JOURNAL OF BACTERIOLOGY, May 1980, p. 694-700
0021-9193/80/05-0694/07$02.00/0
Vol. 142, No. 2

# Purification and Preliminary Characterization of Spiroplasma Fibrils

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Fibrils 3.5 nm in diameter were released from the honeybee spiroplasma (BC3) by treatment with detergents and then purified by isopycnic centrifugation. Purified fibrils were flexuous, of indeterminate length, and had an axial repeat of 8.5 nm. The fibrils were associated in pairs, but in 1 M salt formed aggregates with a marked striated appearance. Pronase completely degraded the fibrils, but trypsin had little effect. The fibrils were composed of a single protein of molecular weight 55,000 which represented about 1% of the total cell protein. A protein of molecular weight 26,000 appeared to be associated with the fibrils. The significance of this in relation to membrane attachment and the possible role of fibrils in maintenance of cell shape and in motility are discussed.

Spiroplasmas are small wall-less microorganisms, closely related to mycoplasmas, but having a characteristic helical morphology (21). They are actively motile by rotary corkscrew-like movements, yet they have no flagella or axial filaments such as those implicated in the motility of spirochetes (5). Isolation of a nonhelical, nonmotile variant of *Spiroplasma citri* (22) indicates that helicity and motility are related functions, but the molecular basis of these functions is unknown.

Cells of *S. citri* contain no elements indicative of supportive structures based on peptidoglycan (2), nor is helicity an inherent property of the cytoplasmic membrane which collapses after cell lysis (18).

Treatment of several different spiroplasmas with the detergent sodium deoxycholate causes cell lysis accompanied by the release of long curvilinear fibrils approximately 3.6 nm in diameter which show a marked tendency to aggregate into doublets and have a prominent axial repeat at 9-nm intervals along their length (23, 24). It has been proposed that these structures may represent part of a supportive or cytoskeletal system (17). Cell shape could be maintained by compression or tensioning of fibrils wound helically around the inner side of the cytoplasmic membrane to which they are anchored by links with integral membrane proteins. Rotary movement might be generated by sequential contraction of these fibrils.

The possibility that mycoplasmas contain contractile proteins closely resembling those of eucaryotic cells has attracted much interest since the reported isolation, from the motile organism *Mycoplasma pneumoniae*, of a protein with molecular weight (MW) 45,000 which has several properties in common with vertebrate muscle actin. It forms curvilinear filaments 5 nm in diameter which bind myosin subunits to give characteristic arrowhead complexes (15). Attempts to purify similar components from other mycoplasmas, including *S. citri*, have confirmed the presence, in these organisms, of proteins with the same MWs as actin and which have similar solubility properties, but which have not been shown to form filaments (9, 11, 20; H. C. Neimark, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D62, p. 61).

Several members of the genus *Treponema* contain bundles of intracytoplasmic microtubules (10) in addition to an axial filament. These fibrils, which appear to vary between 8 and 13 nm in diameter, have recently been purified and shown to be made up of a complex arrangement of thinner strands whose major component is a protein of MW 97,000 (6). The function of these structures has not been resolved, but it has been suggested that they may play a part in maintaining the helical shape of the cell. In view of the morphological similarity between spirochetes and spiroplasmas, the possibility of a relationship between spiroplasm fibrils and these structures is of particular interest.

The current investigation was undertaken to determine the nature of spiroplasma fibrils, to compare them with similar structures occurring in other organisms, and to investigate their function within the spiroplasma cell. This paper describes a method for purifying the fibrils and some of their properties.

## MATERIALS AND METHODS

**Spiroplasmas.** A spiroplasma isolated from honeybees (BC3) (4) was grown in Saglio medium (21)

supplemented with horse serum (10% vol/vol) or fetal calf serum (10% vol/vol). Cells from 1 liter of late-log- or stationary-phase culture were harvested by centrifugation (15,000 × g for 30 min at 4°C) and suspended in 150 ml of 0.1 M phosphate buffer (pH 7.5) containing sucrose (5% wt/vol). The washed organisms were centrifuged (27,000 × g for 15 min at 4°C), and the pellet was suspended in 5 ml of phosphate-buffered sucrose.

**Solubilization of cells.** Washed cells were added to 150 ml of 4 M glycerol in 10 mM Tris buffer (pH 7.5) containing 25 μg of DNase I per ml and either sodium deoxycholate or Triton X-100 (1% wt/vol). After stirring for 2 h at 20°C, the detergent-insoluble components were sedimented by centrifugation (80,000 × g for 3 h at 15°C) and suspended by stirring gently for 16 h at 4°C in 2 ml of 10 mM Tris buffer (pH 7.5) containing detergent (0.1%). In some experiments this solution also contained 1 M potassium chloride and 10 mM EDTA.

