Regulation of Insulin Secretion Studied with Pieces of Rabbit Pancreas Incubated *in vitro*

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The purpose of the present study was to develop a simple system *in vitro* suitable for studies on the control of insulin secretion and based on the rate of release of insulin into the incubation medium from pieces of rabbit pancreas. Rabbit pancreas was chosen for these studies because it is thin (thus permitting ready diffusion of gases and other substances) and because it has a high concentration of insulin relative to that of other species (Marks & Young, 1940). Although the system that has been developed could be used in conjunction with bioassays for insulin, our studies have been greatly facilitated by the immunoassay introduced by Yalow & Berson (1960) and developed in a simplified form in this Laboratory (Hales & Randle, 1963). The latter is more convenient for studies of this sort because of its great sensitivity and specificity and relative simplicity. Earlier studies of the factors affecting insulin secretion have been made both *in vivo* and *in vitro*. In studies *in vivo* the effects of various substances on the concentration of insulin in pancreatic and peripheral venous blood have been investigated with biological and immunological methods of insulin assay (for reviews see Houssay, 1937; Foa, 1956; Randle, 1961; Berson & Yalow, 1962). The most extensive studies *in vitro* have been carried out with perfused rat pancreas in conjunction with biological or immunological methods for the assay of insulin (Anderson & Long, 1948; Grodsky, Bennett, Batts, McWilliams & Vcella, 1962; Grodsky *et al.* 1963). The results of these investigations have led to the generally accepted view that an increase in blood glucose concentration provides the major physiological stimulus for insulin secretion. They have also suggested the possibility that growth hormone and glucagon may have a direct influence on insulin secretion (though evidence on this point has been conflicting) and that hypoglycaemic sulphonamide derivatives may provoke release of insulin.

Evidence is presented below that the release of insulin by pieces of rabbit pancreas *in vitro* provides

a simple and reproducible model for the behaviour of β -cells *in vivo*. The system has been used for a detailed study of factors that may influence insulin secretion and to obtain evidence for the pathway of glucose metabolism in the β -cell that provides the signal for insulin release. Preliminary accounts of some of these findings have been published (Coore & Randle, 1962; Coore, Randle, Simon, Kraicer & Shelesnyak, 1963). Evidence that pieces of pancreas incubated *in vitro* may release insulin (obtained with biological assays for insulin) has also been obtained by Bouman (1960) and Candela (1961).

EXPERIMENTAL

Materials and methods

Rabbits. Pancreas was obtained from male rabbits (1.5–2 kg.) given a diet of oats and kale or cabbage leaves. They were deprived of solid food but allowed free access to 5% (w/v) glucose solution for 16 hr. before removal of the pancreas.

Chemicals. D-Mannoheptulose was given by Professor E. Simon, Rehovoth, Israel. D-2-Deoxyglucose, D-fructose, D-mannose and sodium D-gluconate were obtained from British Drug Houses Ltd., D-ribose from Roche Products Ltd., D-glucosamine hydrochloride (2-amino-2-deoxy-D-glucose) from Hopkin and Williams Ltd., N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) from L. Light and Co. Ltd., D-galactose from T. Kerfoot and Co. Ltd., and 3-O-methyl-D-glucose from Ayerst McKenna and Harrison (New York, U.S.A.). Mannose, fructose, 3-methylglucose and galactose were shown to contain less than 1% of glucose by assay with glucose oxidase (Huggett & Nixon, 1957); 2-deoxyglucose was shown to contain less than 1% of glucose by descending paper chromatography with pyridine-ethyl acetate-water (1:2:2, by vol.) (Jermyn & Isherwood, 1949) followed by detection of the sugars with aniline hydrogen phthalate (Partridge, 1949). Phlorrhizin was obtained from British Drug Houses Ltd., and recrystallized twice from the minimum volume of hot water (70°) with filtration through activated charcoal. Tolbutamide was obtained from Burroughs Wellcome and Co. Phenazine was obtained from British Drug Houses Ltd. and converted into the methosulphate by the method of Hillemann (1938); *p*-phenylenediamine was also obtained from British Drug Houses Ltd.; recrystallized 2,4-dinitrophenol was given by Dr G. D. Greville. Bovine plasma albumin (fraction V) was obtained from the Armour Pharmaceutical

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Co. Ltd., and ergotamine tartrate from Sandoz Products Ltd., London.

Ox insulin (six-times recrystallized) used as a secondary standard for immunoassay of rabbit insulin was given by Boots Pure Drug Co. Ltd. or by Burroughs Wellcome and Co. The ^{125}I -labelled insulin for immunoassay was either prepared as described by Hales & Randle (1963) or obtained from The Radiochemical Centre, Amersham, Bucks. Ox growth hormone and human growth hormone were prepared in this Laboratory by Mr B. R. Slater from ox or human pituitaries by modifications of the methods of Wilhelmi, Fishman & Russell (1948) (for ox) or of Raben (1957) (for human). Adrenaline was obtained from British Drug Houses Ltd., and adrenochrome prepared from it by the method of Feldstein (1958). L-Thyroxine was obtained from British Drug Houses Ltd., and dexamethasone 21-phosphate was given by Merck, Sharp and Dohme Ltd. Acetylcholine and eserine were obtained from British Drug Houses Ltd. Pure pig corticotrophin A_1 was given by Dr H. B. F. Dixon. Crystalline glucagon was a gift of Eli Lilly and Co., Indianapolis, U.S.A.

All other chemicals were either of A.R. grade or (where this was not available) of the purest grade commercially available.

Rabbit insulin. For immunoassay of insulin released by rabbit pancreas, ox insulin was used as a secondary standard. The ox insulin was calibrated against a pure sample of rabbit insulin prepared from rabbit pancreas by the method of Davoren (1962). Rabbit insulin prepared in this way ran as a single band after electrophoresis on Whatman 2MM paper in barbitone buffer, pH 8.9 (Flynn & de Mayo, 1951), made 7M with respect to urea, for 3 hr. at 400V followed by staining of protein with bromocresol green (Fenton, 1959). The rabbit insulin had the same potency as six-times-recrystallized ox insulin by the epididymal-fat-pad assay method of Renold *et al.* (1960) and by the immunoassay method of Hales & Randle (1963).

For the assay of insulin in rabbit-pancreas pieces the insulin was extracted as follows. Pancreas (300–600 mg.) was blended with 2.5 ml. of ethanol-orthophosphoric acid-water (80:1:19, by vol.) in a small Waring Blender (MSE blending assembly 77312) at 14 000 rev./min. for 3 min. The blades were washed with a further 1 ml. of extraction fluid, and the combined extract was filtered through Celite and washed through with 0.5 ml. of bovine plasma albumin (25 mg./ml.) in extraction fluid (the albumin also serves as a carrier in the subsequent precipitation). The filtrate was passed through Celite once more and the latter washed with 1 ml. of ethanol-orthophosphoric acid-water (62:0.1:37.9, by vol.). The insulin was then precipitated by the addition of acetone to the filtrate to give a final concentration of 85% (v/v). The precipitate was separated by centrifugation and, after being washed with diethyl ether, dried *in vacuo*. For assay of insulin it was dissolved in water (20 ml.) and diluted with incubation medium.

