Cytoplasmic Fractions of Rat Myometrium

1. GENERAL DESCRIPTION AND SOME ENZYMIC PROPERTIES

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The technique of differential centrifuging has been widely used on many of the softer tissues for the isolation of cell components. However, centrifugal fractionation has not been used as extensively on muscle tissue. Skeletal muscle has been fractionated by Perry (1952), who found besides the myofibrils an array of granules of different sizes. Skeletal-muscle mitochondria have been further studied by Chappell & Perry (1953, 1954); the mitochondria showed adenosine-triphosphatase activity and were also capable of oxidative phosphorylation. Cardiac muscle has similarly been fractionated by Cleland & Slater (1953). The properties of cardiac and liver granules were later compared by Holton, Hülsmann, Myers & Slater (1957). There is no account in the literature, however, of any detailed fractionation of non-striated involuntary muscle. The smooth muscle of the uterus is of special interest because of the great changes the organ undergoes in structure and metabolism in connexion with its different functional states. The present work therefore is an attempt to isolate from the uterine muscle or myometrium its various cell constituents, and to compare these with the corresponding cell components of other tissues.

The electron micrographs of Shoenberg (1958) have helped to elucidate the intracellular structure of the myometrium. In the gravid uterus the greater part of the cell is occupied by the myofilaments, not much space being left for the remaining structures of the sarcoplasm. Mitochondria, and a sarcoplasmic reticulum from which originate the submicroscopic particles or microsomes of Claude (1941) (see Palade & Siekevitz, 1956*a*), are all lying between the strands of myofilaments.

Four fractions were obtained by differential centrifuging: a fraction consisting of a mixture of cell nuclei, myofilaments, connective tissue and unbroken cells; two fractions of cytoplasmic particles (the mitochondrial and microsomal fractions); and a final supernatant consisting of the soluble elements of the tissue. In all these experi-

ments, unless it is otherwise stated, gravid uteri were used since little material is available from non-pregnant animals.

Some properties of the uterine myofilaments have been studied by Needham & Williams (1959). The present paper deals with some aspects of the remainder of the cytoplasm. The essential features studied are those properties which, for the isolated mitochondria, are regarded as indicating how nearly their condition corresponds to the native condition. Among the criteria which have been used for morphological integrity of the mitochondria are: their ability to show reversal of swelling (Chappell & Perry, 1954; Ernster & Löw, 1955); their ability to exhibit adenosine-triphosphatase activation with low concentrations of dinitrophenol (Lardy & Wellman, 1952; Potter, Siekevitz & Simonson, 1953); their ability to esterify inorganic phosphate while oxidizing substrates of the Krebs cycle (Kielley & Kielley, 1951; Lardy & Wellman, 1952). The effects of different suspension media are also considered.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents were of analytical grade and made up with water distilled in glass.

2-Amino-2-hydroxymethylpropane-1:3-diol (tris) was recrystallized from ethanol. Succinic acid and fumaric acid, 2:4-dinitrophenol and creatine were recrystallized from water. 2-Oxoglutaric acid and L(-)glutamic acid were from Roche Products Ltd., and osmic acid was from Hopkin and Williams Ltd. Sucrose was deionized by passing 500 ml. of a molar stock solution through a column (2 cm. x 5 cm.) of packed, moist Amberlite Monobed Resin MB, analytical grade (British Drug Houses Ltd.), at a rate of about 1 l./hr. The Araldite resins used in tissue embedding were obtained from Aero Research Ltd., Duxford, Cambridge. Janus Green B was from George T. Gurr Ltd. Heparin B.P., dry powder, was from Evans Medical Supplies Ltd. Adenosine monophosphate (AMP) and the crystalline sodium salts of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were from Sigma Chemical Co. Solutions of the nucleotides were neutralized with NaOH. Crystalline bovine-plasma albumin was from The Armour Laboratories, England. Cytochrome *c* and hexokinase were the generous gifts of Dr Malcolm Dixon and Dr Sidney P. Colowick respectively.

