

# Characterization of the Immature Secretory Granule, an Intermediate in Granule Biogenesis

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**Abstract.** The events in the biogenesis of secretory granules after the budding of a dense-cored vesicle from the *trans*-Golgi network (TGN) were investigated in the neuroendocrine cell line PC12, using sulfate-labeled secretogranin II as a marker. The TGN-derived dense-cored vesicles, which we refer to as immature secretory granules, were found to be obligatory organellar intermediates in the biogenesis of the mature secretory granules which accumulate in the cell. Immature secretory granules were converted to mature secretory granules with a half-time of  $\approx 45$  min. This conversion entailed an increase in their size, implying that the maturation of secretory granules includes a fusion event involving immature secretory granules. Pulse-chase labeling of PC12 cells followed by stimulation with high

K<sup>+</sup>, which causes the release of secretogranin II, showed that not only mature, but also immature secretory granules were capable of undergoing regulated exocytosis. The kinetics of secretion of secretogranin II, as well as those of a constitutively secreted heparan sulfate proteoglycan, were reduced by treatment of PC12 cells with nocodazole, suggesting that both secretory granules and constitutive secretory vesicles are transported to the plasma membrane along microtubules. Our results imply that certain membrane proteins, e.g., those involved in the fusion of post-TGN vesicles with the plasma membrane, are sorted upon exit from the TGN, whereas other membrane proteins, e.g., those involved in the interaction of post-TGN vesicles with the cytoskeleton, may not be sorted.

THE regulated pathway of secretion begins with the formation of a dense-cored vesicle from the TGN and ends with the fusion of the secretory granule with the plasma membrane (for review see Burgess and Kelly, 1987). We have previously identified, by biochemical criteria, the first vesicular product of the regulated secretory pathway, the TGN-derived dense core vesicle, in the neuroendocrine cell line PC12 (Tooze and Huttner, 1990). The experimental approach involved pulse-labeling secretogranin II (SgII), a secretory protein specific for neuroendocrine secretory granules (Rosa et al., 1985), with radioactive sulfate, a process known to selectively occur in the TGN (Bauerle and Huttner, 1987), and following the fate of the labeled SgII. Within 15 min of chase, virtually all the labeled SgII was recovered in dense-cored vesicles which were distinct from the TGN by various criteria, including the lack of TGN marker enzymes. These rapidly formed, TGN-derived dense-cored vesicles have been referred to as immature secretory granules (Tooze and Huttner, 1990).

Little is known about which of the characteristic properties of the final, mature secretory granule are already present in the immature secretory granule. Characteristic features of

mature secretory granules include a specific composition of secretory and membrane proteins and the ability to fuse with the plasma membrane in response to an appropriate stimulus (for review see Burgess and Kelly, 1987). Whereas it is known that secretory proteins are sorted into immature secretory granules upon their exit from the TGN (Tooze et al., 1987; Orci et al., 1987; Tooze and Huttner, 1990), it is unclear when secretory granules acquire their final membrane protein composition. It is also not known whether the ability for regulated exocytosis is specific for mature, as opposed to immature, secretory granules.

In PC12 cells, the appearance of sulfate-labeled SgII in immature secretory granules is sufficiently synchronous to allow us to study, using chase times longer than 15 min, their relationship to mature secretory granules. Employing this approach, we describe here the conversion of immature secretory granules to mature secretory granules and compare several of their properties.

## Materials and Methods

### Cells and Reagents

PC12 cells were cultured in growth medium (DME, 10% horse serum, 5% FCS) and passaged as previously described (Tooze and Huttner, 1990). Nocodazole was obtained from Sigma (Munich, Germany) and solubilized in DMSO as a 10-mM stock solution, and stored at  $-20^{\circ}\text{C}$ . Carrier-free

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1. *Abbreviations used in this paper:* hsPG, heparan sulfate proteoglycan; SgII, secretograinin II; TGN, *trans*-Golgi network.

[<sup>35</sup>S]sulfate, [7,8-<sup>3</sup>H]norepinephrine, and [<sup>125</sup>I]protein A were purchased from Amersham Buchler (Braunschweig, Germany).

## Radiolabeling and Pulse-Chase Analysis of PC12 Cells

**Labeling with [<sup>3</sup>H]norepinephrine.** PC12 cells, grown in 15-cm dishes, were labeled for 16 h with 25  $\mu$ Ci [7,8-<sup>3</sup>H]norepinephrine per dish in growth medium. A post-nuclear supernatant was prepared from the cells and subjected to velocity sucrose gradient centrifugation (see below). 100- $\mu$ l aliquots of the fractions (1 ml each) collected from the velocity gradient were subjected to liquid scintillation counting.

**Pulse-Chase Labeling with [<sup>35</sup>S]sulfate.** PC12 cells were pulse labeled with [<sup>35</sup>S]sulfate and chased at 37°C as described previously (Tooze and Huttner, 1990; see figure legends for details), except that the media contained an additional 10 mM Hepes, pH 7.2, and were supplemented with 0.1% horse serum and 0.05% FCS.

**Stimulation of Cells.** After the indicated pulse labeling, or after the chase periods, the labeled PC12 cells were washed once with medium containing 5 mM K<sup>+</sup> (127 mM NaCl, 5 mM KCl, 2.2 mM CaCl<sub>2</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 5.6 mM Glucose, 10 mM Hepes, pH 7.4) (Rosa et al., 1985). In the experiments shown in Figs. 8 and 9, the cells were then incubated for 5 min at 37°C with rocking in medium containing 5 mM K<sup>+</sup>, and the wash was discarded. In all stimulation experiments, the cells were then treated either with medium containing 5 mM K<sup>+</sup> (control) or, for stimulation, with medium containing 55 mM K<sup>+</sup> (77 mM NaCl, 55 mM KCl, 2.2 mM CaCl<sub>2</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 5.6 mM Glucose, 10 mM Hepes, pH 7.4) (Rosa et al., 1985) for 10–20 min at 37°C, as indicated.

**Processing of Samples.** Except for experiments involving velocity and equilibrium sucrose gradient centrifugation (see below), PC12 cells, after labeling, chase, and stimulation, were transferred to ice, and the medium was removed and kept at 4°C. The cells were washed three times with calcium- and magnesium free-PBS at 4°C, and removed from the dish either manually by scraping (Tooze and Huttner, 1990) or with TNTE buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% Tween-20, 5 mM EDTA, 0.5 mM PMSF). The cell lysates were boiled for 3 min, centrifuged for 5 min in an Eppendorf microfuge at room temperature, and the heat-stable supernatants were collected. The labeling, chase, and stimulation media were centrifuged for 5 min in an Eppendorf microfuge at 4°C. Aliquots of the heat-stable supernatants and media were either subjected to acetone precipitation, SDS-PAGE, and fluorography as previously described (Tooze and Huttner, 1990) or to immunoprecipitation as described below. Although not shown, the heat-stable supernatants derived from the cell lysates, which contain virtually all of the SgII present in the cell (Rosa et al., 1985), were always analyzed to check the overall validity of the experiments.

**Immunoprecipitation of [<sup>35</sup>S]sulfate labeled SgII.** Equal aliquots (300  $\mu$ l) of either heat-stable supernatants from cell lysates, chase media, and stimulation media were adjusted to a final concentration of 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 0.5 mM PMSF, incubated for 15 min at 4°C, and centrifuged for 5 min at 4°C in an Eppendorf microfuge. The supernatant was incubated for 120 min at 4°C with 5  $\mu$ l of an antiserum raised against SgII from PC12 cells (Rosa et al., 1985), after which 100  $\mu$ l of a 10% (wt/vol) suspension of protein-A Sepharose, swollen and washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA), was added, and the mixture was incubated with rocking for 60 min at room temperature. The immunocomplexes were pelleted, and washed three times with wash buffer by centrifugation at room temperature at 1,000 rpm in an Eppendorf microfuge, followed by resuspension. After the final wash, the immunocomplexes were washed once in 10 mM Tris-HCl, pH 7.4, solubilized in 150  $\mu$ l of SDS-PAGE sample buffer, and analyzed by SDS-PAGE followed by fluorography.

## Velocity and Equilibrium Sucrose Gradient Centrifugation

After pulse-chase labeling with [<sup>35</sup>S]sulfate or after [<sup>3</sup>H]norepinephrine labeling of PC12 cells as described above, a postnuclear supernatant was prepared as previously described (Tooze and Huttner, 1990). The postnuclear supernatant was applied to a sucrose gradient (0.3 M to 1.2 M) and centrifuged for 15 min as described (Tooze and Huttner, 1990) (velocity sucrose gradient centrifugation). Fractions (1 ml each) were collected from the top of the velocity gradient and an equal aliquot of each was analyzed after acetone precipitation by SDS-PAGE and fluorography. The fractions containing the immature secretory granules (fractions 1–4) or the mature

secretory granules (fractions 4–6) were collected, pooled, applied to a second sucrose gradient (0.5–2.0 M), and centrifuged for 5 h as described (Tooze and Huttner, 1990) (equilibrium sucrose gradient centrifugation). Fractions (1 ml each) were collected from the top of the equilibrium gradient and an equal aliquot of each was analysed after acetone precipitation by SDS-PAGE and fluorography.

