Studies on the Secretion of Pancreatic Glucagon

Thomas McC. Chesney, M.D., and J. G. Schofield, M.A., Ph.D., Bristol, England

SUMMARY

Glucagon secretion was studied in isolated pancreatic islets, thus avoiding interference from immunologically cross-reactive polypeptides of gut origin. Glucagon release was measured by radioimmunoassay. High concentrations of glucose were shown to inhibit glucagon release. Certain amino acids and ouabain were found to stimulate glucagon release, but adrenergic agents and tolbutamide were without effect. Theophylline was found to be a potent in vitro stimulus to glucagon release, suggesting that the intracellular concentration of cyclic adenosine 3′,5′-monophosphate may play a role in the regulation of glucagon secretion. DIABETES *18*:627-32, September, 1969.

Recently there has been increased interest in the regulation of glucagon secretion, particularly owing to the development of specific and sensitive radioimmunoassays for the hormone[1,2] and to the finding that glucagon can play a role in the secretion of insulin.[3] Immunological measurement of pancreatic glucagon is complicated, however, by the existence of gut "glucagons" which cross-react with pancreatic glucagon.[4]

Three experimental approaches are available for assessment of the relative rates of release of pancreatic glucagon and the gut glucagon-like immunoreactivity (GLI). One involves radioimmunoassay of samples utilizing antisera with different affinities for the two polypeptides.[5] In in vivo studies of this type, the difference between total plasma glucagon and GLI is assumed to represent pancreatic glucagon. A second approach, developed by Ohneda and coworkers,[6] involves triple catheterization of animals and comparison of glucagon levels in vena caval, pancreaticoduodenal, and mesenteric venous plasma during in vivo experiments. The third approach involves in vitro use of only one of the glucagon-secreting tissues, and has been used for the study of pancreatic glucagon secretion[7] and jejunal GLI release.[8]

The last approach, using an isolated mouse islet preparation, has been used in the present studies of pancreatic glucagon secretion. Islets were incubated in bicarbonate buffered salt solution containing Trasylol, a proteinase inhibitor,[9] in order to minimize glucagon breakdown by proteinases from the exocrine pancreas and the blood. Glucagon released into the medium was measured by a double antibody radioimmunoassay.

MATERIALS AND METHODS

Our procedures for the preparation of isolated islets of Langerhans and radioimmunoassay are not greatly different from those previously published,[2,10] and only important differences in detail will be presented. The immunoassay for glucagon is based on that for growth hormone of Schofield.[11]

Glucagon: Crystalline beef-pork glucagon (Eli Lilly Lot No. 258-234B-167-1) was used throughout these experiments for induction of antibodies, preparation of radioiodinated glucagon, and as standards in the assay procedure.

Antisera: Antibodies to beef-pork glucagon were raised in guinea pigs by the polyvinyl pyrrolidone technic of Assan et al.[12] Anti-guinea pig γ-globulin sera were produced in rabbits by the procedure of Hales and Randle.[13]

I-125-glucagon: I-125-glucagon was prepared by the method of Greenwood et al.[14] Glucagon (10 μg.) was initially dissolved in buffer containing glycine (25 mM.) and NaCl (20 mM.), pH 10.5, and after iodination was separated from the other reactants by gel filtration through a 25 × 2.3 cm. column of Sephadex G-25 (coarse) equilibrated with buffer (25 mM. glycine, 20 mM. NaCl; 10 mg./ml. bovine plasma albumin, and 1:4,000 thiomersal, pH 10.5). The same buffer was used for elution from the column. Fractions containing I-125-glucagon were pooled, mixed, and stored frozen. Before use in the assay, I-125-glucagon was diluted 1:100 in phosphate buffer and filtered through an Oxoid* cellulose acetate membrane.

Preparation and incubation of islets: Islets were prepared from four-week-old male white mice which had been starved eighteen to twenty hours. Four to six mice

From the Department of Biochemistry, University of Bristol, Bristol, BS8, ITD, United Kingdom. Dr. Chesney's present address is Vanderbilt University, Medical School, Nashville, Tennessee 37203.

