# Insulin Content of Fetal Rat Pancreases Grown in Organ Culture and Subsequently Transplanted into Maternal Hosts

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SUMMARY

Portions of pancreases from 19.5-day fetal rats were grown in organ culture on two types of liquid media: low glucose, containing 162 to 170 mg. glucose/100 ml.; high glucose, containing 1,031 to 1,073 mg. glucose/100 ml.

Following four days of organ culture, the tissues were extracted and assayed for insulin content (immunoassay). Other cultures were transplanted to maternal hosts at two sites: beneath the capsule of the kidney and into the anterior chamber of the eye. Ten days later, the transplants were removed and assayed for insulin content.

Large quantities of immunologically active insulin were present in the culture media following forty-eight and ninety-six hours of incubation. Explants exhibited a four- to sixfold increase in insulin content following four days in vitro. Pancreatic explants cultured on the high glucose medium contained less extractable insulin than similar explants cultured on the low glucose medium.

When cultured explants were transplanted to hosts with normal blood glucose levels, the total extractable insulin content of the grafts was 55 per cent to 130 per cent greater than that at the time of transplantation. When cultures were transplanted to alloxan diabetic hosts, the total extractable insulin content of the grafts was less than that found in grafts to normal hosts.

A small but statistically significant drop in blood glucose level was observed in hyperglycemic diabetic maternal hosts receiving transplants of organ cultured pancreas.

These results indicate the continued growth and functional responsiveness of the islet beta cells during organ culture and following subsequent transplantation. DIABETES *21*:193-202, April, 1972.

Previous studies from this laboratory have reported the cytologic changes of fetal pancreas during organ culture and following subsequent transplantation to maternal hosts.[1] The degree of beta cell granulation in the islets of transplants varied with the blood glucose level in the maternal hosts. Organ cultures with heavily granulated islets lost their granulation when transplanted to hyperglycemic diabetic hosts. Organ cultures with degranulated islets (grown on high glucose medium) developed heavy beta cell granulation when transplanted to maternal hosts with normal blood glucose levels.

The present paper reports our findings concerning the extractable insulin content of organ cultures and of transplants of such cultures.

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## MATERIALS AND METHODS

Fetuses of rats from the Sprague-Dawley strain were used (Simonsen Laboratories, Inc.). Female rats were between 100 and 150 days at the time of conception. The age of the fetuses was calculated from the time of witnessed mating. The methods of organ culture and transplantation were carried out in the manner previously described.[1]

Maternal diabetes was induced by a single intravenous injection of 2 per cent alloxan (35 mg./kg.) on day 11.5 postcoitum. Blood samples were obtained from the tail vein in the morning beginning on Day 13 of pregnancy and throughout the period of observation. The glucose levels in the blood and in the culture media were determined by a modification of the method of Hoffman[2] as applied to the Technicon AutoAnalyzer.

