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# Metabolic Abnormalities in Liver and Pancreas of a Diabetic (Toronto-KK) Strain of Mouse

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# METABOLIC ABNORMALITIES IN LIVER AND PANCREAS OF A DIABETIC (TORONTO-KK) STRAIN OF MOUSE

by

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Michael C. Appel

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# SURVEY OF THE LITERATURE

## Spontaneous Diabetes in Laboratory Animals

Progress in understanding the pathogenesis of diabetes mellitus and improved methods of therapeutic control have dramatically reduced the morbidity and mortality resulting from the disease. However, the precise etiological factors responsible for the development of diabetes mellitus presently remain obscure. The inability to pursue extensive biochemical and physiological experimentation using diabetic humans has produced a need for animal models which manifest comparable genetically inherited diabetic syndromes. The use of such animal models in diabetes research offers numerous advantages including: strict environmental regulation, dietary manipulation, selective inbreeding with relatively rapid generation of progeny and an ability to initiate in-depth biopathological investigations. At present, numerous strains of laboratory animals have been successfully inbred which manifest a wide variety of diabetic syndromes and are described in Table 1. Reports concerning spontaneous diabetes in animals are surprisingly numerous and several excellent reviews have been described (Sirek and Sirek, 1970), (Stauffacher and Renold, 1971), (Bray and York, 1971), as well as two symposium issued (Renold and Dulin, 1967), and (Renold, et al., 1970).

TABLE 1

VARIOUS TYPES OF SYNDROMES ASSOCIATED WITH SPONTANEOUSLY DIABETIC MICE (*MUS MUSCULUS*) AND OTHER SMALL LABORATORY ANIMALS

A. *Mus musculus*

| Name | Gene Symbol | Transmission | Type of Syndrome | | |
|---|---|---|---|---|---|
| | | | MOD* | JOD** | Obesity |
| Lethal Yellow | $A^y$ | Autosomal dominant | + | - | + |
| Viable Yellow | $A^{vy}$ | Autosomal dominant | + | - | + |
| Intermediate Yellow | $A^{iy}$ | Autosomal dominant | + | - | + |
| Obese-Hyperglycemic | ob | Autosomal recessive | + | - | + |
| Adipose | ad | Autosomal recessive | + | - | + |
| Diabetes | db | Autosomal recessive | + | + | + |
| New Zealand Obese (NZO) | | Inbred strain (polygenic) | + | - | + |
| Japanese KK | | Inbred strain (polygenic) | + | - | + |
| Toronto KK Hybrid | | Inbred strain (polygenic) | + | - | + |
| Wellesley Hybrid | | Inbred strain (polygenic) | + | - | + |

B. Other Species

| Name | Common Name | Transmission | Type of Syndrome | | |
|---|---|---|---|---|---|
| | | | MOD | JOD | Obesity |
| *Acomys cahirinus* | Spiny mouse | ? | + | + | + |
| *Genomys tolarium* | Tuco-Tuco | ? | + | - | + |
| *Psammomys obesus* | Sand rat | ? | + | + | + |
| *Gricetulus griseus* | Chinese hamster | Polygenic | - | + | - |
| | Fatty rat | Autosomal recessive | - | - | + |

* - Maturity-Onset Type Diabetes
** - Juvenile-Onset Type Diabetes

## The Japanese KK Mouse

The Japanese KK strain of mouse was first inbred by Kondo (1957) and Nakamura and Yamada (1967) subsequently confirmed the diabetic character of the animal. The mode of inheritance of diabetes mellitus in this animal remains controversial and is poorly understood. Inheritance of the diabetic traits were first thought to be polygenic (Nakumura, 1962), however, more recently it was postulated that diabetes in this animal results from a single dominant gene with reduced penetrance (Butler and Gerritsen, 1970). The onset of diabetes in KK mice is often variable but usually occurs near three months of age. For reasons as yet unclear, incidence of diabetes is less frequent and more varied among female KK mice than among males. Metabolic anomalies in KK mice which occur with the onset of diabetes include: hyperphagia, obesity, intermittent glucosuria, hyperglycemia and hyperinsulinemia (Nakamura, 1967), (Dulin and Wyse, 1970), (Matsuo, 1971). Morphological studies on islets of Langerhans from KK mice have shown hyperplasia and hypertrophy of the islet with frequent β-cell degeneration (Yamada and Nakamura, 1969), (Shino and Iwatsuka, 1970). Glomerulosclerosis has been reported in kidneys of KK mice (Camerini-Davalos et al., 1970) and lenses from the eyes of these animals have been found to develop cataracts (Federman, 1970). Morphologic abnormalities in other organs (adenohypophysis, liver, adrenal and parathyroid) have also been reported (Nakamura and Yamada, 1967). Dulin and Wyse (1970) reported a decreased sensitivity of adipose and muscle tissues in these animals to exogenous insulin and postulated that diabetes in the KK mouse, "probably begins

with increased dietary intake, which results in increased fat deposition, decreased sensitivity of adipose tissue and muscle to insulin and a decreased tolerance to glucose."

## The Toronto-KK Hybrid Mouse

Because of the apparent complexity of the mode in inheritance and variation in the degree of homozygosity of the genes responsible for diabetes in the KK mouse, considerable variation exists with respect to the severity of the diabetic syndrome. Further, it was reported that inadvertent selection against the genes responsible for diabetes may have occurred during inbreeding of KK mice at The Upjohn Company (Dulin and Wyse, 1970). Consequently, reduced numbers of progeny displaying abnormalities associated with diabetes resulted. In an attempt to generate greater numbers of mice with increased homogeneity in the expression of diabetic characteristics, KK mice were crossed with C57BL/6J mice (The Jackson Laboratories, Bar Harbour, Maine) at the University of Toronto. The hybrid progeny resulting from this crossing were then designated as Toronto-KK mice. Incidence of diabetic characteristics was increased among Toronto-KK mice and the syndrome displayed was more homogeneous and predictable.

Diabetes in Toronto-KK hybrid mice appears to manifest the same abnormalities characteristic of diabetic syndromes of KK mice; increased food consumption, obesity, glucosuria and elevated levels of blood glucose and plasma immunoreactive insulin. In addition, similar morphologic abnormalities have been observed in islets of Langerhans of Toronto-KK mice (Soret, 1971), (Appel, et al., 1972). However, at present

little information concerning the pathogenesis of diabetes in the Toronto-KK mouse has appeared in the literature. Therefore, the investigations described in this thesis represent an attempt to more clearly define the diabetic syndrome of this animal.

## Etiology of Hyperglycemia in the Toronto-KK Mouse

Levels of blood glucose reflect the dynamic equilibrium between its utilization and production. Therefore, hyperglycemia in Toronto-KK mice may result from a decreased utilization of glucose by tissues such as adipose and muscle and/or an overproduction of glucose synthesized by tissues such as liver and kidney. As evidence that underutilization is involved in hyperglycemia, Dulin and Wyse (1970) reported a decreased rate of glucose oxidation and uptake of glucose by adipose and muscle tissues of Japanese KK mice. It is tempting to postulate, therefore, that glucose utilization might be impaired in similar tissues of Toronto-KK mice due to the similarity in genotype and phenotypic expression of diabetic syndromes in these two animals. Although underutilization of glucose may be involved, numerous types of genetically diabetic laboratory animals and chemically induced diabetic laboratory animals have also displayed abnormalities in glucose production by liver and other tissues. Hyperglycemia was accompanied by an increased activity of hepatic gluconeogenic enzymes and a subnormal or normal activity of hepatic glycolytic enzymes in obese-hyperglycemic (ob/ob) mice (Seidman, 1970), diabetic (db/db) mice (Chang, 1970), Chinese hamsters (Chang, 1970) and in Alloxan-induced

(Wagle, 1963) (Weber, 1965) and streptozotocin-induced (Chang, 1971) diabetic rats. An increased rate of glucose production would thus result from an imbalance between glycolytic and gluconeogenic processes. One may postulate, therefore, that hyperglycemia in Toronto-KK mice may also result in part from abnormalities in the activities of respective glycolytic and gluconeogenic enzymes.

## Etiology of Hyperinsulinemia Toronto-KK Mice

The elevated levels of plasma immuno-reactive insulin observed from Toronto-KK mice may result from either a diminished metabolism of endogenous insulin and/or an increased rate of insulin secretion from beta cells of the islets of Langerhans. Two lines of evidence suggest that hyperinsulinemia in these animals results from the latter condition. First, insulin extracted from pancreata of diabetic Toronto-KK mice has been shown to have a biological activity similar to that of normal mice (Wyse and Warfield, 1971). Secondly, adipose tissues from Toronto-KK mice have been shown to be responsive to stimulation by insulin in vitro (Appel and Dulin, 1971). Furthermore, morphologic anomalies from islets of Langerhans of Toronto-KK mice (Soret, 1971)(Appel et al., 1972) suggest that concomitant functional disorders may be present in insulin synthesizing and secretory components. Hyperinsulinemia associated with elevated insulin secretory activity has been reported in several diabetic laboratory animals [New Zealand obese mice (Larkins and Martin, 1972), Spiny mice (Junod et al., 1969), Obese-hyperglycemia mice (Sodoyez

et al., 1971), Diabetic mice (Malaisse et al., 1968) and the Zucker obese rat (Stern et al., 1972)] and in obese humans [(Karam et al., 1968) and Grey and Kipnis, 1971)]. The most compelling evidence favoring the hypothesis that hyperinsulinemia results from excessive insulin release, however, stems from the finding that islets of Langerhans from Toronto-KK mice have significantly greater levels of RNA and insulin biosynthetic activity than normal islets (Appel et al., 1972). Since islets of Toronto-KK mice synthesize increased quantities of insulin in response to glucose stimulation, one may infer that these islets may similarly release exaggerated quantities of insulin when stimulated by glucose.

