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# THE 'HOLLOW CYLINDER' PROTEIN OF ERYTHROCYTE MEMBRANES

M.D. WHITE and G.B. RALSTON *

*Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006 (Australia)*

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## Summary

The 'hollow cylinder' protein (Harris, J.R. (1968) Biochim. Biophys. Acta 150, 534—537) has been purified from human erythrocyte membranes. The molecular weight of the native protein, as determined by analytical ultracentrifugation, was found to be 747 000. By means of sodium dodecyl sulphate gel electrophoresis, the purified protein was shown to be composed of three different low molecular weight polypeptides of average molecular weight 25 000. This study provides convincing evidence that the spectrin tetramer is not responsible for the characteristic electron microscopic appearance of the hollow cylinder protein.

## Introduction

A heterogeneous mixture of proteins may be solubilized from erythrocyte membranes by dialysis against low ionic strength buffers [1—3]. In a series of publications, Harris and coworkers [4—7] reported the presence of a 'hollow cylinder' protein in this extract; the protein was reported to be the fastest sedimenting component in the extract and could represent up to 5% of the protein of the intact ghost. The hollow cylinder protein was described as a stacked aggregate of at least four single ring structures. Each ring or torus was said to consist of ten smaller subunits. In the electron microscope the negatively stained protein appeared either as an annular ring of external diameter 110 Å, or presented a rectangular profile of dimensions 180 Å by 110 Å.

Similar structures were seen in micrographs by Haggis [8] and Kirkpatrick et al. [9]. The molecular weight of the hollow cylinder protein has been reported

* To whom correspondence should be sent.

to be approx. 900 000 [7] but the report did not mention the way that this value was obtained.

Although the hollow cylinder protein and the single torus were examined by electrophoresis in acrylamide gels containing urea [5], there are as yet no reports of their mobilities on gels containing dodecyl sulphate, the electrophoresis system that has become a reference standard for the proteins of the erythrocyte membrane [16].

The conditions for the extraction of the hollow cylinder protein and the spectrin tetramer are similar. The spectrin tetramer also has a molecular weight near $1 \cdot 10^6$ [10]. Hoogeveen et al. [11] proposed that the torus and hollow cylinder protein may be different aggregation states of spectrin. Ralston [12] also suggested that the spectrin tetramer and dimer may be stacking states of the torus protein. However, recent studies by Ralston [10] showed that the sedimentation coefficient of the spectrin tetramer was 12 S, whereas the computed sedimentation coefficient for the hollow cylinder protein, based on Harris' model, was 26 S [10]. Harris [6] reported the sedimentation coefficient of the hollow cylinder protein to be 22.5 S. Ralston [10] also indicated that the purified spectrin tetramer, when examined in the electron microscope, showed no structures similar to those seen by Harris [4]. These observations suggested that the hollow cylinder structures reported by Harris [4] were likely to be due to some protein component in the low ionic strength extracts other than spectrin.

In other studies carried out in this laboratory [13,14], it was shown that, apart from the spectrin tetramer, the low ionic strength extract of erythrocyte membranes contained another protein of apparent molecular weight approximately 800 000. We report the purification of this protein which is shown, by mean of electron microscopy, to contain structures identical to those reported by Harris [4,15].

## Methods

*Preparation of membranes.* Fresh human packed cells in citrate anticoagulant were obtained from the Blood Transfusion Service, Sydney. The packed cells were washed three times with 10 mM Tris-HCl buffer, pH 7.7, containing 150 mM NaCl and 20 mM glucose. After each centrifugation at $3000 \times g$ for 5 min, the layer of white cells on the surface of the packed red cells was removed by aspiration.

The washed cells were hemolysed in 10 mM Tris-HCl buffer, pH 7.7, and the membranes pelleted by centrifugation at $25\,000 \times g$ for 15 min. The pelleted membranes were washed two more times with the same buffer and once more with 0.1 mM EDTA, pH 7.5. The membrane preparation thus obtained still retained much haemoglobin and appeared pink or light red in colour.

*Low ionic strength extraction of membranes.* The pink membrane preparation was dialysed without further dilution against 10 vols. of 0.1 mM EDTA, pH 7.5, for $16 \pm 4$ h. The contents of the dialysis tubing were centrifuged in a Beckman L2-65B Preparative Ultracentrifuge at $100\,000 \times g$ for 1 h. The pellet was usually discarded, while the protein-rich supernatant was concentrated to a tenth of its original volume by dialysis against Aquacide III (Calbiochem).

