

TISSUE CULTURE OF A₂ CELL-RICH PANCREATIC ISLETS ISOLATED FROM GUINEA PIGS

ARNE ANDERSSON, BIRGER PETERSSON, JAN WESTMAN, JOHN EDWARDS, GUDMAR LUNDQVIST, and CLAES HELLERSTRÖM. From the Departments of Histology and Anatomy, University of Uppsala, Uppsala, Sweden

INTRODUCTION

Tissue culture of pancreatic B cells has been a matter of increasing interest during recent years and a number of problems related to the biosynthesis and release of insulin are now being approached with the aid of this technique (1-8). By comparison, little is known about the behavior in tissue culture of the A₁ and A₂ cells, mainly because of the small number of these cells in normal mammalian islets. The recent introduction of techniques for production of A₂ cell-rich islets in guinea pigs (9, 10) and for tissue culture of intact, isolated islets (11) seems, however, to have opened up new possibilities for a close study of the structure and metabolism of the glucagon-producing A₂ cells during prolonged culture *in vitro* (12).

In the present investigation, isolated A₂ cell-rich islets were maintained in culture for up to 6 days and then investigated with regard to both light and electron microscopical appearance, glucagon content, glucagon release, and rate of oxygen consumption. It was found that after the culture period the A₂ cells exhibited an excellent morphologic preservation and retained a number of their characteristic metabolic responses.

MATERIALS AND METHODS

Animals

Altogether 43 male guinea pigs weighing about 200 g were injected intraperitoneally with Streptozotocin (Upjohn Co., Kalamazoo, Mich.) in a single dose of 70 mg/animal. Immediately before the injection, the drug was dissolved in citrate buffer, pH 4.5, at a concentration of 35 mg/ml.

Tissue Culture

About 1 wk after the injection, the animals were killed by decapitation and the pancreas was removed under sterile conditions. Small pieces of the gland were immediately fixed in Zenker's formol solution for further histological examinations and the remaining pancreatic tissue was used for isolation of

pancreatic islets according to Howell and Taylor (13). The isolated islets were transferred to a plastic Petri dish containing 80% (vol/vol) TCM 199, 20% (vol/vol) calf serum, antibiotics, and glucose at a final concentration of 1.2 mg/ml. The cultures were subsequently maintained for 6 days. For further details of the culture technique, reference should be made to Andersson and Hellerström (11).

Morphological Studies

For light microscopy the cultured islets were fixed in Zenker's formol solution and embedded in Epon (Epikote). Sections, 1 μ m thick, were cut on an ultramicrotome and stained with chrome-hematoxylin Ponceau fuchsin or aldehyde fuchsin trichrome (14, 15). Fresh living islets, still suspended in the culture dish, were also examined in the phase-contrast microscope. For ultrastructural studies the cultured islets were fixed for 1 h in a mixture of 1% (vol/vol) formaldehyde and 2% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate buffer adjusted to pH 7.4. Postfixation was performed in 1% (wt/vol) osmium tetroxide in 0.06 M phosphate buffer adjusted to pH 7.4. After dehydration the islets were embedded in Epikote. Thin sections were prepared on an LKB ultratome (Laboratorie och Kemikaliska Produkter, Stockholm) and picked up on one hole grids and stained with uranyl acetate followed by lead citrate. The electron microscopy was carried out with a JEOL TEM 100 B.

Assay of Islet Respiration

Groups of five to ten cultured islets were incubated at 37°C in Cartesian divers according to Hellerström (16). The incubation medium consisted of Krebs-Ringer phosphate buffer pH 7.4, and the rate of oxygen consumption was measured either in this buffer alone or after addition of 10 mM sodium succinate.

Determination of Glucagon Content and Release

Groups of 20 islets, either cultured for 6 days or freshly isolated from Streptozotocin-injected guinea

pigs, were homogenized in 50 μ l double-distilled water by means of a Perspex microhomogenizer. Duplicate samples, 10 μ l each, were taken for protein determination according to Lowry et al. (17). The remaining homogenate was extracted with 200 μ l acid ethanol (15 ml 12 N HCl/liter 70% ethanol) for 24 h at 4°C and stored at -20°C before glucagon assay (see below).

