

# High-purity Isolation of Bullfrog Hair Bundles and Subcellular and Topological Localization of Constituent Proteins

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**Abstract.** The small number of hair cells in auditory and vestibular organs severely impedes the biochemical characterization of the proteins involved in mechano-electrical transduction. By developing an efficient and clean "twist-off" method of hair bundle isolation, and by devising a sensitive, nonradioactive method to detect minute quantities of protein, we have partially overcome this limitation and have extensively classified the proteins of the bundles.

To isolate hair bundles, we glue the saccular macula of the bullfrog to a glass coverslip, expose the tissue to a molten agarose solution, and allow the agarose to solidify to a firm gel. By rotating the gel disk with respect to the fixed macula, we isolate the hair bundles by shearing them at their mechanically weak bases. The plasma membranes of at least 80% of the stereocilia reseal. To visualize the proteins of the hair bundle, we covalently label them with biotin, separate

them by SDS-PAGE, and transfer them to a charged nylon membrane. We can detect <500 fg of protein by probing the membrane with streptavidin-alkaline phosphatase and detecting the chemiluminescent product from the hydrolysis of the substrate 3-(4-methoxyspiro-{1,2-dioxetane-3,2'-tricyclo-[3.3.1.1<sup>3,7</sup>]decan}-4-yl) phenyl phosphate (AMPPD).

These techniques reveal a distinct constellation of proteins in and associated with hair bundles. Several proteins, such as calmodulin, calbindin, actin, tubulin, and fimbrin, have previously been described. A second class of proteins in the preparation appears to be derived from extracellular sources. Finally, several heretofore undescribed bundle proteins are identified and characterized by their membrane topology, subcellular localization, and glycosidase and protease sensitivities.

**T**HE hair bundle is the mechanoreceptive organelle of a hair cell, the sensory receptor of the internal ear and lateral-line organ (for reviews, see Howard et al., 1988; Roberts et al., 1988; Hudspeth, 1989). The bundle, which protrudes from the apical surface of a hair cell, is usually a conical structure with a bevelled top surface. Each of a bundle's tens to hundreds of stereocilia consists of a cytoskeletal core of actin microfilaments ensheathed by the plasma membrane. A bundle ordinarily also includes a single true cilium, the kinocilium, which occurs at the tall edge of the beveled array of stereocilia.

The hair bundle is a sensitive biological strain gauge that detects mechanical forces arising as a result of acoustical or accelerational stimulation. Recordings of putative single-channel currents (Ohmori, 1985), ensemble-variance analysis of receptor currents (Holton and Hudspeth, 1986), and mechanical measurements of hair bundle stiffness (Howard and Hudspeth, 1988) indicate that very few mechanically sensitive ion channels, perhaps only one per stereocilium, are responsible for mechano-electrical transduction. Records of extracellular potentials during stimulation place the site of transduction at the distal end of the hair bundle (Hudspeth, 1982), where electron micrographs show that the tip of each stereocilium is connected to the side of its longest neighbor

by a fine, filamentous tip link (Pickles et al., 1984). These and other observations have led to a model for transduction wherein the tip link is taken to be an elastic gating spring that controls the activity of transduction channels at one or both of its ends (Corey and Hudspeth, 1983b; Howard et al., 1988; Roberts et al., 1988; Hudspeth, 1989). Deflecting the hair bundle in the direction of the tallest stereocilia is thought to stretch the tip links, opening the transduction channels; moving the bundle in the opposite direction presumably slackens the tip links, leading to channel closure.

Confirmation of the model expounded above ultimately necessitates an understanding of the physical-chemical basis for mechano-electrical transduction in terms of the stress sensitivity of the channel molecule. Because of the paucity of transduction channels and tip links, and because most auditory and vestibular organs contain only a few thousand hair cells, nothing is yet known of the molecular nature of the transducer. Immunochemical and cytochemical studies have localized several proteins to the hair bundle, including calmodulin (Shepherd et al., 1989), calbindin (Sans et al., 1986; Shepherd et al., 1989), actin (Flock and Cheung, 1977; Flock et al., 1982; Drenckhahn et al., 1982), and fimbrin (Flock et al., 1982; Slepecky and Chamberlain, 1985; Drenckhahn et al., 1985; Tilney et al., 1989). Tubu-

lin, which should occur in the axonemal microtubules of kinocilia, is also apparently present. To date, however, only two reports have described preparations that are potentially appropriate for further biochemical characterization of the components of transduction. Tilney et al. (1989) isolated from hair cells of the chick's basilar papilla a preparation of "hair borders," detergent- and high-salt-resistant bundle remnants that contained actin and fimbrin. Because this preparation required protease treatment of the papilla to release hair cells and consisted of proteins resistant to extreme experimental conditions, it is unlikely to contain the transduction apparatus in an intact state. Shepherd et al. (1989) described an ingenious preparation of hair bundles, the "bundle-blot," in which bundles were isolated by adhesion to a nitrocellulose membrane. Although the protein complement of bundles isolated by this method was clearly distinct from that of the whole macula, many of the more abundant proteins clearly had counterparts in the macula. Because nitrocellulose avidly binds proteins, some of the bands observed in the hair bundle fraction could have been soluble proteins of the macula, derived from cells that were leaking soluble proteins before or during the isolation procedure. This preparation nevertheless allows the isolation of hair bundles in a relatively intact state and may be useful for further studies.

We believe that neither of the previously mentioned hair bundle preparations is sufficient for the complete characterization of the molecules involved in mechano-electrical transduction. As a first step towards identifying transduction channels, tip links, and other components of the transduction apparatus, we have devised an efficient "twist-off" method for hair-bundle isolation that eliminates most potential sources of contamination (Gillespie, P. G., and A. J. Hudspeth. 1990. *Soc. Neurosci. Abstr.* 16:1078.). We have additionally developed a highly sensitive, chemiluminescent system for protein detection that allows us to visualize femtogram amounts of proteins on an electroblot (Gillespie and Hudspeth, 1991). Using these techniques, we have extended the analysis of the hair bundle's protein constituents initiated by Shepherd et al. (1989) and Tilney et al. (1989). We have detected numerous proteins in and associated with the hair bundle, determined the relations of many to the plasma membrane and cytoskeleton, and ascertained the glycosidase and protease sensitivities of the extracellularly exposed proteins.

## Materials and Methods

### Materials

Low melting point agarose was obtained from BRL (Gaithersburg, MD); Cell-Tak was obtained from Collaborative Research (Bedford, MA). Recombinant *Streptomyces plicatus* endo- $\beta$ -N-acetylglucosaminidase H (endo H<sup>1</sup>), *Flavobacterium meningosepticum* endo- $\beta$ -N-acetylglucosaminidase F (endo F)/peptide:N-glycosidase F (PNGase F), *Vibrio cholerae* neuraminidase, pepstatin, and leupeptin were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Sigma Chemical Co. (St. Louis, MO) was the source for the following compounds:  $\alpha_1$ -acid glycoprotein, actin, 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), casein, DNase I, fetuin, hemoglobin, laminin, lysozyme, 2-(N-morpholino)ethanesulfonic acid (MES), myoglobin, PMSF, polyvinylpyrrolidone-40, saponin, subtilisin BPN', and Triton X-114. Pierce Chemical Co. (Rockford, IL) was the supplier of N-hydroxysuccinimidobiotin (NHS-biotin), N-hydroxysulfosuccinimidoacetate (sulfo-NHS-acetate), and N-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin). All electrophoresis reagents were from BioRad

Laboratories (Richmond, CA), as were Zeta-Probe nylon blotting membranes and all molecular weight markers except laminin. Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR); Amicon (Danvers, MA) was the supplier of Centricon-10 centrifugal concentration units.

### Physiological Recording

The saccular receptor organ, or macula, is broadly defined as a sensory epithelium of hair and supporting cells, an overlying otolithic membrane, and a subjacent layer of connective tissue interpenetrated by nerve fibers and capillaries. The otolithic membrane includes a compact portion, which is readily removed by dissection, and subotolithic filaments that extend from the compact component to the apical surfaces of supporting cells. Maculae from the inner ear of the bullfrog *Rana catesbeiana* (Western Scientific, West Sacramento, CA) were dissected into a standard saline solution that contained 110 mM NaCl, 2 mM KCl, 4 mM CaCl<sub>2</sub>, 3 mM D-glucose, and 5 mM HEPES at pH 7.25. Each macula was glued across a hole in a plastic disk and mounted in a two-compartment recording chamber (Kroese et al., 1989). A piezoelectrical stimulator applied a 25-Hz, sinusoidal, nearly saturating stimulus to the compact otolithic membrane, upon which the distal ends of the hair bundles insert. A voltage-clamp circuit was used to measure the resultant transepithelial current (Corey and Hudspeth, 1983a), the combined receptor currents of the ensemble of stimulated hair cells.

### Twist-off Procedure

For each hair bundle preparation, 6–20 saccular maculae were dissected free of surrounding tissue. During the dissection and all steps of the bundle isolation, the maculae were maintained in standard saline solution with protease inhibitors (SSPI), which consisted of standard saline solution to which was added 1  $\mu$ M pepstatin A, 1  $\mu$ M leupeptin, and 200  $\mu$ M PMSF. Pepstatin and PMSF were freshly dissolved in ethanol and diluted into standard saline solution; the ethanol was removed by evacuating the buffer solution for 15–20 min. Because the half-life of PMSF at pH 7.5 is  $\sim$ 1 h (James, 1978), this solution was used promptly.

The maculae were cemented to the bottom of a chamber (see Fig. 1) consisting of a glass coverslip to which was glued a 2-mm-high nylon washer 12.5 mm in internal diameter. The washer allowed exchange of the solution covering the hair bundles and contained the disk of agarose used in bundle isolation. The glass surface of this chamber was coated with Cell-Tak, a solution of marine mussel glue, which was allowed to dry completely. For firm adherence of the connective-tissue surfaces of the saccular maculae, it was necessary to expose a coverslip to four treatments of 1 g/l Cell-Tak, each of 20  $\mu$ l.

After the compact otolithic membranes overlying the hair bundles were removed with fine forceps, and following biotin labeling of proteins when appropriate, the maculae were subjected to the twist-off procedure. In this isolation step, the coverslip holding the maculae was transferred to a Petri dish containing SSPI at 34°C. After 5 s, the buffer was aspirated away, save for the small amount overlying the bundles and trapped by the nylon washer. Molten 3% agarose solution, in SSPI and warmed to 35°C, was streamed onto the bundles with a 5-ml micropipettor (Gilson, Worthington, OH); 20–25 ml of solution were ordinarily used. The coverslip was immediately removed from the solution, carrying with it a small pool of agarose solution retained by the nylon washer, and was incubated at 34°C for 1 min without agitation. The agarose solution was then allowed to solidify to a firm gel in 10 min at 4°C.

After trimming of excess agarose gel outside of the washer, the gel disk was separated from the inner circumference of the washer with a tungsten needle. A 15-gauge needle stub, plunged through the gel to the coverslip, was used as a handle to twist the disk relative to the coverslip through 10–30° of rotation about a vertical axis. Although the extent of rotation was not critical, it was important to twist the disk relatively quickly and to avoid lifting the disk. After the gel disk was loosened, it was inverted onto a glass slide and superfused with a stream of filtered SSPI to wash away any soluble contaminants on the surface of or trapped within the gel. With dark-field illumination, individual hair bundles were apparent under the dissecting microscope as a few thousand points of light within the gel (see Fig. 2). Because the bundles could readily be seen, it was easy to dissect away areas of poor recovery and contaminating cellular debris. The recovery of hair bundles from each sacculus was estimated by visual inspection and was typically  $\sim$ 90%. Estimation of recovery allowed equal number of hair bundles subsequently to be loaded on SDS gels; the results are accordingly reported in terms of saccular equivalents, adjusted for differences in recovery among

collected bundle samples. During the 15–20 min of superfusion, a tungsten needle was used to circumscribe the gel containing the bundles from each sacculus and to pare away obvious cellular debris (see Fig. 1). The gel block containing the bundles (100–200  $\mu\text{g}$  per sacculus) was then undercut with the tungsten needle and was speared and placed into a microfuge tube.