The above procedures for the preparation of membranes and for the extrac-

tion and concentration of water-soluble proteins were usually carried out at 0—4°C.

*Polyacrylamide gel electrophoresis.* Disc gel electrophoresis in the presence of 1% sodium dodecyl sulphate was carried out as described by Fairbanks et al. [16]. In some experiments 7.5% polyacrylamide gels were used instead of the 5.6% gels. The protein bands in the 7.5% gels were quantitated by means of scanning in a Gilford spectrophotometer with a linear transport accessory.

Disc gel electrophoresis in the absence of detergents was carried out in 4% polyacrylamide gels with the use of a discontinuous buffer system, but without sample or spacer gels. The separating gel buffer was 0.375 M Tris-HCl, pH 9.0. The electrode buffer was 0.375 M Tris, 0.1 M glycine, pH 9.4, and contained 0.5 mM thioglycollic acid. Electrophoresis was carried out at room temperature and 100 V (4—6 mA/gel) till the narrow dye front (bromophenol blue) was about 1 cm from the bottom of the gel tube.

Electrophoresis in 3—26% continuous gradients of polyacrylamide gel was carried out as described by Margolis and Kenrick [17]. Electrophoresis at 0—4°C in 0.1 M Tris/borate/EDTA buffer, pH 8.3, usually took 16—20 h.

The gels were stained with 0.01% Coomassie blue in a solution of 10% isopropanol and 10% acetic acid immediately after electrophoresis. Gels were destained in 10% acetic acid. Gels containing sodium dodecyl sulphate were first washed for 6—8 h in a solution of 25% isopropanol and 10% acetic acid prior to staining.

*Column chromatography.* Gel filtration at room temperature was carried out in 600-ml columns of BioGel A15m Agarose beads. The buffer used was 0.05 M Tris-HCl, pH 7.7, containing 0.1 M NaCl, 5 mM EDTA and 5 mM mercaptoethanol. Elution of proteins at a flow rate of 30—40 ml/h per $cm^2$ was monitored at 280 nm in a Varian Superscan spectrophotometer.

Ion-exchange chromatography was carried out at 0—4°C in 40-ml columns of DEAE-Sephadex A-25. The starting buffer was 0.05 M Tris-HCl, pH 8.3, and contained 0.15 M NaCl. Adsorbed protein was eluted at a flow rate of 20 ml/h per $cm^2$ by means of a 140 ml linear gradient of 0.15—0.25 M NaCl.

*Ammonium sulphate fractionation.* Protein solutions were adjusted to the required percentage (w/v) of ammonium sulphate by the slow addition of the solid to the gently stirred protein solution. After standing for 15 min at room temperature the turbid material was collected by centrifugation at 8000 × *g* for 10 min. The pellet was redissolved in 2—3 ml buffer and the supernatant adjusted to a higher concentration of ammonium sulphate as before.

*Analytical ultracentrifugation.* Analytical ultracentrifuge experiments were performed at 20°C in a Spinco model E analytical ultracentrifuge fitted with Schlieren and Rayleigh interference optics. Equilibrium experiments were performed on purified protein solutions by means of the meniscus-depletion method of Yphantis [18] at rotor speeds of 8000 rev./min.

Sedimentation velocity experiments were conducted at 40 000 rev./min and 20°C, with the use of a double-sector cell fitted with a filled epon centrepiece.

## Results

Water-soluble membrane protein extracts prepared by ultracentrifugation contained 0.4—0.8 mg protein/ml. Electrophoresis in detergent-free disc gels

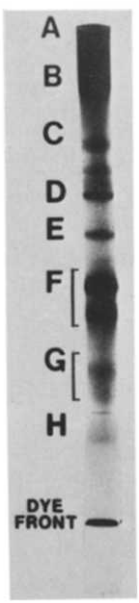
yielded a pattern of Coomassie blue bands designated A—H in order of increasing mobility (Fig. 1). The sharp band at the bottom represents the dye front.

The labelling of the bands is based on earlier work from this laboratory [14]. In the present study, improved resolution of the bands previously labelled 'F' and 'G' has revealed that these two zones appear to consist of two separate bands each. Band 'H' is only visible in more heavily loaded gels.

*Purification of the band D protein*

The steps in the purification procedure for the band D protein were monitored by noting the intensity of band D in detergent-free disc gels.