Immunoassay of insulin. Insulin was assayed in duplicate samples by method A or B of Hales & Randle (1963). The concentrations of insulin in the incubation media were higher than those encountered in blood plasma and it was convenient to use ^{125}I -labelled insulin at a concentration of 20 $\mu\text{mg./ml.}$ and standard solutions of ox insulin in the range 3–160 $\mu\text{mg./ml.}$

PROCEDURE

Incubation media. The medium for all incubations of pancreatic pieces was a bicarbonate-buffered salt solution (Krebs & Henseleit, 1932) supplemented in most experiments with the sodium salts of pyruvic acid, glutamic acid and fumaric acid at concentrations of approx. 5 mM (Krebs, 1950) and containing bovine plasma albumin (2 mg./ml.). Further additions to the medium (e.g. glucose) were as described in the text or in the Tables. The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5).

Incubation of pancreas. Each rabbit was killed by the intravenous injection of 300–400 mg. of hexobarbitone sodium, and the entire pancreas was rapidly removed without damage to intestines and dropped into oxygenated medium containing glucose (0.6 mg./ml.) and washed free of adherent blood. The pancreas was then displayed in a Petri dish containing incubation medium, and adherent fat and blood vessels were dissected away. The pancreas was then divided into about 18 pieces (70–100 mg. each), which were preincubated together in 20 ml. of medium (0.6 mg. of glucose/ml.) at 37° for 30 min. with shaking. The purpose of this preincubation was to permit fading of possible insulin release in response to the terminal hyperglycaemia of the animal. After preincubation the pieces were washed with medium on filter paper in a large Buchner funnel and distributed among six incubation vessels (25 ml. conical flasks or 5 cm. $\times$ 3½ cm. tubes) each containing 2–4 ml. of medium. The pancreatic pieces (two or three in each vessel) were then taken separately through a series of incubations of 15–30 min. duration. Before each individual incubation the pancreatic pieces were rinsed for a minute or so in a small quantity of the medium in which they were to be incubated. Transfer of the pieces to fresh medium was facilitated by the use of small rings of plastic with vertical handles and covered with nylon gauze which could be slipped into the incubation vessel and on which the pancreatic pieces rested. By this technique the amount of insulin released under different experimental conditions could be ascertained for the pieces of pancreas in a single flask (i.e. each such group of pieces serves as its own control). Thus, for example, in studying the effects of anoxia on insulin release the pieces were incubated in separate vessels for 15 min. in $\text{O}_2 + \text{CO}_2$ at low and high glucose concentrations (0.6 and 3.0 mg./ml.), then rested for 30 min. at low glucose concentration in $\text{O}_2 + \text{CO}_2$, and finally incubated for 15 min. in $\text{N}_2 + \text{CO}_2$ at low and high glucose concentrations. Insulin was then estimated in the medium from the first two and last two periods of incubation. In this way the rate of release of insulin at low and high glucose concentrations in the presence and absence of oxygen could be ascertained for each group of pancreatic pieces within a single flask. In some experiments a cross-over design was also employed. Thus, in studying the effects of anoxia, a further series of pancreatic pieces were incubated in $\text{N}_2 + \text{CO}_2$ in the first two periods and in $\text{O}_2 + \text{CO}_2$ in the last two.

Calculation and expression of results. The rates of release of insulin have been calculated and expressed in the Tables as $\mu\text{mg. of insulin/min./flask.}$ The output of insulin has not been corrected for the weight of islet tissue because the latter accounts for a small and unknown proportion of the weight of each piece of pancreas. Because of the experimental design employed (i.e. with each piece of pancreas serving as its own control) qualitative changes in the rate of

insulin release will not be influenced by the weight of islet tissue. Variations in the latter may, however, increase the variance when means are calculated for a number of pieces of pancreas. It has been assumed that this source of variance will be minimized by expressing a particular rate of insulin release as a multiple or fraction of the control rate. This form of calculation has been used in testing the statistical significance of any alteration in rate. The levels of statistical significance were obtained by calculating *t* values.

RESULTS

Quantitative recovery of insulin and reproducibility of the system. In these experiments rabbit insulin has been assayed immunologically and by reference to standard solutions of ox insulin. Since it is known that, with some antisera to insulin, insulins of different species are not equivalent in the assay, it was important to standardize the ox insulin used for standard solutions against pure rabbit insulin. With the antiserum that we have used the two insulins were equivalent (potency ratios, rabbit:ox, 1.05:1 and 0.97:1 at 25 and 50 μ g./ml. respectively). To test the extent to which insulin is destroyed or otherwise lost from the medium experiments were carried out as described at the top of Table 1. In the first series of experiments (columns A, B and C) it was found that during 30 min. of incubation with pancreas pieces approx. 30 % of 100 μ g. of insulin added to the system was lost (see Table 1). Assuming that the rate of loss of added insulin is similar to that of insulin released from the pancreas, then loss of the latter will be no greater than 30 %. This loss is small in relation to changes in the rate of release of insulin induced by agents such as, for example, glucose or tolbutamide (average 1000 % and 600 % respectively), and it seems reasonable to ignore it in interpreting these results. In further experiments (columns D and E of Table 1) a similar but somewhat smaller (approx. 20 %) loss of insulin occurred when insulin was incubated for 30 min. at 37° in medium in which

pieces of pancreas had previously been incubated. The loss of insulin is presumably due to the destruction of insulin by substances released from the pancreas, but these have not been identified. It seems unlikely, however, that chymotrypsin or trypsin was responsible because these enzymes exist as inactive precursors in pancreas, and the observations of Ravin, Bernstein & Seligman (1954) failed to reveal activation of these enzymes in saline extracts of pancreas. Moreover, concentrations of di-isopropyl phosphorofluoridate (DFP) that are inhibitory towards trypsin and chymotrypsin (400 μ M) (Jansen, Fellows-Nutting, Jang & Balls, 1949) did not prevent loss of insulin when incubation medium in which pieces of pancreas had been incubated was pretreated for 30 min. at 23° with 400 μ M-DFP before incubation with insulin at 37°.

The effects of the composition of the incubation medium on the release of insulin at low and high glucose concentrations is shown in Table 2. The small release of insulin observed at low glucose concentrations (0.6 mg./ml.) was not affected by the presence or absence of organic acids in the medium. On the other hand, the increased rate of release induced by a high concentration of glucose (3 mg./ml.) was very much greater in a medium supplemented with glutamate, fumarate and pyruvate (each at 5 mM) than in medium lacking these substrates. The relative importance of the three acids was not ascertained, though the second experiment in Table 2 shows that glutamate and fumarate had a substantial effect. In studying the effects of other substances on the rate of insulin release, incubations were made in medium supplemented with glutamate, fumarate and pyruvate unless otherwise stated. The effects of fatty acids and ketone bodies, also given in Table 2, are described below.

The reproducibility of the response of this preparation to stimulation of insulin release by glucose at different periods of incubation is shown in Tables 3

Table 1. *Destruction of insulin by pancreas pieces in vitro*

Incubations were made for 30 min. at 37° with glucose (0.6 mg./ml.). Experimental details are given in the text. In column A pancreas was incubated without added insulin. In column B incubations were made in medium containing 100 μ g. of insulin/ml. In column D, 1 ml. of medium from A was incubated with 1 ml. of medium containing 200 μ g. of insulin/ml. The recovery in column C was calculated from $B/(100 + A)$ and in column E from $D/0.5(A + 200)$. Assays of media were made in quadruplicate. The values for the concn. of insulin are given as means $\pm$ S.E.M.

