CALCIUM-BINDING PROTEINS IN THE VORTICELLID SPASMONEME

Extraction and Characterization by Gel Electrophoresis

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ABSTRACT

The proteins of the contractile spasmoneme from *Vorticella convallaria, Carchesium polypinum*, and *Zoothamnium geniculatum* have been extracted in the detergent, sodium dodecyl sulfate (SDS), as well as urea and guanidine hydrochloride (GuCl). After SDS extraction, the molecular weight distribution of the proteins was examined by means of SDS-polyacrylamide gel electrophoresis. Significant amounts of material corresponding to the contractile proteins actin and tubulin are not present.

The contractile organelles in the three species examined contain a group of closely related proteins of molecular weight near 20,000, which constitute a major part (40–60%) of the dry mass. The 20,000 mol wt proteins in *Zoothamnium* bind calcium with high affinity (pK $\simeq$ 6) and are termed "spasmins." By means of urea polyacrylamide gel electrophoresis, it is demonstrated that in *Carchesium* and *Zoothamnium* certain spasmin components bind calcium even in the presence of 6 M urea. The binding of calcium in 6 M urea suggests a functional relationship between the spasmins and the calcium-binding proteins of striated muscle which behave similarly. The calcium binding in urea also indicates that the spasmins within a single spasmoneme have different calcium affinities, and this difference in calcium-binding properties may be an important factor in the physiological function of the organelle.

KEY WORDS contraction · calcium · *Vorticella* · spasmoneme

Several species of ciliated protozoa exhibit a rapid form of contraction. This is particularly clear in the stalked peritrichous ciliates, *Vorticella, Carchesium,* and *Zoothamnium,* which, on contraction, throw their stalks into tight helical coils or folds. Within the stalk is the contractile organelle, often referred to as the myoneme (1), though the term spasmoneme (2) is preferable since recent studies indicate that the organelle is biochemically distinct from the myosin- and actin-based contractile systems (3).

The spasmoneme contracts within 2–10 ms (4, 5), and the shortening velocity may be as high as 172 lengths/s, whereas the extension phase is of the order of seconds. The contraction velocity of the spasmoneme in *Zoothamnium,* (measured in lengths/second), is thus nine times greater than in

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the extensor digitorum longis muscle of the mouse, the fastest reported striated muscle (6).

The glycerinated spasmoneme can be induced to contract and (unlike glycerinated muscle) to extend actively. The contraction of the spasmoneme, also in contrast to that of muscle, can be induced without ATP and it is not inhibited by ATPase inhibitors (7, 8). Increasing the calcium ion concentration in the bathing solution from 10^{-8} to 10^{-6} M is sufficient to produce and maintain contraction (8). Many cycles of contraction and extension can be produced by changing the calcium ion concentrations within the range 10^{-8}–10^{-6} M. Owing to the absence of ATP hydrolysis during the contraction cycle in the glycerinated spasmoneme, Amos (8) suggested an alternative source of energy for contraction. The difference in the chemical potential of calcium ion in the bathing solution could drive the reaction, and in order to account for the mechanical work done against the viscosity of the medium during contraction, 44 mg calcium/kg wet weight would have to be bound. The amount of calcium bound to the spasmoneme during contraction was measured (9) and was 360 mg calcium/kg wet weight. The great excess of the bound calcium over the calculated minimum figure can be explained in terms of additional forms of work during contraction (e.g. against the elasticity of the extracellular sheath) or thermodynamic inefficiency.

A preliminary study of the biochemical composition of the spasmoneme from *Zoothamnium geniculatum* has been reported (3). Neither actin nor tubulin was present to any significant extent. The power output of the spasmoneme is too high (8, 10, 11) to be explained by traces of these proteins. The contractile function was therefore presumed to reside in the major portion of the spasmoneme proteins which in *Z. geniculatum* was found to consist of a class of proteins of near 20,000 mol wt. These proteins were subsequently found to bind calcium in the same range as the intact organelle and were given the generic name spasmin.

In this paper, the presence of spasmin proteins in the related species *Carchesium polypinum* and *Vorticella convallaria* also will be described. The binding of calcium to the isolated spasmins was examined by means of polyacrylamide gel electrophoresis at different calcium ion concentrations. By the use of native as well as partial denaturing conditions during electrophoresis, differences were noted in the calcium-binding properties of the spasmin components.

MATERIALS AND METHODS

Collection and Maintenance of Vorticellids

Specimens of *Vorticella convallaria* L., *Carchesium polypinum* L., and *Zoothamnium geniculatum* Ayrton were collected from East Anglian rivers. The distribution of giant colonial vorticellid, *Z. geniculatum,* was particularly patchy. The colonies were found attached to the leaves of the water lilies *Nuphar* and *Nymphaea* in certain regions only, and not to other aquatic plants. Not all water lilies even in the same river had *Z. geniculatum* on them, and from season to season the distribution of the organism varied. Mature colonies of *Z. geniculatum* were found from June to November. The peak population density appeared to be in September and October, after which increasing numbers of resting spores were formed and the population density declined rapidly. Similar observations were made by Wesenberg-Lund (12) on *Z. geniculatum* found in a small lake in Denmark.

Cultures of *Vorticella* and *Carchesium* were maintained for long periods in a sterile, filtered soil extract (40 g/liter) supplemented with *Aerobacter aerogenes.* Cultures of *Z. geniculatum* could only be maintained for a few generations in this medium, and other standard protozoan culture conditions were equally unsuitable.

Glycerination of Vorticellids

The *Vorticella* and *Carchesium* colonies grown on glass slides were rinsed briefly in distilled water and glycerinated at 0°C in a solution containing 50% glycerol vol/vol, 2 mM Na_2-EDTA, 100 mM KCl, and 10 mM histidine buffer, pH 7, for 2 h. They were then stored in a freezer, at between −15°C and −20°C, for at least a month before use.

