A Rat Pancreas-Small Gut Preparation for the Study of Intestinal Factor(s) and Insulin Release

Juan C. Penhos, M.D., Ching-Hui Wu, M.D., Juan C. Basabe, M.D., Nestor Lopez, M.D., and Frederick W. Wolff, M.D., Washington, D.C.

SUMMARY

A technic for in situ perfusion of rat pancreas alone or with a small intestine preparation, which is suitable for studying insulin and/or glucagon secretion and the role intestinal factor(s) has upon it, is described. Krebs-Ringer bicarbonate buffer, containing 4.0 per cent bovine albumin and 80 mg. per 100 ml. glucose with pH adjusted to 7.4, was used as perfusate.

When the pancreas was perfused with a nonrecirculated buffer there was no significant change in the levels of glucose or insulin in the perfusate, while with recirculated buffer, there was a decrease in both measurements.

When additional glucose was added to the perfusate, there was an increase of insulin levels, more marked in the pancreas perfusion with recirculated buffer (possibly pancreatic glucagon plus glucose effect) than in nonrecirculated ones (glucose effect).

In pancreas-intestine perfusion with recirculated buffer, there was a decrease of the levels of insulin and glucose, and when additional glucose was added to the perfusate, there was an increase of the levels of insulin and glucose (possibly pancreatic glucagon and glucose effect).

When glucose was passed through the intestine, there was a marked increase of the levels of insulin which was significantly higher than the previous group studied, probably due to the effects of glucose, pancreatic glucagon and intestinal factors.

It may be possible by the results obtained to separate the effects of the three factors on the release of insulin: glucose, glucagon and intestinal factor(s). DIABETES *18*:733-38, November, 1969.

In 1964, Dupre[9] revived interest in the possible existence of an intestinal hormone affecting glucose metabolism. Since then several studies have followed, demonstrating the relationship between intestinal factor(s) and insulin or glucagon release.[10, 13, 14] More recently, it has been shown that secretin, pancreozymin and gastrin influence insulin and glucagon secretion,[11, 12, 15] which suggested to Unger et al.[12] the existence of an "entero-insular axis."

The possibility of using a preparation, where the pancreas and small intestine were isolated, to study the relationship between the pancreas and intestine stimulated our interest. Several investigators[3-5] have studied insulin secretion by utilizing perfusions of the isolated rat pancreas alone or with the duodenum,[5] or with the spleen, stomach and duodenum[3,4] perfused with blood diluted either with buffer[4,5,8] or pure albumin buffer.[6,7] This paper describes a new technic by which the isolated rat pancreas, alone or with the small intestine, is perfused in situ. This method gives the option of adding a substrate, drug, or hormone to the perfusate, mimicking an intravenous injection, or through the intestine, mimicking an oral ingestion. Preliminary information about the effect of glucose on insulin secretion by the isolated pancreas with, and without, the small intestine using an open and closed circulation is presented to appreciate the characteristics of the preparation.

METHODS AND MATERIALS

Perfusion apparatus

The perfusion apparatus, with the exception of minor modifications, is based on that previously described for perfusions of an isolated liver[1] and that of the hind limb of the rat.[2] The entire apparatus (figure 1) is contained within a wooden box [2.0 × 0.5 × 0.5 m.] except for the perfusion pump (Aminco Peristaltic Pump) which protrudes from one side [1].* The apparatus consists of the following components: a blood filter (Saftilene-Cutter) [2] in which is inserted a medium size polypropylene T connector [3] (H-19606, Federal Scientific Co., Kensington, Maryland), which connects the filter to the bypass [4], such that a pressure head

From the Research Foundation of the Washington Hospital Center and the Division of Clinical Pharmacology, Department of Medicine, The George Washington University School of Medicine, Washington, D.C.

Present address and reprints request: J. C. Penhos, M.D., Diabetes Research Laboratory, Veterans Administration Hospital, Washington, D.C. 20422.

*Numbers in brackets refer to points within figure 1.