## Methodological Approaches to Measurements of Insulin Secretion

Determinations of insulin secretion based upon measurements made in vivo are semiquantitative and often misleading. Interpretation of data obtained in vivo is complicated by the fact that the effects of various stimulating or inhibiting agents upon insulin secretion may not be due to the direct action of these agents upon islet beta cells but rather due to secondary mediating factors. Therefore, in vitro systems used for measurements of insulin secretion afford considerable advantage over in vivo systems because the direct action of substances upon insulin release mechanisms may be observed and possible secondary factors are eliminated.

Several techniques are available to measure insulin secretion in vitro. The first technique introduced by Anderson and Long (1947) and

subsequently modified by Grodsky et al. (1963) involved perfusion of the entire isolated pancreas with various bicarbonate-buffered media and an estimation of the insulin concentration in the effluent collected at various time intervals. Another technique introduced by Coore and Randle (1964) involved incubation of small pieces of pancreatic tissue in various bicarbonate-buffered media with measurement of insulin contained in the medium over various time periods. These two methods are unsatisfactory due to the face that the exocrine pancreas (Acinar tissue) comprises most of the tissue investigated and releases lytic enzymes that degrade the secreted insulin. To obviate the difficulties experienced with proteolytic degradation of insulin and to make direct investigations of islet function, methods have been devised to isolate intact islets of Langerhans from the exocrine parenchyma. Hellerstrom (1964) and Keen (1956) described methods for free-hand microdissection of metabolically intact islets of Langerhans from the pancreas. More recently Lacy and Kostianovsky (1967) reported a procedure for obtaining isolated islets using crude collagenase. This enzyme digested the interstitial collagen matrix releasing islets from the pancreatic stroma and the islets were subsequently harvested by differential gradient centrifugation.

The advent of these methods has made possible measurement of insulin release from isolated islets of Langerhans. This not only provides for optimal exchange of oxygen and nutrients, but also allows for direct and precise stimulation of islet tissues, of correlating insulin secretion with metabolic events in the beta cells, and avoids

the lytic action of exocrine proteases.

## Statement of the Problem

Although the diabetic syndrome in Toronto-KK mice has been partially characterized and presented above, little is known concerning the etiological factors responsible for the observed hyperglycemia and hyperinsulinemia. The investigations described in this thesis were conducted in order to elucidate those mechanisms responsible for these two biopathological conditions and to more clearly define the diabetic syndrome of Toronto-KK mice. Pursuant to this objective, the following investigations were initiated.

The first series of investigations were conducted to explain the seemingly paradoxical physiological condition of having elevated levels of blood glucose coincident with the exorbitant levels of plasma immunoreactive insulin. Since the liver is one of the major organs responsible for the homeostatic regulation of blood glucose, the glycolytic and gluconeogenic capacity of the liver was studied in vitro.

The second series of investigations were conducted to determine if hyperinsulinemia was the result of an excessive rate of insulin release in response to glucose stimulation. To accomplish this, a "perifusion" system was designed to measure insulin release from isolated islets of Langerhans. Also a preliminary microscopic examination of pancreata from Toronto-KK mice was undertaken to correlate the biologic data with previously observed morphologic abnormalities. Finally, numerous other determinations were made (liver protein and

glycogen concentrations, plasma and pancreatic insulin concentrations and islet insulin content) to quantitatively define the metabolic disorders associated with diabetes mellitus of the Toronto-KK mouse and to relate these results with other findings.

## METHODS AND MATERIALS

### Selection of the Animals

The Toronto-KK mice were obtained from the Upjohn colony (Dulin and Wyse, 1970) and were selected on the basis of their fulfilling the following criteria: body weights equal or greater than 35 g., blood glucose concentrations equal to or greater than 200 mg. % and plasma immunoreactive insulin levels equal to or greater than 1000 μU/ml. Male animals of approximately six-months of age were used for the experimentation. Male animals were selected because the diabetic characteristics are more uniform than in female mice and six-month old animals were selected because the diabetic syndrome appears to be most pronounced at this stage of development. C57BL/6J mice (The Jackson Laboratory, Bar Harbour, Maine) of the same age and sex served as controls. All animals were housed in stainless steel cages and were provided with food (Purina Mouse Breeder Chow) and water ad libitum. All blood sampling and sacrificing of animals were performed between the hours of 8:00-10:00 A.M.

### Blood Glucose Determination

Blood samples were obtained from the orbital venous sinus using heparinized capillary tubes and 0.05 ml. aliquots were transferred into Auto Analyzer® vials containing 1.95 ml. of 0.06 M NaF. Glucose concentrations were then determined by the Auto Analyzer® microglucose procedure described by Hoffman (1937).

## Preparation of Liver Fractions

Animals were sacrificed by exsanguination and decapitated. The livers were excised, rinsed in ice-cold 0.15 M KCl (adjusted to pH 7.4 with 0.02 M $KHCO_3$), blotted on filter paper and portions of liver were weighed in tared beakers containing 5.0 ml. of chilled 0.15 M KCl buffer. Additional quantities of 0.15 M KCl buffer were added to make a final volume nine times the weight of the liver tissue. The liver tissue was then homogenized in a glass homogenizing vessel with a Teflon® pestle until a uniform suspension was achieved. The mixture was transferred to a glass centrifuge tube and centrifuged (8,000 x g.) at 4°C. for ten minutes. Following centrifugation, the surface lipid phase was carefully removed and the supernatant transferred to polycarbonate centrifuge tubes and further sedimented (100,000 x g.) at 0° for seventy minutes. The supernatant thus obtained was carefully removed avoiding the surface lipid phase and assayed for glucokinase, hexokinase and phosphoenol-pyruvate carboxykinase. Aliquots of the 100,000 x g. supernatant were collected and frozen immediately in an acetone-dry ice bath and were used to assay pyruvate kinase and fructose-1,6-diphosphatase the following day. The remaining microsomal fraction was rinsed with 1 ml. of 0.15 M KCl buffer (pH 7.4) and the pellet agitated and transferred to a glass homogenizing vessel. The microsomal fraction was then homogenized thoroughly using a Teflon® pestle and additional 0.15 M KCl buffer was added so that the final volume of the microsomal suspension was one-fifth of the original volume of the crude liver homogenate. Aliquots

of the microsomal fraction were then assayed for glucose-6-phosphatase activity.

## Liver Protein Determination

One ml. aliquots of the crude liver homogenates described above were hydrolyzed by addition of one ml. of 2M NaOH and incubation in a water bath at 100°C. for ten minutes. The 2M NaOH hydrolysate was then centrifuged at 8000 x g. for five minutes and the supernatant fraction removed and assayed for protein content according to the Lowry procedure (1951). This procedure indirectly estimates protein content with the use of phenol reagent (Folin-Ciocalteau, Fisher Scientific Co., Pittsburg, Pa.) which undergoes a colorimetric reaction directly proportional to the relative number of tyrosine phenol groups contained per sample. The specific absorbance of this reaction was monitored at 600 nm. Bovine serum albumin (Sigma, St. Louis, Missouri) was used as a standard.

## Liver Glycogen Determination

Approximately 100 mg. of fresh liver tissue was placed in 30 ml. Corex® centrifuge tubes and digested by addition of 30% KOH (0.005 ml./mg. of tissue) and incubation in a water bath of 100°C. for twenty minutes. Water (0.025 ml./mg. tissue) and 95% EtOH (0.06 ml./mg. tissue) were added to the alkaline digest and the mixture was allowed to stand for 20-24 hrs. at 0°C. The mixture was centrifuged at 8,000 x g. for five minutes, washed twice with 66% EtOH and allowed to dry. The sediment was dissolved by addition of two ml. of water and glycogen con-

tent was determined using the phenol-sulfuric acid method of Dubois et al. (1956). Glycogen concentration using this method was estimated spectrophotometrically by measuring the specific absorbancy of the reaction sample at 490 nm. Purified rat liver glycogen (Sigma, St. Louis, Missouri) was used as a standard.

## Liver Enzyme Assays

Liver microsomal and supernatant fractions were prepared as described above. The following enzymes were assayed by methods indicated in the references: glucokinase-hexokinase (Sharma et al., 1963), pyruvate kinase (Shonk et al., 1964), phosphenolpyruvate carboxykinase (PEP-CK) (Wagle and Ashmore, 1963), glucose-6-phosphatase (Nordlie and Snoke, 1967) and fructose-1,6-diphosphatase (Weber and Cantero, 1959). Inorganic phosphate concentrations were estimated according to the method of Nordlie and Snoke (1967). Assays for PEP-CK, glucose-6-phosphatase and fructose-1,6-diphosphatase were initiated at 37°C. following three minutes of preincubation at the same temperature. The remaining enzymes were assayed at room temperature. All assays were performed in duplicate for each animal. The activities of all enzymes excluding PEP-CK were determined spectrophotometrically using a Beckman DB Spectrophotometer. Activity of PEP-CK was determined isotopically using a Packard Tricarb Liquid Scintillation Spectrophotometer. Radioactivity was measured in 15 ml. of Bray's scintillation fluid (Bray, 1960) and count rates were converted to absolute activity using the channel-ratio method

of quench correction. The activities for all enzymes assayed were expressed as millimicromoles of substrate turnover per minute per milligram of total liver protein.