Colonies of *Z. geniculatum* were detached from water lily leaves at their point of attachment with the leaf. Several hundred colonies could be found on a single leaf at the maximum population density, and were visible to the naked eye. The colonies were washed in distilled water and glycerinated. The washed colonies were allowed to settle slowly in vials, through a distilled water layer, into a deeper layer of the glycerination medium at 0°C. After 2 h, the vials were transferred to a freezer and stored as described previously.

Colonies of *Z. geniculatum* grown in the laboratory in culture for several generations were not different in biochemical composition from those collected in the field.

Extraction of Spasmoneme Proteins

The spasmoneme proteins were extracted from several different preparations of glycerinated vorticellids. In

some experiments in which the spasmoneme alone was required, *Zoothamnium* was used, and the organelles were dissected individually from the colonies (10). In other experiments with *Zoothamnium,* the zooids (cell bodies) were removed by cutting off the branches of the colony. The main stalk, containing the large common spasmoneme, was then subjected to chemical extraction. Larger amounts of spasmoneme protein were obtained by a third method, which could be applied in bulk. In this method, the zooids were removed by a shearing process, consisting of repeated pipetting in a dilute solution of sodium dodecyl sulfate (SDS) (0.01%). The denuded stalks were then extracted chemically. This method gave a higher yield of protein.

The bulk of the spasmoneme proteins could be solubilized only in strongly denaturing solutions. These included the ionic detergent, SDS, and the protein denaturants, guanidine hydrochloride (GuCl) and urea. For routine extraction, the solvents were: (*a*) 2% SDS, 60 mM Tris-HCl, pH 6.8, with 1% vol/vol mercaptoethanol, or (*b*) 3 M GuCl with 20 mM sodium cacodylate at pH 6, or (*c*) 6 M urea with 20 mM cacodylate, pH 6. The other extraction solutions used are noted in the text.

Before electrophoresis, the urea- and GuCl-extracted proteins were dialyzed against distilled water or buffer in a Colover ultramicrodialysis cell (Electrothermal Engineering, London). The dialyzed protein was then concentrated to a volume of 5–25 μl in the same apparatus by absorbing the water into 30% polyethylene glycol (mol wt 20,000). Both operations were performed at 4°C.

SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were run using a microslab gel apparatus (13). Six or more samples were run in parallel on this apparatus which can be used to measure as little as 0.01 μg of protein. Discontinuous SDS gels were made according to the formulae of Laemmli and Favre (14) except that the ionic strength was reduced. The stacking gel was 3% acrylamide, 0.08% bis-acrylamide, 0.1% SDS, 62.5 mM Tris-HCl, pH 6.8. The separation gel was typically 15% acrylamide, 0.4% bis-acrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.6. In gels in which the high molecular weight proteins were examined, the concentrations in the separation gel were reduced to 7½% acrylamide, 0.2 bis-acrylamide. The electrode buffer was 26 mM Tris, 100 mM glycine, pH 8.3, with 0.1% SDS. Standard SDS tube-gels (internal diameter, 6 and 2.5 mm) were run, using several dozen vorticellid colonies, to check that no artifacts were produced by the use of the microslab gel apparatus.

The molecular weights were calibrated using standard proteins: lysozyme (14,500), rabbit troponin C (18,500), carbonic anhydrase (29,000), bovine serum albumin (68,000), catalase (60,000 and 120,000) and phosphorylase *a* (98,000) run in adjacent sample wells in the same electrophoresis gel. The gels were stained with 0.2% Coomassie Blue or Fast Green in 50% methanol with 7% acetic acid and destained in 5% methanol with 7% acetic acid. They were scanned directly with a Joyce-Loebl microdensitometer (Gateshead-on-Tyne, England), and the relative amounts of protein were estimated from the areas under the peaks.

Polyacrylamide Gels with Controlled Levels of Divalent Cations

Non-SDS gels were run under the same conditions as the SDS gels except that the pH of the separation gel was reduced to 8. The divalent cations magnesium and calcium were added directly as chlorides or maintained at low levels by the use of EDTA and EGTA (ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid). To maintain known levels of free calcium ions at each pH, calcium and EGTA were mixed in the appropriate ratios calculated from the data given by Sillen and Martell (15) as tabulated by Amos et al (16). In practice, the total concentration of calcium (Ca^{2+} and Ca-EGTA) was kept constant and the amount of EGTA was varied to give the required free calcium ion concentration. In other experiments, the levels of free Mg^{2+} were similarly controlled by the use of EDTA.

Urea-Polyacrylamide Gels

Urea-polyacrylamide gels were also run on the microslab gel electrophoresis apparatus according to the formula of Head and Perry (17). A discontinuous pH gradient was not used. The sample gel contained 3% acrylamide, 0.08% bis-acrylamide, 25 mM Tris, 80 mM glycine, pH 8.5, with 6 M urea. The separation gel was 8% acrylamide, 0.45% bis-acrylamide, 25 mM Tris, 80 mM glycine, pH 8.5, with 6 M urea. The electrode buffer contained 25 mM Tris, 80 mM glycine, pH 8.5, with 6 M urea. Calcium and magnesium were again added as chlorides or maintained at low levels by the use of EGTA and EDTA. No attempt was made to maintain urea gels at a fixed divalent cation concentration because of the unknown effect of strong urea solutions on the binding constants of EGTA and EDTA.

Two-Dimensional Microslab Gel Electrophoresis

To discover which proteins changed their mobility in different concentrations of free calcium ion, the spasmoneme proteins were separated by electrophoresis in one dimension and then run in a second dimension at a different calcium ion concentration. A gel strip containing the proteins separated along the first dimension was cut from the slab and laid horizontally in the sample gel compartment on the second dimension gel, where an acrylamide solution was allowed to polymerize around it. Any protein, whose mobility does not change in the different calcium buffers, will migrate a similar distance in each gel and fall on a diagonal line through the origin. A protein whose mobility varies according to the Ca^{2+}

level will be displaced from this diagonal and so can be identified easily. This method could be useful in recognizing many metal-binding proteins from a mixture of other proteins, and by the use of the microslab apparatus, can be applied to very small amounts of material.

