Insulin Content of Microdissected Fetal Islets Obtained from Diabetic and Normal Rats

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SUMMARY

1. "Insulin" content of microdissected islets was determined using the epididymal fat pad assay method. Measurements were made on fetal islets obtained from both normal and diabetic mothers and from normal postnatal rats up to five weeks of age.

2. The insulin content of the normal fetal islets, expressed as units "insulin" per gram dry weight of tissue, increases progressively with increasing fetal age; at birth the value approximates that of the adult.

3. The insulin content of the fetal islets obtained from diabetic mothers is between 7 and 19 per cent of the corresponding normal value.

4. In the acid-alcohol extracts of the pancreas of seventeen-day-old fetuses, small though measurable amounts of "insulin" were detected viz.: 0.23 units per gram wet weight of normal pancreas. There was a progressive increase in the extractable "insulin" content of the fetal pancreas with age; the "insulin" content of the twenty-one-day normal fetal pancreas was 5.3 units per gram of tissue. In the pancreas of the fetuses from diabetic mothers, the values were about one third of those in the normal.

5. Thus measurable amounts of insulin-like activity were found in the pancreatic tissue of seventeen-day-old fetuses; this is prior to the time that beta cell granules could be detected cytologically using the aldehyde fuchsin staining method.

6. The insulin content of the islet tissue remained relatively constant during the first five weeks of postnatal development.

The cytological observations of Frye[1] and Kim et al.[2] suggested that the fetal islets in the rat begin functioning at the seventeenth or eighteenth day of gestation. Although there was no definite evidence of beta cell granulation in the fetal islets at the seventeenth day, beta granules were seen by the eighteenth day of gestation. Munger,[3] using electron microscopy, stated that he was able to identify beta granules in the "immature" mouse islets by the thirteenth or fourteenth day of embryonic development.

Frye[1] and Kim et al.[2] observed that both the size and number of islets, as well as the number of recognizable beta cells within the islets, increased with increasing age of the fetus. This cytologic observation suggests that the insulin content of the islets should likewise increase with the age of the fetus. It is important, therefore, to correlate the amount of insulin in the islets as determined by the bioassay method with the number of beta cell granules and their time of appearance. Direct determination of the insulin content of fetal islets had not been reported previously.

In this study we have measured the insulin content of (a) microdissected islets and of whole pancreas removed from fetuses of normal and diabetic mothers, and (b) islets from neonatal rats. The epididymal fat pad method used in this study measures insulin-like activity; other hormones[4-7] and unrelated substances[8] likewise stimulate the in vitro glucose oxidation by the fat pad. However, the insulin-like activity measured in the microdissected islets is primarily due to insulin for the following reasons: (a) The biological activity can be completely blocked by the addition of small amounts of anti-insulin serum from guinea pigs (AIS-GP). (b) The amount of other hormones necessary to elicit an equivalent insulin-like activity would greatly exceed the actual weight of islet tissue taken for analysis.

MATERIALS AND METHODS

Three- to five-month-old virgin female rats of the Holtzman strain, weighing about 250 gm., were fed a diet of Purina Fox Chow ad libitum. The females were mated with vigorous young males of the same strain. The length of gestation was determined by witnessing mating or by examining the vagina each morning for the presence of sperm.

On the tenth day of pregnancy the rats were fasted overnight and given a single intravenous injection of alloxan (40 mg. per kilogram of body weight).[9] Most animals used had a blood glucose level greater than 300 mg. per 100 ml. (after the twelfth day of pregnancy). Two animals had blood glucose levels between 250 and 300 mg. per 100 ml. at the time of sacrifice, but the values were higher at earlier times. The rats were bled from the tail vein, and blood glucose was determined by the Folin-Malmros method.[10] The animals were decapi-

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tated by guillotine, the abdomen opened and the fetuses removed. The pancreatic tissue was quickly excised and frozen in liquid nitrogen at −196° C. The frozen pancreas was mounted on blocks at −20° C., using a 4 per cent gum tragacanth solution in 0.9 per cent NaCl.[11] The pancreas was sectioned at 20 microns and the frozen sections lyophilized at −40° C.[12] We have sectioned the embryonic tissue within a few days after removal in order to avoid dehydration which may produce distortion in morphologic details.