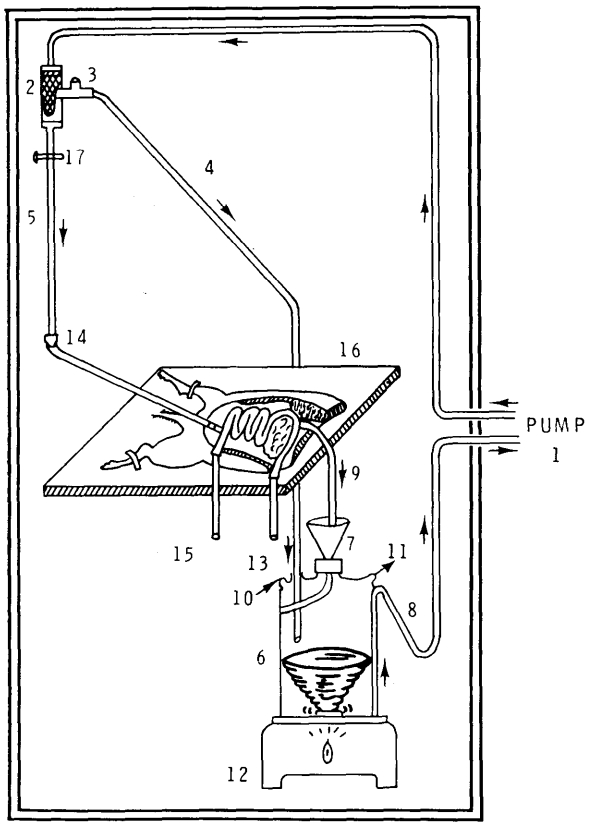

FIG. 1. Rat pancreas-small gut perfusion apparatus. For details see Methods.

of 40 to 45 cm. of buffer pressure is obtained [5]. The excess of buffer is directed through the bypass [4] by plastic tubing (Tygon Tubing Surgical Formulation 5221, ¼" x 1/6") into a 500 ml. polyethylene washing bottle [6]. This bottle has five holes in its superior part; one is in the center of the cap which allows the introduction of the stem of a polypropylene funnel [7] of 65 mm. diameter to collect the perfusate output of the portal vein [9]. A second hole allows the introduction of the inferior end of the bypass [4]. It is directly opposite the dispensing tube of the wash bottle [8] which is connected to the filter [2] with a plastic tube similar to the one available with the filter set which passes through the pump [1]. A fourth hole [10] allows the entrance of gas mixture (O_2 = 95 per cent + CO_2 = 5 per cent), and the fifth one [11] permits the output of gas and sampling of the buffer that also could be done from the polypropylene T connector [3].

The peristaltic pump [1] recirculates the perfusate from the bottom of the bottle to the filter. The perfusate is stirred with the help of a magnetic stirrer [12] obtaining its correct oxygenation. After reaching the filter the perfusate enters a tube [5] which connects with a needle one inch in length, number 18 [14] attached to a polyethylene cannula that will be inserted into the aorta.

The temperature of the box is maintained at 37°C. ± 0.5 by means of a thermoregulator (Bimetal Thermoregulator, No. 545 and Supersensitive Relay, No. 506—The American Instrument Co., Silver Spring, Maryland). A hair dryer and a regular heater bulb are located in the floor of the perfusion box to circulate warm air as a source of heat.

Surgery

Male albino rats (Wistar Strain), weighing 350 to 400 gm., with access to food and water ad libitum were used as pancreas donors.

Sodium amytal (Lilly), 4 mg./100 gm. body weight, was injected intraperitoneally as the anesthetic. A midline abdominal incision from the xiphoid to the pubis was made. The testicles and epididymal fat pads were removed to have an ample surgical field. The rectum was exposed and cut between two ligatures. Next, the large intestine was carefully removed from its anatomical connection and discarded. Then the branches of lineal vessels were ligated and transected and the spleen removed. After careful dissection, the esophagus and attached vessels were severed from their anatomical connections to the stomach. All vessels reaching the stomach, branches of left and right gastric vessels, were tied leaving only those that enter the duodenum and pancreas.

The connective tissue between the aorta and inferior vena cava was carefully dissected by means of a blunt curved forcep. Other vessels were ligated bilaterally and severed in the following order: iliolumbar, renal, adrenal and lumbar. A ligature was placed just above the bifucation of the aorta and inferior vena cava into the iliac vessels, interrupting circulation to the pelvis and hind limbs. A thread was passed around the aorta, just below the diaphragm, ready to close it at the proper moment. Heparin solution 0.25 ml. (1.750 U.) was injected into the neck in the jugular vein, and within two minutes the inferior vena cava was tied above the renal veins.

An incision was made into the stomach near the cardia and another at the end of the small intestine, and both organs were washed with warm saline. A polyethylene cannula, 20 cm. in length (3 mm. i.d. × 4 mm. o.d.) was inserted into the pylorus through the incision made in the stomach (figure 1, [13]). Another

similar cannula was inserted into the end of the ileum (figure 1, [15]).

