

# Insulin, Not C-Peptide (Proinsulin), Is Present in Crinophagic Bodies of the Pancreatic B-Cell

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**ABSTRACT** We have obtained evidence by autoradiography and immunocytochemistry that mature secretory granules of the pancreatic B-cell gain access to a lysosomal compartment (multigranular or crinophagic bodies) where the secretory granule content is degraded. Whereas the mature secretory granule content shows both insulin and C-peptide (proinsulin) immunoreactivities, in crinophagic bodies only insulin, but not C-peptide, immunoreactivity was detectable. The absence of C-peptide (proinsulin) immunoreactivity in multigranular bodies, i.e., in early morphological stages of lysosomal digestion, was compatible with the ready access and breakdown of C-peptide and/or proinsulin by lysosomal degrading enzymes, while the insulin crystallized in secretory granule cores remained relatively protected. However, in the final stage of lysosomal digestion, i.e., in residual bodies where the secretory granule core material is no longer present, insulin immunoreactivity became undetectable. Lysosomal digestion thus appears to be a normal pathway for insulin degradation in the pancreatic B-cell.

The pancreatic B-cell shares with other polypeptide-secreting cells the ability to store its secretory products within cytoplasmic granules available for release by exocytosis (1, 2). However, the totality of insulin manufactured is not destined to be secreted, since it has been shown that the B-cell can degrade a significant portion of its own secretory product (3, 4). One commonly assumed mechanism of intracellular degradation of insulin by the B-cell is the fusion of secretory granules with primary lysosomes (5, 6), a mechanism called granulolysis or crinophagy and initially described in the mammothroph cells of the anterior pituitary (7) and pancreatic A-cells (8). In the B-cell, morphological evidence for crinophagy consists of cytoplasmic organelles (secondary lysosomes) that present a very variable morphology but usually contain dense masses resembling  $\beta$ -secretory granule cores. The lysosomal nature of these structures (also called crinophagic or multigranular bodies) is evidenced by their content in marker lysosomal enzymes such as acid phosphatase or arylsulfatase (9), but whether the content of the granule core-like material is related to insulin polypeptides has not been ascertained. For this purpose, we have followed, by electron microscopic autoradiography, the labeling of the multigranular bodies in pulse-chase experiment with tritiated leucine, and determined their

immunoreactive content using anti-insulin and anti-C-peptide antisera revealed by the protein A-gold method.

## MATERIALS AND METHODS

Rat islets of Langerhans in sections of pancreatic tissue or isolated by collagenase digestion (10) were studied throughout. The material examined originated from the tissue store of the laboratory. All blocks were from five untreated normal rats, the pancreas (or isolated islets) of which were fixed in 2.5% glutaraldehyde alone or with a mixture of 1.25% glutaraldehyde, 1% paraformaldehyde, and 0.02% trinitroresol (11), then postfixated in osmium tetroxide and embedded in Epon or Epon-Araldite. Beside Epon-embedded material, isolated islets embedded at low temperature in Lowicryl resin (12) or lightly-fixed (1% glutaraldehyde alone) tissue processed by ultracyotomy (13) were studied.

**Autoradiography:** The pulse-chase labeling of isolated islets with tritiated leucine was carried out as described previously (14). In this case, batches of freshly isolated islets from three normal untreated rats were maintained in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum and 8.3 mM glucose for 72 h (equilibration period) at 37°C. This 3-d equilibration period allows full recovery from possible damage caused by the isolation procedure. Islets were then transferred in 0.5 ml of Krebs-Ringer bicarbonate-HEPES buffer at 37°C and labeled for 30 min with [<sup>3</sup>H]leucine (1 mCi/ml, 62 Ci/mmol). At the end of the labeling period, the isolated islets were returned to the Dulbecco's modified Eagle medium at 37°C and further incubated for 3- and 24-h chase periods. Prior to fixation, at the end of the labeling period or following the chase, aliquots of islets were washed in Krebs-Ringer bicarbonate-HEPES buffer and fixed with 2.5% glu-

taraldehyde in cacodylate buffer, postfixed in osmium tetroxide, and embedded in Epon. Thin sections were prepared and autoradiographed with the loop method of Caro and van Tubergen (15) using Ilford L<sub>4</sub> emulsion. Exposure time was ~6 wk. Following development of the emulsion, sections were photographed systematically to record autoradiographic grains. The distribution and frequency of the grains over the various intracellular compartments was evaluated quantitatively by the probability circle method (16) coupled to the morphometry of the cell compartments (17) in six different islets for each time point.

