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Glucagon Immunoassay Using Polyethylene Glycol to Precipitate Antibody-Bound Hormone

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Summary. The use of polyethylene glycol 6000 to separate free and antibody-bound ligand has been applied to the radioimmunoassay of glucagon. Equalization of protein content in all tubes before precipitation of the glucagon-antibody complex was required. Time between addition of the polymer and centrifugation had no detectable effect. Degradation of ^{131}I-glucagon during incubation was best prevented by a combination of benzamidine (5 mM) and Trasylol® (500 KIE/tube). Sensitivity of the assay permitted discrimination of buffer or plasma samples (100 µl) whose glucagon contents differed from 25 pg/ml, under 100 pg/ml, and 35 pg/ml, under 200 pg/ml. Reproducibility was 9.5% (coefficient of variation) for plasmas with glucagon concentrations ranging from 100 to 300 pg/ml. Recovery of exogenous glucagon added to plasma was satisfactory. Measurement of glucagon was possible in fasting plasma samples diluted up to 1/8. The separation method described appears to be easy and reliable, especially when large numbers of samples are routinely handled.

Key words: Glucagon, radioimmunoassay, polyethylene-glycol, proteolytic enzymes inhibitors.

Since the initial description of glucagon immunoassay by Unger *et al.* [34, 35], a number of improvements have been introduced (see ref. 21 for a review). Besides utilization of antisera specific for pancreatic glucagon and adequate prevention of labelled glucagon degradation during incubation, most of the refinements of the technique are dealing with the separation of free and antibody-bound hormone.

Three major groups of methods are available. Separation may be carried out by paper chromatography [3, 19, 27, 34]. The glucagon-antibody complex may be precipitated by Na_2SO_4 [14], a second antibody [15, 29], or ethanol [10, 16]. Finally, free glucagon may be selectively adsorbed by an ionic exchange resin [38], cellulose powder [26] or dextran-coated charcoal [2, 20].

Recently, the use of polyethylene glycol 6000 (PEG) to precipitate antibody-bound ligand from the free compound was reported by Desbuquois and Aurbach [8]. So far, this separation technique has been successfully used in radioimmunoassays of insulin, growth hormone, parathyroid hormone [8], arginine-vasopressin [8, 13], cyclic AMP [8, 28], angiotensin I and digoxin [6].

The purpose of this report is to describe the application of this new separation method to glucagon immunoassay.

Materials

1. Buffers

A sodium phosphate buffer (0.04 M, pH 7.4) with 0.1 g bovine serum albumin/100 ml and 0.02 g Merthiolate®/100 ml *(buffer GA)* was used for the dilution of labelled glucagon. Working standards were prepared and unknown diluted (when required) in the same buffer supplemented with NaCl 6 g/100 ml *(buffer GB)*. At the time of the assay, Trasylol® was added to buffer GA *(buffer GA-TRAS)* to reach the concentration of 5000 KIE/ml. This buffer was used to dilute the antiserum or for the blanks (see assay procedure).

2. Glucagon Standards

Monocomponent pork glucagon (Novo, lot MC 6770) was used as standard. Stock solutions of 50 ng/ml were stored in aliquots at −25°C. Each aliquot was thawed once for the preparation of the working standard solutions (0, 50, 100, 200, 400, 600, 1000 and 2000 pg/ml) by volumetric dilution with buffer GB.

3. Labelled Glucagon

^{131}I-glucagon was purchased from the Centre National de Transfusion Sanguine, Paris. The specific activity varied from 600 to 850 µCi/µg. Frozen on arrival, labelled glucagon was thawed immediately prior to use and diluted with buffer GA, to provide a solution of 250 pg ^{131}I-glucagon/ml.

4. Anti-Glucagon Serum

Anti-pork glucagon rabbit serum K814 (from L.G. Heding, Novo Research Institute, Copenhagen, Denmark) was used. It is considered as specific for pancreatic glucagon [17].

5. PEG Solutions

A 23.75% (w/w) solution of PEG (providing a 12.5% final concentration in assay tubes) was freshly prepared before use by dissolving the polymer in distilled water pre-chilled to 4°C. After dissolution, continuous stirring was not required to keep homogeneity.