Wt. of pancreas (mg.)	Estimated concn. of insulin (μ g./ml.)		Recovery C	Estimated concn. of insulin (μ g./ml.) D	Recovery E
	A	B			
250	8.5 $\pm$ 1.5	106 $\pm$ 3	0.98	82 $\pm$ 2	0.79
365	6.5 $\pm$ 0.6	74 $\pm$ 2	0.70	84 $\pm$ 1	0.82
215	6.5 $\pm$ 0.4	68 $\pm$ 0.6	0.64	79 $\pm$ 1	0.77
318	7.0 $\pm$ 0.6	64 $\pm$ 1	0.60	95 $\pm$ 5	0.92
280	11 $\pm$ 0.3	68 $\pm$ 1	0.61	72 $\pm$ 5	0.69
337	14 $\pm$ 0.8	68 $\pm$ 0.8	0.60	86 $\pm$ 2	0.80
Mean recovery			0.69		0.80

and 4. In the experiment described in Table 3 the outputs of insulin during four successive incubations of 15 min. duration was measured at a high glucose concentration (3 mg./ml.). The rate of insulin release was essentially constant during the

last three periods but somewhat lower (though not significantly so) during the first 15 min. There was thus no evidence in this experiment for any decline in the rate of insulin release during 60 min. of incubation. The experiments described in Table 4

Table 2. *Effect of 5 mM-pyruvate, 5 mM-L-glutamate, 5 mM-fumarate, 5 mM-DL-β-hydroxybutyrate, 5 mM-trisodium citrate, 4 mM-acetoacetate and 3.1 mM-n-octanoate on the rate of insulin release at low and high glucose concentrations*

Experimental details are given in the text. The acids were added as sodium salts with omission of an osmotically equivalent amount of NaCl. There were six observations in each group.

Additions to medium	Mean insulin output (μmg./min./flask)		
	With glucose (0.6 mg./ml.)	With glucose (3 mg./ml.)	Difference
None	2.6	12.6	10.0
Pyruvate + glutamate + fumarate	2.6	21.8*	19.2*
Pyruvate	0.6	5.0	4.4
Pyruvate + glutamate + fumarate	0.6	21.3*	19.7*
None	< 1	4.0	> 3.0
n-Octanoate	< 1	3.9	> 2.9
None	0.9	4.5	3.6
β-Hydroxybutyrate	0.6	5.7	5.1
None	1.8	5.3	3.5
Trisodium citrate	1.6	6.6	5.0
Glutamate + fumarate	1.9	10.0	8.1
Glutamate + fumarate + octanoate	2.4	11.3	8.9
Glutamate + fumarate	1.2	8.4	7.2
Glutamate + fumarate + β-hydroxybutyrate	1.2	11.4	10.2
Glutamate + fumarate + pyruvate	1.6	5.4	3.8
Glutamate + fumarate + pyruvate + acetoacetate	1.4	6.2	4.8

* $P < 0.05$ for difference from appropriate control. For other differences $P > 0.05$. Details of the statistical calculations are given in the Procedure section.

Table 3. *Outputs of insulin in four successive 15 min. periods by individual pieces of rabbit pancreas incubated in medium containing glucose (3 mg./ml.)*

Experimental details are given in the text. It is assumed that 23 i.u. $\equiv$ 1 mg. of insulin.

Expt. no. Period of incubation...	Insulin output (μmg./15 min./flask)				Total insulin output in 60 min.	
	0-15 min.	15-30 min.	30-45 min.	45-60 min.	(μg.)	($10^{-3} \times$ i.u.)
1	390	420	430	550	1.79	41
2	230	420	490	520	1.66	38
3	360	670	520	690	2.24	51
4	490	650	590	650	2.88	55
Total (μmg.)	1470	2160	2030	2410		
Mean $\pm$ s.e.m.	367 ± 52	540 ± 70	507 ± 30	602 ± 40		
Cumulative total:						
(μg.)	1.47	3.63	5.66	8.07		
($10^{-3} \times$ i.u.)	33.9	83.6	130.4	185.9		

Table 4. *Insulin output at low and high glucose concentrations (0.6 and 3 mg./ml.) at different periods of incubation*

Experimental details are given in the text. The ratios of insulin output for high:low glucose concentration in the first experiment were 7.9:1 and 7.4:1 and in the second experiment 4.8:1 and 1.6:1.

Concn. of glucose (mg./ml.)	Insulin output (μmg./min./flask)			
	0.6	3.0	0.6	3.0
Period of incubation	0-30 min.	31-61 min.	92-122 min.	123-153 min.
Insulin output (36 observations)	2.4 ± 0.6	$18.0 \pm 1.7^*$	2.7 ± 0.6	$20.0 \pm 1.7^*$
Period of incubation	0-20 min.	21-41 min.	42-62 min.	63-83 min.
Insulin output (four observations)	3.5 ± 1.1	$16.9 \pm 2.4^*$	$10.9 \pm 1.2^*$	$17.9 \pm 2.1^*$

* $P < 0.05$ for difference from appropriate control. For other differences $P > 0.05$. Details of the statistical calculations are given in the Procedure section.

show that the stimulating effect of a high concentration of glucose (3 mg./ml.) may be elicited twice in the same pieces of pancreas with quantitatively reproducible results provided that the pancreatic pieces are rested for 30 min. at low glucose concentration between test periods (to permit fading of the first response to a high glucose concentration). In the experiment shown in the first row of Table 4 the pieces of pancreas were subjected to a cycle of five incubations at glucose concentrations of 0.6 mg./ml. (30 min.), 3 mg./ml. (30 min.), 0.6 mg./ml. (30 min.), 0.6 mg./ml. (30 min.) and 3 mg./ml. (30 min.). The release of insulin was measured in the first two and last two periods, the third period being the rest period. The responses to a high concentration of glucose (measured as the differences in outputs between the first and second periods and the fifth periods) were not significantly different and neither were the individual outputs at a particular glucose concentration. These observations provide experimental justification for the design used in these investigations in which the outputs at low and high glucose concentrations in the first two periods are compared with the outputs in the last two periods with the factor under investigation (see the Procedure section). Essentially similar results are obtained whether the individual periods of incubation are 15 or 30 min. The preparation is thus capable of showing a consistent response to glucose for periods of incubation of at least $2\frac{1}{2}$ hr. The need for a rest period of 30 min. at low glucose concentration is emphasized by the experiment, shown in the second row of Table 4, in which this period was omitted. The output of insulin in the second incubation at a low glucose concentration was abnormally high and this we attribute to persistence of the effect of the high glucose concentration in the preceding period of incubation.

The proportion of pancreatic insulin released during 1 hr. of incubation in medium containing 3 mg. of glucose/ml. was measured by assay of the insulin released into the medium and of the insulin in the pancreas (after extraction, see the Experimental section). In four such experiments the average output was $2.7 \pm 0.22\%$ of the pancreatic insulin (mean $\pm$ s.e.m.). The glucose uptake of pancreatic pieces incubated under these conditions has also been measured and in eight experiments averaged 0.58 ± 0.10 mg. of glucose/g. of pancreas/hr. of incubation (mean $\pm$ s.e.m.). The concentration of glucose in the medium is thus effectively constant during the incubation of pancreatic pieces.

