# pH-Independent and -Dependent Cleavage of Proinsulin in the Same Secretory Vesicle

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*Abstract.* By quantitative immunoelectron microscopy and HPLC, we have studied the effect of disrupting pH gradients, by ammonium chloride, on proinsulin conversion in the insulin-producing B-cells of the islets of Langerhans. Proinsulin content and pH in single secretory vesicles were measured on consecutive serial sections immunostained alternately with anti-proinsulin or anti-dinitrophenol (to reveal the pH-sensitive probe DAMP) antibodies. Radioactively labeled proinsulin, proinsulin cleavage intermediates, and insulin were quantitated by HPLC analysis of extracts of islets treated in the same conditions. Cleavage at the C-peptide/A-chain junction is significantly less sensitive to pH gradient disruption than that of the B-chain/C-peptide junction, but the range of pH and proinsulin content in individual vesicles indicate that both cleavages occur in the same vesicle released from the TGN.

ACIDIFICATION of many of the intracellular membrane bounded compartments that are created on the endocytotic and secretory pathways is coupled to orderly protein processing and membrane traffic. For example, during receptor-mediated endocytosis, low pH promotes the dissociation of the ligand from its receptor before fusion of the endosome with the lysosome. Acidification of vesicles originating from the TGN activates proteolytic enzymes that generate functional hormones by cleaving precursor proteins (e.g., prohormones) as they emerge from the Golgi (for review see Anderson and Orci, 1986; Mellman et al., 1986).

Acidification is mediated by a membrane ($H^+$)-ATPase that is able to maintain variable concentrations of protons within the vesicle lumen. As a consequence, the pH of sequential compartments in an endocytic or secretory pathway can change from 7.0 to as low as 4.5 (for review see Al-Awqati, 1986). There is also clear evidence that in certain cells the pH of a compartment is tightly regulated (Mallya et al., 1992). Therefore, pH is often used to modulate chemical reactions that take place within intracellular vesicles.

In the insulin-producing B-cell of the islet of Langerhans, biochemical and immunocytochemical studies have shown that the synthesis of proinsulin in the endoplasmic reticulum and its transport to the TGN occur in a relatively neutral pH environment. The conversion of proinsulin to insulin, however, is initiated in acidic (~pH 6.0) clathrin-coated secretory vesicles, just after they bud from the TGN. These vesicles mature through further progressive acidification and continued conversion of proinsulin, and the loss of the clathrin coat. Mature secretory vesicles are thus markedly acidic (~pH 5.0) and contain essentially only insulin and C-peptide (Orci et al., 1985, 1986, 1987*a*).

Proinsulin is a single polypeptide chain composed of the B- and A-chains of insulin joined together by the C-peptide. The conversion process requires two enzymes, PC1 (3) and PC2 (Bailyes and Hutton, 1992; Bailyes et al., 1992; Bennett et al., 1992; Davidson et al., 1988). These enzymes are members of a mammalian family of endoproteases related to yeast KEX-2 (for review see Steiner et al., 1992). PC1(3) cleaves at the B-chain/C-peptide junction and is strictly dependent on a low pH for activity in vitro. PC2, by contrast, cleaves at the C-peptide/A-chain junction. Although this enzyme has an acidic pH optimum, it remains quite active even at neutral pH in vitro (Davidson et al., 1988). The kinetics of cleavage at the two proinsulin junctions may thus be expected to display a differential sensitivity to pH, but this has not been investigated in the natural setting of the secretory vesicle in vivo.

To address this problem, we have used glucose to regulate insulin biosynthesis in B-cells, and ammonium chloride to raise the pH during processing. The processing of the prohormone was measured by immunocytochemistry (Orci et al., 1985) and HPLC (to distinguish between the two partially processed intermediate forms of proinsulin) (Sizonenko and Halban, 1991), while the vesicle pH was monitored by exposing B-cells to 3-(2,4-dinitroanilino)-3'-amino-*N*-meth-

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yldipropylamine (DAMP)[1], a basic congener of dinitrophenol that concentrates in acidic compartments and is retained there after aldehyde fixation (Anderson et al., 1984). We found that the vesicle population where the bulk of proinsulin conversion takes place, the immature clathrin-coated vesicle (Orci et al., 1985, 1986, 1987*a*), can vary in pH from 7.0 to 5.5 (mean ~6.3). Intravesicular splitting of proinsulin at the C-peptide/A-chain junction appears to be relatively independent of pH, whereas that at the B-chain/C-peptide junction shows a marked acidic pH dependence. Both cleavage events, however, occur in the same vesicle compartment.

## Materials and Methods

### Materials

Ammonium chloride was purchased from Merck Sharp & Dohme (St. Louis, MO). DAMP was from one of us (R. G. W. Anderson). Protein A was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Gold chloride was from Fluka Chemicals (Buchs, Switzerland). Lowicryl $K_4M$ resin was purchased from Reactolab (Servion, Switzerland).

[$^{35}$S]methionine was obtained from Amersham International (Amersham, Bucks, UK); Lumaflow II liquid scintillant was from Lumac (Groningen, The Netherlands). All other chemicals were analytical grade from Sigma Chem. Co. (St. Louis, MO) or from Fluka Chemicals.