**Isolation of fibrils.** Samples (0.3 ml) of detergent-insoluble material were layered onto preformed 5-ml linear Urografin (Schering, Berlin) gradients (32 to 64% wt/vol in 10 mM Tris buffer, pH 7.5) containing detergent (0.1%) which were centrifuged in a swinging bucket rotor (190,000 × g for 2 h at 4°C). Visible bands were removed through the side of the tubes, and complete gradients were fractionated by dripping out through the bottom of the tube. The densities of fractions were determined from measurements of their refractive indices. Fractions were made up to 8 ml with 10 mM Tris buffer (pH 7.5) and centrifuged (80,000 × g for 2 h at 20°C) to collect separated components. Resulting pellets were suspended in small volumes of 10 mM Tris buffer, pH 7.5.

**Electron microscopy.** Small droplets of sample were applied to carbon-coated grids, washed with several drops of distilled water, and negatively stained with ammonium molybdate (3% wt/vol; pH 6.5) for electron microscopy. In some instances, material was fixed before washing by the application of glutaraldehyde (3.5% wt/vol). Attempts were made to analyze the substructure of the fibrils by studying the optical diffraction pattern obtained from electron micrographs (3).

**Gel electrophoresis.** The composition of fractions was analyzed by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis performed by the method of Laemmli (12), using a stacking gel of 4% (wt/vol) acrylamide and a separating gel of 12 or 15% (wt/vol) acrylamide. Approximately 10 μg of sodium dodecyl sulfate-solubilized protein was loaded per channel and separated by applying a potential difference of 10 V $cm^{-1}$ for 6 h. Gels were stained with Coomassie brilliant blue or periodic acid-Schiff reagent (8). Determinations of MWs were made by reference to the distances migrated by standards of known MWs run on the same gel. Estimates of the relative concentrations of separated components were made from densitometer scans of stained gels with reference to the staining intensity of known concentrations of fibril protein.

Two-dimensional polyacrylamide gel electrophoresis was performed by the method of O'Farrell (16). Samples of whole cells and fibrils were solubilized in Nonidet (1% wt/vol) (Imperial Shell, United Kingdom), with 8 M urea and β-mercaptoethanol (2% vol/vol) or in sodium dodecyl sulfate (1% wt/vol) which was subsequently displaced by the addition of excess Nonidet (1). The first-dimension separation by isoelectric focusing was made on linear pH gradients from pH 4.5 to 8.5. The second dimension was performed under the same conditions as one-dimensional polyacrylamide gel electrophoresis.

**Effect of chemical and enzymatic treatments.** Samples (25 μl) containing approximately 50 μg of purified fibrils were added to an equal volume of test solution to give the concentrations indicated (see below). After 5 to 60 min, drops of solution were applied to grids, fixed, negatively stained, and examined in the electron microscope for the presence of fibrils.

**Protein determinations.** The amount of protein in washed whole cells grown in fetal calf serum and purified fibril preparations was determined by the method of Lowry after solubilization in 0.1 N sodium hydroxide (13).

## RESULTS

**Cell lysis.** Preparations of spiroplasmas lysed by four cycles of freezing and thawing showed many long flexuous fibrils 3.5 nm in diameter with an axial repeat of 8.5 nm, apparently released from ruptured cells and nearly always associated with pieces of membrane. Large numbers of similar structures were present in the detergent-insoluble fractions. The fraction derived from sodium deoxycholate-treated cells contained a high concentration of membrane fragments with which the fibrils continued to associate. However, solubilization with Triton resulted in the complete dissociation of the cytoplasmic membrane.