The warm embedding step was unavoidable in our procedure; all the other steps could be conducted at 4°C. When dissection, biotinylation, and superfusion were performed at this lower temperature, however, the pattern of proteins labeled with biotin in the presence of detergent was indistinguishable from that observed after treatment at room temperature. For this reason, we ordinarily performed at room temperature the dissection, labeling, and isolation steps.

### Labeling with Rhodamine-Phalloidin

Hair bundles were isolated by the twist-off procedure without biotinylation. Plugs holding the bundles from individual sacculi were removed from the disk of gelled agarose by a 1-mm sample corer (Fine Science Tools, Belmont, CA) and transferred to the well of a 3-mm-thick, two-compartment culture slide (Carolina Biological Supply, Burlington, NC). After cleaning by immersion in SSPI, the cores were labeled for 20 min, at room temperature or 4°C, with 660 nM rhodamine-phalloidin in SSPI. In some cases, the membranes of the bundles were permeabilized by the inclusion of 40 mg/l saponin in the labeling solution.

The cores containing labeled bundles were placed on their sides in SSPI, after which the bundle-bearing face of each was cut free with a microscalpel, washed with SSPI, and positioned face-up on a glass microscope slide with #2 coverslip slivers as spacers. A #0 coverslip was dropped into place and was sealed with nail polish. Hair bundles were photographed with a microscope (Universal, Carl Zeiss, Inc., Oberkochen, FRG) equipped for differential-interference-contrast and for epifluorescence observation with respectively a 63 $\times$ , Planapochromatic objective lens of NA 1.4, and a 63 $\times$ , Plan-Neofluor objective lens of NA 1.25.

### EM

Saccular maculae were dissected and maintained in standard saline solution with protease inhibitors. After hair bundles were isolated by the twist-off procedure, the gel block containing the bundles was fixed for 60 min at 4°C and pH 7.4 in a solution containing 200 mM glutaraldehyde and 5 mM  $\text{CaCl}_2$  in 80 mM sodium cacodylate buffer. After a brief wash in buffer solution, each specimen was postfixed, again for 60 min at 4°C and pH 7.4, in a solution containing 40 mM  $\text{OsO}_4$  and 5 mM  $\text{CaCl}_2$  in 80 mM sodium cacodylate buffer. The bundles were then partially dehydrated in ethanol and stained en bloc for 60 min with 0.5% uranyl acetate in 95% ethanol. Hair bundles whose membrane integrity was to be tested were treated in a similar fashion except that the primary fixation, intermediate wash, and postfixation solutions all contained 3 g/l purified Ruthenium red (Luft, 1971). The postfixation was extended to 120 min duration, and en bloc staining was omitted.

After complete dehydration in ethanol and propylene oxide, specimens were embedded in epoxy plastic. Cut at a thickness of 100 nm on a diamond knife, sections were collected on uncoated grids and stained with uranyl acetate and lead citrate. Specimens were examined and photographed in an electron microscope (JEM-1200EX; JEOL Ltd., Tokyo, Japan) operated at an acceleration voltage of 80 kV.

### Biotinylation of Bundle Proteins

Two approaches were used to label hair bundle proteins with biotin. In the first procedure, the bundles were labeled before isolation; the maculae could then be thoroughly washed with buffer after labeling to remove contaminants such as human skin keratin. In the second procedure, the bundles were labeled in the agarose gel after isolation by the twist-off procedure. Although it increased the likelihood of contamination, this method allowed examination of the integrity of the membranes on the isolated stereocilia. The protein constituents of the hair bundle appeared similar whether stained with silver (see Fig. 5) or labeled with sulfo-NHS-biotin (see Fig. 7).

For labeling before isolation, the compact otolithic membranes were removed and the mounted maculae were washed thoroughly with biotin labeling buffer (HSSP; standard saline solution containing 200  $\mu\text{M}$  PMSF and 25 mM HEPES at pH 8.0). The maculae were then incubated at room temperature for 15 min with HSSP containing 2.5 mM sulfo-NHS-biotin or NHS-biotin; these reagents covalently link biotin to lysyl and other side chains on proteins (Anjaneyulu and Staros, 1987). The latter reagent was

dissolved in a small volume of DMSO, the final concentration of which was 2.5%. It was necessary to readjust the pH to 8.0 with NaOH after addition of sulfo-NHS-biotin. In some experiments, 40 mg/l saponin was included in the labeling solution to permeabilize the membranes. Although the addition of saponin occasionally reduced the recovery of hair bundles, the pattern of biotinylated proteins was unchanged. After labeling, the maculae were washed with 1 mM lysine in SSPI to terminate the biotinylation, rinsed in SSPI, and then subjected to the twist-off procedure.

For labeling after isolation, the agarose blocks containing the bundles were placed into 10  $\mu\text{l}$  of HSSP with or without 40 mg/l saponin. After a 10-min incubation at room temperature, 2  $\mu\text{l}$  of 15 mM sulfo-NHS-biotin in HSSP was added and the reaction was allowed to proceed for 15 min. The labeling was stopped by the addition of 1  $\mu\text{l}$  of 1 M lysine. When labeling was conducted after bundle isolation, it was critical that all buffers were filtered through protein-binding filters and that extreme care was taken to avoid contaminating the reaction mixtures with human skin keratin or other substances. To achieve the minimal contamination reported here, we found it necessary to wear gloves and a cleanroom hood and face mask and to perform the dissection, bundle isolation, and labeling in a laminar-flow hood (model 1839; Forma Scientific, Marietta, OH).

### Triton X-114 Extraction

The stereocilia in isolated, biotin-labeled hair bundles were broken open by three cycles of freezing and thawing in 200  $\mu\text{l}$  of lysis buffer (150 mM KCl, 1 mM EDTA, 1 mM EGTA, 3 mM  $\text{MgCl}_2$ , 25 mM HEPES at pH 7.4, 1  $\mu\text{M}$  pepstatin A, 1  $\mu\text{M}$  leupeptin, 200  $\mu\text{M}$  PMSF, and 0.1 g/l lysozyme or myoglobin). This procedure released soluble proteins. Control experiments demonstrated that soluble proteins readily diffused out of the gel blocks. The agarose and bundles were sedimented at 20,000  $g$  for 20 min. The supernatant containing soluble proteins was removed and recentrifuged at 150,000  $g$  for 15 min in an airfuge (Beckman, Fullerton, CA). The proteins in the supernatant from this centrifugation were precipitated at  $-20^\circ\text{C}$  for 1 h with 6 vol of acetone, centrifuged at 16,000  $g$  for 10 min, and dissolved in SDS sample buffer. With vigorous vortexing every 10 min, the pellet containing nonsoluble bundle proteins was then incubated on ice for 60 min in lysis buffer containing 1% Triton X-114 (precondensed through three cycles as described in Bordier, 1981). The extract was centrifuged at 20,000  $g$  for 20 min. The pellet from this centrifugation, which contained insoluble cytoskeletal cores, was washed once with 1% Triton X-114 in lysis buffer and dissolved in SDS sample buffer. The supernatant, which contained Triton X-114-soluble proteins, was recentrifuged to remove residual cytoskeletal cores, then was subjected to phase separation at 34°C as described in Bordier (1981) except that a sucrose cushion was not used. The aqueous and detergent phases were back-extracted with 8% Triton X-114 in lysis buffer and 0.06% Triton X-114 in lysis buffer, respectively, and both aqueous and both detergent fractions were pooled. Each of the phases was then washed three times with 8% Triton X-114 in lysis buffer (aqueous phase) or 0.06% Triton X-114 in lysis buffer (detergent phase). The proteins were precipitated with acetone.

Because the small amount of protein extracted from the hair bundles readily adhered to surfaces, we performed the detergent extraction and phase partitioning in the presence of 0.1 g/l unlabeled carrier protein. The extraction of bundle proteins in buffer or detergent was similar when performed in the presence of either lysozyme or myoglobin; in addition, the phase partitioning of bundle proteins was comparable with either carrier protein.

### Enzymatic Treatment of Hair Bundles

For endoglycosidase and neuraminidase treatment, bundles were labeled before isolation with sulfo-NHS-biotin without saponin permeabilization. The bundles were isolated and placed into tubes containing 20  $\mu\text{l}$  of endo H (0.02 U), endo F/PNGase F (0.40 U), or neuraminidase (0.14 U). The enzymes had previously been concentrated several times with centrifugal concentration units (Centricon-10); after each concentration, the remaining solution was diluted with MES-EDTA (all three enzymes; 2 mM KCl, 110 mM NaCl, 3 mM D-glucose, 20 mM EDTA, 25 mM MES at pH 6.0, 5  $\mu\text{M}$  pepstatin A, 5  $\mu\text{M}$  leupeptin, and 1 mM PMSF) or MES-Ca (neuraminidase only; same as MES-EDTA without EDTA but with 4 mM  $\text{CaCl}_2$ ). Digestion was allowed to proceed for 16 h at 34°C. Reactions were terminated by the addition of SDS sample buffer. The activities of endo H and neuraminidase were assessed by their effects on  $\alpha_1$ -acid glycoprotein and fetuin; DNase I was the substrate for control deglycosidation by endo F/PNGase F. These controls indicated complete or nearly complete

deglycosidation. In addition, the presence of a block of agarose gel had no effect on deglycosidation of control proteins by any of the enzymes. The protease activity of the endo F/PNGase F preparation was examined with a variety of substrates, including lysozyme, carbonic anhydrase, and bovine serum albumin.

Hair bundles were treated with subtilopectidase BPN' using previously published conditions (Howard and Hudspeth, 1987). After labeling with sulfo-NHS-biotin in the absence of saponin, the bundles were thoroughly washed with standard saline solution lacking protease inhibitors. Subtilopectidase BPN' (50 mg/l) was added and digestion was allowed to proceed for 40 min at room temperature. Digested maculae did not adhere readily to the Cell-Tak-covered coverslips; in addition, recovery of the bundles declined to ~50%.

### SDS-PAGE and Electrophoresis

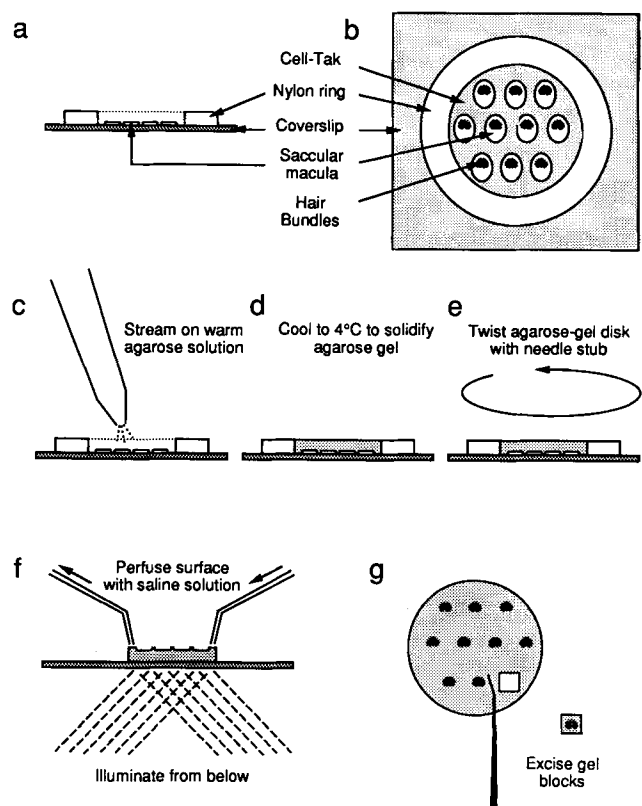
SDS-PAGE was performed with resolving gels of 7.5% acrylamide (3.3% cross-linker) containing 375 mM Tris at pH 8.9, 0.1% SDS, and 1 mM EDTA, or with 3-17% acrylamide gradient gels (Jule Inc., New Haven, CT). Stacking gels were of 3% acrylamide (3.3% cross-linker) and contained 125 mM Tris at pH 6.7, 0.1% SDS, and 1 mM EDTA. The anode and cathode buffers contained 1 mM EDTA; the anode buffer also contained 1 mM thioglycolic acid. Use of low-percentage resolving gels, short stacking gels, fresh acrylamide, EDTA, and thioglycolic acid all reduced aggregation of extracellularly labeled proteins. Mini-gels were run at 200 V for 40 min; standard-format gels (140 × 160 mm) were typically run at 30 mA for 2.5 h. Molecular mass markers (biotinylated for blots and unbiotinylated for silver-stained gels) included: lysozyme (14.3 kD), soybean trypsin inhibitor (20.1 kD), carbonic anhydrase (28.9 kD), ovalbumin (42.7 kD), BSA (66.2 kD), phosphorylase b (97.4 kD),  $\beta$ -galactosidase (116 kD), skeletal muscle myosin (220 kD), and laminin heavy chain. Although one estimate places the molecular mass of the laminin heavy chain at 440 kD (Juliano, 1987), we assigned to this marker the more recently determined mass of 400 kD (Sasaki et al., 1988). The molecular masses reported here for those proteins larger than myosin (220 kD) must therefore be viewed with caution. For use as a marker with chemiluminescent blots, laminin was biotinylated with sulfo-NHS-biotin by the methods described above.