The concentrated water-soluble protein extract (approx. 750 mg) was applied to the 600 ml column of Agarose beads. The material emerging between 380 and 480 ml (fraction 3, Fig. 2) was found to be enriched in the band D protein. Fraction 3 (approx. 50 mg) was then adjusted to 15% (w/v) ammonium sulphate; the pellet, containing mostly spectrin and component 5, was discarded. The supernatant obtained after centrifugation was adjusted to 30% (w/v) ammonium sulphate and allowed to flocculate overnight at 0—4°C. The precipitate containing the band D protein was collected by centrifugation and resuspended in 3—4 ml 0.05 M Tris-HCl buffer, pH 8.3, containing 0.15 M NaCl. After exhaustive dialysis against this buffer, the protein solution (3—



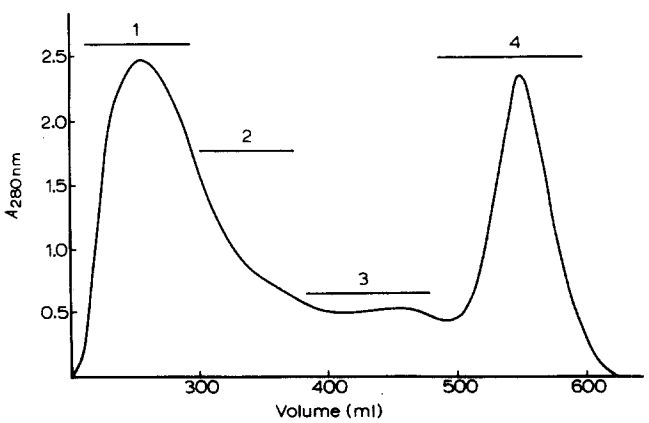




**Fig. 1. Detergent-free disc gel electrophoresis of water-soluble proteins. A sample of crude water-soluble proteins from erythrocyte membranes was electrophoresed in detergent-free disc gels. The 4% polyacrylamide gel was stained with Coomassie blue and the protein bands labelled A—H in order of increasing mobility. The protein of interest corresponded to band D.**

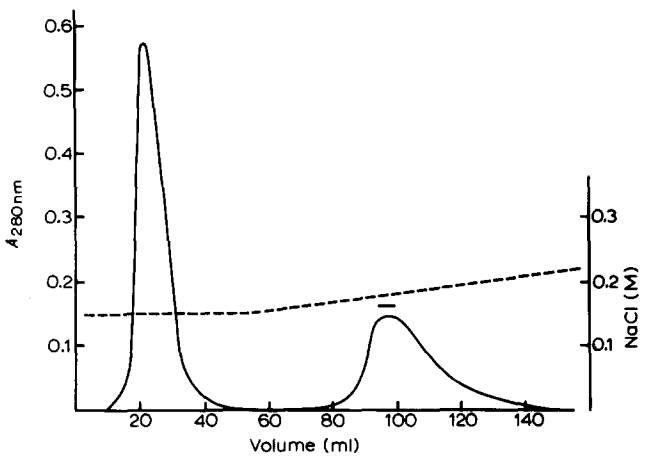
**Fig. 2. Gel filtration of water-soluble proteins. A concentrated water-soluble protein extract was applied to a column of BioGel A15m Agarose beads. Fraction 3 was enriched in the band D protein.**

4 mg/ml) was applied to a DEAE-Sephadex A-25 column equilibrated with the same starting buffer. Protein was eluted from the column by means of a linear gradient of 0.15—0.25 M NaCl. Band D protein was eluted in a single peak near 0.18 M NaCl (Fig. 3). Only those fractions indicated by the bar were used for further analysis.

The band D protein preparation was shown to be homogeneous with respect to electrophoresis at two pH values. In Fig. 4, the purified protein (gels 1—3) was examined in detergent-free disc gels, pH 9.4. A sample of the crude water-soluble protein extract is shown in gel 4. Electrophoresis on acrylamide gradient gels at pH 8.3 also showed only a single component at protein loadings up to 4 μg, with only faint traces of a higher molecular weight contaminant at higher loadings.

*Electron microscopy*

The purified band D protein (approx. 0.1 mg/ml) was examined by means of negative contrast with 2% phosphotungstic acid, pH 7.0, in a Philips EM-201 electron microscope at routine magnifications of 45 000 and 75 000 diameters. One of the micrographs is presented in Fig. 5. The purified protein presented either a rectangular profile of approximate dimensions 180 Å by 110 Å or appeared as an end-on ring (circled). These structures appeared to be identical with the hollow cylinder structure described by Harris [4], suggesting that the band D protein preparation contained the hollow cylinder protein.