**Isolation of fibrils.** Isopycnic centrifugation of the sodium deoxycholate-insoluble material produced several diffuse bands in the Urografin gradients. Fibrils were seen in the material banding at a density of around 1.220 g/$cm^3$, but most remained associated with membrane fragments at the top of the gradient. Triton-insoluble material separated into three distinct bands, the lowest of which formed at a density of 1.225 g/$cm^3$ and contained large concentrations of fibrils and other particulate material. Fibrils were also present in the middle band but were not apparent in the uppermost band or on top of the gradient. All three bands contained a complex mixture of components in which a protein of MW 67,000 predominated (Fig. 1). The middle and lower bands also contained large quantities of proteins with approximate MWs of 55,000 and 26,000. Harvesting the spiroplasmas before they produced sufficient acid to cause precipitation of horse serum proteins or growing the cells in fetal calf serum, which reduced precipitation to a minimum, resulted in the virtual elimination of the 67,000-MW protein (Fig. 2). When 1 M po-

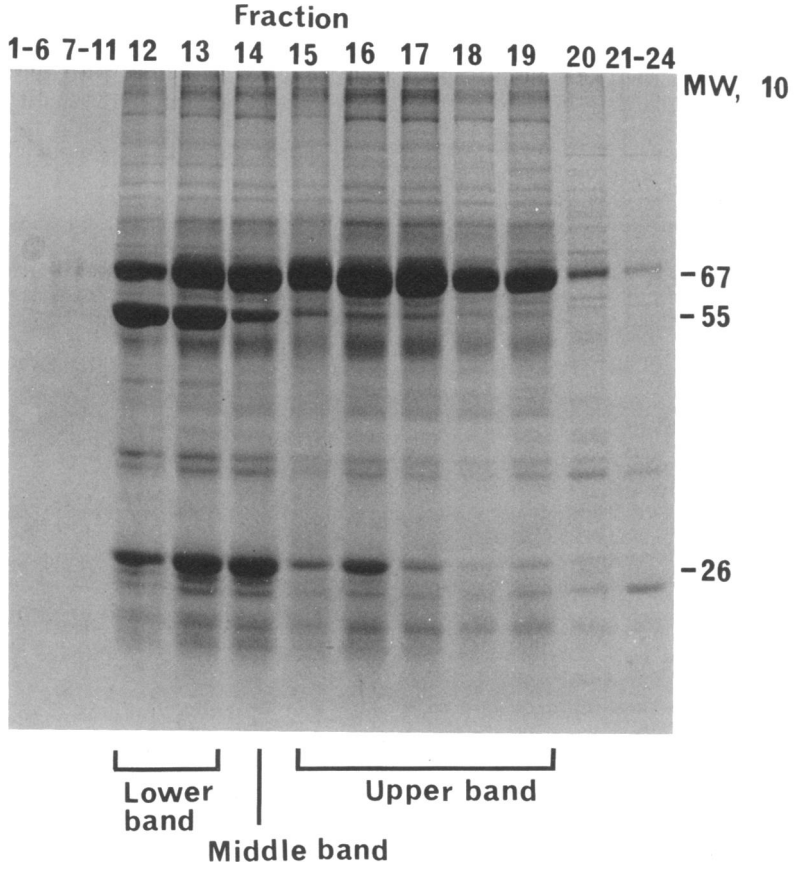


FIG. 1. *Analysis by one-dimensional polyacrylamide gel electrophoresis of 0.2-ml fractions (1 to 24) from a Urografin gradient after isopycnic centrifugation showing the distribution of Triton-insoluble components of cells grown in a medium supplemented with horse serum.*

tassium chloride and 10 mM EDTA were added before isopycnic centrifugation, the lower band formed at a density of 1.270 g/cm$^3$ and was a homogeneous preparation of fibrils as judged by electron microscopy. Only one major protein, MW 55,000, was present (Fig. 2). Trace amounts of 26,000-MW protein were sometimes detected, but nearly all this component was now found in the uppermost band together with significant quantities of 55,000-MW protein but no visible fibrils.

**Morphology of fibrils.** Fibrils prepared without high-salt treatment had a diameter of 3.5 nm ± 0.2 nm, an axial repeat of 8.5 nm ± 0.2 nm, and were of indeterminate length. They were extensively aggregated and showed a marked tendency to associate in pairs giving the 7.5-nm-wide duplex a beaded appearance (Fig. 3). Some fibrils gave the appearance of being helically coiled, but their small diameter and state of aggregation made it impossible to confirm this with certainty by optical diffraction analysis.

Fibrils isolated from material subjected to high salt and EDTA were tightly aggregated into bundles up to 35 nm wide. The fibrils were stacked in a highly ordered manner so that repeating structures were vertically aligned producing marked striations across the aggregates (Fig. 4). The repeat interval remained at 8.5 nm. The absence of marked constrictions in the bundles despite the obvious twisting of component fibrils indicated that they represented flattened hollow tubules rather than twisted ribbons of fibrils.

