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THE MECHANISM OF GLUCOSE-INDUCED INSULIN SECRETION OF 2,4-DIAMINO-5-CYANO-6-BROMOPYRIDINE IN RAT PANCREAS ISLETS

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THE MECHANISM OF GLUCOSE-INDUCED INSULIN SECRETION OF 2,4-DIAMINO-5-CYANO-6-BROMOPYRIDINE IN RAT PANCREAS ISLETS

by

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STATEMENT BY AUTHOR

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David G. Johnson

DAVID G. JOHNSON
Professor of Pharmacology

April 18, 1983
Date

To Pat and Brian

two of the most important people in my life

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ABSTRACT

The effect of 2,4 diamino-5-cyano-6-bromopyridine (Compound I) on glucose-induced insulin secretion was studied using isolated rat pancreatic islets. Glucose concentrations of 100 mg/dl, 300 mg/dl and 600 mg/dl were used. The results for 100 mg/dl glucose were inconclusive. At 300 mg/dl glucose, insulin secretion was significantly increased ($p << 0.001$) by Compound I (0.1 mM). There was no significant difference in insulin secretion between Compound I and the control at 600 mg/dl glucose. Compound I did not augment arginine-stimulated insulin release and the combination of 3-isobutyl 1-methylxanthine (MIX), a phosphodiesterase (PDE) inhibitor and Compound I showed a significantly lower insulin secretion than MIX alone. The effects of Compound I on islet glucose metabolism, on islet PDE activity and on cAMP content were also studied. There was no significant difference in glucose metabolism by isolated islets incubated with glucose alone (300 mg/dl and 600 mg/dl) or glucose and Compound I. Compound I was shown to be a potent PDE inhibitor in isolated islets (control PDE activity was 336 pmol/mg/min supernatant and 94 pellet; IC_{50} with Compound I was 42 ± 6 uM supernatant and 43 ± 18 uM pellet). Neither MIX (0.1 mM) (a PDE inhibitor) nor Compound I (0.1 mM) increased the cAMP content of isolated islets.

INTRODUCTION

The physiologic function of the B-cells of the islets of Langerhans is to synthesize, store and release insulin at the appropriate time and in the required amounts. The B-cells contain all the basic enzymic machinery for the assembly and packaging of insulin into a granular storage form (Ashcroft and Randle, 1975). These cells also contain receptors which function as monitors of extracellular concentrations of glucose that determine when and how much insulin should be released and a system for the coupling between the reception of the secretory signal and the secretion process (Ashcroft and Randle, 1975). The transduction of receptor activation leads to changes within the B-cell that apparently function as a coupling system between stimulus and response (Ashcroft, 1976).

Glucose is one of the major physiological regulators for insulin secretion (Zawalich, 1979; Ashcroft, Bassett and Randle, 1972). Other compounds which affect the release of insulin from the B-cell can be divided into two broad categories: 1) the initiators, which in themselves can cause the release of insulin, and 2) the potentiators, which have little or no effect on insulin release by themselves but can increase the secretory response of an initiator.

Initiators include glucose, mannose, leucine, sulphonylureas, barium ions and an increased concentration of potassium ions (Milner, 1969; Ashcroft, Bassett and Randle, 1972). Potentiators include amino acids other than leucine, such as alanine; the hormones glucagon, gastrin,

and GIP; agents which increase cyclic 3',5' AMP concentrations, such as caffeine, theophylline, and other methylxanthines; and sugars other than glucose and mannose, for example fructose (Ashcroft and Randle, 1975).

Glucoreceptor Models

Two glucoreceptor models have been proposed to explain the regulation of insulin secretion by glucose (Randle et al., 1968). These are the regulator site model and the substrate-site model. In the regulator-site model, glucose binds to a cell surface receptor or receptors, to form a complex which either activates insulin release directly, or perhaps catalyzes the formation of an intracellular messenger which, in turn, activates insulin release. The internal messenger could possibly be Ca^{2+} either alone or in combination with cyclic 3',5' AMP. An uptake of Ca^{2+}, an intracellular redistribution of Ca^{2+} or both appears to be a universal requirement for agents that stimulate insulin release (Malaisse, 1973). Cyclic 3',5' AMP may either potentiate Ca^{2+} uptake or supplement Ca^{2+} in the excitation of the insulin release system.

Evidence for the regulator site model has been difficult to obtain directly, due to the extremely small volumes that would be involved in the detection of membrane-associated, glucose-binding activity in islet extracts (Ashcroft and Randle, 1975) and indirectly, since isolated islet cultures consist of both α and B cells. Experiments using difference spectrometry (Price, 1973) lend support to the regulator site model. In these experiments a perturbation of the absorbance at 283 nm by islet membranes was noted upon the addition of

glucose or mannose (Price, 1973). Other investigators have reported that insulin release can occur independently of changes in the level of intermediates and cofactors in the B-cell (Matschinsky et al., 1971), and that sugars which are not metabolized and are potential inhibitors of glucose metabolism could stimulate insulin release under certain experimental conditions (Landgraft, Kotler-Brajtburg and Matschinsky, 1971). A regulator-site model more readily explains the anomeric specificity of glucose-stimulated insulin release: α-D-glucose is more effective than B-D-glucose in eliciting insulin release but is apparently less effective as a metabolic substrate (Idahl, Sehlin and Taljedal, 1975).

Arguments have been presented against all of the above experiments. In the experiments by Price (1973) the possibility of the interaction noted being due to binding by a transport system is not excluded (Ashcroft and Randle, 1975). Ashcroft et al. have demonstrated increases in hexose phosphate concentration within 5 minutes of increasing extracellular glucose concentration (Ashcroft, Capito and Hedeskov, 1973) and the report that non-metabolized sugars could stimulate insulin release has not been confirmed by other investigators (Ashcroft, Bassett and Randle, 1972; Coore and Randle, 1964; Lacy, Young and Fink, 1968).

The substrate-site model implies that a metabolite of glucose, or perhaps, a cofactor generated during glucose catabolism, and not the intact glucose molecule, is the trigger for insulin secretion (Zawalich, 1979). This line of investigation developed from the observation that

sugars which are easily metabolized by mammalian tissues (e.g. glucose and mannose), also elicit insulin release; while those sugars that are not metabolized to any great extent (e.g. galactose), do not stimulate insulin release (Randle and Hales, 1975; Ashcroft and Randle, 1975; Jarrett and Keen, 1968; Malaisse, Sener and Mahi, 1974). There is considerable experimental evidence to support the substrate-site model: 1) increased catabolism of glucose parallels increased rates of insulin secretion (Ashcroft, Bassett and Randle, 1972; Hellman et al., 1974; Ashcroft, Hedeskov and RAndle, 1970; Zawalich and Matschinsky, 1977), 2) metabolic inhibitors (e.g. mannoheptulose, 2-deoxy-D-glucose and iodoacetate), dramatically reduce stimulated insulin release (Coore and Randle, 1964a,1964b; Kilo et al., 1967; Georg et al., 1971); 3) glyceraldehyde, which is phosphorylated and enters glycolysis, mimics the secretory efficacy of glucose (Hellman et al., 1974; Jain, Logothetopoulos and Zuker, 1975; Malaisse et al., 1976), and 4) sugars which are poorly metabolized are also weak or inefficient insulin release stimulatants (Grodsky et al., 1963; Jarett and Keen, 1968; Malaisse, Sener and Mahi, 1974; Ashcroft, Bassett and Randle, 1972; Ashcroft, Weerasinghe, Bassett and Randle, 1973).

The discovery that both glyceraldehyde and dihydroxyacetone possessed the ability to augment insulin release independently, provided considerable support for the substrate-site theory (Hellman et al., 1974; Jain, Logothetopoulos and Zuker, 1975; Malaisse et al., 1976; Zawalich et al., 1978). D-glyceraldehyde enters the glycolytic pathway at the triose phosphate level and its metabolism is not inhibited by mannoheptulose (Ashcroft, Weeransinghe and Randle, 1973). It has been

verified that the insulin secretion caused by glyceraldehyde is also not inhibited by mannoheptulose (Ashcroft, Weeransinghe and Randle, 1973; Hellman et al., 1974). This observation is not easily explained by the receptor-site theory without the suggestion that there is a receptor site distinct from that for glucose (Ashcroft, 1976). It has been suggested that both the receptor-site model and the substrate-site model are partially corect (Ashcroft, Weeransinghe and Randle, 1973; Davis and Lazarus, 1976).

Role of Calcium Ions and 3' 5'-cyclic AMP in Insulin Release

Once the increased extracellular glucose concentration is recognized by the B-cell, the information must be transmitted further within the cell. Inhibitor studies indicate that irrespective of the stimulus, Ca^{2+} and perhaps 3' 5' cyclic adenosine monophosphate (cyclic-AMP) are essential to excitation and that ATP is necessary for release (Randle and Hales, 1972). As with other secretory cell types, it is thought that an increase in the cytosolic concentration of Ca^{2+} activates the insulin release mechanism and the presence of extracellular Ca^{2+} is, in fact, required (Ashcroft and Randle, 1975; Grodsky and Bennett, 1966). Evidence supporting the role of Ca^{2+} in insulin is varied and considerable. The amount of insulin released by the perfused rat pancreas during a 2-minute pulse was proportional to the medium Ca^{2+} concentration (range 0.25 mM-2 mM) reaching a plateau at approximately 5 mM Ca^{2+} (Curry, Bennett and Grodsky, 1968). It has also been observed that increased extracellular Ca^{2+} concentration up to a maximum of 2.6 mM increased insulin release (Milner and Hales, 1967).

Studies of the processing of $^{45}Ca^{2+}$ by isolated islets have shown a direct relationship between the rate of insulin release and the accumulation of $^{45}Ca^{2+}$ when insulin release was varied by different stimulatory and inhibitory agents (Malaisse, 1973). These and other studies lead to the postulation that glucose increases the intracellular concentration of Ca^{2+} by inhibiting the outward transport of Ca^{2+} across the cell membrane (Malaisse, 1973; Melaisse et al., 1973).

Other studies showing the role of Ca^{2+} are as follows: 1) Ni^{2+}, which inhibits cardiac muscle contraction by interfering with the uptake and/or action of Ca^{2+} on excitation-contraction coupling, is also a potent inhibitor of insulin secretion (Dormer et al., 1974); 2) local anesthetics, such as tetracaine, which interfere with Ca^{2+} uptake by the B-cell, also inhibit insulin release (Brisson et al., 1971); 3) ruthenium red, which may inhibit Ca^{2+} movement across plasma and mitochondrial membranes, is a powerful inhibitor of insulin release (Ashcroft and Randle, 1975); and 4) electrophysiological measurements of B-cell membranes have shown that stimulation of insulin release evoked by electrical activity in the membrane was due primarily to the entry of Ca^{2+} (Dean and Matthews, 1970).