Islets can be identified easily in both stained and unstained lyophilized sections of fetal pancreas by the nineteenth day. In the eighteen-day normal fetus, sites of islet differentiation could be recognized in some instances; these were isolated for insulin assay. The islets were microdissected under a binocular microscope at a magnification of 60×; they were freed of acinar tissue. The samples were weighed on a quartz fiber balance[13] with a sensitivity of 0.020 μg./division.

In fetuses and newborns, approximately four islets, weighing between 0.1 and 0.4 μg., were pooled for insulin assay, while in the neonatal animals individual islets were used. The weighed samples of microdissected islets were added directly to the incubation medium. Samples of whole pancreas (containing both islet and acinar tissue), weighing between 10 and 50 μg.,* were likewise added to the incubation medium.

Insulin activity was determined using the epididymal fat pad method of Renold et al.[14] Epididymal adipose tissue was removed from rats (Holtzman strain) weighing about 250 gm., and gently floated in a petri dish on bicarbonate buffer medium. Care was taken to avoid excessive handling of the fat pads. Uniformly thin portions of both epididymal fat pads were cut into six or seven pieces of equal size. Careful selection of the fat pads reduced the variation in the response. Each piece was then placed in a separate 35-ml. vial containing the following: one milliliter of bicarbonate buffer (*p*H 7.4), 2 mg. gelatin; 1 mg. glucose; and 0.2 μc. glucose-1-C-14 (specific activity about 15 μc. per milligram dissolved in 0.1 ml. water).

Three pieces of the epididymal fat pad from each rat were used for the three insulin standards, i.e., 0, 25, and 500 μU. per milliliter of buffer; the remaining pieces were used for the analysis of the unknowns. The vials were stoppered, equilibrated with 95 per cent oxygen plus 5 per cent carbon dioxide, and incubated for three hours at 37° C. in a Dubnoff shaker (90 to 100 oscillations per minute).[15] The reaction was stopped by adding 2 ml. of 2 N H_2SO_4 to each vessel; 0.5 ml. hyamine was likewise introduced into a microbeaker suspended above the medium. During these manipulations, care was taken to keep the vessels tightly stoppered. The vials were incubated and shaken for another hour. The hyamine containing the trapped C-14-O_2 was quantitatively transferred to the counting vessel with three rinses of scintillation fluid [0.4 per cent 2,5 diphenyloxazole (PPO) plus 0.01 per cent 1, 4-bis 2-5-phenyloxazolylbenzene (POPOP) in toluene]. Each vial, containing 10 ml. fluid, was counted for ten minutes in an automatic counter* at an efficiency of 51 per cent. The insulin content of the unknown was calculated using the individual standard assay curves, and expressed as units of insulin per gram (dry weight) of tissue.

The extractable insulin in the fetal pancreas was also determined by freezing the tissue in liquid nitrogen. Pancreas from three to eight fetuses (obtained from a single mother rat) was weighed in a frozen state on a torsion balance and then homogenized with 10 per cent trichloracetic acid (TCA). The homogenate was centrifuged, the supernatant decanted and the residue extracted two or three times with 0.1 to 1.0 ml. quantities of acid alcohol (15.0 ml. concentrated HCl per 1 liter of 75 per cent ethanol). The extracts were pooled and diluted to a known volume (0.25 to 10 ml.).

The extracts of fetal pancreas from both normal and diabetic mothers were analyzed on the same day. Each extract was assayed at two different concentrations: viz: by adding 5, 10, or 20 μl. aliquots of the extracts to the incubation vials. The alcohol was removed under nitrogen, and 1 milliliter incubation medium was added. The fat pad pieces from six rats were used. The response of the two dilutions of each extract was compared with that obtained with the insulin standards using the tissue pieces obtained from a single rat. The insulin content was expressed as units per gram (wet weight) of pancreas.