**Properties of fibrils.** The MW of the fibril monomer was determined by polyacrylamide gel electrophoresis to be 55,000 ± 550. No staining was seen with periodic acid-Schiff reagent, indicating the absence of detectable glycoprotein. The fibrils were completely destroyed after 5 min of exposure to pronase (1 mg/ml) but were

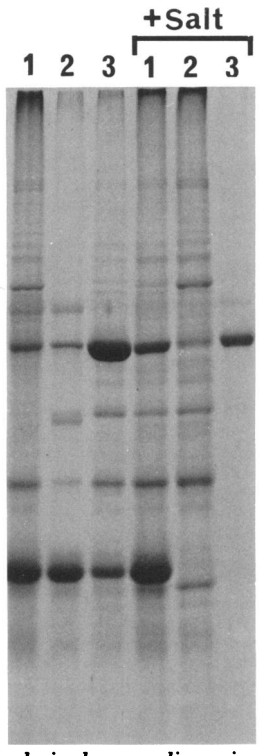


FIG. 2. *Analysis by one-dimensional polyacrylamide gel electrophoresis of 1, upper; 2, middle; and 3, lower bands from Urografin gradients after isopycnic centrifugation, showing the distribution of Triton-insoluble components of cells grown in a medium supplemented with fetal calf serum and the changes in the distribution of these components caused by the addition of 1 M potassium chloride and 10 mM EDTA (plus salt).*

apparently unaffected by 1 h of exposure to trypsin (1 mg/ml). Addition of urea to an 8 M solution destroyed the structural integrity of the fibrils, but amorphous strands of material could still be visualized in the electron microscope indicating incomplete denaturation. Fibrils were unaffected by extensive dialysis against 2.5 mM ATP in 10 mM Tris buffer (pH 7.5).

Addition of potassium chloride to a 1 M solution caused the fibrils to form striated aggregates which could not be dissociated even by extensive dialysis against 1 mM Tris buffer (pH 7.5). Incubating the aggregated fibrils in 10 mM phosphate buffer (pH 6.5) containing 1 mM EDTA and 0.5 mM magnesium chloride for 1 h at 0°C followed by the addition of ice-cold 2 mM colchicine also had no effect. During all treatments the axial repeat interval remained 8.5 nm.

**Identification of the fibril protein.** A protein band of MW 55,000 was present in the one-dimensional pattern of sodium dodecyl sulfate-solubilized whole cells (Fig. 5). Two-dimensional separation revealed several proteins of similar MW and significant quantities of 55,000-MW material which had failed to enter the first-dimension gel (Fig. 6a). Purified fibril protein also failed to enter the gel unless first solubilized in sodium dodecyl sulfate, after which treatment a single major spot was observed near the cathodic end of the gel (Fig. 6b). This protein had no counterpart in the pattern of cells solubilized in nonionic detergent and urea, but patterns obtained after prior solubilization of cells in sodium dodecyl sulfate, although greatly inferior in resolution, did show the presence of a protein with the same electrophoretic properties as the fibril monomer.

**Concentration of fibril protein in whole cells.** For every 100 μg of whole-cell protein starting material, between 0.8 and 1 μg of purified fibril protein banded at a density of 1.270 g/cm$^3$. On the basis of staining intensity of protein bands on polyacrylamide gels, it was estimated that a further 0.75 to 1 μg of 55,000-MW protein was present in the middle and upper bands.

## DISCUSSION

The small diameter of spiroplasma fibrils, the MW of the subunits, and the maintenance of a fibrilar morphology in low-ionic-strength salt solutions, even in the presence of ATP, clearly distinguish them from actin filaments and show that they are not analogous to the actin-like filaments isolated from *M. pneumoniae*. They are also unrelated to the cytoplasmic fibrils in *Treponema* which are readily solubilized by trypsin and are composed of a higher MW protein. Although the fibrils form ordered aggregates, these are clearly different from the striated ρ-fibers occurring in some strains of *Mycoplasma mycoides* (19).

