

# Survival of Isolated Human Islets of Langerhans Maintained in Tissue Culture

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**ABSTRACT** Transplantation of human pancreatic islets to diabetic patients may require that donor islets be kept viable *in vitro* for extended time periods before transfer to the recipient. We have maintained isolated pancreatic islets obtained from the human cadaveric pancreas in tissue culture for 1–3 wk, after which we studied the structure and function of the islets. Electron micrographs of the cultured islets showed a satisfactory preservation of both  $\beta$ -cells and  $\alpha$ -cells. After culture for 1 wk, the islet oxygen uptake proceeded at a constant rate at a low glucose concentration (3.3 mM) and was significantly enhanced by raising the glucose concentration to 16.7 mM. Likewise, after culture for 1 wk, the islets responded with an increased insulin release when exposed to 16.7 mM glucose with or without added theophylline (10 mM). Islets cultured for 1–3 wk were able to incorporate [ $^3$ H]leucine into proinsulin, as judged by gel filtration of acid-alcohol extracts. Glucagon release from the cultured islets was reduced significantly by 16.7 mM glucose alone, but stimulated by glucose (16.7 mM) plus theophylline (10 mM).

It is concluded that viable pancreatic islets can be isolated from the pancreas of adult human donors and maintained in tissue culture for at least 1 wk without loss of the specific functions of the  $\alpha$ - and  $\beta$ -cells. It remains to be established whether such islets will survive and remain functionally competent after transplantation to human recipients.

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## INTRODUCTION

In recent years, pancreatic transplantation has been attempted in some 40 patients with severe, complicated diabetes mellitus (1–5). So far, long-term graft survival has been achieved only in a few instances. Deterrents to success include vascular thrombosis, pancreatic fistula, and graft rejection.

Some of these complications could obviously be avoided if isolated pancreatic islets were utilized as the graft. In rodents, lasting amelioration of experimental diabetes has been achieved after transplantation of isologous islets (6–10). For these experiments, large quantities of well-defined, viable islets were prepared. Attempts at isolating human pancreatic islets have been less successful; islet purity has been unsatisfactory and the yield has been disappointing (11–13).

This communication reports on the isolation of human pancreatic islets and on attempts at maintaining such islets in tissue culture. Successful storage of islets in a tissue culture system would make it possible to collect islets for transplantation from several subsequent donors. Moreover, it would facilitate functional assays of the islets before transplantation and offer a means of sterility control. The system used is particularly suited for recovery of the cultured islets for subsequent transplantation.

## METHODS

**Isolation and culture techniques.** Pancreatic glands were obtained from 14 cadavers, ranging in age from 23 to 64 yr. After surgical exposure, the pancreas was perfused intrarterially *in situ* with chilled (+5°C) electrolyte solution containing dextran 40 (Perfadex, Pharmacia AB, Uppsala, Sweden) for about 15 min. In all but one instance, time elapsing from donor cardiac arrest to cooling was between 5 and 14 min (mean 10 min). In the remaining case the time was 27 min. The body and tail of the pancreas was

excised and transferred to the laboratory in chilled (+5°C) Hanks' solution buffered with 25 mM Hepes. Aseptic isolation of islets began in 13 instances within 6 h and in one instance within 22 h after circulatory arrest. The pancreatic tissue was divided into pieces of about 2×2×2 cm, each of which was distended by injection of Hanks' solution. After the pieces were minced into small fragments (1–2-mm thick), the suspension was washed several times with Hank's solution to remove as much fat as possible. Collagenase (10 mg/ml) was added and the suspension was subdivided into several batches shaken by hand for about 30 min in sterile test tubes, permitting a continual microscopic examination of the progress of the digestion. The digest was then washed three times with Hanks' solution before the isolated islets were collected under a stereomicroscope.

Sterile braking pipettes (14) were used for collecting the isolated islets from the washed suspension of pancreatic tissue. This procedure, together with the preceding collagenase treatment, required about 1½ h. The islets were transferred directly to plastic Petri dishes containing 5 ml of tissue culture medium (TCM 199), supplemented with 20% calf serum and antibiotics (penicillin 100 U/ml; streptomycin 0.1 mg/ml), and with a final glucose concentration of 5.6 mM (15). The culture medium was changed on the 3rd day of culture and then every 2nd day. From the 3rd day onwards the calf serum concentration was decreased to 10%. On either the 7th or on the 21st day of culture, the islets were carefully scraped from the bottom of the culture dishes with the aid of a thin rubber policeman. Groups of islets were studied *in vitro* according to techniques described below.

