

ORIGINALS

Long Term Production of Insulin by Adult Rat Pancreas In Vitro

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Summary. Adult-rat islets of Langerhans were grown in organ culture for a period of one month. Prior to explantation exocrine parenchyma was made atrophic by ligation of the pancreatic duct. Insulin was determined by immunoassay. The fluid phase was Eagle's medium with 10% calf serum. The gas phase was either 5% CO₂ in air or 5% CO₂ in oxygen. With CO₂/air small amounts of insulin averaging about 40 μ U/ml were found in the medium during the experimental period. With CO₂/oxygen the average concentration after 30 days was 70 μ U/ml which was not significantly different from the starting value. Around day 15 a peak was reached with an average of 160 μ U of insulin per ml medium. — In CO₂/oxygen, medium had to be changed daily otherwise the islets succumbed rapidly *in vitro*. — Moderate degranulation of β -cells was found in CO₂/air already on day 1 and remained approximately unchanged until day 30. In CO₂/oxygen, degranulation did not generally start until 4–7 days after explantation and cells with normal granularity persisted until day 30. — Degenerative changes such as fuzzy cell borders, pyknotic nuclei and decreased stainability were already seen in the CO₂/oxygen and the CO₂/air group on the first day after explantation. These alterations were more pronounced in the latter group where the degeneration caused a loss of many islets during the first 4 days. After this a more stable state was reached where remaining islets showed only slight degenerative changes. In CO₂/oxygen the degenerative changes were in general slight during the entire experimental period leading to loss of comparatively few islets.

Production d'insuline à long terme du pancréas de rat adulte in vitro.

Résumé. Des cultures d'organe d'ilots de Langerhans de rats adultes ont été maintenues pendant des périodes d'un mois. Avant leur explantation le parenchyme exocrine était devenu atrophique à la suite de la ligature du canal de Wirsung. L'insuline a été mesurée par la méthode immunologique. Le milieu de culture était celui d'Eagle avec 10% de sérum de veau. La phase gazeuse était 5% de CO₂ dans de l'air ou dans de l'oxygène. Avec le CO₂ dans de l'air, les quantités d'insuline trouvées dans le milieu ont été en moyenne de 40 μ U/ml. En présence de CO₂ et oxygène la concentration moyenne dans le milieu après 30 jours était de 70 μ U/ml et ne différait pas significativement de la valeur au départ. Vers le 15ème jour un maximum était atteint avec, en moyenne, 160 μ U/ml de liquide. En présence de CO₂ et oxygène, le milieu de culture doit être changé quotidiennement sans quoi les îlots succombent rapidement *in vitro*. — Une dégranulation modérée des cellules β a été observée dès le premier jour de l'incubation et s'est maintenue jusqu'au 30ème jour en présence de CO₂ et d'air. En présence de CO₂ et oxygène cette dégranulation n'a débuté que vers 4 à 7 jours après l'explantation et un certain nombre de

cellules normalement granulees ont persisté jusqu'au 30ème jour. — Des indices morphologiques de dégénération ont été observés tant en présence de CO₂ et oxygène qu'en présence de CO₂ et air dès le premier jour après l'explantation. Les indices de dégénérescence étaient l'aspect flou des limites cellulaires, la pycnose de certains noyaux et une diminution de la coloration. Ces altérations étaient plus prononcées pour le groupe d'incubation en présence de CO₂ et air et de nombreux îlots ont disparu par dégénération durant les 4 premiers jours. Par la suite la préparation est devenue plus stable et les signes de dégénérescence des îlots restant ont été modestes. En présence de CO₂ et oxygène les modifications dégénératives ont été bien moindres pendant toute la période expérimentale et peu d'ilots seulement ont été perdus.

Langanhaltende Insulinbildung durch Pankreasgewebe ausgewachsener Ratten in vitro.