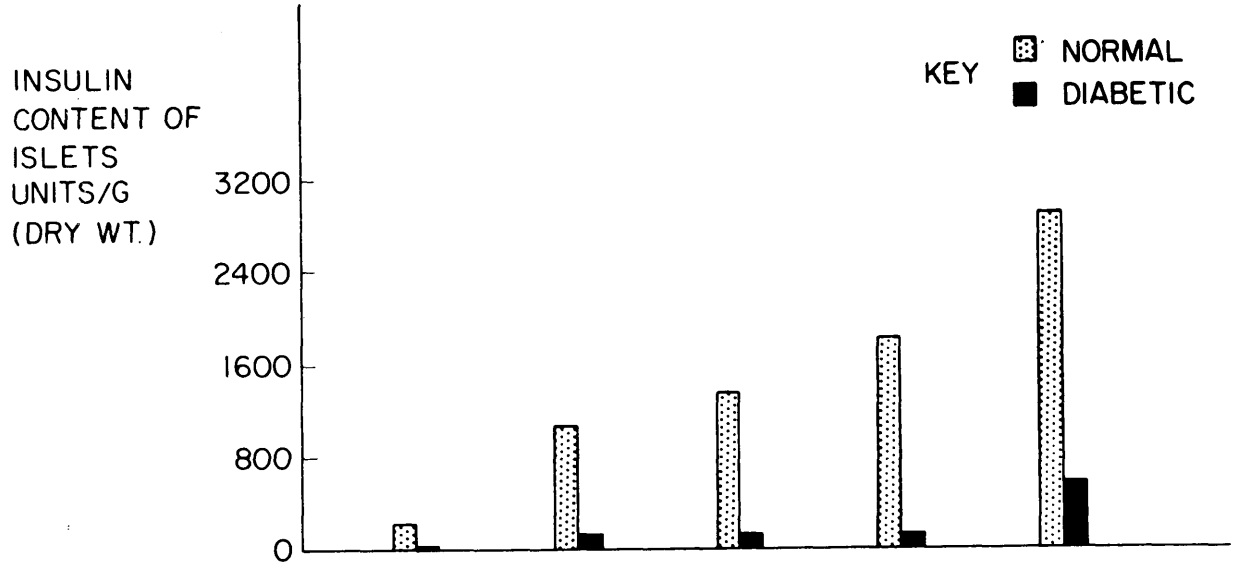
RESULTS

Insulin content of microdissected islets

The insulin content of islets from fetuses of normal and diabetic rats at various stages of gestation is shown in figure 1. In the normal fetus, the insulin content increases progressively with the age of the embryo. The linear regression of the slope of change in the insulin content with age is expressed by the equation $y = 611x - 10756$. The observed increase was highly significant ($p = <0.001$). The insulin content of the

*Weighed on Cahn Electrobalance.

*Tri-carb Automatic Scintillation Counter, Packard Instrument Company, LaGrange, Illinois.



DAY OF GESTATION	18	19	20	21	AT BIRTH
NO. OF LITTERS	3 1	3 4	3 3	3 4	2 2
NO. OF DETERMINATIONS	10 2	14 12	13 9	19 18	7 8
INSULIN CONTENT U/G (DRY WT.)	216 35	1060 120	1339 121	1804 123	2900 561
S.E.	52 —	228 32	273 51	202 17	389 149
P(N VS. D)	—	< 0.0001	< 0.0001	< 0.0001	< 0.0001
RATIO D/N x 100	16.2	11.3	9.0	6.8	19.3

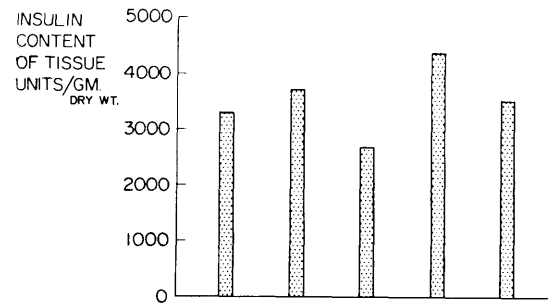
FIG. 1. Insulin content of islets — fetal tissue from normal and diabetic mothers. The linear regression of the increase in the insulin content of normal fetal islets with increase in fetal age is expressed by the slope $y = 611x - 10756$ with x = day of gestation. The standard deviation of the regression coefficient is 64; and the test of significance $t = 9.6$; degrees of freedom (d.f.) 4; ($p = <0.001$).

pancreatic islets obtained from eighteen-day embryos averaged 216 units per gram dry weight, while the value was 1,060 units per gram in the nineteen-day embryo. At birth the insulin content of the microdissected islets averaged 2,900 units per gram (dry weight), a value approaching that found in the adult.[16]

In evaluating the sampling procedure, we found that the variability in the insulin content within the islets of the same pancreas was similar to the variability between animals within the same age group.