Tissue samples (fetal pancreas, organ cultures and

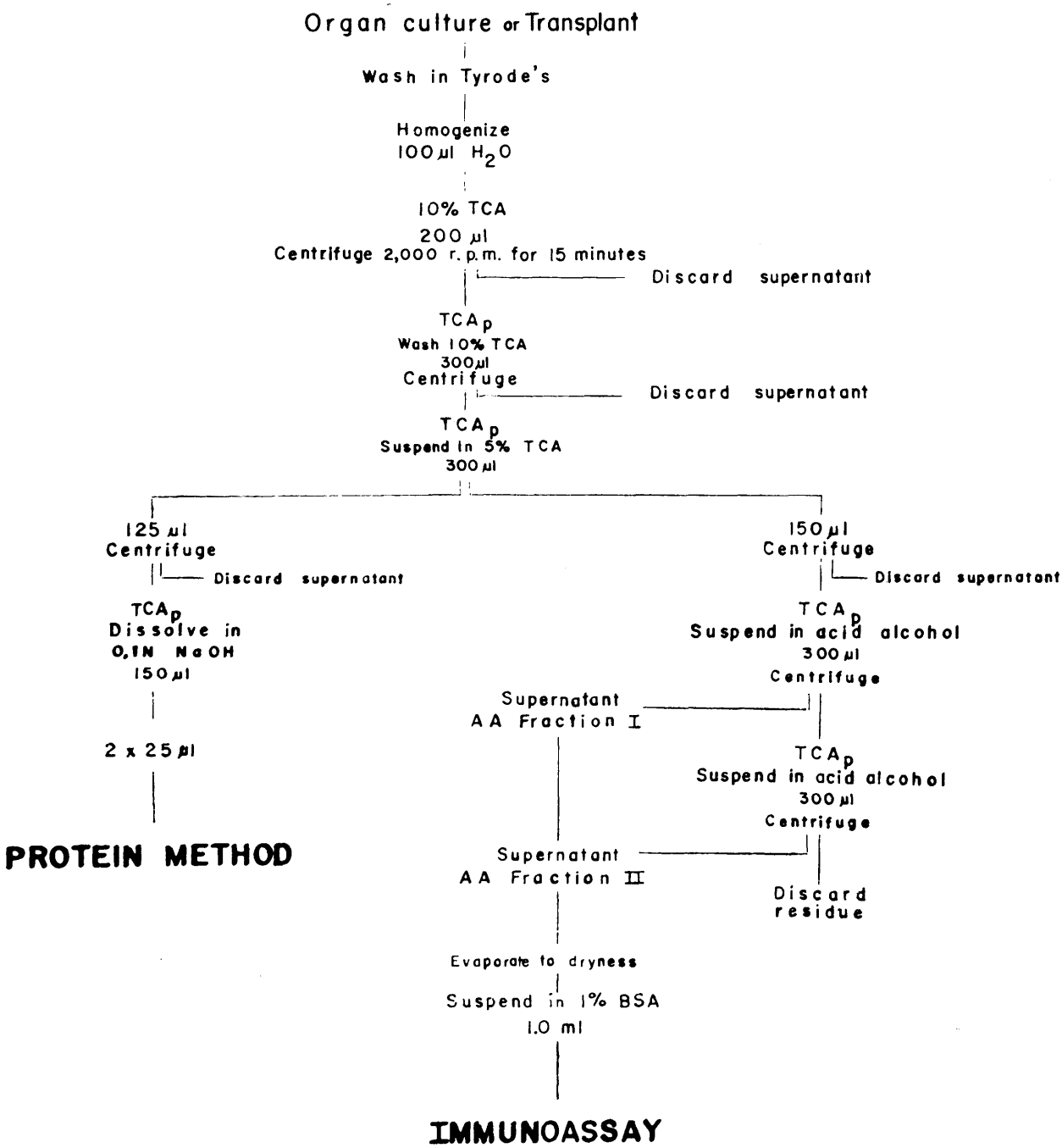


FIG. 1. Insulin and protein determination procedure.

transplants) were homogenized in 100 µl. of triple distilled water and the protein precipitated with 10 per cent trichloroacetic acid (TCA) (figure 1). The precipitate was washed with 10 per cent TCA and resuspended in 300 µl. of 5 per cent TCA. A 125 µl. aliquot of the suspension was used for protein determination and a 150 µl. aliquot was assayed for insulin.

Protein determination: The 125 µl. aliquot was centrifuged and the precipitate dissolved in 150 µl. of 0.1 N NaOH (figure 1). Aliquots of 25 µl. were analyzed in duplicate using a modification of the method of Lowry.[3]

Insulin assay: The 150 μl. aliquot was centrifuged and the precipitate was extracted with 300 μl. of acid alcohol solution (15 ml. 12 M HCl per liter 70 per cent ethanol) for ninety minutes (figure 1). Following centrifugation, the acid alcohol soluble fraction (supernatant) was saved. The precipitate was re-extracted with 300 μl. of acid alcohol for sixty minutes, centrifuged and the acid alcohol soluble supernatants combined and evaporated to dryness in an air stream. The entire extraction procedure was carried out at 4° C. The acid soluble protein was dissolved in 1 per cent bovine serum albumin in borate buffer (pH = 8.4) and assayed for immunologically active insulin using the two antibody method of Morgan and Lazarow.[4,5] The insulin content of the culture media was determined using 0.2 ml. of the sample diluted to 1.0 ml. with 1 per cent bovine serum albumin (BSA) and frozen until assayed.

Because rat insulin standards were not available at the time of this study, endogenous rat insulin values were determined against bovine insulin standards, utilizing I-131-bovine insulin and are expressed as bovine insulin equivalents (μU.).

A concentration-related artifact in this immunoassay method has recently been reported.[6] The net effect of this artifact is an under-estimation of unknown insulin values when higher concentrations of insulin are analyzed. We were aware of a similar dilution nonlinearity