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Preparation of the cytoplasmic fractions

Albino rats, 4–5 months old and weighing 250–300 g., were used. As the rat uterus continues its growth until the very end of pregnancy (Harkness & Harkness, 1954) the animals were killed (by exsanguination) 1 or 2 days before parturition. All subsequent operations were carried out in the cold (0–4°). The horns of the uterus were slit open along the mesometrial attachment and freed of the placentae. The endometrium was removed by alternately scraping with a scalpel and rinsing in ice-cold 0.3M-sucrose in a Petri dish, until the tissue was free from mucosa and mucus. The organ then weighed about 2.5 g. after blotting. It was finely minced with scissors before being homogenized with about 8 vol. of the sucrose solution in an all-glass Potter–Elvehjem homogenizer having ground-glass surfaces (Potter & Elvehjem, 1936). As a routine about 20–30 strokes were adequate. In later experiments it was found easier to use two pestles: a loosely fitting one, followed by a more tightly fitting one. This stage was reached within 20 min. after the death of the animal.

The homogenate was then centrifuged at 800 *g* for 10 min. in the refrigerated MSE centrifuge with horizontal yoke. The supernatant *S*₁ was carefully pipetted out with a Pasteur pipette, to avoid debris floating with trapped air bubbles. The residue, *N*, was broken up with a glass rod, resuspended by shaking in additional sucrose solution, and recentrifuged at the same speed for the same duration. The washing thus obtained was added to the first supernatant. This in turn was centrifuged at 12 000 *g* for 15 min. with the high-speed head of the International refrigerated centrifuge to yield a mitochondrial fraction, *M*, which was also washed. No 'fluffy' or poorly sedimented layer was observed. The resulting supernatant was finally centrifuged at 90 000 *g* for 60 min. in the Spinco ultracentrifuge, model *L*, to yield a microsomal fraction *P*, and the final supernatant, *S*. This last residue, being compact, was not washed by resuspension, but the centrifuge tube was gently rinsed with sucrose solution. Centrifugal forces are calculated from the centre of the tube. The symbols used for the various fractions were adopted from de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The isolated fractions were stored in ice and were used as soon as possible after the completion of fractionation.

When ovariectomized rats were used, 10–12 uteri (each only about 0.1 g.) were pooled. They were used whole after being freed from connective tissue and fat. For these experiments virgin rats (150–200 g.) were killed 3–4 weeks after ovariectomy. Use of the whole uterus after ovariectomy does not provide a strict comparison with the gravid myometrium as such. However, the myometrium constitutes the great bulk of the organ, especially in the castrate state; indeed, in cases where scraping was tried with the latter, the amount of material removed was not considered significant.

Electron microscopy of the cytoplasmic particles. The pellets obtained by centrifugal fractionation were broken into small fragments in a solution of 1% (w/v) osmic acid and 0.44M-sucrose (for the microsomes buffered at pH 7.4 with veronal-acetate buffer according to Palade & Siekevitz, 1956*a*). Fixation was from 2 to 4 hr. at 0°. Small pieces of cellophan (about 3 mm. square) were added to the mixture before centrifuging to prevent the pellet from

adhering to the bottom of the centrifuge tube. After dehydration through ascending concentrations of ethanol (with resuspension and centrifuging at each stage) the material was stained overnight for further contrast in 1% (w/v) phosphotungstic acid dissolved in absolute ethanol. For embedding of the final pellet H. E. Huxley's modification (unpublished work) of the method of Glauert & Glauert (1958) was used. Sectioning was done with the H. E. Huxley microtome (Huxley, 1957) and photomicrography was performed on a Metropolitan–Vickers EM6 microscope.

Enzyme assays

Adenosine triphosphatase. This was assayed at 20° in the presence of 0.05M-tris chloride buffer, pH 7.4, 5 mM-ATP, 0.04M-KCl and tissue suspension corresponding to about 0.1 mg. of nitrogen; the final volume was 2.5 ml. For Mg-activated adenosine triphosphatase 5 mM-MgSO₄ was used. Dephosphorylation, which is linear during the first 6 min. of incubation, was stopped by adding 0.5 ml. of 48% (w/v) trichloroacetic acid and, after centrifuging, inorganic phosphate was estimated on a sample of the acid supernatant.

