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Histochemical and Ultrastructural Studies on Pancreatic A Cells. Evidence for Glucagon and Non-Glucagon Components of the α Granule

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Summary. Glucagon-storing cells were detected in the pancreatic islets of horse, guinea pig, rabbit, rat, dog and man by means of indirect immuno-histochemical procedures using fluorescein or peroxidase as markers of anti-glucagon sera. By subsequently staining horse islets with selective staining techniques for A, B or D cells, it was directly ascertained that only A cells reacted to antiglucagon sera. A comparison of methods known to stain α granules with immuno-histochemical methods, as well as *in vitro* experiments on natural and synthetic glucagon, showed that the xanthydrol method, and perhaps also the *o*-phthalaldehyde method, should be regarded as specific histochemical tests for glucagon whereas the HCl-basic dye technique and phosphotungstic haematein should be considered as unspecific glucagon-staining methods, and Grimelius' silver as a method likely to be unrelated to glucagon. In ultrastructural investigations on the human pancreas, the core of the α granule heavily reacted to phosphotungstic acid and peroxidase-marked antiglucagon antibodies, while being unreactive to Grimelius' silver; conversely, the peripheral halo of the α granule heavily reacted to Grimelius' silver, while being poorly reactive or unreactive to phosphotungstic acid and anti-glucagon antibodies. Thus, both light and electron microscopy findings point to a structural and chemical hetereogeneity of the α granule.

Etudes histochimiques et ultrastructurales des cellules pancréatiques A. Preuves de la présence de constituants glucagoniques et non-glucagoniques dans les granules α

Résumé. Des cellules stockant du glucagon ont été détectées dans les îlots pancréatiques du cheval, du cobaye, du lapin, du rat, du chien et de l'homme au moyen de procédés immuno-histochimiques indirects utilisant la fluorescéine ou la peroxydase comme marqueurs des sérums anti-glucagon. En colorant les îlots des chevaux avec des techniques de coloration sélectives pour les cellules A, B ou D, il a été directement prouvé que seules les cellules A réagissent aux sérums anti-glucagon. Une comparaison entre les méthodes connues pour colorer les granules α et les méthodes immuno-histochimiques, ainsi que les expériences *in vitro* sur le glucagon naturel et synthétique, ont montré que la méthode au xanthydrol et peut-être également la méthode à l'*o*-phtalaldéhyde, doivent être considérées comme des tests histochimiques spécifiques pour le glucagon; par contre la technique à l'HCl-colorant basique et l'hématéine phosphotungstique doivent être considérées comme des méthodes de coloration non spécifiques pour le glucagon, et la méthode à l'argent de Grimelius probablement comme ne concernant pas le glucagon. Au cours d'investigations ultrastructurales sur le pancréas humain, le centre des granules α réagissait fortement à l'acide phosphotungstique et aux anticorps anti-glucagon marqués à la peroxydase, alors qu'il ne réagissait pas à l'argent de Grimelius; inversement le halo périphérique des granules α réagissait fortement à l'argent de Grimelius, et réagissait peu ou pas à l'acide phosphotungstique et aux anticorps anti-glucagon. Ainsi, les résultats à la fois de microscopie optique et électronique indiquent une hétérogénéité structurale et chimique des granules α.

Histochemische und ultrastrukturelle Untersuchungen über die A Zellen der Bauchspeicheldrüse. Nachweis von Glucagon und nicht-Glucagon Anteilen des α-Granulums

