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Structural Lability of Zinc-Containing Secretion Granules of Pancreatic β-Cells after Exposure to Hydrogen Sulphide*

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Summary. Treatment of vertebrate pancreatic islet tissue with hydrogen sulphide, thus initiating the sulphide-silver procedure for ultrastructural visualization of heavy metals in tissues, evoked marked swelling in zinc-containing secretion granules of β-cells in several species rapidly leading to disruption of many granules. Other organelles of the β-cells were unaffected, as also were the secretion granules of adjacent zinc-containing α_2-cells. Likewise, β- and α_2-granules with no heavy metals or only traces of them, like those of the guinea pig and the coypu, were all well preserved after gassing with hydrogen sulphide. It is suggested that this observation may give some information on insulin-zinc-sulphydryl relationships *in vivo*.

Labilité structurale des granules de sécrétion contenant du zinc des cellules β du pancréas après gazage avec de l'hydrogène sulfuré

Résumé. Le traitement des îlots pancréatiques de plusieurs espèces de vertébrés avec de l'hydrogène sulfuré, constituant ainsi la phase initiale de la méthode sulfure-argent pour la visualisation ultrastructurale des métaux lourds dans les tissus, provoquait un gonflement marqué des granules de sécrétion zincifères des cellules β dans plusieurs espèces, conduisant rapidement à la rupture de beaucoup de granules. D'autres organelles des cellules β restaient intacts, ainsi que les granules de sécrétion des cellules α_2 zincifères voisines. De même, les granules β et α_2 sans ou avec seulement des traces de métaux lourds, comme ceux du cobaye et du nutria, étaient bien conservés après le gazage avec l'hydrogène sulfuré. — On suggère que cette observation peut donner quelques aspects sur les relations insuline-zinc-sulfhydryl *in vivo*.

Strukturelle Labilität von Sekretionsgranula mit Zinkgehalt in pankreatischen B-Zellen nach Begasung mit Schwefelwasserstoff

Zusammenfassung. Behandlung mehrerer Arten von pankreatischem Inselgewebe von Wirbeltieren mit Schwefelwasserstoff, womit die Sulfid-Silber-Methode zur ultrastrukturellen Darstellung von Gewebe-Schwermetallen eingeleitet wird, verursacht eine ausgesprochene Anschwellung der zinkhaltigen Sekretionsgranula der β-Zellen, die schnell zum Zerfall der Granula führt. Andere Zellorganellen der β-Zellen waren nicht beschädigt, auch nicht die Sekretionsgranula der benachbarten α_2-Zellen. Ebenfalls waren β- und α_2-Granula mit keinen oder nur Spuren von Schwermetallen, wie die des Meerschweinchens und der Nutria, alle wohlbehalten nach der Behandlung mit Schwefelwasserstoff. — Als eine Arbeitshypothese für zukünftige Untersuchungen wird angedeutet, daß diese Beobachtung von Bedeutung in Bezug auf die Insulin-Zink-Sulfhydryl-Verhältnisse sein kann.

Key-words: Pancreatic islets, zinc, insulin, sulphydryl compounds, sulphide-silver procedure, insulin release, α_2-cells, β-cells, secretion granules, guinea pig, coypu, man, cat, dog, teleost fish, Chinese hamster.

Introduction

In trials to test the so-called sulphydryl (SH) and zinc theories of the pathogenesis of alloxan diabetes, and possibly also of diabetes mellitus in general (cf. FALKMER, 1967), we recently observed some peculiar histochemical reactions in the secretion granules of some kinds of β-cells. They occurred so constantly that we thought it might be worth while to report them briefly. Another reason to give a note on our observations is that they may be of biological significance.

The peculiar reactions observed comprised fairly marked differences in the ultrastructural preservation of the β-granules of various species after H_2S gassing, and also often a variation in this respect between α_2- and β-granules of the same species.

Material and Methods

Pancreatic islet tissue from the following species was used: the bony fish *Cottus quadricornis* (the long-horn sculpin), man, the rabbit, the Chinese hamster, the rat, the guinea pig, the coypu, the cat, and the dog. In this pilot investigation at least two well preserved islets were studied in each animal. In each species the number of animals varied from two to 10. From man, altogether 3 biopsy specimens were obtained from 3 patients during operation for carcinoma of the stomach or the kidney. No patients with overt diabetes were included. Details on the animal and human material will be given in a more comprehensive report that is presently being prepared (PIHL, 1968b).

