THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

FUNCTIONAL AND HISTOLOGICAL OBSERVATIONS ON ISLET TISSUE GROWN IN VITRO

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1955

FUNCTIONAL AND HISTOLOGICAL OBSERVATIONS

ON ISLET TISSUE GROWN IN VITRO

APPROVED BY

THESIS COMMITTEE

ACKNOWLEDGMENTS

The writer wishes to take this opportunity to express his appreciation to Dr. K. M. Richter, who suggested and directed this investigation, and to the members of the Anatomy Department for advice and criticism.

Thanks are also due Mr. Gerbert Rebell for the facilities of his animal house and to the Physiology Department for the use of equipment. Gratitude is also expressed to the following people: P. B. McCay, Georgia Stathos, and N. W. Woodward, Jr. for technical assistance, and especially to H. F. Timmons and Neldagae Smith without whose help and encouragement this thesis would not have been completed.

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FUNCTIONAL AND HISTOLOGICAL OBSERVATIONS ON ISLET TISSUE GROWN IN VITRO

CHAPTER I

INTRODUCTION AND STATEMENT OF PROBLEM

The treatment of diabetes mellitus with pancreatic extracts became a reality more than thirty years ago after the preparation of a physiologically active pancreatic extract by Banting and Best. Even though diabetes can be controlled by dieting and the daily injection of insulin, a more efficient and permanent treatment is to be desired. This can be attained only if some method can be found for inducing regeneration within the pancreas of the non-functioning islets of Langerhans, or if the degenerate tissue is replaced with functioning islet tissue from some other source. Attempts to induce regeneration have been, for the most part, unsuccessful. Experimental transplantation of tissues has been equally disappointing. Recently, however, as more has been learned about immunology and the specific problems involved, more satisfactory results have been obtained. The discovery by Stone, Owings, and Gey (1933) that foetal and newborn tissues could, in some cases, be successfully transplanted, introduced a new field of therapeutics. These tissues which are determined histologically but which are not irreversibly determined biochemically can be transplanted from one

individual to another without the usual foreign body response of the recipient. Later these same workers found that by culturing these tissues in media containing increasing amounts of host serum a higher percentage of "takes" could be obtained when the explants were transferred to intact animals. In this way some of the cultures seemed to be adapted to the body fluids of the recipient. Tissue culture appears to be an important tool in the study of tissue replacement problems.

The present work is part of a more general problem concerning the possibility and practicability of establishing cultures of endocrine pancreas for possible use in the treatment of diabetes mellitus. Specifically, the problem here is an attempt to induce formation of histologically differentiated islet tissue from undifferentiated pancreatic primordia and to establish by insulin assay the functional capacities of these cultures.

CHAPTER II

REVIEW OF LITERATURE

Pancreatic Cultures

The first reported attempt to grow pancreatic tissue *in vitro* was published in 1926. Kapel, in studies of chick hepatic explants, reported the growth of pancreatic tissue which had been accidentally included. Two additional reports on the successful culture of pancreatic tissues have appeared since that time (Chlopin, 1930; Amoroso, 1932). Attempts to grow islet tissue have been equally rare. In 1935, two groups of investigators, working independently, reported the successful cultivation of islet tissue (Selle, 1935; Murray and Bradley, 1935). Selle's work represents the only instance in which completely normal islet tissue (foetal and adult) was utilized for islet culture studies, but his results were never published in detail. Murray and Bradley were able to maintain two highly differentiated islet cell adenomas in culture for approximately eight weeks. No attempts were made either by Murray and Bradley or by Selle to assay the media in which their islet cultures were grown for insulin activity.

Murray and Bradley (1935) made one unsuccessful attempt to treat a human diabetic with transplants derived from neoplastic cultures. However, this does not represent the first attempt at tissue replacement therapy using tissues grown *in vitro*. Stone, Owings, and Gey

(1934, 1935) reported the successful transplantation of embryonic thyroid and parathyroid (dog and human) to their respective species after cultivating the glands for some time in media containing portions of the recipient's serum. By this means they hoped to "acclimatize" the donated tissues to the body fluids of the host. Lux and associates (1936) reported the successful transplantation of homologous adrenal tissues, grown in vitro, to rabbits, rats, and guinea pigs. The most spectacular results with transplantation of homologous endocrine tissues grown in culture were obtained by Kooreman and Gaillard (1950). Using parathyroids taken from human foetuses and newborns and cultivated in recipient serum, they were able to secure eighty percent "takes" in persons suffering from postoperative tetany. Successful culture transfers were obtained only in adolescents and young adults. Since this time, successful transplants have been made with other endocrine organs grown in culture (Gaillard, 1948, 1953, 1954; Jones, 1954; Dameron, 1950, 1951).

Insulin Assays

Although the importance of developing an accurate method for the estimation of blood insulin was realized early, the apparent difficulties involved in detecting a substance normally present in a concentration of only 2×10^{-5} units/ml blood[1] seemed so overwhelming that serious work was not undertaken in this direction until about fifteen

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[1]The unit was at one time defined as one-third of the amount which will, in five hours, lower the blood sugar of a fasting rabbit to the convulsive level (45 mgm per 100 ml), but assay is now conducted by comparing the activity of an unknown sample with that of crystalline insulin, either in lowering the blood sugar of rabbits or in inducing convulsions in mice: crystalline insulin is reckoned at 22 units per mgm (Hawk, Oser, and Summerson, 1947).

years ago. It had been established previously that 1×10^{-2} units of insulin are convulsive for mice and less than this will lower the blood sugar significantly (Wilder, 1952). Hemmingsen and associates, in 1938, found that adrenalectomy in mice increased the sensitivity to 2×10^{-3}. This level is much above the normal estimated blood level for insulin, but it approaches the degree of sensitivity required for assay methods. Later Gelhorn et al (1941) found that hypophysectomy, adrenomedullectomy in rats increased the sensitivity so much that 2×10^{-4} units were detectable. Anderson (1947), to remove the complications induced by the animal's own pancreas, used alloxanized rats which were then hypophysectomized and completely adrenalectomized rats, he could detect 5×10^{-6} units. For the first time assay methods were available which possessed the required sensitivity to detect normal amounts of insulin. The above methods are based on changes in the blood sugar level. These methods are extremely tedious and involved and are not accurate in the hands of an inexperienced investigator. Later Groen and associates (1952) described a method for the detection and assay of insulin in human plasma which was based on the glucose uptake of isolated rat diaphragm surviving <u>in vitro</u>. The sensitivity, while not as great as that previously described, is within the range for accurate determinations and has the added advantage of being rapid, inexpensive, and readily adaptable for routine use.

CHAPTER III

MATERIALS AND METHODS

Introduction

In these studies foetal and adult pancreata of chicks and mice[1] were used. Adult pancreata of both mouse and chick were examined during attempts to perfect reliable staining procedures. Embryonic chick pancreas was used for preliminary studies in developing culture techniques for pancreatic tissue and for most of the histological observations on cultivated pancreas. The foetal mouse pancreas was used exclusively for insulin assay studies.

A series of developing pancreata from the ninth day of incubation to hatching was collected in order to determine when islets could first be identified morphologically and tinctorially.

Histological Technique

All pancreatic cultures were fixed either in Zenker-formol or Spüler-Maximow's fluid to retain the specific granulation of the islet cells. In preliminary studies using adult and developing pancreata, 10 percent formalin and Bouin's fluid were tried, but the results obtained

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[1]The mice used in this study were C_3H and C_{57} brown, inbred strains obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

were less satisfactory than was fixation for ten to twelve hours in either Zenker's or Spüler-Maximow's solutions. After fixation, the tissues were washed for eight to ten hours in running tap water. After dehydration, the tissues were cleared in toluol and embedded in 56° C paraffin, sectioned at four to six micra, and attached to slides using an albumin affixative. Mounted sections were allowed to dry for at least twenty-four hours before staining.

The staining procedures of Bensley (1911), Bayley (1937), Bowie (1924), Gomori (1939, 1939a, 1941, 1949), Lane (1907), Romeis (1948), Heidenhain (1915), Mallory (1900), and Masson (1928) were tried. Of the various staining procedures used, Goldner's and Bencosme's modifications of the Masson trichrome technique seemingly gave the most consistent and satisfactory results for both avian and mammalian tissues. The Goldner modification has the advantage of being a very rapid procedure but the delicate cytological structures are not always shown. The Bencosme modification gives the better results, but this technique requires almost two weeks for completion.