Two-dimensional urea gels were run with EGTA present in the first dimension to maintain low levels of free calcium, and with calcium chloride (0.1–2 mM) in the second dimension. Ovalbumin was used as a marker protein and did not appear to change its mobility under these conditions.

Dry Mass Determinations

The dry mass of the dissected spasmonemes before and after protein extraction was measured by means of a Zeiss Universal microscope fitted with Jamin-Lebedeff interference optics. The approximate retardation was calculated from the interference colors using white light. The retardation (in nanometers) was then measured accurately at 546 nm using de Senarmont's method. The dry mass in g/100 ml was calculated from the formula:

$$\text{dry mass} = \frac{\text{retardation (nm)}}{\text{thickness (nm)} \times 0.0018},$$

where 0.0018 is the specific refractive increment (18). The total mass was calculated after correcting for swelling and shrinkage of the organelle in the different solvents. All measurements were made at × 400 with carbon particles used as surface markers on the organelle.

RESULTS

SDS Extraction and Polyacrylamide Electrophoresis of Spasmoneme Proteins

After shearing in 0.01% SDS, the zooids (cell bodies) are removed from the vorticellids. Fig. 1 *a* is a light micrograph of a glycerinated colony of *Z. geniculatum* after shearing. The spasmoneme can be seen attached to its tendon within the main stalk and continuing up into the branches. Fig. 1 *b* is a higher magnification phase-contrast micrograph of part of the same colony showing the retraction of the spasmoneme from the stalk at the normal point of zooid attachment. The stalk spasmonemes are normally continuous with longitudinal spasmonemes in the zooid (19), and this attachment is presumably weakened by the 0.01% SDS treatment. Little or no material is removed from the spasmoneme by the dilute SDS, as measured by interference microscopy. The dilute SDS does, however, cause a volume change in the spasmoneme, and the organelle is unable to re-

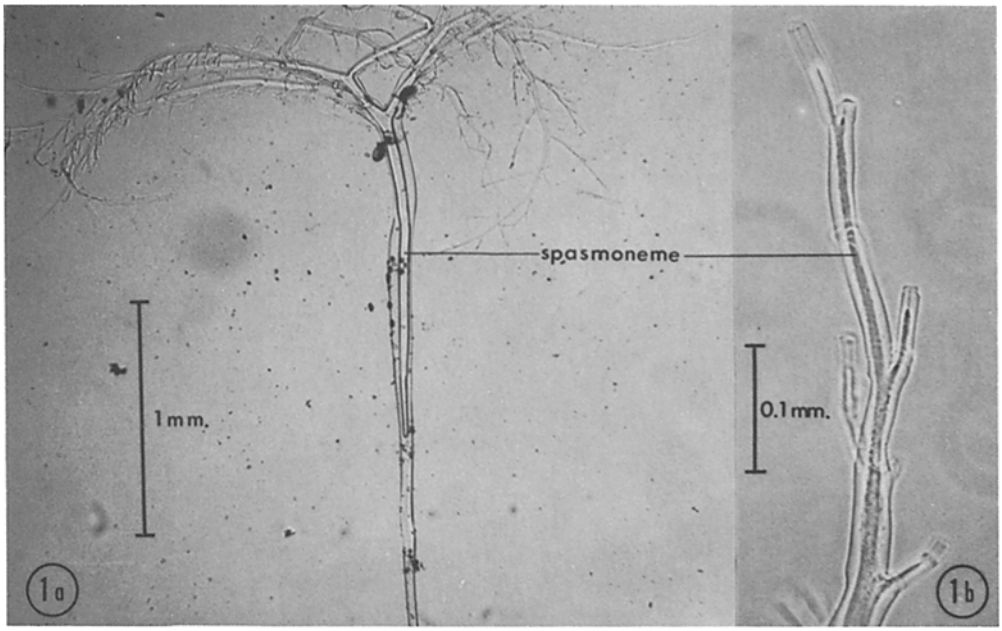

FIGURE 1 (*a*) Low magnification light micrograph of a mature colony of *Z. geniculatum* after removal of the cell bodies (zooids). × 32. (*b*) Higher magnification light micrograph showing a branch from the same colony. Taken with phase-contrast microscopy. × 220.

spond to calcium after this treatment. A similar irreversible effect of detergents binding to the spasmoneme in *Vorticella* was also noted by Hoffman-Berling (7).

When the dissected organelle or the zooid-free stalks were extracted in 2% SDS containing 1% vol/vol mercaptoethanol and 60 mM Tris-HCl, pH 6.8, the spasmoneme swelled within a few seconds and dissolved completely. No material appeared to be extracted from the stalk sheath of *Zoothamnium* during this process. To test whether it is valid to regard the SDS extract of whole zooid-free stalks as a solution of spasmoneme proteins, the SDS gel pattern of this extract (Fig. 10*d*) was compared with that of an extract of individually dissected spasmonemes (Fig. 3*b*). The SDS stalk extract (Fig. 10*d*) was more heavily loaded than the dissected spasmonemes and shows some of the minor protein bands clearly; however, there was no significant difference in the proteins extracted.

By measuring densitometer tracings, it was found that the major peak in *Zoothamnium* contained 50–60% of the total stainable protein, and the bulk of the protein was found in two components of this peak. These two, at apparent molecular weights of 18,000 and 20,000, were called spasmins A and B (3). Satellite peaks at 16,000, 17,000, and 22,000 were also present.

