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SUMMARY

THE ROLE OF GLUCAGON IN HEALTH AND DISEASE

by

KEITH D. BUCHANAN

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The construction of a reliable immunoassay method for glucagon has formed the basis of this thesis. The major factor in the building of a good assay was the recognition of the susceptibility of glucagon to attack by proteolytic enzymes, a fact which at first escaped the notice of earlier investigators. Trasylol, a proteolytic enzyme inhibitor, has proved to be an efficient means to prevent this enzyme degradation. The assay was able to detect as little as 20 µµg. of glucagon.

The application of the assay to measurement of immunoreactive glucagon (IRG) in tissues and body fluids, revealed that IRG was not only present in the pancreas but was also detected in much of the alimentary tract, mainly the small and large intestines. It was apparent also that circulating IRG was derived from both enteric and pancreatic sources. Clearance studies of glucagon showed that glucagon disappeared rapidly from the body and that the liver was a major site for the degradation of glucagon. No difference in the clearances of enteric and pancreatic glucagons was noted. However immunological differences appeared to be present between the enteric and pancreatic glucagons, enteric glucagon reacting less strongly with glucagon antibody than pancreatic glucagon.

When assessing the factors which might affect the release of glucagon, consideration had to be given to the factors cited above which would complicate the studies. It was realised that an assessment of factors affecting the release of glucagon from both the pancreas and gut would have to be undertaken separately. A direct assessment of factors affecting the pancreatic release of glucagon was made possible by the application of/

/of a method for isolating the islets of Langerhans of rat pancreas. Additional use was made of dogs with venous catheters situated in pancreatic and gut veins, so that a separate measurement of IRG changes from blood draining the pancreas and gut could be made. Studies of animals in whom the pancreas was removed were also made, in order that factors affecting gut IRG release could be assessed, in the absence of the pancreas.

It was realised also that studies of peripheral circulating IRG levels may fail to record changes in gut and pancreatic IRG secretion as much of the IRG would be removed in its passage through the liver. Thus many of the measurements of IRG were done in pancreatic, jejunal, colonic or portal blood prior to its passage through the liver. Wherever possible experiments were duplicated both by "in vitro" pancreatic islet studies, and "in vivo" dog or human studies, in order that observations could be confirmed by different approaches.

Because of certain already established pharmacological actions of glucagon on carbohydrate metabolism, the effect of changing glucose concentrations on glucagon release was first of all studied. IRG secretion from the pancreas was found to increase during acute hypoglycaemia whereas no effect was noted on gut IRG secretion. Hyperglycaemia in contrast inhibited IRG secretion from the pancreas but once again had no effect on gut IRG secretion. Oral glucose did, however, stimulate gut IRG release but did not influence pancreatic IRG release.

A study of the effect of the enteric hormones on IRG release was made, because of previous reports of the effect of these hormones on insulin release. Pancreozymin was found to exert a potent effect on both IRG and insulin release from the pancreas, although they had no effect on IRG release/

/release from the gut. Secretin and gastrin did not however possess any IRG releasing properties from either gut or pancreas.

It was considered probable that glucagon might be an important hormone in the control of the metabolic changes during starvation particularly through its pharmacological actions on gluconeogenesis and lipolysis. However contrary to this hypothesis, circulating levels of IRG were found to fall during starvation in human subjects. That this fall might be due to diminished pancreatic IRG secretion was suggested by the finding that the release of IRG from pancreatic islets of starved rats was also reduced.

Amongst the other factors tested on glucagon release, results were obtained to suggest that some of the pituitary-adrenal hormones may influence IRG release from the pancreas. In particular adrenocorticotrophic hormone probably increases IRG release.

Studies were also made of the inter-relationship of glucagon and insulin secretion. Glucagon stimulated insulin release both "in vivo" and "in vitro". However simultaneous measurements of glucagon and insulin release from pancreatic islets seldom produced a direct correlation in the amounts of each hormone released as one might expect if endogenous glucagon were stimulating insulin release.

Clinical studies were performed on a family suffering from multiple endocrine adenomatosis. Immunoassays revealed that all islet cell pancreatic hormones - insulin, gastrin, and glucagon - were involved by the process. It was considered that excess glucagon secretion might have contributed/

/contributed to glucose intolerance and also hypercalcaemia.

It is concluded that pancreatic glucagon's physiological role may be in the prevention of hypoglycaemia which occurs during short periods of fasting. It may act via pancreozymin to prevent reactive hypoglycaemia after a meal, especially one which contains a large amount of protein. Glucagon's effect on insulin secretion may only operate at near fasting or low blood sugar concentrations. It is considered too that glucagon is probably a hormone which is of more importance during acute metabolic events rather than chronic. This is suggested by the lack of influence of chronic starvation on glucagon secretion.

No conclusions could be drawn as to the physiological role of enteric glucagon. Enteric glucagon did not respond to the same stimuli as pancreatic glucagon. The rise of enteric glucagon seen after oral glucose may suggest that its role lies in the stimulation of insulin secretion.

The clinical studies in the thesis suggest that abnormalities of glucagon secretion may be sought in subjects showing any of the following features:- glucose intolerance, hypercalcaemia, and reactive hypoglycaemia.

THE ROLE OF GLUCAGON IN HEALTH AND DISEASE

by

KEITH DEANS BUCHANAN

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To

Maureen

"To travel hopefully is a better thing than to arrive and the true success is to labour".

From "El Dorado" by Robert Louis Stevenson (1850 - 1894).

PREFACE

The work on which this thesis is based was carried out from September 1966 to February 1968 during the tenure of a research fellowship in the Division of Endocrinology and Metabolism, the Department of Medicine, University of Washington, Seattle, Washington, U.S.A., under Professor Robert H. Williams. Because of my previous work on the radio-immunoassay of insulin in Dr. A. H. Imrie's unit of the Royal Infirmary, Glasgow, during 1963 - 1966, I found the study of glucagon by a radio-immunoassay technique a natural extension of my previous interests.

Some of the data contained in this thesis has already been published.

"Rise in Serum Immunoreactive Glucagon after Intrajejunal Glucose in Pancreatectomised Dogs". Proc. Soc. Exp. Biol. and Med., 126 : 813 (1967) (with J.E.Vance, T.Aoki and R.H.Williams).

"A Double Antibody Immunoassay for Glucagon". Diabetes, 17 : 179 (1968) (with W.R.Hazzard, P.M.Crockford, J.E.Vance, R.Chen and R.H.Williams).

"Effect of Glucose Concentration on Insulin and Glucagon Release from Isolated Islets of Langerhans of the Rat". Diabetes, 17 : 187 (1968) (with J.E.Vance, D.R.Challoner and R.H.Williams).

"The Physiologic Role of Glucagon". Proceedings of a Symposium on Radio-isotopes in Medicine, Oakridge, Tennessee (1968) (with J.E.Vance and R.H.Williams).

"Glucagon"/

/"Glucagon Clearance by the Isolated Perfused Rat Liver". Proc. Soc. Exp. Biol. and Med., June (1968) (with S.S.Solomon, J.E.Vance, H.P.Porter and R.H.Williams).

The following papers have been accepted for publication and are "in press".

"Effect of Pancreozymin on Insulin and Glucagon Levels in Blood and Bile". Amer. J. Physiol. (with J.E.Vance, A. Morgan and R.H.Williams).

"Effect of Starvation and Refeeding on Serum Immunoreactive Glucagon and Insulin Levels". J. Lab. & Clin. Med. (with J.E. Vance and R.H.Williams).

"Effect of Starvation on Insulin and Glucagon Release from Isolated Islets of Langerhans of the Rat". Metabolism. (with J.E.Vance and R.H.Williams).

I have been primarily responsible for the work which is in this thesis. However most work in medical research to-day is the result of enlightened collaboration. Especially would I like to record thanks to Dr. James E. Vance who was my colleague in many of the projects recorded in this thesis. I am most grateful for the opportunity I had to work in the department of Professor Robert H. Williams, a man of brilliant inspiration and magnificent enthusiasm. Mrs. Barbara Hickernell and Mrs. Susan Page provided technical assistance of the highest class. Doctors A. Morgan, T. Aoki and K. Dinstl, from the Department of Surgery, University/

/University of Washington, made their surgical expertise available to me during the many dog experiments which required surgical skill. I would like to thank Dr. A. H. Imrie and Professor E. M. McGirr who provided me with the necessary opportunities and encouragement which enabled me to pursue my research interests. Throughout the thesis I have tried to make it clear to whom I owe gratitude. Finally the thesis would not have been possible if it were not for the able assistance of members of my family which resulted in the typing and final preparation of the manuscript.

TABLE OF CONTENTS

INTRODUCTION

Although glucagon has been available in relatively pure form for some years, and its primary structure determined as long ago as 1956[1], there remains controversy as to the physiological role of this hormone. Glucagon's pharmacological actions have been well studied. Glycogenolysis[2], gluconeogenesis[3], insulinogenesis[3], and lipolysis[4] have all been defined as actions of the hormone. However, although these properties are seen under certain pharmacological and experimental conditions, it is less certain what role glucagon may have in the intact organism. The lack of a precise and reproducible assay method for the hormone halted progress along these lines. When Yalow and Berson[5] in 1959 first described the immunoassay technique for insulin, the way was clear for the application of the method to other polypeptide hormones. Unger et al (1961)[6] first described an immunoassay method for glucagon but application of the method to the assay of the hormone in biological fluids was made difficult by the instability of glucagon when exposed to proteolytic enzymes, and by the minute amounts of glucagon present in the circulation which called for high degrees of sensitivity. Hence progress has been slow in accumulating data from the immunoassay of glucagon, and partly due to a lack of appreciation of the difficulties encountered in the assay much misleading and often erroneous/

/erroneous information has been obtained.

It is the purpose of this thesis to report the construction of a reliable immunoassay for glucagon with a sensitivity beyond any yet reported. Utilising this assay studies have been made of the role of glucagon in health and disease.

Chapter 1 deals with the original methods used in the thesis. The construction of the immunoassay for glucagon is described and discussed in detail. A method for isolating islets of Langerhans from rat pancreas is also described and the usefulness of this model in the study of glucagon metabolism is stressed.

Chapter 2 is concerned with the measurement of glucagon in body fluids and tissues. Factors affecting the clearance and degradation of glucagon in the body are studied. The presence of glucagon in the gut as well as the pancreas is described.

Chapter 3 is the main body of the thesis. Here, various factors affecting the release or secretion of glucagon are studied. These factors include glucose concentration, various enteric hormones, starvation and some pituitary-adrenal hormones. In this chapter too, is described the effect of glucagon in stimulating insulin secretion.

In chapter 4 a family is described with multiple endocrine adenomatosis, where all the islet cell hormones, insulin, gastrin and/

/and glucagon are affected by the process.

In chapter 5 the author attempts to summarise the work in the thesis and draw broad conclusions.

HISTORICAL REVIEW OF GLUCAGON

In 1924 Kimball and Murlin[7] noted that when most commercial insulin preparations are injected intravenously, the typical hypoglycaemic effects were preceded by a short period of hyperglycaemia. This fleeting rise in glucose concentration was due to the presence of an impurity named "glucagon" or "mobiliser of sugar" by Murlin and collaborators[8], who first investigated its properties and attempted its purification in 1929. During the same year, Burger and Kramer[9] in Germany performed studies to show that the transient hyperglycaemia that followed injection of certain insulin preparations was due to a glycogenolytic action on the liver.

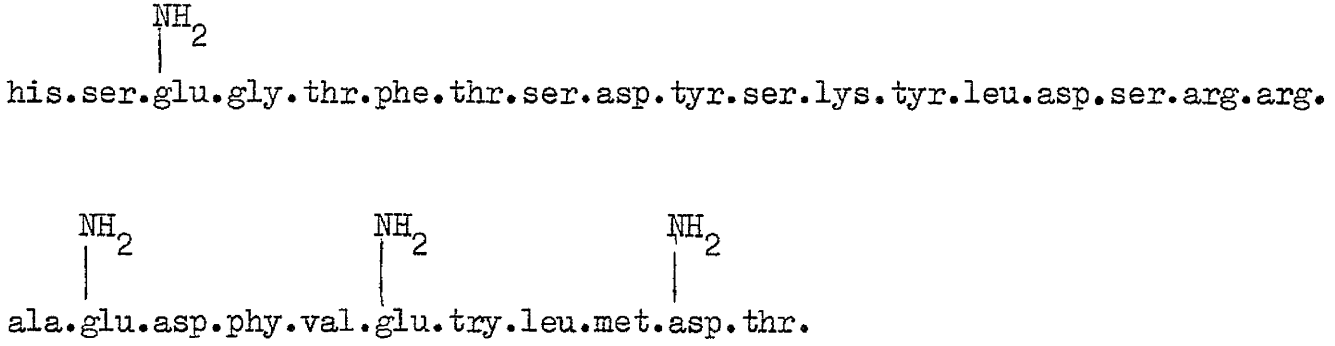
The findings of these workers were confirmed and extended in several laboratories, and soon it became apparent that glucagon was a substance endowed with characteristic physiological and chemical properties[10,11]. This led finally to the announcement by Staub, Sinn and Behrens[12] in 1955 that they had purified and crystallised glucagon.

Glucagon has a molecular weight of 3485 and is a straight-chain polypeptide containing 29 amino acid residues with histidine at the N terminal and threonine at the C terminal (Figure 1).

Site of Origin of Glucagon

The hyperglycaemic action of pancreatic extracts[7,14,15], immediately calls attention to the pancreas as the site of origin of glucagon. The A cells of the pancreatic islets appear to be the source/

FIGURE 1



The primary structure of glucagon is a single polypeptide chain.

/source of glucagon. This has been demonstrated by fluorescent antibody studies[16]. In addition tryptophane has been demonstrated histochemically in the A cells of rabbit pancreas[17], while B cells give a negative reaction. These findings suggest that this method demonstrates glucagon within the A cells since it has a high tryptophane content, whereas this amino acid is absent from the insulin molecule.

Glucagon has also been detected in the alimentary tract by bioassay methods[18,19]. Since the introduction of the immunoassay methods this work has been confirmed[20,21]. Evidence is beginning to accumulate that glucagon extracted from the alimentary tract may be different from pancreatic glucagon. Unger et al[20], Samols et al[21] and Schopman et al[22] all suggest an immunological difference. Unger et al[23] in a recent study suggested that glucagon from the gut had biological differences with pancreatic glucagon in addition to immunological ones, and concluded that glucagon from the gut should be referred to as "glucagon-like immunoreactivity".

Biological Properties of Glucagon

Studies to discover the biological properties of glucagon have been based on the injection of pure preparations of glucagon from the pancreas into intact animals or experimental models. This work although providing fundamental knowledge about glucagon suffers from the fact that often very large and pharmacological amounts of glucagon were used to produce effects. Therefore it is not often possible/

/possible to extrapolate the results to the physiological state.

Injection of glucagon produces hyperglycaemia[8]. This effect is probably mediated via glycogenolysis as simultaneous glucose determinations in the blood of the suprahepatic vein, the portal vein, and the aorta in the dog have shown that glucagon causes an increase in net hepatic glucose output[24,25]. In liver perfusion systems glucagon has been shown to have a profound glycogenolytic effect. Indeed, Sokal et al[2] have found the glycogenolytic effect of glucagon to be greater than that of epinephrine, and have shown that glucagon even in the very low concentration of 0.4 µg.per L of plasma, which may in fact lie in the physiological range, still leads to glycogenolysis. Glucagon possibly initiates glycogenolysis by stimulating the formation of 3', 5', cyclic adenosine monophosphate, and hence the conversion of inactive phosphorylase B to the active phosphorylase A[26]. While the effects of glucagon on liver phosphorylase, liver glycogen, and glucose output have been reasonably well established, its effect on skeletal muscle have been more difficult to ascertain. Evidence does appear to indicate, however, that glucagon has no direct effect on phosphorylase activity, glycogen content, and glucose metabolism of skeletal muscle, although it has been reported that glucagon causes a reduction in the glycogen content of the perfused rat heart[27,28].

Glucagon/

/Glucagon has been shown in the rat isolated liver perfusion system to promote gluconeogenesis[3]. This is supported by other work which shows that glucagon reduces blood amino acid concentration[29,30,31], and increases the incorporation of C^{14} from labelled glycine into liver glycogen[32].

Most reports suggest that glucagon may inhibit fat synthesis and accelerate fat mobilisation. For example, it has been reported that glucagon inhibits the incorporation of acetate, glucose, and fructose into fatty acids and cholesterol of liver[33] and adipose tissue[34], decreases liver fat, promotes the release of fatty acids and glycerol from adipose tissue[35,36,37], and increases the concentration of free fatty acids in plasma[38].

Perhaps the most exciting and even surprising new property for glucagon has been its ability to stimulate insulin secretion, independent of any hyperglycaemia produced[39,40]. Pharmacological doses of glucagon have been used in these experiments, but recently Ketterer et al[41] have shown that physiological amounts of glucagon, infused into the portal vein of dogs, will cause an insulin rise.

Glucagon Excess and Deficiency States

Sometimes the role of a hormone can be deduced from states known to be associated with excess or deficiency of the hormone. Pure glucagon deficiency has not been documented in man, mainly due to an inability to recognise the condition. Pancreatectomy in man/

/man results not only in diabetes mellitus due to the absence of the beta cells, but also insulin sensitivity and low insulin requirements which may be due to glucagon lack[42]. Mirsky et al[43] found that pancreatectomy in the fowl produced no appreciable change in the blood sugar concentration and Mikami and Ono[44] removed the alpha cell containing pancreatic lobes in chickens, and progressive hypoglycaemia developed after several hours, the birds dying in hypoglycaemic convulsions 12 to 36 hours after surgery.

Glucagon excess states can be artificially produced by the repeated injections of large doses of glucagon and this has been done in both experimental animals[45] and man[46]. A mild diabetic state of maturity onset type results. Glucagon excess states have also been reported in association with some pancreatic tumours. One patient with a pancreatic tumour has been documented with high levels of glucagon by immunoassay in her plasma, tumour and metastases[47]. This patient presented with mild diabetes mellitus.

The Assay of Glucagon

The above studies, although emphasising the potency of glucagon as a hormone, fail to define the physiological role and behaviour of the hormone. The construction of a reliable assay method is necessary for further understanding. Bioassay methods for the hormone are tedious and often imprecise and insensitive. Recent methodological advances in the field of immunology[48] have made/

/made possible the development of highly specific and sensitive techniques for identification of various peptide hormones, and for their measurement in plasma[49,5,6,50,51].

Because of the relatively weak antigenicity of glucagon, its rapid degradation in plasma[52], and the demonstration of immunological glucagon-like activity in gastro-intestinal extracts[20,21], the glucagon radioimmunoassay has presented major problems in technique and interpretation to potential investigators. In addition it is possible that even a most sensitive radioimmunoassay may be unable to detect possible physiological changes in glucagon in peripheral blood, for Ketterer et al[41] have shown that when glucagon was injected into the portal veins of dogs in amounts sufficient to produce biological effects, changes in serum glucagon could not be detected in the peripheral blood. This reduction in the peripheral compared to portal blood levels being due mainly to hepatic degradation of glucagon[53,54], and dilutional effects.

Thus, although Unger et al[6] described a glucagon radio-immunoassay as long ago as 1961, much of the earlier work utilising this assay may have given misleading data, because the destructive effect of serum on glucagon whether labelled or not, was not fully appreciated. It was in fact Unger and his colleagues[55] in 1967 who first called attention to the use of Trasylol (Bayer Ltd) a proteolytic enzyme inhibitor, to protect against the degradation of/

/of glucagon by serum. Studies utilising the radioimmunoassay of glucagon are few, and because the majority of these assays were performed without Trasylol and without the knowledge that there were 2 circulating types of immunological glucagon reactivity, it is impossible to accept many of these results as showing completely reliable data. The work of Samols et al[56] who reported a rise in peripheral blood glucagon levels in man after oral glucose is open to the criticisms quoted above. There is only a single study by Unger et al[57] employing the immunoassay to assess the effect of hypoglycaemia on glucagon secretion, but this study which shows a rise in glucagon levels during hypoglycaemia also did not incorporate Trasylol within the assay. A study by Unger et al[58] reporting a rise in glucagon levels during starvation in man is open to the same criticism. Recent work by Unger et al[55] cannot be faulted in these respects, and shows that pancreozymin stimulates glucagon release, which opens up anexciting relationship between the pancreatic and enteric hormones.

MATERIALS AND METHODS

The following methods and materials which were not original were used throughout the Thesis and will not be referred to again. The methods and materials which were peculiar to each particular chapter will be described in these chapters.

Insulin in plasma or in incubation media was assayed by a modification[59] of the double antibody radioimmunoassay technique of Morgan and Lazarow[60] using a human insulin standard (Novo Terapeutisk Laboratory) for assay of samples containing human or rat insulin, and canine insulin standard for assay of samples containing canine insulin.

Plasma glucosé was measured by the ferricyanide method in a Technicon Autoanalyser. Plasma free fatty acids were assayed by a microcolorometric method[61]. Serum acetoacetate was measured by the method of Rosenthal[62] as modified by Walker[63].

C H A P T E R 1

DESCRIPTION OF ORIGINAL METHODS USED IN THESIS

C H A P T E R 1

SECTION 1

A DOUBLE ANTIBODY RADIOIMMUNOASSAY FOR GLUCAGON

SUMMARY

A brief introduction to radioimmunoassays in general is given. This is followed by a detailed description of a double antibody glucagon radioimmunoassay. Guinea pig antiserum allowing the assay of 2500 unknowns per ml. of undiluted serum was induced by biweekly immunisation. Glucagon was iodinated with I^{125} and purified by elution from a cellulose column, maximal immunoprecipitability approximating to 80%. Standard curves in buffer were sensitive to purified glucagon in amounts of less than 100μμg.

Rapid degradation of labelled glucagon added to human blood was demonstrated. Trasylol was confirmed as an effective inhibitor of this degradation. Endogenous plasma immunoreactive glucagon (IRG) appears less labile and may have interfered with the quantitative detection of unlabelled exogenous beef-pork glucagon in plasma recovery studies.

In order to study the behaviour of glucagon, it was considered essential to construct a reliable assay method for the hormone. The introduction of a radioimmunoassay method for insulin by Yalow and Berson[5] in 1960 opened the way for adaptation of the method to the assay of other polypeptide hormones.

Radioimmunoassays have as their basis free competition between isotopically labelled and unlabelled hormone for binding sites to an antibody produced against the hormone. The antibody titre remains constant and is chosen so as to only bind part of the labelled and unlabelled hormone. The concentration of labelled hormone also remains constant, so that the only variable is the concentration of unlabelled hormone. As the concentration of unlabelled hormone rises so will this displace labelled hormone from the binding sites, resulting in loss of radioactivity by the antigen-antibody complex. With this knowledge a standard curve can then be constructed.

The construction of a radioimmunoassay therefore requires:-

(1) a pure supply of standard hormone,

(2) a hormone labelled with a suitable radioactive isotope in such a way that the hormone remains stable, relatively undamaged and retains immunological reactivity, and

(3) the production of an antibody against the hormone.

In/

/In addition the conditions of the assay have to be such as to permit the antigen-antibody reaction to proceed in an optimal way, and precautions must be taken to prevent damage to the hormone during the procedure.

During the assay the "free" hormone and the hormone bound to antibody (the "bound" hormone) must be separated. The original method which accomplished this was the chromato-electrophoresis technique of Yalow and Berson[5]. This method, although an excellent one, is rather tedious and requires elaborate apparatus. A method which enjoys greater simplicity and speed is the "double antibody" technique introduced by Morgan and Lazarow[60] and Hales and Randle[64] in 1963. This method employs a second antibody raised against the gamma globulin of the animal in which the hormone antibody was produced. When this anti-gamma globulin or second antibody is added to the hormone - antibody complex, a precipitate results which can be separated by centrifugation as in the Morgan and Lazarow technique or by filtration as in the Hales and Randle method. The double antibody technique of Morgan and Lazarow for insulin was adapted here for the glucagon assay.

Methods and Materials

(A) Glucagon The glucagon used for immunisation of guinea pigs, preparation of standards, iodination, and addition to blood or serum was of beef-pork origin and was kindly supplied by Dr. Walter Shaw/

Dr. Walter Shaw, Eli Lilly and Company (Lot 258-234B-167-1).

(B) Immunisation Glucagon antibodies were produced in 500-800 gm. guinea pigs by bi-weekly axillary and inguinal subcutaneous injection of 1 mg. of crystalline glucagon dissolved in borate buffer, pH 9.2 and emulsified in 1 ml. of Freund's complete adjuvant. Antiserum (anti-guinea pig serum in rabbits - AGPS-R) was prepared in adult white rabbits by monthly injection of partially purified guinea pig globulin.

(C) Iodination and purification of glucagon I^{125} Crystalline glucagon, 10 µg. dissolved in 0.1 N Na OH and diluted in veronal buffer was iodinated with 2 mC.I^{125} by the method of Hunter and Greenwood[65].

Of the several methods of purification tried, the cellulose column method proved the simplest, quickest and best. Dialysis for 2 hours in 18 X 32 Visking casing, boiled and acetylated, resulted in significant loss through the casing. Sephadex G - 10 and G - 25 yielded 2 and 3 peaks of radioactivity respectively; however the immunoprecipitability and yield was inferior to the simpler cellulose column technique.

The glucagon - I^{125} was purified by elution from a 3.5 cm. cellulose column[66] with serial veronal and 5% bovine serum albumin/

/albumin washes of 1 ml. each. The subsequent 4 aliquots were pooled, diluted 1:40 with veronal buffer, and frozen until use, 50 µl. of the diluted glucagon - I^{125} containing approximately 625 µµg. with an estimated specific activity of 68 mC/mg. The purified glucagon - I^{125} was 70 - 90% immunoreactive with 1:50 guinea pig antiserum (GPAS).; the use of more concentrated antiserum did not appreciably increase immunoprecipitability above this level, the remaining radioactivity not precipitated by antibody presumably representing glucagon altered during iodination to a degree preventing immunoprecipitation with GPAS-AGPS-R. Each iodination provided sufficient glucagon - I^{125} for more than 50 100 - tube assays. Immunoprecipitability of the glucagon - I^{125} declined by approximately 10% over a six week period.

(D) The immunoassay An adaptation of the double antibody technique of Morgan and Lazarow[60] was employed. The buffer utilised was 0.05 M veronal, pH8.6, with merthiolate 1:10,000, to which bovine serum albumin was added to a concentration of 0.25% to prevent glassware absorption of glucagon. Sodium versenate, 0.01 M was added to the medium to inhibit an inactivator of the second antibody system[67]. All reagents, unknowns, and glucagon standards in/

/in buffer were stored at - 20°C and thawed immediately prior to use.

During preparation of the assay all reagents and reaction tubes were maintained in ice baths.

To the tubes were added sequentially:

(1) Buffer with EDTA, 350 µl;

(2) Glucagon-I^{125}, 50 µl;

(3) Trasylol, 1000 KIU in 50 µl; (vide infra);

(4) Standard or unknown, 250 µl;

(5) GPAS 50 µl. in suitable concentration which usually was such as to be able to precipitate approximately 30% of the labelled glucagon in the absence of competing unlabelled glucagon. The titre varied from 1:100 with some antibodies to 1:500 with other antibodies. The final dilution of the GPAS in the assay therefore varies from 1:1500 to 1:7500.

After incubation at 4°C for 24 to 36 hours, second antibody was added and a further 6 hour period of incubation at 4°C was allowed prior to cold centrifugation and removal of the supernatant. Precipitates were then washed with buffer and their radioactivity counted in a well-type auto-gamma spectrometer.

Results

(A) Immunisation Guinea pigs proved reliable producers of glucagon antibodies, the titres achieved within 2 to 3 months/

/months in 3 out of 4 of the animals allowing the assay of between 2000 and 8000 unknowns per ml. of undiluted GPAS. Monthly booster injections a week prior to bleeding maintained adequate titres for over 6 months. Although with protracted immunisation several animals developed insulin antibodies due to the minute quantities of insulin contaminating the glucagon, a total lack of insulin cross-reactivity in the glucagon assayed allowed the continued use of such antisera.

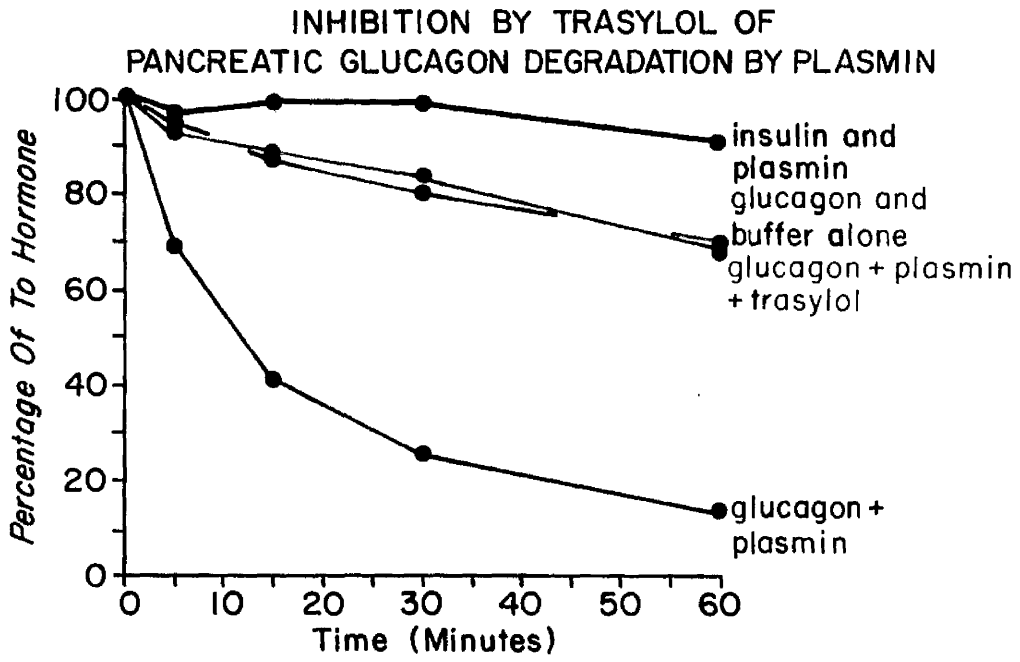
(B) The assay in buffer for the construction of a standard curve.

(1) The role of Trasylol in the standard curve system

Trasylol (F.B.A. Pharmaceuticals) is a basic polypeptide which has been shown to inhibit plasminogen and plasmin, principal degraders of glucagon in plasma[52]. Loss of immunoreactive glucagon (IRG) is demonstrated in figure 1 on incubation with plasmin, but the destruction is inhibited by Trasylol.

In the chromato-electrophoretic immunoassay system, Eisentraut, Whissen and Unger[68], have demonstrated the inhibition by Trasylol of glucagon degradation during incubation. The effect of Trasylol in preserving glucagon-I^{125} immunoprecipitability in the double antibody standard curve system (containing no serum or plasma unknown) is/

FIGURE 1



Incubation of glucagon with plasmin (2.5 µg./ml.) results in loss of glucagon, whereas Trasylol (1000 KIU) prevents this loss. Insulin is not affected by plasmin. Incubations were conducted in veronal buffer pH 8.6 at 37°C. in a shaking water bath.

/is illustrated in Figure 2a, which depicts curves prepared with and without added Trasylol, a small but consistent loss of precipitable radioactivity being recorded at all levels of added unlabelled glucagon in the absence of Trasylol.

(2) Duration of the Incubation The initial reaction reached a plateau of maximal immunoreactivity at 24 hours of incubation at 4°C., no significant change being detected over the ensuing 72 hours (Figure 3). This plateau reflected the stability of the glucagon-antibody complex (in the presence of Trasylol) over prolonged periods of incubation. The presence of unknown serum in the medium did not affect the time at which the plateau was reached. Consequently at least 24 hours were routinely allowed for the first antibody reaction.

The second antibody reaction reached a plateau of maximum immunoprecipitability by 2 hours of incubation at 4°C. A minimum of 6 hours for the second antibody reaction was allowed for the present studies in order to maximise agglomeration of the precipitate for optimal separation by centrifugation and decantation.

Both first and second antibodies may proceed simultaneously in the presence of the required reactants[64]. Therefore, certain studies were conducted in which both

GPAS/

FIGURES 2a and 2b

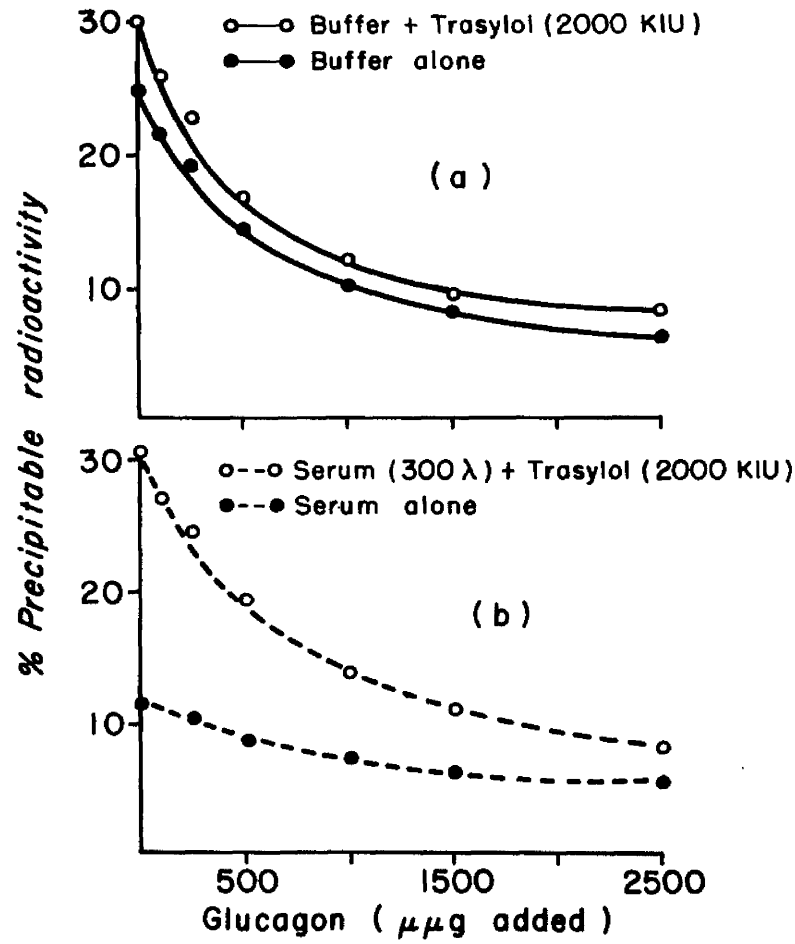
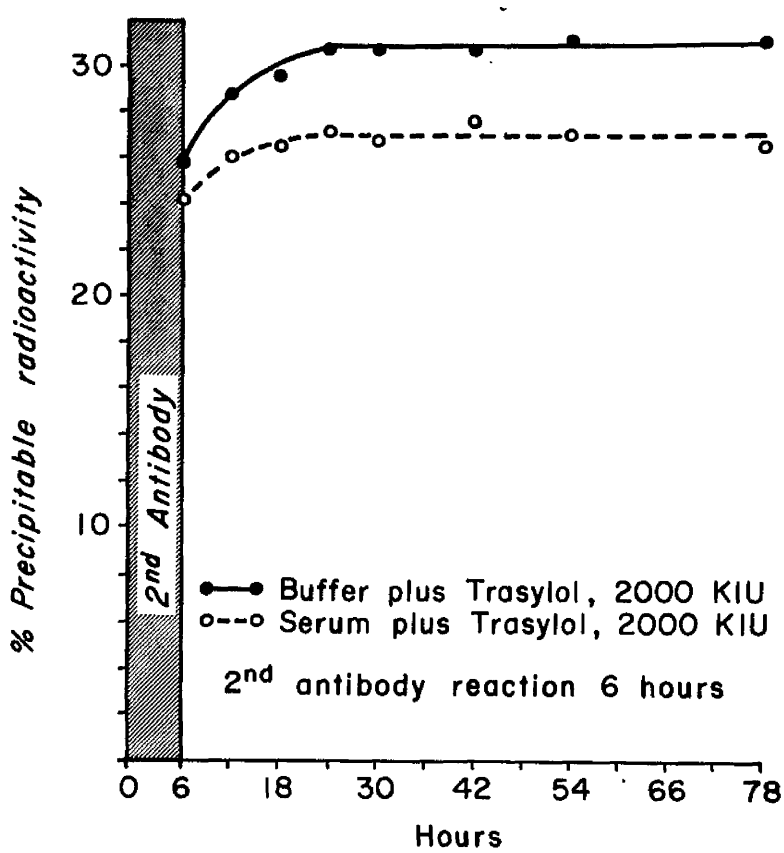


Figure 2a The effect of Trasylol upon the standard curve. The loss of precipitable glucagon-I^{125} in the absence of Trasylol maybe attributed to the degrading factors in the GPAS and AGPS-R.

Figure 2b Parallel studies in the presence of added human serum. The marked additional glucagon degradation introduced by serum is completely prevented by the presence of Trasylol in adequate amounts.

FIGURE 3



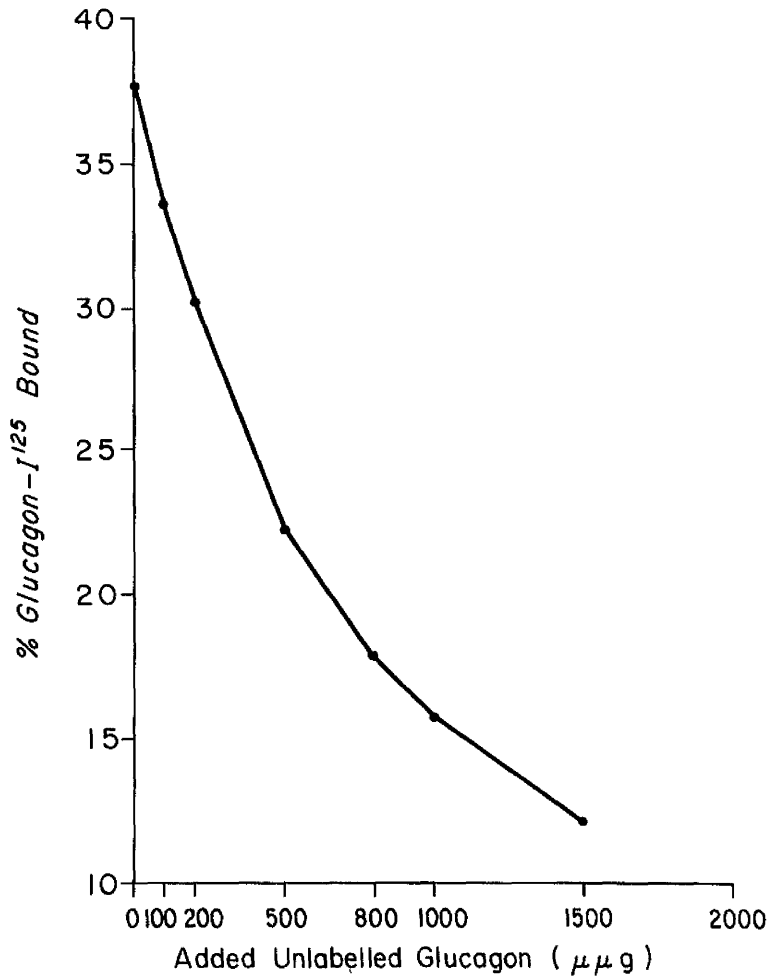
A study of the duration of the first antibody reaction. The distance between the origin and the initial values of the curve represents the time allowed for the second antibody reaction. (the first antibody reaction proceeding concomitantly during this interval).