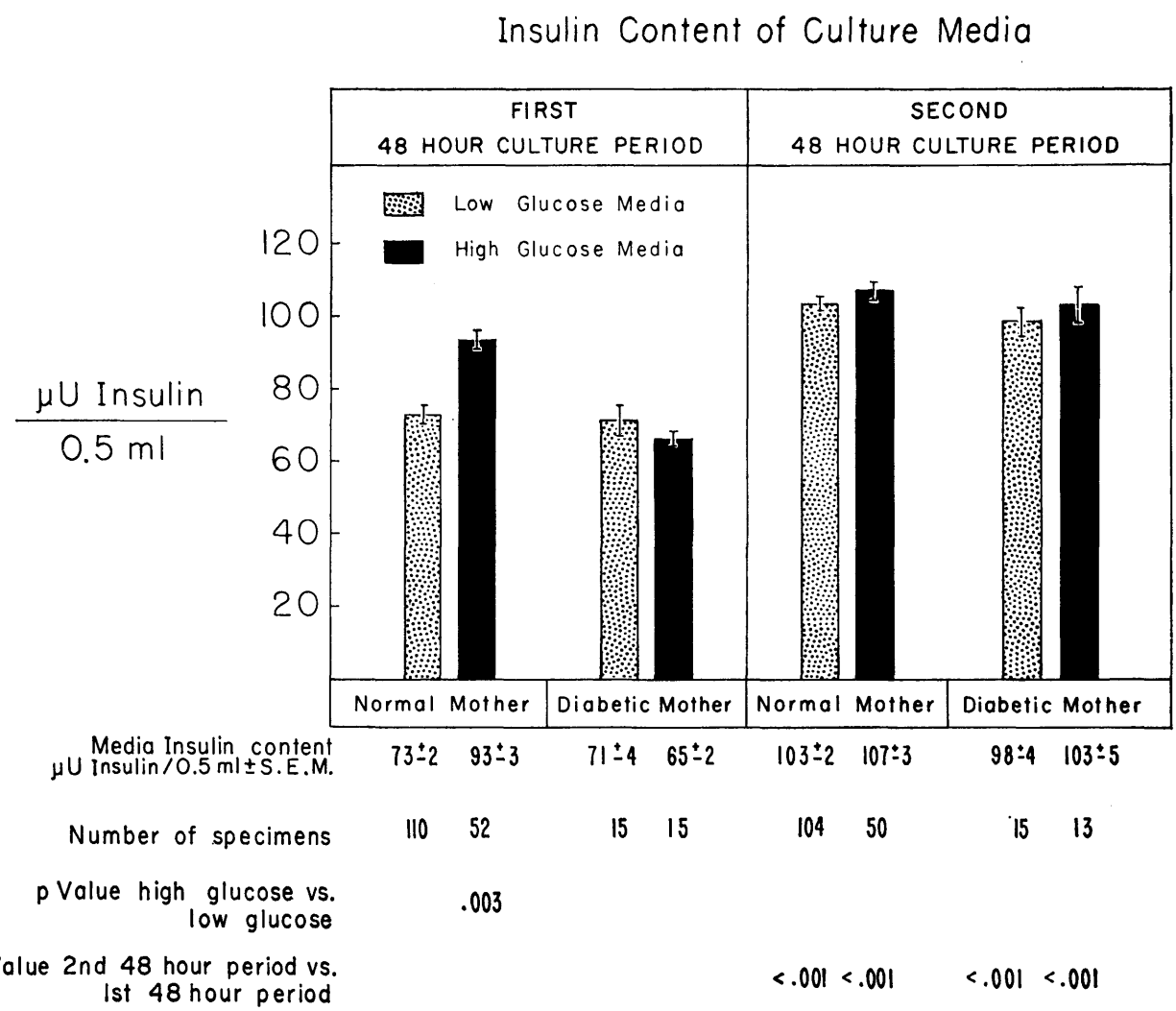


FIG. 2. Insulin content of the culture media. Explants were grown in 0.5 ml. of media. The height of each bar gives the average insulin content (beef insulin equivalents) of each type of culture medium following the first and second forty-eight-hour incubation periods.

under the conditions of this study. Consequently, (1) all tissue extractions and (2) all media samples were assayed at a constant dilution to enable comparability of the observed values.

Index of beta granulation: For fetal pancreas, organ cultures and transplants, the index of beta granulation was calculated by multiplying the percentage of samples by their islets corresponding degree of aldehyde fuchsin positive beta granulation (i.e., 75, 50, 25 or 0-10). These values were summed and averaged. For example, if all the samples in a group (100 per cent) had islets in which 75 per cent of the cells contained beta granules, the index would be 75. The details and data have been reported.[1]