There is considerable evidence that 3' 5' cyclic AMP is involved in the stimulation of insulin release (Randle and Hales, 1972). Agents which can stimulate adenylcyclase such as glucagon, B-adrenergic effectors and corticotropin, or which inhibit cyclic-AMP phosphodiesterase like caffeine or theophylline, may increase the concentration of c-AMP in isolated islets and stimulate insulin release (Malaisse,

Malaisse-Lagae and Mayhew, 1967; Turner and McIntyre, 1966; Turtle and Kipnis, 1967; Turtle, Littleton and Kipnis, 1967). It has also been reported that the dibutyrl derivative of cyclic-AMP (db-cAMP) or c-AMP itself can also stimulate insulin release (Malaisse, Malaisse-Lagae and Mayhew, 1967). Inhibitors of adenylcyclase such as α-adrenergic effectors can inhibit the insulin secretory response to a variety of initiators (e.g. glucose, leucine, and Ba^{2+} (Coore and Randle, 1964a; Malaisse and Malaisse-Lagae, 1970; Porte, 1966). This evidence is not conclusive, however, since high concentrations of 5' AMP, ADP, and ATP can also stimulate insulin release (Malaisse and Malaisse-Lagae, 1970; Iverson, 1970).

It has been reported that glucose increases the levels of cAMP in the cell but the changes of cAMP concentration did not correlate with the rates of insulin release (Grill and Cerasi, 1973,1974; Charles et al., 1973; Hellman et al., 1974). Current evidence suggests that cAMP does not directly mediate the initiating action of glucose on insulin release and that increased cAMP concentration is neither necessary nor sufficient for the stimulation of insulin release but may amplify the response to an initiator (Montague and Cook, 1971; Cooper, Ashcroft and Randle, 1973; Charles et al., 1973; Hellman et al., 1974).

Glucose does not seem to activate adenylate cyclase (Hellman et al., 1974; Howell and Montague, 1973) or have any effects on phosphodiesterase (AShcroft, Randle and Taljedal, 1972) or cAMP dependent protein kinase (Montague and Howell, 1973). Glyceraldehyde and dihydroxyacetone can also increase cAMP concentrations (Hellman et al.,

1974) and, therefore, it may be that the metabolism of these compounds activate adenylate cyclase (Ashcroft, 1976).

Cerasi (1975) reports that the rate-limiting factor in insulin secretion is glucose-induced cAMP generation rather than glucose as such. Cerasi (1975) also suggests that the B-cell contains two pools of adenylate cyclase, only one specific for insulin release and stimulated by glucose. Cyclic-AMP is one of the factors that cause Ca^{2+} to be liberated from intracellular stores and thus increase the cytosolic Ca^{2+} level (Rasmussen et al., 1974). This is seen in the pancreatic islets, and it has been postulated that the amplification of insulin release due to theophylline may be due to the mobilization of calcium from intracellular stores (Brisson et al., 1971).

The following hypothesis for glucose-induced insulin release has been proposed by Cerasi (1975): Glucose increases cAMP, which in turn, causes the increase of cytosolic calcium by freeing bound ions (glucose also limits the loss of cytosolic calcium through the cell membrane). The actual release of insulin is either controlled by Ca^{2+} alone, or by cAMP and Ca^{2+} acting synergistically. There is evidence for both the above possibilities: insulin can be released by a calcium ionophore in the absence of glucose (Zawalich et al., 1974; Wellheim et al., 1975) and a calcium ionophore can increase the islet cAMP content in a glucose free medium (Zawalich et al., 1974).

Cyclic-AMP's effect on ion flux (in particular Ca^{2+}) which, in turn, can effect insulin release, may be considered in relation to the effects of cAMP on the ion flux in other tissues. A close relationship between the effects of cAMP and calcium in secretory and other systems

has been shown by Rasmussen and Tenenhouse (1968). They suggested that cAMP can increase the concentration of ionized calcium in the cytoplasm. The c-AMP-Ca^{2+} relationship is also shown in the inotropic effect of adrenaline (Entman, Levey and Epstein, 1969) but the mechanism is uncertain. From cAMP's action on other tissues, two models for its role in insulin release have been proposed (Randle and Hales, 1972): 1) a cooperative effort exists between Ca^{2+} and cAMP in that increases in intracellular Ca^{2+} trigger the release of insulin and the effects of cyclic-AMP are only seen in cooperation with the effect of the calcium (c-AMP potentiates Ca^{2}- uptake), and 2) cyclic-AMP alters the tissue distribution of calcium, possibly by working on the cell membrane or endoplasmic reticulum, in such a way that the cytoplasmic ionized calcium level is increased (c-AMP supplements or augments the action of Ca^{2+} in exciting the release system).

Arginine and Methylxanthines

Methylxanthines (theophylline, caffeine and 3-isobutyl-1 methylxanthine) are cyclic AMP phosphodiestrase (PDE) inhibitors and are, therefore, often used to help determine if a hormone's action is mediated through the production of cAMP (McDaniel et al., 1977) or used as potentiators to magnify the effect of glucose-induced secretion (Ashcroft, Bassett and Randle, 1972).

Theophylline can cause the release of insulin from islets in the absence of glucose but cannot sustain this release apparently due to its effect on Ca^{2+} movement to the cytosol. Without glucose the Ca^{2+} is lost from the cell (Malaisse et al., 1971; Ashcroft and Randle, 1975).

Caffeine and 3 isobutyl methylxanthine (MIX) are potentiators of insulin release in that they inhibit cyclic-AMP phosphodiesterase, which markedly elevates islet cAMP, but these compounds do not cause insulin release alone (Montague and Cook, 1971; Cooper, Ashcraft and Randle, 1973; Howell and Montague, 1973). In the absence of glucose, 4 mM caffeine (1,3,7 trimethylxanthine) has no effect on insulin secretion in mouse islets and in rat islets only has a small (two-fold) effect (Ashcroft, Bassett and Randle, 1972). In the presence of glucose, the maximum insulin response to glucose was increased approximately forty-fold by caffeine without the alteration of the Km or the sigmoid nature of the response curve (Ashcroft, Bassett and Randle, 1972).

According to Cerasi (1975), low concentrations of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at low glucose concentrations had only moderate effects on the islet cAMP but still enhanced the insulin response to glucose. Thus, the enhancement of the glucose effects by methylxanthines may be caused by magnification of glucose-induced cAMP generation.

Arginine does not appear to act through cAMP since its maximal augmentation of glucose-induced insulin secretion is not associated with an elevation in islet cAMP levels (Charles et al., 1976). Randle and Hales (1972) propose that arginine may act by potentiating Ca^{2+} flux through co-transport with Na^{+}.

Compound I (2,4, diamino-5-cyano-6-bromopyridine)

2,4 Diamino-5-cyano-6-bromopyridine (Compound I) (Fig. 1), as well as other substituted pyridine derivatives, has been shown to inhibit glucose transport in human erythrocytes (Hershfield and Richards, 1976). These derivatives are similar to glucose in hydrogen bonding possibilities, and the inhibitory properties of these compounds have been attributed to this fact (Hershfield and Richards, 1976).

In studies by Johnson and De Haen (1979) using perfused rat pancreas, it was shown that Compound I could stimulate pancreatic insulin secretion 7 to 20 fold in the presence of 300 mg/dl glucose. No effect was seen at 100 mg dl glucose. Compound I requires a glucose concentration higher than the normal fasting concentration to produce an observable increase in insulin secretion. In this respect, Compound I resembles hormonal potentiators of insulin release (Peterson and Brown, 1976; Schauder et al., 1977). As stated previously, requirement for elevated glucose is also a characteristic of agents that elevate B-cell cyclic-AMP (Malaisse, Malaisse-Lagae, and Mayhew, 1967; Johnson, Fujimoto and Williams, 1973). Agents that promote increased insulin secretion through cyclic-AMP rarely give increases of more than 3-fold (Johnson, Fujimoto and Williams, 1973), while that of Compound I was reported as 7 to 20-fold (Johnson and De Haen, 1979).

Because of its resemblance to glucose, Compound I may interact with a cell membrane glucoreceptor. Studies done by De Haen (personal communication, 1981) have identified the major metabolite of Compound I as the sulfuric acid ester, suggesting that the biotransformation (in

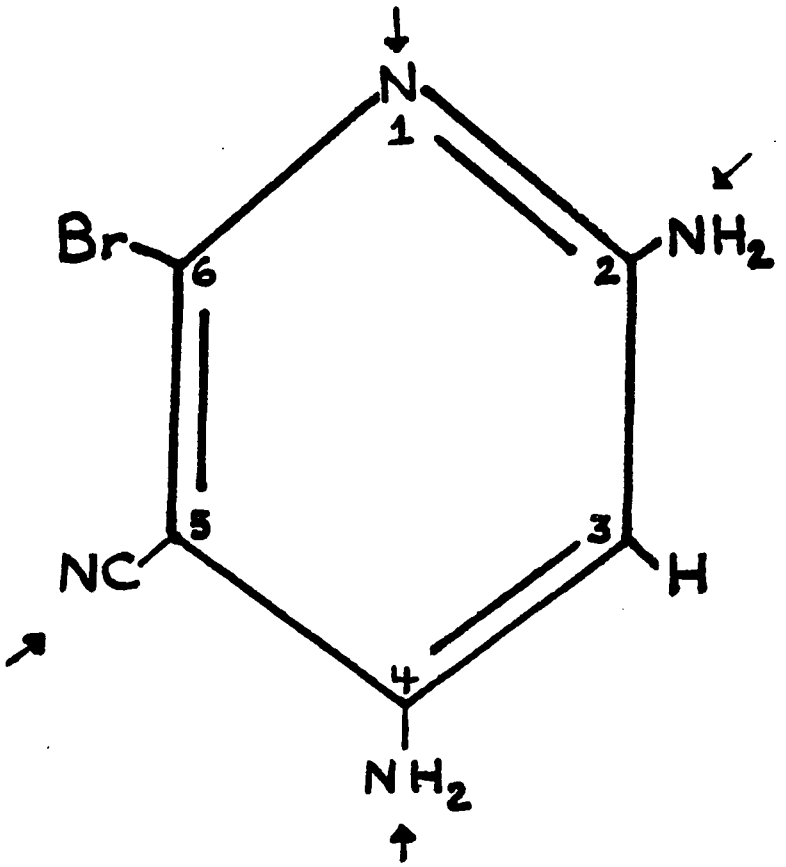

Figure 1. 2,4 Diamino-5-cyano-6-bromopyridine (Compound I). -- The arrows indicate where the hydrogen bonding is similar to glucose.

Wistar rats) of Compound I is similar to phenobarbital: hydroxylation of the 3 position, presumably by the p450 system, followed by the formation of the sulfuric acid ester (De Haën, 1981). The metabolites of Compound I, therefore, do not resemble those of glucose, and the high glucose requirement necessary for the stimulation of insulin release by Compound I suggests that activation of the cell membrane glucoreceptor requires higher levels of glycolytic metabolites intracellularly (Johnson and De Haën, 1979). These previous studies suggested that further study of Compound I and its mechanism of action might help clarify the mechanism of glucose induced insulin release in the pancreatic B-cell.

