CALCIUM AND PANCREATIC ALPHA CELLS IN METABOLISM

Thesis

by

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PREFACE

Fifty years of sustained effort by numerous workers have not sufficed to furnish a complete understanding of the problem of carbohydrate metabolism or its most prevalent disturbance, diabetes mellitus. Although many factors are still unexplained, this disease is, however, adequately dealt with by modern therapeutics. Until thirty years ago, diabetes mellitus was a hopeless disease; nowadays, diabetics, with insulin treatment, enjoy a nearly normal life.

The many obscure points that still exist relative to that syndrome have given rise to various hypotheses. With the introduction of insulin in the therapy of diabetes mellitus, a new experimental fact came forth suggesting the existence of a pancreatic substance other than insulin and possessing hyperglycemic properties. Some workers proposed the Alpha cells of the Islets of Langerhans as the source of this substance. While insulin, the hormone said to originate from the Beta cells of islet tissue of the pancreas, lowers the blood sugar level, the hypothetical hormone, designated as the hyperglycemic-glycogenolytic factor of the pancreas, would increase the blood glucose and prevent ketosis in the insulin-deficient animal.

The present thesis reports in its experimental part:
(a) a search of the dialyzable degradation products resulting from the partial alkaline hydrolysis of the hyperglycemic protein

of the pancreas. This was done in an attempt to isolate and characterize the degradation product - said to be a polypeptide - which causes an increase of glucose output in liver slices. The results of this search gave rise to a short <u>in vitro</u> study of the influence of calcium ions in shifting carbohydrate metabolism towards symptoms of diabetes mellitus.

(b) an investigation of the effects on glucose tolerance and insulin sensitivity, and on insulin content of pancreatic tissue, caused by influencing the development of Alpha cells and Beta cells of the pancreas.

This work is complemented, first, by a survey of the evidence in favor of a hyperglycemic hormone of the Alpha cells of the pancreas and, second, by discussions on the possible rôle of pancreatic Alpha cells in metabolism.

GENERAL INTRODUCTION

DIABETES MELLITUS

The close relationship existing between the work reported in this thesis and the disease, diabetes mellitus, renders a short survey of this syndrome necessary.

Diabetes mellitus is extensively described in all text-books of medicine and physiology (1) (2) (3). Its main symptoms may be summarized as follows:

The glucose content of the blood rises to abnormal values producing a state of hyperglycemia. Some of the excess glucose is excreted into the urine by the kidney thus causing glycosuria.

Acetone, hydroxybutyric acid and acetoacetic acid, the so-called ketone bodies, appear in elevated amounts in the blood - a condition known as ketosis. Some of the ketone bodies pass into the urine thus causing ketonuria.

Acidosis is present as a consequence of ketosis. Polydypsia and polyphagia are occasionally noticed. In spite of great appetite, patients may lose weight.

Sometimes diabetic patients become gangrenous at their extremities.

Diabetes mellitus is treated with insulin and controlled

diets. If untreated, diabetics may die in hyperglycemic coma and acidosis.

ENDOCRINE TISSUE OF THE PANCREAS

STRUCTURE: In mammals, the pancreas is an organ situated near the duodenal loop of the intestine. In some species like man and dog, it is well defined into head, tail and body. The head adheres to the middle portion of the duodenum and the tail extends to the spleen. In rat and rabbit, the pancreas is much less defined and is located between the stomach and the first duodenal loop (1). Complete extirpation in these two species is accordingly very difficult.

Histologically, the pancreas is an alveolar gland of racemose pattern (2). The alveoli produce the external secretion and the cells located between the alveoli furnish the internal secretion. The latter cells constitute the Islets of Langerhans.

The Islets of Langerhans are made up of two main types of cells, the Alpha and the Beta cells. According to Gomori (4), who has examined the pancreas of many species, the two types of cells are invariably present in species possessing islet tissue. The islet cells are distributed in an irregular fashion. Their shape may be cuboidal, columnar or wedge like.

INTERRELATIONSHIPS AND FUNCTIONS: According to Gomori (4), there is no transition between Alpha and Beta cells. In most species, the Beta cells are in majority. In normal humans,

Beta cells form from 60 to 90% of islet tissue, the rest being Alpha cells. The two types of cells are all heavily granulated, a usual characteristic of secretory activity. Only the Beta cells, however, have been proved to possess an endocrine function. Banting, Best and Macleod (5) (6), in 1921, extracted insulin from pancreas and, ever since, indications that the Beta cells are responsible for the secretion of this hormone have accumulated. The Alpha cells have not until recently been assigned a secretory function, but lately, some work has appeared suggesting that these cells are endocrine. The conclusions derived from the work presented in this thesis support this view.

EXPERIMENTAL DIABETES

The condition of diabetes mellitus can be duplicated experimentally by pancreatectomy, by repeated injections of extracts of the anterior lobe of the pituitary, and by treatment with alloxan. Such means of inducing a diabetic state have proved of great value in the study of this disease.

PANCREATECTOMY: In 1889, Minkowski and von Mering (7) extirpated the pancreas of dogs and observed that shortly afterwards the dogs exhibited the symptoms of diabetes mellitus. This classical experiment suggested that the pancreas secretes a carbohydrate-metabolyzing hormone. This indication was later confirmed when in 1921 Banting and Best (5) isolated insulin from pancreas. Experimental animals, having lost the site of insulin production by pancreatectomy, become diabetic. Treated properly with insulin, they may live for a long time; untreated, they will die in a few days.

PITUITARY DIABETES: Houssay and others (9) (10) have shown that repeated injections of crude extracts of the anterior lobe of the pituitary gland produce in the animal a state of diabetes. This phenomenon has been repeatedly studied and confirmed (11) (12) (13) (14) (15) (16) (17). Diabetogenic effect has been observed only on adult animals; young animals respond to the injections by an increased rate of growth (18). The crude extracts of pituitary gland administered by Houssay a few years

ago have nowadays been replaced by nearly pure preparation of single pituitary hormones. Of the six known hormones of this gland, the growth hormone, or somatotrophin, has been shown to be the most diabetogenic one. The diabetes produced by injections of other pituitary hormones, such as ACTH by Conn (19) was probably due to the presence of growth hormone as an impurity.

A few theories have been proposed to explain the diabetogenic effect of somatotrophin. Cori (20) claimed that the pituitary hormone inhibits hexokinase, the enzyme regulating the entry of glucose into glycolysis. This inhibition would be relieved by insulin. Thus, such extracts would impose a strain on the insulin-producing cells so that these cells would eventually become exhausted (8). This theory has been much discussed and attacked because of serious objections to it. Repeated injections of glucose cause a prolonged state of hyperglycemia but fail to produce exhaustive degeneration of insulin-producing cells (21). This objection, however, is not shared by every worker. Baron and State (22) claim that a glucose treatment of 9 days produced in the dog a degranulation of the Beta cells and a sustained hyperglycemia after cessation of the treatment.

The theory of Cori, though much criticized at first, seems to have regained the favor of some authorities. Chaikoff (23) (24) also believes that, in diabetes, there exists a block at the hexokinase level preventing the entry of glucose in metabolism.

Hoffman (25) sees, in the fact that administration of hexose diphosphate to diabetics decreases their insulin requirements, an indication of hexokinase deficiency. A few other theories have been suggested but no one is yet firmly established. Young (18) believes that growth hormone when secreted in the young animal protects from oxidation foodstuffs that would otherwise, in the adult animal, be oxidized. In the adult, slight and prolonged overproduction of growth hormone would result in diabetes, a condition in which the material preserved from oxidation but not utilized would be excreted in the form of sugar. De Jongh (26) claims that the increase of protein metabolism, as demonstrated by increased nitrogen retention on administration of growth hormone to hypophysectomized animals, interferes with carbohydrate metabolism - thus causing diabetes.

The problem of the interdependence of pituitary hormones and diabetes mellitus stands out, at present, as one of the most important and most interesting of all.

ALLOXAN DIABETES: Jacobs, in 1937 (27), observed that the intravenous injections of alloxan produced in the animal a temporary hyperglycemia followed by hypoglycemia and death in hypoglycemic convulsions. The significance of Jacob's observation was not evaluated until 1943 when Dunn, Sheehan and McLetchie (28) attempting to cause kidney lesions with alloxan noted that alloxan produced selective necrosis of the Beta cells of the Islets of Langerhans.

Following injection of alloxan, the blood glucose passes through a triphasic reaction. Up to 5 hours, there is a hyperglycemia followed by a marked decrease in the blood sugar lasting a day or so, after which, a gradual rise brings the animal to a permanent diabetic condition (29).

The mode of action of alloxan is not yet satisfactorily explained. All workers, however, are unanimous in asserting that the final diabetic state of the animal is due to the destruction of the Beta cells of the Islets of Langerhans, thus preventing further production of insulin. The necrosis of the pancreatic Beta cells is the most striking damage caused by alloxan, but it is by no means, the only one: injections of this drug have been observed to cause also lesions in the adrenals, heart and liver (1). Female animals are said to be more susceptible to the diabetogenic action of alloxan than males (30). According to Ridout, Ham and Wrenshall (31) the hypoglycemic phase is due to the leaching of preformed insulin into the blood, but Bhattacharya (32), on the other hand, believes that this phase is due to an increased glycogen content of liver. It has been shown that hepatic glycogen is higher in alloxan hypoglycemia than in insulin hypoglycemia (32), and that the glycogen concentration in the livers of alloxan diabetic animals fasted for 24 hours is higher than that of the correspondingly fasted normal animals (33). Gemmill (34) has observed a blockage of glycolysis in frog muscle extract by alloxan, the degree of inhibition being proportional

to the concentration of alloxan.

The effect of alloxan has been shown to be duplicated by a number of other compounds. Patterson has established the diabetogenic effect of dehydroascorbic acid and of dehydroisoascorbic acid. These compounds, whose chemical structure is somewhat similar to that of alloxan, produce in the experimental animal, the triphasic response of alloxan injection (35) (36) (37) (38). Lately, other organic substances have been added to this list. These substances include sodium diethyl dithio carbamate, potassium ethyl xanthate and thiosemicarbazide (39).

Lazarow and many others claim that the susceptibility of an animal to alloxan or to dehydroascorbic acid is directly influenced by its pancreatic sulfhydryl groups. They maintain that injection of compounds such as glutathione, cysteine, BAL, barbituric acid, 2-5, piperazine, nicotinamide, thiouracil, thioactic acid, thiomalic acid, thiothymine, immediately before administration of alloxan protects the animal from diabetes; if injected after alloxan, this latter drug produces diabetes (40) (41) (42) (43) (44) (45). Gemmill showed that the inhibition of glycolysis produced by alloxan on muscle glycogen is reversed by cysteine (34).

COMPARISON OF THE VARIOUS DIABETIC STATES: The three main types of experimental diabetes differ with respect to the immediate action of their causal agents - presumably in the following way:

(a) pancreatectomy removes the organ responsible for the secretion of insulin,

(b) injections of anterior pituitary extracts exhaust insulin-producing cells, by blocking insulin action,

(c) injections of alloxan destroy the insulin-secreting cells.

These three forms have significant differences in the resulting symptoms. Alloxan diabetes, on the one hand, shows more severe hyperglycemia and glycosuria than pancreatectomy (46), but on the other, exhibits much less ketone body production. The amount of ketone bodies in alloxan diabetes is even sometimes normal (46) (47) (48) (49). Values for ketone bodies in pituitary diabetes are intermediate between those due to pancreatectomy and alloxan injections. Probably because of low ketonemia, alloxan diabetic animals can live for long periods without insulin treatment (46) (50).

OTHER TYPES OF DIABETES: Apart from clinical diabetes and the three types of experimental diabetes described above, there are various other types of lesser importance to the present work but nevertheless deserving mention. These are reviewed by Lazarow (51).

(a) Adrenal cortical diabetes, due to excessive activity of the adrenal cortex,

(b) Thyroid diabetes, produced experimentally by the administration of thyroid hormone to partially depancreatized

dogs. Thyroidectomy, known to be followed by an increase in sulfhydryl groups, if performed previously to alloxan injection, protects the animal against diabetes (52).

(c) Liver and muscle diabetes, due to a decrease in the activity or the amount of enzymes resulting from an endocrine imbalance,

(d) Diabetes due to an abnormal insulin sensitivity or destruction.

HISTORICAL REVIEW

EARLY PERIOD
(1921 - 1937)

INSULIN PRIMARY HYPERGLYCEMIA: In 1921, Banting and Best published their celebrated paper on the first successful isolation of insulin from pancreas (5). At the news of this discovery, investigations began in many countries into the nature and the mode of action of this new hormone, and attempts were made to improve the method of its isolation. The main effect of injection of insulin is its hypoglycemic action but it was soon noted that prior to the lowering of blood sugar there was a transient rise of glucose. It is difficult to assert who first noticed this phenomenon, the literature being uncertain on this point.

Macleod in 1922 (53), studying the effects of extracts of pancreas of certain bony fish, in which the endocrine and exocrine parts are separated, noted that the extracts of the exocrine part produced prolonged hyperglycemia. Macleod did not investigate further this phenomenon.

Murlin, Gibbs and Root (54) (55), as early as 1923, prepared from beef pancreas, by acid extraction, neutralization, and alcohol and acetone treatment, a substance which, when injected into pancreatectomized dogs, reinforced their hyperglycemia within the next three hours. A similar but weaker effect was

observed on rabbits. These workers coined the term "glucagon", and applied it to the hyperglycemic substance extracted from tissues. This hyperglycemic factor was termed "anti-insulin" by Grewenstuck and Laqueur (56).

Burger and Kramer (57) (58), who made important contributions to this subject, noted that the hyperglycemic effect of insulin, known as insulin primary hyperglycemia, took place in the first ten minutes following the injection. They reported experiments (59) (60) (61) showing that the rise in blood glucose was maximum when insulin was injected into the portal vein. They concluded that insulin exerted its action on hepatic cells. The fact that the rise in blood sugar was accompanied by a fall in liver glycogen reinforced this belief. Since Burger observed the hyperglycemia to be very low in cirrhosis of the liver, he suggested the use of this phenomenonon as a test of liver function (62).

The views of Burger and Kramer were supported by the experiments of Ionesco, Cosmulesco and Tomesco (63). Rathery and his colleagues (64) (65) observed insulin primary hyperglycemia but did not believe this action to be directly related to the variations of liver glycogen.

Ionesco et. al. (66), studying the specificity of the liver in the phenomenon, observed insulin hyperglycemia after sectioning of the splanchnics and after paralysis of sympathetic nerve endings with ergotamine. They concluded accordingly that the liver was the site of action of the insulin relative to the hyperglycemic

phenomenon. Rathery, Wichels and Lauber (67) and also Burger and Kramer (68), observed independently that double adrenalectomy in dogs did not prevent the occurrence of hyperglycemia.

Burger and Kramer thought at first that the hyperglycemia produced by insulin was an intrinsic property of the hormone. They considered the action of insulin to be diphasic. Geiling and de Lawder (69), working with Abel who first crystallized insulin, demonstrated that the primary hyperglycemia decreased as the purity of insulin increased, crystalline insulin exhibiting the lowest rise in blood glucose. Burger, testing crystalline insulin, did not observe the same rise in blood glucose that he observed previously with amorphous insulin. This behaviour of insulin led Geiling and de Lawder (69) to conclude that the pancreas contains a hyperglycemic substance extracted along with insulin.

DIRECT ATTEMPTS AT EXTRACTION OF HYPERGLYCEMIC SUBSTANCES FROM PANCREAS:

(a) MURLIN'S GLUCAGON- The work of Murlin et. al. (55) (70) (71) (72) constitutes the first isolation of a hyperglycemic substance from pancreas. In an attempt to improve Banting's method of extraction of insulin, these workers obtained an extract which on injection into depancreatized dogs largely increased their hyperglycemia. Murlin obtained his extract as follows: Pancreas was treated with a solution of 0.2N HCl. To this, 5 volumes of 95% ethanol were added causing precipitation. The supernatant

liquid was defatted with chloroform. A hyperglycemic substance was precipitated by the addition of five volumes of acetone. This precipitate, dissolved in water, was injected subcutaneously into dogs. Their blood glucose increased by 80% in three hours. Murlin et. al. also isolated from cat muscle and brewer's yeast a substance exhibiting a similar action. These authors suggested the name "glucagon" to describe this blood-sugar-raising substance. The same term was used later by Burger.

(b) FISHER'S TOXIC FRACTION- Fisher (73) (74), working on the extraction of insulin by the Somogyi method (75), noted that fractional precipitation with ethanol yielded a dark precipitate. On intraperitoneal injection of an aqueous suspension of this precipitate into rabbits, the animals died in a few minutes with blood sugar values up to 350mg. per cent.

(c) PETSCHACHER'S COMPOUND- Also working on the isolation of insulin, Petschacher (76) isolated a crystalline substance from horse pancreas. This substance produced a significant rise of blood sugar when injected in rabbits. His method of extraction consisted of repeated treatments of pancreas with 95% ethanol, first cold and then hot, followed by hot water. The combined extracts were concentrated and the hyperglycemic substance crystallized out in the cold. It melted at 265^{o} and contained: carbon, 52.0%; nitrogen, 13.0%; hydrogen, 9.7%. Sulphur, phosphorus and chlorine were absent.

No further work was reported on this crystalline material by Petschacher. Stewart at McGill (77), in 1948, repeated Petschacher's work. He extracted pancreas of slaughter house animals and obtained a crystalline compound whose melting point, carbon and hydrogen content were identical with that of Petschacher's compound. The nitrogen content was, however, 7%. Stewart failed to obtain such a substance on extraction of spleen and liver. The chemical and physical tests performed by Stewart on the compound from the pancreas suggested a glycopeptide.

(d) WOHLENBERG'S COMPOUND- Wohlenberg et. al. published papers (78) (79) dealing with the effects of ultrafiltrates of pancreatic juice on blood sugar level. The juice was obtained by a pancreatic duct fistula from dogs and by duodenal tube from humans. It was filtered through porcelain cones to yield filtrates free of protein and enzymes. The injection of such filtrates into rabbits is reported to have caused increases in blood sugar of up to 200 mg. per cent in 30 minutes to 3 hours. Juice from diabetic patients showed more pronounced effect than that from normal persons.

(e) STAHL'S COMPOUND- Another paper reporting the extraction of an ultrafilterable substance from pancreas is that of Stahl (80). This worker based his method of extraction on the insolubility of this substance in 80% ethanol, a concentration at which insulin is soluble. Moreover, this substance was thermostable, and was not precipitated by picric acid or trichloroacetic acid.

Stahl did not report the doses he employed when testing this material on rabbits, but Burger (81) mentioned that Stahl obtained hyperglycemia after injecting doses of three grams into his test animals. If this statement is accurate, the value of Stahl's experiments is greatly diminished.

(f) TANGL AND THAN'S COMPOUND- In 1933, Tangl and Than (82) extracted pancreas with carbon tetrachloride, shook the tetrachloride extract with acidified water and evaporated the water extract to dryness. Testing the product on dogs, they recorded increases of 100 mg. per cent in the blood sugar in 20 minutes after the injection of 80 mg. Stewart (77) attempted to reproduce Tangl and Than's work. Two fractions were obtained. One was very toxic and the rabbits died within short intervals after injections; the other did not produce hyperglycemia.

(g) BURGER'S GLUCAGON- Among all the early workers who studied the phenomenon of hyperglycemia caused by pancreatic extracts, Burger stands out as the one who made the largest contribution. His first reaction to the observation of insulin primary hyperglycemia was to consider this effect as an intrinsic property of insulin. As previously mentioned, the crystallization of this hormone by Abel using pyridine as solvent made the students of this phenomenon abandon the diphasic concept of insulin action.

In 1930, Burger (83) reported the very important and extremely

useful finding that commercial insulin boiled for one-half hour with 1/200N sodium carbonate exhibited only hyperglycemia, the hypoglycemic action of the hormone being destroyed. This observation of Burger, supported by the suggestion of Geiling and de Lawder that hyperglycemia was not caused by insulin proper, paved the way for Burger's important work on isolation of "glucagon".

Burger dissolved dry amorphous "Wellcome insulin" in acetic acid and added various amounts of pyridine, precipitates being collected at different pH's. The purest precipitate was once more submitted to the same treatment. This fraction was treated with brucine, precipitated with ammonia and dried under reduced pressure. This material, called "glucagon" after Murlin (71), caused an average blood sugar increase of 50 mg. per cent in a dose level of 20 gamma per kg. It showed no hypoglycemic effect, gave a weak Millon's test and a pale biuret reaction. Burger's paper of 1935 (81) described it as follows (translated). "Glucagon, the hyperglycemic principle of the pancreas, is exceedingly similar to insulin in physical and chemical properties. It is a protein-like material. Its content of carbon, nitrogen, and sulphur is almost the same as that of insulin. Its isoelectric point is a fraction of a pH unit below that of insulin. Glucagon is insoluble in water, alcohol and organic solvents, but its salts are soluble in water, dilute alcohol and dilute acetone. It is not dialyzable and may be adsorbed on various adsorption media.

It is not inactivated by dilute soda solution or by cystein under conditions which inactivate insulin. Through long contact with alcohol, it is possible to obtain a fraction with higher activity than that of the starting material. Our active glucagon preparation raises the blood sugar of fasted rabbits on injection of 20 gamma per kg. of body weight, 50% (average) of the initial value."

Burger also stated that the liver was the immediate site of action of glucagon, and that the increase in blood sugar could be accounted for by the breakdown of hepatic glycogen. This conclusion is supported by the fact that carbonate-treated insulin produced primary hyperglycemia in normal humans but not in patients suffering from cirrhosis of the liver (84).

Although subject to criticism, the work of Burger is important, especially because of its pioneer nature. In 1937, Burger published a review of his work on glucagon and the hyperglycemic phenomenon, but for the following eight years, the scientific literature makes no mention of work done on this topic. The late war was undoubtedly the reason, diverting the efforts of workers to other fields of activity. In 1933 Hennequin (85) reviewed the literature pertaining to the subject.