***Antibodies.*** Monoclonal anti-DNP (dinitrophenol) antibody was from one of us (R. G. W. Anderson). Anti-proinsulin antibody (monoclonal GS-9A8, Madsen et al., 1984), was obtained from Dr. O. D. Madsen (Gentofte, Denmark). Rabbit anti-mouse IgG was from Miles Yeda (Rehovot, Israel); rabbit anti-mouse IgG coupled to FITC was from Biosys (Compiègne, France).

### Methods

***Immunocytochemistry.*** Islets freshly isolated (Lacy and Kostianovsky, 1967) from Sprague-Dawley rats, strain SIVZ, were preincubated in the presence of 16.7 mM glucose for 1 h at 37°C. At the end of the preincubation, aliquots of islets were maintained in high glucose concentration, while others were transferred for 1 h to a medium containing 1.67 mM glucose in the presence or absence of 25 mM ammonium chloride. After this second incubation, all islets were exposed to 30 μM DAMP for an additional 30 min. The islets were fixed with 1% glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.4. The samples were subsequently dehydrated in ethanol and embedded either in Epon 812 for immunofluorescence, or in Lowicryl $K_4M$ (Armbruster et al., 1982) at low temperature for electron microscope immunocytochemistry.

For light microscopy, semithin sections (0.5–1 μm) of Epon-embedded islets were collected on glass slides. After Epon removal (Maxwell, 1978), sections were incubated with the anti-proinsulin monoclonal antibody GS-9A8 diluted 1:100 for 2 h at room temperature, washed with PBS and exposed to FITC-conjugated rabbit anti-mouse IgG (1:100) for 1 h at room temperature. The sections were washed with PBS, counterstained with 0.03% Evans blue and examined with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

For electron microscopy, thin sections of Lowicryl-embedded islets were picked up on parlodion-coated nickel grids. Immunolabeling was made with the monoclonal anti-proinsulin GS-9A8 antibody diluted 1:3,000, or the monoclonal anti-DNP antibody to reveal DAMP, at 0.4 μg IgG/ml. Incubation for 2 h at room temperature with the respective antibodies was followed by exposure to rabbit anti-mouse IgG (1:400), then to the protein A–gold (gold particles diameter 10 nm) for 1 h at room temperature, with one washing in distilled water between each incubation step (Roth et al., 1978). Sections were contrasted with uranyl acetate and lead citrate and observed in a Philips electron microscope (Philips, Eindhoven, The Netherlands).

***Quantitative Evaluation.*** The labeling density of anti-DNP and anti-proinsulin antibodies was evaluated on pairs of consecutive ultrathin sections. In this way, the respective DAMP and proinsulin content of the same individual secretory vesicle could be estimated. For each vesicle, the number of protein A–gold particles present over the core of the vesicle was recorded with the aid of an electronic pen connected to a personal computer programmed to calculate the number of particles per unit surface area of the core. $NH_4Cl$ treatment induced an enlargement of the clear halo surrounding the dense core but this did not influence the measured numbers of gold particles since they were all related to the unit area (1 $\mu m^2$) of the core. The estimation of pH values on the basis of the numbers of DAMP immunogold particles was carried out as described previously (Orci et al., 1986). In brief, this estimation was made by calculating the relationship $pH = 7.0 - \log D_1/D_2$ where 7.0 = pH of neutrality, $D_2$ = density of DAMP immunogold particles in a neutral compartment (background labeling over the nucleus or mitochondria for example), and $D_1$ = density of DAMP immunogold particles in the secretory vesicle compartment. This method of calculation does not, by definition, yield pH values above 7.0.

***Biochemistry.*** Freshly isolated islets were maintained overnight in tissue culture to allow for recovery from the isolation procedure, and then used for the pulse–chase experiments, according to the following protocols.

Islets were labeled for 1 h at 37°C in 200 μl Krebs-Ringer-bicarbonate buffer, 10 mM Hepes, 0.5% BSA (KRB-Hepes-BSA), 16.7 mM glucose containing 0.1 mCi [$^{35}$S]methionine (specific activity >1,000 Ci/mM). After three washings with KRB-Hepes-BSA containing 1.67 mM glucose, islets were transfered to 1.5-ml microfuge tubes (200–400 islets/tube). One group of labeled islets was retained for analysis, whereas the remainder were incubated for 1 h at 37°C in 250 μl KRB-Hepes-BSA, 1.67 mM glucose with or without addition of 25 mM ammonium chloride. At the end of this chase period, the tubes were centrifuged (100 rpm; 2 min) to pellet the islets. The supernatant was transferred to a separate tube and 100 μl 0.1 M HCl, 0.1% BSA added to the pellet to extract cellular proteins as described previously (Rhodes and Halban, 1988). Cell extracts and incubation buffer were centrifuged (40,000 *g*; 10 min; 2–4°C) to remove cell debris and the supernatants stored at −20°C for analysis.

