THE EFFECTS OF ADENOSINE TRIPHOSPHATE ON THE FIBRE VOLUME OF A MUSCLE HOMOGENATE

by

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Observations in many laboratories during recent years leave little doubt, even if they provide no final proof, that actin, myosin, and adenosine triphosphate (ATP) are the essential components for muscular contraction. The striking contraction-like synaeresis caused by addition of ATP to actomyosin gels or threads at low salt concentration (SZENT-GYÖRGYI[1]), and, on a more organised system, the rapid shortening of glycerol-treated muscle fibres in presence of ATP (KOREY[2]), have been taken to support those theories which assume that relaxation of muscle coincides with energy release to the contractile machine by the enzymic dephosphorylation of ATP. On the other hand, the heat measurements of HILL (summarised by HILL[3]) cannot easily be reconciled with an active relaxation theory, while several observations on model systems would appear to find a more ready interpretation in terms of active, rather than passive contraction; in particular there may be noted the prevention of shortening of washed fibres (KOREY[2]), and of "contraction" of actomyosin gels (KUSCHINSKY AND TURBA[4]), by sulphydryl reagents, and the lengthening of loaded actomyosin threads caused by inorganic pyrophosphate and other pyrophosphate compounds which are not enzymically split (ENGELHARDT AND LIUBIMOVA[5]). The relation between contraction-like effects and the presence or decomposition of ATP is thus obscure, and indeed the supposedly intimate connection between synaeresis of actomyosin and contraction of muscle is by no means firmly established (PERRY, REED, ASTBURY AND SPARK[6]).

The present investigation was commenced to study the effect of ATP on the water retention of a muscle homogenate, and was suggested by the familiar observation that, when fresh muscle is minced, the sarcoplasmic fraction becomes more readily extractable as glycolysis proceeds, while the extraction of fibrillar protein becomes more difficult. Although fluid retention is greatly diminished by a fall in pH (EMPEY[7]), this does not appear to be the whole explanation since the expression of muscle juice is facilitated by post-mortem glycolysis even when acid production is minimised; furthermore, the striking effect of ATP on actomyosin suggests that hydrolysis of ATP might alter the water relations of muscle during the onset of rigor mortis. The investigation has proved unexpectedly fruitful in revealing the presence in muscle of a labile factor which profoundly affects the volume response of muscle fibres to added ATP. A brief account of this work has already appeared[8].

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EXPERIMENTAL

The modified centrifuge

It was suggested by Dr K. BAILEY that a laboratory centrifuge might be modified in some way to permit continuous reading of the volume of the fibre layer of a muscle brei during centrifugation. This was achieved quite simply in the following manner.

A 60 watt lamp was placed below a slit in the base of the centrifuge, vertically below the horizontal position of the spinning tube, and a corresponding slit (for viewing) was cut in the lid of the machine, vertically above the light source. Two slits were made in a metal centrifuge cup, each about 5 cm × 1 cm, diametrically opposite and in line with the axis of the cup, and in such positions that, when the cup was spinning horizontally, light passed from the source, through both slits and out of the viewing slit in the lid, once during each revolution. The counter-balancing cup was not modified.

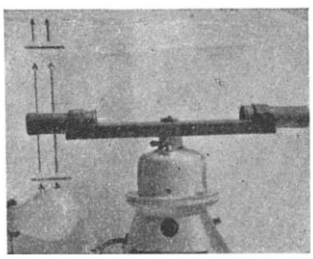
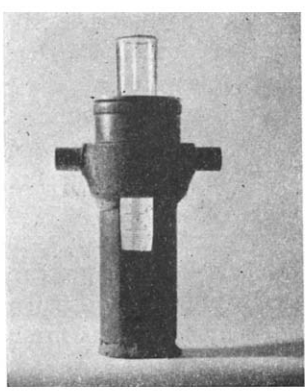
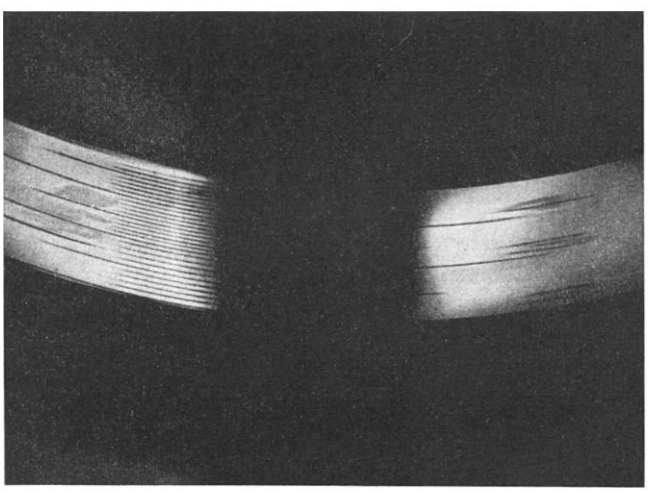


Fig. 1. The modified centrifuge. Casing removed to illustrate position of light source and path of light beam.

When a graduated centrifuge tube containing liquid was spun in this apparatus, not only the graduation lines, but also the etched numbers were clearly visible through the fluid. When the tube contained both solid and liquid layers, the heavier material appeared as a uniform black area while the supernatant fluid allowed the transmission of light to the observer. There was a very sharp boundary between the two phases, and the volume of the more solid phase could be read continuously by reference to the lowest graduation line visible through the liquid layer. The effect was independent of centrifuging speed, and was as clear when direct current replaced A.C. mains to supply the light source. The apparatus bears no resemblance to the stroboscopic microscope-centrifuge of HARVEY[9].





a b

Fig. 2. a: The centrifuge cup, showing position of the slits relative to the graduations on the centrifuge tube; b: Appearance of the same cup when spinning at 1000 r.p.m. The direct beam from the light source has been removed by a shield (central black area) during photographic exposure.