6. Reagents

Salts of analytical grade used for buffers, trichloracetic acid (TCA) and PEG 6000 were purchased from Merck A.G. (Darmstadt, Germany), benzamidine hydrochloride from Schuchardt (München, Germany), bovine serum albumin from Poviet (Amsterdam, Netherlands), Merthiolate® from Eli Lilly and Co. (Indianapolis, U.S.A.), epsilon-aminocaproic acid (EACA) from Koch-Light Ltd. (Colnbrook, England) and heparin from F. Hoffman-La Roche and Co. Ltd. (Basel, Switzerland). Trasylol® powder (5000 KIE/mg) was obtained from Bayer A.G. (Leverkussen, Germany).

Methods

1. Blood Collection

10 ml venous blood were collected into 100 µl buffer GB containing 250 U heparin and 5000 KIE Trasylol®. After centrifugation, the plasma was stored at −25°C. The samples were thawed at room temperature, mixed and centrifuged at 4°C just prior to the assay.

2. Assay Procedure

During the preparation of the assay, all reagents and reaction tubes were maintained in melting ice baths. Into the glass incubation tubes (10 × 55 mm) were pipetted sequentially:

- 100 µl of standard solutions or unknown samples;
- 100 µl of either antiserum diluted (1/1200) in GA-TRAS, or buffer GA-TRAS alone.

After 40 h of incubation at 4°C, 100 µl ^{131}I-glucagon (25 pg) were added for a second incubation of 72 h at 4°C. The incubation times chosen enabled the system to reach equilibrium and provided the best slope of the standard curve. Triplicates were prepared for the standard solutions and their control containing neither unlabelled glucagon nor antiserum *(blank)*; a set of tubes contained only the labelled glucagon *(total radioactivity)*. Each unknown was assayed in duplicate with two blanks.

The standard and unknown incubation tubes were brought to the same final protein concentration by addition of 150 µl of cold human plasma, non-diluted and diluted 1/3 with buffer GB, respectively. Without delay, 0.5 ml chilled PEG (12.5% final concentration) was dispensed in all assay tubes, that were carefully shaken. This concentration of PEG was shown to ensure complete precipitation of antibody-bound hormone [8]. After 30 min incubation at 4°C, the tubes were centrifuged at 4°C for 45 min, at 1500 × g. The supernatant was decanted and the tubes left upside down on soft paper in order to complete removal of the liquid phase. Radioactivity of the precipitate was then counted for 4 min in a well-type Automatic Gamma Analyser (Philips).

3. Statistical Analysis

Statistical analysis of the results was performed according to Snedecor and Cochran [31]. The results are given as the mean ± estimate (s) of the standard deviation (S.D.) obtained from $s = \sqrt{[\Sigma (x - \bar{x})^2/(n-1)]}$ when the number of determinations (n) is greater than 2. Use is also made of the relationship $s = \sqrt{[\Sigma\ d^2/2N]}$, where d is the difference between the two results in a duplicate determination, and N the number of duplicate determinations performed [30]. Calculation of s provides an evaluation of the extent to which repeated measurements of the same sample agree with their mean, in a single assay *(precision)* or in different assays *(reproducibility)*. This evaluation is given by the coefficient of variation $C.V. = s/\bar{x}$, expressed as a percentage of the mean ($\bar{x}$), or the fiducial range which estimates the limits inside which the real value is included. The fiducial range is derived from $M \pm ts/\sqrt{n}$, where M is the mean of n determinations.

Whether two samples significantly differ from each other, and hence the least difference (d) required between two samples to be statistically different *(quantitative sensitivity)* is calculated from $d = t\sqrt{[(s^2_1/n_1) + (s^2_2/n_2)]}$, where s_1 and s_2 are estimates of the two S.D., n_1 and n_2 the number of measurements in each case (i.e. 2 when the determinations are made in duplicate). Determination of the least value distinguishable from zero *(qualitative sensitivity)* is derived from $ts/\sqrt{n}$, where s is the estimate of the S.D. made on n determinations. t values used in these formulae are read at a level of significance $P = 0.05$.

Statistical significance of differences between groups of samples was assessed by Student's *t* test.

4. Experimental Design

Effect of Time and Protein Concentration Upon ^{131}I-Glucagon Precipitation by PEG. Series of tubes containing buffer or plasma samples, with or without antiserum, were run as described under "Assay procedure". The effect of time was studied by allowing variable delays to elapse between completion of PEG distribution and centrifugation. Meanwhile the tubes were kept at 4°C. The effect of protein concentration was studied by adding various amounts of plasma before PEG precipitation.