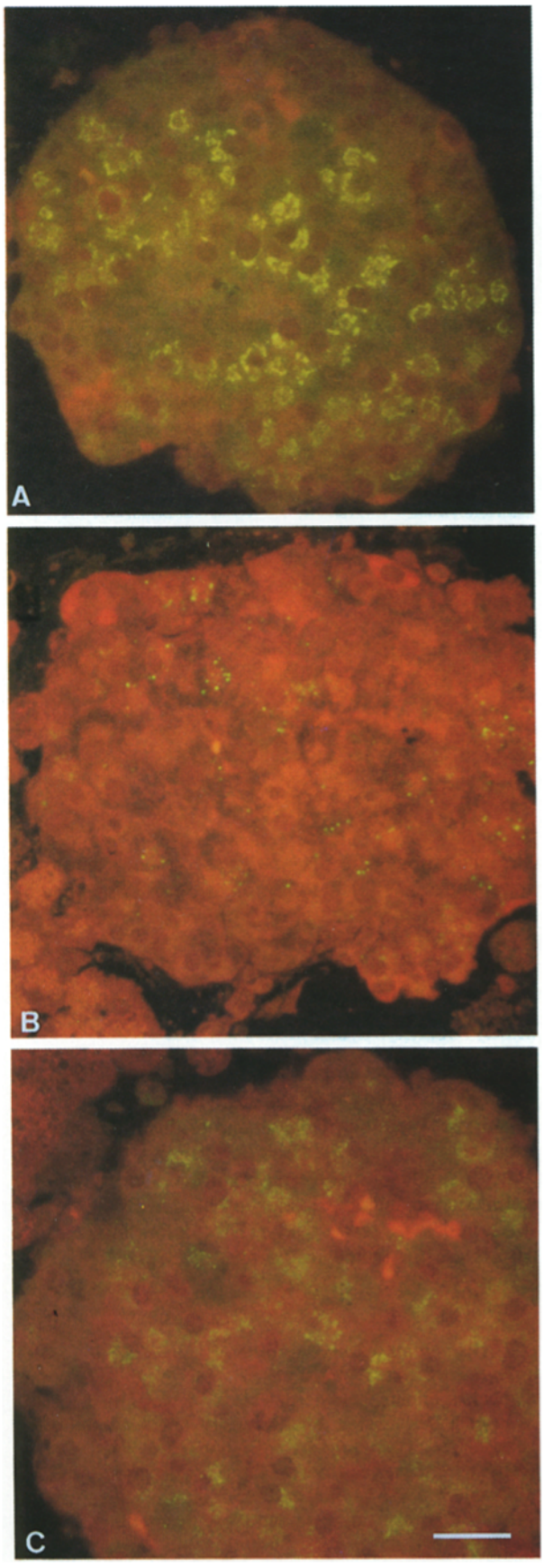
In order to separate radiolabeled proinsulin from insulin, aliquots of islet extracts or of incubation buffer were analyzed by reversed phase HPLC as described previously (Halban et al., 1986; Gross et al., 1988; Sizonenko and Halban, 1991). In brief, samples were injected onto a Beckman Ultrasphere ODS 5 μm column (dimensions 4.5 × 250 mm) connected to a Beckman System Gold HPLC apparatus, using TEAP (20 mM triethylamine, 50 mM phosphoric acid, 50 mM perchloric acid, pH 3.0) as buffer A and 90% acetonitrile/10% $H_2O$ as buffer B. The flow rate was 1 ml/min and 1-min fractions were collected. Radioactivity in each fraction was measured in a liquid scintillation counter after the addition of 3.5 ml Lumaflow II. Rat insulins were first eluted isocratically at 34% buffer B, followed by a linear increase in buffer B to 36% over 55 min to elute proinsulins and their conversion intermediates. Peak identity is based upon our previous characterization (Sizonenko and Halban, 1991). The samples were injected without prior immunoprecipitation in accordance with our established methodology for analyzing radiolabeled proinsulin and insulin related peptides in islets (Halban et al., 1986). The total radioactivity associated with insulin, conversion intermediates and proinsulin was >50,000 CPM for each sample injected into HPLC. As a control of the morphological counterpart of the studies, we added DAMP (30 μM) during the last 30 min of the chase incubation at low glucose. The same HPLC profile of proinsulin conversion was found in the presence or in absence of DAMP (data not shown).

## Results

### Relationship between Vesicle pH and Conversion of Proinsulin to Insulin

During the first hour of incubation in high glucose (16.7 mM) medium, the Golgi and immature secretory vesicles become loaded with proinsulin. Further incubation for one hour in low (1.67 mM) glucose medium allows one to follow the fate of preformed proinsulin in the absence of new proinsulin synthesis. An antibody that recognizes proinsulin but not insulin was used for localization and quantification. This antibody, targeted against the B-C junction of proinsulin, will recognize intact proinsulin and *des 64,65* (C-A split) proinsulin, but will not detect *des 31,32* (B-C split) proinsulin. It

1. *Abbreviations used in this paper*: DAMP, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine, HA, hemagglutinin A, KRB-Hepes-BSA, Krebs-Ringer-bicarbonate buffer, 10 mM Hepes, and 0.5% BSA.




cannot distinguish, therefore, between the two conversion intermediates which were analyzed separately by HPLC.

***Immunofluorescence.*** At the end of the 1-h incubation in high glucose, numerous islet cells showed proinsulin-specific staining (Fig. 1 *A*). The fluorescence in positive cells was prominent in the perinuclear region. Only weak punctate fluorescence was present in islet cells that had been further incubated in low glucose medium (Fig. 1 *B*), which indicates that proinsulin had been converted to insulin (Orci et al., 1985). The loss of proinsulin-specific immunofluorescence was markedly inhibited when the low glucose incubation contained 25 mM ammonium chloride to elevate intravesicular pH (Fig. 1 *C*).