In the experiments in which the equilibrium density of immature and mature secretory granules was determined, the postnuclear supernatant was prepared from PC12 cells [<sup>35</sup>S]sulfate-labeled for 10 min and chased for 30 min or labeled for 6 h and chased for 12 h, and directly subjected to centrifugation at 100,000 g for 3 h on a sucrose gradient ranging from 0.6 to 2.0 M, followed by analysis as above.

## Immunoblotting of SgII

Aliquots of the fractions collected from the velocity gradient were subjected to acetone precipitation followed by SDS-PAGE and immunoblotting using a rabbit antiserum (kindly provided by Dr. H.-H. Gerdes, European Molecular Biology Laboratory [EMBL], Heidelberg, Germany) raised against a peptide corresponding to the amino terminal sequence of SgII (Gerdes et al., 1989). SgII immunoreactivity was revealed using [<sup>125</sup>I]protein A and quantitated by  $\gamma$ -counting of the relevant nitrocellulose pieces.

## Analytical Differential Centrifugation

After pulse-chase labeling of PC12 with [<sup>35</sup>S]sulfate cells as described above, a post-nuclear supernatant was prepared as previously described (Tooze and Huttner, 1990). The postnuclear supernatant was diluted 1:10 with centrifugation medium (0.25 M sucrose containing 1 mM EDTA and 1 mM Tris-HCl, pH 7.4; density = 1.034 g/cm<sup>3</sup>) and subjected to analytical differential centrifugation at 4°C essentially as described (Slinde and Flatmark, 1973), using a TLS-55 rotor and polycarbonate tubes (1.1 cm outer diameter, 3.4 cm length,  $r_{\max}$  = 7.52 cm,  $r_{\min}$  = 5.35 cm) in a Beckman TL-100 ultracentrifuge at revolutions per minute values ranging from 5,000–35,000 for times ranging from 10–35 min, with the brake off. After centrifugation, the pellets were subjected to SDS-PAGE and fluorography, and [<sup>35</sup>S]sulfate-labeled SgII was quantitated as described below.

The “centrifugal effect”

$$\int_0^t \text{rpm}^2 dt \ (\times 10^{-9}) \ \text{min}^{-1}$$

was calculated as described (Slinde and Flatmark, 1973). For each individual condition of centrifugation, the fraction of the [<sup>35</sup>S]sulfate-labeled SgII that had sedimented was determined in duplicate, and the mean value was expressed as:

$$\log_{10} \left[ 1 - \left( 1 - \frac{r_{\min}}{r_{\max}} \right) \frac{Y}{100} \right],$$

where  $r_{\max}$  and  $r_{\min}$  are the distances (in centimeters) from the axis of rotation to the bottom of the tube and the surface of the fluid column, respectively; 100 represents the maximum amount of [<sup>35</sup>S]sulfate-labeled SgII that could be sedimented; Y is the percentage of this value which sedimented at lower centrifugal effect values. Sediterms were constructed from the values obtained as described previously (Slinde and Flatmark, 1973), and for both immature and mature secretory granules, the average sedimentation coefficient (s) was calculated according to the rate equation:

$$\log_{10} \left[ 1 - \left( 1 - \frac{r_{\min}}{r_{\max}} \right) \frac{Y}{100} \right] = - \frac{\bar{s}_0 \int_0^t (\text{rpm})^2 dt}{3.5 \times 10^{13}}$$

## Quantitation of Radioactivity in SgII and hsPG

After SDS-PAGE and fluorography, the quantitation of the radioactivity in the SgII band or the hsPG was performed either by densitometric scanning or by counting, after digestion of the radioactive molecules with pronase and elution from the gel, as described previously (Tooze and Huttner, 1990).

## Electron Microscopy and Morphometry

Aliquots of fractions collected from equilibrium gradients (see above) containing immature (fractions 6–8) or mature (fractions 9+10) secretory

granules were diluted 1:1 with Karnovsky's fixative, pelleted, processed, embedded in Epon, and stained with uranyl acetate and lead citrate as previously described (Tooze and Huttner, 1990). The size of the dense cores was measured from negatives taken at 17,000 $\times$  magnification. Only those dense cores were measured where the limiting membrane was completely visible around the whole granule. The mean "measured" and "true" dense core diameters were calculated using standard techniques and equations (Aherne and Dunnill, 1982) and take into account the "optically lost caps", i.e., the smallest transections through the dense core, which cannot be accurately measured on the negative.

### Indirect Immunofluorescence Labeling

PC12 cells, grown on polylysine-coated coverslips (Rosa et al., 1989), were extracted with 80 mM PIPES-KOH, pH 6.8, 5 mM EGTA, 1 mM MgCl<sub>2</sub>, 0.5% Triton X-100, to remove the soluble pool of tubulin from the cytoplasm, and the remaining microtubules were fixed, exactly as described by Kreis (1987). The fixed cells were labeled with the mAb 1A2, kindly provided by Dr. T. Kreis (EMBL, Heidelberg, Germany), and visualized using a rhodamine-conjugated goat anti-mouse IgG antibody as previously described (Kreis, 1987).

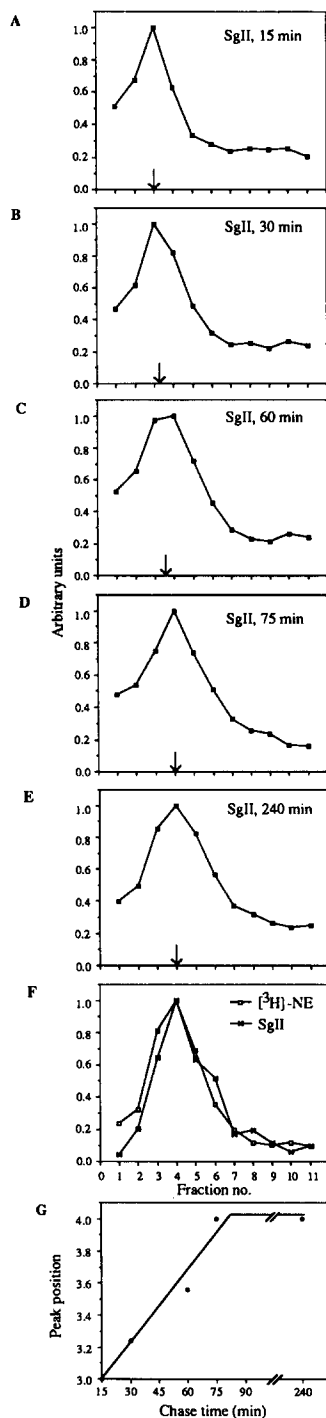
## Results

### Maturation of Secretory Granules in PC12 Cells

We have previously shown that in PC12 cells, the first vesicular product in the regulated pathway of secretion formed from the TGN is a granular structure whose dense core has a diameter ( $\sim$ 80 nm) similar to dense-cored material found within the lumen of the TGN (Tooze and Huttner, 1990). This first product is referred to as an immature secretory granule because, as will be described below, it represents a vesicular intermediate in the formation of the final organelle of the regulated pathway, the mature secretory granule which accumulates in the cell. In PC12 cells, immature secretory granules can be identified using SgII, labeled for 5 min with [<sup>35</sup>S]sulfate in the TGN and chased for 15 min, as a marker (Tooze and Huttner, 1990). In the present study, we have used [<sup>35</sup>S]sulfate-labeled SgII chased for prolonged periods (4–16 h) to identify mature secretory granules and to investigate whether they actually differ from immature secretory granules. Velocity sucrose gradient centrifugation revealed that immature secretory granules peaked in fraction 3 (Fig. 1 A), whereas mature secretory granules peaked in fraction 4 (Fig. 1 E). This allowed us to investigate the time course of maturation of immature secretory granules.

When SgII labeled for 5 min in the TGN was chased for 30 min (Fig. 1 B), 60 min (Fig. 1 C), and 75 min (Fig. 1 D), the peak of the [<sup>35</sup>S]sulfate-labeled SgII shifted gradually from fraction 3 to fraction 4. Between 75 min (Fig. 1 D) and 240 min (Fig. 1 E) of chase, the peak of the labeled SgII remained in fraction 4. These results show that the immature secretory granules, as defined by SgII sulfate labeled for 5 min and chased for 15 min, are an intermediate in the formation of mature secretory granules, and that the conversion of immature secretory granules to mature secretory granules is complete after 75 min of chase (Fig. 1 G), i.e., 75 min after exit of SgII from the TGN.