Statement of Problem

The pancreatic hormone insulin has been used to relieve the symptoms of diabetes for many years. Glucose is well known as the primary physiological regulator of insulin secretion. There are two proposals as to how glucose regulates insulin release: (1) glucose attaches to a cell membrane receptor, or (2) a metabolite of glucose stimulates the release of insulin from the B-cell. A better understanding of glucose-induced insulin release would be helpful in the design of new therapeutic drugs.

Hershfield and Richards described a series of substituted pyridine compounds that inhibited glucose transport in erythrocytes and have structures that show similarities in hydrogen bonding possibilities with glucose. Johnson and De Haën (1979) discovered that these compounds

also were insulinotropic, especially 2,4 diamino-5-cyano-6-bromopyridine (Compound I).

The purpose of this study was to characterize the mechanism of action of Compound I and perhaps help elucidate the mechanism of glucose-induced insulin release in the B-cell. With these goals in mind, the effect of Compound I on insulin secretion, glucose metabolism, phosphosdiesterase activity and cyclic AMP was studied using isolated rat pancreatic islets.

MATERIALS AND METHODS

Male Wistar rats weighing 250-350 gm were used for all experiments. The rats were obtained from Hilltop (Chatsworth, CA), housed two per cage in wire bottom cages and fed ad libitum with Purina Rat Chow and water. 2,4-Diamino-5-cyano-6-bromopyridine (Compound I) was synthesized and kindly supplied by Dr. C. de Haen (Department of Internal Medicine, Endocrine Section, University of Washington, Seattle, WA).

Isolation and Perfusion of Rat Pancreatic Islets

Pancreatic islets were isolated using a modification of Lacy and Kotianovsky's technique (1967). Male Wistar rats were anesthetized with an intraperitoneal injection (57 mg/kg) of sodium pentobarbital (Harvey Laboratories, Inc., Philadelphia, PA; 65 mg/ml). The abdomen was opened with scissors, the portal vein located and clamped at the bifurcation using a hemostat. This caused the islets to become engorged allowing for ease in location and identification later (Aleyassine and Gardiner, 1972). The pancreatic duct was clamped at the point it enters the duodenum. The common bile duct was clamped at the hepatic end, cannulated and the pancreas infused with 1 ml synthetic interstitital fluid (SIF) (Appendix A) containing 100 mg% glucose and 2 mg per ml collagenase (Worthington Biochemical, Freeport, NJ). This was followed by an infusion of approximately 4 ml of SIF (100 mg% glucose). The pancreas was removed from the rat, cleaned of fat and lymph nodes and minced with scissors. The pieces were placed in a 50 ml Erlenmeyer

flask along with 4 ml of the SIF, 100 mg% glucose, and 2 mg/ml collagenase solution for connective tissue digestion. The tissue was continuously kept under an atmosphere of 95% oxygen and 5% carbon dioxide while incubated for 12 to 15 minutes in a 37°C shaking water bath. After incubation, the tissue was disrupted by aspiration through a No. 16 gauge needle and the digestion terminated by pouring the suspension into a 40 ml conical glass centrifuge tube and adding approximately 30 ml of ice-cold SIF containing 100 mg% glucose.

The islets were allowed to settle for about 2 minutes and 20 ml of the solution was drawn off and replaced with fresh SIF (100 mg% glucose). This washing was done a total of three times, each time using fresh cold SIF with 100 mg% glucose.

Approximately 25 ml of the solution was removed and the suspension of islets poured into a small petri dish. One hundred islets were selected, using a dissecting microscope and a small mouth pipette and transferred to two petri dishes containing fresh SIF with 100 mg% glucose (50 islets in each). The perfusion apparatus consisted of two 25 mm Swinnex filter holders (Millipore Co., Bedford, MA) fitted with filters of 14 μpore size (Millipore). The islets were placed on the filter in the chamber with a No. 16 gauge needle and syringe. Tubing from Travenol Solution Administration Sets 2C0001 (Travenol, Deerfield, IL) was used with a Buchler Polystatic Pump (Buchler Instruments Inc., Fort Lee, NJ) to pump the perfusion medium from a beaker through the perfusion chamber to the collecting tubes. The perfusion medium consisted of SIF and glucose for the controls and SIF, glucose and

Compound I for experimental. The islets (50 per chamber) were perfused for 60 minutes. The perfusion medium was SIF with 30 mg% glucose for the first 40 minutes, and SIF with 100 mg%, 300 mg% or 600 mg% glucose for the remaining 20 minutes (control). The experimental medium was the same as the control, except for the addition of 0.1 mM Compound I.

The perfusion chambers and medium were submerged, side by side, in a 37°C water bath and the medium gassed with 95% O_2, 5% CO_2 throughout perfusion. The pump was set to deliver about 1 ml of medium per minute. One minute samples were taken in 12x75 mm tubes after 10 minutes and every 5 minutes thereafter through 40 minutes. This 40 min. time period allowed the islets to return to a basal level of insulin release. Samples for the 41 through 60 minute time period were taken every minute. The samples were then corked and frozen until assayed for immunoreactive insulin (IRI) content using radioimmunoassay.

Radioimmunoassay of Insulin

In order to determine the amount of insulin released by the islets at every time point, a radioimmunoassay was performed (Zaharko and BEck, 1968). The materials used to test for the immunoreactive insulin included:

1. Tris-BSA buffer pH 7.4, 1.8 ml per tube (Trizma^R base, Sigma, St. Louis, MO 63178; BSA - bovine Serum Albumin Fraction V, Miles Laboratory, Elkhart, IN) (Appendix A)

2. Antibody I-35 Guinea Pig Anti-Insulin Serum Antibodies (GPAIS) made in 1967 in the laboratory of R. H. Williams, Seattle, WA.

3. Porcine insulin labeled with ^{125}I according to the Chloramine T procedure (Greenwood, Hunter and Glover, 1963)

4. Rat insulin for preparation of standards 10 mU per ml (Gift of Novo Lab., Copenhagen, Denmark)

5. Cellulose 300MN (Brinkmann Instruments, Westbury, NY). A 10% slurry in Tris-BSA buffer.

For each IRI assay performed, twelve tubes were used. These tubes were set up in triplicate. The first tube was the tracer tube and contained only buffer and ^{125}I insulin. The tracer tube gave the amount of radioactivity that was pipetted into each tube. The second tube was the damaged insulin or DI tube and contained only buffer and ^{125}I insulin but was treated with a 10% cellulose slurry. The cellulose bound all free insulin except insulin that was damaged. Therefore the DI tube gave the amount of insulin that appeared bound to antibody because it is damaged (not separated out by the cellulose). The third tube was the overdose antibody or OD tube. This tube contained buffer, ^{125}I insulin, and a 1:150 dilution of I-35 GPAIS. This amount of antibody should bind all the insulin in the tube except the damaged insulin. Therefore the OD tube is a check of the effectiveness of the antibody and a check of the DI tube. Tubes 4 through 12 contained buffer, ^{125}I insulin, antibody diluted 1:3000, and rat insulin in the following amounts: 0_uU, 1_uU, 2_uU, 3_uU, 4_uU, 6_uU, 10_uU, 15_uU, and 20_uU. All sample tubes were prepared in duplicate and contained 1.8 ml buffer, 50 ul ^{125}I insulin (20,000 cpm), 50 ul 1:3000 antibody, and 50 ul or 100 ul of the sample. In the first stage of the assay, buffer, samples,

and the appropriate dilution of antibody, were added to all tubes. These tubes were then treated in one of two ways: allowed to sit for 1 hour in a 30°C water bath and then in a 4°C refrigerator for 24 hours, or placed in a 4°C refrigerator for 48 hours. Tracer, ^{125}I-20,000 cpm, was then added to all tubes and they were again placed in a 4°C refrigerator overnight.

For the final stage of the assay 1 ml of a 10% slurry of cellulose which had been stirred on ice for 30 min., was added to all tubes except the tracer tubes. The cellulose was allowed to react for 10 minutes. This was done to separate the bound insulin from the unbound. The tubes were then centrifuged for 5 minutes at 2000 rpm (Beckman TJ-6 with Refrigerator Unit, Beckman, Spinco Division, Palo Alto, CA). The supernatant was decanted into clean tubes, corked, and counted for 1 minute on a Tracor gamma counter (Tracor Analytic, Des Plaines, IL).

The data was fed into a HP 9845 desk top computer (Hewlett Packard Desktop Computer Div., Loveland, CO), using a packaged program for radioimmunoassay, modified for the standards used and programmed to give a standard curve of B/B_0 vs units per ml insulin and a Logit B/B_0 vs units per ml insulin curve. Graphs were drawn to reflect uU/ml insulin vs time in minutes and compared (Fig. 2).

Glucose Metabolism

Male Wistar rats 300-450 gm were used for all experiments. The islets were isolated and perfused as previously described using SIF

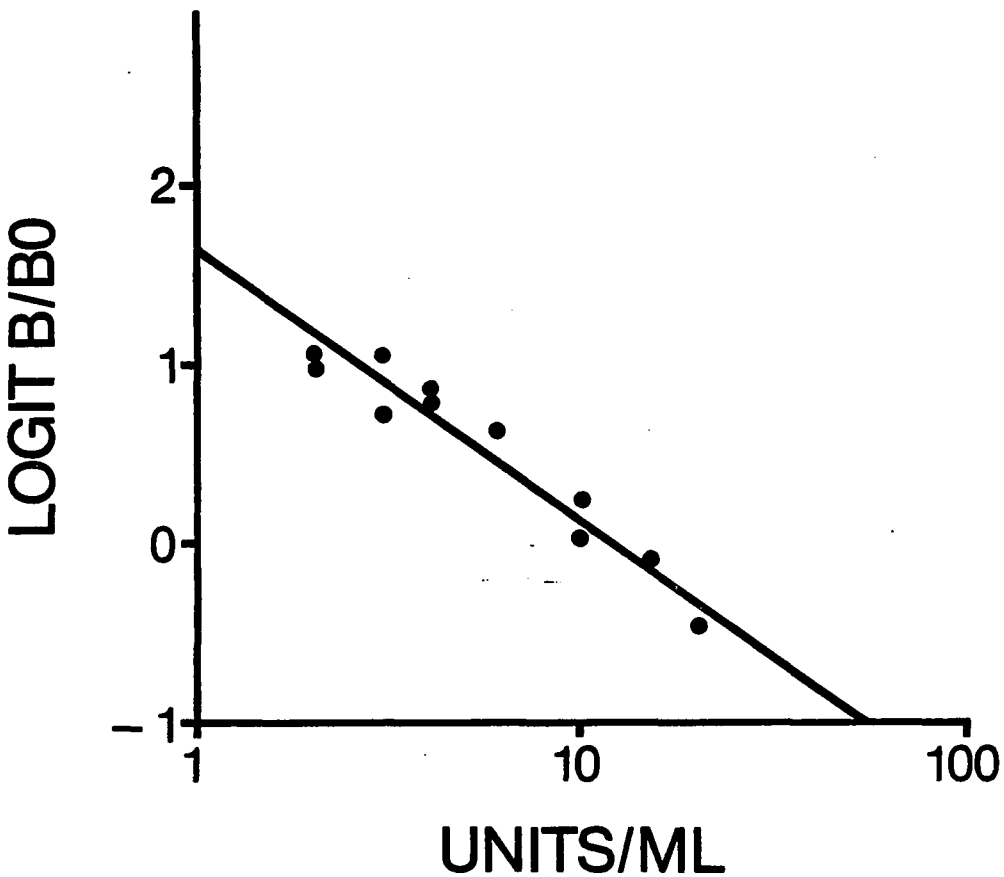

Figure 2. A typical standard curve produced by an HP 9845 desk top computer (Hewlett Packard Desktop Computer Div., Loveland, CO), using a program for radioimmunoassaym modified for the standards used and programmed to give a Logit B/BØ vs Units/ml insulin curve.

with 30 mg% glucose for 40 minutes and SIF with either 300 mg% or 600 mg% glucose. Two filters per perfusion chamber were used. One filter held the islets, the second was used as a blank.