/GPAS and AGPS-R were added on the first day of incubation. This modification did not affect precision or the nearly quantitive glucagon recovery from plasma (vide infra).

(3) The standard curve Glucagon standards containing from 0 to 1500 μμg. inhibited the immunoreactivity of glucagon-I^{125} in a manner so as to allow the construction of a smooth standard curve, as depicted in Figure 4. The curve illustrated was drawn through points of mean per cent glucagon-I^{125} precipitated in 6 consecutive assays performed in triplicate.

The standard curve assays were analysed as to the error of replicate determinations within a single assay (precision) and that introduced by day to day variability, as represented by the standard deviation of the mean slopes between points on each curve and of the mean percentage of labelled glucagon bound at each level of added unlabelled hormone (Table 1). The precision was calculated among triplicates according to the method of Youden[69]. The sensitivity of the system to purified glucagon was then determined as that amount significantly different ($p = .05$) from zero (or lower standard at other points of the curve) as given by $t(S_1^2 + S_2^2)^{\frac{1}{2}}$ where t is 1.96 and S_1 and S_2 are the expression of precision (standard deviation) for the points bounding/

FIGURE 4



The glucagon immunoassay curve. The curve depicted was drawn through points representing the mean percentages of glucagon-I^{125} bound in 6 consecutive assays.

TABLE 1

Slope, precision, and sensitivity of the standard curve for purified beef-pork glucagon*

Amount of glucagon added ($\mu\mu$g.)	Percentage glucagon-I-125 bound (mean ± S.D.)	Precision (S.D.) per cent	Slope (per cent) (± S.D.)	Sensitivity over given range ($\mu\mu$g.)
0	37.7 ± 1.8	± 0.8		
			4.1 ± 1.1	20
100	33.6 ± 2.1	± 0.6		
			3.3 ± 1.1	30
200	30.2 ± 1.2	± 0.3		
			8.1 ± 0.6	40
500	22.2 ± 0.9	± 0.7		
			4.4 ± 0.5	90
800	17.8 ± 0.8	± 0.4		
			2.0 ± 0.5	70
1,000	15.8 ± 0.5	± 0.4		
			3.7 ± 0.4	110
1,500	12.1 ± 0.8	± 0.4		

*Six curves were obtained from consecutive assays performed in triplicate. The precision is an expression of the error of replicate analysis within each assay, whereas the standard deviations of the slopes and mean percentages bound express the variation from assay to assay at each level of added glucagon. The differences between the mean percentages bound were all significant ($p < .001 - < .05$), as were the slopes of the curves between all adjacent points (vs. zero slope, $p < .001$). The sensitivity of the system is expressed as the least amount of beef-pork glucagon which is significantly different ($p = 0.05$) from zero (represented by the lower standard) over each range of added glucagon.

/bounding the range of added glucagon in question. The value of this expression was then referred to the mean curve to allow translation into μμg. of added glucagon[65]. The resulting calculated sensitivity for triplicate analysis varied from a low of 20 μμg. for 0 μμg. added glucagon to 110 μμg. for 1500 μμg added glucagon.

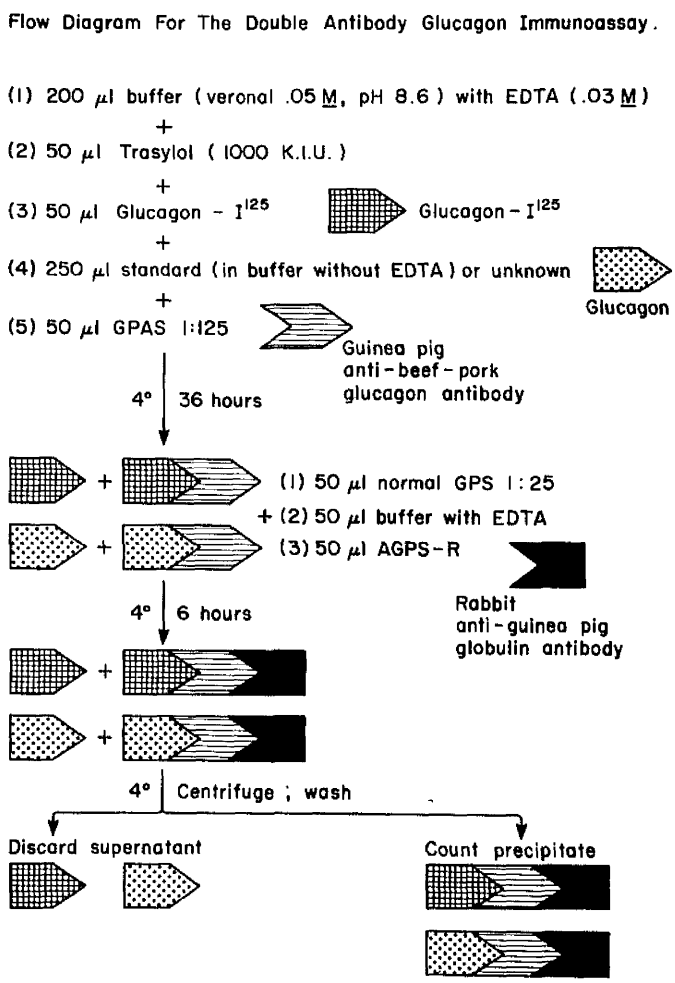
A flow diagram for the glucagon radioimmunoassay is depicted in Figure 5.

(C) Application of the assay: The measurement of IRG in blood.

(a) The requirement of Trasylol. The addition of serum or plasma to incubates containing glucagon-I^{125} greatly enhances the glucagon-degrading capacity of the system, thereby increasing the requirement for Trasylol to a level which will provide maximal inhibition enzymes. In the absence of an adequate Trasylol concentration, much of the labelled glucagon becomes non-immunoreactive. Figures 2b and 6 show the effect of Trasylol in preventing loss of immunoprecipitability when exposed to human sera. Figures 7 and 8 show the effect of Trasylol on increasing the recovery of insulin-I^{125} and glucagon-I^{125} respectively when exposed to serum and pancreatic juice respectively. Serum has little effect on insulin-I^{125} but has a marked destructive effect on glucagon-I^{125}.

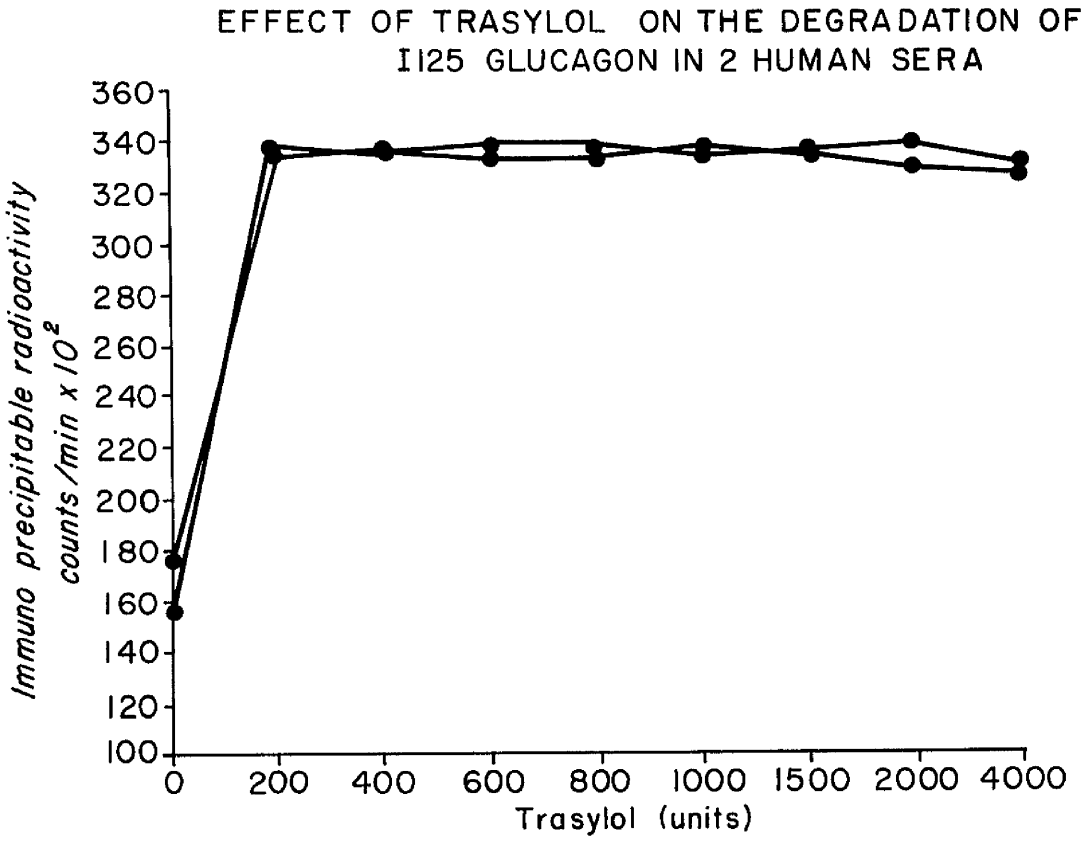
As a result of damage to glucagon-I^{125} immunoreactivity by serum, the apparent immunoreactive glucagon (IRG) concentration/

Figure 5



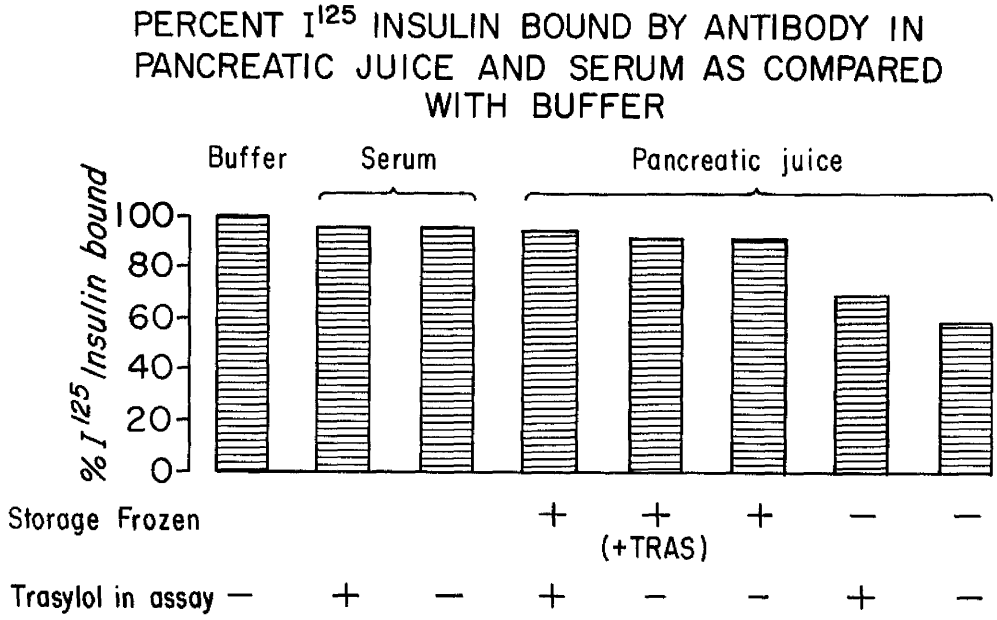
A flow diagram for the double antibody glucagon radioimmunoassay.

FIGURE 6



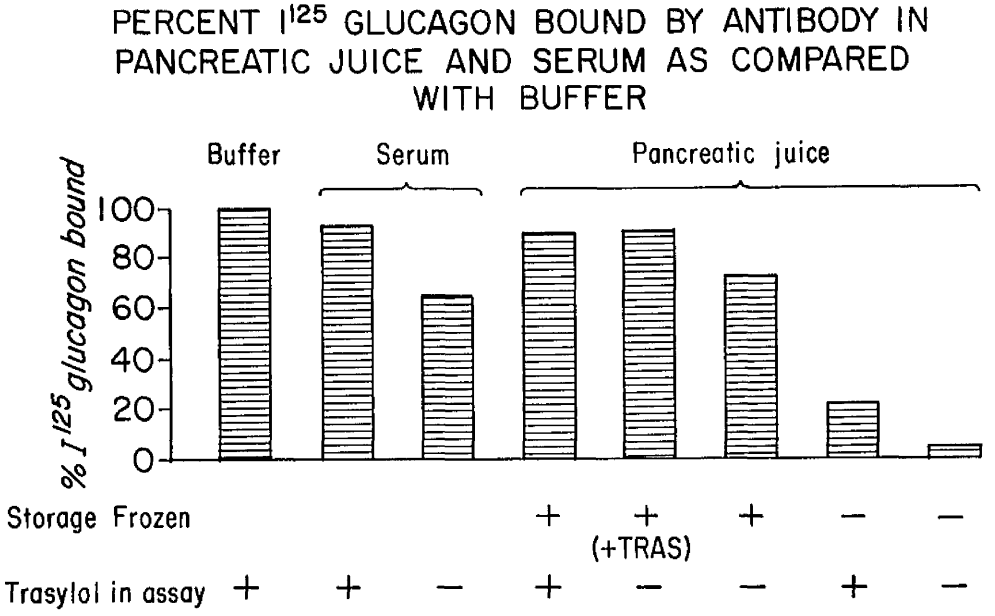
The amount of glucagon-I^{125} precipitated in the presence and absence of Trasylol. Maximum immunoprecipitability was achieved in these two human sera by as little as 100 KIU Trasylol.

FIGURE 7



Insulin antibody binds almost all of insulin-I^{125} in the presence of serum and pancreatic juice stored frozen (inactivated). When pancreatic juice is activated by allowing to stand at room temperature much of the insulin-I^{125} is degraded.

FIGURE 8



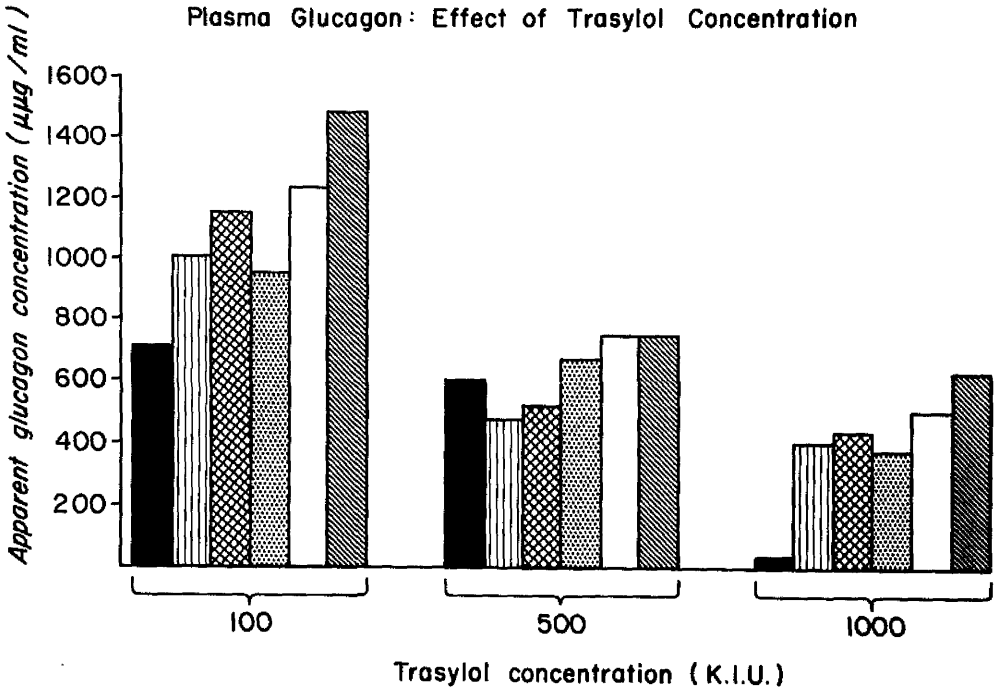
There is much loss of glucagon-I^{125} when exposed to serum but this is almost completely overcome by the presence of Trasylol. Trasylol also helps to overcome the degrading effect of pancreatic juice on glucagon-I^{125}.

/concentration becomes falsely elevated when the precipitable radioactivity in a serum or plasma unknown is compared to the much greater level counted in the standards whose glucagon-I^{125} has been left virtually intact. This effect is prevented by the presence of Trasylol in adequate concentration. This role of Trasylol in the measurement of serum IRG was demonstrated in a study of 19 fasting human sera assayed in the absence as well as in the presence of Trasylol. In those sera an apparent glucagon concentration of 3690 ± 250 μμg.(S.E.M.) per ml. measured without Trasylol was reduced to 256 ± 117 μμg. per ml. by its presence.

Plasma glucagon-degrading activity was variable; consequently the use of less than optimal amounts of Trasylol affected relative as well as absolute levels of IRG measured in plasma of different individuals (Figure 9). However, in studies utilising up to 4000 KIU Trasylol, no additional effect beyond that achieved with 1000 KIU per 250 μl. plasma was detected. Therefore a level of 1000 KIU per 250 μl. plasma or serum was adopted as a minimum for the routine assay procedures.

Epsilon-amino-caproic acid was found to be ineffective in preventing glucagon-I^{125} degradation by serum. Lima bean trypsin inhibitor was on occasion as effective as Trasylol but its effect was/

FIGURE 9



The effect of Trasylol upon measured plasma glucagon in 6 individuals. The relative IRG concentrations among the individuals varied as the Trasylol concentration was increased, reflecting inequality in their levels of plasma glucagon-degrading factors.

/was never as consistent.

(b) The degradation of glucagon during the collection and processing of blood specimens

The degradation of glucagon-I^{125} during the collection and processing of blood specimens was assessed in 6 individuals utilising labelled glucagon present in the syringe before venepuncture. The contents of the syringe were processed in 6 different ways to determine the effect of time, temperature and Trasylol upon glucagon degradation, the per cent recovery being calculated by comparison with that from a syringe filled with buffer rather than blood. Twice the usual concentrations of both first and second antibodies were used in order to assure maximal immunoprecipitation despite the competition exerted by endogenous glucagon.

As recorded in Table 2 (column 1), the time at which Trasylol was added, as well as the method by which the blood specimens were processed, significantly affected the recovery of labelled glucagon. Expeditious collection and immediate exposure to Trasylol preserved 90% of the immunoprecipitability of labelled glucagon, even if as long as 60 minutes elapsed at ice bath temperatures between collection of the blood and separation of the plasma. In the absence of Trasylol, the short/

TABLE 2

A study of the degradation of added labeled and of native glucagon during collection and processing of blood specimens. The per cent recovery of added glucagon-I-125 was calculated by comparison with recovery from buffer.

Conditions	Recovery of added glucagon-I-125 (per cent ± S.E.M.)	Endogenous glucagon level ($\mu\mu$g./ml. ± S.E.M.)
A. Heparinized blood with Trasylol, centrifuged immediately	90 ± 4.2	676 ± 45
B. Same as *A* but without Trasylol	81 ± 4.0	659 ± 71
	B vs. *A*, $p < .001$*	*B* vs. *A* — n.s.
C. Heparinized blood with Trasylol, iced 60 min., then centrifuged	87 ± 4.2	648 ± 41
D. Same as *C* but without Trasylol	71 ± 5.3 (5)	715 ± 60
	D vs. *C*, $p < .02$	*D* vs. *C* — n.s.
E. Unheparinized blood without Trasylol, clotted 30 min. at 25°, then centrifuged	70 ± 3.6	676 ± 54
F. Same as *E* but iced 30 additional min. before being centrifuged	66 ± 4.3	644 ± 46
	F vs. *E* — n.s.	*F* vs. *E* — n.s.

n = 6 except where indicated (5)

*Levels of significance on basis of paired comparisons. n.s. = not significant ($p > 0.10$).

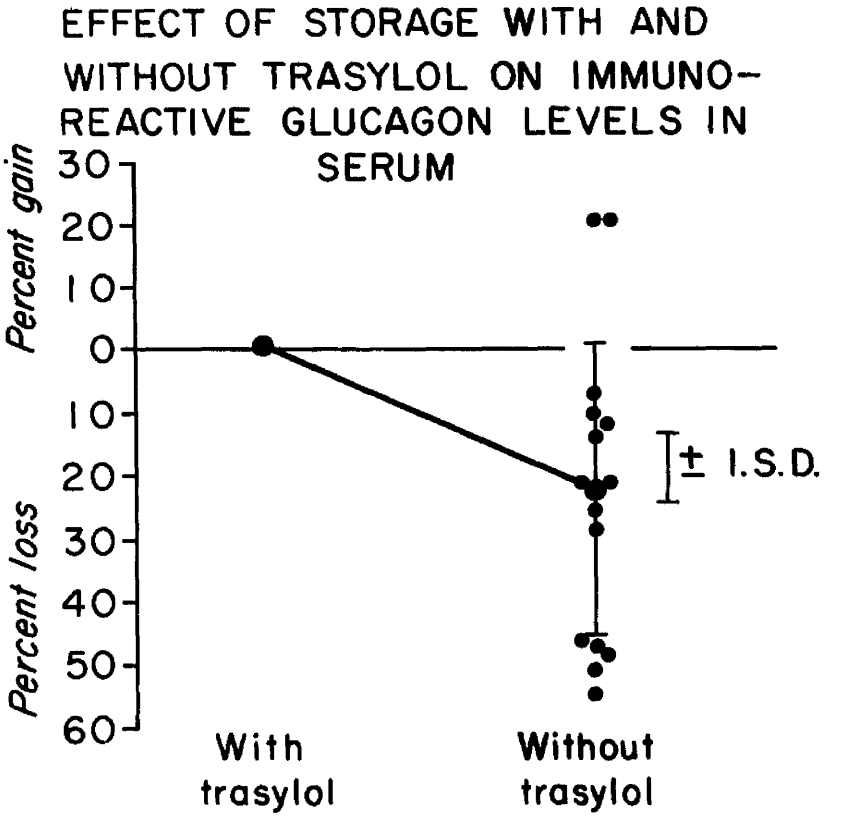
/short period required for centrifugation and separation (less than 30 minutes) resulted in significant ($p < .001$) though minor (9%) degradation with progressive deterioration in immunoprecipitability occurring even at ice bath temperature as the time between collection and exposure to Trasylol increased. Degradation of approximately one third of the glucagon-I^{125} was incurred by allowing the blood to clot at room temperature and separating the serum before its exposure to Trasylol.

However, parallel studies of the native IRG concentration in blood specimens treated indentically to those to which labelled glucagon was added failed to show similar degradation of endogenous IRG despite the absence of Trasylol (Table 2, column 2).

(c) Storage of Specimens A better recovery of plasma glucagon resulted if measured plasma or serum samples were separated into Trasylol (250 µl. plasma per 1000 KIU Trasylol) and stored in the deep freeze until assay, than if the samples were stored without Trasylol (Figure 10).

(d) Plasma recovery studies Recovery of the unlabelled beef-pork glucagon standard, added to human plasma containing Trasylol (1000 KIU per 250 µl.), was measured using a standard curve with a range of 100 to 1500 µµg. In the calculation/

FIGURE 10



The estimates of IRG in serum stored with Trasylol (250 µl. per 1000 KIU Trasylol) were recorded as 100%. The per cent gain or loss for the same sera stored without Trasylol (but Trasylol added at time of assay) was then calculated. Of the 15 sera, 13 recorded lower IRG levels when stored without Trasylol (mean loss 22%).

/calculation of recovery, the amount of endogenous IRG present in the same plasma was subtracted from the total. This method assumes identical reactivity of the endogenous and the added (beef-pork) glucagon towards the guinea pig antibody. When calculated in this manner, recovery varied between 80 and 90% (Table 3).

(e) The assay of serum and plasma IRG An estimate of the precision (s) of the assay when applied to serum was determined from the formula:- $s = (\Sigma\ d^2/2n)^{\frac{1}{2}}$, where"s"is the standard deviation of the difference"d"between a number of duplicate measurements, n^{69}. This figure was calculated over 2 broad ranges of IRG concentration (representing the total of endogenous plus added beef-pork glucagon); it was 216 μμg./ml. for levels less than 1650 μμg./ml. IRG (n = 175), and 409 μμg./ml. for the same range 1650 - 3300 μμg./ml. (n = 43).

The sensitivity was sometimes as low as 20 μμg./250 μl. (80 μμg./ml.).

The concentration of IRG present in fasting human serum was 400 μμg./ml. ± 220 μμg. (S.D) when measured in 43 specimens of peripheral venous blood from 14 healthy young adults (6 male, 8 females).

Discussion The double antibody glucagon assay described above, meets/

TABLE 3

Recovery of unlabeled beef-pork glucagon added to human plasma containing Trasylol.

Exogenous glucagon added to 250 ml. plasma ($\mu\mu$g.)	Total IRG measured ($\mu\mu$g.)	Net glucagon recovered (Total less endogenous) $\mu\mu$g.	per cent
0	120 (endogenous)	—	—
100	200	80	80
500	570	450	90
1,000	990	870	87
2,500	2,210	2,090	84

/meets the standards of precision, reproducability and stability during incubation requisite to a valid and sensitive assay. In addition it offers the advantages of technical efficiency, adaptation to large scale production and economy which have made the double antibody technique so widely accepted for the insulin immunoassay, with which it maybe readily coupled within a single laboratory .

The initial problem of producing glucagon antibodies in adequate titre was overcome by a bi-weekly immunisation schedule. Iodination with I^{125} affords a stable yet highly immunoreactive tracer substance. Purification of glucagon-I^{125} by elution from a cellulose column provides a labelled hormone which competes in a consistent and equal manner with unlabelled glucagon, producing a standard curve which allows the measurement of glucagon in amounts of less that 100 µµg.

Nevertheless when applied to systems containing plasma or serum the assay of glucagon presents a problem in its degradation by plasma as reported by Mirsky et al[51]. Such degradation activity attacks glucagon during incubation. The net result of the degradation is a gross magnification of the apparent IRG concentration due to diminished immunoprecipitability of labelled hormone as compared with the relatively intact level among standards incubated in the absence of plasma.

Trasylol/

Trasylol was confirmed as an effective inhibitor of glucagon-I^{125} degradation. Its place in the measurement of plasma glucagon maybe two fold. A major role is the protection of labelled and probably unlabelled glucagon during the 24 hour incubation with serum or plasma. The amount used must be adequate to overcome differing levels of plasma degrading activity in various individuals, this quantity being determined as 1000 KIU per 250 µl. plasma or serum. Added at an early moment after blood withdrawal it may also prevent the destruction of pancreatic glucagon which may occur in the presence of clotting at room temperature and separation of serum samples. Once exposed to Trasylol, the serum or plasma exhibits no further degradation of IRG during freezing, storage and thawing.

CHAPTER 1

SECTION 2

THE ISOLATED PANCREATIC ISLETS OF LANGERHANS OF THE RAT

SUMMARY

A detailed description of a technique of isolating islets of Langerhans from rat pancreas using collagenase is given. There was no degradation of either glucagon-I^{125} or endogenously released glucagon in incubation vessels containing pancreatic islets when Trasylol was added. Preincubation of islets was found necessary before the effect of test substances could be tried.

The direct measurement of glucagon release from pancreas preparations "in vitro" would enjoy some advantages over "in vivo" studies. The experiments could be rapidly and easily performed, sensitivity would be less of a problem than when the glucagon molecule is distributed throughout the body spaces, mechanisms might be studied, and there would be less chance of substances interfering or cross reacting in the immunoassay as might occur in blood. In addition glucagon release has never been studied "in vitro".

Because of glucagon degradation[70] the pancreas slice technique is not applicable unless a method could be devised for incubation of the slices with glucagon antibody similar to that recently described by Malaisse et al[70] for insulin. The isolated islet technique of Lacy and Kostianovsky[71] seems to be an ideal system to study glucagon and insulin release simultaneously since complete removal of the exocrine pancreatic tissue would presumably free the systems of degrading enzymes.

MATERIALS AND METHODS

Animals

All studies were performed on male albino Wistar rats (350-450 gm). The animals were anaesthetised with ether during preliminary experiments, but later they were killed by decapitation prior to each experiment, since it has been reported that general anaesthesia inhibits insulin release[72].

Incubation media/

/Incubation media

The isolated islets were incubated in 1 ml. of a modified Krebs Henseleit bicarbonate solution (KHB)[73] with dialysed bovine serum albumin (Pentex) (2 per cent) added. The buffer was supplemented with 5 mM of sodium salts of pyruvic acid, glutamic acid and fumaric acid[73,74]. Addition of various concentrations of glucose to the buffer were made just prior to each incubation.

Islet Isolation Procedure

The islet isolation technique of Lacy and Kostianovsky[71] was followed with only a few modifications. Neutral red was not used to stain the islets. The rats were stunned by a blow on the head and killed by decapitation. The abdomen was opened and the common bile duct was cannulated with P.E. 50 polyethylene tubing. The duct was clamped immediately above the cannulation, and also at the entry of the duct into the duodenum. 7-8 ml. of Hanks solution (a physiological salt solution) was injected to distend the acinar tissue. The pancreas was dissected clear of non pancreatic material. Two pancreases so dealt with were minced with scissors and incubated with collagenase 35-50 mg. in 5 ml of Hanks solution at 37°C. for 6 to 15 minutes with constant stirring. The amount of collagenase required varied among the manufacturer's lots. The duration of incubation was also variable and depended on multiple factors including stirring, pH, the batch of collagenese/

/collagenase, temperature and probably other factors which could not be defined. The incubation period was extremely critical because under-incubation resulted in incomplete separation of the islets from the acinar tissue, whereas over-incubation caused destruction of the islets. Aliquots of the collagenase incubation were examined periodically under a dissecting microscope in order to determine when optimum separation of the islets had occurred. This was usually recognised by the preparation becoming finely granular, many single acinar cells being seen, and sometimes isolated islets could be noted.

The islets were then separated from the acinar tissue by a simple sedimentation procedure. The islets sank to the bottom leaving the lighter acinar tissue above. The collagenase-treated material was first diluted with 25-30 ml. Hanks solution and the supernatant, containing predominantly acinar tissue was removed after a 3 - 4 minute sedimentation period. This washing procedure was repeated for a total of eight times, with the sedimentation period progressively shortened to about sixty seconds for the last few washes.

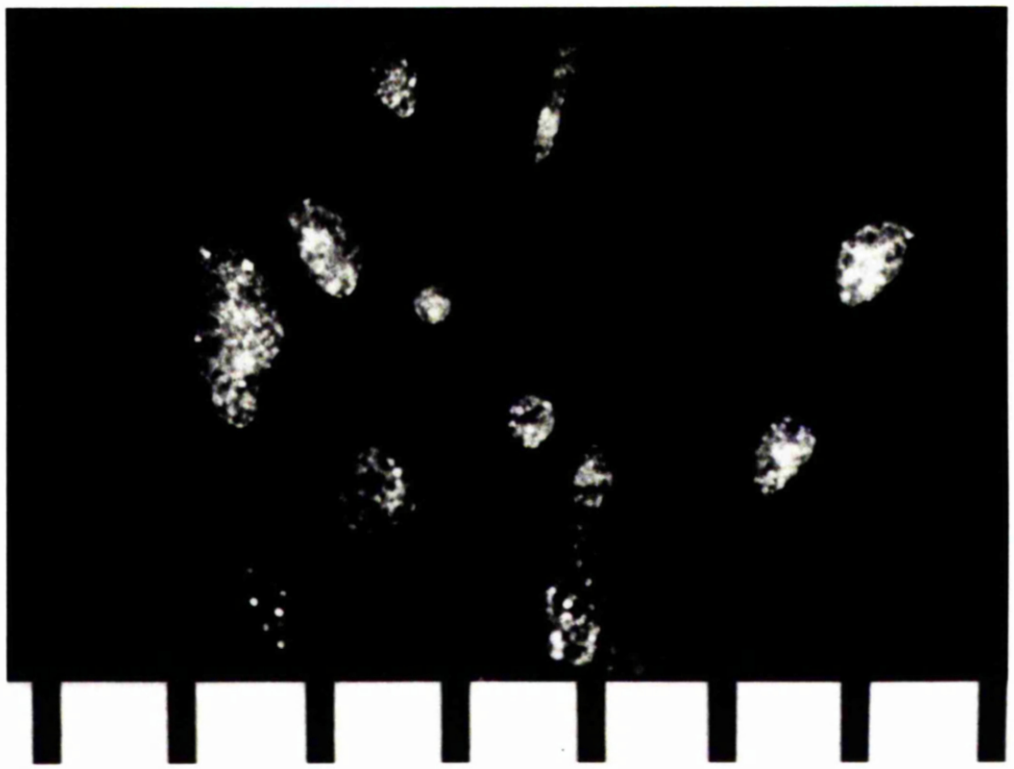
In an average preparation 50 islets per rat could be isolated but sometimes 100 - 150 islets might be found.

Incubation procedure

After the sedimentation process was completed, the sediment was/

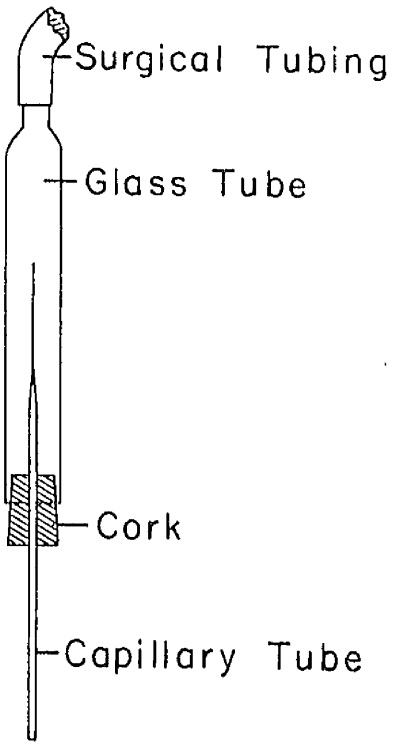
/was placed in a Petri dish surrounded by crushed ice and examined under the dissecting microscope. The free islets were easily recognised as round or oval, densely granular objects which were quite distinct from the feathery and loose acinar tissue(Figure 11). The free islets were then transferred, in groups of ten, with a braking pipette[75] (Figure 12) to individual incubation vessels containing 0.95 ml. of iced KHB with 60 mg. per 100 ml. glucose and 0.05 ml.(1000 KIU) Trasylol. The flasks were then capped, individually gassed for three minutes (95% O_2 : 5% CO_2), and preincubated for thirty minutes in a Dubnoff metabolic shaker at 37°C. Following the preincubation period the buffer was removed with a capillary or Pasteur pipette under the dissecting microscope and discarded and the islets were washed once with 0.9% NaCl solution, taking care not to remove any of the islets in the washing process. At this point, 0.95 ml. of iced KHB containing the test substance (i.e. various concentrations of glucose) and 0.05 ml. of Trasylol were added to the vessels. Samples of 0.150-0.250 ml. were removed with a Hamilton pipette for determination of baseline glucagon and insulin content (t_o). The incubation vessels were recapped, gassed, and incubated for thirty minutes with constant shaking at 37°C. Again, samples were removed for measurement of glucagon and insulin concentrations (t_{30}). In some experiments, in which islets served as their own control/

FIGURE 11



A dark ground photo-micrograph of isolated islets. The islets appear oval and densely granular as compared to the feathery, loose appearance of acinar tissue.

FIGURE 12



Braking pipette used to transfer the isolated islets to the incubation vessels.

/control, the above process was repeated; after a repeat preincubation, the islets were incubated with another test substance and the hormone production during the second incubation compared with that during the first incubation.

Calculation and Expression of Results

The net hormone production during the thirty minute incubation period ($t_{30} - t_0$) was corrected for dilution and expressed, respectively, as the amount of immunoreactive glucagon (IRG) and immunoreactive insulin (IRI) released per 10 islets per 30 minutes. It is realised that the variation in the number of cells per islet, and thus the amount of hormone released per islet increases the scatter of data when the mean hormone production is expressed in terms of a number of islets. Possibly if the results were compared in terms of islet weight this variance could be minimised somewhat, but because of the minute quantity of tissue involved, this form of standardisation of reference was not practical. It was reasoned that the use of ten islets per tube should help to decrease any effect of variability in islet size.

RESULTS

Recovery studies

In order to assess the possible need for Trasylol in the incubation media to prevent glucagon degradation, glucagon-I^{125} was incubated with 8 islets in 0.8 ml.of buffer, with and without the/

/the addition of 1000 KIU of Trasylol to the media. Aliquots were removed at 0, 30 and 60 minutes and reacted first with excess guinea pig glucagon antiserum in order to bind all remaining glucagon-I^{125}. The antibody-bound radioactive hormone mixture was precipitated with excess rabbit antiguinea pig serum. The mean precipitable radioactivity in eight incubation vessels was expressed as a percentage of the value at 0 time ± Standard Error of the Mean (SEM). The mean recovery of glucagon-I^{125} in 6 to 8 flasks containing Trasylol was 88 ± 1.2% after 30 minutes of incubation, and 81 ± 1.5% after 60 minutes. Without Trasylol, the recovery at 30 minutes was 79 ± 5%, a small but significant difference ($p < .05$), and at 60 minutes 56 ± 4.6% ($p < .001$). The addition of Trasylol to control tubes did not affect the antigen-antibody reaction.

To determine if there was degradation of released endogenous hormone in the incubation media, the islets were removed from the media after 30 minutes of incubation and the media were incubated for an additional 30 minutes. The mean glucagon recovered after this additional incubation was 101 ± 0.5% (mean ± SEM of 20 determinations) of the amount present when the islets were removed. Trasylol was added to all incubation vessels in a concentration of 1000 KIU. The insulin recovery under the same conditions was 99 ± 0.75% (mean ± SEM of 7 determinations).