## Plasma and Pancreatic Insulin Determinations

Blood samples obtained from the orbital venous sinus were collected into heparinized tubes and the plasma fraction separated by centrifugation. Plasma insulin concentrations were determined from 0.1 ml. of plasma. Pancreata excised from mice immediately following decapitation were rinsed in ice-cold isotonic NaCl, blotted on filter paper and frozen immediately until date of assay. Extraction of insulin from pancreatic tissue was accomplished by grinding individual pancreata in 1 ml. of ice-cold acid-alcohol (750 ml. absolute EtOH, 15 ml. concentrated HCl and 235 ml. distilled $H_2O$) using a Sorvall Omnimixer. The mixer was rinsed with two ml. of acid-alcohol and the washings were pooled with the original material. The slurry was allowed to incubate for 20 hrs. at 4°C. and was centrifuged. The precipitate thus obtained was resuspended in one ml. of acid-alcohol and centrifuged two hours later. The procedure was repeated twice and the supernatants combined and adjusted to pH 8.0 with concentrated $NH_4OH$. The acid-alcohol extract was assayed at various dilutions prepared with bovine serum albumin (Sigma, St. Louis, Missouri) in 0.1 M Tris-Cl buffer (pH 7.4) (Sigma). All insulin concentrations were estimated according to the radioimmunoassay procedure of Zaharko & Beck (1968).

## Insulin Radioimmunoassay

All reagents used in insulin assays were prepared and diluted in 1% bovine serum albumin in 0.1M Tris-Cl buffer (pH 7.4), hereafter, designated as Tris-Albumin buffer. A 0.1 ml. aliquot of test substance was transferred to a disposable test tube and brought to a volume of 0.5 ml. with Tris-Albumin buffer. 0.5 ml. of 10 μU/ml. $^{125}$I-insulin (Abbott, Chicago, Illinois) with specific activity greater than 50 μc/mg. was added to the test material and the contents were thoroughly mixed. An additional 0.5 ml. of guinea pig anti-insulin serum prepared as described by Morgan and Lazarow (1963) was added to the mixture and the contents were swirled and allowed to incubate at 25°C. for two hours. Following incubation, one ml. of continuously agitated cellulose slurry [prepared by suspension of 10 g. cellulose powder (MN 300 Brinkman, Westbury, New York) in 100 ml. of Tris-albumin buffer] was added, the suspension mixed and centrifuged at 8000 x g. for two minutes. The supernatant was immediately removed and both fractions transferred to Packard counting tubes. Radioactivity was measured in a Packard Auto-gamma Spectrophotometer. Crystalline glucagon-free bovine insulin (Lot number PJ4609, Eli Lilly, Indianapolis, Indiana) was used as a standard.

## Isolation of Intact Islets of Langerhans

Islets of Langerhans were isolated from Toronto-KK and C57BL/6J mice using the method of Lacy and Kostianovsky (1967). Animals were

sacrificed by exsanguination and decapitation. The pancreata were immediately excised and placed into beakers containing ice-chilled Hank's albumin buffer (pH 7.4) prepared as follows:

| | |
|---|---|
| NaCl - - - - - - - - - - | 8.0 g/l. |
| KCl - - - - - - - - - - | 0.4 g/l. |
| $MgSO_4 \cdot 7\ H_2O$ - - - - - | 0.2 g/l. |
| $CaCl_2$ - - - - - - - - - - | 0.14 g/l. |
| $Na_2HPO_4$ (anhydrous) - - | 0.0478 g/l. |
| $KH_2PO_4$ - - - - - - - - - | 0.06 g/l. |
| Bovine Serum Albumin - | 1 g/l. |

Extraneous tissues were trimmed off and the pancreatic tissue was then minced into fine pieces with scissors and the crude tissue transferred to a small glass weighing vessel. The tissue was incubated in Hank's albumin buffer containing 1% collagenase (Nutritional Biochemical Corp., Cleveland, Ohio) with constant stirring for 20-25 minutes at 37°C.

Following incubation of the pancreatic tissue suspension with collagenase, the mixture was collected and diluted to a volume of 25 ml. with ice-chilled Hank's-albumin buffer in a conical graduate cylinder. The diluted suspension was agitated by gentle suctioning and flushing of the mixture with a 25 ml. syringe equipped with a number 15 guage needle and sedimentation of the tissue segments was allowed to proceed for one minute. After sedimentation, the supernatant was removed and was discarded. The sediment was resuspended in 25 ml. of ice-chilled Hank's-albumin buffer and allowed to settle for thirty seconds. This procedure was repeated for a total of eight times using

ice-chilled Hank's-albumin buffer. The final sediment was transferred to a glass Petri dish surrounded by an ice bath and examined with a Zeiss dissecting microscope. (The sedimentation procedure was based on the fact that islets of Langerhans have a greater density than the surrounding exocrine acinar tissues. Islets of Langerhans, therefore, were sedimented first while most of the acinar tissue was discarded with the supernatant.)

Islets of Langerhans were recognized as distinct round or ovoid structures with an opaque greyish-white color. Approximately 90-200 islets were visualized from pancreatic tissues per animal. Those islets which appeared intact and free of acinar and connective tissues were harvested for further use. The time requirement for isolation of intact islets of Langerhans never exceeded one hour.

The perifusion of isolated islets of Langerhans

The apparatus employed for "perifusion" of isolated islets of Langerhans is shown in Figure 1. This filter system consisted of a conical Swinex®-13-Filter Unit (Millipore Corporation, Bedford, Massachusetts) of approximately 2 ml. capacity containing a 0.45 micron cellulose filter and connected at both ends of the chamber of Tygon® tubing (Catalogue number 6408, external diameter 3/8 inch - internal diameter 3/16 inch, Cole-Parmer, Chicago, Illinois). A Cole-Parmer Masterflex microperistoltic pump (Model number 7020C) was used to suction perifusion medium from a reservoir through the tubing and then to perifuse isolated islets contained within the filter unit. The flow rate

FIGURE 1

APPARATUS EMPLOYED FOR PERIFUSION OF ISOLATED ISLETS OF LANGERHANS. (M SHOWS MILLIPORE® CHAMBER WHICH CONTAINED ISOLATED ISLETS. P SHOWS PERIFUSATE RESERVOIRS MAINTAINED AT 37°C. AND GASSED WITH 95% $O_2$ - 5% $CO_2$.)

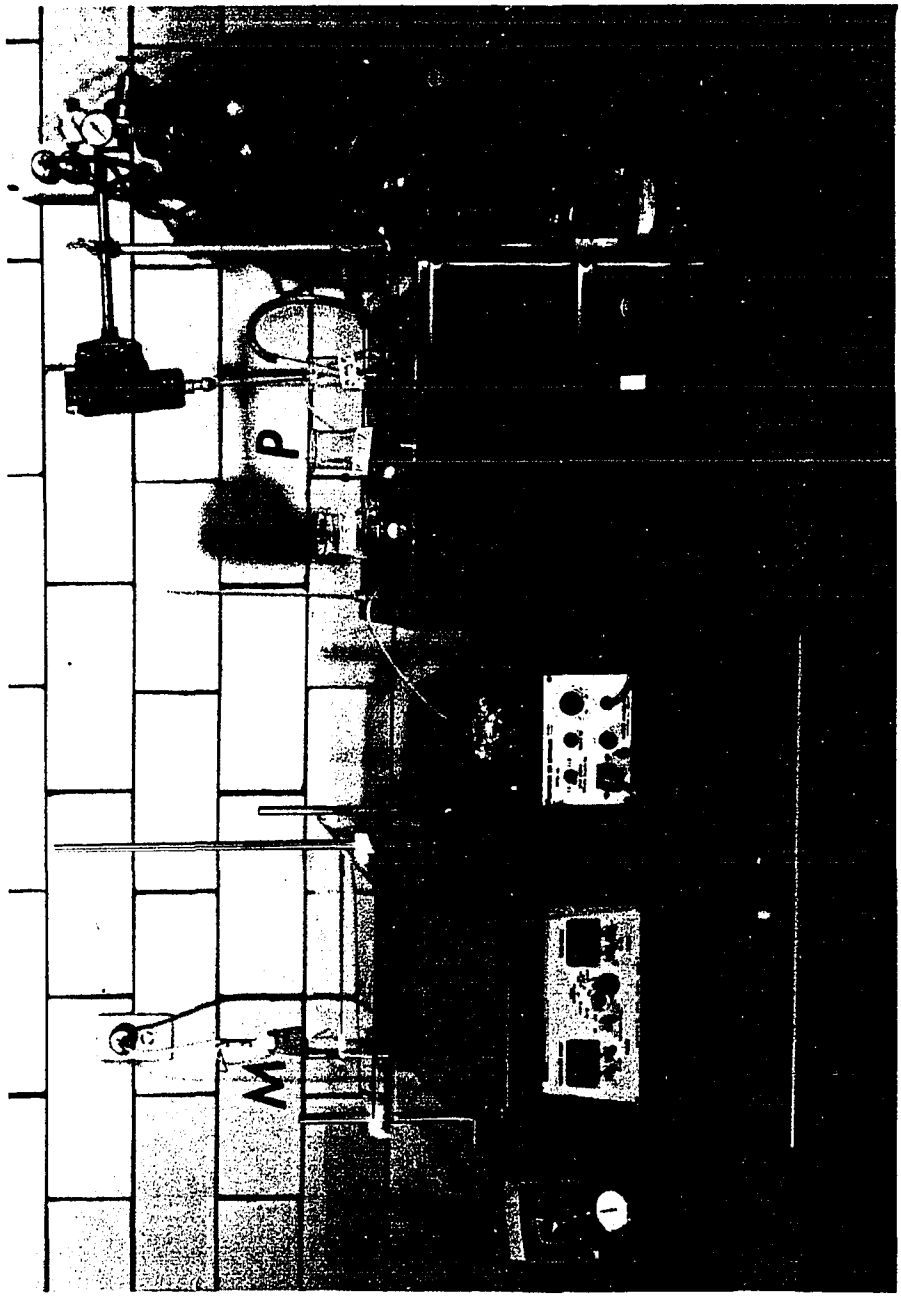



FIGURE 1

remained constant at 2 ml./minute, variation in any one experiment never exceeding 5%. Effluent from the distal portion of the filter unit was then collected using a fraction collector. A medium of the following composition was used as a "perifusate":