The procedure for determining the amount of glucose metabolized by isolated pancreatic islets was that of Zawalich and Matschinsky (1977).

After perfusion the filters were removed from the chambers and each placed in separate 15 x 45 mm vials (Kimble, VWR Scientific, Phoenix, AZ). The incubation fluid was prepared as follows: 3.5 ul of [5-^{3}H] glucose, specific activity 15.6 Ci per mmol (New England Nuclear, Boston, MA) was pipetted into a 10 x 75 mm tube and dried overnight in a dessicator. The next day the tritiated glucose was redissolved with 100 ul of perfusion medium (SIF + 300 mg% glucose). To each vial and filter, 0.250 ml of incubation fluid was added. The vials were gassed with 95% O_2, 5% CO_2 for 30 seconds, stoppered, and placed for one hour in a 37^{o}C, shaking, water bath set at 100 strokes per minute. After one hour the vials were removed from the water bath, unstoppered, and 20 ul of incubation fluid were transferred, in duplicate, to 6 x 30 mm tubes containing 5 ul 1N HCl. These tubes were mixed gently and placed in 20 ml glass scintillation vials containing 0.5 ml distilled water and kept on ice. A 20 ul sample of tritiated water (^3H_2O) was treated in the same way in order to allow for correction for incomplete equilibration during the diffusion step.

For the diffusion step the scintillation vials were wrapped in foil to insure equal heat distribution and kept for 18 hours in a 50^{o}C

oven. After 18 hours the foil was removed, the vials unstoppered and the small 6 x 30 mm tube removed. The outside of the tube was washed with distilled water and the tube dropped into a clean 20 ml scintillation vial containing 0.5 ml distilled water. Ten ml of scintillation fluid made from 95 gm Naphthalene (scintillation grade, Eastman Kodak Co., Rochester, NY), 15.2 gm scintillator (2a70, REsearch Products International, Elk Grove, IL), added to 0.5 gallon of scintillant 1,4-dioxane (Fisher Scientific, Fair Lawn, NJ) were added to all the scintillation vials. The vials were then counted for 10 minutes on a Tracor Analytic Mark III Liquid Scintillation System with a Texas Instruments Silent 700 Electronic Data Terminal (Tracor Analytic, Des Plaines, IL). The data was corrected for diffusion and the filter blanks and the rate of glucose utilization was calculated using the following formula from Ashcroft et al. (1970). Glucose utilization is expressed in picomoles glucose utilized/islet/hour.

$$\text{glucose utilized (pmol)} = \frac{[^3H]\text{ water formed (dpm)}}{\text{sp. radioactivity of } [5\text{-}^3H]\text{ glucose (dpm/pmol)}}$$

Methods for the Study of Other Potentiators of Insulin Release

MIX (3, isobutyl-1 methyl xanthine)

In order to study the combined effect of MIX and Compound I on insulin release from isolated rat pancreatic islets, the following method was used: the islets were isolated and perfused as previously described. The medium used in these experiments consisted of SIF,

30 mg% glucose, and 0.25 mM MIX for 40 minutes and SIF, 300 mg% glucose, and 0.25 mM MIX for 20 minutes as the control. The experimental medium was the same as the control medium with the addition of 10^{-4}M compound I. Samples for the assay of insulin were taken after 10 minutes of perfusion and every 5 minutes thereafter for 40 minutes and every minute for a total perfusion time of 60 minutes. The IRI assay was performed as previously descirbed, data was graphed and compared.

Arginine

For the studies with arginine, islets were isolated and perfused as described previously. They were perfused for 60 minutes in SIF with 100 mg% glucose, SIF with 100 mg% glucose and MIX (controls), or SIF with 100 mg% glucose and Compound I throughout the perfusion. Arginine (20 mM) was added to the medium between 41 minutes and 50 minutes of perfusion. Between 51 minutes and 60 minutes the islets were perfused with the original medium. Samples for insulin assay were taken after 10 minutes of perfusion and every 5 minutes through 40 minutes and then every minute through 60 minutes. The samples were corked and frozen until assayed. The IRI assay as described earlier was used to determine the amount of insulin released at every sample point. The results of the assay were graphed and compared.

Assay for Phosphodiesterase Activity of Compound I in Pancreatic Islets

The method used for the assay of phosphodiesterase activity (PDE) was an ion-exchange resin method described by Thompson et al. (1979). The materials used are as follows: (1) [^{3}H] cyclic AMP (specific activity 21 Ci/mM), [^{3}H]cyclic GMP (specific activity 5.4 Ci/mM); (2) Snake venom: Ophiophagus hannah (Sigma V-0376) 0.5 mg/ml; (3) Anion exchange resin washed and pH adjusted to pH 5; (4) Scintillation cocktail; 2 parts MeOH to 8 parts Aquasol; (5) Assay buffer: 40 mM Tris/HCl (pH 8.0) containing 4 mM 2-mercaptoethanol (freshly diluted each day). This is abbreviated as T/M buffer.

General Assay Procedure

(1) To 12 x 75 mm culture tubes the following solutions were added: 100 ul [^{3}H] cyclic AMP and/or [^{3}H] cyclic GMP in assay buffer (T/M buffer) containing $MgCl_2$; 100 ul c-AMP and/or c-GMP in T/M buffer; enzyme diluted in T/M buffer.

(2) The tubes were incubated for 10 minutes at 30°C.

(3) The tubes were placed in a boiling water bath for 45 seconds and then placed in an ice bath.

(4) 100 ul 0.5 mg/ml snake venom solution was added and the tubes incubated 10 minutes at 30°C.

(5) The tubes were placed in an ice bath and 1 ml of 100% MeOH added.

(6) The entire contents of each tube were transferred to columns (Pasteur pipets containing 1 ml of a 1:4 slurry resin to MeOH). The columns were then placed over scintillation vials.

(7) The reaction mixture was eluted with 1 ml MeOH and the column drained to dryness.

(8) 10 ml scintillation cocktail was added and the samples were counted on a Packard scintillation counter.

PDE Specific Activity

To check the PDE activity of the pancreatic islets, the following procedure was used: (1) to a tube containing approximately 50 islets, 1 ml T/M buffer was added and the islets homogenized (15 strokes); (2) 10 ul homogenate were assayed for cAMP PDE and cGMP PDE; (3) 5 ml T/M buffer were added to 950 ul homogenate and centrifuged at 40K for 60 minutes; (4) the supernatant was assayed for cAMP and cGMP; (5) 1 ml T/M buffer was added to the pellet (to resuspend the pellet) and the pellet was also assayed for cAMP and cGMP. The general assay procedure was followed to assay for cAMP PDE and cGMP PDE. The concentrations of cold cAMP and cGMP used were 1.0 uM and 4.0 uM, respectively. 100 ul of a 0.5 mg/ml solution of snake venom was added to each tube.

The activity of Compound I was assayed using the supernatant and pellet from the PDE activity experiments. Compound I was diluted to the following concentrations (in uM): 1000, 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, and 0.2; and 80 ul of each dilution were tested for PDE inhibition. The IC_{50} for Compound I on cAMP and cGMP was determined using 0.25 uM CAMP and 1.0 uM cGMP and were calculated using the following four parameter logit function:

$$y = \frac{A - D}{L + (x/c) - B} \div D$$

A = 0x

B = Slope

C = midpoint

D = 0 y

Assay of 3'5' cyclic Adenosine Monophosphate (cAMP) Formation from Adenosine

Materials for Incubation

(1) Eight to ten pancreatic islets per tube

(2) Six 12 x 75 mm culture tubes

(3) Solutions:

a. 10 uCi [8-^{14}C] adenosine (NEN, Boston, MA) in 1 ml SIF containing 300 mg/dl glucose and 0.3% bovine serum albumin (BSA)

b. cold SIF (300 mg/dl glucose, 0.3% BSA)

c. incubation solutions:

1. SIF (300 mg/dl glucose, 0.3% BSA)

2. SIF (300 mg/dl glucose, 0.3% BSA) + 0.1 mM Compound I

3. SIF (300 mg/dl glucose, 0.3% BSA) + 0.1 mM MIX

d. Solution of 0.5 mg cold cAMP (carrier cAMP)

Pancreatic islets were isolated from male Wistar rats using the collagenase method (previously described). Eight to ten islets were placed in each of the six 12 x 150 mm culture tubes. 200 ul of the [8-^{14}C] adenosine solution (3a above) was added to each tube. The six

tubes were incubated for 60 minutes in a 37°C shaking water bath with aeration every 10 minutes using 95% O_2-5% CO_2. After incubation, one ml of cold SIF (3b above) was added to each tube. The tubes were centrifuged (5 min 2500 rpm) and the supernatant drawn off and discarded. The six tubes were separated into three groups and 0.5 ml of the appropriate incubation solution added. Two controls received SIF alone, two tubes received SIF + Compound I, and two tubes received SIF + MIX. The tubes were incubated for 30 mintues in a 37°C shaking water bath with aeration every 10 minutes using 95% O_2-5% CO_2. Carrier cAMP (0.1 ml of 0.5 mg/ml solution) was added to each tube and the reaction terminated by placing the tubes in a boiling water bath for 3 minutes. The tubes were sonicated (15 sec) to break the islets and centrifuged to pellet the islets (5 min, 5000 rpm) and the supernatant taken and assayed for cAMP-^{14}C content.