In assessing the effect of various substances on the rate of insulin release by pancreas it was important to know whether the accumulation of insulin in the incubation medium interferes with the further release of insulin. This has been studied by comparing the output of insulin by pieces of pancreas

incubated in 3 ml. of medium with that into 6 ml. of medium (3 mg. of glucose/ml.). The mean rates of output (μ mg./min./flask, means $\pm$ s.e.m. for six observations) were 8.1 ± 1 (for 3 ml.) and 6.5 ± 0.6 (for 6 ml.) and, they did not differ significantly. The insulin concentrations in the larger volume of medium were less than half those in the smaller volume.

Effect of glucose and other sugars and sugar derivatives on the rate of insulin release. The output of insulin by four pancreatic pieces incubated in the absence of glucose was $0.33 \pm 0.02 \mu$ mg. of insulin/min./flask (mean $\pm$ s.e.m.). No increase in output was seen with glucose at a concentration of 0.35 mg./ml. (output $0.27 \pm 0.03 \mu$ mg./min./flask), but the output increased significantly to $0.60 \pm 0.09 \mu$ mg./min./flask when the glucose concentration was raised to 0.7 mg./ml. The effect of increasing concentrations of glucose above 0.7 mg./ml. on the rate of insulin release is shown in Fig. 1. The experiments shown represent the pooled results for pieces from two and three entire pancreases respectively. With increasing concentrations of glucose (between 0.7 and 6 mg./ml. in one experiment and between 1.25 and 20 mg./ml. in the other) there is a progressive increase in the rate of insulin release which tends towards a plateau at higher concentrations of the sugar.

The effects of other sugars and of sugar derivatives on the release of insulin measured at low and high glucose concentrations are shown in Table 5. The ability of these sugars and sugar derivatives to stimulate insulin secretion has been measured at a low concentration of glucose rather than in the complete absence of glucose because the variation in insulin output is much smaller at a low concentration of glucose. The concentration of glucose used (0.6 mg./ml.) induces only marginal stimulation of

Fig. 1. Effect of different concentrations of glucose on the rate of insulin release from rabbit pancreas *in vitro*. ○, Mean of results with two pancreases in one experiment; ●, mean of results with three pancreases in a further experiment.

insulin secretion. The output of insulin was not increased by D-galactose, 3-O-methyl-D-glucose, D-fructose, D-ribose or sodium D-gluconate at 3 mg./ml., nor by D-2-deoxyglucose at 3 and 6 mg./ml. or N-acetyl-D-glucosamine at 15 mg./ml. Glucose at similar concentrations and in the same experiment produced at least a sixfold increase in insulin output. D-Mannose, on the other hand, in the complete absence of glucose accelerated insulin release at 3 and 6 mg./ml. though it was less effective than glucose (the effect of 6 mg. of mannose/ml. was equivalent approximately to that of 3 mg. of glucose/ml.). When insulin release was accelerated by a high concentration of glucose (2 or 3 mg./ml.) the rate of release was neither diminished nor augmented by the inclusion in the medium of D-2-deoxyglucose, 3-O-methyl-D-glucose, D-ribose or N-acetylglucosamine at the concentrations given above. The stimulating effects of mannose and glucose appeared to be additive. The stimulating effect of D-mannose at concentrations of 1 or 6 mg./ml. was not significantly decreased by 2-deoxy-

glucose at 6 mg./ml. (some diminution was noted at 1 mg. of mannose/ml. but the effect was not statistically significant).

D-Glucosamine (4 mg./ml.) stimulated the release of insulin at a low concentration of glucose (0.6 mg./ml.) and inhibited the release of insulin at a high concentration of glucose (3 mg./ml.). The stimulating effect of glucose at 3 mg./ml. on insulin release was unaffected by phlorrhizin at a concentration (1–2 mg./ml.) that markedly inhibits monosaccharide transport in rat diaphragm muscle (Battaglia & Randle, 1960), rat heart muscle (Morgan, Randle & Regen, 1959), rat adipose tissue (Hernandez & Sols, 1963), rat intestine (Newey, Parsons & Smyth, 1959) and human erythrocytes (Le Fevre, 1959).

Effect of anoxia, 2,4-dinitrophenol (250 μ M), salicylate (5 mM), malonate (10 mM), p-phenylenediamine (1 mM), phenazine methosulphate (100 μ M) and tolbutamide (200 μ g./ml.) on insulin release. The results of these experiments are given in Table 6. The stimulating effect of a high concentration of

Table 5. *Effect of sugars and derivatives of sugars on insulin release*

Experimental details are given in the text. The numbers of observations are given in parentheses.

Sugar or sugar derivative Concn. of glucose	Mean insulin output (μ mg./min./flask)		
	0	0.6 mg./ml.	2 or 3 mg./ml. as indicated
None	—	1.4 (4)	27 (4)
D-Galactose (3 mg./ml.)	0.5† (4)	—	—
None	—	1.5 (6)	12.5* (6)
D-2-Deoxyglucose (3 mg./ml.)	—	1.4 (6)	13.8* (6)
None	—	1.2 (6)	7.3* (6)
D-2-Deoxyglucose (6 mg./ml.)	—	3.4 (6)	9.3* (6)
None	—	0.5 (4)	8.9 (4)
3-O-Methyl-D-glucose (3 mg./ml.)	—	0.6 (4)	9.4 (4)
None	—	1.2 (6)	13.6 (6)
D-Mannose (3 mg./ml.)	4.5†† (6)	—	—
None	—	3.2 (6)	24.5 (6)
D-Mannose (6 mg./ml.)	23.5† (6)	—	—
None	—	1 (4)	11.2 (4)
D-Mannose (3 mg./ml.)	—	11.4† (4)	24.1† (4)
D-Mannose (1 mg./ml.)	4.1 (3)	—	—
D-Mannose (1 mg./ml.) + 2-deoxyglucose (6 mg./ml.)	1.5 (3)	—	—
D-Mannose (6 mg./ml.)	29.4 (3)	—	—
D-Mannose (6 mg./ml.) + 2-deoxyglucose (6 mg./ml.)	38.8 (3)	—	—
None	—	3 (6)	22.1 (6)
D-Fructose (3 mg./ml.)	1.6† (6)	—	—
None	—	1.5 (6)	17.3 (6)
D-Ribose (3 mg./ml.)	—	1.2 (6)	20.8 (6)
None	—	0.6 (6)	7.5* (6)
N-Acetyl-D-glucosamine (15 mg./ml.)	—	0.4 (6)	7.8* (6)
None	—	0.6 (6)	4.7 (6)
Sodium D-gluconate (3 mg./ml.)	—	0.7 (6)	—
None	—	1.2 (6)	12.1 (6)
Phlorrhizin (1.2 mg./ml.)	—	—	11.1 (6)
None	—	0.9 (6)	13.9 (6)
D-Glucosamine (4 mg./ml.)	—	2.7† (6)	9.6† (6)

* Concn. of glucose 2 mg./ml.

† $P < 0.05$ for difference from result with 0.6 mg. of glucose/ml. Details of the statistical calculations are given in the Procedure section.

