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THE IN VITRO GROWTH AND IDENTIFICATION OF BETA CELLS OF THE EMBRYONIC CHICK PANCREAS

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CHAPTER I

INTRODUCTION

The endocrine secretions of the pancreas are formed by islets of secretory cells. Each islet is surrounded by the acinar, vascular, neural and connective tissue cells which comprise the bulk of the gland. The Islets of Langerhans constitute 1 to 2% of the total mass of the pancreas of higher vertebrates. The insulin-producing Beta cells make up about 75% of the mass of the million or more islets which are distributed throughout the pancreas (Turner, 1961).

The microanatomy of the pancreas makes it difficult to obtain isolated functional Beta cells in good physiological condition and in quantities that are adequate for the study of their metabolism and function. The study of several aspects of Beta cell function could be greatly facilitated by a readily available source of Beta cells which were largely or entirely free from other components of the pancreas.

As sources of isolated Beta cells there are certain marine teleosts in which the endocrine portion of the pancreas is separate

from the digestive portion. One or more isolated masses of endocrine cells, the principal islets or Brockman bodies, are located in the mesentery. The principal islets of three species have been used for the direct study of the metabolism of islet tissue: the toadfish, Opsanus tau (Lazarow et al., 1957), the short horn sculpin, Cottus scorpius (Falkmer, 1961) and the goosefish, Lophius piscatorius (Bauer, Lindall and Lazarow, 1964). The islets of these marine teleosts contain an insulin which does not differ in its hormonal or antigenic properties from that of man and other higher vertebrates. These similarities suggest that the results of a study of the function of the Beta cells of these animals may have some application to higher vertebrates. However, the use of marine teleosts as a source of islet tissue for metabolic study is restricted to suitably equipped coastal laboratories because of the difficulties encountered in maintaining these animals in laboratories remote from a source of sea water. Results so far reported indicate that these fish respond to experimental manipulation in a manner that is qualitatively similar to responses in man and most laboratory mammals. They show none of the atypical responses to diabetogenic agents seen in a few laboratory mammals and most birds. Guinea pigs (Collins-Williams, Renold and Marble, 1950) and most birds (Scott, Harris and Chen, 1945) are refractory to the diabetogenic action of alloxan. Hamsters become diabetic when injected with alloxan, but recover spontaneously (House and Tassoni, 1957). Study of these atypical responses is of potential value in understanding the typical responses and in understanding the lesions of naturally occurring diabetes mellitus.

Hellerström (1964) has developed a technique for the microdissection of islets from fresh mammalian pancreas. The method has been used for the isolation of relatively large quantities of islet tissue for microchemical analysis. It has been proposed for the direct study of the metabolism of islet cells. However, the technique does not seem to be ideally applicable to metabolic studies since the relatively prolonged dissection procedure probably would not allow the isolation of large quantities of Beta cells in optimal physiological condition. Another disadvantage of the technique is that it is not uniformly successful in the isolation of islet tissue from a variety of different species.

The work presented here is based on the proposition that the methods of tissue culture are potentially applicable to the problem of the isolation of Beta cells from the other cells of the pancreas. Successful application of these methods to the problem requires the establishment of culture conditions which favor the selective growth of Beta cells in cultures of mixed pancreatic cells.

The pancreas alters its secretion of insulin in response to alterations in the level of glucose in the blood perfusing the gland (Grodsky and Bennett, 1963). Continuous injection of high concentrations of glucose into an intact animal causes reduction of Beta cell granulation followed by an extensive proliferation of Beta cells (Woerner, 1938). These responses of Beta cells to glucose form the basis for the development of a tissue culture medium which favors the growth of Beta cells in cultures prepared from the mixed cell populations in tissue fragments dissected from the pancreas. If it is

assumed that the responses of Beta cells to glucose in vivo can be duplicated under appropriate conditions in vitro, an appropriately constituted culture medium supplemented with an optimal concentration of excess glucose should confer a selective advantage upon Beta cells grown in a culture of mixed pancreatic cells. Puck et al. (1957) have developed media which favor the growth of cells with a particular morphology. Human foetal lung tissue grown in Puck's "fibroblastic" medium gives rise to cultures of cells, all of which have the fibroblastic growth habit. The same tissue when grown in Puck's "epithelioid" medium gives rise to cultures which contain epithelioid cells. Puck has shown that cells which show the "fibroblastic" morphology when grown in culture require a medium which is supplemented with embryo extract or which has been conditioned by a "feeder layer" of cells. "Epithelioid" cells on the other hand grow well in a medium containing only serum as a protein source and grow less rapidly in the presence of embryo extract. Puck was careful to point out the fact that the morphology of the cells grown in culture does not necessarily correlate with the morphology of the same cells as they occur in the organism. A tennent of this research was that a cell which undergoes a change in morphology when grown in culture is more likely to show changes in its metabolism than a cell which retains its characteristic morphology in vitro. Consequently, a medium without embryo extract was chosen in an effort to promote the epithelioid morphology which is characteristic of Beta cells as they occur in the organism.

A problem that is invariably associated with the study of a

specific type of cell grown in tissue culture is the positive identification of the type of cell in question. In histological sections, cells tend to have a characteristic morphology and occur in a characteristic association with cells of the same type and with other cell types in the particular organ of which they are a part. Characteristic morphology and histology are such a constant feature of histological sections, that they are commonly taken for granted. However, these features supplement information derived from staining reactions and make it possible to identify a given cell type despite the fact that the stain used may not be specific for the cell type. Thus, Beta cells may be readily identified in histological sections of the pancreas by the staining reaction of their cytoplasmic granules despite the fact that the commonly used Gomori's chrome alum hematoxylin and aldehyde fuchsin methods are not specific for Beta cell granules. Identification based on the staining reaction is confirmed by their occurrence in the Islets of Langerhans which in turn have a characteristic distribution in the pancreas. The problem of identification of cell types is complicated in tissue cultured cells because they occur in tissue culture isolated from their characteristic associations. The tendency of cells to undergo changes in their morphology as a result of minor changes in culture conditions adds to the problem of positive identification of cells grown in culture. Puck concludes from his studies with selective media that "It would be unwise at this stage to attempt to identify cells of the "fibroblastic" morphology <u>in vitro</u> with the specific fibroblasts of mesenchymal origin in the body; or to claim that "epithelioid" cells are directly

descended from body epithelium." Sandström, (1965) cultured embryonic chicken and embryonic rat liver tissue using six different culture techniques and produced four separate cell types. The occurrence of the various cell types varied with the method used and age of the cultures. He used time lapse cinematography and trypan blue-labelled cells and found that three of the four morphologically different cell types were derived from sinusoid endothelium and were modifications of one and the same cell. Penso and Balducci (1963) state that "It is important to realize that all cells in culture have a tendency to assume a similar morphology, so that even for a well trained worker, it is quite difficult to distinguish between cells of epithelial or connective tissue origin. Furthermore, epithelial cells from different parenchymas are essentially similar. It is practically impossible to distinguish liver cells from kidney or conjunctival cells." As a consequence of the tendency of cultured cells to change morphologically, the standard staining methods used for the identification of cells in histological sections usually prove to be inadequate for the positive identification of cultured cells.

In this research, two principal criteria have been adopted to establish the identity of functional Beta cells grown in tissue culture. Beta cells are identified by the fluorescence of their cytoplasmic granules under ultraviolet illumination after staining with anti-insulin gamma globulin conjugated with fluorescien. The aldehyde fuchsin positive granules in the cytoplasm of Beta cells are generally considered to be the precursor of the insulin secreted by these cells. Lacy and Davies (1957) have demonstrated that these granules are anti-

genically similar to insulin and have adapted this property to the fluorescent antibody staining of Beta cells. Beta cells are identified by their synthesis and secretion of insulin. It is assumed that if the Beta cells grown in culture remain physiologically competent, they will continue to produce insulin which, if it is released into the medium, should be detectable by tests with sufficient sensitivity. A variety of methods have been used to detect insulin in the medium of the pancreas cultures.

CHAPTER II

MATERIALS AND METHODS

Preparation of Cultures

All cultures were prepared in a dry box which was equipped with electrical outlets, an electric hot plate, daylight fluorescent lamps and ultraviolet germicidal lamps. The fine dissecting and cutting up of tissue was done with a Bausch and Lomb "StereoZoom" dissecting microscope with the eyepieces projecting through a cutout in the inclined glass front. Polyethylene film was taped to the microscope and to the glass front of the dry box to seal the installation. The microscope focusing control knobs and the two variable intensity Bausch and Lomb dissecting lamps, together with their transformer, were retained at all times completely inside the cabinet. Air was passed from an air line equipped with a diaphragm-type pressure regulator into the cabinet through a cotton-packed bacterial filter. This maintained the cabinet air pressure slightly above atmospheric while the preparation of cultures was in process. Filter-sterilized CO_2 was passed into the cabinet from a gas cylinder. Prior to each culture session, the cabinet was was washed with a surface active bactericidal and mycocidal agent (Cetylcide) and the sterilizing ultraviolet lights were turned on for at least one hour. Access to the cabinet was through sliding panels on the bottom of the

cabinet front. These panels were removed during the preparation of cultures.

All materials used inside the cabinet were sterile. Glassware and heat stable solutions were sterilized by autoclaving at 15 lbs/in^2 pressure and 121° C for 15-60 minutes, depending upon the mass and specific heat properties of the material being autoclaved. Heat labile solutions were sterilized by ultrafiltration (Millipore filter, HA 0,45μ, white, grid, 13 mm). Dissecting instruments were placed in 70% ethanol for 30 minutes and dried with sterile 4 x 4" gauze surgical sponges.

Glassware and instruments were washed in a non-toxic neutral liquid detergent ("7X") solution, rinsed in running tap water, distilled water and three changes of glass distilled water.

Chick tissue was obtained from White Leghorn embryos which had been incubated for fourteen days. Embryos of this age were chosen since the differentiation of the endocrine portion of the pancreas commences on the eleventh day of incubation (Black and Comolli, 1954), Beta cell granulation appears on the twelfth day (Romanoff, 1960) and separation of the islets by a basement membrane is complete by day fourteen (Black and Comolli, 1954). The embryos were removed from the eggs under aseptic condition, and the entire primordium of the pancreas was removed from its position in the loop of the duodenum. The pancreatic primordia were pooled in a small petri dish containing either control culture medium or Hanks' balanced salt solution (Hanks and Wallace, 1949) maintained below 10° C.

During dissection and mincing operations, the tissues were

kept cold by placing them, in their liquid-filled containers, on chilled sealed refrigerant cans of the type used in picnic coolers. The refrigerant cans were chilled by placing them in museum jars filled with 70% ethanol kept in a freezer at -12° C. The cans were removed from the freezer, placed on their sides on a sterile towel inside the dry box, and the petri dishes containing the tissue were placed on them. Tests indicated that under the conditions prevailing inside the dry box, the temperature measured inside a petri dish placed on one of these cans rose from 2° C to 10° C over a period of four hours. During a culture session, the cans were changed frequently enough to maintain the solutions below 10° C. The excised tissues were transferred to a small watch glass filled with Hanks' balanced salt solution or the control medium, placed on refrigerant cans, and dissected under the microscope at 15-35 diameters magnification into roughly cuboidal fragments approximately 0.1 mm^3 in size.

The solution used as culture medium was derived from the "epithelioid" medium described by Puck et al. (1957). The horse serum and human serum in Puck's formula were replaced with gamma globulin-free chicken serum ("Agamma" chicken serum, Hyland Laboratories, Los Angeles, Calif.). Tissue culture medium #199 (Morgan, Morton and Parker, 1950) replaced Puck's synthetic mixture. The control cultures were grown in a medium that had the following composition:

T. C. Medium # 199	10 ml/100 ml
"Agamma" chicken serum	20 ml/100 ml

Hanks' balanced salt solution	70 ml/100 ml
Penicillin	200 U/ml
Strepotomycin sulfate	0.25 mg/ml
Mycostatin	100 U/ml

Hanks' balanced salt solution and T. C. medium #199 contain glucose in a concentration of 100 mg/100 ml; therefore, the control medium contains glucose from these sources in a final concentration of 80 mg/100 ml. This value does not include the glucose which may be present in the chicken serum. This culture medium will be referred to as 1X glucose medium. The experimental cultures were grown in media which had two or four times the glucose concentration of the control medium and the same concentration of all other components. The experimental culture media were made by adding concentrated glucose to the 1X glucose medium. When 1.43 ml of 310 mM glucose were added to 100 ml of 1X glucose medium, the resulting solution was called 2X glucose medium; when 4.30 ml of 310 mM glucose were added, the solution was called 4X glucose medium. The "particle" concentration was used as a measure of the relative tonicity of the media (Stewart and Kirk, 1954). Hanks' balanced salt solution has a "particle" concentration of 309 milliosmoles. Therefore, the addition of 310 mM glucose to the control medium did not change the relative tonicity. The solutions for growing experimental materials had a total tonicity which was equal in all solutions, but there was a slight dilution of the other components of the 1X glucose medium. To test the effect of diluting these other materials a culture medium was made by adding an isosmotic solution of mannitol to the 1X glucose and the 2X glucose

media. The 1X glucose medium had 4.30 ml of isosmotic mannitol added, and 2.87 ml of the isosmotic mannitol was added to the 2X glucose medium. This resulted in solutions in which the components such as salts, proteins and antibiotics were diluted in all cases to the same extent. This medium was used in three culture sessions.