***Immunoelectron Microscopy.*** An example of DAMP and proinsulin-specific immunolabeling on a pair of consecutive thin sections is shown in Fig. 2. These sections are from a B-cell incubated in high glucose medium to load the immature secretory vesicles with proinsulin. Pairs of immunolabeled sections like these for each experimental condition were quantitated to estimate the pH value and proinsulin content of identical secretory vesicles. As previously noted, the clathrin coat that is the morphologic hallmark of immature, proinsulin-rich secretory vesicles, is not easily seen in Lowicryl-embedded material (Orci et al., 1985). Therefore, assignment of individual vesicles to the immature proinsulin-rich category was made on the basis of having a proinsulin immunoreactive content of 300 or more gold particles/$\mu m^2$. Mature vesicles, by contrast, can be recognized because they have a dense core surrounded by a clear halo.

Tabulation of the pH value and proinsulin content for all vesicles with a proinsulin-specific immunogold content of 300 or more particles/$\mu m^2$ is presented in Fig. 3. The number of vesicles per class of pH value and proinsulin content is indicated. The estimation of pH value from DNP immunogold particle content has previously been described (Orci et al., 1986). In high glucose medium, the mean pH value in immature vesicles was ~6.3 and the mean proinsulin content was 927 ± 7 gold particles/$\mu m^2$. In high-then-low glucose medium, the average pH value was unchanged but the content of proinsulin decreased to 484 ± 41 gold particles/$\mu m^2$. The addition of ammonium chloride to the medium caused an increase in average pH to ~6.8; the mean proinsulin content was 760 ± 43 gold particles/$\mu m^2$. Therefore, elevating the intravesicular pH of immature secretory vesicles is correlated with a decrease in proinsulin conversion.

We used the same methodology to evaluate all vesicles with a proinsulin immunogold content of less than 300 particles/$\mu m^2$, corresponding to the mature proinsulin-poor secretory vesicles (Fig. 4). In both high, and high-then-low

*Figure 1.* Proinsulin-specific immunofluorescence in B-cells of isolated islets. (*A*) Incubation in high glucose. Proinsulin staining is predominant in the perinuclear region where the Golgi complex and associated immature secretory vesicles are located. (*B*) After high, then low glucose incubation, the proinsulin staining is limited to a few punctate spots, indicating that most proinsulin has been converted. (*C*) After high-then-low glucose incubation in presence of ammonium chloride, the proinsulin-specific staining is comparable to that seen in high glucose alone (*A*), indicating an inhibition of the conversion process. Bar, 20 $\mu m$.

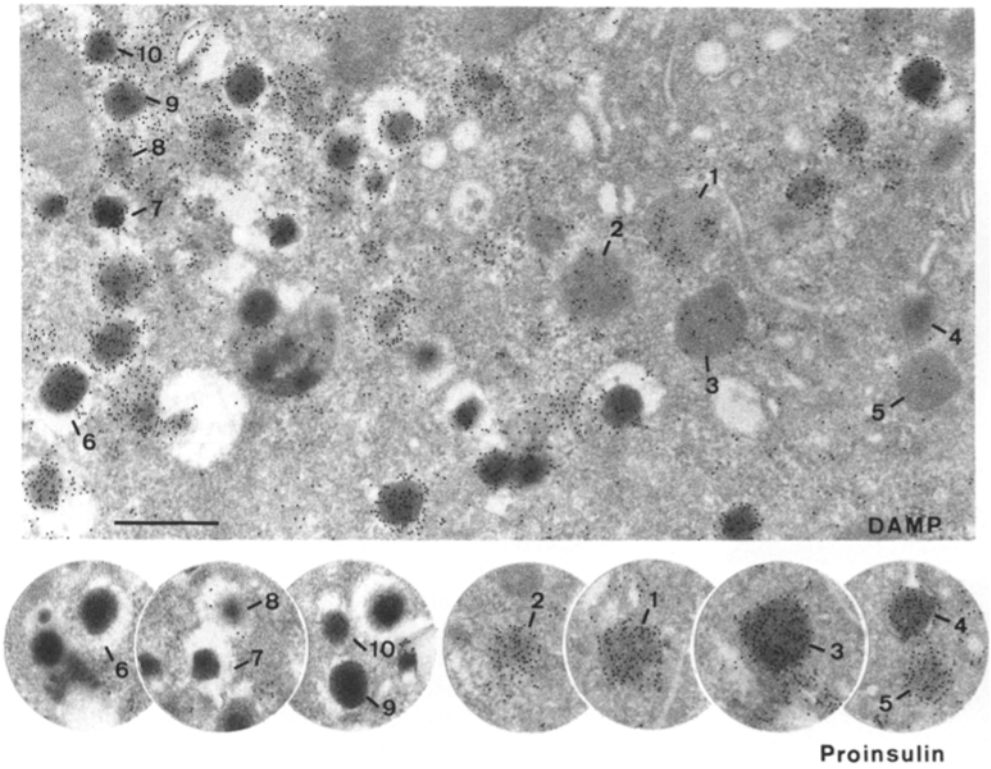
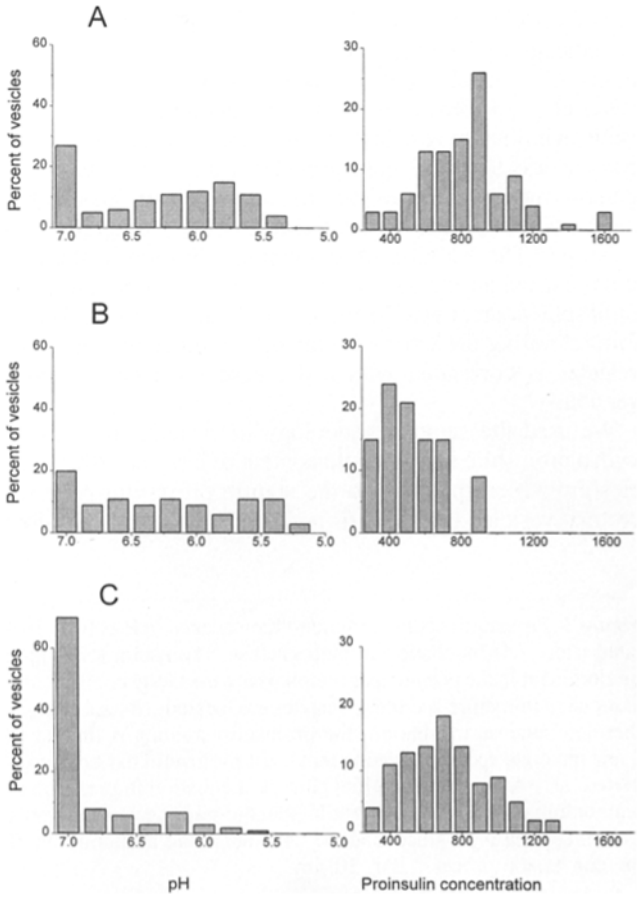