**Immunocytochemistry:** Thin sections of the five normal untreated rat islets variously processed and embedded as described above (this material was distinct from that prepared for autoradiography) were picked up on nickel support grids and prepared for ultrastructural immunocytochemistry by oxidizing (when applicable) excess osmium tetroxide with H<sub>2</sub>O<sub>2</sub> (18) or NaIO<sub>4</sub> (19). Deosmified sections (or nonosmified Lowicryl or ultracryotomy sections) were incubated overnight at 4°C with anti-insulin (lot no. 573; a gift from Dr. Wright, Indiana University, Indianapolis) or with anti-C-peptide (lot no. R-901) antisera at various dilutions (1:400-1:10,000 for insulin; 1:500-1:2,000 for C-peptide). The dilution of the antisera needed to keep the tissular background as low as possible was empirically established for each processing protocol. Following several washes in distilled water, the sections were further incubated with the protein A-gold solution (20), rinsed in distilled water, and stained with uranyl acetate and lead citrate. Immunolabeled sections were systematically photographed in the electron microscope and the distribution of gold particles (revealing antigenic binding sites) was visually assessed. In addition, a quantitative evaluation of the immunolabeling was carried out in samples of islets fixed and embedded according to different procedures (Table I). This was done by recording in each case the number of gold particles per unit area (square microns) of selected cytoplasmic compartments using an electronic pen connected to a microprocessor. The system allowed the measuring the number of single events (i.e., gold particles) together with the surface of the compartments in which they occurred. Specificity of the immunolabeling was assessed by incubation of sections with antisera previously adsorbed with their respective antigens. No specific labeling (i.e., above cellular background) was obtained in these conditions.

**Cytochemistry:** The acid phosphatase and arylsulfatase activities of multigranular bodies were demonstrated in 40- $\mu$ m sections (cut with a tissue chopper) of pellets of glutaraldehyde-fixed, isolated islets of Langerhans according to the methods of Gomori (21), and Bentfeld-Barker and Bainton (22), respectively. Osmium tetroxide postfixation and Epon embedding were carried out after the completion of the cytochemical reactions.

## RESULTS

The intracellular compartments of the pancreatic B-cell thought to be involved in lysosomal degradation of insulin were the following: (a) individual noncoated secretory gran-

ules (Fig. 1 a) considered as the end stage of granule maturation (2, 23, 24); (b) lysosomes identified on both structural (25) and cytochemical (acid phosphatase and arylsulfatase activity) criteria (9). Lysosomes could be further subdivided into (a) multivesicular bodies assumed to be representative of primary lysosomes before they interact with an intra- or extracellular compartment (7, 26); (b) dense bodies with heterogeneous, unclassifiable content often consisting of lipid "droplets" and/or "myelin" figures (Fig. 4 c); these are also called residual bodies and represent the end stage of lysosomal digestion; and (c) crinophagic or multigranular bodies, i.e., membrane sacs of various shapes and sizes containing one or several masses resembling secretory granule cores (Figs. 1 a, 3, a and b). Multigranular bodies are considered secondary lysosomes, as well as multivesicular bodies when they contain granule core-like material (Fig. 4, a and b). Crinophagic bodies show acid phosphatase and arylsulfatase activity (Fig. 1, b and c). Due to the morphological similarity between the content of multigranular bodies and the secretory granule cores, these bodies (and the multivesicular bodies when they contain granule core-like material; see Fig. 4, a and b) are believed to be the sites of lysosomal degradation of insulin. The pattern of autoradiographic labeling of secretory granules in B-cells of isolated islets after a 30-min pulse of tritiated leucine followed by 3 and 24 h of chase is shown in Fig. 2 a, that of multigranular bodies in Fig. 2 b. From 0.5 to 3 h, the radioactivity increased significantly in the secretory granule compartment and slowly decreased from 3 to 24 h. The radioactivity in the multigranular body compartment was low up to 3 h whereas it increased significantly from 3 to 24 h. This pattern, together with the morphological similarity noted above between the cores of secretory granules and those visible in crinophagic bodies, suggested a process of funneling of part of radioactive peptides from the secretory granule compartment into the multigranular body compartment. In the experiments analyzed, the process appeared slow and not to involve newly formed granules since the multigranular bodies were not significantly labeled until 3 h after the pulse. During the period of time studied, the multivesicular body compart-