Effect of Proteolytic Enzyme Inhibitors upon ^{131}I-Glucagon Degradation. To buffer or plasma samples were added buffer GA containing the inhibitor tested, and labelled glucagon, for incubation periods identical to those of the assay. The proportion of non degraded ^{131}I-glucagon was estimated by precipitation with TCA. Standardization of protein content in each tube was carried out immediately before addition of cold TCA (final concentration 5%, w/v). After centrifugation and decantation, the radioactivity of the precipitate was counted. Blood samples utilized for this experiment were not taken into inhibitor.

Evaluation of Precision, Sensitivity and Reproducibility of the Standard Curves. Eleven standard curves were performed over a six months period. For comparison of the different curves together, displacement of ^{131}I-glucagon by unlabelled glucagon was expressed in percentage of the precipitation recorded in the absence of unlabelled glucagon.

For each individual standard curve, the precision and quantitative sensitivity were calculated (see "Statistical Analysis") over each range of unlabelled glucagon addition. The individual values were finally pooled to provide the average C.V. $\pm$ s and average sensitivities $\pm$ s.

Evaluation of Precision, Sensitivity and Reproducibility of the Assay when Applied to Plasma. C.V. of duplicate determinations of the blank (4.28% for 600 cpm during 4 min; n = 50) was not different from that of the counter (4.33% for 500 cpm during 4 min; n = 25). The average blank was therefore substracted from each member of the duplicate measurement of the sample.[1] The two values found served to calculate precision and quantitative sensitivity as already described.

The reproducibility was evaluated by measuring glucagon concentration in 24 plasmas, taken at random, in two different assays. Some of these plasmas were thawed only once, whereas thawing of the others was repeated at the time of the two assays. The two glucagon concentrations found for each sample were compared by Student's *t* test for *paired* data.

Recovery of Glucagon Added to Plasma. Solutions of unlabelled glucagon, with concentrations ranging from 0 to 5 ng/ml were prepared in buffer GB. To 900 µl of several plasmas, containing 1000 KIE Trasylol®, were added 100 µl of the different glucagon solutions. The glucagon concentration in these samples was measured as usual.

Results and Discussion

1. Solubility of Free ^{131}I-Glucagon in PEG

The solubility of ^{131}I-glucagon in PEG concentrations ranging from 2.5 to 15% (w/w) was studied in the conditions of the assay except for the absence of antiserum. The precipitation (mean $\pm$ s, n = 10), expressed as the percentage of total radioactivity, increased from 5.7 $\pm$ 0.3% to 10.0 $\pm$ 0.2% over the range of PEG concentrations studied.

Free glucagon, like growth hormone and parathyroid hormone [8] slightly coprecipitated with serum proteins, whereas insulin, vasopressin [8], and angiotensin I [6] remained virtually fully soluble as PEG concentration was increased.

1 For routine calculation of the results, the corresponding blank values were substracted from the counts given by each standard solution and unknown sample.

2. Effect of Time between Addition of PEG and Centrifugation

As shown in Table 1, delaying centrifugation for as long as 90 min did not significantly modify the percentage of precipitated ^{131}I-glucagon, whether the sample assayed was a plasma or a buffer.

Table 1. *Effect of time between addition of PEG and centrifugation upon ^{131}I-glucagon precipitation*

Time (min)	Antiserum	Buffer sample	Plasma sample
0	+	34.6 $\pm$ 0.1	25.5 $\pm$ 0.6[b]
	−	9.4 $\pm$ 0.4	8.1 $\pm$ 0.5
30	+	36.0 $\pm$ 1.5	27.0 $\pm$ 0.7
	−	9.5 $\pm$ 0.1	8.8 $\pm$ 0.3
60	+	35.6 $\pm$ 1.3	26.1 $\pm$ 1.0
	−	9.4 $\pm$ 0.4	8.3 $\pm$ 0.3
90	+	34.4 $\pm$ 1.1	26.3 $\pm$ 0.9
	−	8.5 $\pm$ 0.3[a]	8.8 $\pm$ 0.2

Precipitation of ^{131}I-glucagon by PEG is expressed in % of total radioactivity. Each value represents the mean $\pm$ s (estimate of S.D.) of 5 determinations. Group a is statistically different ($p < 0.01$) from the corresponding ones at the three preceding times; group b is significantly lower ($p < 0.001$) than the corresponding one at min 30.