The tissue specimens were used for ultrastructural histochemical studies on the occurrence of heavy metals, notably zinc, by our previously described modification of the sulphide-silver procedure (PIHL and FALKMER, 1967; PIHL, 1967).

The modified sulphide-silver method implies initial gassing of the tissue slices immersed in buffered glutaraldehyde. In the present experiments the gassing was performed at temperatures varying from 0°C to about 8°C and at pH from 7.4 to 6.7. Otherwise, the technique closely followed the original description with its latest modifications (PIHL, 1968a) included. The effects of the

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pH variations were not investigated more systematically, *e.g.* by using a graded series of buffers. The ionic strength of the fixative was the same as used previously, *i.e.* essentially 2.5% glutaraldehyde with a 0.1 M phosphate buffer.

The embedding, cutting, staining, and electron microscopic techniques were all those described in detail elsewhere (PIHL and FALKMER, 1967; PIHL, 1967, 1968a).

Results

In experiments performed so far it was observed that whereas the β-granules of islets from the guinea pig, the coypu (Fig. 1), and the sculpin were well preserved after fixation in H_2S-gassed glutaraldehyde, the β-cells of all other species investigated, including man, were often rapidly swollen and emptied by exactly the same treatment. This autolysis selectively struck the β-granules, for other organelles, such as nuclei, endoplasmic reticulum, mitochondria, lysosomes, and cell membranes were all fairly well preserved (Figs. 2—4). Interestingly, the adjacent α_2-cells, including their granules, were well preserved (Figs. 2 and 3) in all species studied. It was also observed that sulphide-silver-positive β-granules close to the nucleus and the Golgi apparatus suffered less from the H_2S gassing than the rest of the secretion granules.

When various stages of the destruction of the β-granules were studied, it was found that initially the granules swelled to two or three times their original size, growing thin centrally (Fig. 3). Then, the granule contents dissolved, sulphide-silver-positive material (probably zinc) being dispersed in all directions. The β-granules of all species were fairly well preserved when H_2S-gassing of the glutaraldehyde was omitted, irrespective of the temperature and pH variations (*vide infra*).

The selective destruction of β-cells by H_2S could be alleviated by keeping the temperature of the glutaraldehyde at 3.5—4.5° C and not allowing the pH to fall below 7.2. As soon as the temperature was allowed to rise to about 6—8° C there was in some species practically no single β-granule left, whereas mitochondria and other cell organelles were still fairly well preserved (Fig. 4).

In specimens kept at 3.5—4.5° C and at a pH above 7.2, and also by the reactivation procedure (PIHL, 1968a), it could be assessed that all the β-granules with a tendency to selective destruction by H_2S contained ample amounts of sulphide-silver-positive material. The β-granules of the guinea pig gave a completely negative sulphide-silver reaction, whereas "reactivation" of ultra-thin sections from coypu islets yielded but traces of metal. In the β-granules of the sculpin only a few seemed to contain sulphide-silver-reactive substance.

The α_2-cells of the guinea pig and the sculpin were sulphide-silver-negative. Those of the coypu, man, and dog were generally positive only after "reactivation" with sulphides; and the α_2-granules of the rat, the rabbit, the Chinese hamster, and the cat, in increasing order, gave clearly positive sulphide-silver reaction.

A more comprehensive report on the sulphide-silver picture of various types of islet parenchyma cells will be given elsewhere (PIHL, 1968b).

Discussion

It is striking that only those β-granules that were found to contain histochemically demonstrable zinc (sculpin, man, rabbit, Chinese hamster, rat, cat, and dog) were destroyed, whereas β-granules which almost lacked such zinc (guinea pig, coypu) retained their structures much better. This observation may at first sight seem to invalidate some of our preceding reports (PIHL and FALKMER, 1967; PIHL, 1967, 1968a). However, this supposed discrepancy is not a real one. The difficulties in obtaining good ultrastructural preservation of heavy metal containing β-granules have been emphasized previously (cf. PIHL, 1968a) and the reasons for the fact that it may be possible to detect heavy metals in fairly well preserved β-granules have been given in the present report. Admittedly, we have, however, no explanation to offer for the finding that heavy metal containing β-granules close to the nucleus and the Golgi region may escape destruction from H_2S gassing better than secretion granules located more peripherally (provided that the temperature and pH are strictly controlled to be 0—4° C and 7.2—7.4, respectively).