Polyacrylamide gels were stained with silver by the method of Blum et al. (1987). Quantitative densitometry of actin bands in silver-stained gels was performed with a laser densitometer (Ultrascan XL; Pharmacia LKB Biotechnology, Piscataway, NJ). Standards of bovine muscle G-actin, whose contents bracketed the amount of actin in hair bundle samples, were run on the same gels.

For detection of biotinylated proteins, we transferred the bundles' proteins to charged nylon membranes (Gershoni and Palade, 1982). After completion of electrophoresis, each polyacrylamide gel was washed with three 5-min changes of 10 mM CAPS at pH 10.8. The separated proteins were then transferred to a membrane (Zeta-Probe) in the same buffer. The transfer was effected in 16 h at 0-4°C with plate electrodes and a high-intensity field of 625 V/m. The use of nylon membranes and a transfer buffer at pH 10.8 (Matsudaira, 1987) reduced the possibility of skewing our results by the inefficient transfer of high molecular weight or basic proteins. Nylon blotting media also enhance the chemiluminescent reaction (Tizard et al., 1990). Over 75% of myosin (220 kD) and laminin heavy chain (400 kD) were transferred from the gel under these conditions.

### Detection of Biotinylated Proteins

Biotinylated proteins were detected by recording on film the chemiluminescence from the product of an alkaline phosphatase reaction. Each nylon membrane was blocked for 2-4 h with 4% bovine casein, 2% bovine hemoglobin, 1% polyvinylpyrrolidone-40, and 3 mM NaN<sub>3</sub> in PBS (initial pH, 7.4; final pH after addition of blocking proteins, ~6.8). The membrane was next incubated for 30 min with streptavidin-alkaline phosphatase (TAGO, Burlingame, CA) diluted 1:10,000 in blocking buffer to a final concentration of ~750 U/l. The membrane was washed with five 5-min changes of 0.3% Tween in PBS and five 5-min changes of assay buffer (1 mM MgCl<sub>2</sub> and 50 mM sodium carbonate-bicarbonate at pH 9.5). The membrane was then incubated for 5 min in assay buffer containing 400  $\mu$ M 3-(4-methoxy-spiro[1,2-dioxetane-3,2'-tricyclo-[3.3.1.1<sup>3,7</sup>]decan]-4-yl) phenyl phosphate (AMPPD; Tropix, Bedford, MA). This compound is quite stable and thus little background arises by nonenzymatic hydrolysis (Bronstein et al., 1989). After dephosphorylation by alkaline phosphatase, however, the product gradually decomposes, and emits a photon with a  $\lambda_{max}$  of 477 nm in solution (Bronstein et al., 1989) or 460 nm on nylon membranes (Tizard et al.,



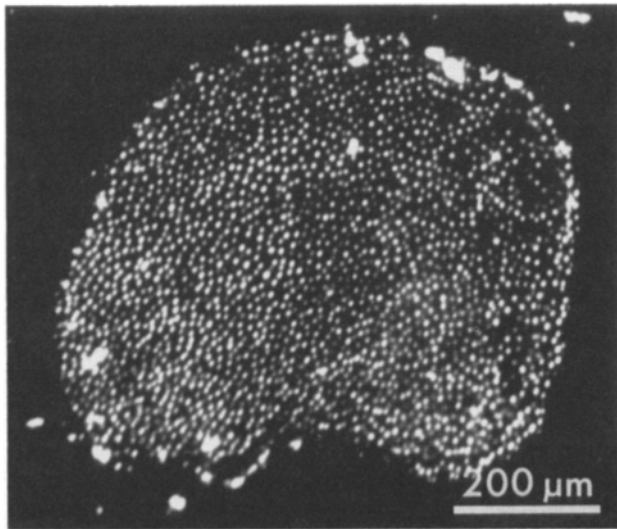
**Figure 1.** Schematic illustration of the twist-off procedure. The isolation chamber consists of a nylon washer glued to a glass coverslip (a, side view; and b, top view). The saccular maculae, attached to the coverslip with Cell-Tak, are bathed with a stream of warm agarose solution (c). When the agarose gel has solidified (d), the stub of a 15-gauge needle is used to rotate the gel disk with respect to the coverslip (e). After the disk is inverted onto a microscope stage, it is superfused with a stream of buffer and viewed with dark-field illumination (f, side view). Gel blocks containing the hair bundles from a single sacculus are excised with a tungsten needle (g, top view). An excised block is depicted to the right of the disk; the needle is poised to dissect the next block.

1990). The membrane was blotted lightly with filter paper to remove surface moisture, encased in plastic wrap, and exposed for 10-1,200 s to X-ray film (XAR or XRP; Eastman Kodak Co., Rochester, NY).

## Results

### Isolation of Hair Bundles by the Twist-off Method

We mechanically isolated hair bundles from the sensory region, or macula, of the bullfrog's sacculus, a vibration-sensitive receptor organ of the internal ear. We immersed dissected maculae in a solution of 3% low-melting-point agarose, which remained liquid at 34°C for the few minutes necessary to infiltrate the extracellular space around, and perhaps within, the bundles. We then cooled the agarose solution until it solidified to a firm gel. When we subsequently applied shear between the agarose gel and saccular maculae, the gel was sufficiently firm to retain the stereociliary shafts. The stereocilia were therefore amputated at their bases, where their attenuated insertions provided a natural abscission point. The twist-off method is diagrammed in Fig. 1.



**Figure 2.** Hair bundle preparation viewed through dissecting microscope. Unlabeled hair bundles were isolated by the twist-off procedure and viewed with dark-field illumination. Each of the  $\sim 3,000$  bright spots represents one hair bundle, which in turn comprises  $\sim 50$  stereocilia and one kinocilium. The particles seen around the periphery of the bundle array are clusters of otoconia.

We inspected the hair bundles after isolation under a dissecting microscope with dark-field illumination (Fig. 2). Observation of the preparation allowed quantitation of the recovery of hair bundles, which routinely surpassed 90% when the canonical procedure was followed. Visual inspection additionally ensured that little cellular debris contaminated the final preparation. The agarose gel was easily washed by superfusion, reducing contamination from soluble proteins released before or during the twist-off step. The procedure for hair bundle isolation was thus both efficient and clean.

Because the elevated temperature employed in the twist-off procedure might have had a deleterious effect on bundle proteins, we used one crucial measure of the functional state of the hair bundles, electromechanical transduction, to judge the effect of the 34°C embedding step. We mounted a saccular macula on a plastic disk in a recording chamber and deflected the hair bundles with a mechanical stimulus applied to the compact otolithic membrane. After recording the control receptor current, we removed the disk from the recording chamber and incubated the macula for 2 min at 34°C. We then replaced the disk in the chamber and measured additional responses. After 30 min recovery, a macula subjected to this procedure retained  $86\% \pm 15\%$  (mean  $\pm$  SD,  $n = 4$ ) of its control response. Although this method did not test the integrity of all of the proteins of the hair bundle, the result demonstrated that the components responsible for membrane stability and transduction were not dramatically affected by the temperature used during the isolation. In addition, immunoblot analysis of the bundle preparation with a polyclonal antiserum directed against chicken fimbrin revealed a single immunoreactive band that comigrated with authentic fimbrin (data not shown). Because hair bundle fimbrin is sensitive to proteolysis (Tilney et al., 1989), the lack of degradation products supports the conclusion that the warming step does not lead to proteolysis.

### ***Integrity of Stereociliary Membranes and Structure of Isolated Bundles***

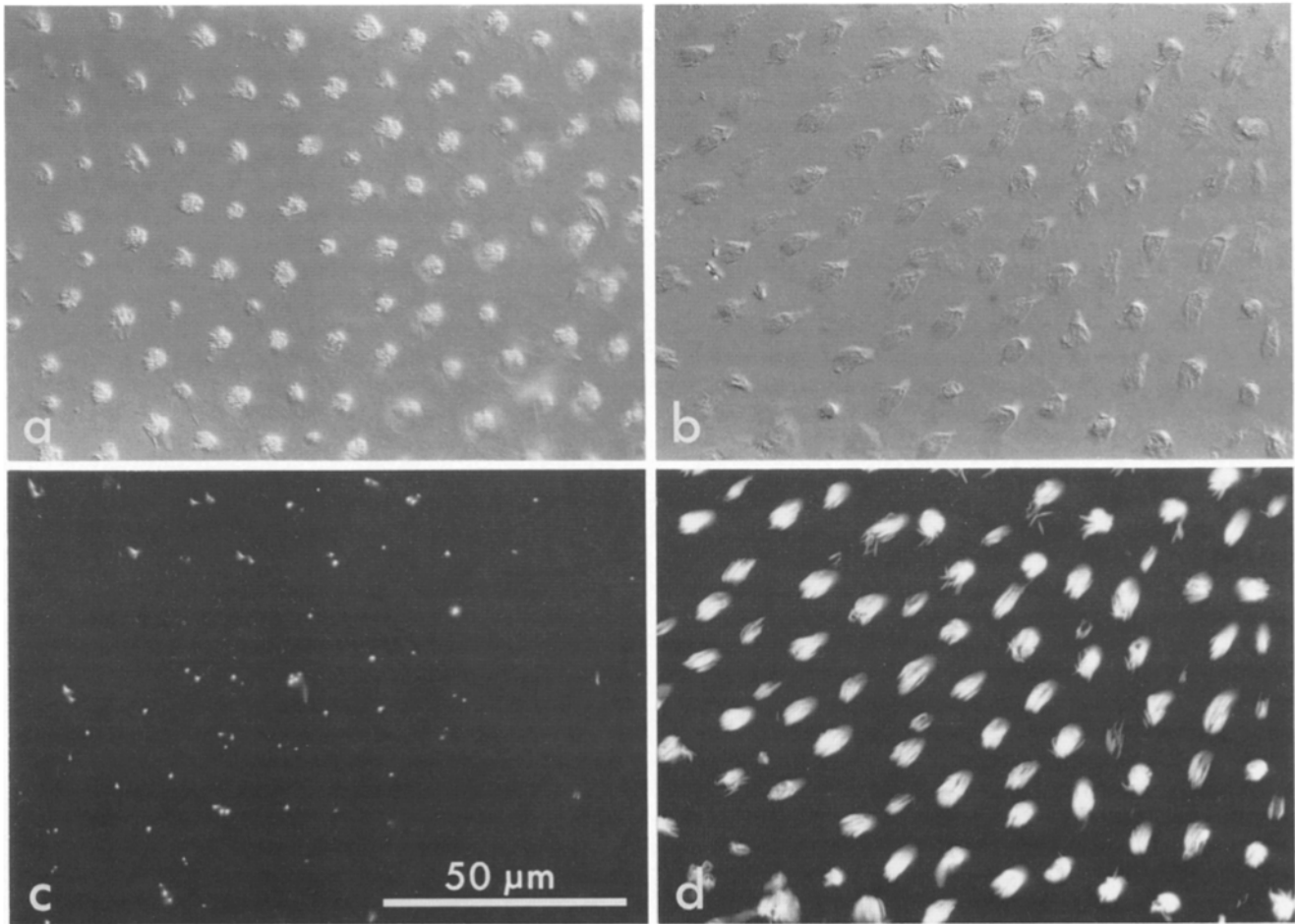
Because we isolated hair bundles under relatively gentle conditions, their plasma membranes might have remained intact and resealed at the severed stereociliary bases. We therefore employed phalloidin, a mushroom toxin that binds tightly to microfilaments (Faulstich et al., 1988), as a reagent to test the integrity of the membranes in the isolated stereocilia. We incubated unfixed hair bundles, embedded in agarose gel, with rhodamine-phalloidin in the absence or presence of 40 mg/l saponin, and observed the bundles with differential-interference-contrast and fluorescence optics (Fig. 3). Few stereocilia were labeled by rhodamine-phalloidin in the absence of detergent (Fig. 3, *a* and *c*). By contrast, when the bundles were exposed to rhodamine-phalloidin in the presence of saponin, all of the stereocilia were intensely labeled (Fig. 3, *b* and *d*). The plasma membranes of most stereocilia therefore resealed after isolation.

