solution and reprecipitation of material. This phenomenon was observed also by Snellman & Tenow (1954) with solutions of dilution precipitates from non-gravid uterus, and by Azzone & Aloisi (1958) with tropomyosin-containing fractions from skeletal muscle. It is probably due to formation and breaking up of unstable complexes in the presence of nucleic acids and tropomyosin. Azzone & Aloisi (1958) found that this difficulty was largely overcome by carrying out the filtrations and optical observations at room temperature rather than at 4° . This was done also in our experiments, but without complete success.

With preparations from non-pregnant uterus a very large decrease in extinction was found between 16 and 22% saturation (F1), with a much smaller decrease in the region of 30% saturation, where actomyosin precipitation may be expected (F2). A large decrease in extinction took place at 35–50% saturation, i.e. in the myosin region (F3). With preparations from pregnant uterus, the main decrease was in the actomyosin zone (F2).

Preparation and examination of F1, F2 and F3 from the first dilution precipitates

In the earlier experiments the precipitation with ammonium sulphate was carried out in the presence of lithium chloride. This was done in the hope that the lithium chloride would have the effect of preventing the precipitation of nucleic acids at con-

centrations of ammonium sulphate below about 50% saturation. This method has been used by J. Marshall (personal communication) for preparation of nucleic acid-free myosin from skeletal muscle. With uterus preparations, which contain much greater amounts of nucleic acid, it was not successful. Thus in one experiment the solution of the first dilution precipitate contained $23.6\text{ }\mu\text{g.}$ of nucleic acid phosphorus/mg. of protein nitrogen, whereas F2 contained $25\text{ }\mu\text{g./mg.}$ of protein nitrogen. Further, the presence of lithium chloride seemed to effect a dissociation of actomyosin. The later experiments were therefore done with ammonium sulphate alone. Table 4 shows the amounts of protein precipitated at the different concentrations of ammonium sulphate, the presence or absence of lithium chloride being indicated, and Table 5 summarizes a number of experiments on the properties of the different fractions.

With non-pregnant uteri, both from pig and rabbit, the large precipitation of protein in F1 is seen. In the non-pregnant rabbit, F2 is negligible and F3 is high. With the non-pregnant pig, a good deal of protein is usually precipitated at 32% saturation, but significant amounts also between 32 and 45%. With the pregnant rabbit, the greatest precipitation is usually at 26–32% saturation.

From Table 5 the following points can be seen. (a) Further examples are given of the low actomyosin content in the dilution precipitates from non-pregnant uteri, the higher content in pregnant uteri and the very low myosin content in almost all cases. (b) In spite of the higher actomyosin content of the pregnant-uterus precipitate, solutions of these had a lower viscosity number after ATP addition than had the solutions from non-pregnant preparations. For the pregnant uteri, Z after ATP addition was higher for F2 than for the first dilution precipitate; but for the non-pregnant it was lower for F2. These observations probably reflect the greater content of salt-soluble collagen in the dilution precipitate of non-pregnant uteri. This protein (assessed by estimation of the hydroxyproline content) was found to be concentrated in F1 (note the high value for Z here) and less remained in F2 (Needham & Williams, 1963a). With the pregnant uteri, the rise in Z , in F2 probably depends on concentration of actomyosin and myosin at the expense of the less viscous associated proteins. (c) With both pregnant and non-pregnant uteri, F2 showed both actomyosin and myosin usually increased in concentration compared with the first precipitate. Much associated protein, however, remained. (d) In F1 from the non-pregnant pig 1, precipitated in the presence of lithium chloride, considerable actin-combining power was found, indicating the presence of myosin; no actomyosin was found in F1 in such cases. In tissues of pigs 2

and 3, precipitated without lithium chloride, some actomyosin (but no myosin) was found in F1. With pig 2 this was confirmed by the expected adenosine-triphosphatase activity. With the non-pregnant rabbit 32 (precipitation with lithium chloride present) there were also clear indications of actin-combining power in F1, though not enough material was available for estimation of the fall in $\log \eta_{rel.}$ with addition of ATP alone. It thus seems likely that the presence of high concentrations of lithium chloride can have a dissociating effect on actomyosin (note also the high actin-combining power in F2 from pig 1). Experiments on F1 described in the next paper (Needham & Williams, 1963*a*) show that some component(s) can form associations with

