Conversion of Proinsulin to Insulin Occurs Coordinately with Acidification of Maturing Secretory Vesicles

Lelio Orci,* Mariella Ravazzola,* Mylene Amherdt,* Ole Madsen,‡ Alain Perrelet,* Jean-Dominique Vassalli,* and Richard G. W. Anderson§

*Institute of Histology and Embryology, University of Geneva Medical School, 1211 Geneva 4, Switzerland; ‡Hagedorn Research Laboratory, 2820 Gentofte, Denmark; §Department of Cell Biology and Anatomy, Southwestern Graduate School of Biomedical Sciences, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Abstract. Proinsulin is a single polypeptide chain composed of the B and A subunits of insulin joined by the C-peptide region. Proinsulin is converted to insulin during the maturation of secretory vesicles by the action of two proteases and conversion is inhibited by ionophores that disrupted intracellular H^+ gradients. To determine if conversion of prohormone to hormone actually occurs in an acidic secretory vesicle, cultured rat islet cells were incubated in the presence of 3-(2,4-dinitroanilino)-3′ amino-*N*-methyldipropylamine (DAMP), a basic congener of dinitrophenol that concentrates in acidic compartments and is retained there after aldehyde fixation. The cells were processed for indirect protein A–gold colocalization of DAMP, using a monoclonal antibody to dinitrophenol, and proinsulin, using a monoclonal antibody that exclusively reacts with the prohormone. The average density of DAMP-specific gold particles in immature secretory vesicles that contained proinsulin was 71/μm^2 (18 times cytoplasmic background), which indicated that this compartment was acidic. However, the density of DAMP-specific gold particles in the insulin-rich mature secretory vesicle averaged 433/μm^2. This suggests that although proinsulin conversion occurs in an acidic compartment, the secretory vesicles become more acidic as they mature. Since the concentration of anti–proinsulin IgG binding in secretory vesicles is inversely proportional to the conversion of proinsulin to insulin, we were able to determine that maturing secretory vesicles had to reach a critical pH before proinsulin conversion occurred.

DURING endocytosis and exocytosis, macromolecules are sequestered in a series of membrane-bound vesicles that migrate to specific intracellular targets. A remarkable feature of these vesicles is that often their interior is maintained at a low pH relative to the surrounding cytoplasm (25, 28). Whereas the pH of vesicles in the endocytic pathway has been measured in situ with pH-sensitive dyes (19, 35), vesicles in the secretory pathway have been isolated and the pH measured directly (4, 10, 11, 40). The pH of these vesicles ranges from pH 4.5 (19) to pH 6.5 (38, 39) depending on the type of vesicle, and there is good evidence that the proton gradient is generated by an ATP-dependent proton pump. Coated vesicles (7, 8, 34), endosomes (9), and lysosomes (20, 30) in the endocytic pathway and the Golgi apparatus (10, 40) and secretory vesicles (4, 11, 29) in the exocytic pathway contain a similar type of proton pump. Thus, acidification of these compartments is an active process that may be regulated in response to specific stimuli.

The function of low pH in these compartments is not entirely understood. When the intraluminal pH of the vesicles is neutralized, either with ionophores or basic amines, several important reactions in both the endocytic and exocytic pathways are inhibited. These include (*a*) receptor recycling during receptor-mediated endocytosis (3); (*b*) degradation of macromolecules in the lysosome (37); (*c*) sorting of macromolecules during secretion (18); and (*d*) proteolytic processing of prohormones (22, 33). Despite the insight provided by this experimental approach, more information is needed about which of these reactions actually do occur in an acidic environment.

To obtain more direct information about the function of acidic compartments in the processing of macromolecules by the Golgi apparatus and secretory vesicles, we have used a newly developed technique for visualizing acidic compartments (2). 3-(2,4-Dinitroanilino)-3′amino-*N*-methyldipropylamine (DAMP)[1], a basic congener of dinitrophenol (DNP), accumulates in acidic compartments and is retained there after fixation with aldehyde fixatives. Sites of DAMP accumulation can be detected in sections of embedded tissue by either light microscopy or electron microscopy with an antibody directed against DNP. Moreover, with suitable antibodies, both DAMP and antigens of interest can be colocalized in the same cell. This technique was used to show that *trans*

1. *Abbreviations used in this paper*: DAMP, 3-(2,4-dinitroanilino)-3′amino-*N*-methyldiproplylamine; DNP, dinitrophenol.

cisternae and vesicles of the Golgi apparatus in human fibroblasts are acidic compartments and that fibronectin resides in acidic *trans* Golgi vesicles (1). We have now used this strategy to study the role of low pH in the proteolytic processing of proinsulin to insulin.

Proinsulin is a single polypeptide chain composed of the B and A subunits of insulin joined by the C-peptide region. Before insulin secretion, the C-peptide and its flanking basic amino acids are proteolytically excised from proinsulin by the combined action of both tryptic and carboxypeptidase-like enzymes (5, 6) that are believed to have an optimum activity at acid pH. Proinsulin can be immunocytochemically distinguished from insulin with a proinsulin-specific monoclonal antibody (15, 16), which allows the immunocytochemical localization of sites of proinsulin cleavage. Therefore, to determine the distribution of acidic compartments relative to the conversion of proinsulin to insulin, islet cells were incubated in the presence of DAMP and processed for immunocytochemical colocalization of either proinsulin or insulin and DAMP.

Materials and Methods

Materials

A mouse hybridoma clone producing a monoclonal antibody directed against DNP conjugated to ovalbumin (clone HDP1) was grown in the peritoneal cavity of mice, and the IgG fraction purified as previously described (2). DAMP was synthesized as previously described (2). Monoclonal anti-proinsulin IgG (GS-9A8 and GS-4G9) was previously characterized (15). Guinea pig anti-porcine insulin serum (lot 573) was provided by Dr. P. H. Wright. Rabbit anti-mouse IgG (cat. No. 65-125) and rabbit anti-mouse IgG coupled to FITC (cat. No. 65-171) were obtained from Miles-Yeda (Rehovot, Israel). Rabbit anti-guinea pig IgG coupled to FITC was obtained from Nordic Immunological Laboratories (Pilburg, The Netherlands). Protein A (17.077001) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Carbowax 20,000 (Peg 20, cat. No. 9732), gold chloride ($HAuCl_4$), trisodium citrate and anhydrous K_2CO_3 (cat. No. 4928) were from Merck Co. (Darmstadt, Germany). Lowicryl K4M (cat. No. 3 15923) was from Polysciences, Inc. (Warrington, PA). Bovine serum albumin (cat. No. 81003) from Miles-Yeda. Epon (cat. No. 45345) was obtained from Fluka (Buch, Switzerland). Monensin (cat. No. 47586) was obtained from Calbiochem-Behring Corp. (Zurich, Switzerland). Other supplies were obtained as previously described (2, 23).