*Oxo Ltd., London S.E. 1.

were decapitated and the pancreases placed in ice-cold incubation medium containing 3 mg./ml. glucose. In all experiments, the incubation medium was Krebs-Henseleit bicarbonate buffer containing defatted, de-insulinized bovine plasma albumin (0.5 mg./ml.) and supplemented with 5.0 mM. pyruvate, glutamate, and fumarate. Additions to this medium were made as described in the text. Islets were prepared by the collagenase procedure described by Howell and Taylor,[10] and transferred by means of a wire loop to centrifuge tubes containing 1 ml. incubation medium containing 500 KIU Trasylol,* and 0.6 mg./ml. glucose, except where otherwise stated. Each tube, containing twenty islets, was then equilibrated with O_2:CO_2 (95:5) and incubated for thirty minutes at 37° C. with shaking. The tubes were then cooled and centrifuged and the supernatants removed with a Pasteur pipette. The islets were resuspended in 1 ml. NaOH (0.02 N), sonicated for ten seconds at position two with a soniprobe (Dawe Instruments Ltd., London) and the sonicate fluid used for assay of residual islet glucagon in some experiments.

Glucagon assay

Procedure: The assay was carried out in 0.05 M. phosphate buffer pH 8.0, containing 1 mg./ml. bovine plasma albumin and 1:4,000 thiomersal. Into 3.5 × 0.8 cm. glass assay tubes were added 100 μl. of standard or unknown solution, 50 μl. of Trasylol (250 KIU), and 50 μl. guinea pig anti-beef-pork glucagon serum (1:500). The tubes were mixed and incubated at 4° C. for eighteen hours. Then 50 μl. of a solution containing rabbit anti-guinea pig γ-globulin (diluted 1:3) and I-125-glucagon (10 mμg./ml.) was added. The tubes were again mixed and incubated for four hours at 4° C. These times were sufficient for equilibration of both the antibody reactions.

The assay was terminated by filtration through Oxoid cellulose acetate membranes, which had been soaked overnight in a solution containing bovine plasma albumin (2 mg./ml.) and thiomersal (1:4,000) at pH 9.0. The same solution was used to wash the assay tube and the membrane, to remove I-125-glucagon. The membranes were placed in scintillation bottles, dried, and counted using a liquid scintillation spectrometer.

Unknown solutions were assayed in quadruplicate at three dilutions and the apparent glucagon concentration determined for each assay tube. These were corrected for dilution and the mean ± standard error of the mean calculated. Statistical significance was calculated by the Student *t* test.

Standard curve and the effect of Trasylol: The effect of the incorporation of Trasylol on the standard curve is shown in figure 1. The upper curve shows the mean (± S.E.M.) for five standard curves carried out with anti-glucagon serum at 1:400 dilution in the absence of Trasylol. The lower curve shows the mean (± S.E.M.) of six standard curves assayed with anti-glucagon serum at 1:500 dilution in the presence of 250 KIU Trasylol per assay tube. Under the latter conditions, the sensitivity of the assay was increased and the standard errors were reduced.

The sensitivity may be calculated using the procedure of Brown et al.[25] An estimate (S) of the standard deviation is calculated from the formula:

$$S = \frac{\Sigma(x-\bar{x})^2}{N(n-1)}$$

where $\bar{x}$ is the mean of n determinations in one of N standard curves. Taking twelve standard curves, 711 cpm. were recovered in the absence of added glucagon and 579 cpm. were recovered at a glucagon concentration of 0.78 mμg./ml. At zero added glucagon, S was 103.4 cpm. and at a concentration of 0.78 mμg./ml., S was 69.7 cpm. The minimum significant drop in radioactivity is given by:

$$t = \sqrt{\frac{s_1^2 + s_2^2}{n}}$$

where $t = 1.96$ ($p < 0.05$). For observations in quadruplicate the minimum significant drop in radioactivity was 121.5 cpm., which was caused by 66 μμg. glucagon in 100 μl.

Specificity of the assay: Dilution curves of media containing mouse glucagon deviated from linearity only at the assay sensitivity limit (figure 2), indicating that mouse and beef-pork glucagon were immunologically similar. All results are expressed as beef-pork glucagon equivalents, as no standard preparation of mouse glucagon was available. Insulin (Boots Pure Drug Co., 6 × recrystalized) in concentrations from 1.0 to 1,000 mμg./ml. was found to have no effect on glucagon binding in the assay.