myosin; this property might account for the presence of myosin and actomyosin in this precipitate. It is also possible that the increase in ionic strength due to the high concentration of lithium chloride might affect the zones of ammonium sulphate salting-out. However, Dr J. Marshall (personal communication) describes the main precipitation of skeletal-muscle myosin at 38–46% saturation of ammonium sulphate in the presence of the concentration of lithium chloride used here; this is similar to the value of 33–45% saturation given by Snellman & Tenow (1954) in the absence of lithium chloride.

An experiment was done on F1 from the dilution precipitate of non-pregnant pig uterus to test for

Table 4. *Ammonium sulphate fractionation of the first dilution precipitate*

For conditions see text.

(NH ₄) ₂ SO ₄ saturation (%) ...	Percentage of protein precipitated			
	0–26 (F1)	26–32 (F2)	26–45 (F2)	32–45 (F3)
Non-pregnant rabbit				
23	32	Trace	—	58
24	41	Trace	—	48
29	34	0	—	35
30	42	Trace	—	48
32	51	0	—	30
Pregnant rabbit				
22 days: 22	10	58	—	—
30 days: 33	13	40	—	—
23 days: 31	19	—	52	—
27 days: 34	22	—	48	—
0–20% saturation				
Non-pregnant pig				
1	25	18	—	16
2*	26	40	—	8
3*	30	36	—	6
17*	30	—	—	—

* (NH₄)₂SO₄ precipitation in the absence of LiCl.

Table 5. *Changes in $\log \eta_{rel.}$ with adenosine triphosphate alone and with adenosine triphosphate after actin, for solutions of dilution precipitate and for fractions made from it*

Fraction 1 was precipitated at 26% saturation for rabbits 30–34; at 20% saturation for pigs 1 and 2; at 20% saturation followed by reprecipitation at 16% for pig 3. Fraction 2 was precipitated at 26–45% saturation for rabbits 30, 31 and 34; at 26–32% for rabbit 33 and for pigs 1–3. All values are calculated for 1 mg. of protein/ml.

	First dilution ppt.			Fraction 1			Fraction 2		
	Δ_{ATP}	$\Delta_{ATP, actin}$	Z_{ATP}	Δ_{ATP}	$\Delta_{ATP, actin}$	Z_{ATP}	Δ_{ATP}	$\Delta_{ATP, actin}$	Z_{ATP}
Non-pregnant rabbit									
30	0.024	0.027	0.112	—	—	—	0.031	0.046	0.085
32*	—	—	—	—	0.057	—	—	—	—
Non-pregnant pig									
1	0.025	0.027	0.122	0.02	0.052	0.14	0.039	0.076	0.094
2*	0.017	0.023	0.09	0.027	0.028	0.14	0.029	0.033	0.071
3*	0.023	0.037	0.110	0.030	0.031	0.24	0.027	0.037	0.099
Pregnant rabbit									
31: 23 days	0.044	0.053	0.078	—	—	—	0.046	0.048	0.115
33: 30 days	0.057	0.063	0.100	—	—	—	0.072	0.099	0.121
34: 28 days	0.048	0.049	0.104	—	—	—	0.073	0.085	0.108

* (NH₄)₂SO₄ precipitation in the absence of LiCl.

the presence of free actin. The method followed that used by Oppel & Serebrenikova (1959) in their demonstration of free actin in extracts of stomach muscle. The precipitate obtained at 20% saturation with ammonium sulphate (no lithium chloride present) was centrifuged down, suspended in 0.01N-sodium hydroxide and dialysed against 0.5M-potassium chloride-0.03M-potassium phosphate, pH 7, containing (per ml.) 40 μ g. each of ATP and neutralized ascorbic acid. The protein solution was centrifuged from insoluble residue and then tested with myosin in concentrations of 1 mg. of F1 and 4.7 mg. of myosin/ml. No increase in viscosity above that to be expected from the mixture was found. A similar experiment with F1 from stomach muscle gave a similar result.

