SELECTIVE EXTRACTION WITH SARKOSYL AND REPOLYMERIZATION IN VITRO OF CYTOSKELETON PROTEINS FROM GIARDIA

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SUMMARY

Sarkosyl has been used to dissociate structures in cytoskeletons isolated from Giardia lamblia. Results from sodium dodecyl sulphate/polyacrylamide gel electrophoresis and from electron microscopy of insoluble residues show that the solubilization of components is partly selective. At low concentrations of detergent (<0·3 %), microribbons and microtubules of the disc cytoskeleton disappear, but doublet microtubules from axonemes resist extraction. Consequently, the microribbon protein giardin is extracted into solution more completely than tubulin.

Soluble proteins in 0·1 % Sarkosyl have been fractionated by gel filtration chromatography in Bio-Gel P300. Giardin elutes in two positions: as a low molecular weight subunit, and in early fractions corresponding to a larger particle size in which subunits might be forming oligomers.

Supernatants prepared in 0·5 % Sarkosyl were diluted with 0·1 M-KCl or 0·1 mM-MgCl₂ to bring about reaggregation of the cytoskeleton proteins. Reassembled structures seen in negatively stained preparations were polymorphic. Some tubulin ribbons of 5 nm protofilaments were identifiable; also there were large fibres and some flat sheets of very thin filaments. Electron micrographs of sheets have been analysed by optical diffraction. The transforms show that the lateral separation of the fine filaments is about 2·5 nm. Axial periodicities from features spaced along filaments were weak. A 3·75 nm layer-line has been detected, corresponding to a similar periodicity found earlier in microribbons.

INTRODUCTION

The disc cytoskeleton of Giardia is a highly ordered protein assembly in the ventral cytoplasm of the trophozoite (Holberton, 1973). The principal components of this structure are tubulin and a smaller protein, giardin, known only from this source. Using the denaturing agent sodium dodecyl sulphate (SDS), giardin has recently been dissociated from isolated cytoskeletons and purified by gel filtration chromatography (Crossley & Holberton, 1983). Giardin is a small structural protein with a chain molecular weight \( (M_r) \) of \( 30 \times 10^3 \), and multiple isoelectric variants. On the basis of its amino-acid composition, which shows a preponderance of helix-forming residues, we have suggested that it may be rod-shaped.

In the cytoskeleton giardin is found in microribbons attached to microtubules (Holberton & Ward, 1981). Microribbons are multilayered; it is thought that giardin forms an extensive scaffold running along the core of the microribbon that serves to bind layers of tubulin in an ordered way. Diffraction studies have shown tubulin subunit lattices at ribbon faces but the internal structure of ribbons is not visible when whole mounts are negatively stained (Holberton, 1981). Thus direct information is
lacking on giardin dimensions, and on the structural arrangements of molecules when associating with tubulin protofilaments.

Another approach is to examine undenatured proteins after purification for reassembly in vitro into ordered structures. In some preliminary experiments on cytoskeletons extracted in low ionic strength Tris/EDTA buffers the yield of soluble protein was low, precluding the successful fractionation of giardin and tubulin. On elution from gel filtration columns, giardin tended to be bound in particles at least twice the chain molecular weight, and co-eluted with tubulin and other larger proteins (Holberton & Crossley, 1981).

In this paper we report the results of experiments using the ionic detergent sodium lauryl sarcosinate (Sarkosyl) to dissociate protein subunits from cytoskeletons. Sarkosyl extraction has the advantage that higher relative yields of giardin are obtained. We have found that this is because, in low concentrations of the detergent, some doublet microtubules from axonemes that remain attached to isolated discs are more resistant than microribbons to dissociation, and can be removed by centrifugation. The soluble fraction is, therefore, a purer preparation of microribbon proteins than it has hitherto been possible to obtain.

Because the effect of Sarkosyl on proteins is mild, its action in dissociating subunits from structural assemblies can be reversed by diluting the detergent. In early studies to characterize tubulin, Stephens (1968) used this behaviour to repolymerize microtubules from sea-urchin sperm tail proteins. By repeating the procedure with Giardia disc proteins, we have obtained polymorphic reaggregation of subunits, including some sheets composed of novel 2-5 nm fine filaments. It is likely that these provide the first images of supramolecular assembly of giardin.