The distribution across the velocity gradient of [<sup>35</sup>S]sulfate-labeled SgII chased for 240 min (Fig. 1 E) was very similar to the distribution of [<sup>3</sup>H]norepinephrine (Fig. 1 F), whose uptake is a characteristic feature of secretory granules in PC12 cells (Greene and Tischler, 1976), as well as to the distribution of SgII immunoreactivity (Fig. 1 F). Because both immature and mature secretory granules contribute to

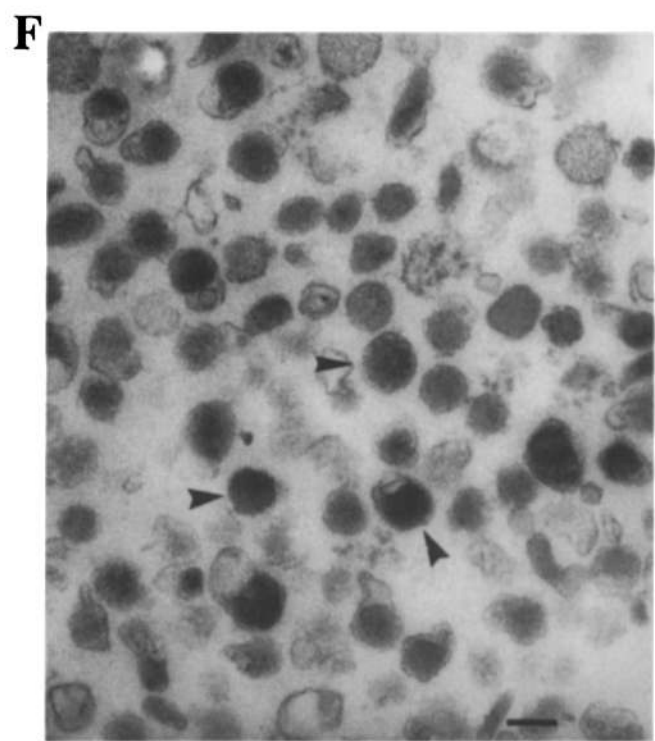
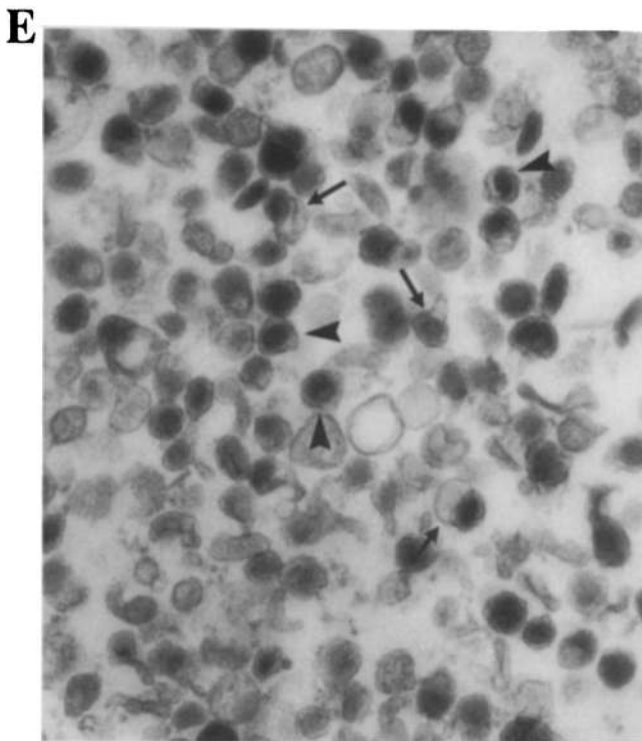
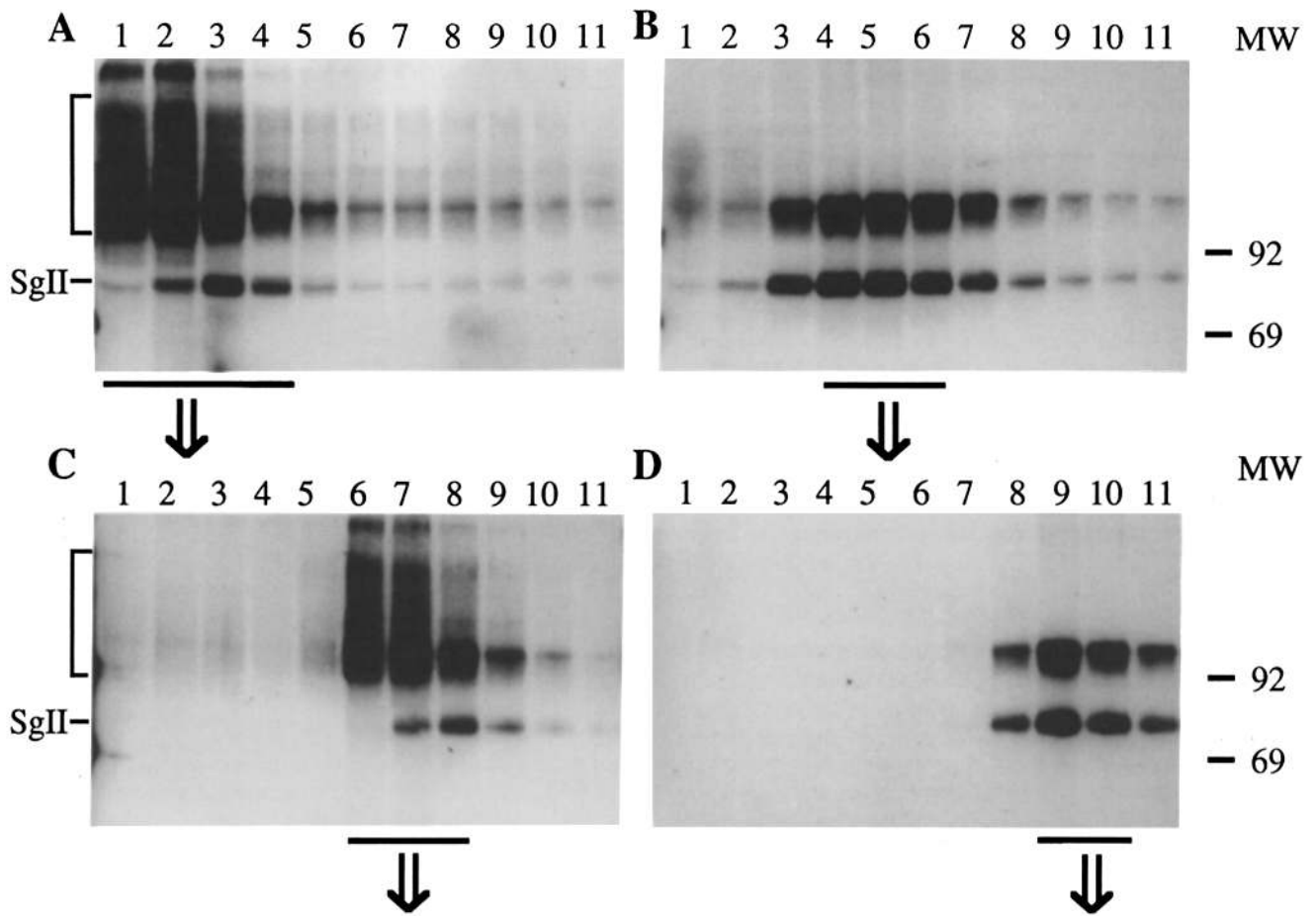


**Figure 1.** Time course of maturation of secretory granules in PC12 cells. PC12 cells were pulse labeled for 5 min with [<sup>35</sup>S]sulfate and chased for 15 (A), 30 (B), 60 (C), 75 (D), and 240 (E) min. Alternatively (F), PC12 cells were labeled for 16 h with 25  $\mu$ Ci of [<sup>3</sup>H]norepinephrine ([<sup>3</sup>H]-NE). A postnuclear supernatant was prepared from the cells and subjected to velocity sucrose gradient centrifugation. Fractions were collected from each gradient (fraction 1 = top; see abscissa on F), and an aliquot of each fraction was either analyzed by SDS-PAGE followed by fluorography and quantitation of the radioactivity in the SgII band (A–E), or counted directly for <sup>3</sup>H-radioactivity (F, open squares). In addition, aliquots of the fractions of a velocity gradient were subjected to immunoblotting for SgII (F, crosses). For each condition, the highest value was arbitrarily set to 1.0. Arrows in A–E indicate the peak, calculated for each curve by determining the intercept of the two slopes of the curve. These peak positions, which shift from fraction 3 (A) to fraction 4 (D and E), are expressed in G as a function of chase time, revealing the kinetics of conversion of immature secretory granules to mature secretory granules.

SgII immunoreactivity, we conclude that mature secretory granules are much more abundant than immature secretory granules in PC12 cells, which is consistent with the relatively short half-life of the latter (Fig. 1 G).