**Electron microscopy.** Islets were fixed for electron microscopy in 2.5% (vol/vol) glutaraldehyde followed by 1% (wt/vol) osmium tetroxide; both fixatives were dissolved in 0.05 M phosphate buffer adjusted to pH 7.4. After dehydration, the islets were embedded in Epon 812 (Shell Chemical Co., New York). Thin sections were prepared on an LKB Ultratome III (LKB-Produkter, Stockholm, Sweden), picked up on one-hole grids, and stained with uranyl acetate (16), followed by lead citrate (17). The electron microscopy was carried out with a JEOL 100 B microscope (JEOL Analytic Instruments, Cranford, N. J.). A number of freshly isolated noncultured islets were examined for comparative purposes.

**Oxygen consumption.** Respiration of the islets was determined with the aid of Cartesian divers as described in detail by Hellerström (18). After being filled, each diver had a gas phase consisting of about 10 µl ambient air, making the system sensitive to changes in the gas volume of 0.9 nL. After culture for 7 days, groups of 5–14 isolated islets were incubated in 1 µl Krebs-Ringer phosphate medium (19), pH 7.4, supplemented with either 3.3 or 16.7 mM D-glucose. The CO<sub>2</sub> evolved during the islet respiration was trapped in 1 µl 0.37 N KOH. After measurements of the oxygen uptake at the lower concentration for about 1 h, more glucose was added to the islet incubation medium from a small side drop (0.5 µl) in the diver, so that the final glucose concentration became 16.7 mM. The respiration rate was subsequently followed for another hour. At the end of the experiment the islets were recovered from the diver and their dry weights determined on an ultra-micro balance (Cahn Electrobalance, Model 4100; Cahn Div., Ventron Instruments Corp., Cerritos, Calif.), sensitive to 0.2 µg. Weight determinations of 7 groups of islets, containing altogether 55 islets, showed a mean dry weight of 0.8 µg/islet.

**Release and content of insulin and glucagon.** Groups of five islets, cultured for 7 days, were incubated at +37°C

during three consecutive 60-min periods in 250 µl bicarbonate-buffered medium (20) supplemented with 2 mg/ml bovine plasma albumin (Fraction V; Armour and Company, Ltd., Eastbourne, England). In the first incubation period the medium contained 3.3 mM glucose, in the second 16.7 mM glucose, and in the third 16.7 mM glucose plus 10 mM theophylline. At the end of each period the incubation medium was immediately frozen and stored at –20°C until radioimmunoassay of the insulin and glucagon concentrations could be accomplished (see below). The islets were recovered, then sonicated in 500 µl acid ethanol (15 ml 12 N HCl/liter 70% ethanol) and finally extracted for 24 h at +4°C and stored at –20°C before radioimmunoassay of their hormone contents.

**Radioimmunoassay of insulin and glucagon.** Immunoreactive insulin was measured in duplicates as described by Wide and Porath (21) with the Phadebas insulin test, kindly donated by Pharmacia AB. Crystalline human insulin was used as a standard.

Glucagon was measured with the radioimmunoassay described by Heding (22). Standards consisted of crystalline pork glucagon and all samples were assayed in duplicate.

**Biosynthesis of insulin.** Groups of 15–25 islets, cultured for either 7 or 21 days, were incubated for 180 min at +37°C in 100 µl bicarbonate-buffered medium (20) supplemented with 2 mg/ml bovine plasma albumin, 16.7 mM glucose, 100 µCi/ml of [4,5-<sup>3</sup>H]L-leucine (sp act 54 Ci/mmol; The Radiochemical Centre, Amersham, England) and 20 µg/ml each of the 12 naturally occurring amino acids used by Eagle (23), except for leucine. Incubations were performed under continuous shaking in a gas phase of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The incubated islets were washed three times in non-radioactive buffer containing cold leucine (10 mM) and homogenized by sonication in 500 µl acid-alcohol containing 1 mg crystalline ox insulin as a carrier protein. After extraction for 20 h in the cold and centrifugation, the acid-ethanol-soluble proteins were precipitated with ether-ethanol (24) and redissolved in 300 µl 1 M acetic acid containing 1 mg bovine plasma albumin. Gel chromatography was subsequently performed in 1 M acetic acid at +4°C on a column (1.6×92 cm) of Sephadex G-50 fine (Pharmacia AB), calibrated with dextran blue 2000, bovine plasma albumin, cytochrome c, bovine insulin, and bovine-porcine glucagon. The optical density of the eluate at 280 nm was recorded continuously (Uvicord II, LKB-Produkter AB, Stockholm, Sweden). A 0.5-ml sample of each fraction (2 ml) was mixed with Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.) and the radioactivity counted in a liquid scintillation spectrometer (Tricarb, Model 3380; Packard Instrument Co., Inc.).