The cannulation of the aorta was done by placing an arterial clamp just below the superior mesenteric, thus allowing cannulation of the inferior part of the abdominal aorta (figure 2), but without interrupting the circulation of the pancreas and small intestine. An incision was then made in the aorta below the iliolumbar vessels. A polyethylene cannula, attached to a needle (No. 18) on the perfusion set (figure 1, [14]), and long enough to reach the surgical table, was inserted into

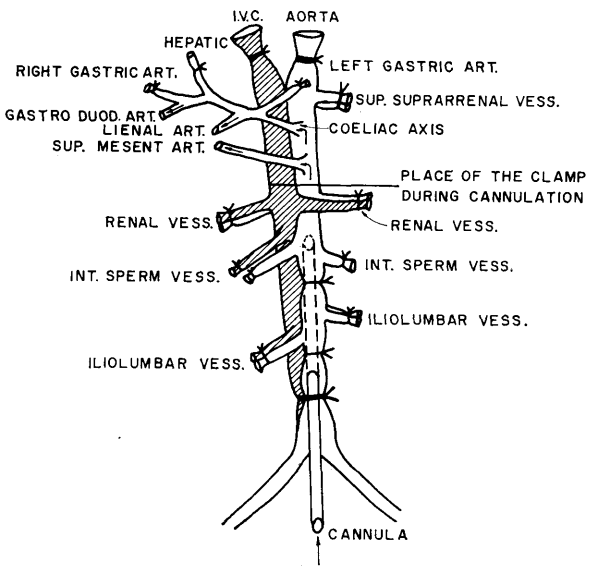

FIG. 2. Placement of aorta and vena cava ligatures. For details see Methods.

the aorta. The cannula was filled with perfusate to prevent air bubble entrance. As soon as the cannula was tied, the arterial clamp was removed, and the aorta was closed above the coeliac axis. At the same time, the perfusate was delivered from the apparatus to the aorta. This maneuver permits isolation of the pancreas and small intestine without the circulation being interrupted. Immediately after the aorta was tied, the middle incision of the skin and muscles was extended to the sides of the animal. Then, with a bone cutter, the vertebral column was severed, and the rat was hemisected below the diaphragm. A free efflux of buffer from the hepatic veins now takes place. The whole hilum of the liver at its highest point was tied. An incision of the portal vein was made and cannulated towards the pancreas with a polyethylene cannula of the same size as that used for the aorta [9].

When the pancreas was perfused alone, the duodenum was dissected and carefully ligated and transected of all vessels connecting the pancreas and the duodenum. The bile and pancreatic ducts must be ligated and severed from their connection with the intestine.

Sequence of manipulation

One hundred milliliters of 0.9 per cent saline were introduced via the funnel into the bottle; then the peristaltic pump was started. Within ten minutes the entire apparatus had been sufficiently wet, and the solution was then drained. With the pump turned off, 150 ml. of buffer (Krebs-Ringer bicarbonate) with glucose (80 mg. per 100 ml.) and 4 per cent bovine albumin, fraction V (Nutritional Biochemical Corp.), with an adjusted pH of 7.4, was introduced via the funnel of the bottle. The $O_2 + CO_2$ mixture (95 per cent and 5 per cent) was then introduced into the bottle through a plastic cannula (figure 1, [10]). The clamp under the filter (figure 1, [17]) was closed to limit the perfusate flow to the bypass.

As soon as the cannula was inserted into the aorta and tied, the aortic clamp was removed (figure 2) and the clamp under the filter was opened. The blood ran up in the cannula and tubing (figure 1, [5]) towards the filter, because of the pressure differential. The pressure was adjusted by lowering or elevating the filter until positioned where the buffer did not push back the blood and the blood did not run up the tubing. At this moment, the pressure of the perfusion set equals the blood pressure of the animal.

After the rat was hemidissected and portal vein cannulated, all the cannulae (aortic, portal, duodenal and ileal) were held to the operating board (figure 1, [16]) with adhesive tape. The preparation was placed in the perfusion box and the portal cannula (figure 1, [9] adjusted to drip into the funnel (figure 1, [7]. The cannula was lifted from the funnel for sampling from the portal vein. Perfusate was sampled from the hole (figure 1, [11]) used for the gas mixture exit.