In the fetuses obtained from diabetic mothers, the insulin content of the microdissected islets was significantly less than that of the corresponding normal. The values averaged between 6.8 and 19.2 per cent of the normal at all the stages of gestation.

The insulin content of the islets of neonatal rats, aged one to five weeks, is shown in figure 2. The values



POSTNATAL AGE	1	2	3	4	5 WEEKS
NO. OF ANIMALS	3	3	3	2	2
NO. OF DETERMINATIONS	10	10	10	6	8
INSULIN CONTENT U/G (DRY WT.)	3291	3737	2679	4373	3532
S.E.	609	579	510	647	944

FIG. 2. Insulin content of islets from postnatal rats.

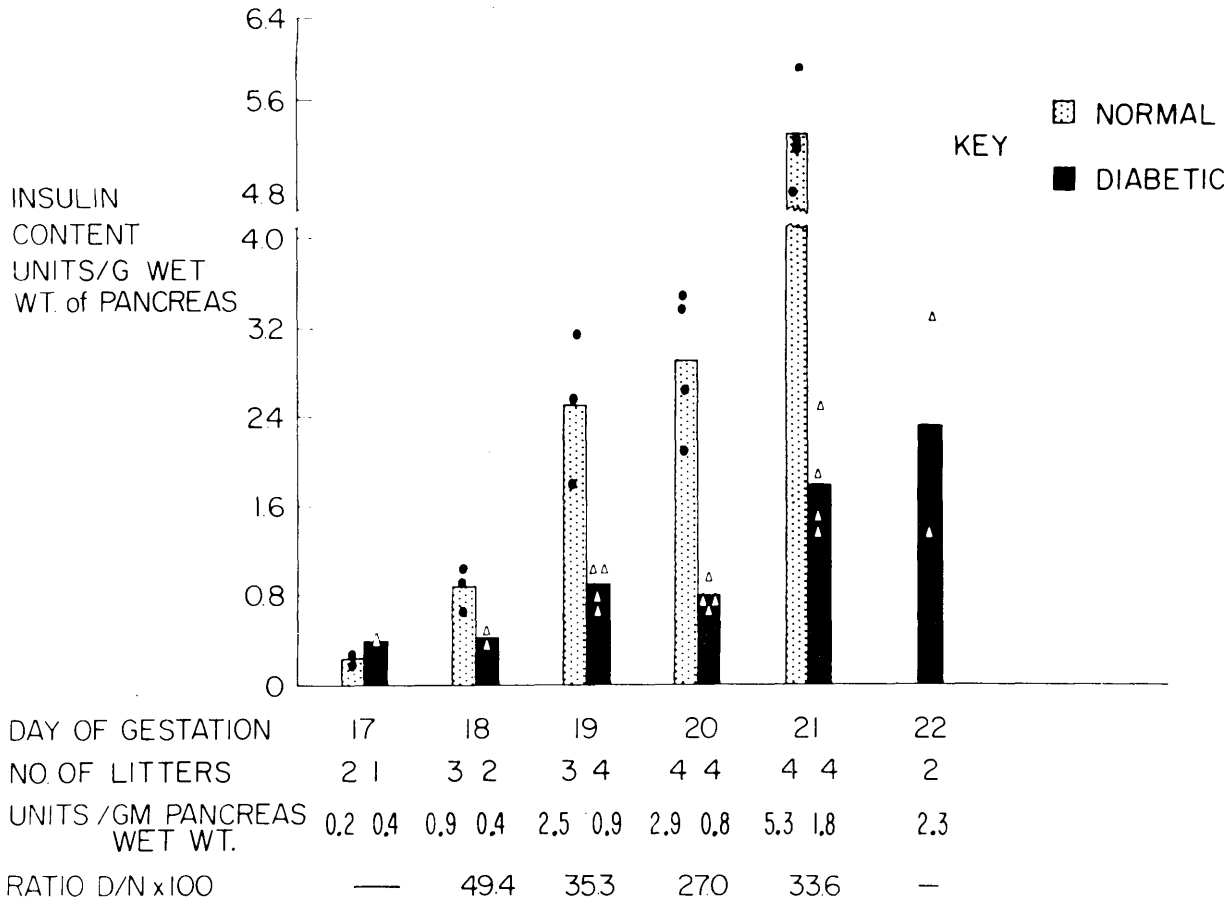


FIG. 3. "Insulin" content of acid-alcohol extracts of pancreas obtained from fetuses of normal and diabetic mothers. The linear regression of the increase in the "insulin" content of pancreas from normal fetuses with age is expressed by the slope $y = 1.22x - 20.78$, with x = day of gestation. The standard deviation of the regression coefficient is 0.149; and the test of significance $t = 8.1$ d.f. $= 4$; ($p = <0.005$).

range between 3,300 and 4,400 units per gram (dry weight). The differences between the various age groups were not statistically significant.

Acid-alcohol extractable "insulin" in the pancreas

As shown in figure 3, the amount of extractable "insulin" from the normal rat pancreas increases with the age of the fetus. The linear regression of the slope can be expressed by the equation $y = 1.22x - 20.78$ ($p = <0.05$). On the seventeenth day, the insulin content averaged 0.23 units per gram (wet weight) of pancreas, whereas, on the twenty-first it was 5.3 units. The acid-alcohol extractable insulin was lower in the fetal pancreas obtained from the diabetic mothers; the average values increased from 0.39 units to 1.78 units per gram during the corresponding time periods. At most instances the fetal pancreas obtained from diabetic mothers contained about one third as much "insulin" as was found in the corresponding normals.

"Insulin" content of the lyophilized pancreatic tissue

The "insulin" content of the lyophilized pancreatic tissue was assayed by adding samples directly to the incubation media; these values likewise increased with embryonic age. In the seventeen-day-old fetal pancreas, the insulin content was 15.7 units per gram (dry weight) of tissue; at birth the value was 31.7 units. This difference was significant ($p = <0.002$).