The SDS-extracted proteins from *V. convallaria* and from dissected spasmonemes of *Z. geniculatum* were run in parallel on 15% SDS-acrylamide gels, and densitometer traces from the two samples are shown in Fig. 2. The patterns are strikingly similar in that the major portion of the spasmoneme material from both organisms is the characteristic near 20,000 mol wt proteins. Some high molecular weight proteins are present in both species.

The SDS-acrylamide gel pattern of *Carchesium* stalk proteins (Fig. 3*c* and *d*) was very similar to that of *Zoothamnium*. In *Carchesium*, two major peaks were present at 20,500 and at 17,500 mol wt with satellite peaks at 16,000, 17,000, and 19,000 mol wt. The low molecular weight region contained 40–60% of the total stainable, with 27% of the protein being present in the two major components.

To examine the possibility that these different components were the products of proteolysis of a single protein, colonies of *Carchesium* were glycerinated in the presence of protease inhibitors. These included *p*-tosyl arginine methyl ester, di-

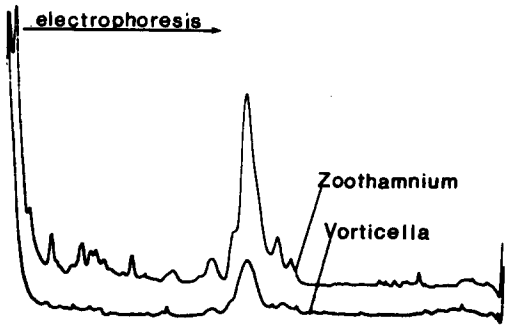

FIGURE 2 Comparison of *Zoothamnium* and *Vorticella* spasmoneme proteins. Superimposed densitometer traces of 15% polyacrylamide-SDS gels. Five dissected spasmonemes from *Z. geniculatum* (upper trace) extracted in SDS. Approx. 200 stalks of *V. convallaria* (lower trace) extracted in SDS. Electrophoresis was from left to right (anodal).

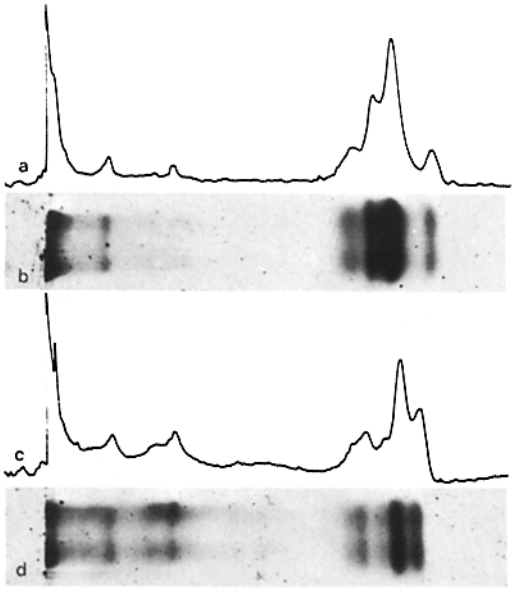

FIGURE 3 Comparison of *Carchesium* and *Zoothamnium* spasmoneme proteins. (*a*) Densitometer trace of 15% polyacrylamide-SDS gel electrophoresis of SDS-extracted proteins from six spasmonemes from *Z. geniculatum*. (*b*) Stained electrophoresis gel as above. Two major protein bands at 18,000 and 20,000 mol wt were present as well as some high molecular weight proteins close to the origin. (*c*) Densitometer trace of electrophoresis gel, run in parallel with Fig. 3*a* but containing 25 zooid-free *Carchesium* colonies. (*d*) Stained electrophoresis gel of *Carchesium* proteins as above. Two major bands at 17,500 and 20,500 mol wt were present as well as some high molecular weight proteins close to the origin. Electrophoresis was from left to right (anodal).

isopropyl fluorophosphate, and sodium mersalyl, all at a concentration of 10 mM. In keeping with previous observations with different inhibitors (8), the colonies were able to contract and extend normally in response to high and low calcium after this treatment. The proteins were then extracted in the 2% SDS medium and run on 15% polyacrylamide gels. There was no change in the proportion of the two major components and the satellite peaks, though there were some small changes in the high molecular weight proteins.

The molecular weight distribution of the high molecular weight proteins in *Zoothamnium* is shown in Fig. 4, which is a densitometer trace of a stained 7½% polyacrylamide gel. At this gel concentration, proteins of below 25,000 mol wt travel with the tracking dye front, and this includes all the spasmin components. The molecular weights of those proteins with molecular weights >25,000 are listed in Fig. 4.

Solubilization of Spasmoneme Proteins in Urea and GuCl

To examine the calcium-binding properties of the spasmoneme proteins, a technique was required which would solubilize the proteins but not render them permanently inactive. High concentrations of KCl did not solubilize the spasmoneme, and urea and GuCl were used instead. In 6 M urea and 3 M GuCl, the spasmoneme was largely extracted, leaving a residue containing 20–30% of the dry mass as measured by interference microscopy. The urea- and GuCl-extracted proteins were dialyzed and run on 15% polyacrylamide-SDS gels and were identical to those extracted directly with 2% SDS.

The solubilization of the spasmoneme in the urea and GuCl solutions was quantitated by use of acrylamide gels. Zooid-free colonies of *Zoothamnium* were extracted in different concentrations of urea or GuCl for 1 h at 4°C. The residue remaining was rinsed in distilled water, extracted with 2% SDS, and then electrophoresed. From the stained gels, the amount of spasmoneme proteins remaining in the colonies was measured. The extraction of *Zoothamnium* proteins in GuCl at pH 6 is shown in Fig. 5. The amount of protein extracted increased almost linearly with GuCl concentration up to 3 M, at which concentration 5% of the extractable protein remained undissolved. The low molecular weight proteins were more soluble than the high molecular weight proteins. Thus, at 2 M GuCl, 95% of the low molecular weight proteins and only 66% of the high molecular weight proteins were solubilized.

