it is possible that pronase enhances the ability of the pituitary to respond to secretory stimuli.

The mechanism of this enhancement is not clear, and the biochemistry of the events which intervene between the addition of prostaglandin E₂ or increase in the K⁺ concentration and the release of growth hormone is equally not known. Prostaglandin E₂ may act by increasing the concentration of cyclic AMP in the pituitary (2), and high K⁺ leads to depolarization (6) and increased Ca²⁺ incorporation (11) without changing the cyclic AMP concentration (10). Since pronase potentiates the effect of both stimuli it is probable that it affects a fundamental property of the secretory system, for example, membrane Ca²⁺ permeability.

Alternatively, since the release of growth hormone is known to be inhibited by the hypothalamic peptide somatostatin (1), pronase could increase release by destruction of somatostatin present in the tissue at the time it was removed from the animal.

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TANNIC ACID-STAINED MICROTUBULES WITH
12, 13, AND 15 PROTOFILAMENTS

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Subunit structure in the walls of sectioned microtubules was first noted by Ledbetter and Porter (6), who clearly showed that certain microtubules of plant meristematic cells have 13 wall protofila-

ments when seen in cross section. Earlier, protofila-

ments of microtubular elements had been de-

scribed in negatively stained material, although exact counts of their number were difficult to
obtain. In microtubular elements of axonemes, some success has been achieved in visualizing protofilaments in conventionally fixed and sectioned material (8, 10); much less success has been achieved in identifying and counting protofilaments of singlet cytoplasmic microtubules. By using glutaraldehyde-tannic acid fixation, as described by Misuhira and Futaesaku (7), Tilney et al. (12) studied microtubules from a number of sources and found that all have 13 protofilaments comprising their walls. These authors note that "...the number of subunits and their arrangement as protofilaments appear universal...".

Preliminary studies of ventral nerve cord of crayfish fixed in glutaraldehyde-tannic acid indicated that axonal microtubules in this material possess only 12 protofilaments (4). On the basis of this observation, tannic acid preparations of several other neuronal and non-neuronal systems were examined. Protofilaments in microtubules from these several cell types are clearly demonstrated, and counts have been made which show that some kinds of microtubules have more or fewer protofilaments than the usual 13 and that at least one kind of microtubule has an even rather than an odd number.

MATERIALS AND METHODS

Material was fixed in glutaraldehyde containing tannic acid and processed in much the same manner as described elsewhere (7, 12). Fixatives were prepared by dissolving tannic acid (Merck Chemical Div., Merck & Co., Inc., Rahway, N.J.) in 0.2 or 0.5 M phosphate solution of the fixative, and just before use, the final pH was adjusted with saturated sodium hydroxide to give the pH of the phosphate buffer at concentrations of 2%, 4%, and 8% at a pH of 6.2, 6.5, 6.8, or 7.0. In some cases, calcium chloride was used to dilute 25% glutaraldehyde to give a 3% solution of 0.5% or 1%. The buffered tannic acid solution was used to dilute 25% glutaraldehyde to give a 3% solution of the fixative, and just before use, the final pH of the fixative solution was adjusted with saturated sodium hydroxide to give the pH of the phosphate buffer used in the original dilution. A precipitate formed in the fixative during preparation, particularly when digitonin was used, and it was used both with and without filtration with no detectable difference in quality of fixation.

After fixation for 1 h at room temperature, material was rinsed in buffer and secondarily fixed in 1% osmium tetroxide in phosphate buffer for 1 h, then rapidly dehydrated through an alcohol or acetone series, and embedded in Epon or Araldite. Sections were obtained with a diamond knife and captured on uncoated 400-mesh specimen screens, and then stained with 2%–2.5% alcoholic uranyl acetate (10 min) followed by lead citrate (4 min), as described by Reynolds (9). Sections were examined and micrographs obtained with either a Philips 300 or a Hitachi HU-11 microscope.

Microtubules Polymerized from Beef Brain Tubulin

Microtubules polymerized from beef brain tubulin were obtained from Dr. L. L. Houston, Department of Biochemistry, The University of Kansas. The tubulin had been isolated and purified by the methods of Weisenberg (13). The supernatant buffer fluid was poured off the sedimented microtubules, and glutaraldehyde-tannic acid fixative (pH 6.2 or 6.8) was added, after which the pellet was teased into suspension intact and further processed. The fixative did not contain digitonin, and better results were obtained if calcium chloride was also omitted.