Manometry and oxidative phosphorylation. This was carried out in Warburg flasks having a capacity of about 20 ml. A system similar to that of Lardy & Wellman (1952) was used. Mitochondrial suspension (equivalent to 0.1–0.2 mg. of nitrogen) was added to a mixture having the following final concentrations: 5–15 mM-phosphate buffer, pH 7.4, 7.5 mM-MgSO₄, 2.5 mM-ATP, 0.01 mM-cytochrome *c*, 10 mM-substrate brought to pH 7.4 with KOH and 0.05M-KCl. The gaseous phase was air, and incubation was at 20° or 25°. For oxidative phosphorylation ADP was added at a final concentration of 10 mM, the reaction being stopped with 0.2 ml. of 48% (w/v) trichloroacetic acid placed in the side arm. After centrifuging, samples of the supernatant were taken for inorganic phosphate estimations.

Analytical methods

Phosphate. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925) for routine analysis, and by the method of Martin & Doty (1949) when labile esters such as creatine phosphate were present. For total phosphorus the method of Allen (1940) was used. Colour intensity was measured at 660 mμ in a Unicam SP. 500 spectrophotometer.

Total nitrogen. This was estimated by the Kjeldahl method. Digestion was effected with the mixture of Umbreit & Bond (1936) and ammonia was estimated according to Ma & Zuazaga (1942). Protein nitrogen, wherever mentioned, was taken as the nitrogen content of the precipitate obtained with 8% (w/v) of trichloroacetic acid.

Collagen. In the different fractions collagen was estimated by a procedure of Needham & Cawkwell (1957). This consisted in determining nitrogen in the residue insoluble after standing overnight at 0° in 0.1N-NaOH.

RESULTS

Effect of different suspension media

The use of 0.3M-sucrose as a medium for fractionation procedures was arrived at after trying the following media, all of which had undesirable effects.

Table 1. *Effect of additives to the fractionating medium on the distribution of total nitrogen*

A homogenate of gravid rat uterus in 0.25 M-sucrose was divided into two equal portions. Heparin (or ATP and EDTA) was added to one of the portions, and parallel centrifugal fractionations were carried out on both portions. The results are expressed as mg. of total N per uterus.

Fraction	Expt. 1		Expt. 2	
	0.25 M-Sucrose	0.25 M-Sucrose, 0.035 % heparin	0.25 M-Sucrose	0.25 M-Sucrose, 1 mM-ATP, 1 mM-EDTA
M	0.56	0.49	1.56	1.90
P	1.90	1.33	2.04	1.30
S	8.28	9.20	8.76	9.64
Total	10.74	11.02	12.36	12.84

The medium of Birbeck & Reid (1956), which contains 6% (w/v) of dextran, is so viscous that with a tough tissue like the myometrium homogenization is almost impossible.

The use of heparin, an anti-agglutinant (Hoster, McBee, Rolnick, van Winkle & Hoster, 1950; Birbeck & Reid, 1956), was tried as an additive to iso-osmotic sucrose. Parallel fractionations were performed on two equal portions of the same homogenate, one of the portions containing heparin at a concentration of 0.035%. The results on total nitrogen distribution (Table 1) indicate that heparin has a solubilizing effect on some of the microsome proteins. The solubilized protein reappears as extra nitrogen in the final supernatant. Heparin also causes a 15% inhibition of succinic dehydrogenase.

As shown below (see also Table 4) the tris-KCl medium of Chappell & Perry is not as beneficial to myometrial mitochondria as is to skeletal-muscle mitochondria. In addition, it appears to cause a shift in the distribution of Mg-activated adenosine triphosphatase. With sucrose as a fractionating medium this enzyme is mainly concentrated in the microsomal fraction (Table 3; see also Wakid & Needham, 1960). However, the tris-KCl medium increases the amount in fraction M at the apparent expense of fraction P (Table 4). The presence of Mg^{2+} ions in this medium may be the cause of this aberration, since addition of Mg^{2+} ions is known to cause aggregation of microsomal material (Jeener, 1948; Siekevitz, 1952; Rendi & Campbell, 1959).