The absence of disturbance in the bound-free hormone equilibrium by PEG seems to be a characteristic of immunoassays using this polymer [6, 8]. This is in contrast with separation techniques using particulate adsorbants, which require careful control of time exposure. Nonaka and Foa [26] reported indeed that adsorption of free glucagon by cellulose powder, for more than 5 min, rapidly decreased the amount of antibody-bound glucagon. In our assay procedure, a 30 min delay at 4°C, starting at the completion of PEG distribution, was routinely adopted.

3. Effect of the Protein Concentration upon PEG Precipitation

Immunoglobulins are almost totally precipitated with PEG [9] and their presence in the incubation medium is required in a sufficient concentration to ensure complete precipitation of antibody-bound hormones [8].

As illustrated in Fig. 1A, raising the volume of plasma added to buffer samples from 0 to 100 µl induced a two-fold increase in ^{131}I-glucagon precipitation in the presence of antibody. Higher volumes of plasma were much less efficient. As the volume of plasma was augmented, a slight increase of precipitated ^{131}I-glucagon was also observed in the absence of antibody. In plasma samples (Fig. 1B), raising the protein content above 100 µl progressively increased ^{131}I-glucagon precipitation by PEG, both in the presence and the absence of antiserum.

Yet, the precipitation due to antibodies remained fairly constant, provided a plasma volume of at least 100 µl was present in the assay tubes, before PEG addition. Hence, the final plasma volume of all incubation tubes was brought to 150 µl immediately before precipitation by PEG (see "Assay procedure").

4. Effect of Proteolytic Enzymes Inhibitors upon ^{131}I-Glucagon Degradation

This series of experiments is based on the assumption that only undamaged ^{131}I-glucagon is precipitable by TCA.

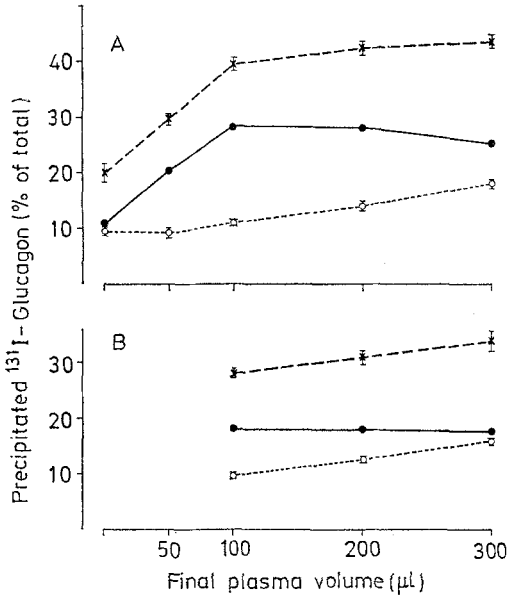

Fig. 1. Effect of protein concentration upon ^{131}I-glucagon precipitation by PEG. Increasing amounts of plasma were added immediately before PEG either to buffer (A) or to plasma (B) samples (100 µl) incubated with (x---------x) or without (o--------o) antiserum. The solid line (●—●) represents the precipitation due to antibodies; it is obtained from the difference between the two other lines. Each point is the mean ± s (estimate of S.D.) of 5 determinations

Table 2 shows that after incubation in buffer, 92 to 93% ^{131}I-glucagon remained precipitable by TCA, whether the assay medium contained a proteolytic enzyme inhibitor or not. In contrast, after incubation with plasma, only 69% labelled glucagon were still precipitable. The presence of Trasylol® (500 KIE/tube) almost completely prevented ^{131}I-glucagon degradation. Three concentrations of benzamidine tested (5, 10 and 16.5 mM) appeared to be nearly as efficient as Trasylol® alone. EACA, known to inhibit plasmin [1], showed a lower protective effect than that of the former drugs. The combination of Trasylol® (500 KIE/tube) and benzamidine (5 mM) exhibited the greatest efficiency, totally preventing glucagon destruction by plasma.