In order to measure glucagon release, groups of ten cultured islets were incubated for 30 min at 37°C in 250 μ l bicarbonate-buffered medium (18) supplemented with 2 mg/ml albumin and 5.5 mM D-glucose. In some experiments 5 mM L-arginine was added to the medium. The glucagon content of the incubation medium was measured with a solid phase radioimmunoassay recently described by Lundqvist et al. (19). Standards were prepared from beef-pork glucagon dissolved in the bicarbonate-buffered medium.

RESULTS

Light microscope examination of pancreatic sections from the Streptozotocin-injected guinea pigs confirmed that in all the animals the islets were composed mainly of A₂ cells and only a few B cells. Isolated islets from these animals were

therefore regarded as suitable for culture of A₂ cell-rich pancreatic islets. During the first days of the culture period the isolated islets attached to the bottom of the culture dish. At this and later intervals a few fibroblastoid cells could be found in the surroundings of the cultured islets, but closer to the center the islets appeared entirely made up of epithelioid cells as revealed by phase-contrast microscopy.

Examination of stained sections of cultured islets with the light microscope showed a normal appearance of the islet cells (Fig. 1). No necrotic areas were found in any parts of the islets and there were very few contaminating fibroblastoid cells. Differential staining of the islet cells revealed a great majority of A₂ cells, but also a few B cells could be detected. Several mitoses, presumably in A₂ cells, were observed (Fig. 1).

In the electron microscope most of the islet cells appeared polyhedral with central nuclei containing one or two distinct nucleoli (Fig. 2). The cytoplasm contained numerous rod-shaped mitochondria, and the rough endoplasmic reticulum was arranged as parallel membranes of

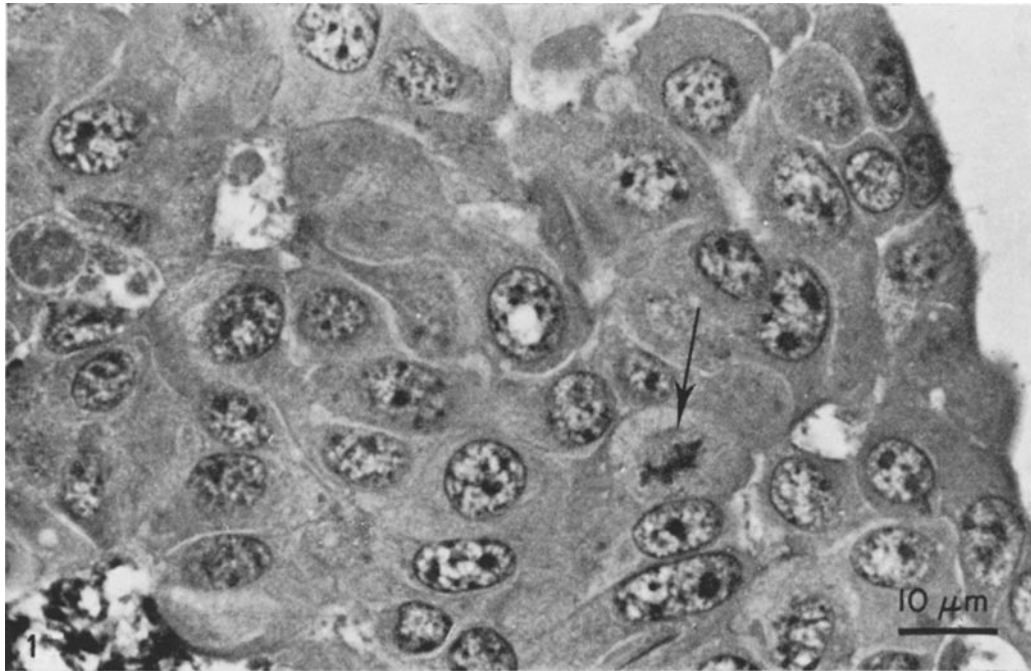


FIGURE 1 Light microscope view of a chrome-hematoxylin Ponceau fuchsin-stained section from an islet cultured for 6 days. The periphery of the islet is seen to the right. In the central part of the picture there is a mitotic figure in an islet cell (arrow). $\times 1,200$.