Since the neutral gentian technique was utilized in the original description of islet cells, slides were prepared by this method, then destained and restained by the Bencosme and Goldner modifications in an effort to establish the relative specificity of the latter methods.

Culture Methods

Chick

Explants of pancreatic tissue for culturing were removed from chick embryos of nine and ten days incubation. As a general rule these

primordia were removed from a number of embryos at one time and "pooled" temporarily in small petri dishes containing Tyrode's solution. The pancreatic primordia were then subdivided into pieces not larger than 0.5 mm^3. Ten to fourteen of these tissue fragments were then transferred to an 11 x 50 mm coverslip and either 1) embedded in a drop of heparinized chicken plasma, subsequently clotted by the addition of chick embryo juice, or 2) placed directly on the surface of a plasma clot. The coverslips bearing the explants were then placed in roller tubes, sealed, inserted in a roller drum rotating at 12 revolutions per hour and incubated at 37.5° C for various lengths of time. In initial experiments, supernate was added immediately after preparation of the cultures. In later experiments the fluid nutrient was not added until the third day of incubation.

The fluid nutrient consisted of 2 ml of a mixture of fifty percent human ascitic fluid,[1] forty-five percent Tyrode's solution and five percent chick embryo extract which was prepared by the conventional method. The first fluid nutrient added to the cultures contained chick extract made from the remains of the embryos from which the pancreatic primordia were taken. The supernates and gaseous phases were changed at five day intervals using media containing tissue extract from embryos three days older than embryos from which the preceding extract was made.[2]

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[1] Ascitic fluid from the same cirrhotic patient was used throughout this investigation.

[2] Progressive increase in age of embryo extract has been shown by Verdon (1944), Gaillard (1948) and Fell (1929) to be important in embryogenesis, and continuous change in composition of the "body fluids" is important for the phenomena of cellular and tissue differentiation.

Extract from embryos older than nineteen days incubation was not used. After the cultures had been transferred twice to supernate containing 5 percent nineteen day embryo extract, they were fed for the remaining period of cultivation with nutrient composed of 50 percent Tyrode's and 50 percent human ascitic fluid. Antibiotics were not used in the preparation of media for chick pancreatic cultures.

Microscopic observations were made daily without removing the cultures from the roller tubes. Gross changes in growth characteristics, as well as changes in cellular constituents of the growth region, were noted. Explants were removed at one week intervals and fixed for histological observations.

Mouse

Since the embryogenesis of the pancreas in C_3H and C_{57}brown mice has been carefully studied by Browning and Resnik (1951), a complete series of developmental stages was not prepared. Representative stages were, however, taken to verify their findings.

The pancreatic primordia were removed from embryonic mice on the fourteenth to sixteenth day after mating. The primordia were removed from all foetuses in a given litter and placed in a petri dish containing Tyrode's solution. One pancreas, picked at random, was selected for histological study; the remaining pancreata were retained for cultivation. The method of preparation of the explants was essentially that used for the preparation of chick cultures.

Explants of hepatic tissue were made (from the same animals) at the same time to furnish control supernate for the insulin assay

studies.

The fluid supernate for both pancreatic and hepatic cultures was the same as that used for chick cultures. Antibiotics were not used routinely in the preparation of media. In a few cases, one hundred units of penicillin per ml of embryo extract were added as a precautionary measure when feeding very old, therefore very valuable, cultures. The final concentration, after dilution, was never more than five units of penicillin per ml of nutrient.

The gaseous and fluid phases of cultures were changed at five day intervals, as for the chick, but the supernates of these cultures were preserved and utilized in insulin assay studies.

Observations were made daily during the first four weeks of cultivation. After this time, observations were made only when the supernates were changed. Neutral red in a dilution of 1:10,000 in Tyrode's was used in an attempt to identify islet tissue within the cultures. Because of the limited number of mouse pancreatic explants, cultures were not sacrificed at regular intervals during cultivation.

Assay for Insulin Activity

Three groups of mouse pancreatic cultures were set up to furnish supernate for the insulin assay studies. Each group consisted of five roller tubes containing a minimum of fourteen explants per tube. A single group (five tubes) of liver cultures was used to supply control supernate for all experimental groups. The method of preparation of cultures was as described on page nine. The fluid nutrient was prepared in large lots, so that all cultures could be given identical media.

New supernate was prepared and added to all cultures each time after the preceding nutrient was withdrawn for assay. The first assay was undertaken seven days after preparation of the cultures and successive experiments were done at five day intervals for the first four nutrient changes. The sixth and last assay was performed after the explants had been cultivated for fifty-eight days.

Assays of culture supernates were carried out using a modification of Randle's rat diaphragm technique (1954). The method generally was as follows: Supernate from experimental (pancreas) and control (liver) cultures were placed in separate containers at the time of withdrawal. For each 2.5 ml of pooled supernate, 0.5 ml of a 0.6 percent glucose solution in Tyrode's was added. Aliquots were taken from both lots for total glucose determinations before incubation with the diaphragms. Three ml of either experimental or control supernate containing the added glucose were placed in each Warburg vessel and stored in the refrigerator until the diaphragms had been prepared.

Albino rats weighing 100-150 grams were fasted for twelve to thirty-six hours prior to the time of assay. Animals were stunned by a blow on the head and then decapitated. Diaphragms were removed, hemisected, and stored in cold Tyrode's until all had been removed. After all diaphragms had been removed, at least four hemidiaphragms were placed in each Warburg vessel. The vessels were placed in the water bath, oxygenated and incubated for four hours at 38° C using a shaker speed of 120 per minute. At the end of four hours, the diaphragms were removed, weighed, and samples of supernate taken from each vessel for glucose determination. An index of insulin activity was obtained by comparing

the amounts of glucose utilized in experimental and in control vessels.

Sugar determinations were made using a modified anthrone technique (Roe, 1955). The technique was as follows: Immediately before use, 200 mgm of anthrone reagent were dissolved in 100 ml of concentrated sulphuric acid (analytical grade). The supernate from the cultures was deproteinized by adding 0.1 ml to 5 ml of a five percent aqueous solution of trichloracetic acid. After centrifugation, four ml of the clear protein-free supernate were withdrawn and placed in a glass stoppered tube. To each of these tubes eight ml of the anthrone-acid mixture were added and mixed thoroughly by agitation. The tubes were placed in a boiling water bath for five minutes to intensify and develop the color. After removal from the water bath, the tubes were allowed to cool and optical density read in a spectrophotometer using a wave-length of 620 mu.

CHAPTER IV

RESULTS

Histological Description of the Chick Pancreas

The pancreas of the nine day chick embryo consists of only a few generations of tubular or solid outgrowths from the original pancreatic diverticula (Plate III, fig. 5). At this time the primitive acini are widely separated by the surrounding mesenchyme. Lumina are present in some of the outgrowths, especially those near the main stem of the gland. Secretory granules have not yet made their appearance in any of the acinar cells.

Zymogen granules appear in the acinar cells in abundance about the eleventh day of incubation. The chromidial substance first becomes noticeable at about this same time. Small masses of non-granular cells can be seen among the zymogen filled acinar cells on the twelfth day of incubation. These areas represent the first indication of the developing islands of Langerhans (Plate III, fig. 6). They may be identified at this time even though the specific granulation is not present. A study of later developmental stages confirms the identification. From this time until hatching, the pancreas increases in size and density as more acini are added. The differentiation of developing islets into discrete alpha and beta islets is not completed until after hatching. Specific

granulation in alpha islets appears at least three days later than in beta islets.

The pancreas of the adult bird is peculiar in many of its features. The most outstanding peculiarity is the almost complete separation of the specific cell types to form alpha and beta islets. This separation is so complete that it is unusual to find both alpha and beta cells in the same islet (Plate I, fig. 1 and 2). In some cases the islets are extremely large and appear to be regionally distributed.

The exocrine portion of the chick pancreas differs from the descriptions given for other animals only in the lack of connective tissue septa dividing the parenchyma into lobes and lobules.

Histological Description of the Mouse Pancreas

The pancreas of the fourteen day foetal mouse is comprised of several generations of acini. At this time, it displays some of the diffuse characteristics of the adult pancreas. The primitive zymogen granules have made their appearance in some of the acini. The central area of each lobule contains the main excretory duct surrounded by dense connective tissue. In cross sections of some of the lobules, large masses of stromal tissue appear isolated and bear a superficial resemblance to the islets of Langerhans, but the lumen of the centrally located duct can usually be found (Plate III, fig. 7).