TABLE I  
Number of Gold Particles

	Processing protocol	Secretory granules	Multigranular bodies*	Background
Anti-insulin	A	185.7 $\pm$ 14.1 (39)	166.3 $\pm$ 11.8 (37) <sup>‡</sup> 101.6 $\pm$ 8.2 (37) <sup>§</sup>	1.44 $\pm$ 0.23 (8)
	B	61.3 $\pm$ 5.6 (17)	51.5 $\pm$ 8.8 (18) <sup>‡</sup> 27.7 $\pm$ 4.9 (20) <sup>§</sup>	0.54 $\pm$ 0.10 (5)
	C	124.6 $\pm$ 6.0 (49)	73.8 $\pm$ 7.0 (25)	0.40 $\pm$ 0.19 (19)
Anti-C-peptide	A	188.6 $\pm$ 9.2 (33)	10.4 $\pm$ 2.5 (33) <sup>‡</sup> 8.5 $\pm$ 1.5 (35) <sup>§</sup>	4.14 $\pm$ 0.69 (8)
	B	34.1 $\pm$ 4.0 (19)	2.4 $\pm$ 1.31 (16) <sup>‡</sup> 1.7 $\pm$ 0.80 (17) <sup>§</sup>	0.84 $\pm$ 0.29 (8)
	C	120.0 $\pm$ 4.2 (51)	4.0 $\pm$ 1.28 (27)	1.08 $\pm$ 0.34 (18)

Quantitative evaluation of the number of gold particles over secretory granules and multigranular bodies of the B-cell following incubation with anti-insulin and anti-C-peptide antisera revealed by the protein A-gold technique. Data are means  $\pm$  SEM. Values in parenthesis are the number of pictures evaluated in each condition. None of the values of C-peptide immunolabeling in multigranular bodies is significantly different from cellular background. Processing protocol: A, glutaraldehyde-paraformaldehyde-trinitroresol-osmium fixation, Epon-Araldite embedding (one rat, three islets in situ). Antibody dilution: insulin = 1:400; C-peptide = 1:1,000; B, glutaraldehyde-osmium fixation, Epon embedding (one rat, three isolated islets). Antibody dilution: insulin = 1:400; C-peptide = 1:500; C, glutaraldehyde alone (1%), low temperature embedding in Lowicryl resin (pool of three rats, nine islets). Antibody dilution: insulin = 1:10,000; C-peptide = 1:2,000.

\* These comprise multivesicular bodies with granule core-like material.

<sup>‡</sup> Values determined over the granule core-like material alone.

<sup>§</sup> Values determined over the entire multigranular body.