MODERN PERIOD
(1945-1952)

The later period of the history of the phenomenon of hyperglycemia produced by pancreatic extracts began with strong evidence in favor of the hypothesis of a second hormone of the pancreas. In fact, the two workers Thorogood and Zimmermann (46) in a series of ingenious experiments obtained results which they could only interpret in terms of such a hypothesis. Their work is classic in the field and will be reviewed in some detail.

Thorogood and Zimmermann injected intravenously into dogs of the "hound" type an alloxan solution in doses varying from 55 to 75 mg. per cent. The gravity of the resulting diabetes was measured in terms of the daily excretion of sugar. Any animal excreting less than 5 grams of glucose per kg. body weight per day was rejected. Dogs which developed kidney damage due to alloxan were also discarded. Of the original 43 dogs, only 7 were suitable for the investigation. The following summary of the description of the dogs is reproduced from Stewart (77).

Dog 1: Lived for 114 days after alloxan, 91 without insulin; 40 units of insulin controlled an average glycosuria of 67 gms. per day. Urinary ketone bodies averaged 36mg. per day; weight loss, 5 kg.

Dog 2: Duct ligation 27 days after alloxan. Insulin requirement, 35 units before ligation, 30 units after.

Dog 3: Duct ligation. Before ligation, excreted 50 gms. glucose in 25 hours; after, 83 gms. Urinary ketone bodies, 200 mg. per day. Animal alive 90 days after cessation of insulin.

Dogs 4, 5, 6 and 7 were treated with alloxan and then depancreatized. The following is a summary of the results obtained.

Dog	Insulin requirement Before Panx.	After Panx.	Glycosuria in gms/24hrs Before	After	Ketonuria in mg./24hrs Before	After	Survival after Panx.
4	34 u.	12 u.	---	62	---	400	5 days
5	76 u.	30 u.	157	53	7	1400	5 "
6	60 u.	20 u.	128	53	34	1400	7 "
7	36 u.	12 u.	---	---	20	710	5 "

These authors interpreted their results in the following terms quoted from the original paper.

"From these data it is apparent that the diabetes of alloxan treated dogs is characterized by a very severe glycosuria as well as by an extraordinarily high insulin requirement. In terms of these criteria, alloxan diabetes is considerably more severe than that which follows total pancreatectomy in the same species. Alloxan treated dogs, on the other hand, live a great deal longer than do depancreatized ones in the absence of insulin treatment. They maintain excellent health for long periods and fail to go into diabetic coma. Their survival appears to be related to a

very low urinary ketone body excretion. Pancreatectomy in these animals results in a reduction of insulin requirement to about one third the preoperative value. Ligation of the pancreatic ducts has little or no effect on the course of alloxan diabetes."

The results of this work strongly suggest the following points.

1- The pancreas secretes a carbohydrate-metabolizing hormone other than insulin.

2- This other pancreatic hormone causes blood glucose to rise by increasing total glucose production.

3- It decreases ketosis and prevents coma in the insulin-deficient animal.

4- It appears that the site of its production is the Alpha cells of the Islets of Langerhans.

The work of Thorogood and Zimmermann seems to have stirred the interest of other workers, for shortly after the publication of their report, papers dealing with this topic began to reappear. The intensity of interest grew to a point that, nowadays, the existence of a pancreatic hyperglycemic-glycogenolytic factor found as a contaminant in commercial insulins has been largely confirmed and specific aspects of the problem widely studied (86) (87) (88) (89) (90) (91) (92) (93) (94).

The method of assaying hyperglycemic effect has been changed by Shipley and Hümel from tests on intact animals to the liver slice technique (95). They showed that addition of insulin to

rat liver slices with high glycogen content incubated in the rat's serum causes an increase in sugar output when compared with liver slices incubated without insulin. This observation was confirmed by Sutherland and Cori (96) who modified the assay method by replacing the animal's serum as incubation medium by a saline-phosphate buffer. Confirming the suggestion derived from the work of Geiling and de Lawder that purification of insulin liberated the hormone from its primary hyperglycemic effect, de Duve, Hers and Bouckaert (97), and Olsen and Klein (98) reported that a Danish insulin from the Novo Laboratories produced no initial rise in blood glucose, whereas crystalline and amorphous insulins from other sources exhibited primary hyperglycemia. The hyperglycemic phenomenon was thus proved to be definitely dissociated from insulin proper.

Sutherland and Cori (96), studying the factors influencing glycogenolysis caused by pancreatic extracts <u>in vitro</u>, reported the following observations.

1- The cellular organization of the hepatic tissue must be intact; no glycogenolysis is produced in homogenates.

2- Phosphate must be present in the incubation medium.

These requirements led the authors to conclude that the hyperglycemic-glycogenolytic factor exerted its action on the phosphorylase system of the liver. Since the hypoglycemic action

of insulin has no effect in liver slice technique, the presence of this hormone as an impurity in the incubation medium does not interfere with the assay of the hyperglycemic-glycogenolytic activity by this method. In their opinion, the liver slice technique is a sensitive and reproducible test system for the study of glycogenolysis induced by "insulin".

To determine the specificity of the pancreatic factor as the cause of hyperglycemia in intact animals and glycogenolysis in liver slices, these authors applied the liver slice assay method to a large number of substances. These included casein, triose dehydrogenase, zein, commercial hydrolysate of wheat gluten, ribonuclease, dialyzed rabbit serum, egg albumin, cysteine, cystine, adenylic acid, coenzyme 1, riboflavin, nicotinamide, and folic acid. None of these materials caused glycogenolysis. They concluded accordingly that pancreatic extract is specific in that respect.

The studies of the physico-chemical properties of the pancreatic factor by Sutherland and Cori (96) supported the early findings that the hyperglycemic-glycogenolytic principle is a protein, non-dialyzable, and stable to alkali treatments that destroy the hypoglycemic action of insulin. They made an observation which is of special interest to the present work. Insulin (exhibiting primary hyperglycemia) was treated with 0.08N KOH for 3 hours at

37°. This treatment released a dialyzable material which increased the glucose output in liver slices. The fact that this dialysate was strongly biuret-positive led Sutherland and Cori to suggest that the hyperglycemic protein could be degraded to some extent by alkali to yield a glycogenolytic peptide. This particular topic of their work will be reviewed more thoroughly later.

The origin and the distribution of the hyperglycemic-glycogenolytic factor were studied by Sutherland and de Duve (99) in 1948. Their paper reported the following points.

1- The glycogenolytic factor extracted from pancreas is identical with the hyperglycemic "impurity" contained in commercial insulin.

2- Although pancreas is the main source of the factor in all the species examined, gastric mucosa, duodenum, and to a lesser extent, ileum of the dog contained some of it.

3- The distribution of factor roughly follows the distribution of islet tissue.

4- Normal amounts of factor are extracted from the pancreas of alloxan-diabetic rabbits.

Sutherland and de Duve accordingly were led to conclude that the Alpha cells of the pancreas were secreting the hyperglycemic-glycogenolytic factor. Simultaneously and independently, Heard, Lozinski, Stewart, and Stewart (100) reported a similar conclusion

based on observation of the specificity of pancreatic extracts as the cause of hyperglycemia. Preparations from liver and spleen proved to be inert. Sokal (101), on the other hand, claims to have isolated from liver a dialyzable compound causing a fall in liver glycogen.

Successful isolation of highly potent pancreatic substances, apparently the first since Burger's extraction of glucagon (81), was reported in 1949 by Sutherland, Cori, Haynes and Olsen (102). These workers submitted pancreas preparations to ethanol precipitation at pH 3.7 and removed an inactive precipitate at 71% ethanol. Similarly, at 83% ethanol content, the precipitate immediately formed was inactive whereas that obtained after one day in the cold, and also that thrown down at 85% ethanol concentration were highly potent. The potency of these fractions was measured by the liver slice method. Sutherland et. al. reported half-maximal glucose output at dose levels of 0.7 to 1.0 gamma per milliliter of incubation medium. On slow precipitation, this active material deposited as tiny spheroids or plates.

Another interesting experiment of Cori and his group is the study of the electrophoretic patterns of insulin from different sources (102). The schlieren diagrams showed that amorphous insulins and, to a lesser extent, crystalline zinc insulins, carry an impurity of a protein nature as indicated by a secondary peak

quite distinct from that of insulin. On the other hand, Novo insulin, which is claimed to cause no primary hyperglycemia when injected into animals (97) (98), showed only the main peak of insulin. The results of this study constitute, according to these workers, another proof that the hyperglycemic-glycogenolytic principle of the pancreas is a distinct entity of a protein nature.

The relationship existing between the hyperglycemic factor of the pancreas and insulin has been the subject of a short study by Zimmermann and Donovan (103). The results obtained by these authors support the conclusion that the hyperglycemic principle influences the blood sugar and blood ketone levels only through its action on the hepatic glycogen reserves. This conclusion is identical with that arrived at by Burger in the early period. Zimmermann and his colleague also believe the action of the hyperglycemic principle to be independent of that of insulin. The hypoglycemic effect of this hormone should not be therefore neutralized directly by the hyperglycemic action of the factor.

The foregoing survey of previous work dealt with either pancreatic fractions or insulin as the source of the hyperglycemic factor. Foà, Weinstein and Smith (104), attempting to demonstrate the hormonal rôle of the hyperglycemic factor, investigated the presence of this principle in the blood. By means of cross-circulation transfusion experiments on dogs, they observed hyperglycemia in the animals receiving pancreatic blood

from an alloxan-diabetic donor. These workers also suggested that this hyperglycemic substance is similar to that found in most insulins. Experiments somewhat similar to those of Foà and colleagues were carried out in 1947 by Burger and Klotzbücher (105). Inducing alimentary hyperglycemia in normal healthy humans by the oral administration of glucose, Burger and Klotzbücher observed that when blood was transfused from these subjects to other fasting healthy humans during the rise and height of the hyperglycemia of the donors, the blood sugar of the receptors increased. These results led Burger and his colleague to conclude that glucagon is present in blood and is responsible for hyperglycemia.

Saka (106), working on diabetic men and alloxan-diabetic animals, isolated from their urine and blood, by fractional ethanol precipitation at various pH's, a substance causing hyperglycemia and glycogenolysis in vivo and on liver slices, according to the method of Shipley and Hümel (95). He also demonstrated its presence in the blood of alloxan-diabetic animals and human diabetic subjects by the intravenous injection of a small quantity of blood of the diabetic men or animals in normal rabbits.

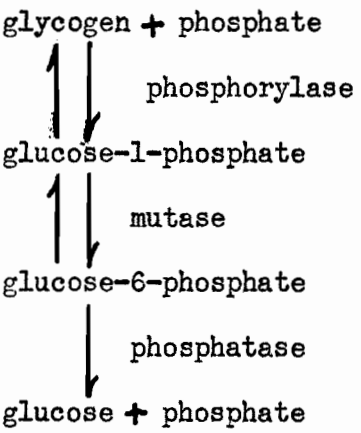
Moya and Hoffman (107) observed that the non-dialyzable concentrates from the urine of normal human subjects, at a dose of 20 mg./kg. body weight cause hyperglycemia in the rabbit. In vitro, these concentrates increase the glucose output of liver

slices but do so without causing a corresponding decrease in glycogen content. They note that the response of liver slices to these preparations is lower than that caused by pancreatic hyperglycemic protein.

Proposed Theories of Action of Hyperglycemic Factor

Despite the large volume of work done on the subject of the hyperglycemic factor, there is, at present, only one theory attempting to explain the mode of action of this principle. This theory has been proposed by Sutherland and Cori (108).

It has been agreed, as seen previously, that the site of action of the factor is the liver glycogen. Muscle glycogen, on the other hand, is unaffected by this substance (91). In the liver, the exchange glycogen-glucose includes three reactions (108) (109):



Of these three reactions, Sutherland and Cori found the exchange glycogen-glucose-1-phosphate to be the rate-limiting one. Working with radioactive phosphorus (P^{32}), they also rejected the possibility that the hyperglycemic factor may increase cell permeability to phosphate. On the basis of these observations and from the facts that cell structure must be intact and phosphate present for

activity of the factor (p.24), Sutherland and Cori proposed the phosphorylase theory of hyperglycemic factor action. This agent, they claim, increases the concentration of active phosphorylase both in vitro and in vivo, an effect which is of sufficient rapidity and magnitude to explain its hyperglycemic action. In other words, the hyperglycemic factor would influence the system that keeps a balance between the active and the inactive form of phosphorylase. They supplemented their work on this topic by a study on the effect of adrenalin on glycogenolysis to discover that this drug exerts on phosphorylase an action similar to that of hyperglycemic factor. Adrenalin, however, possesses other effects not shared by the factor. The main difference between the action of these two agents is that adrenalin, in vivo, causes an elevation of blood lactic acid whereas the factor does not.

This theory of Sutherland and Cori has not been much discussed by other workers; lack of experimental observations, pro or con, being presumably the reason. Some authors, including Levine (110), do not, however, believe in such a direct action and suggest that some factor from the adrenal glands is necessary for the action of the pancreatic principle.

Although not specifically proposed as such, another theory suggests itself from an experiment of Polis, Polis, Kerrigan and Jedeikin (111). On the basis of experiments done on a reaction

coupling oxidation with phosphorylation, the hyperglycemic factor would possess an ATP-ase action. In a system comprising liver homogenate, glucose, ATP, α-ketoglutarate and yeast hexokinase, phosphate esterification was accelerated by insulin and inhibited by the hyperglycemic factor. This suggestion cannot, however, be critically discussed because of lack of experimental data and confirmation. However, it remains a good working hypothesis for future work.

CLINICAL EVIDENCE- A detailed survey of human diabetes mellitus is beyond the scope of the present work. A few points should, however, be mentioned to complete the review of the evidence for the secretion of an Alpha cell hormone.

Human diabetes may be divided into two classes (3):

(a) One class is characterized by relatively high blood sugar and severe glycosuria controlled by moderate doses of insulin. In the absence of insulin therapy, patients of this class exhibit ketosis and die rapidly in coma. This class comprises mainly young people.

(b) The other class is composed of older people who show high blood glucose levels and glycosuria, but fail to exhibit ketosis. Patients of this class usually require very large doses of insulin to control their glycemia.

It appears therefore that condition (a) is the clinical equivalent of pancreatectomy in experimental animals, whereas condition (b) may be compared to alloxan diabetes. In other words, (a) would be due to an insufficient secretion of insulin and Alpha cell hormone, while (b) would be caused by an overproduction of hyperglycemic hormone of the Alpha cells.

Three other points deserve mention.

(a) Histological examinations of pancreas showed that diabetes mellitus is not characterized by any consistent change in islet tissue (112) (113).

(b) Pancreatectomy in the human results in insulin requirements lower than the requirements of most diabetics (114) (115) (116).

(c) Cases of insulin resistance are sometimes met in clinical practice (3) (112). It is suggested that the serum of such patients contains a factor antagonistic to insulin (117).

The various points reviewed are now summarized in order to demonstrate the value and the scope of the evidence supporting the hypothesis that the Alpha cells of the pancreas secrete a hyperglycemic-glycogenolytic hormone.

SUMMARY OF EVIDENCE

Biochemical evidence-

1- Pancreatectomy in alloxan-poisoned dog results in a fall of insulin requirement, but causes severe ketosis and death in the untreated animal.

2- Most of the commercial insulins show contamination with a hyperglycemic substance. Highly purified insulins, on the other hand, do not cause hyperglycemia.

3- Several papers reporting extraction of hyperglycemic substances from pancreas have been published.

4- Increased glycogenolysis in liver slices seem to be caused specifically by pancreatic extracts.

5- Certain experiments suggest the presence of a hyperglycemic substance in blood of animals and humans.

6- The site of action of the hyperglycemic factor appears to be the liver.

7- The physico-chemical properties of the hyperglycemic substance suggest that it is a protein.

8- The ligation of the pancreatic duct and consequent degeneration of the acinar tissue has no effect on the course of alloxan diabetes.

9- Normal amounts of hyperglycemic factor can be extracted from

pancreas of alloxan-poisoned animals or after degeneration of acinar tissue caused by duct-ligation.

Histological evidence-

1- The Alpha cells of the Islets of Langerhans present the histological picture of secretory cells, and are situated in an endocrine organ.

2- The distribution of the hyperglycemic substance roughly follows the distribution of islet tissue in the dog.

3- All species possessing islet tissue possess Alpha cells.

4- The Alpha cells of alloxan-poisoned animals appear to be normal.

5- The ligation of the pancreatic duct and consequent degeneration of the acinar tissue have no effect on the histological appearance of the Alpha cells.

These observations have led some workers to propose the hypothesis that the Alpha cells of the Islets of Langerhans secrete a hormone playing the following rôle:

1- It increases the blood sugar level and the total sugar production,

2- It decreases ketosis and prevents coma in the insulin deficient animal,

3- It acts on the glycogen reserves of the liver.

CHAPTER I

INVESTIGATION OF THE DEGRADATION PRODUCTS OF THE HYPERGLYCEMIC-GLYCOGENOLYTIC FACTOR OF THE PANCREAS

BASIS OF THE PRESENT INVESTIGATION

The pertinent literature previously surveyed contained frequent references to the greater stability of the hyperglycemic factor to alkali as compared to the stability of insulin. Treatments destroying the hypoglycemic property of insulin were said to be without effect on the hyperglycemic component of the commercial hormone (81).

Sutherland and Cori (96) observed that alkaline digestion of "insulin" released dialyzable materials which induced the breakdown of glycogen in liver slices. Quoting from their original paper: "When insulin solutions after incubation in 0.08N KOH were adjusted to pH 5.8 HCl, hydrogen sulfide was released and some protein was precipitated. The precipitate was removed and trichloroacetic acid was added to the clear supernatant fluid to give a final concentration of the acid of 5 per cent. A second precipitate formed which was removed and was found to be active in the glycogenolytic test. The filtrate was shaken with ether to remove the trichloroacetic acid. On the basis of quantitative biuret determinations it contained about 15 per cent of the alkali-treated insulin protein and per unit of protein was about one-half as active in

the glycogenolytic test as the original insulin. The trichloroacetic acid filtrate of native insulin did not contain material giving the biuret reaction and did not accelerate glucose output in liver slices."

"When alkali-incubated insulin was neutralized and dialyzed in a cellophane sac for 24 hours at 5°, 2 to 3 per cent of the protein was recovered in the dialysate on the basis of biuret determinations. The dialysate was about as active per unit of biuret reading as the undialyzed material. The dialysate of native insulin did not contain glycogenolytically active material. The change in the properties of the glycogenolytic factor after treatment with alkali (incomplete precipitation with trichloroacetic acid, partial diffusibility through a cellophane membrane) suggests that it can be degraded by alkali to some extent without losing its activity. The glycogenolytic activity of insulin was lost after more rigorous treatment with alkali (40 minutes at 100° in 0.08N KOH). The fact that the glycogenolytic factor in alkali-treated insulin lost its activity on incubation with trypsin suggests that it is a protein or polypeptide."

This suggestion, no doubt justified by the behavior of the dialysate, was not, however, supplemented by attempts to isolate such a polypeptide. The knowledge that a biologically active substance can be degraded to yield a simpler fraction retaining the activity of the original substance is in itself valuable. The

fact that Sutherland and Cori did not report attempts to isolate and characterize a substance the existence and activity of which they suggested themselves appears strange. Similarly, other workers of this field remained silent on this topic. The present work therefore reports, as far as is known, the first isolation and identification of a glucose-elevating breakdown product obtained from the digestion of the hyperglycemic factor of the pancreas.

A point of difference characterizing the work of the McGill group is the source of hyperglycemic factor. Stewart (77), in order to assure a generous supply of pancreatic extract, investigated the hyperglycemic property of the supernatant liquor from the first isoelectric precipitation of insulin obtained by the insulin-extraction method of Romans, Fisher and Scott (118). This solution, he noted, increased the blood glucose level of rabbits. Accordingly, in much of his work he used this liquor as a source of the factor whereas the other workers relied on commercial insulin. Moya (119) later observed, simultaneously with and independently of Sutherland and Cori, that an appreciable purification of the hyperglycemic factor could be achieved by fractional precipitation with trichloroacetic acid. Applying this treatment to the supernatant liquor from the first isoelectric precipitation of insulin, Moya found that most of the active protein could be precipitated by making the solution 1.6% with respect to this acid. The protein remaining in solution retained little activity and was

accordingly discarded.

In brief, the suggestion of Sutherland and Cori that alkaline partial hydrolysis of the hyperglycemic protein of the pancreas released a glycogenolytically active polypeptide served as the working hypothesis for this investigation. The search for this polypeptide and its eventual characterization have been the immediate purpose of the work reported in this chapter. Unlike Sutherland and Cori, who performed the hydrolysis on "insulin", the present investigation used the supernatant liquor from the first isoelectric precipitation of insulin.

EXPERIMENTAL

METHOD OF ASSAY: Shipley and Hümel (95) had shown that incubation of rat liver slices in serum in presence of insulin caused an increase in the glucose released when compared to liver slices incubated without insulin. In order to make use of this experiment as a dependable and reproducible method of assaying the hyperglycemic factor of the pancreas, Sutherland and Cori (96) modified the incubation technique by replacing serum as the medium with a saline-phosphate buffer of pH 7.4.

Since the work of isolation is made up of a series of various treatments, the assay of a large number of fractions is necessary. Such assays are required to detect the activity rather than to obtain quantitative data. The liver slice technique of Sutherland and Cori was accordingly made the criterion of activity of the various fractions investigated. Liver slice technique was reported by these authors to be insensitive to the hypoglycemic action of insulin (96), a behaviour favorable to the assay of glycogenolytic substances since traces of insulin often were present in some of the fractions studied.

Young rabbits of about 3.0 kg. body weight were anesthetized with nembutal, the central lobes of their livers excised, cut into 3 or 4 pieces and placed in cold physiological saline for 10 minutes. Pieces held firmly between two ground glass plates were sliced with razor blades.

