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Proinsulin modified by analogues of arginine and lysine is degraded rapidly in pancreatic B-cells

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Modified cytosolic proteins are known to be degraded more rapidly than their native counterparts. In order to determine whether the same applies to a modified protein within the potentially protective environment of secretory granules, rat islets were labelled ([^{3}H]leucine) in the presence or absence (controls) of 3mM-canavanine and 3mM-thialysine (analogues of arginine and lysine respectively), followed by a 24h 'chase' period without analogues. The results showed the following. (1) Incorporation of the analogues into newly synthesized labelled proinsulin inhibited its conversion into insulin during the chase period. (2) Despite this block in conversion, the modified proinsulin was released from islets at the same rate as native proinsulin and insulin from control islets. (3) Morphometric analysis of high-resolution autoradiographs showed that products labelled in the presence of analogues were sequestered into secretory granules at the same rate as native products in control B-cells. (4) Only 7% of prelabelled proinsulin had been degraded within islet cells during the chase period in control islets, compared with 36% for proinsulin prelabelled in the presence of analogues. (5) Control experiments showed that the analogues had no effect on the release or intracellular degradation of unmodified stored insulin (present in islets before exposure to the analogues). (6) Despite sequestration into secretory granules, modified proinsulin, if not released from B-cells, is thus degraded more rapidly than native products.

We have found that stored insulin can be degraded within pancreatic B-cells and that such degradation is important for the regulation of B-cell insulin content (Halban & Wollheim, 1980; Halban & Renold, 1983). In other cell types, it has been found that damaged, or modified, proteins are degraded more rapidly than native proteins (Knowles *et al.*, 1975; Knowles & Ballard, 1976; Klemes *et al.*, 1981), but these studies were concerned for the most part with cytosolic proteins, or with secretory proteins not stored for long periods in secretory granules (Berg *et al.*, 1980). We wished to determine whether a modified polypeptide hormone within the potentially protective environment of a secretory granule is also degraded at an accelerated rate. To this end, we modified nascent proinsulin in isolated rat islets of Langerhans by exposure of the islets to analogues of arginine (canavanine) and lysine (thialysine), thereby preventing conversion of the modified proinsulin into insulin (Halban, 1982*a*). Arginine and lysine are the two residues which link the C-peptide of proinsulin with the insulin A- and B-chains (Steiner *et al.*, 1974); modification of these residues prevents cleavage by the converting enzyme(s) (Noe, 1981; Halban, 1982*a*). The fate of the modified proinsulin was then studied. We show that, if not released from B-cells (the release of the modified proinsulin occurring at the same rate as that for native proinsulin and insulin), the modified proinsulin is degraded more rapidly than native insulin, and that such degradation arises after sequestration into secretory granules.

Part of this work was presented at the Nineteenth Annual Meeting of the European Association for the Study of Diabetes, held at Oslo, Norway, on 14–17 September 1983, and has been published in abstract form (Halban *et al.*, 1983).

Materials and methods

Rat pancreatic islets were isolated and subsequently maintained in tissue culture for 3 days as described previously (Halban, 1982*a*). The islets were then preincubated (2h) and labelled (30min, [^{3}H]leucine) at 37°C in the presence of 3mM-*S*-2-aminoethylcysteine (4-thialysine) and 3mM-canavanine (both obtained from Sigma) as described previously (Halban, 1982*a*). Control islets were handled in parallel in the absence of the amino acid analogues. After washing (Halban, 1982*a*), the islets were placed in fresh culture medium (Dulbecco's modified Eagle's medium/10% (v/v) newborn-calf serum/8.3mM-glucose) for a 24h post-labelling (chase) incubation [37°C, humidified atmosphere of air/CO_2 (19:1)], in the absence of the analogues, which were thus only present during the prelabelling and labelling periods. At the end of the chase period, the islets were washed (Halban, 1982*a*) and then kept for subsequent analyses; the culture media were also collected.

