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Ultrastructural Comparison of the Tailed and Tailless P-protein Crystals Respectively of Runner Bean *(Phaseolus multiflorus)* and Garden Pea *(Pisum sativum)* With Tilting Stage Electron Microscopy

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Summary

The periodicity of striation, discovered to measure 12 nm in the tailless P-protein crystal of *Pisum sativum*, is compared with that known in the central body of the tailed P-protein crystal of *Phaseolus multiflorus*. A preliminary model advanced to explain the coarse striation in tails accounts for both the wavy outline in longitudinal section and the near-circular outline in transverse section.

1. Introduction

In general there seems to be one P-protein crystal in each sieve element of the *Papilionaceae* except perhaps for the early root protophloem where crystals have not been reported (see ESAU and GILL, 1971). Of the papilionaceous tribes described by BENTHAM (1865) the species dealt with in the present paper are contained in tribe 7 *Viceae* (*Pisum*) and tribe 8 *Phaseoleae* (*Phaseolus*). These tribes are somewhat unusual in that, so far at least, they have been found to be monotypic with regard to the presence or absence of tails in P-protein crystals.

The P-protein crystal (modern term) of *Pisum sativum* is merely listed by MRAZEK (1910). SALMON (1946/47) divides into several categories the P-protein bodies of some papilionaceous species and other dicotyledons. She puts the crystalline P-protein body of *P. sativum* into a miscellaneous (non-defini) category along with the P-protein bodies of six other species outside the *Papilionaceae*. Her three drawings of slime (P-protein) bodies of *P. sativum* (her Fig. 54) range from one which is more or less globular to one which is nearly spindle shaped. WARK and CHAMBERS (1965) divide the development of stem sieve elements of *P. sativum* into four stages. They write "Pisum stage 3 sieve elements possess one or more fibrous slime bodies (P-protein crystals) which are generally spindle shaped and orientated along

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the long axis of the cell". Their drawing of a P-protein crystal, in longitudinal section, differs from their electron micrographs in that it has deep indentations. They did not observe the P-protein crystal at stage 4 and suggest that dispersal occurs. BOUCK and CRONSHAW (1965) described P-protein crystals of *P. sativum* as "irregular bodies" and wrote of them that "their larger size in sieve elements seems to be unmatched in companion and parenchyma cells". The crystals which they depict are in transverse section and have some nearly square corners, but others which appear greater or less than a right angle. According to the classification by LAWTON (1978) of the P-protein crystals of the *Papilionaceae*, those of *P. sativum* belong to group 2, a category comprised of those crystals which lack tails. Group 2 contains both members of the *Viceae* that have been examined, *Pisum* (see above); *Vicia*, MRAZEK (1910), ZEE (1969); and some genera from other tribes.

Of the attention given to the ultrastructure of P-protein crystals the tailed crystals (group 1) have received the most: *Phaseoleae;* LAFLÈCHE (1966), ESAU (1978) *Phaseolus vulgaris*, WERGIN and NEWCOMB (1970) *Glycine max.*, LAWTON (1978) *Phaseolus multiflorus;* Tribe 6 *Hedysareae;* PALEVITZ and NEWCOMB (1971) *Desmodium canadense*, *Coronilla varia.* Group 1 crystals, in general, consist of a four sided blunt spindle (the central body) which, at each end, has a long thin conical tail. The tail has been found to have a coarse striation with a periodicity of about 45 nm and authors have measured a fine striation of about 12 nm in the tail as well as in the central body; LAFLÈCHE (1966), WERGIN and NEWCOMB (1970), PALEVITZ and NEWCOMB (1971), see also CRONSHAW (1974) and ESAU (1978).

The present paper discusses the difference in diameter of P-protein tubules in pea and bean in relation to the periodicities of striation in their respective crystals. A model is presented which can explain the occurrence of the coarse striation and wavy outline in tails of group one crystals without the need to include more than one component in the structure.