Zusammenfassung. Die Glucagon enthaltenden Zellen werden in der Bauchspeicheldrüse von Pferd, Meerschweinchen, Kaninchen, Ratte, Hund und Mensch durch immunhistochemische, indirekte Techniken mit Fluorescein- oder Peroxidase-Markierung von Anti-Glucagon-Antikörpern lokalisiert. Durch aufeinanderfolgende Färbungen der Langerhansschen Inseln von Pferden mit spezifischen für die A, B und D Zellen histologischen Methoden, wurde bewiesen, daß nur die A Zellen mit Anti-Glucagon-Antikörpern reagierten. Eine Gegenüberstellung der für die Färbung des α-Granulums bekannten Methoden, mit den immunhistochemischen Methoden, und den als *in vitro* Untersuchungen über synthetisches und natürliches Glucagon, haben bewiesen, daß die Xanthydrol-Methode, und vielleicht auch die o-Phtalaldehyd-Methode, für das Glucagon spezifische Methoden darstellen, während die HCl-basischen Farbstoffe und die Phosphorwolframsäure-Hämatin Methoden, als unspezifische Färbemethoden für das Glucagon angesehen werden müssen; die Silbermethode nach Grimelius scheint nicht zum Glucagon in Beziehung zu stehen. In ultrastrukturellen Untersuchungen über die menschliche Bauchspeicheldrüse, reagierte das Zentrum (*core*) des Granulums stark auf die Phosphorwolframsäure und auf die mit Peroxidase markierten Anti-Glucagon-Antikörper, dagegen reagierte es nicht mit der Silbermethode nach Grimelius. Andererseits reagierte der periphere Hof des α-Granulums wenig, oder überhaupt nicht mit der Phosphorwolframsäure und den Anti-Glucagon-Antikörpern. Daher zeigen die Ergebnisse von Licht- und Elektronenmikroskopie eine chemische und strukturelle Ungleichartigkeit des α-Granulums.

Key-words: Pancreatic islets, A cells, glucagon, immuno-histochemistry, α-granule stains, xanthydrol, *o*-phthalaldehyde, HCl-basic dye, phosphotungstic acid, Grimelius' silver, electron histochemistry.

The production of glucagon by the non-B cells of the pancreatic islets has been fully proven; moreover, the involvement of A cells has been suggested, and that of D cells excluded, on the ground of several histochemical and experimental findings (Bencosme *et al.*, 1955; Glenner and Lillie, 1957; Baum *et al.*, 1962; Petersson and Hellman, 1963; Cavallero *et al.*, 1968; Lomsky *et al.*, 1968; Okada *et al.*, 1968; Lange, 1970). However, an extensive direct comparison on the same section of the immuno-histochemical patterns with those of selective stainings for the three islet cell types has not been performed. Of the various staining methods for α granules, only the immuno-histochemical one is usually regarded as fully specific for glucagon. The other methods are considered to be either of limited specificity (as for instance the "tryptophan" tests) or unspecific in respect to the glucagon molecule (as the HCl-basic dye method, phosphotungstic haematein and acid dyes) or of obscure histochemical meaning (as the *o*-phthalaldehyde and silver impregnation techniques).

An investigation has been undertaken on: 1) the staining of glucagon-storing cells with specific immunohistochemical techniques and the identification of these same cells with methods known selectively to detect islet cell types, and 2) the significance of the various methods for α granules in respect of the histochemistry of glucagon and the fine structure of α granules.

Material and Methods

a) Immuno-histochemical investigations

Anti-glucagon antibodies were obtained from 2 adult albino rabbits injected 6 times, at 3 week intervals, with glucagon (beef-pork, crystalline, supplied by E. Lilly, Indianapolis — lot No. 258—234 B-167-1). The first 4 subcutaneous injections were made according to the technique previously employed by Okada *et al.* (1968). The last 2 injections were done intravenously, by administering 3 mg of glucagon in 1 ml of physiological saline buffered to pH 8, +0.05 ml of a solution containing 11% poly-methyl-metacrilate particles (Bofors). The animals were bled 5 days after the final injection.

In addition, we employed an anti-glucagon rabbit serum, kindly supplied by Dr. R. Lomsky (Charles University, Hradec Kralové, Czechoslovakia).

Indirect "sandwich" immunofluorescence tests were performed using a fluorescent goat anti-rabbit gamma globulin serum, prepared as previously described (Bussolati and Pearse, 1970). Controls were made by omitting the anti-glucagon serum or substituting normal rabbit serum.

Absorption tests were done, to test the specificity of the immunological reaction, by absorbing the anti-glucagon serum with 1 mg/ml of insulin (Squibb) or glucagon (E. Lilly). In immunofluorescence experiments the sera absorbed with insulin gave results similar to those of non-absorbed sera, whereas absorption with glucagon resulted in complete inhibition of the A cell staining.