### Density Comparison of Immature and Mature Secretory Granules

In Fig. 2 (A and B) fluorograms are shown which reveal the distribution across the velocity sucrose gradient of [<sup>35</sup>S]sulfate-labeled material after either a 5-min pulse followed by a 15-min chase (Fig. 2 A) or a 6-h labeling followed by a 16-h chase (Fig. 2 B). Confirming our previous results (Tooze and



Huttner, 1990), most of the hsPG and SgII after the 5-min pulse and 15-min chase are found in an overlapping but non-identical distribution in the top four fractions of the gradient (Fig. 2 A). After the 6-h labeling followed by a 16-h chase, only a minor portion of the hsPG was recovered in the post-nuclear supernatant subjected to centrifugation because of the constitutive secretion of the hsPG during the chase (Tooze and Huttner, 1990; also see below). After the 16-h chase, consistent with the results shown in Fig. 1, the peak of SgII (and also that of secretogranin I which could be detected after the secretion of the hsPG) was found in fractions 4–5 (Fig. 2 B), i.e., deeper in the gradient than immature secretory granules.

The observation that mature secretory granules migrated more deeply into the velocity gradient than immature secretory granules could be due to an increase in density, size, or both. To investigate a possible increase in density, immature secretory granules and mature secretory granules were compared using equilibrium sucrose density gradient centrifugation (Fig. 2, C and D). The equilibrium sucrose gradient centrifugation was performed on fractions collected and pooled from the velocity gradients (immature secretory granules, fractions 1–4, see Fig. 2 A; mature secretory granules, fractions 4–6, see Fig. 2 B); this allowed a better resolution, and enrichment, of the immature and mature secretory granules than was achieved by subjecting the post-nuclear supernatant only to equilibrium sucrose gradient centrifugation (data not shown). Upon equilibrium centrifugation, the peak of immature secretory granules was found in fraction 8 (Fig. 2 C), and was separated from the constitutive secretory vesicles identified by the presence of the hsPG (fractions 5 and 6), as shown previously (Tooze and Huttner, 1990), whereas the peak of mature secretory granules was found in fractions 9–10 (Fig. 2 D). To exclude the possibility that these two peaks reflected only the fact that different fractions from the velocity gradient had been subjected to equilibrium centrifugation, and to determine the buoyant density of immature and mature secretory granules, post-nuclear supernatants containing either [<sup>35</sup>S]sulfate-labeled immature or [<sup>35</sup>S]sulfate-labeled mature secretory granules were directly subjected to equilibrium centrifugation on a sucrose density gradient. Immature and mature secretory granules were found to have buoyant densities of 1.148 g/cm<sup>3</sup> and 1.178 g/cm<sup>3</sup>, respectively (mean of two independent experiments). Thus, secretory granules of PC12 cells (at least in the hypertonic sucrose gradients described above) increase in density during maturation.

**Table 1. Comparison of the Diameter of the Dense Core of Immature and Mature Secretory Granules Isolated from PC12 Cells**

Secretory granule type	Core diameter	
	Measured mean (nm)	True mean (nm)
Immature	79.9 ± 12.8 (n = 120)	101.6
Mature	120.0 ± 11.7 (n = 134)	152.9

Immature secretory granules and mature secretory granules were isolated from PC12 cells using sequential velocity and equilibrium gradient centrifugation as described in Fig. 2. The core diameter was measured from micrographs of the granule preparations taken at a magnification of ×17,000. Only those dense cores were measured where the granule membrane was visible surrounding the entire core. The calculations made to obtain the measured and true core diameter of the immature and mature secretory granule were performed according to Aherne and Dunnill (1982).

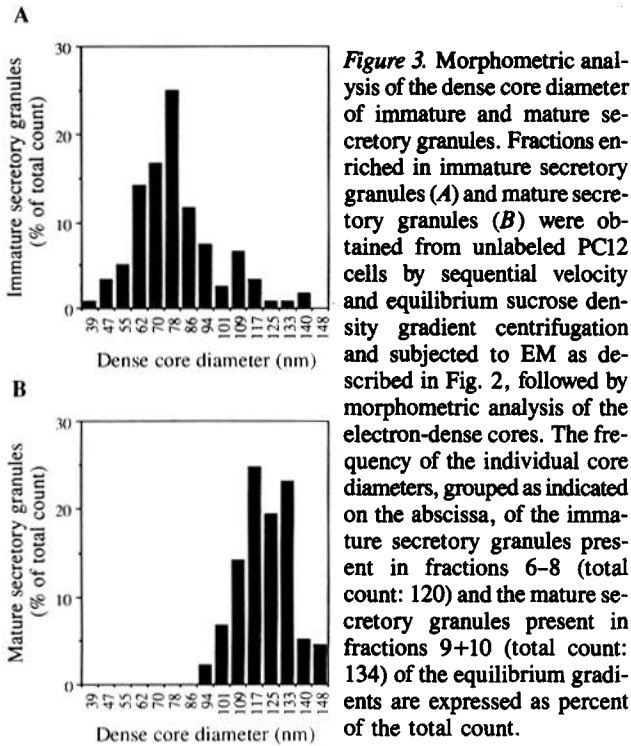
### Size Comparison of Immature and Mature Secretory Granules

We used both a morphological and biochemical approach to investigate whether, in addition to the observed difference in density, immature and mature secretory granules also differed by size. The fractions collected from the equilibrium gradients containing either immature secretory granules (fractions 6–8) or mature secretory granules (fractions 9–10) were pooled and processed for thin section EM (Fig. 2, E and F). Both preparations were enriched in vesicles containing dense-cored material. However, a major difference between the secretory granules in the two preparations was the size of their dense cores; the measured diameter of the dense core was on average ~80 nm for the secretory granules present in fractions 6–8 and on average ~120 nm for the secretory granules present in fractions 9–10 (Table I).

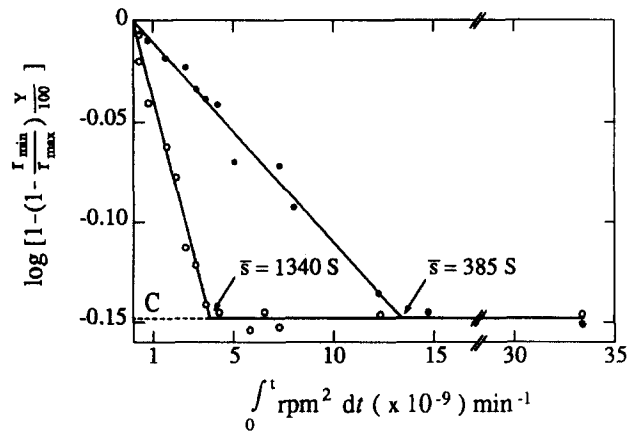
Analysis of the frequency of the individual dense core diameters revealed that the secretory granules present in fractions 6–8 of the second, equilibrium gradient showed a fairly symmetrical distribution around the major peak value of 78 nm (Fig. 3 A), indicating the presence of one major population of secretory granules in these fractions. This secretory granule population was distinct from that found in fractions 9+10 of the equilibrium gradient, which had a peak ranging from 117 to 133 nm dense core diameter (Fig. 3 B).

To investigate biochemically a possible difference in size between immature and mature secretory granules, we determined their sedimentation coefficient in isotonic sucrose using analytical differential centrifugation (Slinde and Flat-

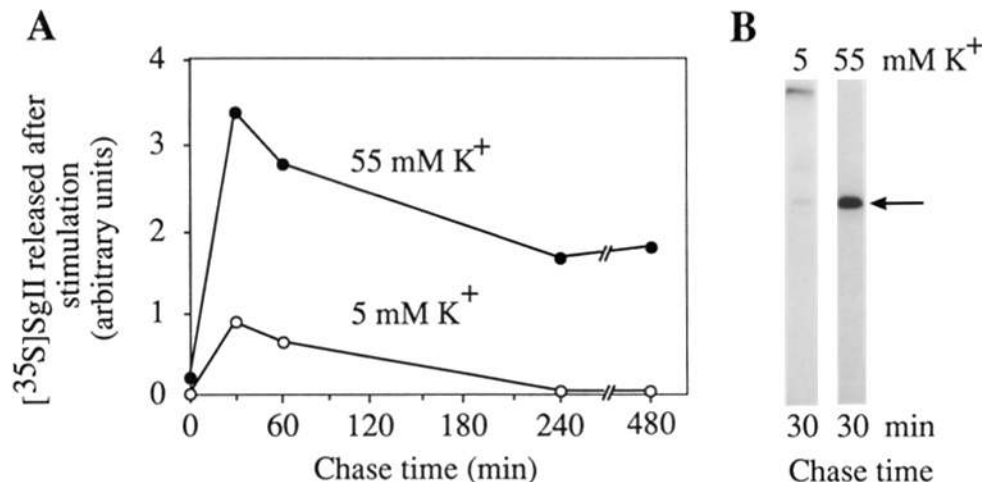
**Figure 2.** Biochemical and morphological comparison of immature and mature secretory granules of PC12 cells. PC12 cells were either pulse labeled for 5 min and chased for 15 min (A), or labeled for 6 h and chased for 16 h (B), followed by the preparation of a post-nuclear supernatant, velocity sucrose gradient centrifugation, and analysis of the fractions by SDS-PAGE and fluorography (fraction 1 = top). The fractions containing the peak of the radioactive hsPG and SgII (fractions 1–4 in A and 4–6 in B; bars) were pooled and subjected to equilibrium sucrose gradient centrifugation followed by analysis of the fractions by SDS-PAGE and fluorography (C and D, respectively). In B and D, the doublet above SgII which comigrates with SgII is secretogranin I. Fractions including the peak of the radioactive SgII (bars) were then collected, pooled and processed for EM; these were; fractions 6–8 in C, chosen to minimize the contamination of immature secretory granules by mature secretory granules; and fractions 9+10 in D, chosen to minimize the contamination of mature secretory granules by immature secretory granules. Representative thin section electron micrographs are shown in E and F, respectively. Note that the cores of the immature secretory granules (E, arrowhead) are smaller than the cores of the mature secretory granules (F, arrowhead), and that the membrane of the immature secretory granules is more often irregularly shaped and loosely surrounding the core (E, arrow), in contrast to the mature secretory granules whose membrane is more uniformly surrounding the core. Bar, 100 nm.