Duration of Incubation and Preincubation

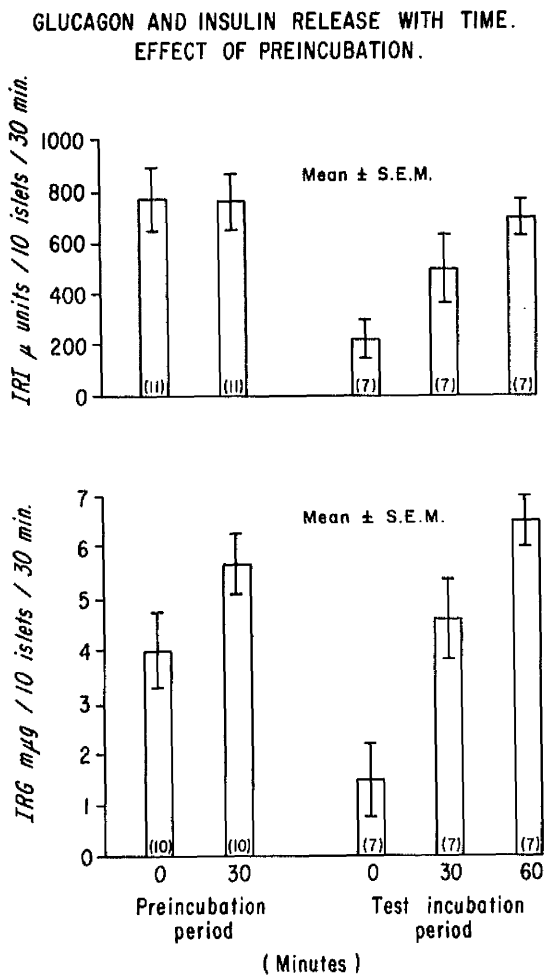
The/

/the release of hormone during a preincubation period in buffer with 60 mg. per cent glucose is shown in Figure 13. The baseline (t_0) insulin and glucagon levels before the preincubation period were high. The mean insulin level at the end of the preincubation period did not change whereas the glucagon level was higher, although the difference was not statistically different from the t_0 value. After changing the buffer and washing the islets once with normal saline solution, the baseline hormone levels were much lower and there was significantly greater insulin ($p < .01$) and glucagon ($p < .05$) released at 30 minutes. At 60 minutes the insulin and glucagon release was not significantly greater than at 30 minutes ($p < .01$). Therefore, for subsequent experiments the islets were preincubated for 30 to 60 minutes in 60 mg. per cent glucose. They were then incubated for an additional 30 minute period in which hormone production was measured and the effects of the various test substances compared.

Discussion

The isolated pancreatic islet system of Lacy and Kostianovsky[71] has proven to be a satisfactory system to study simultaneously factors affecting glucagon and insulin release. There was only a small amount of insulin and glucagon degradation in the system, but if the proteolytic enzyme inhibitor, Trasylol, was added to the incubation media, this degradation could be prevented. Presumably Trasylol acts on contaminating proteolytic enzymes of exocrine pancreatic/

FIGURE 13



The effect of the duration of incubation on insulin and glucagon release. Islets were preincubated for 1 hour in 60 mg. per cent glucose followed by a buffer change and an additional one hour incubation in 60 mg. per 100 ml. glucose. The total amount of hormone present at the various times is expressed as mean ± SEM. The number in parentheses refer to the number of incubation vessels.

/pancreatic origin which are incompletely removed during the sedimentation and washing process. Alternatively, the islets themselves may release proteolytic enzymes.

It is not certain why hormone release from the islets during a first or preincubation period was so poor. Presumably the islets have been subjected to numerous insults during the separation and sedimentation procedures, and they therefore require a "rest" period, before they will respond predictably to stimuli. The high t_0 hormone levels during preincubation might represent a rapid, non specific, release of hormone due to damage sustained during preparation. However, histology (kindly performed by Dr. Stanley L. Erlandsen) of isolated islets prepared as above showed them to be remarkably free of damage, and the granulation of beta cells in freshly isolated islets and in preincubated islets was not altered substantially from the granulation of islets in pancreases which were fixed immediately after death of the animal.

CHAPTER 2

GLUCAGON IN BLOOD AND TISSUES

C H A P T E R 2

SECTION 1

DISTRIBUTION OF GLUCAGON IN THE BODY

SUMMARY

Acid-alcohol extractions of tissues revealed immunoreactive glucagon (IRG) to be present in the pancreas and the alimentary tract of 4 species of animal tested, but not in any other tissue. Following pancreatectomy in 5 dogs, circulating levels of IRG fell only slightly suggesting that circulating IRG was being contributed to from an extra pancreatic source, presumably the gut.

Although it has long been established that the pancreatic A cells are a major source of glucagon[16,17], it is less certain whether glucagon is found elsewhere in the tissues. It seems likely that glucagon however is found in the alimentary tract as it has been detected by bioassay[18,19] and immunoassay methods[20,21].

METHODS

Tissue extractions

Mongrel dogs, male wistar rats, and a squirrel monkey were studied. Tissues were taken immediately after death and rapidly frozen by dry ice in ethanol. The tissues were homogenised using a ground glass homogeniser, and glucagon was then extracted by acid ethanol using the method of Kenny[76]. Care was taken to prevent degradation of glucagon by keeping all extraction processes at 4^{o}C. or ice temperature. The final extract was lypholised, stored at -20^{o}C. and reconstituted in veronal buffer, pH 8.1 for assay.

The single human subject was a 24 year old man, in apparent good health, killed as a result of an automobile accident. His organs were removed one hour after death.

"In vivo" studies

Mongrel dogs were anaesthetised by barbiturate and venous catheters inserted at laparotomy into a peripheral vein, a large jejunal/

/jejunal vein, and the pancreatic-duodenal vein.

RESULTS

Table 1 shows the amount of immunoreactive glucagon (IRG)in the tissues. Results are expressed in mμg. of IRG per gm. of wet organ. Large amounts of IRG were present in the pancreas of all species of animals. Smaller amounts of IRG were present in the alimentary tract, although none was found in the stomach or duodenum (one dog only tested here). A rather surprising finding was the presence of IRG in the colon and even in the rectum. The concentration of IRG in the pancreas was greater than that in the gut, but when the size of the alimentary tract is considered, then the total amount of IRG in the intestine may equal that in the pancreas.

The result of pancreatectomy on circulating IRG levels is shown in Figure 1. Following pancreatectomy in 5 dogs peripheral vein levels of immunoreactive insulin (IRI) fell from a mean of 21 μ units/ml. to 5 μ units/ml. in 30 minutes. Plasma glucose levels rose from a mean of 110 mg./100 ml. to 163 mg./100 ml. in 90 minutes. IRG levels measured in both a peripheral vein and a jejunal vein showed only a very slight fall. This suggests that a large amount of circulating IRG is made up from extra pancreatic sources presumably enteric in origin. Figure 2 shows the results of a more extensive experiment on a single dog.

In/

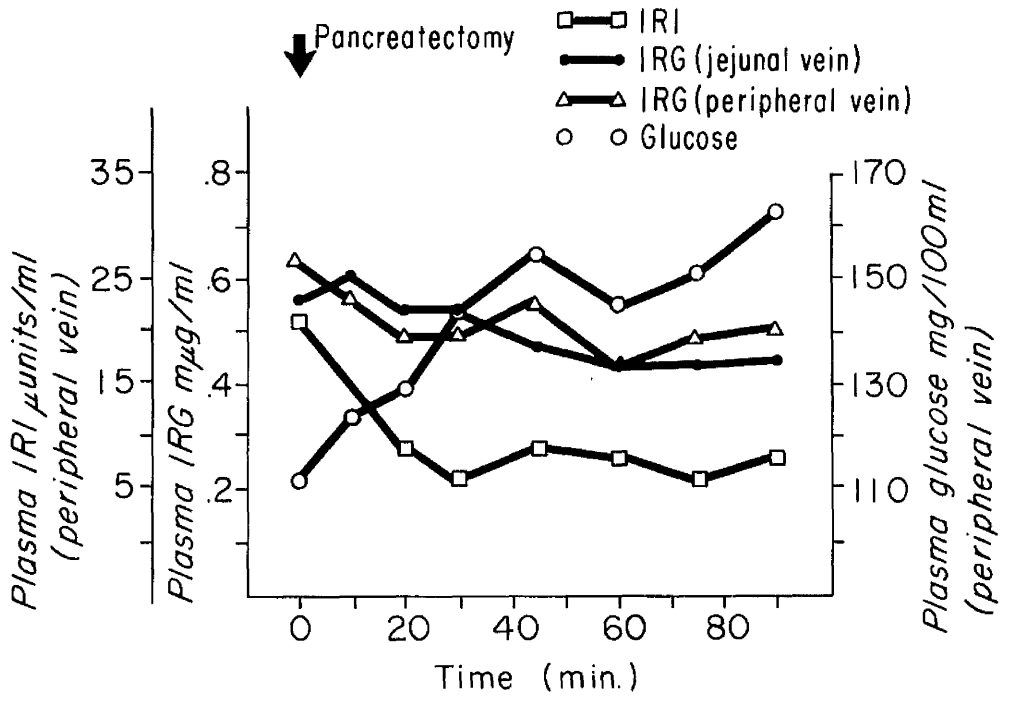
TABLE 1

Immunoreactive Glucagon in Tissues.

	mμg/g wet organ			
	Human	Dog	Monkey	Rat
Pancreas	3700 (1)	2200 (3)	2500 (1)	275 (6)
Stomach	—	0 (2)	—	
Duodenum	—	0 (2)	—	
Jejunum	290 (1)	43 (3)	—	44 (3)
Ileum	—	—	—	
Colon	—	99 (2)	—	
Rectum	120 (1)	—	—	58 (3)
Adrenal	—	0 (1)	—	—
Kidney	—	0 (2)	—	0 (1)
Liver	—	0 (1)	—	0 (1)
Spleen	—	—	—	0 (1)
Heart	—	—	—	0 (1)
Diaphragm	—	—	—	0 (1)
Thymus	—	—	—	0 (1)

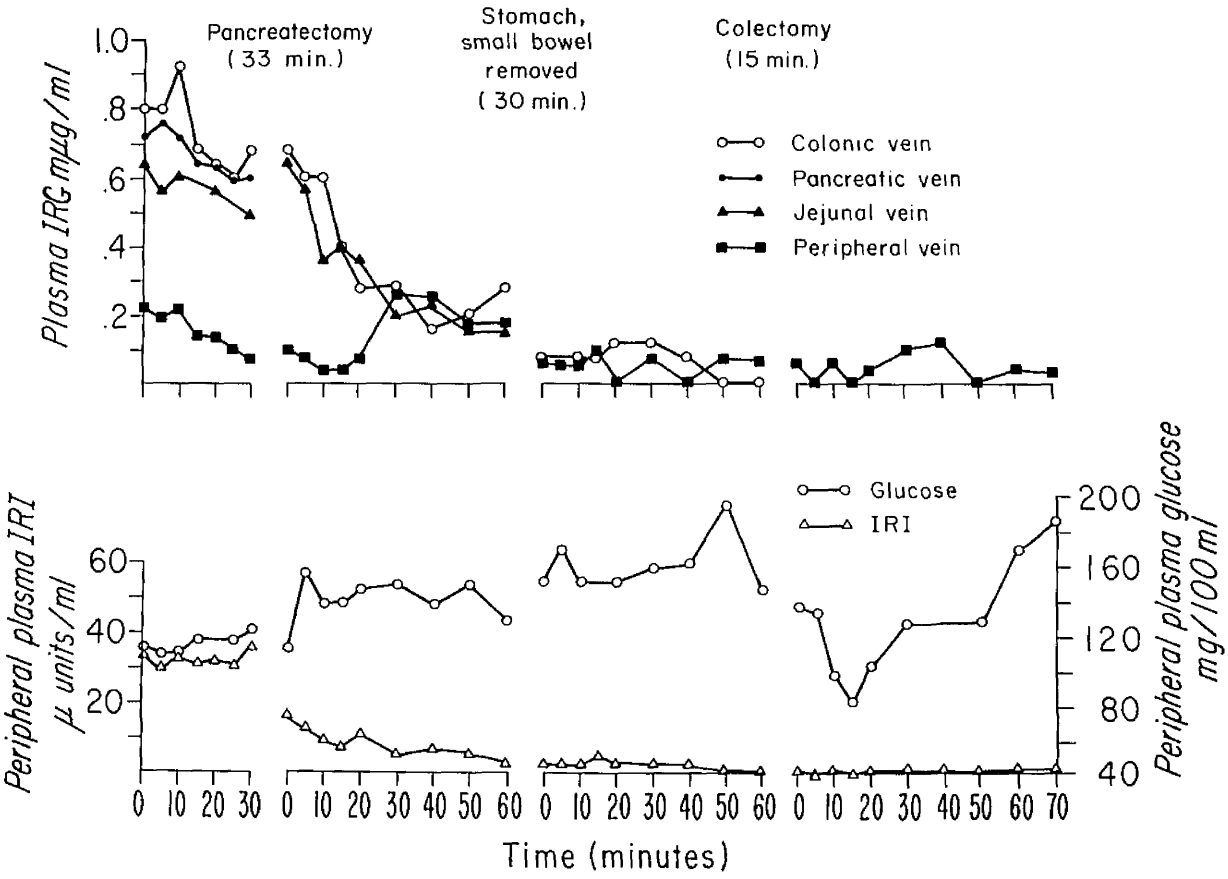
No. of organs assayed is shown in parentheses.

FIGURE 1



Following pancreatectomy in 5 dogs under barbiturate anaesthesia, there was a sharp fall in circulating IRI levels and a rise in plasma glucose levels. IRG levels in peripheral and jejunal veins changed little however.

FIGURE 2



In a single dog colonic vein and jejunal vein, IRG levels were similar to pancreatic vein IRG levels. Following pancreatectomy circulating IRG although falling did not disappear. When the small bowel and stomach were removed, circulating IRG levels virtually fell to zero.

/In this dog, catheters were inserted into a jejunal, a colonic, a pancreatic and a peripheral vein. It is interesting to note that jejunal and colonic vein IRG levels in the intact animal were at least equal to pancreatic vein levels. This particular dog was unusual in this respect, for as will be shown in later parts of this thesis, pancreatic vein levels usually exceeded jejunal vein levels in the basal state. Following pancreatectomy there was a fall in the IRG levels in colonic, jejunal and peripheral veins but there still remained measurable levels of IRG. It was not until the stomach and small bowel were also removed that circulating levels of IRG virtually reached zero. Removal of the colon had no further effect. The experiment suggests that the gut contributes IRG to the circulation.

Discussion

The results strongly suggest that circulating IRG is a product of both the pancreas and the gut. The gut seems to contribute not only from the small bowel but from the large bowel too. Unless experiments are so designed to measure secretion of IRG from different sites of the body (multiple venous catheters, or the pancreatectomised animal) there is no way of distinguishing the relative contribution to circulating IRG from the pancreas and from the gut. The fact that there is a material in the bowel which cross reacts with glucagon antibody, gives no assurance that the cross reacting material is identical with standard pancreatic glucagon/

/glucagon. It might only mean that part of the molecule is similar in that it reacts with antibody. For future reference therefore it would be better to consider the gut material as gut immunoreactive glucagon (GIRG) to distinguish it from pancreatic immunoreactive glucagon (PIRG).

CHAPTER 2

SECTION 2

WHOLE BODY AND LIVER CLEARANCE STUDIES OF GLUCAGON

SUMMARY

The half life for beef-pork glucagon in the intact dog was 3.5 minutes. In the isolated perfused rat liver the biological half life for pork immunoassayable insulin was determined to be 17 minutes. The corresponding figures for beef-pork, rat pancreatic and rat gut immunoassayable glucagon were 19, 23 and 27 minutes respectively. The data are in keeping with the liver being a major site for the degradation of insulin and glucagon.

Samols et al (1966)[77] quote a half life of 10 minutes for glucagon in the circulation of man. The half life of a hormonal substance such as glucagon is a product of two factors:

(1) its distribution within the body spaces, and

(2) its clearance or removal by specific tissues or organs.

The liver is often quoted as a major site for the degradation of glucagon[78,79]. However the evidence for this is only indirect, including destruction of I^{131} glucagon by liver homogenates[54], loss of hyperglycaemic properties of insulin after liver perfusion[53], and trans hepatic gradients in glucagon levels in the intact animal[80]. The introduction of the immunoassay technique now allows a more direct appraisal of the situation.

In addition because of the finding of immunoreactive glucagon (IRG) in both pancreas (PIRG) and gut (GIRG), an attempt was made to compare the clearances of these two substances. Experiments were designed to study the whole body clearance of IRG. The clearance of PIRG and GIRG in the liver perfusion system was also studied. Because of the already well established hepatic clearance of insulin[81], this hormone was also studied concomitantly to provide a comparison for the glucagon values obtained.

METHODS AND MATERIALS

For the whole body clearances two mongrel dogs were anaesthetised/

/anaesthetised by barbiturate, and catheters inserted into the portal, hepatic and peripheral veins. Crystalline beef-pork glucagon was infused into the superior mesenteric vein at a constant rate of .38 µg./minute for 10 minutes using a constant infusion pump. Bloods from portal, hepatic and peripheral sites were withdrawn simultaneously during the infusion and following cessation of the infusion.

Intact livers weighing 14-15 gm. from male Wistar rats (450 gm.) fed ad libitum, were perfused cyclically according to the method of Miller et al[82]. Because haemolysis from red cells in the perfusion system was found to interfere with the immuno-assay systems for insulin and glucagon, the medium used consisted of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 100 mg. of glucose and 1 gm. of albumin per 100 ml. The total perfusate volume was 80 ml. Blood flow through the liver was maintained at 35-40 ml./min. and the perfusions were terminated after one hour. Sampling was done from the hepatic effluent and not more than 10 ml. (12%) were removed during any single perfusion.

Acid-alcohol extracts of rat gut (jejunum and colon) and of rat pancreas were prepared according to the method of Kenny[76]. The gut extracts contained 8 mµg. IRG equivalents and 20 µ units immunoreactive insulin (IRI) per mg. of lypholised weight; the pancreatic extracts had 20 mµg. IRG and 10,000 µU IRI per mg.

After/

/After a 10 minute control perfusion period, the material to be tested was added to the perfusate, and samples were taken over an hour period. PIRG was added in two forms:-

(1) crystalline beef-pork IRG (1.2 µg.) (Eli Lilly), and

(2) rat PIRG 0.4 ug. IRG in 20 mg. extract.

GIRG was added as the rat gut extract (0.4 µg. IRG equivalents in 50 mg. extract). Measurements of IRG were made following the injection of rat pancreatic extract. Simultaneous with the rat GIRG clearances, pork insulin (Eli Lilly) (50,000 µ units added) clearances were measured in the same perfusions.

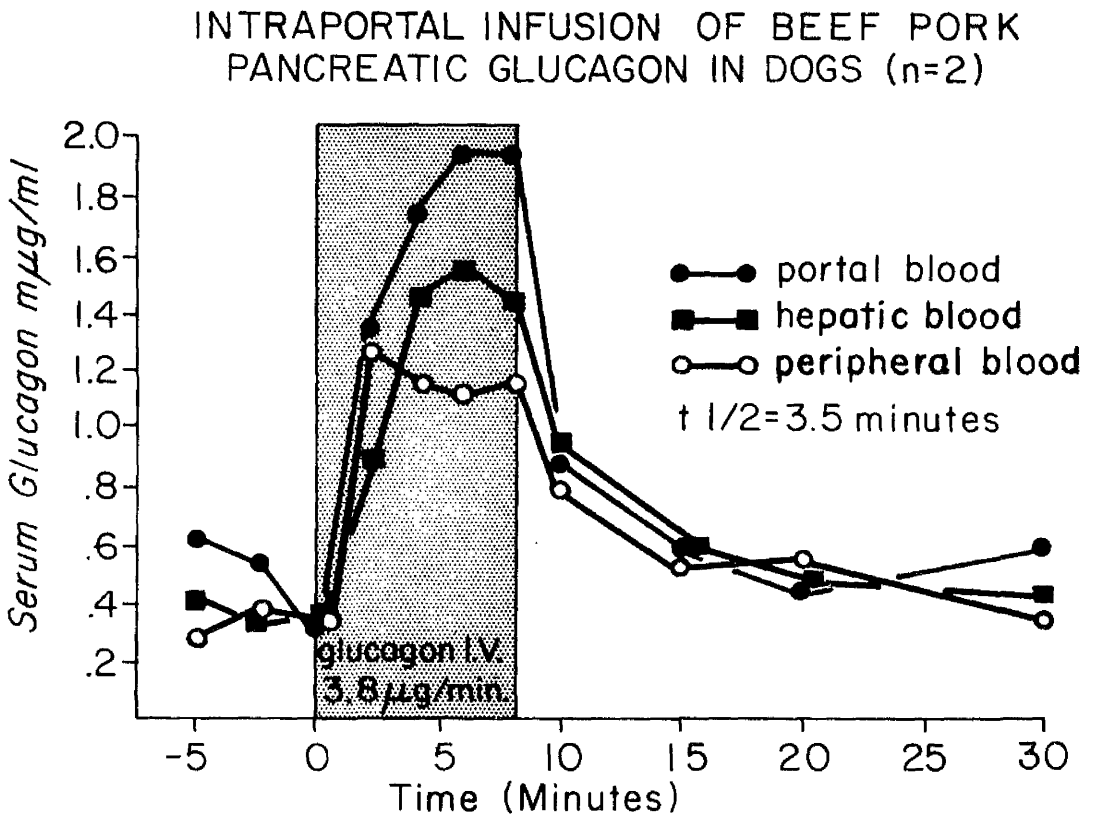
The results were plotted as per cent of maximal (initial) concentration determined 6 minutes after the insulin or glucagon had been added to the bath, when complete mixing had occurred[12]. From semi-logarithmic plots of this data, the half lives ($t\ \frac{1}{2}$) of IRI and IRG in this system were estimated from the linear portion of the graph. No correction was made for the error introduced by the decreasing perfusate volume as this did not exceed 12%.

RESULTS

Glucagon infusion into 2 dogs

The results of this experiment are shown in Figure 3. The $t\ \frac{1}{2}$ for glucagon in the 2 dogs was 3 and 4 minutes respectively which gives a mean value of 3.5 minutes. Portal vein levels were higher than hepatic, and hepatic vein levels higher than peripheral vein/

FIGURE 3



0.38 μg/minute of beef-pork glucagon was infused constantly into the portal vein of 2 dogs. Portal vein levels are higher than hepatic suggesting liver clearance of the hormone. The t $\frac{1}{2}$ for glucagon in the 2 dogs was 3 and 4 minutes respectively.

/vein levels. The portal-hepatic gradient suggests liver clearance of the hormone, but complex blood flow factors complicate the analysis. The portal-peripheral gradient will be partly due at least to dilution effects.

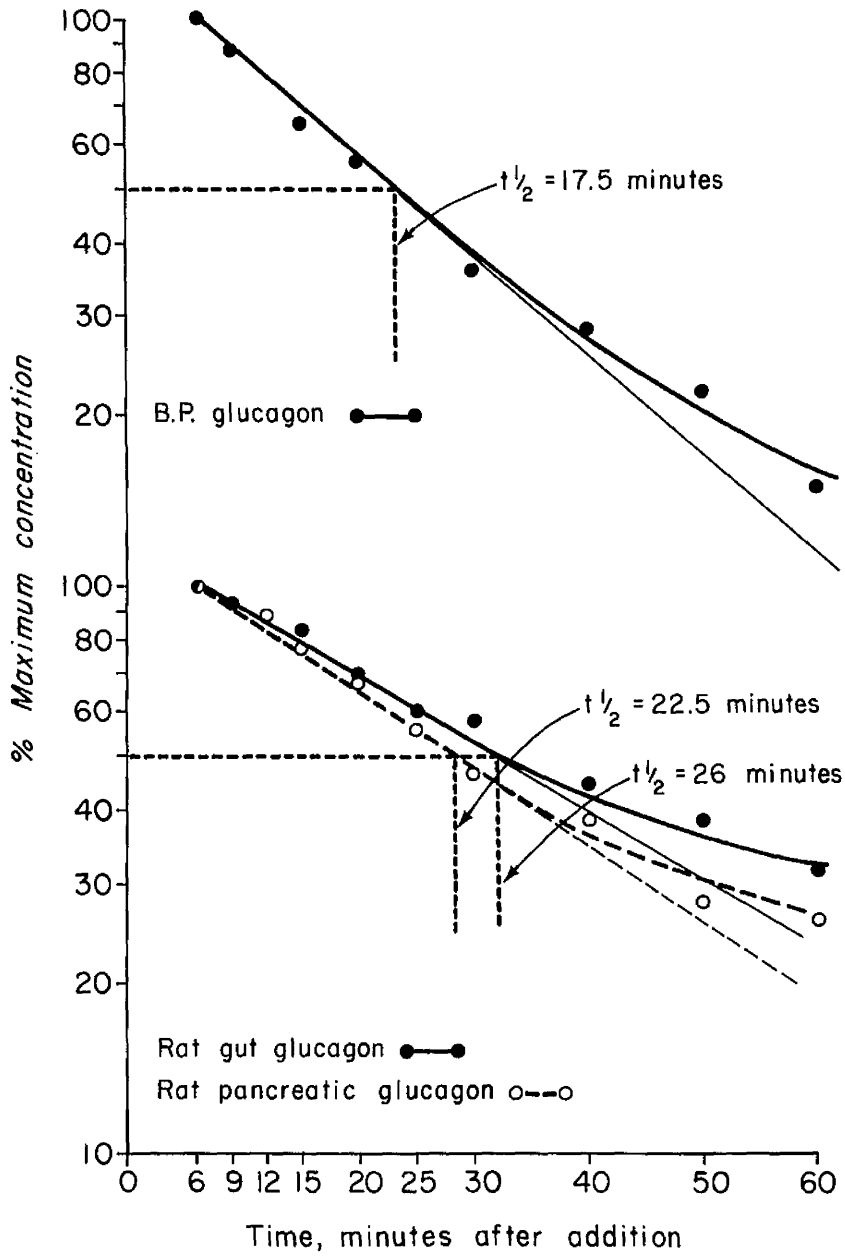
Isolated liver perfusion

The data for all hormone clearances are presented in Table 2, and IRG clearances are also shown graphically in Figure 4. The $t\frac{1}{2}$ values in Table 2 are expressed as mean $\pm$ S.D. of the $t\frac{1}{2}$ for each individual experiment performed for that hormone. The $t\frac{1}{2}$ values in Figure 4 are calculated from the single mean graph of all experiments for the hormone. The 2 methods give fairly similar results. The $t\frac{1}{2}$ values referred to below are those which appear in Table 2. Clearance was mainly by first order kinetics, but there was a tendency for a slower phase of clearance to appear later. For all clearances, the initial linear phase of the logarithmic plot was used to calculate the $t\frac{1}{2}$.

The $t\frac{1}{2}$ for crystalline beef-pork IRG was 19 $\pm$ 2.4 min. (mean $\pm$ S.D.) (Figure 4). The clearances of rat PIRG and rat GIRG are shown in Figure 4, and are similar, 23 $\pm$ 3.6 and 27 $\pm$ 9.3 minutes, respectively. Pork IRI had a $t\frac{1}{2}$ of 17 $\pm$ 4.2 minutes. There were no statistically significant differences between any of the clearances.

The hepatic clearances (k) of the hormones were also calculated/

FIGURE 4



Hepatic clearance of crystalline beef-pork glucagon (upper figure), and rat gut glucagon and rat pancreatic glucagon (lower figure)are plotted as the percentage of the T_6 maximum (initial) concentration of immunoreactive glucagon.

TABLE 2

Hormone	No. of Experiments	Hormone concentration per ml. at 6 min (mean ± S.E.M.)	Minutes after hormone injection 6'	9'	12'	15'	20'	25'	30'	40'	50'	60'	t½(mi
Beef-Pork IRG	3	7750±1350 µµg	100	87±3	81±4	65±2	56±5	-	36±9	28±4	22±5	15±4	19±2.4
Rat PIRG	6	1795±102, µµg	100	93±2	73±4	78±3	68±4	56±7	47±6	39±5	28±6	26±5	23±3.6
Rat GIRG	6	1240±93 µµg	100	94±4	87±5	84±6	70±7	60±7	58±7	45±7	39±8	32±5	27±9.3
Pork IRI	6	303±57 µunits	100	93±4	76±2	66±3	59±6	46±6	41±5	33±6	30±6	26±6	17±4.2

Results are expressed as the percent (mean ± S.D.) of hormone concentration measured 6 minutes after injection.

Hormone clearances in the isolated rat liver perfusion.
IRG = Immunoreactive Glucagon. PIRG = Pancreatic IRG
GIRG = Gut IRG. IRI = Immunoreactive Insulin.

/calculated from the formula (A) of Mortimore et al[83] and Burgi et al[84].

$$(A)\quad k = \frac{2.3 \times V}{\Delta t} \times \text{Log}\ \frac{Ca}{Cf}$$

where k = hepatic clearance of reaction velocity constant.

V = volume of perfusion fluid during sampling time Δt.

Ca = hormone concentration at beginning of time interval Δt.

Cf = hormone concentration at end of time interval Δt.

The k values for pork IRI, beef-pork IRG, rat PIRG and rat GIRG are shown in Table 3.

Discussion

The $t_{\frac{1}{2}}$ for pork IRI of 17 minutes in the liver perfusion system agrees well with the figure of 14 minutes obtained by Solomon et al[81], using the identical liver perfusion technique in the same laboratory as the author, but using a red cell containing media as the perfusate. This would appear to indicate that the substitution of albumin buffer in the system still results in a liver perfusion system adequate for hormone clearance studies.

Our data for glucagon clearance in the liver confirm previous reports[54,53,85] suggesting that the liver is a major source of glucagon degradation. Several workers have, however, suggested that glucagon may be cleared more rapidly by the liver than insulin[54,86], but there was no evidence from the results in the present report that this was the case.

Based/

TABLE 3

HEPATIC CLEARANCE OF GLUCAGON OF VARIOUS SPECIES AND TISSUES

Hormone	Beef-pork IRG	Rat PIRG	Rat GIRG	Pork IRI
k(hepatic clearance ml/min)	2.91	2.40	2.04	3.24

For definition of k see text.

/Based on the assumption that hepatic inactivation is primarily responsible for clearance of these hormones in the intact rat, a theoretical t $\frac{1}{2}$ for glucagon can be calculated using the approximation of Farris and Griffith[87] and formula B of Burgi et al[84]. By calculation this value is 5.1 minutes for beef-pork IRG, 6.2 minutes for rat PIRG, and 7.3 minutes for rat GIRG. These approximate values are a little lower than the estimated t $\frac{1}{2}$ for glucagon in man of 10 minutes[77]. However the values are somewhat greater than the t $\frac{1}{2}$ values for glucagon of 3 and 4 minutes respectively in the intact dogs. The shorter half life in the dogs was presumably partly caused by the large distribution volume into which the rather small molecule of glucagon would go.

Unger et al[55] postulated that IRG extractable from the gut differs from pancreatic IRG. The similar clearances of the two materials which we obtained in these studies, at least suggest identical handling by the liver. The rapid clearance by the liver is in keeping with the quick rise and fall in concentration noted in GIRG after oral glucose[56].

C H A P T E R 2

SECTION 3

IMMUNOLOGICAL STUDIES OF GLUCAGON

SUMMARY

Immunological studies of different species of glucagon showed immunological identity among beef-pork, rat, dog, human and monkey pancreatic glucagons. However dog pancreatic extract was different from dog jejunal extract and human pancreatic extract differed from human jejunal but resembled human rectal extract. It is suggested that jejunal glucagon may differ immunologically from pancreatic glucagon.

Antibodies raised against a hormone of one species will often react differently with the hormone of another species due to slight differences in the physico-chemical structure of the hormone among different species. Insulin has been studied most in this respect. Among 11 insulins from 10 different mammalian species[88] only those from dog[88], pig[89] and sperm whale[90,91] contain identical amino acid sequences, the others being distinguished by differences in one or more residues. Yet all possess essentially identical hormonal potency in all species tested. If antibodies are raised for example against species A insulin some may react very similarly with species A and species B insulin, but others, known as discriminating antibodies will react very differently with species A insulin as compared with species B. The use of a discriminating antibody allowed Samols and Ryder[92] to differentiate exogenously administered beef insulin from endogenous insulin in human subjects.

Hunter and Greenwood[65] described tests of parallelism where it could be calculated whether hormones or unknowns reacted similarly with respect to antibody. If the substances inhibited the reaction between labelled hormone and the antibody with parallel stops then it could be assumed the substances were immunologically indistinguishable.

Although the chemical structure of glucagon has been described[1]/

/described[1] possible variations in structure amongst species have not been studied in the same way as insulin. It thus seemed of interest to test under immunoassay techniques, whether glucagons from different species showed differences in their immunological behaviour. In addition it seemed also pertinent to study the immunological behaviour of pancreatic glucagon as compared with gut glucagon.

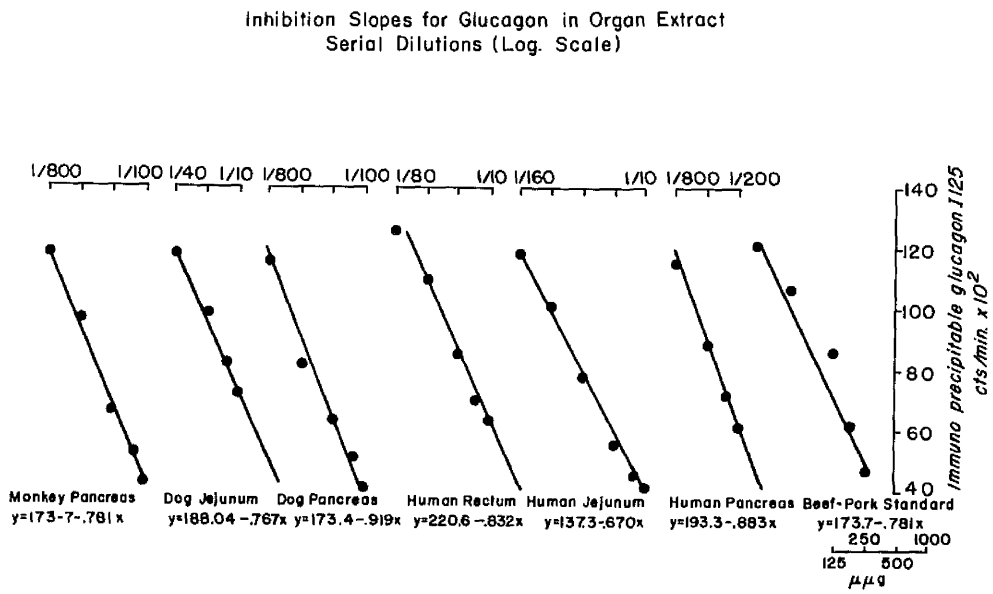
Methods and materials

Tissue extracts from mongrel dogs, male wistar rats, a squirrel monkey, and a human subject were studied. Tissues were extracted by the method of Kenny[76] which has been described elsewhere (Chapter 2, Section 1).

Results

Two antibodies were used in the studies - antibody A and antibody B. The results with antibody A (used at a final dilution of 1:1875) are shown in Figures 5 and 6. Close parallelism existed among all the pancreas extracts - monkey, human, rat, dog and the beef-pork glucagon standard. However, although human rectal extracts were parallel with the human pancreatic extracts, the human jejuanl extracts were shallower than the human pancreas ($p < .01$). Similarly dog jejunal extracts were shallower than dog pancreatic extracts ($p < .01$). Rat pancreatic extracts, however, were not distinguishable from rat small and large gut extracts/

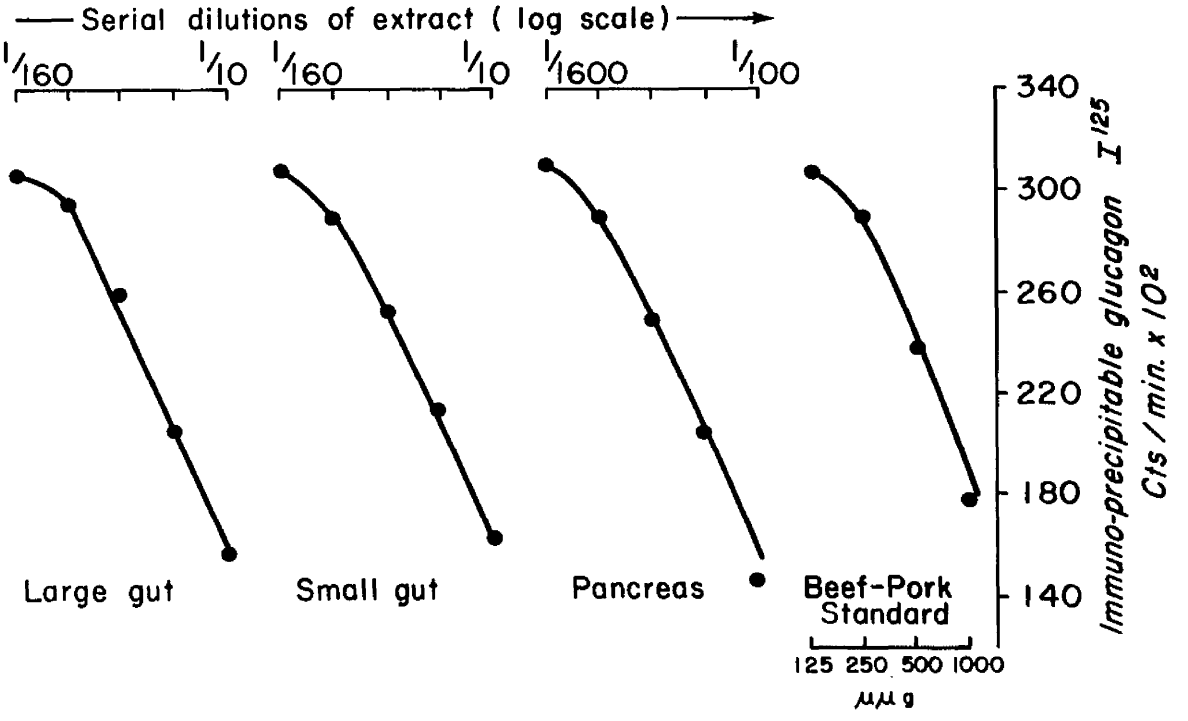
FIGURE 5



Tests of parallelism among organ extracts. All pancreatic extracts were parallel with one another, but human pancreatic extract and dog pancreatic extract were not parallel with their respective jejunal extracts ($p < .01$). Human rectal extract was parallel, however, with human pancreatic extract. (Antibody A used in studies).

FIGURE 6

INHIBITION SLOPES FOR GLUCAGON IN RAT ORGAN EXTRACTS



Tests of parallelism among rat organ extracts. No immunological differences were found among rat small and large gut extracts, rat pancreatic extract and beef-pork glucagon standard. (Antibody A used in studies).

/extracts respectively, and all showed parallelism with the beef-pork glucagon standard.