‡ $P < 0.05$ for difference from result with 3 mg. of glucose/ml.

glucose (3 mg./ml.) on insulin release was abolished by anoxia and by 2,4-dinitrophenol (250 μ M) under aerobic conditions and markedly decreased by salicylate (5 mM) under aerobic conditions. These observations suggest either that respiratory-chain phosphorylation and perhaps ATP are involved in the stimulation of insulin release induced by glucose or that acceleration of glycolysis impairs insulin release induced by glucose. Use of a cross-over design (see the Procedure section) showed that the inhibitory effect of anoxia can be completely removed by 30 min. of incubation under aerobic conditions. The slower rate of release of insulin seen at a low concentration of glucose (0.6 mg./ml.) was not significantly changed by 2,4-dinitrophenol or salicylate under aerobic conditions. Malonate (10 mM) was without influence on the rate of release of insulin at either low or high glucose concentrations. If malonate penetrates the β -cell (and we have no experimental evidence that it does) then this would suggest that inhibition of succinate dehydrogenase and the accumulation of citric acid-cycle intermediates has no influence on insulin release. At a low concentration of glucose (0.6 mg./ml.) *p*-phenylenediamine (1 mM) and phenazine methosulphate (100 μ M) had no significant effect on insulin release but these agents abolished the stimulating effect of a high concentration of glucose (3 mg./ml.). A cross-over design showed that the effects of *p*-phenylenediamine and phenazine methosulphate were not reversible, presumably

because the dyes could not be removed from the tissue by washing (this was obvious on visual inspection in the latter case). The effects of these agents were investigated because it is known that in other tissues they stimulate the reoxidation of NADPH₂ and the oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Wenner, 1959; McLean, 1960). The existence of these enzymes and the oxidation of glucose through this pathway in mammalian β -cells has been described by Field, Johnson, Herring & Weinberg (1960) and Lacy (1962).

The effects of tolbutamide on insulin release were investigated because of its known hypoglycaemic effect in man and experimental animals and because experiments *in vivo* have suggested that it and related compounds may stimulate insulin secretion (Loubatieres, 1959; Yalow, Black, Villazon & Berson, 1960). At a low glucose concentration (0.6 mg./ml.) tolbutamide (200 μ g./ml.) stimulated insulin release markedly, and it was relatively more effective at this low concentration of glucose than in the absence of the sugar or at glucose concentrations of 1.5 mg./ml. or above. The concentration of tolbutamide used in these experiments was less than the peak plasma concentration (250 μ g./ml.) after the oral administration of tolbutamide in man (Berson & Yalow, 1957).

Effect of D-mannoheptulose on release of insulin induced by glucose, mannose and tolbutamide. It was

Table 6. *Effect of anoxia, 2,4-dinitrophenol (250 μ M), salicylate (5 mM), malonate (10 mM), p-phenylenediamine (1 mM), phenazine methosulphate (100 μ M) and tolbutamide (200 μ g./ml.) on insulin release*

Experimental details are given in the text. The numbers of observations are given in parentheses.

Experiment	Mean insulin output (μ mg./min./flask)		
	With glucose (0.6 mg./ml.)	With glucose (3 mg./ml. or as indicated)	Difference
Control	1.3 (6)	23.8	22.5
Anoxia (30 min.)	—	1.7*	0.4*
Control	4.8 (4)	19.4† (6)	14.6
2,4-Dinitrophenol (250 μ M)	6.0 (4)	5.4*† (4)	-0.6*
Control	2.2 (6)	39.8† (6)	37.6
Salicylate (5 mM)	4.4 (4)	18.8*† (4)	14.4*
Control§	1.2 (6)	4.8 (6)	3.6
Malonate (10 mM)§	0.9 (6)	5.8 (6)	4.9
Control	0.8 (6)	6.8 (6)	6.0
<i>p</i> -Phenylenediamine (1 mM)	1.2 (6)	1.0* (6)	-0.2*
Control	0.7 (6)	6.7 (3)	6.0
Phenazine methosulphate (100 μ M)	0.7 (6)	0.4* (3)	-0.3*
Control	8.4 (6)	36.2† (6)	27.8
Tolbutamide (200 μ g./ml.)	48.2 (6)	50.2*† (6)	2.0
Control (3.1 mM-octanoate)	2.5 (4) (no glucose)	—	—
Tolbutamide (200 μ g./ml.)	5.4 (4) (no glucose)	—	—

* $P < 0.05$ for difference from appropriate control. For other differences $P > 0.05$. Details of the statistical calculations are given in the Procedure section.

† Concn. of glucose 1.8 mg./ml.

‡ Concn. of glucose 1.5 mg./ml.

§ Krebs bicarbonate medium without glutamate, fumarate and pyruvate.

decided to investigate the effects of D-mannoheptulose on insulin release because of its ability to induce hyperglycaemia on injection into rats and rabbits and because experiments with depancreatized animals suggested that it might exert its diabetogenic effect by inhibiting insulin secretion (Simon & Kraicer, 1957; Simon, Scow & Chernick, 1961; Coore *et al.* 1963). Mannoheptulose at 3 mg./ml. has no effect on the rate of release of insulin at a low glucose concentration (0.6 mg./ml.) but it completely abolishes the stimulating effect of a higher concentration of glucose (3 mg./ml.) on insulin release (Coore *et al.* 1963; see also Table 7). The stimulating effect of D-mannose on insulin secretion at 6 mg./ml. was similarly abolished by mannoheptulose (Table 7). The effect of mannoheptulose on insulin release induced by tolbutamide is also shown in Table 7. In this experiment the output of insulin induced by tolbutamide in a number of pieces of pancreas was compared with that induced by tolbutamide in the presence of mannoheptulose in different pieces of pancreas. To correct for quantitative differences in output in the two series of pieces the response to a high concentration of glucose was measured initially in each. When allowance is made for the fact that the output of insulin at 2 mg. of glucose/ml. was lower from those pieces subsequently used to test the combined effect of tolbutamide and mannoheptulose, the results show that mannoheptulose had no inhibitory effect on the release of insulin induced by tolbutamide. This is in clear contrast with the inhibitory effect of mannoheptulose on insulin release induced by glucose and by mannose.

Effect of other hormones, acetylcholine and carbamoylcholine on insulin release. These results are given in Table 8. At low glucose concentrations adrenaline (200 µmg./ml.) added *in vitro* had no effect on insulin output but it completely abolished the stimulating effect of a high concentration of glucose (3 mg./ml.) on insulin release. This inhibitory effect of adrenaline was removed by ergotamine tartrate (2.8 µg./ml.). Adrenochrome (200 µmg./ml.) was without effect. Acetylcholine (3 µM) had no effect on insulin output at low or high (0.6 or 3 mg./ml.) concentrations of glucose in the presence of eserine (4 µM). Similarly carbamoylcholine (1 mM) added *in vitro* in the presence of soya-bean trypsin inhibitor (0.4 mg./ml.) had no effect on insulin release.

The output of insulin at low and high glucose concentrations (0.6 and 3 mg./ml.) was not influenced significantly by additions *in vitro* of ox growth hormone (200 µg./ml.), corticotrophin A₁ (8 µg./ml.), dexamethasone 21-phosphate (5 µg./ml.), L-thyroxine (100 µM) or glucagon (5 µg./ml.). Human growth hormone at a high concentration (200 µg./ml.) suppressed the stimulating effect of a high concentration of glucose on insulin release but the effect was not seen at a lower concentration (20 µg./ml.) of the hormone.