The cells were cultured as monolayer outgrowths from the explants on the surface of 30 ml plastic tissue culture flasks (Falcon plastics) or on the glass coverslips of Maximow deep well slides.

The Maximow deep well slides were used for cultures in cases where microscopic examination was required. Such cultures were maintained for 48 hours or less. The deep well preparations were prepared by suspending 15 to 20 tissue fragments in the appropriate medium contained in the well of the slides. The well was covered with a 40 mm square # 2 glass coverslip and the edges were sealed with melted paraffin applied with a camel's hair brush. The slides were incubated in an inverted position, and the explants settled and attached to the surface of the coverslips. Cultures in flasks were prepared by suspending approximately 30 explants in 5 ml of the appropriate medium. The flasks were incubated with their plane surfaces down. The explants settled and attached to the surfaces. The cultures grown in plastic flasks were maintained up to six weeks by changing the medium every 48 hours.

All cultures were kept in an incubator maintained at 37° C.

In preliminary experiments, pancreas tissue was embedded in an agar matrix or in a plasma clot (Diffco T. C. chicken plasma).

The medium for these cultures was made from Simms' balanced salt solution (Simms, 1942) and embryo extract (Bast and Mills, 1963). The clots were formed on the surface of 40 mm square glass coverslips by stirring a drop of Simms' balanced salt solution containing 15-20 fragments into a drop of reconstituted lyophilized chick plasma. A drop of embryo extract was stirred into the resulting mixture to initiate clot formation. The cultures grown in a plasma clot showed an abundant outgrowth of cells with a fibroblastic appearance and very few cells showing the epithelioid growth habit. Explants embedded in an agar matrix did not grow.

The cultures were examined with a Bausch and Lomb phase contrast microscope equipped with a long working distance condenser, a triocular head and lens combinations giving magnifications of 70X dark field, 200X phase contrast and 420X phase contrast. Photomicrographs were taken with Exakta VX and VXIIa cameras on Kodak high contrast copy film. The magnifications indicated on the photomicrographs in figure 3 are absolute magnifications measured from photomicrographs of a stage micrometer.

The relative number of cell types appearing in cultures grown in each of the different glucose concentrations was estimated by counting the cells on photomicrographs taken with an "unselected field" technique. Phase contrast photomicrographs were taken at 200X magnification of several fields on each of the slides or culture flasks. An explant was selected at random and an area in the zone of outgrowth was centered under the 3.5X objective (70X magnification). The 10X

objective was then rotated into position. Since the microscope used was not parfocal between these objectives, it was now out of focus and cell morphology could not be discerned. Without focusing or looking through the microscope, an area of reasonably uniform distribution of cells was projected on the ground glass of the camera by slight movements of the mechanical stage. The microscope was then carefully focused, and the photograph was taken without further adjustment of the mechanical stage. All of the cells whose limits could be determined on the photomicrographs (excluding the mass of the explants themselves) were counted and tabulated according to morphological type.

Fluorescent Antibody Techniques

Production of Antibody

Anti-insulin antibodies were produced by the method of Maloney and Coval (1955) as modified by Armin, Grant and Wright (1960). Guinea pigs were immunized by injecting 1 mg of crystalline insulin[1] in Freund's adjuvant (Freund and McDermott, 1942). Ten mg of crystalline insulin was dissolved in 10 ml of distilled water that had been adjusted to pH 2.5 with 1 N HCl and contained 0.03 g of phenol. This solution was added to 10 ml of 70% mineral oil and 30% anhydrous lanolin and emulsified in a Waring blender. Two ml of this emulsion was injected subcutaneously into a guinea pig, one ml between the shoulders and one ml in the flanks. Three injections of the mixture were given at

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[1]Crystalline beef insulin kindly provided by Dr. W. R. Kirtley of the Eli Lilly Co., Indianapolis, Indiana. Lot #795372, 25.6 U/mg, glucagon content < 0.1%.

four week intervals. Thereafter, the titer was maintained by injecting 1 mg of insulin in Freund's adjuvant per animal every six weeks. During the first 12 hours following injection of antigen, animals sometimes showed symptoms of hypoglycemia in which case they were injected intraperitoneally with 2 ml of sterile 25% glucose solution. Animals which showed signs of anaphylaxis from the later injections of antigen during the course of building and maintaining the anti-insulin titer were treated with 20 mg of diphenhydramine (Benadryl).

Preparation of Labelled Gamma Globulin

Gamma globulin labelled with fluorescien isothiocyanate (FITC) was prepared by a modification of the method described by Kologlu et al. (1963). Blood was drawn by cardiac puncture from the immunized guinea pigs starting two weeks after the third injection. Ten ml of blood was drawn from each of four immunized animals and from each of four untreated guinea pigs. The blood from each animal was placed in a 12 ml conical centrifuge tube and allowed to clot in the slanted tube at room temperature. The tubes were placed in the refrigerator and the clot was allowed to retract overnight. The blood was centrifuged at 6000 xg for 15 minutes in a Servall model RC-2 automatic refrigerated centrifuge held between zero and two degrees C. The serum was pooled as anti-insulin lots and control lots and recentrifuged. Each lot of pooled sera was diluted with three volumes of carbonate-bicarbonate buffer, pH 8.5. Approximately 1 mg of fluorescien isothiocyanate adsorbed on celite was added to each ml of diluted serum. The mixture was stirred taking care not to cause

frothing, and conjugation was allowed to proceed overnight in the refrigerator. The mixtures were centrifuged at 0 to 2° C and 3000 xg for 5 minutes to remove the celite. The supernatent was adjusted to pH 7.6 to 7.8 with sodium bicarbonate or acetic acid, and chilled to 0° C in an ice bath. The gamma globulin precipitation was carried out on a magnetic stirrer kept in the freezer of a refrigerator at -4° C. An equal volume of 50% ethanol cooled to -26° C was added dropwise from a capillary pipette to the conjugated sera. The sera were stirred constantly during the addition of alcohol, and stirring was continued for 20 minutes after all of the alcohol had been added. The resulting fluorescien-labelled gamma globulin precipitate was separated from the supernatent by centrifugation at 28,000 xg and -4 to -6° C for 10 minutes. The centrifuge tubes were inverted and allowed to drain for ten minutes after which the gamma globulin was redissolved in a volume of cold buffered saline (0.85% NaCl, pH 7.0) equal to one-third the volume of the original serum. The labelled gamma globulin was frozen in a thin layer on the sides of shell vials swirled in a dry ice-ethanol mixture and stored in 0.5 ml lots in a freezer at -20° C. The gamma globulin fractionation was routinely checked by electrophoresis on cellulose acetate strips and showed traces of serum albumin remaining in the preparations. The insulin binding potency was checked by determining the antibody preparation's ability to block the movement of crystalline insulin on exchange resin impregnated chromatography paper (Kologlu et al., 1963).

Fluorescent Staining of Cultures

Anti-insulin antibody binds to the insulin precursor granules

in the Beta cells (Lacy and Davies, 1957). Thus anti-insulin gamma globulin conjugated with a fluorescent dye might be used as a specific stain for the identification of the Beta cells grown in culture.

Coverslips were removed from the deep well slide cultures and dipped into absolute methanol for one minute. They were washed in Hanks' balanced salt solution and placed in large covered petri dishes containing a disc of filter paper moistened with water to prevent drying of the cells and the culture was "stained" by covering the cells with a drop of labelled gamma globulin for 30 minutes. The preparations were washed for 10 minutes in Hanks' balanced salt solution to remove unbound fluorescent material.

The specificity of the staining reaction was assured by staining a series of experimental and control preparations. Experimental pancreas cultures grown in 2X or 4X glucose medium were stained with FITC-labelled anti-insulin gamma globulin. Such cultures were also treated with FITC-labelled non-immune gamma globulin. Non-specific adsorption of the fluorescien label would be detected in the latter preparations. A true immune staining reaction can be blocked by binding either the antigen or antibody; consequently, two blocking tests were incorporated. First: experimental pancreas cultures were treated for 30 minutes with unlabelled anti-insulin serum washed with Hanks' solution, then stained with FITC-labelled anti-insulin gamma globulin. The unlabelled anti-insulin serum binds the antigen in the Beta cell granules and thereby reduces or eliminates their ability to bind the labelled antibody when subsequently exposed. Second: FITC-labelled anti-insulin gamma globulin

was mixed with crystalline insulin 30 minutes before it was used to stain experimental pancreas cultures. The insulin binds the antibody reducing the amount of labelled antibody that insulin containing granules can bind, thereby eliminating the staining reaction. Cultures of embryonic chick cardiac tissue were covered with a solution of crystalline insulin for 30 minutes then washed and treated with FITC-labelled anti-insulin gamma globulin. This checked the possibility that cells in the cultures other than Beta cells will be stained after absorbing insulin released into the medium by Beta cells. Chick cardiac cultures which had not been pretreated with insulin were also stained with FITC-labelled anti-insulin gamma globulin. This check tested the possibility that cultured chick tissues in general have an affinity for the labelled anti-insulin gamma globulin.

The preparations were mounted in glycerol buffered at pH 7.0 and the fluorescence was examined with a Leitz ultra-violet microscope at magnifications of 200X and 400X.

Physical Methods of Insulin Detection

Extraction of Insulin from Medium

Insulin could not be detected by direct electrophoretic or chromatographic examination of the medium taken from the experimental cultures. It was assumed that the concentration of insulin released into the medium was too low to be detected by these means. Consequently, the insulin was concentrated by extracting it from the culture medium. The extraction also provided samples that were free from components such as serum proteins, that interfere with the insulin

determinations. The medium from flask cultures grown in 1X glucose, 2X glucose and 4X glucose concentrations was collected at 48 hour intervals, pooled in three batches based on glucose concentration and frozen. When about 200 ml had been collected, insulin was extracted from the pooled media. Extractions were also made from two control media. First, "unused medium" and second, unused medium to which 5 mg/100 ml of crystalline insulin had been added. "Unused medium" was prepared at the time that the culture media were changed by pooling that part of the 1X, 2X and 4X glucose media which was not used for the growth of cells. "Unused medium insulin" was prepared by adding 10 mg of crystalline insulin to 200 ml of the unused medium. The extraction of the unused medium detected insulin present in the agamma chick serum. Insulin recovered from the "unused medium" + crystalline insulin was the extracted insulin standard. Insulin was extracted from the various solutions using a modification of the method of Grodsky and Tarver (1956). One hundred ml of the pooled solution was precipitated with an equal volume of 6% trichloracetic acid (TCA). The mixture was centrifuged at 6000 xg and 0 to 2° C for 10 minutes, and the precipitate was washed several times in TCA. The precipitate was suspended in 15 ml of an acidic solvent composed of ethanol, distilled water and concentrated HCl (15:5:3 by volume) and extracted with constant shaking for two hours at 37° C. The suspension was centrifuged at 6000 xg and 0 to 2° C for 10 minutes; the supernatent was decanted and saved and the precipitate was re-extracted overnight in an additional 15 ml of the acidic solvent. The extracts were combined, and the pH was adjusted to 8.5 to 9.0 with concentrated NH_4OH.

The extracts were refrigerated at -1° C for two hours to allow contaminants to precipitate. The preparations were centrifuged at 6000 Xg and 0-2° C. The precipitates were discarded after washing with ammoniacal alcohol. Crude insulin was precipitated from the combined washings and supernatent by the addition of four volumes of a mixture of 3 parts ethanol to 5 parts diethyl ether. The mixture was allowed to stand in the refrigerator at 7° C until precipitation was complete; the supernatent was decanted; and the crude insulin precipitate was washed with the alcohol-ether mixture. The insulin was dissolved in 2.0 ml of acidic ethanol containing 0.5 ml of 0.1N HCl, in distilled water acidified to pH 2.5 with HCl or in physiological saline at pH 8.5 to 9.0.

Electrophoretic Detection

Extracts of the various culture media were electrophoresed on cellulose acetate strips (Aronsson and Grönwall, 1958). Electrophoresis was carried out with a Beckman Durrum type cell modified to use 14 cm cellulose acetate strips ("Oxoid" strips, Consolidated Laboratories, Chicago Hts., Ill.). The details of the modification are shown in figure 1. The advantage of the shorter strip is that the sample is applied closer to the cathode. Faster separation is achieved because this is the region of greatest mobility. Further, a greater voltage drop per centimeter is accomplished with a given voltage when the strip is shorter. These factors make it possible to achieve an adequate separation in a shorter time and result in a sharper band since diffusion time is reduced.