The slices weighed from 50 to 100 mg. and were about 1 mm. thick. Not more than 20 slices were prepared from the liver of one rabbit. After blotting gently with filter paper, the slices were weighed on a torsion balance, matched as closely as possible for duplicates, and placed in the incubation tubes. In any one experiment, the slices were from the same section of the liver. Previous to placing the slices in the incubation medium, tubes were prepared by adding 2 ml. of isotonic saline-phosphate buffer of pH 7.4*, containing the required amount of test material. In all cases, the amount of material added to the buffer was small enough to maintain the tonicity and the pH close to those of physiological media. The control tubes contained only 2 ml. of saline-phosphate buffer. In a number of experiments, the amount of test material added per tube was expressed as micrograms of nitrogen per milliliter of incubation medium. As the isolation of the glucose-forming substance proceeded, the amount of material tested was expressed as micrograms of such material. The slices were incubated at 37° for 45 minutes or 60 minutes in open tubes, which were constantly agitated.

After incubation, 1 ml. was pipetted out of each tube into 10 ml. of tungstic acid solution for deproteinization. After standing for 15 minutes, the tungstic mixtures were centrifuged, and an appropriate sample

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* - Isotonic saline-phosphate buffer: 20 ml. 0.11M phosphate buffer of pH 7.4 (made by adding 15 ml. 0.11M KH_2PO_4 to 85 ml. of 0.11M K_2HPO_4) plus 100 ml. 0.9% NaCl.

of each supernatant solution was taken for glucose determinations by the Folin-Malmros method (120). The glucose elevation induced by the substances was measured as milligrams of glucose output per gram of tissue and was expressed as per cent and calculated as follows:-

$$\frac{\text{Glucose output of test samples} - \text{Glucose output of controls}}{\text{Glucose output of controls}} \times 100$$

PREPARATION OF THE DIALYSATE: The glycogenolytically active dialysate investigated in the present work was obtained by a process essentially the same as that applied on insulin by Sutherland and Cori (p.38) (96). The alkaline digestion was not, however, performed on the supernatant liquor from the first isoelectric precipitation of insulin as such but on a protein fraction derived from it and judged to be more potent than the original solution.

A volume of supernatant liquor from the first isoelectric precipitation of insulin was made 1.6% with respect to trichloroacetic acid by the dropwise addition of a 10% solution of this reagent (119). This treatment precipitated most of the glycogenolytic protein, leaving much of the inactive matter in solution. After standing in the ice box for 6 to 12 hours, the liquor was centrifuged and the supernatant phase discarded. The protein precipitate was dissolved in a small volume of water and its solution brought to pH 4.8 with 1N sodium hydroxide. Any precipitate forming at this pH was centrifuged off and discarded. The clear protein solution was placed in a cellophane sac and dialyzed

against cold running tap water for twenty-four hours. The protein solution thus free from diffusible impurities was then made 0.08N with sodium hydroxide and incubated at 37° for three hours with constant shaking.

The incubation completed, the pH of the solution was brought to 5.8 with hydrochloric acid, and the solution was dialyzed in the refrigerator against generous amounts of distilled water. At this pH, a strong odor of hydrogen sulfide was given off, indicating the splitting-off of sulfhydryl groups and/or -S-S- groups. A precipitate forming at this point was also discarded since it proved to be inactive. After dialysis, the residual protein solution was rejected and the combined dialysates taken to dryness on the steam bath under reduced pressure, using capryl alcohol as an antifoaming agent.

The dry dialysate gave a strongly positive biuret reaction, proving the presence of polypeptides. Much inorganic matter, mostly sodium chloride, was also present. Biological tests on rabbit liver slices indicated the glycogenolytic activity of this dialysate. (Table 1).

Table 1

Glycogenolytic effect of dialysate on rabbit liver slices

Experiment No.	Additions per cc. in gamma nitrogen	Increased output of glucose in per cent
1	100	72
		56
2	100	102
		71

The large amounts of test material used in the above experiment and in a few subsequent ones were necessary because of the presence of large quantities of inactive matter in the dialysate. With further purification of the active material, the amounts could be reduced.

INCREASE IN YIELD OF GLYCOGENOLYTIC DIALYSATE: The minute quantities of glycogenolytic dialysate resulting from the application of such a mild process of hydrolysis on the one hand, and the large amounts of material required for isolation work on the other, necessitated an increase in the yield of the digestion products. The first attempt to improve the process consisted of extending the digestion time from 3 to 8 hours. This modification proved to be unsuccessful, the amount of material liberated by an eight-hour hydrolysis being the same as that released by a three-hour one, as shown by the following experiment.

Table 2

Effect of digestion time on yield of nitrogenous products from glycogenolytic protein fraction

Incubation time in hours	Total dialyzable nitrogen in gamma
3	660
8	684

Twenty three milliliters of a protein solution containing 2.5 mg. nitrogen per ml. were divided in two equal parts. The two portions were submitted to the digestion process under the same conditions

except that the digestion time was 3 hours for one portion and 8 hours for the other. The dry dialysates were dissolved in 12 ml. of water each and analysed for nitrogen (Table 2).

The close agreement between the two yields seemed to be due to the fact that after three hours of digestion and equilibrium was reached between the protein and its breakdown products. To obtain further hydrolysis the products had therefore to be removed.

In order to achieve this purpose, multiple digestions of three hours each followed by dialysis were performed. The active protein solutions was incubated in 0.08N sodium hydroxide for 3 hours, the pH

Table 3

Glycogenolytic activity of fractions obtained by multiple digestions and dialyses

Exper. No.	Fractions tested	Additions per cc. in gamma nitrogen	Increased output of glucose in per cent
1	Protein before hydrolysis	100	105
	First hydrolysis	100	39
	Second hydrolysis	100	23
	Third hydrolysis	100	20
	Fourth hydrolysis	100	0
	Protein after the fourth hydrolysis	100	0
2	Protein before hydrolysis	100	134
	First hydrolysis	100	-
	Second hydrolysis	100	50
	Third hydrolysis	100	24
	Protein after third hydrolysis	100	0

adjusted to 5.8, the formed precipitate discarded, and the clear solution dialyzed in the cold against distilled water. The residual protein was then retreated in the same way. Four digestions and four dialyses were at first performed on the same portion of a protein solution. Table 3 indicates the results. These figures show that the first three digestions liberated active material whereas the fourth one was without effect. Consequently, three digestions of three hours each were performed on all the subsequent preparations. This experiment also proved that the initial protein is more potent than the first dialysate.

Another attempt to increase the yield of dialyzable active material was performed by subjecting the glycogenolytic protein to acid hydrolysis. The protein fraction obtained by precipitation with trichloroacetic acid was dissolved in water, the solution adjudted to pH 5.8, centrifuged and the supernatant liquid made 2N with respect to hydrochloric acid. This solution was then incubated at 37° for 24 hours. The acid was neutralized with sodium hydroxide and the solution dialyzed against distilled water in the refrigerator. The residue, collected on evaporation of the dialysate, was inactive, being mainly inorganic material and biuret-negative. Hence no further application was made of this process.

TREATMENT OF THE DIALYSATE WITH METHANOL: The first step towards isolating the active component released by the alkaline digestion of

the hyperglycemic protein was extracted with various organic solvents. It was hoped that this treatment applied to the active dialysate would separate the active substance from most of the inorganic matter. Normal butanol was ineffective, 95% ethanol retained minute amounts of active material, whereas absolute methanol extracted nearly all of the glycogenolytic material and left much of the inert matter undissolved.

Table 4

Extraction of the glycogenolytic component from the dialysate by alcohols

Fractions tested	Additions per cc. in gamma nitrogen	Number of slices averaged	Increased ouput in per cent
n-Butanol extract	100	4 slices from 2 rabbits	0
Ethanol extract	100	10 slices from 3 rabbits	4
Methanol extract	57	2 slices from 1 rabbit	81
Methanol extract	57	1 slice	123
Methanol-insoluble residue	100	4 slices from 1 rabbit	42

Accordingly, the dry dialysate was treated with three portions of absolute methanol (from 10 to 100 ml. each depending on the weight of the dialysate), the extracts combined and the methanol removed with suction. Some of these extracts were taken up in saline-phosphate buffer and tested on rabbit liver slices. Table 4 indicates the

selectivity of methanol as a solvent of the glycogenolytic component of the dialysate.

Both the methanol extract and the insoluble residue showed positive biuret reactions.

TEST OF THERMOSTABILITY: Some of the methanol extract taken up in water was boiled for one hour and tested on liver slices for possible loss of glycogenolytic potency. As shown in Table 5 the active component is thermostable.

Table 5

Effect of boiling on the glycogenolytic activity of the methanol extract of the dialysate

Fractions tested	Additions per cc. in gamma nitrogen	Number of slices averaged	Increased output in per cent
MeOH extr.	57	2 slices from 1 rabbit	31
MeOH extr. boiled for 1 hour	57	2 slices from same rabbit	31

TEST OF NON-REDUCING PROPERTY: The measurement of glucose output resulting from the incubation of test material with liver slices was based on the reducing property of the released glucose. It was then feared that an appreciable part of the reducing matter measured as glucose was in fact made up of the amount of added test material. To clarify this point, some of the methanol extract, in a quantity equivalent

to the amounts used in the biological tests, was submitted to the Folin-Malmros method for glucose determination. The following results were obtained.

Amount of methanol extract used with one rabbit liver slice	63 gamma nitrogen
Reducing power of this amount measured as glucose	12 gamma glucose
Range of extra glucose output produced by this amount of methanol extract	126 to 256 gamma glucose

The above experiment proved that the excess glucose produced by this extract could not be due to its own reducing property.

ATTEMPTED ISOLATION OF THE GLYCOGENOLYTIC FACTOR: Chromatography being considered as an appropriate means for the concentration of the active substance, fractionation of the methanol-extracted matter was accordingly attempted by various chromatographic processes. Though many trials were unsuccessful, one method finally led to a concentration of activity in one fraction.

(a) Chromatography on charcoal- An aqueous solution of the methanol extract was percolated, with the aid of suction, through a bed of charcoal (1gm. of charcoal Merck mixed with 4 gm. of powdered glass to assure porosity), the filtrate was collected and found to be biuret-negative but chloride-positive. The bed was washed with successive small (4 ml.) portions of distilled water, each portion being collected separately and tested for chloride. When the washings were chloride-free,

attempts were made to remove the adsorbed nitrogenous matter. Of the eluents tested, n-butanol, found to be the best one, eluted it only in part. One third of the nitrogenous material only was recovered. This eluate showed a weak biuret reaction.

Although the separation of sodium chloride from the biuret-positive matter could be easily achieved, this method had to be abandoned because of the difficulty in recovering the polypeptide compounds. No biological tests were done on the filtrate and washings.

(b) Chromatography on starch- Adsorption of the methanol extract on starch, followed by elution with n-butanol saturated with 0.1 N hydrochloric acid, was tried without success because of the extreme slowness of the percolation and the difficulty of tracing back all the biuret-positive substances. No biological tests were done on fractions collected by this method.

(c) Chromatography on ion-exchange resin- Amberlite MB1(formerly XE81) is a mixture of cation and anion exchange resins said to adsorb ionic substances of molecular weight lower than 200 to 300, allowing the heavier ones to pass through (121). It was thought that the inorganic matter and the amino acids might be adsorbed from the methanol extract, leaving the desired polypeptide in solution.

Five milliliters of an aqueous solution of the methanol extract, containing a total of 4.25 mg. nitrogen, were percolated successively through three columns of Amberlite MB1 of one gm. each. The test for chloride was negative after the third column. About 60% of the

nitrogenous material was retained by the resin. The filtrate, still strongly biuret-positive, was biologically inactive.

The fact that the mass of polypeptides, separated from very small peptides, amino acids and inorganic matter, did not promote glycogenolysis in liver slices, seemed to indicate that the active component was a substance of very low molecular weight, and not, as assumed in the beginning, a polypeptide.

FRACTIONATION OF THE METHANOL EXTRACT:- The fractionation of the active methanol extract of the dialysate resulting from the alkaline hydrolysis of glycogenolytic protein was successfully performed by submitting it to the chromatographic method of Fromageot, Jutisz and Lederer (122).

According to these authors, solutions of amino acids and peptides can be separated into four groups when percolated successively through columns of silica, alumina, and charcoal. The silica is said to retain the basic amino acids; the alumina, the dicarboxylic amino acids; and the charcoal, the aromatic amino acids; the neutral non-aromatic amino acids pass through all these adsorbents. The peptides are distributed in accordance with their amino acid contents. Elution with appropriate agents permits the recovery of the adsorbed material. Two modifications were made to this method in order to adapt it to the present need. Firstly, whenever the use of water saturared with hydrogen sulfide was prescribed, pure water was used because of the toxicity of this reagent to tissues. Secondly, the slow percolation of solutions through charcoal

was hastened by the application of suction.

After preparation of the adsorbents according to the above-mentioned procedure, an aqueous solution of 20 ml. containing approximately 20 mg. of the methanol-extracted material was first adjusted to pH 7.0, then percolated through silica, and the column was washed with water. The combined filtrate and washings were evaporated to 20 ml., the pH readjusted to 7.0 and the solution passed through the alumina column, which was then washed with water.

TABLE 6

Glycogenolytic effect of the chromatograms on liver slices

Fractions tested (groups)	Additions per cc. in gamma nitrogen	Increased glucose output in per cent Rabbit 1 2 slices averaged	Rabbit 2 2 slices averaged
silica eluate (1)	46	38	56
alumina eluate (2)	52	0	0
charcoal eluate (3)	48	77	130
charcoal filtrate (4)	45	28	66
MeOH extr. before chromatogr.	57	-	41

The volume of the mixed filtrate and washings was again evaporated to 20 ml., and glacial acetic acid added to a concentration of 5%. The solution was then passed through charcoal that had been deactivated

with ephedrine and the column washed with a 5% acetic acid solution. Group 1 was obtained by elution from silica with 0.1 N HCl; group 2, by elution from alumina with 1 N HCl; group 3, by elution from charcoal with water saturated with ethyl acetate; and group 4 consisting of the charcoal filtrate and washings. The four fractions were taken to dryness and redissolved in a few milliliters of water and assayed. Table 6 shows the results of the glycogenolytic tests; Table 7 compares the glycogenolytic activity of the four fractions with their biuret reactions.

TABLE 7

Comparison of biuret reactions with glycogenolytic activity of the four chromatograph fractions

Tests	Silica eluate (gp.1)	Alumina eluate (gp.2)	Charcoal eluate (gp.3)	Charcoal filtrate (gp.4)
Biuret	+ +	+ +	—	—
Glycogenolytic activity	+	—	+ +	+

Ephedrine, a sympathomimetic drug, is known to increase the blood sugar of intact animals (123). It was feared that the glycogenolytic activity of the charcoal eluate might be due to some ephedrine eluted during the process. To test this possibility, a blank was run in which charcoal that had been partially deactivated with ephedrine was percolated with water saturated with ethyl acetate, the eluate taken

down to dryness, and the hypothetical residue, dissolved in saline-phosphate buffer, tested on liver slices. At the same time, ephedrine dissolved in saline-phosphate buffer was tested on liver slices (50 gamma ephedrine per ml.). None of these two preparations showed activity.

The possibility that ephedrine was the cause of the activity of the charcoal eluate thus being eliminated, glycogenolysis produced by this fraction was therefore due to active material eluted from this adsorbent. The fact that the active charcoal eluate was biuret-negative indicated that the active material was not a polypeptide. The glycogenolytic activity might then be due to a dipeptide or even an amino acid.

STUDY OF GLYCOGENOLYTIC ACTIVITY OF AMINO ACIDS AND BIOLOGICAL COMPOUNDS:

The results of the last experiments showing that a biuret-negative fraction could stimulate glucose formation prompted the study of the action of some amino acids and biological compounds on liver slices. The availability of the substances tested was the main factor in their selection. The results of these experiments are listed in Table 8.

This table shows clearly that while most amino acids have little effect on the glucose output when incubated in high concentrations with liver slices, l-cystine, and to a lesser extent, l-cysteine showed significant activity. Glutathione and ACTH also induced appreciable glycogen breakdown*.

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*- Conn et. al. have produced a temporary diabetes in man by the injection of purified ACTH (124).

This effect of cysteine and cystine is at variance with the findings of Sutherland and Cori (96) who, testing different substances in order to demonstrate that glycogenolytic activity was specific to the hyperglycemic factor of pancreas, reported that cysteine and cystine caused no glucose increase in liver slices.

TABLE 8

Glucose elevating activity of some amino acids and biological compounds on rabbit liver slices

Substances tested	Additions per cc. in gamma nitrogen	Increased glucose output in per cent (2 slices averaged)
Glycine	60	13
Glycylglycine	60	27
l-Leucine	60	12
l-Arginine	60	12
l-Tyrosine	saturated	0
l-Phenyl alanine	21	7
l-Tryptophane	15	17
l-Glutamic acid	15	16
l-Cystine	15	65
l-Cysteine	19	58
ACTH	15	44
Glutathione	15	47
Cortisone	(25 ug. C.A./ml.)	29
Initial protein	19	50
Charcoal eluate	60	54

The suggestion of the above experiments that cystine or cysteine could be the cause of the activity found in the charcoal eluate was not considered to be serious because of the lability of cystine and cysteine to alkaline digestion. The evolution of hydrogen sulfide during the hydrolysis is an indication of this lability. However, the glycogenolytic effect of cystine was taken advantage of in using this amino acid as a standard of comparison for glycogen breakdown activity in many of the subsequent experiments.

ACID HYDROLYSIS OF CHROMATOGRAPH FRACTIONS: The glucose-forming activity of some amino acids and biological compounds suggested the study of the behavior of the chromatographic fractions after acid hydrolysis. If the activity were due to a peptide, acid hydrolysis, by splitting the peptide into its constituent amino acid would destroy the action of the peptide leaving only the intrinsic activity of the component amino acids. If the activity were retained after acid hydrolysis, it would not be due to the presence of an active peptide but may reside in an amino acid or in a substance of an entirely different chemical nature.

A few milligrams of each of the silica and charcoal eluates and of charcoal filtrate (alumina eluate was ignored in this experiment because of its lack of activity) were placed into flasks. Two ml. of water and 2 ml. of concentrated hydrochloric acid were added making the solutions 6 N with respect to HCl. The flasks were fitted with condensers, placed on a sand bath and refluxed for 20 hours. After this time, the solutions were taken to dryness with suction at room

temperature, and the suction left on to remove the excess of HCl. The nitrogenous substances were then present as their hydrochlorides.

TABLE 9

Effect of acid hydrolysis on the glycogenolytic activity of silica eluate, charcoal eluate and charcoal filtrate

Fractions tested	Additions per cc. in gamma nitrogen		Increased glucose output in per cent	
	Exper. 1	Exper. 2	Exper. 1	Exper. 2
Non-hydrolysed silica eluate	50	45	17	17
Hydrolysed silica eluate	50	45	21	11
Non-hydrolysed charcoal eluate	45	45	64	50
Hydrolysed charcoal eluate	45	45	0	18
Non-hydrolysed charcoal filtrate	-	45	-	13
Hydrolysed charcoal filtrate	-	45	-	0

The residues were dissolved in saline-phosphate buffer to yield solutions of 50 gamma nitrogen per ml. The pH of the solutions was close to 7.4. These were then tested on rabbit liver slices along with samples of the same fractions not submitted to acid hydrolysis. Table 9 summarizes the results.

The behavior of these fractions after acid hydrolysis as compared

to their behavior previous to this treatment seemed to indicate that the activity of the charcoal eluate was due to a peptide made of inactive amino acids whereas the effect of the silica fraction was due to some amino acid or to an acid-stable substance. If the results of this experiment were entirely reliable, very attractive conclusions could be drawn by a pursuit of this line of thought. According to Fromageot et. al., charcoal is supposed to adsorb aromatic amino acids. Glycogenolytic tests on amino acids showed that the two aromatic ones, tyrosine and phenylalanine, possess no intrinsic glycogenolytic activity. This might suggest that the activity of the charcoal fraction was due to a peptide composed of aromatic amino acids. Though attractive, this conclusion proved erroneous.

FRACTIONAL ELUTION FROM CHARCOAL: The impure state of the charcoal eluate rendered the analysis for active material unreliable or even impossible. In order to attempt the further purification and possibly the isolation of the active component, two treatments were applied to the charcoal eluate. Since the activity could be concentrated on charcoal, the subsequent preparations of active material from the supernatant liquor of the first isoelectric precipitation of insulin were performed without eluting the material adsorbed on silica and on alumina.

It was thought that fractional elution from charcoal could produce a concentration of activity. To that effect, an alumina filtrate made 5% with respect to acetic acid was run through charcoal and washed

with 5% acetic acid as described previously (p.55), leaving adsorbed on charcoal an amount of material corresponding to 14.3 mg. of nitrogen. This was eluted by running 5 portions of 30 ml. each of water saturated with ethyl acetate, each fraction being collected

TABLE 10

Glycogenolytic activity of the fractions collected by fractional elution of active material from charcoal

Fractions	Nitrogen in mg.	Additions per cc. in gamma nitrogen	Increased glucose output in per cent (2 slices averaged)
1	8.2	12	0
2	2.2	12	7
3	1.7	12	0
4	0.8	12	24
5	0.7	12	11
Total N recovered	13.6	-	-
Total N adsorbed	14.3	-	-
cystine	-	12	28

separately. This elution removed about 95% of the adsorbed matter as shown in Table 10. Assays on liver slices showed that the fractions richest in nitrogenous matter were devoid of glycogenolytic activity; fraction 4 with low nitrogen being the most potent. Its potency approached that of cystine. Once more, glycogenolytic activity did not run parallel to nitrogen content. Although this method appeared

fairly promising, it was put aside with the intention of returning to it if no better method of purification could be found.

TREATMENT OF CHARCOAL ELUATE WITH ORGANIC SOLVENTS: Methanol, as seen before, dissolved, among other substances, the glycogenolytic factor of the dialysate resulting from the alkaline hydrolysis of active pancreatic extracts. It was reasoned that since the composition of the active mixture had changed, the solubility of the components would have also been modified, some of the constituents at first soluble in methanol being insoluble at this point.

