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A Solid Phase Radioimmunoassay for Pancreatic Glucagon

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ABSTRACT

A solid phase radioimmunoassay procedure has been applied for the determination of glucagon and some variables of the assay procedure were studied. The sensitivity of the assay was 10 pg of glucagon for samples assayed in triplicate. This assay method which has a high precision and is technically simple as compared with other radioimmunoassays, was found to be suitable for studies on glucagon release in vitro.

INTRODUCTION

Since the presentation of the first radioimmunoassay method for glucagon (19, 20), several reports have been published on the measurement of this hormone using different techniques for the separation of antibody-bound from unbound hormone in the assay procedure (1, 8, 12, 18, 15, 13, 4, 9, 2). A particular complication in all glucagon immunoassays is the susceptibility of glucagon, and especially iodinated glucagon, to enzymatic degradation. The enzymatic damage can be reduced by the addition of Trasylol, a proteinase inhibitor, to the incubation mixture (5). However, there is still a demand for shorter incubation times in the assay procedure to reduce the exposure of glucagon to the degrading enzymes.

A solid phase radioimmunoassay procedure, in which antibodies are chemically coupled to an insoluble polysaccharide, has been introduced for the assay of protein and polypeptide hormones (22, 24). In the present investigation this radioimmunosorbent technique was applied for the determination of glucagon and some variables of the assay procedure were studied. This assay method which produces a simple and efficient separation of antibody-bound from unbound glucagon was found to be suitable for studies on glucagon release in vitro.

MATERIAL AND METHODS

Chemicals

Crystalline pork glucagon (kindly supplied by Dr L. Heding, Novo Industri, Denmark, Lot. Nr. B66) was used for the induction of antibodies, and beef-pork glucagon (Sigma Chemical Co., St. Louis, USA) was used for the preparation of I^{125}-glucagon and for the standards in the assay procedure.

Synthesized human gastrin I (Imperial Chemical Industries Ltd., England), crystalline pork insulin (Novo Industri, Denmark, Lot. Nr. S21166), pork secretin and cholecystokinin-pancreozymin (kindly supplied by Prof. V. Mutt) were used in the cross-reactivity studies.

I^{125} was obtained as NaI^{125} in NaOH solution from the Radiochemical Centre, Amersham, England. Rabbit albumin (Fraction V) was obtained from Mann Research Lab., New York, USA, bovine albumin (Fraction V) from Armour Pharmaceuticals Ltd., Eastbourne, England and carbodiimide (morpho-CDI) was from Aldrich Chem. Co., Milwaukee, USA. "Trasylol" was supplied by Bayer, Leverkusen, West Germany. Freunds adjuvant was obtained from Difco Labs., Detroit, USA, and Tween 20 was obtained from Sigma Chemical Co., St. Louis, USA. Microcrystalline cellulose and other reagents of analytical grade were from E. Merck AG, Darmstadt, W. Germany.

Preparation of antisera

Pork glucagon coupled to rabbit albumin by carbodiimide was administered to rabbits in the antibody production process (7, 6). The animals were immunized by series of 3 weekly intramuscular injections of antigen containing 5 mg glucagon. This procedure was repeated every 2 months for a period of 6–12 months. Antisera were stored at −20°C until the preparation of the immunosorbent.

Preparation of I^{125}-glucagon

Beef-pork glucagon was labelled with I^{125} using the chloramine-T method of Hunter & Greenwood (10) as modified by Edwards et al. (4). Separation of the iodinated hormone from free I^{125} was performed on a Sephadex G25 column (25×1 cm).

It has been shown that glucagon and labelled glucagon normally remain at the point of application after paper chromatography on Whatman 3MM paper with veronal buffer, pH 8.6 (20). This method was used in order to

estimate the immunoreactivity of the labelled glucagon. Normal serum and glucagon antiserum (1 : 5 dilution) were incubated for 24 hours with the labelled glucagon (100–200 pg) and Trasylol (1 000 KIU/ml). After chromatography of the incubation mixtures, the papers were cut into 1 cm strips and the distribution of radioactivity was then determined.