Immunofluorescence tests were performed on paraffin sections obtained from blocks of horse, guinea pig, rat, dog and human pancreas fixed a) with 4% formaldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C for at least 24 h, or b) with Carnoy's fluid at room temp. for 1 h.

An indirect immuno-histochemical procedure employing *Peroxidase* as a marker, similar in principle to that described by Avrameas (1969) was carried out by the successive exposure of sections to: 1) rabbit antiglucagon sera; 2) goat anti-rabbit gamma globulin sera; 3) pure rabbit anti-peroxidase antibodies binding free antibody sites of the anti-rabbit gamma globulin antibodies); 4) peroxidase (grade 1, Boehringer and type VI, Sigma), diluted as suggested by Mason *et al.* (1969). After staining with 3—3' diamino-benzidine (Sigma) according to the method of Graham and Karnovsky (1966) for peroxidase activity, sections were treated with 1% OsO_4, and either a) mounted in balsam for light microscopic examination, or b) observed unmounted under the light microscope and then resin-embedded for electron microscopy. Controls were performed by substituting normal rabbit sera for the anti-glucagon sera. Paraffin (5 μ thick) or cryostat (20 μ thick) sections of human, horse and guinea pig pancreas fixed at 4°C in 4% formaldehyde buffered to pH 7.4 were used in this procedure.

Staining procedures. Horse pancreatic islets, after immunofluorescence staining, were photographed. The sections were subsequently refixed for 24 h at room temperature with 4% formaldehyde or 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 or with GPA mixture (Solcia *et al.*, 1968) and restained with aldehyde fuchsin for B cells (Scott, 1952), coryphosphine O for D and B cells (Bussolati *et al.*, 1969; Bussolati, unpublished observations) HCl-toluidine blue (HCl-TB) and HCl-lead-haematoxylin (HCl-PbH) for A and D cells (Solcia *et al.*, 1968 and 1969 b), oxidation-phosphotungstic haematein (ox-PTH) for A cells (Caramia *et al.*, 1965; Cavallero and Solcia, 1968), Grimelius' silver for A cells (Grimelius, 1968) or xanthydrol test for A cells (Cavallero *et al.*, 1968).

b) Histologic investigations

Samples from pancreas of horses, guinea pigs, rats, rabbits, dogs and men were fixed for 1 to 4 days at 4°C or at room temperature with 4% formaldehyde, 5% glutaraldehyde or 2.5% formaldehyde +2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, and with Bouin's fluid, Carnoy's fluid or GPA mixture (Solcia *et al.*, 1968). To paraffin sections from such material, the following α granule methods were applied: "indole" tests with special reference to the xanthydrol method (Lillie, 1957; Solcia *et al.*, 1969a), phosphotungstic haematein, with or without previous acid permanganate oxidation of tissue sections, known to stain basic groups of polypeptides and proteins

(Terner *et al.*, 1964), toluidine blue, coryphosphine O and lead-haematoxylin, with or without previous HCl treatment of sections, known to stain acid groups, particularly side-chain protein or polypeptide carboxyls (Solcia *et al.*, 1968 and 1969; Bussolati *et al.*, 1969) and Grimelius' silver impregnation (Grimelius, 1968). The *o*-phthalaldehyde (OPT) method of Takaya (1970) was applied to both fresh cryostat sections and formaldehyde- or Carnoy-fixed cryostat or paraffin sections.

c) Electron microscopy investigations

Small samples of human pancreatic tissue were fixed at 4°C for 3 to 24 hours in 4% formaldehyde, 2.5% glutaraldehyde or 2.5% formaldehyde +2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, postfixed in osmium tetroxide and embedded in Epon 812 (Luft, 1961). Thin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Some aldehyde-fixed specimens were silver impregnated according to the Grimelius technique (Vassallo *et al.*, 1971) or stained 2 hours at 37°C with 2% phosphotungstic acid (PT) in distilled water; then they were dehydrated and resin-embedded. To localize glucagon at E.M. level, an indirect immuno-histochemical procedure using peroxidase as marker was applied to cryostat sections of formaldehyde-fixed specimens (see *a*).