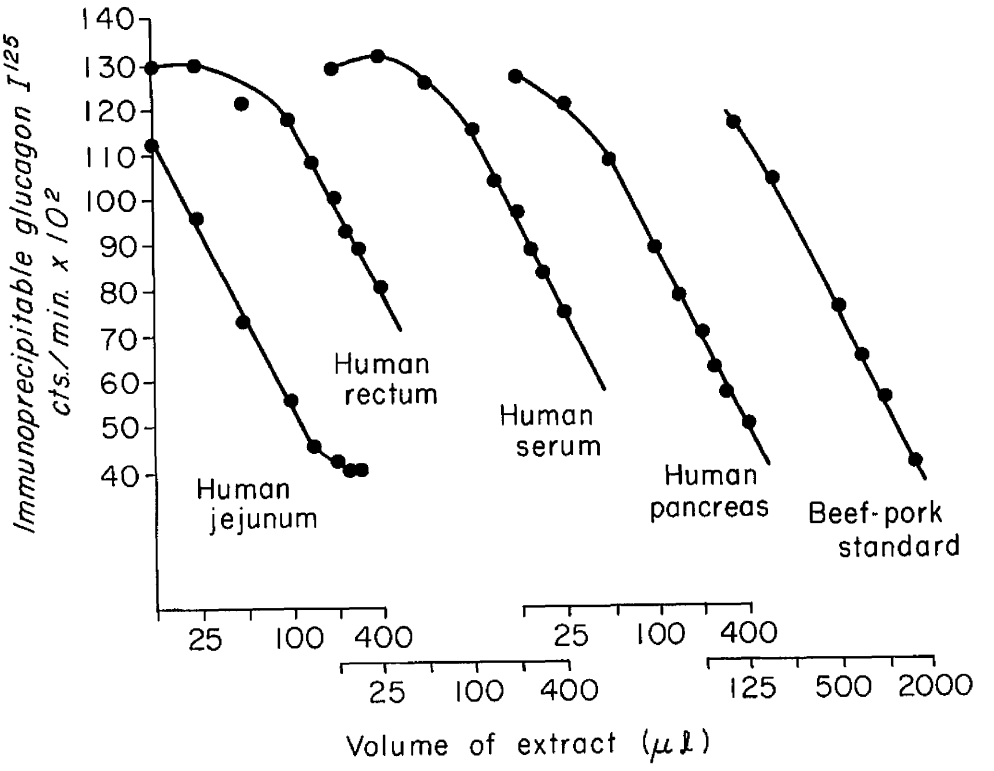
Glucagon antibody B (final dilution 1:4500) showed no discrimination among human jejunum, human rectum, a concentrated acid-alcohol extract of human serum, human pancreas, and beef-pork glucagon standard (Figure 7). Similarly this antibody was unable to discriminate among beef-pork standard, rat gut extract and rat pancreatic extract (Figure 8).

Discussion

These studies suggest that although there appears to be close immunological similarity amongst the pancreatic glucagons of several species of animals, there might be immunological differences in the glucagons found in the pancreas and gut. The use of an antiserum which does not discriminate between pancreatic glucagons of various species and the beef-pork standard glucagon has the advantage that the reading of unknowns gives results in actual glucagon content.

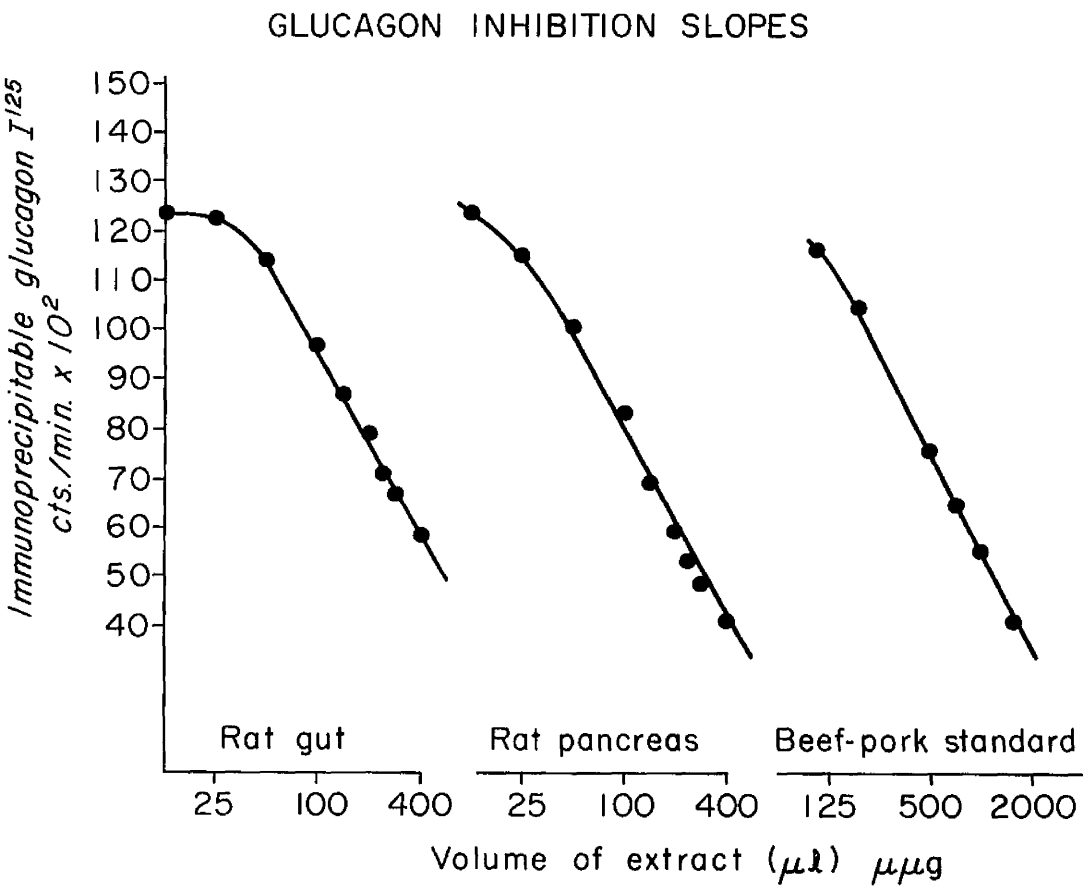
These results showing non parallelism between jejunal and pancreatic extracts agree with those of Samols et al[21] and Schopman et al[22]. In addition both of these authors found perfect parallelism between pancreatic and colonic extracts as did this study. A discriminating antibody is therefore able to detect an immunological difference between small gut glucagon and pancreatic glucagon/.

FIGURE 7



Glucagon antibody B did not discriminate among extracts of human jejunum, rectum, pancreas and serum, and the beef-pork glucagon standard.

FIGURE 8



Glucagon antibody B did not discriminate between extracts of rat pancreas and gut and the beef-pork standard.

/glucagon. This may suggest that gut glucagon is a different substance from pancreatic glucagon. Why colonic glucagon rather than jejunal glucagon should more resemble pancreatic glucagon is impossible to answer. It remains possible, however, that during the preparation of these acid alcohol extracts of tissues that damage may occur in their preparation to make results of the above studies misleading. However, independently 3 groups of workers, including the author, have obtained similar results.

CHAPTER 2

SECTION 4

GLUCAGON AND INSULIN IN BILE

SUMMARY

Immunoreactive insulin and immunoreactive glucagon were detected in bile, the hormone levels in bile corresponding with blood levels. It is suggested that the bile may form an excretory route for these hormones.

Bile has been reported to serve as an excretory route for insulin[170,171], and therefore it was decided to study bile for the possible presence of glucagon.

METHODS AND MATERIALS

The experimental model was identical to the one described in greater detail in Chapter 3, section 3 of this thesis.

In 3 anaesthetised mongrel dogs (20-30 kg.), a catheter was passed into the common hepatic duct, and the bile collected for 10 minute periods. Hormone levels in bile are expressed per 10 minute volumes of bile. Catheters were also passed into the cranial pancreatico-duodenal vein, and a peripheral vein.

Bile was stored frozen for assay of immunoreactive glucagon (IRG) and immunoreactive insulin (IRI).

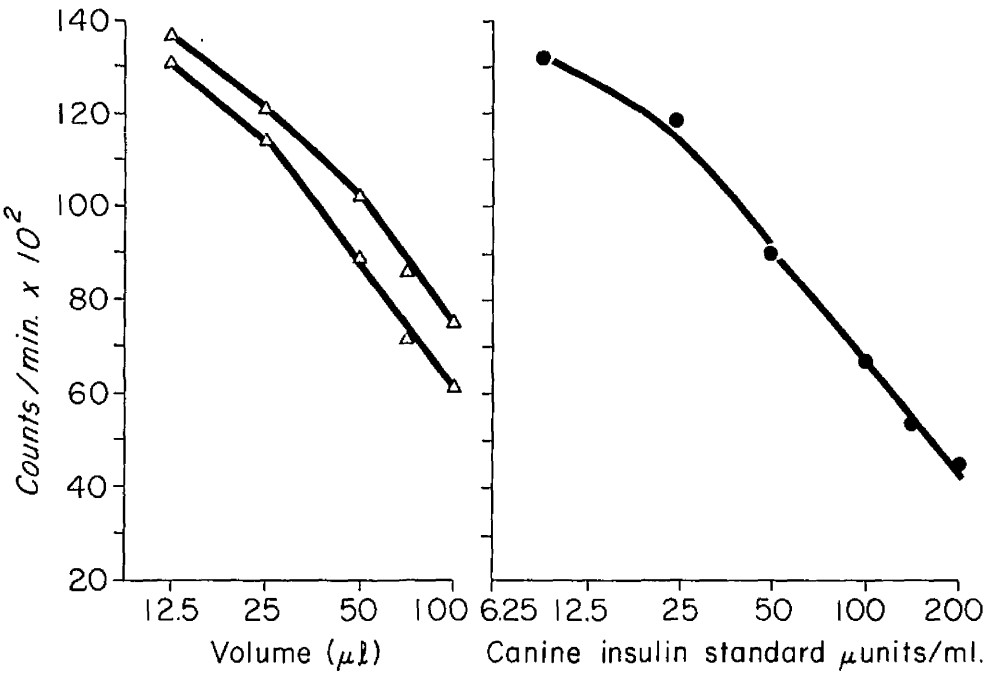
The secretin and pancreozymin used in the experiments were identical to those described in Chapter 3, section 3 of this thesis.

RESULTS

IRI and IRG were detected in bile and inhibition slopes[65] of IRI and IRG (Figures 9 and 10, respectively) in bile indicated close parallelism with standard hormones.

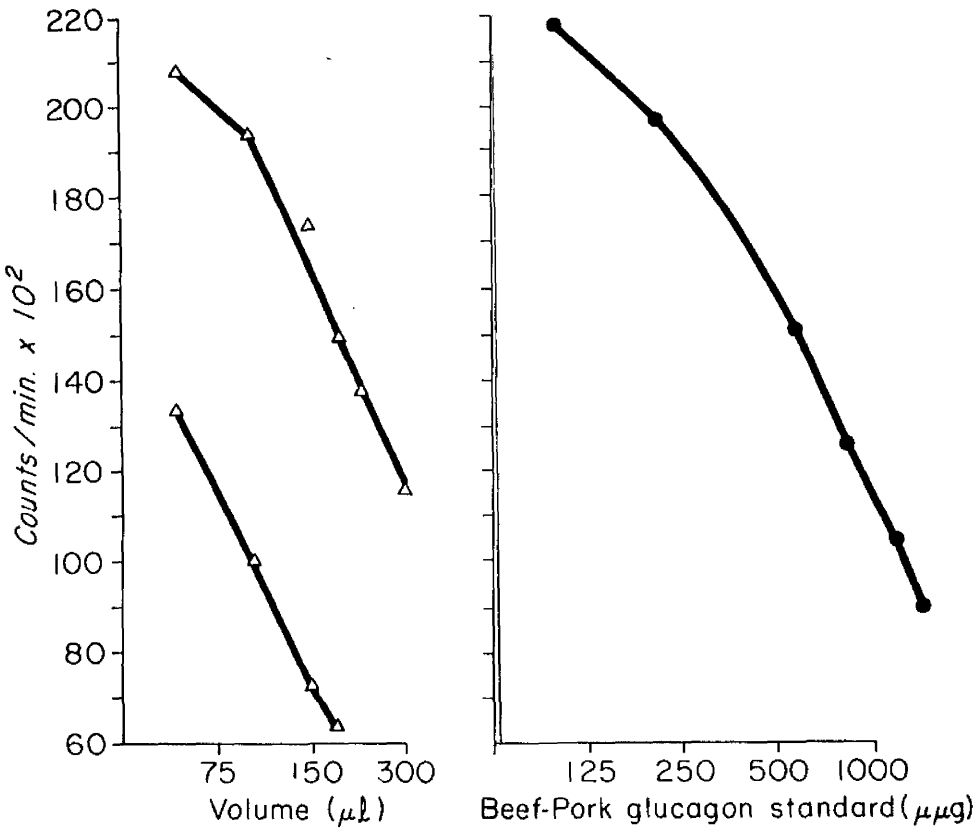
During a secretin-pancreozymin infusion, bile levels of IRG (Table 4) and IRI (Table 5) were recorded. The results are graphically represented in Figure 11. The changes in blood levels of IRI and IRG are fully described in Chapter 3, section 3, and will/

FIGURE 9



Serial dilutions of bile (on left) showed parallelism with standard canine insulin.

FIGURE 10



Serial dilutions of bile (on left) showed parallelism with standard beef-pork glucagon.

TABLE 4

IRG in bile and pancreatic vein blood.

Dog	Site	Minutes 10	20	30	32	35	40	45	50	60	70	80	90
21	P.V.	.99	1.65	1.82	2.67	3.86	3.07	2.84	3.37	3.83	2.97	2.81	2.87
	B.	6.40	6.40	6.40			4.58		7.62	4.75	4.93	3.40	3.40
22	P.V.	1.05	.96	1.14	1.95	1.80	1.29	1.20	.99	1.05	1.05	1.05	1.20
	B.	2.66	2.66	2.66			11.16		6.17	5.21	70.38	19.78	19.78
23	P.V.	.48	.39	.69	1.14	.78	1.10	1.38	1.38	1.35	1.59	-	-
	B.	4.10	3.15	2.37			4.65		4.22	4.22	4.54	4.50	4.50

IRG (mμg) was measured per ml in pancreatic vein (P.V.) blood, and per 10 minute volume in bile (B.). A continuous infusion into a peripheral vein of secretin (0.25 U/min) was begun at 0 time, and 100 units pancreozymin infused into a peripheral vein at 30 minutes. The bile values are shown under the last minute of a 10 minute collection.

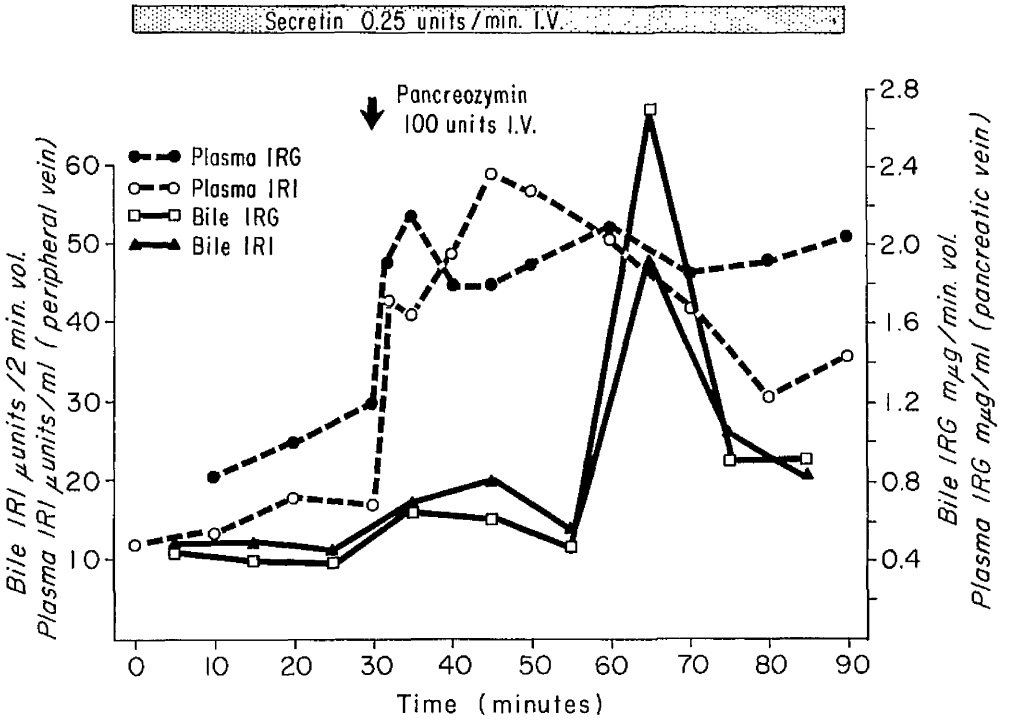
TABLE 5

IRI in bile and superficial vein blood.

Dog	Site	Minutes 10	20	30	32	35	40	45	50	60	70	80	90
21	S.V.	32	43	48	48	52	48	52	58	68	66	73	70
	B.	129	129	129			110		176	105	155	135	88
22	S.V.	15	18	19	34	57	-	77	83	59	32	32	17
	B.	21	21	21			92		64	60	510	204	179
23	S.V.	25	25	33	49	74	181	133	81	43	40	-	-
	B.	36	31	17			54		54	55	46	44	41

IRI (μU) was measured per ml in superficial vein (S.V.) blood, and per 10 minute volume in bile (B.). A continuous infusion into a peripheral vein of secretin (0.25 U/min) was begun at 0 time, and 100 units pancreozymin infused into a peripheral vein at 30 minutes. The bile values are shown under the last minute of a 10 minute collection.

FIGURE 11



Comparison of bile and blood hormone levels in 3 dogs during secretin pancreozymin infusion.

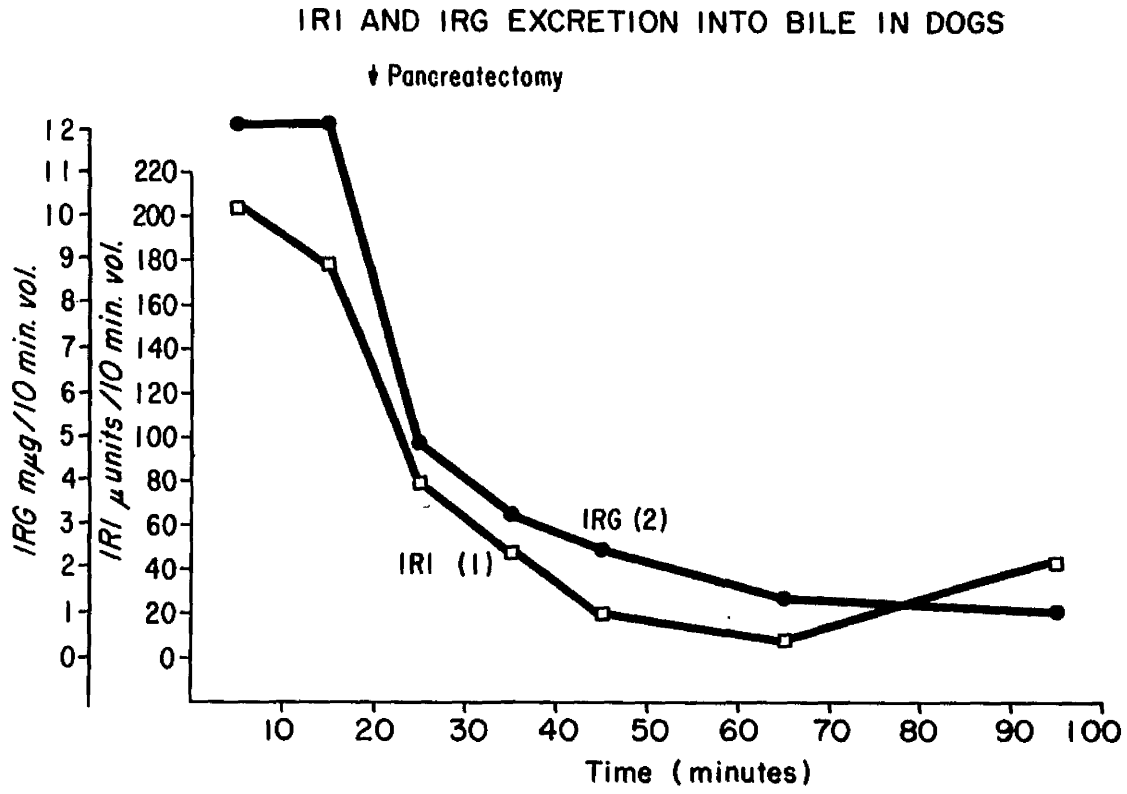
/will not be discussed in detail here. Levels of IRI in bile increased in all 3 dogs during the first or second 10 minute bile collection period following the pancreozymin infusion, and were concomitant with the rise of the hormones in the blood. The IRG levels in bile after pancreozymin were more variable in 2 dogs. In one dog (No.22), however, there was an unexplained enormous rise of both IRI and IRG in bile during the 30-40 minute collection period following pancreozymin. Following pancreatectomy there was a fall in the levels of both hormones in bile (Figure 12).

DISCUSSION

We have confirmed the previous reports of the presence of IRI in bile[170,171], and now find that IRG can also be detected in bile. As the liver is an important site for the degradation of glucagon (Chapter 2, section 2), it is not surprising that it should be found in bile. Bile insulin levels tended to be correlated with blood levels as noted by Lopez-Quijada et al[172]. The fall in hormone levels in bile following pancreatectomy is consistent with the removal of the only source of IRI and one source of IRG. Presumably bile acts as an excretory route for these hormones.

It might be suggested that the bile may be a source of the IRG/

FIGURE 12



Following pancreatectomy, there was a fall in IRG and IRI excretion into bile.

/IRG present in the gut. However, the author was unable to detect any difference in the amount of IRG in the gut when the bile ducts were ligated in rats for 3 days.

CHAPTER 3

FACTORS AFFECTING THE RELEASE OF GLUCAGON

CHAPTER 3

SECTION 1

GLUCOSE CONCENTRATION AND GLUCAGON RELEASE

SUMMARY

Moderate hypoglycaemia (insulin induced) and smaller falls in blood glucose (tolbutamide induced) were equally effective in increasing pancreatic IRG secretion in laparotomised dogs under barbiturate anaesthesia. Hyperglycaemia inhibited pancreatic IRG secretion. Changes in IRG levels in peripheral and jejunal veins during hypoglycaemia were unremarkable. In the pancreatectomised dog no change in IRG secretion from the jejunum was noted. During the tolbutamide experiments slight rises in IRI secretion were related to rises in pancreatic IRG secretion. A rise in serum IRG was demonstrated in conscious dogs after oral glucose. A marked rise in serum IRG occurred after intrajejunal glucose in pancreatectomised dogs, suggesting its enteric source.

In the isolated islets of Langerhans of the rat, the results were consistent with the "in vivo" findings in the dog. Glucose exerted an opposite effect on insulin and glucagon release. As the glucose concentration in the incubation media was raised from 30 to 300 mg. per 100 ml., there was a decreased secretion of IRG and an increased secretion of IRI. The addition of 2-deoxyglucose to the media inhibited IRI release but had no effect on IRG.

It is concluded that hypoglycaemia is a stimulus, and hyperglycaemia is an inhibitor, of pancreatic IRG secretion but

a/

/a changing blood glucose concentration has no effect on enteric IRG secretion. Enteric IRG is stimulated by oral glucose and an insulinogenic role for enteric IRG is considered.

The classical experiments of Foà and associates[10,93,94] appeared to establish the function of glucagon as a hormone of glucose need through its established actions of glycogenolysis[2] and gluconeogenesis[3]. The advent of the immunoassay for glucagon[6] made possible the direct measurement of glucagon during hypoglycaemic states and Unger et al[57] showed an increase in glucagon secretion when the blood glucose concentration fell. Thereafter, however, difficulties with the assay were reported, principally involving the degrading effect of serum on labelled glucagon within the assay, which necessitated the addition of Trasylol (Bayer Ltd.), a proteinase inhibitor, to the assay tubes[55]. The finding, too, of a material in the gut which cross-reacted with glucagon antibody[20,21], and which contributed to circulating glucagon levels, confused the measurement of glucagon in peripheral blood. In addition, the reports of the insulin-stimulating properties of glucagon[39,40,41] have tended to conflict with the classical role of glucagon in providing glucose. Consequently, it was decided to reassess the effect of blood glucose concentration on glucagon secretion from both the pancreas and the gut.

MATERIALS AND METHODS

The studies were performed on overnight-fasted mongrel dogs (20-30 kg.) under barbiturate anaesthesia. Catheters were passed through an abdominal incision into the cranial pancreaticoduodenal vein, a large jejunal vein, and into a peripheral vein. Care was taken/

/taken not to obstruct the pancreatic vein during the experiments and best results were obtained with a 21 guage thin-walled needle inserted in the direction of blood flow. At the time of blood sampling, the pancreatic vein at its entry into the portal vein was occluded with a simple thread snare to prevent reflux from the portal vein.

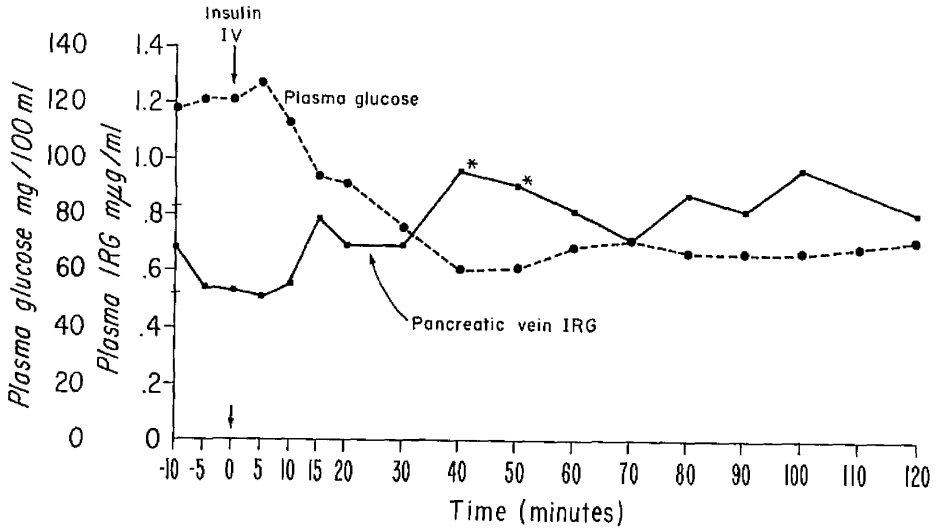
Experiments were also performed on isolated pancreatic islets of Langerhans from male wistar rats (350-450 gm.) prepared as described elsewhere (Chapter 1, section 2).

RESULTS

Insulin Infusions

The effect of insulin induced hypoglycaemia on immunoreactive glucagon (IRG) release is shown in Figure 1. Insulin was injected rapidly (0.5-0.7 units beef-pork regular insulin/kg.) into a peripheral vein in 5 dogs. Fuller details, including individual pancreatic IRG, peripheral vein plasma glucose values, and the mean value for jejunal and peripheral vein IRG levels are presented in Table 1. Pancreatic vein IRG began to rise concomitantly with the fall in plasma glucose, and the rise was significant by the paired t-test at 40 and 50 minutes after the insulin injection ($p < .05$, $< .05$ respectively). A first peak in the IRG secretion occurred at 40 minutes which coincided with the nadir of the plasma glucose values. In 3 of the dogs rises/

FIGURE 1



Hypoglycaemia was induced in 5 dogs by insulin (0.5 - 0.7 U/kg.) injected into a peripheral vein. The asterisks represent statistically significant rises in mean pancreatic IRG levels over basal.

TABLE 1

Dog No.		Control Period -10	-5	0	Minutes after insulin intravenously 5	10	15	20	30	40	50	60	70	80	90	100	120
27	PV IPG†	.44	.32	.20	.52	.40	.68	.32	.32	.47	.48	.28	.32	.32	.76	.52	.52
	PG‡	138	140	143	145	151	-	144	108	100	108	100	110	110	96	82	86
28	PV IPG	.34	.52	.64	.32	.36	.30	.48	.36	.58	.58	.76	.72	.72	.36	.32	.44
	PG	101	101	100	98	92	82	78	66	52	52	52	56	58	68	75	81
29	PV IRG	.52	.30	.32	.31	.32	.30	.40	.52	.96	.96	.48	.40	.40	.35	.42	.74
	PG	106	106	99	119	114	93	80	54	41	41	51	52	52	56	60	59
30	PV IRG	1.18	.80	.86	.66	.80	1.48	1.40	1.40	1.30	1.42	1.58	1.44	2.16	1.84	3.00	1.71
	PG	128	128	132	141	121	121	121	87	68	66	74	71	75	71	72	84
31	PV IRG	.96	.78	.64	.74	.68	1.18	.86	.86	1.44	1.09	.97	.68	.76	.77	.55	.60
	PG	118	131	131	132	89	81	66	-	46	44	-	-	44	44	44	44
Pancreatic Vein IRG (mug./ml.) (mean ± SEM)		.69±.17	.54±.11	.53±.17	.51±.09	.55±.12	.79±.24	.69±.20	.69±.20	.95±.18*	.91±.17*	.81±.23	.71±.20	.87±.34	.81±.28	.96±.52	.80±.24
Jejunal Vein IRG (mug./ml.) (mean ± SEM)		.39±.03	.41±.05	.37±.04	.38±.05	.38±.05	.41±.05	.39±.05	.37±.08	.45±.17	.31±.08	.30±.16	.37±.07	.38±.12	.30±.08	.32±.05	.27±.07
Peripheral Vein IRG (mug./ml.) (mean ± SEM)		.32±.04	.29±.02	.30±.03	.36±.04	.34±.02	.32±.04	.32±.04	.27±.02	.42±.10	.39±.07	.33±.06	.28±.03	.28±.03	.31±.06	.29±.05	.33±.06
Peripheral Vein Plasma Glucose (mg./100 ml.) (mean ± SEM)		118±7	121±8	121±9	127±9	113±11	94±9	91±14	76±12	61±11	62±12	69±11	72±13	67±12	67±9	67±7	71±8

In 5 dogs, insulin (0.5-0.7 U/kg.) was given at 0 time into a peripheral vein.

*Rise over basal $p < .05$ †PV IRG Pancreatic vein IRG (mug./ml.) ‡PG Plasma glucose mg./100 ml. (peripheral vein)

Acute Insulin Hypoglycaemia.

/rises in plasma glucose followed shortly after the peaks of pancreatic vein IRG (dogs 28 - 30). Dog 27 had modest falls in plasma glucose following the insulin, and in this dog the lowest plasma glucose values were not recorded until 90 minutes when pancreatic vein IRG began to rise. IRG levels were higher in the pancreatic vein, in both basal and hypoglycaemic states, than in the jejunal or peripheral veins which both showed similar values (Table 1). The hypoglycaemia did not influence plasma IRG levels in either peripheral or jejunal veins.

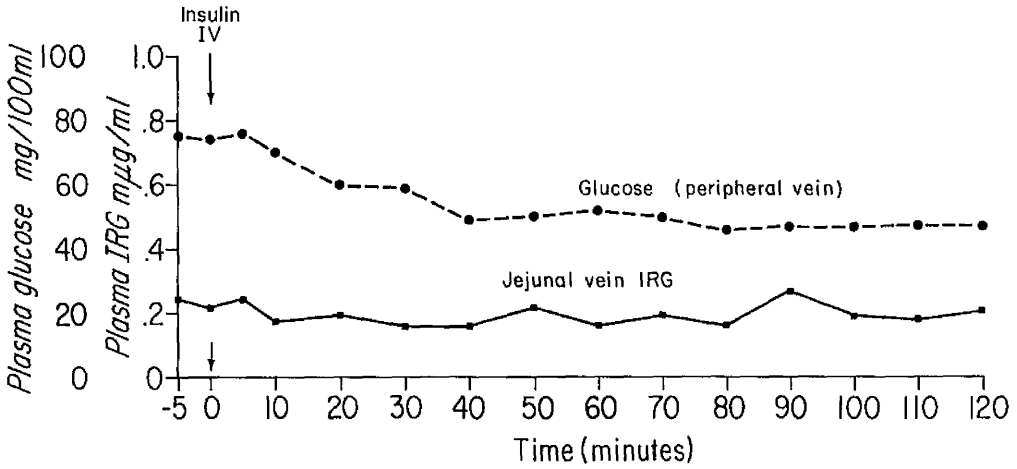
At the conclusion of the above experiments a pancreatectomy was performed in the same 5 animals, and the same dose of insulin as before pancreatectomy was rapidly injected into a peripheral vein. Despite much greater hypoglycaemia than in the intact animal, IRG levels in a jejunal vein remained unchanged (Figure 2). There was also no tendency for the plasma glucose to rise again after it had reached its nadir at 40 minutes.

Tolbutamide Infusion

The production of profound hypoglycaemia by massive doses of insulin can scarcely be described as physiological; such doses may possibly depress glucagon secretion. Therefore, the effect on IRG secretion of milder falls in circulating glucose concentration was assessed in 4 dogs, using tolbutamide. It also provided an opportunity to correlate endogenous IRG

and/

FIGURE 2



Following pancreatectomy in 5 dogs, insulin (0.5 - 0.7 U/kg.)-induced hypoglycaemia had no effect on mean IRG levels in a jejunal vein.

/and immunoreactive insulin (IRI) release. Tolbutamide 1 gm. was given over one minute into a peripheral vein. The results are shown in Table 2, and include individual values for the dogs for IRG in the pancreatic vein, IRI and plasma glucose in a peripheral vein, and the mean IRG values for jejunal and peripheral veins. Pancreatic vein IRG levels in relationship to peripheral vein IRI and glucose values are illustrated in Figure 3.

Following the tolbutamide injection, there was only a modest rise in peripheral IRI levels associated with a gradual descent of the plasma glucose. The general anaesthesia may have rendered the dogs tolbutamide resistant. Pancreatic IRG levels began to rise 20 - 30 minutes after the tolbutamide, and this was significant at 40, 50, 70 and 80 minutes ($p < .05$, $< .05$, $< .05$, $< .01$ respectively). The IRG levels peaked initially at 50 minutes and then again at 80 minutes. It is noteworthy that associated with the rise in IRG levels, there appeared to be a slight rise in peripheral vein IRI levels in 3 dogs (dogs 32, 33, 35). As in the insulin studies, changes in the jejunal and peripheral veins were less than that observed in the pancreatic vein, although inconsistent and insignificant rises in the jejunal vein levels occurred between 20 and 50 minutes following tolbutamide/

TABLE 2

Dog No.		Minutes of control period 50	55	60	Minutes after 1 G tolbutamide I.V. 2	5	10	20	30	40	50	60	70	80	90
32	PV IRG†	.32	.36	.32	.28	.32	.32	.36	.44	.72	.64	.52	.64	.72	.54
	IRI‡	15	19	20	25	31	49	45	40	43	60	65	57	61	61
	PG§	123	122	124	123	122	121	116	112	110	106	103	100	97	93
33	PV IRG	.48	.48	.36	.32	.20	.32	.32	.32	.86	.64	.56	.56	.88	.64
	IRI	15	15	11	26	-	24	24	27	21	24	24	29	23	23
	PG	114	112	110	112	-	108	100	92	90	90	86	87	89	89
35	PV IRG	.36	.36	.36	.26	.28	.26	.52	.72	.66	.78	.20	.58	.54	.60
	IRI	20	19	20	24	22	24	29	9	13	13	22	33	39	44
	PG	113	111	111	131	119	117	108	108	107	107	107	115	101	96
36	PV IRG	.52	.68	.68	1.08	.88	.80	.88	1.04	1.58	1.82	1.72	1.60	2.00	2.30
	IRI	10	11	10	23	16	14	13	16	20	13	16	13	12	10
	PG	108	111	111	111	98	95	93	91	88	97	87	84	85	79
Pancreatic Vein IRG (mμg./ml.). (mean ± SEM)		.42±.05	.47±.07	.43±.08	.48±.20	.42±.15	.42±.12	.52±.13	.63±.16	.95±.21*	.97±.29*	.75±.33	.84±.25**	1.0±.33***	1.04±.43
Jejunal Vein IRG (mμg./ml.) (mean ± SEM)		.35±.05	.34±.05	.35±.05	.29±.03	.35±.08	.37±.06	.75±.37	.55±.16	.70±.30	.52±.13	.44±.10	.46±.07	.46±.10	.45±.07
Peripheral Vein IRG (mμg./ml.) (mean ± SEM)		.28±.02	.24±.02	.24±.02	.25±.007	.26±.04	.24±.03	.31±.01	.32±.02	.35±.03	.32±.02	.28±.02	.28±.04	.25±.01	.23±.02
Peripheral Vein IRI (μunits/ml.) (mean ± SEM)		15±2	16±2	15±3	25±1	23±4	28±7	26±7	23±7	24±6	28±11	32±11	33±9	34±11	35±11
Peripheral Vein Plasma Glucose (mg./100 ml.)		115±3	114±3	114±3	119±5	113±8	110±6	104±5	101±3	99±6	100±4	96±5	97±7	93±4	89±4

*Rise over basal $p < .05$ **Rise over basal $p < .02$ ***Rise over basal $p < .01$

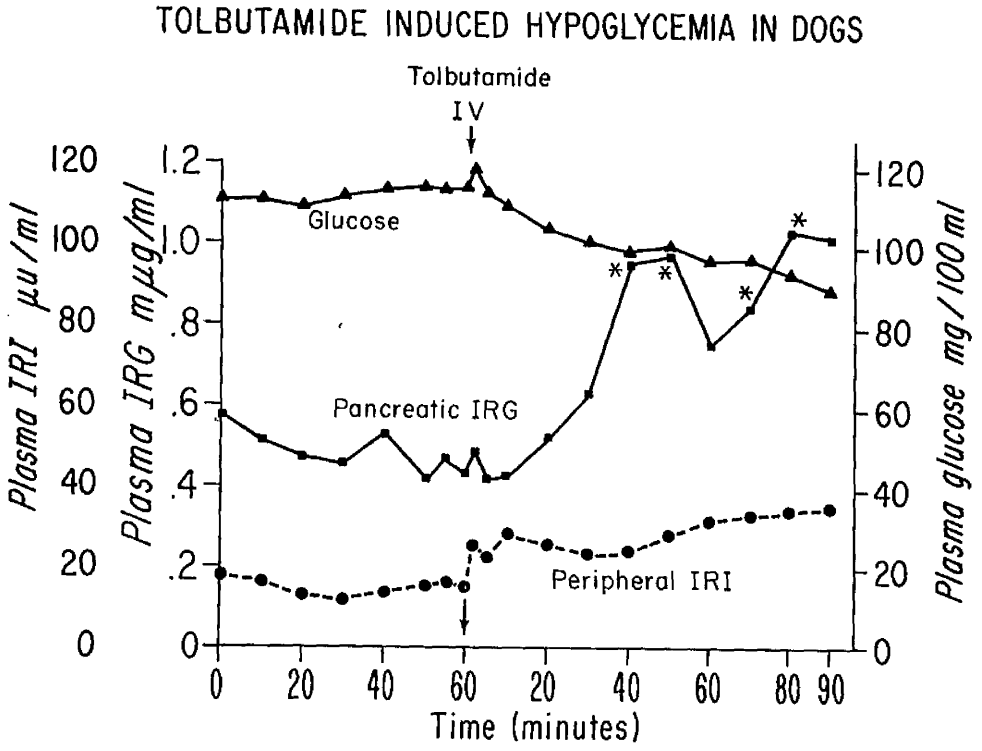
†PV IRG Pancreatic vein IRG (mμg./ml.) ‡IRI IRI (peripheral vein) μunits/ml.