*Figure 2.* Consecutive thin sections of a B-cell immunolabeled with dinitrophenol (DNP) to reveal DAMP (*top*), or proinsulin (*circular insets*) antibodies. High glucose incubation. Ten secretory vesicles (numbered 1 to 10) with varying degrees of DAMP labeling are identified (*top*). Their respective proinsulin labeling is shown on the corresponding circular insets taken from the consecutive serial section immunostained with the proinsulin antibody. Overall, the secretory vesicles with a high degree of DAMP labeling, acidic vesicles (vesicles 6 to 10, mature vesicles), show a very low proinsulin content; the reverse is seen for the secretory vesicles with a relatively low DAMP labeling, less acidic vesicles (vesicles 1 to 5, immature vesicles): these vesicles show a high level of proinsulin labeling. The immunolabeling of such pairs of vesicles on consecutive sections was quantitated in the three experimental conditions studied, and their individual values are plotted in Fig. 5. The DAMP content of the vesicles represents an estimation of the pH in the vesicles: the highest DAMP content, the lowest pH (see Materials and Methods for the mode of calculation). Total number of pairs of vesicles quantitated in the three conditions is 559. Bar, 0.5 $\mu$m.




glucose incubations, the pH of mature vesicles was much less heterogeneous than that of immature vesicles (mean pH value ~5.4). The presence of ammonium chloride in high-then-low glucose medium raised the mean pH in these proinsulin-poor vesicles to ~6.4. The mean number of proinsulin-specific immunogold particles in this population of vesicles under the three incubation conditions was 22 ± 4, 10 ± 2, and 12 ± 2 particles/$\mu m^2$, respectively.

We next determined on pairs of consecutive sections, immunostained alternately for DAMP or proinsulin, the exact relationship between the pH value and proinsulin content of individual vesicles (Fig. 5). Each point in this figure corresponds to a single immature or mature vesicle where the proinsulin content and its pH value are plotted on the vertical and horizontal axis, respectively. These plots indicate that when islets are incubated in the presence of high glucose

*Figure 3.* Histograms of classes of proinsulin-rich (immature) secretory vesicles, arranged on a pH , and a proinsulin concentration (number of immunogold particles/$\mu m^2$) scale. The percent of vesicles in each class is indicated. Secretory vesicles were defined on the basis of their typical morphology in the insulin cell (Fig. 2), i.e., a dense core separated from the limiting membrane by a clear halo of variable width. As a rule, the core of the proinsulin rich (immature) vesicle is relatively pale and close to the limiting membrane with a very narrow (or absent) halo; the core of the proinsulin-poor (mature) vesicle is darker with a wider halo. (*A*) High glucose incubation (81 vesicles evaluated); (*B*) high then low glucose incubation (72 vesicles evaluated); (*C*) high then low glucose incubation in presence of $NH_4Cl$ (112 vesicles evaluated).