Zusammenfassung. Langerhans'sche Inseln ausgewachsener Ratten wurden einen Monat lang in Organkulturen gezüchtet. Vor dem Ansetzen der Kulturen war das exokrine Parenchym durch Ligatur des Ductus pancreaticus zur Atrophie gebracht worden. Das Insulin wurde mit der immunologischen Methode bestimmt. Eagle's Medium mit 10% Kälberserum diente als flüssige Phase, entweder 5% CO₂ in Luft oder 5% CO₂ in Sauerstoff als Gasphase. Mit CO₂/Luft wurden im Medium während der Versuchsdauer kleine Insulinmengen von durchschnittlich etwa 40 μ E/ml gemessen. Mit CO₂/Sauerstoff war die durchschnittliche Konzentration nach 30 Tagen 70 μ E/ml; dieser Wert unterschied sich nicht signifikant vom Ausgangswert. Etwa nach 15 Tagen wurde ein Maximum mit einem durchschnittlichen Wert von 160 μ E Insulin pro ml Medium erreicht. In CO₂/Sauerstoff mußte das Medium täglich erneuert werden, da sonst die Inseln *in vitro* rasch zugrunde gingen. — In CO₂/Luft trat schon am ersten Tag eine mäßige Degranulation der β -Zellen ein, die fast unverändert bis zum 30. Tag bestehen blieb. In CO₂/Sauerstoff kam es im allgemeinen erst nach 4–7 Tagen nach der Gewebsauspflanzung zu einer Degranulation, und Zellen mit normaler Granulierung blieben bis zu 30 Tagen bestehen. — Degenerative Veränderungen wie aufgefärbte Zellränder, pyknotische Kerne und herabgesetzte Färbbarkeit wurden in CO₂/Sauerstoff und CO₂/Luft bereits am ersten Tag nach der Auspflanzung beobachtet. Diese Veränderungen waren in der letzteren Gruppe stärker ausgeprägt, bei der die Degeneration den Verlust vieler Zellen während der ersten 4 Tage verursachte. Danach wurde ein stabileres Stadium erreicht, wobei die erhaltenen Inseln nur leichte degenerative Veränderungen zeigten. In CO₂/Sauerstoff waren während des gesamten Versuches die generativen Veränderungen im allgemeinen leicht und führten vergleichsweise nur zum Verlust weniger Inseln.

Studies of endocrine pancreas in tissue culture have almost entirely been limited to fetal organs. Insulin-like activity has been detected in the culture fluids even after rather long periods of cultivation [1]. MURREL [9], in a recent quantitative study, showed that fetal rat islets synthesized insulin for at least 10 days after explantation.

Experiments with adult islets should give additional information since embryonic tissue may not properly reflect the conditions in the fully differentiated organ. Work with adult pancreas is however rendered difficult by the presence of enzyme-liberating, exocrine parenchyma, which exerts a destructive effect in isolated pieces. Experiments *in vivo* with transplants to the anterior chamber of the eye have shown that this difficulty can be overcome if exocrine parenchyma is selectively destroyed by duct ligation before transplantation [5].

The present communication describes a method whereby islets from adult, duct-ligated rats can be maintained in organ culture for at least 30 days, apparently without losing their morphologic or functional integrity. No studies on long-term insulin production by an adult organ *in vitro* seem to have been published previously. Morphologically intact islets obtained from adult guinea pig and freed of exocrine parenchyma by collagenase treatment, have been found to survive at least 14 days of cultivation *in vitro* [8].

Material and methods

Animals. Four month old Wistar rats of both sexes were used.

Ligation of the pancreatic duct. Atrophy of the lienal part of the exocrine parenchyma was achieved by applying a ligature between the middle and lienal third of the organ — "partial ligature" [4].

Tissue culture. Organ cultures. Pieces of pancreas were aseptically removed 30 days after the pancreatic duct had been ligated. At this stage the organ consists of islets of LANGERHANS embedded in vascular connective tissue, where ducts but no exocrine cells remain [4].

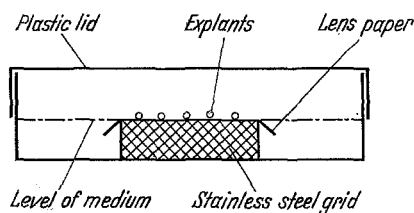


Fig. 1. Construction of the organ culture vessel

The excised tissue was divided with a scalpel into small pieces measuring about one mm³. Within one minute of removal, the explants were introduced into organ cultures (Fig. 1) set up essentially according to JENSEN et al. [7]. Incubation was done at 37°C in a continuous flow of humidified gas, either 5% CO₂ in air or 5% CO₂ in oxygen.

Each culture was nourished by 5 ml Eagle's minimal essential medium [13] with 10% calf serum, penicillin (100 U/ml) and streptomycin (50 µg/ml). Phenol red was omitted because of its interference with the colorimetric determinations of glucose. The pH was indirectly checked by keeping medium with indicator in the same box as the organ cultures. Removed medium was stored at -18°C and later assayed for insulin and glucose. When more than one rat was used for a particular experiment, pooled tissue from all animals was randomly distributed among the individual culture vessels. The time interval between excision of the pancreas and start of incubation of the explants was less than 30 minutes.

Monolayer cultures. The human diploid cell strain WI-38 [2] was used in passage 20–30. Media and conditions of culture were the same as for the organ cultures.