Before analysis, islets (taken either at the end of the labelling period, or at the end of the chase period) were extracted in 0.2M-glycine/human serum albumin (2.5mg/ml), pH8.8 [sonication followed by ultracentrifugation as described previously (Halban *et al.*, 1980; Halban, 1982*a*)] and samples of 24h-chase culture media were ultracentrifuged to remove any cell debris (Halban *et al.*, 1980). The ultracentrifugation step (30000*g* for 30min) resulted in no loss of immunoreactive insulin from the samples (Halban *et al.*, 1980) and no immunoprecipitable radioactive products could be detected in the cell-debris pellet, even after extraction with 1M-acetic acid/0.5% (v/v) Nonidet P40 (Fluka). Radioactively labelled proinsulin or insulin in these samples was measured by quantitative immunoprecipitation, using guinea-pig anti-insulin serum (Miles) and protein A–Sepharose (Pharmacia) to precipitate immune complexes as described in detail previously (Halban & Wollheim, 1980; Halban *et al.*, 1980; Halban & Renold, 1983). In order to separate labelled proinsulin from insulin, samples were first subjected to immunoprecipitation, and the precipitable material then displaced from the immune precipitates and chromatographed (Sephadex G-50) (Halban, 1982*a*; Trimble *et al.*, 1982). This method has been shown previously (Halban, 1982*a*) to accurately reflect the relative percentage of immunoprecipitable radioactive material in the form of proinsulin (both native and modified by the analogues) or of insulin.

The above protocol was modified in order to observe the effects of analogues on the release and intracellular degradation of unmodified islet insulin stores. Islets were cultured for 3 days in the presence of [^{3}H]leucine (Halban & Wollheim, 1980). The cultured islets, with prelabelled insulin stores, were washed in culture medium and then incubated for a total of 2.5h with 3mM-thialysine and 3mM-canavanine, but without [^{3}H]leucine. Controls were incubated in parallel without analogues. The islets were then replaced in culture for a 24h chase period without analogues. The islets in this protocol were thus handled exactly as those in the previous protocol, with the exception that insulin stores had been prelabelled before exposure to the analogues rather than newly synthesized proinsulin being labelled in the presence of analogues.

Since it has been shown previously that there is only minimal degradation of immunoreactive products once released into the culture medium during a 24h chase period (Halban & Wollheim, 1980; Halban *et al.*, 1980; Halban & Renold, 1983), the intracellular degradation of immunoprecipitable radioactive products was estimated as follows. The total recoverable immunoprecipitable radioactivity (i.e. that recovered from islets and from the culture medium) at the end of the 24h chase period was compared with the content of immunoprecipitable radioactive products in the corresponding prelabelled islets. The difference between these two values, expressed as a percentage of the prelabelled islet content, is taken as the percentage of prelabelled immunoprecipitable products degraded intracellularly during the chase period (Halban & Wollheim, 1980; Halban *et al.*, 1980; Halban & Renold, 1983).

All data have been corrected for non-specifically precipitated radioactivity in the immunoprecipitation step (Halban *et al.*, 1980) and for the net change in leucine residues arising from the conversion of proinsulin into insulin (Halban, 1982*a*).

Radioactivity incorporated into total islet proteins was estimated by precipitation with trichloroacetic acid (Halban *et al.*, 1980). In order to monitor the fate of total islet proteins, excluding proinsulin or insulin, the radioactivity incorporated into immunoprecipitable products was subtracted from that found in trichloroacetic acid-precipitable material.

Total (labelled and unlabelled) insulin was determined by a standard radioimmunoassay (Halban *et al.*, 1980). All data are presented as means $\pm$S.E.M. The level of significance for differences between groups was assessed by Student's *t* test for unpaired groups.

For autoradiography, islets were fixed with 2.5% glutaraldehyde in cacodylate buffer, postfixed in OsO_4, and embedded in Epon resin. Thin sections were processed for autoradiography (Caro & van Tubergen, 1962) using Ilford L4 emulsion. The distribution and frequency of the autoradiographic

grains over the rough endoplasmic reticulum and secretory granules was evaluated quantitatively (Whur *et al.*, 1969; Stäubli *et al.*, 1977).

Results

Incorporation of [^{3}H]leucine into proinsulin and trichloroacetic acid-precipitable products

Exposure of islets to the analogues during the 2h prelabelling and 30min labelling periods resulted in a parallel decrease in the incorporation of [^{3}H]leucine into both immunoprecipitable products (proinsulin) and into total islet proteins other than proinsulin (trichloroacetic acid-precipitable radioactivity less immunoprecipitable radioactivity) (Table 1).

For the determination of the rate of release and intracellular degradation of prelabelled products during the 24h chase period, the values presented in Table 1 for islet radioactivity at the end of the 30min labelling period were taken as 100% in order to be able to compare directly data for control islets and for islets exposed to analogues.