The destruction of ^{131}I-glucagon during incubation with human plasma, first reported by Mirsky *et al.* [24], is thought to be due to plasmin. The damage of labelled glucagon during immunoassay was recognized in 1963 [36] and suggested as a cause of erroneously high plasma glucagon values.

Eisentraut *et al.* [11] demonstrated that Trasylol®, an inhibitor of kallikrein, plasmin, trypsin and chymotrypsin [33], efficiently prevented ^{131}I-glucagon degradation during incubation. Other agents, such as mercaptoethanol, mercurophyllin [5], EDTA and Mg^{2+} [32], were also shown to be efficient. More recently, Ensinck *et al.* [12] suggested the use of benzamidine, a competitive inhibitor of plasmin, trypsin and thrombin [23] as a cheap substitute for Trasylol®.

The efficiency of proteolytic enzymes inhibitors was studied in absence of antibodies as they protect ^{131}I-glucagon by themselves [35]. Our results are in agreement with those of Eisentraut *et al.* [11] con-

Table 2. *Effect of proteolytic enzymes inhibitors upon degradation of ^{131}I-glucagon during incubation*

Line No	Inhibitor added	Concentration	TCA precipitable radioactivity (% of total)					
			Buffer samples	P		Plasma samples	P	
1	Nil	—	92.9±3.9 (65)			68.9±8.9 (92)		
2	Trasylol®	500 KIE/tube	94.9±4.3 (30)	vs. 1	< 0.05	87.9±5.2 (74)	vs. B vs. 1	< 0.001 < 0.001
3	Benzamidine	5 mM	93.9±5.6 (14)	vs. 1 vs. 2	N.S. N.S.	85.8±3.4 (26)	vs. B vs. 1 vs. 2	< 0.001 < 0.001 < 0.02
4	Benzamidine	10 mM	93.1±4.7 (14)	vs. 1 vs. 3	N.S. N.S.	88.2±4.1 (26)	vs. B vs. 1 vs. 3	< 0.005 < 0.001 < 0.05
5	Benzamidine	16.5 mM	94.9±4.5 (26)	vs. 1 vs. 3	N.S. N.S.	89.3±4.0 (60)	vs. B vs. 1 vs. 4	< 0.001 < 0.001 N.S.
6	EACA	10 mM	—			78.4±7.3 (18)	vs. 1 vs. 2	< 0.001 < 0.001
7	Trasylol® + Benzamidine	500 KIE/tube 5 mM	94.5±4.0 (14)	vs. 1 vs. 2 vs. 3	N.S. N.S. N.S.	93.3±2.7 (14)	vs. B vs. 1 vs. 2 vs. 3	N.S. < 0.001 < 0.001 < 0.001

Results are expressed as the mean ± s (estimate of S.D.) of the number of samples shown in parentheses. Buffer samples were assayed singly and plasma samples in triplicate. vs. B refers to the statistical comparison between plasma and buffer samples on the same line. Comparison between two groups, in the same column, is indicated by vs. line No of the second group. N.S. means not significant.

cerning Trasylol® protective role, with those of Assan *et al.* [5] regarding the poor effect of EACA and with those of Ensinck *et al.* [12] as to the use of benzamidine. Coincidence of standard curves performed in presence or absence of 5 mM benzamidine (data not shown) ruled out a possible interference of the inhibitor with the antigen-antibody reaction. The same observation was made by Ensinck *et al.* [12] using Unger's 30K antiserum.

Henceforth 5 ml blood samples are routinely collected into 50 µl buffer GB, containing heparin and benzamidine, to achieve a concentration of respectively 15 U/ml and 7.5 mM (to reach a final concentration of about 15 mM in plasma, assuming a 50% hematocrit value). 500 KIE Trasylol® are still added to each tube for the assay.

5. *Precision, Sensitivity and Reproducibility of the Standard Curves*

An estimate of the precision of standard curves is given in Table 3. The average coefficient of variation remained lower than 3.2% up to 400 pg unlabelled glucagon/ml. Differences of less than 25 pg/ml for glucagon concentrations lower than 100 pg/ml and of 33 pg/ml for concentrations between 100 and 200 pg/ml could be discriminated with 95% confidence.