Detection of antibodies

A solid phase "sandwich-technique" (25) was used as a sensitive test of the antisera. Five mg of glucagon was coupled to 100 mg of cyanogen bromide-activated cellulose (using the same method as for the coupling of antibodies, see below), and 0.2 ml of this cellulose-glucagon suspension (5 mg/ml) was incubated for 24 hours with 50 μl of a 1 : 10 dilution of the test antisera. After centrifugation and washing, the cellulose-glucagon-antibody complex was incubated with 100–200 pg of I^{125}-glucagon in a second incubation of 24 hours. The cellulose-glucagon-antibody-I^{125}-glucagon complex was then separated from the free I^{125}-glucagon by centrifugation and washing before determination of the radioactivity of the cellulose-bound I^{125}-glucagon. Antisera which resulted in more than 50 per cent binding of I^{125}-glucagon to the cellulose particles were selected for further studies.

To decide which one of the antisera would give the highest sensitivity in the assay system, titration curves were made with and without the addition of a constant amount of the unlabelled antigen as described by Hurn & Landon (11). When 20% of the I^{125}-glucagon was bound by each antiserum, the antiserum which showed the largest reduction in antibody-bound I^{125}-glucagon in the presence of the added unlabelled glucagon was subsequently used in the assay.

Activation of cellulose and coupling of antibodies

Antibodies were coupled to cyanogen bromide-activated microcrystalline cellulose as described in detail previously (23, 24). The sodium sulphate precipitated gamma globulin fraction from 0.1 ml of antiserum was incubated with 300 mg of the activated cellulose in the coupling procedure. A suspension of 5 mg/ml of the cellulose-bound antibody could then be used in the assay system. The amount of immunosorbent was chosen so that in the absence of unlabelled glucagon, about 20 per cent of the I^{125}-glucagon was bound to the immunosorbent during an incubation time of 16–18 hours.

Immunoassay procedure

Standards and samples were assayed in triplicate. Each assay tube contained 200 μl of a suspension of the immunosorbent (5 mg/ml) in 0.1 M phosphate buffer, pH 7.5 containing 0.5 mg/ml bovine plasma albumin and 0.5% Tween 20, approx, 100 pg of I^{125}-glucagon in 100 μl of the same phosphate buffer, and 200 μl of sample or standard. Trasylol (100 μl of a solution containing 5 000 KIU/ml saline) was added to each tube to prevent degradation of glucagon during the assay. The tubes were then incubated for 16–18 hours at room temperature with constant rotation in a "Rotamix" (Heto, Birkeröd, Denmark) to keep the cellulose particles in suspension. Separation of the glucagon-antibody-cellulose complex from unbound glucagon was performed by centrifugation at 3 000 rpm for one minute in an MSE Super Medium centrifuge (MSE, London, England). The cellulose particles were then washed three times with 1.5 ml volumes of saline before determination of their radioactivity in a gamma counter. In order to test the non-specific binding of I^{125}-glucagon to the cellulose-globulin complex, the sodium sulphate precipitated gamma globulin fraction of normal rabbit serum was coupled to cellulose as described for the preparation of the immunosorbent, and used in the assay conditions.

Isolation and incubation of guinea-pig islets of Langerhans

Islets of Langerhans were isolated from guinea-pig pancreas using collagenase, and incubated in groups of ten using methods described in detail elsewhere (4). The islets were incubated in the presence of 5.5 mM glucose and, in order to inhibit the glucagon release from the islets, with the addition of 5 mM octanoic acid to the incubation medium. Samples of the incubation medium were stored at −20°C until the determination of their glucagon content. Medium taken before the incubations served as controls in the assay.

RESULTS

Iodination of glucagon

The specific activity of iodinated glucagon was 250–300 μCi/μg, while the iodination damage was about 15% shown by TCA precipitation and paper chromatography. Comparisons between the paper chromatography of I^{125}-glucagon incubated with normal serum and antiglucagon serum showed that 80% of the radioactivity, which remained at the origin with normal serum, migrated in the presence of antiglucagon serum. The results indicated that at least 80% of the I^{125}-glucagon was immunoreactive. The iodinated hormone was stored at −20°C and could be used for 4 to 6 weeks.

Antibody production and selection of antiserum

Samples of antisera from 3 of the 6 animals used in the immunization procedure showed sufficient binding capacities of labelled glucagon to be used in the assay. 1 ml of the antiserum with the highest binding capacity could be used for about 3 000 analyses. From the titration curves with and without the addition of unlabelled glucagon it was found that with different antisera reduction of 10–24% of the antibody-bound radioactivity were caused by addition of 200 pg unlabelled glucagon.