A fifteen-minute equilibration period followed the installation of either preparation, pancreas alone or pancreas with the intestine, into the circuit. This period is necessary for the stabilization of the dripping from the portal cannula. Sampling began after this fifteen-minute period (0 minute) and continued at fifteen and thirty minutes. Glucose was added to the perfusate (0.5 mg./ml. of perfusate) or passed through the intestine (0.25 ml./min from a 5 per cent solution) after the thirty-minute sample. When glucose was added to the perfusate, a thirty-five minute sample was taken to check the rise in the insulin released. Another two samples were taken at sixty and ninety minutes. Before

ending the experiment, a 2 ml. solution of the 10 per cent glucose was added to the perfusate to control the islets' survival.

Samples of 1 ml. of perfusate were taken from the pool (arterial influx) and from the portal cannula (venous efflux) to measure levels of glucose[25] and insulin.[26] A determination of the oxygen tension was made from the aortic and portal cannulae at the end of the experiment.

In table 2, the level of insulin (μEq./min.) represents the difference between the concentrations of insulin in the portal perfusate and the aortic pool.

Four groups of perfusions were done: (a) perfusion of the isolated pancreas with nonrecirculating perfusate; (b) the same as (a) with recirculation of perfusate; (c) perfusion of the isolated pancreas and small intestine with glucose added to the perfusate; and, (d) the same as (c) but with glucose passing through the intestine (table 1).

At the end of the perfusion, the pancreas was dissected from the preparation and weighed. Average weight of twenty-four pancreases from rats weighing 350 to 400 gm. was 3.2 gm. (S.E. $\pm$ 0.5).

Oxygen consumption in the preparation was verified by demonstrating a fall in oxygen tension, comparing arterial and venous perfusates (decrease of 160 to 120 mm. Hg).

The flow rate was 0.5 to 1.0 ml. per minute per gram of pancreas in the preparation of the pancreas alone and of 1.5 to 2.0 ml. per gram of pancreas in the pancreas-intestine preparation.

RESULTS

The difference in the results obtained when pancreas alone was perfused in an open or closed circuit can be seen in table 2. When the perfusate was nonrecirculated (a), the levels of glucose and insulin were kept without significant change from 0 to 30-minute samples. When glucose was added to the perfusate, there was a rise in the levels of insulin that remained without significant change during the rest of the observation period.

In the pancreas perfused with recirculating perfusate (closed circuit) (b), the levels of glucose and insulin decreased up to thirty minutes (table 2). After adding glucose, there was an increase in both parameters at thirty-five minutes followed by a decrease at sixty and ninety minutes, although the levels of insulin were higher than in the pancreas perfused in the open circuit. The difference in the levels of insulin between the groups (a) and (b) are statistically significant ($p < 0.001$) at thirty-five and sixty minutes, but not at ninety minutes, possibly due to insulin degradation by pancreatic enzymes in recirculation experiments.

The perfusion of the pancreas and small intestine with recirculating perfusate showed a decrease in the glucose and insulin levels. When glucose was added to the perfusate (c), there was a rise in both parameters at thirty-five minutes followed by a decrease in the glucose levels. When glucose was added through the intestine (d), the decrease of glucose in the perfusate was marked at sixty minutes, followed by an increase which was, however, not significantly higher than the value obtained at thirty minutes. Although the level of glucose in the perfusate was lower, the release of insulin increased tremendously after the sugar was passing through the intestine, compared to the perfusion of both organs with glucose added to the perfusate. The differ-

TABLE 1

Organs perfused	Circulation	Substrate added to	Factors stimulating insulin release	Type	Δ
Pancreas	Open	Perfusate	Substrate	a	a = substrate
	Closed	Perfusate	Substrate glucagon*	b	b-a = glucagon*
Pancreas +intestine	Closed	Perfusate	Substrate glucagon*	c	c-a = glucagon*
	Closed	Intestinal lumen	Substrate glucagon intestinai factor(s)	d	d-b or d-c = intestinal factors

*See discussion.

TABLE 2

Glucose and insulin levels in the perfusate of pancreas (groups a and b) or pancreas-small intestine (groups c and d) with nonrecirculated (group a) or recirculated perfusate (groups b, c, and d). Glucose (G) levels expressed as mg./100 ml. of arterial perfusate and insulin (I) concentration expressed in μU./min. as the difference between its venous and arterial levels. Each figure represents the average (V-A) of six perfusions with its corresponding standard error (S.E.M.). Glucose added after thirty minutes.