Effects of fatty acids, ketone bodies and pyruvate on insulin release. The results are given in Table 2. As mentioned above the stimulating effect of a high concentration of glucose (3 mg./ml.) on insulin release is enhanced by pyruvate, glutamate and fumarate (each at 5 mM), and this effect is due largely to glutamate and fumarate. The output of

Table 7. *Effect of D-mannoheptulose on the release of insulin induced by glucose, mannose and tolbutamide*

Experimental details are given in the text. In series 1 the media at the times shown contained: at 0–30 min. 0.7 mg. of glucose/ml.; at 31–61 min. 2 mg. of glucose/ml.; at 92–122 min. 0.7 mg. of glucose/ml. + 0.2 mg. of tolbutamide/ml.; at 93–123 min. 2 mg. of glucose/ml. + 0.2 mg. of tolbutamide/ml. In series 2 the media at the times shown contained: at 0–30 min. 0.7 mg. of glucose/ml.; at 31–61 min. 2 mg. of glucose/ml.; at 92–122 min. 0.7 mg. of glucose/ml. + 0.2 mg. of tolbutamide/ml. + 3 mg. of mannoheptulose/ml.; at 93–123 min. 2 mg. of glucose/ml. + 0.2 mg. of tolbutamide/ml. + 3 mg. of mannoheptulose/ml. The numbers of observations are given in parentheses.

Experiment	Mean insulin output (μ mg./min./flask)			Difference	
	Without mannoheptulose	With mannoheptulose (3 mg./ml.)			
Glucose (0.6 mg./ml.)	3.5 (14)	3.5 (14)	0		
Glucose (3 mg./ml.)	12.5* (14)	3.5 (14)	-9*		
Mannose (1 mg./ml.)	0.6 (6)	0.6 (6)	0		
Mannose (6 mg./ml.)	3.6* (6)	0.7 (6)	-2.9*		
Insulin output (μ mg./min./flask)					
Period of incubation	...	0-30 min.	31-61 min.	92-122 min.	93-123 min.
Series 1 (3)		0.5	11.1	11.1	9.2
Series 2 (3)		0.6	6.3	7.6	5.7

* $P < 0.05$ for difference from appropriate control. For other differences $P > 0.05$. Details of the statistical calculations are given in the Procedure section.

Table 8. *Effect of hormones and acetylcholine and analogues on insulin release*

Experimental details are given in the text. The numbers of observations are given in parentheses.

Experiment	Mean insulin output ($\mu\text{mg./min./flask}$)		
	With glucose (0.6 mg./ml.)	With glucose (3 mg./ml.)	Difference
Control	0.4 (6)	6.0 (6)	5.6
Ox growth hormone (200 $\mu\text{g./ml.}$)	0.8 (6)	5.9 (6)	5.1
Control	1.2 (12)	8.5 (12)	7.3
Human growth hormone (200 $\mu\text{g./ml.}$)	1.5 (12)	2.3* (12)	0.8*
Control	0.5 (6)	14.7 (6)	14.2
Human growth hormone (20 $\mu\text{g./ml.}$)	0.9 (6)	10.5 (6)	9.6
Control	0.7 (6)	20.6 (6)	19.9
Adrenaline (200 $\mu\text{mg/ml.}$)	0.9 (6)	1.1*	0.2*
Adrenaline (200 $\mu\text{mg./ml.}$)	1.4 (3)	1.4 (3)	0
Adrenaline (200 $\mu\text{mg./ml.}$) + ergotamine tartrate (2.8 $\mu\text{g./ml.}$)	1.2 (3)	14.1* (3)	12.9*
Control	0.3 (6)	8.7 (6)	8.4
Adrenochrome (200 $\mu\text{mg./ml.}$)	0.5 (6)	8.8 (6)	8.3
Control	1.7 (6)	10.3 (6)	8.6
Corticotrophin A ₁ (8 $\mu\text{g./ml.}$)	2.3 (6)	9.6 (6)	7.3
Control	3.3 (6)	25.6 (6)	22.3
Dexamethasone 21-phosphate (5 $\mu\text{g./ml.}$)	4.7 (6)	30.8 (6)	26.1
Control	< 1 (4)	15.0 (4)	> 14
Glucagon (5 $\mu\text{g./ml.}$)	< 1 (4)	25.2 (4)	> 24.2
Control	0.5 (6)	8.9 (6)	8.4
L-Thyroxine (100 μM)	0.7 (6)	8.3 (6)	7.6
Control	1.3 (6)	11.1 (6)	9.8
Acetylcholine (3 μM) + eserine (4 μM)	2.0 (6)	13.6 (6)	11.6
Control [soya-bean trypsin inhibitor (0.4 mg./ml.)]	2.7 (6)	12.0 (6)	9.3
Carbamylcholine (1 mM) + soya-bean trypsin inhibitor (0.4 mg./ml.)	3.7 (6)	14.1 (6)	10.4

* $P < 0.05$ for difference from appropriate control. For other differences $P > 0.05$. Details of the statistical calculations are given in the Procedure section.

insulin at low or high glucose concentrations (0.6 or 3 mg./ml.) was not altered by addition to the Krebs-Henseleit medium of *n*-octanoate (3.1 mM), DL- β -hydroxybutyrate (5 mM) or trisodium citrate (5 mM). When pieces of pancreas were incubated in medium supplemented with glutamate and fumarate the rate of insulin release at either glucose concentration was not significantly changed by 3.1 mM-*n*-octanoate or 5 mM-DL- β -hydroxybutyrate or 5 mM-pyruvate. Similarly 5 mM-acetoacetate had no influence on insulin release in the presence of glutamate, fumarate and pyruvate.

DISCUSSION

The present studies were prompted by the need for a simple system to study *in vitro* the influence of various factors on the output of insulin by the β -cells and to obtain information about the reactions in glucose metabolism involved in the release of insulin induced by glucose. These studies have provided a number of reasons for believing that the behaviour of rabbit pancreas *in vitro* may provide a satisfactory model for the behaviour of pancreatic β -cells *in vivo*. Insulin secretion *in vivo* is known to be accelerated by an increase in plasma glucose concentration (above starvation concentrations of

0.7–0.9 mg./ml.) and by tolbutamide. The present studies have shown a similar response of rabbit pancreas *in vitro*. There is evidence that mannoheptulose injected *in vivo* inhibits insulin secretion induced by glucose (Simon & Kraicer, 1957; Simon *et al.* 1961); but persistence of the hypoglycaemic effect of tolbutamide in animals treated with mannoheptulose suggests that the latter may not inhibit insulin secretion induced by tolbutamide (E. Simon, personal communication). The present studies have shown that mannoheptulose blocks the release of insulin induced by glucose in rabbit pancreas *in vitro* but not that induced by tolbutamide. In these instances the response of the pancreas *in vitro* was qualitatively similar to that of the pancreas *in vivo*. These studies have shown, moreover, that the response to glucose is graded, that the rate of output at a particular glucose concentration does not vary significantly for at least 1 hr. and that the ability to respond to glucose, tolbutamide or mannoheptulose is undiminished after 2½ hr. of incubation (the longest period for which the preparation has been used). Further, on removal of the preparation from medium containing a high glucose concentration to one with a low glucose concentration the rate of insulin release associated with the former was diminished after a

lag period. The preparation thus appears to be suitable for quantitative studies. Further points are the low rate of release of insulin at low glucose concentrations in the presence of 2,4-dinitrophenol, the inhibitory effects of anoxia and 2,4-dinitrophenol on the stimulation of insulin release induced by high concentrations of glucose, and the failure of a number of other sugars and sugar derivatives to stimulate insulin release. These observations suggest that the release of insulin by this preparation is due not, for example, to efflux of insulin from damaged β -cells but to a process involving cell metabolism, and that the stimulation of insulin release by sugars is specific for glucose and mannose (of the sugars tested).