Glucagon recovery: When beef-pork glucagon (50 mμg./ml.) was added to a batch of twenty islets and incubated for thirty minutes at 37° C., 96 per cent was recovered, corrected for glucagon release by a control batch of twenty islets incubated in the absence of added glucagon.

*We are indebted to Dr. H. B. Allen, FBA Pharmaceuticals, Haywards Heath, Sussex, for provision of Trasylol.

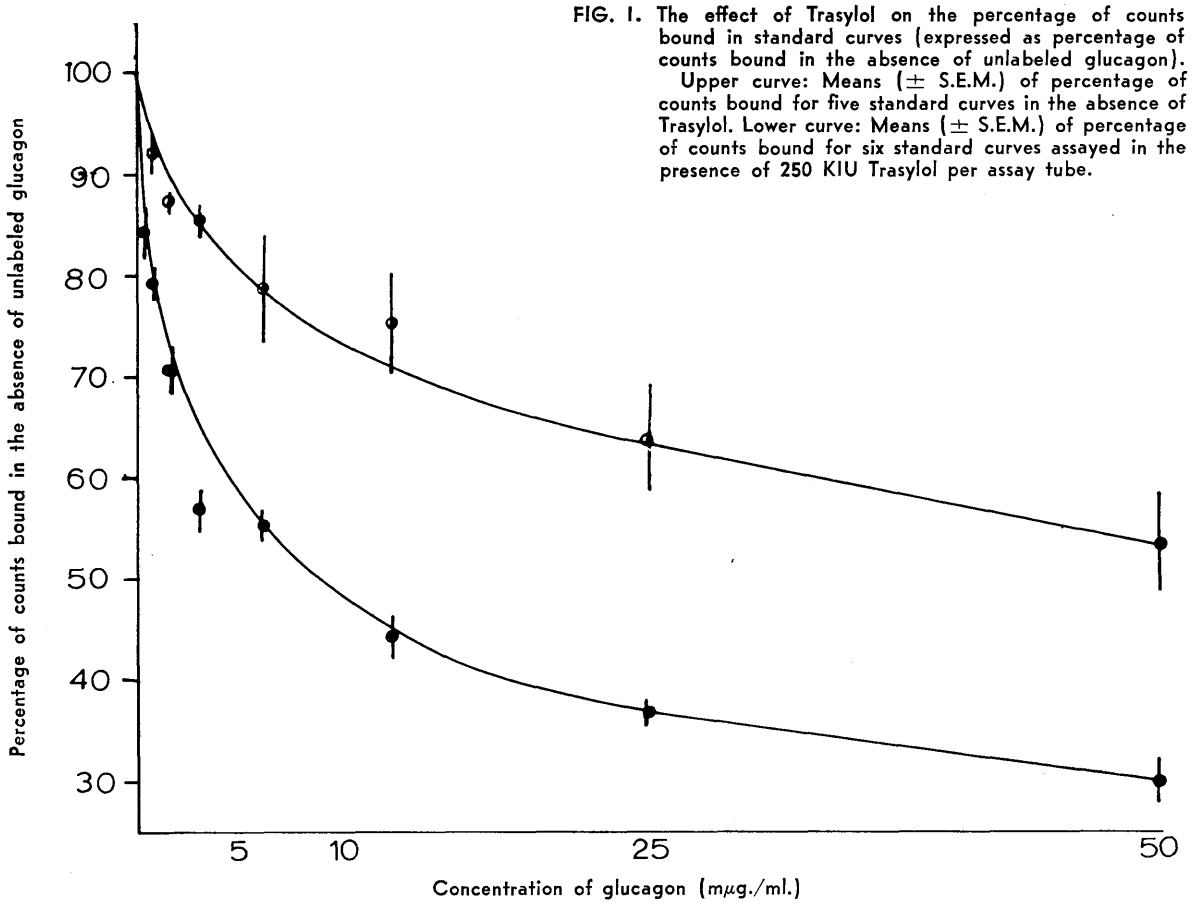

FIG. 1. The effect of Trasylol on the percentage of counts bound in standard curves (expressed as percentage of counts bound in the absence of unlabeled glucagon). Upper curve: Means (± S.E.M.) of percentage of counts bound for five standard curves in the absence of Trasylol. Lower curve: Means (± S.E.M.) of percentage of counts bound for six standard curves assayed in the presence of 250 KIU Trasylol per assay tube.