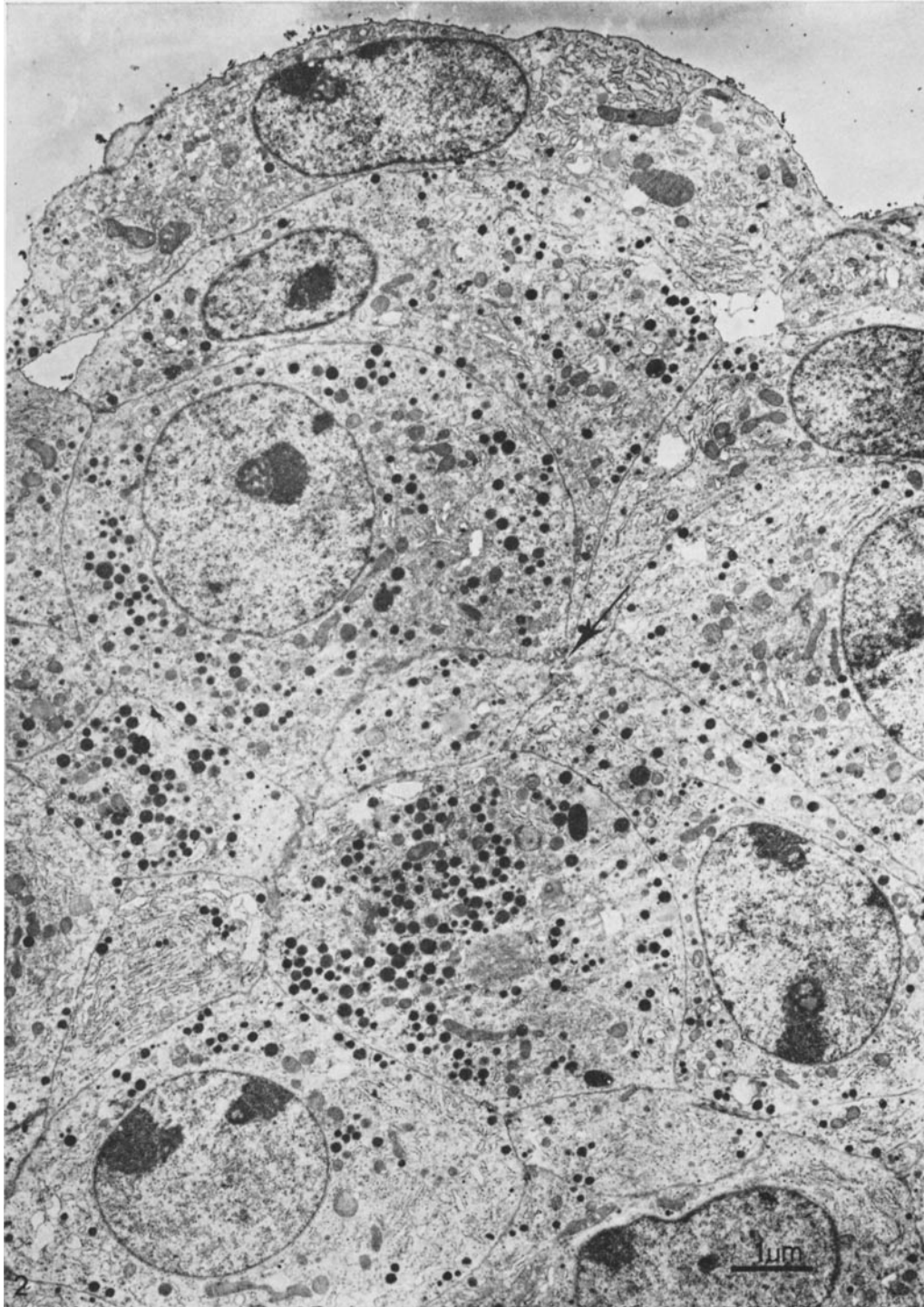


FIGURE 2 Electron microscope view of the peripheral part of an islet cultured for 6 days showing A₂ cells with typical round electron-opaque secretory granules. The granule content of the cell profiles shows some variability. Villous protrusions (arrow) from adjacent cells can be observed in the intercellular spaces. $\times 12,000$.