Effects of analogues on the conversion of proinsulin into insulin

In order to determine the extent of conversion of prelabelled proinsulin into insulin during the chase period, samples of culture medium and of islet extracts taken at the end of the chase period were subjected to immunoprecipitation and gel chromatography. The relative percentage of radioactivity in the form of insulin is shown in Table 1 (right-hand column). For control islets, no labelled proinsulin was detectable in the islets at the end of the chase period, and of the immunoprecipitable products released into the chase medium, only 33% remained as proinsulin, 67% having been converted into insulin during the 24h chase. For islets prelabelled in the presence of the analogues, the situation was quite different. Of the immunoprecipitable products released into the chase culture medium, only 6% had been converted to insulin, and only 42% of the immunoprecipitable labelled products remaining in the islets at the end of the chase period were found to be in the form of insulin.

Labelling of islets in the presence of the analogues thus resulted in an inhibition in the conversion of newly synthesized labelled proinsulin into insulin during the chase period. These data are taken to reflect modification of the proinsulin molecule by the analogues and are in agreement with previous data which showed a block in proinsulin conversion during a 3h, rather than a 24h, chase period (Halban, 1982*a*).

Fate of native and modified proinsulin and labelled proteins during the 24h chase period

Exposure of islets to the analogues during the 2h prelabelling and 30min labelling periods had no significant effect on the percentage of prelabelled proinsulin subsequently released from the islets during the 24h chase period (Fig. 1*a*). Thus approx. 60% of prelabelled proinsulin had been released from both groups of islets, despite the block in the conversion of the analogue-modified proinsulin into insulin. However, exposure of the islets to the analogues had a marked effect on the amount of prelabelled proinsulin remaining in the islets at the end of the chase period (Fig. 1*b*). Whereas 30% of the prelabelled immunoprecipi-

Table 1. *Effects of analogues on the incorporation of [^{3}H]leucine into labelled products and their subsequent fate during a 24h chase period*

Islets were preincubated (2h) and labelled (30min) in the presence ('Analogues') or absence ('Control') of canavanine and thialysine, and then subjected to a 24h chase period without analogues. To estimate the extent of conversion of labelled proinsulin into insulin during the chase period, samples were immunoprecipitated and the immunoprecipitable products then chromatographed (Sephadex G-50). The radioactivity eluted as native insulin was expressed as a percentage of that eluting as proinsulin plus insulin. The data are expressed as means ± S.E.M., with the number of independent observations in parentheses. No significant radioactivity above background was eluted from the columns in the position of: * insulin; † proinsulin.

		Immunoprecipitable radioactivity (d.p.m./islet)	Trichloracetic acid-precipitable less immunoprecipitable radioactivity (d.p.m./islet)	Percentage conversion of proinsulin to insulin
Prelabelled islets	Control	665 ± 62 (8)	2360 ± 240 (8)	–*
	Analogues	365 ± 42 (9)	1320 ± 90 (9)	–*
Islets after 24h chase	Control	114 ± 14 (8)	1180 ± 70 (8)	100†
	Analogues	25 ± 2 (9)	199 ± 7 (9)	42 ± 6 (4)
24h chase medium	Control	307 ± 43 (8)	921 ± 85 (8)	67 ± 4 (5)
	Analogues	193 ± 14 (9)	477 ± 45 (9)	6 ± 1 (4)

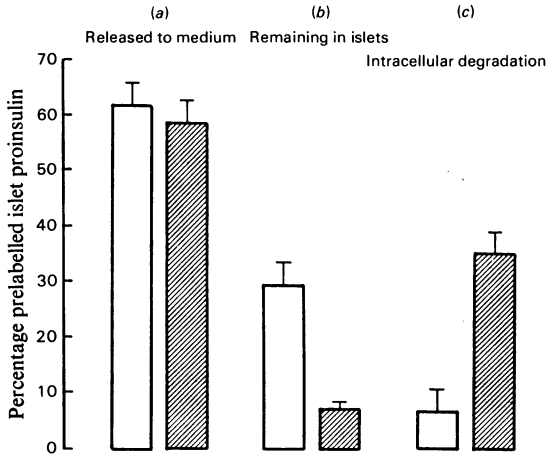

Fig. 1. *Fate of proinsulin prelabelled in the presence* ■ *or absence (control,* □*) of analogues during the 24h chase period*

The islet contents of labelled proinsulin at the end of the 30min labelling period with [^{3}H]leucine (Table 1) for controls and for islets labelled in the presence of analogues were taken as 100% for the determination of the percentage of labelled proinsulin which had been released during the chase period (*a*), which remained in the islets at the end of the chase period (*b*), or which had been degraded within islet cells during the chase period (*c*). The extent of conversion of labelled proinsulin into insulin during the chase period (Table 1) has been taken into consideration for the calculation of these data in order to account for the loss in leucine residues which arises during such conversion (see the Materials and methods). The results are expressed as means$\pm$S.E.M. ($n = 8$ for controls and $n = 9$ for islets preincubated and labelled in the presence of analogues).