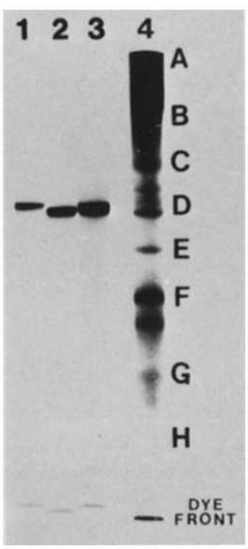




Fig. 3. Ion-exchange chromatography. The protein solution insoluble in 30% (w/v) ammonium sulphate was applied to a 40 ml column of DEAE-Sephadex A-25 maintained at 0—4° C. After the unadsorbed protein had eluted (40 ml), the column was eluted by means of a linear gradient of 0.15—0.25 M NaCl. The band D protein eluted in a single peak near 100 ml. Only those fractions indicated by the bar were used for further analysis.

Fig. 4. Electrophoresis of band D protein preparation. Up to 10 μg of the purified band D protein was applied to detergent-free disc gels, pH 9.4 (gels 1—3). Gel 4 represents the crude water-soluble extract in which the bands have been labelled A—H in order of increasing mobility.

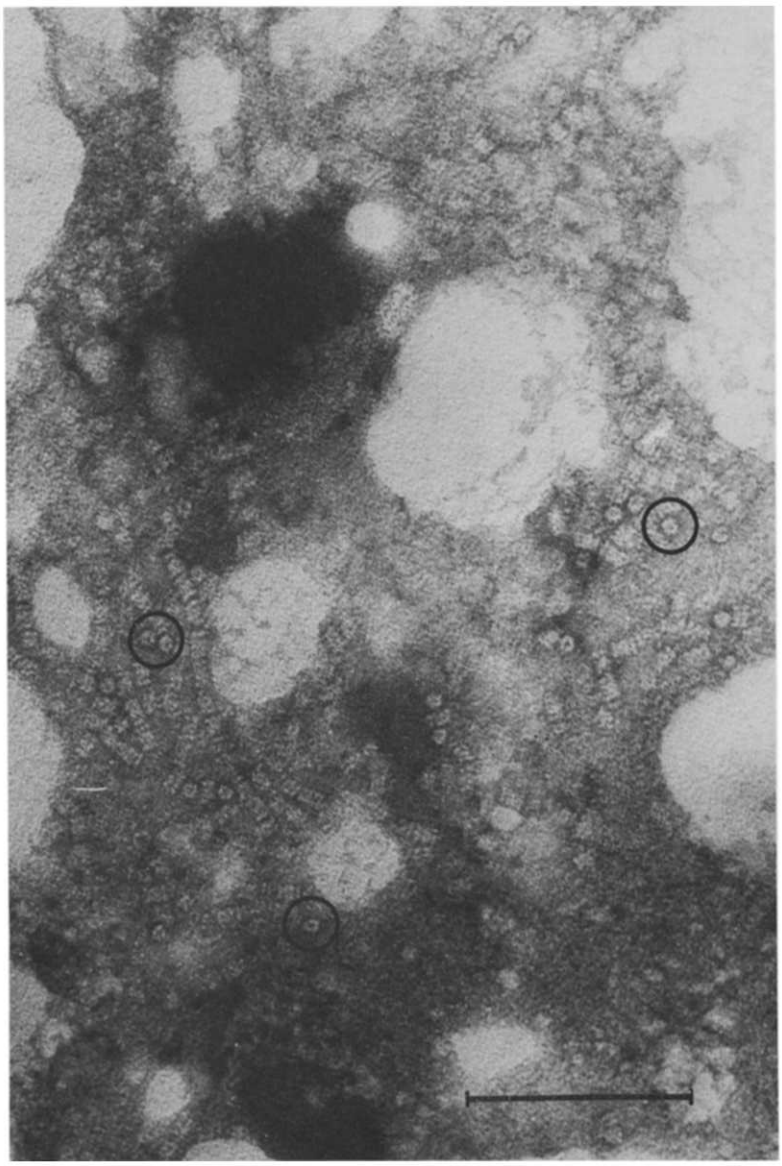


Fig. 5. Negative contrast electron micrograph of band D protein. The purified band D protein presented a rectangular profile or appeared as an end-on ring (circled). These structures appeared identical to the 'hollow cylinder' described by Harris [4]. The bar represents 1000 Å.