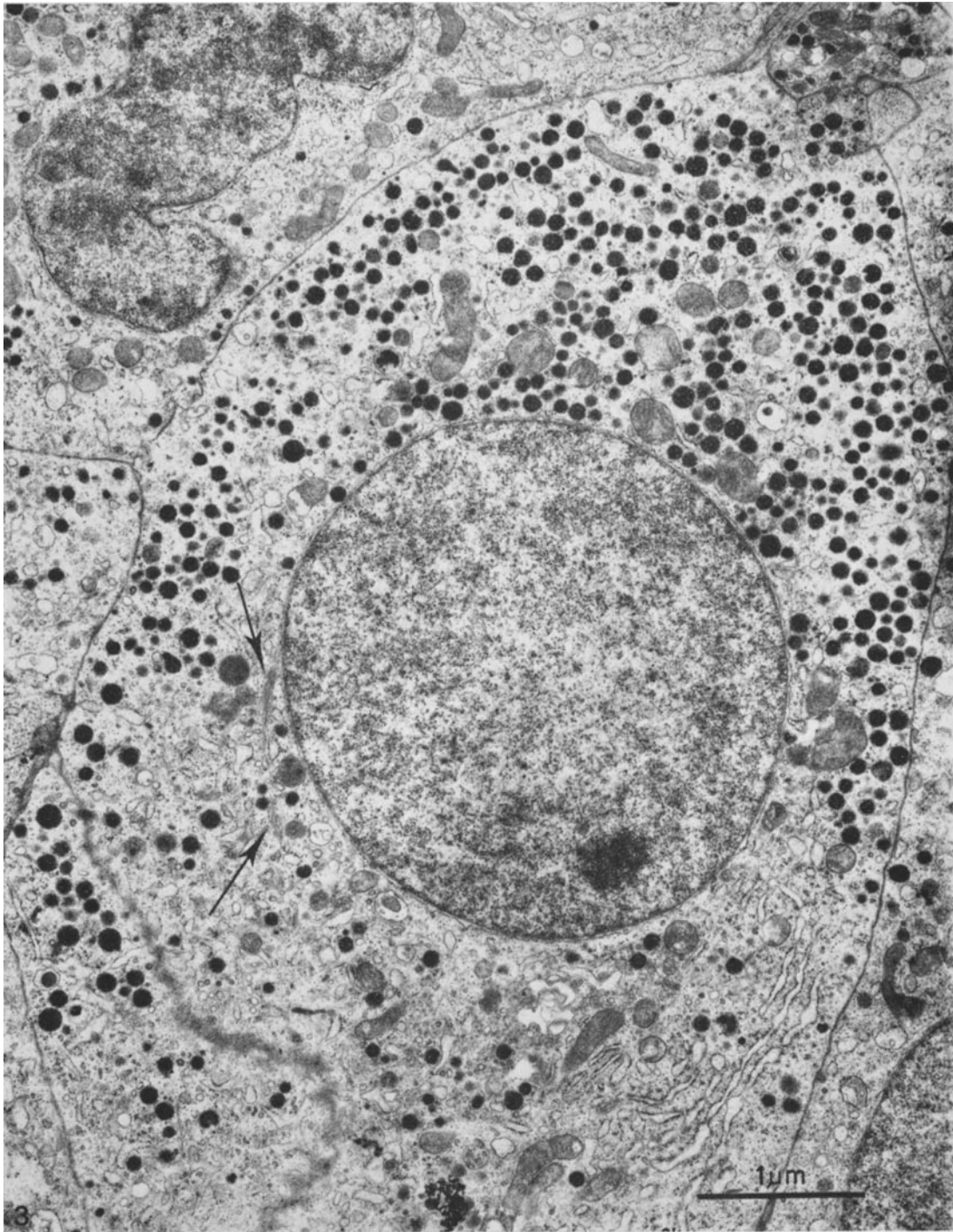


FIGURE 3 An A₂ cell from an islet cultured for 6 days with numerous secretory granules and a prominent endoplasmic reticulum in the lower right part. Note the presence of filamentous or tubular structures within the cell (arrows). $\times 26,000$.

varying length. The intracellular spaces were sometimes widened to small cisternae, where microvilli from adjacent cells could be observed (Fig. 2). The majority of islet cells contained secretory granules of A₂ type with tightly fitting sacs and high electron density (Fig. 3). The number of granules in each cell profile varied a great deal and some of the A₂ cells appeared degranulated (Fig. 2). Only a few B cells were recognized. Necrotic or fibroblastoid cells were seldom observed.

As can be seen in Fig. 4, the respiratory rate of islets cultured for 6 days remained constant for at least 60 min. The oxygen uptake (mean \pm SEM) in the presence of 5 mM glucose was calculated as $3.13 \pm 0.27 \mu\text{l/h}$ per islet ($n = 5$). Addition of 10 mM succinate to the incubation medium significantly stimulated the respiration, which now became $5.61 \pm 0.69 \mu\text{l/h}$ per islet ($n = 4$; $P < 0.025$).