**Data analyses.** Experimental results have been expressed as means±SEM. The *P* values were obtained by Student's *t* test for the significance of the difference between paired individual observations (25).

## RESULTS

In nine experiments, the isolation procedure yielded satisfactory islets. When viewed in the stereomicroscope, such islets were virtually free of exocrine cells and had a spherical or ellipsoid shape with smooth contours and a yellowish-white color, which made them distinguishable from the exocrine fragments. In all these instances, the pancreatic tissue had been harvested from donors aged 45 yr or less. In the other five experiments,

no satisfactory islets could be identified. In this group the age of the donors was between 45 and 65 yr. The pancreatic glands of these older individuals were usually fibrotic and infiltrated with fat, which made the isolation procedure particularly difficult. In two of five unsuccessful experiments, there were additional adverse facts: in one instance the pancreas had been stored in the cold for 22 h and in another the warm ischemia lasted for 27 min. In those cases in which satisfactory islets were obtained, however, there was no correlation between the ischemic period and the success of the isolation procedure or functional behavior of the islets. No attempts were made in this series of experiments to maximize the yield of islets and no more than 400 islets were utilized from any pancreas.

During the culture period, the majority of the explanted islets gradually became attached to the bottom of the culture dish, where they attained a more flattened shape. Some islets never became attached and were washed away when the culture medium was changed. Although a few fibroblastoid cells appeared in the periphery of the attached islets, there was no overgrowth of such cells even after 3 wk in culture, and the islets generally resumed their spherical shape when recovered at the end of the culture period.

The electron microscopic examination of the cultured islets showed a satisfactory ultrastructural preservation, with few necrotic or degenerating cells irrespective of whether the culture period lasted for 1 or 3 wk. As can be seen in Fig. 1, the cells were arranged in an epithelioid manner and the nuclei, mitochondria, and cellular membranes were of normal appearance. The great majority of the cells could be recognized as either  $\beta$ -cells or  $\alpha$ -cells by the appearance of their secretory granules.

However, in the cultured  $\beta$ -cells the granules were rounder with more tightly fitting sacs compared to those of freshly isolated islets. Comparisons of the ultrastructure of the cultured islets with that of the freshly isolated ones showed a much higher frequency of damaged and disintegrated cells in the latter islet group. Obviously such cells had either disappeared or recovered during the culture period.

The oxygen consumption of islets incubated in diver experiments after 7 days of culture remained linear for well over 2 h. This is illustrated in Fig 2, where the linear oxygen uptake was, however, increased to a new (linear) level by addition of glucose to the medium. The islet respiratory rate was  $6.3 \pm 0.6 \mu\text{l} \cdot \text{mg dry wt}^{-1} \cdot \text{hr}^{-1}$  with 3.3 mM glucose in the medium and  $7.7 \pm 0.6 \mu\text{l} \cdot \text{mg dry wt}^{-1} \cdot \text{hr}^{-1}$  with 16.7 mM glucose ( $n = 7$ ). The observed increase by 22% of the oxygen consumption at the higher glucose concentration was significant ( $P < 0.01$ ).

Table I shows the release of insulin and glucagon at various glucose concentrations and in the presence of theophylline. A glucose concentration of 16.7 mM significantly increased the release of insulin but depressed that of glucagon. Addition of theophylline (10 mM) in the presence of 16.7 mM glucose further increased the insulin release and also stimulated glucagon secretion. The values for the hormone content after incubation of the cultured islets indicate a proportionately high mobilization of both insulin and glucagon during the incubation periods.

The radioactive Sephadex G-50 elution profile of partially purified proteins from isolated human islets, cultured for 7 days in 6.1 mM glucose and subsequently incubated for 3 h in 16.7 mM glucose, is given in Fig.