Labeling Islet Cells with DAMP

Islets of Langerhans from normal adult rats (Sprague Dawley, SIVZ strain) were isolated by collagenase digestion (13). Freshly isolated islets were incubated for 1 h at 37°C in calcium-free Krebs-Ringer solution that contained 0.5% BSA and 16.7 mM glucose, bubbled with O_2/CO_2 (95:5 vol/vol). At the end of the incubation period, 30 μM DAMP was added to the medium and the islets were incubated for 1 h at 37°C. The islets were washed and either fixed immediately, incubated an additional 30 min at 37°C in the presence of 25 μM monensin or incubated an additional 30 min at 37°C in the absence of monensin. The islets were then fixed for 2 h at room temperature with 1% glutaraldehyde in 0.1 M Na phosphate buffer (pH 7.3), dehydrated, and embedded in either Epon or Lowicryl K4M.

Indirect Immunofluorescence

Sets of three serial sections (0.5 to 1.0 μm thick) of Epon-embedded islet cells were collected on glass slides and processed to remove Epon (17). All antibodies were prepared in PBS. Individual sections in this series were incubated either with guinea pig anti-insulin serum (diluted 1:400 vol/vol), monoclonal anti-DNP IgG (5 μg/ml), or monoclonal anti-proinsulin IgG (0.1 to 0.2 μg/ml) for 2 h at room temperature in a moist chamber. After washing in PBS two times for 5 min each, the sections were incubated for 1 h at room temperature with the appropriate FITC-labeled anti-IgG (diluted 1:100 vol/vol). Sections were then washed with PBS, counterstained with Evans Blue (0.03% wt/vol) and examined by transmitted fluorescence using a Leitz Ortholux fluorescence microscope.

Immunolocalization in Lowicryl Thin Sections

Colloidal gold particles (10-nm diameter) were prepared by the method of Slot and Geuze (32) and conjugated to protein A by the method of Roth et al. (27). Serial thin sections (80–100 nm thick) of Lowicryl-embedded islet cells were collected on nickel grids and processed for immunolabeling. To localize DAMP, sections were incubated for 2 h at room temperature in a moist chamber with anti-DNP IgG (0.1 μg/ml) followed by rabbit anti-mouse IgG (12 μg/ml) and protein A-gold (diluted 1:70 vol/vol) for 1 h at room temperature. Between each antibody application, the sections were washed extensively with distilled water. Proinsulin was localized by the same procedure using monoclonal anti-proinsulin IgG (GS-4-G9, 0.6 μg/ml). Insulin was localized by incubating sections for 2 h with guinea pig anti-insulin serum (diluted 1:10,000 vol/vol) and protein A-gold (diluted 1:70 vol/vol). Sections were stained with 5% aqueous uranyl acetate and lead citrate before examination with the electron microscope.

Quantitative Evaluation of Protein A–Gold Labeling

To quantify the distribution of DAMP and proinsulin-specific gold particles in the same secretory vesicles, two consecutive serial thin sections of Lowicryl-embedded islet cells were alternately stained with anti-proinsulin IgG or anti-DNP IgG. For each of three islets, 8 to 12 cells were photographed at a calibrated magnification of 12,000 in both sections of the set. When the same secretory vesicle was present in the two serial sections, the number of gold particles per μm^2 was determined for each antigen. Golgi apparatus cisternae and multigranular bodies were morphologically identified. The surface area of the different compartments as well as the number of gold particles present were recorded with an electronic pen on a Tektronix graphic tablet (type 4933) connected to an IMSAI microprocessor system (type 8080) that was programmed to calculate the number of particles per μm^2 in the compartment. A similar method was used to assess the labeling intensity over the following compartments: *cis* Golgi cisternae, *trans* Golgi cisternae, multigranular bodies (lysosomes), and nucleus. Data are presented as a mean ± SEM and a statistical comparison of the values was done with the Student's *t* test.

Estimation of pH

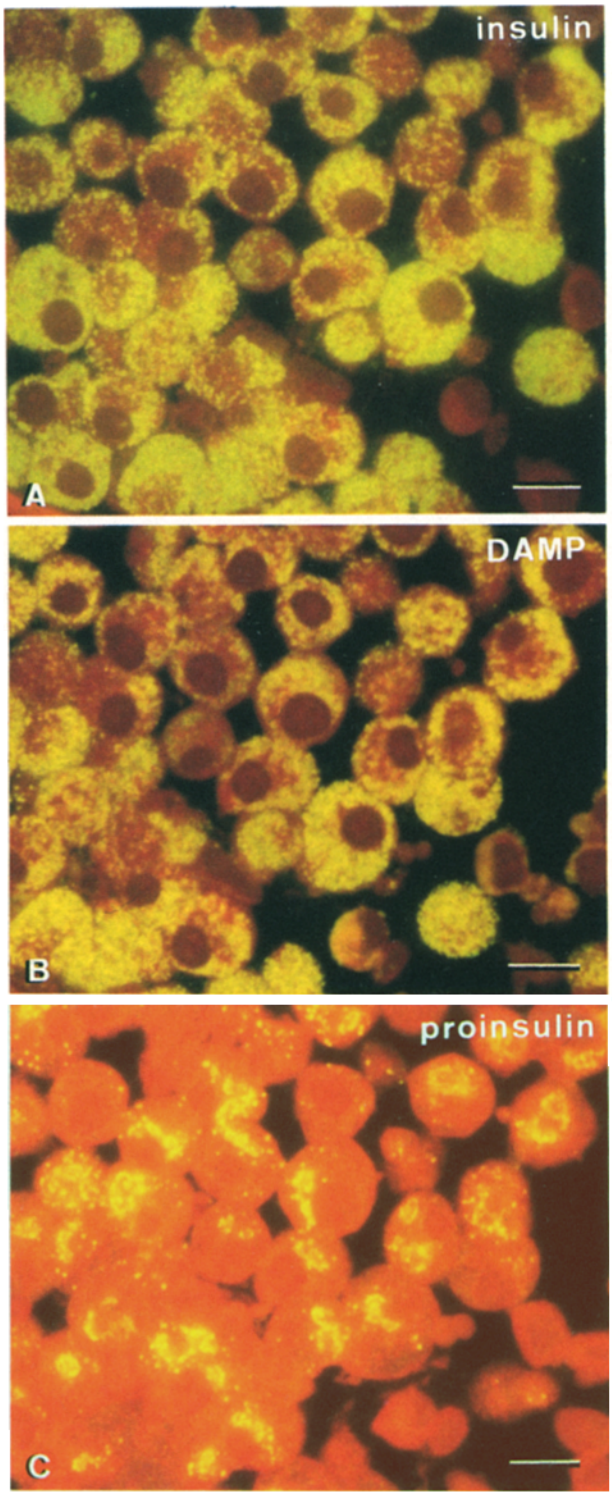
To estimate the pH of the various secretory vesicles, we assumed that DAMP accumulation in these compartments was directly proportional to the H^+ concentration and that aldehyde fixation quantitatively retained DAMP at its site of accumulation. Two important properties of DAMP support these assumptions. (*a*) Titration experiments indicate that DAMP behaves as an amine with a pK of 10.6 where protonation of each molecule involves the interaction of a single H^+ with both the primary and the tertiary amine (2). (*b*) Fixation most likely involves the cross-linking of the primary amine with proteins in the matrix of the secretory vesicle. Since each type of vesicle has a similar amount of protein, DAMP should be retained equally well in all compartments analyzed after fixation.