Compound microscopic observation confirmed the lack of contamination seen with the dissecting microscope; although cuticular plates and nuclei were sporadically observed, for the most part the only features seen in the microscope were hair bundles (Fig. 3, *a* and *b*). Both stereocilia and kinocilia were present in isolated bundles. The bundles varied in their state of dishevelment, due in part to variability in the shear imposed during isolation and to the pressure applied by the coverslip overlying the bundles during microscopic observation.

Transmission EM of the preparation showed that the isolated stereocilia were remarkably well preserved (Fig. 4 *a*). The microfilament cores resisted fragmentation and retained cross-links between actin filaments, lateral attachments of cytoskeletal cores to plasma membranes, and submembrane densities at the stereociliary tips (Fig. 4, *b-d*). The plasma membranes remained intact and resealed at the severed bases of the stereocilia (Fig. 4 *d*). When the isolated bundles were incubated in the presence of Ruthenium red, none of the tracer was found inside stereociliary membranes (data not shown); this result confirmed that the membranes resealed. The preparation also included occasional microvilli from hair or supporting cells, as well as subotolithic filaments that extend from supporting cells to the compact otolithic membrane (Fig. 4 *a*). The subotolithic filaments probably contributed a substantial mass to the preparation and thus complicated interpretation of the SDS gels.

### ***Visualization of Bundle Proteins by SDS-PAGE and Silver Staining***

Examination of the proteins from isolated hair bundles by SDS-PAGE and silver staining allowed us to determine some of the constituents of the hair bundle preparation (Fig. 5). On the basis of stereociliary structure (Flock and Cheung, 1977; Tilney et al., 1980), the principal bundle protein is probably actin. The prominent bundle constituent with an apparent molecular mass of 44 kD, which comigrated with authentic bovine muscle actin, is presumably hair bundle actin. On the assumption that the silver staining of bovine muscle actin is similar to that of frog stereociliary actin, we employed quantitative densitometry to estimate the amount of actin present. In 14 independent preparations, consisting of the hair bundles from 1-50 sacculi per SDS-PAGE lane, we



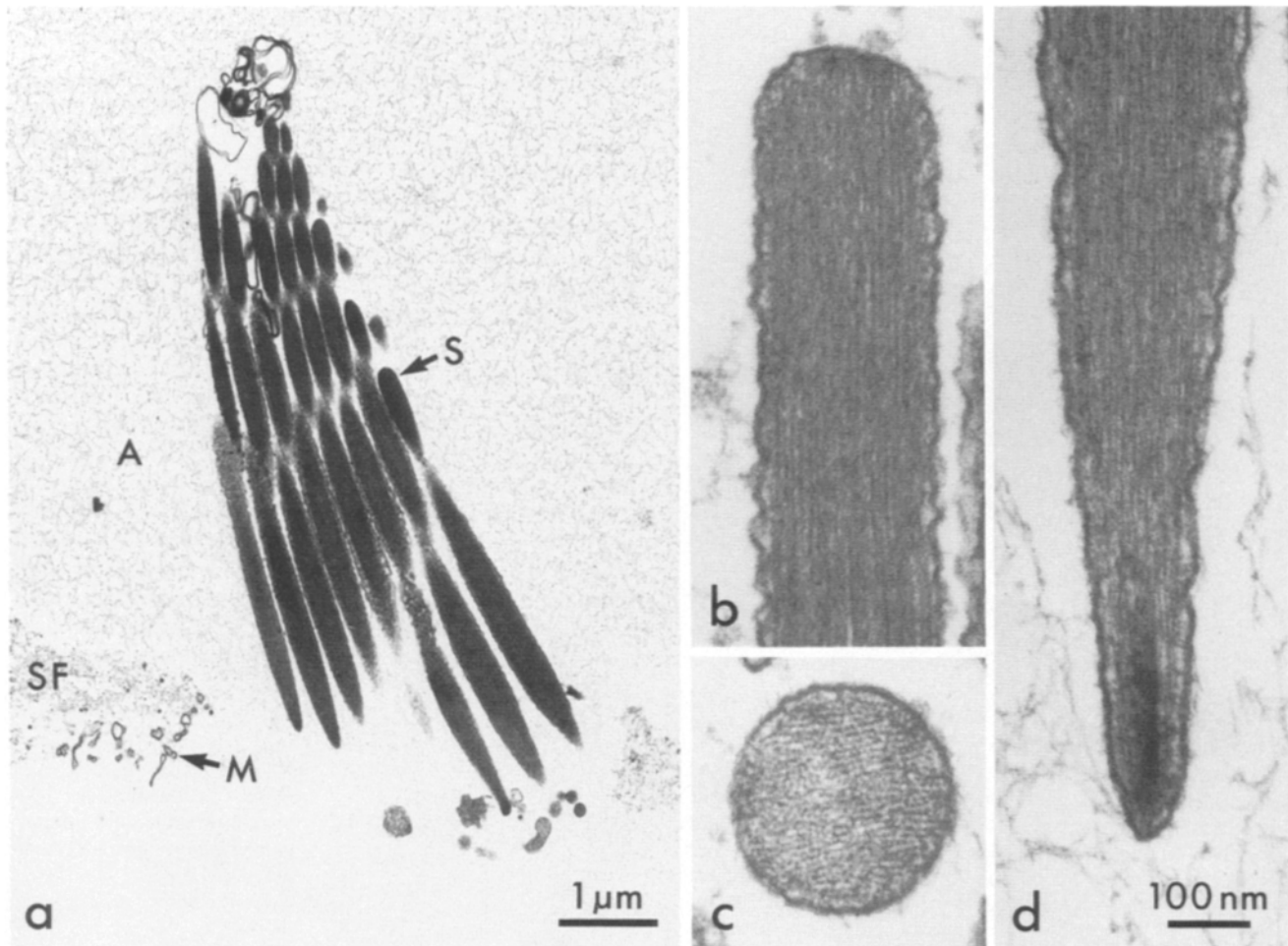
**Figure 3.** Rhodamine-phalloidin labeling of isolated hair bundles with or without membrane permeabilization. Bundles were isolated by the twist-off procedure, then were labeled with 660 nM rhodamine-phalloidin in the absence (*a* and *c*) or presence (*b* and *d*) of 40 mg/l saponin. Hair bundles were observed with differential-interference-contrast optics (*a* and *b*); labeled stereocilia were visualized with fluorescence optics (*c* and *d*).

found  $19 \pm 7$  ng (mean  $\pm$  SD) of actin per saccular equivalent of hair bundles. This value agrees well with the value of 13 ng estimated from a morphometric analysis of the length and number of microfilaments in saccular hair bundles (Jacobs and Hudspeth, 1990). In addition to actin, the preparation appeared to contain several other proteins previously detected in hair bundles, including those of molecular mass 19 kD (calmodulin), 29 kD (calbindin), 55 kD (tubulin), and 67 kD (fimbrin).

A number of other proteins constituted a substantial fraction of the bundle protein; we identified these components on the basis of their apparent molecular masses as determined by SDS-PAGE. Two high molecular mass proteins of 265 and 337 kD each contributed 5–10% of the total protein. These proteins may not have been hair bundle-specific: they comigrated with polypeptides in the peripheral gelatinous matrix that overlies the nonsensory epithelium. It is possible that constituents of the peripheral gelatinous matrix are also components of the subotolith filament, which are present in the isolated preparation (Fig. 4). Other proteins seemed to occur in both the peripheral-gelatinous-matrix fraction and the bundle fraction, including those at 95, 161, and 187 kD, as well as the four proteins larger than 400 kD (Fig. 5).

Commonly used methods for determination of protein content are not sufficiently sensitive to quantify accurately the limited amount of protein in the bundle preparation. Although well aware that proteins vary widely in their silver-staining characteristics, we nevertheless estimated the preparation's protein content by densitometry of silver-stained gels. Under conditions in which the silver staining of actin was linear with respect to the amount loaded, actin appeared to constitute 50–75% of the protein in the preparation (data not shown). It is therefore unlikely that the hair bundles of each sacculus contain a total of  $>40$  ng of protein.

We evaluated the extent to which our hair bundle preparation was contaminated by saccular proteins, other than those of hair bundles, and by exogenous substances. Four sources might have contributed contaminating proteins: the agarose used to isolate the bundles; the hair cells and supporting cells of the residual sensory epithelium after bundle isolation; the otolith membrane; and the peripheral gelatinous matrix. Fig. 5 shows the proteins from the hair bundles of 20 sacculi, from an equivalent amount of agarose, and from the residual maculae, compact otolith membranes, and peripheral gelatinous matrices of two sacculi. The agarose sample examined in the gel of Fig. 5 was from a section of



**Figure 4.** Transmission EM of the hair bundle preparation. Hair bundles were isolated by the twist-off procedure and fixed in the agarose block. (a) Low-power view of an isolated bundle. The agarose gel (A) entraps the cluster of stereocilia (S). Extraneous components of the preparation include subtolithic filaments (SF) and microvilli (M). (b) The tip of a stereocilium displays an intact cytoskeleton with cross-bridges, lateral membrane attachments, and tip density. (c) A cross-section shows the regular disposition of actin microfilaments. (d) The plasma membrane is intact, not only along the shaft of an isolated stereocilium, but also around the severed rootlet. The scale bar in d applies to the high-magnification micrographs of b-d.

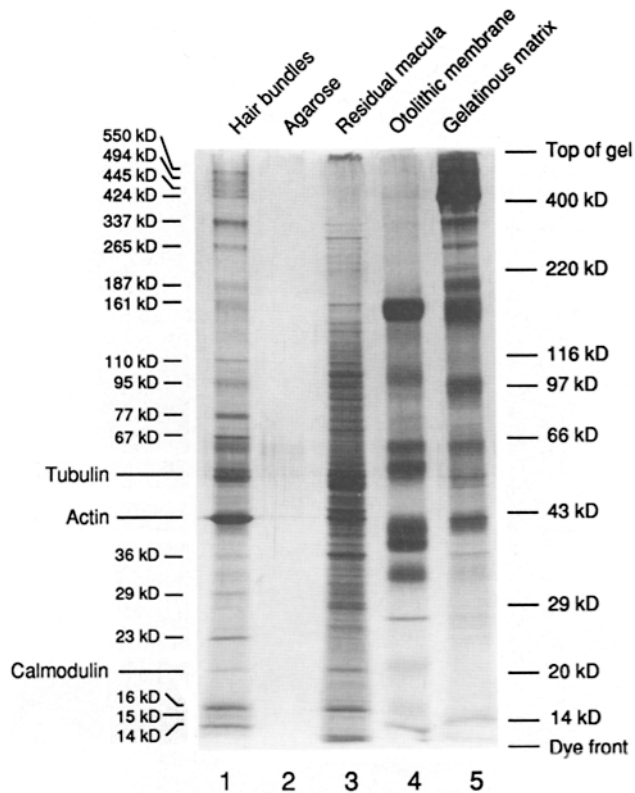
the same gel disk used to isolate the bundles; no protein contaminants were observed, indicating both that the agarose itself did not contain detectable quantities of protein, and that superfusion removed any soluble proteins that were introduced during dissection or released from cells during bundle isolation. Although some proteins present in the residual macula, compact otolithic membrane, and peripheral gelatinous matrix also appeared to occur in the bundle preparation, most were absent. Because the ensemble of bundle proteins differs from that of potential contaminants, the twist-off procedure is clearly effective at isolating hair bundles from the adjacent tissue.