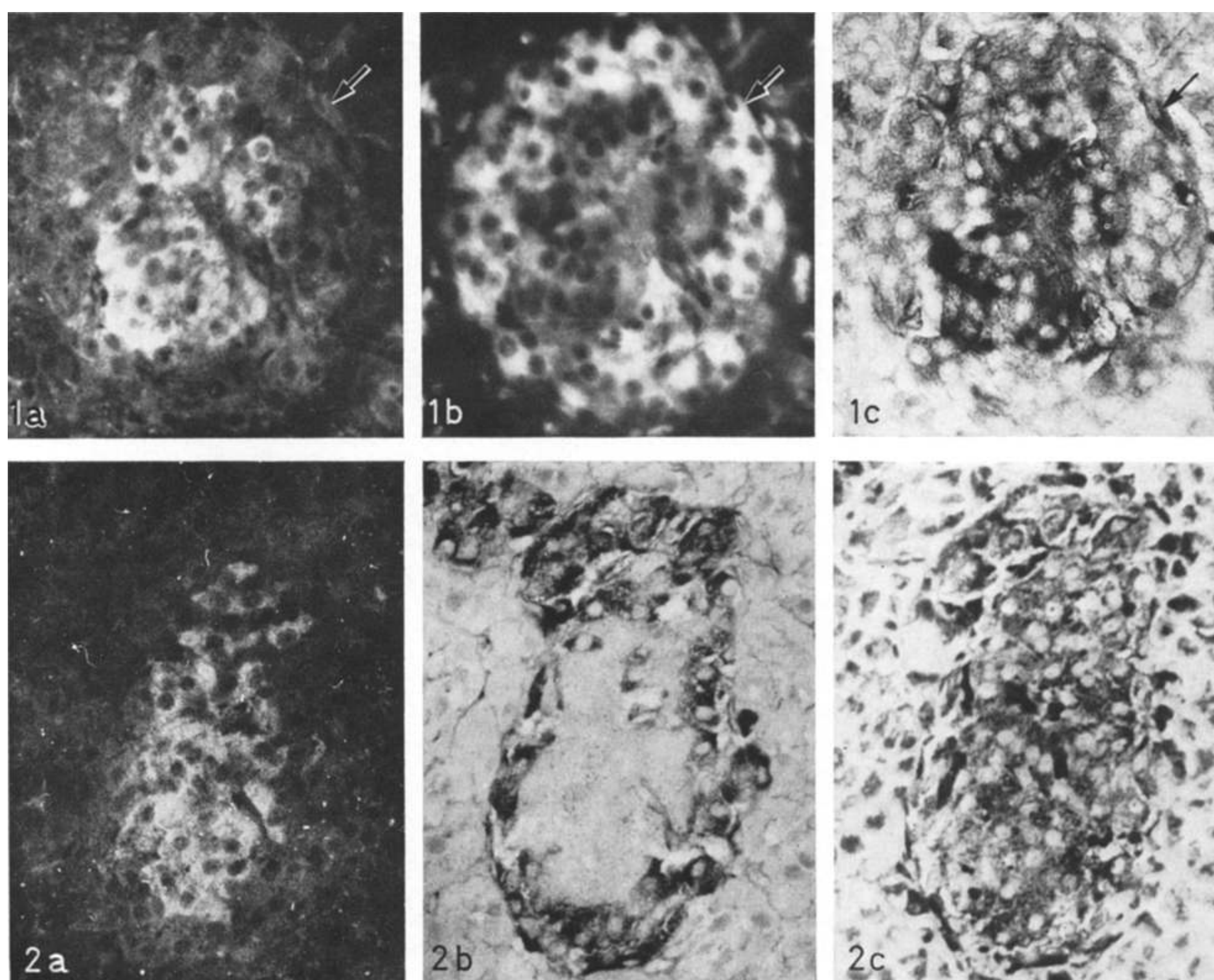

Fig. 1a, b and c. Formaldehyde-fixed horse pancreatic islet successively stained with fluoresceinated anti-glucagon antibodies (a), coryphosphine 0 (b), and HCl-toluidine blue (c). Centrally located A cells are reactive in *a* and *c* (blue), practically unreactive in *b*; a D cell at the periphery of the islet (arrow) is reactive in *b* and *c* (red) but fails to react to anti-glucagon antibodies in *a*. X 280