The MW of the fibril monomer is similar to that of the tubulin subunit (MW 55,000) which occurs in all eucaryotic cells and polymerizes to produce the 5-nm-diameter protofilaments from which microtubules are formed (7). Spiroplasma fibrils appear significantly different from tubulin protofilaments since they do not dissociate when cooled to 0°C in the presence of the alkaloid colchicine, conditions which cause the dissociation of microtubules and prevent their reassembly. However, the possibility that spiroplasma fibrils aggregate to form hollow tubes is an interesting parallel with the microtubule, particularly since it has been reported that structures resembling microtubules, and distinct from those seen in *Treponema*, occur in some large spirochetes inhabiting the hindgut of certain

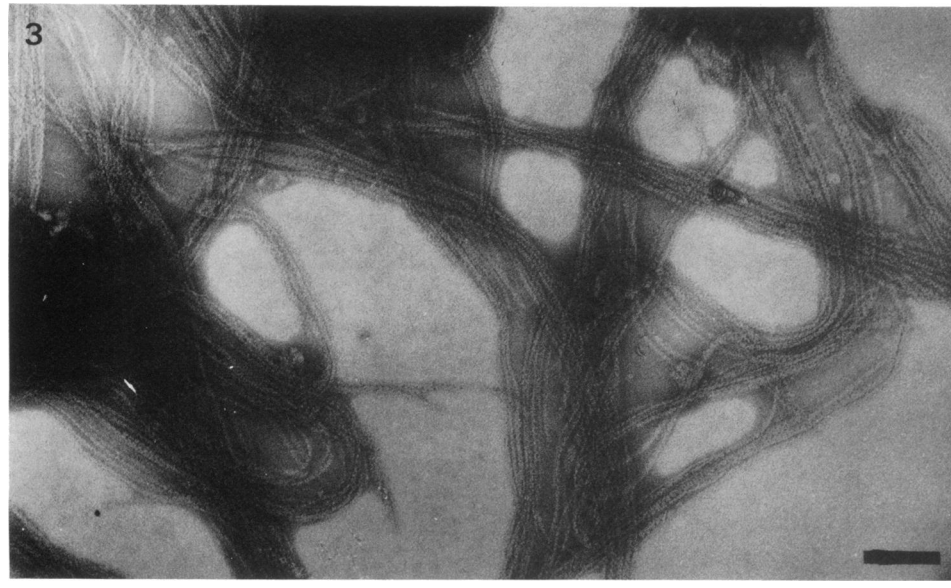


FIG. 3. *Partially purified spiroplasma fibrils prepared without high salt. The fibrils show a marked tendency to associate in pairs. Bar represents 100 nm.*

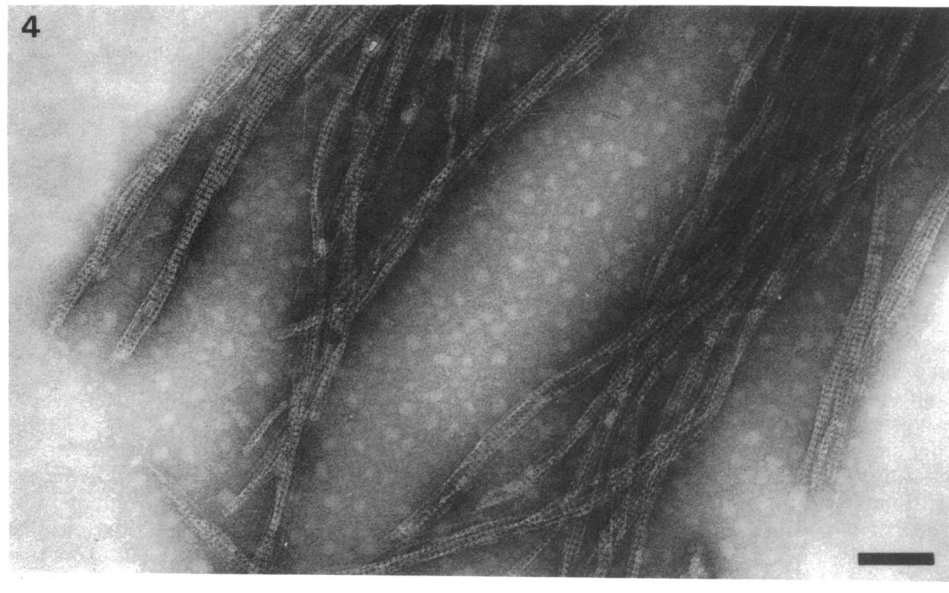


FIG. 4. *Purified spiroplasma fibrils prepared with high salt and EDTA. The fibrils form ordered aggregates with a marked striated appearance. Bar represents 100 nm.*

termites. These bacteria contain significant amounts of a 55,000-MW protein and can be stained with fluorescent antibodies to tubulin (14).