Small discrete clumps of non-granular cells appear on the sixteenth day; these represent the first definite islets of Langerhans (Plate III, fig. 8). In most areas these clumps appear to be arising directly from the duct system. The connecting piece can often be found

which may or may not possess a lumen. From the seventeenth day to the time of parturition, on the twenty-first day after mating, the islets increase in size and in number. The specific granulations of the islet cells begin to appear on the third day after birth.

The islets of the adult mouse are very similar to those of any other mammal. They differ microscopically in that the alpha cells form an interrupted shell or ring around the periphery of the islet leaving a core composed almost exclusively of beta cells (Plate II, fig. 3 and 4). The gross morphology of the rodent pancreas is different from that of other vertebrates in that it is diffuse, i.e. it is distributed throughout the mesentery.

Observations on Chick Pancreatic Cultures

The behavior of the culture varied according to the time of addition of supernate. After twelve hours incubation, both types of explants (cf. page 8) sans supernate showed outward growth of fibroblast-like cells. In most cases, this initial "growth" was almost explosive and appeared to be a migration rather than an actual multiplication of cells. The situation is probably a combination of these two factors. In all cases, this spreading of cells leaves the epithelial component of the explant "exposed" as a system of tubules. This spreading reaction occurs only in cultures not being bathed in a supernate. After another twelve hours incubation, the epithelial tubes, which had been exposed by the exodus of surrounding tissue, begins to grow out as irregular epithelial sheets. The epithelial outgrowth is not always easily visualized due to the profuse growth of surrounding

non-epithelial cells. The epithelial growth varies somewhat from explant to explant even within the same roller tube. In some cases, it presents the form of a distinct pavement epithelium. Other explants show epithelial cells which are elongated and separated from adjacent cells by irregular spaces (Plate V, fig. 11).

Cultures which are allowed to grow for seventy-two hours sans feeding begin to degenerate and fat droplets begin to accumulate first in the fibroblast-like cells and later in the epithelial cells (Plate IV, fig. 10). Such cultures can be maintained almost indefinitely if the liquid nutrient is added at this time. Addition of supernate causes a rapid change in the nature of the explant growth. Spreading stops almost immediately and the cultures begin to round-up. This rounding-up of the entire culture into a compact tissue mass makes microscopic examination of the living cultures for cytological detail difficult or impossible. The explant, after rounding-up, usually has a mass slightly over 1.0 mm^3. A few cases were found in which the final mass was over 1.5 mm^3. The central area of these larger cultures was usually dark and suggested necrosis. The outer portion of the culture was, however, still viable as evidenced by continued luquefaction of the clot and slight fibroblastic activity.

Cultures to which the supernate was added before the roller tubes were incubated showed limited outward migration. Most of the cultures appeared to round-up within twelve to fourteen hours and then resembled those cultures which had been incubated for seventy-two hours before addition of the supernate.

If explants are to be maintained for more than one week, the

clots must be repaired by the addition of plasma and embryo juice or the explants must be subcultured. Cultures of chick pancreas in this condition have been maintained for periods as long as four weeks before fixation and sectioning.

Explants from nine day embryos cultured on double coverslips with Maximow slides pass through stages similar to those described above. Double coverslip preparations whenever kept for more than ten days were washed in Tyrode's to remove metabolic wastes. After washing, the clots were repaired and fresh nutrient added. The initial spreading reaction occurred as described for the roller tube cultures, but since the explants were never bathed by large amounts of fluid nutrient or subjected to the constant agitation of the roller drum, the spreading reaction continued. The epithelial sheets reached their best development in cultures of this type and resembled, grossly, cultures of any other epithelial organ.

Because of the rounding-up process described above, no structural detail could be revealed after staining with neutral red. Cultures growing on double coverslip preparations, in some cases, revealed small discrete areas of cells which were intensely stained; however, these regions were always located near the site of the original explant.

Observations on Mouse Pancreatic Cultures

Explants of mouse pancreas placed on top of the clot soon liquefy the substrate to such an extent that they become free in the nutrient; those placed between the clot and the coverslip exhibited the spreading phenomenon described for the chick. Cultures of mouse

pancreas differed from those of the chick in that this behavior continued even after addition of the nutrient. Later the epithelial tubes exposed by the spreading begin to lose their sharp outlines, and large sheets of epithelial cells appear surrounding the explant. The epithelium produced is typically of the pavement type. Some cultures showed typical epithelial cords and what appeared to be isolated pseudopodial epithelial cells. The edges of the epithelial sheets did not appear to be continuous along their entire border and most often presented a serrated edge. The epithelial growth of these cultures apparently takes place on the surface of the coverslip below the clot. Epithelial outgrowth persists as long as these explants are maintained in vitro. Mouse pancreas explants apparently liquefy the supporting clot quite slowly and to a very limited extent. Cultures of mouse pancreas have been maintained for four to five weeks before it became necessary to replace the clot. One exceptional group of cultures was maintained in the original clots for nine weeks. Usually, by the time the clot needed to be repaired, the epithelial outgrowths of adjacent explants had become confluent and had spread laterally to cover the surface of the entire coverslip (Plate IV, fig. 9).

Neutral red applied according to Bensley's technique for the demonstration of islets in mature pancreas showed areas of brightly staining cells. Areas stained with neutral red were restricted to the thicker areas of the culture near the original explant. Occasionally diffuse areas in the epithelial outgrowth areas stained with the neutral red, but these regions were never intensely stained. If the neutral red technique of Bensley is selective for the specific granules of the

islet cells, the appearance of these vitally stained cultures suggests at least some differentiation of the epithelial outgrowth areas. Almost all of the cells in the growth regions appeared to be completely unaffected by the neutral red. All vitally stained cultures showed some isolated cells containing very large neutral red granules. These cells have been described by Murray in her islet cultures as Mast cells.

The epithelial outgrowth was undiminished after maintenance in culture for five or six weeks, but the site of the original explant could still be determined. Nests of cells, which are so compact that distinct cell boundaries can not be discerned by microscopic examination, sometimes persist at this site.

Explants which become detached or are floating free in a pool of liquefied substrate are the only ones which exhibit the rounding-up process described for the chick.

Histological Appearance of Chick Pancreatic Explants Grown In Vitro

Histological preparations of chick pancreatic cultures were remarkably uniform in appearance. Typically, the cultures were spherical and surrounded by a capsule of some hyaline-like material. The fibrous nature exhibited in certain areas suggested connective tissue. Cultures possessing this shell-like structure exhibited a more nearly typical "islet arrangement" than those in which the encapsulating mass was lacking. The alpha and beta islets are clearly indicated in many of the preparations. This is most evident when both types of structures have differentiated in the same explant. In this situation the darkly

staining alpha cells are contrasted against the more lightly staining beta cells. (Plate VI, fig. 13 and 14) In cultures where both types of cells have not appeared, it is more difficult to determine whether differentiation has occurred. Criteria for differentiation are based almost exclusively on staining reactions. Cultures minus the capsule do not appear to be as well organized histologically as those cultures just described. Central necrotic areas are no more apparent in cultures containing capsules than in cultures in which this structure is missing. This suggests that the capsule itself offers no particular barrier to the diffusion of gases and nutrient materials. A few of the cultures exhibited tubular or cyst-like structures which probably represent remnants of the duct system (Plate V, fig. 12). Typical acinar tissue is never represented in these cultures. Cells which may represent degenerating or atrophic acinar cells can sometimes be located in cultures which have been maintained for less than two weeks in culture. These are identified as acinar cells only by the appearance of scattered persistent granules (zymogen?).

Histological Appearance of Mouse Pancreatic Explants Grown In Vitro

Cultures of mouse pancreas prepared for histological examination did not reveal the high degree of morphological differentiation exhibited by the chick pancreatic cultures. In the sections prepared from the limited material available, differentiated islets could not be identified. Viable areas did not show the capsular enclosure characteristic for the chick cultures. Cells possessing specific granulations peculiar

to the islet cells could never be identified in these preparations.

Results of Assays for Insulin Activity

Assays for insulin activity were performed using only the supernates of hepatic and pancreatic explants of the mouse. The first assay was performed after the explants had been cultivated for seven days. The supernate, which was added on the second day of cultivation, had been in contact with these explants for five days. The next four assays were done at five day intervals, i.e. each time the supernate was changed. The last assay was performed on the fifty-eighth day of cultivation. Glucose uptake of diaphragmata in liver and in pancreatic supernates for each assay are represented graphically on page 23. Specific data from each assay are included in tables on pages 24, 25, 26, 27, 28, 29; the results in most cases are clear and require little explanation.