| | |
|---|---|
| NaCl - - - - - - - - - - - - | 8.0 g/l. |
| KCl - - - - - - - - - - - - | 0.4 g/l. |
| $MgSO_4 \cdot 7\ H_2O$ - - - - - - - - | 0.2 g/l. |
| $CaCl_2 \cdot 2\ H_2O$ - - - - - - - - | 0.31 g/l. |
| $Na_2HPO_4$ (anhydrous) - - - - | 0.0478 g/l. |
| $KH_2PO_4$ - - - - - - - - - - - | 0.06 g/l. |
| Bovine serum albumin - - - | 1.0 g/l. |
| Pancreatic trypsin inhibitor (Sigma, St. Louis, Miss.) | 2,000 μu/l. |

either with or without glucose in one of the following concentrations:

| | | |
|---|---|---|
| | glucose - - - - - - - - - - - | 0.9 g/l. |
| or | glucose - - - - - - - - - - - | 3.75 g/l. |

The perifusate reservoirs were maintained in a water bath at 37°C. with constant agitation and were gassed continuously with 95% $O_2$- 5% $CO_2$. The entire perifusion system was completely jacketed to maintain a closed system and a constant temperature of 37°C.

Fifty intact islets of Langerhans were isolated as described previously and were transferred to the conical portion of the Swinex® filter unit. The two halves of the unit were then connected and Tygon® tubing was attached to both ends of the unit. Perifusion of the islets was then initiated at flow-rate of 2 ml./minute. The perifusion

system utilizes the principle that isolated islets of Langerhans may be gently forced against the filter contained within the Swinex® unit but remain unable to penetrate through the filter pores. The product released from the islets (i.e., insulin), however, passes freely through the filter pores and may be assayed from the effluent. Islets were perifused without glucose for a fifteen minute equilibration period and at the end of fifteen minutes were stimulated with perifusate containing 0.9 g/l. glucose for a total of twenty minutes. Following this stimulation for twenty minutes, islets of Langerhans were perifused with media containing 3.75 g/l. glucose for an additional seventy minutes. The amount of time elapsed during transition from one media to another never exceeded two seconds. Effluent was collected at five minute intervals and samples were then immediately frozen in an acetone-dry ice bath and stored until time of assay. At the date of assay, 0.1 ml. aliquots from each fraction were removed and assayed for insulin concentration using the radioimmunoassay procedure previously described. All assays were performed in duplicate. The glucose-induced insulin release from isolated islets of Langerhans was expressed as microunits of insulin released per minute of perifusion.

Determination of Insulin Content in Isolated Islets of Langerhans

Insulin concentrations were estimated from both perifused and non-perifused islets of Langerhans of the same animal. Following the ninety minutes of perifusion, islets were examined under a dissecting microscope to determine their structural integrity and were

then transferred into plastic test tubes. The islets were sonicated thoroughly and aliquots of the sonicate were assayed for insulin content at various dilutions by the methods previously described. Insulin content was estimated from non-perifused isolated islets of Langerhans in an identical manner.

## Miscellaneous

Liver and pancreatic tissues prepared for histological demonstration were fixed in Bouin's solution and were stained with hematoxylin and eosin (Humason, 1962) and/or with Gomori's aldehyde Fŭchsin-ponceau stain (Gomori, 1950). All substrates, enzymes and coenzymes used in assays were purchased from Boehringer-Mannheim (New York, New York) or Sigma (St. Louis, Missouri). Natt $^{14}CO_3$-(0.1 μCi/μm) used in the phosphoenolpyruvate carboxykinase assay was purchased from New England Nuclear (Boston, Massachusetts).

The numerical values represent the mean of results obtained from studies on N animals. Standard deviation of the mean and probability coefficients (the probability of the difference between C57BL/6J and Toronto-KK mice as determined by T-test) were calculated using methods described by Snedecor and Cochran (1967). Numerical differences of means between Toronto-KK and C57BL/6J mice were regarded as statistically significant when the probability coefficient (P) was less than 0.05.

# RESULTS

## General Properties of C57BL/6J and Toronto-KK Mice

A photograph of six-month old control and diabetic mice typical of those animals used throughout the experimentation is shown in Figure 2. It is evident that Toronto-KK mice may be either black or white and are conspicously larger than control C57Bl/6J mice. Various characteristics of Toronto-KK and control mice are summarized in Figure 3. At six months of age, Toronto-KK mice are significantly heavier than controls and are markedly obese as evidenced by the significantly increased weight of their epididymal fat pads. Non-fasting blood glucose of Toronto-KK mice was significantly higher than controls and plasma immunoreactive insulin was more than thirty times that of controls. Pancreata of Toronto-KK mice contained nearly twice as much insulin per unit tissue weight than controls.

## Livers of Toronto-KK and C57BL/6J Mice

Livers of Toronto-KK mice were found to be greatly enlarged and pale yellow-brown in color. Livers of Toronto-KK mice weighed more than twice that of control livers (Figure 4), accounted for a significantly greater percentage of total animal body weight than livers from control animals and contained significantly less total protein and glycogen per unit tissue weight than controls.

