CHARACTERIZATION OF PROTEINS FROM THE CYTOSKELETON OF GIARDIA LAMBLIA

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SUMMARY

Proteins from the axonemes and disc cytoskeleton of Giardia lamblia have been examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. In addition to tubulin and the 30 × 10^3 molecular weight disc protein, at least 18 minor components copurify with the two major proteins in Triton-insoluble structures. The most prominent minor bands have the apparent molecular weights of 110 × 10^3, 95 × 10^3 and 81 × 10^3.

Protein of 30 × 10^3 molecular weight accounts for about 20% of organelle protein on gels. In continuous 25 mM-Tris-glycine buffer it migrates mostly as a close-spaced doublet of polypeptides, which are here given the name giardins.

Giardia tubulin and giardin have been purified by gel filtration chromatography in the presence of sodium dodecyl sulphate. Well-separated fractions were obtained that could be further characterized.

Both proteins are heterogeneous when examined by isoelectric focusing. Five tubulin chains were detected by PAGE Blue 83 dye-binding after focusing in a broad-range ampholyte gel. Giardin is slightly less acidic than tubulin. On gels it splits into four major and four minor chains with isoelectric points in the pH range from 5.8 to 6.2.

The amino acid composition of the giardin fraction has been determined, and compared to Giardia tubulin and a rat brain tubulin standard. Giardins are rich in helix-forming residues, particularly leucine. They have a low content of proline and glycine; therefore they may have extensive α-helical regions and be rod-shaped.

As integral proteins of disc microribbons, giardins in vivo associate closely with tubulin. The properties of giardins indicate that in a number of respects — molecular size, charge, stoichiometry — their structural interaction with tubulin assemblies will be different from other tubulin-accessory protein copolymers studied in vitro.

INTRODUCTION

The α and β subunits of tubulin, the protein of microtubules, have been shown to exist in a number of forms with subtly different compositions. Multiple tubulins have been demonstrated in protozoan cells of one strain (Lefebvre, Silflow & Rosenbaum, 1980), in purified sperm axonemes (Kobayashi & Mohri, 1977), and in a single mammalian neuron (Gozes & Sweadner, 1981). Recent studies have been directed towards the question of whether different tubulin species in the same cell are used during the assembly of dissimilar microtubules; for example, in the cytoplasm and in flagellar axonemes. Analysis on two-dimensional gels has shown that microtubular organelles have characteristic differences in the patterns of subunits incorporated into their structure, at least for electrophoretic variants of the α protomer (McKeithan & Rosenbaum, 1981). On the other hand, it is also true that microtubules can be polymerized in vitro, using mixtures of tubulin dimers from different organisms.
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(Sheir-Neiss, Lai & Morris, 1978; Water & Kleinsmith, 1976) and different organelles (McKeithan & Rosenbaum, 1981), showing that normally segregated tubulin species are competent to co-assemble.

To explain structural diversity of microtubules in vivo, one possibility is that assembly is specifically modified by other proteins associated with tubulin. Accessory proteins might exert an effect in a number of ways: by direct structural interactions with tubulin; by selecting appropriate subunits from among tubulin species; or, after incorporation of common tubulin precursors, by allowing chemical modification of dimers in organelle- or site-specific patterns.

Giardia is an endoparasitic flagellate with a number of microtubular organelles. Extraction with Triton isolates from this organism the eight flagellar axonemes and a large disc cytoskeleton that normally supports the cell's adhesive sucker. Although there are many microtubules in the disc, its volume is composed overwhelmingly of large microribbons, each one of which is seamed along its length to a single microtubule. In these preparations, tubulin has been found in such excess as to suggest that the protein is a constituent of microribbons as well as of microtubules (Holberton & Ward, 1981). Moreover, the flat faces of microribbons are made of ordered arrays of subunits with tubulin protomer dimensions (Holberton, 1981).

A second protein of discs with a molecular weight of $30 \times 10^3$ is also likely to be a microribbon protein. It has been suggested that this protein first associates with disc microtubules, and then binds additional tubulin to build up the multilayered structure of the microribbon (Holberton, 1981). Thus the behaviour of $30 \times 10^3$ molecular weight ($M_r$) protein may be an important factor in understanding tubulin polymorphism in this organism.

The present work was undertaken in order to examine: (1) the composition and properties of $30 \times 10^3 M_r$ protein, and (2) to what extent Giardia tubulin is heterogeneous.

By column fractionation of disc proteins dissolved in sodium dodecyl sulphate (SDS), a pure sample of $30 \times 10^3 M_r$ protein has been obtained in sufficient yield to allow the first characterization of this protein. This approach was used after initial experiments had shown that cytoskeleton proteins were not well-separated by column chromatography without using denaturing agents (Holberton & Crossley, 1981).

**Materials and Methods**

**Preparation of Cytoskeletons**

To isolate cytoskeletons from *Giardia lamblia*, trophozoites were extracted in a detergent medium described previously (Holberton & Ward, 1981), and now called TEDAMP + Triton. TEDAMP + Triton contains: 10 mM-Tris-HCl buffer (pH 8.3), 2 mM-EDTA, 2 mM-dithiothreitol, 1 mM-ATP, 2 mM-MgSO$_4$, 150 mM-KCl and 0.5% Triton X-100.

The *G. lamblia* cells used were an axenic strain adapted to grow in Diamond's TPS-1 medium (Visvesvera, 1980). Cells were grown in 100-ml or 300-ml medicinal flat bottles completely filled with medium, which was changed at the time of subculturing (3 days) when cells were confluent or had reached high numbers.

For extraction, harvested cells were washed in cold Hanks' salt solution or TEDAMP, then suspended in TEDAMP + Triton at room temperature at a final density of $10^6$-$10^7$ cells ml$^{-1}$. With
vortex agitation, trophozoites were demembranated within 2 min. Membrane-free cytoskeletons were pelleted from suspension at 4 °C by centrifuging at 15,000 g for 10 min. Pellets were washed three times by suspension and repelleting in cold TEDAMP without Triton.