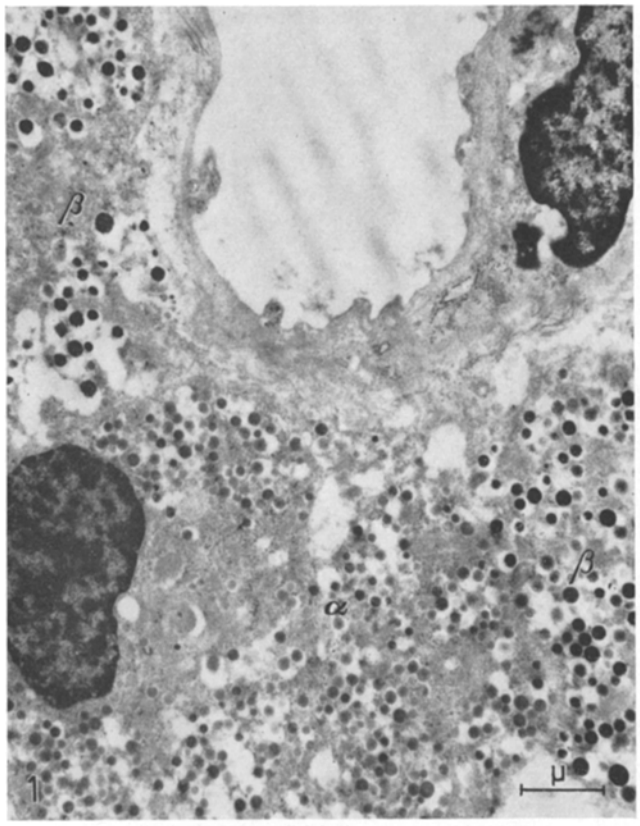
From comparative studies on the amino acid composition of various mammalian insulins, together with biochemical investigations on the mode of binding of zinc to the insulin molecule (cf. SMITH, 1966, 1968), it has been suggested that it is the imidazol group of the histidine molecule at B_{10} position that binds the zinc. The insulins from the guinea pig and the coypu have at the B_{10} position aspartic acid and glutamic acid, respectively (SMITH, 1968; DAVIDSON et al., 1968). Nei-

Fig. 1. Part of a coypu pancreatic islet treated with hydrogen sulphide during fixation. The granules of both the α_2- and β-cells are fairly well preserved and do not differ from those of cells fixed in plain glutaraldehyde. There is no silver precipitation from physical development of the actual cells. (Stained with uranyl acetate alone, as are all the subsequent figures.)

Fig. 2. Human pancreatic islet cells treated as in Fig. 1. The β-cell granules (top) are in a state of dissolution. Some of them (arrows) show silver precipitates. The α_2-granules (below) are not affected by hydrogen sulphide.

Fig. 3. Part of a rat pancreatic islet treated as in Fig. 1. The β-cell granules (top, right) have swollen, being in a state of dissolution and have reacted with silver. Around destroyed granules the reaction product can also be seen in the cytoplasm. The α_2-granules are unaffected by the gassing, and only few granules show silver precipitates.