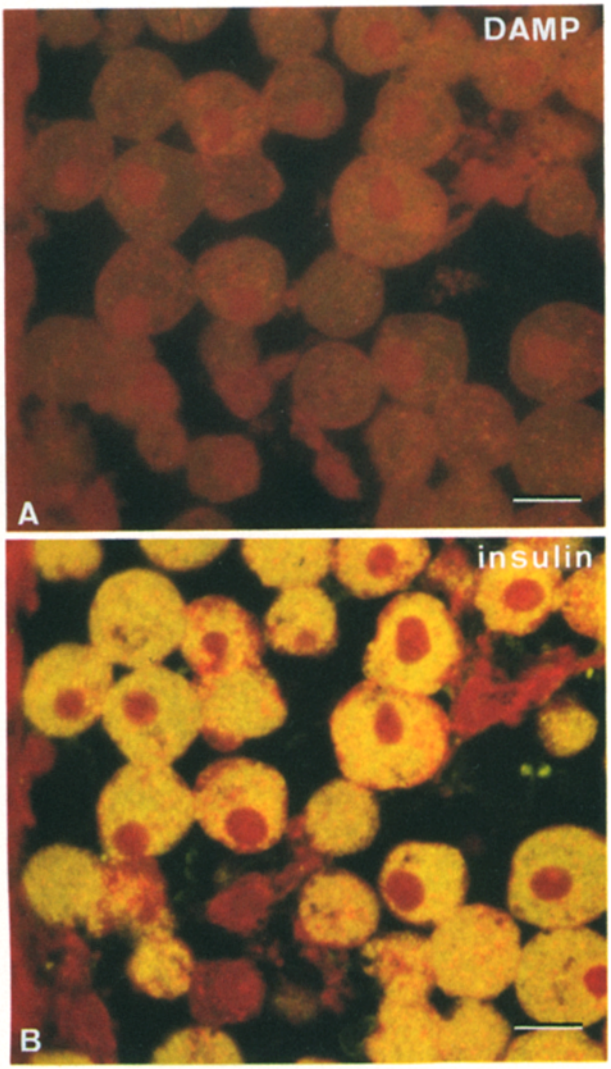
The density of gold particles due to anti-DNP IgG binding can be used to calculate pH if the number of gold particles is proportional to the H^+ concentration (14, 26). The number of gold particles in the compartment being analyzed was divided by the number of gold particles in a compartment of pH 7.0, which corresponds to the fold increase in H^+ concentration in the unknown compartment. This ratio was used to calculate pH according to the formula: $pH = 7.0 - \log \frac{D}{4}$, where 7.0 = pH at neutrality, 4 = density of gold particles in a pH 7.0 compartment (density of DAMP gold particles over the nucleus), and D = density of DAMP-specific gold particles in compartment being analyzed.

Results

Insulin-rich Secretory Vesicle Is a Low pH Compartment

Sites of DAMP accumulation in cells and tissues can be readily identified by indirect immunofluorescence (2). This affords the opportunity to survey the distribution of DAMP





Figures 1 and 2. (Fig. 1 [*left*]) Indirect immunofluorescence localization of insulin (*A*), DAMP (*B*), and proinsulin (*C*) in the same cells. Isolated islet cells were incubated for 1 h at 37°C in medium that contained 30 μM DAMP. After fixation and embedding, three serial sections, 0.5–1.0 μm thick, were prepared and each was stained to localize the indicated antigen as described in Materials and Methods. Bar, 10 μm. (Fig. 2 [*right*]) Effect of monensin on immunofluorescence localization of DAMP. Islet cells were incubated for 30 min at 37°C in the presence of 30 μM DAMP followed by an additional 30-min incubation in the presence of 25 μM monensin. After fixation and embedding, consecutive sections were stained with either anti-DNP IgG (*A*) or anti-insulin serum (*B*). Bar, 10 μm.

in large numbers of cells. In addition, indirect immunofluorescence can be used to determine the distribution of DAMP relative to other antigens of interest in the same cell by preparing consecutive semi-thin sections and staining one of them with anti-DNP IgG and the other with the antigen-specific antibody. This approach was used to map the distribution of DAMP relative to the location of insulin and proinsulin in the same cell.