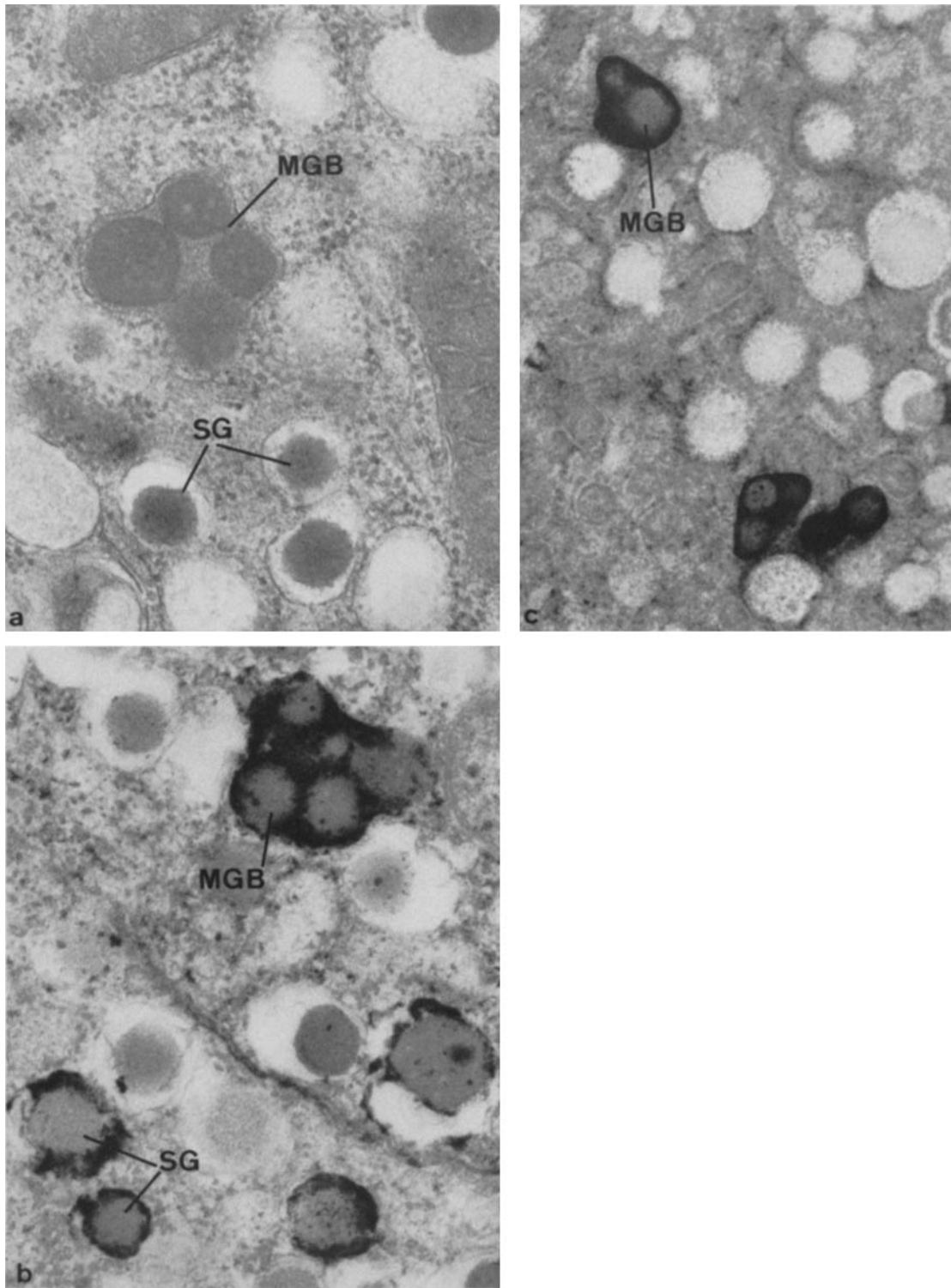


FIGURE 1 (a) Thin section of part of a B-cell cytoplasm illustrating the two compartments thought to be involved in lysosomal digestion of insulin polypeptides. These comprise secretory granules (SG) and multigranular (crinophagic) bodies (MGB). Note the morphological similarity between the secretory granule core and the dense, round masses within the multigranular body. Glutaraldehyde-osmium fixation; Epon embedding. (b) Field of a B-cell cytoplasm showing secretory granules (SG) and a multigranular (crinophagic) body (MGB) after cytochemical demonstration of acid phosphatase. The dense reaction product filling the multigranular body outlines the secretory granule core-like material. As described previously, a positive acid phosphatase reaction is present in the halo of some granules (32). Glutaraldehyde fixation, osmium postfixation (following cytochemistry); Epon embedding. (c) Field of a B-cell cytoplasm as in a but following cytochemical demonstration of arylsulfatase. The three multigranular bodies (MGB) present in the field are cytochemically labeled, but unlike the situation with acid phosphatase, none of the secretory granules is reactive. The incubation in the cytochemical medium for arylsulfatase induced some loss of contrast in the secretory granule cores. Glutaraldehyde fixation, osmium postfixation (following cytochemistry); Epon embedding.  $\times 57,000$  (a);  $\times 51,000$  (b);  $\times 27,000$  (c).