§PG Plasma glucose (peripheral vein) mg./100 ml.

The complete values for the 1 hour control period are not listed.

Tolbutamide Effect.

FIGURE 3



Tolbutamide, 1 gm., was injected into a peripheral vein. Significant rises in pancreatic IRG levels over basal are represented by asterisks. Each point represents the mean of 4 dogs.

/tolbutamide (Table 2).

Glucose Infusion

In the same 4 dogs, two hours after tolbutamide, 25 gm. of glucose was infused over 4 minutes. At this time pancreatic IRG levels were still elevated over the basal in all 4 dogs. The ensuing hyperglycaemia resulted in a rise in IRI secretion but a fall in pancreatic vein IRG secretion ($p < .05$ at 2 minutes after glucose), although IRG levels rose markedly in 1 dog (dog 36) at 50 and 60 minutes after the glucose. The levels, however, remained depressed in the other 3 dogs (Table 3 and Figure 4).

Effect of oral glucose

Following oral glucose, 2 gm./kg. body weight, portal vein IRG rose significantly in conscious dogs (Figure 5). After pancreatectomy in two anaesthetised dogs (Figure 6) serum IRI fell to near zero, and did not rise after 50 gm. intrajejunal glucose. Serum IRG fell only slightly in a periperhal vein following pancreatectomy but not at all in the portal vein (Figure 7). Following intrajejunal glucose, there was a dramatic rise in IRG in both dogs (Figure 7), which was greater than that seen in the intact conscious dog. Prior to pancreatectomy the highest levels of serum IRI and IRG were recorded in the pancreatic vein. There was a distinct portal-peripheral vein IRI/

TABLE 3

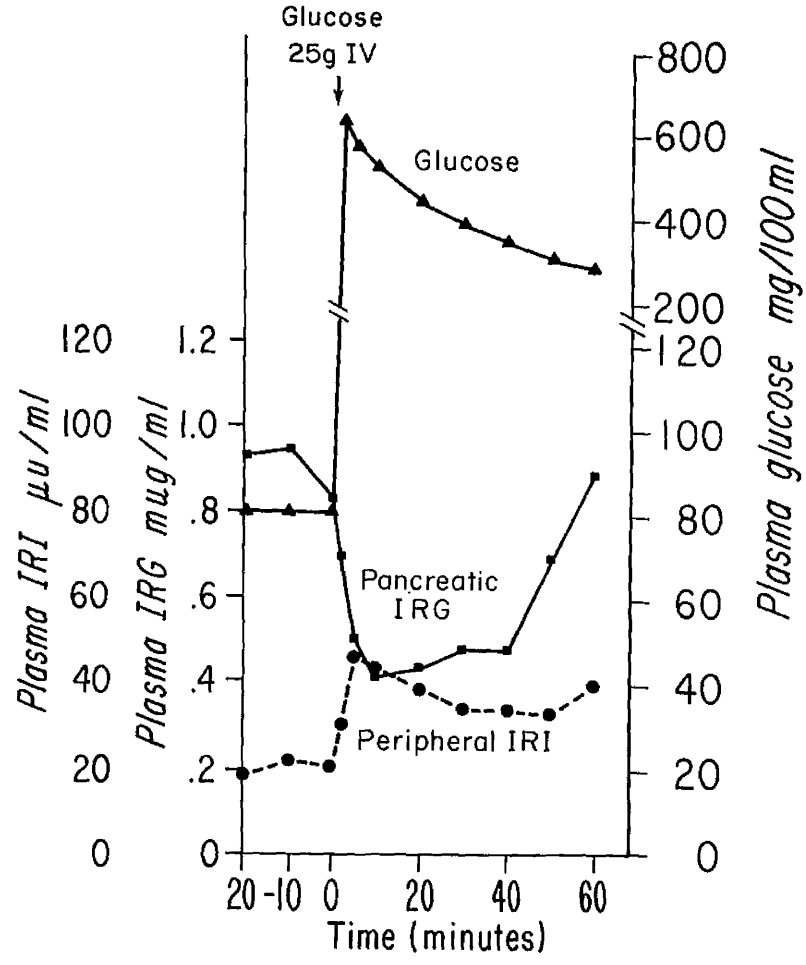
Dog No.		Control Period -20	-10	0	Minutes after I.V. glucose 2	5	10	20	30	40	50	60
32	PV IRG†	.68	.68	.64	.48	.32	.24	.24	.36	.32	1.04	2.32
	IRI‡	34	45	42	26	38	35	22	24	24	28	35
	PG§	80	81	80	600	600	528	414	366	315	300	285
33	PV IRG	.56	.56	.52	.32	.12	.08	.12	.24	.20	.28	.32
	IRI	9	10	10	16	20	20	19	20	22	26	43
	PG	85	85	85	600	600	600	600	582	537	453	441
35	PV IRG	.66	.48	.48	.42	.50	.44	.52	.30	.42	.40	.30
	IRI	25	25	25	65	97	99	97	74	67	57	60
	PG	86	86	86	732	620	568	440	352	312	252	224
36	PV IRG	1.88	2.12	1.72	1.60	1.08	.92	.88	1.04	1.00	1.08	.64
	IRI	8	8	8	15	27	24	16	18	23	20	21
	PG	73	73	74	640	496	436	340	292	264	224	208
Pancreatic Vein IRG (mμg./ml.) (mean ± SEM)		.94±.31	.96±.39	.84±.29	.70±.30*	.50±.21	.42±.18	.44±.17	.48±.19*	.48±.18	.70±.21	.89±.48
Jejunal Vein IRG (mμg./ml.) (mean ± SEM)		.35±.07	.34±.04	.30±.06	.29±.11	.27±.10	.30±.09	.33±.09	.37±.13	.38±.09	.44±.08	.48±.10
Peripheral Vein IRG (mμg./ml.) (mean ± SEM)		.30±.04	.28±.02	.26±.06	.22±.03*	.17±.06*	.17±.04*	.19±.03*	.18±.03*	.21±.02	.21±.04	.20±.04
Peripheral Vein IRI (μunits/ml.) (mean ± SEM)		19±6	22±8	21±8	31±12	46±17	44±19	39±19	34±13	34±11	33±8	40±8
Peripheral Vein Plasma Glucose (mg./100 ml.) (mean ± SEM)		81±3	81±3	81±3	643±31	579±28	533±35	449±55	398±63	357±61	307±51	290±53

*Fall in IRG after glucose $p < .05$ †PV IRG Pancreatic vein IRG mμg./ml. ‡IRI (peripheral vein) μunits/ml.
§PG Plasma glucose (peripheral vein) mg./100 ml.
Control period refers to time 100-120 minutes following tolbutamide.

Effect of 25 gm. Intravenous Glucose on

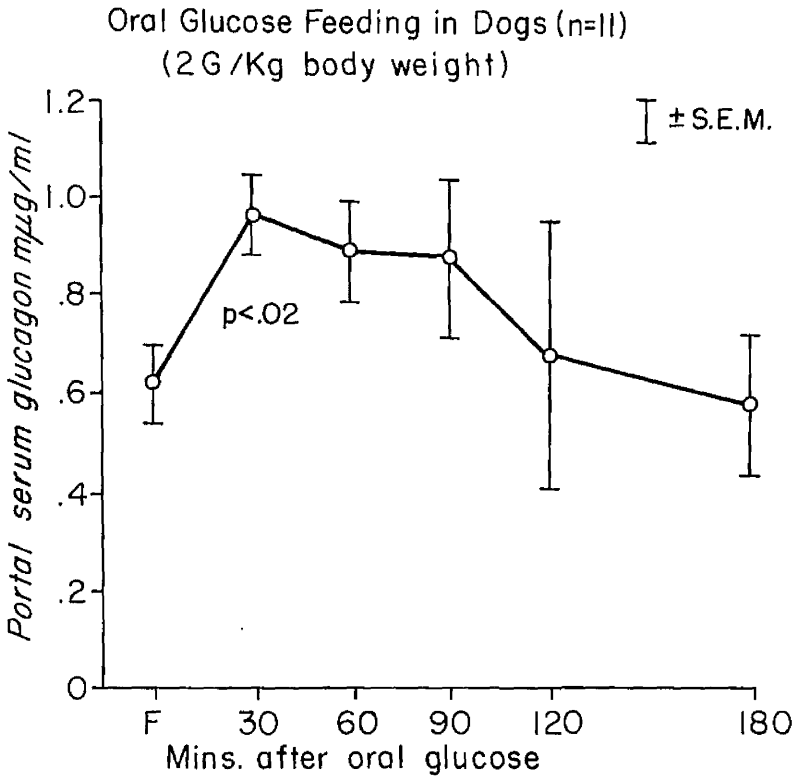
Immunoreactive Glucagon Levels.

FIGURE 4



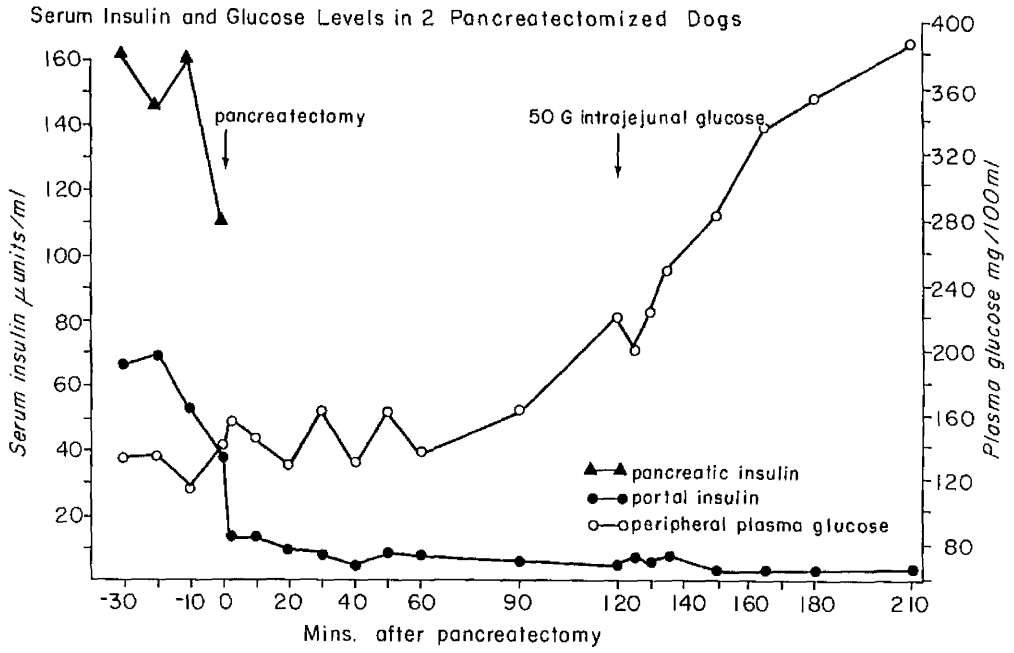
Glucose, 25 gm., was given to 4 dogs 2 hours after tolbutamide administration and the effect on the mean plasma IRI, IRG, and glucose is illustrated.

FIGURE 5



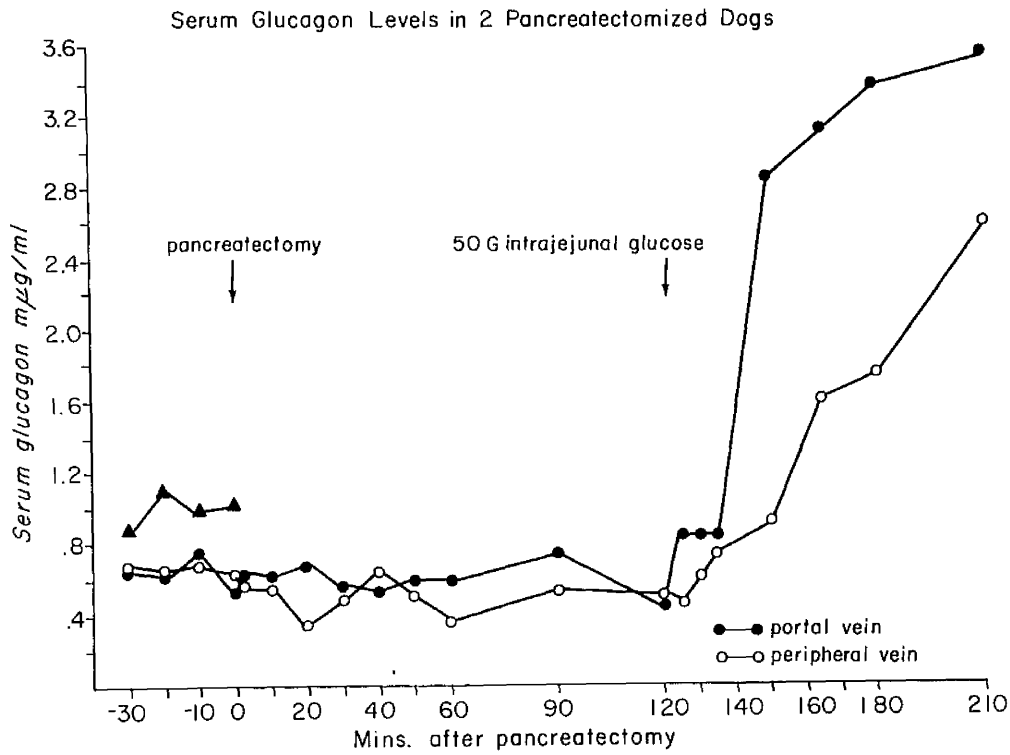
Oral glucose feeding (2 gm./kg. body weight) in 11 dogs resulted in a significant increase in portal vein serum glucagon ($p < .02$).

FIGURE 6



After pancreatectomy in 2 anaesthetised dogs serum insulin fell to near zero and did not rise after 50 gm. intrajejunal glucose.

FIGURE 7



Serum glucagon fell only slightly in a peripheral vein following pancreatectomy in 2 anaesthetised dogs. Following intrajejunal glucose, there was a dramatic rise in IRG in both dogs.

/vein IRI gradient, but no portal-peripheral vein IRG gradient. Following intrajejunal glucose, the level of IRG in the portal vein, which in the dog drains the gastrointestinal tract apart from the anal canal, was higher than in the superior mesenteric vein which drains only the jejunum and ileum.

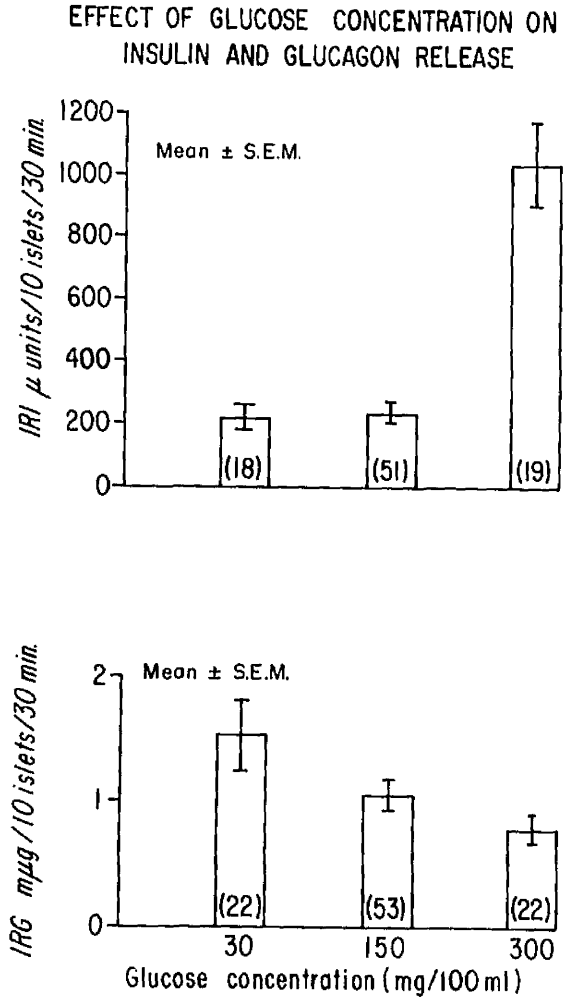
Effect of glucose concentration on IRI and IRG release from isolated islets

The effects of various glucose concentrations on hormone release are compared in Figure 8. IRI release was augmented by increasing the glucose from 30 to 300 mg. per 100 ml. IRG release (mean ± S.E.M.), however, was greatest at a glucose concentration of 30 mg. per 100 ml.(1.53 ± 0.28 mμg./10 islets/30 min.), and the output was diminished by increasing the glucose concentration to 150 mg. per 100 ml.(1.06 ± 0.13 mμg./10 islets/30 min.), although this difference did not achieve statistical significance ($p < .01$). There was a slight further decrease in the IRG release when the glucose concentration was raised to 300 mg. per 100 ml.(0.78 ± 0.12 mμg./10 islets/30 mins.) and this amount was significantly less than that produced with 30 mg. per 100 ml. glucose ($p < .02$). Thus the effect of glucose concentration on IRI release is opposite to its effect on IRG release.

Effect of glycolytic block on IRI and IRG release from islets

It is known that 2-deoxyglucose and mannoheptulose, both incompletely metabolisable sugars, will block the release of insulin/

FIGURE 8



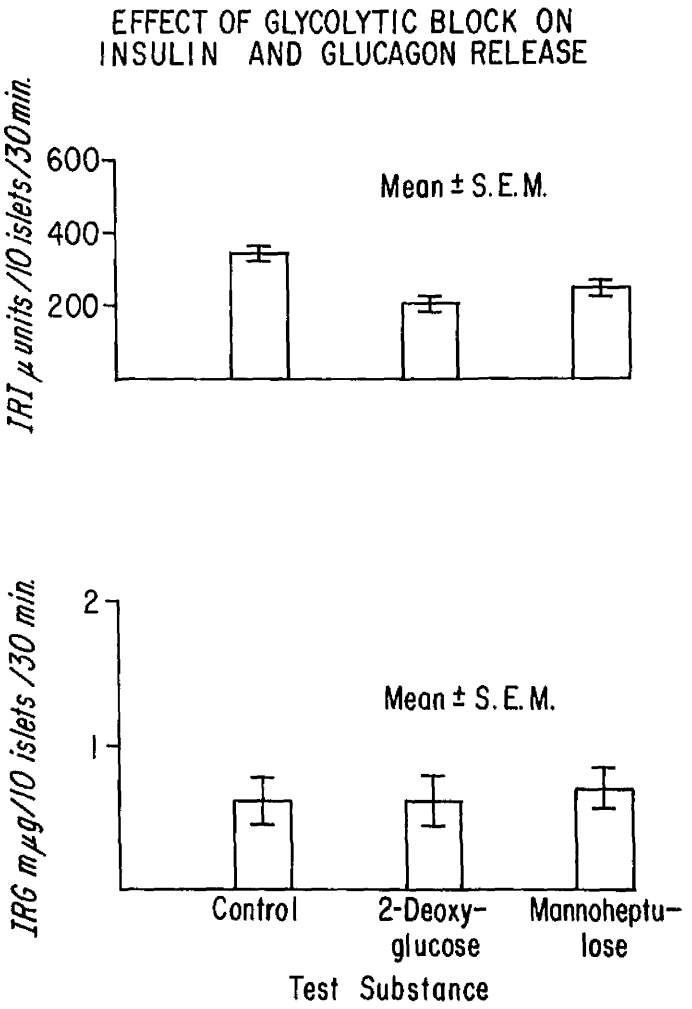
The effect of glucose concentration on insulin and glucagon release. The net hormone production during 30 minute incubations in various glucose concentrations is compared. Each incubation was preceded by a 1 hour preincubation in 60 mg. per 100 ml. glucose. Results are expressed mean ± S.E.M.

/insulin in response to a glucose stimulation[74]. To determine the effect of these sugars on glucagon release, islets were incubated with 150 mg. per 100 ml. glucose and the IRI and IRG response compared to other islets incubated with 2-deoxyglucose (150 mg.per 100 ml.) or mannoheptulose (150 mg. per 100 ml.) plus 150 mg. per 100 ml. glucose. As seen in Figure 9 the addition of nonmetabolisable sugars had no effect on IRG release but caused the expected inhibition of IRI release.

DISCUSSION

Both the isolated islet studies and the dog studies confirm the "in vivo" findings of Unger et al[57] of a rise in pancreatic vein IRG during insulin induced hypoglycaemia. Certain quantitative differences, however, exist between the data of Unger et al[57], and the present results. Unger et al showed a slow steady rise in pancreatic IRG which did not become significant until 2 hours after the insulin infusion, and was still rising at 3 hours, whereas this study shows peak levels which coincide with the nadir of the glucose values at 40 minutes, and thereafter there was no significant further increase. The negative effects of hypoglycaemia on IRG secretion reported by Lawrence[95] and Samols et al[96] are probably due to the fact that peripheral blood measurements of IRG were made in their studies. In the present study/

FIGURE 9



The effect of glycolytic block on insulin and glucagon release. After an initial preincubation for one hour in 60 mg. per 100 ml. glucose, a comparison was made between the net hormone production from islets subsequently incubated for thirty minutes in 150 mg. per 100 ml. glucose, 150 mg. per 100 ml. glucose + 150 mg. per 100 ml. 2-deoxyglucose, and 150 mg. per 100 ml. glucose + 150 mg. per 100 ml. mannoheptulose. The results are expressed as mean ± SEM.

/study no changes in peripheral vein levels of IRG occurred despite a rise in pancreatic vein levels.

It is of interest that a slight fall in plasma glucose produced by tolbutamide was equally as effective in promoting pancreatic IRG secretion as the greater degree of hypoglycaemia produced by large doses of insulin. The study has confirmed too the report by Unger et al[57] that hyperglycaemia depresses pancreatic IRG secretion. It might be postulated that glucagon sensitively responds to small changes in circulating glucose concentration, being stimulated by small falls, and inhibited by rising levels, and so producing blood glucose homeostasis. The data are consistent with glucagon as a hormone of glucose need as originally proposed by Foà and co-workers[10,93,94] and with the established glycogenolytic[2] and gluconeogenic[3] properties of glucagon.

In these experiments no effect of hypoglycaemia on the secretion of IRG from the jejunum was detected, and the results are similar to the effects of pancreozymin on pancreatic and gut IRG secretion[55]. This would be in agreement with the observation of Unger et al[23] who were unable to show glycogenolytic effects in a liver perfusion system of IRG extracted from the gut.

The finding of a rise in IRG after intrajejunal glucose in pancreatectomised dogs confirms Unger et al's[23] finding that the glucagon rise after oral glucose is enteric in origin in dogs, but is/

/is contradictory to Samols et al's[21] findings in man. Because of the much higher level of IRG after glucose in the portal vein than in the superior mesenteric vein, it appears that the colon and/or rectum may contribute to the rise in IRG seen after oral glucose. It is interesting to speculate as to what the role of intestinal IRG may be. The rise of circulating gut IRG after oral glucose would certainly be consistent with an insulin stimulating role. It was interesting to note that in the experiments in the pancreatectomised dog, the rise in gut IRG was more dramatic than in the intact animal, suggesting that insulin deficiency may augment its release.

It is well known that 2-deoxyglucose and mannoheptulose sugars which are incompletely metabolised and which block the intracellular metabolism of glucose, inhibit the release of insulin both "in vitro" and "in vivo"[113,114]. It has been proposed, therefore, that the metabolism of glucose within the islet in some way serves as the signal for insulin release[97,98]. The question is then raised as to what is the mechanism of the stimulation of glucagon release by a low glucose concentration. The reciprocal release of glucagon and insulin demonstrated in Figure 8, suggested the possibility of a negative feedback from the same effector mechanism: glucose utilisation. The studies using 2-deoxyglucose and mannoheptulose in these experiments were designed/

/designed to see if blocking glycolysis within the islet would stimulate glucagon release. The results, however, showed that the inhibitors had no effect on glucagon release. Thus, it appears that energy production from glycolysis is necessary for optimum release of insulin whereas it is not essential for glucagon release.

C H A P T E R 3

SECTION 2

EFFECT OF STARVATION ON GLUCAGON RELEASE

SUMMARY

The effect of starvation on circulating immunoreactive glucagon (IRG) levels was reinvestigated, employing new techniques which prevent degradation of glucagon in the assay. In addition isolated islets of Langerhans of the rat were used to study the effect of starvation on the direct release of IRG and immunoreactive insulin (IRI) from the pancreas, in order to overcome the confusing contribution to circulating IRG from intestinal sources. During a 3 day starvation period, peripheral venous IRG levels fell in 6 normal male subjects, but in 11 mongrel dogs starved for a similar period IRG levels remained unchanged. Serum immunoreactive (IRI) levels declined during the starvation in the humans and dogs. Acute refeeding following starvation demonstrated carbohydrate intolerance, and in the human subjects this was associated with a delayed and excessive IRI response. The serum IRG responses to a carbohydrate meal before starvation were variable and statistically insignificant in the human. After starvation, carbohydrate ingestion elicited a slow steady rise in circulating IRG levels. The demonstrated fall in circulating levels does not necessarily reflect the actual secretory status of pancreatic glucagon, since serum glucagon levels are probably derived from more than one source, including the pancreas and intestine. However, isolated islets of Langerhans from 3 day starved/

/starved rats released less IRI and IRG in various glucose concentrations, than control rats fed "ad libitum". It does appear, therefore, that the release of pancreatic IRG is decreased by starvation. It is suggested that glucagon may play a role in the regulation of insulin secretion during starvation and after carbohydrate ingestion.

Glucagon has been shown to have powerful glycogenolytic[2], gluconeogenic[3], and lypolytic[99] actions. During starvation, when all of these processes are accelerated, increased glucagon secretion might be expected. Although the behaviour of serum insulin during starvation has been well documented[100,101], much less is known about the changes in serum glucagon. Indeed, there appear to be only two studies[58,95] in which circulating glucagon levels have been measured in man during starvation; each demonstrated a rise. The scarcity of reports on serum glucagon has been due largely to difficulties encountered in the assay of this hormone, due principally to degradation of glucagon by serum (Chapter 1, section 1). Since the previous studies reporting a rise in serum glucagon were conducted before recognising the extent of glucagon degradation during the assay, the effect of starvation on circulating glucagon levels was re-examined, utilising an immunoassay for glucagon with Trasylol added to the sera after blood centrifugation (Chapter 1, section 1).

It has recently become evident that immunoreactive glucagon (IRG) from the intestine, as well as the pancreas, contributes to the measured circulating IRG levels[23] (Chapter 2, section 1). Thus, changes in plasma IRG levels "in vivo" do not necessarily reflect changes in pancreatic IRG release, because of the unavoidable simultaneous measurement of gut glucagon. In addition/

/addition, therefore, to "in vivo" studies performed on humans and dogs, the release of IRG and immunoreactive insulin (IRI) from isolated islets of Langerhans of starved and fed rats, was studied in order to directly study the release of pancreatic glucagon under these conditions.

MATERIALS AND METHODS

Six healthy lean male volunteers between 25 and 33 years of age, who had normal glucose tolerance, were studied. After overnight fasting, blood was withdrawn daily before breakfast for 3 or 4 days prior to the starvation period (control), for determination of serum IRG, IRI, serum acetoacetate, and plasma glucose. On the morning of the first day of starvation, 5 of the 6 subjects were given a meal containing a calculated 100 gm. of carbohydrate and blood was withdrawn serially for 3 hours thereafter for determination of the IRG, IRI, and glucose responses to a carbohydrate load in the control state. The subjects were then starved for the remainder of the day and for the next 2 days and allowed nothing by mouth except water, 2 cups of black unsweetened coffee per diem, sugarless gum, and sodium chloride ad libitum. The starvation was terminated on the morning of the fourth day by a similar breakfast in the same 5 subjects and blood was withdrawn as mentioned.

Also, two young obese, but otherwise healthy males were starved/

/starved in the same fashion for 14 days and blood samples drawn daily for IRG, IRI, glucose and acetoacetate determinations, for several days prior to the starvation, during starvation, and during the refeeding period. One hundred gm. carbohydrate meals were given prior to starvation, and a similar meal to terminate the starvation. Bloods were withdrawn during these tests as in the lean subjects.

Eleven mongrel dogs were studied in a similar fashion and were allowed only water during the 3 day starvation period. Indwelling portal vein catheters were inserted at laparotomy, beyond the entry of the pancreatico-duodenal vein. Studies were done only when the dog was again eating normally, and in no case sooner than 5 days postoperative. Portal blood was withdrawn on the day prior to the fast and daily during the starvation period for determination of IRG, IRI, and glucose. On the first day of starvation, the dogs were given an oral glucose tolerance test (GTT) by stomach tube (2 gm. glucose per kg. body weight), and a similar test was performed to break the fast on the morning of the fourth day.

Male wistar rats (400-500 gm.) were allowed to eat "ad libitum" (controls) or were denied all oral intake except water for 72 hours (starved) prior to study. Islets were isolated as described elsewhere in this thesis (Chapter 1, section 2) for measurement/

/measurement of IRI and IRG release. For blood collection a laparotomy was performed under ether anaesthesia and blood for measurement of circulating hormone levels and plasma glucose was obtained by exsanguination from the vena cava. For tissue extraction, control and 3 day starved rats were decapitated and the pancreas was removed and extracted for IRI and IRG content as described in this thesis (Chapter 2, section 1).

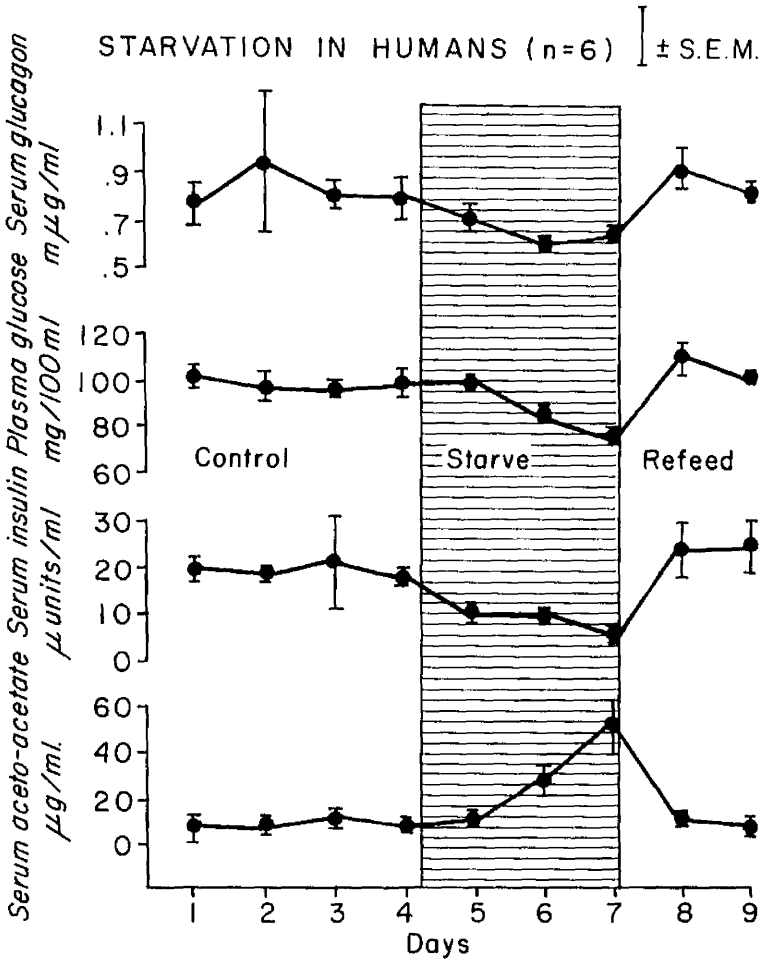
RESULTS

Starvation in humans and dogs

The mean serum IRG, IRI, acetoacetate, and plasma glucose concentrations in the human subjects are shown in Figure 10. The acetoacetate concentration rose markedly and was still on the upward trend on the third day of the fast. The glucose concentrations declined progressively during the starvation. The mean IRI concentrations fell significantly on the first day ($p < .01$) and reached the lowest level on the third starvation day.

The IRG concentrations, which averaged 830 μμg. per ml. before starvation, fell slightly ($p < .05$) to 710 μμg. per ml. on the first day of the starvation, and continued to fall on the second and third days of starvation to 610 μμg. per ml. ($p < .01$) and 640 μμg. per ml. ($p < .01$) respectively. (Significance tested by the paired t - test). With refeeding there were rises in IRI glucose, and IRG to above control levels, but the decreases in the serum/

FIGURE 10



Effect of starvation on plasma glucose, serum IRG, IRI, and acetoacetate in 6 lean human subjects. The fall in IRG was significant on each of the 3 starvation days ($p < 0.05, < 0.01, < 0.01$, respectively).

/serum acetoacetate.

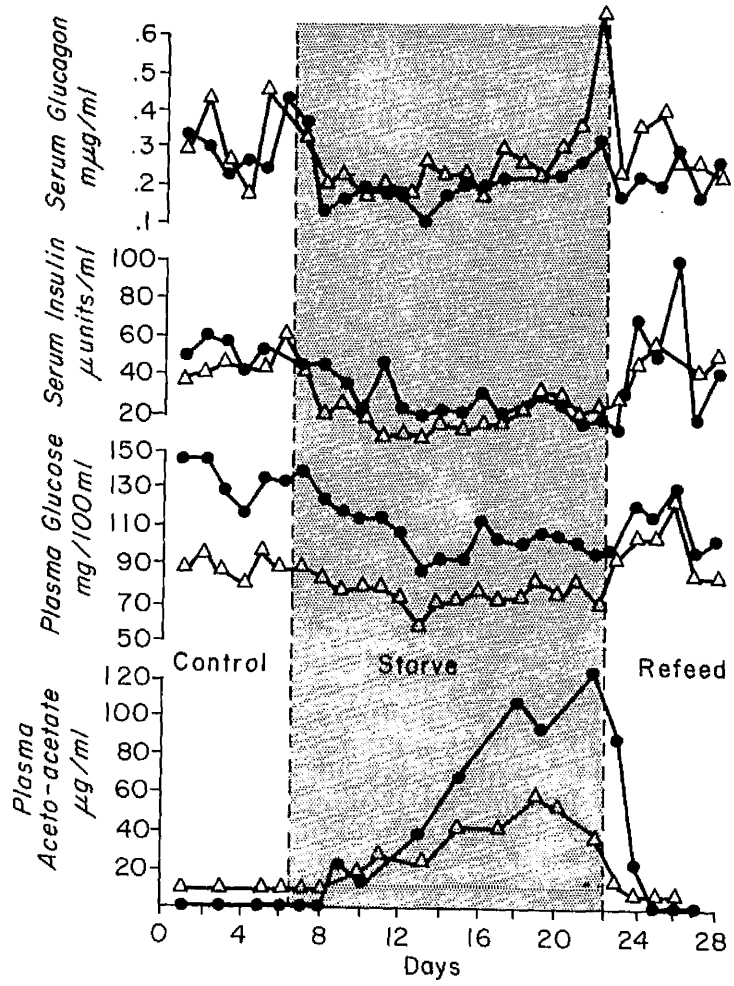
During 14 days fast periods for obese subjects glucose, IRI, and IRG levels fell but there was a tendency for IRG levels to rise during the last few days of the fast (Figure 11).

The changes measured during starvation in the dogs (Figure 12) were less than those observed in the humans. The plasma glucose remained unchanged although the serum IRI fell during the starvation. Serum levels of IRG showed no significant change during the 3 day starvation period.