The extraction of the spasmoneme proteins in urea at pH 6 was similar to that in GuCl and is shown in Table I. Higher concentrations of urea were required to give a comparable degree of solubilization. Thus 6 M urea was needed to extract 92% of the total extractable proteins of the spasmoneme. The residue after extraction in 6 M urea contained 24% of the original dry mass, and no further material was extractable with the 2% SDS medium. For reasons which are outlined in the Discussion, this residue is not believed to contain the essential elements of the contractile mechanism.

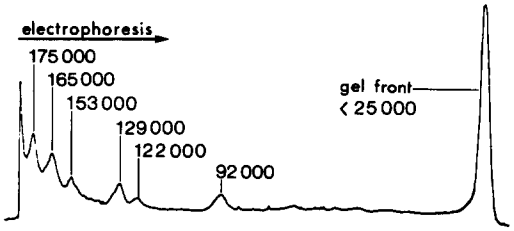

FIGURE 4 Densitometer trace of a 7½% polyacrylamide-SDS gel of *Zoothamnium* spasmoneme proteins showing the molecular weight distribution of the larger proteins.

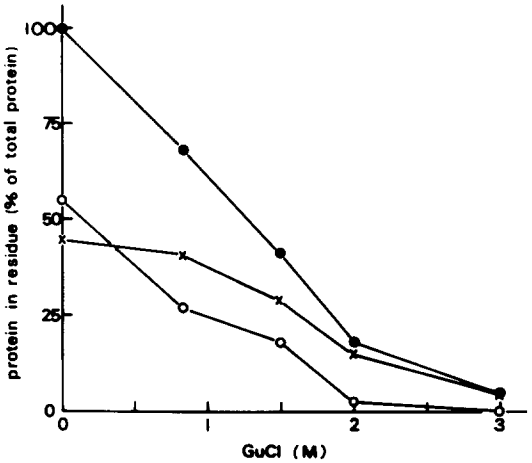

FIGURE 5 Extraction of spasmoneme proteins in GuCl. The amount of protein remaining after extraction was determined by quantitative densitometry on SDS-polyacrylamide gels. Total remaining proteins ●; high molecular weight proteins ×; spasmin components ○.

Interaction with Calcium on Polyacrylamide Gels

The GuCl-solubilized proteins from *Zoothamnium* were dialyzed to remove the salt and then electrophoresed under non-SDS conditions in a microslab gel. In a 15% polyacrylamide gel at pH 8.8, there were two major bands migrating close to the front. When electrophoresed in a second dimension in 15% polyacrylamide SDS, these bands were found to correspond to spasmins A and B, with the first band on the non-SDS gel being spasmin A. The spasmins are acidic, and this was confirmed by isoelectric focusing which showed two major bands between pH 4.7 and 4.8.

The glycerinated spasmoneme contracted at 10^{-5} M calcium and extended at 10^{-8} M calcium in the pH range 6–9, though the rate of contraction was slow at pH 9. The effect of calcium on the electrophoretic mobility of the isolated spasmins was examined in non-SDS gels at pH 8.0. Densitometer traces of the gels are shown in Fig. 6 with 10^{-5} M calcium chloride (high calcium) and with 1.0 mM EDTA (low calcium). In the low calcium gel, there was a large peak close to the front with several trailing peaks incompletely separated from it. With added calcium, the pattern was completely altered: two groups of protein with reduced mobilities were present.

The threshold for this effect was examined by the use of calcium EGTA buffers. At 10^{-8} M free calcium, the electrophoretic pattern was similar to that in EDTA, but two distinct spasmin peaks were resolved. At 10^{-6} M free calcium, the mobility of both spasmin peaks decreased. Subsidiary peaks were seen as trailing shoulders on both spasmin peaks. Free magnesium ion concentrations between 10^{-8} and 10^{-5} M had no effect on the mobility of the spasmoneme proteins, the electrophoretic pattern being identical to that at low calcium levels.

TABLE I

Urea Extraction of Spasmoneme Proteins from Zoothamnium geniculatum

Urea	Protein extracted	Spasmin proteins in residue	High mol wt proteins in residue
(mol/liters)		*% total protein*	
0	0	59	41
1.5	32	32	36
3.0	51	32	17
6.0	92	3	5

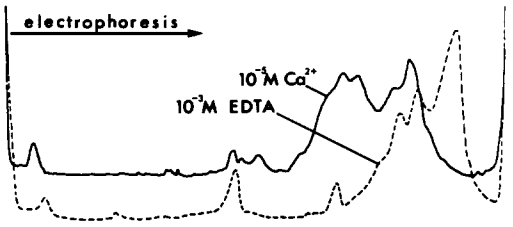

FIGURE 6 The effect of calcium on the electrophoresis of spasmin. Superimposed densitometer traces of *Zoothamnium* spasmoneme proteins electrophoresed in non-SDS polyacrylamide gels; with 10^{-5} M added calcium (solid line), with 1 mM EDTA (broken line).

Effect of Calcium in Urea Gel Electrophoresis

The spasmoneme proteins from *Carchesium* and *Zoothamnium* were electrophoresed in 6 M urea at pH 8.5 in the presence and absence of free calcium ion. When free calcium ion was reduced to low levels (by the use of EGTA), some of the spasmoneme proteins migrated with a mobility 75% that of the ovalbumin marker (Figs. 7 and 8, broken line). When calcium was added to the electrophoresis buffer (0.1–2 mM as $CaCl_2$), several of these protein peaks were lost and new protein peaks appeared which had mobilities similar to that of ovalbumin (Figs. 7 and 8, solid lines). The position of ovalbumin relative to the fluorescein dye front was not affected by the presence or absence of calcium. It was concluded that certain spasmoneme proteins in *Zoothamnium* and *Carchesium* were able to interact with calcium even in the presence of 6 M urea.