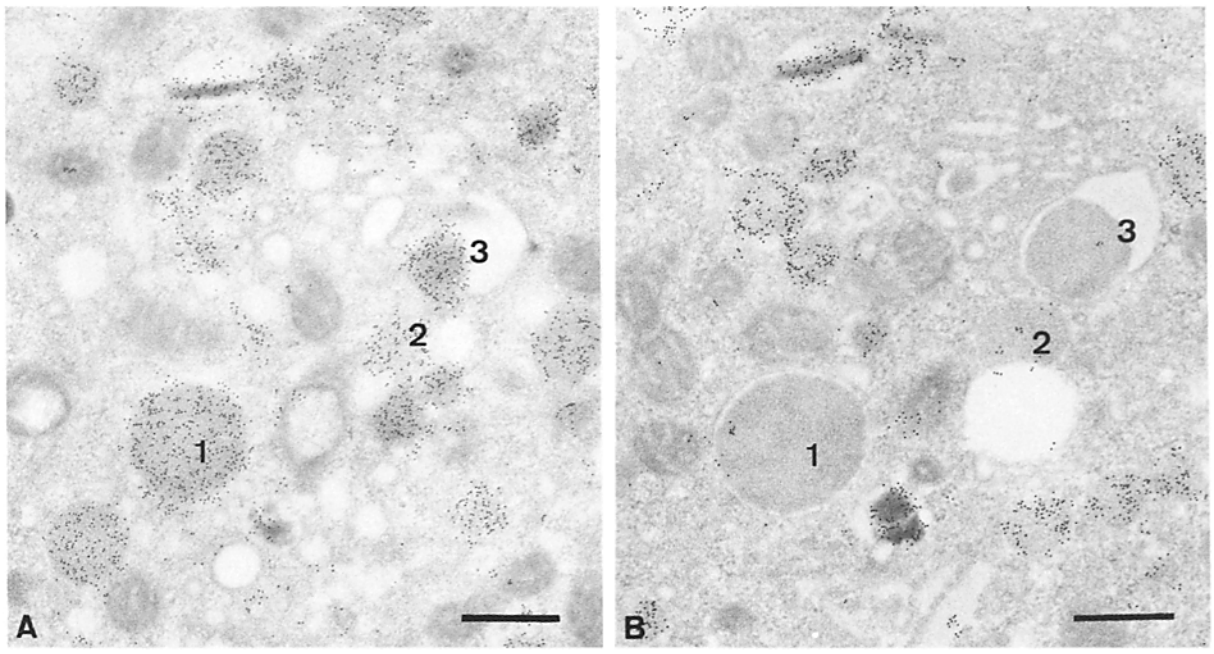


Figure 3. Localization of insulin (*A*) and DAMP (*B*) in consecutive serial thin sections. Islet cells were incubated in the presence of 30 μM DAMP for 1 h at 37°C. After fixation, the cells were embedded in Lowicryl K4M, serial sectioned (80–100 nm thick), and processed for protein A–gold localization of insulin (*A*) or DAMP (*B*). Vesicles numbered *1*, *2*, and *3* have a relatively low density of DAMP-specific gold particles. Bar, 0.5 μm.

Fig. 1 shows three serial sections (0.5–1 μm thick) through a population of cultured islet cells that had been incubated in the presence of 30 μM DAMP for 60 min at 37°C. The first section (Fig. 1 *A*) was stained with anti–insulin serum and numerous fluorescent vesicles were seen distributed throughout the cytoplasm of these cells. The second section (Fig. 1 *B*) was stained with anti–DNP IgG; the fluorescent pattern in this section was strikingly similar to the anti–insulin serum pattern (compare Fig. 1 *A* with 1 *B*). The final section in the series (Fig. 1 *C*) was used to localize proinsulin; proinsulin was confined to the perinuclear area of the cell, a distribution that corresponds to the Golgi apparatus (23). In contrast to the insulin-rich vesicles, there appeared to be little DAMP accumulation in the proinsulin-rich compartment (compare Fig. 1 *B* with 1 *C*).

DAMP accumulation was dependent on the presence of a proton gradient in the insulin-rich secretory vesicles. When DAMP-treated islet cells were post-incubated in the presence of 25 μM monensin, which dissipates proton gradients, anti–DNP IgG binding was abolished (Fig. 2 *A*). However, the anti–insulin serum staining pattern on the adjacent section was unchanged (Fig. 2 *B*).

Proinsulin Secretory Vesicle Is Less Acidic Than Insulin Secretory Vesicle

By electron microscopic immunocytochemistry, proinsulin is found principally in the Golgi cisternae and immature clathrin-coated secretory vesicles (22, 23). To determine the relationship between secretory vesicle acidification and their content of either proinsulin or insulin, we incubated cultured islet cells in the presence of 30 μM DAMP and then fixed and embedded them in Lowicryl K4M. Serial sections were prepared (80–100 nm thick) and alternate sections were used to localize either DAMP and proinsulin or DAMP and insulin with the protein A–gold technique. The same secretory vesicle was identified in both sections and assessed for the presence of DAMP and the respective antigen.

Fig. 3 shows two consecutive sections that were alternately stained for insulin (Fig. 3 *A*) and DAMP (Fig. 3 *B*). Many of the secretory vesicles contained a high concentration of both antigens, as judged from the density of gold particles. A subpopulation of these vesicles, however, had a high concentration of insulin but a low concentration of DAMP (compare density of gold particles in vesicles labeled *1–3*, Fig. 3, *A* and *B*). Since anti–insulin antibodies bind to proinsulin (23), the DAMP-poor vesicles could contain proinsulin. Therefore, we prepared a second set of serial sections and alternately stained these for proinsulin and DAMP (Fig. 4). A visual inspection of these images (compare density of gold particles in vesicles labeled *1–6* in Fig. 4, *A* and *B*) shows that the proinsulin-rich vesicles had relatively little DAMP as compared to the insulin-rich vesicles (vesicles that don't bind anti–proinsulin IgG). However, compared to the Golgi apparatus (*G*, Fig. 4 *A*), which also contained proinsulin, there appeared to be a significant amount of DAMP accumulation in the proinsulin-rich secretory vesicles. By this analysis, there appeared to be an increase in the amount of DAMP that accumulated as the secretory vesicles matured, which indicates a progressive acidification of this compartment.

These visual impressions were confirmed by a quantitative analysis of the density of DAMP-specific gold particles in the various compartments of the secretory pathway (Table I). The density of particles in the *cis* and the *trans* Golgi cisternae were not significantly above background (background = number of gold particles/μm^2 over the nucleus); however, we detected a weak but significant labeling of the forming secretory vesicles that appeared to be budding from the *trans*-most cisternae. By contrast, the density of gold parti-

cles in the proinsulin-rich vesicles and the insulin-rich vesicles averaged 71/μm^2 and 433/μm^2, respectively. Labeling in all compartments was abolished if the cells were post-incubated in the presence of monensin. Therefore, the density of gold particles due to DAMP reflected the proton concentration in the compartment.

Proinsulin-to-Insulin Conversion Occurs in an Acidic Compartment

The majority of secretory vesicles in a B-Cell either contain proinsulin, insulin, or both. Since anti-proinsulin IgG only recognizes the prohormone, the protein A-gold density due to binding of this antibody is a measure of proinsulin concentration in the secretory vesicle. Therefore, a decrease in the density of proinsulin-specific gold particles in an individual vesicle is a function of the amount of proinsulin that has been converted to insulin. We used this knowledge to determine the relationship between vesicle acidification (density of DAMP-specific gold particles) and the conversion of proinsulin to insulin in individual secretory vesicles.

Cultured islet cells that had been incubated in the presence of 30 μM DAMP were embedded in Lowicryl K4M. Consecutive thin sections were prepared and alternately stained with anti-proinsulin IgG or anti-DNP IgG. We identified 200 secretory vesicles that were present in both sections and determined the density of DAMP-specific and proinsulin-specific gold particles in each vesicle. In Fig. 5, the proinsulin gold density is plotted on the ordinate and the DAMP gold density on the abscissa. The density of DAMP-specific gold particles ranged from 0–800 per μm^2 in the various secretory vesicles. The vesicles that contained a high density of proinsulin-specific gold particles, however, had a DAMP gold density in the lower range of this distribution (0–200 per μm^2).

If acidification of secretory vesicles is required for conversion of proinsulin to insulin, then there may be a critical pH required for conversion to take place. This would be reflected by the concentration of DAMP in each of the secretory vesicles that contained either proinsulin or insulin. Therefore, from the data in Fig. 5, the secretory vesicles were subdivided into eight groups covering a DAMP concentration from 4 to 800 gold particles/μm^2 and the average density of proinsulin-specific gold particles for each group was calculated (Fig. 6). Each of the groups from four DAMP gold particles/μm^2 to 127 DAMP particles/μm^2 had the same average density of proinsulin-specific gold particles, which indicates that the concentration of proinsulin was the same in these compartments. However, in the sets of vesicles that had a higher density of DAMP-specific gold particles, there was a marked decline in the concentration of proinsulin. This suggests that the pH of the vesicles must decline to a critical level before proinsulin is converted to insulin. Moreover, once this pH is reached, prohormone conversion occurs very rapidly.