Materials for cAMP-^{14}C Content Assay

1) Columns 0.6 x 13 cm

2) 50% (u/v) suspension of Dowex 50W-X4 200-400 mesh H form (Bio-Rad Lab, Richmond, CA)

3) 0.25 M solutions of $ZnSO_4$ and $Ba(OH)_2$

4) Scintillation fluid (8 g/l Omnifluor^R, NEN, Boston, MA in 1,4 Dioxane)

Cyclic-AMP was isolated by ion-exchange chromatography columns prepared by pipetting 2 ml of a 50% (v/v) suspension of Dowex 50-H^+ in water. The columns were prepared by washing the resin with distilled

water several times. The samples were poured over the columns, eluted with H_2O and two ml fractions were collected. According to Krishna et al. (1968) 75% of the cAMP-^{14}C should be in the third fraction and most of the ATP and ADP in the first fraction. Each fraction was tested for the absorbance of carrier cAMP on an ultraviolet spectrophotometer at 260 mu to verify that the majority of the cAMP-^{14}C was in the third fraction. Each cAMP fraction tube was treated in the following manner in order to remove the nucleotides and inorganic phosphates: 1) 0.2 ml $ZnSO_4$ (0.25M) and 0.2 ml $Ba(OH)_2$ (0.25M) added; 2) the tubes were mixed and centrifuged (5 min. 2500 rpm); 3) the supernatant was decanted to clean tubes and 4) the $ZnSO_4$-$Ba(OH)_2$ precipitation (steps 1 and 2) was repeated, 5) the supernatant was centrifuged and a 0.5 ml aliquot added to 15 ml scintillation fluid. Radioactivity was measured on a Tracor Mark III Scintillation counter. The cAMP-^{14}C accumulation was calculated as pg/8 islets/30 min using the following formula:

$$pg = \frac{dpm}{dpm/pCi} \quad \frac{\text{MW adenosine in pg/pmol}}{\text{specific activity of adenosine in pCi/pmol}}$$

Statistical Analysis

Statistical analysis of the data was performed using the Student's t-test for significance (Hodges, Krech and Crutchfield, 1975).

RESULTS

100 mg/dl Glucose

These results were inconclusive. The amount of insulin released was too low to be detected by the IRI assay used to calculate the amount of insulin in each sample. A 50 ul aliquot and a 100 ul aliquot were both assayed to try and bring the numbers to a detectable level. It could not be determined if Compound I had any effect on insulin secretion at 100 mg/dl glucose concentration.

300 mg/dl Glucose

With Compound I (0.1 mM) present in the perfusion medium, a significant increase ($p << 0.001$) in insulin secretion was observed at this glucose concentration (Figure 3). The large standard errors can be attributed to the wide variation in the insulin content of pancreatic rat islets (even those of the same size) (Steinke, Patel and Ammon, 1972). The increase in insulin secretion was observed for all time points through 55 minutes. Between 55 and 60 minutes perfusion the insulin secretion for Compound I was not significantly different from the control. The increase in insulin secretion was biphasic and ranged from a 2-fold to a 4-fold increase.

600 mg/dl Glucose

There was no significant difference between the control and Compound I at a glucose concentration when observed as a whole (Fig. 4).

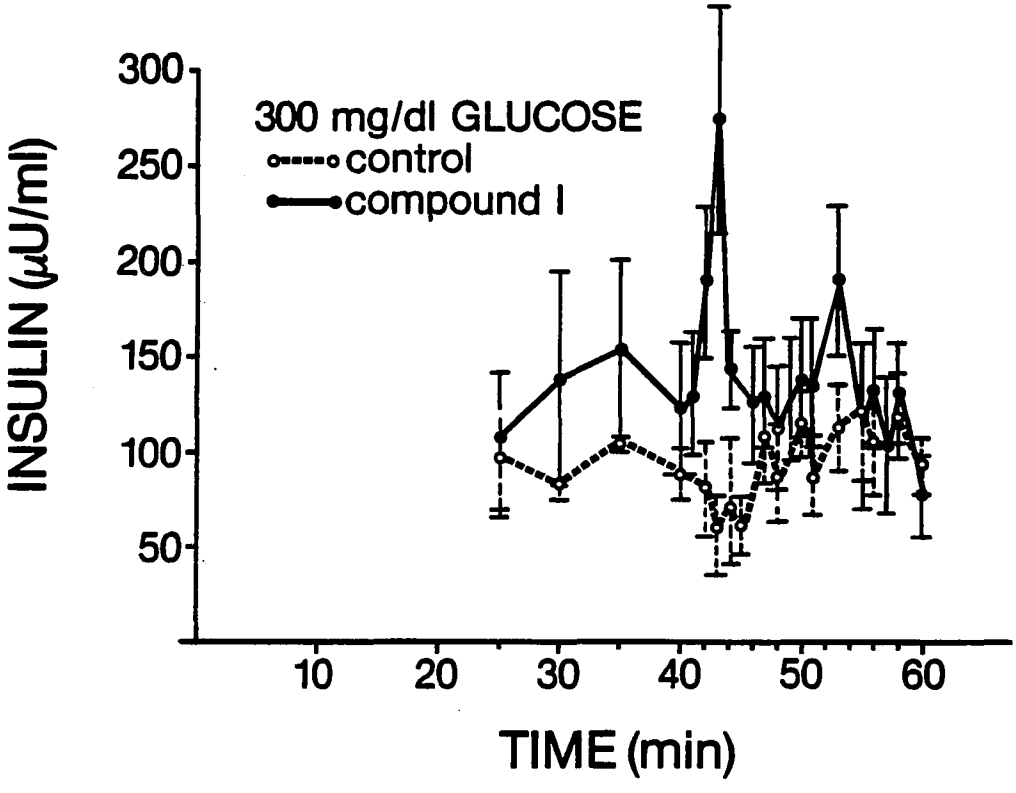

Figure 3. Effect of Compound I on glucose-stimulated insulin release at glucose concentration of 300 mg/dl. -- Each point is the mean ± SEM of four (4) Compound I experiments (•) and five (5) controls (o).

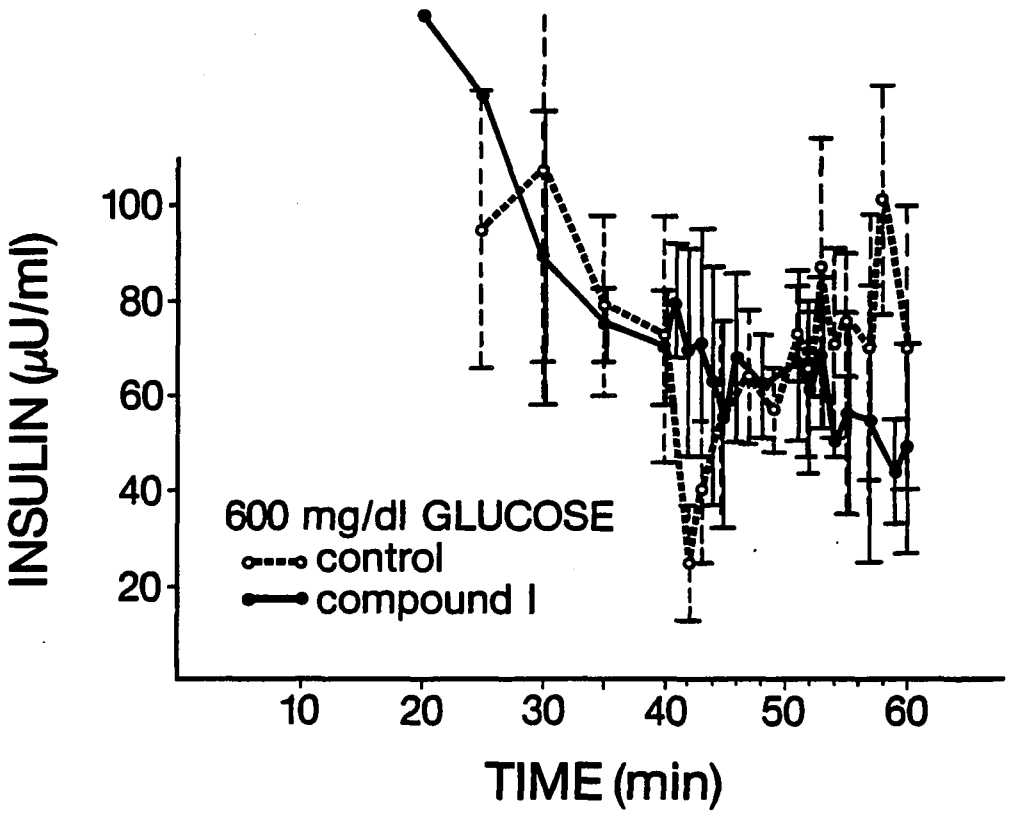

Figure 4. Effect of Compound I on glucose-stimulated insulin release. -- Glucose concentration of 600 mg/dl. Each point is the mean ± SEM for four (4) controls (o) and four (4) Compound I experiments (●).

However, if only the last 9 minutes of perfusion are examined, the control is significantly higher than Compound I. This drop in insulin secretion by Compound I was also observed in other experiments (Fig. 3) but since those values were originally significantly greater than the control, the drop was not significantly different.

Compound I Effects on Glucose Metabolism

Since Compound I has hydrogen bonding possibilities similar to those of glucose (Hershfield and Richards, 1976), but was not metabolized like glucose (De Haen, 1981), experiments were performed to determine if the increase in insulin secretion observed in the presence of Compound I was due to an increase in glucose metabolism in the islet.

At 300 mg/dl glucose, the control islets metabolized [5-^{3}H]-glucose to ^3H_2O at the rate of 35.09 ± 25.37 pmol/islet/hr. At the same glucose concentration with the addition of Compound I (0.1 mM), the islets metabolized [5-^{3}H] glucose to ^3H_2O at the rate of 42.49 ± 29.94 pmol/islet/hr (Table 1). Using 600 mg/dl glucose the controls averaged 39.92 ± 22.36 pmol/islet/hr and Compound I averaged 49.58 ± 15.50 pmol/islet/hr. There was no significant difference between the islets incubated with Compound I or the controls of either 300 mg/dl glucose or 600 mg/dl glucose.

3-Isobutyl-1-methylxanthine (MIX)

MIX is a known phosphodiesterase (PDE) inhibitor that increases the levels of insulin secretion, apparently by preventing the breakdown of cyclic-AMP. Experiments were performed to determine if Compound I

Table 1. Effect of Compound I on islet glucose metabolism *in vitro*.

Glucose concentration (mg percent)	Compound	Glucose metabolism (pmol/islet/hr)
300	Control (12)	35.09 ± 25.37
	Compound I (12)	42.47 ± 29.94
600	Control (8)	39.92 ± 22.36
	Compound I (8)	49.58 ± 5.50

Values are expressed as the means (± SEM) for vials containing 15 islets each. The number in parentheses is the number of repetitions.

augmented the MIX-stimulated insulin release (Fig. 5). Compound I not only did not augment the MIX-stimulated insulin release, it significantly inhibited this release ($p << 0.001$).