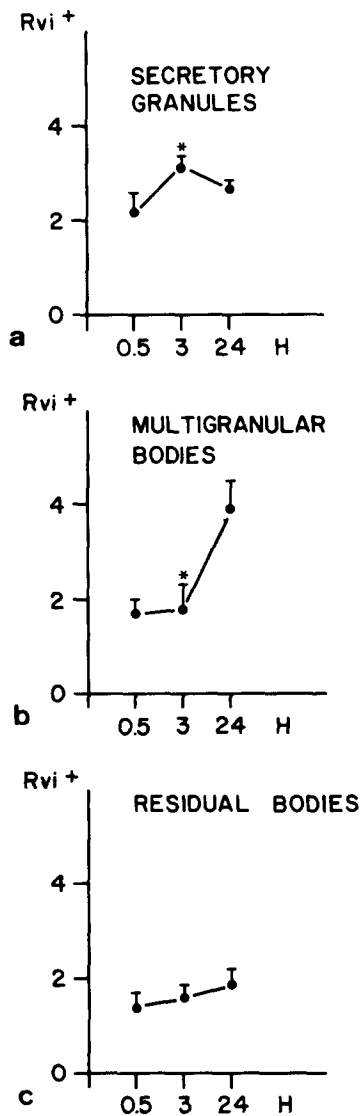


FIGURE 2 Quantitative evaluation of the autoradiographic reaction in three compartments of the B-cell thought to be involved in lysosomal degradation of insulin. The radioactivity in each compartment is expressed as the specific radiation label density ( $Rvi^+$ ) which represents the number of autoradiographic grains per unit volume of the compartment (16, 17). The experiment involved a pulse of 30 min (0.5 h) followed by chase periods of 3 and 24 h. The first time point evaluated is at the end of the 0.5-h pulse. The asterisks at 3 h indicate in a, a value significantly different ( $p < 0.05$ ) from that at 30 min, and in b, from that at 24 h. Each time point is the result of the evaluation of six different isolated islets and represents a mean  $\pm$  SEM (vertical bar). Glutaraldehyde-osmium fixation. Epon embedding. The three curves derive from the analysis of a total number of 1,778 autoradiographic grains.

ment without granule-core like material, i.e., representative of primary lysosomes before interaction with endogenous or exogenous material (7, 26), showed a pattern of autoradiographic labeling that was the inverse of that of crinophagic bodies, namely a high level of radioactivity at 30 min, decreasing markedly and significantly by 3 h, and remaining low up to 24 h (data not shown). This labeling was assumed to be due to the radioactivity incorporated into lysosomal enzymes. The pattern of autoradiographic labeling of residual bodies is shown in Fig. 2c. It revealed a constant level of radioactivity over the period of time studied.

The qualitative assessment of immunocytochemical staining after application of insulin or C-peptide antisera showed the following constant pattern: all secretory granules appeared labeled with both anti-insulin and anti-C-peptide antisera (Fig. 3, a and b). Multigranular bodies showed by contrast a marked immunoreactivity towards the anti-insulin antiserum (Fig. 3a), but virtually no labeling with the anti-C-peptide antiserum (Fig. 3b). Multivesicular bodies containing granule core-like material (Fig. 4, a and b) were also different in immunoreactivity as multigranular bodies. The dense residual bodies without identifiable granule core-like material (Fig. 4c) presented, by contrast, no immunolabeling either with anti-insulin or with anti C-peptide antisera. The tissue embedded

at low temperature in Lowicryl resin and that lightly fixed with glutaraldehyde and submitted to ultracyotomy showed a pattern of labeling comparable with that described above. The results of the quantitative evaluation of the protein A-gold immunolabeling in samples of islets fixed and embedded following various procedures is shown in Table I. Irrespective of the processing protocol used, the quantitative data revealed a comparable difference (although the values varied in absolute terms) in the immunolabeling of secretory granules and crinophagic bodies when insulin and C-peptide antisera were used.