RESULTS

Effect of glucose on glucagon release: The effect of variation of glucose concentration in the incubation medium on release of glucagon was studied and the results are depicted in figure 3. Glucagon release at 1.67 or 3.3 mM. glucose was significantly greater than that at 16.7 mM. ($p < .001$). The residual glucagon of islets incubated in 1.67 mM. glucose was 68 per cent greater ($p < 0.02$) than that of islets incubated in 16.7 mM. glucose (table 1). The percentage of total glucagon released in the presence of 1.67 or 3.3 mM. glucose was twice that released at 16.7 mM. glucose.

Effect of amino acids on glucagon release: The effect of arginine, lysine, histidine, and leucine on the release of glucagon from islets was studied (table 2). Glucagon release was increased by arginine ($p < 0.05$) and by lysine ($p < 0.10$), but histidine and leucine were without effect. The effect of arginine was repeated in five further experiments; the mean stimulation by arginine was 62 per cent ± 14 per cent ($p < .05$).

TABLE 1

Glucagon contents of batches of twenty islets after incubation for thirty minutes at different glucose concentrations

Glucose concentration of incubation	Glucagon content mμg./twenty islets Mean ± S.E.M.	Total glucagon (content + secreted) mμg./twenty islets Mean	Per cent release
1.67 mM.	47.7±3.9	54.0	11.6
3.3 mM.	29.1±5.8	33.8	13.9
16.7 mM.	28.4±6.8	30.4	6.7

TABLE 2

Effect of amino acids on glucagon release. Batches of twenty islets were incubated in the presence of glucose (3.3 mM.) for thirty minutes.

Addition to medium	Glucagon release (mμg./twenty islets/30 min.) Mean ± S.E.M.	Per cent of control
None	4.7±0.8	
Arginine (mM.)	9.4±1.9	198 ($p < .05$)
Lysine (mM.)	8.2±1.9	173 ($p < .10$)
Histidine (mM.)	5.9±0.9	126
None	5.8±1.4	
Leucine (mM.)	5.0±0.9	86

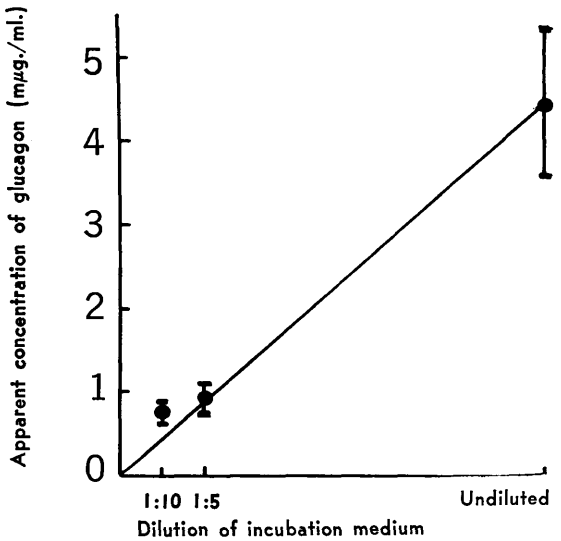

FIG. 2. The effect of dilution on apparent glucagon concentration in incubation media. Mouse glucagon was assayed in medium in which twenty islets had been incubated, and at three dilutions. The results are expressed as mμg./ml. beef-pork glucagon.

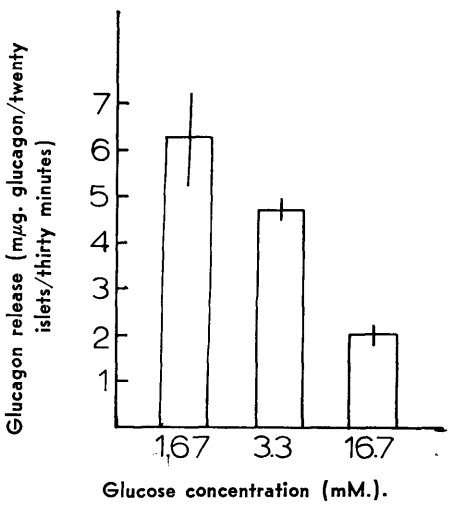

FIG. 3. Effect of glucose on glucagon release.