Group	Circuit or system			Minutes −15	Minutes 0	Minutes 15	Minutes 30	Glucose added	Minutes 35	Minutes 60	Minutes 90
a	Open	G	V-A	79	76	74	74	To the perfusate (0.5 mg./ml.)	114	113	111
			S.E.M.	1.1	1.9	2.9	1.9		2.2	2.8	2.5
		I	V-A	—	67	66	70		120	124	123
			S.E.M.	—	4.1	5.5	5.4		6.6	5.2	4.1
b	Closed	G	V-A	79	71	66	60		103	93	86
			S.E.M.	1.3	2.4	1.1	1.6		1.2	1.1	2.0
		I	V-A	—	57	46	37		168	154	135
			S.E.M.	—	4.0	3.7	3.5		4.9	3.7	4.2
c	Closed	G	V-A	78	61	50	41		87	53	26
			S.E.M.	0.9	3.5	3.6	3.8		3.3	3.9	1.9
		I	V-A	—	59	48	39		162	144	126
			S.E.M.	—	5.3	4.6	4.5		6.4	5.7	6.4
d	Closed	G	V-A	79	58	48	39		—	27	41
			S.E.M.	1.3	2.4	3.2	3.9	Passed through small intestine	—	2.0	1.5
		I	V-A	—	57	46	36	(Sol. 5%	—	281	263
			S.E.M.	—	4.9	5.0	4.7	0.25 ml./min.)	—	8.9	19.3

ence in the levels of insulin between groups (c) and (d) is statistically significant ($p < 0.001$) at sixty and ninety minutes.

DISCUSSION

Four different preparations were perfused in order to separate the effects on insulin release of three important factors: substrate, pancreatic glucagon and intestinal factor(s). When the perfusion was done, using the isolated pancreas in an open circuit with the perfusate nonrecirculated (group a), the only stimulus to insulin release is the substrate (glucose in this case) added to the perfusate (table 1). When the perfusate was recirculated (group b), we must add to the effect of glucose the effect of glucagon released into the perfusate by the alpha cells[23] and possibly other islet "hormones." The difference in values obtained in groups (a) and (b) may be considered to demonstrate the effect of glucagon. Of course, in recirculating experiments we do not know whether the insulin or material from delta cells released in the perfusate has a further effect on the beta cells.

In perfusions of pancreas and intestine with glucose added to the recirculated perfusate (group c), the factors that affect the insulin release are again glucose and glucagon. When glucose solution was passed through the small intestine (group d) the factors that affect insulin secretion were glucose, glucagon and intestinal factor(s). The difference between the insulin levels of groups (c) and (d) or (b) and (d) may be considered to demonstrate the effect of intestinal factor(s) alone on insulin release.

Although the glucose levels in our experiments are difficult to keep equal, due to the different conditions used, it is still more remarkable that when glucose was passed through the intestine, there was a greater release of insulin with lower levels of glucose in the perfusate. These results confirm the studies done by several investigators where a higher level of insulin in man and animals evoked by oral glucose loading as compared with intravenous administration[16-22] was found.

The pancreas without the intestine was perfused with recirculated perfusate and with nonrecirculated perfusate. The difference in the insulin levels between both technics could be possibly attributed to pancreatic glucagon potent insulin releasing activity.[23-24]

Although we did not identify the enteric hormones, it is evident that they have, like glucagon, an important

role in the secretion of insulin. Substrates in the lumen of the small intestine release beta-cytotropic hormones which then cause release of insulin before the blood levels of the substrate rise sufficient to produce[12] an excessive hyperglycemia or hyperaminoacidemia. Recently[27] evidence has been presented with electron microscope studies of rat gastrointestinal mucosa that suggest the existence of a substance in the intestine very similar to glucagon, biologically and immunologically, secreted by a type of cell which resembles the alpha cell of the pancreas.

Further studies are now in progress to observe the effect of other sugars, as well as of known insulin-stimulating drugs, on these factors.

REFERENCES

[1] Penhos, J. C., Wu, C. H., Daunas, J., Reitman, M., and Levine, R.: Effect of glucagon on the metabolism of lipids and on urea formation by the perfused rat liver. Diabetes *15*:740, 1966.

[2] Mahler, R. J., Szabo, O., and Penhos, J. C.: Antagonism to insulin action on the perfused hind limb of the rat by a reduced insulin-B chain—albumin complex. Diabetes *17*:1, 1968.

[3] Anderson, E., and Long, J. A.: The effect of hyperglycemia on insulin secretion as determined with the isolated rat pancreas in a perfusion. Endocrinology *40*:92, 1947.