Fig. 2a, b and c. Formaldehyde-fixed horse pancreatic islet successively stained with fluoresceinated anti-glucagon antibodies (a), aldehyde fuchsin (b), and phosphotungstic haematein (c). A cells are reactive in *a* and *c*, unreactive in *b*; B cells are reactive in *b*, keep in *c* part of their aldehyde-fuchsin staining of step *b*, but are unreactive to anti-glucagon antibodies in *a*. X 280

d) In vitro tests

These were done by scattering synthetic (both crystalline and lyophilized synthetic glucagon, kindly supplied by Dr. E. Wunsch and coworkers, Max-Planck-Institut fur Eiweiss- und Lederforschung, München) or natural glucagon (beef-pork crystalline glucagon, lots No 258—561 b-180 and No 258—234 B-167-1, kindly supplied by E. Lilly, Indianapolis, and twice crystallized pork glucagon lot No B 66, kindly supplied by Novo Terapeutisk Laboratorium, Copenhagen) in a gelatine-sorbitol film on slides; part of these slides were plunged in the same fixing mixtures used in histological procedures. Both fixed and unfixed slides were stained just as tissue sections.

Results

A) *Reaction of horse islets cells to antiglucagon sera.* As already noted, (Hellman *et al.*, 1962; Solcia and Sampietro, 1965), horse pancreatic islets usually showed groups of A cells in the centre, surrounded by B cells; elongated D cells were scattered at the extreme periphery of the islets. This peculiar pattern was of great help in identifying fluorescent and non-fluorescent cells of restained sections. As shown in Table 1 and Figs. 1 and 2, neither B nor D cells were stained by the immuno-histochemical methods employing antiglucagon sera. Groups of immunofluorescent, glucagon-storing sells were located in the centre of the islets; the same cells were subsequently stained blue with ox-PTH, blue-violet with HCl-TB, blue-black with HCl-PbH, black with Grimelius' silver and blue-gray with xanthydrol. Such staining pattern is known to be peculiar to A cells. Immunofluorescence controls gave completely negative results.

A cells were also stained with the immuno-histochemical method employing peroxidase as marker. The reaction product was dark brown and apparently granular (Fig. 7).

B) *Comparison of glucagon stains to other α-granule methods.* A cells of the various species investigated showed different degrees of reactivity to granule-staining methods. For instance, human, rat and dog α granules reacted poorly to indole tests, whereas horse, guinea-pig or rabbit granules gave heavier staining; conversely, Grimelius' silver gave intense impregnation in all species, perhaps even more intense in man and dog than in the other mammals. Immunofluorescence, OPT, PTH with or without previous oxidation, HCl-TB and HCl-PbH, although always giving heavier stainings than indole tests, seemed to parallel better the behaviour of the last methods than that of Grimelius' silver (Figs. 3—6).

The behaviour of immunofluorescence, OPT, PTH and Grimelius' silver in staining formaldehyde- or Carnoy-fixed horse A cells is illustrated in Table 2; results obtained by staining guinea pig A cells in Bouin-fixed sections treated with hot HCl are reported in Table 3. In both cases Grimelius' silver behaved quite differently from other methods.

C) In experiments *in vitro* both fixed and unfixed natural or synthetic glucagon reacted blue-gray to xanthydrol and blue-violet to PTH, and fluoresced intensely with OPT, just as pancreatic α granules did. Conversely, glucagon failed to react to Grimelius' silver.

Table 1. *Reaction of formaldehyde-fixed horse islet cell types to fluorescein-labelled antiglucagon serum and selective granule stains*

Cell types	Aldehyde-fuchsin	Corypho-sphine	HCl-TB	HCl-PbH	Oxidation-PTH	Grimelius' silver	Xanthydrol	Antigluca-gon sera
A cells	—	—	+++ blue-violet	+++	+++	+++	++	+++
B cells	+++	++	—	—	—	—	—	—
D cells	—	+++	+++ red	+++	—	±	—	—

Table 2. *Influence of formaldehyde or Carnoy fixation on the staining of horse α granules*