Upon histological examination, livers of Toronto-KK mice were shown

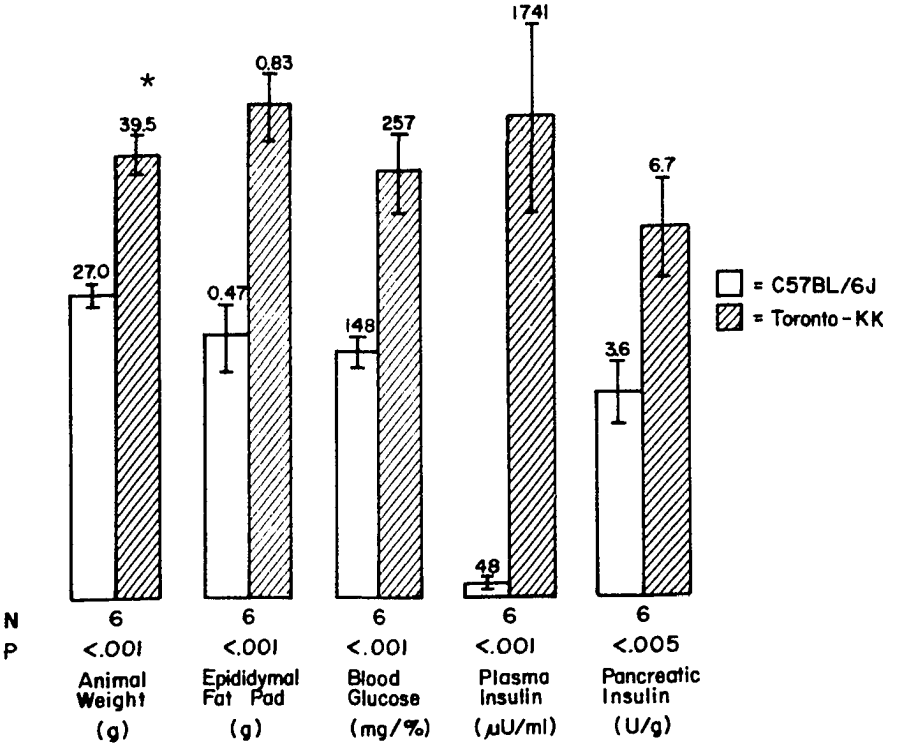
FIGURE 2

SIX-MONTH OLD MALE TORONTO-KK HYBRID (T) AND C57BL/6J (X) MICE


FIGURE 2

FIGURE 3

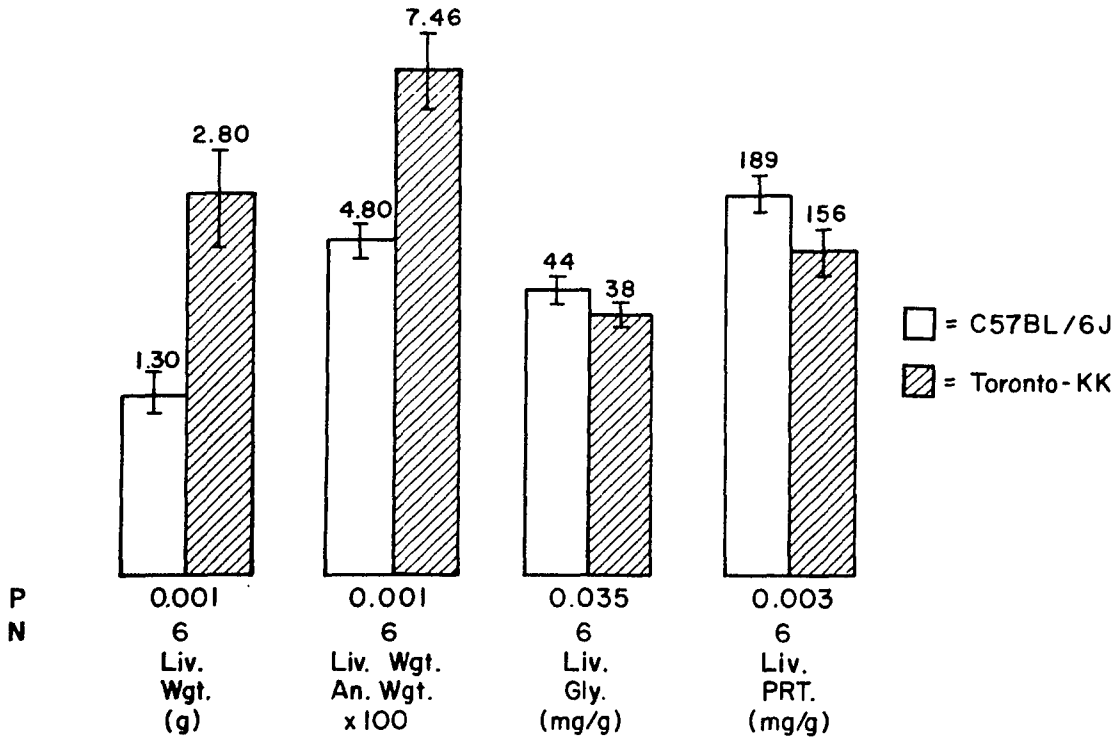
VARIOUS CHARACTERISTICS OF SIX MONTH OLD C57BL/6J AND TORONTO-KK MICE




* In all the bar graphs presented the numerical values represent the mean of results obtained from studies on N animals. The standard deviation of the mean is indicated by brackets and (p) equals the probability of the difference between C57BL/6J and Toronto-KK mice as determined from T-test.

FIGURE 4

VARIOUS CHARACTERISTICS OF LIVERS OF SIX-MONTH OLD TORONTO-KK AND C57BL/6J MICE



to have large intracellular deposits abundant throughout the hepatocytes casting a "moth eaten" appearance to the microscopic liver parenchyma (Figure 5). These deposits most likely were large inclusions of lipid materials which dissolved with routine histological preparation. Identification of these inclusions as lipoid substances, however, would require the use of special staining techniques not undertaken in this research.

The activities of six hepatic "regulatory" enzymes were assayed (Figure 6). No significant difference in the activities of glycolytic enzymes (glucokinase, hexokinase and pyruvate kinase) was observed between liver fractions from Toronto-KK and control mice. However, activities of glyconeogenic enzymes (phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and fructose-1,6-diphosphatase) was significantly elevated in livers from Toronto-KK mice.

## Insulin Secretion Studies

Since islet tissues were perifused for relatively prolonged periods of time in a highly dynamic and artificial environment, post-perifusion examinations of islet tissues were undertaken to assess their structural integrity. Examination of these tissues under the Zeiss dissecting microscope indicated that islets remained intact with no gross structural damage.

Figures 7 and 8 show results obtained from typical experiments in which the effects of "high" and "low" concentrations of glucose upon insulin release were studied. The patterns of glucose-induced insulin release were generally similar from islets of Toronto-KK

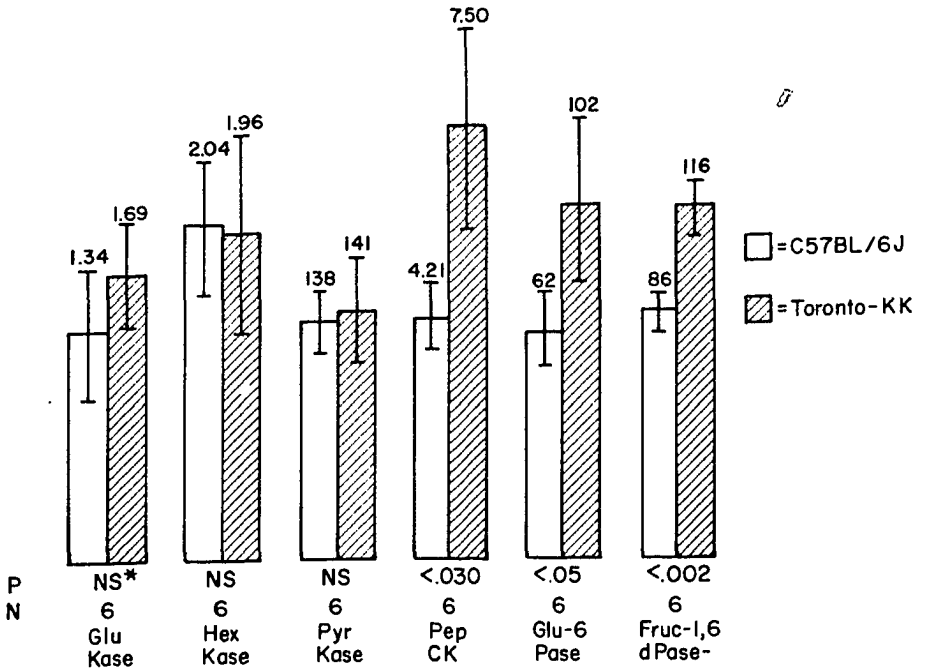
FIGURE 5

LIVER OF A DIABETIC TORONTO-KK HYBRID MOUSE. INTRACELLULAR LIPID INCLUSIONS (L) ARE ABUNDANT THROUGHOUT THE PARENCHYMA.

FIGURE 5

FIGURE 6

ACTIVITIES OF SIX HEPATIC GLYCOLYTIC AND GLUCONEOGENIC ENZYMES OF NON-FASTED TORONTO-KK AND C57BL/6J MICE




*NS = the difference is not significant (P>0.05)

FIGURE 7

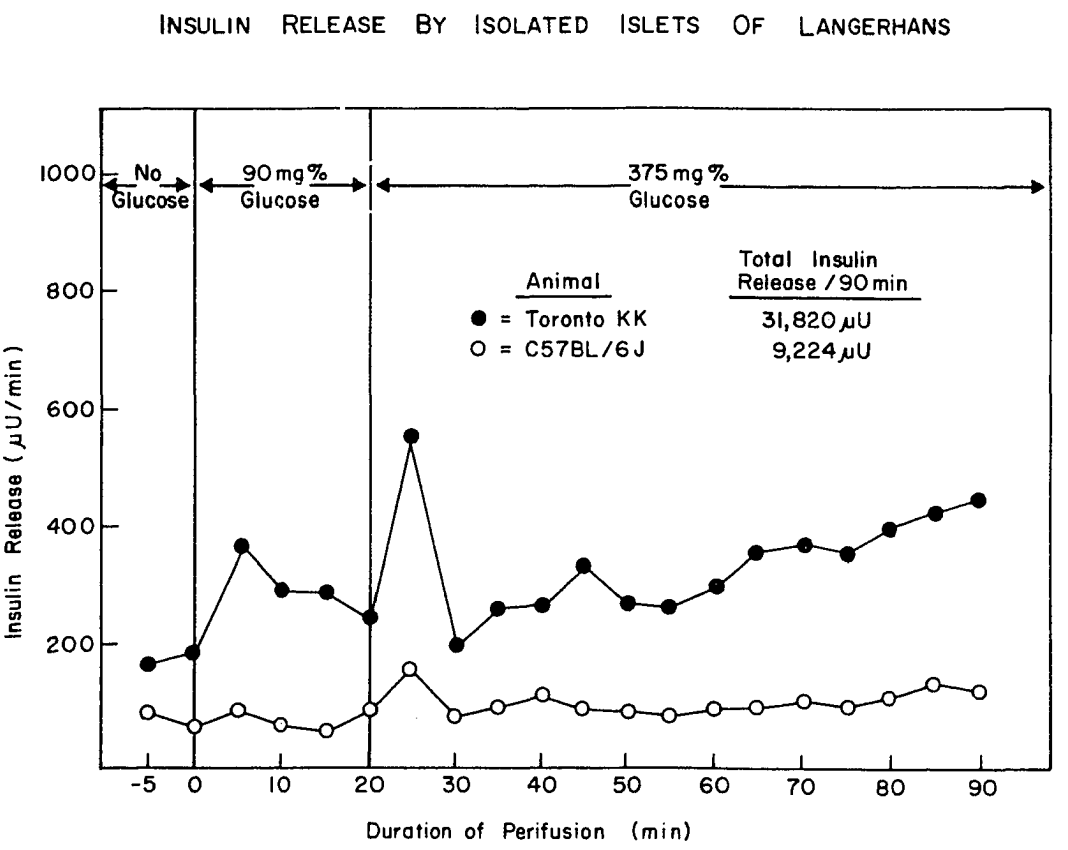
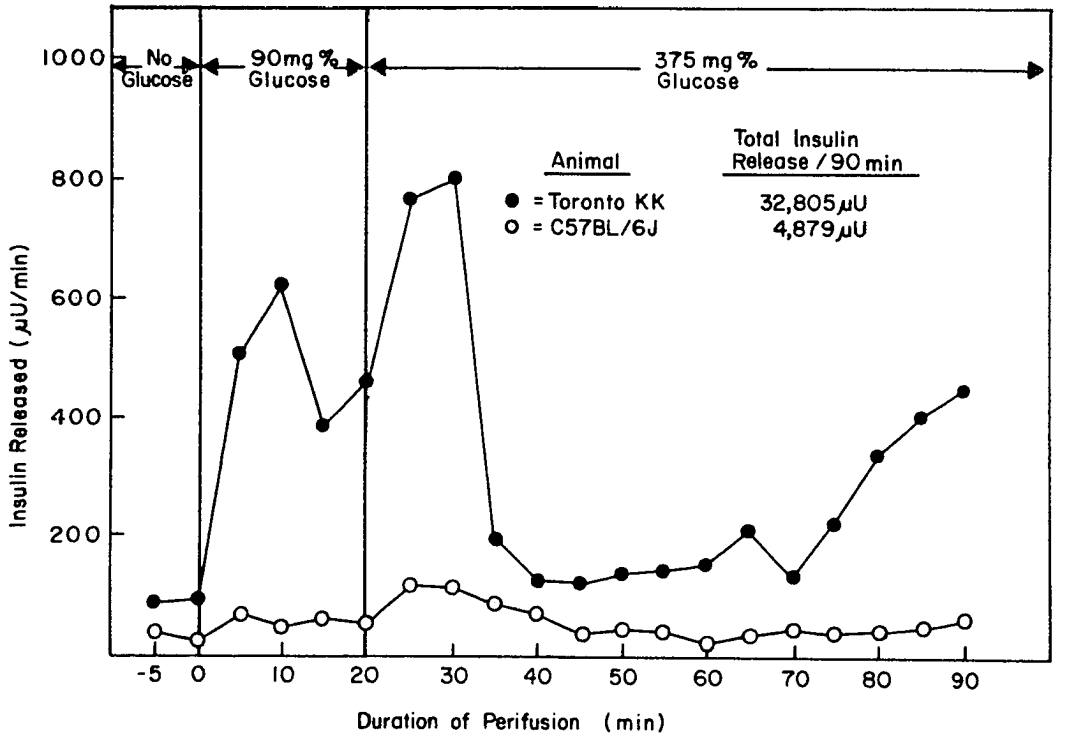