## DISCUSSION

The present data offer evidence that, in the pancreatic B-cell, insulin polypeptides are introduced into a lysosomal compartment; this process may represent, at least in part, the substratum for intracellular insulin degradation demonstrated in B-cells by biochemical experiments (3, 4). Lysosomal degradation of secretory polypeptides would thus appear to be a normal component of the insulin cycle.<sup>1</sup> This conclusion was based upon autoradiography, which showed the kinetics of the migration of tritiated polypeptides between intracellular compartments, and immunocytochemistry, which allowed the identification of the content of these compartments. The difference in the pattern of radioactive labeling of multigranular bodies and residual bodies is particularly interesting with respect to the hypothesis that the former are a site of insulin degradation. Multigranular bodies would represent an early stage of the degradation process at which the characteristic granule core-like structures are still detectable together with their associated radioactivity. That autoradiographic grains of multigranular bodies reveal insulin polypeptides rather than lysosomal enzymes themselves is suggested by the time course of the labeling. The increase in multigranular body labeling occurs at a time (between 3 h and 24 h) when the radioactive content of multivesicular bodies (representative of primary lysosomes) is already low; this increase would result from the uptake of additional radioactive material, most likely insulin polypeptides, by multigranular bodies. In residual bodies, recognizable structures that resemble granule cores are no longer present and it can be assumed that radiolabeled fragments originating from insulin breakdown have diffused across the lysosomal membrane to reach the cytosol (26), keeping therefore a constant low level of radioactivity in the residual body itself. This hypothesis may be confirmed by the immunocytochemical data of the present investigation showing that multigranular bodies contain insulin immunoreactive sites, whereas residual bodies do not. The finding that crinophagic bodies, unlike secretory granules from which they are assumed to derive, were not labeled at significant levels by C-peptide antiserum can be interpreted as follows: in mature, dense-core secretory granules, insulin may form crystals involving zinc ions (27) and probably excluding C-peptide (28, 29). Upon fusion of one or several granule(s) with a primary lysosome to form a multigranular body, C-peptide is thus likely to be more easily accessible and digestible by lysosomal enzymes than the compact and relatively insoluble (27) insulin crystals. This was evidenced by the link between the presence of insulin immunoreactivity and the granule core

<sup>1</sup> The material analyzed was not intended to assess the magnitude of this process, nor whether it can be experimentally modulated by the stimulation of the B-cell secretion.