Soluble proteins associated with actomyosin in the uterus

Soluble proteins in the dilution precipitate. The protein precipitated on dilution to 0.04M of a 0.5M-potassium chloride extract of uterus changed its composition very little on repeated precipitation in this way (Table 3). However, it was found that precipitation in stages (with removal of the precipitate at each stage) by dialysis against 0.27, 0.1 and 0.04M-potassium chloride led to separation of soluble proteins. This was the case whether the starting material was the first dilution precipitate itself, this precipitate after removal of F1, or F2. Table 6 summarizes these results and some of the properties of the various fractions. In all cases one-half to one-third of the protein was not precipitated

after final dialysis against water. All the fractions tested contained nucleic acid. With pig 9, precipitation at 0.25M gave a fraction with greatly increased actomyosin content. With pig 13, little protein was precipitated at 0.29M, but the sedimentation coefficients of the fraction precipitated at 0.03M suggest the presence of both actomyosin and myosin (see Portzehl *et al.* 1950).

With pig 17, the fraction soluble in water was freeze-dried and redissolved. It had a low viscosity when tested in 0.02M-potassium phosphate, pH 7; this fell still lower when potassium chloride was added to 0.5M. The value of the fall in $\log \eta_{rel.}$ from 0.060 to 0.011 for a solution containing 2 mg./ml., when compared with the values given for tropomyosin B solutions under similar conditions by Tsao, Bailey & Adair (1951), suggests that 12-15% of tropomyosin B may be present.

The electrophoretic mobilities (values for ascending mobilities are given) for this same water-soluble fraction cannot be clearly interpreted (Fig. 2a). The ionic strength used was 0.35, and the value -3.5×10^{-5} cm.²/v/sec. might in these conditions be due to tropomyosin B or to denatured actin (Perry & Corsi, 1958); the value -1.8×10^{-5} is similar to that given by the myogen group of proteins, and also by Tsao's water-soluble protein isolated from washed myofibril preparations (Tsao *et al.* 1958). This Tsao protein had very low viscosity and sedimentation coefficient, $10^{13} S_{20,w}$ 6 at a concentration 5 mg./ml. Similar mobilities (-3.5×10^{-5} and -2.0×10^{-5} at pH 7.1) were also obtained with a soluble fraction from another pig preparation

Table 6. *Some properties of the proteins obtained by dilution in stages*

For the ATP effects on $\log \eta_{rel.}$ (Δ_{ATP} and $\Delta_{ATP, actin}$), solutions in 0.5M-KCl-0.04M-potassium phosphate, pH 7.0, were used; results are calculated for 1 mg./ml. For sedimentation coefficients solutions about 6 mg. of protein/ml. were used. All animals were non-pregnant.

Pig	Material used	Precipitated on dialysis		Soluble in water
		0.25M-KCl	0.03M-KCl	
9	120 mg. of F2 Δ_{ATP} 0.025 $\Delta_{ATP, actin}$ 0.03	41 mg. Δ_{ATP} 0.05 $\Delta_{ATP, actin}$ 0.056	20 mg.	Much protein, but not estimated
13	38 mg. of first dilution precipitate after removal of F1	Very little precipitate*	15 mg. 40 μ g. of nucleic acid P/mg. of protein N $10^{13} S_{20,w}$ (I 0.6, pH 7): 20.6, 4.6	18 mg.
13	94 mg. of first dilution precipitate	22 mg.	32 mg. 36 μ g. of nucleic acid P/mg. of protein N $10^{13} S_{20,w}$ (I 0.6, pH 7): 7.2, 2.9 Δ_{ATP} 0.005 $\Delta_{ATP, actin}$ 0.012	28 mg. 45 μ g. of nucleic acid P/mg. of protein N
17	275 mg. of first dilution precipitate after removal of F1	130 mg.	33 mg.	85 mg. $10^{13} S_{20,w}$ (I 0.35, pH 7): 6.5, 0.83

* 0.29M-KCl used for dialysis instead of 0.25M.