Control vessels of the first assay, containing liver supernate which assayed negatively for insulin, used slightly more glucose than experimental vessels containing pancreatic supernate. The second assay, made on the twelfth day of cultivation, showed an almost equal amount of glucose uptake in both control and experimental vessels, while the last four determinations showed a significant increase in the glucose uptake of experimental over control vessels.

All glucose utilization values were corrected to a uniform time of incubation (four hours). The percent utilization of glucose induced by the insulin or insulin-like substance in experimental pancreatic cultures was calculated as follows:

P - pancreas supernate
L - liver supernate
$Glucose_1$ - supernate before incubation
$Glucose_2$ - supernate after incubation

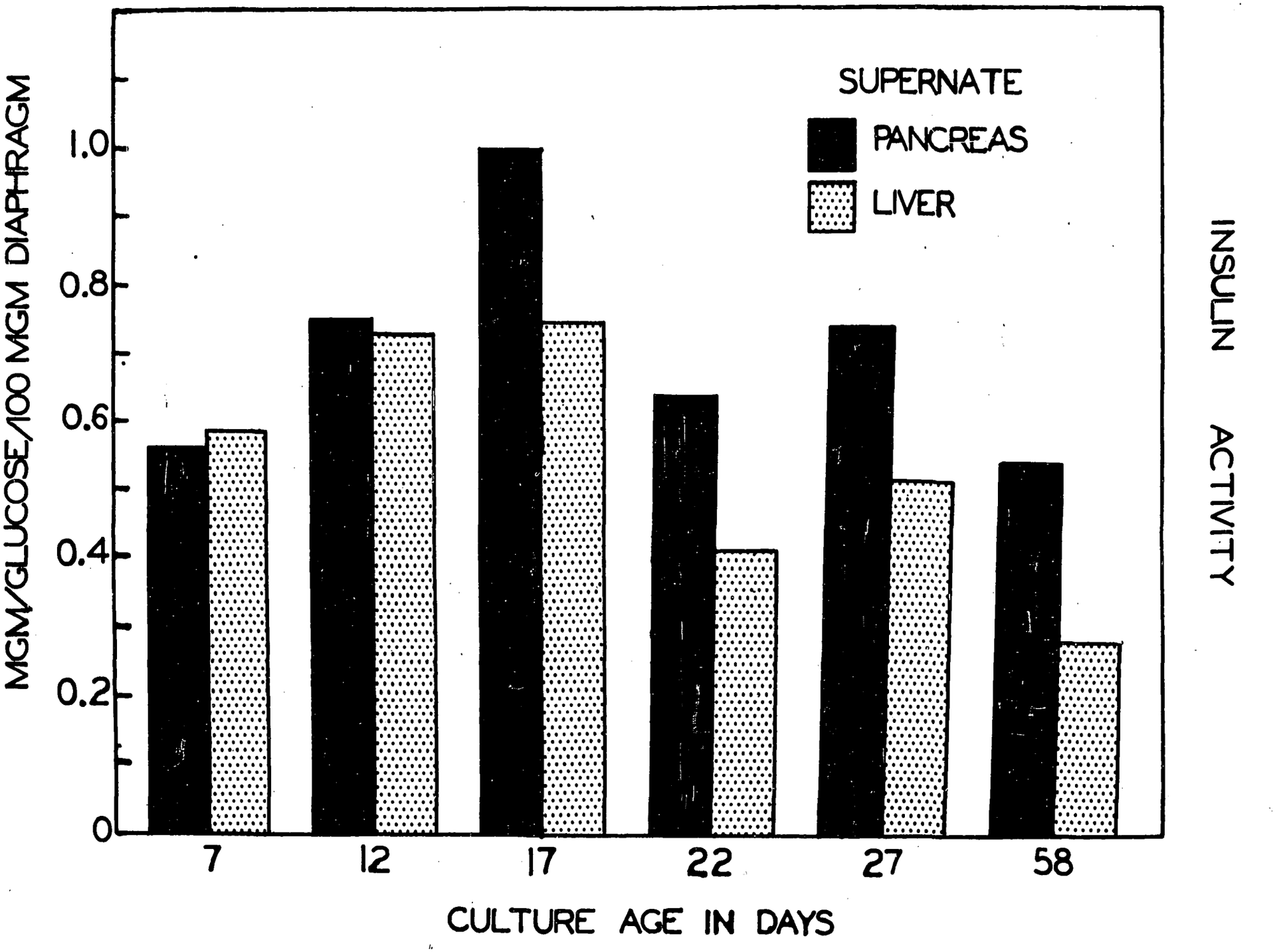
I $\quad Glucose_1^P - Glucose_2^P = Glucose^P$ utilized

$\frac{Glucose^P \text{ utilized}}{\text{Diaphragm wt. mgm}}$ = $Glucose^P$ utilized per mgm diaphragm

II $\quad Glucose_1^L - Glucose_2^L = Glucose^L$ utilized

$\frac{Glucose^L \text{ utilized}}{\text{Diaphragm wt. mgm}}$ = $Glucose^L$ utilized per mgm diaphragm

III $\quad \frac{Glucose^P \text{ utilized}}{Glucose^L \text{ utilized}} \times 100 = \%$ increase in glucose utilization induced by insulin or insulin-like substance


SUPERNATE
PANCREAS
LIVER
MGM/GLUCOSE/100 MGM DIAPHRAGM
0
0.2
0.4
0.6
0.8
1.0
7
12
17
22
27
58
CULTURE AGE IN DAYS
INSULIN ACTIVITY

INSULIN ASSAY #1
Culture Age 7 days

Glucose before incubation mgm/ml	Glucose after incubation mgm/ml	Corrected for 4 hrs	Diaphragm weight mgm	Glucose used/100 mgm diaphragm/4 hrs
Pancreas				
2.71	2.51	1.60	284.0	0.571
	2.34	2.96	455.0	0.657
	2.42	0.72	156.0	0.450
Average				0.559
Liver				
3.15	3.04	0.80	166.0	0.470
	2.97	1.44	237.0	0.600
	2.96	1.52	241.0	0.633
Average				0.567

INSULIN ASSAY #2
Culture Age 12 days

Glucose before incubation mgm/ml	Glucose after incubation mgm/ml	Corrected for 4 hrs	Diaphragm weight mgm	Glucose used/100 mgm diaphragm/4 hrs
Pancreas				
2.10	0.78	3.66	487.0	0.751
	0.73	4.11	538.0	0.764
Average				0.757
Liver				
2.23	0.76	4.41	588.0	0.747
	0.68	4.65	602.0	0.775
	0.72	4.53	612.0	0.742
Average				0.754

INSULIN ASSAY #3
Culture Age 17 days

Glucose before incubation mgm/ml	Glucose after incubation mgm/ml	Corrected for 4 hrs	Diaphragm weight mgm	Glucose used/100 mgm diaphragm/4 hrs
Pancreas				
2.38	0.66	5.16	515.0	1.012
	0.54	5.52	508.0	1.104
Average				1.058
Liver				
2.01	0.62	4.17	565.0	0.744
	0.57	4.32	571.0	0.757
	0.60	4.23	565.0	0.748
Average				0.749

INSULIN ASSAY #4
Culture Age 22 days

Glucose before incubation mgm/ml	Glucose after incubation mgm/ml	Corrected for 4 hrs	Diaphragm weight mgm	Glucose used/100 mgm diaphragm/4 hrs
Pancreas				
1.60	1.47	0.48	116.0	0.443
	0.97	2.50	335.0	0.759
	1.00	2.56	350.0	0.732
Average				0.644
Liver				
1.93	1.70	0.92	176.0	0.542
	1.62	1.24	408.0	0.310
	1.61	1.26	343.0	0.373
Average				0.408

INSULIN ASSAY #5
Culture Age 27 days

Glucose before incubation mgm/ml	Glucose after incubation mgm/ml	Corrected for 4 hrs	Diaphragm weight mgm	Glucose used/100 mgm diaphragm/4 hrs
Pancreas				
1.89	1.58	1.24	148.0	0.826
	1.63	1.04	156.0	0.650
Average				0.738
Liver				
2.03	1.63	0.53	116.0	0.441
	1.47	0.74	116.0	0.600
Average				0.520

INSULIN ASSAY #6
Culture Age 58 days

Glucose before incubation mgm/ml	Glucose after incubation mgm/ml	Corrected for 4 hrs	Diaphragm weight mgm	Glucose used/100 mgm diaphragm/4 hrs
Pancreas				
2.09	1.30	2.70	598.0	0.457
	0.83	4.32	658.0	0.654
	0.99	3.77	709.0	0.531
Average				0.547
Liver				
1.44	1.01	1.47	623.0	0.237
	0.84	2.05	621.0	0.330
Average				0.283

CHAPTER V

DISCUSSION

Embryogenesis of the pancreas of C_3H and C_{57} brown mice has been adequately described by Browning and Resnik (1951); the descriptive histology has been presented by Ferner (1952), De Witt (1906), Glaser (1926), and Volk, Lazarus and Goldner (1954). An account of the pancreatic development in the chick can be found in standard vertebrate embryology texts. Histological descriptions of the chick islets have been made by Clara (1924), Volk, Lazarus and Goldner (1947), Trautmann and Fiebiger (1952), and Sturkie (1954).