Several trials at extracting the active component with n-butanol and 95% ethanol from the solid charcoal eluate proved unsuccessful as shown in Table 11. Faint activity was noted in methanol extracts, but the main part of the active material remained undissolved by this alcohol. Moreover, experiment 4 proved definitely that the glycogenolytic potency of the fraction investigated was dissociated from nitrogenous matter. It thus confirmed the hint suggested by the experiments on fractional elution (p.61) and on Amberlite MB1 (p.52) that the nitrogen level and glycogenolytic activity did not run parallel. The initial working assumption that an active polypeptide was the cause of increased glucose accumulation, later changed to that of a biuret-negative dipeptide or amino acid, had to be modified once more to the assumption of a nitrogen-free substance as the factor inducing glycogen breakdown in liver slices.

Up to the present stage of the work, relatively small volumes of supernatant liquid from the first isoelectric precipitation of insulin were used in the attempt to isolate and identify the diffusible glycogenolytic factor of its alkaline digestion. This restraint was imposed

TABLE 11

Glycogenolytic activity of the fractions obtained by extraction of dried charcoal eluate with methanol

Exper. No.	Fractions tested	Additions per cc. in gamma nitrogen	Increased glucose output in per cent (2 slices averaged)
1	MeOH extract	30	8
	MeOH residue	30	26
2	MeOH extract	51	7
	MeOH residue	83 ug. residue	53
3	Intact charcoal eluate	44	32
	MeOH extract	46	6
	MeOH residue	46	66
4	MeOH residue	100 ug. N-free residue	56

by the uncertainty of the outcome of a treatment and the limited availability of the starting material. The quantities of liquor at the start were from 200 to 500 ml. yielding from 1 to 3 gm. of solid dialysate, about one fifth of which was methanol-extractable. Consequently, the amounts of impure charcoal eluates collected from such volumes of starting material were minute (about 10 to 20 mg. with each preparation).

Further purification being hindered by such small quantities of working material, a batch was prepared from 4 litres of supernatant liquor from the first isoelectric precipitation of insulin by essentially the same procedure as that described previously. This run produced 26 gm.

of solid dialysate, 6 gm. of which were methanol-soluble. On elution from charcoal, from 1.5 to 2.5 gm. of a syrupy semi-solid mixture were obtained. Treatment of this eluate with methanol removed the nitrogenous matter leaving a nitrogen-free but glycogenolytically active residue, as experiment 4, Table 11, indicates.

TABLE 12

Physical and chemical tests performed on the nitrogen-free, active, charcoal eluate

Tests	Remarks	Results
Nitrogen	According to Nessler	Nil
Reducing property	As glucose, by Folin-Malmros method	Nil
Sulphur	Sample heated on dime	Nil
Carbohydrate	Molish test	Nil
Chloride	Silver nitrate	Positive
Combustibility	Chars a little leaving a residue	-
pH	Aqueous sol'n of syrup	5.8
Thermostability	Sample of syrup in tube placed in boiling water for 1 hr. No decrease in activity	stable
Solubility	In water	Soluble

PHYSICAL AND CHEMICAL TESTS ON NITROGEN-FREE ACTIVE CHARCOAL ELUATE:

The larger supply of working material permitted the application of some physical and chemical tests to the active nitrogen-free eluate with the

hope of gaining some clue to the nature of its active component. Table 12 summarizes the tests with their results.

The great quantity of incombustible material, the absence of nitrogen, and the many negative responses to the applied tests suggested the investigation of the possibility that an inorganic constituent be the cause of glycogenolysis. The following paragraph describes this enquiry.

ISOLATION OF THE GLYCOGENOLYTIC FACTOR: The above table shows that the pH of an aqueous solution of the syrup was on the acid side. The fact that a few metallic hydroxides are insoluble in water suggested the idea of attempting the precipitation of some of the inorganic matter of the syrup by rendering its pH alkaline.

To that effect, a small sample of the syrupy matter, dissolved in a few milliliters of water, was made alkaline by the dropwise addition of a solution of sodium hydroxide. A heavy white precipitate appeared at once. Sodium hydroxide was added until precipitation was complete. After filtration with suction and washing with water, the precipitate was tested for glycogenolytic potency, and compared to the activity of the filtrate. The precipitate showed glycogenolytic action whereas the filtrate was inactive as seen in Table 13. This treatment was then applied to the rest of the syrup to yield a total of approximately 0.5 gm. of a white active powder.

Table 13

Glycogenolytic activity of the precipitate and the filtrate resulting from the alkaline treatment of the syrup

Exper. No.	Fractions tested	Additions per cc.	Slices averaged	Increased glucose output in per cent
1	Pptate	50 gamma	3	58
	Filtrate	50 "	2	0
2	Pptate	52 "	2	43
3	Pptate	52 "	3	35

IDENTIFICATION OF THE GLYCOGENOLYTIC FACTOR: A melting point test and a flame test soon showed that the precipitate obtained by treating the syrup with alkali was inorganic. It failed to melt when heated to 300° and did not burn nor carbonize in the flame. Once more, the working assumption had to be discarded to be replaced this time by that of an inorganic substance as the glycogenolytic factor. Being inorganic, the precipitate could easily be identified. A systematic qualitative analysis (125) of the cations performed on approximately 0.2 gm. of this powder, proved that this substance was a mixture of the hydroxides of calcium and magnesium, calcium hydroxide being predominant.

Which of the two ions was responsible for glycogenolytic activity was demonstrated by testing calcium and magnesium ions separately and together on rabbit liver slices, and comparing their effect to that of the precipitate. Zinc, known to be present in pancreas (126)

and in some commercial insulins, was also tested, as its chloride, for glycogenolytic potency. The three experiments reported in Table 14 revealed that the activity of the precipitate was due to the presence of calcium ions.

SOURCE OF THE ISOLATED CALCIUM: The first question that suggested itself on consideration of the above results was that of the source of the extracted calcium (and magnesium). The solutions investigated had been percolated through columns of silica, alumina and charcoal. The presence of calcium and magnesium as impurities in these adsorbents was a distinct possibility. This objection was easily refuted by making the methanol extract of the dialysate alkaline with sodium hydroxide previously to chromatography. Such a treatment resulted in the precipitation of some inorganic matter identified as calcium and magnesium hydroxides. This precipitation, although definitely observed, took place to a smaller extent than after chromatography. The presence of organic matter was most likely the cause of this inhibition since it is known that such matter prevents calcium and magnesium from precipitating totally (125).

The previous experiment disposing of the objection that calcium and magnesium could have originated in the adsorbents had to be complemented by work establishing the exact source of these two ions. Calcium and magnesium, if present in the initial protein investigated (that is, in the protein fraction derived from the supernatant liquor from the

Table 14

Glycogenolytic activity of some metallic ions as compared to the activity of the precipitate

Exper. No.	Additions per cc.	Slices averaged	Increased glucose output in per cent
1	Mg ions (50 ug. MgO)	2	4
	Zn ions (70 ug. $ZnCl_2$)	2	10
	Ca ions (50 ug. $Ca(OH)_2$)	1	33
	Cystine (50 ug.)	2	25
	Precipitate (50 ug.)	2	27
2	Mg ions (50 ug. MgO)	2	4
	Ca ions (50 ug. $Ca(OH)_2$)	2	41
	Ca and Mg ions (25 ug. $Ca(OH)_2$, 25 ug. MgO)	2	2
	Precipitate (50 ug.)	2	28
3	Mg ions (25 ug.)	2	0
	Ca ions 25 ug.)	2	40
	Ca and Mg ions (12.5 ug. Ca, 12.5 ug. Mg)	2	9
	Precipitate	2	31

first isoelectric precipitation of insulin by treatment with trichloroacetic acid) could exist in two different forms. They could have been present as free and dialyzable ions, or could have occured as components bound to protein. An experiment devised to elucidate this point showed that calcium and magnesium were bound to the original hyperglycemic-glycogenolytic protein mixture.

A sample of the supernatant liquor from the first isoelectric precipitation of insulin was made 1.6% with respect to trichloroacetic acid. The precipitate was dissolved in water, the pH adjusted to 4.8 with sodium hydroxide and the precipitate thus formed was removed by centrifugation and discarded. The clear protein solution, identical to the solution that yielded calcium and magnesium, was then dialyzed against cold tap running water for 24 hours. For another 24 hours, it was dialyzed against distilled water in the cold, the water being frequently changed. The last lot of distilled water, allowed to remain in contact with the dialyzing sac for 9 hours, was taken to about 25 ml. with suction and heated on the steam bath. The concentrate of this dialysate, made ammoniacal, failed to yield a precipitate on addition of ammonium oxalate. Similarly, addition of disodium monohydrogen phosphate had no effect. Calcium and magnesium did not accordingly occur in a free state.

The protein solution thus thoroughly dialyzed was then evaporated to dryness and burnt totally over a flame in a platinum crucible.

The white ash, dissolved in water, was tested for the presence of calcium and magnesium. Made ammoniacal, the solution yielded, on addition of ammonium oxalate, a white crystalline precipitate insoluble in acetic acid and soluble in hydrochloric acid, tests characteristic of calcium (125). The ammoniacal filtrate resulting from the removal of the calcium oxalate yielded, on addition of disodium monohydrogen phosphate, a white crystalline precipitate thus indicating the presence of magnesium.

CALCIUM AS THE ONLY GLUCOSE-ELEVATING COMPONENT OF THE DIALYSATE: The hyperglycemic activity of calcium ions observed on addition of a calcium salt to the incubation medium in presence of rabbit liver slices proved by no means that the glucose-forming action of the protein dialysate was entirely caused by the presence of calcium. A sample of calcium-free dialysate was then assayed in order to determine if it would still exhibit a glucose-rising action. The calcium content of a preparation of protein dialysate was first determined and found to be 80 micrograms per milliliter. After the pH had been taken to 9 with ammonia, an amount of ammonium oxalate exactly equivalent to the amount of calcium present was then added to an aliquot of this dialysate solution, in order to avoid residual calcium or excess oxalate. After standing overnight in the cold, the calcium oxalate precipitate was centrifuged off and the clear supernatant liquid divided in two equal portions. To one was added solid

calcium chloride to a concentration of 80 micrograms of calcium per milliliter, to other portion being kept for assay of calcium-free dialysate. The three portions, untreated dialysate, calcium-free dialysate and calcium-free dialysate to which calcium was added, were then assayed for effect on rabbit liver slices in saline-phosphate buffer. Table 15 proves that the glucose-forming action of the dialysate was entirely

Table 15

Effect of removing the calcium of the dialysate on the glucose output of rabbit liver slices

N content: 95 ug./ml.: Ca; 16 ug./ml. of incubation media

Exper. No.	Increased glucose output in per cent		
	Untreated dialysate	Ca-free dialysate	Ca-free dialysate with Ca added
1	88	2	97
2	36	0	38

due to its content of calcium ions. Thus was rejected the idea of the possible presence of another active component in the dialysate.

CALCIUM AS THE ACTIVE COMPONENT OF THE HYPERGLYCEMIC FACTOR OF THE PANCREAS: Since the glucose-rising effect of the dialysate of the hyperglycemic protein was found to be due to the presence of calcium ions, it was thought desirable to find out whether the activity of the original hyperglycemic protein is due to its content of bound calcium. Unfortunately, this question cannot be answered since bound calcium

cannot be removed from the protein molecule without denaturing it. An attempt was made to precipitate the bound calcium butaddition of ammonium oxalate to the well dialyzed active protein solution, but no precipitate of calcium oxalate formed.

Table 16

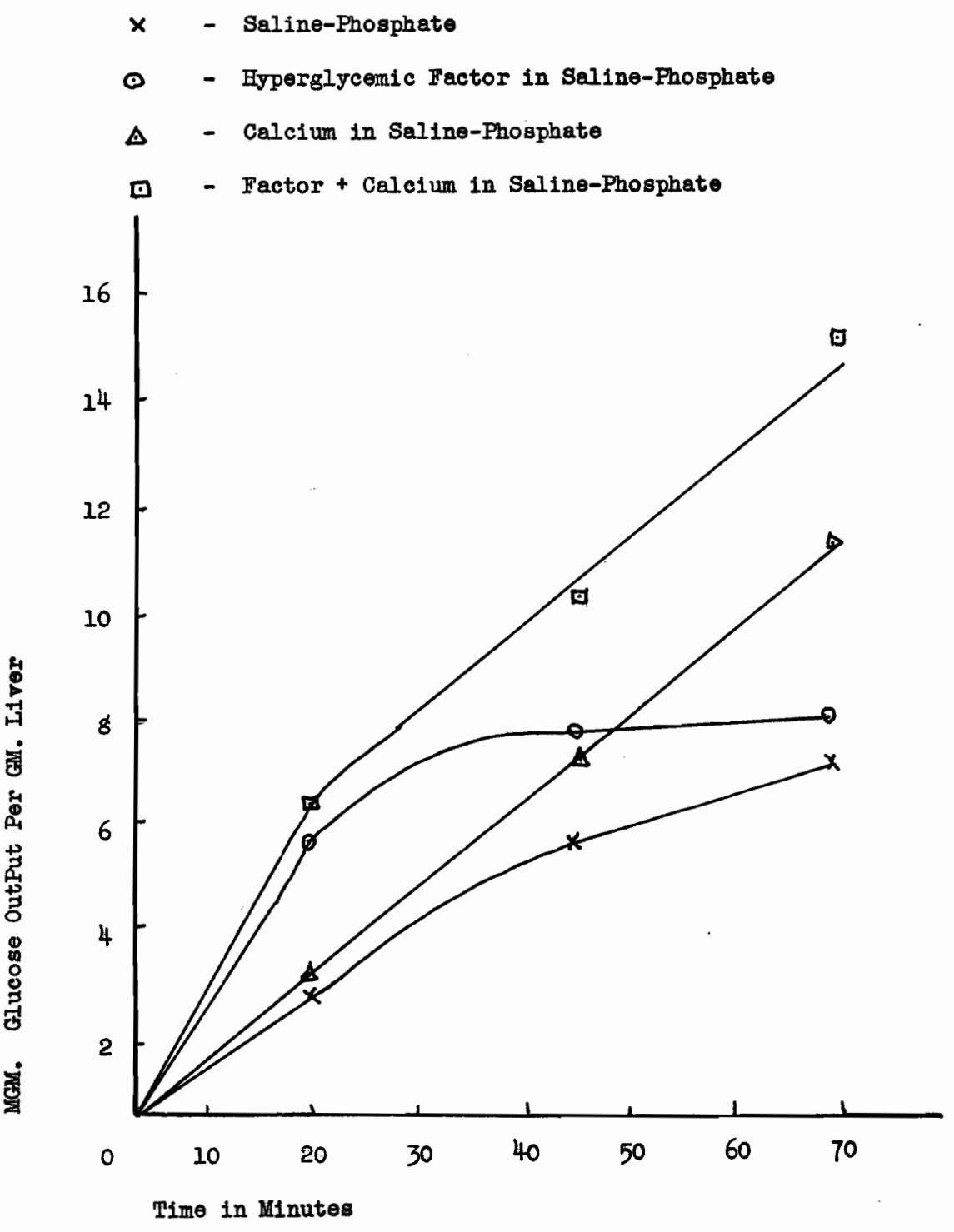
Combined effect of hyperglycemic factor and calcium on glucose output of rabbit liver slices

	Increased glucose output in per cent (2 slices aver.)		
Exper. No.	Hyperglycemic factor	Calcium M/1600	Hyperglycemic factor with calcium at M/1600
1	64	45	100
2	57	49	73

The unsuccessful attempt to remove the bound calcium from the active protein preparation was replaced by a study of the combined action of both the hyperglycemic factor and calcium. It was found that the presence of calcium at a level of M/1600 in the incubation buffer increased significantly the glucose-rising effect of the protein. The results are seen in Table 16. Moreover, time curves (fig. 1) studying the progress of the glucose accumulation caused by calcium and the factor individually, and by both combined, almost suggested that calcium was necessary for the activity of the hyperglycemic factor. Further experiments studying glycogen disappearance caused by each of these agents showed, however, that their mode of action is different.

Figure 1

Glucose Output of Liver Slices Caused by Different Agents

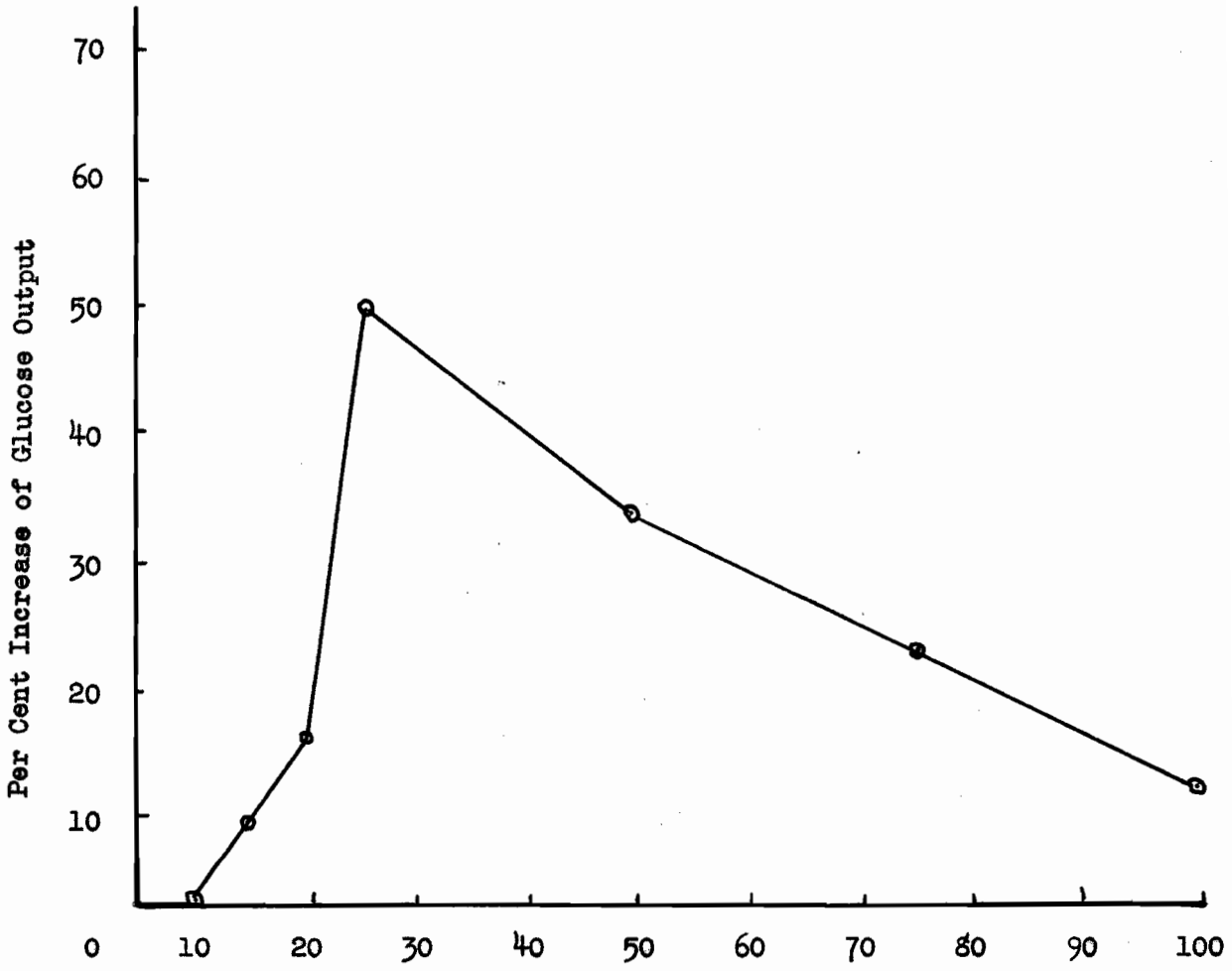


RELATIONSHIP BETWEEN THE GLUCOSE OUTPUT AND CALCIUM CONCENTRATION OF THE INCUBATION MEDIUM: The experiments done on the active dialysate from the hyperglycemic protein showing the glucose-promoting action of calcium on liver slices were obviously done at an unknown concentration of calcium, since this ion was not suspected to be the active agent. Most of the planned experiments were, however, done at a level of 25 micrograms per milliliter of incubation buffer. Experiments were performed in the usual way to establish the relation between calcium level and the degree of glucose accumulation. Figure 2 places the height of activity at a calcium concentration of 25 micrograms per milliliter and indicates that the activity of calcium in promoting glucose formation is not at all proportional to its concentration. On the contrary, concentrations of calcium higher than 25 micrograms per milliliter produce a declining glucose accumulation. It may be noted that a concentration of 25 micrograms (M/1600) is within physiological limits, since plasma and interstitial fluid contain 10 milligrams per cent, that is 100 micrograms per milliliter (M/400) of this ion. This point will be considered more fully in the discussion.

CALCIUM AS THE POSSIBLE ACTIVE COMPONENT OF THE DIALYSATE FROM ALKALI-TREATED COMMERCIAL INSULIN: The suggestion of Cori (96) that the pancreatic hyperglycemic factor could be degraded to yield a dialyzable product exhibiting the hyperglycemic property of initial

Figure 2

Glucose Output of Liver Slices at Different Calcium Concentrations



protein, a suggestion accepted as the working hypothesis of the present work, was derived from experiments done on commercial insulins. It could be argued that the work reported here, leading to the observation that calcium was the component of the dialysate responsible for increase in glucose output of liver slices, was performed, not on insulin, but on the supernatant liquor from the first isoelectric precipitation of insulin. Calcium, present in this liquor as protein-bound, was never reported to be present in insulin. In order to reject this last objection, two insulin samples of different degree of purity were analyzed for calcium by the method of Sendroy (127) (128). The two samples were obtained through the courtesy of The Connaught Laboratories of Toronto. One was an impure protein taken during the process of insulin production. The other was a sample of the purest crystalline zinc-insulin prepared by this firm. Calcium analysis performed on 50 milligrams of each of these two samples showed the presence of this material at a concentration of 10 micrograms per milligram of the impure insulin and of 2/3 microgram per milligram of the very pure crystalline zinc-insulin. The definite presence of calcium in samples of pure commercial insulin indicates that the increased glucose output observed by Sutherland and Cori on liver slices, when small amount of dialysate was added, may very well have been due to the presence of calcium. It may be remarked that a quantity of 2/3 microgram calcium per milligram of insulin protein is

not too small to produce a glucose-elevating calcium concentration in the incubation medium since the dialysate tested for activity is prepared from larger amount of alkali-treated insulin. In other words, Cori and his colleague could very well have attained a concentration of 25 micrograms per milliliter of incubation medium by concentrating a dialysate originating from, say, half a gram of insulin protein.