Fig. 4. Part of a human pancreatic β-cell treated as in Fig. 1. The large vacuoles (V) are thought to represent granules which are almost dissolved. One large aggregation of silver precipitates is seen within the upper vacuole. The mitochondrion (M) is fairly well preserved.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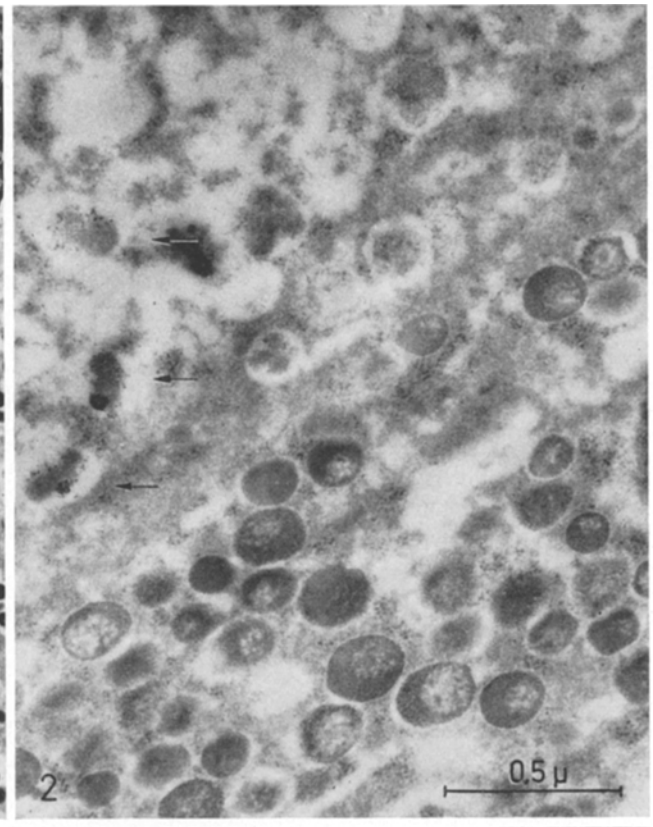
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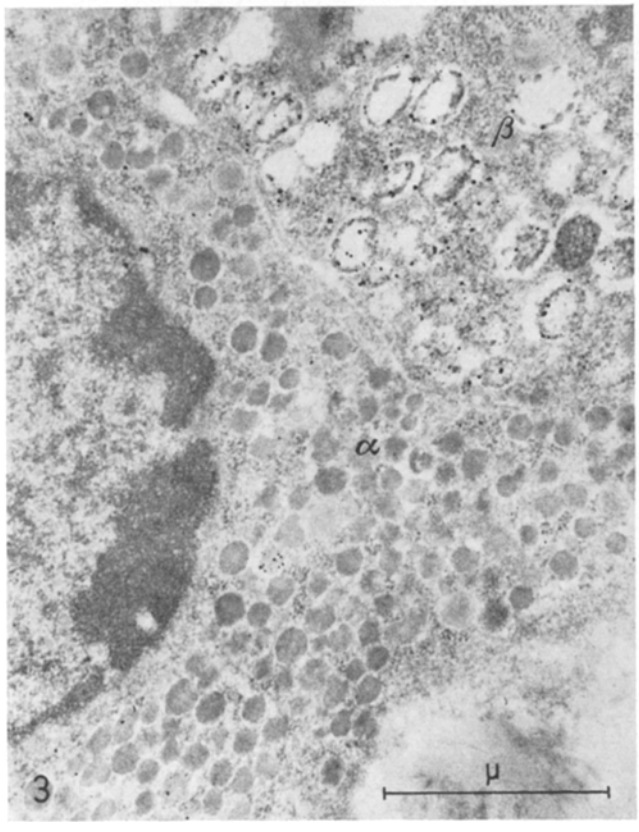
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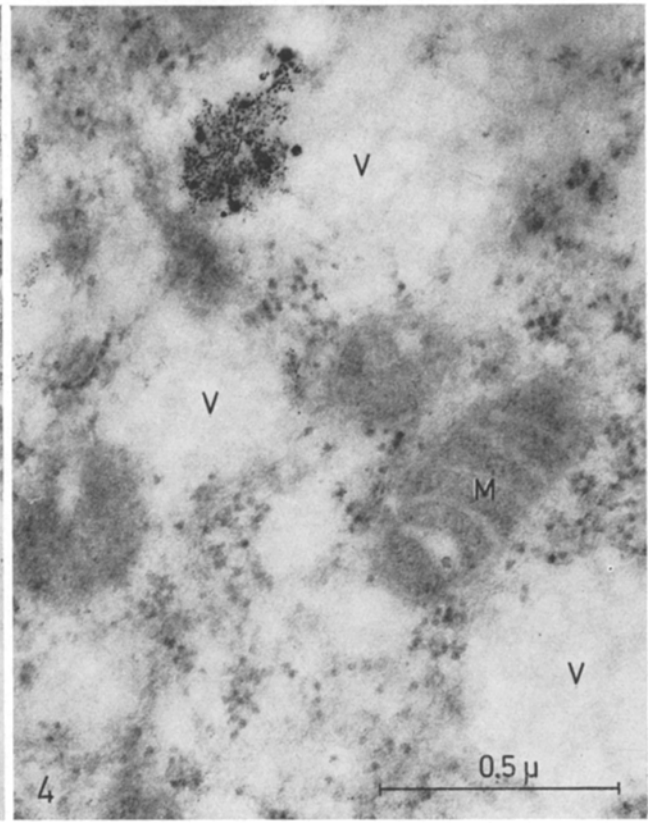
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ther of these two amino acids contains such a potent zinc binder as the imidazol group of histidine. pK_{im} is around 6 and pK_1 of H_2S is around 7 under the actual conditions. Provided that pK_{im} is not seriously displaced by the presence of the zinc atom, by far the major part of the imidazol should be in the unchanged form at pH 7.2 and hence suitable as a ligand to zinc. At pH 6.7 part of the zinc-imidazol bond should be broken. The presence of SH^- as a counterion to H^+ would reinforce, synergistically, the effect of lowering pH by forming zinc sulphide, and the joint effect of H^+ and SH^- on the granules should therefore become more marked than would be anticipated from the pK_{im}. A similar mechanism has been found for the effect of HCl on the Fe^{3+} -imidazol bonds in Cytochrome C (BOERI et al., 1953). The disruption of such a stabilizing bond in a protein molecule as Zn-imidazol will almost certainly give rise to alterations in the tertiary structure.

