Effects of carbohydrates on secretion of insulin from isolated rat pancreas

GEROLD M. GRODSKY, ADRIENNE A. BATTs, L. BENNETT, CARL VCELLA, NANCY B. McWILLIAMS, AND DESMOND F. SMITH

Metabolic Research Unit and Departments of Biochemistry and Physiology, University of California School of Medicine, San Francisco, California

GRODSKY, GEROLD M., ADRIENNE A. BATTs, LESLIE L. BENNETT, CARL VCELLA, NANCY B. McWILLIAMS, AND DESMOND F. SMITH. Effects of carbohydrates on secretion of insulin from isolated rat pancreas. Am. J. Physiol. 205(4) : 638-644. 1963.—The effect of carbohydrates on the secretion of immunochemically measurable insulin was studied in an isolated perfused pancreatic preparation from the rat. Degradation of circulating insulin (as measured by chromatographic examination of added insulin-1131) was less than 15% during the 4-hr experimental period. Without the addition of glucose, or at glucose concentrations of less than 50 mg/100 ml, insulin secretion was not detectable. At glucose concentrations of 50-150 mg/100 ml, insulin secretion occurred immediately and persisted throughout the experimental period. Insulin secretion was further increased by increasing glucose concentration to 150-500 mg/100 ml. The incidence of islet cell degranulation increased with increasing insulin secretion, suggesting that glucose stimulated secretion of stored insulin faster than synthesis of insulin de novo. Galactose, xylose, L-arabinose, pyruvate, and 2-deoxyglucose in concentrations of 600 mg/100 ml did not stimulate insulin secretion. Mannose stimulated the pancreas equally as well as glucose. Fructose was also active, but was less effective than glucose. Neither 2-deoxyglucose nor galactose blocked the insulin secretion by glucose. The data suggest that secretion of insulin is stimulated by a metabolite or a product resulting from the metabolism of glucose which can also be supplied by other metabolizable sugars.

MATERIALS AND METHODS

Bovine insulin-1131 (approximately 5 mc/mg) was obtained from Abbott Laboratories, 2-deoxyglucose and pyruvate from Nutritional Biochemicals, fructose and xylose from the California Foundation for Biochemical Research, galactose and L-arabinose from Pfanstiehl Laboratories, Inc., and mannose from Eastman Organic Chemicals.

The perfusion apparatus used was a modification of the one described by Anderson and Long (1), except that the dialyzer was omitted and a more efficient oxygenator was introduced. The latter consisted of a glass tube, about 18 in. long, set at an angle to permit the blood to run down the side while a humidified mixture of O2 and CO2 (95:5) passed up the tube. The perfusion apparatus was assembled shortly before use and filled with a mixture of 2% gelatin in saline, which was circulated at 37 C 1 hr before the blood was added and the pancreas was attached.

The perfusate was a 1:1 mixture of 2% gelatin in 0.9% NaCl and of fresh heparinized rat blood. Donor...
rats of the Long-Evans strain, weighing 300 g, were fasted overnight, anesthetized with sodium pentobarbital, 6 mg/100 g body weight administered intraperitoneally, and bled by aortic puncture. Penicillin G and streptomycin sulfate were added to the perfusate to a concentration of about 100,000 U/100 ml and 0.28 g/100 ml, respectively. The total volume of perfusate was about 160 ml.

A rat of about 250 g body wt., fasted overnight, and anesthetized with sodium pentobarbital, was used as the pancreas donor. The object of the dissection was to remove in one block the stomach, spleen, pancreas, and duodenum and to attach cannulas to the celiac axis and portal vein. The mesenteric artery was doubly ligated and cut, and the entire intestine below the duodenum was separated and removed from the rat to simplify the exposure. The esophagus was ligated as high as possible and cut above the ligature. A loose ligature was placed, but not tied, around the entire gastrohepatic ligament. The aorta was cautiously exposed through the crura of the diaphragm well above the point of origin of the celiac axis. The ligature around the gastrohepatic ligament was then tied as tightly as possible, and the ligament cut above the ligature. The aorta was cut between the double clamps and below the lower clamp, and the preparation was lifted out of the abdomen. The clamps were removed from the aorta which was slit open on the side opposite the origin of the celiac axis, revealing the opening of the latter. The arterial cannula was inserted and tied in place. An opening was made close to the ligature, as near the end of the portal vein as possible. Circulation through the preparation was initiated and blood was observed to flow from the slit in the portal vein. After a minute or two, the flow was stopped and a cannula was inserted into the portal vein and tied in place. Flow was then resumed; the total time blood flow was halted during the surgical procedure was less than 5 min. Flow rates through the pancreas were usually 7–9 ml/min at perfusion pressures of about 100 mm Hg. Oxygen consumption in the preparation was verified by demonstrating a fall in \( \Delta O_2 \), comparing arterial and venous blood using an oxygen electrode.