*Electrophoresis in sodium dodecyl sulphate gels*

The purified hollow cylinder protein preparation was examined by means of gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 6, gels 1—3). Gel 4 represents the crude, water-soluble extract and gel 5 the total membrane protein. The bands have been labelled according to the nomenclature of Fairbanks et al. [16]. The purified protein appears to consist of three low molecular weight polypeptides, which have been designated as components 7.1,

7.2 and 8, as they appeared to correspond to components 7 and 8 of gel 4.

The apparent minimum molecular weights of these polypeptides were estimated by calibrating 7.5% polyacrylamide gels containing 1% sodium dodecyl sulphate using proteins of known molecular weight. These proteins included serum albumin, ovalbumin, carbonate anhydrase, trypsin inhibitor, haemoglobin and cytochrome *c*. The migration distance was plotted as a function of molecular weight, from which plot the position of components 7.1, 7.2 and 8 were found to correspond to apparent molecular weights of 28 000, 25 500 and 22 500. These values are consistent with that of 29 000 determined by Steck [19] for the major band 7 component.

The intensities of bands 7.1, 7.2 and 8, as determined from integration of the densitometer traces, were in the ratios 1.0 : 0.5 : 0.86. For both ratios, the standard deviation from six determinations was 0.06. After dividing these intensities by the respective apparent molecular weights, the molar ratios were found to be 1.0 : 0.53 : 1.03, very close to the integral values 2 : 1 : 2.

*Molecular weight and sedimentation coefficient*

The molecular weight of the purified hollow cylinder protein was determined by the meniscus-depletion sedimentation equilibrium method of Yphantis [18]. Point average, weight average molecular weights were calculated by an unweighted linear regression to the data points taken five points at a time, and these values were plotted against fringe displacement, again by an unweighted least-squares routine. Where the data appeared excessively noisy, the

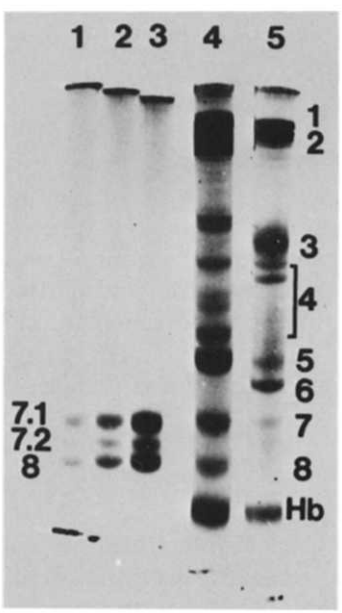



**Fig. 6. Sodium dodecyl sulphate gel electrophoresis. The purified hollow cylinder protein was applied in increasing amounts to 1% sodium dodecyl sulphate disc gels (1—3), along with samples of crude water-soluble proteins (gel 4) and total membrane (gel 5). The bands were labelled according to the Fairbanks et al. [16] scheme. The protein bands in gels 1—3 have been designated as components 7.1, 7.2 and 8, as they appeared to correspond to proteins migrating in the regions of components 7 and 8.**

TABLE I

MOLECULAR WEIGHT OF THE HOLLOW CYLINDER PROTEIN

The meniscus-depletion method of Yphantis [18] was used to determine the molecular weight of the hollow cylinder protein. Three different protein concentrations were used and the results of several determinations are shown. The molecular weight of the purified protein appeared to be 747 000 ± 38 000 (S.D.).

| Run No. | Cell 1 (0.1 mg/ml) | Cell 2 (0.2 mg/ml) | Cell 3 (0.3 mg/ml) |
|---|---|---|---|
| 1 | 729 400 | 727 000 | 666 400 |
| 2 | 795 000 | 712 300 | 735 400 |
| 3 | 788 000 | 775 400 | 794 800 |
| 4 | 725 500 | 775 500 | 767 500 |
| Average ± S.D. | 759 500 | 742 600 | 741 000 |
| | 37 000 | 28 300 | 55 000 |

number of points for the weight average was increased to eleven or fifteen. Number average molecular weights were computed by the method of Yphantis [18].

The number and weight average molecular weights showed very little concentration dependence, and the entire set of log $J$ vs. (radius)$^2$ data for each experiment were fitted by an unweighted linear regression.

The slope of this plot was used as an estimate of molecular weight, and the results of several determinations at various protein concentrations are shown in Table I. The molecular weight of the hollow cylinder protein was found to be 747 000 ± 38 000.

The sedimentation coefficient of the hollow cylinder protein at single protein concentration (approximately 0.5 mg/ml) and at 20°C was found to be 19.3 S.