COMPARISON BETWEEN THE MODE OF ACTION OF CALCIUM AND THAT OF THE HYPERGLYCEMIC FACTOR:

(a) ORIGIN OF THE EXTRA GLUCOSE - The previous work indicating a glucose-accumulating action of calcium finds its logical sequence in a study of the over-all process of this action. Sutherland and Cori had used the term "glycogenolytic" to indicate the action of the component of the dialysate causing a rise in the glucose output on liver slices. They had assumed that the substance was causing an elevation of the medium sugar by increasing the rate of glycogen breakdown. It was thought desirable to investigate the mechanism of action of calcium in this respect in order to know whether calcium was really glycogenolytic and, if not, to know by what process it is acting.

Experiments on rabbit liver slices were performed for the purpose of measuring the glycogen disappearance and to compare it with glucose output in saline-phosphate buffer with and without calcium and hyperglycemic factor. This was done in order to measure to what extent the glucose produced represents glycogen broken down. The method used was

essentially the liver slice technique of Sutherland and Cori used in previous experiments. Measurement of glycogen*before incubation was obtained on two slices assayed immediately after slicing. The other slices were incubated in 2 ml. of their respective medium for 45 minutes at 37°. Then, 1 ml. was withdrawn from each tube for analysis of the glucose output, and the glycogen was determined on the incubated slices. From these data, it was calculated to what extent the glucose output represented the glycogen breakdown, and the influence of calcium or of the hyperglycemic factor on glycogenolysis. Table 17 reveals very interesting relationships which may lead to productive speculations. These experiments show that calcium, unlike the hyperglycemic factor, is not glycogenolytic. Its presence in the incubation medium does not increase the amount of glycogen broken down but causes an elevated accumulation of glucose, as compared to media devoid of it, by partly shifting the orientation of the broken down glycogen towards glucose rather than via glycolysis. The significance of this action will be considered in the discussion.

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* Method of glycogen analysis: To the tube containing the liver slice was added 2 ml. of saturated KOH and the tube placed in boiling water for 45 min. To the hot solution was added 1.3 volumes of 90% EtOH, stirred and allowed to stand in the cold overnight. The glycogen was centrifuged and the precipitate washed twice with 60% EtOH and once with 90% EtOH. The glycogen was then hydrolyzed for 2 hours with 1 ml. of N sulphuric acid, the acid neutralized with NaOH, the volume made up to 25 ml. with water and sugar determined on a 0.5 ml. sample.

Table 17

Effect of hyperglycemic factor and of calcium on the glycogen disappearance and its recovery as glucose

Exper. No (2 slices aver.)	Agents and Medium	ug. glycogen disappeared /100 mgm of liver	per cent recover as glucose
1	saline-phosphate buffer	1568	58
	buffer + hyperglycemic factor	2056	59
	buffer + calcium	1606	82
	buffer + calcium + factor	2081	90
2	saline-phosphate buffer	876	55
	buffer + hyperglycemic factor	1440	57
	buffer + calcium	871	83
	buffer + calcium + factor	1414	86

(b) INFLUENCE OF CALCIUM IONS AND OF HYPERGLYCEMIC FACTOR ON OXYGEN CONSUMPTION OF LIVER SLICES - The above-mentioned work showed that calcium elevated the glucose output of liver slices without

Table 18

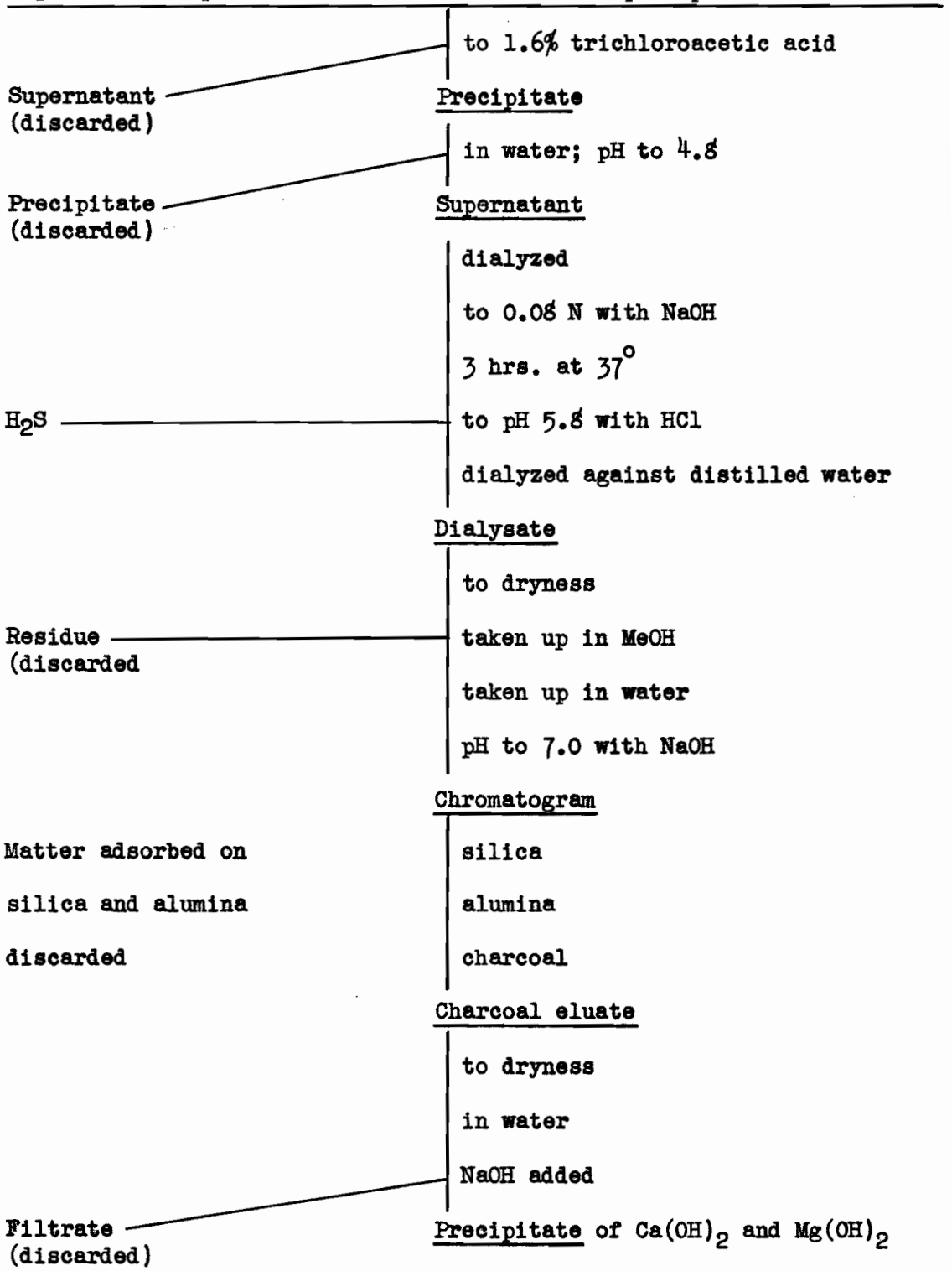
Effect of calcium and of hyperglycemic factor on oxygen consumption of rat liver slices

Exper. No.	Agents tested	$-Q^{O2}$
	saline-phosphate buffer	7.9
1	buffer + calcium at 100 ug./ml.	6.2
	buffer + calcium at 25 ug./ml.	5.2
	saline-phosphate buffer	7.5
	buffer + factor	7.0
2	buffer + factor + Ca at 25 ug./ml.	7.0
	buffer + Ca at 25 ug./ml.	5.1
	saline-phosphate buffer	7.4
	buffer + factor	7.3
3	buffer + factor + Ca at 25 ug./ml.	7.2
	buffer + factor + Ca at 25 ug./ml.	5.2

increasing glycogenolysis. Glucose, in these conditions, should then accumulate at the expense of glycolysis. In other words, calcium, by shifting glycogenolysis towards glucose production should therefore inhibit partly glycolysis with the consequence that oxygen consumption of liver slices should be reduced. This behaviour was in fact observed when rat liver slices were incubated in Warburg vessels in an atmosphere of oxygen at 37° for 45 minutes. The $-Q^{O_2}$'s calculated indicate, on the one hand, a reduced oxidation resulting from a weaker glycolysis due to the presence of calcium ions, and, on the other hand, the reversal of this inhibition by the hyperglycemic factor. It is seen in Table 18 that the factor does not by itself increase the oxygen consumption but, presumably by supplying glycolysis and the tricarboxylic acid cylcle with more fuel, relieves the tissue from the calcium inhibition.

Conclusion: With this set of experiments ends the first part of this thesis. This chapter devoted to the search of the glucose-promoting agent of the degradation products of pancreatic hyperglycemic protein established the absence of a glycogenolytic peptide, on the one hand, and, on the other hand, the influence of calcium ions in the orientation of carbohydrate metabolism in these conditions. The following discussion will now attempt to stress the significance of these findings.

SYNOPSIS OF THE EXTRACTION PROCESS



SUMMARY OF THE EXPERIMENTAL WORK REPORTED IN CHAPTER I

(a) PURPOSE OF PRESENT WORK: An attempted isolation and characterization of the glycogenolytic component (s) of the dialysate collected from the alkaline hydrolysate of a glycogenolytic extract of pancreas.

(b) WORKING HYPOTHESIS: Originally, that a polypeptide was the cause of increased glucose output induced in liver slices; first modified to a biuret-negative peptide or amino acid; then changed to an organic nitrogen-free compound; finally that an inorganic substance was responsible for most of the glucose-elevating action.

(c) SOURCE OF PANCREATIC EXTRACT INVESTIGATED: A protein fraction derived by precipitation with trichloroacetic acid from the supernatant liquor from the first isoelectric precipitation of insulin. This supernatant liquor arose from the insulin extraction method of Romans, Fisher and Scott.

(d) CRITERION OF BIOLOGICAL ACTIVITY: Increased glucose output induced in rabbit liver slices incubated in saline-phosphate buffer, according to the method of Sutherland and Cori.

(e) METHOD OF ISOLATION OF GLYCOGENOLYTIC COMPONENT: See synopsis on previous page.

(f) NATURE AND ORIGIN OF THE GLUCOSE-ELEVATING SUBSTANCE ISOLATED: A calcium salt; calcium ions being the cause of increased glucose accumulation. The calcium was shown to be bound to the protein fraction used as starting

material, and not present as dialyzable ions.

(g) VARIOUS OTHER POINTS: (1) An increase in the yield of dialyzable glucose-elevating matter arising from the alkaline digestion of the active protein fraction could be achieved by applying multiple digestions, each digestion followed by dialysis.

(2) The glucose-rising activity of amino acids and other biological compounds was investigated with the results that cystine, and to a lesser extent, cysteine, glutathione and ACTH were found to cause some increase in the release of glucose by liver slices.

SUMMARY

(a) The evidence pointing to the existence of a hyperglycemic-glycogenolytic principle of the pancreas is reviewed.

(b) The hypothesis according to which the site of secretion of the hyperglycemic-glycogenolytic factor would be the Alpha cells of the Islets of Langerhans is presented.

(c) The reports claiming the isolation of hyperglycemic and glycogenolytic substances from pancreas and from crude insulin are surveyed.

(d) The experiments that led Sutherland and Cori to claim that the hyperglycemic and glycogenolytic protein of the pancreas can, on alkaline degradation, yield a dialyzable product, presumably a polypeptide, exhibiting glycogenolytic activity *in vitro* is described and presented as the basis of the present work.

(e) The alkaline digestion-dialysis procedure that Sutherland and Cori applied to insulin is here applied to a hyperglycemic-glycogenolytic protein fraction derived from the supernatant liquid from the first isoelectric precipitation of insulin to yield a glucose-elevating dialysate showing a positive biuret reaction.

(f) The glucose-elevating dialysate is subjected to a fractionation process, including methanol extraction, successive chromatographic percolations through silica, alumina, and charcoal, further methanol extraction of the charcoal eluate to remove the inactive nitrogenous

matter, and finally, the alkaline treatment of the nitrogen-free mixture, to result in the isolation of a substance elevating glucose output in liver slices and identified as a mixture of calcium and magnesium salts.

(g) The assay of calcium and magnesium salts from other sources showed the glucose-elevating action to be due to the presence of calcium ions.

(h) The analysis of the dialysate preceding the alkaline digestion of the active protein and the analysis of the ash resulting from the total combustion of this protein proved that the calcium and the magnesium were bound to this protein.

(i) Calcium was found to be the only component of the dialysate producing an increased glucose output on liver slices.

(j) The height of the glucose-forming action of calcium on liver slices incubated in saline-phosphate buffer was observed to be at M/1600 calcium, that is, at 25 gamma calcium per ml. of incubation medium.

(k) The glucose-forming action of the calcium on liver slices incubated in saline-phosphate buffer was found to be due to a deviation of the pathway taken by the breakdown products of glycogenolysis whereas the pancreatic hyperglycemic factor was once more seen to cause increased glucose accumulation by stimulating glycogenolysis.

(l) The significance of this last observation is stressed in the discussion where a hypothesis is proposed in which the possible fluctuations of the ionic environment, in general, and of the calcium level of liver cells, in particular, could produce, in the animal, a diabetic-like condition.

DISCUSSION

The isolation of calcium from the dialysate of the supernatant liquid from the first isoelectric precipitation of insulin and the observation that this ion was the agent responsible for the increased glucose output on liver slices do not raise much discussion. On the basis of positive biuret reaction of alkali-treated insulin dialysate, on the one hand, and the increased accumulation of glucose caused by this dialysate, on the other, Cori had concluded that alkali treatment of the hyperglycemic factor of insulin liberates a glycogenolytic polypeptide. Such a conclusion was unjustified, since Cori and his colleague had neglected to measure glycogen disappearance along with glucose increase. Furthermore, the fact that the dialysate exhibited a positive biuret reaction by no means proved that the activity was due to a peptide since this protein hydrolysate comprised constituents other than polypeptides. The possibility of a glucose-elevating peptide being liberated from the hyperglycemic protein of pancreas is not, however, totally ruled out. It has been seen (p.57) that, under the conditions of the experiments, certain amino acids, namely cystine, do cause an elevation of glucose output. However, the fact that the removal of calcium from the active dialysate abolished its glucose-promoting action proved that calcium was, in fact, the active component of the preparation investigated.

The difference in starting materials employed by Cori and the author

does not invalidate the conclusion that calcium was the active component of the insulin dialysate of Cori. Both insulin and the supernatant liquor from the first isoelectric precipitation of insulin have the same origin, show the same glycogenolytic behaviour, both contain calcium and should, on alkali incubation, yield similar products. The quantitative difference in the calcium contents of these two protein preparations seems, at first sight, a more serious objection. But, as was discussed before (p.77), the calcium content of alkali-treated insulin dialysate could have been multiplied manyfold by the concentration of dialysates. Sutherland and Cori did not suspect the activity of the dialysate to be due to calcium and based the quantities of material tested on their biuret reactions. This may have misled them as to the concentration of calcium obtained by addition of dialysate to their incubation buffer. At any rate, the problem proposed here for investigation received an adequate answer.

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During the course of the present investigation, it was repeatedly noted that the hyperglycemic factor of pancreatic origin does, in fact, favor glucose accumulation by true glycogenolysis on liver slices. This is in accordance with the observations of the workers in this field. It was also noted that the increase in glucose output was taking place without derangement of the path of glycogen breakdown. The glucose output,

either with or without hyperglycemic protein represented 55 to 60% of the glycogen broken down under the experimental conditions used. On the other hand, calcium deviated nearly 90% of the glycogen degradation products to glucose. This explains the results obtained in the incubation of slices in presence of both the factor and calcium at 25 gamma per ml. Under these conditions, the glucose output represented about 90% of a larger glycogenolysis. This also shows - and this is of a greater value - that the hyperglycemic factor is not only glucose - promoting but that it also sends more fuel through glycolysis, presumably, the other 40 to 45%. That the factor does act in this manner is shown by the fact that the partial inhibition of oxygen consumption of slices caused by calcium is relieved by the factor (Table 18, p.80). According to the present status of knowledge relative to carbohydrate metabolism (141) (155), low oxalacetate content of liver favors ketone body formation. The 2-carbon fragments originating from fatty acid breakdown, unable to find the required oxalacetate to react with, would combine among themselves to form ketone bodies. On the other hand, adequate reserve of oxalacetate - as shown by administration of oxalacetate to animals made ketotic with butyric acid (129) - keeps the ketone bodies at a low level. Moreover, a generous glycolysis assures an adequate formation of oxalacetate by the combination of pyruvic acid with carbon dioxide. It will be recalled at this time that alloxan-diabetic animals, presumably through the agency of their Alpha

cells secreting the hyperglycemic factor, exhibit a high blood glucose but low ketosis. It can now be suggested why the hyperglycemic factor would prevent the formation of ketone bodies. By increasing glycogenolysis, the factor would, on the one hand, allow more glucose into the blood thus causing hyperglycemia, but on the other, would feed glycolysis more generously. The oxalacetate supply would be preserved by continuous refill thus assuring the utilization of the 2-carbon fragments. This anti-ketotic action of the hyperglycemic factor would also manifest itself in the case of the hexokinase-inhibition theory. Here again, the factor would keep the glycolysis working by supplying it with an adequate level of glycogen breakdown products. This glycolysis-supplying action of the factor would be similar in reducing ketone bodies to the action of feeding carbohydrate in starvation diabetes.

This tentative overall theory of action of the pancreatic hyperglycemic factor is not contrary to facts and thus seems quite acceptable. Previous workers have too often regarded the factor as a glucose-forming agent alone and because of that have called its action antagonistic to that of insulin. This attitude is wrong. Its net effect on glucose level is opposed to that of insulin, but it is, by no means, diabetogenic. It may be well to start considering it as a carbohydrate-metabolizing agent.

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The study of calcium action on liver slices, reported in the experimental part, brought out a state of affairs that may very well bear significance in the etiology of some cases of diabetes mellitus. It was assumed from the beginning, after Cori and his colleague, that the active agent of the alkali-treated insulin performed its glucose elevation through an increase in glycogenolysis. Further work showed that such was not the case. Calcium was found to be glucose-elevating but non-glycogenolytic. Its action is accomplished by means of a deviation of the path of glycogenolysis. Whereas saline-phosphate buffer, with or without hyperglycemic factor, distributed the glycogen breakdown products to the extent of 55 to 60% in favor of glucose, the presence of calcium ions at a level of M/1600 forced about 90% of the glycogen metabolized to appear as glucose. Consequently, about 40% of the glycogen degraded goes through glycolysis in the first case, whereas, in the second, only 10% does, that is, a 4-fold difference. What adds to the significance of this behaviour is that a calcium concentration of M/400 in the incubation medium is physiological and without such a disturbing effect. This observation leads to the attractive hypothesis that a stimulus that could cause either an actual change in the calcium concentration of the interstitial fluid of the liver, or have a similar effect (may be by influencing cell permeability) would produce in the animal a diabetic-like condition. It has been seen in the previous section that low glycolysis results in low oxalacetate which in turn favors formation of

ketone bodies. Therefore, should calcium level of the interstitial fluids of liver fluctuates from the physiological level of M/400 to M/1600, this would cause hyperglycemia at the expense of glycolysis thus, at the same time, producing ketosis. It will be noted again that such an action takes place irrespective of the degree of glycogenolysis. In other words, glycolysis is inhibited by this concentration of calcium, but glycogenolysis is not affected.

That the quantitative composition of the incubation medium is affecting the overall state of carbohydrate metabolism, in vitro, has been shown by various authors. Ostern, Herbert and Holmes (130) studied the formation and breakdown of glycogen in the liver and published results which are partly summarized below.

1- Glycogen synthesis is very small in a bicarbonate buffer without calcium.

2- Glycogen synthesis is small or absent in a phosphate buffer without calcium, but that appreciable synthesis occurs if the phosphate concentration is decreased and calcium added.

3- Varying the concentration of calcium influences the extent of glycogen synthesis. The maximum synthesis is observed when the concentration of calcium is 10 mg. per cent, that is, approximately that found in blood.

Later, Buchanan, Hastings and Nesbett (131) (132) confirmed and extended the work of the above authors. They studied the effect of the

ionic environment on the synthesis of glycogen from glucose in rat liver slices and summarized their results as follows. "Greater glycogen formation by rat liver slices from glucose occurred in a medium rich in potassium ions than in one rich in sodium ions. The presence of calcium or magnesium increases glycogen formation, the calcium being more effective than magnesium." They also brought in the following remark, "It is of interest to note that when calcium is present in concentrations approximately physiological, it is not particularly effective in stimulating glycogen formation in rat liver slices. Only when present in considerably higher concentrations does it serve as an effective stimulant of the glycogenic reaction of the liver." Finally, these authors noted: "The net accumulation of glycogen is the result of two opposing reactions, namely glycogenesis and glycogenolysis. In the absence of glucose in the incubating medium, however, there may result an over-all breakdown of the preformed glycogen stores of the liver slices. Glycogenesis is then best studied in the presence of glucose, and glycogenolysis, in its absence."