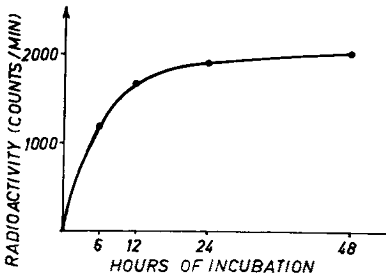

Fig. 1. The uptake of I^{125}-glucagon by cellulose-bound antibody. The immunosorbent was incubated with labelled glucagon under the conditions of the assay and the radioactivity of the cellulose-bound I^{125}-glucagon determined at the times shown.

Assay incubation temperature

In order to study the influence of incubation temperature upon the antigen-antibody reaction, incubations were performed at room temperature and at 4°C. Although the standard curves were parallel, the uptake of labelled hormone was increased when incubated at room temperature and assays were therefore performed at room temperature.

Assay incubation time

In the glucagon assay at room temperature it was shown that the uptake of I^{125}-glucagon on the immunosorbent was rapid during the first twelve hours of incubation with little further increase after this time (Fig. 1). An incubation time of 16–18 hours was subsequently used in the assay system.

Standard curve

A standard curve for determinations in triplicate over the range 0–500 pg glucagon per sample is shown in Fig. 2. The standard curve is presented both as radioactivity bound to the particles vs. dose and after logit transformation according to Rodbard et al. (16). The average of the standard errors of the mean for the triplicate samples was 0.5% of the radioactivity bound at each level of added glucagon. The sensitivity of the assay expressed as the lowest amount of glucagon which could be detected from a sample containing zero glucagon with 95% confidence when the samples were assayed in triplicate, was calculated to be approx. 10 pg of glucagon. Twenty pg of glucagon could be detected for samples assayed in duplicate. The coefficient of variation for means of triplicate within an assay was calculated to be about 3% at a glucagon level of 299–400 pg.

Specificity of the assay

Samples containing pork insulin, synthetic human gastrin I, pork secretin or pork cholesystokinin-

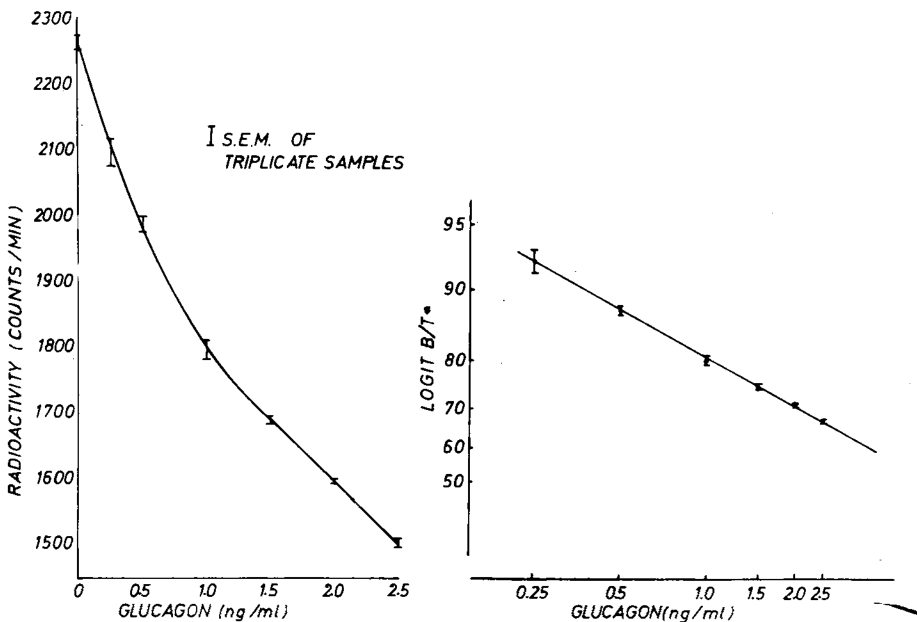

Fig. 2. Glucagon assay standard curve. A single assay was performed as described in the text and the radioactivity of the cellulose-bound I^{125}-glucagon was determined for additions of unlabelled glucagon over the range 0–500 pg. The ratio B/T is expressed as a percentage, where B equals activity bound at each level of added glucagon and T^* equals activity bound in the absence of unlabelled glucagon.

pancreozymin at concentrations from 1 to 1 000 ng/ml were incubated with the immunosorbent and I^{125}-glucagon under the conditions of the assay. It was found that there was no inhibition of the binding of labelled glucagon to antibody by these hormones. Samples containing guinea-pig glucagon and beef-pork glucagon gave parallel dilution curves in the assay procedure.