Contamination of hair bundle samples by human skin keratin was a substantial problem. SDS-PAGE bands present at 55–65 kD appear when samples are contaminated by small amounts of keratin (Ochs, 1983). Observable with other detection methods (see below), these bands are not an artifact of silver staining, as is commonly assumed. Laboratory dust and dandruff from the bearded investigators were found to be common offenders. Upon adoption of sufficiently strin-

gent isolation conditions, however, the amount of keratin contaminating the bundle preparation could be reduced to a minimum (Fig. 5).

#### *Detection of Proteins by Chemiluminescence*

The small amount of protein present in the hair bundle preparation necessitated a sensitive method of labeling for routine detection. The method described here employs biotinylation of the proteins, SDS-PAGE and electroblotting, binding of streptavidin-conjugated alkaline phosphatase, and the subsequent detection of hydrolysis of the substrate AMPPD. A full description of the technique will be provided elsewhere (Gillespie and Hudspeth, 1991). In control experiments, we could detect <500 fg of BSA that was labeled with 10 mol biotin per mole of BSA, or <75 amol of biotin per band. This heightened sensitivity allowed us to detect numerous hair bundle proteins on a standard-format SDS polyacrylamide gel with the hair bundles from only two sacculi per lane (Fig. 6). Substantially fewer hair bundles were



**Figure 5.** Silver-stained gel of hair bundles and associated structures. Saccular maculae were dissected and the compact otolithic membranes removed. Hair bundles were isolated using the procedure described in the text. (lane 1) Hair bundles (20 saccular equivalents); (lane 2) 3 mg of agarose (an amount similar to that loaded in lane 1); (lane 3) residual sensory epithelium after bundle isolation (from two sacculi); (lane 4) compact otolithic membranes (from two sacculi); and (lane 5) peripheral gelatinous matrix overlying nonsensory epithelium (from two sacculi). Samples were run on a 3–17% acrylamide gradient gel with a 3% stacking gel. The apparent molecular masses of major hair bundle constituents are listed on the left, as are the migration positions of purified bovine calmodulin, actin, and tubulin; the molecular masses of calibration proteins are indicated to the right of the gel.

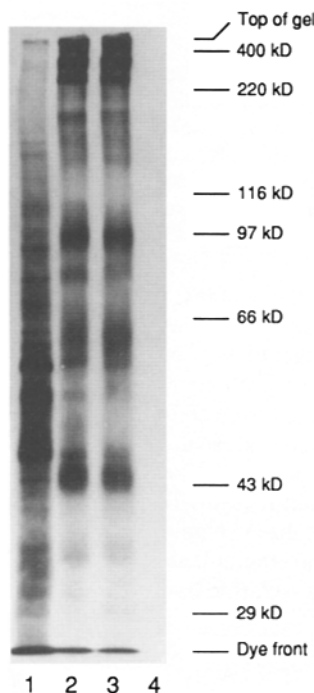
necessary when mini-gels were used; for example, 0.2 saccular equivalent, or ~600 bundles, sufficed in Fig. 7 *a*.

In this report, we identified the proteins of hair bundles with molecular masses that were determined by silver staining of unbiotinylated hair bundle proteins after SDS-PAGE. The apparent molecular masses of proteins increased slightly after biotinylation. Although the increase in molecular mass due to biotinylation could have led to errors in comparison of bands, we are confident of our assignments because the overall pattern of bands was similar to that observed upon silver staining. The extent of biotinylation could be roughly estimated both by the chemiluminescent intensity of a band and by its shift in molecular mass; in control experiments, each biotin bound to BSA increased the protein's relative molecular mass by ~400 D (data not shown). Applying this value to Fig. 7 *b*, we found that actin's shift in molecular mass resulted from the addition of ~1.5 mol of biotin per mole of actin.

### Topological Localization of Hair Bundle Proteins by Selective Labeling

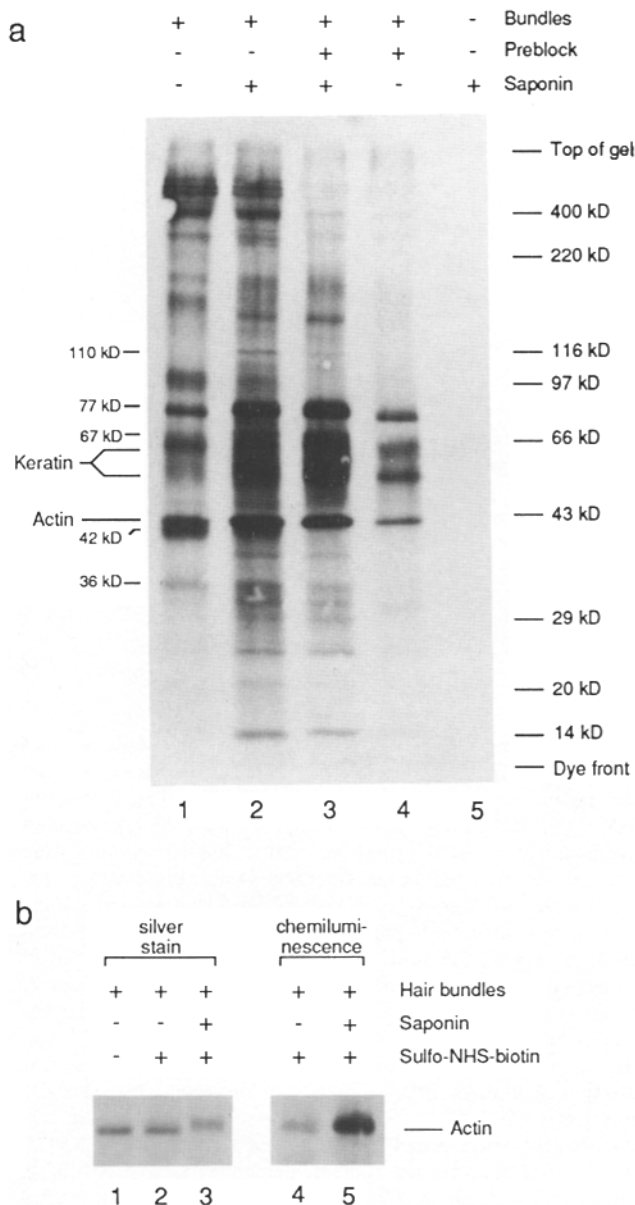
Because the rhodamine-phalloidin labeling experiments suggested that a majority of the isolated stereocilia resealed, we expected to be able by selective labeling to assign the proteins of the preparation to three categories: intracellular, extracellular, and transmembrane. We first sought to label the proteins of hair bundles before isolation. Preisolation labeling was convenient because it eliminated some of the contamination problems that plague silver-staining experiments; any exogenous proteins such as keratin were washed away after labeling, and any contaminants introduced later in the procedure were unlabeled. We initially used membrane-permeant and membrane-impermeant biotinylation reagents, NHS-biotin and sulfo-NHS-biotin, to label bundle proteins (Fig. 6). Unfortunately, NHS-biotin labeled an array of proteins (lane 2) that was similar to that labeled by the membrane-impermeant reagent (lane 3). Most significantly, labeling of the moderately intense band of 44 kD, identified as actin by its migration, was only two- to threefold as great when the membrane-permeant reagent was employed, much less than would be expected. This result suggested that NHS-biotin was not an effective labeling reagent for intracellular proteins.

-	+	+	+	Hair bundles
+	-	-	-	Residual macula
+	+	-	-	NHS-biotin
-	-	+	-	Sulfo-NHS-biotin



**Figure 6.** Labeling of hair bundle proteins with biotinylation reagents. Saccular maculae were isolated, glued to a coverslip with Cell-Tak, and thoroughly washed with pH 8.0 buffer. Biotinylation reagents were dissolved in DMSO (NHS-biotin) or saline solution (sulfo-NHS-biotin) immediately before use, then were added to the chamber containing the sacculi. After a 15-min incubation at room temperature, the chamber was washed with 1 mM lysine in pH 7.4 buffer; the bundles were then isolated with the twist-off method. The sensory epithelium, excluding isolated bundles and connective tissue, was also isolated. (lane 1) Residual sensory epithelium proteins labeled with NHS-biotin before bundle isolation (from 0.5 sacculus). (lane 2) Bundle proteins labeled before isolation with NHS-biotin (two saccular equivalents). (lane 3) Bundle proteins labeled prior to isolation with sulfo-NHS-biotin (two saccular equivalents). (lane 4) Unlabeled bundle proteins (two saccular equivalents). Notice the absence of keratin labeling in these samples. Note also the separation of actin and the diffusely migrating 42-kD protein, which are less clearly resolved on mini-gels (Figs. 7*a*, 8, 9). Lane 4 shows that hair bundles contained insignificant quantities of biotin-bearing proteins. The film was exposed for 1 min.





**Figure 7.** Identification of intracellular, extracellular, and transmembrane hair bundle proteins. (a) Selective labeling of hair bundle proteins. Saccular maculae were isolated, glued to a coverslip with Cell-Tak, and washed with buffer. In some cases (lanes 3 and 4), the externally exposed reactive sites were blocked with 10 mM sulfo-NHS-acetate at room temperature for 20 min (pH 8.0). Bundles were isolated by the twist-off method, then labeled for 15 minutes with sulfo-NHS-biotin (pH 8.0) in the presence or absence of 40 mg/l saponin. (lane 1, extracellular) Unblocked; labeled with sulfo-NHS-biotin after isolation. (lane 2, total) Unblocked; labeled with sulfo-NHS-biotin in the presence of saponin after isolation. (lane 3, intracellular) Blocked with sulfo-NHS-acetate before isolation; labeled with sulfo-NHS-biotin in the presence of saponin after isolation. (lane 4, control) Blocked with sulfo-NHS-acetate before isolation; labeled with sulfo-NHS-biotin after isolation. (lane 5, agarose control) Agarose from twist-off disk labeled with 2.5 mM sulfo-NHS-biotin in the presence of saponin. Lanes 1-4 each contained 0.2 saccular equivalent, while lane 5 had approximately the same amount of agarose (30  $\mu$ g). The migration positions of several polypeptides of interest are indicated on the left of the figure. If the isolation procedure did not entail some disruption of stereocilia and some introduction of keratin, no labeled bands would appear in lane

As an alternate means of selectively labeling intracellular and extracellular proteins, we labeled bundles after isolation with sulfo-NHS-biotin in the presence or absence of 40 mg/l saponin. At this low concentration, saponin forms pores by complexing with cholesterol in membranes (Bangham and Horne, 1962; Glauert et al., 1962), thereby permeabilizing but not solubilizing most cellular membranes. In addition, we blocked reactive groups on the surfaces of some hair bundle preparations by labeling before isolation with a nonbiotinylated reagent, sulfo-NHS-acetate. Isolated bundles blocked with this substance were subsequently labeled with sulfo-NHS-biotin in the presence or absence of saponin.