The tris-KCl medium also produces changes in the distribution of total nitrogen similar to those produced by heparin. It appears that the ATP and the ethylenediaminetetra-acetate (EDTA) present in the tris-KCl medium are the components responsible for the changes in nitrogen distribution. When either ATP or EDTA or both are added to iso-osmotic sucrose as a fractionating medium the same effect is observed (Table 1). Similar effects on protein-nitrogen distribution were also obtained with liver tissue. In all fractionations with ATP and EDTA the final supernatant was more reddish,

Table 2. *Percentage of collagen nitrogen in the cytoplasmic particles of rat myometrium*

Collagen estimations are described in the Materials and Methods section. With pregnant rats one organ was used for each fractionation, but with ovariectomized animals batches of 10-12 were used. The results are expressed as means $\pm$ s.e.m. and the numbers of experiments are given in parentheses. Fraction S is devoid of collagen.

Fraction	Pregnant rat	Ovariectomized rat
M	24.2 $\pm$ 1.6 (4)	36.0 $\pm$ 6.4 (2)
P	5.1 $\pm$ 0.4 (4)	17.8 $\pm$ 2.5 (2)

and the cytoplasmic particles were paler than the corresponding fractions isolated with sucrose alone. Korff (1957) has noticed similar effects with liver tissue when using 0.01 M-EDTA.

The use of sucrose solutions as a medium for centrifugal fractionation has been so extensive in the past (Schneider & Hogeboom, 1951; de Duve & Berthet, 1954) that its use is justified here for the sake of comparison. A concentration of 0.3 M was chosen because it approaches more closely the osmolarity of the cell (Conway & McCormack, 1953).

General description of the cytoplasmic fractions

The first residue, fraction N, sediments as a pinkish pellet which consists mainly of a viscous mass of entangled myofilaments and connective tissue. It agglutinates slightly on standing and cannot be uniformly resuspended. Microscopically there can also be seen unbroken cells, nuclei, erythrocytes and fragments of glass. Thus although fraction N would correspond to the nuclear fraction of other tissues, it has so many other inclusions that such a title is hardly suitable.

The mitochondrial fraction, M, sediments as a whitish pellet. Electron micrographs of the pellet show mitochondria of 0.7-1.3 μ in length. The chief contaminant appears to be collagen fibres (Table 2). Although most of the connective tissue has settled in fraction N, contamination of subsequent fractions occurs, due to fragmentation of the collagen

fibres during homogenization. Microsomal material and what may be disintegrated myofilaments are among other contaminants appearing in electron micrographs of the mitochondrial fraction.

The microsomal fraction, P, sediments as a translucent rose-coloured pellet. Electron micrographs show that it consists of at least two kinds of vesicle which look like the vesicles of the sarcoplasmic reticulum reported by Shoenberg (1958) in sections of whole tissue. Most of the vesicles have dense particles attached to their walls, but others are smooth-walled (see Plate 1). Palade & Siekevitz (1956*a, b*) have reported similar observations with liver and pancreas microsomes, and they present evidence that the dense bodies consist of ribonucleoprotein. Mitochondria and small fragments of collagen fibres contaminate fraction P.

The final supernatant, S, is a clear straw-coloured liquid which usually has (especially with ovariectomized rats) a thin layer of lipid floating on the surface.

When Janus Green B is added to the fractionating medium at a concentration of 0.005% the dye is so well adsorbed by all the particles that they sediment as deep-blue pellets leaving almost colourless supernatants. With liver and brain the dye is rapidly reduced to red diethyl safranin if the pellets are warmed (Potter, Recknagel & Hurlbert,

1951; Lazarow & Cooperstein, 1953; Wakid & Kerr, 1955). Chick-embryo mitochondria (Carey & Greville, 1959) will also slowly reduce the dye over a period of 24 hr. in the presence of succinate when oxygen is excluded. However, no such change was observed in the blue pellets of cytoplasmic particles from the myometrium.