**Separation of extracted proteins**

Cytoskeletons were totally soluble in SDS at concentrations of 1-2%. Protein extracts for gel filtration were prepared by dissolving cytoskeletons at a protein concentration of 3-6 mg ml⁻¹ in 1-2 ml of an SDS buffer. After heating in a boiling water bath for 4-8 min, any insoluble aggregates were removed by centrifugation at 48,000 g for 1 h.

Samples to be run on Bio-Gel P300 (Bio Rad Laboratories) were extracted in 1% SDS, 10 mM Tris-HCl (pH 7.5), to which sodium azide (NaN₃) was added to a final concentration of 0.02%. The same buffer was used to equilibrate and elute the column.

A different procedure was used to separate proteins on Ultrogel AcA34 (LKB Instruments), which is not stable in concentrations of SDS above 0.1%. For these experiments the extracting solution was 2% SDS in 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), with 0.02% NaN₃. Columns were run in the same buffer with a lower concentration (0.1%) of SDS. Before running, the soluble proteins were first equilibrated with the running buffer on a short column of Bio-Gel P2.

**Concentrating column fractions**

Column fractions were concentrated by centrifuging in CF25 Centriflow cones (Amicon Corporation) at 900 g for 30-60 min until the sample size had reduced to 50-100 μl. For electrophoresis, an equal volume of SDS diluting solution was added to each sample. Protein adhering to the cone was brought into solution by touching the cone support against a vortex mixer for 5-10 s.

For some experiments, protein was precipitated directly from column fractions in nine parts of acetone (Weiner, Platt & Weber, 1972). After pelleting at 1000 g for 10 min, precipitates were washed three times in 90% acetone and once in absolute acetone, then stored desiccated at −20 °C.

**Protein assay**

Protein in samples was measured from peptide amino groups using the Biuret method of Gornall, Bardawill & David (1949), or the micro-Biuret method of Goa (1953).

**Preparation of brain tubulin**

Tubulin was purified from a homogenate of fresh rat brains following the thermal polymerization method of Shelanski, Gaskin & Cantor (1973). Before reassembly, tubulin supernatants contained 0.5 mM-GTP, and were made 4 M in glycerol. After two cycles of assembly and cold depolymerization, preparations were stored at −20 °C in buffer made 8 M in glycerol, or were lyophilized. Tubulin of this purity was used as a mobility standard on electrophoresis gels. For amino acid analysis preparations were further purified by gel filtration chromatography.

**SDS/polyacrylamide gel electrophoresis (SDS/PAGE)**

Proteins were analysed on vertical slab gels by electrophoresis in the continuous 25 mM-Tris-glycine buffer system used previously (Holberton & Ward, 1981). Gels were prepared as described by Stephens (1975), with final acrylamide concentrations from 7.5-15%. They were routinely cast to 1 mm thickness in glass gel-formers 72 mm long, and either 47 or 63 mm wide. For the preparation shown in Fig. 2, a sample was run on a longer separating gel of final track length 150 mm.

Proteins were dissolved in 2% SDS, 10% (v/v) glycerol, 0.5% (v/v) mercaptoethanol in 0.025 M-Tris-HCl (pH 8.3), with 0.005% Bromophenol Blue as a tracking dye. To prevent endogenous proteolysis, samples were made 0.005% in phenylmethylsulphonyl fluoride, and were heated in a boiling water bath for 3-6 min.

Electrophoresis was carried out for 2-3 h at room temperature at a constant current of 6 mA for smaller gels, and 8 mA for longer gels.

Gels were stained for 30-60 min at 60 °C in 0.25% PAGE Blue 83 (BDH), dissolved in 45.4% (v/v) methanol/9.2% (v/v) acetic acid. Gels were destained at 60 °C in the same methanol/acetic acid almost to transparency, then transferred to 7.5% methanol/5.0% acetic acid at room temperature.
Peptide mapping

Peptide mapping was carried out in one dimension using the limited proteolysis method of Cleveland, Fischer, Kirschner & Laemmli (1977). Proteolytic products from papain and \( \alpha \)-chymotrypsin digestion were separated in 0.1% SDS on 12.5% and 10% polyacrylamide gels. For small quantities, proteolysis was initiated in the sample wells of a stacking gel. Polypeptide substrate (2-4 \( \mu \)g) was introduced into each well and overlaid with enzyme in 10% (v/v) glycerol in constant volume (1 \( \mu \)l). To vary the extent of digestion in different wells, serially diluted solutions of enzymes in 10% glycerol were loaded in sequence, starting with the lowest concentration. Electrophoresis was begun within 1 min of completing loading. Running conditions were as for continuous electrophoresis.

Isoelectric focusing

Proteins purified by gel filtration were focused on 5% polyacrylamide gels with 8 M-urea, and 2% ampholytes (LKB Instruments) ranging in pH from 3.5 to 10 (Danno, 1977). Gels were cast in a 120 mm \( \times \) 220 mm gel-former, and run on an LKB Multiphor apparatus. Samples in 100-200 \( \mu \)l of urea-Ampholytes were applied to the surface of the gel on 10 mm \( \times \) 15 mm strips of Whatman GF/B papers, some 4-5 cm from the cathode. Electrode wicks were soaked in 1-7% phosphoric acid (anolyte) or 2% ethylene diamine in 6 M-urea (catholyte). Gels were run for 2 h at 25 W constant power, with a voltage ceiling of 1500 V. To measure the pH gradient, a strip was removed after the run from the edge of the gel parallel to the protein separation. Sections (5 mm) of the strip were eluted separately for 4 h in 2 ml of deionized distilled water in capped test tubes, then the pH was measured.

The focused gel was soaked overnight in three changes of 12.5% trichloroacetic acid, washed briefly in distilled water, and stained with PAGE Blue 83 as for electrophoresis.