mark, 1973). Analysis of the post-nuclear supernatant of PC12 cells labeled for 5 min with [<sup>35</sup>S]sulfate and chased for 15 min showed that the vesicles containing labeled SgII, i.e., the immature secretory granules, exhibited a sedimentation behavior characteristic of a single, homogeneous population of particles (Fig. 4). The *s*-value was calculated from the logarithmic expression of the rate equation and found to be 385 ± 19 S. When PC12 cells were labeled for 6 h with [<sup>35</sup>S]sulfate followed by a 16-h chase, the vesicles containing labeled SgII, i.e., the mature secretory granules, also exhibited a sedimentation behavior characteristic of a single,



homogenous population of particles (Fig. 4). However, their *s*-value (1340 ± 73S) was 3.48-fold greater than that of the immature secretory granules. This increase in sedimentation rate cannot be explained by a difference in density between mature and immature secretory granules because the greater density of mature secretory granules in hypertonic sucrose gradients was calculated to account for at most a 1.26-fold



regulated secretion of SgII from immature secretory granules. PC12 cells were either pulse labeled for 10 min with [<sup>35</sup>S]sulfate (*t* = 0), or pulse labeled and chased for 30, 60, 240, or 480 min. At each time point, the cells were then incubated for 15 min in medium containing either 5 or 55 mM K<sup>+</sup>, followed by immunoprecipitation of SgII from this medium, SDS-PAGE and fluorography of the immunoprecipitates and quantitation of the SgII band by densitometric scanning (A). Optical density values are expressed as arbitrary units. B shows fluorograms of immunoprecipitates obtained after 30 min of chase and incubation in either 5 or 55 mM K<sup>+</sup> medium. The arrow indicates SgII.

increase in *s*-value. We therefore conclude that in PC12 cells, mature secretory granules differ from immature secretory granules by an increase in size.

### Regulated Exocytosis of Immature Secretory Granules

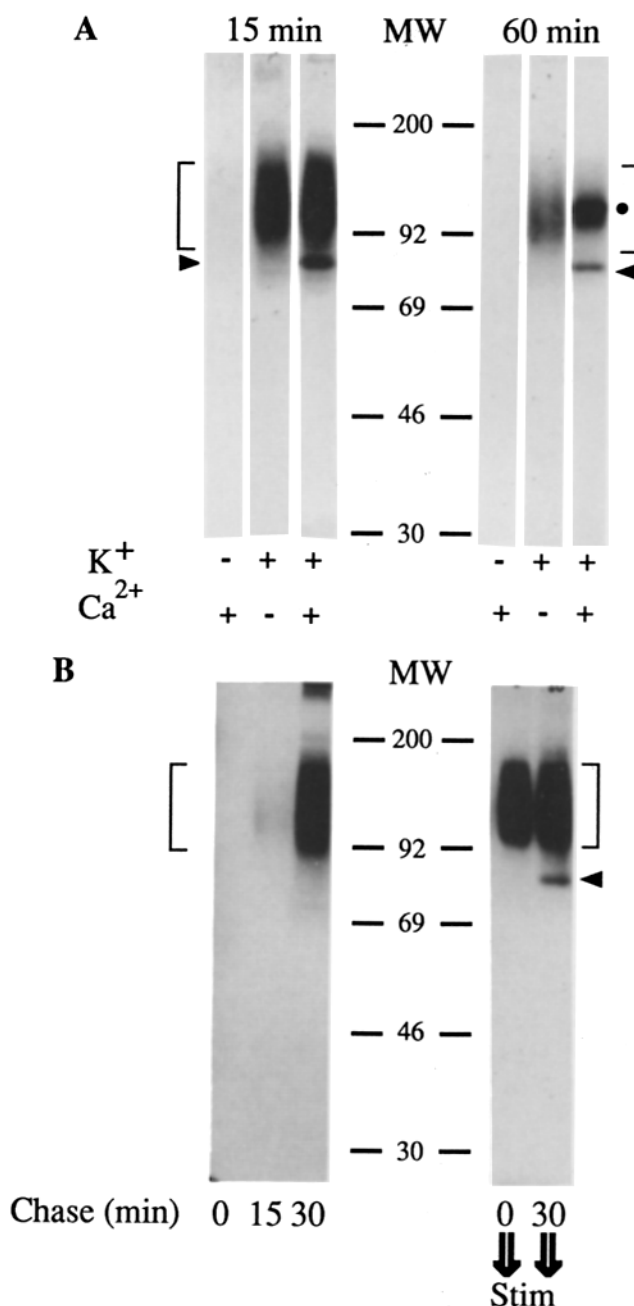
A characteristic feature of secretory granules is their ability to fuse with the plasma membrane and to release their contents in response to an appropriate stimulus (for review see Burgess and Kelly, 1987). It was of interest to determine if immature secretory granules also can undergo regulated exocytosis. This fusion process can be triggered in PC12 cells by depolarization of the plasma membrane with high (55 mM)  $K^+$  in the presence of extracellular  $Ca^{2+}$  (Schubert and Klier, 1977). As shown in Fig. 5, after a 10-min pulse with [ $^{35}S$ ]sulfate followed by a 30-min chase, labeled SgII was released from the cells after stimulation with 55 mM  $K^+$ . (The length of the stimulation period itself [15 min in Fig. 5] has been ignored because it has been shown that the peak of regulated release occurs 2 min after the addition of high  $K^+$  medium [Lowe et al., 1988].) In contrast, very little SgII was released after treatment with low (5 mM)  $K^+$ . Stimulation of cells at the end of the pulse resulted in no significant release of labeled SgII. Interestingly, the amount of labeled SgII released in response to stimulation decreased after 30 min of chase (Fig. 5 A). The time course of the regulated release of labeled SgII clearly was distinct from the appearance of labeled SgII in mature secretory granules (Fig. 1 G) and suggested that immature secretory granules have the ability to undergo regulated exocytosis.

To obtain further support for this conclusion, the release of labeled SgII after stimulation was investigated using both a shorter pulse with [ $^{35}S$ ]sulfate (5 min) and a shorter chase time (15 min) than in the experiment shown in Fig. 5. Indeed, as shown in Fig. 6 A, more labeled SgII was released in response to stimulation after 15 min of chase, i.e., when the labeled SgII was entirely in immature secretory granules (cf. Fig. 1 A), than after 60 min of chase, i.e., when the majority of the labeled SgII was in mature secretory granules (cf. Fig. 1 C). This release was dependent on the presence of  $Ca^{2+}$  in the medium (Fig. 6 A).