**MATERIALS AND METHODS**

**Extraction of proteins from cytoskeletons**

Methods used for axenic culture of Giardia lamblia trophozoites in TPS-I medium (Visvesvera, 1980), and for preparing demembranated cytoskeletons in TEDAMP+ Triton medium were described in an earlier paper (Crossley & Holberton, 1983). Suspensions of cytoskeletons at a total protein concentration of 0-4–0-6 mg ml⁻¹ were extracted at 0°C in concentrations of Sarkosyl from 0-0125–0-3 % (v/v). Sarkosyl (Ciba-Geigy) was made up in 10 mM-Tris-phosphate buffer (pH 7-8) with 0-1 mM-dithiothreitol (Linck, 1976). Control samples were extracted in buffer without Sarkosyl. In a duplicate series some samples contained 50 μg ml⁻¹ phenylmethylsulphonyl fluoride (PMSF) and 0-1 mM-benzethonium chloride as checks against endogenous proteolysis.

Material pelleted after extraction was washed briefly in 10 mM-Tris-phosphate buffer to remove excess Sarkosyl. Final residues were divided so that the effects of extraction could be assessed both by electron microscopy and by SDS/polyacrylamide gel electrophoresis (SDS/PAGE).

For gel filtration chromatography, proteins from 5 mg ml⁻¹ of cytoskeletons were extracted overnight in 0-1 % Sarkosyl at 0°C. Insoluble fragments were pelleted by centrifuging at 100,000 g for 1 h. A sample of 3-2 mg of soluble proteins was then run on a column of Bio-Gel P300 (Bio Rad Laboratories) in Tris-phosphate buffer.

**SDS/PAGE**

Soluble proteins in column fractions were mixed with an equal volume of an SDS sample solution that, after dilution, contained 2 % SDS, 10 % (v/v) glycerol, 1 % mercaptoethanol in
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25 mM-Tris-glycine buffer (pH 8.3) with 0.005% PMSF (Pringle, 1970). Insoluble residues were dissolved in the same SDS solution.

Protein samples were separated in a continuous buffer system on small vertical slab gels (Amos, 1976) and stained using identical procedures as before (Holberton & Ward, 1981; Crossley & Holberton, 1983). The running buffer was the same low ionic strength buffer used in preparing gels: 25 mM-Tris-glycine (pH 8.3) with 0.1% SDS (Stephens, 1975).

Electron microscopy

Residues from Sarkosyl extraction and control pellets were fixed, embedded and sectioned as described previously (Crossley & Holberton, 1983).

Protein aggregates diluted out from a Sarkosyl supernatant were collected on grids and negatively stained. Material from the precipitates was applied as a suspension to colloidon-coated 200 or 300 mesh copper grids using the drop method (Haschemeyer & Meyers, 1972). Grids were stained with either 0.5% uranyl formate or 0.5% uranyl acetate. The wetting agent Bacitracin was added to all stains at a working concentration of 50 µg ml⁻¹ (Gregory & Pirie, 1973). Immediately before use stains were passed through a GS filter (Millipore Ltd).

Grids were viewed and photographed at an accelerating voltage of 80 kV in a JEOL 100C electron microscope. To detect periodicities in protein aggregates micrographs were analysed by optical diffraction. For these studies, specimens were photographed at low beam current and at an instrument magnification of ×26 000 or ×50 000. Micrographs of repolymerized brain microtubules taken on the same occasion were used to calibrate diffraction spacings. A single diffracting lens was used on a ≈5 m horizontal bench (Markham, 1968). To record diffraction patterns a focusing screen was replaced by a parfocal camera back and exposures made on either Polaroid type 53 P/N film, or Panatomic X 35 mm film (Kodak Ltd).

RESULTS

Sarkosyl extraction

Washed Giardia cytoskeletons were extracted for 1 h at 0°C as a series of samples in different Sarkosyl concentrations, buffered at pH 7.8 in 10 mM-Tris-phosphate.