2. Materials and Methods

For Fig. 1 a living stem was sectioned in a shallow dish containing 0.2 M aqueous mannitol. For sectioning a small scalpel was made from a fragment of razor blade wired into a split matchstick. The micrograph was taken on an Olympus BHC microscope with an Olympus PM6 photomicrography unit using Ilford FP-4 film developed in Ilford Microphen.

All micrographs except Fig. 1 are of material conventionally fixed and embedded (see LAWTON 1978). Briefly the method consisted of fixation in buffered glutaraldehyde and osmium tetroxide, dehydration in acetone and propylene oxide, and embedment in Epon resin. Thin sections collected from the trough of the diamond knife on Formvar-coated grids were post-stained with uranyl acetate and lead citrate, and were examined in an AEI 801 electron microscope.

Semi-thin sections (0.5 μm thick) were collected from the trough of a glass knife (trough made of "Sleek" tape made by S & N bought at Boots the Chemist, filled with water) with a dissecting needle (see AMEELE 1976) and were dried down from a drop of aqueous

acetone on a hotplate set at 80 °C. The slide was allowed to cool and the sections were stained on a 40 °C hotplate for one minute in 1% toluidine blue in saturated aqueous sodium tetra borate. Excess stain was rinsed away and a cover slip was attached with immersion oil.

To produce Fig. 18 the twisted thread shown in Fig. 16 was embedded in resin and a rough transverse section about 4 mm thick, cut with a hacksaw, was rubbed down to a thickness of about 0.5 mm on a file.

3. Results

Papilionaceous P-protein crystals are genuine structure that can be demonstrated unstained in longitudinal fresh hand sections (Fig. 1) with a student microscope. In *Pisum sativum* the crystal is usually spindle-shaped, 33 μm long and 2.1 μm wide (Fig. 1), sometimes with a slight central constriction. No sign of tails has been detected in *P. sativum*. In transverse section the crystal is shown to be constructed to a square format (Fig. 2, inserts) but new planes of growth often arise and the final shape may be highly irregular (Figs. 6–8). On fixation the crystals seem liable to split and it could then begin to appear that some sieve elements contained more than one P-protein crystal (Fig. 2, lower insert, dart). The crack-like faults, visible in the mature P-protein crystal (Fig. 8) are shown also in the immature phase (Fig. 2). Although the immature crystal is in contact with some of the irregular clouds of amorphous (P-3) protein (Fig. 2) there is no evidence either for or against the conversion of such amorphous protein into the crystalline form.

In *Phaseolus multiflorus* fairly often two tails develop at one of the ends of a crystal (Fig. 4) instead of the more usual single tail. The view shown in Fig. 4 raises the question of whether tails could come to be joined to the central body some time after the synthesis of tails has begun. SALMON (1946/47, her Fig. 51 b) drew three "fines baguettes" or long thin structures judged to be parts of a young P-protein crystal, in an immature sieve element of *Phaseolus vulgaris*. The "fines baguettes" lie near to one another but are not mutually joined nor aligned in any specific orientation. Their appearance is similar to that now shown in Fig. 4. The area in between the upper end of the central body and the ends of the two tails is shown at higher magnification in Fig. 3. Two profiles of endoplasmic reticulum become indistinct soon after their appearance in the bottom left hand corner (double dart). Much of the amorphous material here is probably membranes sectioned not at right angles. A structure near the right hand tail (arrow) appears to be composed in some places of filaments, and apparently similar filaments occur near the end of this tail (darts) and in other places. ESAU (1978), however, shows an electron micrograph of a P-protein crystal, apparently with an attached tail, in a very young sieve element of *P. vulgaris* (her plate 1); a sieve element so young that neither the darkly staining secondary layer of the wall has yet begun to develop, nor have the callose platelets in the sieve plate (compare with Fig. 4). The balance of evidence favours the idea that tails develop in contact

with central bodies and that a section which shows such a view as that depicted in Figs. 3 and 4 has missed the point of junction.