FIGURE 8

INSULIN RELEASE BY ISOLATED ISLETS OF LANGERHANS



and C57BL/6J mice. Isolated islets from both Toronto-KK and C57BL/6J mice released very small and approximately equal amounts of insulin with no glucose present in the perifusate. Islets from Toronto-KK and control mice responded to stimulation at a low glucose concentration (0.9 g/l.) although the magnitude of the insulin secretory responses differed. In the five perifusion studies conducted, islets from Toronto-KK mice released significantly larger quantities of insulin in response to a low glucose concentration. In four of the five paired studies, insulin release appeared to be multiphasic as was previously reported by Grodsky et al. (1967). This multiphasic pattern of insulin release was most dramatic from Toronto-KK islets and was characterized by the following secretory events:

i - an initial secretory response phase of approximately 10 minutes duration.

ii - a decline in insulin release for approximately 5 minutes duration.

iii - a second secretory response phase of lesser magnitude approximately 5-10 minutes subsequent to the initial phase.

Isolated islets of Langerhans from Toronto-KK and control animals released more insulin when stimulated by a high glucose concentration (3.75 g/l.) than by previous stimulation with low glucose. The quantities of insulin released in response to the high glucose stimulus, however, were again much greater by Toronto-KK islets than by controls in all studies conducted. Stimulation of islets with a high glucose

concentration also resulted in a multiphasic insulin secretory pattern in four of the five paired studies undertaken. This multiphasic secretory response pattern was characterized as follows:

i - an initial secretory response phase of approximately 10 minutes duration.

ii - a decline in insulin release for approximately 10-15 minutes duration.

iii - a second secretory response phase 10-15 minutes subsequent to the initial phase during which irregular but gradually increasing quantities of insulin were released until termination of the perifusion.

The total amount of insulin released from islets of Toronto-KK mice during ninety minutes of perifusion was from three to six times greater than that of controls.

Table 2 summarizes the results of the insulin secretion studies and shows some quantitative variances between Toronto-KK and C57BL/6J islets. Islets of Langerhans of Toronto-KK mice contained more than twice as much insulin per islet than control islets either before or after perifusion. Further, Toronto-KK islets released more than four times as much insulin during the ninety-minute perifusion period than control islets when stimulated with the same concentrations of glucose. Finally, the percentage of insulin released per islet relative to the total insulin contained per islet was more than two times greater from Toronto-KK islets than from islets of controls during the ninety minute perifusion period.

TABLE 2
COMPARISON OF ISLET INSULIN CONTENT AND INSULIN RELEASE BETWEEN SIX-MONTH OLD TORONTO-KK AND C57BL/6J MICE

| Type Mouse | Islet Insulin Non-Perf.[1] (μU/islet) | Islet Insulin Post-Perf. (μU/islet) | Total Insulin Released (μU/50 islet/ 90 min.) | % Islet Insulin Released[2] |
|---|---|---|---|---|
| C57BL/6J | 2318±536[3] (4) | 1760±578 (4) | 6866±1944 (5) | 6.77±3.10 (4) |
| Toronto-KK | 4988±969 (4) | 4067±1105 (4) | 29,556±13,601 (5) | 14.00±2.95 (4) |
| (P)[4] | <.005 | <.010 | <.005 | <.01 |

1 Values were obtained using islets other than those used in perifusion studies. (Toronto-KK:Islet insulin content was determined from 25-100 islets per animal. C57BL/6J:Islet insulin content was determined from 35-50 islets per animal.)

2 Values were derived by the following expression:

$$\frac{\text{Insulin content per islet following 90 minutes of perifusion}}{\text{Insulin content per non-perifused islet}} \times 100$$

3 Values represent means ± S.D. Number of mice used are in parenthesis.

4 P is the probability for the difference between C57BL/7J and Toronto-KK mice as determined from T-test.

Histological Examination of Pancreatic Islets of Langerhans

Preliminary light microscopic observations of islets of Langerhans of Toronto-KK and C57BL/6J mice are shown in Figures 9-13b. Islets of Toronto-KK mice show marked hyperplasia which appears to result chiefly from beta-cell proliferation. Fasting appears to increase aldehyde fuchsin granulation in beta cells of both animals, although Toronto-KK islets were considerably less granulated than control islets in either fasted or fed states. Hypertrophied beta cells with enlarged nuclei were frequently observed throughout the endocrine parenchyma of Toronto-KK mice and some islets showed pronounced cavitation. The ovoid or spherical islet typical of C57BL/6J mice contrasted sharply with the diabetic Toronto-KK islets appearing frequently as irregularly contoured structures. Though not demonstrated in the light micrographs presented in this section, Toronto-KK mice had an increased number of islets per unit area of pancreas.

FIGURE 9

PANCREATIC ISLET OF A SIX-MONTH OLD C57BL/6J MOUSE FED AD LIBITUM. BLOOD GLUCOSE 105 MG %, PLASMA IMMUNOREACTIVE INSULIN 41 μU/ml. HEMATOXYLIN AND EOSIN STAIN. 350X.

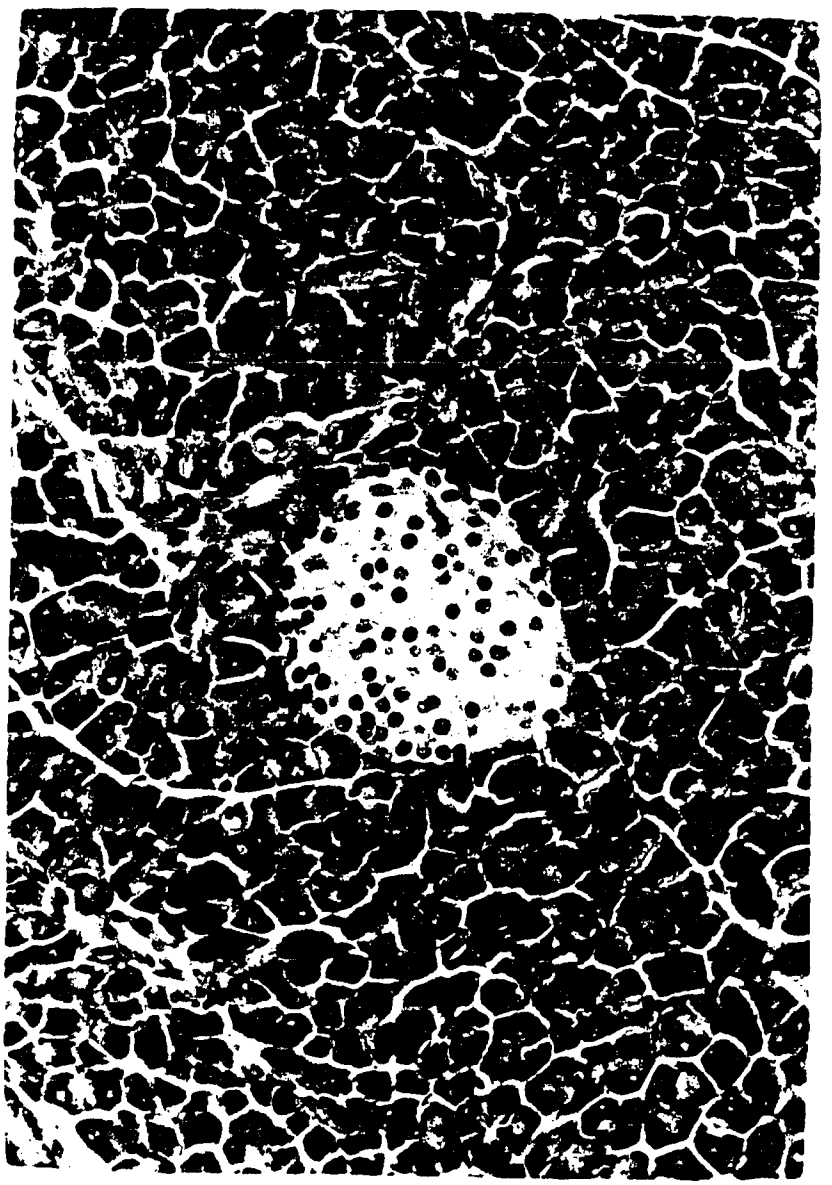


FIGURE 9

FIGURE 10

PANCREATIC ISLET OF A SIX-MONTH OLD TORONTO-KK HYBRID MOUSE FED AD LIBITUM DEMONSTRATING HYPERPLASIA. BLOOD GLUCOSE 294 mg %, PLASMA IMMUNOREACTIVE INSULIN 2560 μU/ml. SEVERAL ADJACENT ACINAR CELLS (A) ARE HYPERTROPHIED. HEMATOXYLIN AND EOSIN STAIN. 350X.