TABLE I  
*Secretion of Insulin and Glucagon by Isolated Human Pancreatic Islets Maintained in Tissue Culture for 1 wk at a Glucose Concentration of 5.6 mM*

| Hormone  | 3.3 mM glucose     | 16.7 mM glucose                                       | 16.7 mM glucose<br>+ 10 mM theophylline | Content               |
|----------|--------------------|---|---|-----------------------|
|          |                    | <i>ng · five islets<sup>-1</sup> · h<sup>-1</sup></i> |   | <i>ng/five islets</i> |
| Insulin  | $2.5 \pm 0.6$ (36) | $4.5 \pm 1.0$ (36)                                    | $10.9 \pm 2.3$ (36)                     | $24 \pm 4$ (38)       |
| <i>P</i> |                    | $< 0.02$  | $< 0.001$                               |                       |
| Glucagon | $1.2 \pm 0.1$ (15) | $0.9 \pm 0.1$ (15)                                    | $1.8 \pm 0.2$ (15)                      | $13 \pm 2$ (30)       |
| <i>P</i> |                    | $< 0.025$   | $< 0.001$                               |                       |

The islets were incubated consecutively at three glucose concentrations for 60 min in each incubation period. They were then recovered and extracted in acid ethanol before assay of the insulin and glucagon content. Both the insulin and glucagon release have been expressed as nanograms per five islets per hour, and the hormone content as nanograms per five islets. Levels of significance refer to comparisons between the hormone release in incubation periods II or III with that in period I. Figures are given as the means  $\pm$  SEM with the number of observations in parentheses. Each mean value is based on observations from four to six pancreatic glands.