Glucagon release from isolated guinea-pig islets

The rate of glucagon release from guinea-pig islets incubated in vitro in medium containing 5.5 mM glucose was found to be 1.50±0.12 ng/islets/30 min (11 observations).

When 5 mM octanoic acid was added to the incubation medium, glucagon release from the isolated islets was markedly inhibited. The rate of glucagon release in the presence of 5.5 mM glucose and 5 mM octanoic acid was 0.44±0.18 ng/10 islets/30 min (9 observations). Possible interference by the incubation media was excluded by the use of control samples.

DISCUSSION

Several methods have previously been reported for the separation of antibody-bound glucagon from unbound glucagon in radioimmunological assay methods. When comparing their relative merits, the efficiency in separation of free from bound hormone as well as the technical simplicity and speed of the analyses have to be taken into consideration. For glucagon measurements the ability of the method to avoid interference from damaged hormone is also important, since glucagon is very unstable to enzymatic degradation.

In this respect, the radioimmunosorbent techniques offer some advantages over other methods of radioimmunoassay (25). An almost complete separation of free hormone from bound is obtained and the reported rapid dissociation of the antibody-antigen complex (15) was not observed during the separation procedure of this system. Proteolytic enzymes in the antisera are removed before the immunosorbent is used in the assay and the amount of proteolytic enzymes in the assay incubation mixture are therefore very small.

The binding of glucagon to antibody could be temperature dependent for some antisera (9). When this was investigated in our assay system only small differences were found between the ratio of antibody-bound glucagon to unbound in incubations at room temperature and at 4°C with the particular antisera used.

The non-specific adsorption of radioactive material to the cellulose particles in the assay was very low, below 0.5% of added radioactivity. The background radioactive contamination of the antibody-bound hormone was therefore small, contributing to the high precision of the assay. Interference by damaged I^{125}-glucagon which has been shown to cause errors in other assay methods (2), was negligible.

In this radioimmunosorbent glucagon assay the sensitivity obtained was 10 pg of glucagon per sample, comparable to the sensitivity of the ethanol precipitation assay described by Heding (9). In other assay systems 20–200 pg could be detected with the same confidence (3, 8, 15, 18, 21). The uptake of the labelled glucagon by the immunosorbent was rapid during the first 12 hours of incubation, and longer assay incubation times were unnecessary. The high sensitivity could therefore be obtained in spite of the relatively short incubation time of 16–18 hours as compared with other analyse methods where incubation times of up to 72 hours are used.

The cross-reaction of the glucagon antisera with other pancreatic hormones or hormones with similar structure to glucagon was studied. Since purified hormones of guinea-pig were not available, samples of hormones from other species were used. No cross-reaction was observed with pork insulin, human gastrin I, pork secretin or pork cholecystokinin-pancreozymin.

The separation of antibody-bound hormone from free is technically simple and rapid, and using automatic techniques (24), it is possible to perform the separation procedure for 200 tubes in 45 min.

When glucagon release from isolated guinea-pig islets was measured, an inhibition of glucagon release was obtained by octanoic acid. It has been found previously that fatty acids inhibit glucagon release both from isolated islets incubated in vitro (3) and from the pancreas in vivo (14). The levels of glucagon release from the isolated guinea-pig islets incubated in the presence of glucose and of octanoic acid compare favourably with those found using ethanol precipitation assay method (4). The basal glucagon secretion in the presence of glucose also

corresponds to that from isolated rat islets assayed using a double-antibody assay (21).

This assay has primarily been developed for studies on glucagon release in vitro. The specific problems with plasma measurements of pancreatic glucagon (reviewed by Heding 1971) and the additional difficulties caused by the presence of gut glucagon-like immunoreactivity (17) have not yet been studied in this system.