The histological observations on pancreatic structure of adult chickens and mice noted in this investigation were made during attempts to perfect reliable staining techniques and are not intended as detailed re-investigations of these works. Preparations were made during the developmental stages of these animals merely to verify the findings of the above workers and to preclude the possibility of differentiated islets being present in the pancreatic primordia used for culturing. The morphological observations, regarding the time of islet development, are in complete agreement with the findings of these authors. However, during attempts to demonstrate specific cell types in the adult and developing islets, numerous staining procedures had to be tested (cf. page 7).

Although some of these procedures were stated to be highly specific for islet cell types in all classes of vertebrates, the results obtained here are not in complete agreement. Almost all of the techniques tried differentially stained the alpha and beta cells of the mammalian pancreas, but the same techniques when applied to avian pancreas did not permit their identification. Slides prepared here and slides borrowed from other workers using the same staining procedures have not substantiated the claims made for the various techniques. Because of this confusion concerning the specificity of staining reactions, it was necessary to apply the technique used by Lane and Bensley for the original description of these cells. Slides were first stained with neutral-gentian, destained, and then restained with either the Bencosme or the Goldner modification of the Masson technique to verify the selectivity of these stains.

Clot liquefaction is a property common to all actively growing cultures, but the rapidity with which it occurs varies from tissue to tissue. The observation that tissues grown in homologous plasma tend to liquefy clots more rapidly than tissues grown in non-homologous plasma has been made by many workers (Murray and Bradley, 1935; Fischer, 1949; Cameron, 1950). The extremely rapid softening reported by Murray for her islet cultures, grown in homologous plasma could have been aggravated by the neoplastic nature of her cultures[1]. To combat this objectional feature, Murray found it necessary to use non-homologous plasma during the later culturing stages. The use of homologous plasma for cultivation

[1]The degree of malignancy of a neoplasm can be determined in tissue culture by comparing the rates of clot liquefaction in normal and in neoplastic cultures (Cameron, 1950).

of chick pancreas could explain the accelerated clot liquefaction in this study, as well as results obtained by previous workers using chick pancreas. Although it is commonly believed that rapidly growing tissues tend toward early clot liquefaction, it would appear that the use of non-homologous plasma counteracts this influence, since mouse cultures could be maintained for four to eight weeks in the same non-homologous clot.

The tendency for chick pancreas to round-up into compact cell masses, as noted in this work, is in contrast to the reports of Chlopin (1930) and Amoroso (1932). These investigators reported that cultures of pancreas grow wildly and resemble neoplastic tissues with the ability to invade and destroy adjacent explants. A possible explanation for this difference is that the behavior of the explant is influenced by both the age of the pancreas cultured and the individual animal species utilized. The pancreata used by these workers were taken from nineteen and twenty day chick embryos (Chlopin and Amoroso) and from foetal guinea pigs just before parturition (Amoroso). These animals are older than those used in this series of experiments (Chick - nine and ten day; mouse - fourteen and sixteen day). That differences exist between different animal species can be shown by comparing the behavior of mouse and chick explants as reported in this study. Explants of mouse pancreas spread and continue to spread as long as they are maintained in culture, whereas chick pancreatic explants can not be induced to spread when cultivated under the same conditions. Conversely, mouse explants could be made to round-up only if they were allowed to float freely in the fluid nutrient.

The mouse pancreas grown here appears to be comparable in

growth characteristics to the highly differentiated islet cell adenomas cultivated by Murray and Bradley. The growth rate of both groups of cultures was good, but the rate was slow when compared to non-specialized or undifferentiated tissues such as heart fibroblast cultures. In both cases, the explant behavior is like that of any epithelial organ grown in vitro. The only apparent difference between cultures of mouse pancreas in this work and Murray's human adenomas, is the degree of clot liquefaction. The adenomas, although in non-homologous plasma, liquefy the clot rapidly whereas the mouse explant can be grown for weeks in the original clot.

The tendency of chick pancreas to round-up into compact cell masses offers a possible explantation for the high degree of differentiation obtained with explants grown in culture. The rounding-up may influence differentiation in vitro, since only rounded explants possessing hyaline capsules showed cytologically differentiated islet cells. This finding is in agreement with the observations of Gaillard and Kooreman (1950) and Gaillard (1954) for parathyroid gland cultures. They found that organized growth and differentiation occurred only when an encapsulating shell was present and intact. Examination of sections prepared from mouse cultures lends additional evidence to the role of a capsule in promoting organized growth and cytological differentiation as emphasized by Kooreman and Gaillard. As stated previously, mouse cultures continue to spread throughout the duration of cultivation, an encapsulating shell does not form, and histological or cytological differentiation does not occur.

Chen, Oldham, and Geiling (1940) and Rahn and Drager (1941)

were able to demonstrate that the melanophore expanding hormone was present in the intact chick pituitary at least five days before cytomorphic differentiation had become evident[1]. The results of these workers indicate clearly that cytological differentiation, as indicated by tinctorial methods, is neither a prerequisite for, nor an indication of, physiological activity. Microscopic examination of mouse pancreatic explants made after the last insulin assay did not show cytomorphic differentiation, i.e. the cells appeared to be completely undifferentiated. Despite the lack of cellular differentiation in mouse explants, the insulin assay studies indicate a functional differentiation of islet tissue. An examination of table 1, which shows that more glucose was utilized in the control than in the experimental vessels, indicated the absence of insulin activity. This difference in glucose utilization in the experimental vessels could be the result of changes brought about in the media when acinar tissue, which had developed before culture, begins to degenerate and release the primitive zymogen granules which are known to be present. Results of the second assay, made on the twelfth day after cultivation, show an almost equal amount of glucose used by diaphragm in experimental and control vessels. Although the actual value is somewhat greater in the experimental vessels it is not considered significant[2]. Examination of tables three through six shows a progressive increase in glucose utilization from approximately 30 percent in the third assay

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[1] Two attempts to demonstrate insulin activity in aqueous extracts of fourteen and sixteen day mouse embryos were unsuccessful.

[2] Increases of less than 20 percent were not considered significant; a margin of safety this large should place the readings beyond the range of experimental error of the sugar determinations.

to slightly less than 100 percent in the sixth which was made on the fifty-eighth day of cultivation. From these data it could be inferred that insulin or an insulin-like substance first appears in pancreatic supernate, in amounts detectable by this procedure, between the twelfth and seventeenth days of incubation. The rise in glucose consumption found in the later assays may be attributable to two factors, 1.) improvement in assay techniques, and 2.) formation of new islet tissue in culture resulting in the formation of more of the insulin-like substance per unit volume of nutrient. It should be emphasized that the graphic representation on page 23 can not be considered as a quantitative curve because of variations in, 1.) rat populations, 2.) fasting time of individual rats and 3.) media; therefore, valid comparisons can be made only between control and experimental vessels of the same assay. These determinations are entirely qualitative; no attempt was made to determine quantitatively the insulin content of the fluid nutrient.

These results indicate the presence, in the supernate of pancreatic tissue grown <u>in vitro</u>, of some substance having an insulin-like action. This substance cannot be demonstrated in the supernate from hepatic cultures.

In view of the previous work of Gaillard, Stone, Lux, Jones and Dameron on transplantation of cultured endocrine organs, the observations recorded here on the production of insulin or an insulin-like substance by pancreatic cultures strongly suggests the possibility of utilization of pancreatic cultures in replacement therapy in selected cases of diabetes mellitus. It was initially planned to investigate this possibility by producing diabetic mice experimentally with alloxan and correlating the

physiological effect of pancreatic culture transplants *in vivo* with the results of insulin assay determinations. Since the alloxan diabetes produced was of a transitory nature and the endogenous insulin produced by the recovering pancreas would invalidate such an experiment, this aspect of the problem was postponed.