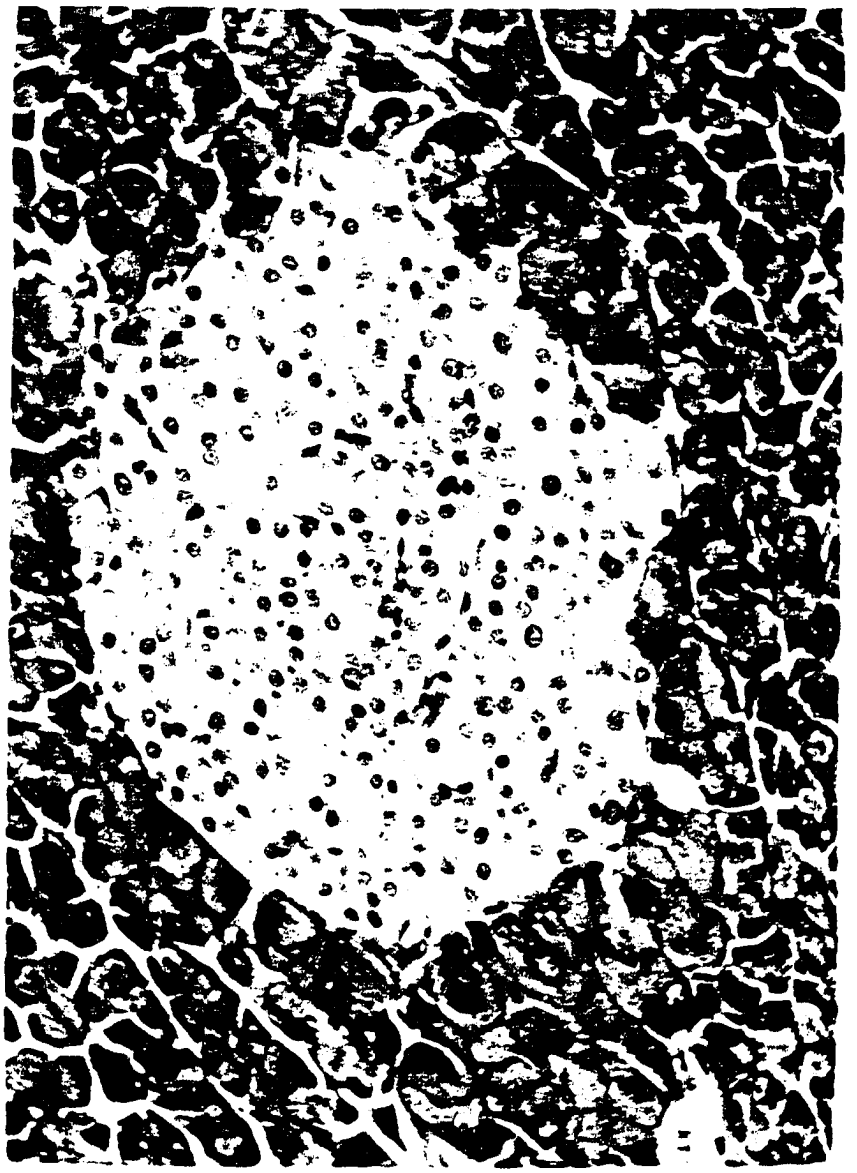


FIGURE 10

FIGURE 11

HYPERPLASTIC ISLET OF SIX-MONTH OLD TORONTO-KK MOUSE FASTED FOR 20 HOURS. BLOOD GLUCOSE 84 MG %, PLASMA IMMUNOREACTIVE INSULIN 81 μU/ml. BETA CELLS ARE WELL GRANULATED. ADJACENT ACINAR CELLS (A) ARE ENLARGED. ALDEHYDE FUCHSIN-PONCEAU STAIN. 300X.

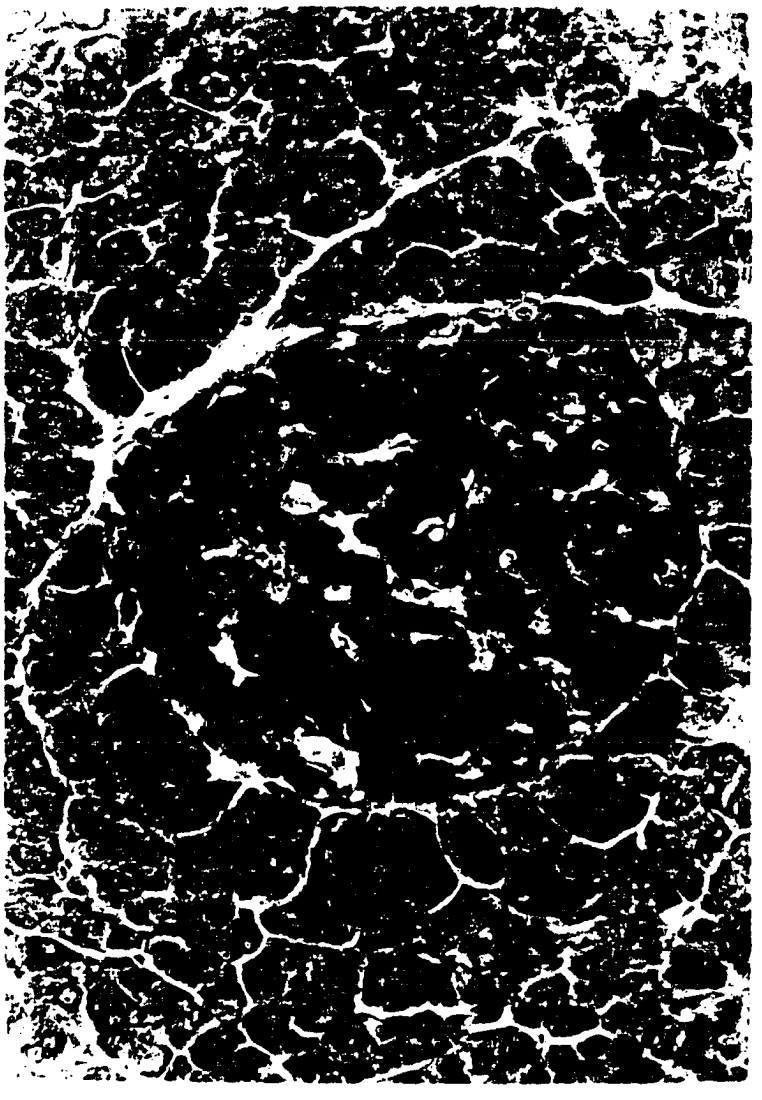


FIGURE 11

FIGURE 12

HYPERPLASTIC ISLET OF A SIX-MONTH OLD TORONTO-KK HYBRID MOUSE FED AD LIBITUM. BLOOD GLUCOSE 272 MG %, PLASMA IMMUNOREACTIVE INSULIN 2560 μU/ml. LINES DEMARCATE THE BOUNDARIES OF THE ISLET TISSUE. ABUNDANT AREAS OF BETA CELL DEGRANULATION (D) ARE SHOWN. ALDEHYDE FUCHSIN-PONCEAU STAIN. 300X.

FIGURE 12

FIGURE 13a

PANCREAS OF A SIX-MONTH OLD TORONTO-KK MOUSE FED AD LIBITUM. BLOOD GLUCOSE 247 MG %, PLASMA IMMUNOREACTIVE INSULIN 1650 μU/ml. THE HYPERPLASTIC ISLET SHOWS MARKED BETA CELL DEGRANULATION WITH CENTRAL CAVITATION (C). ALDEHYDE FUCHSIN-PONCEAU STAIN. 350X.