Histological methods. Pieces were carefully removed from the organ cultures, fixed in Bouin's solution, embedded in paraffin, cut into serial sections and stained with paraldehyde-fuchsin-Ponceaufuchsin.

Glucose determinations. The glucose oxidase method as described by HJELM and DE VERDIER was followed [3].

Insulin determinations. INSULIN was immunoassayed essentially according to YALOW and BERSON [14]. Antiserum was produced in guinea-pigs immunized by monthly injections of recrystallized beef insulin in FREUND's incomplete adjuvant. Antiserum was collected 2 weeks after last injection. Insulin-¹³¹I was prepared with a slight modification [14] of the method of HUNTER and GREENWOOD [6] for labeling growth hormone.

50 µl of the culture medium sample together with 1 µU of insulin-¹³¹I and insulin antiserum (dilution 1:80000) was incubated at 4°C for 3 days in a total volume of 0.5 ml. All dilutions were made in 0.1 M Veronal buffer, pH 8.6, containing 0.125% human serum albumin. After incubation bound and free insulin were separated by paper chromatography in 0.1 M Veronal buffer after addition of 50 µl of human plasma. The strips were cut into two parts and the radioactivity of the two fractions measured in a well-counter.

A standard curve based on analysis of 1–12 µU of Novo recrystallized human insulin was always included. All samples were assayed in duplicate. The standard error of the mean was less than ± 4 µU/ml.

Experimental

1. **Adult rat pancreas incubated in air-CO₂.** Six organ cultures were incubated in 5% CO₂ and 95% air. Medium was changed twice a week. Pieces were removed for histological examination 1, 2, 4, 7, 15, and 30 days after explantation. A few viable islets were found on all these occasions. However in most there were rather severe degenerative changes already on day 1, which did not diminish with time.

2. *Effect of high oxygen concentrations on adult rat pancreas.* These experiments were motivated by the results of MURREL [9] who could demonstrate synthesis of insulin and cell differentiation of fetal rat tissue incubated in the presence of a high oxygen concentration. Organ cultures were incubated in 5% CO₂/95% O₂. Medium was changed twice a week. Pieces were removed for histological examination 1, 2, 4, 7, 15, and 30 days after explantation. It was found that extensive necrosis had already occurred by day 2 and that no recovery took place with longer periods of incubation.

Repeated determinations showed a rapid consumption of glucose (Fig. 2). Three to four days after

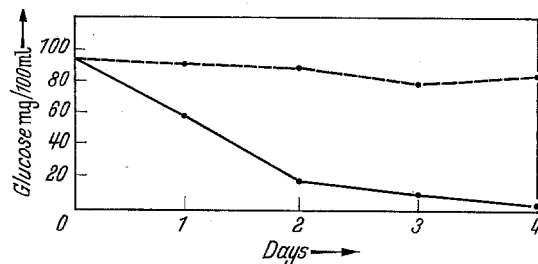


Fig. 2. Comparison of glucose-consumption in cultures containing islet tissue (uninterrupted line) and control cultures with no tissue (dotted line). Gas phase: 5% CO₂/95% O₂

explantation and before medium was changed virtually no glucose remained. Since this depletion might be responsible for extensive necrosis, the effect of adding extra glucose was studied. Pilot experiments were carried out with human fibroblasts in monolayer cultures.

3. *Effect of high oxygen concentrations on fibroblast monolayers.* WI-38 cells were kept as monolayers without subcultivation under conditions listed in table 1. Four Petri dishes were used in each group. The

Table 1. Viability of human diploid fibroblasts (strain WI-38) under different conditions *in vitro*. All cultures kept in Eagle's medium (13) with 10% calf serum

| group no. | conditions of culture | | | extra glucose added | survival time *** (days) |
|-----------|--------------------------------------|----------------|------|---------------------|--------------------------|
| | gas phase | medium changes | | | |
| 1 | 5% CO ₂ in air | every 3rd day | no | | > 20 |
| 2 | 5% CO ₂ in O ₂ | every 3rd day | no | | 5 |
| 3 | 5% CO ₂ in O ₂ | every 3rd day | yes* | | 7 |
| 4 | 5% CO ₂ in O ₂ | daily** | yes* | | > 20 |

* addition of 2.5 mg daily

** half of the volume of medium (2.5 ml) was replaced by 2.5 ml of fresh medium at intervals of 24 hours.

*** interval between start on experiment and detachment and death of at least 90% of the cells.

observation period was 20 days. Toxic effects resulting in cell death were noted in groups 2 and 3, whereas the other two groups were unaffected within the experimental period (Table 1). The toxic effect of oxygen in

group 2 was comparable with that in the rat organ cultures (see above). This is in accordance with previous observations on the effect of high oxygen concentrations on animal cells [2, 10, 12, 13]. It was possibly partially reversed by addition of glucose in amounts sufficient to compensate for the rapid consumption (group 3) as shown by a small lengthening of the survival of the cultures. If, however, complete medium was added daily (group 4), the fibroblasts showed no degenerative changes and still had a viable appearance after 20 days in the high oxygen atmosphere.