Refeeding studies in humans and dogs

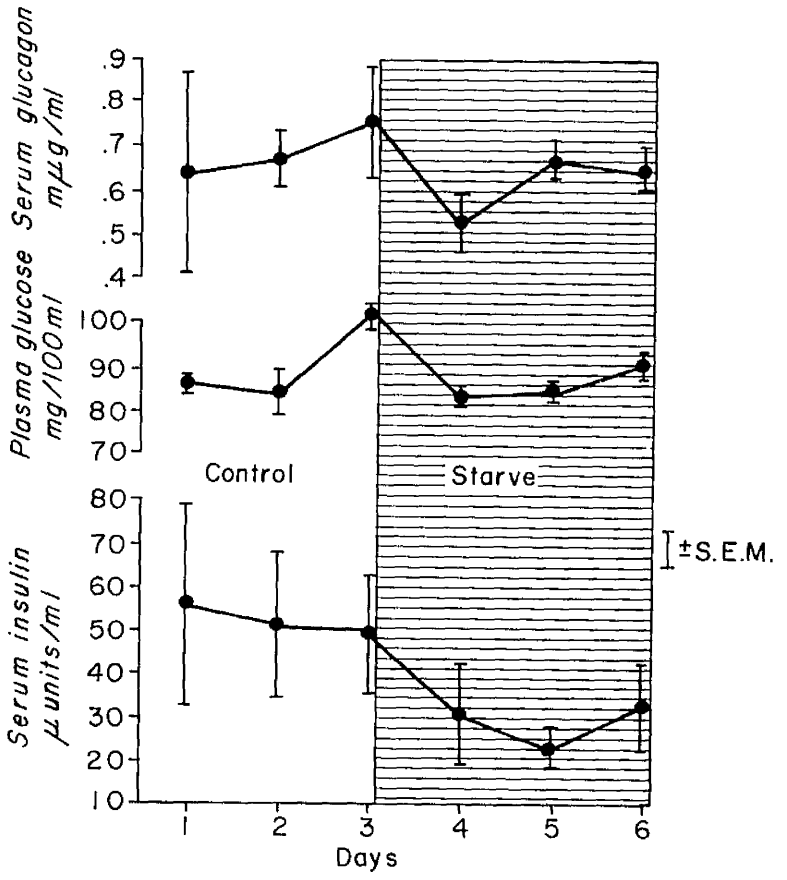
In 5 lean male subjects, the effects of a 3 day fast upon metabolic responses to an oral carbohydrate load are shown in Figure 13. All subjects developed carbohydrate intolerance and hypernormal IRI responses. The IRI response in the first 30 minutes, however, appeared inadequate, as it was no greater than in the control state, despite much higher glucose levels. Four of the 5 subjects in the control test showed a slight rise in serum IRG and one subject showed an enormous rise. The apogées were transient and variable in timing, although they tended to occur in the first hour. The increase over the fasting level did not achieve significance at any time. In contrast to the control test, after the 100 gm. carbohydrate meal, administered at the termination of the 3 day starvation, the serum/

FIGURE 11



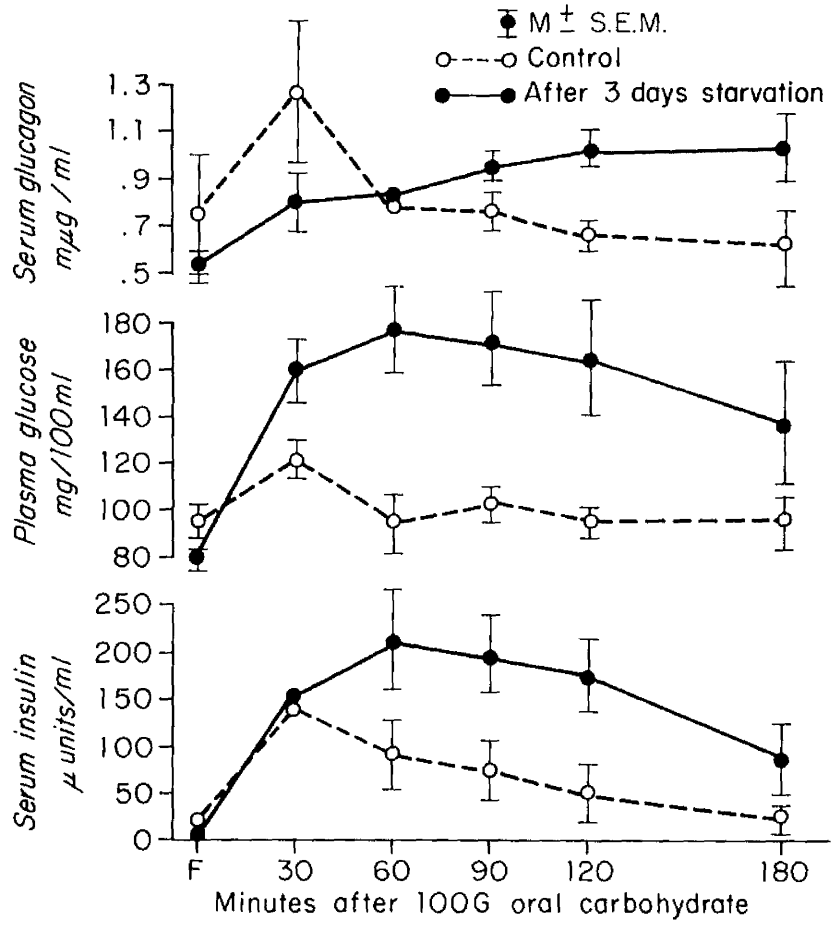
Effect of prolonged starvation on plasma glucose, serum IRG, IRI, and acetoacetate in 2 obese human males. Serum IRI and IRG both fell, although there was a tendency for the IRG to rise towards the end of the prolonged starvation.

FIGURE 12



Effect of starvation on portal vein plasma glucose, serum IRG and IRI in 11 dogs. There was no significant change in the glucose or IRG level but IRI levels progressively fell.

FIGURE 13



The response of plasma glucose, serum IRG, and IRI levels to oral 100 gm. carbohydrate meals in 5 human subjects before and after 3 days fasting. Glucose intolerance and an increased insulin response developed following starvation. The IRG rise over the fasting level achieved significance at no time during the control test, but in the poststarvation test the IRG rose steadily and was significantly higher than the fasting value at 30, 60, 90, 120 and 180 minutes after the CHO ($p < 0.02$, < 0.05, < 0.02, < 0.01, < 0.05, respectively). At only the 120 minute value did the starvation test reach higher levels than the control test ($p < 0.02$).

/serum IRG on all subjects rose steadily to reach a peak at 3 hours. The rise over the fasting level was significant by the paired t-test at each time ($p < .02$, $< .05$, $< .02$, $< .01$, $< .05$ at 30, 60, 90, 120 and 180 minutes respectively) and became significantly higher than the corresponding control test at 120 minutes ($p < .02$). The early glucagon rise in the control tests matched the early glucose and insulin rise whereas the late glucagon rise in the poststarvation test was concurrent with the delayed insulin and glucose rise.

In the 2 obese subjects the carbohydrate meal produced greater IRG rises poststarvation than prestarvation (Table 4). Theydid, however, show different behaviours in glucose tolerance and insulin responses. Subject S, who had normal glucose tolerance prior to starvation, showed very slight deterioration following starvation, with a delayed insulin response similar to the pattern of the lean subjects. Subject B, on the other hand, had abnormal glucose tolerance with a delayed insulin response prior to starvation, and both abnormalities disappeared after the 14 day starvation period.

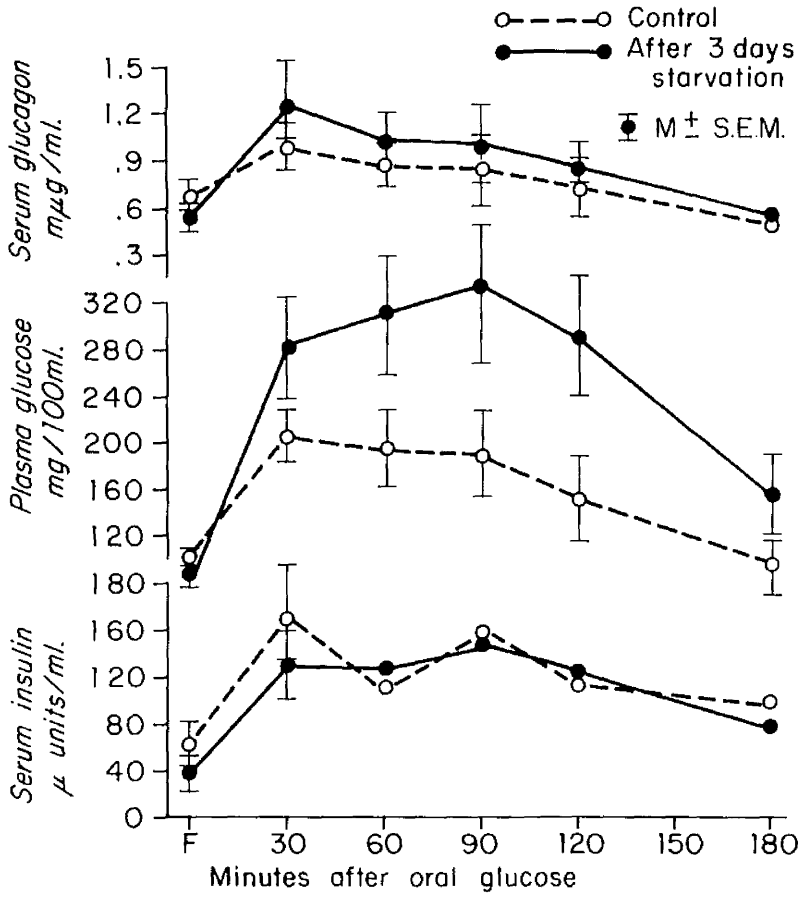
Starvation in the dogs also produced intolerance, but without a change in IRI levels (Figure 14). A rise in IRG to oral glucose in the control test ($p < .02$) remained unchanged by the starvation/

TABLE 4

		Control 100 g. CHO Test						Post Starvation 100 g. CHO Test					
	Minutes:	0	30	60	120	180	240	0	30	60	120	180	240
SUBJECT B	Plasma Glucose mg./100 ml.	134	156	168	190	138	102	98	142	177	106	77	68
	IRI μU/ml.	61	74	89	147	114	82	16	117	110	73	28	29
	IRG mμg/ml.	.43	.50	.26	.73	.30	.23	.33	.63	.83	.56	.40	.26
SUBJECT S	Plasma Glucose mg./100 ml.	88	119	101	83	90	81	68	83	90	98	105	102
	IRI μU/ml.	62	180	160	96	112	37	24	121	123	115	136	98
	IRG mμg/ml.	.40	-	.23	.23	.30	.30	.66	3.30	2.15	.30	.26	.33

The glucose, IRI and IRG responses to 100 g. CHO meal in 2 obese subjects (B amd S) are shown before and after a 14 day starvation period. Subject S shows a marked imcrease in IRG response associated with a more sustained IRI response following the starvation. Subject B has only a slight increase in IRG response but shows improved glucose toleramce and a normal insulin response following starvation.

FIGURE 14



The response of portal vein plasma glucose, serum IRG, and IRI levels to oral glucose (2 gm. per kg. body weight) in 11 dogs before and after 3 days of starvation. Following starvation, the dogs developed glucose intolerance without a change in insulin secretion. The IRG response to glucose was significant in both the control and starvation tests ($p < 0.02$ in both instances) but the starvation test did not become significantly higher than that of the control.

/starvation (Figure 14).

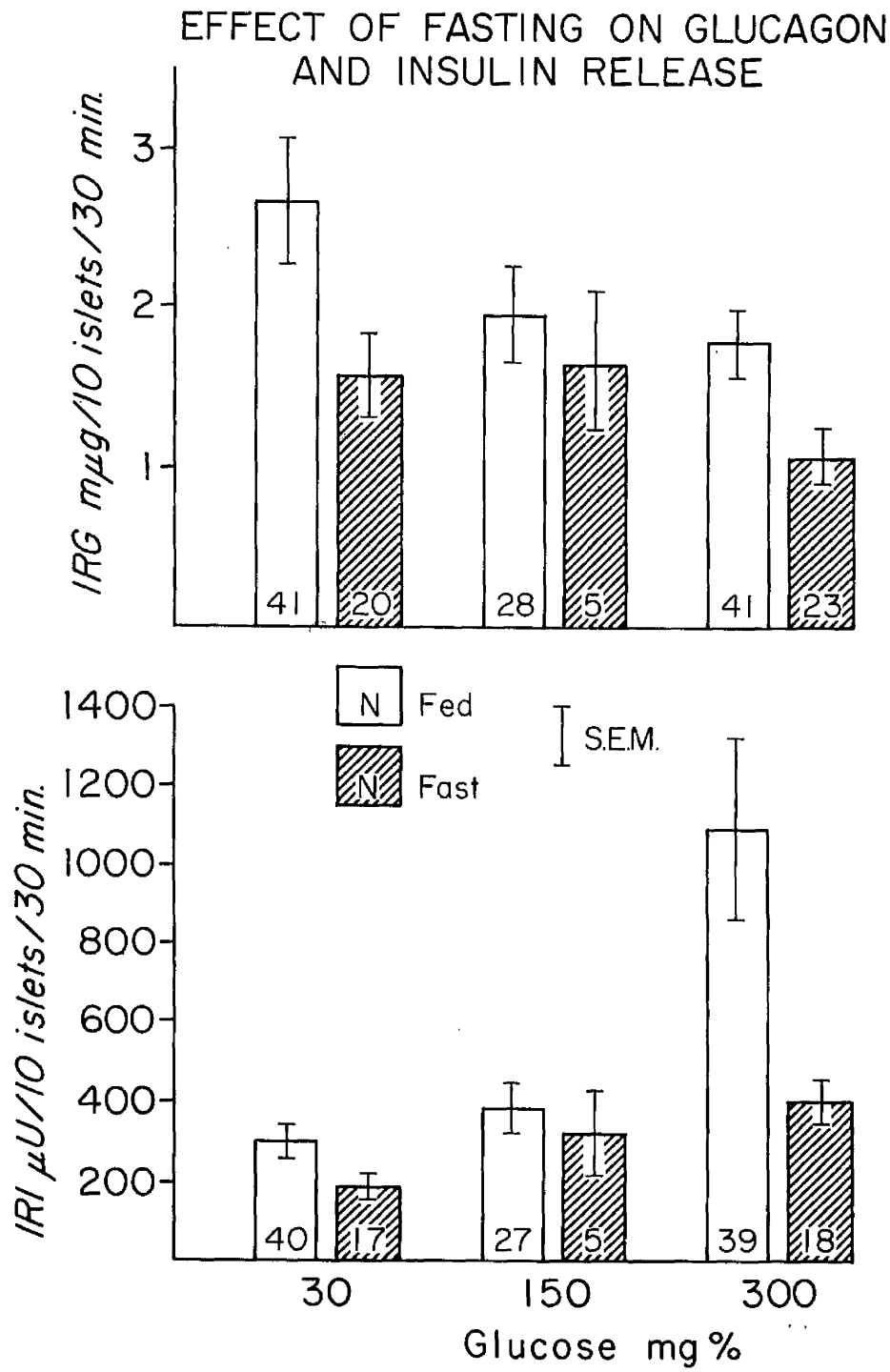
Isolated islet and rat studies

The release of IRI (μU/10 islets/30 min. mean ± SEM) from control rat islets was higher ($p < .001$) during incubation in 300 mg.% glucose (1096 ± 225) than in 30 mg.% (300 ± 42) as illustrated in Figure 15. Glucose concentrations of 30 and 150 mg.% were not significantly different in effect on insulin release ($p < .2$) from control rat islets. Starved rat islets responded qualitively the same as controls, but significantly less insulin was released during incubations with 30 mg.% (186 ± 33) ($p < .01$) and 300 mg.% (403 ± 56) ($p < .05$).

Figure 15 also shows that increasing the glucose concentration of the incubation buffer results in less release of IRG (mμg./10 islets/30 min.mean ± SEM) from control islets incubated with 300 mg.% (1.88 ± .32) than it does from islets incubated with 30 mg.% glucose (2.67 ± .40). This difference, however, did not achieve statistical significance ($p < .2$). Glucagon release from starved rat islets was diminished, achieving a significance from controls with both 30 mg.% (1.58 ± ,27) ($p < .05$) and 300 mg.% glucose concentrations (1.07 ± .17) ($p < .05$).

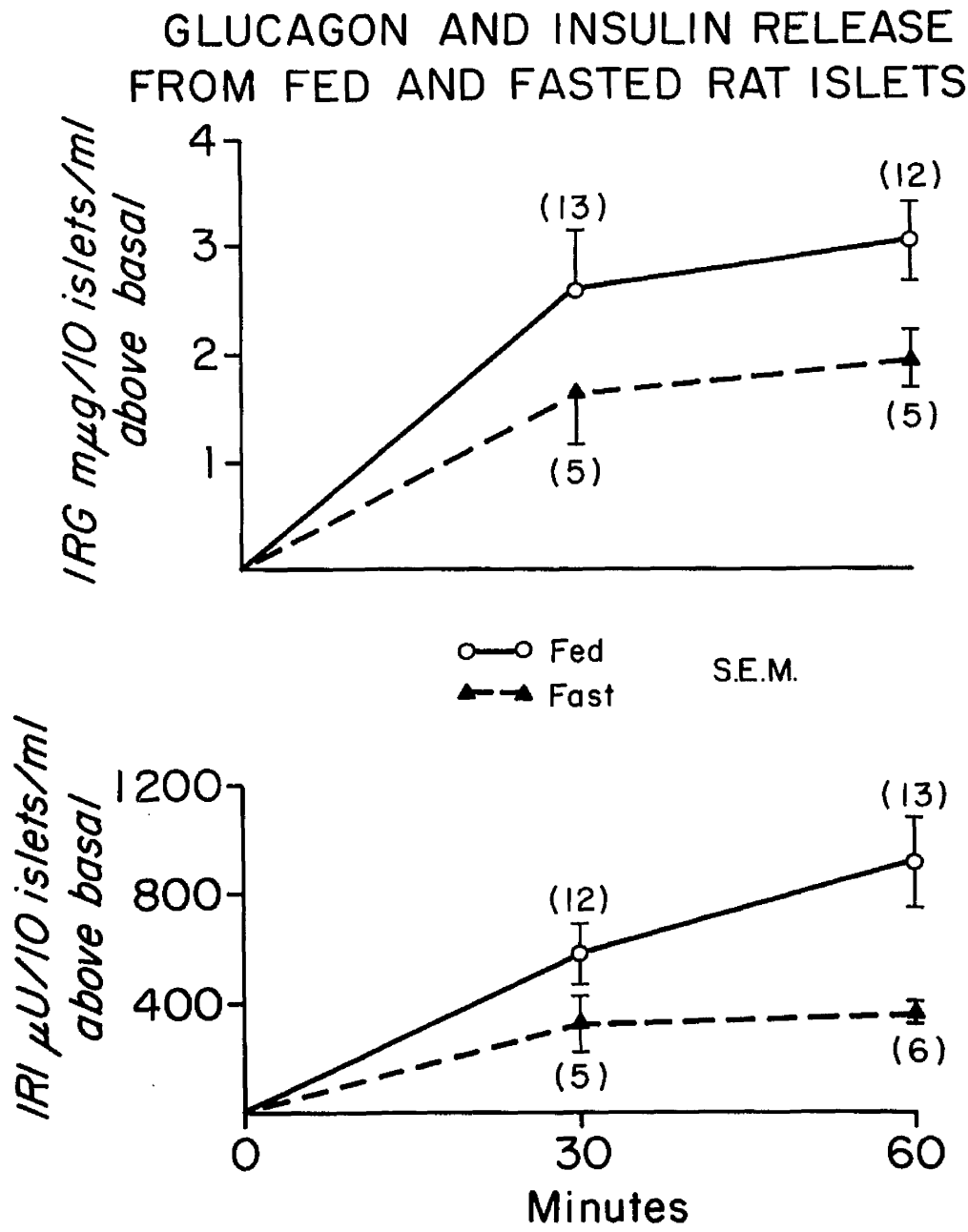
Extending the incubation period to one hour resulted in continued impaired IRI release from the islets of the fasted animals, with 150 mg.% glucose in the medium (Figure 16) IRG release from starved/

FIGURE 15



The effect of starvation on IRG and IRI release from isolated islets. The net hormone released during the 30 minute incubation period is expressed as the mean ± SEM. The number of incubation vessels for each bar is shown at the base of the bar.

FIGURE 16



The effect of the incubation time on the release of IRG and IRI. The points represent the net hormone above the basal level at the beginning of the incubation. The number of incubation vessels for each point is shown in parenthesis.

/starved rat islets was also less than controls, but the difference at 60 minutes was not statistically significant ($p < .1$).

The extractable IRI and IRG content of fed and starved rat pancreases is illustrated in Table 5. Starvation had no significant effect on the concentration of either hormone in the pancreas.

The serum IRI and plasma concentrations in the vena cava were significantly less in fasted rats than in controls, but serum IRG levels were the same (Table 6).

DISCUSSION

On first consideration it seems surprising that glucagon, which is a powerful gluconeogenic hormone, should fall or remain unchanged during starvation. It has been suggested that glucagon may augment substrate availability during starvation through glycogenolysis and gluconeogenesis[58]. The results of our studies indicate that glucagon may not contribute significantly to glucose mobilisation during fasting. Cahill and co-workers[100], however, presented evidence that starvation results in decreased glucose mobilisation, whereas lipid mobilisation and utilisation are increased. Glucagon is a prompt, potent, hepatic glycogenolytic agent[2,79], thus explaining its possible role in acute hypoglycaemic states. Since fasting rapidly depletes rat liver glycogen stores[102] and muscle glycogen serves as the main source of carbohydrate/

TABLE 5

EFFECT OF STARVATION ON EXTRACTABLE GLUCAGON AND INSULIN CONTENT OF RAT PANCREAS

	Insulin m units/gm. wet weight	Glucagon m μg/gm wet weight
Control	15.6 ± 3.7 (6)	27.5 ± 7.0 (6)
Starved 3 days	15.3 ± 2.1 (5)	20.5 + 4.5 (5)

Means ± S.E.M.

N in parenthesis

TABLE 6

SERUM IRG, IRI, AND PLASMA GLUCOSE

BEFORE AND AFTER 3-DAY STARVATION

	Control	3-day Starvation
Serum IRI μU/ml	27 ± 3.8 (13)	8 ± .8* (13)
Serum IRG mμg/ml	.801 ± .087 (20)	.876 ± .099 (22)
Plasma Glucose mg.%	153 ± 7.1 (8)	85 ± 4* (10)

All values represent mean ± S.E.M.

Numbers in parentheses refer to number of observations.

*$p < .001$ compared with control.

/carbohydrate (pyruvate) during starvation[103], then the demand for glucagon as a carbohydrate mobiliser in the starved animal is probably not as great as in the fed.

Although glucagon is lipolytic in pharmacological amounts[104], its ultimate physiological effect on lipolysis in the intact animal is debatable[99,105]. Other hormones with potent lipolytic properties, i.e. epinephrine and growth hormone, are secreted in greater quantities during starvation[106,107] and may stimulate the increased lipolysis which occurs during starvation. Elevation of free fatty acid levels has recently been shown to depress circulating glucagon levels[108]. Elevated free fatty acids probably contribute to the impairment of glucagon release during fasting.

The studies of Cahill and colleagues[100] implicated an insulin-glucose feedback mechanism in the regulation of substrate utilisation during fasting. It maybe that other hormones, such as glucagon, also help to regulate insulin secretion. Samols and associates[39] and Crockford and co-workers[40] have shown that glucagon is a powerful stimulator of insulin secretion "in vivo". Possibly glucagon plays a greater role in the regulation of insulin secretion than in the reutilisation of energy substrates during starvation. This would help to explain the observed parallel fall in blood glucagon and insulin levels during starvation.

Interpretation/

/Interpretation of changes in circulating peripheral or portal vein blood IRG levels must be made with caution because of a contribution to these levels from more than one source. Specifically, an intestinal mucosa extract has been found to contain biological[18,19] immunological properties[20,21,22] similar to glucagon. Circulating IRG levels do not fall to zero after pancreatectomy in dogs (see chapter 2, section 1) indicating another (presumably intestinal) source of circulating IRG. Also the studies of Ketterer and co- workers [41] suggest that changes in the secretion of glucagon could have biological effects, such as stimulation of insulin release, without causing a detectable change in immunoassayable peripheral IRG levels. In the absence of a method of differentiating the relative contributions from the pancreas and the intestine to circulating serum IRG levels, it must be concluded that circulating IRG is a measure of IRG from both sources. It is possible, therefore, that actual changes in pancreatic IRG secretion during starvation might differ from the changes that are reflected in the peripheral and portal veins of starved man and dog, respectively. However the finding that the release of glucagon was impaired by starvation, does suggest that some of the changes in circulating IRG in the human studies might be due to diminished pancreatic release of glucagon.

The diminished insulin release of starved rat islets in this study/

/study was most pronounced during incubation in a high glucose concentration. Since the pancreatic insulin content was not altered, starvation appears to have impaired the insulin secretory process more than the synthetic and storage mechanisms. The mechanism of the suboptimal insulin release from the fasted rat islets cannot be explained. Malaisse et al[109], who made similar observations with a pancreas slice technique, theorised that diminished activity of islet cell glycolytic enzymes maybe responsible for this blunted insulin release following fasting. This diminished insulin release "in vitro" is consistent with the fall in vena cava IRI levels.

The results of this study, showing a fall in circulating glucagon levels during starvation, maybe opposite those of Unger and associates[58] and of Lawrence[95] because of differences in technique. At the time of their study, Trasylol was not incorporated in the glucagon assay system and, therefore, degradation of labelled hormone in the assay and changing blood proteolytic activity during the starvation may have contributed to their results. Recently, however, Samols[110] has reported no change in circulating IRG levels in fasted human subjects.

This study has confirmed the previous reports of the glucose intolerance and insulin resistance with hypersecretion of insulin that are observed after oral glucose administration to subjects starved/

/starved for short periods[101,111]. Species variability may explain the failure of the starved dog to develop hyperinsulinism after oral glucose, since the acetoacetate response of dogs to fasting is also known to be different from the human[112]. However, the 3 day starvation in the dog may have resulted in an absolute insulin deficiency, a phenomenon thought to occur in humans after longer periods of starvation[101].

The significance of the post starvation IRG rise after oral carbohydrate is not known. A similar response in non fasted humans[95,56] has been observed previously by others. An attractive hypothesis would be to consider that the IRG rise is from an intestinal origin[23], that it stimulates insulin release[23], and that it stimulates larger amounts of insulin during a meal following starvation, in order to overcome the insulin resistance and glucose intolerance of starvation.

C H A P T E R 3

SECTION 3

THE EFFECT OF ENTERIC FACTORS ON GLUCAGON AND INSULIN RELEASE

SUMMARY

The effect of the enteric factors secretin, and pancreozymin (PPZ) were studied on the secretion of immunoreactive insulin (IRI) and immunoreactive glucagon (IRG), utilising anaethetised mongrel dogs with multiple venous catheters "in situ", and isolated pancreatic islets from the rat. Secretin by continuous infusion (0.25 U/min.) produced no change in the levels of IRI and IRG in the pancreatic venous blood of dogs despite causing a copious flow of pancreatic juice. PPZ, however, caused a dramatic increase in both hormone levels. Concomitant with these effects there was a rise in the plasma glucose and a fall in free fatty acids (FFA). Following PPZ, the concentration of IRG in a jejunal vein was much less than for the pancreatic vein, and in dogs after pancreatectomy, PPZ was without effect on IRG secretion, suggesting that the IRG in the gut was not influenced by PPZ. PPZ, secretin, and gastrin, failed to cause a release in IRI and IRG release from isolated pancreatic islets. It is suggested that enteric factors may mediate their effects on IRI and IRG release by indirect means, or that the isolated islets are insensitive to factors which rapidly release IRI and IRG.

For many years the enteric hormones have been recognised to be of considerable importance in the regulation of exocrine pancreatic secretions. The possibility that these enteric hormones may also play a role in controlling the endocrine pancreas was suggested by the demonstration that an oral glucose load is disposed of more rapidly than an intravenous load[115] and that the oral route of administration is associated with a greater rise in circulating insulin levels[116]. These observations prompted subsequent studies to define the effect of various gut factors on islet cell hormone release. Secretin[117,55,118] and pancreozymin[119] have each been reported to stimulate insulin secretion. Unger et al[55] have also shown that pancreozymin stimulates glucagon release. Gastrin has slight and transient effects on insulin secretion[55]. Another enteric factor has been identified which has biological properties and immunological properties similar to glucagon (Chapter 2, section 1). It has, however, recently been suggested that the immunoreactive material from the gut does not in fact have the biological properties of pancreatic glucagon and that the function of this "gut glucagon" maybe to stimulate insulin secretion after eating[23].

The interpretation of changes in peripheral IRG levels is difficult because of the contribution to these levels from at least two sources, the intestine and the pancreas. Therefore, it was the purpose of this study to examine the effect of pancreozymin/

/pancreozymin and secretin on immunoreactive insulin (IRI) and IRG secretion and to identify changes in circulating IRG levels with an origin in the pancreas, intestine, or both. In addition it was considered that the study of enteric factors on IRI and IRG release from isolated pancreatic islets (Chapter 1, section 2) may give some insight into the possible role of these factors in controlling the endocrine pancreas and their mechanism of action.

MATERIALS AND METHODS

Using mongrel dogs (20 - 30 kg.) under barbiturate anaesthesia, catheters were passed through an abdominal incision into the craniopancreatico-duodenal vein, a large jejunal vein, and into a peripheral vein; care was taken not to obstruct the pancreatic vein. For the pancreatic vein it was found desirable to insert a 21 guage paediatric thin-wall needle in the direction of blood flow. At the time of blood sampling, the pancreatic vein at its entry into the portal vein was occluded with a simple thread snare to prevent reflux from the portal vein. The main pancreatic duct was catheterised and the accessory duct ligated.

The islets were prepared from 200 - 300 gm. male albino Wistar rats (fed ad libitum) by the technique described in Chapter 1, section 2 of this thesis. Ten per cent pure cholecystokinin-pancreozymin (PPZ) (No.26741) and purified secretin (No.16741) were obtained through the kindness of Professor D.E.Jorpes, G.I.H. Research/

/Research Unit, Chemistry Department, Karolinska Institutet, Stockholm. Gastrin prepared by a modification of the method of Gregory and Tracy[120] was kindly supplied by Dr. T. Aoki of the Department of Surgery, University of Washington, Seattle. Two µg. of this gastrin preparation were necessary to produce maximum effects on biological assay. The amounts of IRI and IRG present in these substances are shown in Table 7.

RESULTS

Dog Experiments

The effects of PPZ and secretin on the levels of IRI and IRG in pancreatic blood, and of plasma glucose and free fatty acids (FFA) in peripheral blood are shown in Table 8 and graphically in Figure 17. After a 10 minute control period, secretin was constantly infused into a peripheral vein at a rate of 0.25 U/min. which was sufficient to produce a copious flow of pancreatic juice in all dogs. Following 30 minutes of secretin infusion, 75-100 Ivy units of PPZ were rapidly infused into a peripheral vein. During secretin infusion alone there were no significant changes in the levels of IRG, glucose, IRI and FFA. Following PPZ, the IRI rose from a pre-injection level of 1089 $\pm$ 777 µU/ml. (mean $\pm$ S.D.) to a level at 2 minutes of 3390 $\pm$ 1819 µU/ml. ($p < .02$) and these values were maintained for 15 minutes. IRG levels also rose from 1.04 $\pm$ .49 mµg./ml. to a peak/

TABLE 7

	IRI (μ units)	IRG (m μg.)
Pancreozymin (per Ivy unit)	4	.24
Secretin (per unit)	0	0
Gastrin (per μg.)	0	0

The amounts of immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) in the test substances.

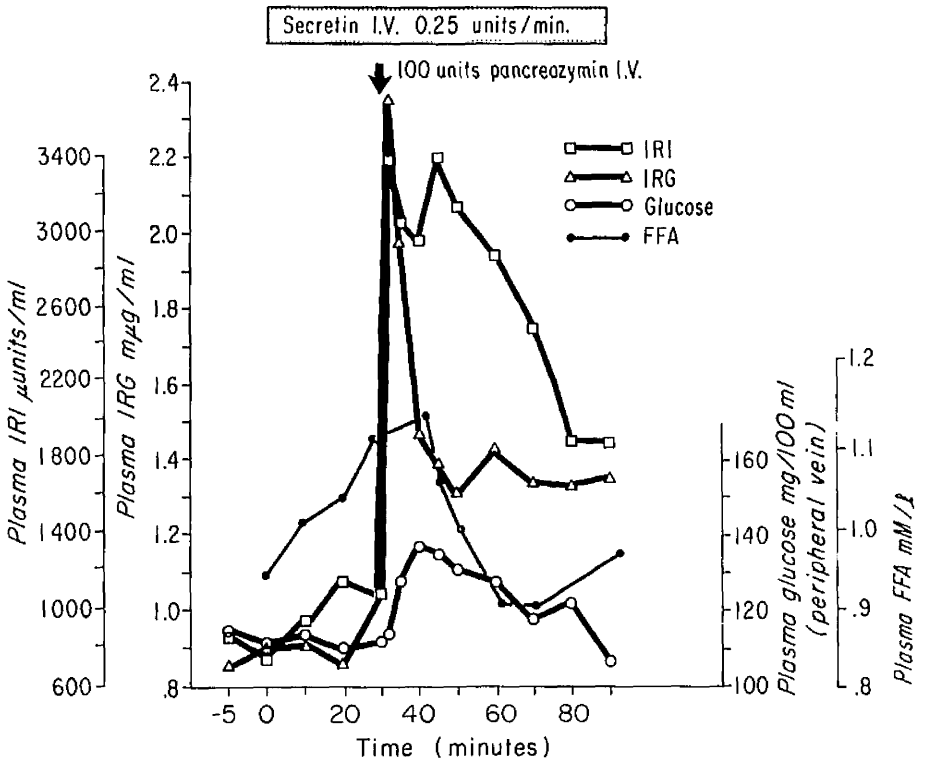
TABLE 8

THE EFFECT OF PANCREOZYMIN (PPZ) AND SECRETIN ON INSULIN AND GLUCAGON SECRETION

	Minutes													
	-5	0	10	20	30	32	35	40	45	50	60	70	80	90
Pancreatic Vein IRG mμg/ml.	.85±.17	.90±.17	.91±.14	.86±.21	1.04±.12	2.35±.38	1.98±.49	1.47±.33	1.39±.30	1.31±.42	1.43±.48	1.34±.34	1.33±.50	1.35±1.0
Pancreatic Vein IRI μU/ml.	869±300	754±226	927±225	1158±446	1089±311	3390±727	3038±640	2974±1071	3404±1220	3124±1182	2889±1130	2505±1060	1900±725	1898±926
Plasma Glucose mg/100 ml. Peripheral Vein	114±5.8	111±3.8	114±5.2	110±4.0	112±6.4	114±6.7	128±8.0	137±7.2	135±6.6	131±5.1	128±9.3	118±6.9	122±7.8	107±10.
FFA mEq./l Peripheral Vein	-	.94±.09	1.05±.07	1.08±.06	1.18±.16	-	-	1.19±.10	1.07±.06	.96±.06	.89±.06	.89±.12	-	.99±.16

Plasma glucose, FFA, IRG and IRI levels in 6 dogs. The time scale shows minutes after the start of a continuous infusion of secretin 0.25 U/ml minute into a peripheral vein. 100 Ivy units of PPZ were given rapidly into a peripheral vein at 30 minutes. All values show mean ± S.E.M.

FIGURE 17

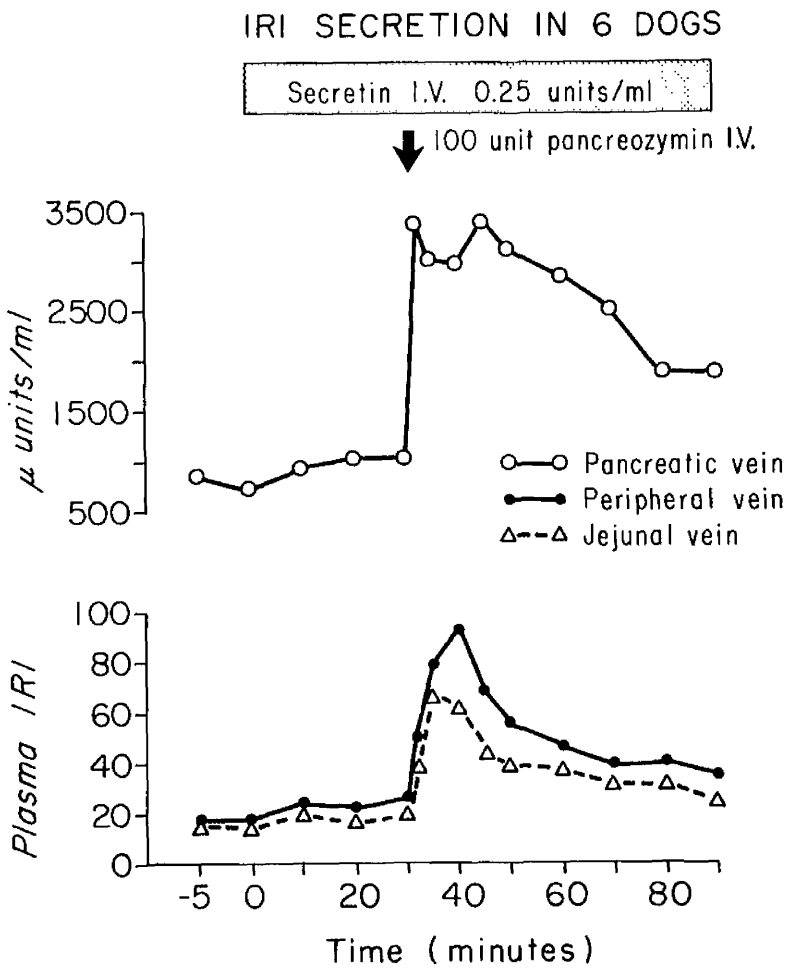


The effect of secretin and PPZ on IRI and IRG levels in the pancreatic venous effluent and on plasma glucose and FFA in the peripheral vein. The experiments were performed on 6 dogs under anaesthesia at laparotomy. There was no change following secretin infusion alone, but following PPZ there were dramatic increases in IRI ($p < .02$), IRG ($p < .02$) and plasma glucose ($p < .01$). FFA levels fell but this fall did not achieve significance.

/peak at 2 minutes of 2.35 ± .94 mμg./ml. ($p < .02$). Plasma glucose rose at 10 minutes (from 112 to 137 mg./100 ml.) ($p < .01$) suggesting a glycogenolytic effect of the endogenously released glucagon, but FFA levels fell (1.18 ± .39 mEq./L to .89 ± .14 at 30 minutes), although this did not achieve statistical significance.

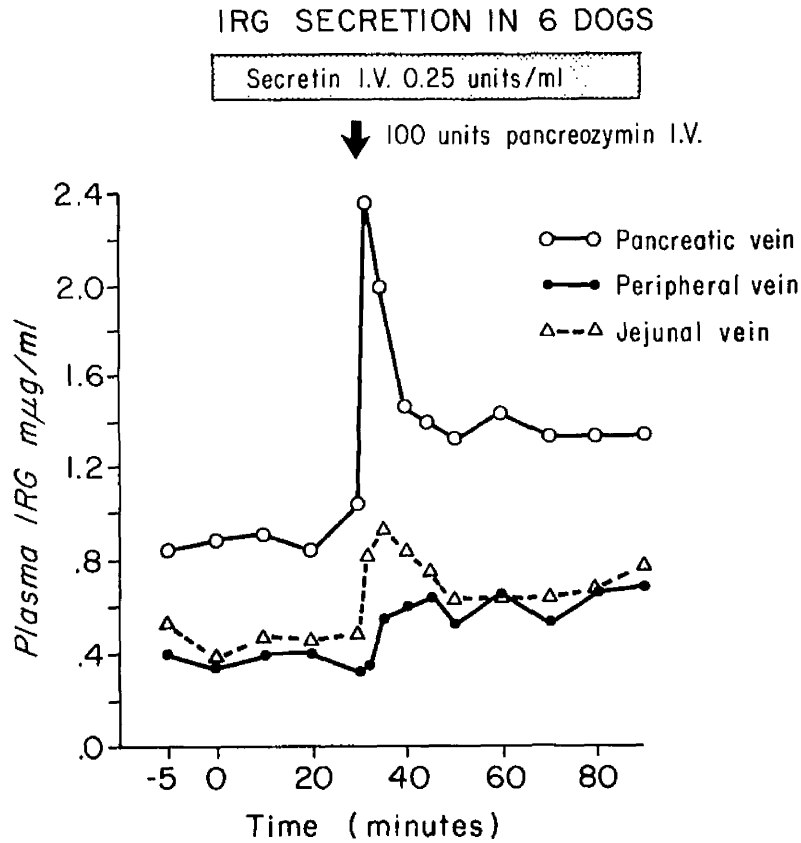
The relative values for IRI and IRG in the pancreatic, jejunal, and peripheral veins are shown in Figures 18 and 19 respectively. The changes in pancreatic vein insulin were similarly reflected in both the peripheral and jejunal veins, although peak values in the peripheral and jejunal veins occurred in the 5 or 10 minute sample rather than the 2 minute. Values for the pancreatic vein blood were 30 - 70 times greater than those for peripheral blood. (Canine insulin standard was used in these studies which resulted in a correct dilutional effect). IRG changes in the peripheral and jejunal veins were of small degree (Figure 19). The peak value in the jejunal vein occurred at 10 minutes, but only very small increments in the peripheral vein values were found from 5 to 60 minutes. Jejunal vein levels were slightly greater than peripheral vein levels: jejunal vein: peripheral vein ratios at 2, 5, 10, 15 and 20 minutes after PPZ were found to be: 2.2, 1.6, 1.4, 1.2 and 1.2 respectively. However, there were much higher ratios of pancreatic vein: peripheral vein levels at 2, 5, 10, 15 and 20 minutes after PPZ; these were 6.3, 3.6, 2.4, 2.2 and/

FIGURE 18



Comparative values for IRI in the pancreatic, jejunal and peripheral veins in 6 dogs during secretin-PPZ infusion.