Table 1. Differential extraction by Sarkosyl of proteins from cytoskeletons

<table>
<thead>
<tr>
<th>Sarkosyl concentration (% v/v)</th>
<th>Total protein solubilized* (%)</th>
<th>Amount of protein in residues after extraction†</th>
<th>Relative composition of insoluble residues: giardin/tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tubulin (%)</td>
<td>Giardin (%)</td>
<td></td>
</tr>
<tr>
<td>Control: 0.0</td>
<td>0</td>
<td>17.5</td>
<td>19.8</td>
</tr>
<tr>
<td>0.017</td>
<td>0</td>
<td>18.1</td>
<td>20.2</td>
</tr>
<tr>
<td>0.033</td>
<td>0</td>
<td>16.4</td>
<td>19.1</td>
</tr>
<tr>
<td>0.075</td>
<td>16</td>
<td>18.3</td>
<td>20.0</td>
</tr>
<tr>
<td>0.17</td>
<td>18</td>
<td>18.4</td>
<td>19.3</td>
</tr>
<tr>
<td>0.25</td>
<td>23</td>
<td>19.3</td>
<td>17.7</td>
</tr>
<tr>
<td>0.33</td>
<td>47</td>
<td>20.7</td>
<td>15.4</td>
</tr>
</tbody>
</table>

* Soluble protein was determined according to the following protocol: a suspension of cytoskeletons of known protein concentration was dispensed in 0.1 ml portions, and to each was added 0.9 ml of Sarkosyl medium. After extraction, samples were centrifuged at 48 000 g for 15 min to remove residues. Protein in the supernatant fractions was assayed by a microbiuret method (Goa, 1953).
† Residue proteins were determined after dissolving in 2% SDS from densitometry of microgels stained in PAGE Blue 83 (Holberton & Ward, 1981).
Table 1 shows that more protein was solubilized as the concentration of Sarkosyl was increased above 0.07%. Below 0.07% Sarkosyl, the proteins did not dissolve in quantities large enough to be detected by the microbiuret assay.

The appearance of Triton-insoluble cytoskeletons before Sarkosyl extraction is shown in Fig. 1. After extraction, the insoluble residues were pelleted from suspension, then fixed, embedded and sectioned. When examined in the electron microscope, the sectioned material was now found to be mainly unstructured filamentous aggregates. Complete axonemes were not seen, but doublet microtubules were common in the pellet (Fig. 2). These were often in groups of nine, but had lost their usual cylindrical arrangement. Also, central pair microtubules were missing from the cross-sections. Both in longitudinal and transverse section the doublets appeared smooth, so that dynein arms and nexin links were not visible. No complete discs were seen; in the few disc fragments that were present the microtubules had been lost from microribbons.

**SDS/PAGE**

SDS/PAGE of the cytoskeletons showed that tubulin, giardin, high and intermediate molecular weight polypeptides of bands 1–8, and low molecular weight polypeptides of bands 16–20 (Crossley & Holberton, 1983), were all present in the residue after extraction. However, certain of these proteins appear to be solubilized preferentially at the higher Sarkosyl concentrations (Fig. 3). The extraction was most marked for the polypeptides of band 6, and for giardin. On the other hand, band 5 protein and band 10 protein (which has a mobility similar to actin) were clearly less soluble at high concentrations of Sarkosyl.

The relative amounts of the two major proteins present in the residues pelleted after extraction, as calculated from peak areas of densitometer traces, are given in Table 1. For these two proteins there were slight differences in the composition of cytoskeletons exposed to low concentrations of Sarkosyl. Since there was no measurable extraction of protein at these concentrations, the variation is probably the error in the estimate from densitometry of nearly identical samples at different sample loadings. However, the mass ratios of the two proteins were almost the same as that of a control sample. With Sarkosyl concentrations higher than 0.075%, the mass ratio shows that relatively more giardin was extracted. Tubulin, then, accounted for an increasing...
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Figs 1–2
proportion of the residues when extraction was more complete, which is consistent with the presence of persistent microtubule doublets seen by electron microscopy.