The centrifuge is illustrated in Fig. 1, and the appearance of a tube during and after spinning is shown in Fig. 2.

Materials

The psoas and adductor muscles of rabbits were used throughout the investigations. Most of the animals were well fed and were killed by decapitation after thirty minutes' complete relaxation produced by intraperitoneal or intravenous injections of myanesin (BATE-SMITH AND BENDALL[10]).

Adenosine triphosphate (ATP) was prepared by the method of LOHMANN as described by NEEDHAM[11].

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Technique

Following the example of the SZEGED school[1], potassium chloride (0.16 *M*) was the medium generally used in the present work. About 2 g muscle were homogenized in 8 ml of this solution for ½–3 minutes, using the apparatus of MARSH AND SNOW[12], and the brei was quickly transferred to a graduated centrifuge tube (10 ml, 0.1 ml divisions) in the modified centrifuge, which was rapidly accelerated to about 2,800 r.p.m. (relative centrifugal force at tip of tube about 1750). Taking zero time at the commencement of centrifuging, the volume of the heavier fibre layer was recorded against time as its surface level reached each graduation mark.

A considerable decrease in fibre volume occurred due merely to centrifugal packing, but this could be practically eliminated in one of two ways, depending on the initial state of the muscle. (a) When fresh, pre-rigor muscle was examined, the complex curve relating volume to time of centrifuging was "corrected" for the simple packing effect by dividing the observed volume by that of a brei of rigor muscle at the same time of spinning. Trials showed that the fibre volume of a homogenate of muscle in full rigor decreased smoothly with time of centrifuging, no further change being detected after about 80 minutes, and the volume at any other time was expressed as a multiple of the 80-minute volume (*e.g.* 3-minute volume = 1.20; 20-minute volume = 1.09, etc.). This series of values was used to "correct" other curves for simple centrifugal packing. The method is illustrated in Fig. 3. (b) When the effect of added reagents, notably ATP, was examined on muscle homogenized after the development of rigor mortis, it was found simpler to obtain first a "control curve" for that particular brei. The small fibre plug was then re-incorporated with the supernatant fluid by stirring, the reagent was added to the suspension of fibre pieces, and the fibre volume was again observed in the centrifuge. The second curve obtained was then corrected for simple packing by dividing the observed values by those of the control curve at corresponding times of spinning. This is illustrated in Fig. 4. It will be observed that, while method (a) provides a final curve

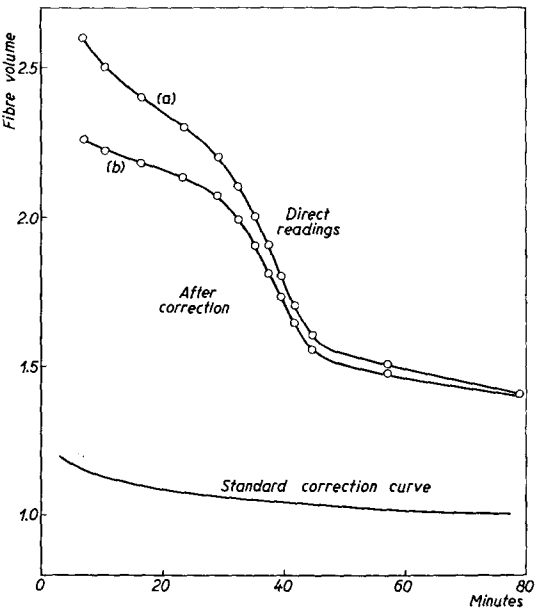

Fig. 3. The fibre volume of a homogenate of fresh muscle as a function of time of centrifuging. a: curve obtained by direct observation; b: the same after dividing each observed value by a correction factor (derived from the standard correction curve) to eliminate simple centrifugal packing.

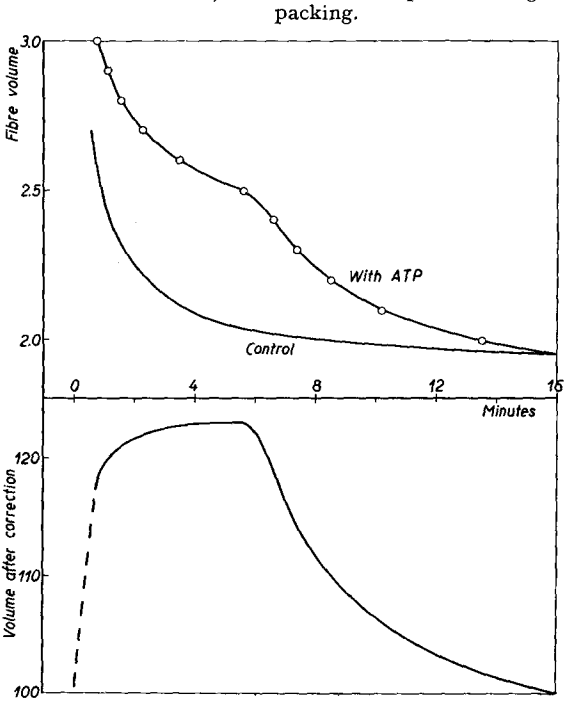

Fig. 4. The swelling effect on fibre volume of added ATP. Upper: Fibre volume before and after addition of ATP to a brei of rigor muscle. Lower: Volume behaviour after correcting for simple centrifugal packing. 1 mg ATP–P added at time 0.

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expressing volume in ml, method (b) gives a curve in which volume is represented as a percentage of that in absence of the added reagent.