Amino acid analysis

Acetone powders of protein fractions were hydrolysed in 6.1 N-HCl in pyrex tubes sealed under nitrogen for 16 h in a fan oven at 110\(^\circ\)C (± 1 deg. C). The cooled hydrolysate was vacuum-desiccated over \( \text{P}_2\text{O}_5 \), washed in a small quantity of deionized distilled water, and dried again. Amino acid analysis was carried out on a Locarte 4 analyser. Samples of 5-6 nmol were loaded in 0.2-N-sodium citrate (pH 2.2), with 24.5 nmol of norleucine as an internal standard. Acids were separated on a single 25 cm column of Locarte resin in sodium citrate buffers (pH 3.25 to pH 6.65) by a three-step elution programme (Moore & Stein, 1963). The amounts of amino acids were assayed by the ninhydrin reaction. The elution position and colour factor for each amino acid were determined from standard mixtures run in an identical way.

Electron microscopy

Following extraction with Triton, some of the pelleted material was fixed for electron microscopy as described previously (Holberton & Ward, 1981). After embedding in E mix medium resin (EMScope), silver and grey sections were stained with uranyl acetate and lead citrate. Sections were examined and photographed at 80 kV in a JEOL 100C electron microscope.

RESULTS

Composition of isolated cytoskeletons

The cytoskeletons isolated by Triton from \( G. \) lamblia were essentially the same as those from \( G. \) duodenalis illustrated in an earlier paper (Holberton & Ward, 1981). Electron microscopy of structures recovered from suspension by a brief centrifugation showed large numbers of ventral discs and flagellar axonemes, with little contaminating material (Fig. 1).
Giardia structural proteins

Fig. 1. Thin section of cytoskeletons pelleted at 15,000 g after extraction of G. lamblia in TEDAMP + Triton. Near the centre of the pellet, discs (d) and axonemes (ax) are closely packed and mostly intact. Discs are made up from a side-by-side arrangement of microtubules (mt) bearing the large microribbons (mr). Bar, 500 nm.

After dissolving pellets in 2% SDS, proteins of the cytoskeletons were separated on polyacrylamide gels by electrophoresis in a low ionic strength, high pH, Tris-glycine buffer that resolves the two subunits of tubulin (Stephens, 1975). Patterns were very similar to those obtained earlier from G. duodenalis (Holberton & Ward, 1981). Since rather more cells of G. lamblia were available from axenic culture, it was now possible to visualize the minor protein bands. From results using gels of different
Table 1. **Molecular weights of components from the Triton-insoluble cytoskeleton of *Giardia lamblia***

<table>
<thead>
<tr>
<th>Band*</th>
<th>Molecular weight†</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(&gt; 120 000)</td>
<td></td>
</tr>
<tr>
<td>2a,b,c</td>
<td>(111 000–115 000)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(106 000)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(101 000)</td>
<td></td>
</tr>
<tr>
<td>5†</td>
<td>95 500</td>
<td></td>
</tr>
<tr>
<td>6a</td>
<td>81 000</td>
<td></td>
</tr>
<tr>
<td>6b</td>
<td>78 500</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>70 500</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>67 000</td>
<td></td>
</tr>
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<td>9a</td>
<td>58 000</td>
<td>α-tubulin</td>
</tr>
<tr>
<td>9b</td>
<td>53 500</td>
<td>β-tubulin</td>
</tr>
<tr>
<td>10</td>
<td>44 000</td>
<td>Actin</td>
</tr>
<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>35 000</td>
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<td>13</td>
<td>33 000</td>
<td></td>
</tr>
<tr>
<td>14a</td>
<td>31 000</td>
<td>Giardin A</td>
</tr>
<tr>
<td>14b</td>
<td>30 000</td>
<td>Giardin B</td>
</tr>
<tr>
<td>15</td>
<td>29 000</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>24 500</td>
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<td>19</td>
<td>17 500</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>17 000</td>
<td></td>
</tr>
</tbody>
</table>

* *The numbered bands are those appearing most reproducibly on SDS/polyacrylamide gels after continuous electrophoresis (Fig. 2).  
† Molecular weights greater than 95 × 10^3 were not calibrated from larger standards.  
‡ On some gels band 5 had multiple close components.*

strengths, over 20 bands were resolved with apparent molecular weights above 15 000 (Table 1). Fig. 2 is a representative pattern in which the positions of the most consistent bands are identified.

Two of the bands are α- and β-tubulin. After staining with PAGE Blue 83, densitometry showed that the tubulins account for 20–26% of the stained material, which is the same result as reported earlier for *G. duodenalis* cytoskeletons. The third prominent band is a 30 × 10^3 M_r protein, which often migrates as a doublet and on this series of gels represents 18–22% of protein stained under these conditions. This is slightly more than is found in *G. duodenalis*, although the staining procedures were not identical in the two studies.

The intensity of staining of the minor components was variable, this being most noticeable for those polypeptides larger than tubulin. However, the single band 1, and the multiple bands 2, 5 and 6, were consistently stronger than the other minor components.

On some gels molecular weights were calibrated from the following mobility standards run in a parallel track: phosphorylase *b* (94 000); bovine serum albumin
Fig. 2. A. Gel pattern from *G. lamblia* discs + axonemes dissolved in 2% SDS and run in a continuous Tris-glycine buffer (pH 8.3) on a 10% polyacrylamide slab. Stained with PAGE Blue 83. Twenty bands appearing reproducibly on gels are labelled in order of increasing mobility. The apparent $M_r$ values of these components are given in Table 1 from calibrated runs. Bands 9a and 9b are $\alpha$-tubulin and $\beta$-tubulin. B. Molecular weight standards (see text) on the same slab.
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(67 000); ovalbumin (43 000); carbonic anhydrase (30 000); soybean trypsin inhibitor (20 100); α-lactalbumin (14 400).