Frog Lung Fluke Sperm

Frog lung flukes (Haematoloechus medioplexus) were isolated from Rana pipiens and that part of their body containing a seminal receptacle or the testes was dissected and placed in fixative. The structure was punctured several times with a microneedle to provide better access for the fixative. Wall substructure of the cortical singlet microtubules was examined.

Crayfish Sperm

Pieces of sperm-containing vas deferens of crayfish (Procambarus clarkii) were placed in 1% digitonin in Van Harreveld's saline for 5 min and 15 min, after which the pieces were fixed in glutaraldehyde-tannic acid without digitonin. Microtubules found in the four arms and in the body of these sperm were examined.

Axons of the Frog Olfactory Nerve

Short pieces of the olfactory nerve of the frog Rana pipiens were isolated in glutaraldehyde-tannic acid without digitonin, and microtubules in axons of the nerve were examined.

Goldfish Brain

Goldfish (Crassius auratus) cerebellum was fixed in glutaraldehyde-tannic acid for 1 h and routinely processed. Microtubules of both neuronal and glial components were examined.

Vinblastine-Treated Aplysia Nerve

Short segments of pleurovisceral connective nerves of Aplysia californica were fixed in glutaraldehyde-tannic acid; before fixation, some nerve segments were incubated at room temperature for 1 h in phosphate buffer (pH 7.0) containing 0.2 mM vinblastine sulfate (Velban: Eli Lilly and Co., Indianapolis, Ind.). Digitonin and calcium chloride were omitted from all solutions. Mi-
crotubules and vinblastine-induced crystalloidal elements in axons were examined.

**Axons of Crayfish and Lobster Nerve Cord**

Interconnectives of the ventral abdominal nerve cord of the crayfish (*Procambarus clarkii*) and lobster (*Homarus americanus*) were isolated and cut into short open-ended segments to allow diffusion of fixative into the axons. Digitonin was not used in the fixative. Axonal microtubules as well as microtubules of surrounding glial or supporting cells were examined.

**RESULTS**

Except in the case of frog olfactory nerve, protofilaments were counted in at least 20 favorably oriented microtubules in each system studied. Olfactory nerve did not fix well, and only six microtubules were seen wherein protofilaments could be counted. The most extensive counts were made of protofilaments in axonal microtubules of crayfish nerve cord, where accurate counts were obtained in a total of 87 microtubules.

Examination of microtubules polymerized from

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**Figures 1 and 2** Microtubules polymerized from beef brain tubulin. × 414,700.

**Figures 3 and 4** Cortical microtubules of frog lung fluke sperm. × 414,700.

**Figures 5 and 6** Microtubules of crayfish sperm showing 15 subunits. Figs. 5 and 6 show arm microtubules, while the inset in Fig. 6 shows a microtubule lying in the "body" of the sperm. × 414,700.
Figures 7 and 8  Microtubules of axons of olfactory nerve of the frog. × 414,700.

Figures 9 and 10  Microtubules of axons of goldfish brain. × 459,000.

Figures 11 and 12  Sections through axons of vinblastine-treated *Aplysia* nerve showing transverse and longitudinal profiles of crystalloidal material. Note the transverse section through a microtubule near the crystalloidal element in Fig. 11. Fig. 11. × 277,000. Fig. 12. × 154,000.
beef brain tubulin showed that their walls are constructed of 13 protofilaments when viewed in cross section (Figs. 1, 2). No exceptions were noted among microtubules from which counts could be obtained, and protofilaments were more distinct in material fixed at pH 6.2 than at pH 6.8. Many of these microtubules appeared in pairs, with both members of a pair having a complete set of protofilaments (Figs. 1, 2). Cortical microtubules of lung fluke sperm are also composed of 13 protofilaments as viewed and counted in cross sections of microtubules (Figs. 3, 4); no exceptions were noted. Although typical 9 + 2 axonemes are not present in sperm of crayfish, microtubules are abundant in the four slender cytoplasmic arms which wrap around the cell bodies of sperm in the vas deferens. Examination of both arm microtubules and those found in the cell body revealed that they are usually made up of 15 protofilaments (Figs. 5, 6). Of over 30 microtubules from which protofilament counts were obtained, only two exceptions were noted: a single microtubule with 14 and another with 16 protofilaments.