The glucagon content of both freshly isolated and cultured pancreatic islets is given in Fig. 5. It is evident that a 6-day culture period did not alter the hormone content of the islets. The glucagon release (mean \pm SEM) of cultured islets to a medium containing 5.5 mM glucose was $0.10 \pm 0.02 \text{ ng/ten islets per 30 min}$ ($n = 10$). When 5 mM arginine was added the glucagon release increased to $0.22 \pm 0.02 \text{ ng/ten islets per 30 min}$ ($n = 11$; $P < 0.001$).

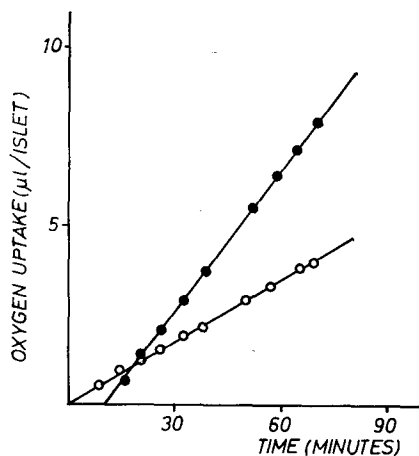


FIGURE 4 Kinetics of oxygen uptake in two divers each containing nine islets cultured for 6 days. The incubation medium in the two divers consisted of Krebs-Ringer phosphate medium alone (open circles) or this medium supplemented with 10 mM succinate (filled circles).

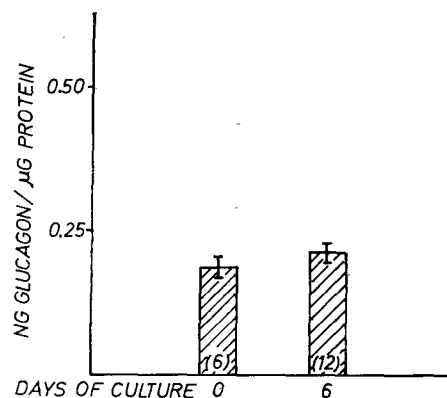


FIGURE 5 Glucagon content (means \pm SEM) of isolated pancreatic islets from Streptozotocin-injected guinea pigs.