Fixation	Immuno-fluorescence	OPT	PTH	Grimelius' silver
Formaldehyde	+++	+++	+++	+++
Carnoy	+++	+++	+++	—

Table 3. *Influence of HCl (0.2 N at 60° C for 3 hours) on the staining of Bouin-fixed guinea-pig α granules*

Pretreatment of sections	Xanthydrol	TB	PbH	Grimelius' silver
—	++	±	±	+++
HCl	++	+++	+++	—

D) *Ultrastructural studies.* The application of Grimelius' silver and PT to specimens processed for ultrastructural investigations gave an opportunity to compare directly light and electron microscopy findings. As shown by Terner *et al.* (1964), light microscopy staining of tissues with PTH is essentially due to PT;

moreover, PT is known to give high electron contrast to glucagon fibrils (Davies, 1968).

The ultrastructural findings in human A cells resembled that already described (Like, 1967; Shibasaki and Ito, 1969). In particular α granules showed two components, the core and the peripheral halo, whose staining patterns (see Table 4 and Fig. 8) were quite different. Despite poor penetration of antibodies

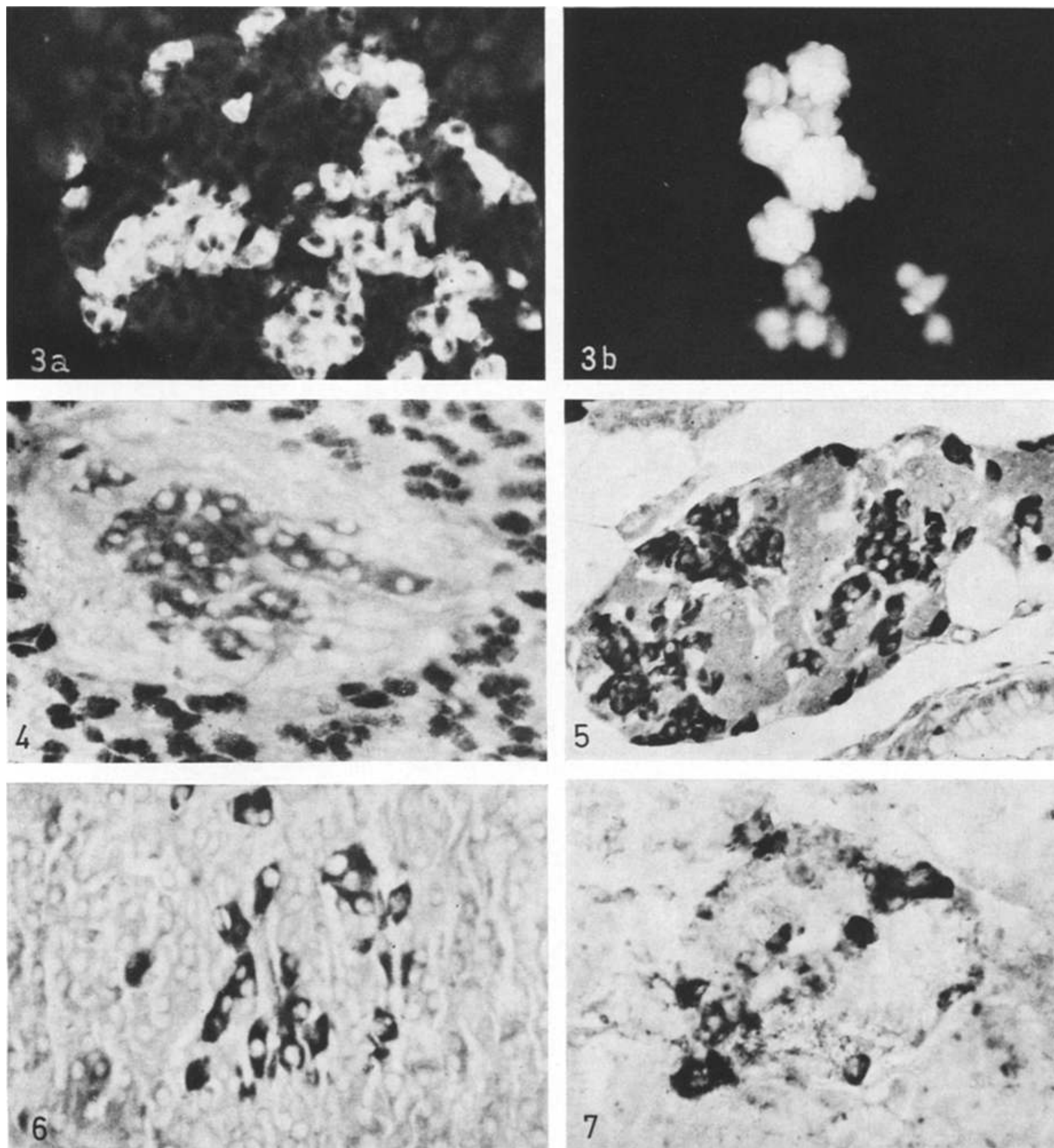

Fig. 3a and b. Formaldehyde-fixed guinea pig pancreas (*a*; cryostatic section) and glucagon crystals (*b*) stained with *o*-phthalaldehyde. x 400

Fig. 4. GPA-fixed horse pancreas stained with xanthydrol: A cells blue-gray, B and D cells unreactive, zymogen granules violet. x 320

Fig. 5. Glutaraldehyde-fixed guinea pig pancreas stained with HCl-lead haematoxylin. Reactive cells are A (many) and D cells (few); B cells do not react. x 220

Fig. 6. Bouin-fixed guinea pig pancreas stained with Grimelius' silver. A cells are blackened. x 360