Band 10 was found to comigrate with actin. Usually it was a weak band, but could be seen when the sample loading was especially high. Feely, Schollmeyer & Erlandsen (1982) have reported that actin is located by immunocytochemistry around the periphery of the ventral disc, and is a component of electrophoresis patterns from whole-cell lysates. When present in cytoskeletons, this is no doubt due to the incomplete removal by Triton of filamentous structures from the edges of some discs.

The leading peptides (bands 17–20) are weakly stained in Fig. 2; they are seen more clearly in overloaded samples after fractionation (Fig. 3b).

**SDS gel filtration chromatography**

For peptide and amino acid analysis, a method was sought to separate pure samples of cytoskeleton proteins on a preparative scale. Experiments to solubilize tubulin and 30 × 10³ Mr protein from isolated cytoskeletons had resulted in low yields. When cytoskeletons were extracted in 2 mM-Tris–EDTA, the detergent Sarkosyl (sodium lauryl sarcosinate) or the chaotropic salts, KCl, KI and KSCN, always less than 50 % of the proteins was solubilized. A further disadvantage was that, during gel filtration chromatography of low-salt or Sarkosyl extracts, 30 × 10³ Mr protein did not migrate in a monodisperse fashion, but aggregated and eluted in more than one peak (Holberton & Crossley, 1981).

Therefore, we chose instead to separate pure cytoskeleton proteins at high yield by gel filtration chromatography in the presence of SDS.

The results from a Bio-Gel P300 column are shown in Fig. 3. Eluting with SDS running buffer, the absorbance profile at 280 nm had four close protein peaks followed by a complex salt peak. When column fractions were individually concentrated and analysed by SDS/polyacrylamide gel electrophoresis, it was apparent that proteins had eluted in the order corresponding to their monomeric molecular weights (Fig. 3b). The first peak, containing more than half the protein, was predominantly of the high Mr and intermediate Mr species (bands 1–8), with some tubulin, and low levels of 30× 10³ Mr protein, which may have been incompletely dissociated from the larger particles. The second peak was poorly separated from the first, but a median sample (fraction 18) showed it to be mostly tubulin. At the leading and trailing edges of this peak the tubulin was significantly contaminated by the other polypeptides. The third peak was much smaller, but was a pure fraction of 30 × 10³ Mr protein. The fourth peak, also a small sample, contained very low Mr polypeptides (bands 17–20).

The elution behaviour was found to be reproducible over a number of subsequent runs. From the absorbance profiles, fractions corresponding to pure samples of the principal polypeptide species were pooled across runs, acetone-powdered, and stored desiccated at −20 °C. From 10 mg of original protein dissolved in SDS, it was possible to prepare powders amounting to 0·9 mg of tubulin, and 0·6 mg of 30 × 10³ Mr protein.

After initial results on Bio-Gel P300, some samples were run on other gel media in an attempt to obtain better separation of tubulin from high Mr components of the early peaks. Also, there were technical disadvantages in the use of Bio-Gel P300. With
repeated runs it was found that elution times became extended and flow-rates dropped to <1 ml h\(^{-1}\).

Ultrogel AcA 34 separated cytoskeleton proteins to higher resolution. There were six well-spaced protein peaks in the absorbance profile (Fig. 4A). Peaks 3 and 4 were shown by gel electrophoresis to correspond to the elution positions of tubulin and \(30 \times 10^3\) \(M_r\) protein. The two proteins eluted separately from each other and from the other polypeptides. In the electrophoresis patterns of peak fractions (Fig. 4B), the tubulin sample was seen to have some high \(M_r\) contaminants, but the \(30 \times 10^3\) \(M_r\) protein sample was essentially pure.

**Isoelectric focusing**

Although it eluted from an SDS gel column in one peak, pure \(30 \times 10^3\) \(M_r\) protein is seen on electrophoresis gels to include at least two closely spaced bands (Fig. 3c). For this reason, the detailed composition of purified *Giardia* proteins was examined by isoelectric focusing to determine the extent of microheterogeneity.

The proteins in fractions from an SDS–AcA 34 gel column were precipitated in acetone, washed, and dissolved in urea–ampholytes. Samples were then run in 5% polyacrylamide gels. Distortion of bands was reduced if, before running, fractions were desalted on a short column of Bio-Gel P2 equilibrated with urea–ampholytes. More tightly focused patterns were then obtained. The pH gradient measured at the edge of gels was linear between pH 4 and pH 9·5.

Proteins of tubulin size and smaller all had acidic isoelectric points. The two major cytoskeleton proteins had multiple bands (Fig. 5). Protein from the tubulin peak fractions focused to two clearly separated groups of bands with isoelectric points covering the range of pI values from 5·6 to 5·75. There appeared to be two of the more acidic bands, while the slightly less acidic group had three components. Protein of \(30 \times 10^3\) \(M_r\) focused at equilibrium in the pI range 5·8–6·2, giving a pattern of eight bands that were not equally spaced. Five bands, one of which was pronounced, were closely grouped around pI 6·0. Two remaining bands were more acidic and clearly separated. The eighth band was focused in a more basic position apart from the main group.

These results show that *Giardia* tubulin purified by gel filtration chromatography has a degree of microheterogeneity comparable to other pure tubulins examined by isoelectric focusing (Witman, Carlson & Rosenbaum, 1972; McKeithan & Rosenbaum, 1981). The result confirms the conclusion reached by Holberton & Ward (1981) from SDS/PAGE that there is no evidence of a unique microribbon protein co-migrating with other *Giardia* tubulins. Protein of \(30 \times 10^3\) \(M_r\) is more heterogeneous, as might be expected from its electrophoretic migration (Fig. 2). The tightly grouped bands around pI 6·0 may comprise one polypeptide class. The remaining components are probably less closely related structurally to this group.