Discussion

Previous studies (22, 23) have documented that proinsulin to insulin conversion is a dynamic process that occurs in a subpopulation of secretory vesicles that are decorated by a clathrin coat. Because monensin inhibits conversion (22), these investigators suggested that the proteolytic processing of the prohormone depends on a low pH environment. In support of this idea, the pH optimum for the enzymes thought to process proinsulin is ~pH 5.5 (5, 6, 33) and isolated insulin-rich secretory vesicles have an acidic interior that is maintained by an ATP-dependent proton pump (11). The application of the DAMP technique has allowed us to show directly that the proinsulin-rich vesicles are acidic but that the pH declines further in the mature insulin-rich secretory vesicle.

The quantification procedure that we have used allows us to estimate the time-dependent change in pH as the secretory vesicle matures (see Materials and Methods for calculations). The immature vesicle can be distinguished from the mature vesicle by their reactivity to anti-proinsulin IgG, and the density of gold particles due to DAMP accumulation can be used to estimate the pH. Using this approach, the average pH of the proinsulin compartment was estimated to be pH 5.7 and the insulin-rich compartment averaged pH 5.0. Our estimate of the pH of mature insulin-containing secretory vesicles (pH 5.0) is in good agreement with the pH measured in isolated insulin-rich vesicles (pH 5.5–5.0) by Hutton (11).

Based on our analysis of the density of anti-proinsulin IgG binding sites in vesicles with different densities of anti-DNP IgG binding sites, there appears to be a critical pH that is required for conversion of proinsulin to insulin to take place. From the DAMP-specific gold particle density in Fig. 6, we estimate this to be pH 5.5. This estimate is in good agreement with the known pH optimum (pH 5.5 [33]) of the peptidases that are thought to cleave the C-peptide. What is puzzling, however, is that our results suggest that cleavage only occurs within a narrow pH "window" yet these peptidases have significant activity above and below their pH optimum (6).

The density of DAMP-specific gold particles in both immature and mature secretory vesicles was quite variable. This may reflect a heterogeneity of pH in these compartments due to different rates of proton pumping. On the average, however, the pH of the mature vesicles appeared to be considerably below that required for the processing of proinsulin to insulin. Quite possibly the additional drop in pH provides an environment that supports other important chemical reactions that must take place for proper secretion. For example, the lower pH may promote the crystallization of insulin (for discussion see reference 11) or play a role in the exocytosis of the secretory vesicle when the cell is properly stimulated. In addition, there may be a relationship between vesicle pH and the targeting of some insulin-rich secretory vesicles to lysosomes for crinophagy (24).

These data suggest a model for how the processing of proinsulin to insulin is regulated. The pH in the cisternae of the Golgi apparatus appeared to be near neutrality; therefore, the proinsulin in this compartment cannot be processed because the appropriate enzymes are not active. Proinsulin, together with the proteolytic enzymes, are packaged into *trans* Golgi vesicles that are surrounded by a membrane that contains the ATP-dependent proton pump. As the proton pump transports H^+ into these vesicles, the pH decreases, the proteolytic enzymes are activated, and the C-peptide is cleaved from the A and the B chains of insulin. Therefore, for proper processing of prohormone, four distinct molecules (the two proteases, the proinsulin, and the proton pump) must be coordinately segregated into the same secretory vesicle as it buds from the *trans* cisternae of the Golgi apparatus.