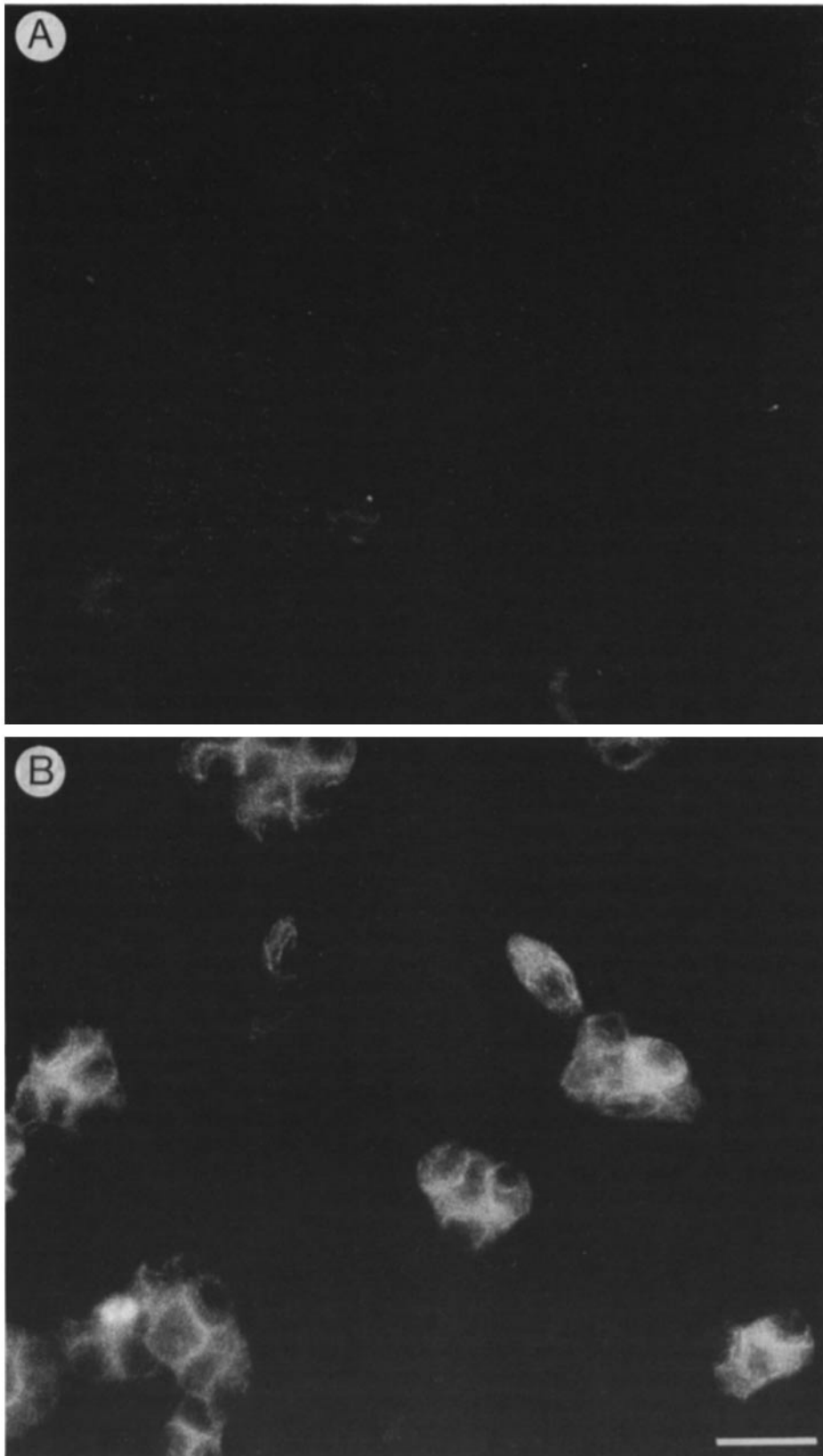
This experiment, in which SgII had been analyzed without prior immunoprecipitation, also led to the unexpected observation that the hsPG, used as a marker for the constitutive secretory pathway (Tooze and Huttner, 1990), was released into the medium after incubation of cells in high  $K^+$ . However, this "stimulated release" of the hsPG differed from the regulated secretion of SgII in two important aspects. First, it was independent of extracellular calcium (Fig. 6 A). Second, it followed different kinetics; when the cells were incubated for 10 min in high  $K^+$  medium immediately after the pulse, labeled hsPG was found in the medium, in contrast to labeled SgII (Fig. 6 B, right). This did not simply reflect the constitutive secretion occurring under resting conditions because very little labeled hsPG was detected in the medium after a 15-min chase in normal medium, i.e., in the presence of a low concentration of  $K^+$  (Fig. 6 B, left). Nevertheless, as will be explained in the Discussion, all of the hsPG was released via the constitutive pathway of secretion.

### Role of Microtubules in the Constitutive and Regulated Secretory Pathway

Immature secretory granules, being derived from the TGN,



**Figure 6.** Differential kinetics and  $Ca^{2+}$  requirement for the secretion of the hsPG and SgII. (A) PC12 cells were pulse labeled for 5 min with [ $^{35}S$ ]sulfate and chased for either 15 (left) or 60 (right) min. The cells were then incubated for 10 min in medium containing either 5 (–) or 55 (+) mM  $K^+$ , without (–) or with (+) 2.2 mM  $Ca^{2+}$ , as indicated. Aliquots of this medium were analyzed by SDS-PAGE followed by fluorography. All six lanes are from the same fluorogram. (B) PC12 cells were pulse labeled for 5 min with [ $^{35}S$ ]sulfate ( $t = 0$ ), or pulse labeled and chased for either 15 or 30 min. Aliquots of the labeling medium (0) and the chase media (15, 30) were analyzed by SDS-PAGE and fluorography for 17 days (left). Alternatively, at the end of the pulse or after 30 min of chase, cells were stimulated (*stim*) by incubation for 10 min in medium containing 55 mM  $K^+$  and 2.2 mM  $Ca^{2+}$ , and equivalent aliquots of this medium were analyzed by SDS-PAGE and fluorography for 7 days (right). Brackets mark the hsPG, part of which comigrates with secretogranin I (●). Arrowheads indicate SgII.

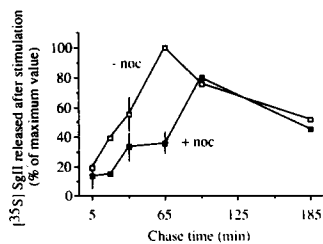


**Figure 7.** Indirect immunofluorescence of PC12 cells treated with nocodazole to depolymerize the microtubule network. PC12 cells were treated for 30 min at 0°C, and then for 30 min at 37°C either with (A) or without (B) 10  $\mu$ M nocodazole. The cells were extracted, fixed, and immunolabeled with an antitubulin antibody. Bar, 10  $\mu$ m.

are initially located in the perinuclear region of cells (Farquhar et al., 1978; Orci et al., 1985; Tooze and Tooze, 1986). Because immature secretory granules can release their contents into the extracellular medium, as shown in Fig. 5, they

must be translocated to the plasma membrane. We were interested in determining whether this translocation involves microtubules. To depolymerize the microtubule network in PC12 cells, it was necessary to cool the cells to 4°C for 30





**Figure 8.** Microtubules enhance the ability of immature secretory granules to undergo regulated secretion. PC12 cells were treated without (–noc) or with (+noc) nocodazole as described in Fig. 7. They were then pulse labeled for 5 min with [<sup>35</sup>S]sulfate (*t* = 0) and chased for 5, 20, 35, 65, 95, or

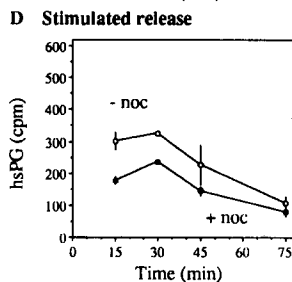
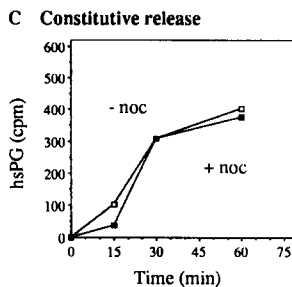
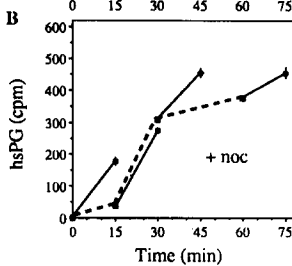
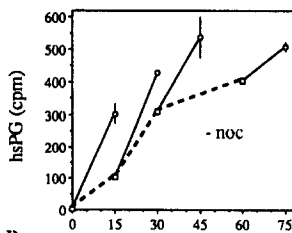
185 min, in all conditions without (–noc) or with (+noc) 10 μM nocodazole. During the last 5 min of chase, the cells were incubated in medium containing 5 mM K<sup>+</sup>. At each time point, the cells were incubated for 10 min in medium containing 55 mM K<sup>+</sup> and 2.2 mM Ca<sup>2+</sup>, either without (–noc) or with (+noc) 10 μM nocodazole. Aliquots of this medium were analyzed by SDS-PAGE followed by fluorography and quantitation of the SgII band. The mean of two experiments is shown; for each experiment, data have been expressed as the percentage of the highest value obtained in the absence of nocodazole. In the case of duplicate data points, bars indicate the deviation of the individual data from the mean.

min and then treat them for 30 min at 37°C with nocodazole. As shown by indirect immunofluorescence using antitubulin antibodies (Fig. 7), this treatment completely depolymerized the microtubule network. The cells rounded up in the presence of nocodazole, but remained attached to the plastic dish.