**Analysis of the Sarkosyl supernatant by gel filtration chromatography**

The cytoskeleton proteins solubilized on ice in 0.1% Sarkosyl were run into a column of Bio-Gel P300 equilibrated with 0.1% Sarkosyl in 10 mm-Tris-phosphate buffer (pH 7.4). The proteins eluted in four peaks (Fig. 4A). The absorbance profile at 280 nm indicated that the majority of the protein eluted in the first peak.

Individual column fractions were concentrated at 900 g in Amicon CF25
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Fig. 4

Absorbance at 280 nm

Fraction number

Bands:

Tubulin

Giardin

Fig. 4
Table 2. Relative change in protein concentration of column fractions before SDS/polyacrylamide gel electrophoresis

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Final concentration ( \times 10^{-2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.56</td>
</tr>
<tr>
<td>17</td>
<td>0.38</td>
</tr>
<tr>
<td>19</td>
<td>0.49</td>
</tr>
<tr>
<td>21</td>
<td>0.82</td>
</tr>
<tr>
<td>23</td>
<td>0.98</td>
</tr>
<tr>
<td>25</td>
<td>1.00</td>
</tr>
<tr>
<td>27</td>
<td>0.67</td>
</tr>
<tr>
<td>29</td>
<td>0.44</td>
</tr>
<tr>
<td>31</td>
<td>1.00</td>
</tr>
<tr>
<td>33</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Centriflow cones (Crossley & Holberton, 1983), and analysed by SDS/PAGE (Fig. 4b). The gels showed that the second peak (fraction 25) corresponded to the elution of tubulin, and the third peak (fraction 31) was giardin. The first peak (fractions 16 and 17) contained high \( M_r \) proteins (bands 1–8), some tubulin, probably in the form of dimers, and band 10 protein. Also, substantial amounts of giardin eluted at the trailing edge of the first peak (fractions 19–21), ahead of the main tubulin elution. The elution profile for this protein therefore had a double peak, suggesting that some of the giardin subunits from discs had behaved in this buffer as oligomers, or were bound to other proteins.

Polyacrylamide gels were loaded with samples of equal volume, but from column fractions that had undergone unequal volume changes during the concentrating step. The staining of proteins in each track is, therefore, not quantitative with respect to the original elution of proteins from the column. From the measured final volumes, Table 2 gives the relative concentration changes that occurred in the fractions used to prepare the gel in Fig. 4b. It is apparent that the fractions corresponding to the elution of low \( M_r \) giardin (fractions 29, 31), and those collected earlier containing this protein (fractions 19, 21), were concentrated to about the same extent before running the gel. The fact that the bands from the early fractions stain more densely in the electrophoresis pattern then indicates that more of the giardin was in the form of larger particles, a smaller proportion eluting as dissociated subunits.

Reassembly of cytoskeleton proteins

Cytoskeletons were not easily dissolved in non-denaturing solutions other than

Figs 5, 6. Structures formed from *Giardia* proteins in 0.5% Sarkosyl-GTP by diluting 1:10 with 0.1 mM-MgCl₂. Negatively stained in 0.5% uranyl formate (pH 4.75).

Fig. 5. Low-power micrograph showing long fibres (arrow) and plaque-like aggregates (p). Bar, 500 nm.

Fig. 6. Detail from Fig. 5 to show substructure at higher magnification. Protofilaments can be detected in the reaggregated long fibres (arrows). Bar, 100 nm.
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Figs 5–6
Sarkosyl. Attempts were made to reassemble cytoskeleton proteins from Sarkosyl supernatants following a method used earlier by Stephens to repolymerize microtubules from sea-urchin sperm tail proteins. Stephens (1968, 1969) reported that in the presence of GTP most of the protein from axonemes was soluble in 0.5% Sarkosyl, but that 10-fold dilution of the soluble fraction in either distilled water, 0.1 M-KCl or 0.1 mM-MgCl₂, resulted in reaggregation of the protein to form filamentous structures, including structures resembling microtubules.