To test for the specificity of divalent cation binding in urea, the spasmoneme proteins for *Z. geniculatum* were electrophoresed in 6 M urea with 2 mM magnesium chloride, 1 mM EGTA. The electrophoretic pattern with an excess of Mg^{2+} present was identical to that in EGTA alone.

To identify unequivocally the proteins which changed their mobility, two-dimensional electrophoresis was performed in which the spasmoneme proteins from *Z. geniculatum* were first electrophoresed at low levels of free calcium ion and then in excess free calcium. In Fig. 9 is shown the two-dimensional electrophoresis pattern of spasmoneme proteins for *Z. geniculatum*. The position of the ovalbumin standard is marked (*X*). Alongside the two-dimensional gel (Fig. 9*c*) are sections

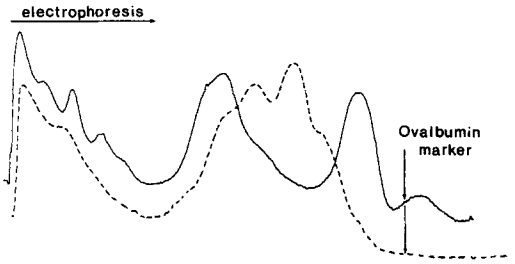

FIGURE 7 Calcium binding to *Zoothamnium* spasmoneme proteins in 6 M urea-polyacrylamide gels. Superimposed densitometer traces of urea-extracted proteins electrophoresed with 2 mM added calcium (solid line) and with 2 mM EGTA (broken line). The position of the ovalbumin marker protein is indicated by the arrow. Electrophoresis is from left to right (anodal).

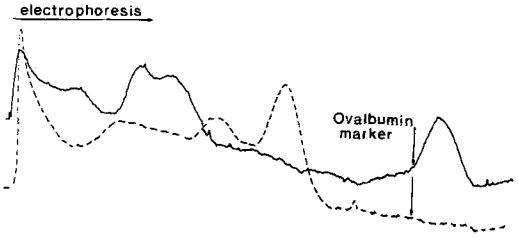

FIGURE 8 Calcium binding to *Carchesium* spasmoneme proteins in 6 M urea-polyacrylamide gels. Densitometer traces of gels run under conditions identical to that in Fig. 7.

from stained gels of spasmoneme protein which were electrophoresed in one dimension only (Fig. 9*a* and *d*). The proteins whose mobility increased in the presence of calcium are marked by arrows, and consisted of a fast major component and an even faster satellite peak. The mobility of the slower component and its satellite peaks did not change in calcium urea gels.

The correspondence between the proteins in urea gels and those observed in the SDS gels was examined by further use of two-dimensional gel electrophoresis (Fig. 10). The spasmoneme proteins from *Zoothamnium* were electrophoresed in urea with 2 mM $CaCl_2$ (Fig. 10*a*) and then in 15% polyacrylamide SDS. Much of the protein at the origin in the first dimension gel remained in the stacking gel in the second dimension (Fig. 10*b*). A small amount of the material did, however, enter the second dimension separation gel and remained close to the origin (Fig. 10*c*), indicating that it was of high molecular weight. The other components in the calcium urea gel all corresponded to proteins in the spasmin region

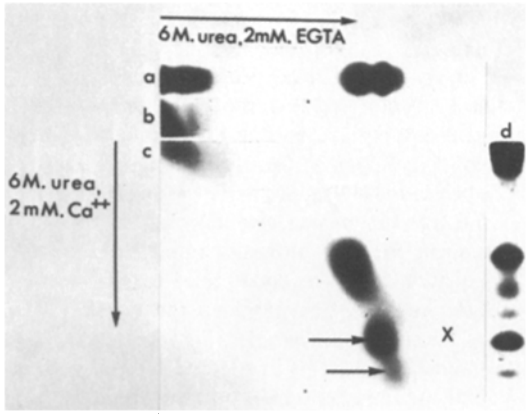

FIGURE 9 Calcium binding in two-dimensional urea-polyacrylamide gels. Stained polyacrylamide microslab gels. (*a*) Urea-extracted spasmoneme proteins from *Z. geniculatum* electrophoresed in 6 M urea with 2 mM EGTA. (*b*) Duplicate gel to Fig. 9*a* after electrophoresis in the second dimension in 6 M urea with 2 mM calcium. (*c*) Proteins electrophoresed from gel (*b*) into the two-dimensional gel. (*d*) Urea-extracted spasmoneme proteins electrophoresed in parallel to gel (*c*). The position of the ovalbumin marker protein in comparable gels is marked (*X*). The proteins with altered electrophoretic ability are marked (→).

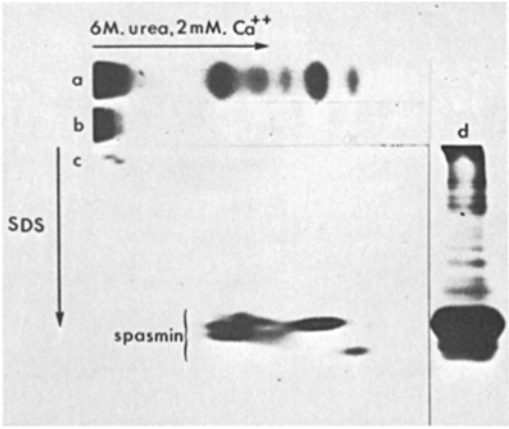

FIGURE 10 Molecular weight distribution of the spasmoneme proteins from 6 M urea gels. (*a*) Urea-extracted *Zoothamnium* spasmoneme proteins electrophoresed in 6 M urea with 2 mM added calcium and stained. (*b*) A duplicate sample to Fig. 10*a* run in a second dimension in a 15% polyacrylamide-SDS gel and then stained. (*c*) Proteins electrophoresed from gel (*b*) into the second dimension gel. (*d*) SDS-extracted *Zoothamnium* spasmoneme proteins run in parallel to Fig. 10*c* in a 15% polyacrylamide-SDS gel. The gel (*d*) is overloaded and shows some of the minor bands present in the spasmoneme.

described previously. The two fast spasmin components observed on calcium urea gels had molecular weights of 20,000 and 16,000, with the 20,000 protein (spasmin B) being the dominant spasmin component overall. The prominent slow moving band in the calcium urea gel was composed of two components with molecular weights of 20,000 and 18,000 and may be a dimer of spasmin A and a protein with a molecular weight similar to that of spasmin B. The slow migrating satellite peaks on urea gels formed a diffuse zone between the two major bands on the SDS polyacrylamide gels.