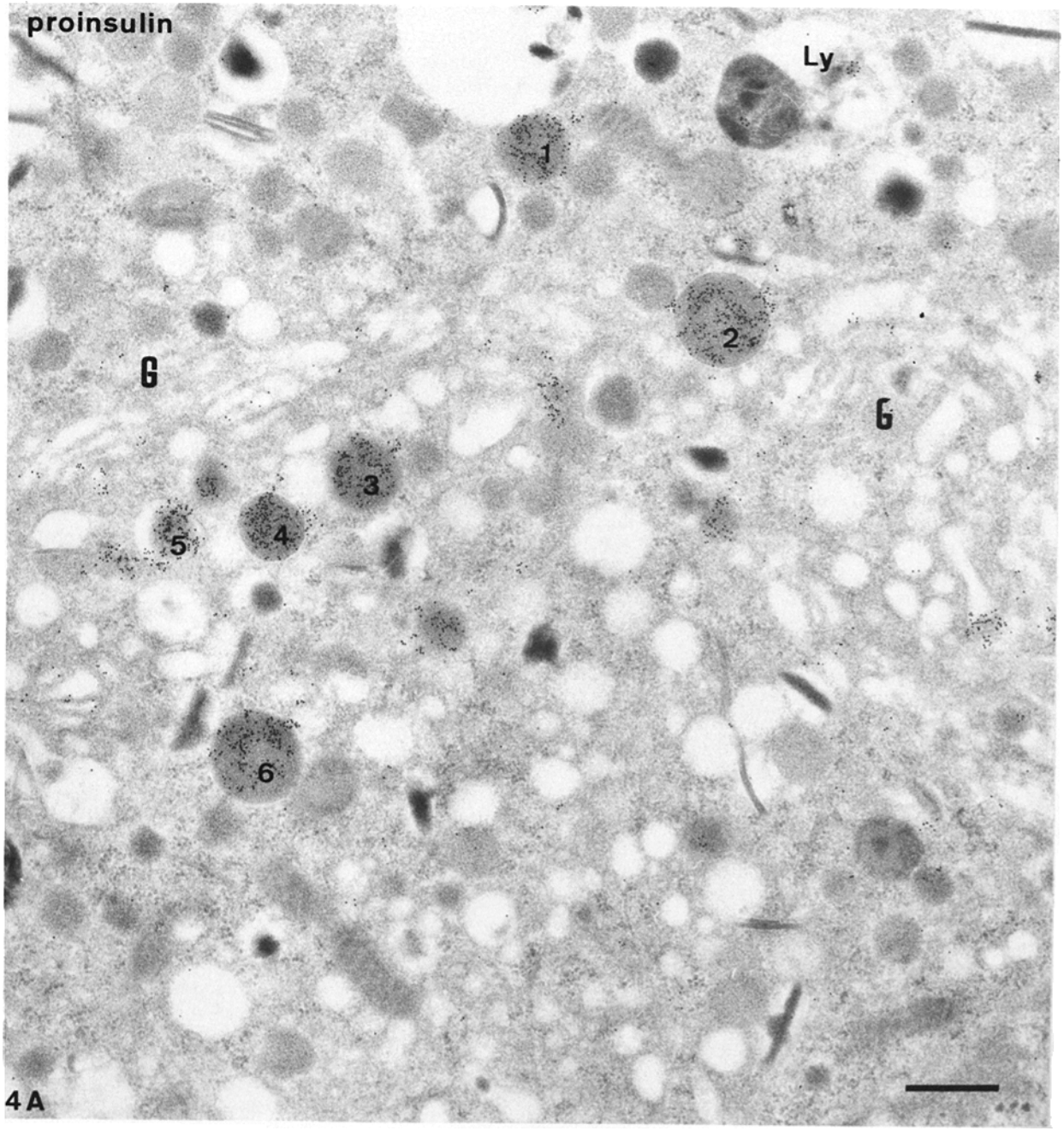


Table I. Density of Monoclonal Anti-DNP Binding Sites

	Number of gold particles/μm^2 of cell compartment		
		Exp. 2	
Cellular compartment	Exp. 1	Control	Monensin
Cis Golgi cisternae	4 ± 1 (17.7)	3 ± 1 (21.7)	–
Trans Golgi cisternae	5 ± 1 (13.9)	4 ± 1 (17.2)	–
Condensing vesicles*	15 ± 4 (3.8)	12 ± 4 (2.6)	–
Total Golgi apparatuses	–	–	2 ± 0.1 (20.4)
Proinsulin-rich vesicles	74 ± 7 (10.8)	68 ± 12 (6.2)	–
Insulin-rich vesicles	380 ± 20 (12.1)	486 ± 39 (6.7)	7 ± 0.2 (5.67)
Multigranular bodies (secondary lysosomes)	245 ± 21 (4.2)	170 ± 16 (5.7)	9 ± 1 (3.7)
Nucleus	5 ± 1 (77)	3 ± 0.3 (62.7)	2 ± 01 (26.6)

Cultured islet cells were incubated in the presence of 30 μM DAMP for 1 h at 37°C and processed directly for immunocytochemistry (Exp. 1). In Exp. 2, the cells were incubated an additional 30 min in the presence or absence of 25 μM monensin at 37°C before processing. Proinsulin-rich verses insulin-rich compartments were distinguished by the presence (>150 gold particles/μm^2) or absence of anti-proinsulin IgG binding. The other compartments were identified by morphology. The data are presented as the mean ± SEM. The numbers in parenthesis indicate the number of μm^2 evaluated for each compartment.
* Proinsulin-rich vesicles aligned with, but separate from, the *trans*-most Golgi cisternae.

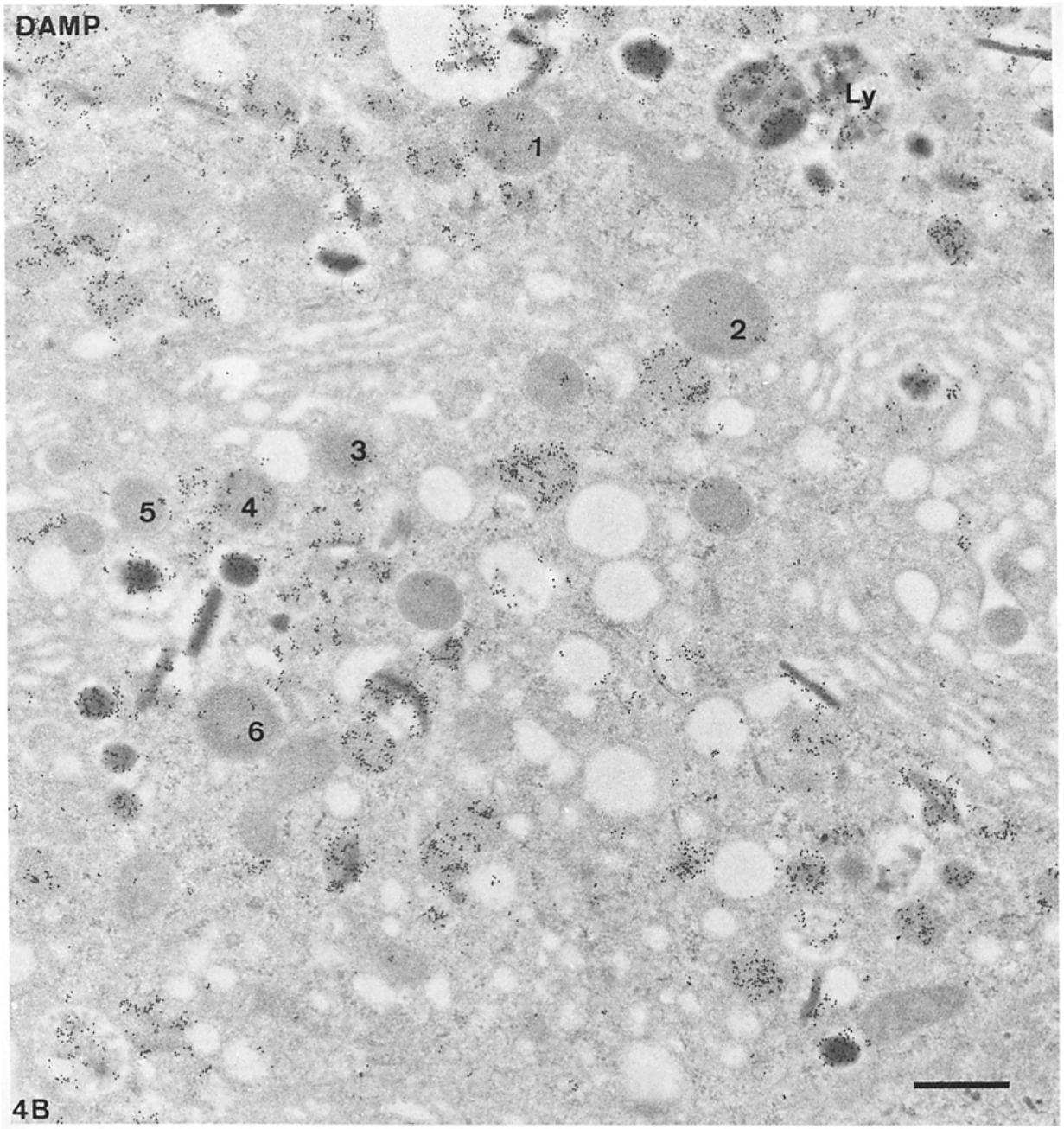


Figure 4. Localization of proinsulin (*A*) and DAMP (*B*) in consecutive serial thin sections. Islet cells were processed as described in Fig. 3. Serial thin sections were prepared and alternately stained with anti-proinsulin IgG (*A*) or anti-DNP IgG (*B*) followed by protein A-gold. Vesicles numbered *1-6* appeared in both sections. *G*, Golgi apparatus; *Ly*, multigranular body. Bar, 0.5 μm.