The insulin content of fetal pancreas obtained from diabetic mothers was less than one half that in the corresponding normal controls. At the seventeenth day, the difference was not significant; however, at all later stages of embryonic development, the insulin content of fetal pancreas from diabetic mothers was significantly lower than the corresponding normals ($p = <0.01$).

DISCUSSION

Although many studies have been carried out on the

differentiation of the embryonic pancreatic islet tissue in the rat,[1,2,17,18] the insulin content has been measured only in the embryonic calf pancreas.[19,20]

Using the epididymal fat pad method, we could detect insulin in the microdissected rat islets on the eighteenth day and in the whole pancreas on the seventeenth day of embryonic development. It was difficult to microdissect the islets on the seventeenth day of gestation, and hence no determinations were made at this time. Bioassays were carried out on the whole pancreas at the sixteenth day, but no detectable "insulin" was found in the small amounts of the tissue available.

Cytological studies[1,2] have demonstrated that morphological differentiation of islets occurs between the seventeenth and eighteenth day. Definitive beta granules, stained by the aldehyde fuchsin method, first appear in the rat about the eighteenth day of gestation. Since the beta granules presumably represent stored insulin,[21] the cytologic observations suggest that insulin secretion may likewise begin at this time. It is possible, however, that insulin may be synthesized and stored in a nongranular form at an earlier stage of fetal islet development. Prior to the eighteenth day, the amount of insulin may be below the limit of its cytologic detection as beta cell granules. It is relevant to point out here that Munger,[3] using the electron microscope, reports that the beta cells begin to differentiate in the mouse at thirteen to fourteen days of gestation. Although beta granules were identified in electron micrographs, "these 'immature' cells did not stain by the aldehyde fuchsin method."