The behavior of the spasmoneme proteins from *Carchesium* with calcium in 6 M urea (Fig. 8) was similar to that of the *Zoothamnium* proteins. In the presence of 2 mM $CaCl_2$, a prominent spasmin band increased in mobility, migrating slightly ahead of the ovalbumin marker protein (Fig. 8, solid line), while the mobility of the slower migrating spasmins did not increase.

DISCUSSION

Comparison of Spasmonemes from Several Vorticellids

The form and size of the contractile stalks in *V. convallaria, C. polypinum,* and *Z. geniculatum* are very different. *V. convallaria* is a solitary organism with a stalk up to 1 mm long, containing a spasmoneme of 1.2 μm diameter (19). In *Carchesium,* the cell body divides many times and the stalk bifurcates after each division, which results in a colony of a hundred or more cell bodies. The spasmoneme associated with each cell body is restricted to a single branch, and can be up to 10 μm in diameter. In *Carchesium,* each branch can contract independently of the rest of the colony. On contraction, the stalk forms tight helical coils as does the individual stalk of *Vorticella.* In the colonial vorticellid *Z. geniculatum,* the spasmoneme is continuous between the branches (Fig. 1*b*) and forms a common main stalk organelle (Fig. 1*a*). This stalk spasmoneme may be 30 μm in diameter and > 1 mm in length. When *Z. geniculatum* is stimulated either mechanically or electrically, the stalk folds at a specialized knee joint to which the spasmoneme is connected by means of a tendonlike structure.

Though the size and form of the spasmoneme differ in the three species, the light microscope suggests a similarity in ultrastructure. Under conditions where extension is restricted, the spasmoneme may appear frayed locally into longitudinal strands (5, 8). The fibrous structure of the spasmoneme was confirmed by electron microscopy: individual filaments with a diameter of 2–3 nm and a periodicity of 3.5 nm have been observed (5). Transverse sections of the spasmoneme in all three vorticellids show, in addition to these filaments, cross sections of a large number of membranous tubules (19, 20), which are thought to store calcium (21). Surrounding the spasmoneme is a layer of cytoplasm containing mitochondria, and in *Z. geniculatum* there are also mitochondria embedded in the interior of the spasmoneme (5).

As the ultrastructure of the spasmoneme is similar in *Vorticella, Carchesium,* and *Zoothamnium,* it was expected that the contractile proteins would be similar also. In all three species, proteins corresponding to actin or tubulin were not detected. If actin or tubulin were present in the spasmoneme, it could constitute no more than 2% for the total protein, which would not be sufficient to account for the measured tension during contraction which approaches that of striated muscle (10, 11).

The proteins which are most obvious and which are consistently present in the spasmoneme of all species are those with molecular weights near 20,000 which constitute 50–60% of the soluble proteins of the organelle. In *Zoothamnium* and *Vorticella,* these proteins showed a very close resemblance not only in the major peak, but also in the satellite peaks associated with it (Fig. 2). With the increased resolution obtained in Fig. 3, only small differences were noted in the electrophoretic mobility of the spasmoneme protein from *Carchesium* and *Zoothamnium* in the 20,000 region. The presence of these major components and satellite peaks with similar molecular weights would seem to be a characteristic feature of this type of contractile system. This collection of isotypes of spasmin may reflect a highly specialized and conserved arrangement of proteins, assembled in such a way as to give directionality to the tension created on calcium binding.

Calcium Binding to the Spasmoneme Proteins

The glycerinated spasmonemes from *Vorticella, Carchesium,* and *Zoothamnium* contract in the presence of calcium ion and extend when calcium is removed (8, 10, 19). Amos (5, 8) found that the calcium threshold for contraction was 5 ×

10^{-7} mol/liter and that this threshold was not affected by magnesium ions even at millimolar concentrations.

The amount of calcium bound, during contraction, to the glycerinated spasmoneme has been measured by means of the X-ray microprobe (9). The amount of calcium bound was equivalent to a 9 mM solution of calcium chloride of the same volume as the spasmoneme and, thus, represents a massive binding of calcium to components in the spasmoneme.

With the extraction techniques used in the present study, 70–100% of the dry mass of the glycerinated spasmoneme was extracted. The nature of the material not extracted in urea or GuCl is unknown, but of the soluble material 50–60% was the high affinity calcium-binding fraction of around 20,000 mol wt. The fact that the 20,000 wt fraction is the major component in the three vorticellids examined and that these proteins bind calcium but not magnesium between 10^{-8} and 10^{-6} mol/liter strongly suggests that these proteins are the functional components of the contractile system.

The function of the high molecular weight components of the spasmoneme (Fig. 4) is unknown but their molecular weights differ from species to species and they do not bind calcium with a high affinity under the conditions used in this study. The ultrastructure of the spasmoneme would suggest that some mitochondrial proteins should be present as well as ATPases involved in calcium sequestering within the tubular system (21). The high molecular weight proteins purified by gel chromatography also do not bind calcium when tested in a membrane filter assay (22), and thus, if these proteins are involved in a calcium sequestering system, calcium affinity has been lost during isolation.