Effect of adrenergic agents and tolbutamide on glucagon release: The effects of epinephrine (100 μM.), norepinephrine (1 mM.), isoproterenol (1 mM.), and phentolamine (100 μg./ml.) on glucagon release from islets were studied (table 3). None of these adrenergic agents had significant effect on glucagon release. Tolbutamide (0.2 mg./ml.) was found to cause no significant alteration in glucagon release from isolated islets (table 3).

Effect of theophylline and ouabain on glucagon release: The suggestion that cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) is effective in stimulating the secretion of other hormones[17,18] led us to speculate that cyclic AMP might also stimulate glucagon release. Theophylline has been shown to increase cyclic AMP concentrations by inhibition of cyclic nucleotide phosphodiesterase,[26] and was therefore used in this study. It should be noted that a related compound, caffeine, has been shown to increase the concentration of free Ca^{2+} in muscle.[27] Islets were incubated in media containing theophylline (1 mM. or 7.6 mM.) or theophylline (7.6 mM.) and phentolamine (100 μg./ml.). The results are shown in table 4. The stimulation by theophylline alone and by theophylline and phentolamine was significant ($p < .01$). Ouabain has been shown to stimulate the release of insulin in vitro,[28] and the effect of ouabain (10 μM.) on glucagon release was therefore studied. The results are shown in table 4. There was an increase in glucagon release ($p < .002$), but no significant change in islet glucagon content, after incubation in ouabain.

TABLE 3

Effect of adrenergic agents and tolbutamide on glucagon release. Glucagon release by batches of twenty islets incubated for thirty minutes was measured in the presence of glucose (3.3 mM.). Other additions were as given in table. None of the differences was significant.

Additions to medium	Glucagon release (mμg./twenty islets/30 min.) Mean ± S.E.M.	Per cent of control
None	4.9±1.3	98
Epinephrine (100 μM.)	4.7±0.9	
None	5.8±1.4	
Norepinephrine (mM.)	5.8±1.3	100
Isoproterenol (mM.)	5.5±1.2	95
Phentolamine (370 μM.)	3.7±0.5	65
Tolbutamide (670 μM.)	3.9±0.5	67

DISCUSSION

The isolated pancreatic islet preparation has been shown to be useful in the study of pancreatic glucagon release in vitro. Mouse glucagon could be assayed by double antibody radioimmunoassay utilizing beef-pork glucagon for production of antiserum, for standards, and for iodination, since mouse glucagon behaves immunologically as beef-pork glucagon on dilution. Insulin, which might be released by the islets, had no effect on the binding of I-125-glucagon to antibody; moreover there was no destruction of glucagon during incubation in the presence of islets in medium contain-

TABLE 4

Effect of theophylline and ouabain on glucagon release. Glucagon release by twenty islets incubated over a thirty-minute period in the presence of glucose (3.3 mM.) is shown and expressed as a percentage of control release.

Additions to medium	Glucagon release (mμg./twenty islets/ 30 min.) Mean ± S.E.M.	Per cent of control	p value
None	5.3±1.1		
Theophylline (1 mM.)	11.0±1.7	207.5	($p < .01$)
None	5.8±1.4		
Theophylline (7.6 mM.)	11.4±1.2	196.6	($p < .01$)
Theophylline (7.6 mM.) and phentolamine (100 μg./ml.)	14.3±1.6	246.6	($p < .001$)
None	3.4±0.08		
Ouabain (10 μM.)	8.6±0.11	253	($p < .002$)

ing Trasylol. The effect of Trasylol on the assay may be due to protection of glucagon from proteinases present in the rabbit anti-serum, which is used at high titer.