RESULTS

Fibre volume and rigor mortis

When the fibre volume of a fresh actively-glycolysing muscle brei was examined as a function of time of centrifuging, a curve resembling (a) in Fig. 3 was obtained, and on correction for packing this was transformed to curve (b) in that diagram. Three more corrected curves are illustrated in Fig. 5, selected at random from over fifty such observations. In all these experiments each curve consisted of three distinct phases which for convenience will be described as pre-rapid, rapid, and post-rapid, respectively. The fibre volume at commencement of the rapid phase may be considered as unity to permit calculation of rate and extent of volume diminution, and to aid these determinations, the three linear phases may be extrapolated to give precise values at their points of intersection for volume and time at the beginning and end of the rapid phase.

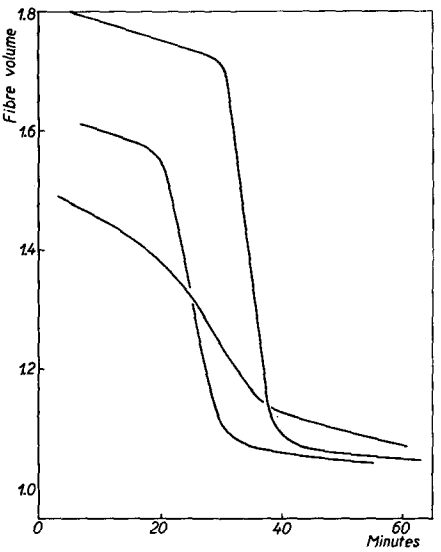

Fig. 5. The fibre volume of a fresh muscle homogenate as a function of time of centrifuging, after application of packing correction curve. Illustrating the three phases invariably seen on centrifugal observation of a brei of fresh muscle in 0.16 *M* KCl.

The pre-rapid phase occupied up to 50 minutes, its duration being markedly affected by glycogen reserves (regulated by control of feeding) and by the degree of exhaustion of the animal at death (controlled by myanesin). In these respects it may be compared with the delay period preceding the elasticity changes which accompany the onset of rigor mortis in whole muscle (BATE-SMITH AND BENDALL[13]). Thus, well-fed animals treated before death with myanesin almost invariably had muscles which, taken soon after death, showed a pre-rapid phase of at least 20 minutes, and occasionally over 40 minutes, duration, while at the other extreme the muscles of two insulin-treated animals with negligible glycogen content (ultimate pH 7.16, 7.18) showed in one case a pre-rapid phase of three minutes and in the other this phase was complete before observations could be commenced. There was little or no volume decrease during the pre-rapid phase, the observed diminution being almost exactly accounted for by the packing effect, and except in two cases the rate of decrease did not exceed 0.5% per minute, calculated on the volume at commencement of the rapid phase.

The rapid phase commenced quite suddenly, the rate of volume diminution sometimes increasing ten-fold within five minutes after a pre-rapid phase occupying several times this period. The rapid phase showed wide variations in velocity and extent, the briefest lasting only 3½ minutes and the longest 21½ minutes, while the volume decrease in the fibres varied from 17 to 46% of the value at commencement of the rapid phase. The rate of volume diminution varied from 1–8% per minute, which, on individual

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curves, represented an increase over the rate of shrinkage in the pre-rapid phase of 5–10 times.

The post-rapid phase. The decrease of fibre volume ceased quite abruptly at the end of the rapid phase, and the volume thereafter remained constant or decreased only slowly at about the same rate as in the pre-rapid phase. No further change in volume behaviour occurred, this phase being linear for as long as observations were continued.

Relation between velocity and extent of synaeresis. A large volume decrease was associated with a rapid phase of short duration, and some degree of control over these variables was possible in that tissue homogenized for a longer period exhibited greater synaeresis, the rapid phase occupying a shorter time interval. Thus a fibre volume decrease of 20% and occupying 20 minutes was observed in a brei, the preparation of which involved homogenizing for 30 seconds; while muscle reduced by treating for 3 minutes diminished in fibre volume by about 40% in 6 minutes. This is better expressed in Fig. 6, in which each of the 36 points represents the relation between the velocity and extent of synaeresis in the rapid phase of one homogenate. Analysis in this way was sometimes impossible since the pre-rapid phase was occasionally too brief to permit accurate determination of its linearity and slope, while sometimes the post-rapid phase was obscured by a light precipitate, presumably denatured myogen, which descended from the supernatant liquid to blur the boundary and prevent further observation. Nevertheless there are sufficient points to indicate a definite relation.

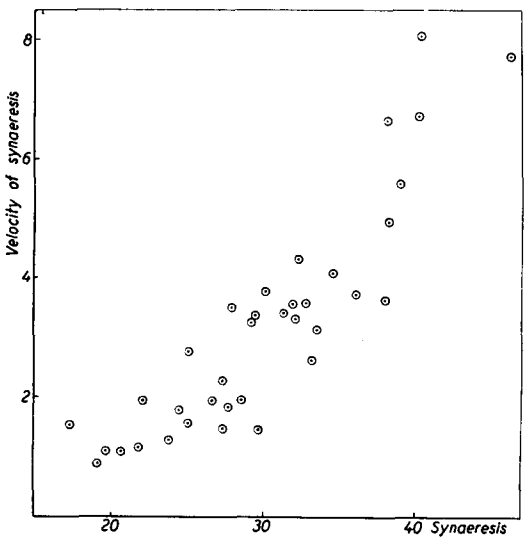

Fig. 6. The extent and velocity of fibre synaeresis in centrifuging homogenates of fresh muscle. Extent: Fibre volume decrease, during rapid phase, as a percentage of initial volume. Velocity: Extent of synaeresis per minute during rapid phase.