Those bundle proteins that occur exclusively intracellularly should have been labeled only when the membrane was permeabilized (Fig. 7 a, lanes 2 and 3), regardless of whether the external reactive sites had been blocked. The most prominent of these proteins was actin. Interpretation of the labeling of actin was complicated by a 42-kD extracellular protein that migrated only slightly faster; this band is evident in lanes 1 and 2 of Fig. 7 a, but its labeling was suppressed by sulfo-NHS-acetate in lanes 3 and 4. Lane 4 shows proteins of preblocked hair bundles labeled in the absence of saponin; because the isolation procedure left some stereocilia permeable to small molecules (Fig. 3), internal proteins such as actin and the 77-kD protein were partially labeled.

The labeling of the 42-kD band in lane 4 was completely suppressed by external preblocking, yet the 44-kD band in this lane was of similar intensity to that in lane 1. The band at 44 kD in lane 1 is therefore likely to represent the actin derived from that small fraction of stereocilia disrupted during isolation. In support of this contention, we observed that eight times the exposure was required for the signal of the 44-kD band in lanes 1 and 4 to match that in lanes 2 and 3. Such an increase would be expected for an intracellular protein. Although it is possible that a 44-kD, extracellular protein may be present as well, we believe that there are only two proteins of molecular mass between 42 and 44 kD: the extracellular, glycosylated, 42-kD protein and actin.

4. Because some stereocilia were ruptured, actin and the 77-kD protein were partially labeled. In addition, the bands corresponding to the two major keratin polypeptides are clearly visible in lane 4, and their migration positions are also indicated on the left of the figure. The keratin contaminants partially mask proteins in the 55-65-kD region in lanes 1-3. The film was exposed for 30 s. (b) Increase in labeling of actin band after permeabilization. Hair bundles were isolated and labeled with sulfo-NHS-biotin in the presence or absence of 40 mg/l saponin. Duplicate samples were run on a standard-format gel, which was necessary to adequately separate actin from the 42-kD extracellular protein. One half of the gel was stained with silver (left; two saccular equivalents per lane), while the proteins of the other half were transferred to a charged nylon membrane and were identified with the chemiluminescence detection method (right; 1.5 saccular equivalents per lane). Only the actin regions of the gel and blot are shown. The 42-kD band is sufficiently separated from the actin band that it does not appear in this figure. (lane 1) No labeling. (lanes 2 and 4) Labeled with sulfo-NHS-biotin in absence of detergent. (lanes 3 and 5) Labeled with sulfo-NHS-biotin in the presence of saponin. Lanes 1-3 are from the half of the gel that was stained with silver; lanes 4 and 5 were from the half that was transferred to a charged nylon membrane, incubated with streptavidin-alkaline phosphatase, and detected by AMPPD hydrolysis. The film was exposed for 20 s.

Lane 4 of Fig. 7 *a* also illustrates the problem of contamination; even after extraordinary precautions, some labeled keratin was introduced. In the absence of such precautions, the keratin signal could completely dominate a blot.

Other intracellular proteins were present in the preparation; the most prominent of these was the 77-kD protein. Although it appeared by silver staining to be present at a level less than one-tenth that of actin (Fig. 5), the 77-kD protein was labeled almost as intensely. A variety of other proteins were significantly labeled only after permeabilization, including those at 14, 20, 23, 29, 39, 110, and 150 kD. Some of these proteins were labeled less in lane 3 than in lane 2; because none of these proteins appeared in lane 1, however, the decreased labeling in lane 3 may have been a result of a lower amount of bundle protein applied to this lane. An intracellular protein that migrated with a relative molecular mass of 67 kD was probably fimbrin; a band comigrating with tubulin (55 kD) was difficult to identify among the keratin contaminants, but was seen clearly in experiments other than that of Fig. 7 *a*.

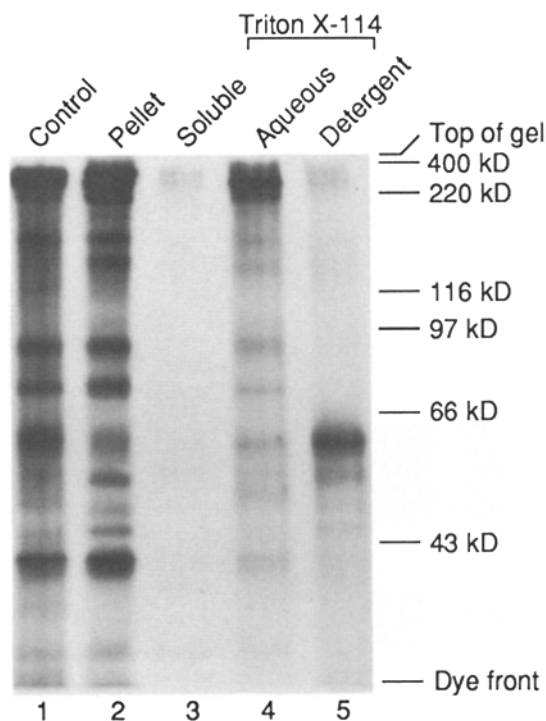
Proteins that are exclusively extracellular, including those in the subtolithic filaments and those on the outer leaflet of the stereociliary plasma membrane, should have been labeled under the conditions of lanes 1 and 2 of Fig. 7 *a* but not those of lanes 3 and 4, in which extracellular reactive sites had been blocked. Most of the proteins that were labeled well in nonpermeabilized bundles were in this class, including those of 42, 95, 161, 265, 337, 424, 445, 494, and 550 kD. Several proteins that were also strongly labeled without permeabilization lay in the region of the gel contaminated by keratin.

Transmembrane proteins should have been labeled under all conditions except those in lane 4 of Fig. 7 *a* (block and no permeabilization); the greatest labeling should have occurred in lane 2 (permeabilization and no block). While no band unequivocally fit these criteria, proteins of molecular mass 36 and 187 kD may have had these properties.

Biotinylation of the isolated bundles in the presence and absence of saponin confirmed the extent of resealing of bundle membranes demonstrated by rhodamine-phalloidin and Ruthenium-red labeling. When biotinylated proteins were examined on standard-format gels that afforded separation of the 42-kD extracellular protein from actin, permeabilization with saponin substantially increased the amount of actin that could be labeled by sulfo-NHS-biotin (Fig. 7 *b*). When the chemiluminescent blot in Fig. 7 *b* was exposed six times as long, the signal of the nonpermeabilized lane matched that of the original exposure of the permeabilized lane. The increase in labeling, however, was not due to different amounts of protein; the silver-stained lanes of Fig. 7 *b* show that the amount of actin in each sample was approximately equal. Note too that the shift in actin's apparent molecular mass occurred only in the sample treated with saponin.

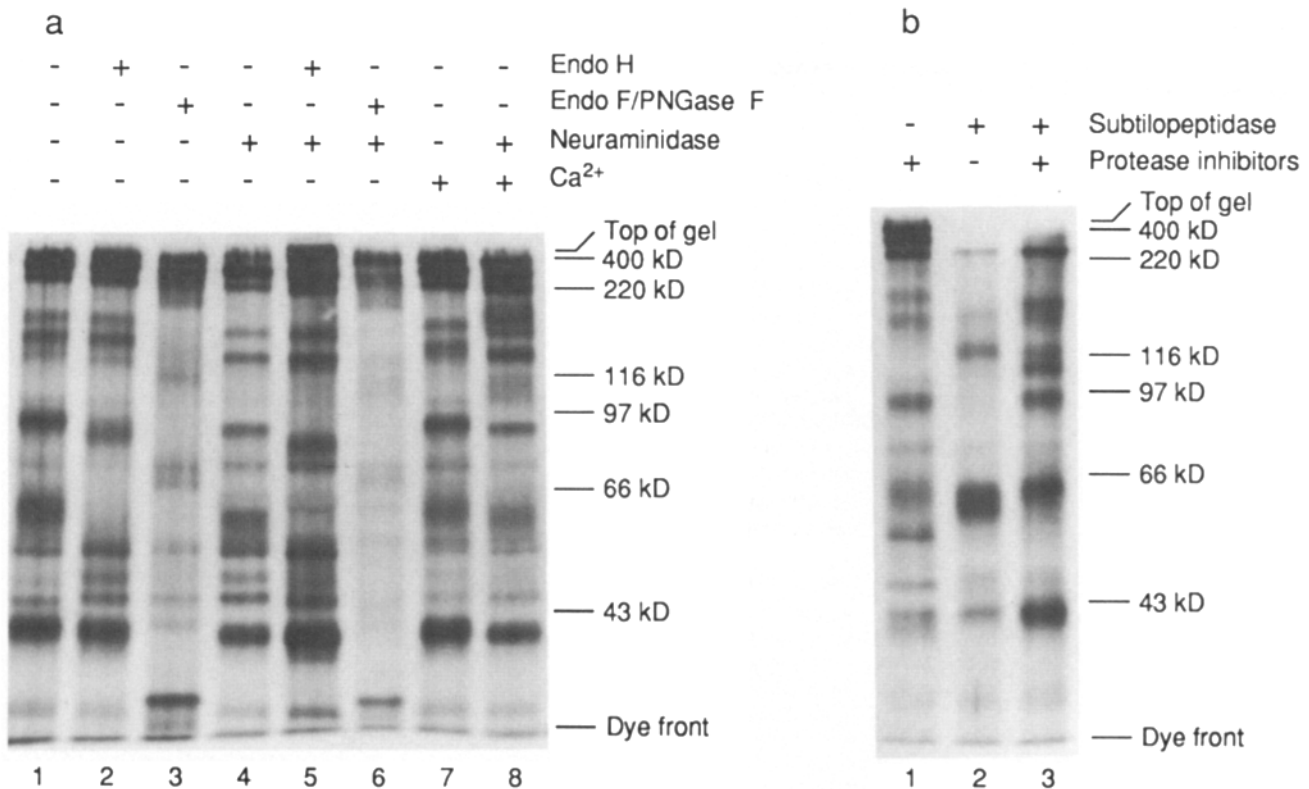
#### Subcellular Fractionation of Bundle Proteins with Triton X-114

The nonionic detergent Triton X-114 has been widely used to identify peripheral and integral membrane proteins by phase separation (Bordier, 1981). We fractionated the hair bundle preparation into four components: a soluble fraction, comprising proteins released after membrane lysis; a cyto-



**Figure 8.** Buffer and detergent extraction of hair-bundle proteins and phase-partitioning with Triton X-114. Before isolation, the saccular maculae were labeled for 15 min at pH 8.0 with 2.5 mM sulfo-NHS-biotin in the presence of 40 mg/l saponin; the hair bundles were then isolated by the twist-off method. Soluble proteins were extracted with lysis buffer, and detergent-soluble proteins were extracted with 1% Triton X-114 in lysis buffer. The detergent-soluble proteins were subjected to phase partitioning. (lane 1, control) Bundles labeled with 2.5 mM sulfo-NHS-biotin in the presence of 40 mg/l saponin before isolation (0.5 saccular equivalent). (lane 2, cytoskeleton and insoluble extracellular material) Proteins insoluble after extraction with lysis buffer and 1% Triton X-114 (0.5 saccular equivalent). (lane 3, soluble) Proteins soluble after permeabilization of bundles in the absence of detergent (two saccular equivalents). (lane 4, aqueous) Detergent-soluble proteins that partition into the Triton X-114 aqueous phase after warming to 34°C (two saccular equivalents). (lane 5, detergent) Detergent-soluble proteins that partition into the Triton X-114 detergent phase after warming to 34°C (two saccular equivalents). The bands seen at 50 and 60 kD in the pellet lane (lane 2) represent contaminating proteins that are occasionally introduced when bundles are labeled before isolation. These bands are not observed in the control lane (lane 1). This film was exposed for 1 min; shorter exposures more clearly show that the 337-kD protein was enriched in the Triton X-114 aqueous phase.