For a valid comparison of measurements of the periodicities of striation, in different kinds of P-protein crystal, to be made in electron micrographs it is important that the specimens should be treated identically. Hence stems of pea and bean were processed for electron microscopy at the same time with the same batch of chemicals. Electron micrographs of thin sections of immature crystals of both species were taken on the same afternoon at the same magnification, and prints from the negatives were made one after the other at the same setting of the enlarger. The results are shown in Figs. 5 and 9. Striations with a periodicity measured to be 12 nm are visible in some parts of the P-protein crystal of pea (Fig. 5). The periodicity of the striation in the central body of the bean P-protein crystal (Fig. 9) also measures 12 nm. The significance of the coincidence of the measurements in both species is discussed. The possibility that differences in orientation of the crystal with respect to the electron beam might alter the periodicity has not been ignored and an experiment is described below which examines the possibility.

The tail of the bean P-protein crystal has an obvious coarse striation, the periodicity of which is about 45 nm (Fig. 9). The fine striation in the depicted tails is, however, much more difficult to detect than in the central body (Figs. 3 and 9–13). The striations in a tail judged to be young are easily detectable, and their periodicity is measured to be about 12 nm (Fig. 9, insert).

An investigation was carried out to determine the effect, on the appearance of the striations, of changing the orientation of the crystal with respect to the electron beam. A crystal was selected whose central body showed a strong striation at the neutral position ($X^{0\circ}\ Y^{0\circ}$) of the tilting stage. The specimen was oriented so that the Y axis ran, as nearly as could be judged, longitudinally through the crystal and micrographs were taken at $X^{0\circ}\ Y^{0\circ}$, $X^{0\circ}\ Y^{-7\circ}$, $X^{0\circ}\ Y^{-14\circ}$, $X^{0\circ}\ Y^{-20\circ}$ (Figs. 10–13). In Fig. 10 at the top left, eleven striations can be measured in the space of 5 · 8 mm and in Fig. 11 the measure-

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Fig. 1. Light micrograph of a hand-cut longitudinal section of unfixed unstained Pea stem. The two immature sieve elements each contain one tailless P-protein crystal. ×1,850

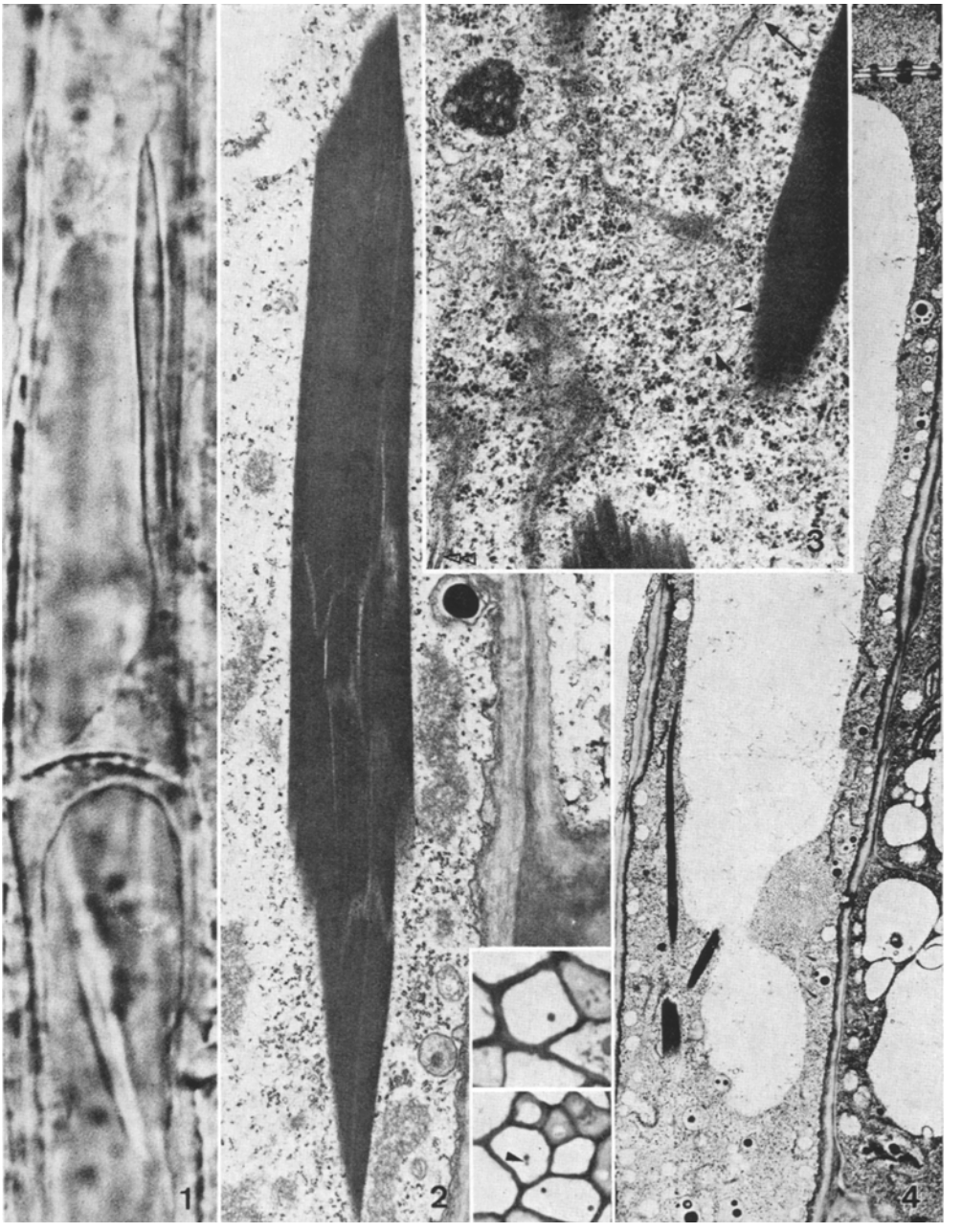
Fig. 2. Electron micrograph of a thin section showing a P-protein crystal of Pea. ×10,580