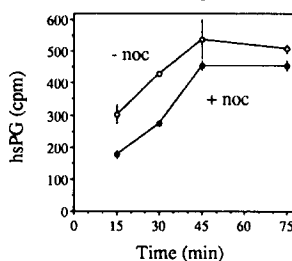
We then compared the regulated release of SgII in nocodazole-treated and control cells. After the 60-min treatment without or with nocodazole, the cells were pulse labeled with [<sup>35</sup>S]sulfate, chased for various times, and then stimulated with high K<sup>+</sup> medium, all in the absence or presence of nocodazole (Fig. 8). Up to 65 min of chase, the amount of labeled SgII released after stimulation was significantly higher in control than nocodazole-treated cells, whereas similar amounts were released after 95 and 185 min of chase. The fact that the peak of release of labeled SgII was seen after 65 min of chase, rather than 30 min as in Fig. 5, was probably due to the cold treatment which was also performed for control cells. Considering the shape of the control curve shown in Fig. 8, we believe that the peak of release of labeled SgII reflects, for the most part, release from immature secretory granules. At any rate, because the difference between control and nocodazole-treated cells was already observed after 20 min of chase, i.e., at a time point when the labeled SgII was clearly released from immature secretory granules, our results suggest that the transport of immature secretory granules to the plasma membrane is enhanced in the presence of an intact microtubule network.

These experiments also provided information as to whether the secretion of the hsPG was affected by microtubules. Confirming previous results (Tooze and Huttner, 1990), the constitutive secretion of the sulfate-labeled hsPG approached a plateau between 30 and 60 min of chase (Fig. 9 A, dashed thick line). This secretion was not significantly affected by nocodazole treatment (Fig. 9 B, dashed thick line), as illustrated by the comparison shown in Fig. 9 C. When PC12 cells were subjected to sequential incubations for 5 min in low K<sup>+</sup> medium and then for 10 min in high K<sup>+</sup> medium, either directly after the pulse or after various times of chase, the amount of labeled hsPG released upon this treatment was maximal directly after the pulse and after the first 15 min of

**A Constitutive and stimulated release**



**E Total: Constitutive plus stimulated**



**Figure 9.** Microtubules enhance the secretion of the hsPG in response to 55 mM K<sup>+</sup>. PC12 cells were treated without (–noc) or with (+noc) nocodazole as described in Fig. 7. They were then either pulse labeled for 5 min with [<sup>35</sup>S]sulfate (*t* = 0) or pulse labeled and chased for 15, 30, and 60 min (constitutive release), in all conditions without (–noc, open symbols) or with (+noc, closed symbols) 10 μM nocodazole. At each time point, the cells were incubated for 5 min in medium containing 5 mM K<sup>+</sup> followed by 10 min in medium containing 55 mM K<sup>+</sup> and 2.2 mM Ca<sup>2+</sup> (stimulated release), either without (–noc) or with (+noc) 10 μM nocodazole. Aliquots of the latter medium as well as of the labeling medium and the chase media were analyzed by SDS-PAGE followed by fluorography and quantitation of the hsPG. (A and B) The secretion of the hsPG during the chase (constitutive release, dashed thick lines and squares) is shown for cells treated without (–noc, A) and with (+noc, B) nocodazole. To these values, the amount of labeled hsPG secreted during the stimulated release from cells treated without (–noc, A) with with (+noc, B) nocodazole has been added (solid thin lines and circles). (C–E) A comparison of the hsPG secreted during the chase (constitutive release) from cells treated either without (–noc) or with (+noc) nocodazole is shown in C. A comparison of the hsPG secreted during the stimulated release from cells treated either without (–noc) or with (+noc) nocodazole is shown in D and E, either without (D) or with (E)

the addition of the chase values shown in C. For the 0, 30, and 60 min chase time points and the subsequent 15 min stimulation, the mean of two experiments is shown; for these data points, bars indicate the deviation of the individual data from the mean and are only shown for the values obtained after incubation in the 55 mM K<sup>+</sup> medium.

chase, and decreased thereafter (Fig. 9 A, solid thin lines), confirming and extending the results shown in Fig. 6 A. This secretion was reduced by nocodazole treatment (Fig. 9 B, solid thin lines), as illustrated by the comparisons shown in Fig. 9 D. Thus, as shown in Fig. 9 E, the total amount of hsPG secreted was reduced by the nocodazole treatment.

## Discussion

### *Immature Secretory Granules—An Obligatory Intermediate in Granule Biogenesis*

Our study shows that the biogenesis of secretory granules in PC12 cells proceeds in two sequential steps, (a) the formation of immature secretory granules from the TGN, and (b) their conversion to mature secretory granules. Under the present conditions, immature secretory granules were found to be distinct from mature secretory granules in terms of size and density, being smaller as well as lighter. Immature secretory granules are obligatory precursors to mature secretory granules, rather than a subpopulation of secretory granules, because virtually all of the pulse-labeled SgII appeared in this organelle immediately after exit from the TGN, and subsequently was found in mature secretory granules. The half-life of immature secretory granules, as defined by pulse-labeled SgII, was found to be  $\approx 45$  min; this is relatively short compared to that of mature secretory granules which, ignoring cell division and in the absence of cell stimulation, can exceed one day.

The intermediate nature of the immature granules, their smaller size and their short half-life reported here for PC12 cells confirm previous observations in mammothrophs of the anterior pituitary using pulse-chase EM autoradiography (Salpeter and Farquhar, 1981). In this system, the [ $^3\text{H}$ ]leucine label incorporated into secretory proteins was, after exit from the TGN, first found in secretory granules characterized by a diameter ranging from 100 to 200 nm, and  $\approx 1$  h afterwards in secretory granules characterized by a diameter ranging from 600–900 nm (Salpeter and Farquhar, 1981). Thus, kinetic studies have shown that in two distinct endocrine cell types, the mammothroph and the chromaffin cell-related PC12 cell, the formation of a secretory granule proceeds via an organellar intermediate, the immature secretory granule. It will be interesting to determine whether this is true for most, if not all, endocrine cells.

### *Secretory Granules Increase in Size During Maturation*

Analytical differential centrifugation revealed that immature and mature secretory granules behaved as two distinct populations of particles, each population being highly homogeneous. Because the increase in the  $s$ -value of the mature secretory granules compared to immature secretory granules was much greater than could be accounted for by the increase in density, the conversion of immature to mature secretory granules also entails an increase in size. These observations are consistent with our morphometric data, which indicated the presence of a homogeneous population of secretory granules with an average core diameter of 80 nm in the fractions containing the [ $^{35}\text{S}$ ]sulfate-labeled SgII chased for 15 min, and the presence of another homogeneous population of secretory granules with an average core diameter of 120 nm in the fractions containing the [ $^{35}\text{S}$ ]sulfate-labeled SgII chased for 16 h. Although we do not have direct evidence that the dense-cored vesicles seen in the electron microscope actually contained the [ $^{35}\text{S}$ ]sulfate-labeled SgII, it can be deduced that this must be the case, for the following reasons. First, dense core formation is known to begin in the TGN (see Farquhar and Palade, 1981, for review; Orci et al., 1987; Tooze et al., 1987); second, [ $^{35}\text{S}$ ]sulfate-labeled SgII

is very efficiently ( $>95\%$ ) stored in PC12 cells; third, SgII is found by immunoelectron microscopy in the dense cores of secretory granules (Bassetti et al., 1991). Thus, any [ $^{35}\text{S}$ ]sulfate-labeled SgII that has exited from the TGN can only be present in dense cored vesicles. In other words, the [ $^{35}\text{S}$ ]sulfate-labeled SgII after the 15-min chase must be present in some of the 80 nm secretory granules seen by electron microscopy in fractions 6–8 of the equilibrium gradient, and the [ $^{35}\text{S}$ ]sulfate-labeled SgII after the 16 h chase must be present in some of the 120 nm secretory granules seen by electron microscopy in fractions 9 + 10. In conclusion, both the biochemical and morphometric data indicate that secretory granules in PC12 cells increase in size during maturation. This conclusion is consistent with previous morphological observations on mammothrophs (Farquhar et al., 1978).

### *Fusion of Secretory Granules during Maturation*

Because an increase in vesicle size is always accompanied by an increase in the surface area of the limiting membrane, a fusion event with another membrane vesicle must be postulated to explain the increase in secretory granule size during maturation, unless one assumes *de novo* membrane synthesis in immature secretory granules. Several previous morphological observations are consistent with the notion that in PC12 cells, immature secretory granules fuse with each other to form larger, mature secretory granules. These include (a) the presence of membrane-enclosed structures containing multiple dense cores with a diameter typical of the dense core of immature secretory granules (Farquhar et al., 1978; Tooze and Tooze, 1986; Tooze and Huttner, 1990), and (b) the presence of mature secretory granules containing both growth hormone and prolactin in which these two hormones are not significantly intermixed (Fumagalli and Zanini, 1985). Fusion of immature secretory granules with each other has also been discussed as one mechanism to explain the difference in size of secretory granules of mast cells as measured by patch clamp techniques (de Toledo and Fernandez, 1990).