*Giardia* cytoskeletons from approximately 3 × 10⁸ of freshly harvested cells were washed by pelleting in 100 ml of 10 mM-Tris-HCl (pH 7.8). They were then suspended at 4 mg ml⁻¹ and extracted on ice for 1 h in 0.5% Sarkosyl, 1 mM-GTP, 0.1% (v/v) mercaptoethanol in 10 mM-Tris-HCl (pH 7.5). Insoluble residues were removed by centrifuging at 35,000 g for 15–30 min. Under these conditions about 50% of protein went into solution, as determined by microbiuret assay (Goa, 1953).

On dilution of the soluble fraction in 10 volumes of cold, degassed 0.1 M-KCl or 0.1 mM-MgCl₂, turbid aggregates were formed, which could be pelleted by centrifuging again at the same speed. The experiment was repeated several times with the same result. The pelleted precipitates were negatively stained in 0.5% uranyl formate or uranyl acetate. The material on all grids had a similar appearance, irrespective of the salt used to dilute the protein samples.

The most extensively formed structures were large granular plaques with a mottled appearance (Fig. 5). It has not been possible to resolve any order within these structures. They seem to represent a compact aggregation of particulate material that was elsewhere spread more unevenly over the same grids. In addition, there were many filamentous structures of different kinds. Some long ribbons of fairly uniform width were seen, at higher magnifications, to be made up of longitudinally arranged protofilaments resembling microtubule protofilaments (Fig. 6). Where it was possible to measure across a number of protofilaments, they were found to be separated by distances of about 5 nm. However, fine details of their structure were partly obscured by disc-shaped platelets, about 15–20 nm across, which extensively decorated many structures in these preparations, including the flat plaques.

Other fibrous structures were broad tactoids, either spindle-shaped or microribbon-like (Fig. 7). Most of the larger ribbons stained densely and were heavily coated with other material. In some areas these ribbons were co-extensive with some more transparent flat sheets that were visibly divided into fine longitudinal filaments (Figs 9–11). The filaments in sheets were obviously smaller and more closely packed than the ≈5 nm protofilaments seen on the same grids. Fig. 8 shows regions in which ordered tracts of fine filaments are apparently continuous with

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Figs 7, 8. Uranyl formate stained structures repolymerized when supernatant proteins in 0.5% Sarkosyl-GTP were diluted 1:10 with 0.1 M-KCl.

Fig. 7. In addition to plaques (↑), there are broad dense ribbons, and thinner sheets and ribbons of various sizes formed from laterally associating narrow filaments. Bar, 100 nm.

Fig. 8. Showing at higher magnification how fine filament tracts are easily disaggregated, some filaments appearing to coalesce with the disordered material in plaques (arrows). Bar, 100 nm.
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Figs 7–8
plaques or with some of the loosely aggregated material covering the surface of the grid.

Some areas of sheets were masked off for optical diffraction. The strongest feature of the diffraction patterns was a pair of equatorial reflections arising from the lateral packing of filaments. The positions of spots varied slightly when diffracting from different sheets in the same preparation, or from separately masked areas of the larger sheets. Diffraction spacings were calibrated from repolymerized brain microtubules photographed in series, using the 4 nm layer-line from the tubulin protomer repeat. The results show that, in different micrographs, the separation of the small Giardia filaments was equivalent to distances of between 2.4 and 2.9 nm.

In most images axial detail along sheets was weak; consequently in diffraction patterns few layer-lines were consistently present that had peaks significantly more intense than the background scatter. There are probably several reasons for this. In these structures, formed de novo from an impure fraction containing a number of protein species, the re-association of filaments might not be precise, except over short distances. Evidence of this is seen in the way splits occur easily between filaments, and at the ragged edges of some sheets where filaments appear to peel away and break down. Also, it is likely that the filament lattice is in many places covered by additional disordered material sticking to the sheets.

Figs 12 and 13 are transforms showing certain spacings that were present in most of the sheets. These are a pair of meridional spots with values between 5.0 and 5.5 nm, and a layer-line at about 3.75 nm. In different patterns, pairs of peaks appeared on this layer-line in various positions off the meridian. These two periodicities fall close to the third and fourth orders of the strong 15 nm repeat that characterizes intact microribbons where, it is believed, several dissimilar protein sheets are superimposed (Holberton, 1981).