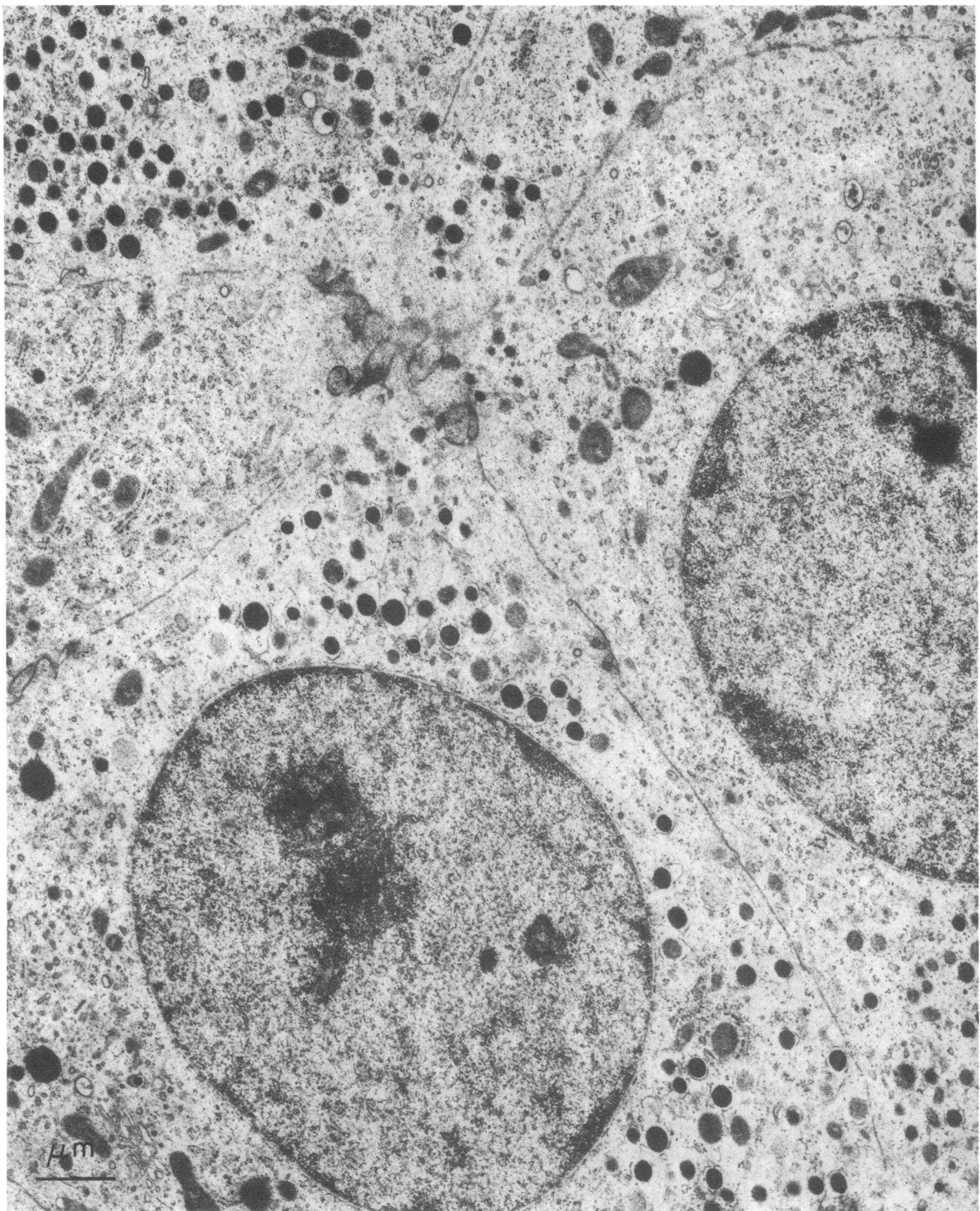


FIGURE 1 Electron micrograph of an isolated human islet maintained in tissue culture for 3 wk at a glucose concentration of 5.6 mM. All the cells can be identified as  $\beta$ -cells by their content of electron-dense secretion granules surrounded by wide sacs. Well-preserved cell nuclei, cytomembranes, and mitochondria are evident.  $\times 10,500$

3. Three distinct radioactive peaks are evident, and of these the first (I) eluted with the void volume and the third (III) with the carrier insulin. The position of the second peak (II) corresponded to what might be expected from a protein with a molecular weight of about 9,500, and has previously been shown to correspond to the elution position of radioactively labeled proinsulin extracted from mouse islets (26). On the basis of these observations, it was concluded that peak II represented human proinsulin and peak III human insulin. When extracts of islets cultured for 21 days were subjected to gel filtration, the distribution of radioactivity was identical to that described above.

## DISCUSSION

We undertook the present investigation with the primary aim of assessing whether adult human pancreatic islets can be isolated in a state compatible with their survival in tissue culture. Initial studies indicated that the isolation procedure was generally more complicated and yielded considerably fewer islets than in laboratory animals, a finding that agrees with previous reports in the literature (11–13, 27). The results were also related to donor age in that only pancreatic glands obtained from donors below 45 yr of age yielded satisfactory islets. Before the isolation procedure, the islets had furthermore been exposed to fairly long periods of ischemia and therefore it seemed particularly important to apply rigid criteria for the assessment of islet cell viability, which included both structural and functional studies. It should be noted in this context that islets seriously damaged during the isolation procedure have been shown to remain suspended in the culture medium and will therefore be washed away when the medium is changed (15). Thus, to a certain extent the culture technique in itself may lead to enrichment of more viable islets, a notion supported by the ultrastructural observation of many more damaged cells in the freshly isolated islets than in the cultured ones. This was why we did not perform metabolic or functional studies on freshly isolated human islets.

It appears from the present observations on the respiration of the cultured islets that viability in terms of oxygen uptake was maintained in the islets throughout the culture period. This was further supported by the observation of a significant stimulation of the oxygen uptake by glucose, which we (18, 28–30) and others (31) have previously shown to be a characteristic property of the islets of other species, including rats, mice, and guinea pigs. The present figures on the respiratory rate of human islets agree well with those previously reported in rodents (18, 28–31), although the stimulatory action of glucose appeared less pronounced. This could reflect the higher proportion of  $\alpha$ -cells in the

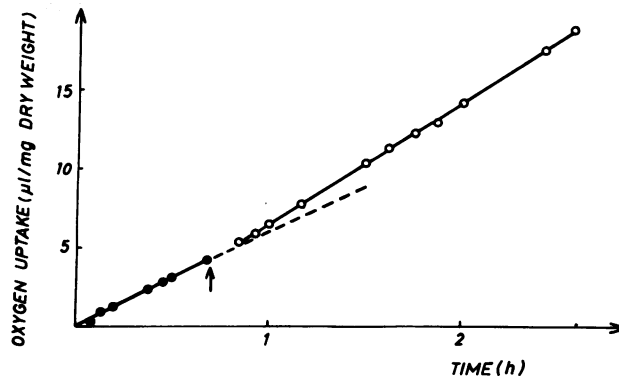


FIGURE 2 Oxygen uptake of a group of five isolated islets (dry wt 8.3  $\mu$ g) cultured for 1 wk at 5.6 mM glucose and subsequently incubated in the Cartesian diver system (18). The first part of the line (●—●) represents the oxygen consumption at 3.3 mM glucose and the second part (○—○) shows the respiration at 16.7 mM glucose, added at the time depicted by the arrow. In this particular experiment, the oxygen uptake increased by 31% after the addition of glucose.

human islets (32), since  $\alpha$ -cells show a considerably lower increase of respiration in response to glucose (30, 33).