Effect of varying KCl concentration. The three-phase diagram relating fibre volume to time of centrifuging was observed when homogenates were prepared in water or in KCl solutions of concentrations up to 0.4 *M*. The turbid supernatant liquid and blurred boundary obtained when molarities of more than 0.3 were used made volume observation difficult, but in all cases the same type of behaviour was recognizable. The pre-rapid phase was shortened considerably when the molarity was low; the duration of this phase in water was only 3½ minutes compared with 12 minutes when another sample of the same muscle was examined in 0.16 *M* KCl.

Effect of other ions. Centrifugal examination of a brei prepared in RINGER-TYRODE solution (Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, $H_2PO_4^-$, HCO_3^-) revealed a volume/time relationship very different from the normal three-phase behaviour. An immediate synaeresis occurred, the fibre volume decreasing very rapidly to only one half the expected value. There was no suggestion of a pre-rapid phase, and within a few seconds of commencement of centrifuging the fibres had packed so tightly that their volume remained almost unaltered despite prolonged spinning. Further investigations, using solutions of TYRODE

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constituents either alone or in various combinations as homogenizing fluid, showed that calcium was the ion responsible for the new type of volume behaviour. When calcium was omitted from the TYRODE solution the usual three-phase diagram was observed, but the presence of 0.02% calcium chloride, either alone or with other salts, invariably permitted only the sudden synaeresis.

Effect of iodoacetate. Several homogenates were examined after preparation in 0.16 *M* KCl + 0.002 *M* sodium iodoacetate to prevent completely the formation of lactic acid. On centrifuging, the three-phase diagram was obtained in all cases, but the duration of the pre-rapid phase was shortened considerably. Thus in one pair of experiments this phase lasted only 10 minutes in the presence of iodoacetate while the control (without inhibitor) showed a delay of 26 minutes. On another occasion the corresponding times were 7 and 22 minutes respectively. The observed synaeresis is not conneced directly, therefore, with post-mortem acid production, but must obviously be intimately associated with a change occurring fairly early post-mortem.

The effect of added ATP on fibre volume

To test the hypothesis that the dephosphorylation of ATP was related to the rapid phase of volume diminution, the ester was added back to the shrunken system in amounts of about 1 mg labile-P (in 1 ml solution) per 10 ml brei (containing about 2 g muscle). One or other of two large and very different effects was caused by this treatment – a freely reversible increase in fibre volume or an irreversible synaeresis.

Reversible volume increase

ATP addition to a muscle brei which had passed through a rapid phase of shrinkage only shortly before almost invariably produced the effect illustrated by Fig. 4. The fibre volume as read, before applying the correction factors for simple centrifugal packing, was observed to remain fairly constant, or to decrease less than that of the fibres of the control brei, for some minutes. This phase was followed by a rapid volume decrease which ceased when the volume was about equal to that of the fibres of the control homogenate after the same time of centrifuging. After applying the control curve to eliminate the packing effect the volume changes resembled the lowest curve of Fig. 4, consisting of a brief period of rapid volume increase of 15–25%, a phase of up to five minutes duration during which this high level was maintained or slightly increased, and another brief period during which the fibre volume decreased rapidly to about its original value. This series of changes usually occurred when ATP was added to a rigor brei prepared on the same day as the animal was killed, and the same effect was observed in about two of every three experiments when this procedure was applied to a brei prepared from rigor muscle after storage for 24 hours at 0° C. Volume reversal could often be repeated several times on the same brei by further additions of ATP, although maxima and minima tended to be slightly lower with each repetition.

The effects of the presence of other salts on this volume behaviour was examined. ATP was added to homogenates prepared in solution of TYRODE constituents, and with one exception, fibre volume increases were observed in all cases where the fibres of a control brei in KCl showed the swelling effect. In the presence of 0.02% $CaCl_2$, however, this was replaced by irreversible synaeresis. The volume response to added ATP therefore paralleled exactly that of homogenates of fresh muscle prepared in various solutions as previously described.

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Homogenates of rigor muscle in isotonic KCl were unaffected by the addition of inorganic phosphate (4 mg P) or of inorganic pyrophosphate (4 mg acid-labile P).

Irreversible synaeresis

Addition of ATP to a brei of *rigor* muscle frequently caused an immediate and considerable synaeresis instead of the fibre swelling previously described. This shrinkage was extremely rapid and was almost invariably complete within about 20–30 seconds. Its onset could often be recognised, before centrifugal observation was recommenced, by a characteristic increased whiteness of the fibre particles. The volume behaviour is illustrated in Fig. 7. The apparent slight swelling which followed the large synaeresis, as shown in the lowest curve of that figure after correcting for centrifugal packing, was observed in all cases of considerable shrinkage, and would appear to be due merely to the extremely "superpacked" nature of the particles. The appearance of these pieces was very different from that of the fibres of the control brei, and their density, after losing almost half their water content during synaeresis, must have been greater than before ATP addition, so it is not surprising that the particles failed to follow exactly a course related linearly to that taken by the fibre pieces of the control homogenate. The slight volume increase following synaeresis is considered, therefore, to be of no real significance.