This study extends our analysis of acidic compartments in the secretory pathway in two ways. First, in human fibroblasts (1) and hepatoma cells (31) the trans Golgi cisternae are clearly acidic; however, we found that in islet cells there was little acidification of any Golgi cisternae. Therefore, acidification of this compartment can be regulated to serve the needs of individual types of cells. Second, we now have localized two secreted proteins, proinsulin and fibronectin (1), to acidic *trans* Golgi vesicles. Whereas the secretion of insulin is strictly regulated, the secretion of fibronectin in human fibroblasts generally is not regulated (36), although secretion can be stimulated by glucocorticoids (21). Therefore, even though there is good evidence that regulated and nonregulated secretory pathways are separate within the same cell (12) and that in some cases the sorting of the two types of secretory proteins may be dependent on low pH (18), our results suggest that both classes of proteins may pass through acidic compartments. Additional studies using the DAMP procedure to colocalize acidic compartments together with regulated and nonregulated secretory proteins in the same cell are needed to better understand the relationship between acidification and secretion.

These studies clearly establish the range of applications of the DAMP technique. Not only can we obtain quantitative in-

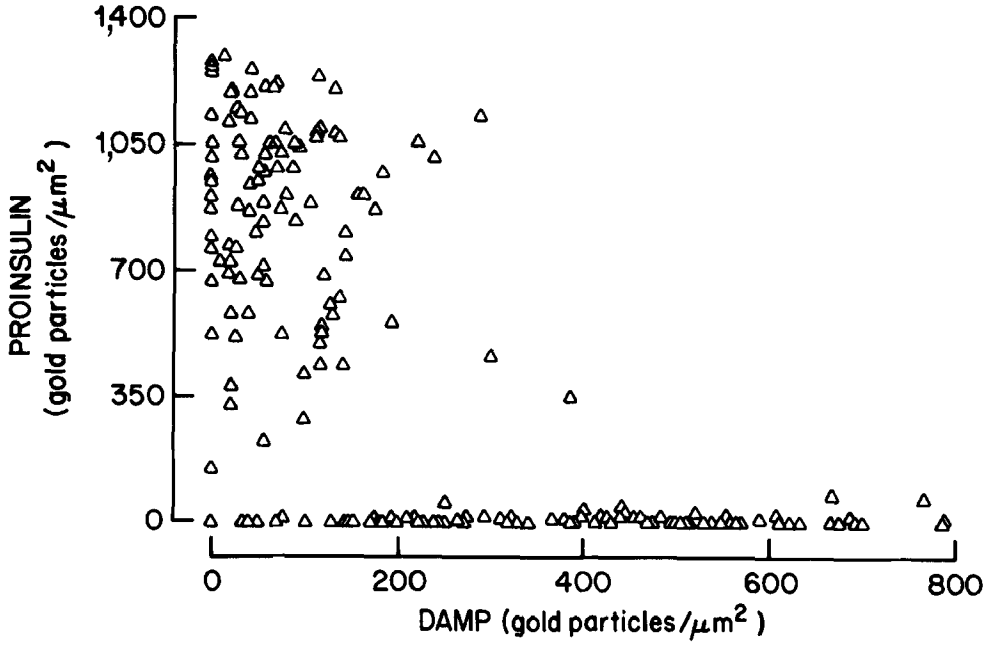


Figure 5. Concentration of proinsulin vs. the concentration of DAMP in the same secretory vesicle. Consecutive serial thin sections of cultured islet cells that had been prepared as described in Fig. 3 were alternately stained with anti-proinsulin IgG or anti-DNP IgG. After the incubation, both sections were processed for protein A-gold localization of the antibodies. 200 secretory vesicles that could be identified in both sections (see Fig. 4) were assessed for the number of gold particles per μm^2 due to the binding of anti-proinsulin IgG and anti-DNP IgG.

formation about the co-distribution of DAMP and specific intracellular antigens but we also can use this information to estimate relative pH values for these compartments. This permits the identification of compartments where the pH is regulated in response to specific stimuli. This should be a powerful adjunct to existing techniques for investigating the function of intracellular low pH compartments in various types of cells.

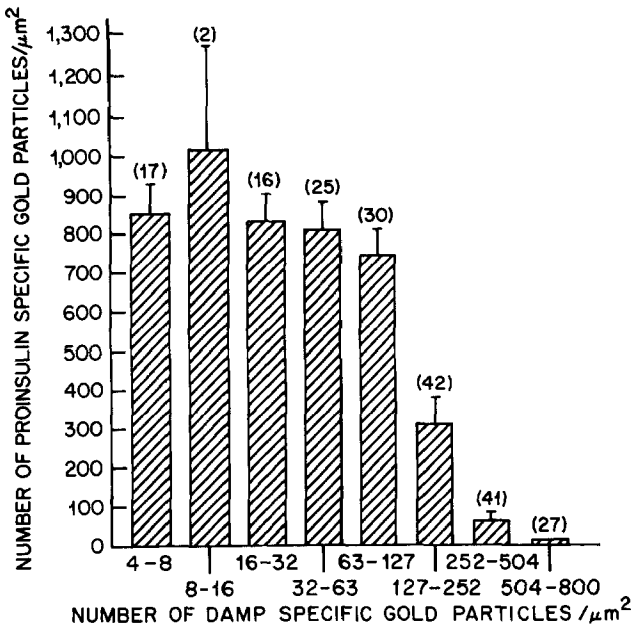


Figure 6. Average density of anti-proinsulin IgG binding sites in secretory vesicles with different densities of anti-DNP IgG binding sites (concentrations of DAMP). From Fig. 5, sets of secretory vesicles were grouped according to the indicated density of DAMP-specific gold particles. The density of proinsulin-specific gold particles for each secretory vesicle in this range was averaged. Data is presented as mean ± SEM.

We wish to thank H. C. Hutton, M. S. Brown, and J. L. Goldstein for critical reading of the manuscript; M. Baumann, A.-M. Lucini, G. Perrelet, and G. Tripet for technical assistance; G. Negro and P.-A. Rüttimann for photographic work; and I. Bernard and M. Surovik for the typing of the manuscript.

This work was supported by the Swiss National Science Foundation Nr. 3.404.86 (to L. Orci) and by grant HL 20948 from the US Public Health Service National Institutes of Health (to R. G. W. Anderson).

Received for publication 2 June 1986, and in revised form 12 August 1986.

References

1. Anderson, R. G. W., and R. K. Pathak. 1985. Vesicles and cisternae in the *trans* Golgi apparatus of human fibroblasts are acidic compartments. *Cell.* 40:635–643.

2. Anderson, R. G. W., J. R. Falck, J. L. Goldstein, and M. S. Brown. 1984. Visualization of acidic organelles in intact cells by electron microscopy. *Proc. Natl. Acad. Sci. USA.* 81:4838–4842.

3. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell.* 32:663–667.