(Fig. 2*b*). In this the water-soluble fraction was treated with dilute hydrochloric acid to the point of maximum precipitation; the precipitate was removed and dissolved in water at pH 7, electrophoresis being carried out at I 0.35.

Starch-gel electrophoresis of the water-soluble fraction from pig 17 showed four bands: three from proteins that had moved rapidly towards the anode and one diffuse band spreading back towards the cathode from the origin. Much protein appeared to remain at the origin, and so it is possible that the four bands are only minor components of the soluble fraction. The central of the three bands running towards the anode corresponded to nucleotropomyosin that had been prepared from pig uterus.

The behaviour of the proteins of a dilution precipitate from pregnant rat uterus was rather different. Out of 45 mg. of protein, 57 % was precipitated at 0.27M-potassium chloride, a further 13 % at 0.1M and only 20 % remained in solution in water. At first sight this high precipitation at 0.27M seems to conflict with the finding (Table 1) that the dilution precipitate from the rat contains so small a percentage of actomyosin. However, about 70 % of the protein precipitated at 0.27M was

found to be insoluble in 0.5M-potassium chloride. This part therefore probably consisted of denatured associated protein.

Soluble protein not removed by exhaustive washing of the tissue. If frozen uterus muscle is comminuted on the freezing microtome, and ground and extracted exhaustively with 0.03M-phosphate, pH 7.4, and then after centrifuging extracted with buffered 0.6M-potassium chloride (containing ATP), it is found that all the protein extracted at the higher ionic strength is not precipitated by dilution to 0.04M. This was shown by Ivanov *et al.* (1959), who used four washings, with 10 vol. each time of the dilute buffer, and found after precipitation by dilution of the extract made at higher ionic strength that 75 % of the extracted protein, the 'T fraction', remained in solution.

We have repeated this experiment with slight modifications. The cooled tissue (from non-pregnant pig) was extracted three times with 10 vol. of the dilute buffer, being ground each time thoroughly with quartz; then extraction took place as usual with 0.5M-potassium chloride containing 0.04M-phosphate, pH 7.1, and ATP. Two experiments were made. In both cases the extract contained about 200 mg. of protein nitrogen/100 g. wet wt.

Fig. 2. (a) Electrophoresis pattern of the water-soluble fraction from pig 17. Electrophoresis for 720 min. at 1.3 v/cm.; medium, 0.22M-KCl-0.05M-sodium phosphate, pH 6.5; concn. 6.8 mg./ml. (b) Electrophoresis pattern of a water-soluble fraction from pig 14; for details see text. Electrophoresis for 720 min. at 1.43 v/cm.; medium, 0.25M-NaCl-0.087M-sodium phosphate, pH 7.1; concn. 8 mg./ml. (c) Electrophoresis pattern of T fraction, after freeze-drying, from pig 20. Electrophoresis for 120 min. at 6.6 v/cm.; medium, 0.035M-potassium phosphate, pH 7.2; concn. 8 mg./ml. Ascending fronts, upper row.

and of this only about 40% was precipitated on dialysis to 0.04M-potassium chloride. In the first experiment the precipitated protein showed little actomyosin content or actin-combining power. There was correspondingly low adenosine-triphosphatase activity. Neither the actin-combining power nor the adenosine-triphosphatase activity could be increased by treatment with cysteine. In the second experiment the actomyosin content and actin-combining power were normal (Δ_{ATP} 0.02; $\Delta_{ATP, \text{actin}}$ 0.032 for 1 mg. of protein/ml.). This difference may depend on the fact that in the first experiment the minced tissue was treated with repeated small volumes of the washing fluid. In the second experiment care was taken to grind the tissue from the beginning with a large volume of the washing fluid. This point will be discussed below.