[4] Grodsky, G. M., Batts, A. A., Bennett, L. L., Vcella, C., McWilliams, N. D., and Smith, D. F.: Effects of carbohydrates on secretion of insulin from isolated rat pancreas. Amer. J. Physiol. *205*:638, 1963.

[5] Sussman, K. E., Vaughan, G. D., and Timmer, R. F.: An in vitro method for studying insulin secretion in the perfused isolated rat pancreas. Metabolism *15*:466, 1966.

[6] Grodsky, G. M., Bennett, L. L., Smith, D. F., and Schmid, F. G.: Effect of pulse administration of glucose or glucagon on insulin secretion in vitro. Metabolism *16*:222, 1967.

[7] Grodsky, G. M., and Bennett, L. L.: Cation requirements for insulin secretion in the isolated perfused pancreas. Diabetes *15*:910, 1966.

[8] Sussman, K. E., and Vaughan, G. D.; Insulin release after ACTH, glucagon and adenosine-3′-5′-phosphate (cyclic AMP) in the perfused isolated rat pancreas. Diabetes *16*:449, 1967.

[9] Dupre, J.: An intestinal hormone affecting glucose disposal in man. Lancet *2*:672, 1964.

[10] Dupre, J., and Beck, J. C.: Stimulation of release of insulin by an extract of intestinal mouse. Diabetes *15*:555, 1966.

[11] Unger, R. H., Ketterer, H., Eisentraut, A. M., and Dupre, J.: Effect of secretin on insulin secretion. Lancet *2*:24, 1966.

[12] Unger, R. H., Ketterer, H., Dupre, J., and Eisentraut, A. M.: The effects of secretin, pancreozymin and gastrin on insulin and glucagon secretion in anesthetized dogs. J. Clin. Invest. *46*:630, 1967.

[13] Pfeifer, E. F., Telib, M., Ammon, J., Melani, F., and Ditschuneit, H.: Letter to the Editor. Diabetologia *1*:131, 1965.

[14] McIntyre, N., Turner, D. S., and Holdsworth, C. D.: Intestinal factors and insulin secretion. Diabetologia *1*:73, 1965.

[15] Dupre, J., Rojas, L., White, J. J., Unger, R. H., and Beck, C. J.: Effects of secretin on insulin and glucagon in portal and peripheral blood in man. Lancet *2*:26, 1966.

[16] Scow, R. P., and Cornfield, J.: Quantitative relations between the oral and intravenous glucose tolerance curves. Amer. J. Physiol. *179*:435, 1954.

[17] Arnould, Y., Bellens, R., Franckson, J. R. M., and Conard, V.: Insulin response and glucose-C^{14} disappearance during the glucose tolerance test in the unanaesthetized dog. Metabolism *12*:1122, 1963.

[18] McIntyre, N., Holdsworth, C. D., and Turner, D. S.: New interpretation of oral glucose tolerance. Lancet *2*:20, 1964.

[19] Grodsky, G. M., Karam, J. J., Pavlatos, F. C., and Forsham, P. H.: Serum-insulin response to glucose in prediabetic subjects. Lancet *2*:290, 1965.

[20] Elrick, H., Stimmler, L., Hlad, C. J., Jr., and Arai, Y.: Plasma insulin response to oral and intravenous glucose administration. J. Clin. Endocr. *24*:1076, 1964.

[21] McIntyre, N., Holdsworth, C. D., and Turner, D. S.: Intestinal factors in the control of insulin secretion. J. Clin. Endocr. *25*:1317, 1965.

[22] Dupre, J.: Effect of route of administration on disposal of glucose loads. J. Physiol. *175*:58, 1964.

[23] Samols, E., Marri, G., and Marks, V.: Promotion of insulin secretion by glucagon. Lancet *2*:415, 1965.

[24] Crockford, P. M., Porte, D., Jr., Wood, F. C., Jr., and Williams, R. H.: Effect of glucagon on serum insulin, plasma glucose and free fatty acids in man. Metabolism *15*:114, 1966.

[25] Faulkner, D. E.: An automated micro-determination of blood glucose with an AutoAnalyzer. Analyst *90*:736, 1965.

[26] Hales, C. N., and Randle, P. J.: Immunoassay of insulin with an antibody precipitate. Biochem. J. *88*:137, 1963.

[27] Orci, L., Pictet, R., Forssmann, W. G., Renold, A. E., and Rouiller, C.: Structural evidence for glucagon producing cells in the intestinal mucosa of the rat. Diabetologia *4*:56-57, 1968.