Axonal microtubules from various sources were examined after fixation in glutaraldehyde-tannic acid. Microtubules in small axons of the olfactory nerve of the frog have walls composed of the usual 13 protofilaments (Figs. 7, 8), as do those found in axons of goldfish brain (Figs. 9, 10). Microtubules of glial cells of goldfish brain were also observed to have 13 protofilaments. Studies of normal and vinblastine-treated Aplysia nerve revealed microtubules with 13 protofilaments, although microtubules are less abundant, as expected, after vinblastine treatment. After such treatment, axons show many characteristic crystalloidal elements apparently stained with tannic acid, but relatively few intact microtubules can be seen (Figs. 11, 12). Tannic acid staining revealed little substructural detail in the crystalloidal elements.

Axonal microtubules of crayfish nerve cord are unusual in that their walls are made up of 12 instead of 13 protofilaments (Figs. 13, 14). Protofilaments were counted in a total of 87 microtubules in nerve cord independently prepared and examined in two separate laboratories, and 12 protofilaments were present in all but two cases, where 13 were counted. Surrounding many axons in the crayfish nerve cord are thin layers of glial or supporting cell cytoplasm in which microtubules occur. This arrangement allows direct comparison between axonal microtubules and those in the layer of supporting cell cytoplasm surrounding the axon. In contrast to axonal microtubules, 13 protofilaments can clearly be seen in the walls of supporting cell microtubules (Figs. 15, 16). To determine whether 12 protofilaments are unique to axonal microtubules of crayfish, nerve cords of a related decapod crustacean, the lobster, were examined and also found to have 12 protofilaments (Figs. 17, 18).

In the microtubules examined, measurements were made of the center-to-center spacing between wall protofilaments, and in all cases the distance was in the 50–60-Å range. In longitudinal view, tannic acid-stained microtubules most often appear as shown in Fig. 19, where two rather thick lines define the limits of the wall. Sometimes, however, the tannic acid appears to lie in longitudinal grooves between adjacent protofilaments so that the protofilaments appear to follow a zigzag path rather than a straight line (Fig. 20). In longitudinal view, the center-to-center spacing be-

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**Figures 13 and 14** Microtubules of axons of crayfish nerve cord showing 12 subunits. Fig. 13, × 414,700. Fig. 14, × 453,000.

**Figures 15 and 16** Glial or supporting cell microtubules of crayfish nerve cord. These microtubules have 13 protofilaments. × 414,700.

**Figures 17 and 18** Axonal microtubules of axons of lobster nerve cord showing 12 subunits. Fig. 17, × 430,000. Fig. 18, × 445,000.

**Figures 19 and 20** Representative longitudinal views of tannic acid-stained microtubules. The most common longitudinal profile is as shown in Fig. 19, which is of a crayfish sperm microtubule. In Fig. 20, the surface of an axoplasmic microtubule from crayfish nerve cord shows longitudinal protofilaments; such longitudinal views are less commonly seen. × 350,000.
tween adjacent protofilaments is about 40 Å, and often the wall subunits in cross sections of microtubules appear radially elongate or oval.

**DISCUSSION**

The diameter of most microtubules is about 250 Å, and it has been well shown that the walls of such microtubules fixed in the presence of tannic acid are made up of 13 subunits when viewed in cross section (12). In microtubules which markedly deviate from a diameter of about 250 Å, it is expected that the number of protofilaments in the wall would vary accordingly. The microtubules of crayfish sperm are reported to be about 300 Å in diameter (1), as confirmed in a more recent study (11). Considering that these microtubules are somewhat greater in diameter than the “typical” microtubule, it is not surprising that their walls contain 15 instead of 13 subunits.

The diameter of axonal microtubules of crayfish nerve cord is in the 235–250 Å range, although precise measurements are difficult to obtain due to material adherent to their surfaces (3). Supporting cell microtubules in crayfish nerve cord measure about 260 Å in diameter (mean = 259 Å of 20 microtubules measured, with a range of 243–285 Å) and have the usual number of protofilaments in their wall, with the difference in diameter between axonal and supporting cell microtubules undoubtedly reflecting the difference in number of protofilaments comprising their walls. Other evidence suggests that there are more subtle differences between axonal and supporting cell microtubules of crayfish nerve cord. For example, supporting cell microtubules are resistant to dissociation at 40°C, while axonal microtubules readily dissociate within 1 h at this temperature (5).