## OBSERVATIONS

*Pancreases from fetuses of normal mothers*

Explants from 19.5-day fetuses of normal mothers

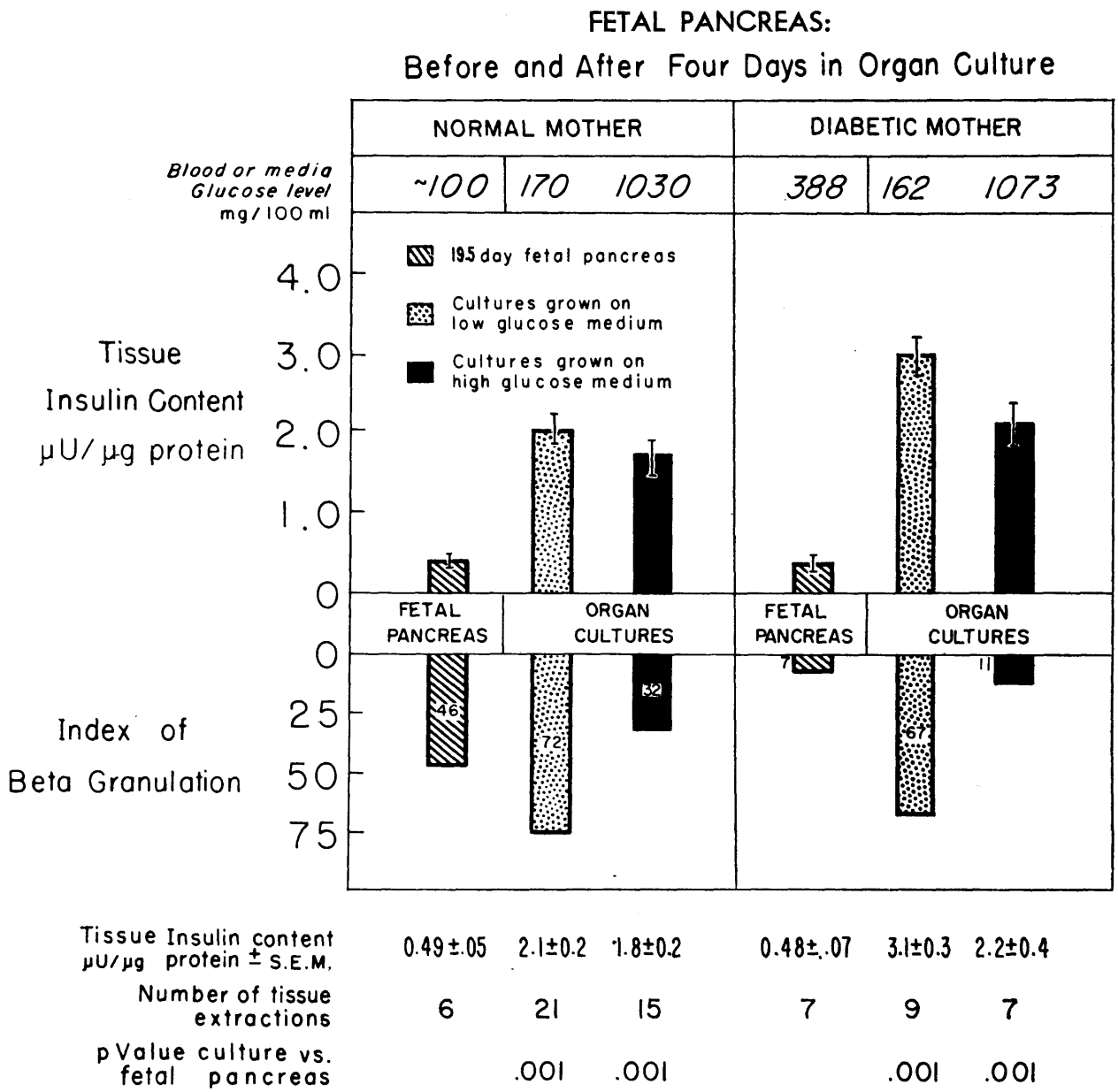


FIG. 3. Fetal pancreas: before and after four days of organ culture. The ascending component of each bar gives the average µU. of extractable insulin per µg. protein for the pancreas or organ culture. The descending component of each bar depicts the beta granulation index exhibited by that tissue as previously determined.[1]

released large amounts of insulin into the media during two successive forty-eight-hour incubation periods (figure 2). The small amounts of insulin present in the fresh media prior to incubation (6-9 μU./0.5 ml.) were presumably due to insulin in the components of the culture media, cock serum and embryo extract. Following the first forty-eight-hour incubation period, the total insulin content of the low glucose and high glucose media averaged 73 ± 2 and 93 ± 3 μU. insulin respectively. Following the second forty-eight-hour incubation period, the low and high glucose media contained an average of 103 ± 2 and 107 ± 3 μU. insulin respectively.

The insulin content of pancreases from 19.5-day fetuses of normal mothers was 0.49 ± 0.05 μU. insulin/μg. tissue protein at the time of explant (figure 3, striped bar). About 50 per cent of the cells in the islets of these pancreases contained beta granules as indicated by a beta granulation index of 46.

When such pancreases were grown in organ culture for four days, there was a marked increase in the insulin content of the tissue. Cultures grown on the low glucose medium (figure 3, stippled bar) contained 2.1 ± 0.2 μU. insulin/μg. tissue protein, an increase of 329 per cent. These cultures developed heavily granulated islets and had a beta granulation index of 72. At the time of transplant, the total insulin present in the low glucose cultures averaged 106 μU. insulin/explant (figure 4, stippled bar).

When such cultures were transplanted to normal maternal hosts, the total insulin content was increased. Following ten days in vivo, transplants to the kidney site