**Amino acid analysis**

*Giardia* tubulin and \(30 \times 10^3\) \(M_r\) protein used for amino acid analysis were purified on Bio-Gel P300. Fig. 6 shows that the proteins were satisfactorily separated in the peak fractions.
Fig. 3A and B
A sample of twice-cycled rat brain tubulin was further purified by SDS/gel filtration chromatography on Bio-Gel A 0.5. The absorbance profile showed that the protein mostly eluted in a single peak. Three of the peak fractions were pooled and examined by SDS/gel electrophoresis. From the gels it was apparent that the sample was quite pure, with low levels of high $M_r$ associated proteins (Fig. 6).

The amino acid compositions of samples of Giardia proteins and brain tubulin after 16 h hydrolysis are given in Table 2.

Giardia tubulin and rat brain tubulin have very similar amino acid compositions according to this analysis. The fact that the two proteins from different sources compare closely is an indication of the purity of the samples. The largest differences are in the content of threonine and the non-polar residues alanine and proline.

Protein of $30 \times 10^3 M_r$ appears in this analysis to be quite different from tubulin in amino acid composition. Of the basic amino acids, it contains nearly twice as much lysine, and slightly more arginine. There is also a higher percentage of acidic aspartate residues, which is partly offset by less glutamate. As a result, the net charge on the
Fig. 4. Fractionation of cytoskeleton proteins in 0.1% SDS on AcA 34. The SDS-soluble supernatant containing 3-4 mg of protein was equilibrated with column running buffer on a short column of Bio-Gel P2, then run on a 1.5 cm × 85 cm column of AcA 34.

A. Elution profile at a flow-rate of 5-6 ml h⁻¹ shows six protein peaks. Fraction size, 2 ml.

B. Acetone-precipitated fractions from peaks 3 and 4 were electrophoresed in SDS on a 10% polyacrylamide gel and stained in PAGE Blue 83. These peak fractions contained relatively pure tubulin and 30 × 10⁵ Mₚ protein.
Giardia structural proteins

Fig. 5. Isoelectric focusing of *G. lamblia* disc proteins. Peak fractions of tubulin and $30 \times 10^3 M_r$ protein were prepared by gel chromatography on AcA34 (Fig. 4). Desalted samples were applied centrally to a flat 5% polyacrylamide gel containing 2% ampholytes, 8M-urea. Focused proteins were fixed in trichloroacetic acid, washed, and stained in PAGE Blue 83.

Fig. 6. Preparation of protein samples for amino acid analysis. Purity of samples was assessed from SDS/polyacrylamide gel electrophoresis of column fractions; 10% polyacrylamide gel, stained in PAGE Blue 83.

First track: rat brain tubulin sample (T), after chromatography on a Bio-Gel A 0-5 column.

Remaining tracks: composition of successive 2-ml fractions from a Bio-Gel P300 column (Fig. 3), loaded with 5-9 mg of *Giardia* disc proteins in 1% SDS. Flow-rate, 4 ml h$^{-1}$. The protein yields in those fractions used for amino acid analysis were 5-5 nmol of *Giardia* tubulin and 6-7 nmol of $30 \times 10^3 M_r$ protein.
protein will be somewhat less acidic than for tubulin, as was found by isoelectric focusing. The neutral amino acid glycine is present at about half its level in tubulin, and there are marked differences in the content of the hydrophobic residues—proline, leucine and valine.

**Peptide maps**

Casual hydrolysis of axonemal tubulin is known to produce a peptide of $30 \times 10^3$ to $35 \times 10^3$ to $35 \times 10^3$ A/µ. A fragment of this size is a fairly common contaminant of aging tubulin preparations. Protein of $30 \times 10^3 M_r$ is stoichiometrically prepared from *Giardia* discs together with tubulin under conditions that inhibit proteolysis, and appears to be a natural component of these structures.

Nonetheless, it was decided to examine the two proteins for similarity under controlled digestion. Cleveland et al. (1977) prepared peptide maps of tubulin in one dimension on polyacrylamide gels, and their method offered a suitable approach to analysing proteins that had been prepared in the presence of SDS.

Reproducible results were obtained from proteolytic cleavage of *Giardia* tubulin and $30 \times 10^3 M_r$ protein by the enzymes papain and α-chymotrypsin, when digestion was carried out *in situ* on gels, or at 37°C in samples before loading. A unique banding pattern resulted from each enzyme–substrate combination. Partial cleavage of tubulin by papain gave rise to five discrete peptide bands, and a diffuse group of leading peptides. The two largest peptides appeared to migrate more slowly than $30 \times 10^3 M_r$.

### Table 2. Comparison of the amino acid compositions of rat brain tubulin, *Giardia* tubulin, and $30 \times 10^3 M_r$ protein

<table>
<thead>
<tr>
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<th>Rat brain tubulin</th>
<th><em>Giardia</em> tubulin</th>
<th>$30 \times 10^3 M_r$ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>31</td>
<td>32</td>
<td>56 (17)</td>
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<tr>
<td>Histidine</td>
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<td>Proline</td>
<td>68</td>
<td>48</td>
<td>21 ( 6)</td>
</tr>
<tr>
<td>Glycine</td>
<td>78</td>
<td>77</td>
<td>39 (12)</td>
</tr>
<tr>
<td>Alanine</td>
<td>63</td>
<td>81</td>
<td>80 (24)</td>
</tr>
<tr>
<td>Hall-cystine</td>
<td>16</td>
<td>18</td>
<td>24 ( 7)</td>
</tr>
<tr>
<td>Valine</td>
<td>63</td>
<td>59</td>
<td>44 (13)</td>
</tr>
<tr>
<td>Methionine</td>
<td>26</td>
<td>29</td>
<td>17 ( 5)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>38</td>
<td>42</td>
<td>45 (13)</td>
</tr>
<tr>
<td>Leucine</td>
<td>65</td>
<td>77</td>
<td>107 (32)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>27</td>
<td>30</td>
<td>20 ( 6)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>37</td>
<td>36</td>
<td>37 (11)</td>
</tr>
</tbody>
</table>

The results are expressed as residues per 100 000 g protein; also for $30 \times 10^3 M_r$ protein as residues per $30 \times 10^3 M_r$ (in parentheses). Colour yields were estimated against 25 nM-norleucine as internal standard. Values are not corrected for partial destruction. Tryptophan was not determined.
Giardia structural proteins protein (Fig. 7A). Protein of $30 \times 10^3 \, M_r$ was more resistant than tubulin to proteolysis; after incubating at 37°C for 20 min with papain at concentrations that digested tubulin, it produced no peptides resolvable on maps (Fig. 7A). When digested in situ on gels by serially increasing concentrations of papain in a number of sample wells, hydrolysis was apparent only at high enzyme concentrations, when a diffuse band of very low $M_r$ products appeared.