Additional work will be required to firmly establish that in PC12 cells immature secretory granules fuse with each other during their maturation, and to determine how many immature secretory granules fuse to form one mature secretory granule. It is, however, interesting to note that the differences between immature and mature secretory granules in core diameter and  $s$ -value (after correction for the maximal possible contribution of density to the different  $s$ -values) would be consistent with the fusion of  $4 \pm 1$  immature secretory granules with each other.

### *Immature Secretory Granules Can Fuse with the Plasma Membrane after Stimulation*

An unexpected finding was that immature secretory granules, in addition to undergoing fusion in the course of their maturation in resting PC12 cells, are capable of undergoing regulated exocytosis at the plasma membrane. After a sulfate pulse and a 15-min chase, at which time labeled SgII was present only in immature secretory granules, labeled SgII was released in response to cell stimulation. This release fulfilled the criteria of regulated exocytosis in that it was dependent on extracellular calcium. The fact that immature secretory granules, in contrast to constitutive secretory vesicles, can undergo regulated exocytosis implies differential

exit from the TGN of the membrane proteins involved in regulated and constitutive exocytosis.

We observed that the amount of [<sup>35</sup>S]sulfate-labeled SgII released in response to stimulation from immature secretory granules (15 and 30 min of chase) was greater than that released from mature secretory granules (60 min chase and thereafter). Because there was no significant degradation of [<sup>35</sup>S]sulfate-labeled SgII during the first few hours of chase, and assuming that the cells were in steady state, i.e., that the total number of SgII molecules released upon stimulation was the same irrespective of the time of chase, the decrease in the amount of [<sup>35</sup>S]sulfate-labeled SgII with time reflects a decrease in the specific activity of [<sup>35</sup>S]sulfate-labeled SgII released upon stimulation. One, though not the only possible, explanation for such a decrease in specific activity could be the preferential exocytosis of immature secretory granules upon stimulation. It has previously been documented that the newly synthesized form of certain hormones, e.g., insulin, appears to undergo exocytosis in preference to the stored form of hormones (see Rhodes and Halban, 1987, and references therein). It will be interesting to determine whether the subcellular basis for this phenomenon is the preferential exocytosis of immature secretory granules.

The observations that immature secretory granules undergo fusion in the course of their maturation and can also fuse with the plasma membrane have implications with respect to the molecular machinery regulating vesicular traffic. GTPases have recently been shown to be part of this machinery, being involved in the fusion of vesicles with their appropriate target membrane (for review see Bourne et al., 1990) as well as in the budding of vesicles from donor compartments (Tooze et al., 1990). Thus, immature secretory granules can be expected to carry, or bind, at least two distinct GTPases, involved in the recognition event preceding (a) the putative immature secretory granule-immature secretory granule fusion, and (b) the immature secretory granule-plasma membrane fusion.

### *Immature Secretory Granules Are Transported Along Microtubules*

Depolymerization of microtubules with nocodazole reduced by ≈50% the amount of labeled SgII released in response to stimulation during the first 65 min of chase, i.e., when SgII was predominantly released from immature secretory granules. Although one cannot exclude an involvement of microtubules in the budding of immature secretory granules from the TGN, a likely explanation of our results is that immature secretory granules require an intact microtubule network for efficient translocation to the plasma membrane. The observation that some translocation to the site of release occurred even in the absence of a microtubule network may be attributed to immature secretory granules moving by diffusion; the distance between the TGN and the site of exocytosis in PC12 cells is relatively short compared to, for example, neurons. This short distance probably also explains why after 95 and 185 min of chase, the amount of labeled SgII released in response to stimulation was unaffected by microtubule depolymerization; after this time, secretory granules presumably had arrived at the plasma membrane independent of an intact microtubule network.

Microtubules have been shown to be involved in the transport of secretory granules to the plasma membrane in endo-

crine (Boyd et al., 1982) and neuronal (Tooze and Burke, 1987; Kreis et al., 1989; Tooze et al., 1989) cells. None of these studies, however, addressed the question whether immature secretory granules can be translocated along microtubules or whether they first have to undergo maturation. Our findings, which suggest that immature secretory granules are translocated along microtubules, imply that the membrane protein(s) involved in this movement exit from the TGN with immature secretory granules.

### *The Role of Microtubules in the Constitutive Pathway of Secretion*

In studying the possible involvement of microtubules in the movement of constitutive secretory vesicles, we obtained the unexpected result that the release of the hsPG occurred not only spontaneously but could also be stimulated by high K<sup>+</sup>. Salton et al. (1983) previously concluded that the hsPG found in the medium after stimulation of PC12 cells with high K<sup>+</sup> reflected release from secretory granules. However, we believe that this stimulated release of the hsPG does not reflect secretion via the regulated pathway, for the following reasons. First, most, if not all, of the hsPG left the TGN in vesicles distinct from those containing SgII (see also Tooze and Huttner, 1990) and, in contrast to SgII, was released from the cells into the medium during the chase without stimulation. Second, the kinetics of responsiveness to stimulated release were distinct for the hsPG and SgII, the former being responsive already at the end of the sulfate pulse. Third, the release of the hsPG after stimulation was calcium independent. We therefore believe that the stimulated release of the hsPG was due to an enhanced release via the constitutive secretory pathway, a conclusion consistent with the following observations. First, only one population of hsPG-containing post-TGN vesicles was observed by equilibrium density gradient centrifugation (see also Tooze and Huttner, 1990). Second, the component of the hsPG that could be released by stimulation decreased concomitantly with the spontaneous, nonstimulated release, indicating that both the spontaneously released hsPG and the hsPG released after stimulation were derived from the same intracellular pool. These results are also consistent with the previous report by Gowda et al. (1989) that PC12 cells contain only one type of hsPG.

Depolymerization of microtubules reduced the amount of the hsPG released in response to high K<sup>+</sup>, but not that released spontaneously. How can this observation be interpreted and reconciled with the above conclusion that the spontaneously released hsPG and the hsPG released after stimulation were derived from the same intracellular pool? In the TGN, the hsPG is tightly bound to the membrane (Chanat and Huttner, 1991); this membrane-bound hsPG needs to be converted to a soluble form to be released into the medium. Because the arrival of sulfate-labeled hsPG at the cell surface ( $t_{1/2} \approx 15$  min; Pimplikar, S. and Huttner, W. B., unpublished data) is faster than its appearance in the medium ( $t_{1/2} \geq 20$  min; Fig. 1 in Tooze and Huttner, 1990), at least part of this conversion occurs after arrival at the cell surface. Thus, the simplest answer to the above question would be that the high K<sup>+</sup> directly stimulates this conversion. In low K<sup>+</sup> medium, in the absence as well as presence of microtubules, the conversion of the hsPG to the soluble form, rather than its transport from the TGN to the plasma

membrane, would be the rate-limiting step for its release into the medium, masking a possible involvement of microtubules in the translocation of constitutive secretory vesicles. In high  $K^+$  medium, the rate-limiting step would no longer be the conversion of the hsPG to the soluble form but its transport from the TGN to the plasma membrane, revealing the involvement of microtubules in the translocation of constitutive secretory vesicles. Considering the distances that proteins delivered in constitutive secretory vesicles must travel, for example in neurons from the perikaryon to the presynaptic membrane, such a translocation might be essential for maintaining cellular function.

In contrast to Rivas and Moore (1989), but in agreement with other investigators (Arnheiter et al., 1984; Matsuuchi et al., 1988), we conclude that not only secretory granules, but also constitutive secretory vesicles are translocated along microtubules. This raises the possibility that, in contrast to membrane proteins involved in vesicle fusion, membrane proteins involved in the interaction of post-TGN vesicles with microtubules may not be sorted upon exit from the TGN. It is unclear whether or not the rates of transport from the TGN to the plasma membrane are different for constitutive secretory vesicles and immature secretory granules. The hsPG was released by incubating PC12 cells immediately after the sulfate pulse for 5 min in low  $K^+$  medium followed by 10 min in high  $K^+$  medium, during which time the constitutive secretory vesicles were transported to, and spontaneously fused with, the plasma membrane. In contrast, SgII was not significantly released under these conditions, probably because release only occurs during the first 2–3 min of incubation in high  $K^+$  medium (Lowe et al., 1988), at which time labeled immature secretory granules had not yet arrived at the plasma membrane. Thus, the differences in the kinetics of release between the hsPG and SgII do not accurately reflect the differences, if any, in the transport of constitutive secretory vesicles and immature secretory granules to, and their fusion with, the plasma membrane. This issue could be investigated using *in vitro* polymerized microtubules as tracks for constitutive secretory vesicles and immature secretory granules as defined in our previous (Tooze and Huttner, 1990) and present studies.

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