The present work extends and cites exceptions to information provided by Tilney et al. (12), where all complete microtubules from a number of cell types showed 13 protofilaments after tannic acid staining. With the exception of axonal microtubules of crayfish and lobster nerve cord, all neuronal microtubules we examined showed the usual 13 protofilaments. Further, although cortical microtubules of lung fluke sperm were reported to have 8 wall protofilaments based on Markham rotation studies (2), 13 are clearly shown to be present in such microtubules after tannic acid staining. It should be noted that microtubules polymerized from beef brain tubulin all showed 13 protofilaments, although the tubulin undoubtedly was derived from microtubules of several different cell types constituting the intact brain. Although this suggests that all these cells have identical microtubules in terms of protofilament number, it is possible that the isolation procedures might select for a given kind of microtubule. In this connection, in two preliminary analyses of tannic acid-stained microtubules polymerized from crayfish nerve cord tubulin, we have observed microtubules with only 13 protofilaments.

**SUMMARY**

Tannic acid-stained microtubules were examined from several cell types, and it was confirmed that most microtubules in the 250-Å size class show 13 wall protofilaments when viewed in cross section. Two exceptional kinds of microtubules were noted: those of crayfish sperm have walls composed of 15 protofilaments, while axonal microtubules of crayfish and lobster nerve cords have but 12 protofilaments.

Parts of this research appear in abstract form in the *J. Cell Biol.* 1974. (2, Pt. 2) 63:44A, 139A (Abstr.).

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SUBSTRUCTURE OF THE GLOMERULAR SLIT DIAPHRAGM IN FREEZE-FRACTURED NORMAL RAT KIDNEY

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In the renal glomerulus, the narrow slits between adjacent epithelial podocytes are bridged by a diaphragm (2, 8, 11). In rat and mouse kidneys fixed by perfusion with tannic acid and glutaraldehyde (TAG), it has recently been discovered that this diaphragm has a highly ordered, isoporous substructure (9). It consists of a regular array of alternating cross bridges extending from the podocyte plasma membranes to a centrally running filament. This zipperlike pattern results in two rows of rectangular pores, approximately 40 × 140 Å in cross section, dimensions consistent with the proposed role of the diaphragm as an important filtration barrier to plasma proteins (6).

In the present study, we have found in freeze-cleaved and in freeze-etched normal rat glomeruli that the surface of the slit diaphragm has an appearance conforming to the pattern found in sectioned material.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.), weighing 125 g, were anesthetized with intraperitoneal sodium pentobarbital (Diabital, Diamond Laboratories, Inc., Des Moines, Iowa) 5 mg/100 g of body weight. Heparin (Liquemarin, Organon Inc., West Orange, N. J.) 100 USP units/100 g of body weight was given intravenously immediately before opening the abdomen. Renal perfusion was performed using the protocol (method 1) and apparatus described by Griffith et al. (4). Blood was rinsed from the kidneys by perfusion (at a pressure of 140 mm of mercury) for approximately 1 min with Hanks' balanced salt solution (Microbiological Associates, Bethesda, Md.), pH 7.3, at room temperature. This was followed by perfusion for 15 min in two animals with TAG solution (3, 9) consisting of 1% tannic acid (reagent grade, Fisher Scientific Co., Pittsburgh, Pa.) and 1% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M phosphate buffer, pH 7.3, at room temperature. In another rat, perfusion was performed with formaldehyde-glutaraldehyde fixative (5) diluted 1:1 with 0.1 M cacodylate buffer, pH 7.3. A portion of the cortex from each kidney was diced with razor blades into pieces 2 mm across and then processed for freeze-fracture. To facilitate the examination of larger amounts of material by freeze-fracture, glomeruli were isolated from the remaining cortex by the method of Burlington and Cronkite (1): tissue was forced by a spatula through a stainless steel screen of no. 66 mesh (250 μm pore) (The W. S. Tyler, Co., Div. of W. S. Tyler, Inc., Mentor, Ohio) and rinsed with 0.1 M phosphate buffer through successive screens of no. 100 mesh (150 μm pore) and no. 200 mesh (75 μm pore) placed in series. Large numbers of glomeruli and some tubular fragments were found on the lowermost fine screen. After immersion in 25% glycerol in 0.1 M cacodylate buffer for 3 h, pieces of tissue and thick suspensions of the isolated glomeruli were transferred to Balzers specimen holders (Balzers AG, Liechtenstein), rapidly frozen in Freon 22 (Dupont Instruments, Wilmington, Del.) cooled by liquid nitrogen, fractured in a Balzers high vacuum freeze-etch unit BA 360 M at −104°C, and, either immediately or after etching for up to 20 min, shadowed with platinum at an angle of 45°. Some specimens were immersed in Tris buffer 0.01 M copy.