FIGURE 19



Comparative values for IRG in the pancreatic, jejunal and peripheral veins in 6 dogs during secretin-PPZ infusion.

/and 2.4 respectively. These results indicate that PPZ had a greater effect on pancreatic IRG secretion than on the release of IRG-like material from other sources, such as the intestine.

To find whether PPZ had any effect on gut IRG secretion, the pancreas was totally excised in 5 dogs, and the secretin and PPZ stimuli repeated. When IRG levels reached a steady state, usually 60 minutes but not greater than 90 minutes after pancreatectomy, secretin and PPZ were once again infused. This time there was no change in IRI, IRG or plasma glucose levels (Figure 20) indicating that PPZ probably influences the release of pancreatic IRG only.

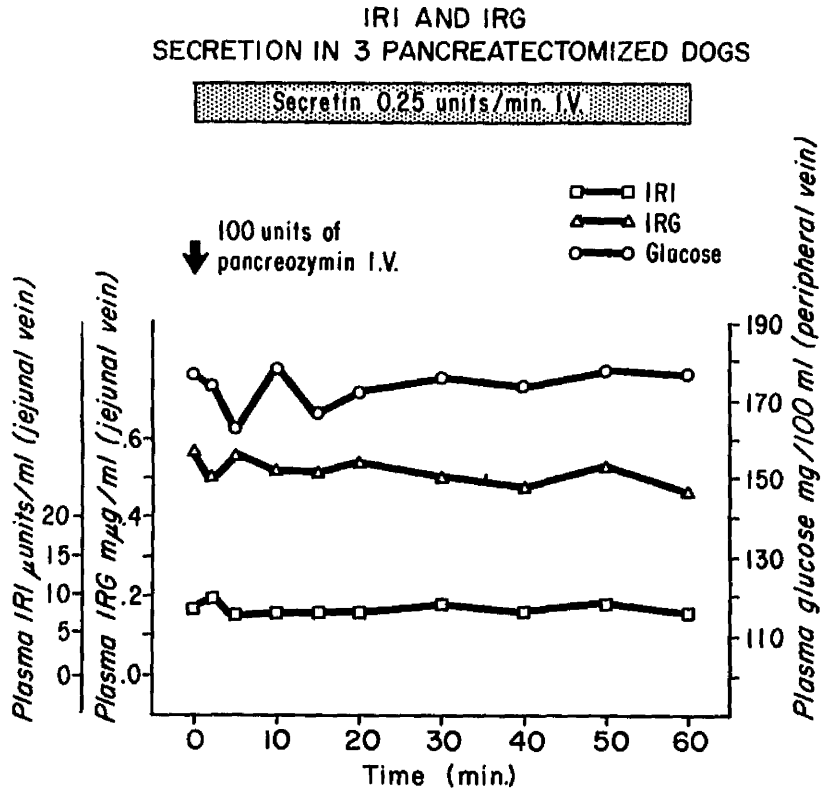
Islet Studies

PPZ (Figure 21), gastrin (Figure 22), and secretin (Figure 23) were all ineffective in promoting either IRI or IRG release at varying concentrations of stimuli and glucose (not all of the concentrations of test substances used are shown in the figures). PPZ at concentrations greater than 5 units/ml. recorded significant amounts of IRG in the t_0 vessels, so as to make interpretation of the results difficult.

DISCUSSION

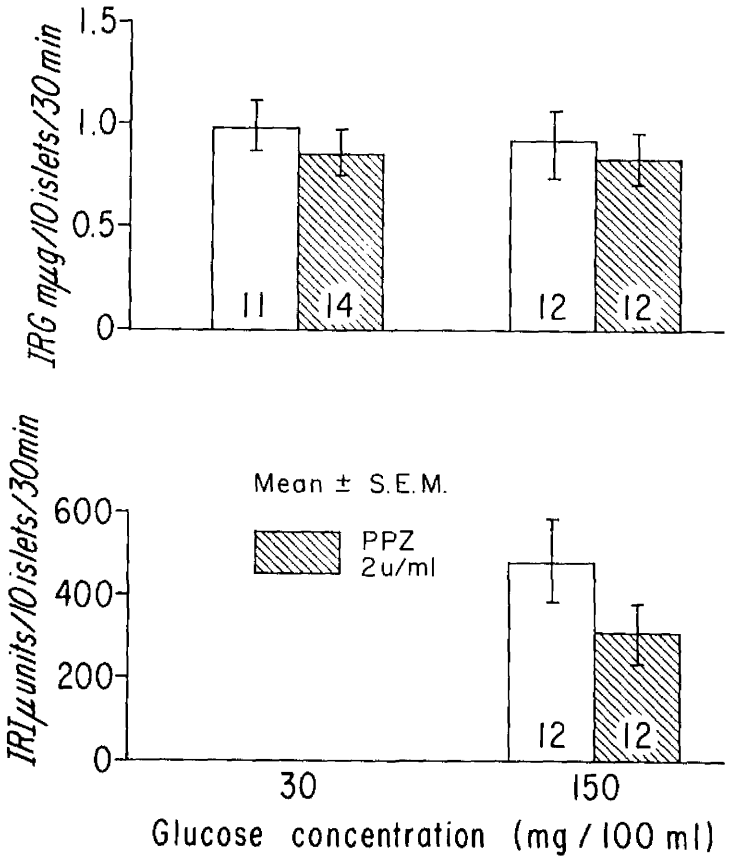
This study has confirmed previous reports of the effect of PPZ on IRI secretion in the dog[55,119] but the results conflict with unsuccessful attempts to promote IRI release with PPZ in man[121,122]. The/

FIGURE 20



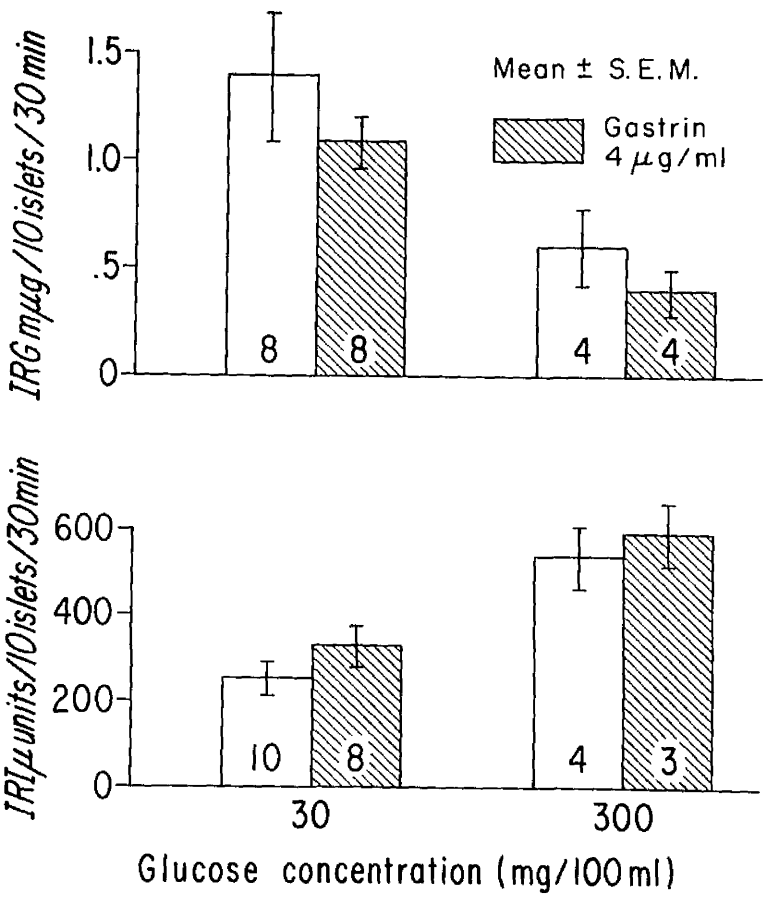
In 5 dogs following pancreatectomy, secretin-PPZ infusion had no effect on peripheral vein glucose and IRI values, and jejunal and peripheral vein IRG values.

FIGURE 21



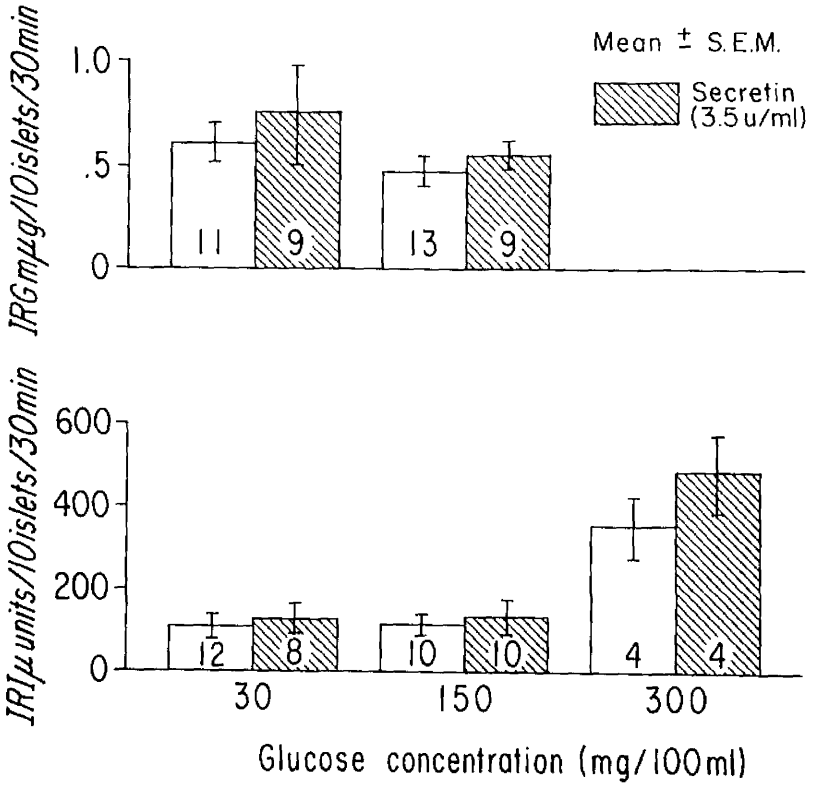
PPZ had no effect on IRI and IRG release from isolated pancreatic islets. Numbers of groups of 10 islets are shown at foot of column. The empty columns are control islets.

FIGURE 22



Gastrin had no effect on IRI and IRG release from isolated pancreatic islets. Numbers of groups of 10 islets are shown at the foot of the column. The empty columns are control islets.

FIGURE 23



Secretin had no effect on IRI and IRG release. Numbers of groups of 10 islets are shown at the foot of the columns. The empty columns are control islets.

/The absence of an effect of PPZ on IRI secretion "in vitro" is in agreement with the results of Turner and McIntyre[123] and Turner[124] who observed no increase in IRI release from rabbit pancreas preparations using Jorpes' PPZ. The conflict may be explained by species variation - that the effect of PPZ may only be seen in the dog, but could also be due to the different preparations of PPZ employed. The workers reporting that PPZ has active insulin stimulating properties have all used purified preparations obtained from Professor D. E. Jorpes[119,55]. Recently, however, Dupré et al[125] have found that PPZ can enhance, in man, the rise in blood IRI concentration that occurs after intravenous infusion of glucose and arginine.

Our inability to stimulate IRI release with a secretin infusion is in contrast to the numerous reports of the promotion of IRI secretion by this hormone[117,55,118]. The small dose and mode of infusion which were employed, although insufficient to promote IRI secretion, were large enough to maintain a flow of pancreatic juice. Turner and McIntyre[123] and Turner[124] also could observe no IRI release from rabbit pancreas "in vitro" incubated with Jorpes' secretin, although Boot's pure secretin had insulin stimulating properties. These results then are in agreement with the negative results of secretin on IRI release from isolated islets, produced in the present study. Pfeiffer et al[126] demonstrated/

/demonstrated stimulation of insulin secretion from pancreas slices using Vitrum secretin. When crude preparations of a stimulus are employed, the possibility exists that impurities may have produced the observed effects. It has been reported that optimal effects of secretin on IRI secretion may only be seen in the presence of hyperglycaemia[127].

It has also not been possible to demonstrate any effect of gastrin "in vitro", on either IRI or IRG release. The effects of IRI release by gastrin reported by Unger et al[55] were only small and transient and may not be specific.

The study has demonstrated that PPZ injection causes a prompt release of IRG, which produced higher levels of IRG in the pancreatic vein than in a gut vein, confirming the observations of Unger et al[55]. It was further established that PPZ does not stimulate the release of IRG in pancreatectomised dogs. This might lead to the conclusion that IRG from pancreas and gut are different molecules as Unger et al[23] suggest. This is not necessarily so, however, as the different responses to stimuli maybe explained by different receptor sites in the intestine and the pancreas. The rise of blood glucose after PPZ is consistent with a glycogenolytic dose-related action of glucagon, and was also seen in the experiments of Unger et al[55]. Meade et al[119] found no change in the blood glucose concentration after PPZ, but they used smaller/

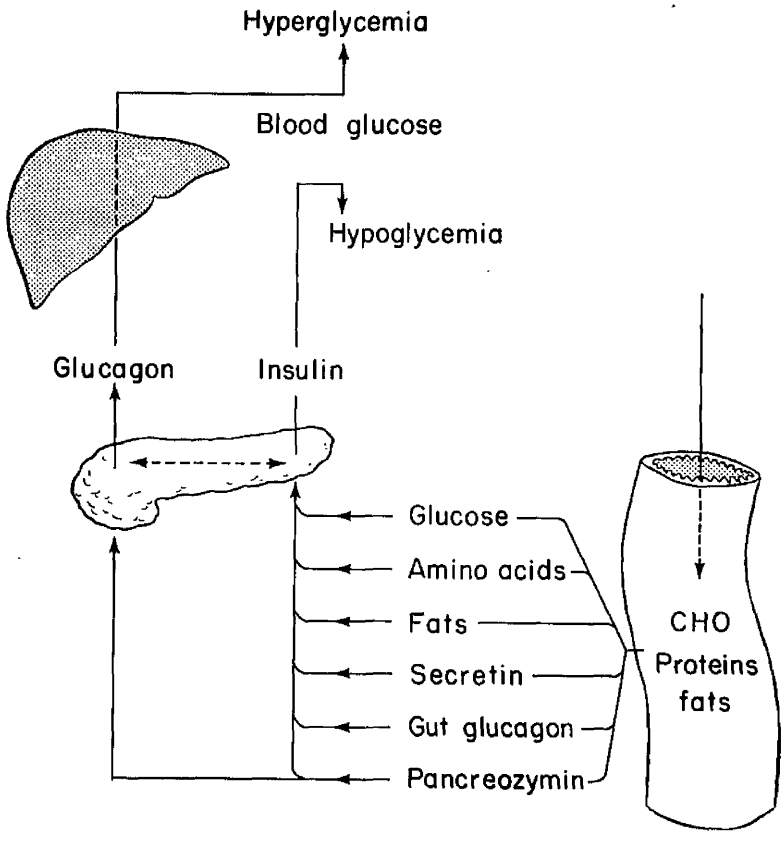
/smaller doses than Unger[55]. The rise in IRG was not associated with a rise in FFA as one might expect from Lefebre's data[99] which showed that small doses of intraportal glucagon were associated with a rather delayed rise in FFA levels. The failure of FFA to rise in our experiments might be explained by the antilipolytic effects of the IRI secreted simultaneously, or the minute amounts of IRG which appeared in the peripheral circulation might have been insufficient to cause lipolysis.

It might be argued that the effect of PPZ was due to the presence of contaminating IRI and IRG in the preparation. The amounts of contaminating IRI and IRG were small, being approximately 24 mμg. IRG and 400 μU. IRI per 100 Ivy units PPZ infusion. An effect of the contaminating IRI can be discounted, as a rise in peripheral circulating IRI levels following PPZ infusion was not seen in the dogs whose pancreas had been removed. (Figure 20). Similarly the PPZ infusion did not cause a rise in circulating IRG levels in dogs following pancreatectomy (Figure 20) so that increased IRG levels in the intact dogs following PPZ must have been secondary to a direct effect on the A cells. It is possible, however, that the contaminating 24 mμg. IRG may have caused the IRI secretion in the intact dogs, as Ketterer et al[41] have reported IRI secretion following the infusion of 100 mμg.IRG into a portal vein. However, any secretion they recorded was slight, transient and/

/and variable, and it seems unlikely, therefore, that 24 mμg. IRG would have caused the large, consistent and rather prolonged effect on IRI secretion observed in the present studies. The contrast between the "in vivo" findings of PPZ being a potent stimulator of IRI and IRG release and the negative effects "in vitro", may suggest that PPZ influences the endocrine pancreas by indirect means, perhaps by changing blood flow to the pancreas[128]. The isolated islets may respond poorly to factors which rapidly release IRI and IRG. The isolated islets have been found to respond reproducably and predictably to changing glucose concentrations in both IRI and IRG secretion (Chapter 3, section 1). A stimulus such as starvation (Chapter 3, section 2) has also produced marked effects on hormone secretion from the isolated islet. It is concluded that the isolated islet may be more responsive to chronically administered stimuli than to substances which produce their effects rapidly.

Apart from its well known function on the exocrine pancreas, it is interesting to speculate what part pancreozymin may play in the control of the endocrine pancreas after ingestion of food. Many factors are now known to cause IRI release after eating. Figure 24 summarises the events which may occur. Amino acids cause IRI release, yet without marked hypoglycaemia[129]. Fajans et al[130] argue that this may be the result of rapid gluconeogenesis from/

FIGURE 24



A schematic representation of metabolic events which might occur after eating. Numerous factors stimulate insulin release. Pancreozymin stimulates glucagon release perhaps to prevent hypoglycaemia after eating protein.

/from the protein or that proteins cause the release of both IRI and IRG. These authors refer to one experiment in which the oral administration of arginine stimulated a rise in circulating IRG levels[130]. In addition IRG stimulation may be mediated via pancreozymin as the ingestion of protein is probably the greatest stimulus for pancreozymin secretion[131]. Thus pancreozymin might be secreted after a large protein meal, not only to stimulate the exocrine pancreas, but also to cause the secretion of IRI and IRG, the IRG acting to prevent hypoglycaemia.

Several factors of intestinal origin, not only secretin and pancreozymin but also gastrin[55], glucagon[132], and other unknown substances[124] have been implicated as mediators in the enhanced insulin response that is seen after oral glucose as compared to intravenous glucose. It is as yet premature to assign any physiological function to these intestinal factors on the endocrine pancreas until more accurate methods for their assay become available. Attempts to produce physiological secretion of secretin and pancreozymin[127] by intestinal acidification have failed to stimulate insulin release.

C H A P T E R 3

SECTION 4

THE EFFECT OF GLUCAGON ON INSULIN RELEASE

SUMMARY

The effect of glucagon on immunoreactive insulin (IRI) release was studied in intact dogs, and isolated pancreatic islets from rats. In the islet studies glucagon was confirmed to be effective in stimulating IRI release but only at the high concentration of 10 µg./ml. and only at a concentration of glucose of 150 mg. per cent, but not at 30 or 300 mg. per cent. However crude acid alcohol extracts of gut containing immunoreactive glucagon were ineffective in promoting IRI release. In the dogs very small doses of glucagon administered intraportally during hyperglycaemia were effective in promoting IRI secretion.

Perhaps the most exciting and even surprising new property for glucagon has been that of its ability to stimulate insulin secretion, independent of any hyperglycaemia produced[39,40]. Pharmacological doses of glucagon have been used in these experiments, but recently Ketterer et al[41] have shown that physiological amounts of glucagon, infused into the portal vein of dogs will cause an insulin rise. Indeed the reports of the insulin stimulating properties of glucagon have tended to conflict with the classical role of glucagon in providing glucose.

The finding of an immunological substance in the gut which resembles glucagon (Chapter 2, section 2) has raised speculation as to its role. Unger et al[23] suggest that it may stimulate insulin secretion and this would be consistent with the finding of a rise in gut glucagon following oral glucose (Chapter 3, section 1).

Consequently the effect of both pancreatic and gut IRG on insulin secretion has been assessed both "in vivo", and also on the isolated pancreatic islet model (Chapter 1, section 2).

METHODS AND MATERIALS

Crude gut immunoreactive glucagon (IRG) was prepared from acid-alcohol extracts of rat gut (jejunum, ileum and colon. (Chapter 2, section 1). There were 8 mμg. of IRG equivalents and 20 μ units of immunoreactive insulin (IRI) per mg. of lypholised/

/lypholised weight of the extract.

Isolated pancreatic islets from male Wistar rats (200 - 300 gm.) were prepared as described elsewhere in this thesis (Chapter 1, section 2).

Mongrel dogs at laparotomy were prepared with multiple catheters "in situ" as described in Chapter 3, section 1.

Crystalline beef-pork glucagon (Eli-Lilly) was used as the pancreatic glucagon preparation throughout the studies. This glucagon has 2 μ units of IRI per μg.

RESULTS

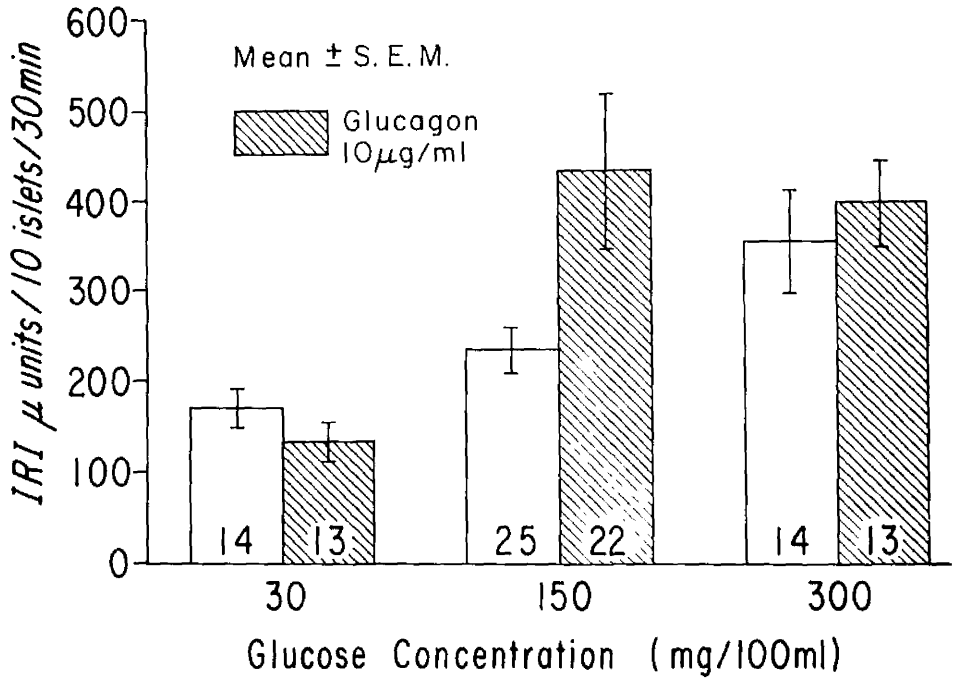
Islet Studies

Crystalline beef-pork glucagon at a concentration of 10 μg./ml. increased IRI release at 150 mg.% glucose ($p < .05$) but not at 30 or 300 mg.% glucose (Figure 25). Lesser concentrations of glucagon, and increasing the concentration to 100 μg./ml., were ineffective in promoting IRI release. At a concentration of 100 μg./ml. of glucagon, there was so much contaminating IRI as to render interpretation of the results difficult. In these experiments in the control vessels without glucagon, a glucose effect on IRI release was noted, there being a greater IRI release at 300 mg.% glucose than 30 mg.% ($p < .01$) (Figure 25).

Crude gut IRG (Figure 26) was however ineffective in promoting IRI release at varying concentrations of glucose and IRG.

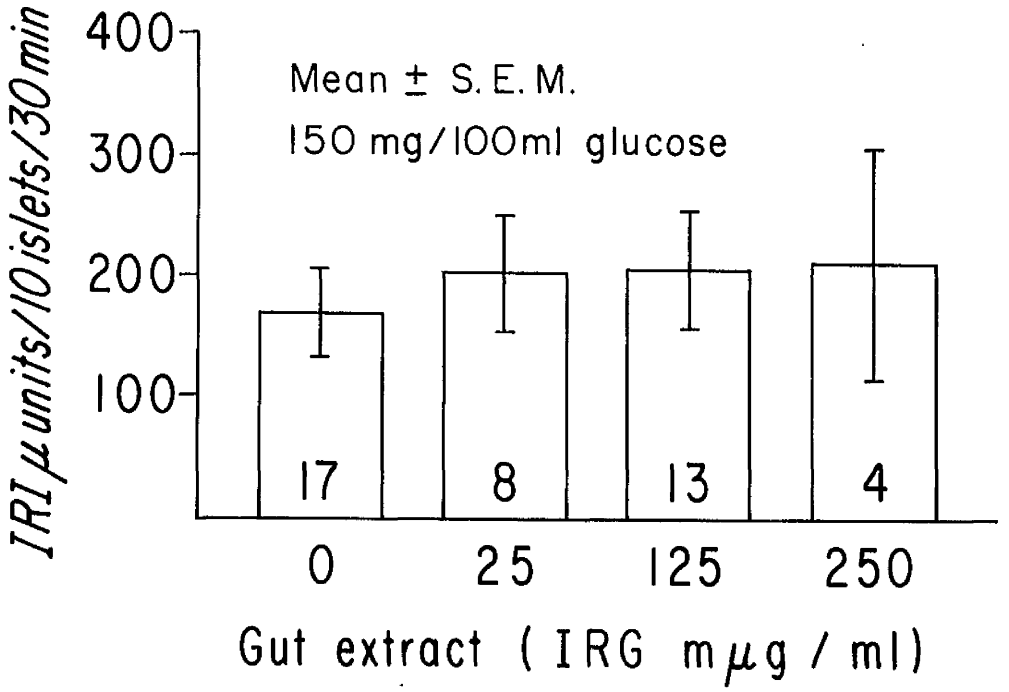
At/

FIGURE 25



The effect of crystalline beef-pork glucagon (10 μg./ml.) on IRI release. Glucagon produced a significant effect ($p < .05$) at 150 mg. per cent. glucose only. The difference between the IRI release in the control 30 mg. per cent. glucose flasks and the 300 mg. per cent. glucose flasks is significant at the $p < .01$ level. Number of groups of 10 islet incubations is shown at foot of columns.

FIGURE 26



An acid alcohol extract of rat gut containing IRG was ineffective in promoting IRI release. Numbers of groups of 10 islets are shown at foot of the columns.

/At the high concentrations of the crude gut preparation there was much contaminating IRI material as to possibly influence the results.

Dog Studies

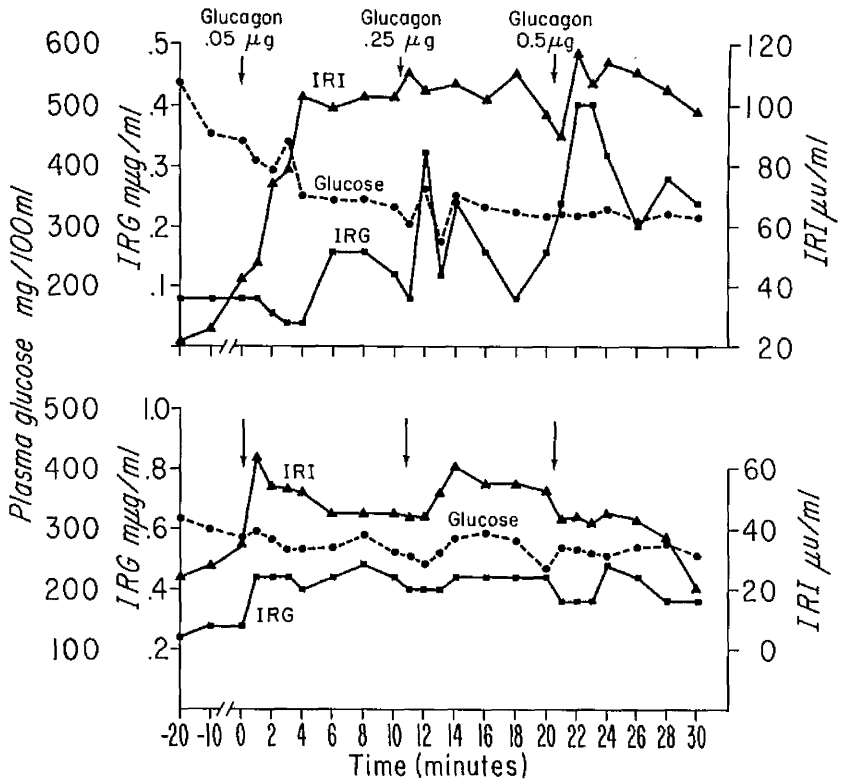
Two dogs in which tolbutamide had been infused followed by glucose (Chapter 3, section 1), were given glucagon intraportally 60 minutes after the glucose when hyperglycaemia was still present. (Figure 27). Glucagon in a dose of 0.05 µg. was sufficient to produce rises in circulating IRI levels in both dogs, although further and larger glucagon infusions (0.25 - 0.5µg.), failed to produce further IRI secretion. Neither dog showed distinct rises in peripheral circulating IRG levels after 0.05 µg. glucagon intraportally, although 1 dog showed clear changes after 0.25 and .5 µg.

It is concluded that the dogs under the conditions of the above experiments can respond by rises of circulating IRI and glucose to intraportal infusions of glucagon in amounts which cannot be detected in the peripheral blood by immunoassay. The physiological doses of intraportal glucagon in a dog may then be less than 0.25 µg., and possibly in the range 0.05 - 0.1 µg. or even less.

DISCUSSION

The "in vivo" experiments have confirmed the insulin stimulating/

FIGURE 27



In 2 intact dogs, 60 minutes after the infusion of 25 gm. glucose intravenously, the effect of small, rapid, intraportal infusions of crystalline glucagon on peripheral vein IRI and IRG levels is shown.

/stimulating properties of glucagon[39,40], and the data agrees with that of Ketterer et al[41] who showed that intraportal doses of glucagon which could barely be detected in the peripheral blood by immunoassay were never the less associated with insulin secretion. The insulin secretion promoted by glucagon in the present experiments was produced at a high glucose concentration and several workers[77,132,133] have reported enhancement of glucagon-stimulated insulin secretion when there is hyperglycaemia.

The "in vitro" experiments show less convincing evidence of an effect of glucagon on insulin release. At a concentration of 10,000 times physiological, an effect of slight significance was produced. Coore and Randle[74] were unable to produce an effect "in vitro", but since then all reports[133,134,135] consistently show an effect, although in the "in vitro" work there is less agreement as to the influence of glucose concentration on glucagon-stimulated insulin release. Malaisse et al[132] were unable to promote insulin secretion with glucagon "in vitro" until the glucose concentration was raised to 150 mg.% and they noted further enhancement at 300 mg.%. Lambert et al[136] observed glucagon-induced insulin release from isolated foetal pancreatic tissue even in the absence of glucose, but the effect was enhanced by the addition of glucose to the media. Devrim and Recant[135], on/

/on the other hand, noted a greater release of insulin by glucagon from rat pancreas slices in the absence of glucose than in its presence.

If glucagon has physiological importance in the control of insulin release, then one might expect a positive correlation in the release of these hormones from the pancreatic islet. However, glucose concentrations appear to have an opposite effect on their release, hypoglycaemia stimulating glucagon release and hyperglycaemia stimulating insulin release. Adrenalectomy (Chapter 3, section 5) stimulated glucagon release from the islets but inhibited insulin release. Theophylline, and tolbutamide both stimulate insulin release from the islets, but have no effect on glucagon release, and diazoxide and epinephrine inhibit insulin release but have no effect on glucagon release (K. D. Buchanan and J. E. Vance,preliminary results). Starvation, however, (Chapter 3, section 2) does inhibit both insulin and glucagon release from the islet, and this is the only condition which the author has met where the 2 hormones have been secreted concomitantly. In addition most reports have indicated that glucagon-stimulated insulin release is enhanced by hyperglycaemia. Yet during hyperglycaemia pancreatic glucagon secretion is inhibited (Chapter 3, section 1). It seems unlikely, therefore, that glucagon/

/glucagon will promote insulin secretion when the blood glucose concentrations are high, although the effect maybe mediated at lower glucose concentrations.

Perhaps, as Unger et al[23] suggest, it is gut glucagon which acts to promote insulin secretion. In the author's study crude gut IRG did not promote insulin release but this lack of effect may simply be related to the small dose employed (0.25 µg. IRG equivalents).

An alternative explanation of this conflicting data which maybe offered, is that because of the proximity of the A and B cells within the islet, the A cells maybe able to affect the B cells without necessarily reflecting changes in the glucagon concentration outside of the islet.

C H A P T E R 3

SECTION 5

THE EFFECT OF PITUITARY - ADRENAL HORMONES

ON GLUCAGON RELEASE

SUMMARY

Adrenalectomy in rats increases immunoreactive glucagon (IRG) release from isolated islets but decreases IRI release. Cortisone therapy prevented these events occurring. The IRG content of the pancreas of the adrenalectomised animals was reduced suggesting that adrenalectomy affected the release of glucagon rather than its synthesis and storage. Rats treated with ACTH also showed increased IRG release from the islets. ACTH may therefore be the mediator of the increased IRG release from isolated islets of the adrenalectomised rat. It is suggested that these various hormones which have potent effects on carbohydrate metabolism may exert control mechanisms over one another.

The adrenal corticosteroids have potent effects on carbohydrate metabolism. They promote gluconeogenesis and hepatic glycogenesis[137,138]. In addition it has long been known that the administration of corticosteroid drugs may produce decreased glucose tolerance which is reversible on discontinuing therapy[139,140,141]. Recently it has been shown[142,143,144] that the insulin response to glucose is increased by pre-treatment with corticosteroids in normal, diabetic and pre-diabetic subjects. Several workers too have reported that adrenocorticotrophin (ACTH) stimulates insulin release in animals[145,146,147,148].

Glucagon also promotes gluconeogenesis[3] and also increases insulin secretion[39,40]. It was considered, therefore, of interest to study the effect of pituitary-adrenal hormones which have a known effect on carbohydrate metabolism on the release of glucagon from the pancreatic islets.

METHODS AND MATERIALS

Pancreatic islets were isolated as described elsewhere in this thesis (Chapter 1, section 2) from male wistar rats (200 - 300 gm.) The rats were adrenalectomised surgically under barbiturate anaesthesia and thereafter were maintained on normal saline drinking water and fed "ad libitum". Control rats were "sham operated", the rats being exposed to the whole surgical procedure, except that the adrenals were not excised. The mortality rate amongst/

/amongst the adrenalectomised animals was only slightly greater than the sham operated, but the adrenalectomised rats lost slightly more weight than the sham operated. Some of the adrenalectomised animals were maintained on intraperitoneal injections of cortisone acetate (1 mg. cortisone per 100 gm. animal).

Studies were performed 4 or 5 days post operatively.

RESULTS

The results are shown in Table 9 and Figure 28. Islets from adrenalectomised animals released less immunoreactive insulin (IRI), but increased immunoreactive glucagon (IRG) ($p < .01$ in both instances) when incubated in 150 mg. per cent glucose. When cortisone was administered to the adrenalectomised animals the increased release of IRG was almost abolished, and the decreased release of IRI was over corrected.

The pancreases from 7 sham operated and 7 adrenalectomised animals were extracted for IRI and IRG content (Figure 29)..The adrenalectomised animals showed a significant reduction ($p < .05$) in the IRG content of the pancreas, but did not differ from controls in IRI content.