Proteolysis of Giardia proteins by $\alpha$-chymotrypsin required high concentrations of this enzyme. With up to 200 $\mu$g ml$^{-1}$ of enzyme in sample wells (which was sufficient for nearly total digestion of ovalbumin), the proteolysis of tubulin was incomplete, and $30 \times 10^3 \, M_r$ protein was largely refractory to cleavage. When substrates were incubated with enzyme for 30 min before loading on to gels, some of the $30 \times 10^3 \, M_r$ protein was digested, and produced a map of relatively few peptides. Peptide positions are shown in Fig. 7B from a density scan of this gel. There is some resemblance to the banding pattern from digestion of tubulin, although the two maps are not identical. Under the same conditions tubulin was almost completely broken down. Six bands are labelled in the tubulin map. The second band had the same mobility as undigested $30 \times 10^3 \, M_r$ protein, but was always more weakly staining than the other cleavage products. The leading peptides were poorly resolved and appeared as a broad peak, with a trailing shoulder in both maps. In addition, there was one other strong band at a similar position in each map (tubulin peptide 3).

DISCUSSION

Gel patterns from G. lamblia cytoskeletons have more than 20 components. Of these the two major proteins, tubulin and a $30 \times 10^3 \, M_r$ protein, are present in almost equal amounts and account for 40–50% of the total stainable protein. These two species have been purified by gel filtration chromatography in SDS to allow their preliminary characterization.

Earlier, Holberton & Ward (1981) considered the smaller protein to be a genuine component of discs, and not a fragment of tubulin, since precautions had been taken in their study to avoid proteolysis. A number of results now show this protein has different polypeptide chains: first, the amino acid compositions of the two proteins are distinct; second, they focus at different positions in an ampholyte gradient; and third, in the presence of SDS they respond differently to experimental cleavage by proteolytic enzymes.

Heterogeneity of tubulin and $30 \times 10^3 \, M_r$ protein

Purified Giardia tubulin from peak fractions focuses in a broad-range ampholyte as two clusters of bands in which a total of five components are distinguishable. Because the first step in obtaining tubulin was to solubilize whole cytoskeletons in SDS, the protein was diverse by source and included subunits from axonemal and disc microtubules, but probably mostly from microribbons (Holberton & Ward, 1981).

There is now much evidence that tubulin from a single microtubular organelle is heterogeneous. When examined by isoelectric focusing, Chlamydomonas flagellar
Fig. 7A and B
Giardia structural proteins

doublets were found to have five tubulins (Witman, Carlson & Rosenbaum, 1972), neurotubules of cloned neuroblastoma or glioma cells had at least five (2β; 3–6α) tubulins (Feit, Neudeck & Gaskin, 1977), and Asterias sperm tails had eight (4α; 4β) species (Kobayashi & Mohri, 1977). In the studies cited above, as in the present study of Giardia tubulin, bands were visualized by dye binding. Minor tubulins have been identified more completely in narrow-range ampholytes by autoradiography after loading gels with [35S]methionine-labelled proteins. By this means, nine (4α; 5β) tubulin species have been found in the flagella of Polytomella (McKeithan & Rosenbaum, 1981), and eight tubulins shown to be present in a single rat sympathetic neuron (Gozes & Sweadner, 1981).

In some cases, heterogeneous tubulin species have distinct amino acid compositions (Stephens, 1978) and, by implication, are the products of alternative tubulin genes. Additionally, multiple tubulin bands within one subunit class may be generated in vitro by modifying side-chains; for example, by phosphorylation or glycosylation (Feit et al. 1977). Recently, Lefebvre et al. (1980) and McKeithan & Rosenbaum (1981) have provided evidence that the predominant α-tubulin (α3) in flagella from Chlamydomonas and Polytomella matures from a cytoplasmic precursor (α1) by a modification occurring post-translationally.

Tubulin heterogeneity may well be more extensive than the variants revealed by isoelectric focusing, since subtle changes in the amino acid composition will be detected only where these: (1) involve charged residues; and (2) result in a net charge difference. Certainly there is evidence from sea-urchin flagella of widespread differences in the tryptic peptides of both α- and β-tubulins when central-pair tubules are compared to outer doublets, and A subfibres are compared to B subfibres (Stephens, 1978).

On this evidence, it is probable that the five Giardia tubulins detected represent the minimum number of species in the cytoskeleton. Nevertheless, it is notable that Giardia tubulins, predominantly microribbon proteins, focus to a pattern that is

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Fig. 7. One-dimensional separation of peptides on SDS/polyacrylamide gels, after limited proteolysis of substrates by papain or α-chymotrypsin. All stained with PAGE Blue 83.

a. Protein samples preincubated with papain for 20 min at 37°C before loading on a 12.5% polyacrylamide slab.