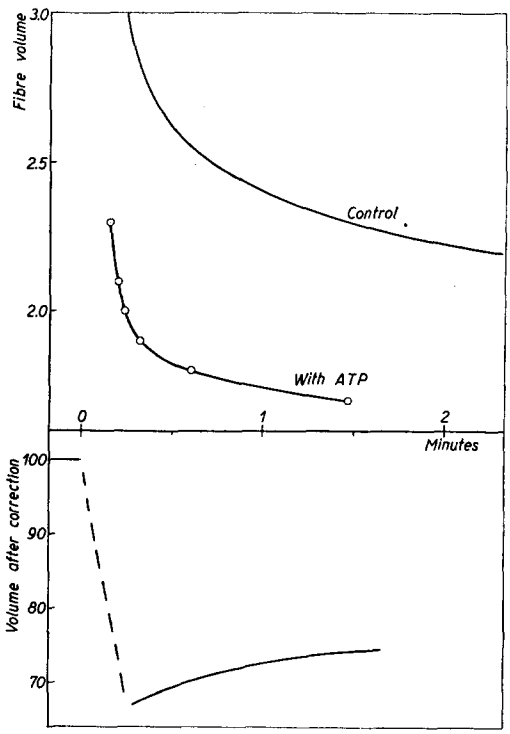

Fig. 7. The shrinking effect on fibre volume of added ATP. Upper: Fibre volume before and after ATP addition to a brei of rigor muscle. Lower: Volume behaviour after correcting for simple centrifugal packing. 1 mg ATP–P added at time 0.

Well over fifty volume shrinkages of this type were observed in the course of the investigation, and by comparing the volumes before and after ATP addition after one minute's centrifuging in each case, a mean volume diminution of 23% was calculated, with extreme limits of 11% and 43%. No method of reversing this shrinkage was found. Washing the fibres to remove ATP or further addition of ATP proved equally ineffective, the volume/time relationship remaining unchanged from that of the first large shrinkage.

Intermediate behaviour

In addition to the very distinct effects described above, many experiments produced volume/time curves of greater complexity. In some, for instance, a volume increase of 5–10% above the control was followed after 2–3 minutes by a rapid phase during which

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the volume decreased to 3–6% below that of the control. In other cases an immediate synaeresis of about 8% was followed by a swelling which increased the fibre volume almost to that of the control, and a second synaeresis then reduced the volume to a steady value about 8% below that of the control. These curves were at first mistaken for other quite distinct types of volume behaviour, but it later became obvious that the systems were all displaying effects intermediate between reversible swelling and irreversible synaeresis. By graphically compounding these two effects in varying ratios it was possible to reproduce even the most complex curves obtained in centrifuge experiments. All the observed effects were therefore the resultant of two volume changes of similar magnitude but opposite in direction and of different rates. While one was clearly a reversal of the synaeresis observed when the fibre volume of a fresh, actively-glycolysing muscle brei was examined, the other appeared to be directly related to the synaeresis of extracted actomyosin and the shortening of glycerol-treated muscle fibres induced by added ATP.

The factor controlling volume changes

To this point, the only clues to the possible nature of the factors influencing the response of the fibres were the apparent sensitivity to calcium of the volume-swelling mechanism, and the increased tendency toward fibre shrinkage with increasing age of the preparation. Other factors were investigated.

The effect of pH. The volume response to added ATP was found to be unaffected by the presence of 0.002 *M* iodoacetate. In order to prepare homogenates of fixed pH a sample of muscle was homogenized in 0.16 *M* KCl, and when the pH had attained the required value iodoacetate was added. Creatine phosphate and ATP decomposition were complete within a few minutes, after which the centrifuge test could be performed with no possibility of pH shift. In the pH range 7.2–5.8, to which observations were confined, pH had no consistent effect on volume response to ATP, and both swelling and synaeresis were found at any pH within this range. However, the *extent* of volume change was markedly dependent on pH, the total change (maximum increase above control + maximum decrease below control) decreasing from 20–25% at pH 6.7–7.2 to 8–10% at pH 5.8–6.3.

The effect of ATP concentration. Although immediate synaeresis could be produced by adding lesser amounts of ATP, it became clear that this was not the essential difference between the two volume effects since on many occasions immediate synaeresis followed the addition of very appreciable amounts of ATP. Nevertheless the results were of some interest; in those systems which displayed fibre swelling on the addition of appreciable amounts of ATP, the minimum concentration of that ester required to cause a full volume increase was found to be 0.07 mg ATP-P per g muscle (assuming that the distribution of added ATP was uniform throughout both fibre pieces and suspending medium). Maximum immediate synaeresis was brought about by the addition of 0.02 mg labile P per g muscle.

The sarcoplasmic factor. Replacement of the supernatant liquid of a centrifuged homogenate by fresh 0.16 *M* KCl solution altered entirely the volume response to ATP. In those systems which swelled on addition of ATP, the intermediate type of volume behaviour was obtained merely by re-suspending the fibres in fresh KCl solution, and when the fibre pieces were twice washed in KCl solution with alternate centrifuging, they responded to ATP addition by shrinking immediately. Some degree of control over

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the volume effects was now possible in that synaeresis could be brought about at will by a standard procedure, as illustrated by Fig. 8, but of greater significance was the indication of the existence of a substance, soluble in 0.16 *M* KCl solution, which must be intimately concerned in volume increase effects. This will be referred to as the "factor"

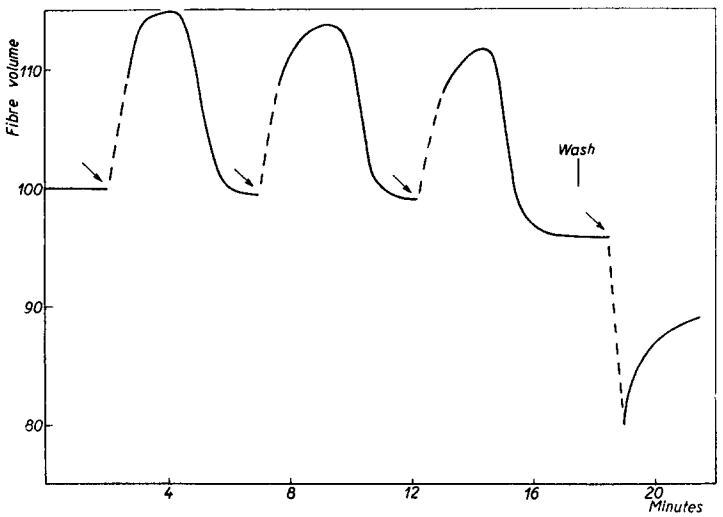

Fig. 8. Reversible volume increase, and the irreversible synaeresis observed after washing the homogenized fibres. Fibre volume expressed as a percentage of the control volume after the same time of centrifuging. 0.76 mg ATP–P added at each arrow.