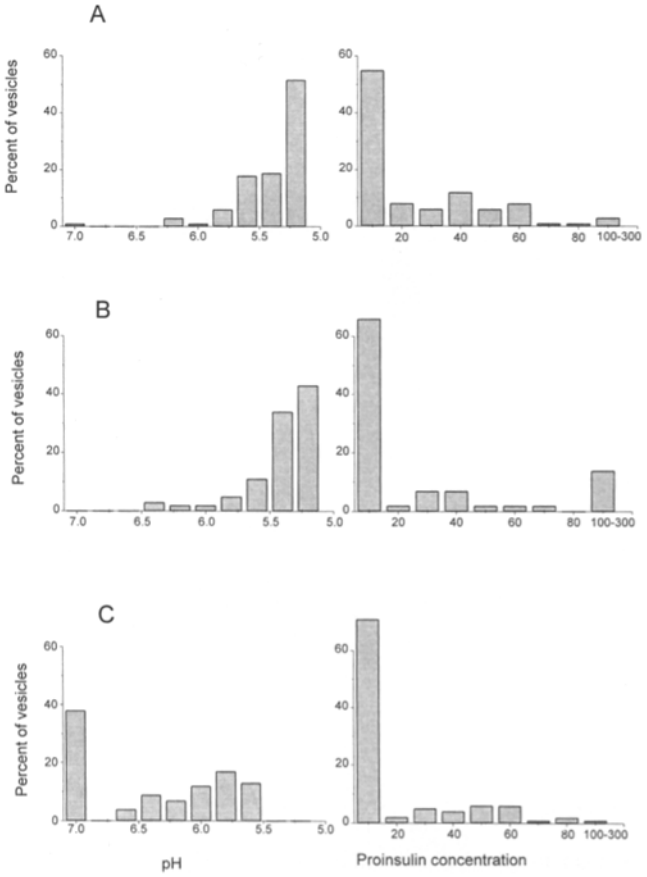



*Figure 4.* Histograms of classes of proinsulin-poor (mature) secretory vesicles, arranged on a pH, and a proinsulin concentration (number of immunogold particles/$\mu m^2$) scale. The percent of vesicles in each class is indicated. (*A*) High glucose incubation (80 vesicles evaluated); (*B*) high then low glucose incubation (76 vesicles evaluated); (*C*) high then low glucose incubation in presence of $NH_4Cl$ (138 vesicles evaluated). See legend of Fig. 3 for the definition of each type of secretory vesicle evaluated.

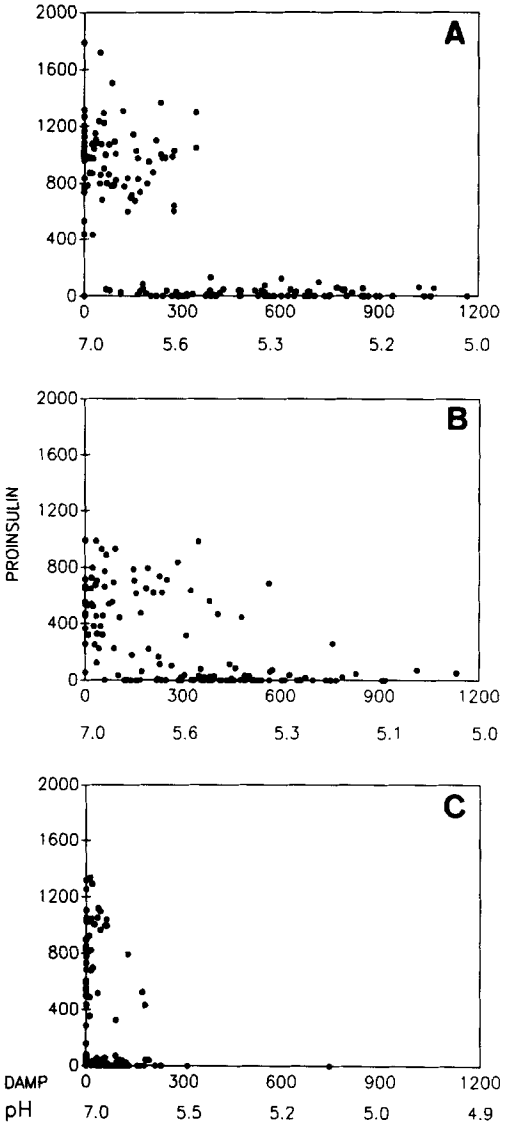



*Figure 5.* Immunolabelings of individual secretory vesicles (proinsulin-rich and proinsulin-poor) expressed as numbers of gold particles per $\mu m^2$ of vesicle's core after labeling with anti-proinsulin or anti-DNP antibodies on alternate consecutive sections. Each vesicle is represented by a dot. (*A*) High glucose; (*B*) high, then low glucose; (*C*) high, then low glucose in presence of 25 mM ammonium chloride. (*A*) 161 vesicles; (*B*) 148 vesicles; (*C*) 250 vesicles. 559 total vesicles evaluated.

alone (Fig. 5 *A*), or in high-then-low glucose (Fig. 5 *B*), proinsulin-rich secretory vesicles are present that have a pH as low as 5.6 to 5.3. On the other hand, individual vesicles with virtually no proinsulin were present that have a pH of 7.0. The major effect of ammonium chloride (Fig. 5 *C*) was to shift pH values to the less acidic range with a majority (72%) of vesicles now having a pH from 6.3–7.0.