Tracks: 1. 10 µg bovine serum albumin (BSA);
2. 10 µg BSA + 36 ng papain;
3. 10 µg BSA + 18 ng papain;
4. 1 µg 30 × 10^5 M_3, protein;
5. 1 µg 30 × 10^5 M_3, protein + 18 ng papain;
6. 1 µg 30 × 10^5 M_3, protein + 36 ng papain;
7. 2.5 µg Giardia tubulin;
8. 2.5 µg Giardia tubulin + 18 ng papain;
9. 2.5 µg Giardia tubulin + 36 ng papain.

b. Densitometer traces from peptide maps of Giardia tubulin (top) and 30 × 10^5 M_3 protein (bottom) on the same 10% polyacrylamide gel. Samples of protein were incubated with α-chymotrypsin at a substrate : enzyme ratio of 10 : 1, for 5 min at 37°C, then boiled for 2 min before loading. Tubulin peptides (Tub) are numbered in order of increasing mobility.
closely similar to, and certainly no more diverse than, the patterns given by microtubular polypeptides on similar gels.

We have not determined directly which of the *Giardia* isotubulins correspond to α and β subunits, as defined by conventional SDS/PAGE. Judging from the behaviour of tubulins of other cells, it is most probable that the group of three bands with the more basic pI values are α-tubulins, and the pair of more acidic components are β-tubulins. Apparently, the β subunit of flagellar tubulin always focuses to the more acidic positions, where this has been determined either on two-dimensional gels (Lefebvre et al. 1980; McKeithan & Rosenbaum, 1981) or after separation of reduced and alkylated proteins on hydroxylapatite columns (Kobayashi & Mohri, 1977).

Protein of $30 \times 10^3 \text{Mr}$ from *Giardia* also shows extensive heterogeneity by isoelectric focusing. The wide spacing of bands in the pH gradient suggests that more than one class of subunit may be present. At equilibrium, four major components are separated from each other by charge differences as great as that distinguishing α-isotubulins from β-isotubulins in the same gel.

Three components have already been discerned in the broad $30 \times 10^3 \text{Mr}$ band of SDS/polyacrylamide electrophoresis patterns from cytoskeletons (Holberton & Ward, 1981). Fig. 2 in the present paper shows two major bands accompanied by two minor polypeptides that are apparently larger and smaller than the doublet protein. Also, Fig. 3c shows that purified protein from the monomeric $30 \times 10^3 \text{Mr}$ peak eluting from Bio-Gel P300 can subsequently be resolved into two components by SDS/PAGE, and PAGE Blue 83 dye binding indicates that these two polypeptides are present in equal amounts.

When cytoskeletons are dialysed against 2 mM-Tris-EDTA, the supernate contains $\approx 30 \times 10^3 \text{Mr}$ proteins with different behaviour in solution. As judged from its elution from Bio-Gel P300 in the absence of SDS, the component with the faster electrophoretic mobility tends to be aggregated (particle $Mr > 100 \times 10^3$), whereas at least some part of the slower component elutes as a dissociated $30 \times 10^3 \text{Mr}$ chain (Holberton & Crossley, 1981).

These results suggest that in vivo polymers of $30 \times 10^3 \text{Mr}$ protein may be structural mosaics of two (or more) similar but essentially anisomeric chains. In terms of microribbon structure, if, as has been suggested earlier (Holberton, 1981), $30 \times 10^3 \text{Mr}$ protein in the core of the ribbon stabilizes the bonding of tubulin in sheets, then the presence of multiple species of $30 \times 10^3 \text{Mr}$ protein provides a basis for specific interactions with α- and β-tubulins.

**Composition of $30 \times 10^3 \text{Mr}$ protein**

Because only a small amount of protein was available, the amino acid analysis of $30 \times 10^3 \text{Mr}$ protein was performed once and not repeated. Nevertheless, its composition is clearly distinctive. As a check on the validity of the single estimation, a rat brain tubulin standard was analysed in series on the same run. There have been a number of previous analyses of tubulin from various sources (Stephens, 1968; Bryan & Wilson, 1971; Everhart, 1971; Lu & Elzinga, 1977) including rat brain (Eipper,
Giardia structural proteins. From these reports, there are measurable differences in the relative levels of different amino acids, particularly when comparing flagellar and brain tubulins. Our analysis of rat brain tubulin agrees closely with the earlier result, except for the amino acids proline, serine and histidine, which are overestimated. The largest discrepancy is for proline, for which the chromatogram gave a broad ninhydrin peak close to the baseline, which was difficult to estimate accurately.

In Giardia tubulin, the analysis shows lower levels of proline and serine, which are more consistent with the composition of doublet tubule proteins from Tetrahymena axonemes and sea-urchin sperm tails (Stephens, 1968).

Against these standards it is possible to comment on the particular composition of $30 \times 10^3 M_r$ protein, and draw some inferences about its structure.

By computing the frequencies with which particular residues occur in alternative secondary conformations in known proteins, it has been shown that probability parameters can be derived that are reasonably predictive of native secondary structure for a given amino acid sequence (Chou & Fasman, 1978). Certain residues are not ambivalent in their effects on chain folding. Proline and glycine are strong helix breakers. They tend, therefore, to define bends in the polypeptide backbone. The total content of these two amino acids in $30 \times 10^3 M_r$ protein is very low (18 out of 258 residues, according to Table 2). Weight for weight, this is less than half the levels found in Giardia tubulin, or other tubulins (Eipper, 1974).

On the other hand, when comparing helix formers, $30 \times 10^3 M_r$ protein has 15–20% more of these residues than does tubulin. Leucine, which is strongly biased to the inner residue positions in $\alpha$-helical domains (Chou & Fasman, 1974), is noticeably prevalent. These results suggest molecules with a high $\alpha$-helix content and few bends. The large amount of leucine implies that helices may be unusually long. It is probable, therefore, that $30 \times 10^3 M_r$ proteins are elongated, perhaps rod-like, molecules.

Hydrophobic amino acids are important in stabilizing tertiary structures. Protein of $30 \times 10^3 M_r$ has approximately 45% of these amino acids, which is very similar to the amount in the tubulins. However, if the molecule is more extended, hydrophobic groups may be less deeply buried than in a globular molecule, so that hydrophobic side-chain interaction at the surface of molecules may be a cause of $30 \times 10^3 M_r$ protein aggregation in polar solvents (Holberton & Crossley, 1981).