Changes in extinction of the mitochondria

Chappell & Perry (1954) have shown that skeletal-muscle mitochondria, isolated in their tris-KCl medium, swelled when diluted at room temperature in 0.1M-KCl, 0.05M-tris, pH 7.4, and 5 mM-MgSO₄. The swelling was followed as a decrease in *E* at 520 m μ . The addition of ATP caused a rise in extinction which was shown to be due to a reversal of the swelling.

In a similar experiment with uterine mitochondria ATP merely arrested the swelling. However, when the mitochondria of the same preparation were diluted in 15 mM-phosphate buffer, pH 7.4, 0.01M-glutamate and 0.1M-KCl the addition of ATP and Mg²⁺ ions (Baltscheffsky, 1957) did cause a gradual but perceptible reversal of swelling. This rather attenuated response of uterine mitochondria is perhaps due both to their slightly damaged condition and to the presence of foreign material such as collagen fibres.

Adenosine triphosphatase and 2:4-dinitrophenol

Table 3 shows the effect of dinitrophenol and Mg²⁺ ions on the adenosine-triphosphatase activity of fresh and aged preparations of fractions M and P. In the absence of Mg²⁺ ions a concentration of 0.1 mM-dinitrophenol activates the adenosine triphosphate of fresh uterine mitochondria to about

EXPLANATION OF PLATE 1

Electron micrograph (magnification $\times 42\,500$) of the microsomal fraction of rat myometrium at the end of pregnancy. C, Fragments of collagen fibres. M, Section of mitochondrion. D, Microsomal vesicles with dense bodies attached to their walls. S, Smooth-walled microsomal vesicles.

Table 3. *Effect of dinitrophenol and Mg²⁺ ions on adenosine-triphosphatase activity of the cytoplasmic particles*

Incubations were at 20° in the presence of 0.05M-tris, pH 7.4, 0.04M-KCl and 5 mM-ATP with additions as indicated. Where sucrose was used KCl was omitted. Aging was carried out by warming the particles for 15 min. at 37° before assay. Activities are in μ moles of inorganic P liberated/hr./mg. of total N. In the columns headed activation the increase in activity due to 2:4-dinitrophenol (DNP) is given as a factor of the activity in its absence.

Additions	Mitochondria				Microsomes			
	Fresh		Aged		Fresh		Aged	
	Activity	Activation	Activity	Activation	Activity	Activation	Activity	Activation
None	10.8	1	13.2	1	13.3	1	7.5	1
DNP (0.01 mM)	21.1	1.9	—	—	—	—	—	—
DNP (0.1 mM)	45.5	4.2	13.2	1	15.6	1.2	7.5	1
DNP (0.5 mM)	52.5	4.8	—	—	—	—	—	—
Sucrose (0.2M), DNP (0.1 mM)	36.0	3.3	—	—	15.6	1.2	—	—
Mg ²⁺ (5 mM)	185.8	17	175	13	390	29	355	47
Mg ²⁺ (5 mM), DNP (0.1 mM)	208.0	19	176	13	413	31	353	47

the same extent as it does that of skeletal-muscle (Chappell & Perry, 1954) or chick-embryo mitochondria (Carey & Greville, 1959). Although activation here is not as great as with liver mitochondria (Lardy & Wellman, 1953) it is still considered an index of freshness and morphological integrity.

Dinitrophenol activates maximally when the KCl in the assay medium is at a concentration of about 0.04M (Fig. 1). If KCl is replaced by 0.2M-sucrose, dinitrophenol activation is slightly inhibited. The inhibitory effect of sucrose on dinitrophenol activation has been demonstrated with liver mitochondria by Potter & Recknagel (1951).

Mg²⁺ ions have a strong activating effect on the adenosine triphosphatase of fresh myometrial mitochondria and this activation greatly surpasses

that due to dinitrophenol. This pattern of behaviour resembles that of heart sarcosomes (Holton *et al.* 1957) but is quite in contrast with that of fresh liver mitochondria (Lardy & Wellman, 1952; Potter *et al.* 1953). However, with aged mitochondria from liver activation by Mg²⁺ ions is greater than dinitrophenol activation.