The pancreas was perfused for an equilibration period of 1 hr. At this time the carbohydrate dissolved in saline was added directly to the perfusate or, in the case of glucose, fructose, and mannose, was infused at a fixed rate into the perfusate after an initial priming dose. At the end of the experiment, sections were taken from the tail of the pancreas and fixed for histologic study.

Insulin in the perfusate was measured immunochemically by the method of Grodsky and Forsham (8, 14), which is based on the decrease in per cent binding of insulin-\(^{131}\)I to insulin antibodies induced by increasing quantities of unlabeled insulin. The per cent of free insulin is measured quantitatively after preferential precipitation of bound insulin with sodium sulfate. The technique was modified to assay perfusate insulin directly without preliminary extraction with acid alcohol. To each 0.5 ml of perfusate was added a 1-ml aliquot of a freshly prepared (1–2 min) mixture containing 10 \( \mu l \) diluted guinea pig sera containing antibodies to bovine insulin in 0.49 ml 0.2 M glycine buffer (pH 8.6) and

### Table 1. Degradation of insulin-\(^{131}\)I added to circulating perfusate

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Per Cent Insulin-(^{131})I Degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st hr / 2nd hr / 3rd hr / 4th hr</td>
</tr>
<tr>
<td>Saline</td>
<td>36 / 78 / 92</td>
</tr>
<tr>
<td>3% Albumin</td>
<td>30 / 38 / 92</td>
</tr>
<tr>
<td>6% Dextran</td>
<td>49 / 73 / 88</td>
</tr>
<tr>
<td>White's solution</td>
<td>60 / 75 / 96</td>
</tr>
<tr>
<td>3.5% Polyvinylpyrrolidine</td>
<td>0 / 10 / 18</td>
</tr>
<tr>
<td>1% Gelatin</td>
<td>0 / 4 / 35</td>
</tr>
<tr>
<td>2% Gelatin</td>
<td>0 / 5 / 11</td>
</tr>
</tbody>
</table>

\* 100 \( \mu U \) insulin-\(^{131}\)I/ml was added to perfusate before it was circulated through the pancreatic preparation. Insulin degradation was determined by hydrodynamic flow chromatography (4). Perfusion fluids were 1:1 mixtures of fresh rat blood and diluent. Results were means of three experiments.

**FIG. 1.** Effect of varying concentration of glucose on insulin secretion. Carbohydrate values are means of all experiments. Shaded area is \( \pm 2 \) SD of per cent change in displacement of insulin-\(^{131}\)I at 1 hr before addition of any carbohydrate. Heavy line is mean per cent displacement of insulin-\(^{131}\)I. A: \( \bullet \) no glucose, \( O \) glucose 30–45 mg/100 ml. B: glucose, 50–150 mg/100 ml. C: glucose, 150–500 mg/100 ml.
TABLE 2. Effect of glucose on islet cell degranulation

<table>
<thead>
<tr>
<th>Glucose Conc., mg/100 ml</th>
<th>No. of Perfusion Exp.</th>
<th>No. of Pancreases With Detectable β Cell Degranulation</th>
<th>Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>170-500</td>
<td>14</td>
<td>9</td>
<td>64</td>
</tr>
<tr>
<td>50-170</td>
<td>12</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>0-45</td>
<td>17</td>
<td>3</td>
<td>18</td>
</tr>
</tbody>
</table>

0.5 ml of 5% albumin containing 30% urea. After incubation for 1 hr at 25°C, insulin bound to antibody was precipitated with the total globulins by addition of 2-2.5 volumes of 25% sodium sulfate solution (exact optimal salt concentration was determined empirically (8)). After 30 min at 25°C, the precipitated proteins were removed by centrifugation and an aliquot of the supernatant containing free insulin-1131 was counted in a well-type scintillation counter.