Insulin extracts used for electrophoretic detection were

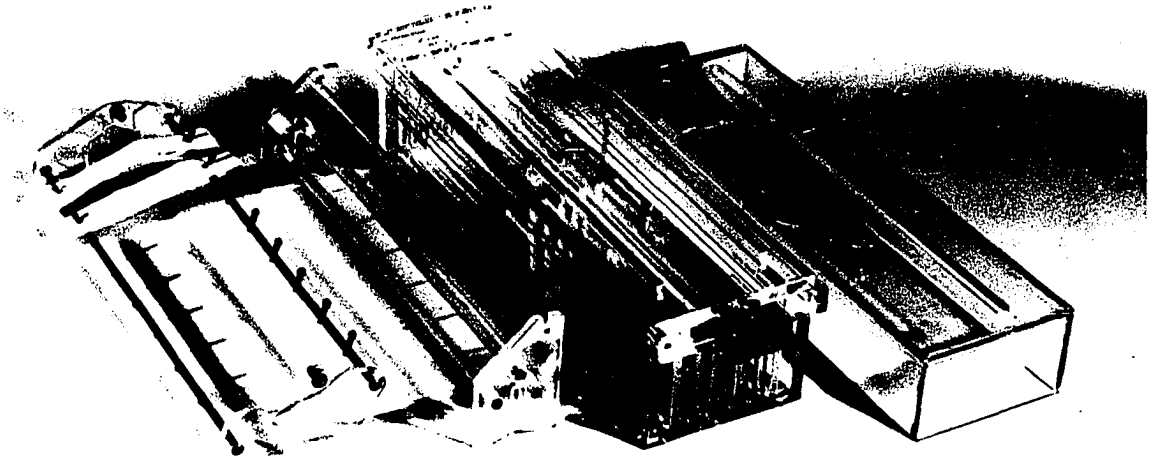

Figure 1a

Modified Beckman Model R Durrum type electrophoresis cell partially disassembled. The new parts are a top, stationary rack and folding rack.

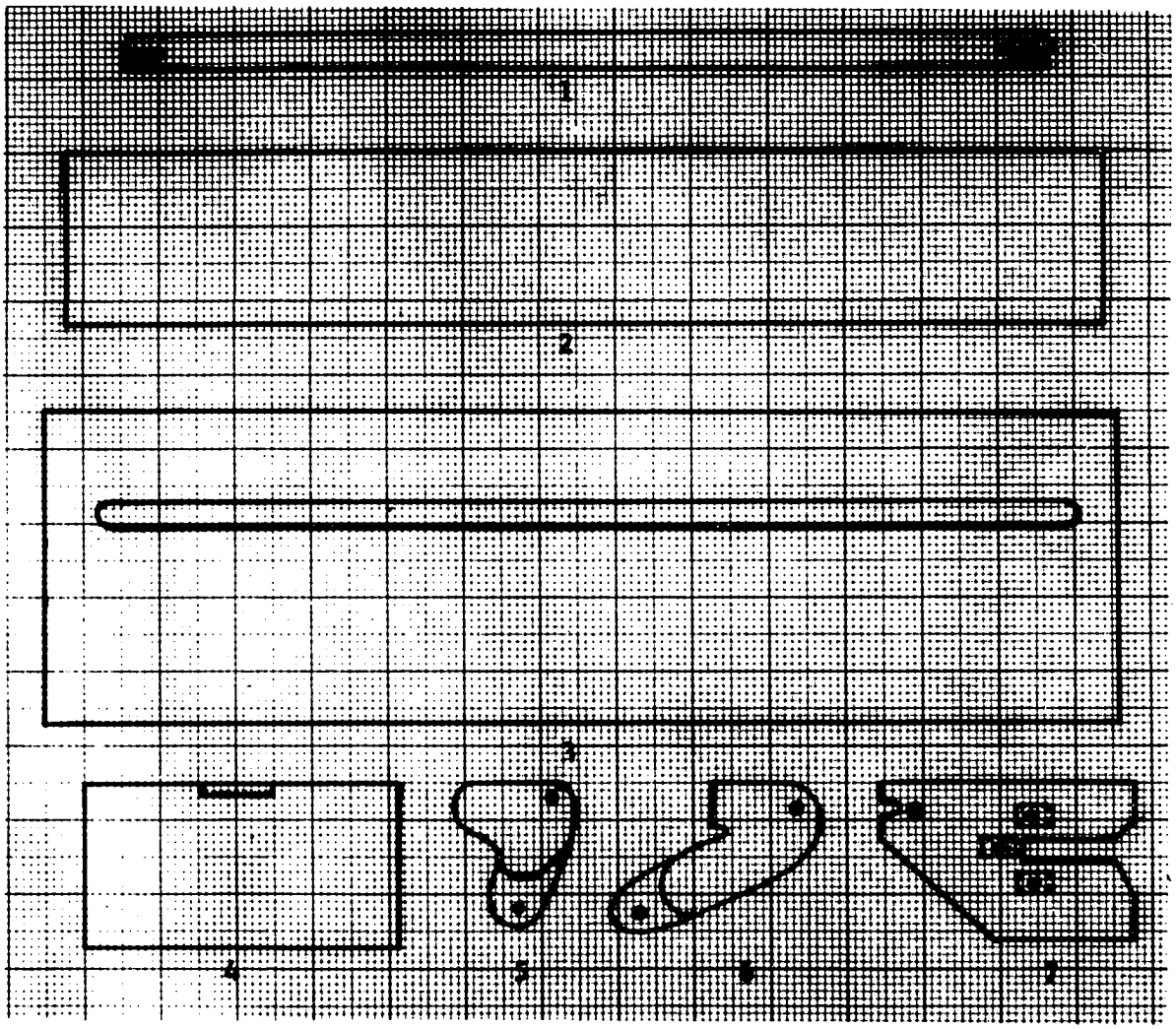

Figure 1b

Scale drawings of the modified parts for the Durrum electrophoresis cell (1 inch squares).

The sides of the lid were made of 1/8" acrylic plastic. The ends of the folding rack were laminated from two pieces of 1/8" acrylic plastic. All other parts were made of 1/4" acrylic plastic. The screws are nylon. Those in the folding rack are 8-32 thread; those in the stationary rack are 6-32 thread.

1. Longitudinal rod of stationary rack (make 3); 2. Side of lid (make 2); 3. Top of lid (make 1); 4. End of lid (make 2); 5. End of folding rack, cathode side (make 2, left and right); 6. End of folding rack, anode side (make 2, left and right); 7. End of stationary rack (make 2).

dissolved in physiological saline adjusted to pH 8.5 to 9.0 with 1 N NaOH. Samples of sixteen lambda of a variety of solutions were applied to individual cellulose acetate strips. Eight samples were electrophoresed simultaneously. Each electrophoretic run included extracts of the media pooled from pancreas cultures grown in 1X glucose, 2X glucose and 4X glucose concentrations and an extract of the unused medium. The remaining four strips were devoted to various standards. These included an extract of the unused medium to which 5 mg/ 100 ml of crystalline insulin had been added; crystalline insulin, 2 mg/ ml extracted from physiological saline; crystalline insulin, 2 mg/ ml dissolved in physiological saline: and an extract of freshly removed rat pancreas.

The samples were electrophoresed for $3\frac{1}{2}$ hours in veronal buffer (pH = 8.6, ionic strength = 0.075). The current drifted and was periodically readjusted to 10 ma. The voltage varied from 130-150 V (approximately 10-12V/cm). At the end of electrophoresis the acetate strips were removed from the apparatus, dried between sheets of blotter paper and stained with Naphthol Blue Black (Colour Index no. 2462, Conn, 1953). The staining solution was made by dissolving 100 mg of the dye in 100 ml of 10% acetic acid. The dried strips were immersed in the dye for 15 minutes. The strips were removed, blotted on paper towels and given two 10 minutes rinses in 10% acetic acid. The stained strips were rinsed for one minute in 95%

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[2]Assigned by the Society of Dyers and Colourists, Bradford, Yorkshire, England, 1924.

ethanol, then were immersed for one minute in a clearing solution composed of 25% glacial acetic acid in 95% ethanol. Glass plates slightly larger than the strips were placed under them, and the strips were lifted from the clearing solution. Excess clearing solution was squeegeed from the strips, and the strips and glass plates were placed in a chromatography oven at 100° C for 30 minutes. The electrophoretic mobility of the experimental extracts was compared with that of the various insulin standards incorporated into each run.

Chromatographic Detection

Although proteins cannot normally be separated by paper chromatography, Wiesel et al, (1963) isolated insulin chromatographically on a specially prepared resin exchange paper (Amberlite IRC 50 H form, Grade WA-2 of Rohm and Haas, Philadelphia). This method was used to compare the chromatographic mobility of the insulin extracts of the experimental media with that of crystalline insulin standards. Samples were dissolved in physiological saline adjusted to pH 8.5 to 9.0 and applied as 10 lambda spots 3 cm from the end of a 7 X 9" sheet. Six spots were applied to each sheet of paper. These included extracts of the culture media pooled from pancreas cultures grown in 1X glucose, 2X glucose and 4X glucose concentrations and an extract of the unused medium. Spots of an extract of the unused medium to which 5 mg/ 100 ml of crystalline insulin had been added and crystalline insulin, 2 mg/ ml dissolved in physiological saline were included as standards on each sheet. The sample spots were dried with a cold air blower and the papers

were suspended in a chromatographic developing chamber until the solvent front descended to within about 2 cm from the bottom of the sheet. The solvent system was a mixture of butanol, distilled water and glacial acetic acid (12:5:6 by volume). The sheets were removed from the chamber, dried in air or in a chromatography oven at 100° C and stained with Napthol Blue Black for 20 minutes. The blue background was rinsed from the sheets with methanol and they were dried in a chromatography oven at 100° C.

A more specific detection technique involves binding insulin by anti-insulin antibodies and then chromatographing on exchange resin impregnated paper. Insulin bound by an antibody does not move from the site of application while insulin mixed with non-immune serum can be separated from it chromatographically (Kologlu et al., 1963). Gamma globulin from immune guinea pigs and non-immune gamma globulin were prepared as described on page 15. Guinea pig anti-insulin gamma globulin was mixed with an equal volume of each of the insulin extracts of the various media which had been dissolved in physiological saline adjusted to pH 8.5 to 9.0. It was similarly mixed with a solution of crystalline insulin in physiological saline so that the final concentration of insulin was 1 mg/ ml. A second series of insulin-gamma globulin mixtures was prepared with non-immune gamma globulin. The mixtures stood overnight at room temperature to complete binding of the insulin by the antibody. Six ten lambda samples were applied to each sheet of resin impregnated paper. These included extracts of 2X glucose medium and 4X glucose medium collected from pancreas cultures. These extracts had been

mixed with anti-insulin gamma globulin. A crystalline insulin standard (2 mg / ml in physiological saline) and an unbound anti-insulin gamma globulin control were also included.

The samples were chromatographed and stained with Naphthol Blue Black. In this procedure the anti-insulin gamma globulin conjugates with insulin and the insulin doesn't migrate. Therefore, there is a reduction of the intensity of the color of the chromatographic spot at the characteristic insulin Rf when insulin is present. Non-specific binding of insulin would be detected by a similar reduction in intensity of the chromatographic spots of those preparations mixed with non-immune gamma globulin.

These physical methods were applied in the detection of insulin in the media collected from three different culture sessions in which the cells were maintained from 4 to 6 weeks.

Bioassay of Insulin

The activity of the insulin extracts of the various culture media was assayed by measuring the ability of the suspected insulin sample to enhance the release of $C^{14}O_2$ from glucose-1-C^{14}. This is accomplished by placing a part of an epididymal fat pad in a mixed solution of glucose—1-C^{14} and non radioactive glucose and measuring the radioactivity trapped in a CO_2 absorber (Renold et al., 1960). The method is capable of detecting 10 μU of insulin per ml. The fat tissue was incubated in a modified Krebs' bicarbonate buffer to which a solution of radioactively-labelled glucose had been added. The modified buffer had the following ion composition in mM/L: Na, 146; K, 5; Ca, 2; Mg, 1; Cl, 114; and HCO_3, 40. Gelatin (200 mg / 100 ml)

was added to prevent the glass from adsorbing the insulin. Glucose was present at a concentration of 250 mg/ 100 ml.

The labelled glucose solution was prepared by dissolving 50 μc of glucose-1-C^{14} (153 mc/ mM) in 12.5 ml of distilled water. Non-radioactive glucose (150.4 mg) was added to bring the final glucose concentration of this solution to 1250 mg/ 100 ml. Thus, 0.1 ml of this solution contained 0.4 μc and 1.25 mg of glucose. Two ml of the complete incubation medium contained 0.4 uc and 6.0 mg of glucose.

Epididymal fat tissue was obtained from albino Sprague-Dawley rats weighing between 158 and 294 grams. The animals were killed by a blow on the head, and the abdominal cavity was opened. The testes were displaced from the scrotum into the abdominal cavity by gentle pressure. The fat pad attached to the epididymis was removed by a cut made just distal to the epididymal vessels. The fat pad was held in forceps, lifted free and three segments of approximately equal size were cut off with sharp scissors. The segment closest to the epididymis was designated "proximal" (P) and was followed by the "medial" (M) and "distal" (D) segments. The portion of tissue which had been handled with the forceps was not included in any of the segments. As each segment was severed, it was allowed to fall into a preweighed 6 dram shell vial. The various vials contained 0.1 ml samples of assorted insulin extracts or insulin standards and 1.9 ml of modified Krebs' bicarbonate buffer that had been gassed for ten minutes with a gas mixture containing 95% O_2 and 5% CO_2 and had been stoppered until used.