Arginine-Stimulated Insulin Secretion

The results of these experiments are shown in Figures 6 and 7. If the whole graph is considered, there was a significant difference between control and Compound I ($p < 0.001$). However, arginine was present in the perfusion medium only between 41 minutes and 50 minutes. A Student's t-test on these means show no significant difference between the control and Compound I. There was a significant difference between the control and MIX ($p << 0.001$) whether the whole perfusion time was considered or only the time points when arginine was present were considered. The difference between Compound I and MIX was barely significant ($p < 0.06$) when the complete perfusion was considered but MIX was significantly greater when only the time when arginine was present was considered. In conclusion, Compound I did not augment arginine-stimulated insulin release, while MIX did augment arginine-stimulated release.

Phosphodiesterase (PDE) Activity of Compound I

The above results suggested that Compound I does not increase insulin secretion simply by elevating cyclic-AMP. However, Compound I is known to inhibit PDE activity in the rat kidney (De Haën, 1981, personal communication). Therefore, the effect of Compound I on rat pancreatic islet phosphodiesterase was studied.

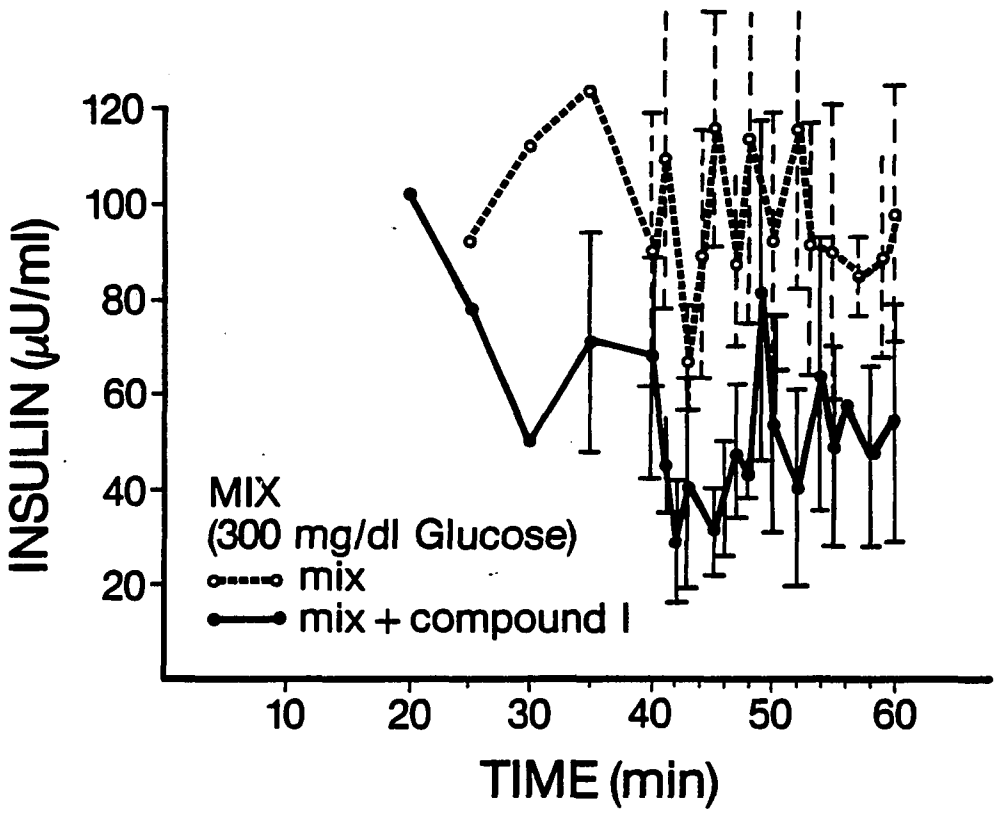

Figure 5. Effect of 3-isobutyl-1-methylxanthine (MIX, 0.25 mM) (o) and MIX (0.25 mM) + Compound I (0.1 mM) (●) on glucose-stimulated insulin release (glucose concentration 300 mg/dl). -- Points are the mean ± SEM for four (4) experiments for MIX alone and four (4) for MIX + Compound I.

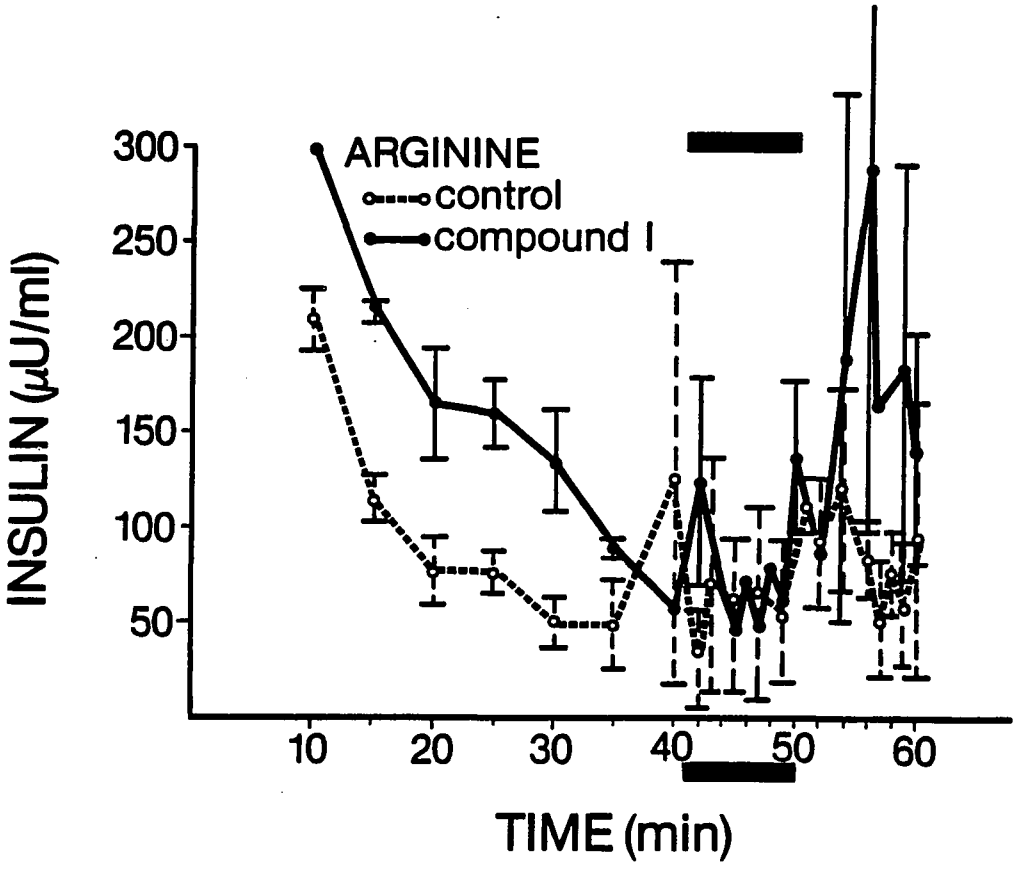

Figure 6. Effect of Compound I (0.1 mM, glucose conc. 100 mg/dl) (●) on arginine-stimulated insulin release. -- The control consisted of SIF + 100 mg/dl glucose (o). The points are the mean ± SEM for four (4) experiments each. The arginine (20 mM) was present only during the time points between the bars (41-50 min).

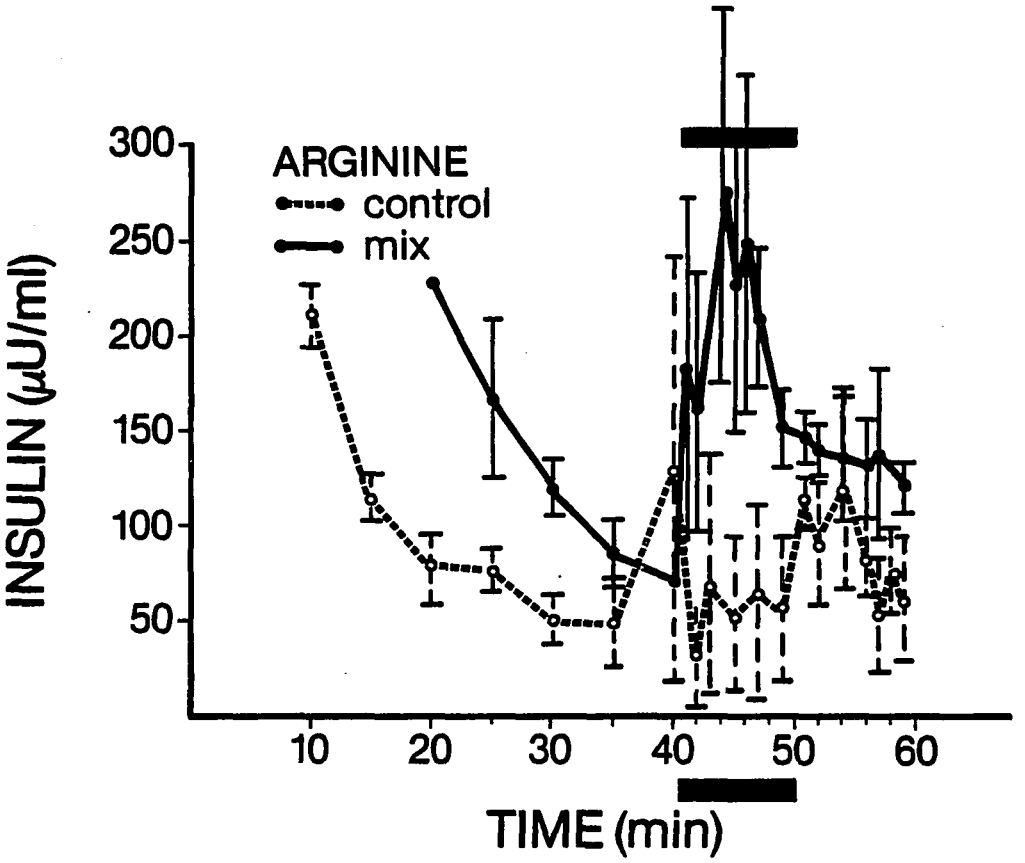

Figure 7. Effect of 3-isobutyl-1-methyxanthine (MIX (•)) (0.25 mM) on arginine-stimulated insulin release. -- The control (o) is the same as the control for Figure 6. The points are the mean ± SEM for four (4) experiments for each group. The arginine was present only during the time points between the bars (41-50 min).

Pancreatic islets were isolated and homogenized. The homogenate was centrifuged at 100,000 x g for 45 minutes, and the PDE activity measured in the supernatant and resuspended pellet. Control PDE activity with 0.25 uM cAMP, was 336 pmol/mg/min in the supernatant and 94 pmol/mg/min in the pellet. The IC_{50} with Compound I was 42 ± 6 uM in the supernatant and 43 ± 18 uM in the pellet (Table 2). These results show Compound I to be a potent inhibitor of pancreatic islet phosphodiesterase.