The glucagon content of mouse islets was found to be 1.69 mμg./islet and the rate of release of the hormone was 0.27 mμg./islet/30 min. at 3.3 mM. glucose concentration. These figures compare with an insulin content of mouse islets of 160 mμg./islet[18] and an unstimulated rate of insulin release of 10 mμg./islet/hour.[18] The percentage release of glucagon was 27 per cent per hour, while for insulin the rate is 6.2 per cent per hour. The high rate of percentage release of glucagon and the relatively low islet content of this hormone may indicate that turnover of stored glucagon is more rapid than that of insulin. The rate of glucagon release observed in this study is in good agreement with that published by Vance et al.[7] for rat islets.

The effect of glucose on the rate of release of glucagon from islets observed in this study agrees with the in vitro findings of Vance et al.[7] with rat islets, and the in vivo findings of Unger et al.[19] The observation that the glucagon content of islets incubated at 1.67 mM. glucose was significantly higher than that of islets incubated at 16.7 mM. glucose may suggest that the rate of glucagon synthesis is increased at the lower glucose concentration, but this point would require further investigation.

The effect of amino acids on glucagon release by islets is similar to their effects on the release of insulin.[20] Thus arginine and possibly lysine stimulate the release of both hormones, and histidine has no effect. It is possible that increased output of pancreatic glucagon explains the increase in plasma glucagon following arginine infusion observed by Lawrence[21] and this would be in accordance with the studies of Luyckx and Lefebvre[8] who showed that amino acids do not induce the release of GLI from rat jejunum in vitro. Leucine, which stimulates the output of insulin in vivo[20] and in vitro,[22] was without effect on the release of glucagon. However, Fajans et al.[20] have shown that the mechanism of leucine stimulation of insulin release differs from that of arginine and other amino acids. It is possible that arginine and possibly lysine act in the same way on alpha and beta cells; an alternate explanation is that the effects of arginine and lysine on insulin release are mediated by an increased output of glucagon, a known stimulus of insulin release.[3,4]

The effects of a variety of other substances known to modify insulin secretion have been studied. It was found that tolbutamide, which stimulates insulin release, and adrenalin, which inhibits release in response to glucose,[23] were without effect on glucagon output in vitro. Ouabain and theophylline stimulate glucagon output, which may indicate that release is dependent on the ionic composition of the intracellular medium, and that cyclic 3′ 5′ AMP may be involved in the release process. Vance and Buchanan[24] did not observe an effect of theophylline on glucagon release in the presence of 16.7 mM. glucose whereas 3.3 mM. glucose was used in our studies.

ACKNOWLEDGMENT

The authors are grateful to S. J. H. Ashcroft and J. R. Gill for advice about the preparation of islets, and Mrs. P. Burd and Mrs. J. E. Eaborn for technical assistance. T. Mc. Chesney was supported by United States Public Health Service Special Fellowship (No. 1-F3-HD-34,071-01) from the National Institute of Child Health and Human Development. Additional funds were provided by The British Diabetic Association to Professor P. J. Randle, whom the authors also thank for much helpful advice.

REFERENCES

[1] Unger, R. H., Eisentraut, A. M., McCall, M. S., and Madison, L. L.: Glucagon antibodies and an immunoassay for glucagon. J. Clin. Invest. 40:1280-89, 1961.

[2] Hazzard, W. R., Crockford, P. M., Buchanan, K. D., Vance, J. E., Chen, R., and Williams, R. H.: A double antibody immunoassay for glucagon. Diabetes 17:179-86, 1968.

[3] Samols, E., Marri, G., and Marks, V.: Interrelationships of glucagon, insulin, and glucose; the insulinogenic effect of glucagon. Diabetes *15*:855-66, 1966.

[4] Samols, E., Tyler, J., Megyesi, C., and Marks, V.: Immunochemical glucagon in human pancreas, gut, and plasma. Lancet *2*:727-29, 1966.

[5] Eisentraut, A. M., Ohneda, A., Parada, E., and Unger, R. H.: Immunologic discrimination between pancreatic glucagon and enteric glucagon-like immunoreactivity in tissues and plasma. Diabetes *17*(Supplement 1):321, 1968 (Abstract).

[6] Unger, R. H., Ohneda, A., Valverde, I., Eisentraut, A. M., and Exton, J.: Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose. J. Clin. Invest. *47*:48-65, 1968.