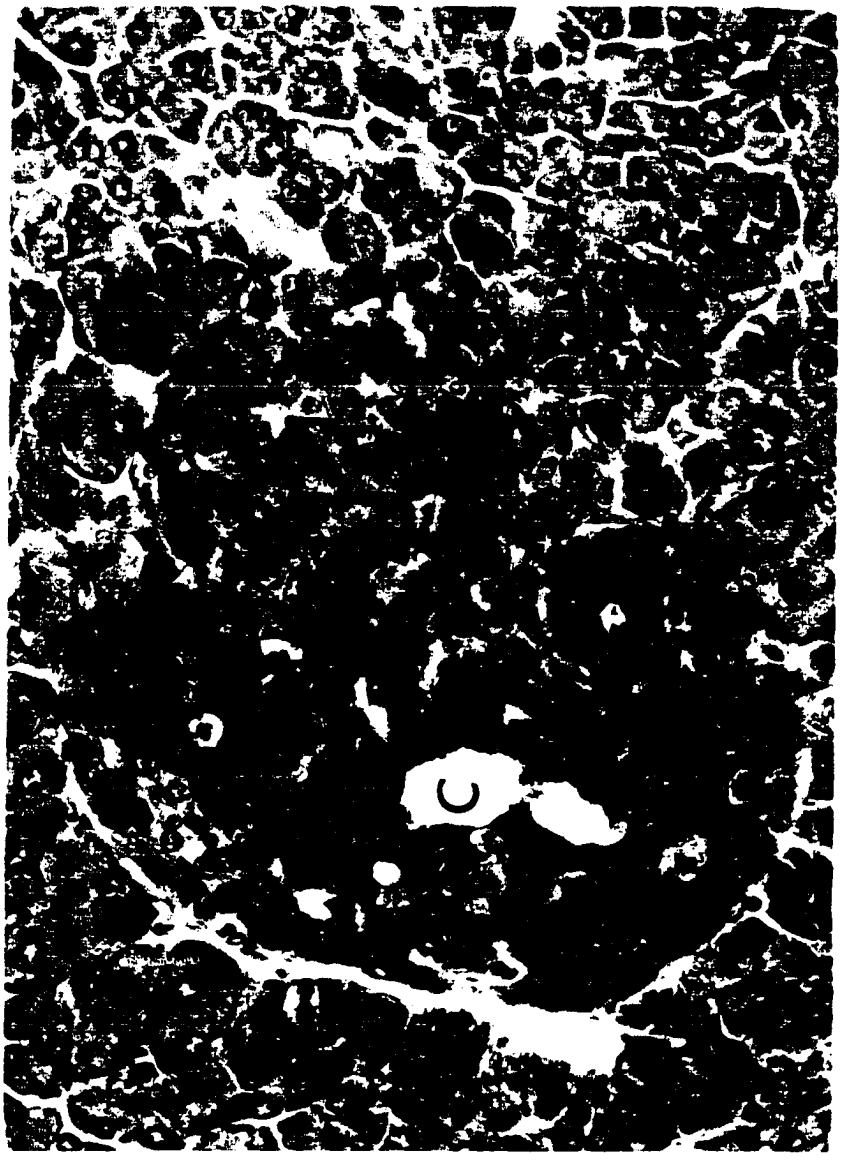



FIGURE 13a

FIGURE 13b

AREA OF CAVITATION WITHIN AN ISLET OF A SIX-MONTH OLD TORONTO-KK MOUSE. THE CAVITY IS FILLED WITH A MATERIAL [GLYCOGEN (GL)] WHICH IN OTHER SECTIONS HAS BEEN DEMONSTRATED TO BE PAS POSITIVE. ALDEHYDE FUCHSIN-PONCEAU STAIN. 800X.


FIGURE 13b

# DISCUSSION AND SUMMARY

The present studies clearly demonstrate a variety of metabolic disorders in the six-month old Toronto-KK mice investigated which appeared consistent with the inherited diabetic syndrome described previously by Dulin and Wyse (1970). In additon, the present studies confirm the notion that hyperglycemia and hyperinsulinemia result, in part, from abnormal metabolism in livers and islets of Langerhans of these animals.

The elevated levels of blood glucose observed from Toronto-KK mice appear to result, in part, from an increased rate of glucose production by the liver. Toronto-KK mice showed significantly increased activities of hepatic gluconeogenic enzymes (phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and fructose-1,6-diphosphatase) but normal activities of glucose catabolizing enzymes (glucokinase, hexokinase and pyruvate kinase). Since it is generally accepted that the rate of metabolic pathway is dependent upon the activities of the enzymes regulating that pathway, one may infer that livers of Toronto-KK mice produce increased quantities of glucose due to an accelerated rate of hepatic gluconeogenesis.

That hepatic gluconeogenesis is accelerated in diabetic Toronto-KK mice is surprising from at least three points of view. Hepatic gluconeogenesis normally becomes active during periods of fasting or starvation in an attempt to accomodate metabolic conditions when no caloric intake is provided (Cahill, 1971). Livers of Toronto-KK mice produce

glucose during fed conditions. Secondly, increased levels of hepatic gluconeogenesis have been reported in human juvenile-onset diabetics (Willms, et al., 1969) and in diabetic Chinese hamsters with similar syndromes (Chang et al., 1970). In such individuals a gross insulin deficiency produces an inability of adipose and skeletal tissues to utilize glucose resulting in glucose deprivation by these tissues. The circulating non-metabolized glucose eventually spills into the urine resulting with a negative energy expenditure. Hepatic gluconeogenesis, therefore, is stimulated in these individuals to supply starved adipose and skeletal tissues with glucose and offset the energy loss. Since Toronto-KK mice have strikingly elevated levels of plasma immunoreactive insulin and are obese, it is surprising that livers of these animals demonstrate an accelerated rate of gluconeogenesis when there is no apparent metabolic demand for glucose. Lastly, insulin has been implicated as having an inductive effect on hepatic "regulatory" glycolytic enzymes and a suppressive effect on hepatic "regulatory" gluconeogenic enzymes based on observations made from Alloxan (Weber, 1965) and streptozotocin (Chang, 1971) diabetic rats. Therefore, livers of Toronto-KK mice do not respond to insulin in the manner expected since elevated activities of hepatic "regulatory" gluconeogenic enzymes were coincident with exorbitantly increased levels of plasma immunoreactive insulin.

The extensive intracellular lipoid inclusions observed from hepatocytes of Toronto-KK mice closely resembles the "fatty liver" condition described of severely obese humans (Anderson, 1971). During this

condition, food consumption grossly exceeds existing metabolic requirements and excess nutritional materials are deposited in normal fat stores (subcutaneous fat, perirenal fat, scapular fat, buttock, etc.), as well as in ectopic sites (liver, renal glomeruli, muscle, etc.). Livers of such individuals appear pale yellow-brown due to intracellular lipoid inclusions in the hepatic parenchyma. Also, due to the disporportional amount of fat contained in Toronto-KK livers, it is not surprising that levels of liver glycogen and protein per unit tissue weight were subnormal.

The data concerning the dynamic aspects of glucose-induced insulin release suggest that insulin secretory mechanisms within the beta cells of Toronto-KK mice are not imparied, but rather are hyperresponsive. Supportive evidence for this notion is based upon several experimental observations. Both Toronto-KK and C57BL/6J islets released minimal and essentially equal quantities of insulin with no glucose present in the perifusate. Therefore, insulin release from Toronto-KK islets does not proceed spontaneously and requires previous stimulation by those physiological agents directing normal insulin release. However, whether this "basal" insulin release represents a physiological metabolic event within the beta cell or represents an artifact induced by the islet isolation procedure is a subject of considerable controversy (Sussman et al., 1969) (Creutzfeldt et al., 1969) and at present is unclear.

Secondly, perifusion of Toronto-KK and control islets with low and high physiological glucose concentrations produced similar biphasic

patterns of insulin release characterized by a brief initial rapid phase of secretion followed with a slower and sustained phase. This biphasic insulin secretory response to glucose has been reported earlier by Grodsky (1967) (1970), who proposed that insulin may exist in two storage pools, a smaller pool being particularly labile to stimulating agents and released from stored materials, and a larger pool composed of newly synthesized insulin which responds more slowly. Since the insulin secretory response patterns were similar from islets of both animals, it is tempting to infer that insulin from Toronto-KK islets is released from similar compartments in response to glucose stimulation and is regulated by similar metabolic components.

Final evidence supporting the notion that hyperinsulinism in Toronto-KK mice is accompanied by hyperresponsive rather than defective insulin release mechanisms rests with the observation that the rates of insulin release from islets of Toronto-KK mice were elevated. Islets from Toronto-KK mice not only released much more insulin than controls during the ninety minute perifusion period but also released a greater percentage of their component insulin reserves. Thus, the total insulin output and the ratio of insulin output to islet insulin content were both increased from Toronto-KK islets upon stimulation with glucose. Also, histological examination of islets of Langerhans from Toronto-KK mice revealed a marked β-cell hyperplasia and a striking loss of aldehyde-fuchsin positive granulation with feeding. Therefore, the exaggerated quantities of insulin released in response to glucose stimulation probably reflect an accelerated rate of insulin

secretion from an increased number of beta cells; the absolute rate of secretion being, in part, dependent upon the insulin reserves contained within the islet.

The precise role of the islets of Langerhans in the diabetic syndrome of Toronto-KK mice remains unclear. The data indicates that hyperinsulinism in Toronto-KK mice is accompanied by hyperresponsive insulin release mechanisms. Similarly, previous studies have shown elevated levels of RNA and insulin biosynthetic activity from islets of Toronto-KK mice (Appel, 1972). However, though insulin biosynthetic and secretory activities are elevated, little is known concerning the causal effect of these mechanisms on the observed β-cell hypertrophy and hyperplasia. The tremendously increased islet volume from pancreata of Toronto-KK mice most likely represents an adaptive response of pancreatic beta cells to an increased demand for insulin. These specific causative factors ultimately responsible for this beta cell proliferation currently remain unknown and will require additional investigation.

The present investigation sought to determine the etiological agents responsible for hyperglycemia and hyperinsulinemia observed from Toronto-KK mice and to more clearly define the diabetic syndrome of this animal. Hyperglycemia appears to result, in part, from excessive and inappropriate hepatic gluconeogenesis. Hyperinsulinemia appears to result from excessive insulin release from the pancreatic islets in response to glucose stimulation. This accelerated rate of insulin secretion from the islets most likely reflects both an increased volume

of beta cells and a concomitant hyperrespondency displayed by these component beta cells. The primary factors directly responsible for this increased beta volume remain unclear.

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