skeletal fraction, consisting of those proteins insoluble after Triton X-114 extraction; the aqueous Triton X-114 phase, containing peripheral membrane proteins; and the detergent Triton X-114 phase, containing integral membrane proteins (Fig. 8). Few soluble proteins were released from the preparation after freezing and thawing; longer exposures of the blot of Fig. 8 revealed, however, that several proteins were found specifically in this fraction. Additional soluble proteins apparently ran at the dye front in this 7.5% gel (data not shown). Although a larger number of proteins were extracted with Triton X-114, the great majority of the bundle



**Figure 9.** Endoglycosidase, neuraminidase, and subtilopectidase treatment of extracellularly exposed hair bundle proteins. Saccular maculae were labeled with 2.5 mM sulfo-NHS-biotin before bundle isolation. (a) Endoglycosidase and neuraminidase treatment. After twist-off isolation, agarose gel blocks containing hair bundles were placed into 20  $\mu$ l of the indicated enzymes and were incubated at 34°C for 16 h in the presence of 1 mM PMSF, 5  $\mu$ M leupeptin, and 5  $\mu$ M pepstatin. The reactions were terminated by heating the samples to 65°C for 20 min in SDS-PAGE sample buffer. (lane 1, EDTA control) No enzyme; MES-EDTA buffer. (lane 2, endo H) 0.02 U of endoglycosidase H; MES-EDTA buffer. (lane 3, endo F) 0.40 U of endo F/PNGase F; MES-EDTA buffer. (lane 4, neuraminidase) 0.14 U of neuraminidase; MES-EDTA buffer. (lane 5, endo H and neuraminidase) 0.02 U of endoglycosidase H and 0.14 U of neuraminidase; MES-EDTA buffer. (lane 6, endo F and neuraminidase) 0.40 U of endo F/PNGase F and 0.14 U of neuraminidase; MES-EDTA buffer. (lane 7, calcium control) No enzyme; MES-Ca buffer. (lane 8, neuraminidase) 0.14 U of neuraminidase; MES-Ca buffer. Each lane contained 0.5 saccular equivalent. In this blot, the diffuse, 42-kD protein is well labeled; because it is digested by endo F, it is clearly not actin. The film was exposed for 30 s. (b) Protease treatment. After labeling, tissue was digested for 40 min with 50 mg/l subtilopectidase BPN'. Bundles were then isolated by the twist-off method. (lane 1) Control (no enzyme). (lane 2) Subtilopectidase treatment in the absence of protease inhibitors. (lane 3) Subtilopectidase BPN' treatment in the presence of 200  $\mu$ M PMSF, 1  $\mu$ M leupeptin, and 1  $\mu$ M pepstatin. Each lane contained 0.5 saccular equivalent. The film was exposed for 1 min.

proteins were found in the cytoskeletal fraction, which should also have included insoluble extracellular proteins trapped in the agarose gel. Of the proteins extracted by detergent, the 337-kD protein was enriched in the aqueous phase and a 62-kD protein in the detergent phase. Both proteins were labeled with biotin from the outside (Figs. 6 and 7 a and data not shown). The 62-kD protein may be a transmembrane protein; the 337-kD protein is probably a membrane-associated, extracellular protein.

Extractions at a high pH (Steck and Yu, 1973) or high salt concentration (Fairbanks et al., 1971) are often used to strip peripheral membrane proteins from membrane fractions. When isolated, labeled hair bundles were frozen and thawed to permeabilize the membranes, then treated with carbonate buffer at pH 11 or with 1 M NaCl, no proteins were quantitatively extracted from the pellet into the supernatant. Polypeptides of 42, 95, 161, 187, 265, 337 kD, as well as several larger proteins, were partially extracted. Other proteins, in-

cluding those of 36, 44, 62, and 77 kD, were extracted to a significantly lesser degree (data not shown).

#### **Glycosidase and Protease Treatment of Extracellularly Exposed Bundle Proteins**

Because stereocilia are enveloped by a glycocalyx (Hirokawa and Tilney, 1982; Santi and Anderson, 1987; Jacobs and Hudspeth, 1990), and because agarose gel-entrapped extracellular material may include glycoproteins, many of the extracellularly exposed proteins in our preparation may be extensively glycosylated. We therefore probed the glycosidation of the proteins in twist-off samples by digesting extracellularly labeled hair bundles with endo H, with a mixture of endo F and PNGase F, or with neuraminidase (Fig. 9 a).

Endo H, which liberates asparagine-linked, high-mannose oligosaccharides from glycoproteins (Trimble and Maley, 1984), reduced the molecular masses of several extracellu-

Table I. Properties of Identified Hair Bundle Proteins

Molecular mass*	In peripheral gelatinous matrix?‡	Inferred subcellular location§	Triton X-114 fraction	Glycosidase sensitivity¶	Possible identity**
kD					
550	yes	extracellular			
494	yes	extracellular			
445	yes	extracellular			
424	yes	extracellular			
337	yes	extracellular	aqueous	F, N	
265	yes	extracellular		F, N	
187	yes	transmembrane?	pellet	H, F, N	
161	yes	extracellular	pellet	H, F, N	
150	no	intracellular			
110	no	intracellular			
95	yes	extracellular	pellet	H, F, N	
77	no	intracellular	pellet		
67	no	intracellular			fimbrin
62	?	transmembrane?	detergent	H, F, N	
55	?	intracellular	pellet		tubulin
44	?	intracellular	pellet		actin
42	yes	extracellular	pellet	H, F, N	
36	yes	transmembrane?	pellet		
29	no	intracellular			calbindin
23	no	intracellular			
19	no	intracellular			calmodulin
16	no				
15	no				
14	no				

\* Estimated from silver-stained gels.

‡ A "yes" entry indicates that comigrating bands are present in hair bundle and peripheral-gelatinous-matrix fractions.

§ Based primarily on labeling of isolated bundles, with and without preblocking with sulfo-NHS-acetate, with and without saponin permeabilization. Characteristics of the three labels are as follows: (*intracellular*) labeled only in the presence of saponin, regardless of whether bundles are preblocked; (*extracellular*) labeled in presence or absence of saponin only when bundles are not preblocked; and (*transmembrane*) labeled in the presence or absence of saponin if bundles are not preblocked, and labeled in the presence of saponin if bundles are preblocked. Labeling of transmembrane proteins is the greatest in unblocked bundles in presence of saponin. Blanks indicate uncertain assignment.

|| Triton X-114 fractions are: (*pellet*) proteins insoluble in Triton X-114; (*soluble*) proteins released after freezing and thawing in absence of Triton; (*aqueous*) Triton X-114-soluble proteins that partition to the aqueous phase after phase separation; and (*detergent*) Triton X-114-soluble proteins that partition to the detergent phase after phase separation. Blanks indicate uncertain assignment.

¶ (H) Endo H-sensitive; (F) endo F/PNGase F-sensitive; (N) neuraminidase-sensitive.

\*\* An entry is provided only for those proteins previously localized to hair bundles by immunochemical or biochemical criteria. Unidentified proteins may comigrate with the identified proteins. Assignments are based primarily on molecular mass; assignment is also based on Triton X-114 fractionation, subcellular localization, glycosidase sensitivity, and immunoreactivity.

larly exposed hair bundle proteins. Endo H slightly lowered the molecular masses of bands of 95, 161, and 187 kD. The band at 42 kD, which migrated just slower than actin, displayed a slight decrease in apparent molecular mass; endo H more extensively increased the migration of a diffuse band at 62 kD.

Digestion with a mixture of endo F, which cleaves asparagine-linked high-mannose and some hybrid oligosaccharides (Elder and Alexander, 1982), and PNGase F, which removes most asparagine-linked oligosaccharides (Tarentino et al., 1985), led to a more profound reduction in mass of nearly all of the extracellularly exposed bundle proteins. A notable exception was the 337-kD protein. Control experiments with a variety of proteins demonstrated that, with the high concentration of protease inhibitors used, the endo F/PNGase F mixture had little or no protease activity. The dramatic reduction in molecular mass of most of the extracellularly exposed proteins of the bundle preparation therefore indicates that they are extensively glycosylated.

Neuraminidase, which removes terminal sialic acid residues, was used to digest bundle proteins in the presence or absence of  $Ca^{2+}$ , which is capable of stimulating the enzyme 5–10-fold (Ada et al., 1961). The pattern of digestion nonetheless appeared to be similar whether or not  $Ca^{2+}$  was present (compare lanes 4 and 8). Several proteins were reduced in apparent molecular mass, most apparently by no more than 10%, including those at 62, 95, 161, 187, 265, and 337 kD. Combinations of neuraminidase and endo H or endo F/PNGase F appeared to have simple, additive effects; no additional endo H or endo F sites were revealed by neuraminidase treatment.

A bacterial protease, subtiloepitidase BPN<sup>1</sup>, has been employed in electrophysiological and anatomical studies to detach the otolithic membrane from hair bundles (Howard and Hudspeth, 1987; Jacobs and Hudspeth, 1990). Enzymatic digestion has no known effect on mechano-electrical transduction, but it degrades the stereociliary glycocalyx and the subotolithic filaments (Jacobs and Hudspeth, 1990). We

therefore labeled saccular maculae with sulfo-NHS-biotin in the absence of detergent, then digested with subtilopectidase BPN'. After bundle isolation by the twist-off procedure, the proteins were separated by SDS-PAGE (Fig. 9 b). Many proteins were digested by the enzyme; by contrast, the 265-kD protein appeared to be resistant, as did the 36-kD, putatively transmembrane protein.

## Discussion

The purification and biochemical characterization of hair bundle proteins is a daunting task because of the limited amount of starting material. The bullfrog's sacculus has only  $\sim 10^3$  hair bundles, each bearing merely  $\sim 10^2$  transduction channels or tip links. By contrast, a vertebrate retina can contain as many as  $10^8$  photoreceptors, and a photoreceptor can hold over  $10^9$  molecules of rhodopsin. Studies of the acetylcholine receptor (Raftery et al., 1980) and the sodium channel (Miller et al., 1983) benefitted from the use of animals whose specialized organs provided large amounts of starting material. Although it would be valuable to identify a readily available species with a large number of hair bundles, no animal has yet been found with as many as  $10^6$  bundles per ear. Perhaps the most promising sources are salt-water fishes, such as rays (Barber and Emerson, 1980), sharks (Corwin, 1981), and catfishes (Popper and Tavolga, 1981), which have tens or hundreds of times as many hair bundles as amphibians. Lacking a more abundant source of hair bundles, we have focused for the present on improved isolation and optimized detection techniques.

### Twist-off Isolation of Hair Bundles

The twist-off method is an efficient, clean, and gentle technique for the preparation of frog hair bundles. On the basis of measurements of bundle dimensions (Jacobs and Hudspeth, 1990), which imply a bundle mass of  $\sim 0.1 \mu\text{g}$ , and of the macula's wet mass of  $500 \pm 100 \mu\text{g}$  (mean  $\pm$  SD,  $n = 6$ ), the isolation method is calculated to effect a 5,000-fold purification in a single step. Because the bundles can easily be observed in the dissecting microscope after isolation, we can readily quantify their recovery. In a typical preparation,  $\sim 90\%$  of the bundles are left embedded in the gel; recovery of 100% of the bundles is not uncommon. The final preparation is very clean: soluble proteins are washed away after isolation, and contaminating cellular debris can be dissected away with a tungsten needle.

Using three independent methods, we demonstrated that  $>80\%$  of the stereocilia reseal after isolation. Most dramatically, detergent permeabilization increases by at least five-fold the amount of actin labeled by rhodamine-phalloidin (Fig. 3) or biotinylation (Fig. 7 b). In addition, when the isolated bundles are incubated with Ruthenium red and subsequently examined in the electron microscope, the tracer is never found inside the stereocilia. In intracellular recordings from a hair cell, no voltage transient is observed when the stereocilia are mechanically sheared off (A. J. Hudspeth, unpublished observation); the apical surface of a hair cell must therefore reseal immediately. If the isolated stereociliary membranes reseal as rapidly, little soluble protein may be lost from the preparation. Although no major proteins of the preparation appear to be soluble (Fig. 8), we suspect that this

is due to their relatively low native abundance in the stereocilia, rather than to their selective loss.