Table 2. *Fate of labelled islet proteins other than proinsulin/insulin during 24h chase period*

Islets were preincubated (2h), labelled (30min) and, then incubated for a 24h chase period as described in Table 1. The radioactivity in trichloroacetic acid-precipitable products, less than in immunoprecipitable material in the prelabelled islets, was taken as 100% (for absolute values, see Table 1) for expression of the percentage of labelled products which were released from islets or degraded during the chase period. The results are expressed as means$\pm$S.E.M., $n = 8$ for control islets ('Control'), and $n = 9$ for islets preincubated and labelled in the presence of analogues ('Analogues').

	Percentage of prelabelled islet proteins (other than proinsulin/insulin)	
	Released during 24h chase	Degraded during 24h chase
Control	38$\pm$3	19$\pm$4
Analogues	36$\pm$7	49$\pm$2

table radioactivity was found remaining in control islets, only 7% was left in islets prelabelled in the presence of the analogues. When the total recoverable immunoprecipitable radioactivity was calculated (the sum of that remaining in the islets and that released into the medium), it was found that only 7% of the prelabelled proinsulin had been degraded in control islets, whereas 36% had been degraded in the islets exposed to the analogues (Fig. 1*c*). The degradation of islet proteins other than proinsulin/insulin was also increased after their labelling in the presence of the analogues, without any change in the rate of release of such products during the 24h 'chase' period (Table 2).

It can be tentatively concluded from these data that the modification to the proinsulin molecule evoked by incorporation of the analogues resulted in an inhibition in the conversion of the modified proinsulin into insulin with an associated increase in the rate of degradation of the modified product compared with that of native proinsulin and insulin. The observation that the modified proinsulin was released at the same rate as native products would suggest that it had been packaged into secretory granules. In order to confirm this important aspect, the passage of radioactive products from the rough endoplasmic reticulum to secretory granules was evaluated by high-resolution autoradiography.

Morphometric analysis of high-resolution autoradiographs

The specific radiation label density of the rough endoplasmic reticulum and secretory granules in B-cells (number of autoradiographic grains per unit volume of compartment) was determined morphometrically. It can be seen (Fig. 2) that during the course of the 24h chase period there was a movement of radioactive products from the rough endoplasmic reticulum to the secretory granules. The rate of transfer between these two compartments was comparable for controls and for islets which had been prelabelled in the presence of the analogues. The lower values for the specific radiation label density of both the rough endoplasmic reticulum and secretory granules in B-cells from islets prelabelled in the presence of analogues is in keeping with the decrease in the incorporation of [^{3}H]leucine into islet proteins and proinsulin in such islets (see Table 1).

On the basis of both the biochemical and the morphological data it is thus concluded that the modified proinsulin is indeed sequestered into secretory granules.

Effects of analogues on unmodified stored insulin

Although the above data are in keeping with a selective effect of the analogues (their incorporation into proinsulin resulting in a block in its conversion into insulin) it cannot be excluded from the data to hand that the analogues exert other effects on islet function as a whole due to modification of non-insulin-related islet proteins synthesized during exposure of the islets to the analogues. Two experimental approaches were used to verify this point. In the first, the influence of analogues on immunoreactive insulin was evaluated. Immunoreactive insulin will consist of both labelled (modified) and, predominantly, unlabelled insulin already present in islets before the relatively brief exposure to the analogues (the total time of exposure being 2h plus 30min). Any effects of the analogues on the handling of immunoreactive insulin would thus reflect a generalized effect on islet function independent of any modification of newly synthesized proinsulin. No significant effects, however, were observed on either islet insulin content or on the amount of immunoreactive insulin released from the islets during the 24h chase period (Table 3). The second approach involved a separate experimental protocol in which the intracellular degradation of islet insulin stores prelabelled before exposure to analogues was determined. To this end, islets were labelled with [^{3}H]leucine for 3 days. The labelled islets were then exposed to the analogues for 2.5h (without [^{3}H]leucine), followed by a 24h chase period without analogues. In this series of experiments the islet insulin stores have thus been labelled rather than newly synthesized insulin as in the previous protocol. Furthermore, the labelled insulin stores will not be modified by the analogues, which were only present after the labelling period. The release and intracellular degradation of the prelabelled insulin stores was monitored during the 24h chase period. It was found that exposure of the islets to the analogues had no effect on either of these parameters (Table 4).