Despite the heterogeneous pH, one trend was apparent from these plots: the vesicles with the lowest pH tended to have the lowest proinsulin content. Furthermore, a few vesicles in islets exposed to ammonium chloride (even 50 mM, data not shown) still had a low pH and these few persistently acidic vesicles invariably had a low proinsulin content. Although this indicates that proinsulin conversion did occur in such vesicles, it is not clear whether conversion took place before or after addition of the weak base to the incubation buffer. In other words, these could be vesicles in which insulin had been stored long before the start of the experimental period. Indeed, it is noteworthy that after the 1-h exposure to ammonium chloride, a population of secretory vesicles with a neutral pH and a low proinsulin content becomes apparent (see Fig. 5 *C*): these are assumed to be mature vesicles in which conversion was already complete and which were neutralized by the weak base.

### *pH Dependence of Endoproteolysis at the Two Proinsulin Junctions*

The immunocytochemical experiments enabled the assessment of the pH and proinsulin content of individual secretory vesicles. However, as mentioned earlier, the proinsulin antibody cannot discriminate between intact proinsulin and one of the two partially converted intermediates. Therefore, we used HPLC to examine the effect of elevated pH on the relative efficiency of cleavage at the junctions between the A- and B-chains and the C-peptide of newly synthesized, $^{35}S$-labeled

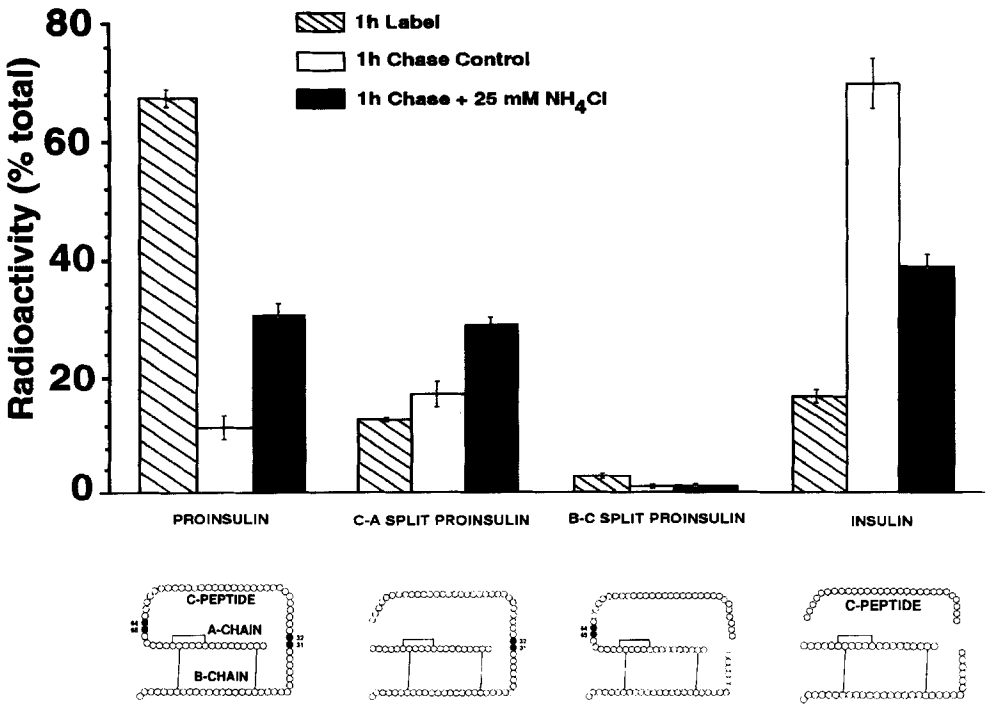



*Figure 6.* HPLC analysis of radioactively labeled proinsulin, proinsulin conversion intermediates, and insulin, in conditions shown in Figs. 3–5. The bars represent mean values ± SEM for four independent experiments.

proinsulin (Fig. 6). Islets were labeled with [$^{35}$S]methionine in high glucose concentration for 1 h and then chased in low glucose medium in the presence or absence of ammonium chloride. Radioactive proinsulin, *des 31,32* (B-C split) proinsulin, *des 64,65* (C-A split) proinsulin and insulin were then separated and quantitated. Only proinsulin (and insulin) II were labeled due to the presence of methionine in their sequence. Since small amounts of labeled, proinsulin-related products were released during the chase, due to the low glucose concentration in the incubation medium (2.8 ± 0.3% and 6.7 ± 1.6% of cell content for control islets and islets exposed to ammonium chloride, respectively), only HPLC analyses of cell extracts are considered here. Some of the proinsulin had been fully converted to insulin during the 1-h labeling period (*hatched bars*). The conversion of proinsulin to insulin during the 1-h chase was markedly reduced in islets that had been incubated in the presence of ammonium chloride (*open bars*) as compared to islets that were not exposed to the drug (*closed bars*). This was accompanied by an increase in the relative amount of C-A split proinsulin. B-C split proinsulin, the other conversion intermediate, was a minor component under all incubation conditions.

If one assumes that the conversion of rat proinsulin II consists of two independent events, the first being cleavage of proinsulin at its C-A junction, and the second, rate-limiting event, being cleavage of the B-C junction of C-A split proinsulin, it is possible to estimate the effect of ammonium chloride on the two cleavage events. Thus, the loss of radioactivity due to intact proinsulin during the chase can be considered to reflect the rate of cleavage at the C-A junction, whereas the appearance of radioactivity due to insulin reflects cleavage at the B-C junction of the C-A split intermediate. Such a calculation reveals that in presence of $NH_4Cl$, cleavage at the C-A junction is inhibited by 33.8 ± 3.5%, whereas that at the B-C junction is inhibited by 57.5 ± 2.4%.