In the β -cell insulin is located mainly in the form of granules though some insulin may also be present in the cytoplasm (for review see Randle, 1961). When insulin release *in vivo* is markedly stimulated by glucose or tolbutamide degranulation of β -cells has been noted, from which it has been inferred that insulin stored in granules has been released into the blood stream. We have no evidence that degranulation occurs in rabbit pancreas *in vitro*, but direct measurements have shown that only a small proportion of insulin (less than 5%) is released, and such a small change in the insulin content of the pancreas is not likely to be detected histologically. There is the possibility that the degree of stimulation of insulin release induced by glucose may be greater *in vivo* than *in vitro*, but there is at present no evidence on the rate of insulin release at various glucose concentrations by rabbit pancreas *in vivo*. The possibility that the insulin released *in vitro* might be entirely newly synthesized insulin (i.e. that changes in the rate of release *in vitro* are really a reflexion of alterations in the rate of synthesis) has been tested in experiments with puromycin. Glucose at a concentration of 3 mg./ml. induced a tenfold increase in the rate of insulin release in pancreas pieces incubated with 340 μ M-puromycin, and this increase did not differ significantly from that of control pieces incubated without puromycin (12-fold increase). This or lower concentrations of puromycin has been shown to be an effective inhibitor of protein synthesis in liver, thyroid and lymph nodes (Goldstein, Stella & Knox, 1963; Field, Johnson, Kendig & Paston, 1963; Helmreich, Kern & Eisen, 1962), but reports on the effects of puromycin on insulin synthesis are lacking.

With the reservation that the quantitative response of β -cells *in vitro* may be different from that of β -cells *in vivo*, it seems reasonable to suggest that rabbit pancreas *in vitro* does provide a satisfactory model for the behaviour of pancreatic β -cells *in vivo*.

Effects of hormones and acetylcholine and sugars and sugar derivatives and of ketone bodies on insulin release. The major finding to emerge from studies of

the effects of hormones has been the inhibitory effect of adrenaline on the release of insulin induced by glucose and its reversal by ergotamine tartrate. It is not known whether adrenaline exerts similar effects *in vivo*, and previous attempts to study effects of adrenaline *in vitro* in perfused rat pancreas were frustrated by the vasoconstriction induced by the hormone (Anderson & Long, 1948). The concentration of adrenaline that we have used (200 μ mg./ml.) is high in relation to the concentrations detected in the circulation *in vivo* (cf. Euler, 1961), and no inhibition was observed in an experiment with a lower concentration of the hormone (10 μ mg./ml.). On the other hand, high concentrations of the hormone are known to be necessary for the demonstration *in vitro* of other effects of adrenaline that are known to occur *in vivo* [70 μ mg./ml. for stimulation of glycogenolysis in liver slices (Sutherland & Cori, 1951), and 180 μ mg./ml. for stimulation of cyclic 3',5'-AMP formation by particle suspensions of liver and heart (Murad, Chi, Rall & Sutherland, 1962)]. If this effect of adrenaline occurs *in vivo* then adrenaline-induced hyperglycaemia would not stimulate insulin secretion as long as the blood adrenaline concentration is high. The mechanism of the adrenaline effect is not known but 10 μ M-cyclic 3',5'-AMP (which mediates the action of adrenaline on glycogenolysis) did not inhibit glucose-induced insulin secretion. Such an experiment does not, however, exclude the possibility that the effect of adrenaline on insulin release is mediated by cyclic 3',5'-AMP formed in the β -cell because it is not known whether the β -cell is permeable to the nucleotide.

The physiological significance of the inhibitory effect of human growth hormone is difficult to evaluate because the concentration required is at least 1000-fold greater than blood concentrations of the hormone (Roth, Glick, Yalow & Berson, 1963) and because of the known inhomogeneity of available preparations. The absence of any effect of ox growth hormone is in keeping with observations made with perfused rat pancreas by Grodsky *et al.* (1962) but not with an earlier report of inhibitory effects by Anderson & Long (1948). The overall effects of treatment with ox growth hormone *in vivo* lead if anything to an increased output of insulin by the pancreas (Randle, 1955). In view of this and earlier considerations it seems unlikely that the inhibitory effect that we have observed with one preparation of human growth hormone is of significance to the physiological action of growth hormone. The absence of any effect of thyroxine or dexamethazone merely suggests that they have no acute effects on the release of insulin by β -cells. Since known effects of thyroxine and dexamethazone on tissues are of long latency (Thibault, 1957; Fain, Scow & Chernick, 1963), the possibility of a

similar type of effect on β -cells *in vivo* is not excluded by these experiments. In a further experiment (not shown) the release of insulin at glucose concentrations of 0.6 and 1.5 mg./ml. was tested after 75 min. of preincubation with $13\text{ }\mu\text{M}$ -dexamethasone 21-phosphate; no effects of the steroid on insulin release were detected.

Of the sugars and sugar derivatives tested only glucose and mannose (and to a much smaller degree glucosamine) stimulated the release of insulin, and no inhibition of the effect of glucose was seen with 2-deoxyglucose, 3-*O*-methylglucose or ribose. In the perfused rat pancreas Grodsky *et al.* (1963) find that insulin release is stimulated by glucose and mannose and inconsistently by fructose at high concentration (cf. Grodsky *et al.* 1962, 1963), but not by galactose, 2-deoxyglucose, D-xylose or L-arabinose, and that insulin release induced by glucose is not inhibited by 2-deoxyglucose or galactose. The two series of observations are in general consistent, though stimulation of insulin release by fructose has not been observed with rabbit pancreas. These findings are in general disagreement with earlier observations *in vivo* in the dog by Pozza, Galansino, Hoffeld & Foa (1958) and by Kilo, Long, Bailey, Koch & Recant (1962), which provided evidence for the stimulation of insulin secretion by galactose but not by mannose and for an inhibitory effect of 2-deoxyglucose on insulin release induced by glucose. Species differences between dog and rat and rabbit are a possibility to be considered. It is possible that species differences are also responsible for the failure of acetoacetate and β -hydroxybutyrate to stimulate significantly the release of insulin by rabbit pancreas *in vitro* whereas they may stimulate output of insulin by dog pancreas *in vivo* (Madison, Mebane & Lochner, 1964).