Also present in a number of transforms were peaks on or near the meridian with the values of 3.0–3.3 nm, and 2.6 nm, which may be higher orders of the same system. On the other hand, there were also weak off-meridional reflections at 4.5 nm that do not belong to the 15 nm orders.

In some transforms there were additional reflections at 7.5 nm and 15 nm, which show weakly in Fig. 13. The structure that gave this pattern is shown in Fig. 11. It has visible near orthogonal lines crossing the filaments but appears thicker than some of the other sheets, suggesting that it may be a composite sheet of more than one ordered subunit layer.

Figs 9, 10. Sheets of fine filaments from the preparation shown in Fig. 7. Filaments run longitudinally and are less than 3 nm wide. The optical diffraction pattern from the framed area is shown in Fig. 12. Bar, 20 nm.

Fig. 11. A sheet repolymerized in 0.1 M-KCl, negatively stained in 0.5% uranyl acetate (pH 4.5). Filaments are longitudinal, but towards the bottom of the sheet widely spaced orthogonal fine lines are also discernible (arrows). The central region (between the arrowheads) appears thin compared to areas of the sheet at top and bottom, which exclude the stain more completely, and are decorated with other material. The region between the horizontal lines was masked for the diffraction pattern in Fig. 13. Bar, 20 nm.
Figs 12–13
Reassociation of giardin

Fig. 14. Composition of structures formed from Sarkosyl-soluble proteins after dilution in 0.1 M-KCl. Reaggregated material was pelleted at 35 000 g, washed, then dissolved in 2% SDS. Gel pattern shows electrophoresis on a 10% polyacrylamide gel, stained in PAGE Blue 83. Both tubulin and giardin are components of reformed structures.

Analysis by SDS/PAGE of a sample of the material re-aggregated in 0.1 M-KCl showed that giardin and tubulin were the principal components (Fig. 14). Band 5 protein and very high Mr components were also present. Compared to the composition of cytoskeletons, giardin accounted for a much larger proportion of the protein in reassociated structures.

Fig. 12. Diffraction pattern from the reconstituted sheet in Fig. 10. The strong reflexions on the equator at 2.7 nm correspond to the lateral spacing of fine filaments. Note weak layer-line reflexions at ±3.75 nm and ±2.6 nm.

Fig. 13. Diffraction pattern from Fig. 11. The centre spot has been attenuated photographically. The strong spots on the equator show that the filaments in this sheet were separated by 2.4 nm. The upper half of the pattern identifies features at 3.75 nm along filaments. Other periodicities in this series of images are indicated by the weak layer-lines marked in the bottom half of the transform.
DISCUSSION

Giardin subunits

Sarkosyl was used at 0-1 % in this study to dissociate cytoskeletons without grossly denaturing constituent proteins. Previously, we had separated the denatured chains of giardin from other cytoskeleton components by gel filtration in SDS. When filtering proteins in Sarkosyl through Bio-Gel P300, the early elution of giardin at high particle weight is an indication that the treatment was mild.

Sarkosyl is an anionic lauryl derivative like SDS, but does not have the same drastic effects on tertiary conformation of protein chains. In a number of studies in which Sarkosyl has been used as a dissociating agent, proteins have been shown to retain native properties. For example, hamster scrapie infectivity was unchanged in Sarkosyl concentrations as high as 5 % (Prusiner et al. 1980), and mouse RNA polymerase II activity was enhanced in isolated nuclei in concentrations of the detergent up to 2 % (Green, Buss & Gariglio, 1975). Also, after dissociating Arbacia punctulata sperm tails in 0-5 % Sarkosyl, then diluting the supernatant in the presence of GTP, Stephens (1968) obtained the reassembly of the soluble protein into microtubules and ribbons of protofilaments.