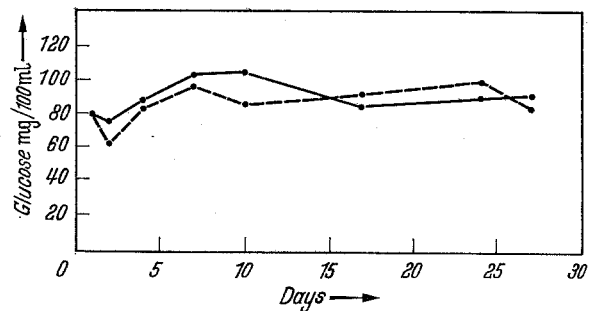


Fig. 3. The mean values of glucose concentration in the medium of islet tissue explants at different intervals. Medium was changed daily
Uninterrupted line denotes 5% CO₂/95% O₂
Interrupted line denotes 5% CO₂/95% air

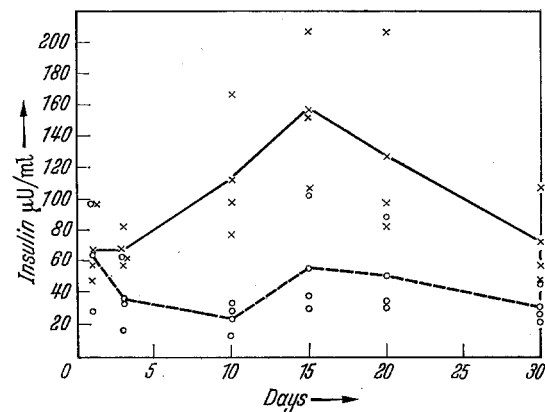


Fig. 4. Concentration of insulin in the medium of organ cultures of adult rat islet tissue during 30 days after explantation. Crosses and uninterrupted line denote 5% CO₂/95% O₂. Circles and interrupted line denote 5% CO₂/95% air

4. *Cultivation of rat islet tissue with addition of glucose and daily changes of medium.* Pancreatic tissue from rats was pooled and divided into two experimental groups each consisting of three cultures. Half of the medium (2.5 ml) was removed daily and substituted with the same amount of fresh medium. In addition 0.3 ml of 30% glucose was added at intervals of 24 hours. One group was incubated in 5% CO₂ in air and the other in 5% CO₂ in oxygen. The experiment was terminated on day 30 when all the explants were fixed and processed for histological examination.

The results of the glucose determinations are given in Fig. 3. It is seen that a stable concentration of about 90 mg/100 ml was maintained throughout the experiment. The insulin determinations are plotted in Fig. 4. It is seen that the values for the first day did

and to retain its capacity to produce insulin. Future use of the method should make it possible to study regulatory mechanisms for insulin production at a level where most of the shortcomings of an *in vivo* approach are avoided.

References

- [1] COALSON, R.E.: Functional and histological observations on islet tissue grown *in vitro*. *Anat. Rec.* **124**, 484 (1956).
- [2] HAYLICK, L.: The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614–636 (1965).
- [3] HJELM, M., and C. H. DE VERDIER: A methodological study of the enzymatic determination of glucose in blood. *Scand. J. clin. Lab. Invest.* **4**, 15 (1963).
- [4] HULTQUIST, G.T., and L.E. JÖNSSON: Ligation of the pancreatic duct in rats. *Acta Soc. Med. upsaliensis* **1–2**, 82–88 (1965).
- [5] —, and J. THORELL: A method for isolating islets of Langerhans by transplantation. *Acta Soc. Med. upsaliensis* **69**, 291–303 (1964).
- [6] HUNTER, W.M., and F.C. GREENWOOD: Preparation of iodine-131 labeled human growth hormone of high specific activity. *Nature (Lond.)* **194**, 495 (1962).
- [7] JENSEN, F.C., R.B.L. GWATKIN and I.D. BIGGERS: A simple organ culture method which allows simultaneous isolation of specific types of cells. *Exp. Cell Res.* **34**, 440–447 (1964).
- [8] MOSKALEWSKI, S.: Isolation and culture of the islets of Langerhans of the guinea pig. *Gen. Comp. Endocrinol.* **5**, 342–353 (1965).
- [9] MURREL, L.R.: Endocrine pancreas of the fetal rat in organ culture: Culture methods, some aspects at histogenesis, insulin synthesis, and release and the incorporation of tritiated leucine into tissue protein fractions. University of Minnesota, Ph. D. Diss., No. 64–9499, Univ. Microfilms, Inc., Ann Arbor, Michigan (1964).
- [10] PACE, D.M., I.R. THOMPSON and W.A. VAN CAMP: Effects of oxygen on growth in several established cell lines. *J. nat. Cancer Inst.* **28**, 897–907 (1962).
- [11] THORELL J. I.: Insulin antibodies in pregnant guinea pigs and in their offspring. *Acta Endocr. (Kbh)* **52**, 255–267 (1966).
- [12] TROWELL, O.A.: The culture of mature organs in a synthetic medium. *Exp. Cell Res.* **16**, 118–147 (1959).
- [13] WILLMER, E.N., et al. Cells and tissues in culture. *Methods, Biology and Physiology* I, II. Acad. Press. London-New York (1965).
- [14] YALOW, R.S., and S.A. BERSON: Immunoassay of endogenous plasma insulin in man. *J. clin. Invest.* **39**, 1157–1175 (1960).