Our bioassay data demonstrating a progressive increase in the insulin content of the islet from the eighteenth day to birth parallels the progressive granulation of the islets observed cytologically.[1,2]

The epididymal fat pad responds to insulin as well as to a number of other hormones. Growth hormone,[4] prolactin,[5] glucagon,[6] and adrenocorticotropin (ACTH)[7] can stimulate the in vitro oxidation of glucose to carbon dioxide by the epididymal fat pad tissue. Although glucagon is presumably synthesized by alpha cells, according to Hard[17] and Nerenberg,[18] the definitive alpha cells do not appear until two to four days after birth. Also, for the reasons stated in the introduction, it is unlikely that the "insulin" activity in the microdissected embryonic islets could be due to glucagon or other hormones.

The pancreas of the eighteen- to twenty-one-day fetuses from diabetic mothers had an insulin content of about one half to one third that found in the corresponding normal controls. (This observation parallels those of Frye[1] and Kim et al.[2] who found diminished numbers of beta granules in the pancreatic islets of fetuses of diabetic mothers.) According to Kim et al.,[2] when the maternal blood glucose was greater than 240 mg. per 100 ml., the beta cells in the fetal islets invariably lacked granulation; only about 0.17 per cent of the islet cells in the diabetic groups were granulated, as compared to 41.6 per cent for the control fetuses.

Thus the diminished insulin content of the islets in the fetuses of the diabetic mothers, as measured by the bioassay technic, correlates with the cytologic studies, which indicated a decreased insulin content on the basis of diminished beta cell granulation. This decrease in the insulin content may result from a release of insulin from the fetal islets in response to maternal (and fetal) hyperglycemia.

There is no reason to believe that the injection of alloxan prior to the time of the differentiation of the islet tissues could have affected the development of the beta cells. The islets in the fetuses of the diabetic mothers were larger than normal. The offspring of these alloxan injected mothers do not show any manifest signs of diabetes in postnatal life.[2] According to Frye,[1] the average number of islets per unit area of the pancreas in the fetuses of normal and diabetic rats is almost identical at eighteen and nineteen days of gestation; Frye was, however, unable to compare the total quantity of islet tissue in the pancreases of these animals "because of the lack of differentiation and definitive organization of the islet tissue at this time." In the twenty-, twenty-one-, and twenty-two-day-old embryos obtained from diabetic mothers, he found a significant (almost two-fold) increase in the total quantity of islet tissue, as compared to the corresponding normal animals. In studies carried out in this laboratory,[22] the volume of islet tissue, in the near-term fetuses (twenty-one days, fifteen hours) from diabetic mothers, was found to have increased from 3.2 to 5.9 per cent of the pancreas, while the per cent of islet cells with beta granules was decreased from 43 to 13.

It should be emphasized here that the insulin content per unit weight of islet tissue in the fetuses of the diabetic mother (expressed as per cent of those of the normal) is decreased to a greater extent than is the amount of insulin extracted from the whole pancreas. Thus, at twenty-one days, the insulin content of the islet tissue from the fetuses of the diabetic mothers was 6.8 per cent of the normal, whereas in the acid-alcohol extracts of the fetal pancreas from the diabetic

mothers it was 33 per cent of the corresponding normal. This discrepancy is only partially explained on the basis of the observed increase in the islet volume in the fetuses of the diabetic animals.

The insulin content of the microdissected dried islets is several hundred times that of the fresh pancreas. This is to be expected inasmuch as the islet tissue comprises 0.5 to 3 per cent of the pancreas and the pancreas contains about 75 per cent water.

The "insulin" content of the lyophilized pancreas corrected for water content is considerably greater than the "insulin" extracted by acid-alcohol. The reason for this apparent discrepancy is not known.

SUMMARIO IN INTERLINGUA

Contento de Insulina in Microdissecate Insulas Fetal, Obtenite ab Rattas Diabetic e Normal

1. Le contento de insulina in microdissecate insulas esseva determinate per medio del methodo de essayage a cossino de grassia epididymal. Le mesurationes esseva facite in insulas fetal, obtenite ab matres normal e diabetic, e in insulas ab normal rattos de etates de usque a cinque septimanas.

2. Le contento de insulina in le insulas fetal, exprimite como unitates de "insulina" per gramma de peso sic de tissu, cresce progressivemente con le avantiamento del etate fetal. A nato, le valor es approximativemente illo trovate in rattos adulte.

3. Le contento de insulina del insulas fetal obtenite ab matres diabetic es inter 7 e 19 pro cento del correspondente valor normal.