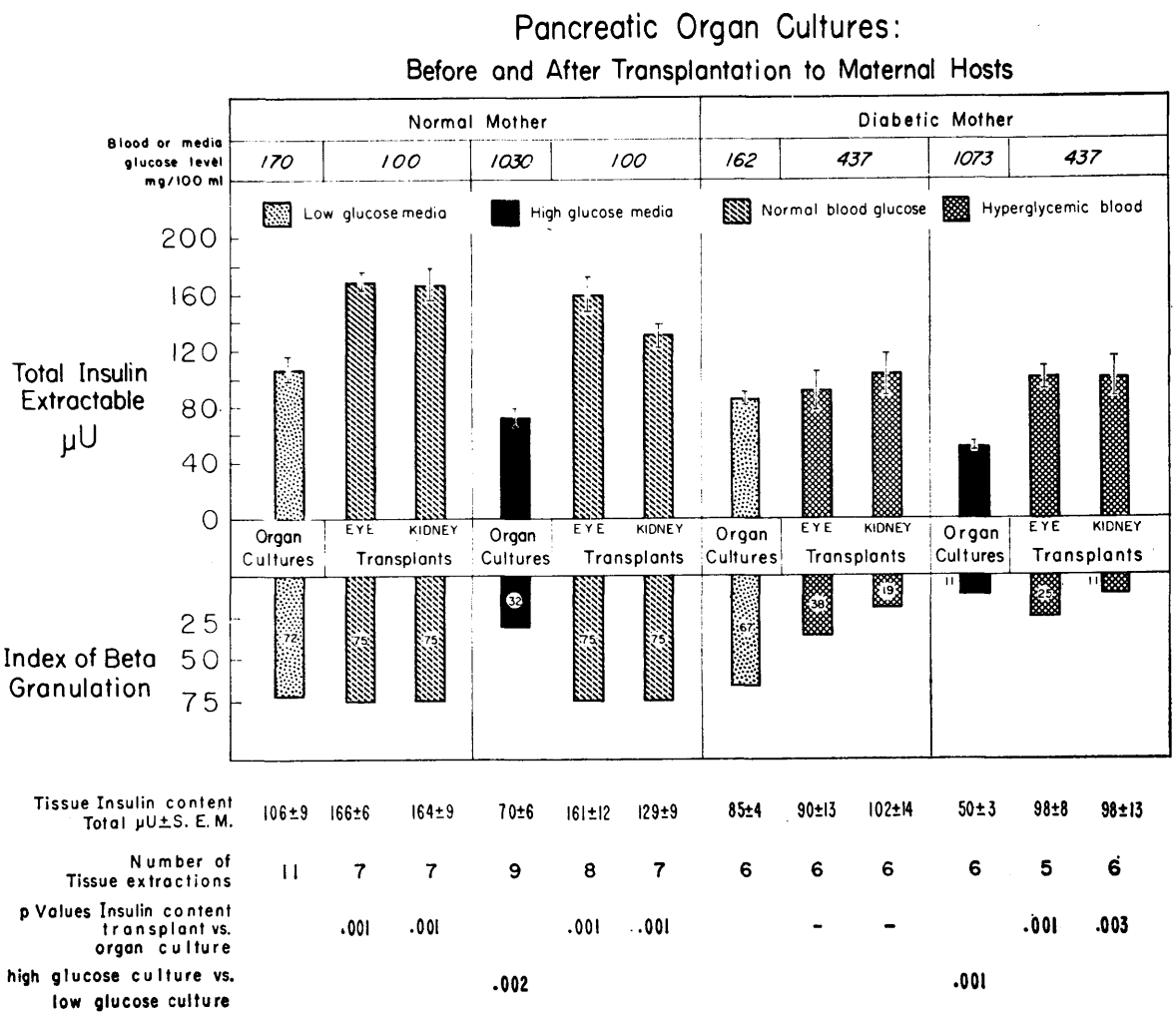


FIG. 4. Pancreatic organ cultures: before and after transplantation to maternal hosts. The ascending component of each bar gives the average total μU. of extractable insulin (beef insulin equivalents) in the organ culture or transplant. The descending component of each bar depicts the beta granulation index exhibited by that tissue as previously determined.[1]

averaged 164 μU. insulin/transplant and those to the eye site averaged 166 μU. insulin/transplant (figure 4, striped bars). This represents a 55 to 75 per cent increase ($p = .001$) in the total insulin content during the transplantation period. The beta granulation index remained high at 75 in transplants to both sites.

When pancreases from fetuses of normal mothers were grown in organ culture on the high glucose medium (figure 3, solid bar), their insulin content increased from 0.49 ± 0.05 to 1.8 ± 0.2 μU. insulin/μg. tissue protein. Although the index of beta granulation exhibited a slight decline from 46 at the time of explant to 32 following organ culture, the insulin content of these cultures increased by 267 per cent for the corresponding time period. At the time of transplant, the total insulin present in the high glucose cultures averaged 70 μU. insulin/explant (figure 4, solid bar), significantly less than that in low glucose culture (106 μU. insulin/explant) ($p = .002$). This difference in total insulin content was also reflected in a correspondingly lower beta granulation index for the high glucose cultures (32 as compared to 72).

When these high glucose cultures were transplanted to normal maternal hosts for ten days, the total insulin content increased significantly. Transplants to the kidney site averaged 129 μU. insulin/transplant and those to the eye site averaged 161 μU. insulin/transplant (figure 4, striped bars). This represented an 84 to 130 per cent increase ($p = .001$) in total insulin content. This was accompanied by an increase in beta granulation index from 32 to 75 at both sites.

*Pancreases from fetuses of diabetic mothers*

Explants from 19.5-day fetuses of alloxan diabetic mothers released large amounts of insulin into the culture media during two successive forty-eight-hour incubation periods (figure 2). Following the first forty-eight-hour incubation period, the total insulin content of the low and high glucose media averaged 71 ± 4 and 65 ± 2 μU. insulin respectively. Following the second forty-eight-hour incubation period, the low and high glucose media contained an average of 98 ± 4 and 103 ± 5 μU. insulin respectively.

At the time of explant, pancreases from fetuses of diabetic mothers contained 0.48 ± 0.07 μU. insulin/μg. tissue protein (figure 3, striped bar). Less than 10 per cent of the cells in the islets of these pancreases contained beta granules (the beta granulation index was 7).