Glucose has previously been shown to stimulate the insulin release from freshly isolated human islets (11, 34, 35), and the present data show that this property was retained by the cultured islets. Indeed, the amounts

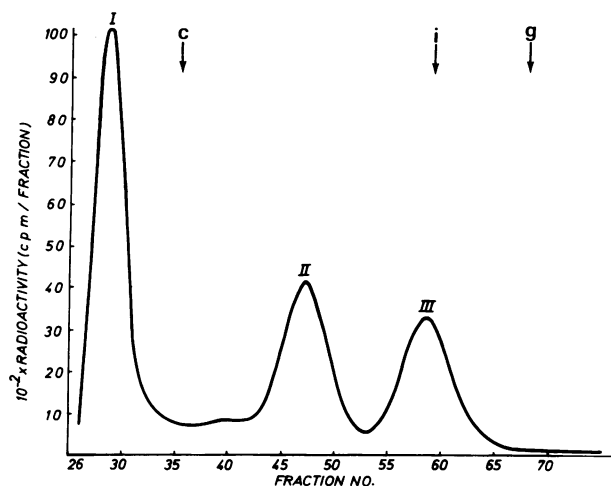


FIGURE 3 Gel filtration profile of [ $^3$ H]leucine incorporation into acid-ethanol-soluble proteins from isolated human islets cultured for 1 wk at 5.6 mM glucose and subsequently incubated for 180 min in a medium containing 16.7 mM glucose and 100  $\mu$ Ci/ml of [4,5- $^3$ H]L-leucine. Gel filtration was carried out on a Sephadex G-50 column (1.6  $\times$  92 cm) in 1 M acetic acid. The volume of each fraction was 2 ml. The elution volumes of the molecular weight markers, cytochrome c (c), insulin (i), and glucagon (g), are indicated by arrows.

of insulin released at the various glucose concentrations and in the presence of theophylline agreed very well with those previously given by Ashcroft et al. (34) in their study of freshly isolated islets from the pancreas of a 4-yr-old girl. A stimulation of the insulin release in response to glucose was recently reported also in a monolayer culture of human islet cells (36). In the present culture system, however, the insulin content of the islets was lower than previously recorded in freshly isolated human islets (34) and the proportion of insulin released during a 60-min incubation was relatively high. The presence of many well-granulated  $\beta$ -cells in the electron micrographs nevertheless confirms that extensive depletion of insulin had not occurred during culture. In addition, the finding of a marked incorporation of labeled leucine into both proinsulin and insulin shows that the diminished insulin content did not reflect a grossly deficient insulin biosynthesis of the cultured islets. The pattern of leucine incorporation observed in the present gel filtration profiles was, in fact, very similar to that recorded previously in extracts of both human insulinomas (37, 38) and of isolated rat and mouse islets (26, 39). The present data, therefore, support the notion that in the normal human  $\beta$ -cell, proinsulin is being synthesized and converted into insulin by the same basic mechanisms demonstrated in the  $\beta$ -cells of laboratory animals (40).

The glucagon response to glucose followed the same pattern in the cultured islets as reported in vivo in man (41) and in vitro in both freshly isolated islets (42-44) and in monolayer cultures of islet cells (45, 46) from laboratory animals. There seem, however, to be no previous data on the glucagon release from human isolated islets. The present findings confirm that glucose suppression of human plasma glucagon-like immunoreactivity at least in part reflects the secretory response of pancreatic  $\alpha$ -cells, although gastrointestinal sources of glucagon may also contribute (47). The stimulation of glucagon secretion by theophylline is noteworthy, since this occurred at a high glucose concentration, inhibitory in itself. Enhancement of the glucagon secretion by theophylline at both a low and a high glucose concentration has recently been reported in the rat (46, 48), and may be ascribed to accumulation in the  $\alpha$ -cells of cyclic AMP and stimulation of cyclic AMP-dependent protein kinase (49).

Altogether, the present data indicate that viable islets of Langerhans can be isolated from the pancreas of adult human donors and maintained in tissue culture for at least 1 wk without loss of specific functions in the  $\beta$ - and  $\alpha$ -cells. These observations support the view that the present culture system may serve as a tissue bank for the collection and storage of human islets intended for transplantation. Islets can be checked with regard to viability and microbial contamination during culture

and they can be easily and rapidly recovered at the end of the culture period. It remains to be established whether cultured islets will survive and continue to manufacture insulin in response to glucose after transplantation to a human recipient.

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