It is concluded that the increased degradation of proinsulin modified by the analogues is not due to

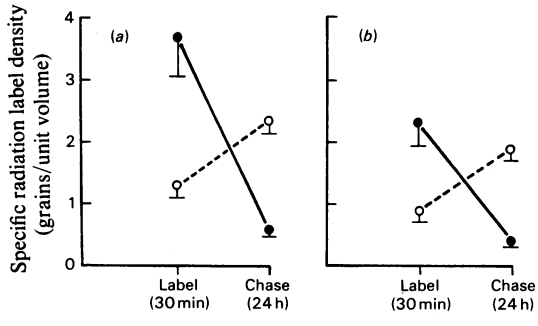

Fig. 2. *Morphometric analysis of autoradiographic grain localization in B-cells of islets prelabelled with (a) or without (control, b) analogues, followed by a 24h chase period*

Islets (three per group for each time point) were fixed and high-resolution autoradiographs analysed morphometrically in order to determine the specific radiation label density (number of autoradiographic grains per unit volume of each compartment) of the rough endoplasmic reticulum (●) and secretory granules (○). The results are presented as means ± S.E.M.

Table 3. *Effects of analogues on islet content and release of immunoreactive insulin*

Data are presented as means ± S.E.M. with the number of independent observations in parentheses. No statistically significant differences were found between control islets ('Control') and those preincubated and labelled in the presence of the analogues ('Prelabel + analogues'), the protocol for these experiments being as given in Table 1.

	Immunoreactive insulin (ng/islet)		
	Islet content before chase	Islet content after chase	Released during chase
Control	30.8 ± 2.7 (9)	30.2 ± 3.3 (8)	21.6 ± 2.3 (8)
Prelabel + analogues	31.7 ± 1.7 (9)	38.2 ± 5 (9)	22.6 ± 2.7 (9)

Table 4. *Effects of analogues on the release and intracellular degradation of labelled islet insulin stores during a 24h chase period*

Islets were labelled with [^{3}H]leucine for 3 days and then exposed to analogues for 2.5h in the absence of [^{3}H]leucine. Islets were taken at this time for analysis (3-day-labelled islets) and the remaining islets were incubated for a 24h chase period in the absence of analogues. The results are expressed as means ± S.E.M., $n = 6$, for each group.

	Immunoprecipitable radioactivity (d.p.m./islet)			Percentage of labelled immunoprecipitable radioactivity	
	3-Day-labelled islets	Islets after 24h chase	Released during 24h chase	Released during 24h chase	Degraded during 24h chase
Control	1430 ± 20	620 ± 30	290 ± 20	20 ± 1	35 ± 4
Exposed to analogues	1440 ± 130	600 ± 50	290 ± 10	20 ± 2	36 ± 8

additional effects of the analogues on B-cell function as a whole but is related to the observed inhibition in the conversion of the modified product into native insulin.

Discussion

The conversion of proinsulin into insulin is initiated during its passage through the Golgi complex (Orci, 1982) and continues thereafter within secretory granules [the converting enzyme co-residing in granules with proinsulin (Docherty *et al.*, 1982)] with a half-time of approx. 60 min (Steiner *et al.*, 1974). The finding that 33% of immunoprecipitable radioactive products released from control islets during the 24 h chase period was still in the form of proinsulin, despite the comparatively short half-time for proinsulin conversion, is in keeping with the observation that newly synthesized proinsulin and insulin are released rapidly, and preferentially, from islets *in vitro* (Gold *et al.*, 1982; Halban, 1982*b*). Any labelled proinsulin released from B-cells before its conversion into insulin would not be so converted in the chase culture medium. By contrast, material remaining in B-cells throughout the chase period was found, as expected, to have been fully converted into insulin in control islets. For islets exposed to the amino acid analogues, only 6% of the immunoprecipitable material released into the chase medium had been converted into insulin, confirming previous findings that incorporation of canavanine and thialysine into proinsulin inhibits its conversion into insulin (Noe, 1981; Halban, 1982*a*). Only 7% of the initial islet content of labelled immunoprecipitable products (proinsulin) remained in islets at the end of the chase period after labelling in the presence of analogues. Of this material, 58% was still in the form of proinsulin. The very low levels of radioactivity available from such material renders chemical characterization impracticable and it cannot thus be excluded that some, if not all, of the lower-M_r immunoprecipitable products were proinsulin degradation products rather than native insulin. Even assuming that 42% of the proinsulin remaining in these islets had been converted into insulin, this still represents less than 5% of the initial islet content of labelled proinsulin.