In the second experiment, after removal of the precipitate formed at 0.04M-potassium chloride, the supernatant was dialysed against water and then freeze-dried. The resulting powder was readily soluble in 0.02M-phosphate, pH 7.0. The value for $\log \eta_{rel.}$ for this solution (at 2 mg./ml.) fell from 0.0445 to 0.0075 on addition of potassium chloride to 0.5M. Comparison of this result with those of Tsao *et al.* (1951) indicates the presence of about 16% of tropomyosin B. Electrophoresis of a solution (8 mg./ml.) in 0.035M-potassium phosphate, pH 7.2, gave two broad-based peaks with mobilities -7.2 and -5.3×10^{-5} cm.²/v/sec. ascending; -5.3 and -3.6×10^{-5} cm.²/v/sec. descending (Fig. 2c). Tropomyosin B is probably the component giving the higher mobility, since this experiment was carried out at low ionic strength (I 0.095); Hamoir & Laszt (1962) give a value -9.6×10^{-5} cm.²/v/sec. for the ascending mobility of tropomyosin B from artery muscle at I 0.075. The asymmetry of the ascending and descending limbs may be connected with protein association.

DISCUSSION

The low actomyosin content of uterus muscle (and also of stomach muscle) is striking. The protein-nitrogen content of the first dilution precipitate varied from about 0.7 to 2 mg./g. wet wt. for rabbit, pig and cow uterus in the present work, and no consistent difference was seen between pregnant and non-pregnant uterus. Values of about 2 mg./g. wet wt. are given by Ledermair (1959) for cow, and by Schwalm & Cretius (1958) for human uterus, whether pregnant or non-pregnant. This is to be compared with about 14 mg./g. wet wt. for skeletal muscle. The difference becomes even more striking when we find that even with pregnant uterus the first dilution precipitate usually contained only some 50% of actomyosin plus myosin, whereas

non-pregnant uterus contained only 30% or less. With pregnant rabbit it was found that about 80% of the actomyosin of the extract was precipitated in the first dilution precipitate. Values calculated for actomyosin per g. wet wt. from the results in the dilution precipitate should therefore be increased by about 25%. With the pregnant uterus of the rat the protein nitrogen of the first dilution precipitate might be about 3.5 mg./g. wet wt., but of this only about 15% was actomyosin. Values found by us for actomyosin content of rabbit and rat extracts agree fairly well with those of Csapo (1950). Uterus muscle contains much more stroma protein intimately associated with the muscle cells than does skeletal muscle; in some types of uterus also collagen sheets, very difficult to remove from the muscle sheets, are found on the exterior surface. Needham & Cawkwell (1957) found 38 mg. of collagen/g. wet wt. in rat uterus in oestrus, 24 mg. at the end of pregnancy. Cretius (1959) showed that the corpus of human uterus has 22 mg. of collagen/g. wet wt. non-pregnant, 14 mg. at term (to be compared with 4.8 mg. in skeletal muscle). If allowance could be made for this collagenous material the actomyosin content representing that of the actual muscle cell would be much higher. The reason for the very high values (7.8 mg./g. wet wt.) for protein nitrogen in the dilution precipitate given by Hollmann & Rösick (1954) for pregnant cow uterus is not clear. The time of pregnancy is not stated: possibly it was much nearer term than was the material used by other workers.

After precipitation with ammonium sulphate the fractions obtained were still very inhomogeneous. F1 (studied from non-pregnant uterus) contained 10–15% of salt-soluble collagen (Needham & Williams, 1963a) and 25–35% of actomyosin or myosin; the nature of the rest is unknown. F2 usually contained more actomyosin than the first dilution precipitate, but still much unidentified protein. It is not clear what part, if any, nucleic acids play in carrying down extra protein in the various precipitates. The question of the presence of free actin in extracts of smooth muscle is being further investigated.

The water-soluble protein associated with actomyosin in the dilution precipitates made at 0.04M-potassium chloride was not carried down with actomyosin precipitated at 0.27M-potassium chloride. By precipitation in stages, with removal of precipitate at each stage, a considerable fraction of water-soluble protein can be separated. The amount of tropomyosin B found in this fraction (10–15% for pig 17) is compatible with the amount (about 4%) found by Needham & Williams (1959) in the whole dilution precipitate from non-pregnant pig uterus, since the soluble fraction constitutes about one-third of the whole. The facts (a) that

uterus actomyosin and myosin have similar viscosity numbers to those of the skeletal-muscle proteins (Huys, 1960; Needham & Williams, 1963*b*), and (b) that the presence of soluble proteins of low viscosity as well as salt-soluble collagen has been demonstrated in the uterus dilution precipitates, show that the different viscous behaviour of the uterus and skeletal-muscle dilution precipitates is due to the presence in the former of other proteins besides actomyosin and myosin, and not to different properties of the actomyosin and myosin themselves [as has been suggested by Hasselbach & Ledermair (1958) and Ledermair (1959)].