Fig. 7. Formaldehyde-fixed human pancreas stained with anti-glucagon antibodies marked with peroxidase. A cells are intensely reactive. x 450

and/or peroxidase into pancreatic tissue, immunocytochemical tests in electron microscopy showed that peroxidase linked to anti-glucagon antibodies was selectively bound to the core of α granules.

Table 4. *Reactivity of the core and the halo of human α granules to fixing and staining methods at the ultrastructural level*

Fixation	Staining	Core	Halo
Osmium	Uranyl-lead	+++	−
Aldehyde-osmium	Uranyl-lead	+++	+±
Aldehyde	PT	+++	+
Aldehyde	Grimelius	−	+++

Besides immunohistochemical methods, the xanthydrol test provides a reliable histochemical detection of glucagon in A cells; the reason for the blue-gray colour of glucagon, instead of the violet staining of other tryptophan-storing structures, remains unexplained (Solcia *et al.*, 1969). The selective fluorescence of A cells stained with Takaya's *o*-phthalaldehyde method appears to be also linked to glucagon, though the exact interrelationship of glucagon with the aldehyde is unknown (Takaya, 1970).

Methods like those involving the use of phosphotungstic acid in light or electron microscopy certainly stain glucagon, as probably do azocarmine and other acid dyes; however, we do not know whether other substances with basic groups are also present in the α granules and contribute to their staining by such methods. The staining by HCl-toluidine blue and HCl-lead-haematoxylin is likely to be mostly due to side-chain protein or polypeptide carboxyls unmasked by hot HCl; aspartic and glutamic acids ("free" carboxyls) as well as asparagine and glutamine (amides) may contribute such groups (Solcia and Sampietro,