DISCUSSION

The data presented here indicate that the A₂ cells of the pancreatic islets retained a number of their morphologic and metabolic characteristics when maintained in tissue culture for up to 6 days. Thus, for a slight and varying degranulation, the cultured A₂ cells were morphologically indistinguishable from those seen in freshly isolated islets or in islets remaining in the intact pancreas (9, 20-22). The additional observation of a high proportion of A₂ cells in the cultured islets lends further support to previous reports suggesting that, in the guinea pig, Streptozotocin is harmless to the A₂ cells whereas most B cells are destroyed by the drug (9, 10).

The predominance of A₂ cells in the cultured islets made it feasible to investigate their survival also in terms of oxygen consumption. These data indicate that cellular respiration continued in vitro at a constant rate for a period of time comparable to that seen in freshly isolated A₂ cell-rich islets (10). Direct comparisons between the $-Q_{O_2}$ (oxygen uptake in microliters/milligrams dry weight) of cultured islets and the corresponding variable of freshly isolated ones were not practical since the dry weight of the small amount of islet cells recovered after culture could not be readily determined. Further support for a normal respiratory behavior of the cultured A₂ cells was, however, provided by the observation of a significant stimulation of the oxygen uptake by the addition of succinate to the medium. A similar respiratory enhancement by succinate has previ-

ously been reported for freshly isolated A₂ cell-rich islets of guinea pigs (10).

In evaluating the behavior of A₂ cells in tissue culture it seemed particularly important to study the specific functions of these cells in terms of glucagon storage and release. The present data suggest that both these variables remained intact throughout the culture period, in that the glucagon content of cultured islets was not different from that of fresh islets and the glucagon release was significantly stimulated by arginine. The magnitude of glucagon release in both the presence and absence of arginine was, however, smaller than previously observed for freshly isolated A₂ cell-rich islets of guinea pigs (9). Whether this discrepancy was due to a lower "baseline" release of glucagon from cultured islets, or to other factors, remains to be clarified. It is worthy of note that the basal release of insulin (i.e., at 0.6 mg/ml glucose) has been reported to be significantly lowered in islets cultured for 6 days (11).

Altogether, the present results suggest that tissue culture of isolated pancreatic islets is compatible with satisfactory survival of glucagon-producing A₂ cells for at least 6 days in vitro. It is suggested that the present approach should be useful for providing new information on the long-term effects in vitro of various factors on the structure and metabolism of the A₂ cells.

SUMMARY

A₂ cell-rich pancreatic islets were isolated from Streptozotocin-injected guinea pigs. The islets were subsequently maintained in tissue culture for 6 days before being studied with regard to morphology and metabolism. As revealed by both light and electron microscopy, there was a satisfactory preservation of the cultured islets. Mitoses were frequently observed among the islets. The oxygen consumption of the cultured islets proceeded at a linear rate and responded to succinate in a manner similar to that in freshly isolated islets. The glucagon content of the islets remained unchanged after the culture period. Arginine stimulated the glucagon release from the cultured islets to the same extent as from non-cultured ones. It is concluded that the present technique for tissue culture of isolated A₂ cell-rich islets is useful for long-term studies in vitro of the morphologic and metabolic behavior of the glucagon-producing A₂ cells.

Financial support from the Swedish Diabetes Association, The Medical Faculty of The University of Uppsala, The Swedish Association for Medical Research, and The Swedish Medical Research Council is gratefully acknowledged. Dr. Edwards was in receipt of a Wellcome-Swedish Travelling Research Fellowship.

Received for publication 25 September 1972, and in revised form 15 November 1972.