When such pancreases were grown in organ culture

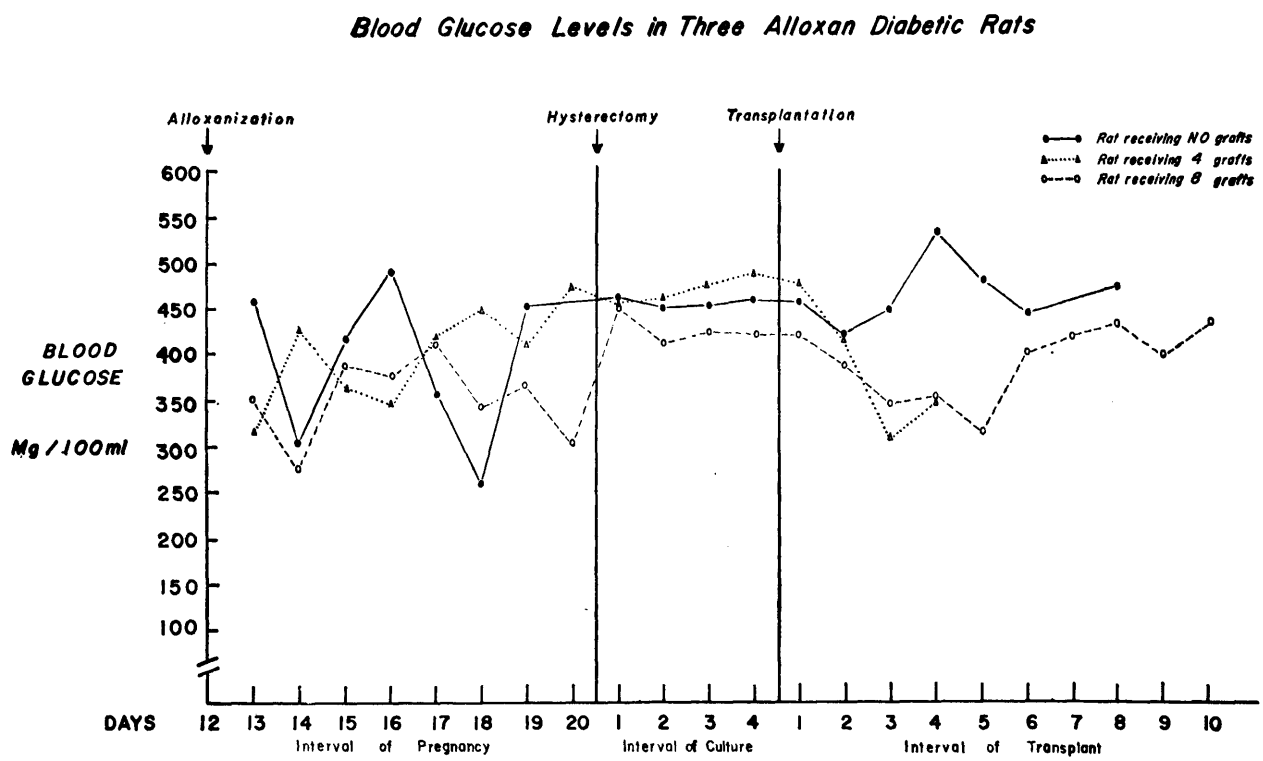


FIG. 5. Changes in blood glucose levels in three typical alloxan diabetic rats: rat receiving no grafts (●———●), rat receiving four grafts (△.....△) and rat receiving eight grafts (○----○).

TABLE 1

Changes in average blood glucose levels of diabetic maternal hosts; before and after transplantation

| Group | Number of animals | Number of transplants per animal | Blood glucose levels* before transplantation mg./100 ml. (± S.E.M.) Day 13-20 of pregnancy prior to hysterectomy | Blood glucose levels* before transplantation mg./100 ml. (± S.E.M.) Day 1-4 following hysterectomy | Blood glucose levels* following transplantation mg./100 ml. (± S.E.M.) Day 5 posthysterectomy to autopsy | Change in blood glucose during transplant period |
|---|---|---|---|---|---|---|
| 1 | 21 | 4 | 391 ± 8 | 427 ± 13 | 410 ± 15 | 17 ↓ |
| 2 | 24 | 8 | 385 ± 7 | 456 ± 8 | 437 ± 8 | 19 ↓† |
| 3 | 5 | 0 | 361 ± 18 | 422 ± 10 | 425 ± 20 | 3 ↑ |

* Determined by the method of Hoffman.[2]

† Analysis by the paired comparison "*t*" test of the changes in blood glucose in Groups 1 and 2 combined indicates that the decrease in blood glucose observed following transplantation is highly significant ($p = .003$).

for four days, there was a marked increase in the insulin content of the tissue. Cultures grown on the high glucose medium (figure 3, solid bar) contained 2.2 ± 0.4 μU. insulin/μg. tissue protein, an increase of 358 per cent. However, the islets in these cultures remained degranulated as indicated by their beta granulation index of 11, essentially unchanged from that at the time of explant. At the time of transplantation, the total insulin present in the high glucose cultures averaged 50 μU. insulin/explant (figure 4, solid bar).

When these high glucose cultures were transplanted to diabetic maternal hosts, the total insulin content increased. Following ten days in vivo, transplants to both the kidney and eye sites contained an average of 98 μU. insulin/transplant (figure 4, cross-hatched bars), representing a 96 per cent increase in the total insulin content ($p = .003$). The islets in these transplants remained degranulated. Transplants to the kidney site maintained a beta granulation index of 11 while transplants to the eye had an index of 25.

When pancreases from fetuses of diabetic mothers were grown in organ culture on the low glucose medium, their insulin content increased from 0.48 ± 0.07 to 3.1 ± 0.3 μU. insulin/μg. tissue protein (figure 3, stippled bar). These cultures had developed heavily granulated islets (beta granulation index of 67). The total insulin present in the low glucose cultures (85 μU. insulin/explant) (figure 4, stippled bar) was significantly more than that in high glucose cultures (50 μU. insulin/explant) ($p = .001$). This difference in total insulin content was reflected by a correspondingly higher beta granulation index for low glucose cultures (67 compared to 11).

When these low glucose cultures were transplanted to diabetic maternal hosts for ten days, the total insulin content remained essentially unchanged. Transplants to the kidney site averaged 102 μU. insulin/transplant and transplants to the eye site averaged 90 μU. insulin/transplant (figure 4, cross-hatched bars). However, the beta granulation index for the transplants at the kidney and eye sites dropped from 67 to 19 and 38 respectively.

*Effect of pancreatic transplants on blood glucose levels in diabetic hosts* (*table* 1)

The average blood glucose level in twenty-one diabetic females (Group 1) from Day 13 to 20 of pregnancy was 391 ± 8 mg./100 ml. During the four days following delivery by hysterectomy and prior to transplantation, the blood glucose level averaged 427 ± 13 mg./100 ml. During the subsequent four-day transplant period for which the diabetic mothers received four transplants of organ cultures, the blood glucose level averaged 410 ± 15 mg./100 ml.