*Diffusion coefficient*

The diffusion coefficient of the purified hollow cylinder protein was estimated by means of gel electrophoresis. Several proteins of known $D_{20,w}$ values ($\beta$-lipoprotein, apoferritin, albumin, ovalbumin, haemoglobin and the spectrin tetramer and dimer), were electrophoresed overnight in a polyacrylamide gradient slab gel. The migration distance of these proteins was measured and plotted as a function of their diffusion coefficients. The diffusion coefficient of the hollow cylinder protein was estimated to be $2.65 \cdot 10^{-7}$ cm$^2 \cdot$ s$^{-1}$. This value, combined with the sedimentation coefficient, yielded a molecular weight of 660 000.

## Discussion

In this study, hemolysed red cells were washed a total of four times, so that there was minimal fragmentation of the membranes. Excessive washing of the membranes in order to obtain haemoglobin-depleted preparations usually resulted in decreased yields of protein. This suggests that the hollow cylinder protein may be either a cytoplasmic component which becomes adsorbed to the membrane during preparation, or is a loosely bound peripheral membrane protein which can be removed from the membrane during the washing proce-

dure. However, water-soluble proteins extracted from creamy-white membranes still contained much of the hollow cylinder protein.

The hollow cylinder protein appears to be composed of at least three different proteins which have been designated components 7.1, 7.2 and 8. Component 7.2 is not usually detected in sodium dodecyl sulphate gels of crude water-soluble proteins. This is probably due in large part to the presence of large amounts of other proteins migrating in the regions of components 7.1 and 8. Two-dimensional electrophoresis of the water-soluble proteins (White, M.D. and Ralston, G.B., unpublished results) indicate that in addition to the three polypeptides comprising the hollow cylinder, another major component of the band 7.1 regions exists as a monomer in the extract, while one from the band 8 region appears to exist as a dimer. These two proteins appear to be quite distinct from the hollow cylinder protein which is resolved as a closely spaced triplet in the two-dimensional gels. The presence of these other proteins increases the total staining in bands 7.1 and 8, and obscures the presence of band 7.2. Since the hollow cylinder protein was shown to be homogeneous with respect to gel electrophoresis at two different pH values, it is unlikely that any of the three components are due to contaminants. Also, there were no traces of lower molecular weight material in sodium dodecyl sulphate gels, indicating that proteolytic activity was not likely to account for the three bands seen. Furthermore, patterns identical with those shown in Fig. 6 have been obtained with samples of the hollow cylinder from several different preparations. This study therefore provides convincing evidence that the spectrin tetramer (components 1 and 2) and the hollow cylinder protein are non-identical.

Harris [5], on the basis of electron microscopic evidence, proposed that the hollow cylinder protein was composed of four single ring or torus structures. Further, each torus was composed of at least ten subunits. Harris [5] suggested, however, that the outer pair of tori may be dissimilar to the inner pair. Harris and Maddy [7] reported the molecular weight of the hollow cylinder protein to be approximately 900 000, but it was not clear whether this value was estimated from apparent dimensions or experimentally determined.

This study has shown that the hollow cylinder protein is composed of nearly equimolar amounts of components 7.1 and 8, and about half as much 7.2. Now if the single torus was composed of ten subunits each of molecular weight near 25 000, it would have a molecular weight of about 250 000. A stack of four such tori, would have a predicted molecular weight near $1 \cdot 10^6$. However, the experimentally determined molecular weight of the hollow cylinder protein was only 747 000, considerably less than the predicted value.

The sedimentation coefficient of the hollow cylinder protein (19.5 S in the present study cf. 22.5 S in Ref. 6) is also considerably less than that expected (26 S, Ref. 10) from the model described by Harris [5].

These results suggest that an alternative model must be proposed for the hollow cylinder protein. From the values of the molecular weights and the molar ratios of the subunits, together with the measured molecular weight of the hollow cylinder protein, the most likely model would comprise 12 chains each of component 7.1 and 8, and six chains of component 7.2. At present, however, there are insufficient data to warrant the proposal of a detailed alternative model.

Harris based his model for the hollow cylinder protein on the symmetry determined for the single torus protein [15], with the assumption that the single torus protein may be a dissociation state of the hollow cylinder. We have not observed the presence of the single torus protein in any of our preparations. Furthermore, there are small, but perhaps significant, differences between the dimensions of the hollow cylinder and those of the single torus [6,15], and it is possible that the two proteins are in fact not related.

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