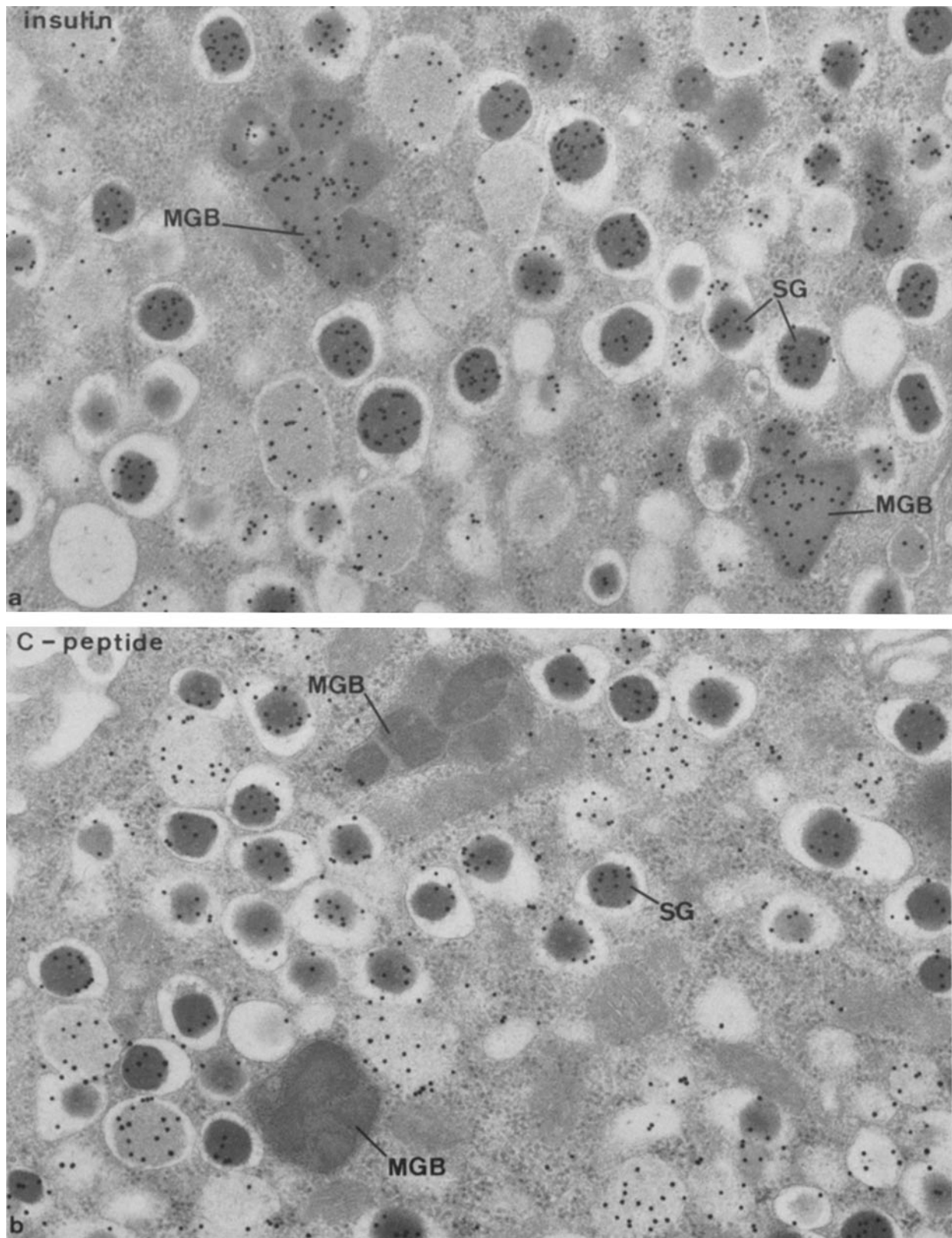
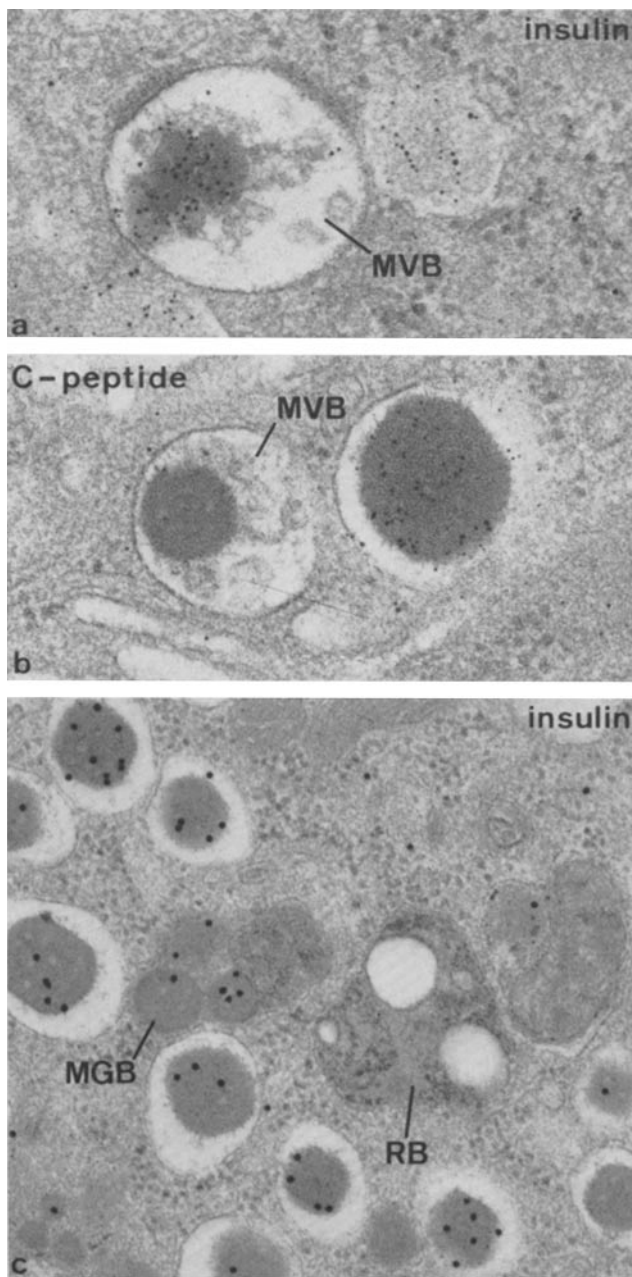