Nature of the factor

Solutions suspected to contain the factor were examined by observing in the centrifuge the volume response to added ATP of washed fibres suspended in the solution. Absence of the factor was shown by an immediate synaeresis, and presence of the factor by reversible volume swelling, while intermediate behaviour—a slight swelling followed by appreciable synaeresis—was interpreted as an indication of the presence of the factor in diminished quantity.

Evidence for protein nature. The constituents of TYRODE solution, either separately or in combination, permitted only irreversible synaeresis when they were tested with washed fibres. The supernatant fluid from a spun homogenate lost all factor activity after removal of heat-coagulated material (2 minutes at 60°), or by removal of the precipitate formed after neutralization to pH 7 of an acidified aliquot (pH 3.6, HCl). These demonstrations of the lability to heat and acid of the factor suggested that it consists, at least in part, of protein material, and extracts (known by the centrifuge test to contain the factor) were therefore dialysed at 0° C for 16 hours in cellophan sacs against either distilled water or 0.16 *M* KCl. Of six such experiments, four showed that no factor activity remained within the sac, while the other two showed partial activity estimated from the positions of their centrifuging curves to be about 25% and 50%. In no case could any activity be detected in the outer dialysing fluid, and when this was concentrated and added to the inner solution no enhancement of activity was observed. In all these experiments a small amount of precipitated protein failed to redissolve in KCl solution. A rapid dialysis (4 hours) against ice-cold distilled water, with frequent changes

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of fluid and continual agitation of the sac did not inactivate the volume-reversing factor, full activity remaining within the sac. On this occasion all the precipitated protein readily redissolved in 0.16 *M* KCl solution.

Fractionation with ammonium sulphate failed to provide any information, possibly because of the necessity of prolonged dialysis, and elution by phosphate buffer of proteins adsorbed on calcium phosphate also failed, no fraction showing any factor activity after dialysis.

The magnesium-activated ATP-ase described by KIELLEY AND MEYERHOF[14] was prepared, but showed no activity. The same result was obtained when tests were made using several relatively pure glycolytic enzymes (phosphorylase, lactic dehydrogenase, aldolase, enolase, creatine phosphokinase, and triose phosphate dehydrogenase) kindly supplied by Dr. B. A. ASKONAS[15].

Function of the factor. It was found that the rate of dephosphorylation of ATP by the fibres of a muscle brei varied over a wide range, being considerably higher, in general, when the fibres were washed before incubation with ATP. A closer examination revealed a correlation between presence of the volume-reversing factor and diminution in the ability to split ATP. Those systems, the fibres of which increased in volume on ATP addition, released inorganic phosphate from excess ATP at a rate of only about 40 μg P/minute/g muscle, while in those homogenates which responded to added ATP by immediate fibre synaeresis, inorganic phosphate appeared at the rate of about 300–400 μg P/minute/g muscle. (All measurements were made at 17° C). Since almost 10 μg P were produced per minute per g muscle by soluble enzymes in the former case, the ratio of fibrillar ATP-ase activities in the two systems was about 1:10. No separation of this apparent ATP-ase inhibition from the fibre-swelling factor has been achieved. The former effect was not due to aerobic resynthesis of ATP since the presence of 0.01 *M* cyanide did not alter the rate of production of inorganic phosphate. Neither was it due to a preferential diversion of ATP to other reactions (for instance, the phosphorylation of fructose-6-phosphate), since more complete analysis showed that the amount of ATP removed during incubation agreed with the amount of inorganic phosphate produced, and no accumulation of other phosphate esters was detected.

Changes in micro-structure

Although the volume changes reported above suggest that changes in fibre length were involved, the swelling and synaeresis might conceivably have been caused merely by changes in rigidity of the fibre pieces, or by alterations in fibre diameter at fixed length. Changes in systems displaying either increased or decreased volume immediately on ATP addition were examined microscopically (× 100). A drop of a suspension of fibre particles in either 0.16 *M* KCl solution or in an extract containing the swelling factor was placed within a wax circle of 4 mm diameter mounted on a glass slide. ATP solution was added by pin-point and stirred into the suspension, and measurements were made at intervals.

Those fibres suspended in KCl solution responded very rapidly to added ATP and within a few seconds had attained their ultimate shape. Their length decreased by about 60% and their breadth increased by about 40%, causing an overall volume decrease of approximately 20%. In presence of the volume-increasing factor the effect was very different, the fibre lengthening by about 20% during the first minute, and by a further 20% in the next ten minutes when shortening did not intervene before this time. Short-

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ening then occurred at a rate of 10–20% per minute till the particle had returned to within 10% of its initial length, after which no further change occurred. No alteration in diameter was detected, but as attention was directed towards length changes, the possibility of breadth alterations is not excluded. The duration of the phase of increasing or high volume was long compared with the corresponding phase in the centrifuge tests, possibly because ATP removal in the former case was achieved solely by enzymic decomposition while in the centrifuge this process was augmented by centrifugal transfer of ATP from the fibres to the supernatant fluid. Apart from this, the observations on micro-structure were entirely consistent with the volume behaviour of the fibres during observation in the centrifuge. In particular, fibre swelling corresponded to lengthening, and synaeresis to shortening.