CHAPTER VI

CONCLUSIONS

1. Complete cytomorphic differentiation of islet cells *in vitro* can be induced from undifferentiated pancreatic primordia of the chick.
2. Insulin activity can be demonstrated in mouse pancreatic cultures which do not show morphological or cytological islet cell differerentiation as demonstrated by tinctorial methods.

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PLATE I

Figure 1

Adult chicken pancreas stained with Goldner's modification of the Masson trichrome technique. A single lightly staining beta islet can be seen near the lower edge of the picture. One large darkly staining alpha islet surrounded by numerous satellite islets occupies the upper portion of this illustration. (X 28)

Figure 2

Adult chicken pancreas stained with Bencosme's stain. A single dark alpha islet occupies the upper left corner. Near the center of the picture two beta islets, one large and one small, can be seen. (X 28)

PLATE I

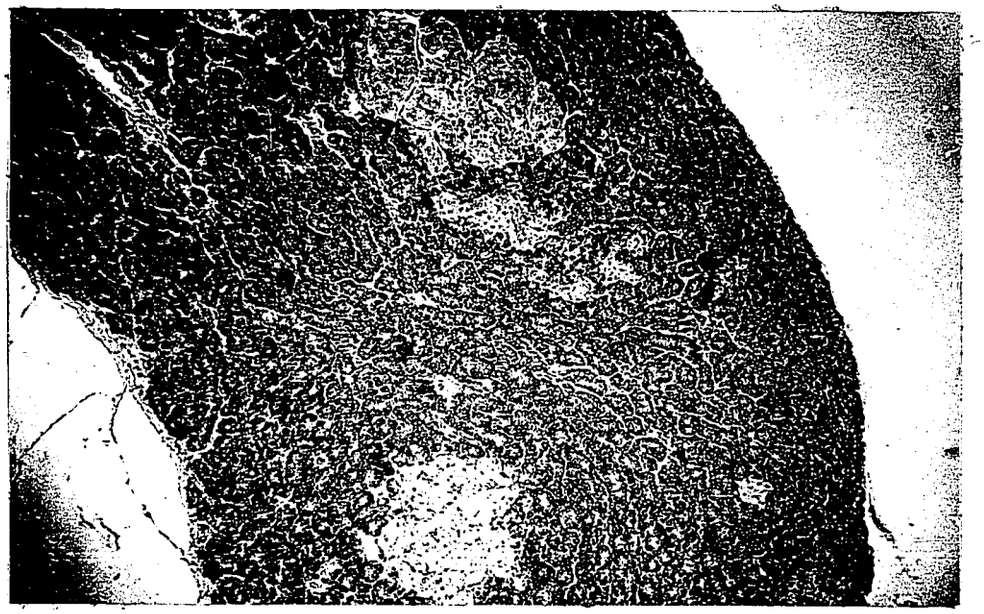

fig. 1

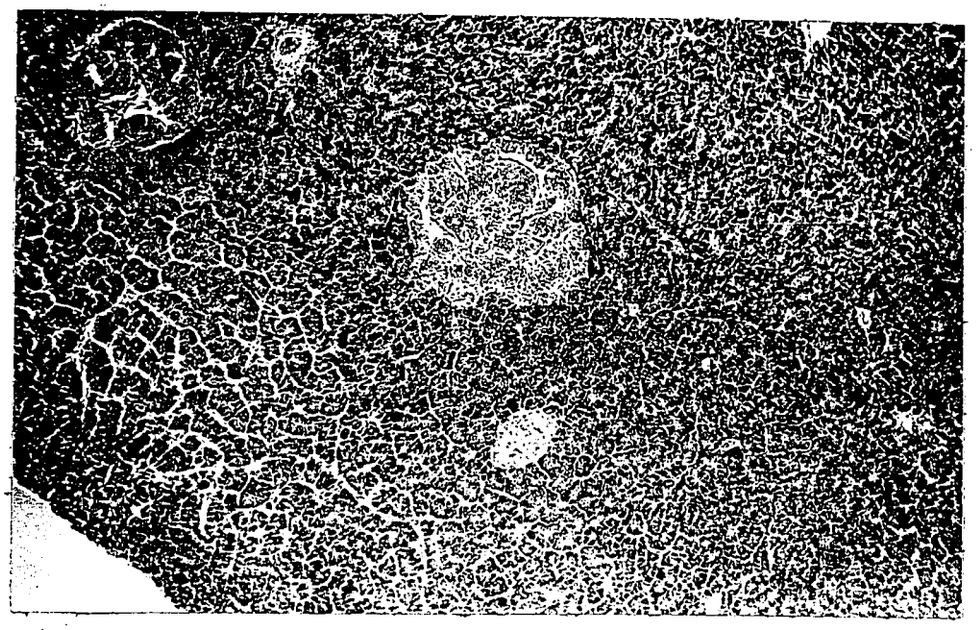

fig. 2

PLATE II

Figure 3

Adult mouse pancreas stained with Bencosme's stain. The top clear area of this islet is composed almost exclusively of beta cells while the lower half is composed almost entirely of alpha cells. (X 1000)

Figure 4

Adult mouse pancreas stained with Goldner's stain. Central area composed almost exclusively of beta cells. A single alpha cell (x) occurs in the central area. Additional alpha cells occur around the periphery of the islet at 12, 3, and 6 o'clock to form an incomplete shell. (X 1000)

PLATE II

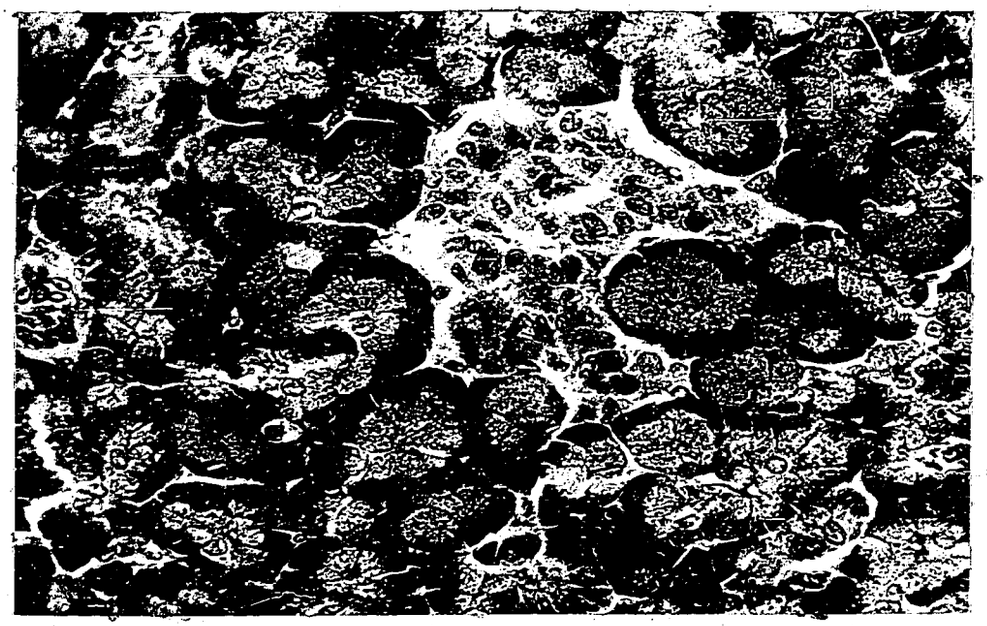

fig. 3

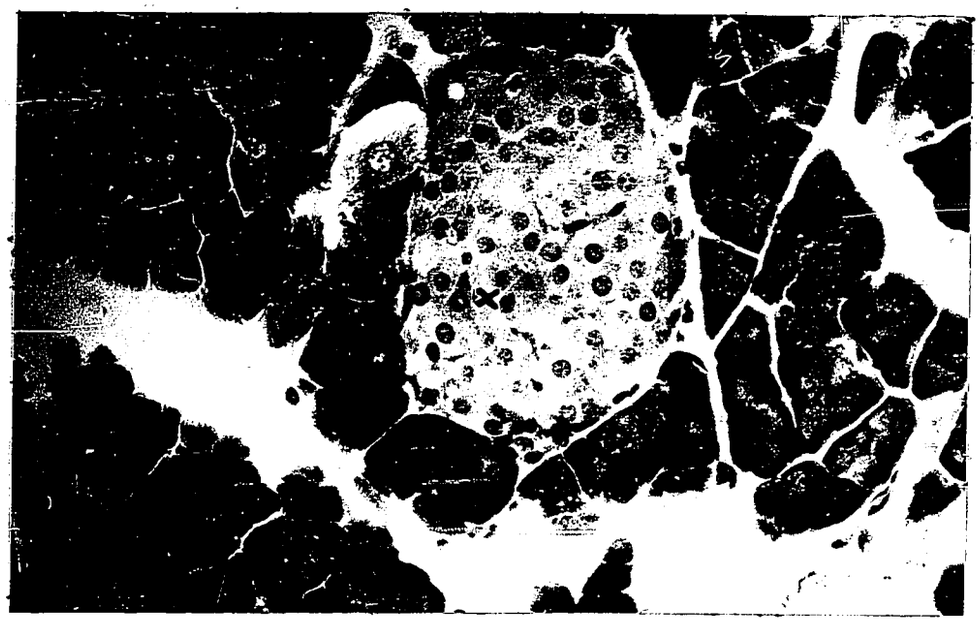

fig. 4

PLATE III

Figure 5

Pancreas of 9 day chick embryo. The dark areas represent generations of tubular or solid outgrowths from the original pancreatic diverticula. The lighter area between the parenchymal elements represents filling mesenchyme. (X 28)