Aging by warming does not significantly stimulate the freshly prepared adenosine triphosphatase as it does with other tissues, nor does it increase the Mg-activated enzyme. However, it abolishes activation by dinitrophenol completely (Table 3).

In a trial to find out whether the tris-KCl isolation medium of Chappell & Perry (1954) would increase dinitrophenol activation, as was seen to be the case with skeletal-muscle mitochondria, it was found that uterine mitochondria have a high native adenosine triphosphatase in the absence of added Mg²⁺ ions or dinitrophenol as compared with those isolated in sucrose (Table 4). The result was an apparently low activation with dinitrophenol. However, this high native adenosine triphosphatase can be explained on grounds that the Mg²⁺ ion present in the isolation medium was itself acting as an activator, its final concentration in the assay medium having become 0.5 mM. This was verified by omitting the Mg²⁺ ions from the tris-KCl medium. In this experiment activation by dinitrophenol was again about as high as in sucrose-isolated mitochondria. It is possible that the residual activity in the absence of added Mg²⁺ ions or dinitrophenol is due to endogenous cations in the sucrose-isolated mitochondria.

The microsomal fraction of rat myometrium does not exhibit any appreciable stimulation of adenosine triphosphatase with dinitrophenol (Tables 3 and 4). Mg²⁺ ions, however, activate the microsomal adenosine triphosphatase even more than that of the mitochondria. A similar situation was found to exist with the particles of rat sartorius (unpublished data). The percentage distribution of

Fig. 1. Effect of KCl and sucrose on the dinitrophenol activation of mitochondrial adenosine triphosphatase from rat myometrium. Incubations were at 20° with 0.05M-tris chloride, pH 7.4, and 5 mM-ATP, in the absence of Mg²⁺ ions. Where used, dinitrophenol was at a concentration of 0.1 mM. A gives the value found with no dinitrophenol; B the value with 0.2M-sucrose added in the presence of dinitrophenol.

Table 4. *Effect of tris-potassium chloride isolation medium on adenosine-triphosphatase activity of the cytoplasmic particles*

The tris-KCl medium (Chappell & Perry, 1954) is a solution of 0.05M-tris chloride buffer, pH 7.4, 0.1M-KCl, mM-ATP, mM-EDTA and 5 mM-Mg²⁺ ions. In a separate fractionation Mg²⁺ ions were omitted from the medium. See Table 2 for conditions. Activities are in μmoles of inorganic P liberated/hr./mg. of protein N. In the columns headed activation the increase in activity due to 2:4-dinitrophenol (DNP) is given as a factor of the activity in its absence.

Additions	Tris-KCl medium (whole)				Tris-KCl without Mg ²⁺ ions			
	Mitochondria		Microsomes		Mitochondria		Microsomes	
	Activity	Activation	Activity	Activation	Activity	Activation	Activity	Activation
None	47	1	25	1	19.5	1	2.4	1
DNP (0.1 mM)	78	1.6	30	1.2	58.6	3	2.4	1
Mg ²⁺ (5 mM)	350	7.4	193	7.7	371	19	500	208
Mg ²⁺ (5 mM), DNP (0.1 mM)	356	7.5	180	7.2	410	21	500	208

Mg-activated adenosine triphosphatase was almost identical with that of the myometrial fractions although the skeletal muscle particles had lower specific activities (150 μ moles of P/hr./mg. of nitrogen for fraction P). The Mg-activated enzyme of myometrial microsomes is also higher than that of brain particles (Jordan & March, 1956), liver mitochondria and heart sarcosomes (Holton *et al.* 1957).

Adenosine triphosphatase and bivalent cations

The effect on activity of adenosine triphosphatase of other bivalent cations besides Mg^{2+} was also determined. The microsomal fraction was taken as a source of enzyme because it shows more activation with Mg^{2+} ions than the mitochondrial fraction. The results are shown in Fig. 2. All the cations studied activate particle adenosine triphosphatase, and in the following descending order: Mg^{2+} , Ca^{2+} , Mn^{2+} , Cd^{2+} . Activation is in every case greater than that due to dinitrophenol. In the presence of Mg^{2+} ions the cytoplasmic particles are capable of dephosphorylating ATP completely. Fluoride inhibits Mg-activated adenosine triphosphatase only partially (maximal inhibi-

tion being 45% at a F^- ion concentration of 0.05 M).