The soluble protein described by Ivanov *et al.* (1959, 1962), unlike the soluble material just described above, is not precipitated by dilution of the salt extract to 0.04M. Our further observation that treatment of the tissue with successive small volumes of the washing fluid led to loss of actomyosin from the final dilution precipitate can probably be explained in the light of the recent demonstration by Huys (1961) of the presence of 'tonoactomyosin' in uterus muscle. This seems to be a form of actomyosin soluble at low ionic strength if a small amount of ATP is present. In our second experiment, the swamping of the tissue's own salts and ATP by large volumes of dilute buffer would have prevented extraction of tonoactomyosin. Jaisle (1960) has suggested that homogenization of human uterus muscle three times in 10 vol. of water extracted the contractile proteins nearly completely, since the extract made subsequently with 0.5M-potassium chloride showed no adenosine-triphosphatase activity and no viscosity fall with ATP.

From their study of the water-soluble T fraction by immunological and electrophoretic methods Ivanov *et al.* (1962) conclude that about 50% consists of tropomyosin B and Tsao protein. The present work confirms that one component is tropomyosin B. Much protein readily denatured during concentration of the supernatant (by evaporation in collodion bags at 12° before a fan) was also found by Ivanov *et al.* (1959). They showed that the T fraction was obtained in a similar way from skeletal and heart muscle. The absolute amount was rather less and a smaller proportion of material denatured on evaporation was contained in it. The ratio of actomyosin to T fraction was very much higher than with uterus. They consider that the method of preparation of the T fraction shows that it must be of myofibrillar origin, and put forward the suggestion that the high proportion of T fraction to actomyosin in smooth muscle indicates that the T fraction has a function in the development of the tonic state. However, the actual form of the contractile mechanism in vertebrate smooth muscle remains obscure (for a discussion of this see

Needham & Williams, 1963*b*) so that, although the presence of soluble proteins in washed skeletal myofibrils has indeed been established (Perry & Corsi, 1958; Perry & Zydowo, 1959), the location of the T fraction in the smooth-muscle cell is difficult at present to envisage. Further, except for the changes in state of aggregation of tropomyosin B solutions with changes in ionic strength (Tsao *et al.* 1951), we have as yet no knowledge of properties of proteins of the T fraction that would seem to fit them for the role suggested.

SUMMARY

1. The presence of actomyosin in dilution precipitates made from extracts of uterus muscle (extracted with buffered 0.5M-potassium chloride-1.5M-ATP, and diluted to 0.04M) was confirmed; little or no free myosin was found. With pregnant-rabbit uteri near term, about 45% of this protein precipitate was actomyosin; with uteri of non-pregnant rabbits or pigs, the amount was only about 25%. The actomyosin content of myometrium is thus less than 10% of that in skeletal muscle.

2. On fractionation with ammonium sulphate, especially with non-pregnant uteri, much of the protein (up to 35%) was precipitated at 16–26% saturation. This precipitate contained some actomyosin or myosin and some salt-soluble collagen, but no free actin.

3. The soluble proteins associated with the actomyosin in the dilution precipitate were studied. These cannot be separated by repeated precipitation by dilution to 0.04M, but can be isolated by dilution in stages. The soluble proteins of the T fraction, prepared as described by Ivanov *et al.*, have also been investigated. This contained material of high electrophoretic mobility (probably tropomyosin B). The soluble protein from both sources gave solutions of low viscosity; in both cases viscosity measurements suggest the presence of 10–15% of tropomyosin B.

4. The nucleic acid content of the dilution precipitates and of the various fractions made from them was estimated. The effect of removal of nucleic acid on the behaviour of the proteins was investigated.

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