Figure 6

Pancreas of 13 day chick embryo (Goldner stain). The boxed in area represents the first indication of the developing island of Langerhans. (X 300)

Figure 7

Lobule of 14 day foetal mouse (Goldner's stain) showing excretory duct surrounded by clear areas of stromal cells. In cross sections these resemble developing islets, cf. figure 8. (X 450)

Figure 8

Pancreas of 18 day foetal mouse (Bencosme's stain). Islets are becoming distinct, but the specific granulations have not begun to appear in either the alpha or the beta cells. (X 950)

PLATE III

fig. 5

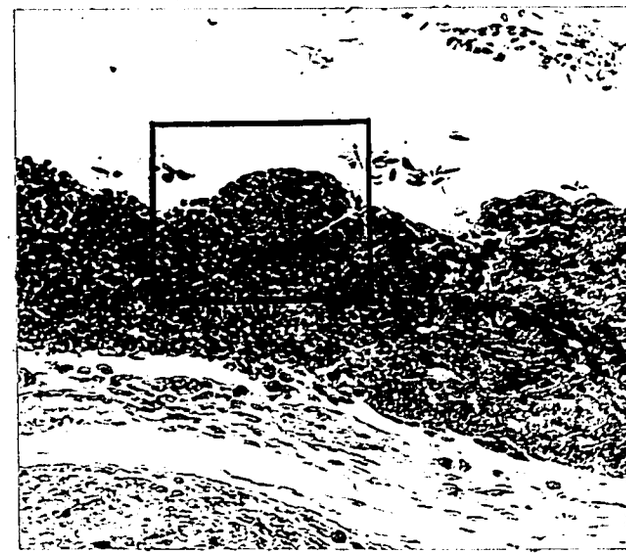

fig. 6

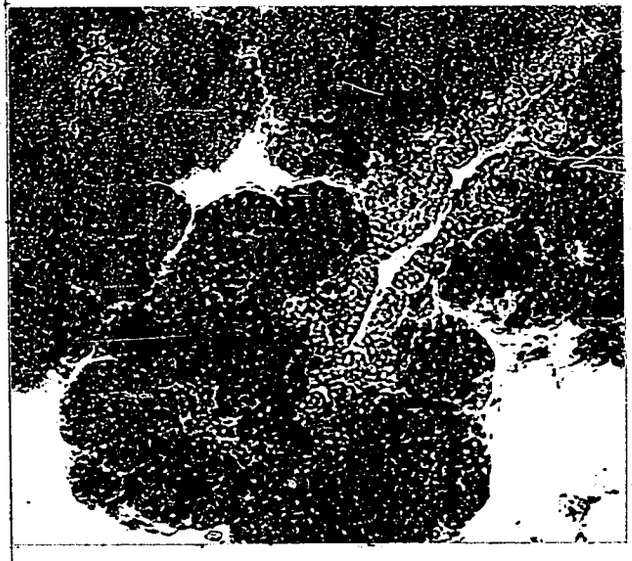

fig. 7

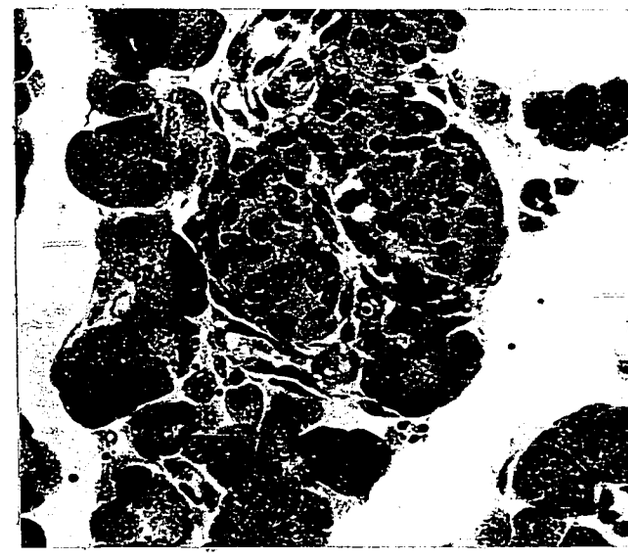

fig. 8

PLATE IV

Figure 9

Photomicrograph of living, unstained mouse pancreatic culture. Note the epithelial outgrowth at the periphery of the explant. (X 90)

Figure 10

Photomicrograph of living, unstained culture of mouse pancreatic tissue grown for 84 hours without nutrient. Fat droplets have accumulated in the epithelial as well as the fibroblast-like cells. (X 600)

PLATE IV

fig. 9

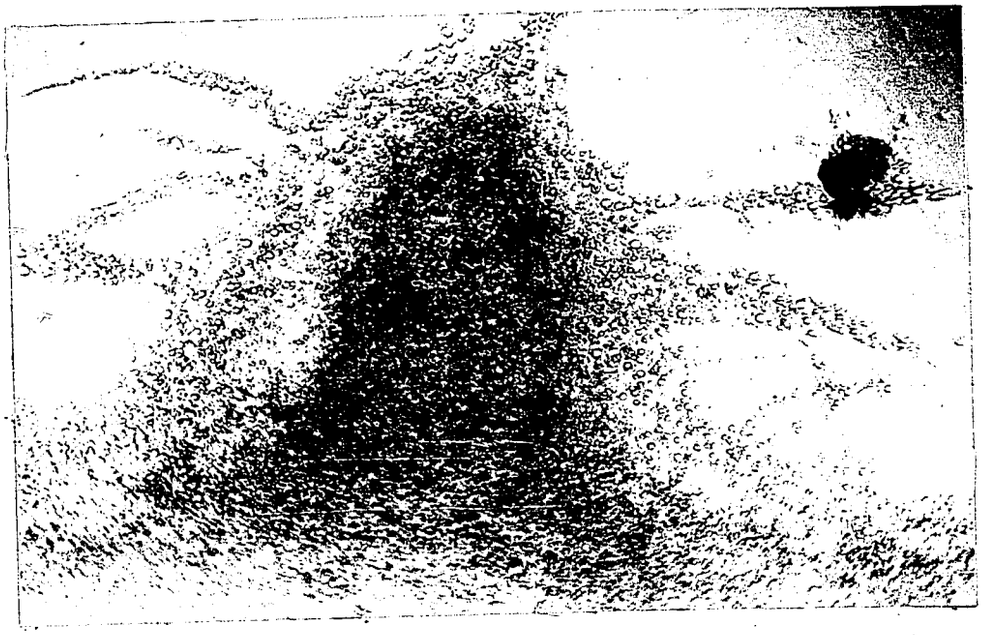

fig. 10

PLATE V

Figure 11

Photomicrograph of living unstained pancreatic culture showing distinct pavement epithelium (cf. Plate IV, figure 10). The epithelial cells at the edge of pavement are elongate and separated from adjacent cells by irregular spaces. (X 600)

Figure 12

Section of chick pancreatic culture grown in roller tube for 21 days. This culture exhibits tubular or cyst-like structures which may represent remnants of the duct system. The staining properties of the dark cells immediately surrounding these cavities suggest the presence of differentiated alpha cells. (X 450)