The assay vials were assembled as shown in figure 2. Each assay consisted of 18 shell vials in a "balanced segment design" (Sheps et al., 1960). In this arrangement, one segment from each rat appears in each of six different "treatments" and proximal, medial and distal segments appear in each treatment. This arrangement orders the metabolic rate differences which occur among segments of the same rat (proximal segments are significantly lower than the other segments) and variations which occur from one rat to another in such a manner that it is possible to remove these differences by statistical methods. A diagram of this distribution is shown below:

	Rat 1	Rat 2	Rat 3
Unknown 1	D	P	M
Insulin standard 1	M	D	P
Unknown 2	P	M	D
Unknown 3	D	P	M
Insulin standard 2	M	D	P
Unknown 4	P	M	D

Balanced segment design

Immediately after the tissues were added, the vials were rapidly stoppered, reweighed and regassed for five minutes through 18 gauge transfusion needles inserted through the sleeve type serum caps. The weight of fat tissue added was calculated by difference. Exactly 0.1 ml of C^{14} labelled glucose solution was injected into the incubation medium through the stoppers, and the vials were incubated for two hours at 37° C with constant shaking at 90 cycles per minute.

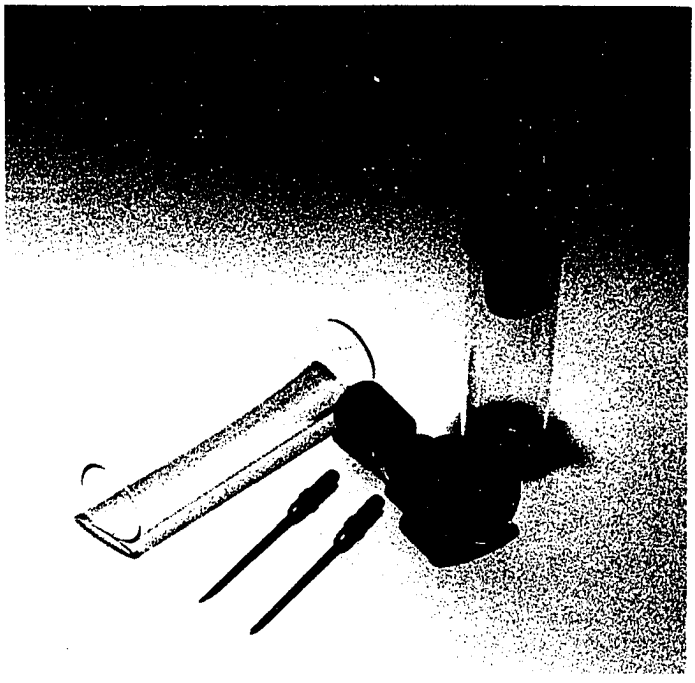


Figure 2

Insulin Bioassay Vial

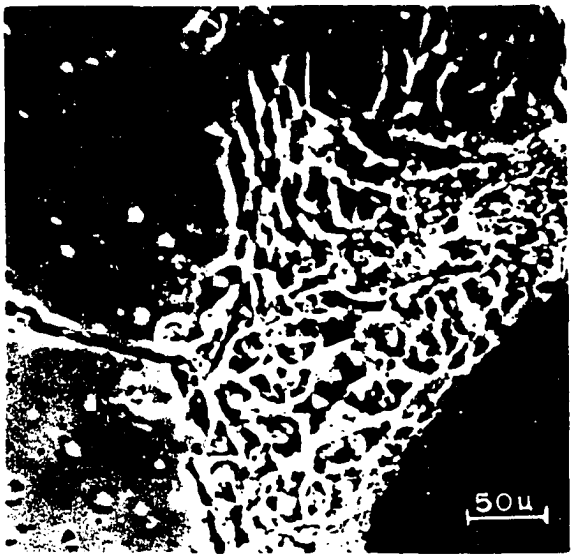
After the incubation period, 0.1 ml of 1.8 N NaOH was injected into a plastic cup suspended inside the shall vials. Two-tenths of a ml of 10 N H_2SO_4 was added to the incubation medium thus driving the CO_2 out of solution. The vials were placed on a shaker for at least 60 minutes at 90 cycles per minute. The CO_2 released from the incubation mixture was absorbed in the NaOH as sodium carbonate. The Na_2CO_3 was transferred from the plastic cups to preweighed stainless steel planchets. The cups were washed twice with seven drops of CO_2-free distilled water and the washings were added to the planchets. The planchets were thoroughly dried on a spin dryer; allowed to cool in a desicator and reweighed. The beta emissions from each planchet was then counted on a Picker compact scaler equipped with a mica end window Geiger-Mueller tube (G-M Detector, Atomic accessories Inc. Bellerose, N. Y. Type EWH-108, 1.4 ml per C_m^2). The scaler and tube were standardized against a calibrated carbon source (C^{14}, 0.15 µc, New England Nuclear Corp.). The results were recorded as the logarithm of number of counts per minute per milligram of adipose tissue. The data were analyzed according to the method of Sheps et al. (1960).

CHAPTER III

RESULTS

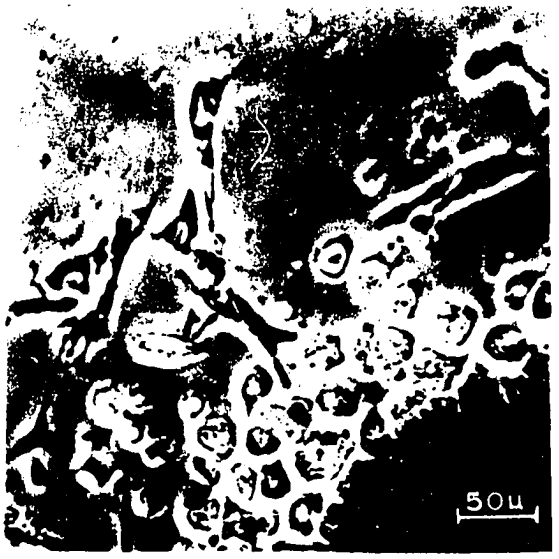
Growth of Beta Cells in Culture

The suspended explants settle onto the bottom of the culture vessels and become attached to the surfaces. There follows a rapid migration and proliferation of cells in a radial pattern about the periphery of the explants. Within 24 hours continuous or broken sheets of cells have begun to be formed around most of the explants in the culture vessels (Fig. 3a-c). Three of the four fundamental cellular types (Penso and Balducci, 1963) can be identified in the cultures: "star-like cells," "fusiform cells" and "round cells." The round cell has a unique set of responses. It is the only cell type in the culture that exhibits fluorescence after being treated with fluorescien labelled anti-insulin gamma globulin. Consequently, in estimating the percentage of Beta cells occurring in the different cultures, all cells of the round type have been considered to be Beta cells. These round cells were 15-20μ in diameter with a centrally located nucleus and coarse cytoplasmic granulation. Photomicrographs were taken of cells growing on the coverslips of the culture slides. When the outline of the cell could be determined, it was



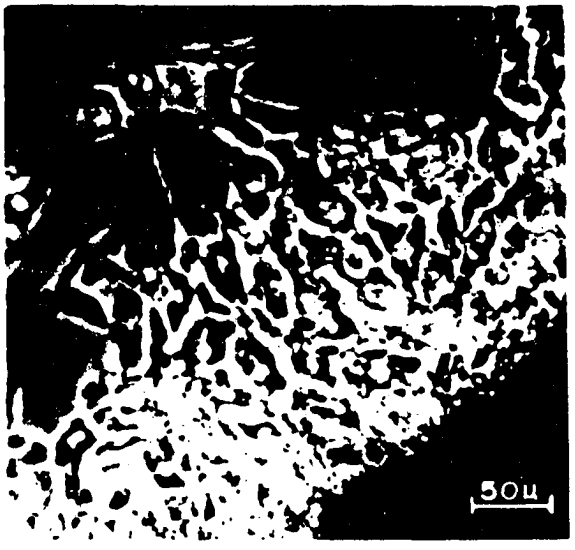
a

Phase contrast photomicrograph of pancreas culture in 1X glucose medium prepared 6/23/65.



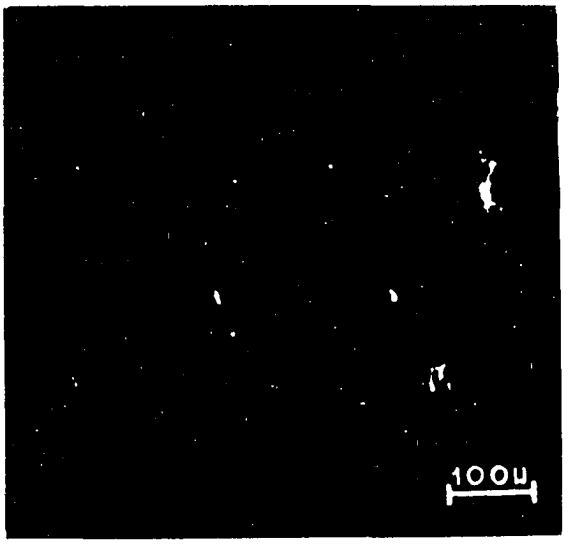
b

Phase contrast photomicrograph of pancreas culture in 2X glucose medium prepared 6/23/65.



c

Phase contrast photomicrograph of pancreas culture in 4X glucose medium prepared 6/23/65.



d

Ultraviolet photomicrograph of pancreas culture stained with FITC-labelled anti-insulin gamma globulin prepared 8/27/65.

Figure 3

counted as a Beta cell or as an "other" type of cell.

Tables 1a through 1c show the results of three culture sessions in which embryonic chick pancreas tissue was cultured in 1X glucose, 2X glucose and 4X glucose media. The results of three culture sessions in which pancreas tissue was cultured in the mannitol dilution test media are presented in tables 1d through 1f. The data presented in Table 1 have been converted by standard methods to the mean percentage of Beta cells $\pm$ 2 standard errors of the mean for each glucose concentration and for each culture session. The mean percentages of Beta cells in cultures grown at the same glucose concentration were compared with unpaired "t" tests. These tests reveal that within any given culture session, there is no significant difference ($p > .05$) in the mean percentage of Beta cells produced in the regular media and the mannitol test media. There is a significant difference in the mean percentage of Beta cells from one culture session to another between the regular media and the dilution test media as well as between media of the same composition.

The mean percentage of Beta cells produced in the 1X glucose media ranges from 25.5 to 37.6%. The range in 2X glucose media is from 60.8 to 74.4% and in the 4X glucose medium is from 32.9 to 47.6%. In some earlier experiments, it was found that glucose concentrations of 8X and 10X cause a general inhibition of the growth of pancreas cultures. It was not determined whether this inhibition was a result of the excess glucose or of the dilution of the culture medium which occurred as a consequence of the addition of the necessary quantities of isosmotic glucose.