REFERENCES

1. MURRELL, L. R. 1966. Mammalian pancreatic islet tissue in organ culture. *Exp. Cell Res.* **41**: 350.
2. HILWIG, I., S. SCHUSTER, W. HEPTNER, and E. VON WASIELEWSKI. 1968. Monolayer culture of pancreatic tissue. I. Methods of culture, morphology, insulin content of tissues and culture medium. *Z. Zellforsch Mikrosk. Anat.* **90**:333.
3. MACCHI, I. A., and E. H. BLAUSTEIN. 1969. Cytostructure and endocrine function of monolayer cultures of neonatal hamster pancreas. *Endocrinology.* **84**:208.
4. LAMBERT, A. E., B. BLONDEL, Y. KANAZAWA, L. ORCI, and A. E. RENOLD. 1972. Monolayer cell culture of neonatal rat pancreas. Light microscopy and evidence for immunoreactive insulin synthesis and release. *Endocrinology.* **90**:239.
5. MOSKALEWSKI, S. 1965. Isolation and culture of the islets of Langerhans of the guinea pig. *Gen. Comp. Endocrinol.* **5**:342.
6. ANDERSSON, A., C. HELLERSTRÖM, and B. PETERSSON. 1967. Phase-contrast microscopy of fresh and cultured pancreatic islet cells of guinea pigs. *Z. Zellforsch. Mikrosk. Anat.* **82**:110.
7. ANDERSSON, A. 1970. Monolayer culture of pancreatic islet cells. In *The Structure and Metabolism of the Pancreatic Islets*. S. Falkmer, B. Hellman, and I. B. Täljedal, editors. Pergamon Press Ltd., Oxford. 73.
8. BODER, G. B., M. A. ROOT, R. E. CHANCE, and J. S. JOHNSON. 1969. Extended production of insulin by isolated rabbit pancreatic islets. *Proc. Soc. Exp. Biol. Med.* **131**:507.
9. HOWELL, S. L., J. C. EDWARDS, and M. WHITFIELD. 1971. Preparation of B-cell deficient guinea pig islets of Langerhans. *Horm. Metab. Res.* **3**:37.
10. PETERSSON, B., C. HELLERSTRÖM, and R. GUNNARSSON. 1970. Structure and metabolism of the pancreatic islets in Streptozotocin-treated guinea pigs. *Horm. Metab. Res.* **2**:313.
11. ANDERSSON, A., and C. HELLERSTRÖM. 1972. Metabolic characteristics of isolated pancreatic

- islets in tissue culture. *Diabetes*. 21(Suppl. 2): 546.
12. HELLERSTRÖM, C., S. L. HOWELL, J. C. EDWARDS, and A. ANDERSSON. 1972. An investigation of glucagon biosynthesis in isolated pancreatic islets of guinea pigs. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 27:97.
 13. HOWELL, S. L., and K. W. TAYLOR. 1968. Potassium ions and the secretion of insulin by islets of Langerhans incubated in vitro. *Biochem. J.* 108:17.
 14. BENCOSME, S. A. 1952. Studies on the methods of staining the islet cells of the pancreas. *Arch. Pathol.* 53:87.
 15. GOMORI, G. 1950. A new stain for elastic tissue. *Am. J. Clin. Pathol.* 20:665.
 16. HELLERSTRÖM, C. 1967. Effects of carbohydrates on the oxygen consumption of isolated pancreatic islets of mice. *Endocrinology*. 81:105.
 17. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.
 18. GEY, G. O., and M. K. GEY. 1936. The maintenance of human normal cells and tumor cells in continuous culture. *Am. J. Cancer*. 27:45.
 19. LUNDQVIST, G., J. C. EDWARDS, and L. WIDE. 1971. A solid phase radioimmunoassay for pancreatic glucagon. In *Dissertations from the Faculty of Medicine, University of Uppsala, Sweden*.
 20. CARAMIA, F., B. L. MUNGER, and P. E. LACY. 1965. The ultrastructural basis for the identification of cell types in the pancreatic islets. *Z. Zellforsch. Mikrosk. Anat.* 67:533.
 21. BJÖRKMAN, N., and B. HELLMAN. 1964. Ultrastructure of the pancreatic A₂-cells in different species. In *The Structure and Metabolism of the Pancreatic Islets*. S. Brolin, B. Hellman, H. Knutson, editors. Pergamon Press Ltd., Oxford 131.
 22. WESTMAN, J., A. ANDERSSON, C. HELLERSTRÖM, and B. PETERSSON. 1970. Ultrastructure of monolayer cultures of pancreatic islet cells. *Acta Diabetol. Lat.* 7:557.