Pure rat insulin was not available as a standard for the insulin assay system. Crude preparations of rat insulin obtained by acid alcohol extraction of rat pancreas react with antibodies to beef insulin in guinea pig serum but not in the same proportion as does beef insulin (19). Similar results were obtained with pork insulin or when antibodies to pork insulin were employed. Therefore, as a measure of the rat insulin in the perfusates, results were reported empirically as the per cent change in supernatant activity, taking the value of the perfusate at zero time as the zero reference point. All samples from a given perfusion were assayed in duplicate and the results were averaged with an identical assay performed on a subsequent day. The standard deviation of the assay determined from a series of duplicate estimations was 4.5%.

Carbohydrate determination. For experiments in which glucose alone was added to the perfusate, glucose was determined by the method of Somogyi-Nelson (18). When xylose, L-arabinose, mannose, galactose, or fructose alone were added, they were determined as reducing sugars using the appropriate standard. Galactose in the presence of glucose was determined as nonfermentable reducing substance. Both alone and in combination with glucose, 2-deoxyglucose was determined by the method of DeMoss and Happel (5).

Histologic procedure. Pancreatic tissue was fixed in Bouin's fluid for at least 24 hr, dehydrated, imbedded in paraffin (mp 56°C), and serially sectioned at 4μ. The slides were stained with Gomori aldehyde fuchsin and chrome hematoxylin and counterstained for 10 min with the following mixture: 0.25 g chromotrope 2R, 0.1 g light green, 0.5 g phosphotungstic acid, 20 ml glycerol, 80 ml water, and 1 ml glacial acetic acid.

RESULTS

It was observed initially that pumping of perfusate of whole blood in saline through the apparatus for 4 hr at 37°C resulted in considerable hemolysis even when the pancreas preparation was not included. Since other studies in our laboratory showed that insulin-1131 is rapidly degraded by hemolyzed serum or plasma during standing (particularly when the temperature is raised to 37°C), it was necessary to ascertain and minimize the degree of degradation of insulin occurring during a perfusion experiment.

An unhemolyzed perfusion mixture of whole blood and saline did not degrade insulin-1131 significantly after 4 hr standing as measured by hydrodynamic flow chromatography (4, 9) or appearance of label in supernatant after precipitation of proteins with 5% trichloroacetic acid. The same mixture, when circulated through the apparatus either in the presence or absence of the pancreas, became hemolyzed and caused rapid destruction of added circulating insulin-1131 (Table 1). Insulin-1131, added to a hemolyzed 4-hr perfusate and allowed to stand at 37°C, was also degraded rapidly. Part but not all of the degraded products were soluble in trichloroacetic acid. Hemolysis and insulin degradation were not decreased by prewashing the red cells with saline or by the addition to the perfusate of antibiotics, soybean trypsin inhibitor (0.4 mg/ml), or tryptophane (2 mg/ml). Ether anesthesia of the donor rats caused even greater hemolysis than sodium pentobarbital. At one stage in the study, it was thought that crystalline hemoglobin could be substituted for the intact red cells as the oxygen...
carrier. Crystalline hemoglobin was prepared by dialyzing hemolyzed red cells against 65% ammonium sulfate. This preparation of crude hemoglobin, when dissolved in saline, still degraded insulin. Only extensive purification by fractionation with ammonium sulfate produced a hemoglobin preparation which was free of this nondialyzable factor in the red cell capable of destroying insulin. Various diluents substituted for saline in the perfusion mixture (Table 1), 6% dextran in saline, 3% human serum albumin in saline, or White's buffer (22), did not prevent insulin degradation. Polyvinyl pyrrolidine (3.5%) minimized hemolysis and insulin degradation but interfered with protein precipitation by salt in the insulin assay. Gelatin, 2% in saline, was finally adopted for the diluent since it also proved effective in reducing hemolysis and insulin degradation in the presence of pancreas to 0-15% and did not interfere in the insulin assay.