Under nondenaturating conditions, all the spasmin components bind calcium between 10^{-8} and 10^{-6} mol/liter on the acrylamide gels. The mobility of the spasmins (anodally) is decreased, and this could reflect a decrease in the effective negative charge on the proteins, an increase in the Stokes radius of the molecules, or a combination of both effects. What can be said with certainty is that calcium induces a significant conformational transition in the spasmin molecules.

A Comparison with Other Contractile and Calcium-Binding Systems

The two contractile systems so far biochemically characterized in animal and plant cells appear to use a sliding filament mechanism to generate movement. These are the actomyosin system (23) and the microtubular-based ciliary and flagellar movement (24, 25). Other contractile mechanisms include the beating rotation of bacterial flagella (26) and the contraction of the T-even phages (27). However, the mechanism and regulation of these latter systems is little understood at present.

The spasmoneme-based contractile system represents a new contractile mechanism which has been characterized biochemically only in the vorticellid ciliate protozoa. Ultrastructural and physiological studies indicate, however, that the spasmoneme-type contractile system is present in other ciliated protozoa including *Stentor* (28–30) and *Spirostomum* (31). Because of their small size and often diffuse nature, the spasmoneme-type filaments can be easily overlooked, and it is not certain whether they exist in organisms other than the ciliated protozoa.

The spasmoneme contraction does not appear to be a sliding-filament mechanism. In the vorticellids (10, 32) and in *Stentor* (30), the birefringence of the extended spasmoneme is high but falls to a very low level when the organism contracts. This is in contrast to the A band in muscle or to flagella axonemes where the birefringence remains high during contraction. The birefringence of the spasmoneme in *Z. geniculatum* is largely form birefringence (16), suggesting that the birefringence in the extended organelle is due to ordered filaments which become folded or coiled when calcium is bound. On the basis of abundance, it seems probable that the protein, spasmin, is the major component of these filaments which themselves compose the bulk of the spasmoneme.

In the actomyosin and microtubular axoneme systems, the energy for contraction is derived from ATP. In the glycerinated spasmoneme, however, ATP is not required for contraction or extension, and metabolic inhibitors do not interfere with this calcium-driven contraction.

The actomyosin ATPase in striated muscle is regulated by calcium by means of the troponin complex. The component in this complex which binds calcium is troponin C which has an overall binding constant of 5×10^{-6} mol/liter (33). Striated muscle also contains a soluble sarcoplasmic calcium-binding protein, parvalbumin, with a binding constant of 1.10^{-7} mol/liter (34).

Both troponin C and parvalbumin are able to interact with calcium even in the presence of high concentrations of urea (17, 35), suggesting that the calcium-protein complex is highly stable and can resist the denaturing effect of strong urea solutions. Troponin C binds calcium in 6 M urea with a small increase in electrophoretic mobility; if, however, the Mg-ATPase inhibitory protein (troponin I) is also present in the calcium-urea gel, the two proteins interact, forming a slower migrating complex (17). Thus, both the calcium-binding site(s) and the troponin I recognition site of the troponin C molecule retain their integrity in 6 M urea. Similarly, parvalbumin undergoes a conformational change on addition of calcium in solutions containing 4 M urea (35).

In the case of the spasmins from both *Zoothamnium* and *Carchesium*, spasmin B and a minor component can bind calcium in 6 M urea, and their electrophoretic mobility increases greatly. In 6 M urea, the polypeptide backbone of the spasmin molecules is likely to be unfolded, yet in spasmin B the integrity of the calcium-binding site is maintained as judged by the retention of specificity for Ca^{2+} rather than Mg^{2+}. The increase in electrophoretic mobility (anodally) of spasmin B is in the direction opposite to that expected from a simple neutralization of net negative charge on the protein. The binding of Ca^{2+} presumably causes the unfolded protein to become more compact, leading to an increase in electrophoretic mobility.

From the homology of amino acid sequences, it has been suggested that the calcium-binding sites in troponin C are similar to those in parvalbumin (36) where Ca^{2+} is coordinated by six oxygen atoms in an octahedral arrangement (37). The amino acid composition of the 20,000 and 18,000 mol wt spasmin in *Zoothamnium* is not identical to that of either troponin C or parvalbumin (3). but the high calcium affinity and binding of Ca^{2+} in strong urea solutions may well indicate a structural relationship between the calcium-binding sites of these proteins.

The mechanism by which calcium produces contraction of the spasmoneme is still not resolved. In *Stentor*, microfilaments of 3–4 nm are observed in the extended organism and a majority of thicker structures (28) in the contracted state, possibly corresponding to helically coiled microfilaments (29, 30). These helically coiled filaments appear as tubules of 8–12 nm diameter with walls of 3–4 nm thickness when examined in cross sections. No helical coiling of filaments has yet been observed in the spasmoneme of contracted vorticellids, though such a mechanism may occur.

Small changes in the conformation or charge or the spasmin molecule (such as observed in gel electrophoresis) could produce the change in the structure of the microfilaments indicated by birefringence measurements. When calcium is removed from the spasmoneme, the organelle is observed to produce a pushing force, as the microfilaments become aligned (10). This may represent an active change in the angle of bonding between the spasmin subunits, which in a coiled or folded filament would produce a large change in the overall length. Such a change in intersubunit bonding may be produced by a calcium-sensitive conformational change in spasmin. Whether the filaments are in fact composed of spasmin has not been directly demonstrated. This will require the reassembly of the spasmins into filaments in much the same way as has been demonstrated for muscle actin. How the different spasmin proteins may be assembled into these filaments is again unknown. However, the fact that a similar range of proteins occurs in three different genera suggests that the filament may consist of a highly organized protein complex which is conserved during evolution.

The mechanism of contraction in the spasmoneme may eventually prove to be as ordered as that of the actin/myosin/troponin/trypomyosin complex in skeletal muscle. The spasmoneme does, however, hold out the possibility of studying directly, at the molecular level, the conversion of chemical potential energy into mechanical work by a biological system.

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