At first sight, the results described in the present work seem contradictory to the experiments cited above. These workers recorded increased glycogenesis with calcium whereas the present work reports glycogenolysis. It may be argued, firstly, that the quantitative content of glycogen and glucose was not the same in both cases. The work of the cited authors involved initial glucose concentration of 1%, a factor in

itself favoring the reaction towards glycogen formation, whereas incubation of liver slices reported here was carried out at zero initial glucose content. Secondly, the qualitative and quantitative composition of the incubating media were not identical in the two sets of experiments. Ostern and colleagues noted that in $NaHCO_3$-Ringer buffer, the physiological concentration of 10 mg. calcium per cent brings the maximum glycogen synthesis. The present work on glycogenolysis involved incubations at 2.5 mg. per cent calcium ions (25 gamma per ml.); that is at subphysiological concentrations. Buchanan, Hastings and Nesbett reported as the best medium for glycogen deposition a calcium concentration of 10 mM per litre, that is four times the physiological value, whereas, in the present work, calcium concentrations of 0.6 mM per litre (25 gamma per ml.) were used.

The nature of the ions present, other than calcium, as well as their concentrations also play a rôle in the direction and rate of the reaction glycogen-glucose. Ostern, Herbert and Holmes claim to have obtained greatest glycogen formation when calcium was present in physiological amounts of 10 mg. per cent in the presence of bicarbonate. Buchanan, Hastings and Nesbett, on the other hand, noted that at physiological concentration, calcium was not very glycogenetic when the medium was rich in potassium.

It is then easily realized from the work of these groups and from the present work that quantitative differences in the incubation media

result in appreciable changes in direction and rate of reactions of carbohydrate metabolism.

The next question to ask is whether the relationship between ionic environment and orientation of metabolism observed in vitro has an in vivo counterpart. A direct answer to this question is not yet at hand. The incubation medium of slice experiments is the interstitial fluid of intact animal, and, at present, analysis of interstitial fluid cannot be done by simple methods but only by the use of the micro pipette. Should a reliable routine method become available, the study of such a relationship should be undertaken.

There exist, however, many hints as to the interdependence of glucose fluctuations and ionic content of body fluids. It has been repeatedly observed (I), for instance, that elevation of blood glucose is accompanied by similar elevation of plasma potassium, and conversely, that serum potassium falls according to the rate of fall of blood sugar in insulin treatment (133). Adrenalectomized dogs treated with cortisone exhibit a lowering of plasma sodium and chloride with a corresponding elevation of potassium and glucose, whereas, when treated with desoxy-corticosterone, the mineral and glucose variation is reserved (134). Such variations of the ions of the blood can legitimately be assumed to originate from or to cause variations in intracellular and extra-cellular fluids. Ionic variations of potassium, sodium or chloride are certainly accompanied by rearrangements of the calcium levels of

the interstitial fluids. However, it is not suggested here that the 4 to 1 change of calcium concentration of incubating buffer of liver slices is actually happening in vivo. It is rather proposed as an example to indicate that carbohydrate metabolism of liver could to a certain extent be regulated by specific modifications of interstitial and intracellular fluids - calcium level of the interstitial fluids being one factor. If true, some ionic combinations would then be diabetogenic, some others would be "normogenic". A comparable state of affairs, though not at all identical, is observed in the physiology of muscle contraction, where the enzymatic (ATP-ase) activity of actomyosin complex is highly dependent on the presence and relative concentrations of calcium and magnesium (135) (136). The importance of such a mechanism is emphasized by Lehninger (137) who claims that this observation of Engelhard and Szent-Györgyi is one of the most significant developments in modern biochemistry.

If it seems reasonable to assume that ionic environment of tissue, in general, controls to a certain extent its metabolism, and is diabetogenic or "normogenic" in case of the liver, one must ask what hint is there as to the primary cause of such ionic variations in tissue fluids. Though no conclusive evidence is available at present, this regulation of ionic environment is most likely to be achieved through hormonal action. As mentioned previously, corticoids and insulin do produce fluctuations in the mineral content of plasma

accompanied by certain diabetic or antidiabetic manifestations. Moreover, it is known that imbalance of adrenal cortex secretions can produce diabetes even in the presence of a normal pancreas (51). Or, again, that desoxycorticosterone renders insulin-sensitive some diabetics found to be insulin-insensitive before corticoid treatment (138) (139).

This hormonal behaviour suggests the hypothesis that some endocrine glands, presumably the adrenal cortex, through the medium of their hormones, modify quantitatively the ionic environment of liver (and may be of other tissues) with the result that metabolism proceeds normally or abnormally. This would furnish, within physiological limits, a mechanism to explain many diabetic manifestations of non-pancreatic origin by dealing with them from basic grounds. This hypothesis does not invalidate theories of diabetes mellitus based on lack of insulin secretion or hexokinase inhibition; it merely offers a possible foundation for cases not explained by these theories. In addition, it supports the hepatic overproduction theory of diabetes, and, inasmuch as liver is a consuming tissue, it links it to the under-consumption concept.

To conclude, the opinion of Potter may be quoted in support of the importance of calcium fluctuations in metabolism. This author writes (140) "Stimulation is assumed to release a cofactor (possibly calcium) for ATP breakdown which furnishes the energy for the work

process and at the same time yields products that stimulate glycolysis". The stimulation that would make calcium available in order to increase its "active" concentration in the interstitial fluid from the diabetogenic level of M/1600 to the "normogenic" level of M/400 would in fact, as observed in vitro, stimulate glycolysis and, in turn, withdraw the diabetic manifestations from the tissues.

CHAPTER II

PANCREATIC ALPHA CELLS IN METABOLISM

DISCUSSION

The extraction of a hyperglycemic factor from pancreas and blood, together with the difference observed in the biochemical picture of the blood of alloxan diabetic animals as compared to that of pancreatectomized animals, leaves no doubt as to the existence of such a substance and suggests that the pancreatic Alpha cells, which are unaffected by this drug, are involved in the secretion of this hyperglycemic-antiketotic principle. If this evidence is not sufficient to earn for the factor the status of legitimate hormone, it nevertheless strongly registers its claim for consideration.

So far, the literature reviewed mentioned mainly work concerned with extraction. To this must be added a few other indications that pancreatic Alpha cells play an endocrine rôle. It must be admitted that in many cases these are mere suggestions. Nevertheless, any observations indicating that certain metabolic or somatic manifestations have their origin in the pancreatic Alpha cells must be taken into consideration if knowledge is ever to be gained about the rôle played by these cells in the general economy of the body.

It might be well to preface the presentation of these few additional ideas by a hypothesis that would in most cases offer an acceptable explanation for the observed facts. This hypothesis would give the Alpha cell secretion the rôle of managing, together with insulin, certain parts of carbohydrate regulation. Insulin would, for instance, control the entry of blood glucose into glycolysis and stimulate fat synthesis from carbohydrate (142), whereas the factor would - by increasing glycogenolysis and thus accelerating glycolysis, (as seen in the previous section) - insure at all time the availability of carbohydrate metabolites for immediate use. The Alpha and Beta cells far from being antagonistic, would thus, by different mechanisms, maintain the carbohydrate metabolism within normal physiological limits. Each of these two hormones would - among other functions - prevent the detrimental extreme of the other to be reached. Severe hypoglycemia would accelerate secretion of the factor whereas exaggerated hyperglycemia would be compensated by greater insulin secretion. This would also mean that insulin would control the external reactions of glycolysis - hexokinase reaction and fat synthesis from carbohydrates - the Alpha cell factor playing an internal function.

It will be recalled that the hyperglycemic factor was said to act at the phosphorylase level (108). It must be emphasized that, as Cori suggests, the factor can accelerate the phosphorylase reaction

in both directions, that is, both in the direction of glycogenolysis and glycogenesis. If the reversed action of the factor occurs, then the carbohydrate-regulating function of this agent would gain in efficiency. Its two-way influence on phosphorylase would, depending on the needs of the organism, insure both the feeding of glycolysis from glycogen and the storage of excess metabolites as glycogen. This means that, with respect to supplying glycolysis with fuel, the factor would maintain a second line of defence - the first being the entry of glucose into the metabolic mill managed by insulin. The wisdom of this dual action of the pancreas would lie mainly in the prevention of ketosis and acidosis - the real threat of diabetes - in the case of failure of insulin action.

It is common knowledge that insulin tends to produce an increase in weight. Normal animals injected with insulin, or psychotics under insulin shock treatment, sometimes exhibit a weight increase which is not seen in control animals or untreated patients. Conversely, in spite of polyphagia, some diabetics become emaciated (3). This effect of insulin can be explained partly by the fact that it favors glucose entry into glycolysis at the hexokinase level thus accelerating the metabolism. Hence, it may be said that any agent that procures more fuel for metabolic reactions - provided these reactions can maintain their normal efficiency - should favor weight increase. On the basis of this explanation, the pancreatic

hyperglycemic factor - if it possesses in vivo the glycolysis-promoting action it exhibits in vitro - should also cause an increase in body development in normal animals.

Experimental evidence supporting a growth-promoting action of the factor is yet meagre since this question has never been studied directly. Sergeyeva (143) claims that animals submitted to operations of their pancreas resulting in a multiplication of Alpha cells exhibit a body development that is suggestive of such an action. The work to be reported in this chapter partly bears on that question.

If the problem of body development as a whole has not been studied in relation to hyperglycemic factor or pancreatic Alpha cells, anabolic effects have been reported and are considered to be due to these causes. Gley, Bernard and Goosens (144) have noted that the healing of standard wounds in rats was accelerated by injection of insulin-free pancreatic extract. This extract may legitimately be considered a preparation of pancreatic hyperglycemic factor. The attempt of the body to repair its injured part through the agency of its pancreatic Alpha cells is again indicated from the work of Pareira and Probstein (145). These workers report that in post-operative state, patients sometimes develop what the authors call a "transient diabetes". This condition of the operated patients is characterized by the clinical syndrome of diabetes mellitus. Such a disturbance in carbohydrate metabolism is restored to normal upon

recovery. This transient diabetes can be considered to mean that the body - in an attempt to insure the availability of carbohydrate metabolites for the cell replacement of its injured part - activates the secretion of its pancreatic Alpha cells - hence the symptom of hyperglycemia.

A relationship between growth and hyperglycemic factor is again reflected in the work of Young (146). This author gave pituitary growth hormone to alloxan-diabetic-hypophysectomized-adrenalectomized rats or to intact cats thus inducing diabetes. Portal blood from these animals was consistently found to exert a hyperglycemic action when administered to untreated recipient alloxan-diabetic-hypophysectomized-adrenalectomized rats. Such a hyperglycemic effect was not observed when growth hormone was administered directly to the recipient rats. Quoting the opinion of Young "These observations are consistent with the view that under the influence of growth hormone the pancreatic islets liberate their contained hyperglycemic substance into the portal blood." Similar conclusions have been arrived at by Ferner (147). It cannot be implied, however, that growth hormone always exerts its action through the medium of pancreatic cells since it is known that growth hormone is (depending on the species and conditions of the animals) still either growth-promoting or diabetogenic in depancreatized animals. But one cannot help being amazed by the similarity of action

of the pituitary somatotrophin and the proposed effects of pancreatic hyperglycemic factor. Both are known to produce hyperglycemia, and the factor is claimed to possess, like somatotrophin, a growth-promoting action.

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Soon after the isolation of insulin from pancreas by Banting, it was proposed that the site of secretion of this hormone is the Beta cells of the Islets of Langerhans. For many years, this concept has been widely accepted and claimed to be highly substantiated by observation. Selye (1) lists the evidence in support of the belief that the Beta cells are the producers of insulin as follows:

(1) "The development of diabetes, following extensive partial pancreatectomy, is proportional to the progressive degeneration of the Beta cells, in particular.

(2) Diabetogenic anterior-pituitary extracts exert a specific damaging effect upon the Beta cells, and simultaneously decrease the insulin content of the pancreas in proportion to the Beta-cell degranulation produced.

(3) The Beta granules, like insulin, are highly soluble in alcohol.

(4) High carbohydrate diets cause degranulation of the Beta cells. This is presumably a sign of compensatory hormone secretion.

(5) Insulin causes degranulation and involution of the Beta cells as well as a decrease in the insulin content of the pancreas. This is presumably as a result of compensatory atrophy.

(6) Alloxan is a drug which damages the Beta cells in a rather specific manner. It causes a type of pancreatic diabetes whose severity parallels the Beta-cell damage produced."

As seen by this list, much of the evidence for the Beta-cell origin of insulin is based on the histological appearance of the Beta-cell granules. If this concept is said to be widely substantiated by observation, there are nevertheless many refractory points that cast doubt over this long accepted idea. For instance, one good source of insulin is pancreas from four months old calf foetus, an organism exhibiting no Beta cells. Or again, the pancreas of newborn animals (puppy) are devoid of visible Beta-cell granules (148). These animals are, however, not at all diabetic. Or, as Gomori reports (149), insulin precipitated from commercial solutions by histologic fixatives does not show the staining properties of Beta granules. Furthermore, tumors with no demonstrable Beta cells were found to have high insulin activity.

But critical minds may say that the inability to detect Beta-cell granules in these cases does not prove that the Beta cells are not present and active. The staining properties of these cells may have been modified or the staining mechanism may have not yet developed. It must be remembered that this argument, which tries to save the status of the Beta cells as the sole source of insulin by claiming that lack of coloration of cells does not prove their absence, is exactly the foundation for the belief that the Beta cells do secrete insulin. If the staining property of these cells is the only test by which their presence may be known, their failure to stain must prove at least their deficiency if not their absence. These objections forced Gomori (149) to conclude, as he words it, "It is by no means proved that they (the Beta cells) actually secrete insulin, or that the Beta granules are the morphologic expression of insulin content."

If these observations lead to the conclusion that the Beta cells of pancreas are not the sole producers of insulin, they are not, however, considered to mean that the Alpha cells - appearing sooner than the Beta cells (148) - do secrete insulin. All that can be said on this point is to suggest that where demonstrable Beta cells are absent in normal cases, the carbohydrate regulation may be achieved to a certain extent by the visible Alpha cells.

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In clinical practice, the first noticeable symptom of diabetes is glycosuria. This is indicative of an elevated blood glucose level. To these symptoms, in order to supplement his diagnosis, the clinician may add the tests of glucose tolerance and insulin sensitivity. The dynamic picture of the blood of the patient as a result of glucose or insulin administration determines his condition. Glysocuria, hyperglycemia, decreased glucose tolerance, and sometimes, decreased insulin sensitivity are, for the clinician, proofs of the diabetic state of the patient.

It may be relevant to mention the opinion of Himsworth (150) that too often an elevated blood glucose level is taken as proof of diabetes whereas in many cases it could simply mean a normal response of the body to increase fuel utilization when so needed. It is unfortunate that workers do not agree on the significance of hyperglycemia. According to the opinion of Wick and Drury (151), an induced hyperglycemia of 1000 mg. glucose per cent cannot increase the fuel utilization in the same manner that insulin injection does. But yet many workers see in hyperglycemia a regulatory mechanism that can accelerate carbohydrate consumption. For instance, Wendt (152) thinks that, in some cases, increased blood sugar represents a system whereby the permeability of muscle cells to glucose may be increased.

This mechanism by which the body would increase its head of blood glucose to insure a greater fuel utilization may again be found

in a more generous secretion of a hyperglycemic Alpha cell hormone of the pancreas. Just as hyperglycemia triggers off insulin secretion so hypoglycemia would trigger off the secretion of hyperglycemic factor. The existence of such a compensatory mechanism for combating hypoglycemia is indicated by the observations of Sergeyeva (143) that, when large doses of insulin are administered to cats, the pancreas of the cats exhibit a tremendous increase in the number of Alpha cells. Compensation is again observed on the animals submitted to glucose tolerance and insulin sensitivity tests. When performing the work to be reported in this chapter, we have observed that, in the majority of cases, the animals respond to glucose administration by an eventual decrease of sugar below the fasting level, and conversely, respond to an injection of insulin by an eventual increase of blood glucose above fasting level. In other words, regulatory mechanisms overshoot their objectives.

It is obvious that the compensatory mechanism that prevents detrimental hyperglycemia is to be found in an increased insulin secretion. Sometimes, however, the blood sugar elevating system is attributed to certain hormones, like those of the adrenals, while it could have been achieved by the pancreas. One such example is taken from the work of Somogyi (153) who observed that when glucose and insulin are administered simultaneously, the alimentary hyperglycemia is suppressed; but if insulin is injected 40 to 50 minutes before

glucose, alimentary hyperglycemia is greatly increased. This, he explains, is a hyperglycemic response of the adrenals to insulin hypoglycemia. Though it is not denied that the adrenals could act in this way, this conclusion seems arbitrary. Such a compensatory action to insulin hypoglycemia could have originated from the pancreatic Alpha cell secretion.

It is true that at present no strong evidence allows us to conclude that the pancreas does secrete a second hormone originating from the Alpha cells and sharing with insulin the duty of carbohydrate regulation, but the sum of suggestions derived from clinical and experimental observations demands that minds of workers should be kept open as to this possibility. The dual function of the pituitary which - as proposed by Selye (154) - would maintain homeostasis in the organism by the opposite actions of somatotrophin and adrenocorticotrophin could be simulated in the pancreas by the secretion of insulin and hyperglycemic factor for the purpose of insuring proper carbohydrate metabolism.

BASIS OF EXPERIMENTAL WORK

The histology of the pancreas has already been summarized in the General Introduction. However, since the work to be reported now arises from histological observations, it is necessary to consider a more detailed description of the morphological development of this gland.

The structure of the pancreas is extensively described in textbooks of anatomy and histology. The following precis of its morphology is taken mainly from "Histology" by Ham (148).

In mammals, the pancreas originates from two different embryonic outgrowths or primordia, one dorsal, and the other ventral. The tail, the body and part of the head develop from the ventral primordium. This dual origin of the organ is emphasized by the persistence of the proximal parts of the primordia to give rise to two ducts opening into the duodenum. The main duct, or pancreatic duct of Wirsung, is made up of tissue coming from both outgrowths whereas the proximal part of the tubule of dorsal origin develops into the accessory duct of Santorini. The ends of the duct system develop into exocrine secretory units resembling grapes in their distribution, hence the term acini (grape-like) is applied to them. In some species, these detach themselves from the duct network to form the Islets of Langerhans. This islet tissue comprises the endocrine units of the pancreas.

The first distinction between acini and islet patches was made by Langerhans. Later, Laguesse noted that the obstruction of the duct resulted in degeneration of the acini while the islands persisted. Laguesse was also the first to suggest the still debated idea that acinous tissue can be transformed into islet cells. Evidence has been presented to support the idea that undifferentiated duct epithelium can give rise to Beta cells. It appears then that both Beta cells and epithelial cords of relatively undifferentiated epithelium can produce new Beta cells. Later, Lane and Bensley devised histochemical methods that enabled them to establish that the granules of islet cells had histochemical properties different from those of zymogen granules. This proved that islet cells were fundamentally different from acinous cells. Furthermore, they established that there were two kinds of islet cells, the Alpha and the Beta cells as indicated by the difference in the chemical properties of their granules.

The morphological development of the pancreas varies greatly in different species. In the puppy - the animal investigated in the present work - the part of the pancreas originating from the dorsal outgrowth is located, at birth, along the periphery of the gland. This part will develop later into the Alpha cell area. In the same young animal, the portion originating from the ventral outgrowth occupies the centre of the gland from one end of the pancreas to

the other. This portion will become, after birth, the area of Beta cells. The normal morphological development of the gland will lead to an eventual interpenetration of these two areas to form a mosaic pattern. Though there exists species variability, the Alpha cells are usually the first cells of the islets to appear. At birth, the pancreas of many animals exhibit only Alpha cells.

EFFECT OF EARLY LIGATURE OF THE PANCREAS. It has been the purpose of many experiments to attempt the separation of the endocrine units of pancreas in order to study the effect of each constituent cell separately. Obliteration of one type of cells leaving the other intact has achieved that aim to a certain extent. Alloxan administration, known to cause the necrosis of the Beta cells, permits the study of the functions of these cells. The achievement of the reverse destruction has unfortunately not been successful. No drug has yet been found to obliterate selectively the Alpha cells. A recent communication (156), however, mentioned that a cobalt salt can necrotize the Alpha cells without damaging the remainder of the gland. No specific information is yet at hand so that the results cannot be evaluated.

If the problem of segregation of the two main types of cells has not yet been solved by pharmacological means, another means of separation has been devised leading to satisfactory and promising

results. Sergeyeva (157) (158), who kindly collaborated in the present work, achieved to a large extent, such a dissociation of the pancreas into its constituent units. By a method of ligature of the pancreas of new-born animals, histological data can be obtained concerning the structure of this organ. On the basis of the dual origin of the pancreas, the mutual interpenetration of the Alpha and Beta areas can be prevented with the result that each area can potentially be studied separately. Depending on the site of the ligature, the ratio between the number and the potency of Alpha cells to Beta cells can be modified. This means that an increase in the number or endocrine potency of either type of cells can be achieved and the effect studied. After ligation of the pancreas - which leaves the animals in a perfectly normal condition - the process of interpenetration is stopped and the acini of both areas are transformed into endocrine tissue. Acini of dorsal pancreas now become the peripheral area composed of Alpha cells. The gradient of these cells is maximal in the splenic end of the organ and minimal, approaching total absence, in the duodenal end. As for Beta area, it will, after the ligature, occupy the centre of the organ showing its peak of concentration in the duodenal end. The great variability in the morphology of the pancreas from different species leads to different patterns of distribution. Though the same general tendency of orientation was observed in the species studied - cats, dogs, pigs - the most

suitable pattern for histological studies is observed in the newborn dog. The age of the animal at the time of operation is an important factor in the sharpness of the area separation. The younger the puppy at the time of operation, the thicker is the Alpha area at the periphery; the older the dog, the greater is the development of Beta area. In the light of her previous experiments on different species, Sergeyeva is of the opinion that the higher the animal stands in the taxonomic scale, the better is the dissociation of the two areas.