Fig. 2, upper insert. Light micrograph of a 0.5 μm thick transverse section of Pea hypocotyl. The P-protein crystal appears approximately square in T.S. ×1,160

Fig. 2, lower insert. Light micrograph of a 0.5 μm thick transverse section of Pea hypocotyl. The P-protein crystal seems to be made of two loosely connected parts (dart). ×1,160

Fig. 3. Electron micrograph. The central area from Fig. 4 at higher magnification. The identity of the filamentous material is uncertain and no straightforward image of tail synthesis emerges. ×30,050

Fig. 4. Electron micrograph. Longitudinal thin section of runner bean stem showing an immature sieve element. A P-protein crystal is developing two tails at, or near, one end. ×5,830



Figs. 1–4

ment is the same. In the same position in Figs. 12 and 13 the striations are not sufficiently distinguishable to be measured. It seems reasonable to conclude that, since the striations disappear outside a small range, the measurement of the periodicity in such sections is not affected by the orientation. It is important to note that the stage was returned to the $X^{0\circ}$ $Y^{0\circ}$ position for a fifth micrograph, which was identical to that shown in Fig. 10, *i.e.*, the loss of striation on tilting is not a secondary effect resulting from prolonged exposure to the electron beam. At no angle of tilt is a striation with a periodicity at or near 12 nm visible in the tail (Figs. 10–13).

Fig. 5. Electron micrograph. Part of the P-protein crystal, at higher magnification, of the Pea shown in Fig. 2. The periodicity of the striation is measured to be 12 nm. ×41,000

Figs. 6 and 7. Light micrographs. Serial transverse sections of an irregular P-protein crystal of pea. ×2,200

Fig. 8. Electron micrograph. Transverse section of a P-protein crystal of Pea. The crystal is clearly constructed to a square packed principle. New planes of growth, however, lead to an irregular outline. One part of the crystal has lost contact with the main body. ×12,970

Fig. 9. Electron micrograph. Longitudinal section of a P-protein crystal of runner bean. The periodicity of the striation of the central body is measured to be 12 nm. The coarse striation in the tail is measured to have a periodicity of 45 nm. No fine striation is detectable in the tail. ×41,000