However, this explanation of the selective β-granule destruction as an ionic and pH effect is not compatible with the recent observation by COORE et al. (1967) and TÄLJEDAL, (1968) that isolated mammalian β-granules are more stable at low pH than at pH 7.2—7.4. Neither does it cover the fact that the glucagon-containing (cf. BROLIN and HELLERSTRÖM, 1967) α_2-granules of the islets of the Chinese hamster, the rabbit, and the rat were not destroyed by the H_2S-gassing despite their zinc content (VOIGT, 1959; PIHL and FALKMER, 1967). A similar — though admittedly not so marked — difference in the preservation of the specific granules after gassing with H_2S was also noted between the mast cells and the eosinophil granulocytes, which both contain zinc (cf. PIHL and GUSTAFSON, 1967; PIHL et al., 1967). So far, we have no data to explain these differences adequately. We can only speculate that zinc must be bound to the granules of mast cells and pancreatic β-cells in another way than in eosinophil granulocytes and pancreatic islet α_2-cells.

It must also be taken into consideration that the surplus amount of SH^- ions might activate a hypothetical SH-dependent lytic enzyme within the granule, or markedly interfere with the function of the perigranular membrane, for it is known that SH^- ions and metals have this effect (ROBINSON, 1966). As a matter of fact we recently observed that the β-granules of the Chinese hamster, but not those of the guinea pig, seemed to contain protein-bound SH groups (PIHL and FALKMER, 1968). However, it does not seem probable that such mechanisms of lytic enzyme activation would be effective at 0—4° C.

At physiological pH insulin is said to be precipitated only by Zn, Co, Ni, or Cd (SCOTT, 1934). Probably the hormone is kept within the β-cell granule in insoluble form (MASKE, 1955). It seems fair to assume that any mechanism quickly mobilizing Zn from insulin would bring about dissolution of the granule. It is known that following administration of the chelating substance dithizone "a large number of β-cells were injured severely" (KAWANISHI, 1966), and that endogenous insulin was mobilized without any morphologie signs of increased activity of β-cells (MOHNIKE et al., 1966). The recent observation by YOSHINAGA and YAMAMOTO (1966) that active sulphonylureas bind zinc, whereas inactive derivatives do not, may also be of some interest here. This finding indicates that insulin becomes released from the secretion granules when the binding to zinc or any other heavy metal is broken.

As a *speculative working hypothesis for future investigations*, it thus seems fair to assume that the effect of SH^- ions on β-granules is due to its breaking of Zn-protein bonds. If this be the mechanism of insulin release in zinc-containing β-cells, it seems reasonable that the content of active SH groups in the β-cell would have to be kept critically low. Actually, this is exactly what has been postulated in LAZAROW's SH-theory on the mechanism of alloxan diabetes, saying that the content of SH groups in the β-cells is much lower than in other cells of the body due to the synthesis of insulin with its high content of cysteine (cf. COOPERSTEIN et al., 1964). It must be admitted, however, that so far we have not obtained any data indicating a particularly low content of active SH groups in isolated pancreatic islet tissue (cf. FALKMER, 1967; HAVU et al., 1968).

It is obvious from what has been said above that many more basic facts about these peculiar reactions must be established before any less speculative discussion on the interpretation of our observation can be given. As noted by us previously (cf. FALKMER, 1967), it may have implications for both insulin release *in vivo*, and the zinc and SH theories of the pathogenesis of some types of diabetes mellitus.

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