Different modifications of this technique can be utilized leading to various morphological pictures of the pancreas. Ligation of the duodenal end results in the proliferation of Beta cells with an almost complete disappearance of Alpha cells, in the ligated segment, whereas ligation of the splenic end results in the appearance of masses of Alpha cells in the ligated portion. According to the opinion of Sergeyeva, the method of early ligature for the purpose of studying the rôle of each type of cell should be considered as experimentally useful as alloxan treatment in the necrosis of Beta cells. Important facts can be revealed in such animals which the surgical operation leaves in a condition closer to normal than does alloxan treatment.

NERVOUS CONTROL OF PANCREAS. The problem of nervous control of pancreatic secretion has been considered by investigators for a long time. Though it is generally accepted that the endocrine functions of the pancreas are not essentially governed by its autonomic nerves, physiologists have nevertheless studied the possible role of these nerves as a refined mechanism of command of the organ and have investigated their distribution in the gland with respect to its various types of cells. Ham (155) writes that the problem of nervous control of insulin secretion - and hence the effect of severe emotional states - has not yet been studied adequately and that the work presented up to now offers only indications of such a possible relationship.

From the work of Babkin (159) (160) it was concluded that, in the cat, the vagus is distributed at the periphery of the lobule, the sympathetic nerves being in the centre. This - transposed to the dog - should mean that the Alpha area is mainly supplied by sympathetic nerves whereas the Beta area is predominently supplied by parasympathetic.

The opinions of investigators of the problem of nervous control of pancreas are not always in agreement but tendencies can nevertheless be suspected. Clark (161) found that vagotomy produced a temporary increase in glucose tolerance gradually declining to a lowered tolerance. Friedenwald (162) observed that vagotomized dogs

metabolize glucose more rapidly, but that this operation is without effect on insulin sensitivity. Quigley (163) could observe no insulin insufficiency after vagotomy. Ranquist (164) noted that the behaviour of vagotomized and sham operated animals was essentially the same.

On the other hand, Cannon (165) and Dworkin (166) claim that sympathectomy in the dog increases significantly its sensitivity to insulin. The conclusions derived from these experiments are that glucose tolerance and insulin sensitivity are very slightly increased, if at all, by vagotomy. However, sympathectomy, though without effect on glucose tolerance increases significantly sensitivity of the animal to insulin.

Houssay (167) (168) and Gayet (169) did not believe in a nervous control of insulin secretion but maintain that insulin works under humoral regulation. Observing a greater lowering of the glycemic level after vagotomy as compared to control animals similarly treated, they concluded that the nervous system exerts a slight inhibitory action on the lowering mechanism in hyperglycemia. Contrary to the opinion of these workers, Zunz and LaBarre (170), from results of complicated cross-circulation experiments, claim that insulin secretion is under nervous regulation. From the results of vagotomy and sympathectomy performed on dogs, Sergeyeva (143) stands with the majority of the workers by stating that ablation of either set of autonomic nerves causes no great variation in the glucose tolerance

of the experimental animals. However, her results indicate, after Cannon and Dworkin, that sympathectomy leads to a permanent and striking increase in sensitivity to insulin.

The studies of Sergeyeva (143) on the relation of nervous connections to the development of pancreatic cells bring results that are of special interest to this work. It had been shown (165) (172) that after insulin administration, the first convulsions were the ones that imposed the greatest strain on the sympathetic nerves. She observed, first, that in all cases of increased activity of sympathetic nerves (injection of adrenalin, anterior pituitary extracts, desoxycorticosterone acetate, vagotomy in the cat) the number of Alpha cells is increased. Second, that insulin injection into the cat produced a large increase of the Alpha cells of the pancreas. As mentioned in the discussion, this speaks strongly in favor of the existence of a compensatory mechanism, involving the Alpha cells, to combat hypoglycemia. As for the nervous regulation of the pancreas, it appears that Alpha cells can be controlled by the sympathetic nerves.

The problem of a possible interdependence between endocrine cells of the pancreas and the autonomic nervous system has been studied by Simard (173) from a different angle. This worker observed in all pancreases examined permanent structures consisting of intimate association of nervous cells and epithelial cells.

These structures are called "neuro-insular complexes". Simard claims that these complexes should be considered as an organ which contributes to the chemical transmission of the nervous influx in the autonomic nervous system or placed in the group of chemo-receptor organs. In his opinion "The reduction and the absence of the neuro-insular complexes of the pancreas might play a rôle in the pathogenesis of diabetes."

If the problem of the relationship between endocrine secretion of the pancreas and the autonomic nervous system has been studied with the above-mentioned results, the effect of denervation of the gland on its histological picture and morphological development has been almost entirely ignored. Sergeyeva (158) found herself in the position of presenting the first account of the resulting manifestations of vagotomy on the structure of this organ. She described the effects of this operation by saying that after vagotomy, the distribution of zymogen is very uneven, some areas being filled, some areas empty. This picture varies appreciably among the animals studied (cat, dog, pig). The areas now denuded of zymogen seem to correspond to areas of the former vagus innervation. As mentioned previously, these studies led her to the observation that increased activity of the sympathetic nerves produces a multiplication of Alpha cells. This massive formation of Alpha cells is dependent on the presence of normal acinous tissue (cat). This adds another point in favor of

the possibility, said to be still discussed, of the direct transformation of acinous cells into Alpha cells. The morphological representative of the endocrine activity of the sympathetic nerves seems therefore to be the Alpha cells. If vagotomy is performed on the new-born animal thus increasing the activity of the sympathetic nerves, the resulting Alpha cell multiplication can be obtained with a corresponding absence of Beta cells. This intervention can therefore produce a pancreas exhibiting almost only Alpha cells. This is the basis underlying some of the experiments to be described now.

PURPOSE OF THE PRESENT WORK

It is easily seen that the technique of Sergeyeva for the decomposition of the pancreas into two groups of its constituent cells possesses a great potential value for physiological analysis. In this way, the "fabrication" of a pancreas or of segments of pancreas free of either Alpha or Beta cells can be achieved. The imbalance between Alpha and Beta cells produced by early ligature of the pancreas at various points or the absence of Beta cells caused by early vagotomy can be thus applied to gain knowledge on the effect of proliferation or involution of one type of cell on the behaviour of the operated animals, compared to that of control animals, in the face of carbohydrate regulation tests. Moreover, the insulin potency of the segments

of pancreas can - by such segregation - be studied in relation to the histological composition of each portion. Finally, further knowledge concerning the histology and morphological development of the pancreas can be gained. For the purpose of studying these points the present work was undertaken.

Despite the potentialities of the method, it must be emphasized that the experiments of the present investigation constitute preliminary tests. Like any preliminary tests, these were performed mostly for the purpose of finding the proper way of employing such a tool with maximum efficiency. This thesis constitutes therefore the first report of the application of this technique to studies extending outside the field of histology.

The sum of work caused by experiments of this type, added to the specialized character of the various steps, necessitated the collaboration of a team. It has been our good fortune to find such a collaboration. Dr. Sergeyeva of L'Universite de Montreal performed the operations on the young dogs and the histological examinations of their pancreases, and Dr. Wrenshall of the Banting Research Institute supervised the insulin assays. The author was given the duty of carrying out the tests of glucose tolerance and insulin sensitivity. It is beyond the scope of this thesis to present a complete report of the microscopical examination of pancreatic tissue and observations that may interest only specialists of this field. However, it is

necessary to connect histological analysis with the results of the biochemical tests. To that extent only, histological data from Dr. Sergeyeva's work will be introduced.

MATERIALS AND METHODS

PROCEDURE. For the reasons mentioned previously, the new-born dog was chosen as the experimental animal. No selection was made as to the breed or to the size of the bitches. Altogether, eight families of puppies born from mongrel bitches, making a total of 40 puppies, were investigated. These animals, obtained together with their mothers at different times, were operated in the first days of their life by Dr. Sergeyeva at L'Universite de Montreal in the manner described in the next section. The puppies were kept with their respective mothers for 5 or 6 weeks. At about this age, they were taken to McGill University and the bitches returned to the owner. In this laboratory, the puppies were fed either Pard or Dr. Ballard's dog food and Carnation milk. Throughout their lives, they were weighed at regular intervals. In all cases, the animals were managed by families. When the puppies were approximately 6 to 8 weeks old, they were submitted to the tests of glucose tolerance and insulin sensitivity. After these tests were completed on any one family, the puppies were sacrificed, their pancreas examined to verify the success of the operations and the duodenal and splenic segments of this organ excised. These segments were examined histologically by Dr. Sergeyeva and the most interesting ones were assayed for insulin content by Dr. Wrenshall of The Banting Research

Institute in Toronto. To insure objectivity in the interpretation of the results, the reports of each of the three collaborators were exchanged on the same day at the completion of the whole experiment.

SURGERY. The surgical operations performed by Dr. Sergeyeva comprised four types of ligatures, vagotomy and sham operations. A different number of puppies was submitted to each type of operation. In all cases of ligation, care was exercised not to obstruct the blood vessels. The animals were operated under ether anesthesia.

Single ligature: This ligature was placed across the gland as indicated by figure b. The duct of Santorini cannot function as an outlet for external secretion and the whole splenic end of the gland was invariably found to be sclerosed. Microscopic examination revealed the disposition of the thick layer of Alpha cells at the periphery whereas the centre was occupied by the Beta cells. Figure a indicates the dual origin of the two cell areas. The dorsal pancreas (dark) is potentially the Alpha area, and the ventral pancreas (pale) is potentially the Beta area.

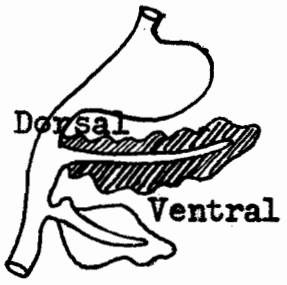

figure a

Dual origin of pancreas

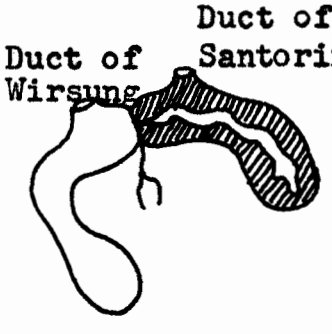

figure b

Single ligature

figure c

Double ligature

Double ligature: This operation (figure c) comprises a single ligature as described above and another ligature on the duodenal side of the duct of Wirsung. The blood vessels were undisturbed and the area left for external secretion was about 1 to 2 sq. cm.. The splenic sclerosed end presented under the microscope the same picture as in the single ligature. The duodenal end, also sclerosed, exhibited under the microscope the same mutual position of the two areas but reversed in their development. The peripheral Alpha area was very thin, sometimes hardly noticeable, whereas the central Beta area was well developed.

Segmental ligature: In this operation (figure d), only the extreme splenic end of the pancreas was ligated thus favoring an intense development of the Alpha area.

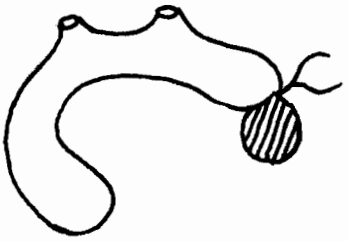

figure d

Segmental ligature

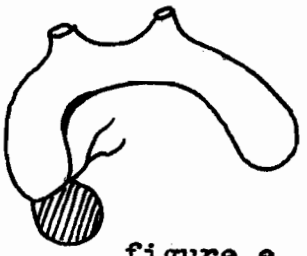

figure e

Duodenal ligature

Duodenal ligature: This (figure e) is the counterpart of the segmental ligature. Here, only the extreme duodenal end was ligated. This operation promotes the extension of the Beta area.

Vagotomy: Bilateral vagotomy above the diaphragm was done by making an incision on the right side of the chest.

GLUCOSE TOLERANCE TEST. A volume of sterile aqueous solution containing 0.5 gm. of glucose per milliliter was injected into each animal by way of its front leg veins so as to inject 0.75 gm. of glucose per kilogram of body weight, that is, 1.5 milliliter per kilogram.

INSULIN SENSITIVITY TEST. Due to individual variations in the fasting glucose level, Himsworth (150) is of the opinion that the method of administration of insulin alone for the purpose of defining the insulin sensitivity of the animal is not reliable. He thus suggests the injection of a solution containing both insulin and glucose. According to this worker "This method does not take into account the height of fasting sugar level at which the tests are performed and therefore remains a measure of sensitivity to insulin at a particular blood sugar level."

The solution thus injected contained - as for the glucose tolerance test - 0.5 gm. of glucose per milliliter of solution to which was added insulin to a concentration of 1 unit per milliliter. An injection of 1.5 milliliter of this solution per kilogram gave the animal 0.75 gm. glucose and 1.5 units of insulin per kilogram of body weight.

SAMPLING FOR GLUCOSE ANALYSES. The work involved in the manipulation of 40 animals submitted to the tests of glucose tolerance and insulin sensitivity was reduced to manageable proportions so as to permit all the members of any one family to be treated simultaneously for either test. Before experiments, the puppies of a family were fasted for 12 to 15 hours. All blood samples were withdrawn from the marginal ear vein. The glucose and insulin-glucose solutions were injected via the front leg vein. Five blood samples were taken from each animal: at 0 minute (fasting level), at 3 minutes (the point assumed to represent the peak of the curve), at 60 minutes, at 120 minutes and at 180 minutes. The glucose analyses were performed according to the method of Folin-Malmros (120).

SLAUGHTER OF THE ANIMALS. After any one family had been tested for glucose tolerance and insulin sensitivity, the puppies were killed with an overdose of nembutal. Their abdomens were then opened, their pancreas examined for the outcome of the surgery, and segments cut for histological examination and for insulin assay.

INSULIN ASSAY. The segments of pancreas assayed for insulin content were, in all cases, adjacent to those kept for histological examination. After removal from the animals, they were prepared and shipped according to the recommendations of Dr. Wrenshall. They were stripped of

surrounding fat and connective tissue, weighed, cut in small pieces, placed in acid alcohol and shipped to him in Toronto. The acid alcohol prepared according to Scott and Fisher (174) was made up of 790 c.c. of 95% ethyl alcohol, 15 c.c. of concentrated hydrochloric acid and 195 c.c. of distilled water. A volume of four c.c. of the acid alcohol was added to each gram of pancreas to be extracted. The insulin assays were done by the mouse-convulsion method (175). These assays were done on 32 samples of pancreatic tissue from 20 animals.

HISTOLOGICAL EXAMINATION. The segments of pancreas cut from every animal for histological examination by Dr. Sergeyeva were preserved in Bouin fluid and their sections stained by a method combining Masson's and Gomori's methods. The Alpha cells stained bright pink and pale pink whereas the Beta cells stained grey and semitransparent.

RESULTS

PRESENTATION OF RESULTS: The inclusion of curves describing the growth rates and the response of each of forty animals to carbohydrate regulation tests would make this report too voluminous and would rather create confusion. It was thought preferable to calculate - by weighing - the areas under the curves and to evaluate from these areas the responses of the animals. The method used here is essentially that of Himsworth (150). In this manner, the data from forty animals are rendered more readily comparable. However, the method suggested by Himsworth for the evaluation of glucose tolerance and insulin sensitivity though in essence applied here had to be modified. It was mentioned in the Discussion (p. 108) that - in support of the existence of compensatory mechanisms to combat hyperglycemia and hypoglycemia respectively - the compensatory mechanisms overshoot their objectives. A few hours after injection of glucose, in nearly all cases, the glycemic level of the blood stands lower than the pre-injection level, and conversely, in a few animals, the glycemic level following administration of insulin climbs higher than the fasting level. Moreover, - what seems to be peculiar to young animals - the fasting levels change appreciably from one day to the next. It was not uncommon to observe on the same animal on two consecutive days a difference of 10 to 25 mgm.

per cent in its fasting sugar level. Consequently, the fasting levels could not be chosen as the axis of abscissa of the curves because in most cases some portions of the curves lay beyond the fasting level.

To overcome these difficulties, the following modifications were introduced. First, all the figures obtained from the glucose analyses were adjusted so that the fasting levels were at 100 mgm. per cent. This was done by either adding or subtracting from the five points of every curve whatever quantity made the difference between the actual value of its fasting level and one hundred. Thus, any one curve remained parallel to what it would have been if plotted before such a correction. Second, in the evaluation of the glucose area, the area under the curve was not taken from the "100 mgm. per cent" line but from the zero base line up. This modification permitted anyone curve to appear in its totality on the graph paper, and also to obtain, by simple subtraction of the area under the insulin-glucose curve from the glucose curve, the true insulin area. This insulin area is the area I of Himsworth.

It is easily understood that the selection of the zero baseline as the axis of abscissa of the glucose tolerance curves gives rise to a "dead space" on every tolerance curve. This "dead space" does not, however, invalidate the results since this method

is devised strictly for comparative purposes. The "dead space" changes the areas of all the tolerance curves to the same extent and therefore enables the comparison to be made. Though the subtraction of this "dead area" was not necessary, it was nevertheless done for the only purpose of rendering the differences in the glucose tolerance areas more striking. The area obtained by subtracting the "dead space" from the original glucose area is designated as area G. It must be emphasized that these modifications change in no way the accuracy of the original method of Himsworth, because the relative values of the figures thus obtained remained unchanged.

With the use of this modified method of Himsworth, the data of the glucose tolerance and of insulin sensitivity tests were obtained. These are listed in Table 1.

Column A represents the areas between the glucose tolerance curves and the zero base line.

Column B represents the areas between the insulin-glucose curves and the zero base line.

Column C represents the new glucose areas found by subtracting the "dead space" from area A. This is area G of Himsworth.

Column D represents the areas obtained by subtracting area B from area A. This is area I of Himsworth and is a measure of the effect of insulin on the animals.

The true insulin action is indicated by the value of the ratio I/G. The higher the ratio, the more sensitive the animal is to insulin, the lower, the less sensitive.

Table 2 indicates the results of insulin assays performed on sections of pancreas by Dr. Gerald A. Wrenshall.

Table 3 is a summary of the histological observations made by Dr. Maria Sergeyeva on the most interesting samples from pancreases assayed for insulin content.

Table 4 gives a list of the weights of the animals at operation and at sacrifice.

Table 1

Tests of glucose tolerance and insulin sensitivity

Family & No.	Type of operation	Sex	Area A**	Area B	Area C	Area D	Ratio I/G	Samples to Banting***
A 1	Control	M	.914	.519	.474	.395	0.83	S + D
2	Single ligature	F	.844	.347	.404	.497	1.23	
4	" "	M	.819	.554	.379	.265	0.70	S + D
5	" "	F	.938	.469	.498	.469	0.94	
6	Control	M	.867	.407	.427	.460	1.08	
B 1	Control	F	.893	.535	.453	.358	0.78	S + D
2	Segmental ligature	F	.637	.449	.197	.188	0.95	
3	" "	M	.882	--	--	--	--	S + D
C 1	Control	M	.909	.657	.469	.252	0.54	S + D
2	Vagotomy	F	.960	--	--	--	--	
3	"	F	.777	.562	.337	.215	0.63	S + D
4	"	F	.880	.529	.440	.351	0.80	S + D
5	Control	F	.961	.471	.521	.490	0.94	
6	Vagotomy	F	--	.497	--	--	--	
D 1	Control	F	.901	.330	.461	.571	1.24	
2	Vagotomy	M	.697	.516	.257	.181	0.70	S + D
F 1	Control	F	.811	--	--	--	--	S + D
2	Double ligature	M	--	.675	--	--	--	
3	" "	M	.767	.699	.327	.068	0.21	S only
4	Vagotomy	M	.778	.598	.338	.180	0.55	S + D
5	"	F	.889	.551	.449	.338	0.75	S + D
6	Control	M	.779	.616	.339	.163	0.49	
7	Duodenal ligature	F	.837	.534	.397	.303	0.76	
8	" "	F	.734	--	--	--	--	
G 3	Control	M	.721	.426	.281	.295	1.05	
4	Vagotomy	M	.575	.387	.135	.188	1.39	S + D
5	Single ligature	F	.715	.424	.275	.291	1.05	S only
6	Double ligature	F	.761	.466	.321	.295	0.92	S only
H 1	Control	M	--	.424	--	--	--	
2	Duodenal ligature	F	.821	.528	.381	.293	0.77	
3	Segmental ligature	F	.827	.490	.387	.337	0.86	
4	Control	F	--	.587	--	--	--	
5	Single ligature*	M	.682	.531	.242	.151	0.62	S + D
6	Segmental ligature*	M	--	--	--	--	--	S + D

Table 2

Determination of Insulin Content on Sections of Pancreas (by Dr. Gerald A. Wrenshall).

Family & No.	Pancreas Section	Gm. of P. Extracted	Extractable Insulin u/gm. P.	No. of mice injected	Approximate % S.E. of assay	Ratio S./D.
4 A	S	0.79	19.3	54	± 9.6	11.4
4 A	D	1.56	1.70	54	± 9.6	
1 A	S	2.16	6.64	45	± 10.5	3.0
1 A	D	1.76	2.22	48	± 10.2	
1 B	S	1.10	2.98	30	± 12.9	17.5
1 B	D	0.80	0.17	24	± 14.4	
3 B	S	0.15	Too small to measure	18	± 16.7	--
3 B	D	0.61	0.19	18	± 16.7	
4 C	S	1.71	3.10	37	± 11.6	5.45
4 C	D	1.52	0.57	14	± 19.1	
3 C	S	2.31	4.61	44	± 10.7	6.10
3 C	D	1.344	0.76	12	± 20.4	
1 C	S	2.35	2.50	37	± 11.6	7.15
1 C	D	1.594	0.35	16	± 17.6	
1 F	S	3.63	2.93	42	± 10.9	7.78
1 F	D	2.62	0.38	12	± 20.4	
5 F	S	2.75	4.29	42	± 10.9	4.31
5 F	D	3.02	0.97	29	± 13.0	
4 F	S	3.08	2.18	40	± 11.2	3.42
4 F	D	3.78	0.64	42	± 10.9	
3 F	S	0.55	12.9	30	± 12.9	--
4 G	S	2.82	3.85	42	± 10.9	3.70
4 G	D	2.91	1.04	48	± 10.2	
2 D	S	3.17	3.38	42	± 10.9	3.62
2 D	D	3.464	0.93	47	± 10.3	
6 G	S	0.53	12.1	48	± 10.2	--
5 G	S	0.454	15.3	42	± 10.9	--
5 H	S	0.794	2.63	35	± 11.9	4.60

TABLE 3

Histological Examination of Segments of

Pancreas Assayed for Insulin Content.