Oxidative phosphorylation

Respiration rates of rat-myometrial mitochondria were not stimulated by dinitrophenol or by phosphate acceptors such as ADP or AMP. Although they can oxidize substrates of the tricarboxylic acid cycle (see Table 5) at rates not very different from those of liver mitochondria (Lardy & Wellman, 1952), it was impossible to detect any phosphate uptake with ADP or AMP as acceptors. On the contrary, inorganic phosphate increased in the medium, presumably through the breakdown of these nucleotides. Since the equilibration period might have deleterious effects on the mitochondria, some experiments were done in which phosphate measurements alone were made. Phosphate increase was found, even when the samples were taken within 8 min. of the beginning of incubation. In one such experiment, when the substrate succinate was omitted from the medium, the increase in inorganic phosphate was 20% higher. This suggests some masked phosphorylation which might be detected with the use of labelled phosphate.

Since the adenosine-triphosphatase activity of fraction P is higher than that of M (see Table 3), reducing microsomal contamination of the mitochondrial fraction was attempted by centrifuging the latter at lower speeds (6 000 g); also, less extensive homogenization was tried, to avoid damage, but still no phosphorylation could be observed. Omission of Mg^{2+} ions and the use of F^- ions to lower the enzyme activity, as well as addition of phosphate acceptors such as creatine or glucose with hexokinase were tried, but without success. Plasma albumin which, with liver mitochondria, prevents action of the naturally occurring inhibitor, mitochrome (Polis & Shmukler, 1957), also had no effect.

It is therefore assumed that, besides unavoidable damage to the mitochondria, the very high adenosine-triphosphatase activity of this fraction is opposing the phosphorylative processes.

Fig. 2. Adenosine-triphosphatase activity of the microsomal fraction at varying concentrations of bivalent cations. For conditions of assay see Materials and Methods section. Curves (A) and (B) are from two different preparations.

Table 5. Oxidation of dicarboxylic acids by mitochondria from rat myometrium

Initial rates of oxygen uptake, given as μ l./hr./mg. of N, were measured at 25° in the presence of 5–15 mM-phosphate buffer, pH 7.4, 7.5 mM- $MgSO_4$, 2.5 mM-ATP, 0.01 mM-cytochrome c, 10 mM-substrate at pH 7.4 and 0.05 M-KCl.

Substrate	Preparation		
	1	2	3
Glutamate	338	—	277
2-Oxoglutarate	—	370	213
Succinate	—	—	289
Fumarate	—	103	—

DISCUSSION

The myometrium is a tough tissue to homogenize. This toughness is perhaps not as much due to a high percentage of collagen in the organ (Needham & Cawkwell, 1957) as it is to the viscosity of the muscle tissue itself. This can be noticed especially when comparing the consistency of the organ during pregnancy and after ovariectomy. The percentage of collagen in the latter state is higher, and yet manipulation of the homogenizer gives the feeling that the tissue is relatively more friable.

Having regard to this toughness it is not surprising that the isolated mitochondria of myometrial tissue do not possess all the properties of the fresh intact particles of liver or skeletal muscle. They are incapable of oxidative phosphorylation, and their ability to show reversal of swelling is not conclusive. However, they are capable of exhibiting activation of adenosine triphosphatase with dinitrophenol. No other case so far as we know has been described where dinitrophenol can activate the adenosine triphosphatase of mitochondria, which are themselves incapable of oxidative phosphorylation. The functional relationship between oxidative phosphorylation and dinitrophenol-activated enzyme has been discussed by Cooper & Lehninger (1957) and by Slater (1958). Since the microsomal adenosine triphosphatase is scarcely affected by dinitrophenol, activation in the mitochondrial fraction could not be due to contamination with microsomes.