Assay of 3'5' Cyclic-AMP Formation from [8-^{14}C] Adenosine

Cyclic-AMP formation in isolated islets were assayed using [8-^{14}C] adenosine. Groups of 8 islets were incubated in SIF containing [8-^{14}C] adenosine alone (control), SIF with [8-^{14}C] adenosine and 0.1 mM Compound I, or SIF with [8-^{14}C] adenosine and 0.1 mM 3-isobutyl-1-methylxanthine (MIX). The control islets accumulated 254 ± 48 pg/8 islets/30 min cyclic-AMP (Table 3). The Compound I and MIX islets produced 236 ± 28 pg and 277 ± 35 pg/8 islets/30 min, respectively. These values were not significantly different from the control or from each other.

Discussion

The ability of 2,4-diamino-5-cyano-6-bromopyridine (Compound I) to increase glucose-stimulated insulin secretion has been demonstrated both *in vivo* and in perfused rat pancreas (Johnson and De Haën, 1979). This ability to increase insulin secretion was also demonstrated using collagenase-isolated rat pancreatic islets.

Table 2. Effect of Compound I on phosphodiesterase activity in pancreatic islets incubated *in vitro*.

	Specific Activity pmol/mg/min	IC_{50} Compound I
Cyclic AMP		
supernatant	336	42 uM ± 6
pellet	94	43 uM ± 18
Cyclic GMP		
supernatant	323	--
pellet	67	51 uM ± 18

Table 3. Effect of Compound I on cyclic-AMP accumulation in pancreatic islets incubated *in vitro*.

Compound (in 300 mg/dl glucose)	Cyclic AMP-14-C accumulation (pg/8 islets; 30 min)
Control	254 ± 48 (5)
Compound I (0.1 mM)	236 ± 28 (7)
3-isobutyl-1-methylxantine (MIX, 0.1 mM)	277 ± 35 (8)

Values are expressed as the mean (± SEM). The number in parentheses is the number of repetitions.

As stated in the introduction, two models have been proposed to explain how glucose increases insulin secretion (Randle, Ashcroft and Gill, 1968; Ashcroft, 1976): 1) a cell membrane receptor recognizes increased glucose concentration, or 2) a metabolite (or metabolites) of glucose regulates insulin release. It has been suggested that both models are partially correct (Ashcroft, Weeransinghe and Randle, 1973; Davis and Lazarus, 1971).

Compound I resembles glucose with respect to hydrogen-bonding possibilities (Hershfield and Richards, 1976), but its metabolites do not resemble those of glucose (De Haën, personal communication).

Compound I apparently requires an elevated concentration of glucose to be active (no results at 100 mg/dl glucose but very positive results were obtained at 300 mg/dl glucose), and, therefore, cannot entirely substitute for glucose in eliciting insulin secretion. The above observations help support the suggestion that both models for insulin release might be partially correct.

It was proposed that although Compound I itself could not be metabolized like glucose, its mechanism of action might be to increase the metabolism of glucose in the islet. Since Compound I did not significantly increase the islet metabolism of glucose either at 300 mg/dl glucose or at 600 mg/dl glucose, this is not the mechanism.

3-Isobutyl-1-methylxanthine (MIX), is a phosphodiesterase inhibitor that apparently increases insulin secretion by increasing cyclic-AMP levels. It was proposed that the mechanism of action of Compound I might also be one of increasing islet cAMP levels. It was

proposed that the mechanism of action of Compound I might also be one of increasing islet cAMP levels. It was thought that if the mechanism of action of MIX and Compound I were the same, Compound I would not increase the insulin secretion produced by 3-isobutyl-1-methylxanthine. Compound I not only did not increase MIX insulin secretion, the combination of MIX and Compound I significantly decreased insulin secretion. It is possible that these results were due to competitive inhibition between two phosphodiesterase inhibitors or some other mechanism.

Arginine increases insulin release, possibly by potentiating calcium ion flux (Randle and Hales, 1972). 3-Isobutyl-1-methylxanthine (MIX) augmented arginine-stimulated insulin release significantly but Compound I did not. It is interesting to note that once the arginine was removed, Compound I significantly increased the insulin secretion almost as if the increase in insulin secretion was present but in some way the combination of arginine and Compound I prevented secretion of the insulin. Since it is thought that arginine works by potentiating Ca^{2+} flux (Randle and Hales, 1972), it might be that Compound I also potentiates glucose-stimulated insulin release through an effect on calcium and that the combination of the two effects is inhibitory.

Compound I was discovered to be a phosphodiesterase inhibitor in rat kidney (De Haën, personal communication). As a result, it was proposed that Compound I might also inhibit phosphodiesterase activity in rat islets. Our experiments showed that Compound I is a potent inhibitor of phosphodiesterase activity in pancreatic islet cells.

In order to try and reinforce the proposal that phosphodiesterase inhibition might be one of the major mechanisms through which Compound I acted, a study was done to determine if Compound I would increase cAMP levels in pancreatic islets. 3-Isobutyl-1-methylxanthine (MIX) was used as a second control since its apparent mechanism of action is through the increase of cAMP by phosphodiesterase inhibition. Neither MIX nor Compound I significantly increase the islet cAMP levels. These results could be due to several factors: 1) possibly the newly synthesized cAMP was degraded nearly simultaneously with formation and therefore the presence of a PDE inhibitor showed no effect, or 2) the presence of a phosphodiesterase inhibitor at high glucose concentration does not necessarily increase cAMP levels. Some investigators have previously reported that cAMP in the presence of high glucose and a phosphodiesterase inhibitor is increased (Charles et al., 1973; Grill and Cerasi, 1973; Hellman et al., 1974). Other investigators did not confirm the cAMP increase (Montague and Cook, 1971; Cooper, Ashcroft and Randle, 1973). These results agree with the latter group of investigators, since neither MIX nor Compound I increased cAMP.

In summary, Compound I was shown to be a potent phosphodiesterase inhibitor in rat pancreatic islets. Although the experiments measuring accumulation of ^{14}C cAMP were inconclusive, it is thought that Compound I may increase glucose-stimulated insulin secretion partially through its phosphodiesterase activity. Since Compound I did not augment either MIX or arginine-stimulated insulin release, it is possible

that a membrane receptor also plays a very important part in insulin release.

Compound I was found to be relatively non-toxic in rats (Johnson and De Haen, 1979) and could be useful as an oral treatment of diabetics that are non-insulin dependent. Since Compound I is such a potent phosphodiesterase inhibitor, it might possibly also be useful in relieving such diseases as bronchial asthma.

A defined mechanism of action for Compound I is still unknown, but a possible important factor in insulin release, is the role of Ca^{2+}. The effect of Compound I on Ca^{2+} levels has not yet been studied, but it is possible that Compound I has a significant effect on Ca^{2+} levels and studies of Compound I and Ca^{2+} interaction would be worthwhile. The changes in Ca^{2+} levels may be the key to glucose-induced insulin release.

Conclusions

1. Compound I increases glucose-stimulated insulin release in isolated rat pancreatic islets.

2. Compound I is a potent phosphodiesterase inhibitor in rat pancreatic islets.

3. The difference between MIX and Compound I as to arginine stimulated insulin release indicates that phosphodiesterase inhibition alone is not the mechanism of action of Compound I.

4. The fact that Compound I has a structure that resembles glucose in hydrogen bonding possibilities and the 3 points above support the suggestion that both a glucoreceptor (with which Compound I may interact)

and some metabolite of glucose might be active in eliciting insulin secretion.

APPENDIX A

PROCEDURES

0.1 M Tris-1% Albumin, pH 7.4

12.1 g Trizma base in approx. 600-700 ml H_2O
pH to 7.4 w/ approx. 7.0 ml conc. HCl
add 0.1g Thimersal
add 10 g BSA (let sit until BSA is dissolved, don't shake)
QS to 1 liter
Check pH

Standard

Stock standard NovoRat Insulin R1676 10 mU/ml
Working standard 0.1 uU/ul
dilute stock 1:100 (store in small quantities)

Antibody

Stock antibody GPais I-35
Overdose dilution 1:150 dilution of stock
Regular dilution 1:3000 dilution of stock

Tracer

dilute newly made Insulin ^{125}I to give 20,000-30,000 CPM, freeze

Cellulose

10% slurry in buffer & mix in ice for at least 30 min before using.
Keep slurry mixing while adding to tubes

Make all standards, antibody tracer and cellulose dilutions in Tris-Albumin buffer

Bretag, A. H., Synthetic interstitial fluid for isolated mammalian tissue. Life Sciences 8:317-329, 1969.

Stock Solns.

A.	NaCl	63 g/L	1077 mM
B.	KCl	6.5 g/250 ml	348 mM
C.	$MgSO_4 \cdot 7H_2O$	4.3 g/250 ml	69 mM
D.	$NaH_2PO_4 \cdot H_2O$	5.8 g/250 ml	167 mM
E.	$CaCl_2 \cdot 2H_2O$	10 g/100 ml	10%
	$CaCl_2$	7.55 g/100 ml	

A.	100 ml/l	BSA 0.3%
B.	10 ml/l	
C.	10 ml/l	
D.	10 ml/l	
E.	1.7 ml/l	

$NaHCO_3$ 2.2 g/l
NaGluconate 2.1 g/l
qs to 1 L

APPENDIX B

300 mg% glucose controls

Time (min)	Insulin uU/ml #1	#2	#3	#4	Mean	SEM
25	71	46	93	178	97.0	28.7
30	61	78	88	104	82.8	9.0
35	103	101	111	97	103.0	2.9
40	91	61	127	78	89.3	14.0
41	99	58	99	82	84.5	9.7
42	152	51	76	42	80.3	25.0
43	118	85	47	29	69.8	21.2
44	141	46	74	36	74.3	33.5
45	90	40	60	52	60.7	15.1
46	143	44	111	81	94.8	21.1
47	142	48	139	94	105.8	22.2
48	42	50	142	120	88.5	25.0
49	113	43	131	104	97.8	19.1
50	93	115	102	151	115.3	18.0
51	143	47	94	68	88.0	20.7
52	127	38	148	126	109.8	24.4
53	128	51	120	152	112.8	21.7
54	177	60	75	137	112.3	27.3
55	184	121	59	119	120.7	36.1
56	175	44	96	108	105.8	26.9
57	127	42	138	152	114.8	24.8
58	161	54	140	119	118.5	23.1
59	127	45	138	112	105.5	20.9
60	92	55	125	94	9.15	14.3

300 mg% glucose + Compound I

Time (min)	Insulin uU/ml #1	#2	#3	#4	#5	Mean	SEM
25	248	64	99	77	43	106.2	36.6
30	304	66	111	73	137	138.5	56.0
35	248	145	104	111	163	154.3	46.9
40	184	112	121	62	132	122.3	35.2
41	207	61	155	97	129	130.0	32.2
42	285	140	107	224	190	189.0	40.4
43	210	479	327	129	230	275.0	60.0
44	191	170	150	67	135	142.6	21.1
45	139	117	288	56	71	134.2	41.3
46	226	124	153	47	73	124.6	31.4
47	212	180	115	92	54	130.6	28.9
48	207	170	88	47	49	112.2	32.5
49	219	176	119	56	65	127.0	31.5
50	210	171	94	138	80	138.8	31.1
51	200	161	135	136	46	135.5	32.7
52	142	201	164	94	-	150.3	22.4
53	187	236	253	82	-	189.5	38.5
54	259	107	274	86	-	181.5	49.4
55	-	122	227	70	29	112.0	42.8
56	151	166	175	42	-	133.5	30.9
57	115	82	236	46	35	102.8	36.2
58	81	123	204	116	-	131.0	26.0
59	36	111	135	69	37	77.6	19.8
60	19	77	149	88	48	76.2	21.8