FIGURE 3 (a) Thin section of a pancreatic B-cell immunostained with the protein A gold technique for the demonstration of insulin. Two multigranular (crinophagic) bodies (MGB) present in this field show numerous gold particles over the secretory granule core-like material. The secretory granules (SG) either with dense or pale core are similarly labeled. The quantitative evaluation of the immunoreaction over multigranular bodies and secretory granules is shown in Table I. (b) Thin section comparable to that shown in a but immunostained with an anti-C-peptide antiserum. Although this antiserum marks the pale and dense-core secretory granules (SG) as the anti-insulin antiserum, the two multigranular bodies (MGB) present in this field appear virtually not labeled. The quantitative evaluation of labeling is shown in Table I. (a and b) Glutaraldehyde-paraformaldehyde-trinitroresol-osmium fixation, Epon-Araldite embedding.  $\times 38,000$ .



**FIGURE 4** Fields of B-cell cytoplasm stained with either anti-insulin (a, c) or anti-C-peptide (b) antisera revealed by the protein A gold technique; a illustrates a multivesicular body (MVB) with its characteristic coated membrane segments and that contains granule core-like material immunostained with anti-insulin antiserum. b shows another example of a multivesicular body (MVB) containing a granule core, but immunostained with anti-C-peptide antiserum. No significant labeling is detectable as compared with the neighboring secretory granule. Multivesicular bodies with granule core-like material are considered secondary lysosomes and were evaluated together with multigranular bodies in Table I. c shows a residual body (RB) where secretory granule core structure(s) are no longer present. At this stage, assumed to represent the terminal stage of digestion, insulin immunoreactivity is absent whereas it remains detectable in a neighboring multigranular body (MGB). Sections in a and b were immunostained with small size gold particles produced with tannic acid (33). Glutaraldehyde-paraformaldehyde-trinitro-cresol-osmium fixation; Epon-Araldite embedding.  $\times 76,000$  (a and b);  $\times 48,000$  (c).

appearance, both of which are lost at the residual body stage when degradation is assumed to have been completed, accompanied by granule core dissolution. It must be emphasized that the quantitative data presented do not allow the estimation of the actual immunoreactive polypeptide content of secretory granules and/or multigranular bodies, since the ratio between the number of antigenic sites and the number of protein A-gold particles is unknown for our antisera in any of the fixation/embedding protocols. In addition, the C-peptide antiserum recognizes both the C-peptide region of proinsulin and the C-peptide molecule itself released upon cleavage of proinsulin into insulin. In this respect, C-peptide antiserum is a marker of proinsulin as well as of C-peptide (30). Likewise, the anti-insulin antiserum reacts primarily with the insulin molecule, but a certain degree of cross-reactivity with proinsulin is also known to occur. These various specificities emphasize the relative significance of the numbers of gold particles measured in the different compartments. A last cautionary note concerns the fixation of the C-peptide molecule: due to the relative small size and limited number of sites available for cross-linking by glutaraldehyde, C-peptide may not be completely retained during tissue processing. If such a wash-out occurs, the observed C-peptide immunoreactivity would thus represent proinsulin which is also present in secretory granules (29) and/or intermediates, rather than C-peptide itself. Although the presence of distinct C-peptide immunoreactivity in ultracryotomy sections that have not undergone dehydration and embedding (i.e., where washing out of poorly fixed molecules should be greatly reduced) speaks in favor of C-peptide being retained in our experimental conditions, the data that shows sequential disappearance of secretory granule immunoreactive content upon encounter with lysosomes remain valid, irrespective of whether C-peptide, proinsulin, or both were labeled.

In summary, we have presented autoradiographic and immunocytochemical evidence that secretory granules of the pancreatic B-cell gain access to a lysosomal compartment where they appear to be morphologically degraded, together with their insulin and C-peptide immunoreactivities. Insulin immunoreactivity disappears at a later morphological stage of lysosomal digestion than C-peptide immunoreactivity, suggesting a greater resistance of the insulin crystal to hydrolytic digestion as compared with C-peptide and/or proinsulin. Lysosomal action would thus account, at least in part, for the normal pattern of insulin degradation in the B-cell. This does not exclude, however, other pathways for degradation as suggested by the presence, in pancreatic islets, of apparently nonlysosomal insulin degrading enzymes (31).

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