[7] Vance, J. E., Buchanan, K. D., Challoner, D. R., and Williams, R. H.: Effect of glucose concentration on insulin and glucagon release from isolated islets of Langerhans of the rat. Diabetes *17*:187-93, 1968.

[8] Luyckx, A. S., and Lefebvre, P. J.: Release of glucagon or a glucagon-like immunoactive material by rat jejunum incubated in vitro. *In* Protein and Polypeptide Hormones, Vol. III, edited by M. Margoulies. Excerpta Medica Foundation, Amsterdam, (in press).

[9] Eisentraut, A. M., Whissen, N., and Unger, R. H.: Incubation damage in the radioimmunoassay for glucagon and its prevention by Trasylol. Amer. J. Med. Sci. *255*:137-42, 1968.

[10] Howell, S. L., and Taylor, K. W.: Potassium ions and the secretion of insulin by islets of Langerhans incubated in vitro. Biochem. J. *108*:17-24, 1968.

[11] Schofield, J. G.: Measurement of growth hormone released by ox anterior-pituitary slices in vitro. Biochem. J. *103*:331-41, 1967.

[12] Assan, R., Rosselin, G., Drouet, J., Dolais, J., and Tchobroutsky, G.: Glucagon antibodies. Lancet *2*:590-91, 1965.

[13] Hales, C. N., and Randle, P. J.: Immunoassay of insulin with insulin-antibody precipitate. Biochem. J. *88*:137-46, 1963.

[14] Greenwood, F. C., Hunter, W. M., and Glover, J. S.: The preparation of I-131-labeled human growth hormone of high specific activity. Biochem. J. *89*:114-23, 1963.

[15] Schofield, J. G.: Role of cyclic 3′,5′-adenosine monophosphate in the release of growth hormone. Nature *215*:1382-83, 1967.

[16] Malaisse, W. J., Malaisse-Lagae, F., and Mayhew, D.: A possible role for the adenyl cyclase system in insulin secretion. J. Clin. Invest. *46*:1724-34, 1967.

[17] Turtle, J. R., and Kipnis, D. M.: An adrenergic receptor mechanism for the control of cyclic 3′ 5′ adenosine monophosphate synthesis in tissues. Biochem. Biophys. Res. Commun. *28*:797-802, 1967.

[18] Coll-Garcia, E., and Gill, J. R.: Insulin release by isolated pancreatic islets of the mouse incubated in vitro. Diabetologia *5*:61-66, 1969.

[19] Unger, R. H., and Eisentraut, A. M.: Glucagon, *in* Hormones in Blood, Vol. 1, 2nd Edition, edited by Grey, C. H., and Bacharach, A. L., New York, Academic Press, 1968, pp. 83-128.

[20] Fajans, S. S., Floyd, J. C., Knopf, R. F., and Conn, J. W.: Effect of amino acids and proteins on insulin secretion in man. Recent Progr. Hormone Res. *23*:617-22, 1967.

[21] Lawrence, A. M.: Unpublished observation, 1966, cited in reference 20.

[22] Milner, R. D. G., and Hales, C. N.: The role of calcium and magnesium in insulin secretion from rabbit pancreas studied in vitro. Diabetologia *3*:47-49, 1968.

[23] Coore, H. G., and Randle, P. J.: Regulation of insulin secretion studied with pieces of pancreas incubated in vitro. Biochem. J. *93*:66-78, 1963.

[24] Vance, J. E., and Buchanan, K. D.: Interrelationship between glucagon and insulin release from isolated islets of Langerhans. Diabetes *17*(Supplement 1):311, 1968.

[25] Brown, J. B., Bulbrook, R. D., and Greenwood, F. C.: An additional purification step for a method of estimating oestriol, oestrone and oestradiol-17β in human urine. J. Endocr. *16*:41-49, 1957.

[26] Butcher, R. W., and Sutherland, E. W.: Adenosine 3′5′-phosphate in biological materials. J. Biol. Chem. *237*:1244-50, 1962.

[27] Bianchi, C. P.: The effect of caffeine on radio calcium movement in frog sartorius muscle. J. Gen. Physiol. *44*:845-58, 1961.

[28] Hales, C. N., and Milner, R. D. G.: The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol. *194*:725-43, 1968.