Mix

Time (min)	Insulin uU/ml #1	#2	#3	Mean	SEM
25	170	32	75	92.1	40.7
30	166	42	127	111.5	36.6
35	193	43	135	123.9	43.6
40	149	59	62	89.3	29.4
41	174	72	83	109.5	32.3
42	104	110	108	107.1	1.6
43	78	67	57	67.4	10.6
44	69	139	57	88.5	25.5
45	135	147	67	116.0	25.0
46	149	109	40	99.1	31.8
47	103	110	53	88.4	18.1
48	176	116	46	112.7	37.8
49	124	132	44	100.0	28.3
50	90	141	46	92.1	27.4
51	113	171	51	111.6	34.8
52	159	131	52	113.8	32.1
53	119	117	38	91.2	26.8
54	92	147	34	9k.2	32.6
55	81	147	42	90.0	30.5
56	72	128	71	90.2	18.8
57	80	100	75	84.6	7.6
58	93	114	57	88.1	16.7
59	108	113	48	89.4	21.0
60	116	132	45	97.5	26.7

Mix + Compound I

Time (min)	Insulin uU/ml #1	#2	#3	#4	Mean	SEM
20	194	11	73	131	102.4	39.2
25	109	54	51	75	78.1	16.8
30	67	52	37	46	50.4	9.4
35	116	71	53	44	71.0	22.8
40	63	74	54	83	68.2	25.9
41	68	30	32	49	44.6	9.8
42	59	37	38	41	43.8	13.0
43	68	28	41	65	50.3	21.8
44	56	46	50	32	45.8	11.4
45	32	47	45	39	40.7	8.5
46	61	40	30	47	44.5	12.0
47	61	38	53	45	48.2	13.7
48	56	37	45	35	43.3	4.8
49	51	53	129	41	68.5	32.9
50	50	17	212	29	53.9	23.4
51	50	26	101	20	49.0	18.4
52	38	24	96	25	40.3	20.6
53	23	35	113	33	51.0	20.8
54	40	41	149	27	63.9	28.5
55	45	26	109	17	49.4	20.8
56	59	24	121	23	56.8	23.1
57	62	12	112	24	52.2	22.6
58	62	11	93	22	46.9	18.7
59	26	26	142	9	50.7	30.5
60	33	26	128	29	54.2	24.8

Arginine Control (100 mg% glucose)

Time (min)	Insulin uU/ml #1	#2	#3	#4	Mean	SEM
10	226	194	189	232	210.2	16.1
15	103	126	98	131	114.4	11.8
20	59	94	52	101	76.5	18.0
25	87	65	59	93	75.9	11.4
30	63	36	26	74	49.8	13.5
35	73	25	41	57	49.0	23.8
40	58	33	43	48	45.7	12.3
41	46	23	26	41	33.5	20.2
42	40	20	25	35	30.1	26.2
43	101	47	67	89	74.3	63.2
44	99	49	59	89	73.9	46.1
45	76	30	44	63	53.3	41.1
46	80	48	42	74	58.0	41.4
47	94	34	49	79	64.0	50.6
48	89	31	45	75	60.1	46.2
49	74	38	55	57	55.8	36.7
50	71	60	51	81	65.8	23.4
51	102	120	105	117	110.9	14.4
52	116	68	81	103	92.0	33.7
53	119	79	83	115	99.0	19.8
54	138	100	98	140	119.1	13.2
55	107	70	78	99	88.4	25.8
56	104	72	80	86	83.2	21.2
57	62	42	41	63	52.0	30.2
58	79	72	74	77	75.5	23.0
59	77	44	78	42	60.2	32.4
60	131	57	139	49	94.0	72.7

20 mM Arginine present

Arginine-Mix

Time (min)	Insulin uU/ml #1	#2	#3	#4	Mean	SEM
20	341	215	253	303	278.3	132.9
25	138	196	89	245	167.2	42.4
30	135	106	87	153	120.4	14.8
35	70	100	58	112	84.9	17.1
40	68	72	55	85	70.1	1.7
41	201	162	132	231	181.6	15.3
42	163	165	162	166	164.4	1.2
43	290	205	282	213	247.6	24.3
44	321	227	303	245	274.3	25.0
45	243	210	255	198	226.6	9.4
46	282	213	288	207	247.7	14.3
47	219	199	232	186	208.8	37.4
48	222	156	229	149	189.3	60.3
49	171	131	188	114	150.9	19.7
50	145	149	150	144	146.1	2.0
51	160	132	164	128	145.8	13.8
52	124	153	131	146	138.5	14.6
53	102	171	116	157	136.4	34.5
54	103	168	117	153	135.1	32.6
55	110	151	119	141	130.1	20.6
56	128	132	141	119	129.8	25.9
57	121	155	141	135	138.1	43.5
58	104	151	123	132	127.5	23.1
59	106	133	128	111	119.5	13.7
60	111	78	134	56	94.8	16.7

20 mM Arginine

Arginine - Compound I

Time (min)	Insulin uU/ml #1	#2	#3	#4	Mean	SEM
10	347	252	291	309	299.8	47.6
15	220	208	131	297	213.9	6.3
20	194	137	162	168	165.1	28.5
25	178	141	146	173	159.5	18.1
30	164	109	158	115	136.4	27.4
35	95	86	96	84	90.3	4.7
40	42	70	37	75	56.0	27.1
41	60	92	63	89	76.0	15.9
42	110	136	118	131	124.7	34.8
43	99	103	95	107	101.0	38.8
44	67	74	60	81	70.4	39.9
45	32	63	17	78	47.6	15.6
57	47	98	52	93	72.7	36.0
48	43	53	40	56	48.3	25.0
48	98	59	62	95	78.5	27.6
49	71	59	56	74	65.1	5.9
50	123	153	114	162	138.2	40.4
51	113	96	104	105	104.5	8.4
52	84	90	78	97	87.3	7.7
53	130	111	113	128	120.5	16.3
54	247	133	237	143	189.9	29.1
55	344	172	326	191	258.4	34.7
56	399	178	368	209	288.7	51.4
57	209	127	152	184	168.1	31.0
58	213	140	139	215	176.9	28.7
59	237	131	173	192	182.3	37.6
60	142	139	85	196	140.5	19.3

600 mg% glucose controls

Time (min)	Insulin uU/ml #1	#2	#3	#4	#5	Mean (using data from #1,2,3 and 4)	SEM
10	260.2	246.4	426.0	583.1	605.4	378.9	79.4
15	185.5	168.3	328.9	345.1	145.8	257.0	46.5
20	82.6	173.1	249.4	107.5	69.3	153.2	37.3
25	37.2	119.5	165.1	59.9	51.9	95.4	29.0
30	34.5	128.9	210.5	53.9	13.8	107.0	40.1
35	62.4	62.3	134.8	57.2	6.1	79.2	18.6
40	22.3	63.8	145.5	54.9	1.4	71.6	26.2
41	23.8	39.0	104.3	98.1	-	66.3	20.4
42	8.0	4.8	28.2	57.5	2.2	24.6	12.1
43	9.9	35.5	32.5	80.8	4.1	39.7	14.9
44	19.1	24.4	55.0	107.7	4.4	51.6	20.3
45	11.4	23.0	79.3	102.8	0.1	54.1	22.0
56	25.4	49.1	68.2	98.1	2.8	60.2	15.4
47	53.8	44.1	53.9	105.9	2.5	64.4	14.0
48	55.1	36.8	54.2	88.1	0.6	58.6	10.7
49	55.7	35.9	53.2	81.0	8.4	56.5	9.3
50	26.2	90.4	58.4	103.7	4.9	69.7	17.3
51	49.1	80.9	63.2	96.8	3.2	72.5	10.4
52	25.6	75.9	54.4	99.9	2.1	64.0	15.8
53	20.1	136.5	67.3	125.8	17.3	87.4	27.1
54	11.7	100.0	85.7	84.5	5.7	70.5	19.9
55	41.8	102.7	78.3	83.2	8.6	76.5	12.7
56	17.0	125.5	57.5	100.3	2.9	75.1	23.9
57	25.2	145.0	39.8	70.6	3.3	70.1	26.7
58	56.9	169.7	80.4	98.7	2.6	101.4	24.3
59	35.4	136.2	50.1	91.2	7.7	78.2	22.6
60	3.7	137.7	42.5	96.2	2.8	70.0	29.5

600 mg% glucose Compound I

Time (min)	Insulin uU/ml #1	#2	#3	#4	#5	Mean (using data from #2, 3 and 4)	SEM
10	204.3	468.4	463.0	285.5	181.8	405.6	60.1
15	106.2	252.6	398.9	278.1	209.5	309.9	45.1
20	76.1	80.2	204.3	135.3	85.3	139.9	35.9
25	62.1	80.3	136.4	153.4	60.3	123.4	22.1
30	30.1	30.0	133.3	103.1	35.4	88.8	30.7
35	6.2	61.9	72.0	90.1	13.5	74.7	8.2
40	22.1	50.0	67.9	92.9	3.8	70.3	12.4
41	7.8	62.5	75.0	103.3	2.9	80.3	12.1
42	25.2	42.3	50.4	112.7	2.3	68.5	22.2
43	18.0	43.0	51.8	119.3	48.2	71.4	24.1
44	-	21.9	56.0	108.1	11.4	62.0	25.1
45	-	18.4	50.8	92.9	16.8	54.0	21.6
46	17.4	37.1	69.3	98.2	34.0	68.2	17.6
47	25.4	31.3	69.7	99.7	20.4	66.9	19.8
48	37.3	44.0	58.9	82.8	14.9	61.9	11.3
49	16.5	27.6	45.6	116.0	16.4	63.1	27.0
50	8.7	24.5	56.7	115.7	14.4	65.6	26.7
51	-	69.4	35.9	97.7	4.1	67.7	17.9
52	-	59.0	31.5	91.4	12.0	60.6	17.3
53	-	64.0	43.6	99.3	14.3	69.0	16.3
54	8.2	46.5	47.2	56.3	15.0	50.0	3.2
55	12.5	38.3	31.1	97.8	4.9	55.7	21.1
56	25.2	31.9	42.7	89.2	10.4	54.6	17.6
57	24.1	21.6	28.1	112.3	28.8	54.0	29.2
58	10.0	31.8	26.2	80.3	8.6	46.1	17.2
59	29.1	42.8	25.6	64.3	7.5	44.2	11.2
60	33.2	28.6	24.2	93.9	12.2	48.9	22.5

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