DISCUSSION

It may be argued, with some justification, that the volume and length changes in muscle fibre pieces described in this work are related only to the onset and reversal of rigor mortis. Certainly the slowness of the changes and the fact that fibre shortening and synaeresis occur only at low ATP concentrations might be taken as evidence that the observations have no connection with muscular contraction. Nevertheless the magnitude of these changes and their obvious dependence on an intimate relation between fibre proteins and ATP suggest that the system may be regarded as a model muscle undergoing contraction and relaxation. Indeed, the physiological completeness, high degree of fibrillar organisation, and ready reversibility of volume changes in the present work suggest that the muscle homogenate is more nearly a true model system than actomyosin threads or glycerol-treated fibres.

From the evidence of electron micrographs, PERRY *et al.*[6] concluded that the synaeresis of actomyosin under the influence of ATP is not the process underlying the act of muscular contraction. The present work seems in no way incompatible with this conclusion. The partial dehydration of the fibres of the muscle homogenate is regarded, not as the fundamental cause, but rather as one effect, of fibre shortening; the volume changes are merely a useful guide to the extent of those fundamental changes in molecular configuration which affect the state of hydration. That such large volume changes are not observed in living muscle is no argument against the validity of comparing the homogenate with intact muscle for, whereas the fibre pieces have been so extensively damaged as to permit the ready outflow of fluid, this change is paralleled in the intact muscle by an appreciable increase in pressure during contraction (HILL)[16]. Thus, while agreeing with PERRY *et al.*[6] that an actomyosin system cannot, "by a process of alternate dehydration and rehydration, reproduce the mechanical properties of the cell", we consider that synaeresis may still provide, in certain circumstances, a valuable index to more deep-seated changes of molecular configuration.

The relaxation-like effect observed in those systems which swelled on ATP addition appears to be a new phenomenon in muscle models, the only comparable effect being a lengthening of actomyosin threads, when loaded, under the influence of the pyrophosphate group. Since fibre swelling has been shown to depend upon the presence of an extremely labile factor, it is not surprising that other model systems show no similar response to ATP; solubility of the factor in water and low salt concentrations would ensure its absence from actomyosin threads or gels, while due to its instability it would be absent

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from glycerol-treated fibres. As reported here, the factor appears to be accompanied by an ability to suppress the ATP-ase activity of the fibril, and it will be assumed that the fibre-swelling effect is associated with this enzymic inhibition. It must be admitted, however, that the only reason for supposing the identity of the volume-increasing factor and the ATP-ase-inhibiting factor is the simultaneous disappearance of both from an extremely complex system, and the components responsible for the two effects may prove to be different substances, alike only in their instability. Should only one component be responsible for both effects, it is suggested that the rate of ATP decomposition will be a much more sensitive guide to presence or absence of the factor than the centrifuge test, which in the present work provided only a semiquantitative determination of factor activity, and might conceivably have failed to record relatively appreciable amounts of the component.

Assuming that fibre-swelling and enzyme inhibition are intimately related—possibly by a masking of essential groups of myosin, or by formation of a "protective conjugation" between factor and ATP, as suggested by ENGELHARDT[17]—how are the changes in fibre length and volume to be explained in relation to the presence or decomposition of ATP? In those systems which displayed fibre swelling on addition of ATP, presence of the ester in appreciable concentration was greatly prolonged, and an increase of volume and length is therefore to be associated with the *presence*, rather than with the rapid breakdown, of ATP. On the other hand, in those homogenates which exhibited immediate synaeresis, the relatively high ATP-ase activity ensured that presence of the ester in appreciable amount was maintained for no more than a very brief period of time. If ATP-ase activity exceeded the rate of ATP diffusion into the fibre pieces, ATP could not attain, even momentarily, a significant concentration at the site of its interaction with the contractile proteins, yet energy release proceeded at a high rate. A volume decrease is thus to be associated with the near *absence* of ATP, coinciding with, or preceded by, its rapid breakdown. This explains why the presence of calcium permitted only synaeresis on ATP addition; calcium is a powerful activator of myosin ATP-ase (BAILEY)[18] and its presence would be expected, therefore, to exert an effect opposite to that of the labile factor.

Similarly the effect of adding smaller amounts of ATP, in presence of the factor, finds a simple explanation. Addition of the ester in large amount causes fibre swelling since an appreciable concentration of ATP can be maintained. Addition of small amounts of ATP causes only synaeresis because, even at a low rate of dephosphorylation, energy is being supplied to the contractile system while at the same time ATP is present in insufficient amount to maintain saturation of the relevant centres. The conditions for synaeresis—low concentration of ATP and breakdown of the ester—are thus satisfied. Since 0.07 mg ATP-P per g muscle is required to permit a complete reversible cycle of fibre swelling, while 0.02 mg ATP-P causes maximum immediate synaeresis in 1 g muscle, it is justifiable, on the above reasoning, to assume that 0.02 mg ATP-P is that amount which just saturates the ATP-reacting groups of 1 g muscle, while 0.05 mg ATP-P is the minimum amount required to maintain this saturation while maximum increase in volume is being attained. Since ATP decomposition proceeds, in presence of the factor, at a rate of about 40 μg P/minute/g muscle, the duration of the phase of rapidly increasing fibre volume should be about $1^1/_4$ minutes, and it was indeed found that this phase lasts about 1–2 minutes in all cases.