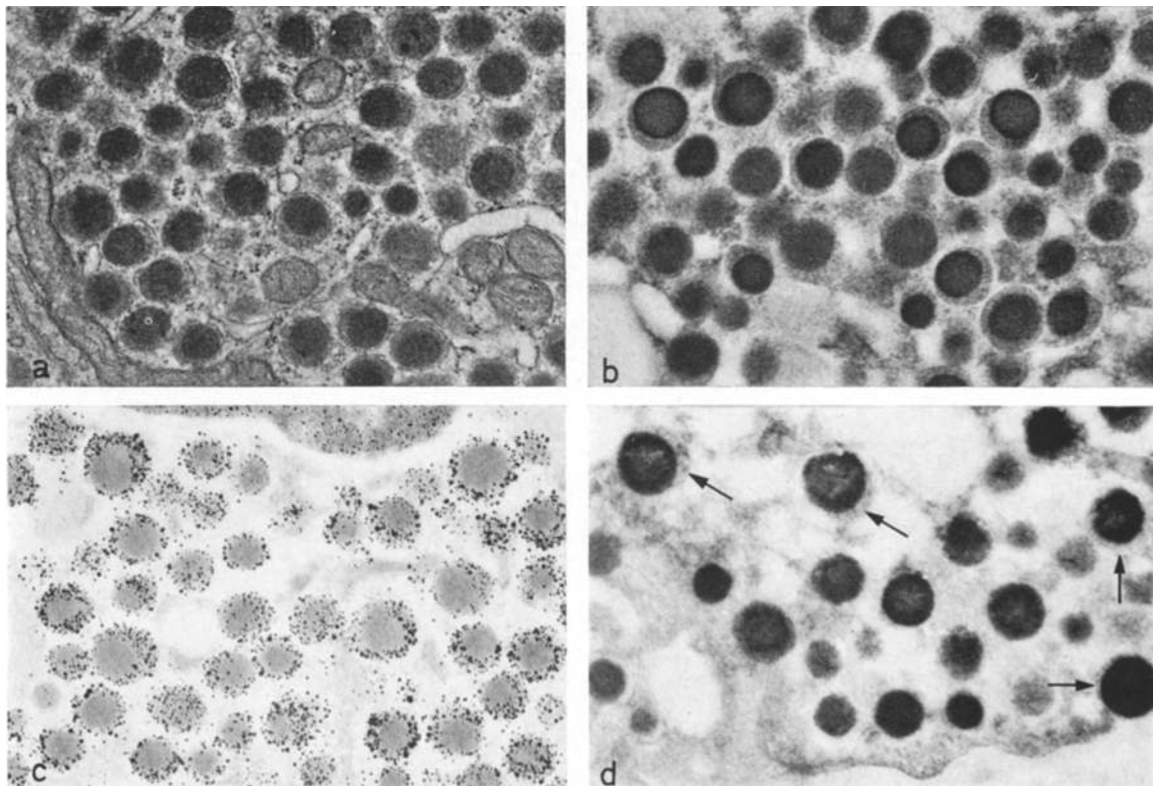

Fig. 8a, b, c and d. Granules of human A cells:
a, formaldehyde-glutaraldehyde mixture, osmium, uranyl, lead.
b, formaldehyde-glutaraldehyde, phosphotungstic acid.
c, formaldehyde-glutaraldehyde, Grimelius' silver, uranyl.
d, formaldehyde, antiglucagon antibodies-peroxidase, benzidine, osmium.
The "core" of the granules is heavily stained in *a*, *b* and *d*, while it lacks silver grains and is only stained by uranyl in *c*. The "halo" is intensely reactive only to Grimelius' silver. X 24000

Discussion

These results confirm that only A cells react to antiglucagon sera, whereas D cells do not react. Thus, although A cells must be considered the source of glucagon, D cells seem unrelated to this hormone; the exact nature of the hormone they produce remains to be ascertained.

1966; Solcia *et al.*, 1968 and 1969; Pearse, 1969). A total of 7 aspartic acid, glutamine or asparagine residues is represented in the glucagon molecule (Bromer *et al.*, 1956). We do not know whether, besides glucagon, other substances bearing side-chain carboxyls contribute to such stainings.

The above data seem to point to the conclusion that in human A cells glucagon is at least mostly located in the core of α granules, which, according to conventional ultrastructural investigations, should be considered as a definite internal structure of the α granule (Like, 1967; Shibasaki and Ito, 1969). It should be added that in some fishes the core frequently displays a crystalline pattern (Bencosme *et al.*, 1965; Thomas, 1970) similar to that shown by glycagon crystals *in vitro* (Staub *et al.*, 1955) and that we found some evidence of crystalline structure in the core of a few human granules.

Grimelius' silver behaves quite differently from glucagon-staining methods; thus, the Grimelius-positive material associated with the α granule "halo" seems to be chemically unrelated to the hormone. As to its exact nature, we have little information. However, it should be noted that its apparent solubility in Carnoy's alcohol-chloroform-acetic acid mixture may be in keeping with the presence of some lipid component (lipoprotein ?). In fact, experiments now in progress suggest binding of silver to ethylene groups of unsaturated fatty acids.

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