There is also a slightly higher proportion of polar amino acids in $30 \times 10^3 M_r$ protein than in tubulin. At physiological pH values the protein would possess a number of charged sites capable of undergoing ionic interactions with other protein subunits.

The resistance of $30 \times 10^3 M_r$ protein to digestion by papain is hard to understand. The same result was obtained when digestion experiments were repeated a number of times on gels. The enzyme cleaves peptide bonds adjacent to most amino acid residues (Hill, 1965), and steric hindrance of the active site would be unlikely for an SDS-denatured substrate. One possibility is that the large active site of papain was product-inhibited by binding tightly, at the $S_2$ subsite, a peptide with a phenylalanine residue. This effect has been observed for substrates in which phenylalanine is the second residue from the C terminus (Berger & Schecter, 1970).
Giardin

We have characterized $30 \times 10^3 M_r$ protein in a preliminary way; consequently, we now propose the name giardin for this protein, understanding that we refer to a group of non-identical polypeptides that may, like tubulin, constitute a class of related structural proteins.

The giardins are residue proteins after Triton extraction of the disc cytoskeleton of Giardia. Both the organelle and its microribbon structures are unusual, so the extent to which similar proteins may be found in other organisms is unknown and difficult to predict. There is evidence that undenatured subunits of giardin associate into oligomeric particles of defined sizes (Holberton & Crossley, 1981), and that they interact strongly with tubulin (Holberton & Ward, 1981); properties that favour their role as structural proteins of the microribbon. Also, we have found that the giardins can form two-dimensional sheet polymers \textit{in vitro} (Crossley & Holberton, unpublished data). Giardin molecules have not been clearly visualized by electron microscopy, so their shape is uncertain. The 3:75 nm periodicity observed in the core of the \textit{Giardia} microribbon (Holberton, 1981) also appears in sheets of protein formed \textit{in vitro}, and may be either a dimension of the molecule or, more probably, a periodic feature of the arrangement of giardin in lattices.

Microribbon structure

In \textit{Giardia} microribbons, tubulin and giardin copolymerize in flat sheets with ordered subunit arrangements (Holberton, 1981). Elsewhere, structural tubulin has been found \textit{in vivo} only in microtubules, although assembly \textit{in vitro} is polymorphic. The present study has shown that \textit{Giardia} tubulin has chains similar to microtubule tubulin from other sources, therefore it is unlikely to have entirely novel properties of self-assembly. Assembly of tubulin into microribbons is probably primarily the result of interactions with the other ribbon proteins, principally giardin.

The effect of binding additional proteins to tubulin has been examined previously \textit{in vitro} for two classes of factors: various foreign basic proteins (RNase A, protamine, histone f1, lysozyme), and native microtubule-associated proteins (MAPs) from brain supernatants.

Basic proteins stabilize tubulin assemblies. Their interaction with tubulin is electrostatic and non-specific; relatively low concentrations will induce polymorphic aggregation (Erickson & Voter, 1976). The simplest explanation of their effect is that they act as macroligands, reducing the high intrinsic charge on tubulin dimer aggregates, thereby discouraging depolymerization (Lee, Tweedy & Timasheff, 1978).

MAPs copurify with cycled brain tubulin. Those of high $M_r$ (HMW/MAPs) associate with microtubules through successive cycles of polymerization and may account for 20–25% of the total protein (Borisy \textit{et al.} 1975). Their molar ratio to tubulin dimers in microtubules is, therefore, of the order of 1:12 (Amos, 1977). In urea, HMW/MAP chains have a net acidic charge and focus alongside tubulin in a
Giardia structural proteins

pH gradient (Berkowitz, Katagiri, Binder & Williams, 1977). However, on ion-exchange columns, the native proteins behave as weak cations, and are retained by the cation-exchanger phosphocellulose (Weingarten, Lockwood, Hwo & Kirschner, 1975). It seems that cationic groups are clustered on these large molecules in regions that will bind to tubulin aggregates. Consequently, they may promote tubulin assembly by the same general mechanism as artificial polycations; certainly the enhancement by poly(L-lysine) of self-association of pure tubulin is quantitatively identical to the effect of endogenous MAPs on cycled tubulin (Lee et al. 1978).

On the other hand, the association with tubulin may be effectively specific if positively charged ends of HMW/MAPs are a complementary fit to acidic domains on the tubulin dimer. The C-terminal peptides of both α- and β-tubulin chains, for instance, are rich in anionic residues (Lu & Elzinga, 1977). On brain microtubules, HMW/MAPs form a helical scaffold over the tubulin dimer lattice, and contribute to long-range order (Amos, 1977). Evidence from electron microscopy has shown that MAPs in vitro will specifically decorate otherwise smooth microtubules with projections at a characteristic 32 nm spacing that is also detected in vivo (Murphy & Borisy, 1975; Kim, Binder & Rosenbaum, 1979).

The role played by giardin in the microribbon is somewhat different, and its interpretation must take account of three observations. First, like HMW/MAPs, the net charge on denatured giardin is acidic. The distribution of charge on the native protein is unknown. Second, giardin appears to be an integral structural component of the ribbon and is present at high concentration, probably >40 % of ribbon protein. Because of its small size, there will be two to three giardin molecules for each tubulin dimer, considerably more than the HMW/MAP - tubulin stoichiometry. Third, it tends to be insoluble and spontaneously aggregates in the absence of dispersing agents.

For these reasons, if the ultrastructural model presented earlier (Holberton, 1981) is correct, then it is probable that tubulin protofilaments are held to an ordered, substantial giardin framework by a specific alignment of sites allowing bonding. Though small, if they are rod-shaped, individual giardin molecules may still extend across a number of dimers in the tubulin lattice. There is also no reason to rule out the possibility that other cytoskeletal proteins participate in this association.

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REFERENCES


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