It would be natural with the present experiments to suspect that this dinitrophenol activation is due to contamination of the mitochondrial fraction with myofilaments. This is possible since the adenosine triphosphatase of uterus actomyosin, like that of skeletal muscle, is also activated by dinitrophenol (Needham & Cawkwell, 1956). However, the following points argue against this view.

(i) To produce only a twofold activation of adenosine triphosphatase in isolated uterine actomyosin a concentration of dinitrophenol 10–50 times as high as that used for mitochondria is required (Needham & Cawkwell, 1956). Isolated myofibrils from the uterus have not been tested, but Perry & Chappell (1957), using isolated myofibrils from skeletal muscle, found no effect of dinitrophenol (1–5 mM) on magnesium-adenosine-triphosphatase activity in media of low potassium chloride concentration (0.05M).

(ii) A marked dinitrophenol activation for the myosin adenosine triphosphatase of both types of muscle is observed only at concentrations of potassium chloride above 0.1M, requiring at the same time the presence of the bivalent cation Ca^{2+} , not Mg^{2+} (Perry & Chappell, 1957; Greville & Needham, 1955; Needham & Cawkwell, 1956).

(iii) The adenosine triphosphatase of skeletal-muscle myofibrils, if affected at all, is slightly inhibited by dinitrophenol in the absence of activating cations (S. V. Perry, personal communication).

(iv) As with mitochondria from other tissues the dinitrophenol effect in myometrial mitochondria is abolished by aging.

The problem of assessing myofilament contamination of fraction M can perhaps be approached by the use of concentration-gradient solutions of sucrose in differential centrifuging, or by extraction

with 0.6M-potassium chloride after prior removal of proteins soluble at lower ionic strengths (Needham & Williams, 1959).

SUMMARY

1. Rat-uterine muscle from pregnant animals was homogenized in 0.3M-sucrose and fractionated by differential centrifuging to yield an easily sedimentable fraction, N, composed of cell nuclei, myofilaments and connective tissue; a mitochondrial fraction, M; a microsomal fraction, P; and a clear final supernatant.

2. Fraction M was contaminated to the extent of about 25% with fragments of collagen fibres. It could oxidize substrates of the tricarboxylic acid cycle, but was incapable of oxidative phosphorylation.

3. In the absence of metal activators the adenosine-triphosphatase activity of fraction M was about 10 μmoles of phosphate/hr./mg. of nitrogen; it was not increased by 'aging' but was stimulated about fourfold by 0.1 mM-dinitrophenol. Mg^{2+} ions produced a 10- to 20-fold stimulation of adenosine triphosphatase. The dinitrophenol stimulation was abolished by aging, but that due to Mg^{2+} ions was only slightly diminished.

4. Fraction P was composed of fragments of the sarcoplasmic reticulum (vesicles, many of which had attached dense bodies). It possessed an adenosine-triphosphatase activity greater than that of M; this activity was almost completely dependent on bivalent cations, and was practically unaffected by dinitrophenol.

5. The choice of medium for centrifugal fractionation is discussed.

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Cytoplasmic Fractions of Rat Myometrium

2. LOCALIZATION OF SOME CELLULAR CONSTITUENTS IN THE PREGNANT AND OVARECTOMIZED STATES

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Wakid (1960) gave a qualitative description of the various cytoplasmic fractions isolated from the myometrium of the pregnant rat. The present study is devoted to some quantitative aspects of changes in intracellular distribution that accompany the physiological changes in this muscle. Since the uterus at the end of pregnancy and after ovariectomy is at its two extremes in size and functional activity the comparison has been carried out on

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these two states in the hope of showing maximum contrast.

Telfer (1953) and Longwell & Reif (1955) have demonstrated that after oestradiol injection the increase in succinoxidase activity of the uterus of castrate rats is faster than the rate of growth of the organ on a dry-weight basis. Telfer surmises that her observed effects could 'well be due to an increase in the mitochondrial mass through duplication or enlargement of these bodies'. It was apparent in this connexion that isolation of the mitochondria by differential centrifuging would