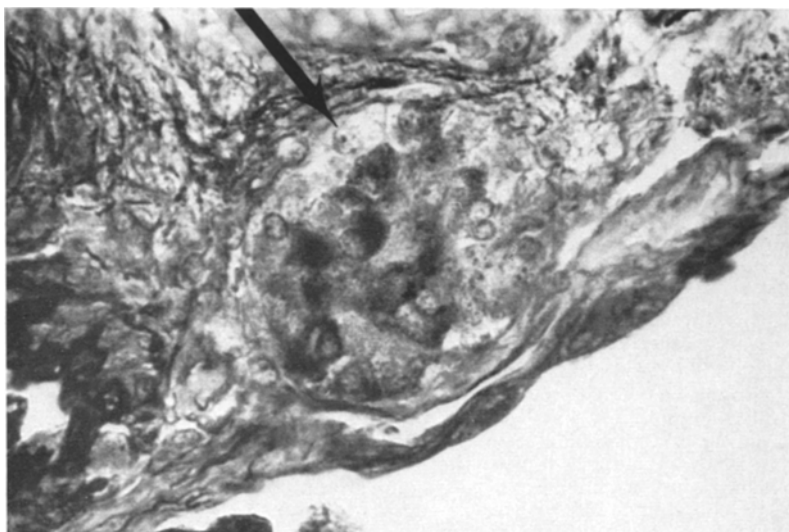


Fig. 5. Islet of Langerhans in 6-day-old explant kept in 5% CO₂/95% O₂ atmosphere with daily changes of medium supplemented with glucose. α -cells are present at the periphery of the islet (arrow)

Paraldehydefuchsin — Ponceaufuchsin. \times 720

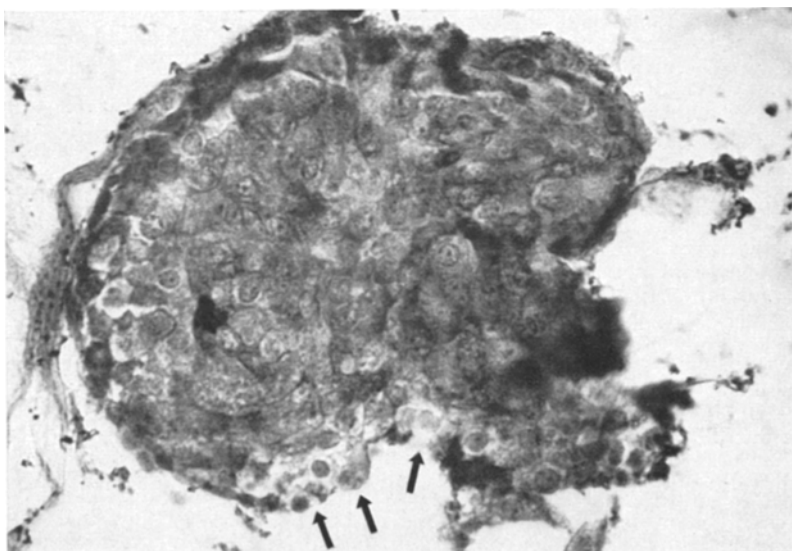


Fig. 6. Islet-tissue in 30-day-old explant kept in 5% CO₂/95% O₂ with daily changes of medium supplemented with glucose. Note presence of α -cells (arrows)

Paraldehydefuchsin — Ponceaufuchsin. \times 720

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