Table 1a

Percentage of Beta cells in pancreas cultures prepared June 23, 1965

1X glucose			2X glucose			4X glucose		
Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells
25	73	34.2	46	88	52.2	95	200	47.5
40	76	52.6	87	140	62.2	99	177	55.9
21	68	30.9	132	186	71.0	77	139	55.4
14	70	20.0	61	118	51.8	92	165	55.8
29	160	18.1	84	150	56.0	112	186	60.2
15	95	15.8	140	224	62.4	74	138	54.4
43	70	61.4	107	159	67.3	32	160	20.0
19	106	17.9	152	217	70.0	84	183	45.9
4	143	2.8	161	215	74.8	66	153	43.2
23	99	23.2	213	258	82.6	38	94	40.5
18	99	18.2	105	193	54.4	28	84	33.4
21	165	12.7	105	162	64.8	83	189	43.9
43	135	31.8	87	131	66.4	119	233	51.0
40	96	41.7	127	200	63.5	87	167	52.0
49	94	52.2	150	208	72.1	85	136	62.5
42	92	45.6	74	109	68.0	82	157	52.2
27	119	22.7	75	153	49.0	77	186	41.4
4	66	6.1	173	240	72.1	24	80	30.0
38	93	40.0	67	113	59.3	90	177	50.8
18	111	16.2	257	316	81.4	75	160	47.8
7	69	10.2	115	146	78.7	110	140	78.4
23	113	20.7	58	111	52.2	31	148	20.9
563	2212	25.5	82	118	69.6	52	107	48.6
			222	303	73.2	105	147	71.4
			94	163	57.6	69	156	44.4
			87	101	86.2	66	149	44.6
			365	457	79.9	74	153	48.4
			125	192	65.1	36	129	27.9
			107	193	55.4	77	154	50.0
			83	161	51.6	36	96	37.5
			151	190	79.4	62	148	41.9
			175	240	72.9	91	188	48.4
			102	151	67.5	137	299	45.8
			96	153	62.8	46	104	44.2
			87	147	59.2	2511	5280	47.6
			4352	6406	67.9			

Variance = 258.85
Std. deviation = 16.09
Std. error = 3.43
$\bar{x} \pm 2SE = 25.5 \pm 6.9\%$
N = 22

Variance = 99.16
Std. deviation = 9.96
Std. error = 1.71
$x \pm 2SE = 67.9 \pm 3.4\%$

Variance = 147.35
Std. deviation = 12.14
Std. error = 2.08
$\bar{x} \pm 2SE = 47.6 \pm 4.2\%$
N = 34

Table 1b

Percentage of Beta cells in pancreas cultures prepared September 17, 1965

1x glucose			2x glucose			4x glucose		
Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells
23	78	29.5	53	84	63.2	16	41	39.0
18	85	21.2	49	78	63.0	16	58	27.5
8	84	9.5	58	96	60.4	37	77	48.1
23	76	30.3	100	159	62.8	21	67	31.4
54	106	51.0	39	54	72.2	9	32	28.1
19	74	25.7	40	68	58.8	4	27	14.8
25	85	29.5	97	158	61.4	15	31	48.4
27	82	33.0	74	112	66.0	2	46	4.3
41	85	48.3	79	124	63.7	18	73	24.7
9	73	12.3	99	155	63.8	30	88	34.2
17	74	23.0	68	114	59.6	9	38	23.7
23	78	29.5	54	97	55.7	10	38	26.3
28	88	31.8	76	141	53.8	27	72	37.5
18	94	19.2	129	187	69.0	24	62	38.7
14	64	21.9	60	91	65.9	16	43	37.2
26	84	31.0	42	121	34.7	15	41	36.6
33	95	34.8	65	117	55.6	23	57	40.4
2	46	4.3	71	105	67.6	17	47	36.2
10	67	14.9	1253	2061	60.8	309	938	32.9
14	56	25.0						
7	66	10.6						
439	1640	26.8						
Variance = 137.28			Variance = 65.27			Variance = 61.54		
Std. deviation = 11.71			Std. deviation = 8.11			Std. deviation = 7.85		
Std. error = 2.56			Std. error = 1.91			Std. error = 1.83		
$\bar{x} \pm 2SE = 26.8 \pm 5.1\%$			$\bar{x} \pm 2SE = 60.8 \pm 3.8\%$			$\bar{x} \pm 2SE = 32.9 \pm 3.7\%$		
N = 21								

Table 1c

Percentage of Beta cells in pancreas cultures prepared October 1, 1965

1X glucose			2X glucose			4X glucose		
Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells
31	69	44.9	59	79	74.7	12	48	25.0
51	101	50.4	251	276	91.0	11	49	22.4
20	52	38.5	256	284	90.1	17	80	21.3
29	58	50.0	84	155	54.2	18	45	40.0
10	47	21.3	50	145	34.5	29	50	58.0
34	70	48.6	249	282	88.3	21	46	45.7
8	31	25.8	136	177	76.8	28	88	31.8
18	33	54.6	48	86	55.8	27	84	32.2
21	68	30.9	104	139	74.8	14	45	31.1
2	18	11.1	188	249	75.4	13	53	24.5
26	75	34.7	231	296	78.0	129	169	76.2
10	43	23.3	234	297	78.8	18	50	36.0
13	53	24.5	39	72	54.2	27	63	42.8
12	38	31.6	15	35	42.8	17	45	37.6
8	23	34.8	124	173	71.6	19	65	29.2
7	60	11.7	64	101	63.3	12	29	41.4
12	60	20.0	136	182	74.7	19	40	47.5
312	899	34.7	69	11	62.2	10	31	32.2
			2337	3139	74.4	19	43	44.2
						48	99	48.5
						508	1222	41.6

Variance = 183.89
Std. deviation = 13.56
Std. error = 3.29
$\bar{x} \pm 2SE = 34.7 \pm 6.6\%$
N = 17

Variance = 285.51
Std. deviation = 16.90
Std. error = 3.98
$\bar{x} \pm 2SE = 74.4 \pm 8.0\%$
N = 18

Variance = 173.77
Std. deviation = 13.8
Std. error = 2.95
$\bar{x} \pm 2SE = 41.6 \pm 5.9\%$
N = 20

Table 1d

Percentage of Beta cells in pancreas cultures prepared August 27, 1965

1X glucose + mannitol			2X glucose + mannitol			4X glucose + mannitol		
Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells
13	53	24.6	55	98	56.2	8	71	11.3
53	98	54.0	53	88	60.2	28	76	36.8
42	78	53.9	80	133	60.1	21	55	38.2
44	92	48.0	54	80	67.5	31	85	36.5
44	117	47.6	81	117	69.0	31	95	32.6
40	82	48.9	39	73	53.5	17	103	16.5
20	55	36.4	39	64	61.0	42	109	38.5
59	141	41.8	111	169	65.7	29	106	27.4
17	67	25.4	63	112	56.3	34	116	29.2
5	68	7.4	114	182	62.6	48	77	62.5
14	59	23.8	146	216	67.5	18	51	35.3
19	74	25.7	835	1332	62.7	307	944	32.9
370	984	37.6						
Variance = 102.36			Variance = 26.93			Variance = 214.30		
Std. deviation = 10.12			Std. deviation = 5.19			Std. deviation = 14.63		
Std. error = 2.92			Std. error = 1.53			Std. error = 4.41		
$\bar{x} \pm 2SE = 37.6 \pm 5.8\%$			$\bar{x} \pm 2SE = 62.7 \pm 3.1\%$			$\bar{x} \pm 2SE = 32.9 \pm 8.8\%$		
N = 12			N = 11			N = 11		

Table 1e

Percentage of Beta cells in pancreas cultures prepared September 17, 1965

1X glucose + mannitol			2X glucose + mannitol		
Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells
3	20	15.0	146	204	71.6
16	53	30.2	73	115	63.4
13	48	27.1	44	101	43.5
20	63	31.7	87	139	62.6
19	63	30.2	79	131	60.3
8	33	24.2	56	100	56.0
2	25	8.0	56	95	59.0
11	30	36.6	86	111	77.5
13	52	25.0	120	157	76.4
28	73	38.4	69	92	75.0
18	62	29.0	49	79	55.1
12	72	16.7	65	92	70.7
27	77	35.1	31	54	57.4
30	83	36.2	65	94	69.2
14	43	32.6	27	35	77.2
6	20	30.0	10	25	40.0
11	49	22.4	18	26	69.2
268	943	28.4	1136	1736	65.6

Variance = 7.43
Std. deviation = 2.73
Std. error = 0.64
$\bar{x} \pm$ 2SE = 28.4 $\pm$ 1.3%
N = 18

Variance = 118.95
Std. deviation = 10.90
Std. error = 2.57
$\bar{x} \pm$ 2SE = 65.6 $\pm$ 5.1%
N = 18

Table 1f

Percentage of Beta cells in pancreas cultures prepared October 1, 1965

1X glucose + mannitol			2X glucose + mannitol		
Beta Cells	Total Cells	% Beta Cells	Beta Cells	Total Cells	% Beta Cells
7	25	28.0	38	56	67.8
10	25	40.0	28	63	44.4
7	31	22.6	30	50	60.0
19	35	54.2	50	73	68.6
8	23	34.8	49	73	67.2
34	70	48.6	76	108	70.4
5	27	18.5	157	197	79.7
10	28	35.7	64	104	61.5
18	70	15.7	53	86	61.6
25	64	39.1	68	100	68.0
18	52	34.6	48	96	50.0
21	54	38.8	63	99	63.6
5	33	15.1	34	59	57.6
6	29	20.7	50	85	58.8
25	69	36.2	91	105	86.6
23	62	37.1	104	131	79.4
18	56	32.1	53	81	65.4
10	35	28.6	19	30	63.3
27	74	36.5	21	39	53.9
296	862	34.3	1096	1635	67.0

Variance = 97.43
Std. deviation = 9.87
Std. error = 2.26
$\bar{x} \pm 2SE = 34.3 \pm 4.5\%$
N = 19

Variance = 103.93
Std. deviation = 10.19
Std. error = 2.34
$\bar{x} \pm 2SE = 67.0 \pm 4.7\%$
N = 19

These results indicate that the differential growth of Beta cells is favored by an increase in the glucose concentration of the culture medium, and that an excess of glucose inhibits the growth of all pancreas cells. Under the conditions prevailing in these cultures, maximum growth of Beta cells occurs at glucose concentrations near 200 mg%.

Fluorescent Antibody Staining

Treatment of the experimental cultures with fluorescien-labelled anti-insulin gamma globulin produced a yellow-green fluorescence in the cytoplasmic granulation of all cells of the round type which could be seen when these preparations were examined under ultraviolet illumination. Figure 3d shows an eight minute time exposure of the fluorescing cytoplasmic granules. No fluorescent granules could be detected in preparations of experimental cultures treated with fluorescien-labelled gamma globulin from non-immune guinea pigs after these preparations had been washed in Hanks' solution. These results indicate that the anti-insulin gamma globulin is bound to the cytoplasmic granules of the round cells while the non-immune gamma globulin is not. The staining reaction was blocked by pretreatment of the experimental cultures with unlabelled anti-insulin serum or by preincubation of the fluorescien-labelled anti-insulin gamma globulin with crystalline insulin. Untreated cardiac tissue was not stained by the labelled immune gamma globulin. The chick cardiac preparations which had been pretreated with crystalline insulin showed a faint overall fluorescence in the tissue fragments which remained attached to the coverslips. These results indicate

that the staining of the experimental cultures is due to a true immune reaction, and that the material of the cytoplasmic granules in the round cells is antigenically similar to insulin. The use of highly purified crystalline insulin minimizes the danger of simultaneously immunizing the guinea pigs against glucagon since less than 0.001 mg of the latter hormone is administered at each injection. The use of labelled gamma globulin instead of labelled whole serum eliminates serum proteins which might otherwise cause non-specific adsorption of the fluorescent label. Consequently, it is highly probable that the cytoplasmic granules in the round cells contain insulin or an insulin procurser that is antigenically similar to it.

Electropheretic Detection

When crystalline insulin dissolved in physiological saline is electrophoresed under the conditions described earlier, it migrates toward the anode at a rate of approximately 0.27-0.30 microns/sec/V/cm. Figure 4 is a photograph of stained electrophoresis strips after electrophoresis of the extracts of the various culture media and standards. The extraction process alters the electrophoretic mobility of insulin (compare the migration of crystalline insulin dissolved in physiological saline to that of insulin similarly dissolved and then extracted from the saline solution). Extracted insulin migrates toward the anode at a rate of approximately 0.12-0.15 microns/sec/V/cm.

Extracts of the 1X glucose, 2X glucose and 4X glucose media produce electrophoretic bands which correspond to that of crystalline insulin which has been extracted after having been added to saline solution. This band is not observed in extracts of the unused medium.

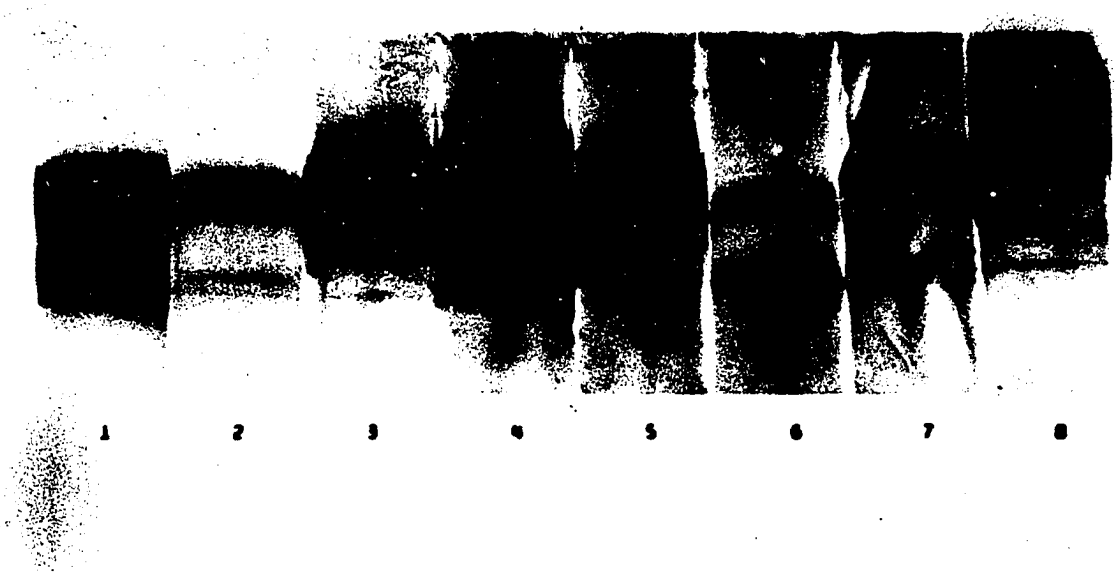


Figure 4

Photograph of electrophoretic analysis of extracts of experimental culture media pooled from pancreas cultures prepared April 1, 1965.