Fig. 9, insert. Electron micrograph. Possibly a young tail in longitudinal section. Only in such sections, in the present study, has a fine striation been detected. ×42,350

Figs. 10–13. Electron micrographs. Four views of the same area of a P-protein crystal of runner bean in longitudinal section. The micrographs were taken at increasing angles of tilt *i.e.* Fig. 10, $X^{0\circ}$ $Y^{0\circ}$; Fig. 11, $X^{0\circ}Y{-}^{7\circ}$; Fig. 12, $X^{0\circ}Y{-}^{14\circ}$; Fig. 13, $X^{0\circ}Y{-}^{21\circ}$, the "Y" axis being approximately parallel with the midline of the tail. The striations in the central body gradually disappeared on tilting but reappeared when the stage was returned to its original position (not shown). ×54,150

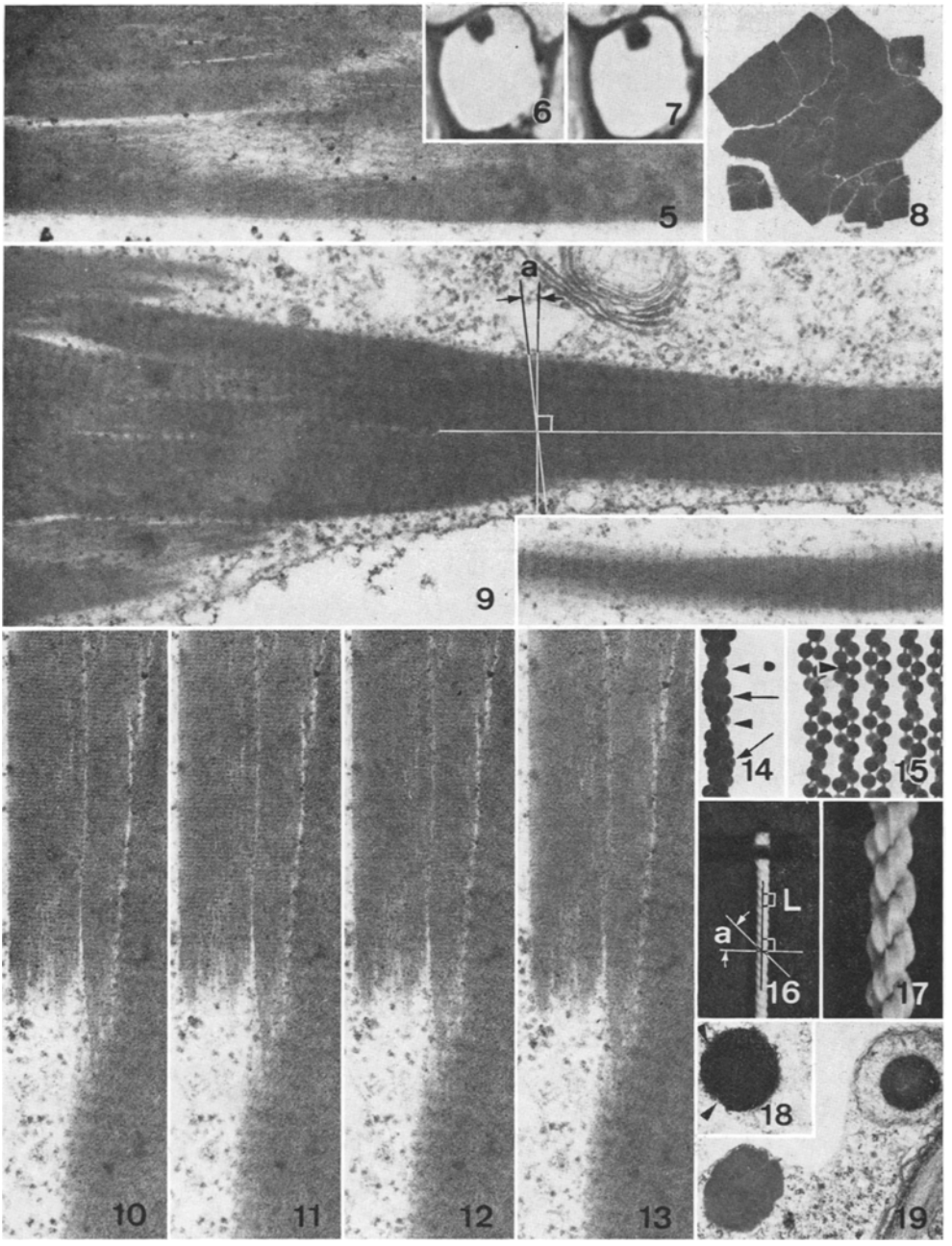
Figs. 14 and 15. A model composed of double helices of beads, as far as possible in register. In "end on" view (Fig. 14) it appears that a striation might appear at the widest points rather than at the narrowest. A marker placed at a "node" (Fig. 14) is seen at an "antinode" when the model is rotated through 90° (Fig. 15, dart)