PLATE V

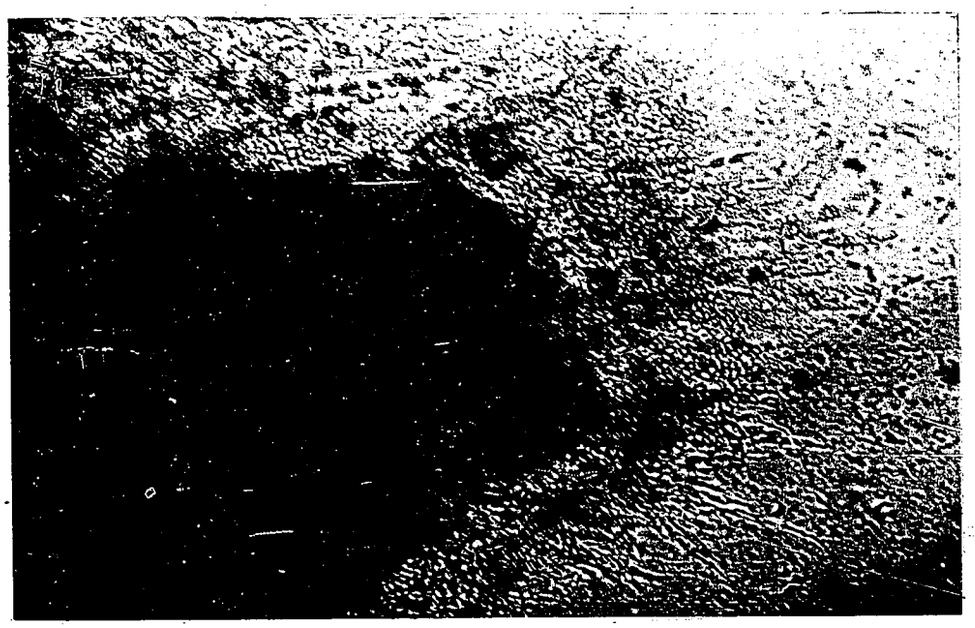

fig. 11

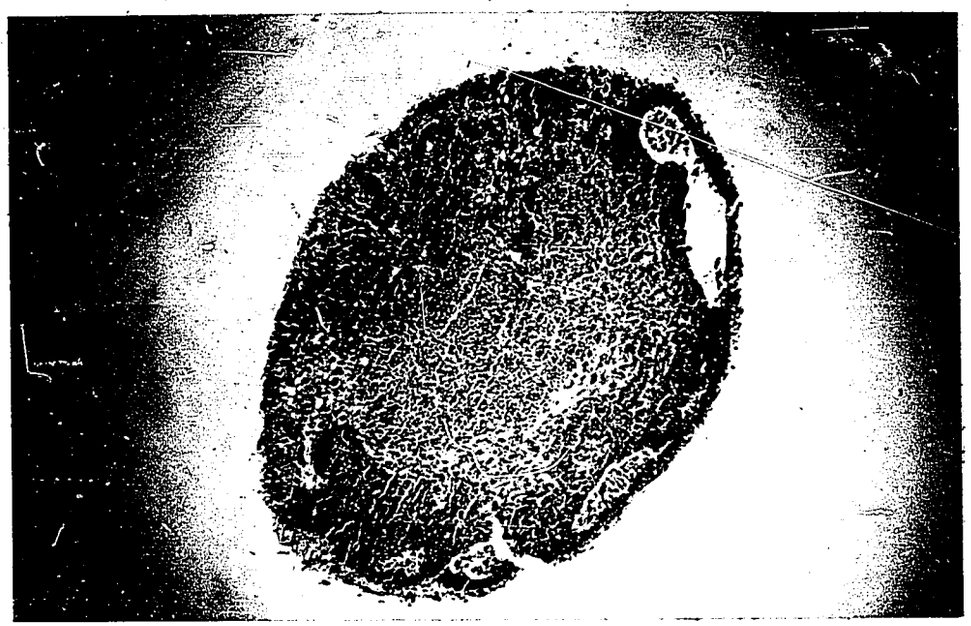

fig. 12

PLATE VI

Figure 13

Section of chick pancreatic culture stained with Goldner's stain. The dark cellular mass occupying the upper portion of this section represents a concentration of alpha cells. (X 450)

Figure 14

Similar to above section, but here alpha cells are more evenly distributed throughout the explant. The clear area toward the right represents a collection of beta cells. Identification of alpha and beta cells is simplified when both cell types have differentiated in the same explant. The dark alpha cells are then contrasted against the lighter staining beta cells. (X 450)

PLATE VI

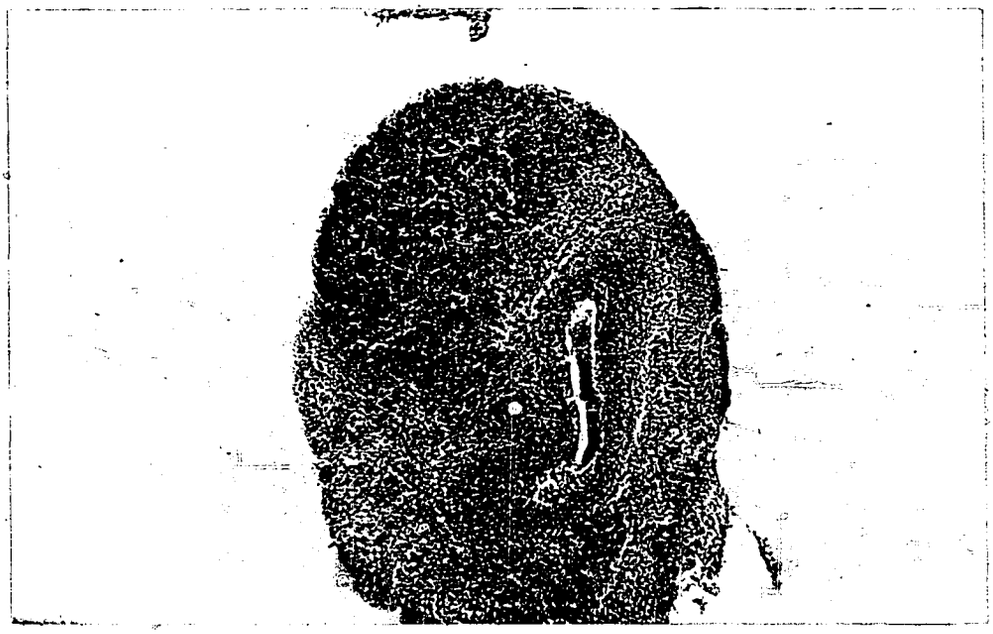

fig. 13

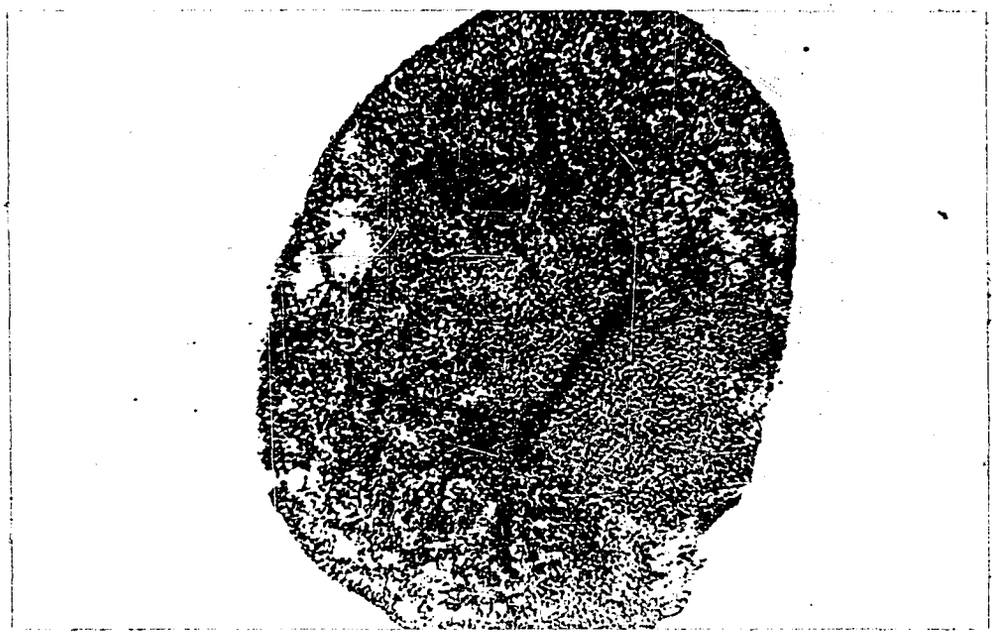

fig. 14