1. Crystalline insulin, 2 mg/ml extracted from physiological saline; 2. Rat pancreas; 3. 1X glucose medium; 4. 2X glucose medium; 5. 4X glucose medium; 6. Unused medium; 7. Unused medium + Crystalline insulin, 5 mg/100 ml; 8. Crystalline insulin, 2 mg/ml.

The material extracted from fresh rat pancreas, from unused medium to which insulin had been added, and from saline to which insulin had been added all have the same electrophoretic mobility. Although no attempt was made to measure insulin quantitatively by electrophoresis, the material extracted from the 2X glucose medium in which pancreas cells had been grown produced an electrophoretic band that stains more intensely than the other extracts. These results indicate that insulin, or a substance with the same electrophoretic mobility as insulin is produced in the pancreas cultures, and that it occurs in a higher concentration in the 2X glucose cultures.

Chromatographic Detection

When the specially prepared resin exchange chromatographic paper is used, insulin will migrate with the solvent front out into the chromatographic field. Figure 5 is a photograph of the results of chromatographing extracts of the various culture media. Crystalline insulin produces a spot which has an Rf value of 0.80 under these conditions. When extracts from the 1X glucose, 2X glucose, 4X glucose media, and an extract of unused medium to which crystalline insulin had been added were tested, a spot with a similar chromatographic mobility was observed. The spot produced by the extract of the 2X glucose medium is larger and more intense than that produced by the other extracts. These results indicate that insulin or a substance which migrates with a similar chromatographic mobility is present in the medium in which pancreas tissue has been cultured. This material occurs in the highest concentration when the growth medium contains twice the normal amount of glucose. There is a general tendency of

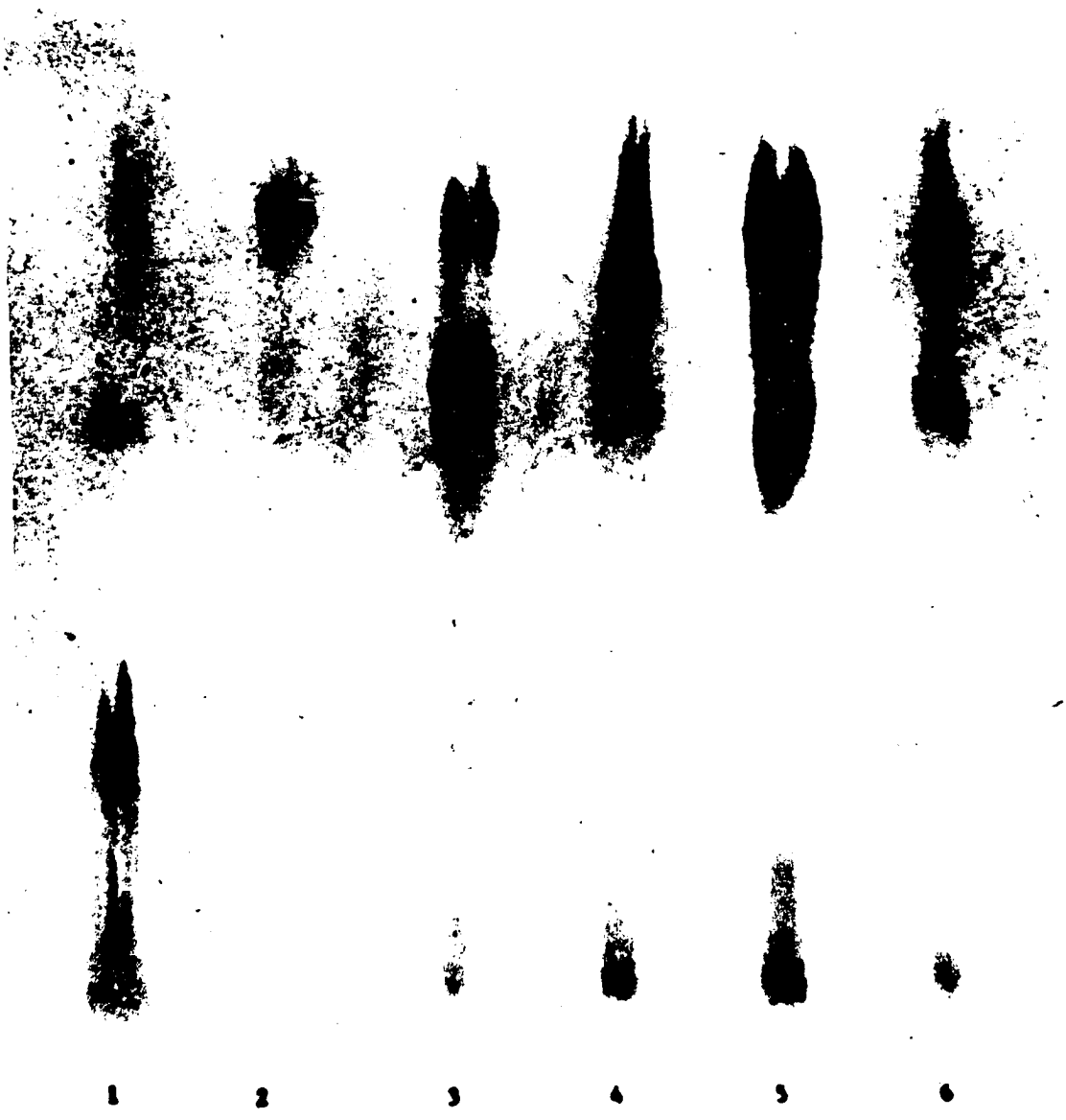


Figure 5

Photograph of a chromatographic analysis of extracts of experimental culture media pooled from pancreas cultures prepared April 1, 1965.

1. Unused medium; 2. Crystalline insulin, 2mg/ml; 3. Unused medium + crystalline insulin, 5 mg/100 ml; 4. 4X glucose medium; 5. 2X glucose medium; 6. 1X glucose medium.

the extracted samples to tail, and a second spot with an Rf value lower than that of the crystalline insulin standard is produced by each extracted sample. The source of this second spot has not been determined, but it is probably a denaturation product of some protein component of the culture medium which was extracted along with the insulin.

Figure 6 shows the results of immune chromatography of extracts of the various culture media. Gamma globulin does not move into the chromatographic field. When crystalline insulin is mixed with non-specific gamma globulin it may be separated from it chromatographically, moving into the field while the gamma globulin remains at the site of application. When crystalline insulin is allowed to stand overnight with anti-insulin gamma globulin it becomes bound to it and the mixture remains at the site of application providing there is sufficient gamma globulin present to bind all of the insulin. Otherwise, excess insulin migrates into the chromatographic field. When extracts of the 2X glucose and 4X glucose media are allowed to stand overnight with non-specific gamma globulin, they may be separated from it chromatographically, migrating into the field with the characteristic chromatographic mobility of the crystalline standard. Extracts of 2X glucose and 4X glucose media similarly mixed with anti-insulin gamma globulin do not show a spot corresponding to crystalline insulin. When equal volumes of extracted medium and immune gamma globulin are mixed, no spot corresponding to the crystalline insulin standard is found above the site of application of the extract of the 4X glucose medium, and the intensity and size of the spot above the 2X glucose

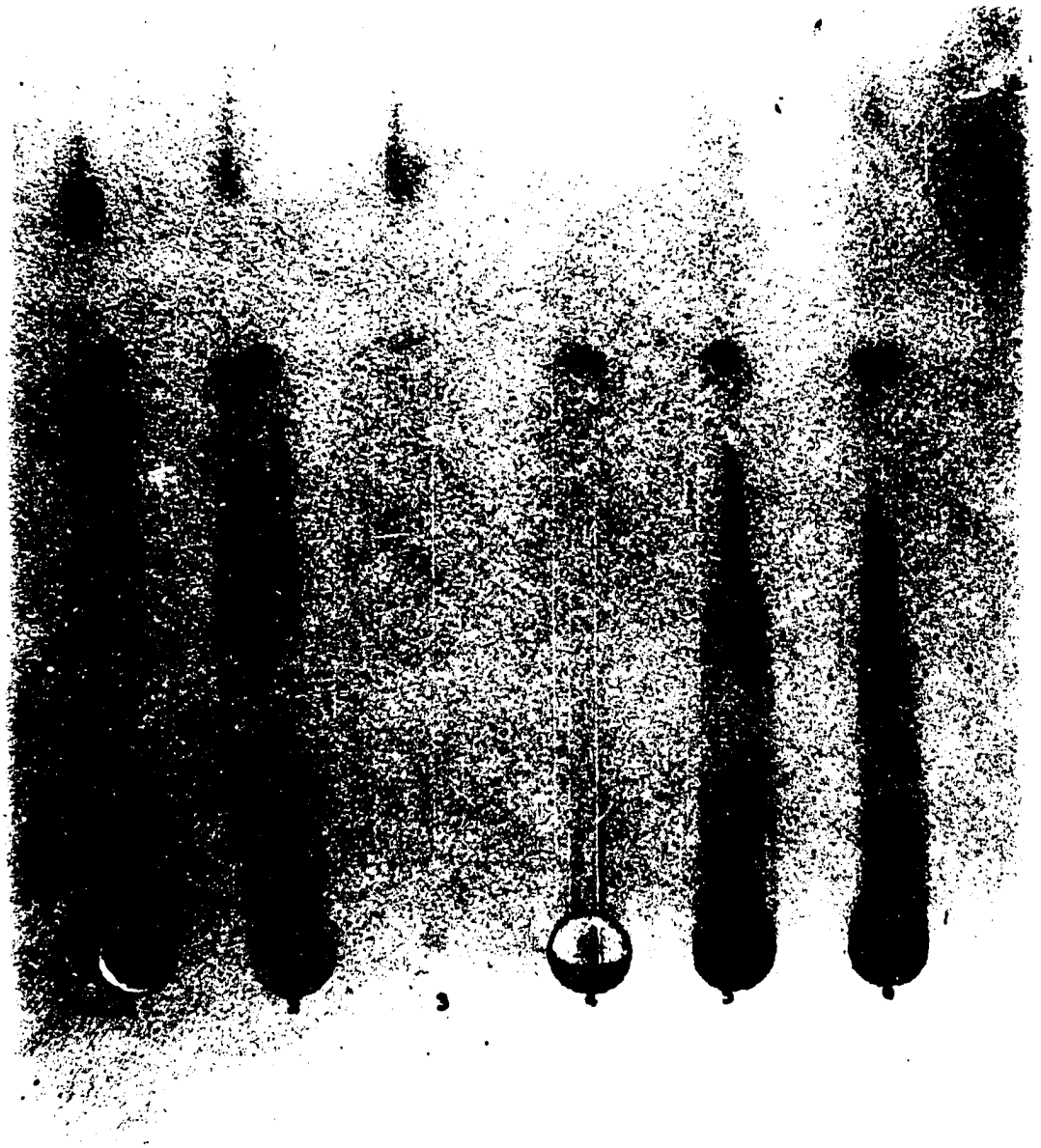


Figure 6

Photograph of an immune chromatographic analysis of extracts of experimental culture media pooled from pancreas cultures prepared May 11, 1965.

1. Control gamma globulin + 2X glucose medium; 2. Control gamma globulin + 4X glucose medium; 3. Crystalline insulin, 2 mg/ml; 4. Anti-insulin gamma globulin; 5. Anti-insulin gamma globulin + 2X glucose medium; 6. Anti-insulin gamma globulin + 4X glucose medium.

extract is considerably reduced.

These results indicate that the material produced in the pancreas cultures behaves antigenically the same as insulin, and that it occurs in a higher concentration in the 2X glucose medium than in the other media.

Bioassay of Insulin

Table 2 presents the raw data from three assays of the extracts of the media pooled from pancreas cultures prepared April 1, 1965 and two assays of the extracts of the media pooled from pancreas cultures prepared on May 11, 1965.

The results were subjected to an analysis of variance and the variance contributed by differences between rats, between segments and between treatments was removed. Standard deviation for the assay was calculated from the residual variance. Missing values were replaced according to the method of Bliss (1952) and a degree of freedom was dropped from the residual mean square for each value replaced. In no case were there fewer than 15 valid values. The slope of the standards was calculated for each assay separately and was used to estimate the insulin-like activity of the unknowns. The standard error of the mean was calculated for each unknown ("Expanded" SEM) and 95% confidence limits were calculated according to the formula: $M_L, M_U = M \pm t\lambda\sqrt{\frac{N_S + N_U}{N_S N_U}}$. The symbols M_L and M_U are the logarithm of the lower and upper limits respectively of the 95% confidence interval, M is the logarithm of the potency estimate for a given unknown, t is the value from Fisher's t distribution at the significance level selected ($p = 0.05$), λ is the index of precision (residual standard deviation divided by the standards

slope) and N_S and N_U are the number of responses to one dose level of a standard or to a specific unknown respectively. A complete calculation from the observations listed in table 2d is presented in figure 7 as an example.