We have previously shown, and have confirmed in the present study, that proinsulin modified by analogues is released from islets at the same rate as native proinsulin and insulin (Halban, 1982*a*). This was taken to indicate that the modified material had been packaged into secretory granules before its release from B-cells. We have now confirmed this by a high-resolution autoradiographic study which showed an apparently normal rate of sequestration of the modified labelled proinsulin into β-granules. We therefore conclude that this material is released from B-cells by the conventional exocytotic pathway (Orci, 1982), and suggest that the same should apply to the rare cases of hyperproinsulinaemic patients with modified proinsulin arising from a mutation in their insulin gene (Gabbay *et al.*, 1979; Robbins *et al.*, 1981).

The degradation of secretory proteins within their cell of origin has been documented for a number of cell types (see Bienkowski, 1983, for review), including the B-cell (Halban & Wollheim, 1980; Halban & Renold, 1983). The mechanism of the intracellular degradation of hormones stored in secretory granules remains to be elucidated, but it is commonly assumed that crinophagy, the fusion of granules with primary lysosomes (Smith & Farquhar, 1966; Chertow, 1981), is the principal pathway. However, identification of proteinases in B-cells which can degrade insulin, but which are not, apparently, of lysosomal origin (Kohnert *et al.*, 1976) implies that a non-lysosomal pathway may also be active in B-cells. For cytosolic proteins in other cell types, the main pathways for the intracellular degradation of endogenous proteins have been shown to be either lysosomal (Dean, 1979; Chertow, 1981) or mediated by neutral cytosolic proteinases (Goldberg & St. John, 1976; Hershko & Ciechanover, 1982).

To date, studies on the intracellular degradation of modified or damaged proteins have been largely concerned with cytosolic proteins (Knowles *et al.*, 1975; Knowles & Ballard, 1976; Klemes *et al.*, 1981), an ATP-dependent proteinase having been implicated in such degradation (Etlinger & Goldberg, 1977; Goldberg & Boches, 1982). It has also been found, however, that enhanced degradation of a modified exportable protein (collagen) most probably occurs by a lysosomal pathway (Berg *et al.*, 1980), but, unlike insulin, collagen is not stored for any length of time in secretory granules. In the present study we found that islet proteins other than proinsulin/insulin (trichloroacetic acid-precipitable less immunoprecipitable radioactive products) were degraded more rapidly after labelling in the presence of analogues. Such products will consist, for the most part, of cytosolic islet-cell proteins. Interestingly, whereas the degradation of modified proinsulin was approx. 5 times that found for native proinsulin/insulin, the degradation of islet proteins which had, presumably, also been modified by the analogues, was only 2.5 times that for native products, suggesting that a different mechanism may have been responsible for the enhanced degradation in the two instances.

We envisage the following alternatives to account for the accelerated degradation of modified proinsulin in B-cells. (1) The modified proinsulin in some way alters the properties of secretory

granules, these alterations are 'sensed' by lysosomes, and such granules are introduced into the digestive compartment (Smith & Farquhar, 1966; Orci *et al.*, 1968; Meda, 1978) at a faster rate than those containing native products; (2) the modified proinsulin, unable to be packed in the form taken by native insulin in mature granules [i.e. the insulin crystal (Greider *et al.*, 1969)] may be more sensitive to proteolytic digestion; (3) a portion of the modified proinsulin is recognized as being abnormal before sequestration into secretory granules and is disposed of by an alternative pathway. The elucidation of which of these pathways is involved will require the characterization of the modified proinsulin-containing compartments within the B-cell. It will then be necessary to determine whether these compartments contain damaged or undamaged modified products (by immunocytochemical criteria), and/or whether the treatment with analogues is accompanied by an increased mobilization of lysosomes.

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