(From Dr. Sergeyeva's Report)

(a) Normal - In the splenic end of the gland, the average number of islands in one field of view (lower power) is from 10 to 20. The cells present are all of the Alpha type. There are no real Beta cells at this age. The duodenal end exhibits rare clumps of Alpha cells.

(b) Single ligature - Macroscopically, the splenic end is thin and sclerosed. The length of this part represents about 1/3 of the total length of normal pancreas at this age. Under the microscope, two areas of endocrine cells are easily discernable in the sclerosed splenic segment. The peripheral thick layer is composed almost exclusively of short cords of Alpha cells. This Alpha cell area envelopes the central Beta cell area which is composed of strands of duct epithelium cells with Beta cells scattered throughout. Endocrine cells of the Alpha cell area show all gradations in color: from deep pink to pale pink and finally to an almost colorless tinge closely resembling that of Beta cells. The fractions of Alpha cells are in the shape of short cords giving the

impression that they are rosettes of acini compressed by ingrowing connective tissue fibres. In earlier periods of sclerosis, fractions of bright pink Alpha cells are prominent. The older the puppy is at the time of operation, the more intricate is the form of the boundary between the two areas. The endocrine potential of the splenic portion of pancreas has been raised by the operations. The ratio of Alpha area to Beta area in puppy A4 is about 3 to 1; in G5, it is about 2 to 1.

(c) Double libature - Macroscopically, both splenic and duodenal ends are found sclerosed. The splenic portion is similar to that described under "single ligature". The duodenal portion is much thinner than the splenic one. The middle portion of the gland, left in a normal state by the operation, occupies about 1/3 of the pancreas at this age. Microscopic examination reveals the presence of the same two areas of endocrine cells - Alpha area and Beta area - in the splenic end. However, on closer examination, it is observed that the Beta area has a greater expansion than in the corresponding cases of single ligature. The duodenal sclerosed end is composed of strands of epithelial cells and agglomerations of Beta cells. The histological structure of this end closely resembles that of the Beta

area in the splenic end. However, in many places at the periphery, short cords of cells closely resembling those of the colorless fraction of Alpha cells of the splenic end are noticed. It seems that this peripheral layer is simply a continuation of the Alpha area from the splenic end to the duodenal end. This layer is very thin in the duodenal end, sometimes being hardly noticeable. Therefore, agglomerations of Beta cells exist here in a practically isolated state. The endocrine potential of both ends is raised. The Alpha to Beta ratio in the splenic end is approximately 3 to 1 in puppy F3 and 3 to 2 in puppy G5.

(d) Segmental ligature (splenic segment) - The ligated splenic segment is about the size of a pea. Microscopically, it is composed of Alpha and Beta cells. The ratio between these cells is very variable. In some cases the Alpha cells make up 80 to 90 per cent of total endocrine cells; whereas in other cases, only 40 to 50 per cent. The rest is composed of Beta cells.

(e) Duodenal ligature - Macroscopic and microscopic pictures correspond to those described for the duodenal end under Double ligature.

(f) Vagotomy - Pancreatic sections from vagotomized new-born puppies show striking acidophily (Alpha cells). The endocrine potential of the gland is raised, that is, islands are more numerous. Even in the duodenal end, islands are seen more frequently than in normals. In the splenic end, the average number of islands is from 12 to 24 in one field of view under lower power. Islet cells exhibit pink granulations characteristic of Alpha cells. There are no real Beta cells. Zymogenic granules are unevenly distributed in a checker-board manner. Some areas are well filled with zymogen and some are depleted. The duodenal end in some cases is completely devoid of zymogen.

Table 4

List of Weights

Family & No.		Type of operation	Sex	Weight in grams at operation	Weight in grams at sacrifice	Age in days at sacrifice
A	1	Control	M	520	5700	63
	2	Single ligature	F	520	4970	"
	4	Single ligature	M	610	6525	"
	5	Single ligature	F	700	5-25	"
	6	Control	M	670	5350	"
B	1	Control	F	290	1260	36
	2	Segmental ligature	F	270	1290	"
	3	Segmental ligature	M	330	1375	"
C	1	Control	M	280	1450	45
	2	Vagotomy	F	270	1200	"
	3	Vagotomy	F	420	1710	"
	4	Vagotomy	F	420	1700	"
	5	Control	F	280	990	37
	6	Vagotomy	F	270	1080	"
D	1	Control	F	460	4360	58
	2	Vagotomy	M	360	4540	"
F	1	Control	F	570	4000	56
	2	Double ligature	M	610	4530	"
	3	Double ligature	M	720	4615	"
	4	Vagotomy	M	810	4780	"
	5	Vagotomy	F	710	4175	"
	6	Control	M	850	5475	"
	7	Duodenal ligature	F	820	4515	"
	8	Duodenal ligature	F	930	5315	"
G	3	Control	M	720	4750	46
	4	Vagotomy	M	690	4070	"
	5	Single ligature	F	720	3960	"
	6	Double ligature	F	690	3270	"
H	1	Control	M	780	2700	51
	2	Duodenal ligature	F	850	2220	"
	3	Segmental ligature	F	740	2050	"
	4	Control	F	510	1580	"
	5	Single ligature*	M	720	2085	"
	6	Segmental ligature*	M	740	2000	"
I	1	Single ligature	F	460	2320	43
	2	Double ligature	F	530	2285	"
	3	Double ligature	F	470	1725	"

EXAMINATION OF RESULTS

A - Effect of the various operations on the tests of carbohydrate regulation. *

(1) Glucose tolerance (columns A or C).

(a) Single ligature.

Family	A,	2-,	1+	
	G,	1^{o}		
	H,	1^{o}		
	I,	no controls.		
Total		2-,	1+	

(b) Vagotomy.

Family	C,	1+,	2-	
	D,	1-		
	F,	1+,	1-	
	G,	1-		
	H,	1-		
Total		6-,	2+	

(c) Segmental (splenic) ligature.

Family	B,	1^{o},	1-	

(d) Double ligature.

Family	F,	1-		
	I,	1-,	3+	no controls
Total		2-,	3+	

(e) Duodenal ligature.

Family	F,	1-,	1+	

---

* Areas of experimental animals compared to areas of control animals. +, - and o mean respectively areas of experimental animal greater than, smaller than or equal to areas of control animals.

(2) Insulin sensitivity (column B).

(a) Single ligature.

Family	A,	1-,	1+,	1^{o}
	G,	1^{o}		
	H,	1^{o}		
Total		1-,	1+,	3^{o}

(b) Vagotomy.

Family	C,	2-	
	D,	2+	
	F,	2-	
	G,	1-	
Total		5-,	2+

(c) Segmental ligature.

Family	B,	1-
	H,	1+

(d) Double ligature.

Family	F,	2+
	G,	1+
Total		3+

(e) Duodenal ligature.

Family	F,	1-
	H,	1^{o}

(3) Insulin sensitivity (ratio I/G).

(a) Single ligature.

Family	A,	1+,	1-,	1^{o}
	G,	1^{o}		
Total		2-,	1+,	1^{o}

(b) Vagotomy.

Family	C,	2+	
	D,	1-	
	F,	2+	
	F,	1+	
Total		5+,	1-

(c) Segmental ligature.

Family B, 1+

(d) Double ligature.

Family F, 1-
G, 1-

(e) Duodenal ligature.

Family F, 1+

The results of these tests can be summarized as follows:

Single ligature, segmental (splenic) ligature and duodenal ligature caused no observable change in the glucose tolerance and insulin sensitivity. Double ligature seems to have produced a very slight reduction of glucose tolerance and of insulin sensitivity. Vagotomy increased both the glucose tolerance and the insulin sensitivity.

The lack of effect of the three first ligatures (single, segmental and splenic) may be explained by the fact that in any one of these ligatures, less than one third of the pancreas becomes sclerosed, with the consequence that the other two thirds are capable of maintaining a normal glycemic response. Or again, the decreased exocrine capacity of the pancreas resulting from sclerosis of the splenic end is compensated by the action of an increase in insulin content in this same segment.

The slight reduction of tolerance to glucose and sensitivity

to insulin as a result of double ligature may be interpreted to mean that the larger tolerance that should be observed as a result of the increased insulin content of the splenic portions has been over-counteracted by the very large inhibition of the exocrine secretion of the gland. This inhibition of enzyme production and secretion is the result of the extensive sclerosis of the gland caused by an operation affecting two thirds of the pancreas. It is important to keep in mind that duct obstruction in the new-born animal produces death whereas the adult animal can live normally after such an operation.

The parallel fluctuations seen in the response of the puppies to both tests seem at first sight contradictory. An increased glucose tolerance after an operation may be interpreted as indicating a larger secretion of insulin as a result of this treatment. This more generous insulin secretion should reflect itself in a lowered sensitivity to exogenous insulin because of adequate amounts or potency of pre-existing insulin. The increased insulin sensitivity caused by vagotomy is therefore at variance with this interpretation. However, this interpretation may very well not apply here. It must be emphasized that the vagotomies performed on the young dogs were general vagotomies and not pancreatic. Many changes other than pancreatic changes may occur in animals submitted to this operation. Moreover, this interpretation of the meaning of variations in insulin sensitivity is not shared by all authorities. Soskin (171) for

instance, rejects it and favors the central role of the liver in the response of an animal to insulin treatment.

B - Insulin content and histological picture of the pancreas.

Normal. Four controls of different families examined histologically had segments of their pancreas assayed for insulin content with the following results.

Puppy No.	Insulin in splenic end	Insulin in duodenal end
A 1	6.64 u/gm.	2.22 u/gm.
B 1	2.98 "	0.17 "
C 1	2.50 "	0.35 "
F 1	2.93 "	0.38 "
Average of B1, C1, + F1	2.8	0.3

The three controls (B1, C1 and F1) showing close agreement in their insulin content also showed morphological similarity. A1, with an exceptionally high insulin content was also histologically exceptional. Both ends of its pancreas contained numerous islets of Langerhans with no real Beta cells, as mentioned in Table 3.

Single ligature. Of all the animals examined, puppy A4 is the most interesting one. A littermate of control A1 possessing a splenic end

of pancreas rich in insulin, A4 exhibits the highest insulin content, a gigantic body development as compared to its littermates and a greatly raised endocrine potential. Its ratio between Alpha-area and Beta-area is 3 to 1. Its insulin content of splenic end was 19.3 u/gm. It seems that in this family (A) the ratio between the two areas was in favor of a greater development of dorsal pancreas with a high endocrine potential. On this hereditary greater development of dorsal pancreas was superimposed the transformation of the splenic end into a mass of endocrine cells with prevalence of Alpha area resulting from single ligature of its pancreas. The physiological effect of both factors (heredity and surgical intervention) acting in one direction was summated with the consequences of considerable insulin content and gigantic body size.

Double ligature. The two splenic samples taken from two different families show close similarity of insulin content (F3 = 12.9 u/gm.; G6 = 12.1 u/gm.). Sample I made up of three splenic ends pooled together gave a value of 13.2 u. insulin / gm. Two of these segments were taken from pancreas of double ligature, the other from pancreas of single ligature. It seems therefore that the value 13.2 is an average of a figure of about 15 for the single ligature and two figures of about 12 for the double ligatures. In this type of operation both ends of the pancreas were ligated leaving about one third of gland for exocrine secretion. Table 3 indicates that in these cases, the Beta area shows a greater expansion than in the corresponding

cases of single ligature. This, together with a decreased insulin content of splenic ends (19.3 and 15.3 compared to 12.9 and 12.1) seems to suggest that the insulin content (if not production) does not run parallel to Beta area development in these young animals.

Vagotomy. Material from six vagotomized puppies were assayed for insulin content. The results are as follows.

Puppy No.	Insulin in splenic ends	Insulin in duodenal ends
C 3	4.61 u/gm.	0.76 u/gm.
C 4	3.10 "	0.57 "
F 5	4.29 "	0.97 "
F 4	2.18 "	0.64 "
G 4	3.85 "	1.04 "
D 2	3.38 "	0.93 "
Average of vagotomised puppies	3.57 "	0.82 "
Average of normal puppies	2.8 "	0.3 "

Comparing these figures with those of normal puppies, one may see that insulin content is increased in both ends as a result of vagotomy. Histologically, the islet areas are also increased towards a greater Alpha cell development. The endocrine potential of the whole gland is increased. There are no real Beta cells.

There again the secretion or content of insulin does not run parallel to the Beta cell development.

C - Body development and histological picture of the pancreas

This experiment was not planned directly for the purpose of studying the relation between pancreatic endocrine cells and body development. Many factors are therefore uncontrolled and render any interpretation about such a relation very difficult. The animals were not of selected size and breed, the puppies were submitted to various types of operations thus reducing for comparison the number of puppies in each group, and altogether too few animals were investigated to permit an evaluation with statistical rigidity.

Only indications can be derived from this investigation with respect to body development. The most striking specimen was puppy A^4, submitted to single ligature operation. This animal exhibited a larger body development than its littermates and an increased expansion of its pancreatic endocrine cells with an obvious prevalence of Alpha area. The vagotomized animals - with no real Beta cells and a multiplication of Alpha cells - in spite of deprivation of parasympathetic nerves seem to show increased growth as compared to controls of their own families. This greater body growth if real could be explained by an increased insulin production resulting in a greater appetite and hence an increase in weight. It seems now that such an insulin action would result in fat accumulation but not in skeleton growth. However, only experiments specifically designed for the study of such a relationship performed on purebred animals could clarify this question.

CONCLUSIONS

The experiments of early ligature of the pancreas of newborn puppies designed to attempt an isolation of the Alpha cells led to the following conclusions. Single ligature, segmental ligature and duodenal ligature cause no observable change in the glucose tolerance and insulin sensitivity. Vagotomy produces an increase in both, whereas double ligature resulted in a slight decrease in the response of the operated animals to these two tests.

From the comparison of the insulin content of ligated segments of pancreas and their histological pictures arises the suggestion that in the young animal, the Beta cells are not the morphologic expression of insulin content. The parallel increase in insulin content and Alpha cells after ligature of splenic end of the pancreas, together with the presence of insulin in pancreas free of Beta cells as is the case for young normal puppies and vagotomized puppies is interpreted by our colleague, Dr. Sergeyeva, as meaning that in these cases insulin is secreted by the Alpha cells.

It is true that the Alpha cells do increase in number and endocrine potency after these operations, but, as mentioned in Table 3, the Beta cells are also more developed. The endocrine potency of the gland is raised with respect to both types of cells. Consequently, the larger amount of insulin may be produced by the newly-developed Beta cells. Examination of duodenal ends

for both insulin and islet cells after duodenal or double ligature would probably establish whether both insulin content and Beta cell proliferation go parallel. Unfortunately, such examinations were not done because of difficulties of time and technique.

However, if, in the animals investigated here, Beta cells are sometimes totally absent but insulin normally secreted, one must conclude that, under these conditions, insulin is secreted by the Alpha cells present.

In the first chapter, it was suggested that the Alpha cells of the pancreas are secreting the hyperglycemic factor. One point of importance should be emphasized here. The belief that the Beta cells are the sole producer of insulin and the hypothesis that the hyperglycemic factor is secreted by the Alpha cells apply to adult animals. But the animals investigated here were very young. Such animals differ in their requirements and stability from adult ones. It is thus possible that the endocrine secretions of the pancreas could differ in new-born animals as compared to adult animals. If the conclusion that Alpha cells seem to produce insulin does not invalidate the old concept of the Beta cells as the origin of insulin and the hypothesis of the Alpha cells secreting the hyperglycemic factor, the observations of the present investigation give rise nevertheless to a new hypothesis. The fact that the first cells to appear in the pancreas are the Alpha cells, together with the observation that young animals are not diabetic because of their lack of Beta cells

suggest that during the development of the foetus and in the early post-natal life, the Alpha cells would secrete insulin. With time, the animal requiring more stability in its glycemic control and metabolism, its pancreas would undergo the transformation to the structure seen in the adult. The Beta cells appearing along with Alpha cells would then take over the role of secreting insulin whereas the Alpha cells would produce the hyperglycemic factor thus controlling - as explained in chapter one - the carbohydrate metabolism from another position.

It has been seen that the ratio of the size of dorsal pancreas to that of ventral pancreas varies with different families. Some families exhibit a large development of dorsal pancreas (Alpha area) as compared to the ventral pancreas development whereas in other families, the importance is shifted towards the ventral pancreas. If such differences exist between the growth of the two primordia of the pancreas, these differences may well play a role in homeostasis. It is obvious then that animals showing predominant dorsal pancreas development are the best material for isolation of Alpha cells whereas those with large ventral pancreas are the best source of Beta cells. By the proper selection of the species or by careful choice of pure-bred dogs, complete isolation of the components of the pancreas may then be achieved.

SUMMARY OF CHAPTER II

Basis of Experimental Work. The pancreas originates from two primordia, a dorsal outgrowth believed to give rise to the Alpha cell area, and a ventral outgrowth said to develop into the Beta cell area. These two outgrowths will eventually interpenetrate so that both types of cells will be scattered throughout the gland. Ligature of the body of the pancreas in the new-born dog prevents the mutual interpenetration of the two cell areas. The dorsal pancreas will form the periphery of the ligated segment exhibiting Alpha cells whereas the ventral pancreas will form the central portion of the ligated segment. This central area will be populated with Beta cells. Moreover, vagotomy in the new-born dog "fabricates" a pancreas showing only Alpha cells.

Early ligature and early vagotomy both produce an increase in the Alpha cells of the ligated portion.

Purpose of Experimental Work. (1) To attempt, by early ligature of the body of the pancreas of new-born dogs, the isolation of the two islet cell areas in order to study the effect of the resulting changes in Alpha cell to Beta cell ratio. (2) To measure the glucose tolerance and the insulin sensitivity of the operated animals and their controls, and to compare the insulin content of portions of pancreas with their histological pictures.

Materials and Methods. The new-born dog was chosen for this experiment. Four types of ligature were performed: single ligature (between the two ducts), double ligature (single ligature plus ligature on duodenal side of the duct of Wirsung), segmental ligature (extreme splenic end) and duodenal ligature (extreme duodenal end). Some were submitted to supra-diaphragmal bilateral vagotomy. In each family some were sham operated to serve as controls. Forty puppies from eight families were investigated for glucose tolerance and insulin sensitivity. Thirty-three samples of pancreatic tissue from twenty animals were assayed for insulin content. The surgical operations and the histological examinations were done by Dr. M.A. Sergeyeva of L'Université de Montréal, and the insulin assays were conducted by Dr. G.A. Wrenshall of The Banting Research Institute of Toronto. The author performed the tests of glucose tolerance and insulin sensitivity on the animals.

Conclusions. The operations of single, duodenal and segmental ligatures produced no observable change in the glucose tolerance and insulin sensitivity of the animals as compared to controls. Double ligature caused a slight decrease in the glucose tolerance and insulin sensitivity, whereas vagotomy produced an increase in both tests.

Single, double and segmental ligatures invariably caused sclerosis of the splenic ligated portion and, at the same time, the histological picture of an increased endocrine potential of the sclerosed portion. The insulin content of these segments has been

increased many-fold by these operations. Vagotomy also increased the endocrine potential of the pancreas but produced pancreases showing only Alpha cells. The insulin content of these glands is slightly raised in both ends by this operation.

These results led to the conclusion that in pre-natal and early post-natal life, the Alpha cells of the pancreas secrete insulin. Later, when Beta cells appear, this function is taken over by these latter cells whereas the Alpha cells would play another role, presumably that of secreting the hyperglycemic factor. At any rate, the results of this work support the statement that the Beta cells are not always the morphological expression of insulin content.

CLAIMS TO ORIGINALITY

The work presented in this thesis is original to the author with respect to the following:

Chapter I

The investigation of the degradation products obtained from the alkaline digestion of the pancreatic hyperglycemic factor carried out for the purpose of isolating and characterizing the component responsible for the glucose-elevating property of the hydrolysate.

The findings that:

there was no glucose-elevating polypeptide in the hydrolysate,

the glucose-elevating action of the hydrolysate was due to the presence of calcium ions liberated from the pancreatic protein by the digestion process,

the glucose accumulation caused by the presence of calcium ions in the incubation medium is not proportional to the concentration of calcium but is maximal at 25 ug. per ml. (M/1600) and minimal at 0 and 100 ug per ml. (M/400).

contrary to the action of the hyperglycemic factor known to exert its glucose elevation by an increased glycogenolysis, calcium does so by shifting the orientation of the glycogen breakdown products towards glucose, at the expense of glycolysis thus leaving the degree of glycogenolysis unchanged.

The proposition of a hypothesis according to which some cases of diabetes mellitus of non-pancreatic origin would be caused by fluctuations of the ionic composition of the interstitial liquid of the liver in general, and of variations of the calcium level of this fluid in particular.

A suggested explanation of the anti-ketotic action of the hyperglycemic factor.

Chapter II

The use, outside the field of histology, of the method of early ligature of pancreas for the purpose of isolating the component islet cells.

The application of the method of early vagotomy as a method of producing pancreas showing only Alpha cells in a more active state.

The findings that:

double ligature of the pancreas in the new-born puppy results in a decrease of the glucose tolerance and insulin sensitivity,

vagotomy in new-born puppies causes an increase in the response of the animals to these two tests,

the Beta-cell-free pancreas obtained by vagotomy in the new-born dog is secreting insulin in a normal manner,

the ligated pancreas of young dogs can secrete insulin independently of the development of their Beta cells.

The hypothesis that places upon the Alpha cells the function of secreting insulin in the new-born animal.

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