Studies of the sedimentation rate of sea-urchin axonemal tubulin in 0-2–0-5 % Sarkosyl were consistent with the protein being totally dissociated to monomers (Stephens, 1968, 1969). However, using a lower concentration of Sarkosyl, we have found that Giardia tubulin elutes from Bio Gel P300 in two positions (peaks 1 and 2, Fig. 4A), which suggests that the higher $M_t$ tubulin particles might be undissociated dimers.

Similarly, the fraction of giardin that eluted early from the column might be explained if the native protein is a low order oligomer of giardin chains. The elution of high $M_t$ giardin was symmetrical about the peak fraction as would be expected for particles of uniform size as opposed to random aggregates. The peak of its elution did not coincide exactly with the main elutions of proteins of higher chain weight, like tubulin, band 5 protein and band 6 protein. Furthermore, from the staining density on gels, giardin was in excess in these fractions over accompanying proteins. For these reasons it seems unlikely that after Sarkosyl extraction, the presence of giardin in early fractions is solely due to binding to other larger proteins.

It is not possible to establish the particle size reliably from gel filtration chromatography, and hence the degree of oligomerization of giardin. Proteins eluting in the same or neighbouring fractions, for which polypeptide chain sizes have been determined were: tubulin (dimer $M_t = 110 000$), band 5 protein ($M_t = 95 500$) and band 6 protein ($M_t = 80 000$). At face value this would indicate a particle of from two to four giardin chains, unless the co-eluting proteins were also aggregated. Since the proteins were incompletely fractionated at the trailing edge of the first peak, this possibility cannot be excluded. Also, the shape of the giardin aggregate is unknown. We have suggested that the molecule of giardin is more likely to be rod-shaped than globular. This conclusion was reached on the basis of its amino-acid composition, but is now supported by images in this paper of 2-5 nm filaments (see below).
Consequently, if the aggregate is filamentous, its elution volume determined by the Stokes radius ($R_t$) of the particle, cannot be calibrated accurately for molecular size ($M_r$) by the elution positions of proteins belonging to a different conformational class, such as tubulin or other globular proteins commonly used as column standards (Le Maire, Rivas & Moller, 1980). Also, it has been shown that large elongated proteins may be retarded because of end-on insertion into gel pores, thereby eluting in positions that correspond to anomalously small $R_s$ values compared to those obtained by viscometry or sedimentation behaviour (Nozaki, Schecter, Reynolds & Tanford, 1976).

Reassembly in vitro

The result of diluting the Sarkosyl supernatant was to produce polymorphic aggregation of cytoskeleton proteins. The heterogeneous composition of precipitates, as shown by SDS/PAGE, accounts for the variety of structures seen in the electron microscope.

There were many tubulin ribbons among the aggregated Giardia proteins, judging from the dimensions of their protofilaments. It is not clear whether these structures were sufficiently numerous to account for the tubulin present; there remains the likelihood that a proportion of the tubulin may have been sequestered by binding to giardin. It might be expected that during polymerization competitive interactions occurring simultaneously between dissimilar proteins, particularly between giardin and tubulin, might interfere with processes of self-assembly. Such effects, leading to low-order closed aggregates, might explain the large amounts of unstructured material on grids. Even for axonemal proteins in Sarkosyl, in which tubulin is the major component, a large part of the protein precipitated amorphously on dilution (Stephens, 1968). Reassociation into ordered protofilaments was only partly successful in those experiments; Stephens (1969) estimated that in 0·1 mM-MgCl$_2$ well-defined structures accounted for about 40% of the protein originally solubilized from Arbacia sperm tails, and a somewhat smaller proportion of the tubulin from axonemes of Strongylocentrotus. In neither case was assembly into complete doublet microtubules observed.

For Giardia proteins, the excess of giardin in the supernatant would favour giardin polymerization, as was apparent from its preponderance also in the newly formed pellet. Other proteins, such as tubulin, which in vivo might modify giardin assembly, would have less influence in this preparation. The 2·5 nm filament sheets were the largest ordered structures found after reaggregation. They were probably composed almost entirely of giardin. Tubulin molecules, because of their larger subunit dimensions (Amos & Klug, 1974), could not be a component of 2·5 nm filaments, although tubulin might be bound to the outside of sheets where these appear more dense than a single layer.