Table 2

Insulin Bioassays of Extracts of Pooled Culture Media and of Extracts of Various Standards

Raw data and calculated estimates of the insulin-like activity of various culture medium extracts and controls are tabulated on the following five pages.

Notations:

1X = extract of pooled 1X glucose medium (three replicates); 2X = extract of pooled 2X glucose medium; 4X = extract of pooled 4X glucose medium; LI = insulin standard, low dose (51.2 μU); HI = insulin standard, high dose (512μU); CI = Crystalline insulin extracted from saline (1024 μU); C = extract of unused medium; C + CI = extract of unused medium to which 5 mg/ 100ml of crystalline insulin has been added; ILA = Insulin-like activity; M_L = lower limit of 95% confidence interval; M_U = upper limit of 95% confidence interval.

The values in boxes have been replaced by the values immediately to the right of the boxes according to the method of Bliss (1952).

Table 2a

Bioassay of extracts of culture media pooled from pancreas culture prepared April 1, 1965

Sample	mg tissue	CPM	Segment	$\frac{CPM}{mg\ tissue}$	$Log\frac{CPM}{mg\ tissue} = y$
1X - 1	118	282	RD	2.390	1.378
LI - 1	140	240	RM	1.714	1.234
2X - 1	149	376	RP	2.2523	[1.402] 1.614
4X - 1	133	398	LD	2.992	1.476
HI - 1	133	622	LM	4.677	1.670
CI - 1	177	230	LP	1.329	1.124
1X - 2	161	322	RP	2.000	1.301
LI - 2	176	418	RD	2.375	1.376
2X - 2	161	677	RM	4.205	1.624
4X - 2	171	282	LP	1.649	1.217
HI - 2	202	1052	LD	5.208	1.717
CI - 2	241	304	LM	1.260	1.100
1X - 3	156	490	RM	3.141	1.497
LI - 3	146	421	RP	2.883	1.460
2X - 3	141	195	RD	3.511	1.545
4X - 3	223	383	LM	1.717	1.235
HI - 3	148	574	LP	3.878	1.589
CI - 3	235	288	LD	1.225	1.088

Potency estimate:	Estimated ILA = R	Lower confidence limit = M_L	Upper confidence limit = M_U
1X glucose	182	56	583
2X glucose	570	177	1832
4X glucose	142	44	456
CI	110	34	354

Table 2b

Bioassay of extracts of culture media pooled from pancreas culture prepared April 1, 1965

Sample	mg tissue	CPM	Segment	$\frac{\text{CPM} \times 10}{\text{mg tissue}}$	$\text{Log}\frac{\text{CPM} \times 10}{\text{mg tissue}} = y$	
1X - 1	368	517	RD	14.0	1.146	
LI - 1	321	452	RM	14.1	1.149	
2X - 1	440	395	RP	9.0	0.954	1.332
4X - 1	362	709	LD	19.6	1.292	1.116
HI - 1	419	615	LM	14.7	1.167	
C - 1	348	300	LP	8.6	0.434	
1X - 2	461	385	RP	8.4	0.924	
LI - 2	466	494	RD	10.6	1.025	
2X - 2	415	717	RM	17.3	1.238	
4X - 2	513	456	LP	8.9	0.494	
HI - 2	477	1226	LD	25.7	1.410	
C - 2	433	508	LM	1.17	1.068	
1X - 3	302	458	RM	15.2	1.182	
LI - 3	482	303	RP	6.3	0.799	
2X - 3	426	898	RD	21.1	1.324	
4X - 3	364	554	LM	15.2	1.182	
HI - 3	348	384	LP	11.0	1.041	
C - 3	279	570	LD	20.4	1.310	.964

Potency estimate:	Estimated ILA = R	Lower confidence limit = M_L	Upper confidence limit = M_U
1X glucose	13.9	Not applicable; the regression F ratio is not significant.	
2X glucose	20.0		
4X glucose	13.5		
C	5.0		

Table 2c

Bioassay of extracts of culture media pooled from pancreas culture prepared April 1, 1965

Sample	mg tissue	CPM	Segment	$\frac{CPM}{mg\ tissue}$	$Log\frac{CPM}{mg\ tissue}$	= y
1X - 1	115	362	RD	3.148	.498	
LI - 1	100	407	RM	4.070	.610	
2X - 1	120	1017	RP	8.475	.928	
4X - 1	122	354	LD	2.902	.463	
HI - 1	80	689	LM	8.612	.935	
C + CI - 1	78	235	LP	3.013	.479	
1X - 2	137	302	RP	2.204	.343	
LI - 2	117	574	RD	4.906	.691	
2X - 2	168	830	RM	4.940	[.694]	.945
4X - 2	94	343	LP	3.549	.562	
HI - 2	174	1042	LD	5.988	.777	
C + CI - 2	175	596	LM	3.406	.532	
1X - 3	177	353	RM	1.994	.300	
LI - 3	134	396	RP	2.955	.471	
2X - 3	138	962	RD	6.971	.843	
4X - 3	153	735	LM	4.804	[.682]	.378
HI - 3	196	702	LP	3.582	.554	
C +CI - 3	142	473	LD	3.331	.523	

Potency estimate:	Estimated ILA = R	Lower confidence limit = M_L	Upper confidence limit = M_U
1X glucose	2.6	Not applicable; the regression F ratio is not significant.	
2X glucose	420.7		
3X glucose	9.1		
C+ CI	16.6		

Table 2d

Bioassay of extracts of culture media pooled from pancreas culture prepared May 11, 1965

Sample	mg tissue	CPM	Segment	$\frac{CPM}{mg\ tissue}$	$Log\frac{CPM}{mg\ tissue} = y$
1X - 1	108	588	RD	5.44	.736
LI - 1	164	375	RM	2.29	.360
2X - 1	92	734	RP	7.98	.902
4X - 1	148	726	LD	4.90	.690
HI - 1	98	572	LM	5.84	.766
C - 1	147	557	LP	3.79	.579
1X - 2	128	587	RP	4.59	.662
LI - 2	142	942	RD	6.63	.821
2X - 2	137	728	RM	5.31	.725
4X - 2	131	480	LP	3.67	.565
HI - 2	98	1029	LD	10.50	1.021
C - 2	133	676	LM	5.08	.706
1X - 3	169	852	RM	5.04	.702
LI - 3	187	496	RP	2.65	.423
2X - 3	160	1335	RP	8.34	.921
4X - 3	191	1072	LM	5.61	.749
HI - 3	139	727	LP	5.23	.718
C - 3	171	1538	LD	8.99	.954 .620

Potency estimate:	Estimated ILA = R	Lower confidence limit = M_L	Upper confidence limit = M_U
1X glucose	182	56	583
2X glucose	570	177	1832
4X glucose	142	44	456
C	110	34	354

Table 2e

Bioassay of extracts of culture media pooled from pancreas cultures prepared May 11, 1965

Sample	mg tissue	CPM	Segment	$\frac{\text{CPM} \times 10}{\text{mg tissue}}$	$\text{Log}\frac{\text{CPM} \times 10}{\text{mg tissue}} = y$	
1X - 1	382	1863	RD	48.8	1.688	1.258
LI - 1	353	287	RM	8.1	0.908	
2X - 1	512	652	RP	12.7	1.104	
4X - 1	370	1121	LD	30.3	1.481	1.155
HI - 1	272	397	LM	14.6	1.164	
C - 1	502	449	LP	8.9	0.949	
1X - 2	479	585	RP	12.2	1.086	
LI - 2	318	160	RD	5.0	1.699	
2X - 2	420	775	RM	18.4	1.265	
4X - 2	519	561	LP	10.8	1.033	
HI - 2	323	339	LD	10.5	1.021	
C - 2	382	393	LM	10.3	1.013	
1X - 3	398	580	RM	14.6	1.164	
LI - 3	409	155	RP	3.8	0.580	
2X - 3	341	639	RD	18.8	1.274	
4X - 3	425	433	LM	10.2	1.009	
HI - 3	456	176	LP	3.9	0.591	
C - 3	344	300	LD	8.7	0.940	

Potency estimate:	Estimated ILA = R	Lower confidence limit = M_L	Upper confidence limit = M_U
1X glucose	900	Not applicable; the regression F ratio is not significant.	
2X glucose	1524		
4X glucose	268		
C	24		

Figure 7

The Statistical Analysis of a Sample Assay

Responses

	Standards		Unknowns					
Rat	Lo: 51.2	Hi: 512	U1 1X	U2 2X	U3 4X	U4 Con	Rat Sum (R)	Seg Sum (S)
1	M .360	M .766	D .736	P .902	D .690	P .579	4.033	M 4.008
2	D .821	D1.021	P .662	M .725	P .565	M .706	4.500	D 4.809
3	P .423	P .718	M .702	D .921	M .749	D .954	4.133	P 3.894
						.620		
Sum (T)	1.604	2.505	2.100	2.548	2.004	1.905	12.666 = (Σy)	
Mean ($\bar{y}$)	.535	.835	.700	.849	.668	.635		

Basic Calculations:

$\Sigma(y^2)$: 9.361 $\Sigma(R^2)/6$: 8.9328

$\Sigma(y)^2/N$: 8.9126 $\Sigma(T^2)/r$: 9.1317

$\Sigma(S^2)/2r$: 9.0009

$$b = \frac{\bar{y}_{Hi} - \bar{y}_{Lo}}{\bar{x}_{Hi} - \bar{x}_{Lo}} = \frac{.300}{1} = .300$$

$\lambda = s/b = .440$

Analysis of Variance

Source	d.f.	SS	MS	F
Total	17	.4493		
Rats	2	.0202	.0101	
Seg	2	.0882	.0441	
Tre	5	.2191	.0438	
Regr	(1)	.1353	.1353	7.77 =SSR $p < 0.05$
Res	7	.1218	.0174 = s^2	
			.1319 = s	

$\bar{y}_s$ = .685

$\bar{x}_s$ = 2.209

$t_{.05}$ = 2.306

$g = s^2t^2/SSR$ = .684

$1/(1-g)$ = 3.165

$$t\lambda\sqrt{\frac{N_S + N_U}{N_S N_U}} = .507$$

Potency estimate:	U1	U2	U3	U4
(1) $\bar{y}_u - \bar{y}_s$	.015	.164	-.017	-.050
(2) (1)/b = M'	.050	.547	-.057	-.167
(3) (2)+x = M	2.259	2.756	2.152	2.042
(4) $\log^{-1}$ (3) = R	181.6	570.2	141.9	110.2
"Expanded" Standard Error of M				
(5) (1)^2/SSR $1/(1-g)$	.005	.629	.007	.058
(6) $1/(1-g)$ $(5) + \frac{N_s + N_u}{N_s N_u}$	1.599	3.527	1.605	1.768
(7) $\sqrt{(6)}$	1.261	1.889	1.265	1.330
(8) λ(7) = "Expanded" SEM	.555	.831	.557	.585

Confidence Limits:		U1	U2	U3	U4
(9)	$t\lambda\sqrt{\frac{N_s+N_u}{N_sN_u}} - (3) = M_L$	1.752	2.249	1.645	1.535
(10)	$t\lambda\sqrt{\frac{N_s N_u}{N_sN_u}} + (3) = M_U$	2.766	3.263	2.659	2.549
(11)	$\log^{-1}(9) = R_{Lo}$	56	177	44	34
(12)	$\log^{-1}(10) = R_{Hi}$	583	1832	456	354

Notations: y= an individual response (log cpm/mg). x= log dose of insulin $\bar{y}$, $\bar{x}$= mean of y and x respectively. D,M,P = distal, medial and proximal segment of fat pad respectively. R = in the analysis of variance, sum of y for one rat. R, R_{Lo}, R_{Hi} in computation of potency and confidence limits = μU ILA. r = number of rats or number of responses to one "treatment" S = sum of y for a segment (D, M, or P). N = 6r, total number of responses. N_s= 2r number of responses to insulin standard. N_u = r, number of responses to a specific unknown. Σ= "the sum of". $t_{.05}$ = value of t at the significance level selected (p = 0.05) for the available degrees of freedom (d.f.) in the residual mean square (s^2). SS = sum of squares. MS = mean square (SS/d.f.). F = variance ratio (specific MS/residual MS). b = standards slope. λ= index of precision. Seg = segments. Tre = treatments. Regr = regression Res = residual

Formulas for the analysis of variance:

Source	d.f.	SS
Total	N-1	$\Sigma(y)^2-(\Sigma y)^2/N$
Rats	r-1	$\Sigma(R^2)/6-(\Sigma y)^2/N$
Segments	3-1	$\Sigma(S^2)/2r-(\Sigma y)^2/N$
Treatments	6-1	$\Sigma(T^2)/r-(\Sigma y)^2/N$
Regression (std)	(1)	$(T_{Hi}-T_{Lo})^2/2r$
Residual	N-r-7	Total SS - (Rat + Segment + Treatment SS)

Test for regression: F = regression MS/residual MS = 7.77. ($p < .05$.)
If the regression F ratio is not significant, potency estimates cannot be made.
Values of y in boxes have been replaced according to the method of Bliss. (1952).