In a second group of twenty-four diabetic females (Group 2) the average blood glucose level from Day 13 to 20 of pregnancy was 385 ± 7 mg./100 ml. During the four days following delivery by hysterectomy and before transplantation, the blood glucose level averaged 456 ± 8 mg./100 ml. During the subsequent ten-day transplant period for which the diabetic mothers received eight transplants of organ cultures, the blood glucose level averaged 437 ± 8 mg./100 ml.

In a control series of five animals (Group 3) the blood glucose level averaged 361 ± 18 mg./100 ml. from Day 13 to 20 of pregnancy. During the first four days following delivery by hysterectomy, the blood glucose level averaged 422 ± 10 mg./100 ml. No transplants were made in these animals and their blood glucose levels were determined for the next four to ten

days. During this period, these animals had a blood glucose level that averaged 425 ± 20 mg./100 ml.

Statistical analysis of the change in blood glucose level (before and after transplantation) of all forty-five animals receiving transplants (utilizing the paired comparison "*t*" test) indicates that the observed decrease, although small, was statistically significant ($p = .003$).

## DISCUSSION

The beta cells in organ cultures of fetal rat pancreas continue to function in vitro, releasing large amounts of insulin into the incubation media. A high level of glucose in the media during the first forty-eight-hour incubation period appeared to stimulate a greater release of insulin (27 per cent) from cultures of normal fetuses when compared to similar cultures grown at low glucose levels ($p = .003$) (figure 2). These pancreases were heavily granulated (representing stored insulin) at the time of explant. A similar stimulation was not observed in cultures from fetuses of diabetic mothers which were predominantly degranulated (little stored insulin) at the time of explant. Following the second forty-eight-hour culture period, an increase in the insulin content of the media was observed in all types of cultures ($p = .001$) (figure 2). Our evidence indicates that this increase is due, in part, to the continued growth of new islet tissue during the in vitro period. At the end of the second forty-eight-hour culture period, the insulin levels in the culture media were similar irrespective of glucose concentration. This lack of an insulin response to high glucose following extended organ culture has been previously reported and suggests an adaptation of the cultured islets to high glucose levels.[7,8]

The observations presented in figure 3 suggest that during the four days in organ culture a large increase occurred in the amount of islet tissue. This is further supported by our quantitative studies,[9] in which the islet volumes of fetal pancreas and organ cultures of fetal pancreas were compared by a linear scanning method,[10] and is consistent with other investigators who have reported endocrine differentiation and growth in vitro.[11-14] When fetal pancreas from normal mothers was grown in organ culture on high glucose medium, the insulin content of the tissue increased by over 250 per cent (from 0.49 to 1.8 μU. insulin/μg. tissue protein). This increase in insulin content cannot be attributed to changes in beta granulation since the islets in these cultures became degranulated (lost stored insulin) during the in vitro period (beta granulation index declined from 46 to 32). The increased insulin content presumably results from the growth of new islets and/or hyperplasia of existing islets during organ culture. Similar results were obtained when fetal pancreas from diabetic mothers was grown in organ culture (figure 3). Following four days on high glucose media, the insulin content of the cultured tissue had increased by over 350 per cent (from 0.48 to 2.2 μU. insulin/μg. tissue protein). Only a small part of this increase in insulin content can be attributed to changes in beta granulation (stored insulin) since the islets remained degranulated during the in vitro period (initial beta granulation index of 7 remained essentially unchanged at 11). An increase in total islet tissue during organ culture would explain the large increase in insulin content. Cultures grown on low glucose media also exhibited large increases in insulin content following four days in organ culture; cultures from fetuses of normal mothers increased by 329 per cent and those from diabetic mothers increased by over 540 per cent. The index of beta granulation also increased greatly in these cultures; from 46 to 72 in cultures from normal fetuses and from 7 to 67 in the diabetic series. It is suggested that the increase in insulin content represents both an increase in stored insulin (beta granulation) and in the total amount of islet tissue.

The total extractable insulin content of the organ cultures reflected the degree of beta granulation in the islets (figure 4). Low glucose cultures from normal fetuses (stippled bar) developed heavily granulated islets (index of 72) and had an insulin content of 106 ± 9 μU. insulin/culture. Similar cultures grown on the high glucose media (solid bar) in which the islet beta granulation was substantially less (index of 32) had a lower insulin content of 70 ± 6 μU. insulin/culture. In the diabetic series, similar results were observed. Here, the heavily granulated low glucose cultures (representing stored insulin) had a 70 per cent higher insulin content than the degranulated high glucose cultures (figure 4).

We observed that the degree of beta granulation in islets of transplanted organ cultures was inversely related to the level of glucose in the host's blood.[1] Ten-day transplants to normal maternal hosts had islets with a beta granulation index of maximum 75. Similar grafts to diabetic hyperglycemic hosts had an index averaging less than 40. It is now clear that the insulin content of such transplants is also inversely related to the blood glucose level in the maternal hosts. Transplants for ten days to both the eye and kidney sites in normal hosts contained from 32-84 per cent more extractable insulin than did similar transplants to diabetic hyperglycemic hosts (figure 4, cf. striped and cross-hatched bars).