Fig. 16. Rubber thread, square in cross section when twisted comes to have a scalloped outline and ridges passing over its surface

Fig. 17. An approximate model of a P-protein tubule. The ridges on a piece of rubber thread are imagined to represent a single chain of subunits. The ridges on one piece of thread tend to slot into the grooves on the other piece. If this were to happen in a real P-protein tubule, images from Markham rotation would be confused unless only very short lengths of tubule were present in the section. See LAWTON and JOHNSON (1976) for an accurate model of a P-protein tubule in *Nymphoides peltata*

Fig. 18. The model (Fig. 16) was embedded in resin and a 0.5 mm thick piece was prepared using a file. An approximately circular profile is displayed with two slight indentations (darts)

Fig. 19. Electron micrograph. Transverse section of a tail of a runner bean P-protein crystal. Unlike the plastid, the tail does not have an encompassing membranous envelope. The tail is approximately circular with indentations. ×17,600



Figs. 5–19

4. Discussion

Although some advances have certainly been made (see SLOAN, SABNIS, and HART 1976) we still do not know the extent of variability of molecular P-protein. It is, however, certainly proper in a first analysis to take a simple hypothesis and assume that the different structures that are observed can be formed by the same molecules arranged in different patterns (see PALEVITZ and NEWCOMB 1971). However, since the resolution afforded by thin sections is limited, evidence on the dimensions of P-protein molecules is virtually unobtainable (see LAWTON and JOHNSON 1976). Two lines of approach can be taken for inference about the probable sizes of molecules, and these lines are now briefly explored.

Table 1

Author	Species	Overall tubule diameter	Wall thickness	Lumen diameter	Ratio of wall thickness to lumen diameter
JOHNSON 1968	*Nymphoides peltata* (Water fringe)	24 nm	9 nm	6 nm	1.5 : 1
WERGIN and NEWCOMB 1970	*Glycine max.* (Soybean)	13.5 nm	5 nm	3.5 nm	1.4 : 1

The first line of approach involves the variation in the dimensions of P-protein tubules. The same author (ZEE 1969) reported the diameters of P-protein tubules not only in bean (*Vicia faba*) to be about 15 nm but also in pea (*Pisum sativum*) reported them to be about 21–23 nm in diameter. As to comparisons, JOHNSON (1968) for tubules in *Nymphoides* reported a diameter of 24 nm whereas WERGIN and NEWCOMB (1970) for tubules of *Glycine max* reported a diameter of 13.5 nm. Although the overall dimensions can thus be quite different, when the ratio of the thickness of wall to lumen diameter is calculated a similarity is revealed (see Tab. 1). The simplest hypothesis with regard to the similarity of the ratios is that the architecture is probably the same but that the scale is different, *i.e.*, that the molecules are of different sizes, namely spheres with respective diameters of 3 nm and 1.7 nm (figures based on the model developed by LAWTON and JOHNSON [1976]) (see Fig. 17 for an approximate model).