In two of the assays of the extracts of culture media pooled from cultures prepared on April 1st (tables 2b and 2c), and one of the assays of the May 11th media (table 2e) the slope of the standards curve was not significant at the significance level selected ($p = 0.05$) as indicated by the regression F ratio. In these assays, the potency estimates are not valid at the 5% level. These assays are included because they indicate the relative potency of the different extracts when compared to each other and in this respect are in agreement with the results of those assays in which the standards regression was significant. Extracts of the media from cultures prepared on April 1st contained 67, 312 and 35 micro units of insulin-like activity (ILA) in 0.1 ml of the 1X glucose, 2X glucose and 4X glucose media extracts respectively. Since the media were concentrated 100 times during the extraction process, this corresponds to 6.7, 31.2 and 3.5 μU of insulin-like activity per ml of the original pooled media. Corresponding values for the cultures prepared on May 11th were 182, 570 and 142 μU ILA in 0.1 ml of the 1X glucose, 2X glucose and 4X glucose extracts respectively and 18.2, 57 and 14.2 μU ILA/ml of the original media. A solution which contained 1 mg of crystalline insulin per ml in physiological saline was prepared and extracted. The extract was diluted with modified Krebs' bicarbonate buffer to a concentration of 1024 μU/ml assuming complete recovery of insulin by the extraction process. This solution was assayed and found to have an insulin-like activity of 7.4 μU/ml. This value represents 0.73% of the activity of the insulin solution before extraction. This corresponds to an activity loss of 137.8X as a result of the

CHAPTER IV

DISCUSSION

A prerequisite to the study of several aspects of pancreatic Beta cell function is the availability of a source of Beta cells that is largely or entirely free from the other cellular elements of the pancreas. The present sources of Beta cells, islets of marine teleosts and islets of other forms obtained by microdissection are not ideally suited for metabolic research.

Pancreas explants grown as tissue cultures under conditions which confer a selective growth advantage upon the Beta cells are proposed here as an alternative source of cells for metabolic studies. Since the scope of the total problem of growth and maintenance of Beta cells and their metabolism is rather large, the current study is limited to the isolation and identification of Beta cells. It is assumed that responses of Beta cells to glucose *in vivo* might be duplicated under appropriate conditions *in vitro*, and that these responses might form the basis for selection. Beta cells respond to excess glucose *in vivo* by increased insulin secretion and by proliferation; thus, it should be possible to increase their relative numbers in tissue culture by subjecting pancreas

explants to optimal concentrations of excess glucose in the medium. It is further assumed that physiologically competent Beta cells present in the cultures should have the specific attributes of Beta cells in vivo and should thus be detectable by similar methods. The demonstration of cytoplasmic granules that are similar or identical to granules of known Beta cells is taken as presumptive evidence for the presence of functional Beta cells in the cultured pancreas cells. The detection of insulin in the culture media is taken as another evidence that the cells are functional Beta cells. The methods of insulin detection in the culture medium are chromatographic, electrophoretic and bioassay, and the identification of the granules of the cultured cells rests upon the antigenic similarities of their protein and that of the granules of known Beta cells.

The results of these experiments strongly indicate that the conditions prevailing in the cultures that contain twice the normal amount of glucose confer a selective advantage upon pancreatic Beta cells. When populations of mixed cells of the whole pancreas are cultured, there is a change in the ratio of types of cells, and the Beta type becomes more abundant. Beta cells comprise approximately 1.5% of the mass of the pancreas (Turner, 1961). In the 2X glucose cultures there is a 40 to 50-fold increase in the concentration of Beta cells since the sheets of pancreas tissue in these cultures are 60.8 to 74.4% Beta cells.

Within any given culture session there is no significant difference in the mean percentage of Beta cells produced in the experimental media and in the mannitol dilution test medium of the

same glucose concentration. This suggests that the slight dilution of the salts and nutrients by the addition of glucose solution to make 2X and 4X glucose media has no effect upon the percentage of Beta cells produced. However, the nature of the medium is such that it is not possible to alter the glucose concentration without simultaneously altering either the concentration of the other components of the medium or the osmolarity. In these experiments the osmolarity has been held constant. The possibility that the dilution of the medium has an effect which is duplicated by the mannitol in the dilution test media is remote, but has not been eliminated. These results indicate that the increase in the abundance of Beta cells in the 2X glucose medium is due to the added glucose and not to the dilution effect.

The estimate of the percentage of Beta cells produced in the various culture media is based on the assumption that all of the round cells seen in the photomicrographs are Beta cells. The identification of this cell type as Beta cells is in turn based on the similarity of the staining reaction of its cytoplasmic granulation and the granulation of known Beta cells when treated with fluorescien labelled anti-insulin gamma globulin. The cytoplasmic granules of pancreatic Beta cells are presumed to be the precursers of insulin and to be antigenically similar to it. Consequently, the validity of this identification is dependent upon the specificity of the presumed antigen-antibody reaction. The staining reaction is blocked by preincubation of the fluorescien-labelled anti-insulin gamma globulin with crystalline insulin or by pretreatment of the experi-

mental cultures with unlabelled anti-insulin gamma globulin. The experimental cultures are not stained by fluorescien-labelled gamma globulin which does not contain the specific anti-insulin antibody. These results support the conclusion that the fluorescent staining is due to a true immune reaction. Immunization to glucagon would result in the erroneous identification of Alpha cells present in the cultures as Beta cells. This possibility is minimized by the use of a highly purified preparation of crystalline insulin which contains negligible quantities of glucagon. Acinar cells, duct epithelium and the endothelial cells of blood vessels are present in the explanted pancreas fragments. The possibility exists that the epithelial morphology of one or more of these cell types may be preserved in the "epithelioid" medium, and that such cells might appear in the photomicrographs as round cells. Pancreatic cells of non epithelial morphology might also appear in the photomicrographs as round cells. The probability of either of these occurrences is low since all the round cells seen in the fluorescent antibody preparations had fluorescent granules. Erroneous identification of pancreas cells of non-endocrine origin as Beta cells would require that they assume the round morphology under the conditions of culture, and that there be a non-specific adsorption of the fluorescent antibody on the surface of their cytoplasmic granules.

Electrophoresis of extracts of the experimental media reveals the presence of a substance that has the same electrophoretic mobility as crystalline insulin which has been subjected to the extraction process. This mobility differs from that of crystalline insulin which

has not been subjected to the extraction process. The nature of the effect of the extraction process upon the electrophoretic mobility of insulin has not been determined, but it appears to be associated with a loss of activity and therefore may represent a denaturation of the insulin molecule by reagents used in the extraction process.

Chromatography of extracts of the experimental media reveals the presence of a substance with the same chromatographic mobility as crystalline insulin. The alteration in electrophoretic mobility does not appear to be reflected in the chromatographic mobility. A second substance is present in the extracts of all of the media which has a lower mobility. Since this substance is present in the extract of the unused medium (which has been shown to contain negligible amounts of insulin-like material by all other means of detection) it probably does not represent a product of insulin denaturation. The lower spot is probably produced by a component of the culture medium which has been extracted with the insulin-like material.

Immune chromatography reveals that extracts of the experimental media are bound by anti-insulin gamma globulin, but not by gamma globulin lacking anti-insulin antibodies. These results are consistent with the results of fluorescent antibody staining and indicate the presence of a material that is antigenically similar to insulin in the experimental culture media.

Insulin bioassays indicate that the pancreas cells grown in the 2X glucose medium produce the highest concentration of a material with insulin-like activity. Bioassay of a crystalline insulin sample of known concentration indicates that less than 1% of the insulin

activity is retained after extraction. This result is similar to that obtained by Leonards, et. al., (1962) who found less loss of activity with a similar but slightly different extraction technique.

None of these experiments provide definitive proof of the presence of Beta cells in the cultures or of the presence of insulin in the culture media. However, the presence of cells in the culture media whose relative abundance parallels the relative concentration of a substance with many insulin-like properties, and whose cytoplasmic granules are stained by fluorescien-labelled anti-insulin gamma globulin strongly suggests that these cells are Beta cells. It seems unlikely that the parallel relationship between the abundance of these cells and the concentration of the insulin-like material would be retained in the presence of any significant errors in the identification of Beta cells. A substance can be detected in the culture medium pooled from all pancreas cultures. This material is antigenically similar to insulin, has electrophoretic and chromatographic mobilities similar to those of insulin and has an insulin-like activity that is highest in the medium from those cultures which have the highest relative abundance of cells which are thought to be Beta cells. The overwhelming implication of these results is that the material is insulin and that the cells are Beta cells.

The results of these experiments indicate that the optimal concentration of glucose for the growth of Beta cells lies closer to that of the 2X glucose medium than to that of any other medium used. A few earlier experiments indicated a general inhibition of the growth of pancreas cultures by glucose concentrations above 4X.

These data do not permit a precise determination of the optimal glucose concentration for the growth of Beta cells, and further experiments specifically designed to determine this concentration are indicated.

Two obvious questions which arise as a consequence of these experiments remain unanswered. First: what is the nature of the selection process operating in these cultures, and second: what is the time course of its action? In the case of the first question, two alternative mechanisms are suggested: (1) The presence of excess glucose in the culture medium causes an increase in the rate of division of Beta cells with a consequent overgrowth of the other cell types by the rapidly-dividing Beta cells; or (2) the excess glucose inhibits the growth of other cell types thus allowing Beta cells to become predominant in the cultures. The data from these experiments do not permit a choice between the two alternatives. In the case of the second question, adequate data on the relative abundance of Beta cells in the cultures are available only for the period 24 hours after the original explantation of the tissue fragments. At this time, vacuolation of the star-like cells and the fusiform cells has commenced. Star-like cells and round cells persist in cultures maintained 4-6 weeks, but no quantitative data on their relative abundance are available. Insulin can be detected in the medium pooled from cultures maintained up to six weeks, but since the medium is pooled throughout this interval, any change in the rate of insulin production would go undetected. Further investigation directed towards obtaining the answer to both of these obvious

questions is indicated.

It is suggested that certain aspects of the design of this research might have more general application to the problem of the isolation of specific cell types from tissues containing mixed cell populations and to the problem of the determination of the lineage of cells grown in tissue culture. In essence, the approach to this problem has been to determine by the method of trial and error those physical and chemical conditions of culture under which a given cell type can be made to exhibit in vitro some of the properties which characterize it in vivo. If it happens that one or more of these properties is characteristic of only one of the possible cell types occuring in a given culture, the property or properties may form the basis for the identification of that particular cell type despite the fact that it may be changed morphologically. This approach has been used by Westfall, Evans and Earle (1953) in the identification of liver parenchymal cells on the basis of their high glycogen content, by Sandström (1965) in the identification of liver endothelial cells on the basis of their phagocytic properties and by numerous workers in the identification of cardiac muscle cells on the basis of their rhythmic contraction.

This approach is particularly well suited to pancreatic Beta cells since they produce a unique and well studied secretion which can be identified within the cells and in the culture medium. The nature of their regulation in vivo has been extensively studied and information derived from these studies forms the basis for the selection in vitro used in this research. Many other cells in the

body, particularly glandular epithelial cells have similarly well studied regulatory mechanisms and produce similarly unique products. These properties may form the basis for the selective growth and identification of these cells *in vitro*.

CHAPTER V

SUMMARY

Beta cells grown in tissue culture under conditions which confer a selective growth advantage upon them have been proposed as a source of Beta cells for metabolic study.

A technique based on known responses of Beta cells to glucose *in vivo* has been developed which promotes the selective growth *in vitro* of Beta cells from explants of whole embryonic chick pancreas.

It has been shown that a culture medium which has been supplemented with a prescribed excess of isosmotic glucose solution confers a selective advantage upon Beta cells growing as monolayers in a culture of mixed pancreatic cells. The culture medium is composed of 20% gamma globulin-free chicken serum, 10% tissue culture medium #199 and 70% Hanks' balanced salt solution. Penicillin, streptomycin and Mycostatin are added to it in concentrations normally used in tissue culture media. Maximum growth of Beta cells occurs in the 2X glucose medium where they comprise 60.8 to 74.4% of the cells present; however, the limited series of glucose concentrations used in these experiments does not permit a precise determination of the optimal glucose concentration for the growth of Beta cells.

Several methods have been employed to overcome the difficulty of the positive identification of cells grown in tissue culture which arises from the fact that cultured cells occur isolated from their usual associations with other cells in the body, and tend to undergo morphological changes.

The presence of Beta cells in the pancreas cultures has been demonstrated by immune fluorescent staining with fluorescien-labelled anti-insulin gamma globulin. A substance has been detected in the medium of cultures maintained as long as six weeks which is electrophoretically and chromatographically similar to or the same as crystalline insulin. The substance is bound by anti-insulin gamma globulin and accelerates the production of $C^{14}O_2$ by fragments of rat epididymal fat pad tissue incubated in the presence of glucose-1-C^{14} in the same manner as does insulin. These results have been taken as presumptive evidence that the substance is insulin, and that the culture method increases the relative abundance of functional Beta cells in cultures prepared from explants of the whole pancreas gland.

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