Thin sectioning of spiroplasmas has not revealed evidence of structures as complex as microtubules, and it is possible that aggregation of fibrils is an in vitro artifact unrelated to their biological function. However, the tendency of fibrils to pair and their highly ordered arrangement when aggregated suggest that interactions between fibrils may be an important feature of their function within the living cell. The most plausible role for spiroplasma fibrils still appears to lie in the maintenance of cell shape and motility, although no shortening or extension, as

measured by changes in the axial repeat interval, occurred under any of the conditions tested. The basis of spiroplasma motility may therefore be more complex than simple contraction and relaxation of individual fibrils.

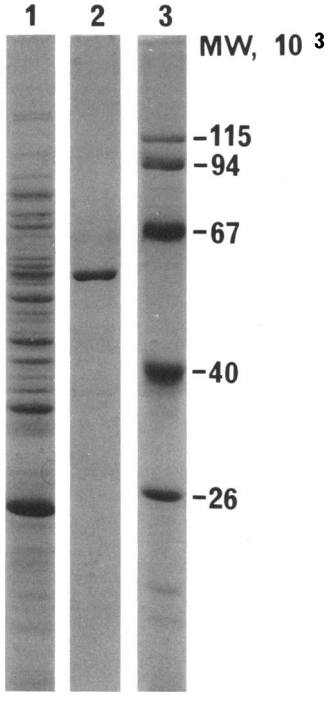


FIG. 5. *One-dimensional electrophoretic pattern of (1) whole cell spiroplasma proteins, (2) fibril protein, and (3) molecular weight standards.*

Estimates of the total amount of fibril protein in each spiroplasma cell show that if the subunits are of the order of 3.5 nm in diameter and are arranged in a simple straight chain, then there is sufficient material to make between 30 and 60 fibrils running the complete length of a 2-μm-long cell. If, as seems probable, the subunit chain is helically coiled, this number will be reduced according to the pitch of the helix. In any case it seems very unlikely that there are sufficient fibrils to form a contractile peripheral sheath around the entire cell.

The association of fibrils with membrane fragments after sodium deoxycholate treatment supports the theory that they are linked to an integral component of the membrane. The major protein component of the cytoplasmic membrane of *S. citri* is spiralin (MW 26,000) (26). A speculative model has been proposed in which dimers of spiralin span the lipid bilayer and aggregate in the plane of the membrane to form larger oligomers (25). Such a transmembrane oligomeric arrangment would be compatible with spiralin, providing the anchorage points for cytoskeletal structures in *S. citri*. This is supported by observations that antiserum prepared against spiralin causes a rapid loss of helical morphology and that some of the antigenic determinants of the molecule, expressed on the cytoplasmic face of the membrane, are shielded by extrinsic components (25). Substantial amounts of a protein, with a similar MW to spiralin (26), copurify with the honeybee spiroplasma fibrils. This protein may be bound ionically to the fibrils since it was removed by high salt with EDTA.

Of the other prominent proteins associated

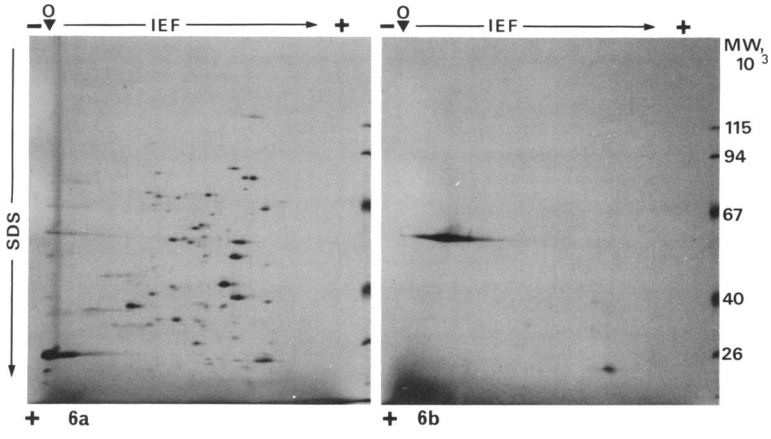


FIG. 6. *Two-dimensional electrophoretic separations of (a) spiroplasma whole cell proteins solubilized in Nonidet and urea and (b) purified fibril protein solubilized in sodium dodecyl sulfate which was subsequently displaced by the addition of excess Nonidet with urea (origin, O). SDS, sodium dodecyl sulfate.*

with partially purified fibrils, two have MWs similar to those of proteins which have previously been implicated in the maintenance of helicity and in motility. One has a MW of 43,000 similar to those of the actin-like proteins and the other (MW 40,000) may be identical to the protein which is absent from the membrane of the nonhelical variant of *S. citri* (22).

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