DISCUSSION

The effect of adrenalectomy on IRI release from the islet confirms the report by Malaisse et al[149], who also showed increased IRI/

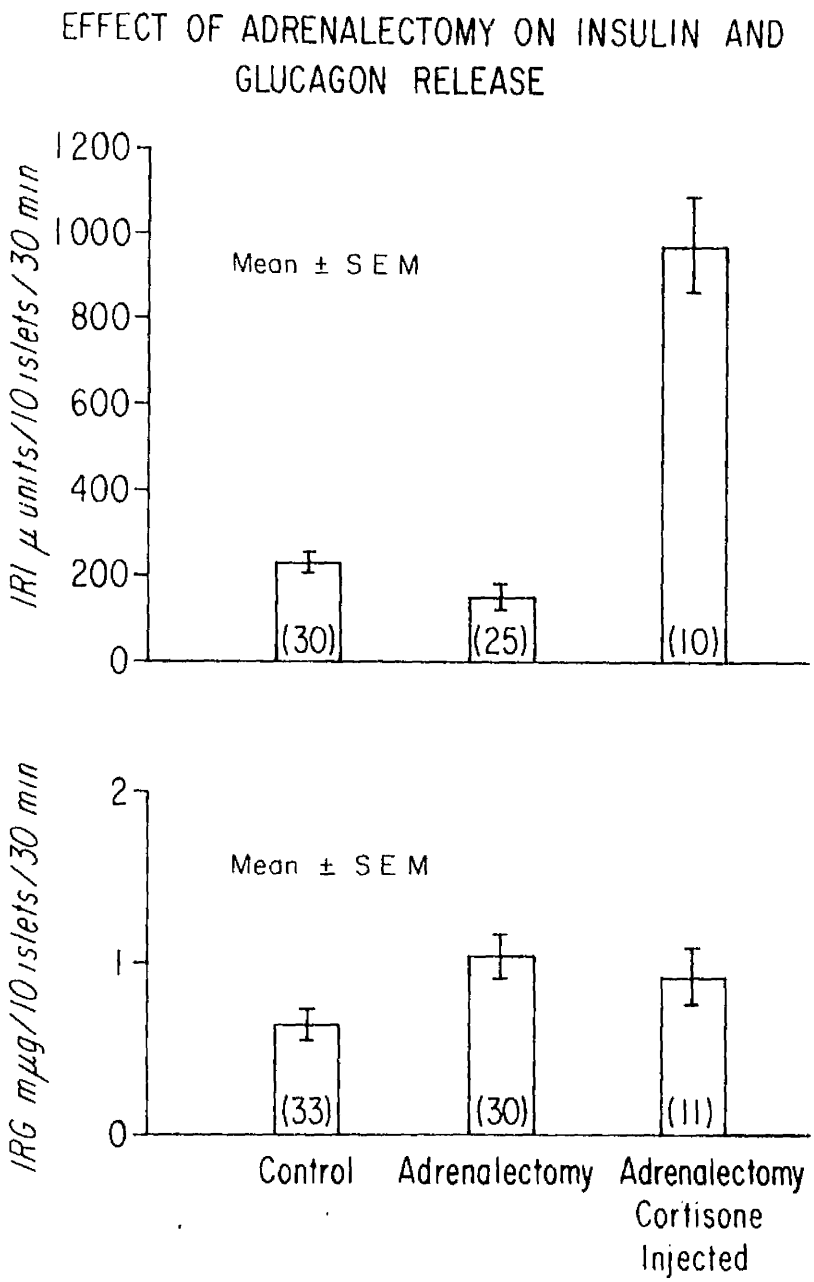
TABLE 9

	IRI (Mean ± SEM) μU/10 islets/ 30 min.	IRG (Mean ± SEM) μμg./10 islets/30 min.
Sham operated	245 ± 28	639 ± 106
Adrenalectomised	157 ± 27*	1238 ± 197*
Adrenalectomised + Cortisone	972 ± 116*	965 ± 272

(*Achieved significance ($p < .01$) in comparison with sham operated).

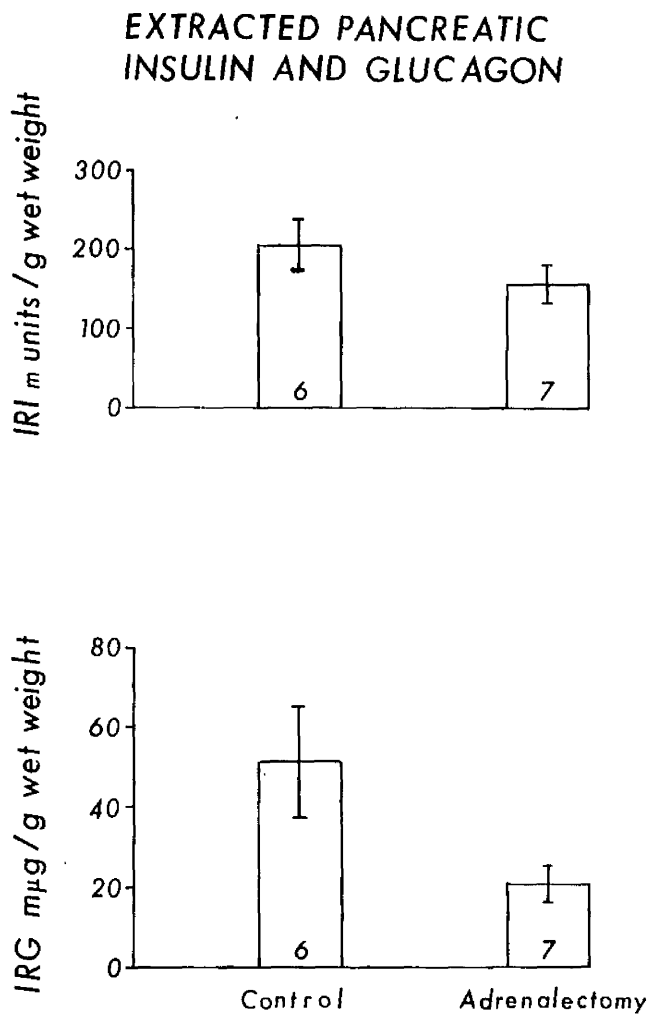
The release of immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) from isolated islets of sham operated and adrenalectomised rats.

FIGURE 28



Control rats were sham operated. Adrenalectomy significantly reduced IRI release ($p < .01$) but increased IRG release ($p < .01$). Cortisone injection reduced the IRG release and over corrected the IRI release. The numbers at the foot of the columns refer to the numbers of groups of 10 islets studied.

FIGURE 29



Adrenalectomised animals showed a reduction in the IRG content of the pancreas ($p < .05$) although IRI content was not affected. The numbers at the foot of the column refer to the numbers of pancreases extracted.

/IRI release from cortisol treated rats. The observation, however, that adrenalectomy augments IRG release from the islet is a novel one. The explanation for this phenomenon could be multiple. Cortisol therapy apparently abolished the increased IRG release but this does not necessarily mean that cortisol lack produced the IRG release. One possibility is that adrenalectomy stimulated ACTH production. ACTH stimulates insulin release and it is possible that itmay stimulate glucagon release. In some preliminary work, ACTH injected animals showed a 274% increase in IRG release, and when ACTH was added "in vitro" to the islets (1 U/ml.) IRG release was also increased by 180% although IRI release was unaffected. (K. D. Buchanan, J. E. Vance, A. E. Kitabchi. Preliminary observations). This might suggest, therefore, that it was ACTH, rather than corticosteroid lack, which stimulated IRG release.

Another explanation which remains is that it is the removal of the adrenal medulla rather than cortex which is the critical factor. The catecholamines exert important actions in carbohydrate metabolism, mainly on gluconeogenesis and glycogenolysis[150,2]. However when aminoglutethimide (Elipten), a drug which inhibits the synthesis of corticosteroids[151], was injected into rats IRG release was increased 101% although IRI release was unaffected. Epinephrine (2 µg./ml.) addition "in vitro" to the islets inhibited IRI release (55%) but had no effect on IRG release.

K. D. Buchanan/

/K. D. Buchanan, J. E. Vance, A. E. Kitabchi. Preliminary observations).

The reduction in the glucagon content of the pancreases from the adrenalectomised animals suggests that although IRG release was increased, synthesis probably was not. The results suggest that the mechanism of the increased IRG release following adrenalectomy may be caused by the resulting stimulation of ACTH, although the results are by no means conclusive in this respect. It does suggest, however, that these various hormones which have potent effects on carbohydrate metabolism may exert control mechanisms over one another.

CHAPTER 4

CLINICAL STUDIES OF A FAMILY SHOWING EXCESSIVE SECRETION OF ALL PANCREATIC ISLET CELL HORMONES

SUMMARY

Symptomatic and asymptomatic members of a family with multiple endocrine adenomatosis (pancreatic islet cell tumours, parathyroid hyperplasia, and hyperadrenocorticism) were studied in detail. The only living member of the first generation, and all five direct descendants studied, had excessive secretion of insulin. Three of these six showed increased levels of plasma glucagon. Of the five members of the second and third generations tested, four had increased fasting plasma gastrin levels. No definite clinical features could be related to the hyperglucagonaemia and the patients are compared with reports in the literature of patients with glucagonoma. A hypothesis is formulated suggesting that the primary defect in the multiple endocrine adenomatosis syndrome may lie in an abnormal proliferation of islet cells which is genetically determined, and the changes in other glands may be secondary to over production of islet cell hormones.

McGavran et al in 1966[47] described a patient considered to be suffering from an alpha-cell carcinoma of the pancreas. The patient had mild diabetes mellitus. Hepatomegaly was present and at laparotomy a tumour of the pancreas was found which on histology proved to be an alpha cell carcinoma. The neoplasm was shown to have large amounts of glucagon by immunoassay and hyperglucagonaemia was present in the patient. Yoshinaga et al (1966)[152] also described a patient with a pancreatic A-cell tumour. The subject had high levels of glucagon-like activity in the serum although immunoassay was not performed.

Both of the above patients presented with diabetes mellitus and both had clinically apparent abdominal tumours. To the author's knowledge these are the only two cases of glucagonoma reported, and possibly only the case of McGavran et al can be fully accepted because of the use in this case of the more specific immunoassay method.

The following is a report of a family with multiple endocrine adenomatosis (MEA) in which studies have revealed that all the hormones of the pancreas - glucagon, insulin and gastrin - have been affected by the process.

METHODS

All subjects consumed a diet providing 300 gm. of carbohydrate daily for 3 days prior to testing. The following tests were/

/were performed in random order on consecutive days in the resting state after an overnight fast: 100 gm. oral glucose tolerance test, 1 gm. intravenous tolbutamide test, and 1 mg. intravenous glucagon test.

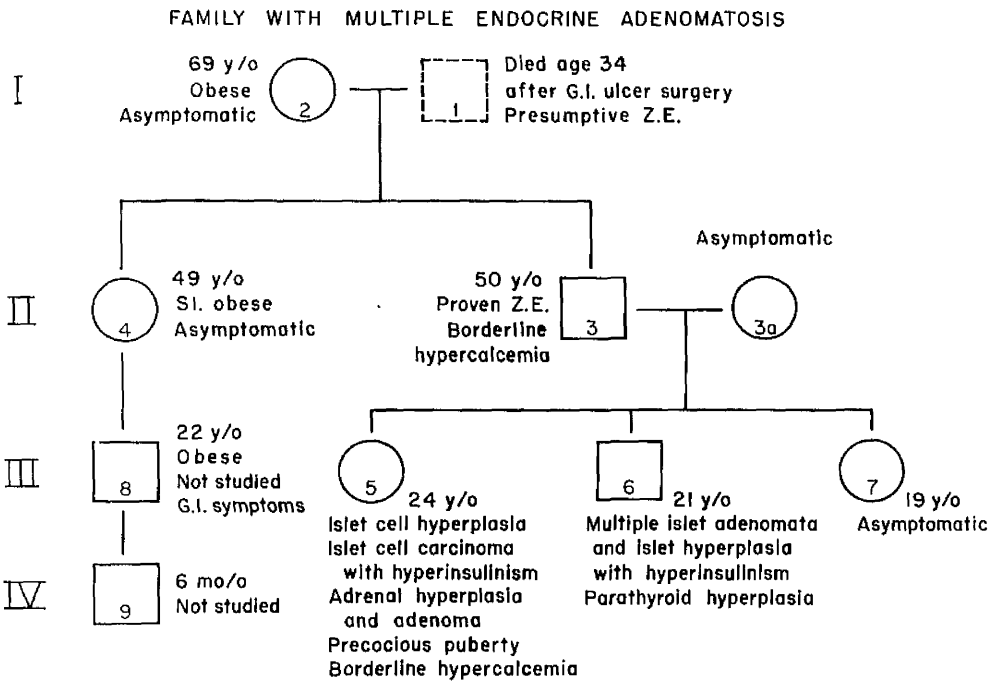
Subject 5 received 300 mg. of diazoxide every 6 hours during most of her tests but some tests were repeated while off the drug. Subject 3 was studied one year following total gastrectomy, and Subject 6 was studied following subtotal parathyroidectomy but prior to pancreatectomy.

Plasma immunoreactive gastrin (IRGa) levels were kindly measured by Dr. James McGuigan, Washington University School of Medicine, St. Louis, Missouri, using a double antibody technique developed in his laboratory[153,154].

CLINICAL SUMMARIES

Figure 1 outlines the pedigree of this family. The propositus (No. 5) was seen at age 6 because of hypoglycaemic seizures. As summarised in Table 1, she had 3 separate pancreatic operations for diffuse islet cell hyperplasia with two micro-adenomata (1950), diffuse islet cell hyperplasia with islet cell adenomata (1955), and metastatic islet cell carcinoma (1964). She also manifested precocious puberty, and underwent a unilateral adrenalectomy for diffuse hyperplasia and a subsequent subtotal removal/

FIGURE 1



Pedigree of a family with multiple endocrine adenomatosis.

TABLE 1

Age (year)	Symptoms, signs & clinical course	Hormone assays	Surgery	Surgical pathology	Postoperative course
6 (1950)	Hypoglycemic seizures, slight obesity, pubic hair	———	2/3 pancreatectomy	Diffuse islet cell hyperplasia & microadenomata	Hypoglycemia treated with ACTH & cortisone for 9 mos.
7 (1951)	Hypoglycemia resolved, steroid treatment stopped; Slight cliteromegally	———	———	———	———
9 (1953)	Further growth of pubic hair, onset of menses, breast development; 36 hour fast caused hypoglycemia	24 hour urine 17-KS, 17-OH g normal	———	———	———
11 (1955)	Hypoglycemic seizures	24 hour urine 17-KS 21.5 mg	Excision of 2 pancreatic adenomas & majority of head of panc. Right adrenalectomy	Diffuse islet cell hyperplasia. 2 islet adenomata; adrenal cortical hyperplasia	Hyperglycemia requiring insulin therapy for 3 months.
14 (1958)	Onset of hirsutism	———	———	———	———
18 (1962)	Heavy beard, shaved daily; cortisone and estrogen therapy started	24 hour urine 17-KS 23.4 mg, plasma cortisol 15 µg/100 ml	———	———	———
20 (1964)	Cortisone and estrogen therapy stopped; hypoglycemic seizures	Hyperinsulinism (See text, Table II)	Subtotal pancreatectomy; excision of metastatic hepatic nodules; removal of 2/3 left adrenal	Islet cell adenomata in pancreas and liver; adrenal cortical adenoma & hyperplasia	Persistent hypoglycemia; diazoxide therapy started
24 (1968)	Relatively asymptomatic on diazoxide therapy; persistent obesity, hirsutism, cliteromegally	See text, Figs. ● ●	———	———	———

Case history of Propositus (No. 5).

/removal of the remaining adrenal because of an adenoma and hyperplasia. Presently, she is maintained relatively free from hypoglycaemic symptoms with diazoxide therapy. A detailed account of her initial response to diazoxide has been published[155]. Patient 6 had intermittent hypercalcaemia for two years and at age 21 subtotal parathyroidectomy revealed hyperplastic changes in all 4 glands. At that time asymptomatic glucose intolerance and fasting hypoglycaemia prompted further studies (vide infra). A total pancreatectomy was performed revealing diffuse islet cell hyperplasia and adenomata. He is presently asymptomatic, receiving insulin and pancreatic enzyme replacement. Patient 3 had a total gastrectomy for the Zollinger Ellison syndrome prior to this study. Pancreatic tumour tissue at that time revealed a gastrin-like material by bioassay, reported in detail elsewhere[156]. He is now asymptomatic but has residual pancreatic and duodenal adenomata. Subject 1 was studied at the Mayo clinic in 1924 for peptic ulcer symptoms. He had gastric hyperacidity and a single duodenal ulcer at surgery. An operation two years later, following intractable abdominal pains and severe diarrhoea, revealed "ulcers at the other end of the stomach". He died soon thereafter and no autopsy was performed. Presumably he also had the Zollinger Ellison syndrome. The other members of the family studied/

/studied (Nos. 2, 3, 5, and 8) have had no symptoms of endocrine dysfunction and have not undergone any operative procedures involving the endocrine glands.

RESULTS

The test results obtained in the study of this family are summarised in Table 2. Values were considered to be increased or decreased if greater or less than two standard deviations from mean control values, respectively. The mean fasting values in our assays of control subjects are for immunoreactive insulin(IRI) 18 ± μU/ml. (Mean ± S.D.), and for immunoreactive glucagon(IRGl) 380 ± 180 μμg./ml., and IRGa 425 μμg./ml. The area under the IRI response curves following each of the test stimuli was calculated for each subject[157]. Of 12 control subjects only one showed a significant rise in IRGl after oral glucose, and in 6 control subjects after intravenous tolbutamide no change in circulating IRGl levels were noted.

As shown in Table 2, two members of the family (Nos. 5, 6) had elevated fasting IRI levels and all subjects in the genetic line of transmission (Nos. 2, 3, 4, 5, 6, 7) had exaggerated IRI responses to oral glucose and tolbutamide. Four of the subjects (Nos. 3, 4, 5, 6) had elevated fasting IRGa levels. One subject (6) had elevated fasting IRGl values (mean of 12 fasting values 771 μμg./ml.). Three subjects (Nos. 3, 4, 6) had elevated IRGl responses/

TABLE 2

Gene-ration	Pt. #	Clinical episodes	Gast. anal. (1 hr. basal)	Fasting blood glucose	Fasting plasma immuno-reactive gastrin	Plasma immunoreactive glucagon **			Plasma immunoreactive insulin **				Surgery	Pancreatic pathology	Associated Disorders
						Fasting	After tolbut. (1 gm i.v.)	After oral glucose (100 gm)	Fasting	After tolbut. (1 gm i.v.)	After oral glucose (100 gm)	After glucagon (1 mgm i.v.)			
I	2*	Asymptomatic	—	N	—	N	N	N	N	Inc.	Inc.	—	Not operated	—	Obese
II	3*	3 years post-op total gastrectomy for ZE	Inc.	N	Inc.	N	Inc.	Inc.	N	Inc.	Inc.	N	Total gastrectomy	Multiple pancreatic and duodenal adenomata	Intermittent hypercalcemia
	4	Episodes of "fainting" years ago. Now asymptomatic.	N	N	Inc.	N	N	Inc.	N	Inc.	Inc.	Inc.	Not operated	Not operated	Slightly obese
III	5	See text, Table I	N	Dec.	Inc.	N	N	N	Inc.	Inc.	Inc.	N	See Table I	Islet cell hyperplasia progressing to carcinoma	Adrenal cortical hyperplasia and adenoma, intermittent hypercalcemia precocious puberty virilization
	6	Emotional lability, easy fatigue	Borderline Inc.	Dec.	Inc. Pre-pancreatectomy N Post-pancreatectomy	Inc.	Inc.	Inc.	Inc.	Inc.	Inc.	Inc.	Total pancreatectomy, subtotal parathyroidectomy	Multiple pancreatic adenomata, islet cell hyperplasia	Increased adrenal cortical secretory rate; chief cell hyperplasia of parathyroids, parathyroidectomy
	7	Asymptomatic	N	N	Borderline Inc.	N	N	N	N	Inc.	Inc.	N	Not operated	Not operated	None

Family with Multiple Endocrine Adenomatosis

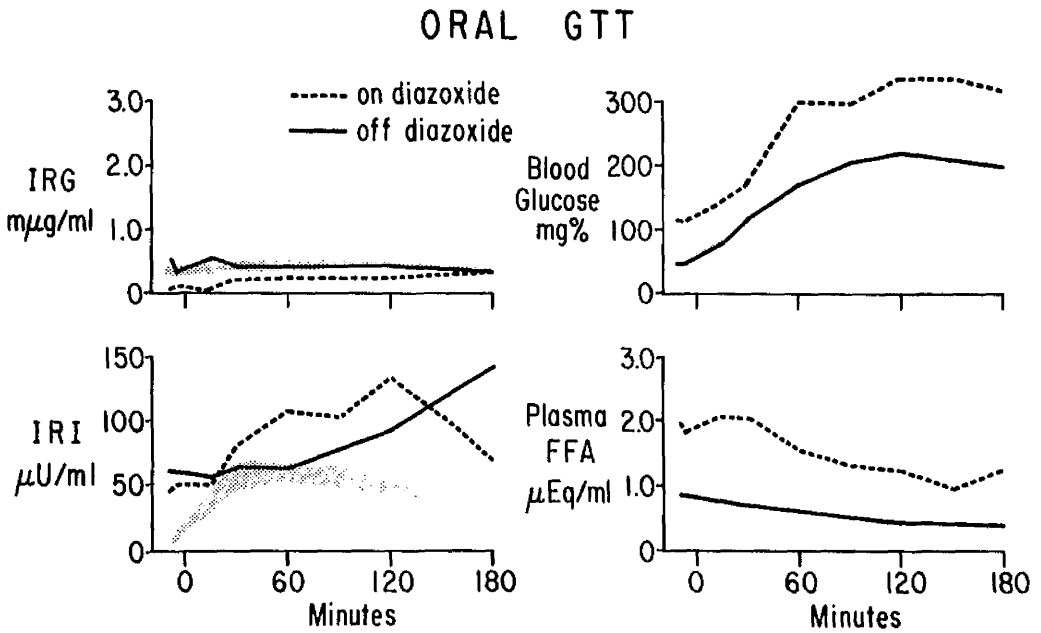
*Subject 1 is deceased and was not studied; Subject 3a had normal responses to all tests, so her data are not included. N = Normal.
Inc.= greater than 2 standard deviations above mean control.
Dec.= greater than 2 standard deviations below mean control.
**The area under the hormone response curve for each individual was calculated and compared with the mean response area for normal controls.

/responses to oral glucose and two (Nos. 3 and 6) had excessive responses to intravenous tolbutamide. Prior to pancreatectomy subject 6 had elevated fasting levels of IRI, IRG1 and IRGa. The pancreatic adenomata found at operation in this subject contained extractable IRI and IRG1 (IRGa not yet measured), and the elevated fasting hormone levels all returned to normal following pancreatectomy. Three members of the family have had intermittent hypercalcaemia (3, 5, 6). Basal hourly gastric acid secretion was increased in subject 3 and showed borderline increased levels in subject 6.

The propositus (No. 5) was studied in January, 1968, while receiving diazoxide therapy and again after stopping the drug (Figures 2 and 3). Oral glucose tolerance, intravenous tolbutamide, and intravenous glucagon tests were performed on consecutive days after overnight fasting while continuing to receive 300 mg. of diazoxide every 6 hours by mouth. The tests were then repeated in the same order after discontinuing diazoxide therapy; the first repeat test was performed 24 hours after stopping the drug. During the 3 day period while off diazoxide, she consumed a 300 gm. carbohydrate diet divided into 8 feedings per 24 hours, with the last feeding given at 3 a.m., or 5 hours before starting the tests, each day.

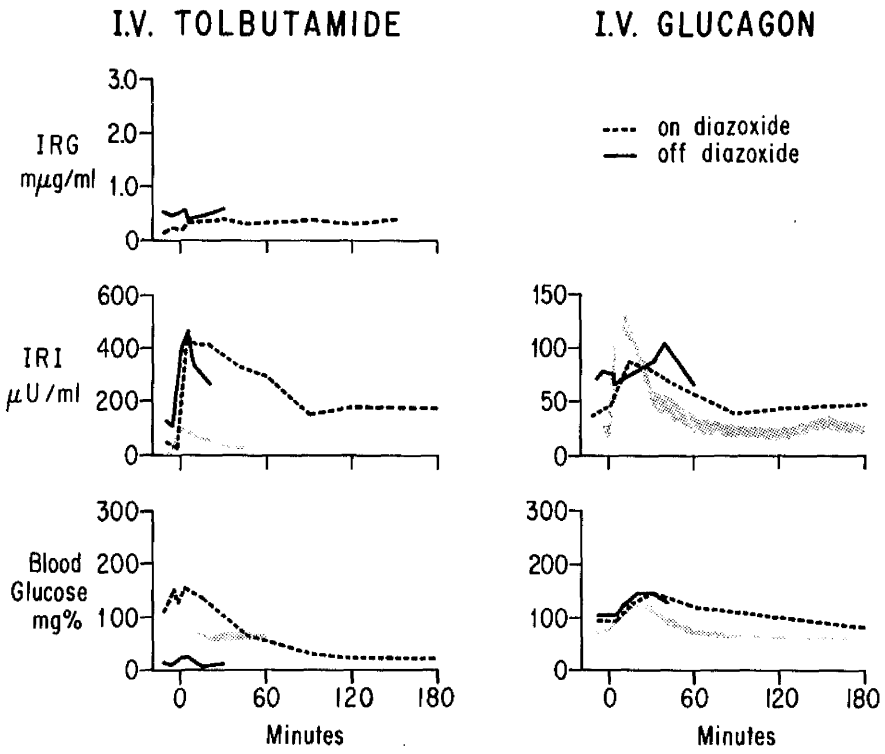
As/

FIGURE 2



Oral glucose tolerance tests performed on propositus (subject 5) while on and off diazoxide. Shaded lines represent the normal mean values.

FIGURE 3



Intravenous tolbutamide and glucagon tests performed on propositus (subject 5) while on and off diazoxide. Shaded lines represent the normal mean values.

/As can be seen in Figures 2 and 3, fasting normoglycaemia associated with, on two out of three occasions, slight elevations of fasting IRI levels was noted while on diazoxide therapy. When diazoxide therapy was discontinued, fasting asymptomatic hypoglycaemia with elevated IRI levels developed. Fasting plasma IRGl levels while receiving diazoxide were approximately 50% lower than the levels while off medication. Elevated plasma IRGl levels in response to hypoglycaemia were not observed. The oral glucose tolerance tests (Figure 2) while on and off diazoxide therapy, elicited qualitatively similar responses: no change in plasma IRGl levels, glucose intolerance associated with delayed but excessive IRI release, and only a slight delayed fall in FFA levels.

Three members of the family have had intermittent hypercalcaemia (Nos. 3, 5, 6). The following tests were normal in all subjects: haematocrit, white blood count and differential; serum sodium, potassium, chloride and carbon dioxide; fasting serum immunoreactive growth hormone, serum thyroxine; 24 hour urinary 5-hydroxyindolacetic acid, 17-ketosteroids and 17-hydroxysteroids; skull x-rays of the sella turcica.

DISCUSSION

It cannot be accurately deduced from these case histories what may be the clinical features of a glucagon excess state. These/

/These patients had abnormalities of several endocrine glands and therefore the clear cut effects of glucagon excess will be confused with the effects produced by excess of other hormones. Two of the subjects (3 and 6) had elevated levels of plasma IRG1 following tolbutamide which might be expected to produce secretion of pancreatic IRG1 (Chapter 3, section 1) whereas three (3, 4 and 6) had elevated plasma levels of IRG1 after oral glucose which promotes secretion of IRG1 from the gut (Chapter 3, section 1). Neither of the two subjects who had elevated levels of pancreatic IRG1 showed any particular features which might be related to this. Subject 3 had normal oral glucose tolerance, but subject 6 had a mild diabetic oral glucose tolerance. However, subject 6 also had hyperinsulinism, and oral glucose intolerance is a well known feature of insulinoma subjects[158]. In addition both subjects with the hyperglucagonaemia of pancreatic origin had hyperinsulinism and hypoglycaemia, so that it might be argued that the hyperglucagonaemia in these subjects might be due to the stimulus of chronic hypoglycaemia. However, patient 5 with severe protracted hypoglycaemia did not have evidence of hyperglucagonaemia.

The only conclusion that may be drawn from the glucagon measurements in this particular family, is that glucagon may be another/

/another hormone which is involved in the multiple endocrine adenomatosis syndrome. It is not possible, however, to conclude what effects the glucagon may have been producing. Reports in the literature[47,152] suggest that glucagon excess states may be associated with diabetes mellitus of a maturity onset type. Although there is in the literature only one accurately documented case of glucagonoma, there are reports of alpha cell carcinomas of the pancreas not associated with the Zollinger Ellison syndrome which have varying aberrations in carbohydrate metabolism, from steroid induced renal glycosuria[159] to overt diabetes[47,152,160,161].

There is, as yet, no evidence to incriminate glucagon excess as a cause of idiopathic diabetes mellitus. Glucagon hypersecretion in the circulation has only been detected in patients with pancreatic tumours as the patients in this report. Because of the inability to accurately assess pancreatic glucagon secretion in human subjects, the question as to whether glucagon plays a role in the pathogenesis of diabetes mellitus remains to be answered.

The most widely accepted theory of the pathogenesis of the multiple endocrine adenomatosis syndrome is that a common genetic defect in each affected endocrine gland leads directly to cellular proliferation/

/proliferation, independent of humoral factors[162]. The predominant involvement of the pancreatic hormones in this study, however, leads to interesting speculation as to another possible explanation for the syndrome. The hypothesis is suggested that the genetic defect involves primarily the pancreatic islets and that involvement of other endocrine glands results from chronic overproduction of islet cell hormones.

Support for this concept is gained from recent provocative clinical associations. Poloyan et al[163] reported two patients with alpha cell hyperplasia, hyperglucagonaemia and hyperparathyroidism. They have shown that glucagon injections in rabbits caused acute hypocalcaemia, and, over a prolonged period, parathyroid hyperplasia[164,165]. Alvioli et al[166] found that after glucagon administration in dogs, a substance with the biological properties of calcitonin was released. The ability of calcitonin to stimulate parathyroid hyperplasia is further supported by the observation that calcitonin can be extracted from medullary thyroid carcinoma[167], a tumour which is associated with parathyroid hyperplasia[168]. It may, therefore, be that alpha cell hyperplasia and chronic hyperglucagonaemia stimulate calcitonin release, thereby producing hypocalcaemia. During prolonged compensatory secretion of parathormone to maintain eucalcaemia, the parathyroids become hyperplastic and eventually develop autonomous function, and clinical/

/and clinical hyperparathyroidism. In an analagous fashion, insulin acts directly or indirectly to stimulate the release of growth hormone, ACTH and glucocorticoids, and epinephrine[169]. Chronic hyperinsulinism might accelerate each of these processes, and over the course of years lead to hyperplasia and subsequently to functioning tumours of the pituitary, adrenal cortex and adrenal medulla.

C H A P T E R 5

SUMMARY AND CONCLUSIONS

The construction of a reliable immunoassay method for glucagon has formed the basis of this thesis. The major factor in the building of a good assay was the recognition of the susceptibility of glucagon to attack by proteolytic enzymes, a fact which at first escaped the notice of earlier investigators. Trasylol, a proteolytic enzyme inhibitor, has proved to be an efficient means to prevent this enzyme degradation. The assay was able to detect as little as 20 μμg. of glucagon.

The application of the assay to measurement of immunoreactive glucagon (IRG) in tissues and body fluids, revealed that IRG was not only present in the pancreas but was also detected in much of the alimentary tract, mainly the small and large intestines. It was apparent too that circulating IRG was derived from both enteric and pancreatic sources. Clearance studies of glucagon showed that glucagon disappeared rapidly from the body and that the liver was a major site for the degradation of glucagon. No difference in the clearances of enteric and pancreatic glucagons was noted. However immunological differences appeared to be present between the enteric and pancreatic glucagons, enteric glucagon reacting less strongly with glucagon antibody than pancreatic glucagon.

When assessing the factors which might affect the release of glucagon, consideration had to be given to the factors cited above which/

/which would complicate the studies. It was realised that an assessment of factors affecting the release of glucagon from both the pancreas and gut would have to be undertaken separately. A direct assessment of factors affecting the pancreatic release of glucagon was made possible by application of a method for isolating the islets of Langerhans of rat pancreas. The use too was made of dogs with venous catheters situated in pancreatic and gut veins, so that a separate measurement of IRG changes from blood draining the pancreas and gut could be made. Studies of animals in whom the pancreas was removed was also made, in order that factors affecting gut glucagon release could be assessed in the absence of the pancreas.

It was realised also that studies of peripheral circulating IRG levels may fail to record changes in gut and pancreatic IRG secretion as much of the glucagon would be removed in its passage through the liver. Thus many of the measurements of IRG were done in pancreatic, jejunal, colonic or portal blood prior to its passage through the liver. Wherever possible experiments were duplicated both by "in vitro" pancreatic islet studies and "in vivo" dog or human studies, in order that observations could be confirmed by these two different approaches.

Because of certain already established pharmacological actions of glucagon on carbohydrate metabolism, the effect of changing glucose/

/glucose concentrations was first of all studied. IRG secretion from the pancreas was found to increase during acute hypoglycaemia whereas no effect was noted on gut IRG secretion. Hyperglycaemia in contrast inhibited IRG secretion from the pancreas but once again had no effect on gut IRG secretion. Oral glucose did, however, stimulate gut IRG release but did not influence pancreatic IRG release.

A study of the effect of the enteric hormones on IRG release was made because of previous reports of the effect of these hormones on insulin release. Pancreozymin was found to exert a potent effect on both IRG and insulin release from the pancreas, although it had no effect on IRG release from the gut. Secretin and gastrin did not however possess any IRG releasing properties from either gut or pancreas.

It was considered probable that glucagon might be an important hormone in the control of the metabolic changes during starvation particularly through its pharmacological actions on gluconeogenesis and lipolysis. However contrary to this hypothesis, circulating levels of IRG were found to fall during starvation in human subjects. That this fall might be due to diminished pancreatic IRG secretion was suggested by the finding that the release of IRG from pancreatic islets of starved rats was also reduced.

Amongst the other factors tested on glucagon release, results were/

/were obtained to suggest that some of the pituitary adrenal hormones may influence IRG release from the pancreas. In particular adrenocorticotrophic hormone (ACTH) probably increased IRG release.

Studies were also made of the inter-relationship of glucagon and insulin secretion. Glucagon stimulated insulin release both "in vivo" and "in vitro". However simultaneous measurements of glucagon and insulin release from pancreatic islets seldom produced a direct correlation in the amounts of each hormone released as one might expect if endogenous glucagon were stimulating insulin release.

It is still premature to confidently attach to glucagon a unified concept of its physiological role. It is probable that like insulin it has many actions and that many factors influence its release. The number of factors which influence insulin release has reached almost embarrassing proportions. These include many of the monosaccharides - fructose, ribose, mannose, galactose and glucose, although glucose still enjoys pride of place -, free fatty acids, octanoic acid, ketone bodies, proteins and amino acids ACTH and glucagon, pancreozymin, secretin and gastrin, theophylline and caffeine, Beta adrenergic blockers such as isuprel, and many others. It seems probable that these many factors which influence insulin secretion do so through a common mechanism such as the cyclic/

/cyclic 3' 5' adenosine monophosphate mechanism as Malaisse et al (1967)[132] suggest. This thesis reports several factors which influence pancreatic IRG release. These include hypoglycaemia, pancreozymin and ACTH. Reports in the literature in either preliminary or abstract form suggest that other factors influence pancreatic IRG release. Ohneda et al (1968)[172] report that amino acids increase the release of IRG from the pancreas and Madison et al (1968)[173] have shown that elevation of free fatty acids inhibit the release of pancreatic IRG. Just as with insulin the further widespread application of the immunoassay method for glucagon is likely to see the growth of a number of factors affecting glucagon secretion.

At the present state of our knowledge one can see pancreatic glucagon as being a hormone of importance in maintaining normoglycaemia either during short episodes of fasting or during the reactive hypoglycaemia which occurs after eating. It is probable that it is just after food that the relationship which the enteric hormones pancreozymin and secretin have with insulin and glucagon plays its role. Following a meal secretin and pancreozymin will be released and this in turn will influence the secretion of insulin and glucagon, the insulin being released to mobilise substrate and the glucagon being more directed towards the prevention of hypoglycaemia which would be prone to occur particularly after/

/after a high protein, low carbohydrate meal. The amino acids may mediate their effects on pancreatic IRG release only via pancreozymin which they are known to influence. N. S. Track (1968)[174] describes fascinating and provocative structural similarities among gastrin, secretin, insulin, glucagon and possibly pancreozymin as well. This structural similarity might lead one to suspect that these hormones might have certain properties in common. Glucagon, pancreozymin, secretin and gastrin all affect insulin secretion. Secretin in addition to gastrin has recently been reported by Lazarus et al (1968)[175] to have hyperglycaemic, glycogenolytic properties. Gastrin has enjoyed eminence ever since its association with the Zollinger Ellison syndrome, but pancreozymin and secretin have to some extent remained metabolically dormant in a little backwater of exocrine pancreatic function. They now emerge with challenging new functions. It seems eminently possible that pancreozymin and secretin will fall victim to the immunoassayist's axe. Until this happens then the full metabolic role of these hormones and their association with insulin and glucagon will remain enigmatic.

It is difficult to assign an exact place to the property of glucagon in stimulating insulin release. It seems contradictory that a hormone which is secreted when there is glucose need, should influence/

/influence the secretion of insulin which is a hormone of glucose abundance. The thesis has demonstrated too that pancreatic glucagon release is inhibited by hyperglycaemia. It may be, however, that glucagon may influence insulin release at relatively low glucose concentrations in order to maintain basal insulin secretion.

In summary then glucagon's physiological role may be in the prevention of hypoglycaemia which might occur during short periods of fasting, or the reactive type which occurs after a meal. Its effect on insulin secretion may only operate at near fasting or low blood sugar concentrations. It is considered too that glucagon is probably a hormone which is of more importance during acute metabolic events rather than chronic. This is suggested by the lack of influence of chronic starvation on glucagon secretion.

The whole problem of the nature and role of the IRG in the gut is even more uncharted than that of pancreatic IRG. Factors which affect the release of pancreatic IRG do not influence the release of the gut material, and the reverse is also true. Similarities concerning its clearance and degradation as compared with pancreatic IRG were found. Immunological dissimilarity between the two IRG materials was however noted, and this work has been extended by Heding (1968)[177] who in a recent communication showed that/

/that by the use of a grossly discriminating antibody pancreatic IRG could be separated from gut IRG in the peripheral blood. The fact that the enteric material rises after oral glucose may suggest that its role lies in the possible promotion of insulin secretion. However the assignment of this material to a role remains purely conjectural. So, too, must its true nature and its relationship with pancreatic glucagon. Unger et al (1968)[23] did consider that the gut IRG possessed none of the biological properties of glucagon but this work was at variance with the earlier reports of Kenny and Say (1962)[18] and Makman and Sutherland (1964)[19] who both reported a glucogenolytic-hyperglycaemic substance in gut. More recently, however, Valverde et al (1968)[176] describe two fractions of glucagon immunoreactivity in the gut, one being of similar molecular weight to pancreatic glucagon and sharing at least some of its biological properties, and the other being a larger molecular weight substance and not possessing the biological properties of pancreatic glucagon. However, neither of these gut fractions reacted with glucagon antibody in a manner identical with pancreatic glucagon. The elucidation of the nature and role of enteric immunoreactive glucagon material remains an exciting challenge for the future.

Glucagon's role in disease is unknown. Apart from the one or two cases of glucagonoma reported (Chapter 4), no clearly defined assocation of glucagon with any disease state has yet been described/

/described. It is highly provocative to consider that an abnormality of glucagon secretion may in fact lead to diabetes mellitus. This possibility is not so remote as the association which was once thought to occur between growth hormone secretion and diabetes mellitus. It remains, however, a possibility which still has to be tested. So too are the thoughts that glucagon deficiency may play a role in certain hypoglycaemic syndromes.

There remain certain technical difficulties which lie in the way of the elucidation of these problems. Application of the glucagon immunoassay to human subjects remains unsatisfactory but recent advances in methods of improving immunoassay sensitivity (Miles and Hales, 1968[178]) and in the differentiation of pancreatic and gut glucagon (Heding, 1968[177]) promise that these difficulties will soon be overcome. The future of glucagon is only just being realised.

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