Effect of glucose on insulin secretion. When glucose was not added to the circulating perfusate, the endogenous glucose from the donor rat blood rapidly disappeared (after 2 hr the perfusate contained less than 15 mg glucose/100 ml), presumably due to utilization by the tissues. The insulin levels after 4 hr of perfusion were unchanged when compared to those at the 1-hr equilibration period. When glucose was perfused to maintain a level of 25-45 mg glucose/100 ml, there was still no evidence of insulin secretion (Fig. 1A). The amount of insulin secreted was, however, sensitive to glucose concentration as glucose levels were raised above 50 mg/100 ml. In eight perfusions, a rise in mean cumulative insulin after 4 hr was observed when blood sugar was maintained at 50-150 mg/ml. A significant rise in circulating insulin was detectable within 60 min after addition of glucose (P = 0.01) (Fig. 1B) and the insulin levels (representing cumulative insulin) rose progressively throughout the succeeding 2 hr. Insulin in the perfusate at the 4th hr displaced an average of three times more bovine insulin-131 from antibody than comparable perfusates from experiments in which glucose was not added. Maintaining the glucose level of the perfusate at 150-500 mg/100 ml elicited further insulin secretion (Fig. 1C). Insulin was detected within 30 min and by 4 hr, the secreted insulin displaced a mean of five times more bovine insulin-131 than the controls. The responses in this range were variable and proved indistinguishable from each other regardless of the individual glucose levels employed.

Effect of carbohydrates on β-cell degranulation. Degranulation of the islet cells was generally detectable in experiments in which insulin secretion had occurred but was a less consistent finding than the rise in circulating insulin. In a series of experiments at glucose levels of 170-500 mg/100 ml glucose, at which concentrations a rise in circulating insulin occurred in all experiments, 64% of
the pancreases had observable degranulation. At the intermediate glucose range of 50–150 mg/100 ml, 33% of the pancreases had observable degranulation. The incidence of degranulation in these experiments was significantly greater ($P = <0.05$) than in those performed at 0–45 mg glucose/100 ml, where insulin secretion was not stimulated (Table 2).

Efects of various carbohydrates on insulin secretion. The specificity of the secretory phenomenon for glucose is shown in Figs. 2–6. The addition of the carbohydrates galactose, xylose (Fig. 2), L-arabinose, and 2-deoxyglucose (Fig. 3) at concentrations of 400–600 mg/100 ml produced no secretion of measurable insulin. The concentration of these sugars was at least five times the glucose concentration which was previously shown to cause a detectable rise in circulating insulin (Fig. 1B).

Fructose stimulated a significant secretion of insulin; however, the levels of insulin achieved by 500 mg/100 ml fructose were less than those resulting from perfusion of 50–150 mg/100 ml glucose (Fig. 4). There was no glucose detectable by glucose oxidase (20) in the perfusates after 4 hr of fructose perfusion ($<10$ mg/100 ml).

In five perfusions, mannose (400 mg/100 ml) caused an immediate and sustained secretion of insulin to levels comparable to those after stimulation with glucose (Fig. 5). Glucose at 4 hr varied from undetectable to a maximum of 25 mg/100 ml.

In the presence of pyruvate, 500 mg/100 ml, mean insulin secretion at 4 hr was somewhat greater than insulin levels at 4 hr in control experiments, but the difference was not significant ($P = >0.05$) (Fig. 6).

Effect of galactose on stimulation of insulin secretion by glucose. Insulin secretion, which was marked at 70–100 mg/100 ml glucose, was not depressed by the addition of a five- to tenfold excess of galactose (Fig. 7). A 50% reduction of the effective glucose concentration to 35–45 mg glucose/100 ml would have been sufficient to completely suppress insulin secretion.

Effect of 2-deoxyglucose on stimulation of insulin secretion by glucose. Insulin secretion resulting from the perfusion of 150 mg/100 ml glucose was not reduced when 2-deoxyglucose, 400 mg/100 ml, was simultaneously perfused (Fig. 8).

**DISCUSSION**

The application of an immunochemical assay to the measurement of insulin secreted from a perfused rat pancreas offers advantages of precision, sensitivity, and particularly a high degree of specificity when compared with most existing biologic assays. Nonspecific factors arising from pancreatic secretions (particularly glucagon as well as substances from the stomach, spleen, and duodenum included in the perfusion preparation) do not interfere with the insulin determination (10). The
nonproportional cross reaction of rat pancreas extracts with antibodies to beef or pork insulin indicates that the chemical structure of this hormone may be different from most of the mammalian insulins or that something in extracts of rat pancreas interferes with the immunologic reaction (extracts of beef pancreas or human pancreas prepared identically cross reacted quantitatively with these antibodies (8, 13)).

The isolated perfused pancreas preparation appears to reflect closely the physiologic state of the pancreas. Secretion of immunologically measurable insulin did not occur when glucose was absent or when glucose was maintained at hypoglycemic levels less than 50 mg/100 ml. At glucose concentrations approximating the physiologic range in the unfasted rat (80–120 mg/100 ml) insulin secretion first became detectable.