The second line of approach involves a comparison of the periodicities of striation in P-protein crystals of pea (tubules 21–23 nm in diameter, ZEE 1969), and runner bean (tubules about 15 nm in diameter, LAWTON and NEWMAN 1979). As stated in the results, the periodicities of striation are found to be the same in both pea and bean, namely 12 nm. The first line of approach might have led one to expect a periodicity of striation in pea,

proportionally larger than in runner bean, of about 18 nm. Since this expected larger periodicity of striation has not been found to exist, the simplest hypothesis based on the second line of approach is that the molecules of P-protein in both species are the same size.

Two quite different hypotheses thus emerge. It is concluded that evidence, from a technique with increased resolution, must be obtained before it is possible to distinguish between the two hypotheses.

The construction of the tail has received little attention (see PALEVITZ and NEWCOMB 1971). The present results show that in *P. multiflorus* the periodicity of the coarse striation is about 45 nm and that of the fine striation in young tails is about 12 nm. These figures are similar to those given by prior authors; WERGIN and NEWCOMB (1970) *Glycine max*, PALEVITZ and NEWCOMB (1971) *Desmodium canadense*. In a proven mature sieve element LAWTON (1978) shows a P-protein crystal of *P. multiflorus* in which the tail is shown to have a fine striation periodicity of about 12 nm (plate 5 *b*). In the present investigation, however, fine striations were not detected in tails except for possibly younger tails (Figs. 9–13). A possible explanation is that near the periphery of tails the striation is detectable but that it is somehow obscured towards the centre.

As to the origin of the coarse striations in the tail, a model has been developed to account for them. Rubber thread with a square cross-section, when twisted, comes to have the four hitherto straight edges passing along in a helical path (Fig. 16). Considerable twisting is required to give helices which have a declination from a perpendicular to the midline of the tail of even 45 ° (angle a, Fig. 16). Such a declination does seem to exist in sectioned tails and despite the difficulty of knowing the position of the midline of the tail, the angle of declination is measured at between 5 and 10 ° (angle a, Fig. 9). A length of twisted rubber thread proportional to the length of tail in a thin section 60 nm thick is marked "L" in Fig. 16. In transverse (axial) view such a length of twisted rubber thread shows a slightly indented outline (Fig. 18) more regularly circular, however, than the appearance of real tails in transverse section (Fig. 19) (see also LAWTON 1978, LAWTON and NEWMAN 1979).

The model (Fig. 16) shows a wavy, scalloped outline similar to that shown by tails in longitudinal section (Fig. 9), see WERGIN and NEWCOMB (1970).

A brief review of the origin of striations in P-protein will allow a fuller explanation of the fine striations in crystals. Avoiding a requirement for the periodic occurrence of a different kind of molecule, PARTHASARATHY and MÜHLETHALER (1969) suggested that striations in disaggregating tubules arise at points where one of the two helical components of a tubule crosses the other, *i.e.*, at "nodes" (Fig. 14, darts). The essence of the idea is acceptable and yet models photographed in silhouette give the impression that the striations could be at "antinodes" (Fig. 14, arrows). A marker placed at an

antinode in a face view of a model (Fig. 15, dart) is found to be at a node when the model is turned through a right angle (Fig. 14). A rotation of 90 ° thus produces an apparent shift in the model of a quarter of a wavelength. With the 40 ° of tilt available on some electron microscopes an apparent shift of 0.1 of a wavelength should be obtainable. Such a result would provide strong support for the (double) helix as the unit of construction of P-protein crystals. However, the fact that the shift is small and, in addition, the requirement for a fiduciary reference object have both conspired to prevent the development of a straightforward analysis.

It has been suggested that tails are constructed from the same kind of molecular subunits as is the central body (PALEVITZ and NEWCOMB 1971). It is difficult for the present model to accommodate this possibility. A flexible model of a central body when twisted would come not only to have a wavy outline but the constituent helices would be tightened or slackened depending on the direction of twisting, the striations coming closer together or going further apart. A requirement for the periodicity of the striation to be the same after twisting as it was before the model was twisted is an unsatisfactory complication.

The present model for the structure of the tail is a useful advance since it can explain both the scalloped outline, the coarse striation and the near-circular outline in transverse section. Whether the tail is formed throughout from identical molecules and whether the tail and the central body are formed from identical molecules are questions to be actively pursued.

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