A growth of islet tissue during the transplantation period is indicated by the changes in total insulin content of the transplants following ten days in vivo (figure 4). When low glucose cultures from normal fetuses (stippled bar) were transplanted to normal maternal hosts (striped bars) the insulin content of the tissue increased by 54 to 57 per cent (from 106 μU. insulin/culture to 164-166 μU. insulin/transplant). Since the degree of beta granulation in the islets remained essentially unchanged during the transplantation period (72 for cultures and 75 for transplants), the increase in insulin content presumably resulted from a proliferation of beta cells as new islets and/or from hyperplasia of existing islets. Fetal pancreatic explants from diabetic mothers grown on low glucose media, contained an average of 85 ± 4 μU. insulin/culture (figure 4, stippled bar) and had a beta granulation index of 67. Following ten days of transplantation to diabetic hyperglycemic hosts, the insulin content of the grafts had increased slightly to 90 to 102 μU. insulin/transplant (cross-hatched bars). However, the degree of beta granulation (stored insulin) had dropped in grafts at both sites; from 67 to 38 at the eye and from 67 to 19 at the kidney. A large decrease in stored insulin accompanied by an increase in total insulin is again, indicative of an increased islet volume in the transplant. This suggestion is supported by further quantitative studies (in progress) in which the islet volumes of organ cultures and transplants of organ cultures are compared. Preliminary results indicate that the transplants do have an increased islet volume (~ 50 per cent).

In the hyperglycemic diabetic hosts, a measurable drop in their average blood glucose level was observed during the transplant period when compared to the four-day period just prior to transplantation (table 1, figure 5). The drop averaged 17 to 19 mg./100 ml. (4 per cent) and was statistically significant approaching the 99 per cent confidence level. A similar drop was not observed in a control series of diabetic animals which did not undergo transplantation. Examination of the blood glucose profiles of the transplanted hosts (figure 5) indicates little effect of the transplant during the first forty-eight hours, presumably when vascularization of the grafts occurred. Lowest blood glucose levels (maximum effect of the transplant) were found between Days 3 and 5 following transplantation and postoperative recovery of the animals. This finding further suggests that the beta cells grown for a period in organ culture and subsequently transplanted to diabetic rats, remain functionally active i.e., they synthesize and release insulin into the host's blood stream. Quantitative studies of the organ cultures prior to transplant indicate that islet tissue represents about 6 per cent of explant volume.[9] It can be estimated that the alloxan diabetic hosts in this study received islet tissue equivalent to about 2 to 4 per cent of the islet volume of an adult rat. Partial pancreatectomy studies indicate that 10 per cent of the pancreas is necessary for blood glucose homeostasis.[15] Therefore we could not expect large decreases in host hyperglycemia. Further studies are underway in which larger numbers of organ cultured pancreatic explants are transplanted to diabetic hosts.

The results reported indicate (1) that the insulin content of pancreatic organ cultures and transplants of organ cultures is reflected by the degree of islet beta granulation in the tissue; (2) that differentiation and growth of the islets of Langerhans occurs during organ culture and continues during subsequent transplantation of the cultures to diabetic and normal maternal hosts; and (3) that transplants of organ cultures are functionally competent and release insulin in response to host hyperglycemia.

## ACKNOWLEDGMENT

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## *Galactose Toxicity and Alterations in Brain Metabolism in the Chick*

*Chicks fed a diet containing 40 per cent galactose incorporated less P-32 into brain phosphatidylinositol than did controls. The levels of brain glucose and ATP were also significantly reduced in the groups fed galactose, and galactose levels were greatly increased. The reduction in glucose level and in the synthesis of phosphatidylinositol may be related to the convulsive seizures in chicks fed galactose. The chick appears to be a sensitive model system for study of metabolic changes produced by high tissue levels of galactose.*

In the inherited metabolic condition of galactosemia in human beings, galactose cannot be converted to glucose because of the low levels of the enzyme galactose-1-phosphate uridyltransferase, which catalyzes the conversion of galactose-1-phosphate to glucose-1-phosphate. As a consequence, galactose, galactitol, and galactose-1-phosphate accumulate. If lactose (or galactose) is not removed from the diet of affected infants, development of cataracts and mental retardation may occur, provided that the child survives the initial acute illness and liver damage which the disease causes (D. Y.-Y. Hsia and M. E. O'Flynn, in *Modern Nutrition in Health and Disease,* Fourth Edition, M. G. Wohl and R. S. Goodhart, Editors, p. 1058. Lea and Febiger, New York, 1968).

Despite the knowledge of the enzymatic pathways in the conversion of galactose to glucose, there is still little information on the biochemical mechanisms by which accumulation of galactose or its metabolites produces the symptoms observed, especially those of mental retardation. Changes in the metabolism of myo-inositol in galactose toxicity in rats and human beings have been reported (H. J. Wells and W. W. Wells, Biochemistry 6:1168, 1967; R. Quan-Ma et al. Am. J. Dis. Child. *112*:477, 1966). Cataracts and hepatic cirrhosis may even be present at birth in infants (Nutrition Reviews *28*:55, 1970), and cell division in the fetal rat brain can be reduced when the maternal diet is high in galactose (Ibid., loc. cit.). Neurological degeneration in the brain of chicks fed galactose has been reported (R. H. Rigdon, J. R. Couch, C. R. Creger, and T. M. Ferguson, Experientia *19*:349, 1963), and the chick is even more sensitive to dietary galactose than

(*Continued on page* 208)