It is also of interest that, assuming 100 mg myosin/g muscle, 0.02 mg ATP-P per g

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muscle corresponds to 1 mole of ATP per 310,000 g myosin. This may be compared with the ratio of 1 mole of ATP per 100–300,000 g myosin required to dissociate completely the actomyosin complex in 0.5 *M* KCl (MOMMAERTS)[19]. Interpreting these observations on the intermolecular level, it is considered that the contractile proteins of relaxed muscle are maintained in the dissociated (actin + myosin) state because of the greater affinity of the sulphydryl groups of myosin for ATP than for actin (BAILEY AND PERRY[20]). Because of the presence of the labile factor reported above, the rate of ATP breakdown is slow, and consequently a state where ATP is temporarily absent does not occur; hence actomyosin cannot be formed. Contraction involves a transformation to the actomyosin condition, due to (a) an increased rate of energy release from ATP, and (b) a temporary absence of ATP from the vicinity of the –SH centres of myosin—these changes are caused by increased ATP-ase activity, as a result of either Ca^{++} release[18], or a temporary inactivation of the labile factor, or both effects. Relaxation is regarded as a return to a low ATP-ase activity by reversal of factor inactivation or by removal of Ca^{++}, this permitting accumulation of ATP which dissociates actomyosin to its component proteins, and the structure imbibes water. This swelling may be due to the greater water-retaining ability of the dissociated protein, or to the relatively greater freedom of the component proteins, or perhaps to the elastic properties of the sarcolemma which, on disruption of the actomyosin structure by ATP, might attempt to regain its normal resting length.

The above demonstration that ATP-ase activity in muscle fibres can be greatly diminished by the factor directs attention to the relatively high rate at which ATP dephosphorylation proceeds in other model systems. Analysis merely of the medium containing such systems after "contraction" induced by ATP might well indicate that only a small amount of the ester has been decomposed, but it by no means follows that "contraction" is due to the presence (as distinct from the decomposition) of ATP. Provided that, in the model itself, and not in the medium, ATP dephosphorylation can keep pace with inward diffusion of the ester, contraction-like effects will necessarily be observed.

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SUMMARY

1. By means of a centrifuge simply modified to permit continuous reading of the fibre volume of a spinning muscle brei, a decrease in the fluid-retaining ability of fresh homogenized fibres has been detected. This synaeresis, amounting to 17–46% of the initial volume, occurs after an interval of up to 50 minutes commencing when ATP has virtually disappeared.

2. Addition of ATP to the shrunken system causes either a volume increase of 10–25% followed by a decrease to about the original volume, or an immediate irreversible synaeresis of 10–40%. The former effect is accompanied by reversible fibre lengthening, and the latter by irreversible shortening.

3. The essential difference between the two effects is due to the presence in the former system of a labile component, probably a protein, in the absence of which ATP addition invariably causes irreversible synaeresis.

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4. The labile factor appears to exert its effect by diminishing greatly the ATP-ase activity of the homogenized fibres.

5. The results are discussed in relation to the behaviour of other model systems and to muscular contraction.

RÉSUMÉ

1. Une modification légère apportée a une centrifugeuse nous a permis de mesurer sans interruption le volume pendant sa centrifugation des fibres d'un "brei" de muscle. On signale par ce moyen une synérèse de fibres fraîches et homogénéisées qui varie de 17% a 46% de leur volume primitif; cette synérèse commence lorsque l'ATP est virtuellement éliminé et s'accomplit en 50 minutes au plus.

2. L'addition d'ATP aux fibres rétrécies cause, soit une augmentation en volume de 10–25%, que suit un retour, ou à peu près, au volume primitif, soit une synérèse nouvelle de 10–40%, celle-ci instantanée et irréversible. De ces résultats, le premier s'accompagne d'un allongement réversible, le second, d'un raccourcissement des fibres.

3. La différence capitale entre ces résultats s'explique par la présence, dans le premier cas, d'un constituent labile (et probablement, protéinique): en l'absence de ce constituant l'ATP cause toujours une synérèse irréversible.

4. Il paraît probable que l'action de ce constituant labile consiste en une importante réduction de l'activité ATP-asique des fibres homogénéisées.

5. Ces résultats sont discutés par rapport au comportement d'autres systèmes modèles et au problème même de la contraction musculaire.

ZUSAMMENFASSUNG

1. Durch eine geringe Onderung an einer Zentrifuge konnte das Volumen der Fasern eines Muskelbreies kontinuierlich abgelesen werden. Auf diese Weise wurde eine Verminderung der Flüssigkeits-Retention von frisch homogenisierten Fasern festgestellt. Diese Synerese, welche 17–46% des Anfangsvolumens beträgt, beginnt, wenn das ATP praktisch verschwunden ist und dauert bis zu 50 Minuten.

2. Zugabe von ATP zu dem geschrumpften System bewirkt entweder eine Zunahme des Volumens von 10–25%, welche von einer Abnahme bis zum ursprünglichen Volumen gefolgt ist, oder eine sofortige irreversibele Synerese von 10–40%. Der erstgenannte Effekt ist von einer reversibelen Faserverlängerung, der letztgenannte von einer irreversibelen Faserverkürzung begleitet.

3. Der wesentliche Unterschied zwischen den zwei Effekten wird durch die Gegenwart einer labilen Komponente, wahrscheinlich eines Proteins, im ersten System, bewirkt, in Abwesenheit dieser Komponente hat die Zugabe von ATP immer eine irreversibele Synerese zur Folge.

4. Der labile Faktor scheint zu wirken, indem er die ATP-ase-Aktivität der homogenisierten Fasern bedeutend vermindert.

5. Die Ergebnisse werden im Verhältnis zu dem Verhalten anderer Modellsysteme und zur Muskelkontraktion erörtert.

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