Stimulation of insulin release by glucose. In the absence of detailed knowledge of the enzymes involved in the metabolism of glucose in β -cells only a few general comments can be made. The observation that phlorrhizin does not prevent glucose from accelerating insulin release suggests that the β -cell may be freely permeable to the sugar. This raises the possibility that the phosphorylation of glucose and a pathway of glucose 6-phosphate metabolism within the β -cell may provide the signal for insulin release induced by glucose. Two observations are consistent with this view. Of the sugars tested mannose alone shared with glucose the property of stimulating insulin release. Mannose is the only sugar apart from glucose that is known to be phosphorylated by both glucokinase and hexokinases of animal tissues and that is readily metabolized beyond the hexose monophosphate stage (2-deoxyglucose is phosphorylated but 2-deoxyglucose 6-phosphate is not metabolized to any appreciable

extent). Moreover, mannoheptulose, which inhibits release of insulin induced by glucose and mannose, has been shown to inhibit the phosphorylation of glucose and mannose in rat-liver slices and to inhibit rat-liver glucokinase and the hexokinases of rat liver, brain and adipose tissue (Sols & Crane, 1954; Hernandez & Sols, 1963; Coore & Randle, 1964). To be set against this evidence is our failure to demonstrate inhibitory effects of 2-deoxyglucose and *N*-acetylglucosamine on insulin release induced by glucose (2-deoxyglucose is a substrate for enzymes phosphorylating glucose in other animal tissues, and *N*-acetylglucosamine is an inhibitor of these enzymes). Clearly there is a need for further information about the properties of enzyme(s) phosphorylating glucose in rabbit β -cells before firm conclusions can be drawn about the role of this process in insulin release.

The known pathways of glucose 6-phosphate metabolism that might be involved in insulin release are glycolysis followed by pyruvate oxidation, the pentose phosphate pathway and the metabolism of UDP-glucose. In our experiments and those of Grodsky *et al.* (1963) the failure of pyruvate to stimulate insulin release suggests that pyruvate oxidation is unlikely to be involved in the release induced by glucose. In other tissues anoxia accelerates glycolysis and phenazine methosulphate accelerates the oxidation of glucose 6-phosphate through the pentose phosphate pathway, and in rabbit pancreas these factors inhibited insulin secretion. One interpretation of these findings could be that glycolysis and the pentose phosphate pathway do not provide the signal for insulin release, and that these agents inhibit insulin secretion by diverting glucose residues to these pathways (e.g. from a pathway of metabolism of UDP-glucose). It is, however, questionable whether the effects of these agents on metabolism are sufficiently specific for these conclusions to be sustained. They may, for example, be exerting their effects by lowering intracellular concentrations of ATP or other high-energy phosphate compounds.

This discussion emphasizes that the value of preparations such as the isolated rabbit pancreas for studying biochemical mechanisms involved in the control of insulin secretion by glucose (and other factors) is limited by lack of knowledge of glucose metabolism in the β -cell. The major disadvantage of our preparation in this respect lies in the fact that the β -cells are dispersed among the acinar cells and that they form only a small proportion of the total pancreatic mass. The present studies do appear to show, however, that isolated rabbit pancreas is a useful preparation for studying the influence of various factors on the rate of insulin release and that it can provide a satisfactory model for the behaviour of β -cells *in vivo*.

SUMMARY

1. The effect of various factors on the rate of release of insulin from pieces of rabbit pancreas incubated *in vitro* has been studied by estimating the insulin concentration in the medium after incubation by immunological assay with insulin-antibody precipitate, and the conditions necessary for eliciting consistent responses have been defined.

2. Insulin release was accelerated by D-glucose at concentrations above 0.35–0.7 mg./ml. and by D-mannose (3 or 6 mg./ml.), but not by D-galactose, 3-O-methyl-D-glucose, D-fructose, D-ribose or sodium D-glucuronate (3 mg./ml.), D-2-deoxyglucose (3 or 6 mg./ml.), N-acetyl-D-glucosamine (15 mg./ml.) or D-mannoheptulose (3 mg./ml.). The stimulating effect of glucose (3 mg./ml.) on insulin release was abolished by mannoheptulose but not by 2-deoxyglucose, 3-O-methylglucose, ribose or N-acetylglucosamine.

3. Insulin release at a low glucose concentration (0.6 mg./ml.) was accelerated by tolbutamide (200 µg./ml.) but not by anoxia, 2,4-dinitrophenol, salicylate, *p*-phenylenediamine or phenazine methosulphate. The rate of insulin release at a high glucose concentration (3 mg./ml.) was markedly diminished by anoxia, 2,4-dinitrophenol (250 µM), salicylate (5 mM), *p*-phenylenediamine (1 mM) and phenazine methosulphate (100 µM), but not by malonate (10 mM). The stimulating effect of tolbutamide (unlike that of glucose) was not influenced by mannoheptulose.

4. The stimulating effect of glucose (3 mg./ml.) on insulin release was augmented by the presence in the medium of glutamate, fumarate and pyruvate. At a low glucose concentration (0.6 mg./ml.) neither these acids nor octanoate, acetoacetate or β -hydroxybutyrate influenced the rate of insulin release.

5. The stimulating effect of glucose (3 mg./ml.) on insulin release was abolished by adrenaline (200 µg./ml.), and the effect of adrenaline was suppressed by ergotamine tartrate (2.8 µg./ml.).

6. Evidence is presented that the release of insulin by rabbit pancreas *in vitro* provides a suitable model for the behaviour of β -cells *in vivo* and for studying the influence of various factors on insulin secretion.

7. The possible roles of glucose phosphorylation and of pathways of metabolism of glucose 6-phosphate in β -cells in the stimulation of insulin release induced by glucose are discussed.

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Nucleotide Metabolism in Germinating Seeds

THE RIBONUCLEIC ACID OF *PISUM ARVENSE*

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Preliminary studies have been reported (Barker & Douglas, 1960) of the variations of RNA, ribonuclease and protein in the cotyledons and in the root and shoot of the developing pea seedling, and a possible mechanism was suggested for the translocation of RNA from the cotyledon to the growing parts of the plant. This possibility is now being investigated. Earlier experiments were carried out with *Pisum sativum* (var. English Wonder), but in subsequent batches of seed, germination and growth under the cultural conditions used showed considerable variation between individual plants, and it was not possible to obtain reliable values for the RNA content of the plants in the early stages of growth. Much more uniform results have been obtained with seed of *Pisum arvense*. The present paper records experiments designed to establish conditions for studying translocation of nucleotide material from cotyledon to the growing seedling. The RNA content of the cotyledons and the course of the incorporation of [³²P]phosphate during the early stages of growth of *P. arvense* have been measured. A satisfactory method is described for the determination of RNA in cotyledon tissue.

METHODS AND MATERIALS

Total phosphate was determined by the method of Fiske & Subbarow (1925). Difficulty was experienced in decolorizing the solution after incineration, but this was achieved by the addition of 1 drop of 60% (w/v) perchloric acid followed by 1 drop of 100-volume hydrogen peroxide. Pentose was determined by the method of Drury (1948). All analyses were carried out in duplicate.

Ribonucleic acid of Pisum arvense. Peas were soaked for 36 hr. in water at 22°, removed from the water and cooled to 0°. A sample (200 g.) was ground with solid CO₂, and crude RNA was prepared by the method of Markham (1955). The crude material was dissolved in dilute NaOH at pH 8 (50 ml.) and brought to pH 7. A small residue was removed by centrifugation and pure RNA was extracted with 90% (w/v) phenol by the method of Kirby (1956). The purified material had a value for $\epsilon(P)$, as defined by Chargaff & Zamenhof (1948), of 8100. The $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$ ratio was 0.49. Warburg & Christian (1941–42) give a value of 0.49 for the $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$ ratio for yeast RNA. This value for the ratio indicates the presence of 0.1% of protein by Layne's (1957) method of calculation; according to Kalckar's (1947) equation the value corresponds to a complete absence of protein.

Determination of ribonucleic acid. The method of Smillie & Krotkov (1960) was modified to make it suitable for cotyle-