4. Cidon, S., H. Ben-David, and N. Nelson. 1983. ATP-driven proton fluxes across membranes of secretory organelles. *J. Biol. Chem.* 258:11684–11688.

5. Docherty, K., and D. F. Steiner. 1982. Post-translational proteolysis in polypeptide hormone biosynthesis. *Annu. Rev. Physiol.* 44:625–638.

6. Docherty, K., R. Carroll, and D. F. Steiner. 1982. Conversion of proinsulin to insulin: involvement of a 31,500 molecular weight thiol-protease. *Proc. Natl. Acad. Sci. USA.* 79:4613–4617.

7. Forgac, M., and L. Cantley. 1984. Characterization of the ATP-dependent proton pump of clathrin-coated vesicles. *J. Biol. Chem.* 259:8101–8105.

8. Forgac, M., L. Cantley, B. Wiedenmann, L. Altstiel, and D. Branton. 1983. Clathrin-coated vesicles contain an ATP-dependent proton pump. *Proc. Natl. Acad. Sci. USA.* 80:1300–1303.

9. Galloway, C. J., G. E. Dean, M. Marsh, G. Rudnick, and I. Mellman. 1983. Acidification of macrophage and fibroblast endocytic vesicles *in vitro*. *Proc. Natl. Acad. Sci. USA.* 80:3334–3338.

10. Glickman, J., K. Croen, S. Kelly, and Q. Al-Awqati. 1983. Golgi membranes contain an electrogenic H^+ pump in parallel to a chloride conductance. *J. Cell Biol.* 97:1303–1308.

11. Hutton, J. C. 1982. The internal pH and membrane potential of the insulin-secretory granule. *Biochem. J.* 204:171–178.

12. Kelly, R. B., T. L. Burgess, and H.-P. H. Moore. 1985. Sorting of secretory proteins in an endocrine cell line studied by DNA transfection. *In* Protein Transport and Secretion. M. J. Gething, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 156–161.

13. Lacy, P. E., and M. Kostianovsky. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 16:35–39.

14. Lyman, G. E., and W. J. Waddell. 1977. pH gradients in the developing

teeth of young mice from autoradiography of [^{14}C]DMO. *Am. J. Physiol.* 232:F364–F367.

15. Madsen, O. D., R. M. Cohen, F. W. Fitch, A. H. Rubenstein, and D. F. Steiner. 1983. The production and characterization of monoclonal antibodies specific for human proinsulin using a sensitive microdot assay procedure. *Endocrinology.* 113:2135–2144.

16. Madsen, O. D., B. H. Frank, and D. F. Steiner. 1984. Human proinsulin-specific antigenic determinants identified by monoclonal antibodies. *Diabetes.* 33:1012–1016.

17. Maxwell, M. H. 1978. Two rapid and simple methods used for the removal of resins from 1.0 μm thick epoxy section. *J. Microsc.* 112:253–255.

18. Moore, H.-P., B. Gumbiner, and R. B. Kelly. 1983. Chloroquine diverts ACTH from a regulated to a constitutive secretory pathway in AtT-20 cells. *Nature (Lond.).* 302:434.

19. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.

20. Ohkuma, S., Y. Moriyama, and T. Takano. 1982. Identification and characterization of a proton pump on lysosomes by fluorescein-isothiocyanate-dextran fluorescence. *Proc. Natl. Acad. Sci. USA.* 79:2758–2762.

21. Oliver, N., R. F. Newby, L. T. Furcht, and S. Bourges. 1983. Regulation of fibronectin biosynthesis by glucocorticoids in human fibrosarcoma cells and normal fibroblasts. *Cell.* 33:287–296.

22. Orci, L., P. Halban, M. Amherdt, M. Ravazzola, J.-D. Vassalli, and A. Perrelet. 1984. A clathrin-coated, Golgi-related compartment of the insulin secreting cell accumulates proinsulin in the presence of monensin. *Cell.* 39:39–47.

23. Orci, L., M. Ravazzola, M. Amherdt, O. Madsen, J.-D. Vassalli, and A. Perrelet. 1985. Direct identification of prohormone conversion site in insulin-secreting cells. *Cell.* 42:671–681.

24. Orci, L., M. Ravazzola, M. Amherdt, C. Yanaihara, N. Yanaihara, P. Halban, A. E. Renold, and A. Perrelet. 1984. Insulin, not C-peptide (proinsulin), is present in crinophagic bodies of the pancreatic B-cell. *J. Cell Biol.* 98:222–228.

25. Reeves, J. P. 1984. The mechanism of lysosomal acidification. *In* Lysosomes in Biology and Pathology. Vol. 7. J. T. Dingle, R. T. Dean, and W. Sly, editors. Elsevier Science Publishers, New York. 175–199.

26. Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.

27. Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1074–1081.

28. Rudnick, G. 1986. ATP-driven H^+ pumping into intracellular organelles. *Annu. Rev. Physiol.* In press.

29. Scherman, D., J. Nordmann, and J.-P. Henry. 1982. Existence of an adenosine 5′-triphosphate-dependent proton translocase in bovine neurosecretory granule membrane. *Biochemistry.* 21:687–694.

30. Schneider, D. L. 1981. ATP-dependent acidification of intact and disrupted lysosomes: evidence for an ATP-driven proton pump. *J. Biol. Chem.* 256:3858–3864.

31. Schwartz, A., G. J. A. M. Strous, J. W. Slot, and H. J. Geuze. 1985. Immunoelectron microscopic localization of acidic intracellular compartments in hepatoma cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:899–904.

32. Slot, J. W., and H. J. Geuze. 1985. A new method for preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 38:87–93.

33. Steiner, D. F., K. Docherty, and R. Carroll. 1984. Golgi/granule processing of peptide hormone and neuropeptide precursors: a minireview. *J. Cell. Biochem.* 24:121–130.

34. Stone, D. K., X.-S. Xie, and E. Racker. 1982. An ATP-driven proton pump in clathrin-coated vesicles. *J. Biol. Chem.* 258:4059–4062.

35. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α-2-macroglobulin. *Cell.* 28:643–651.

36. Uchida, N., H. Smilowitz, P. W. Ledger, and M. L. Tanzer. 1980. Kinetic studies of the intracellular transport of procollagen and fibronectin in human fibroblasts. *J. Biol. Chem.* 255:8638–8644.

37. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B. *J. Cell Biol.* 63:430–440.

38. Yamashiro, D. J., and F. R. Maxfield. 1984. Acidification of endocytic compartments and the intracellular pathways of ligands and receptors. *J. Cell. Biochem.* 26:231–246.

39. Yamashiro, D. J., B. Tycko, S. R. Floss, and F. R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell.* 37:789–800.

40. Zhang, F., and D. L. Schneider. 1983. The bioenergetics of Golgi apparatus function: evidence for an ATP-dependent proton pump. *Biochem. Biophys. Res. Commun.* 114:620–625.