We believe that our preparation has significant advantages over the bundle-blot method (Shepherd et al., 1989). With that technique, the yield and purity of hair bundles cannot be routinely assayed, as they can through the dissecting microscope in our preparation, and any soluble proteins leaking from damaged cells or released as the bundles are isolated will also adhere to the nitrocellulose membrane. In the twist-off preparation, such soluble proteins are washed away during superfusion. In addition, the integrity of the hair bundle membrane after the twist-off isolation allows selective labeling and could be useful for ion-flux and electrophysiological studies of isolated stereocilia. The demonstration that hair cells retain mechano-electrical transduction after a heat treatment similar to the one used in the isolation procedure indicates that such studies are plausible.

We recognize that the twist-off method also has several drawbacks. Despite a number of efforts, we have not been able to extract the bundles from the agarose gels without heating to over  $60^\circ\text{C}$ . Agarose gels that melt at less than  $40^\circ\text{C}$  unfortunately lack sufficient gel strength for efficient shearing of the bundles. Agarose in the gel disk may prove troublesome if trace contaminants inhibit bundle enzymes, as can occur with other enzymes (Sambrook et al., 1989). Although transduction remains intact, it is also possible that our  $34^\circ\text{C}$  treatment of the macula leads to proteolysis. We note, however, that plasma membranes in the bundles are intact during this warming step. At the time when proteases might be released, the twist-off step, the bundles are cooled to  $4^\circ\text{C}$  and are bathed in protease inhibitors. The demonstrated lack of degradation of fimbrin provides direct evidence that proteolysis is not a significant problem in our procedure. Another drawback to the preparation is revealed by EM: specific contaminants occur in the preparation. The agarose gel traps not only some of the microvilli found on hair and supporting cells, but also the subotolithic filaments that extend from the apical surface of the supporting cells to the overlying, compact otolithic membrane (Jacobs and Hudspeth, 1990). We suspect that the proteins of hair bundle preparations that comigrate with the proteins of the peripheral gelatinous matrix represent components of these strands.

The twist-off method can be applied to other auditory and vestibular organs and to other species. In preliminary experiments, we have isolated hair bundles from the utriculus of fresh-water catfish and have begun characterization of their constituent proteins. While the bundle recoveries are more variable than those from the bullfrog, further refinement of the technique may lead to more consistent results.

### Detection of Proteins by Biotinylation and Chemiluminescence

The bundle preparation described here yields  $<0.04 \mu\text{g}$  of protein per frog sacculus; by comparison, a single chicken intestine yields 5,000–10,000  $\mu\text{g}$  of microvillar protein (Bretscher and Weber, 1978), a single bovine retina yields at least 400  $\mu\text{g}$  of photoreceptor protein (Papermaster, 1982), and a bullfrog olfactory epithelium yields  $\sim 200 \mu\text{g}$  of olfactory ciliary protein (Anholt et al., 1986). We have circumvented the difficulty in working with this small amount of protein by developing a sensitive detection

method. With a detection limit of <500 fg (8 amol) of BSA, this chemiluminescence detection method is 1,000-fold as sensitive as commonly used silver-staining techniques (Merril et al., 1981; Merrill, 1990), and 10–100-fold as sensitive as the most sensitive silver stains (Merril, 1990) and colorimetric assays. The biotinylation and chemiluminescence detection system therefore allows a more extensive characterization of hair bundle proteins than would be possible with conventional techniques, such as silver staining.

Enhancement of the detection method's sensitivity increases the difficulties due to contamination. We found that the signal due to small amounts of hair bundle protein can be overwhelmed by that due to ubiquitous human skin keratin. By using stringent conditions when labeling after isolation, or by labeling before isolation so that labeled contaminants can be washed away, we reduced the interference by keratin to an acceptable level.

### **Proteins of the Hair Bundle**

On the basis of their relative molecular masses and biochemical fractionation (Figs. 7 and 8), several proteins previously identified in hair bundles by cytochemical and biochemical techniques appear to be present in our preparation (Table I). Although strict confirmation of their identities awaits quantitative immunoblotting or immunoprecipitation, proteins in our preparation exhibit the same molecular masses as calmodulin, calbindin, actin, tubulin, and fimbrin. Under the low-Ca<sup>2+</sup> conditions in which the electrophoresis for Figs. 5 and 7 *a* was performed, calmodulin and calbindin, respectively, should exhibit apparent molecular masses near 19 kD (Glenney and Glenney, 1985) and 29 kD (Oberholtzer et al., 1988); proteins of these molecular masses are indeed present in the hair bundle preparation. If fimbrin is the major cross-linking protein of the stereociliary actin core (Flock et al., 1982; Slepecky and Chamberlain, 1985; Drenckhahn et al., 1985; Tilney et al., 1989; Shepherd et al., 1989), one would expect it to be present at a high concentration. We were surprised that the 67-kD protein, identified by immunoblotting as fimbrin, appears to be present at much less than one-tenth the amount of actin (Fig. 5). The signal may be low because fimbrin binds silver poorly, because the frog's stereociliary microfilaments are not extensively cross-linked, or because other cross-linking proteins are also present.

We identified several novel proteins that are likely constituents of the hair bundle (Table I). As judged by Triton X-114 extraction, a 62-kD glycoprotein appears to be the predominant integral membrane protein without a strong cytoskeletal attachment. Because this protein migrates as a diffuse band near the upper band of the keratin artifact, it is difficult to determine whether its labeling increases after membrane permeabilization, and thus whether it is a transmembrane protein. The only other protein that is likely to be a transmembrane protein is the 36-kD polypeptide, which is labeled well in the presence or absence of permeabilization. Although the 187-kD protein might also be a transmembrane protein, it shares many characteristics with the extracellular proteins thought to originate in the subtolithic filaments, and is therefore unlikely to traverse the membrane. Another polypeptide that shares characteristics with the proposed subtolithic-filament proteins is that of 337 kD. Because it is readily extracted with Triton X-114 and partitions into the

aqueous phase, however, this protein is more likely associated with the extracellular leaflet of the stereociliary membrane. Although the 265-kD protein of the frog's sacculus might be related to the detergent-soluble, 275-kD protein that occurs on the chicken's stereociliary plasma membranes (Richardson et al., 1990), the results of Triton X-114 extraction suggest otherwise. One of the most striking components of the preparation is a 77-kD protein, which is so highly labeled by sulfo-NHS-biotin that its intensity on chemiluminescent blots approaches that of actin. By silver staining, however, it appears to be present at one-tenth or less the concentration of actin. The high degree of biotin labeling suggests that this protein has an abundance of lysyl side-chains exposed on its surface. The 77-kD protein is apparently an intracellular protein that is associated with the cytoskeleton; definition of its function awaits further study.

Although we cannot exclude their presence on the basis of silver staining and biotin labeling, three proteins of the cuticular plate are not prominent in our preparations. These include  $\alpha$ -actinin (~105 kD; Drenckhahn et al., 1982; Slepecky and Chamberlain, 1985), spectrin/fodrin (~240 kD; Drenckhahn et al., 1985; Scarfone et al., 1988), and a protein of molecular mass ~400 kD (Tilney et al., 1989). In support of our light- and electron microscopic observations, the apparent absence of these proteins provides evidence that the twist-off procedure cleanly separates hair bundles from the apical portions of hair cells.

The structural and developmental similarity of stereocilia to microvilli (Hirokawa and Tilney, 1982; Tilney and DeRosier, 1986) prompts a comparison of the major proteins of the twist-off preparation with those of brush-border microvillar preparations. Protein bands in the hair bundle preparation exhibit the same apparent molecular masses as calmodulin, actin, and fimbrin, all major components of the microvillus. Immunocytochemical evidence (Flock et al., 1982; Drenckhahn et al., 1982) suggests that hair bundles lack villin (Bretscher and Weber, 1980); in our preparation, the 95-kD glycoprotein makes it impossible to ascertain whether villin is present. We observe a protein of 110 kD, the same molecular mass as brush-border myosin I (Howe and Mooseker, 1983). Because Shepherd et al. (1989) did not note such a band, it is possible that the 110-kD protein in twist-off preparations is derived from microvilli contaminating the hair bundle sample (Fig. 4). If that is the case, these microvilli must reseal, for the 110-kD protein of the bundle preparation is labeled only after saponin permeabilization (Fig. 7 *a*). A myosin I would be a candidate to mediate mechanical adaptation in the hair bundle (Howard and Hudspeth, 1987; Assad et al., 1989). Our preliminary experiments with [<sup>32</sup>P]-8-azido-ATP labeling, ATP-dependent cytoskeleton binding, and immunoblot analysis have as yet failed to confirm the 110-kD protein as a myosin I (D. Raizen, P. G. Gillespie, and A. J. Hudspeth, unpublished data). Thus, while the hair bundles appear to contain several microvillar proteins, others are absent; in addition, the bundle contains many nonmicrovillar polypeptides.

Although the hair bundle's protein complement is unique, we suspect that some of the preparation's proteins, including those of 95, 161, 187, 265, 424, 445, 494, and 550 kD, derive from the subtolithic filaments attached to supporting cells. The 42-kD protein may also be derived from the subtolithic filaments. These proteins share several characteristics. All

are labeled well in the absence of detergent but poorly if external sites are blocked before labeling (Fig. 7 a). Glycosidase digestion indicates that each of these polypeptides is heavily glycosylated; with the notable exception of the 265-kD protein, each is readily proteolyzed by extracellular application of subtiloepitidase BPN' (Fig. 9). Finally, all of these proteins are extracted from the agarose-gel blocks at high pH or in high-salt conditions, which suggests that they are less tightly associated with the agarose gel than are actin and other cytoskeletal proteins. These results together indicate that these polypeptides form a distinct class and that they are probably components of the subtotolithic filaments.

### Potential Isolation of Transduction Elements

Although fura-2 measurements of Ca<sup>2+</sup> accumulation have been interpreted as situating mechano-electrical transduction channels at the bases of the stereocilia (Ohmori, 1988), recent evidence supports the earlier contention (Hudspeth, 1982) that the channels occur at the tip of the bundle. Studies with the Ca<sup>2+</sup> indicator fluo-3 (Huang, P. L., and D. P. Corey. 1990. *Biophys. J.* 57:530a) suggest that Ca<sup>2+</sup> flows through transduction channels into the tips of stereocilia. Moreover, transduction is most sensitive to focal ion-trophoretic application of channel blockers when they are applied precisely at the stereociliary tips (Jaramillo, F., and A. J. Hudspeth. 1991. *Biophys. J.* 59:184a). We therefore expect that the transduction apparatus is present in our isolated hair bundle preparation.

Although the transduction channels and the tip links are exposed extracellularly and thus are likely to be labeled with sulfo-NHS-biotin, the small number of these components, no more than a few hundred per hair bundle, makes their detection difficult. The biotinylation and chemiluminescence detection scheme presented here may nevertheless have sufficient sensitivity to detect these components. Suppose that the channel consists of a single protein whose mass exceeds 100 kD; such a molecule may be derivatized with 10 or more biotin moieties (Guesdon et al., 1979). If the average hair bundle has 100 transduction channels (Holton and Hudspeth, 1986; Howard and Hudspeth, 1988), the ~3,000 hair bundles of a single frog's sacculus (Lewis and Li, 1973; Jacobs and Hudspeth, 1990) contain 300,000 molecules of channel protein, or ~0.5 amol. At the current detection limit of 75 amol of biotin, proteins of this low abundance could be detected with as few as 15 sacculi. If the background associated with the chemiluminescence detection method could be further diminished, the minimum detectable amount of protein could be reduced to a tiny fraction of that in this study.

The ability to detect transduction channels and tip links is not sufficient to ensure their isolation; methods must also be devised to identify these molecules. Because no toxins or high-affinity ligands have been found for the mechano-electrical transduction channel, alternative approaches may be necessary. Molecular-genetic and immunological approaches may prove fruitful. To obtain more extensive biochemical information about hair bundle proteins, sensitive methods must ultimately be applied to a more abundant source of hair bundles.

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