Insulin secretion did not occur as a single exhaustive spurt when glucose was perfused, but continued throughout the 3 hr of the stimulation. Increases in concentration of glucose elicited an immediate and progressive rise in insulin secreted from pancreas in vitro, suggesting that insulin secretion is directly and continuously stimulated as a function of glucose concentration and is not simply a threshold effect in which insulin is secreted completely at a critical glucose concentration. These findings are in agreement with those of Metz (17) and of Seltzer (21), who found the rate of appearance of insulin from pancreas of the dog perfused in situ increased with increased glucose concentration.

Apparantly, there is an insulin “reserve” capable of responding to the continued challenge of a specific glucose concentration. Since increased insulin secretion in the presence of glucose may be the result of a stimulation of insulin synthesis as well as secretion, part of the pancreatic reserve could arise from insulin synthesized de novo. The depletion of the granules in the islet cells usually observed during perfusion with high levels of glucose indicates that in our experiments insulin secretion was occurring at a greater rate than insulin synthesis. Current studies in progress show that glucose still stimulates insulin secretion from a pancreas in which insulin synthesis from amino acids has been completely blocked with dinitrophenol.

The mechanism by which glucose stimulates insulin secretion is unclear. A common isomeric structure at carbon atoms 1–3 apparently is not important since galactose, xylose, and L-arabinose did not stimulate significant secretion although they are structurally identical to glucose in this portion of the molecule.
Pozza et al. (19), in cross-circulation experiments in dogs, found that galactose administered to the donor dog caused hypoglycemia in the recipient animal. Galactose also causes hypoglycemia in certain subjects with galactosemia (12), a condition resulting from a defect in galactose-1-phosphate uridyl transferase activity. Our in vitro experiments, which indicate that galactose does not directly stimulate the pancreas to secrete insulin, agree with the finding that glucose stimulates the utilization of glucose-C\textsubscript{14} in man, but that galactose does not (3) and that galactose, in contrast to glucose, does not raise the immunochromically measured insulin levels in obesity (14). Possibly, hypoglycemia after administration of galactose is primarily a hepatic effect.

The present studies indicate a close correlation between stimulation of insulin secretion by a specific sugar and its known rate of metabolism to intermediates in the glycolytic or oxidative pathway, suggesting that a metabolite or a product resulting from the metabolism of glucose rather than the glucose molecule itself is the causative factor of insulin secretion. Galactose, xylose, L-arabinose, and 2-deoxyglucose, which are poorly metabolized in most tissues (with the possible exception of galactose in the liver), did not affect insulin release. Pyruvate, which did not effect significant secretion of insulin, is poorly metabolized in fish islets (6). In contrast, mannose and, to a lesser extent, fructose were active stimulators. Hellman and Larsson (11) have shown that fructose is incorporated into the protein of the fish although less effectively than glucose. Mannose metabolism has not been reported in islets and although extrapolating rates of metabolism of carbohydrates established in other tissues to pancreatic islets is hazardous, it is noteworthy that Ball and Cooper (2) and Wood, Cahill, and Renold (23) found in adipose tissue that mannose can be metabolized at rates comparable to glucose.

Both fructose and mannose can enter the glycolytic or oxidative pathways (the presence of glucose 6-phosphate dehydrogenase (15, 16) and the process of C\textsubscript{1} oxidation of glucose (7) have been demonstrated in islets) and thus supply glucose metabolites, TPNH, or other cofactors which may be required. It is also possible that both sugars could be converted to glucose in the islets which, in turn, directly stimulates the pancreas. However, intracellular glucose concentration would have had to be 3-5 times higher than the extracellular concentrations observed in the perfusate with these sugars (< 25 mg/100 ml) for insulin secretion to have occurred as a result solely of glucose levels.

Glucose stimulation of the pancreas was not inhibited by either galactose or 2-deoxyglucose, although the latter compound is an effective inhibitor of glycogen uptake in muscle. Our observations are consistent with the hypothesis that carbohydrates may enter islet cells by noncompetitive free diffusion as they do in hepatic and other visceral cells. Alternatively, if a subsequent step in glucose metabolism were rate limiting in the islets, partial inhibition of glucose uptake may have occurred but was not sufficient to reduce glucose metabolism and depress insulin secretion rate.

REFERENCES