2·5 nm filaments

The way in which giardin molecules might be organized in the 2·5 nm filaments is not obvious from the electron micrographs. This is partly because the images of
filament sheets give variable diffraction patterns, but also because the most likely dimensions of giardin molecules are not readily matched to those spacings that have been detected.

Given a chain $M_r$ of $30 \times 10^3$, giardin molecules packed end to end into a 2.5 nm filament would repeat at intervals greater than 7 nm, assuming the molecules are compact ellipsoids with a protein density of 1300 daltons nm$^{-3}$ (Mikhailov & Vainshtein, 1971). But they could extend for considerably longer distances if individual molecules do not completely fill the lattice volume implied by the centre-to-centre spacing of filaments in sheets. Micrographs give the impression that filaments are not of uniform width; they touch periodically, but otherwise stain penetrates easily between them. Sheets also split and fray easily, so the lateral bonding of filaments seems to be relatively weak.

For filaments arranged in sheets, optical diffraction analysis has resolved a series of short periodicities in the direction of the filament axis, which arise from features spaced closer than 5.5 nm. None of these spacings is likely to coincide with the dimensions of the chemical subunit. Longer periodicities are seen in some transforms but not consistently. Those images giving layer-lines closer to the equator are usually found to be rather thick sheets, like that shown in Fig. 11. This raises the possibility that the most visible long spacings result where there is optical interaction between superficial material and the underlying thin filaments, so the diffraction peaks are not a reliable indication of intrinsic giardin packing.

For sheets that are apparently thin and well-flattened, the information on the 3.75 nm layer-line is a reasonably pronounced morphological feature (Fig. 13). A weak 3.75 nm axial periodicity was found earlier in microribbons sectioned horizontally, and was ascribed provisionally to structures in the ribbon core after it was found that masking out the ribbon face layers did not weaken the layer-line (Holberton, 1981).

It is likely that the sheets of giardin formed here in vitro provide a reasonable model of the exposed microribbon core. In the earlier study of isolated intact microribbons, the extent of resolution was only about 2.5 nm when diffracting through ribbon faces; thus it is not surprising that structures the size of giardin filaments were not detected. Recently we have found that fine filaments become visible where microribbons break down. This is seen especially following dialysis of cytoskeletons against a 10 mM-HEPES/EDTA buffer at pH 8.6 (unpublished results). Amongst the residues are ribbons that have frayed longitudinally, and which give rise to fields of filaments 2–3 nm wide. These are of indeterminate length, but are often aligned in pairs.

Morphologically, the fine filaments from Giardia are similar to 2–3 nm filaments described recently in sea-urchin sperm flagella after tubulin had been extracted by sodium thiocyanate or urea (Linck & Langelin, 1982). These authors showed by SDS/PAGE that the insoluble sperm residues contain a set of polypeptides that they had earlier named 'tektins' (Linck, Albertini, Kenney & Langelin, 1982), having apparent molecular weights between $M_r = 47 \times 10^3$ and $M_r = 55 \times 10^3$; i.e. somewhat larger than giardin. Tektin is apparently insoluble in Sarkosyl, whereas we have shown that giardin is at least partly soluble in this detergent. In some other respects:
high α-helix content, isoelectric focusing patterns, the two proteins resemble one another. Both might belong to a more widespread family of filamentous proteins of relatively low molecular weight that can combine with tubulin protofilaments. Linck & Langevin (1982) have suggested that other members of this family might be the intermediate filament proteins (see Lazarides, 1982). The availability of alternative tubulin/low molecular weight protein interactions might be one factor governing the apparent structural diversity of microtubules, i.e. as between flagellar microtubules, cytoplasmic microtubules or modified tubules such as those giving rise to micro-ribbons in the *Giardia* disc.

This work was funded by a grant from the Science and Engineering Research Council, whose support we gratefully acknowledge. We also thank Roland Wheeler-Osman for preparing photographs of gels.

REFERENCES


(Received 4 January 1983—Accepted 5 March 1983)