## Discussion

Electron microscopic detection of the pH sensor, DAMP, was used previously to show that rat proinsulin is converted to insulin coordinately with the acidification of the immature secretory vesicle (Orci et al., 1986, 1987*a*). We now have determined that low pH controls the conversion of C-A split proinsulin to insulin in situ. The pH at which the converting enzyme PC1 works (responsible for cleavage at the B-C junction) is sufficiently narrow (Davidson et al., 1988) that within the confines of the secretory vesicle, the production of mature hormone is inhibited at neutral pH even though the first stages of proteolytic processing have begun.

The kinetics of PC1 and PC2 dependent conversion of proinsulin have been extensively studied in vitro, using human proinsulin as the substrate (Davidson et al., 1988; Bailyes et al., 1992; Bailyes and Hutton, 1992; Bennett et al., 1992; Rhodes et al., 1992). A comparison of the processing of human, rat I and rat II proinsulin in islet B-cells, has shown that a basic residue in the −4 position preceding the two proinsulin dibasic cleavage sites is critical for conversion (Sizonenko and Halban, 1991; Sizonenko et al., 1993). In primary and transformed rat B-cells, high levels of PC1 are required for conversion to proceed normally (Neerman-Arbez et al., 1993), while relatively low levels of PC2 are found in primary B-cells (Neerman-Arbez et al., 1994). Thus, proinsulin conversion is a complex process, but no amount of biochemical analysis can reveal exactly how prohormones are converted to hormones within the microenvironment of an individual secretory vesicle.

In the present study, we have focused on the conversion of rat proinsulin II since it can be selectively labeled with [$^{35}$S]methionine. The first event in the conversion of this proinsulin molecule, unlike human or rat I proinsulin, is cleavage at the C-peptide/A-chain junction, leading to generation of the C-A split intermediate (Sizonenko and Halban,

1991, Sizonenko et al., 1993). When vesicular pH was raised by ammonium chloride, there was increased accumulation of radioactivity in the form of C-A split proinsulin. There was also more labeled proinsulin remaining unprocessed and less fully processed insulin. These data suggest that disruption of pH gradient only slightly impairs cleavage at the C-peptide/A-chain junction, while the B-chain/C-peptide cleavage is markedly inhibited. This is in keeping with the pH dependency of the two conversion endoproteases in vitro: both enzymes display an acidic pH optimum, but only PC2 remains active even at neutral pH (Davidson et al., 1988). Our results thus indicate for the first time that cleavage at the two junctions displays differential pH-dependency in the intact B-cell. Our results do not support, however, the previous suggestion that cleavage at the C-A junction is a Golgi event, and B-C junction cleavage a post Golgi event (Davidson et al., 1988). The data showing a wide range of pH in individual secretory vesicles indicate, on the contrary, that each secretory vesicle can build up the required ionic environment for optimal activity of the two proteases involved in the processing of proinsulin. The use of site-specific antibodies targeted against the C-A (Steiner et al., 1987), or the B-C junction (this work, and Orci et al., 1985, 1986, 1987*a*) of proinsulin, respectively, had previously suggested that no detectable amounts of partially-cleaved proinsulin intermediate appear before the prohormone is segregated to the clathrin-coated, immature secretory vesicles.

It has been reported that the weak base chloroquine diverts the secretory peptide ACTH from a regulated to a constitutive pathway in At-T20 cells (Moore et al., 1983), a finding not confirmed in subsequent work (Mains and May, 1988). The B-cell contains a sparse population of 100–300-nm vesicles with an electron-lucent content which were shown to carry constitutively-secreted hemagglutinin A (HA) en route to the plasma membrane in influenza virus-infected cells (Orci et al., 1987*b*). In control and $NH_4Cl$-treated B-cells, no vesicular compartment other than typical dense core secretory vesicles was detectably labeled with proinsulin antibody. This seems to rule out the possibility that our immunocytochemical data were influenced by the quantitation of proinsulin putatively missorted to constitutive vesicles. In both control and $NH_4Cl$ conditions, the absolute amount of newly synthesized (labeled) proinsulin which is released remains very low and the difference in percentage observed can in no way account for the measured difference in conversion kinetics.

There is no evidence that the pH of secretory vesicles in islet cells is ever regulated, which suggests that these cells do not use pH to control the production of mature hormone. Nevertheless, our findings do have implications for disease processes that affect the function of the acidification apparatus. Barash et al. (1991) reported that acidification of intracellular organelles of cells from patients that have cystic fibrosis is impaired and that, as a consequence, intravesicular pH is elevated. If such changes in intravesicular pH also occur in secretory vesicles of the B-cells, they may have a profound effect on the efficiency of prohormone processing in these patients.

In conclusion, the results of this combined morphological and biochemical study confirm that the bulk of proinsulin conversion arises in immature secretory vesicles and show that intravesicular pH affects cleavage at the two proinsulin junctions differentially.

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