4. In extractos a acido-alcohol ab le pancreas de fetos de dece-septe dies de etate, micre sed mesurabile quantitates de "insulina" esseva detegite, specificamente 0,23 unitates per gramma de peso humide de pancreas. Esseva notate un augmento progressive in le extrahibile contento de "insulina" del pancreas fetal con le avantiamento del etate. Le contento de "insulina" del normal pancreas fetal post vinti-un dies de gestation esseva 5,3 unitates per gramma de tissu. In le pancreas del fetos ab matres diabetic, le valores esseva circa un tertio de illos trovate in normales.

5. Assi mesurabile quantitates de activitate insulinosimile esseva trovate in le tissu pancreatic de fetos de dece-septe dies de etate. Isto precede le tempore al qual granulos de cellula beta poteva esser detegite cytologicamente per medio del methodo tincturatori a fuchsina aldehydic.

6. Le contento de insulina del tissu insulari remaneva relativemente constante durante le prime cinque septimanas del disveloppamento postnatal.

ACKNOWLEDGMENT

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Enzymatic Defects in the Hereditary Fructosurias

. . . Hereditary fructose intolerance is characterized clinically by manifestations of hypoglycemia after the ingestion of fructose-containing foods or the parenteral administration of fructose (E. R. Froesch, A. Prader, H. P. Wolf, and A. Labhart, Helv. Paed. Acta *14*:99, 1959). The symptoms may include nausea, vomiting, sweating, somnolence, and coma. An excessive elevation of the blood and urine fructose concentrations, a decrease in blood glucose, and a prolonged lowering of the serum inorganic phosphate occur after fructose administration in patients with this condition. The normal pathway of fructose utilization in the liver, and possibly in other tissues, involves a phosphorylation to fructose-1-phosphate which is catalyzed by fructokinase and a splitting of fructose-1-phosphate to a triose and a triose phosphate under the influence of an aldolase. Since hereditary fructose intolerance is associated with a diminished rate of fructose utilization and a prolonged lowering of the serum inorganic phosphate after fructose administration, a deficiency of hepatic fructose-1-phosphate aldolase activity with a consequent accumulation of fructose-1-phosphate was postulated.

E. A. Nikkila, O. Somersalo, E. Pitkanen, and J. Perheentupa (Metabolism *11*:727, 1962) have measured the activities of several enzymes involved in fructose and glucose metabolism in the liver, muscle, and erythrocytes of two sisters with hereditary fructose intolerance and two healthy children of corresponding ages. In liver tissue from the affected siblings, fructose-1-phosphate aldolase activity was almost absent and fructose diphosphate aldolase activity was reduced to about 10 per cent of that found in the normal subjects. No enzymatic abnormality was discovered in muscle tissue or erythrocytes. Other investigators have also found diminished hepatic aldolase activities in patients with hereditary fructose intolerance (H. G. Hers and G. Joassin, Enzymol. Biol. Clin. *1*:41, 1961); F. Schapira, G. Schapira, and J. C. Dreyfus, Ibid *1*:170, 1961). Hepatic fructokinase activity was normal or only moderately reduced.

Benign fructosuria is an asymptomatic condition which is usually discovered during routine testing of the urine for reducing substances. The majority of patients with this disease have only traces of fructose in the urine and blood in the fasting state, but following the ingestion of fructose or fructose-containing polysaccharides, there is an abnormally large increase in the concentration of fructose in the blood and urine. Whereas only 1 or 2 per cent of an ingested fructose load is excreted in the urine of normal subjects, 10 to 20 per cent appears in the urine of patients with benign fructosuria. Fructose administration is followed by a small decrease rather than a rise in the blood glucose concentration, the increases in the respiratory quotient and blood lactic acid concentration are less than normal, and the normal fall in the serum inorganic phosphate concentration is absent.

These observations indicated a diminished utilization of fructose by the usual pathways and led to the hypothesis that benign fructosuria resulted from a deficiency of hepatic fructokinase. This hypothesis has been confirmed by Schapira, Schapira, and Dreyfus who found an absence of fructokinase activity in the liver biopsy specimen of a twenty-one-year-old male patient with asymptomatic fructosuria. Aldolase activity in this patient was normal. . . .

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