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REFERENCES

1. Assan, R., Rosselin, G., Drouet, J., Delais, J., Tchobroutsky, G. & Derot, M.: Dosage radioimmunologique du glucagon plasmatique chez l'homme. Ann Endocr *27:* 690, 1966.
2. Buchanan, K. D. & McCarroll, A. M.: Comparison of methods of separation of free from bound hormone in the radioimmunoassay of insulin and glucagon. *In* Radioimmunoassay methods (ed. K. E. Kirkham and W. M. Hunter), p. 266. Churchill Livingstone, Edinburgh and London, 1971.
3. Edwards, J. C., Howell, S. L. & Taylor, K. W.: Fatty acids as regulators of glucagon secretion. Nature *224:* 808, 1969.
4. — Radioimmunoassay of glucagon released from isolated guinea-pig islets of Langerhans incubated in vitro. Biochim Biophys Acta *215:* 297, 1970.
5. Eisentraut, A. M., Whissen, N. & Unger, R. H.: Incubation damage in the radioimmunoassay for human plasma glucagon and its prevention with "Trasylol". Amer J Med Sci *225:* 137, 1968.
6. Goldfine, I. D. & Ryan, W. G.: Rapid production of glucagon antibodies. Horm Metab Res *2:* 47, 1970.
7. Goodfriend, T. L., Levine, L. & Fasman, G. D.: Antibodies to bradykinin and angiotensin: A use of carbodiimides in immunology. Science *144:* 1344, 1964.
8. Hazzard, W. R., Crockford, P. M., Buchanan, K. D., Vance, J. E., Chen, R. & Williams, R. H.: A double antibody immunoassay for glucagon. Diabetes *17:* 179, 1966.
9. Heding, L. G.: Radioimmunological determination of pancreatic and gut glucagon. Diabetologia *7:* 10, 1971.
10. Hunter, W. M. & Greenwood, F. C.: Preparation of iodine[131]-labeled growth hormone of high specific activity. Nature, London *194:* 495, 1962.
11. Hurn, B. A. L. & Landon, J.: Antisera for radioimmunoassay. *In* Radioimmunoassay methods (ed. K. E. Kirkham and W. M. Hunter), p. 121. Churchill Livingstone, Edinburgh and London, 1971.
12. Lawrence, A. M.: Radioimmunoassayable glucagon levels in man: Effects of starvation, hypoglycemia, and glucose administration. Proc Nat Acad Sci *55:* 316, 1966.
13. Leclercq-Meyer, V., Mialhe, P. & Malaisse, W. J.: Assay of glucagon using dextran-charcoal. Diabetologia *6:* 121, 1970.
14. Luyckx, A. S. & Lefebvre, P. J.: Arguments for a regulation of pancreatic glucagon secretion by circulating plasma free fatty acids. Proc Soc Exp Biol Med USA *133:* 524, 1970.
15. Nonaka, K. & Foà, P. P.: A simplified glucagon immunoassay and its use in a study of incubated pancreatic islets. Proc Socc Exp Biol Med *130:* 330, 1969.
16. Rodbard, D., Rayford, P. L., Cooper, J. A. & Ross, G. T.: Statistical quality control of radioimmunoassays. J Clin Endocr *28:* 1412, 1968.
17. Samols, E., Tyler, J., Megyesi, C. & Marks, V.: Immunochemical glucagon in human pancreas, gut and plasma. Lancet *2:* 727, 1966.
18. Shima, K. & Foà, P. P.: A double antibody assay for glucagon. Clin Chim Acta *22:* 511, 1968.
19. Unger, R. H., Eisentraut, A. M., McCall, M. S., Keller, S., Lanz, H. C. & Madison, L. L.: Glucagon antibodies and their use for immunoassay for glucagon. Proc Exp Biol Med *102:* 621, 1959.
20. Unger, R. H., Eisentraut, A. M., McCall, M. S. & Madison, L. L.: Glucagon antibodies and an immunoassay for glucagon. J Clin Invest *40:* 1280, 1961.
21. Vance, J.E., Buchanan, K. D., Challoner, D. R. & Williams, R. H.: Effects of glucose concentration on insulin and glucagon release from isolated islets of Langerhans of the rat. Diabetes *17:* 187, 1968.
22. Wide, L. & Porath, J.: Radioimmunoassay of proteins with the use of Sephadex-coupled antibodies. Biochim Biophys Acta *130:* 257, 1966.
23. Wide, L., Axen, R. & Porath, J.: Radioimmunosorbent assay for proteins. Chemical couplings of antibodies to insoluble dextran. Immunochemistry *4:* 381, 1967.
24. Wide, L.: Radioimmunoassays employing immunosorbents. *In* Immunoassays of gonadotrophins (ed. E. Diczfalusy), Acta Endocr *63,* Suppl. 142: 207, 1969.
25. — Solid phase antigen-antibody systems. *In* Radioimmunoassay Methods (ed. K. E. Kirkham and W. M. Hunter), p. 405. Churchill Livingstone, Edinburgh & London, 1971.

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