Comparison of the 11 standard curves performed during successive assays was hampered by the use of different ^{131}I-glucagon batches. In these assays, the percentage of total ^{131}I-glucagon precipitated in the absence of unlabelled glucagon amounted to 38.1 ± 9.7 (mean ± s (estimate of S.D.); range: 22.6 to 52.1%). Fig. 2 demonstrates, however, a high reproducibility of the standard curves, when expressed as percentages of the radioactivity precipitated in absence of unlabelled glucagon. Furthermore, the slope of the curve was such that less than 40 pg unlabelled glucagon (400 pg/ml) were sufficient to cause a 50% fall in labelled glucagon binding by the antiserum.

Table 3. *Estimation of precision and sensitivity of standard curves for pure glucagon*

Amount of glucagon added (pg/ml)	Average C.V. (%)	Average quantitative sensitivity over given range (pg/ml)	Range of quantitative sensitivity (pg/ml)
0	2.15±0.94		
		23.7± 9.6	11— 42
50	2.50±0.81		
		21.6± 5.9	13— 31
100	2.37±0.74		
		33.0±10.5	16— 48
200	2.74±1.20		
		51.3±10.2	32— 64
400	3.16±0.78		
		86.6±34.8	46—136
600	4.90±2.38		
		179.6±67.3	100—332
1000	7.12±3.06		
		532.3±199.1	200—930
2000	8.59±4.33		

Results, calculated for 11 individual standard curves, are expressed as the mean ± s (estimate of S.D.). For details in method of calculation, see section "Experimental design".

6. *Precision, Sensitivity and Reproducibility of the Assay when Applied to Plasma*

Estimation of precision and sensitivity was performed on 200 plasma samples taken at random and distributed over 5 ranges of glucagon content (Table 4).

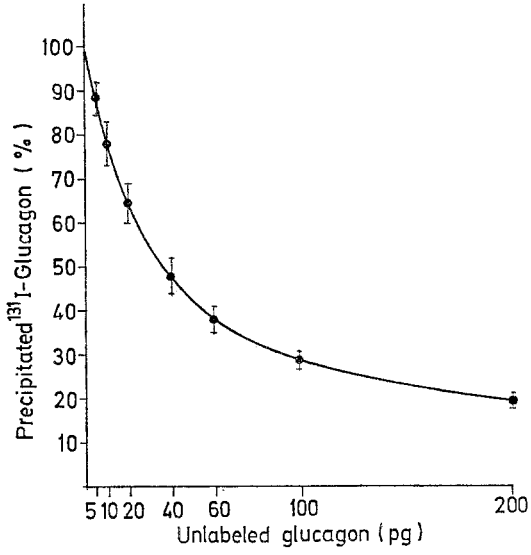

Fig. 2. Glucagon immunoassay standard curve. Each point represents the mean ± s (estimate of S.D.) of 11 different assays

Table 4. *Precision and sensitivity of the assay estimated by analysis of 200 duplicate determinations in plasma samples*

Concentration range of glucagon (pg/ml)	Mean ± s (pg/ml)		C.V. (%)	M ± fiducial range[a] (pg/ml)	Quantitative sensitivity over the range considered (pg/ml)
<100	71.0±11.9	(32)	16.8	M±17.2	24.3
100—150	126.9±17.8	(74)	14.1	M±25.2	35.6
150—200	177.1±18.7	(47)	10.5	M±26.6	37.6
200—300	240.0±22.4	(35)	9.3	M±32.2	45.6
300—450	347.7±24.5	(12)	7.0	M±38.1	53.9

The number of samples is indicated in parentheses. s is an estimate of S.D.

[a] M is the mean of any duplicate determination; over each range of glucagon concentration, the fiducial range of M is calculated at a level of 95% confidence (for details see "Statistical Analysis").

Plasmas, whose glucagon contents (ranging from 100 to 200 pg/ml) differed from about 35 pg/ml could be distinguished from each other with 95% confidence.

This quantitative sensitivity was still greater for lower glucagon concentrations, and remained satisfactory for plasma glucagon contents above 300 pg/ml. The qualitative sensitivity enabled us to distinguish a glucagon concentration of 17 pg/ml from zero.

The quantitative sensitivity of the assay in plasma was similar to that of the standard curve for purified glucagon (Table 3). The method and the antibody used provided a quantitative sensitivity (with 95% confidence) comparable with the best one recently reported: 30 pg/ml [7, 25]; a qualitative sensitivity of 10 pg/ml was mentioned by Iversen [18] and by Manns [22].

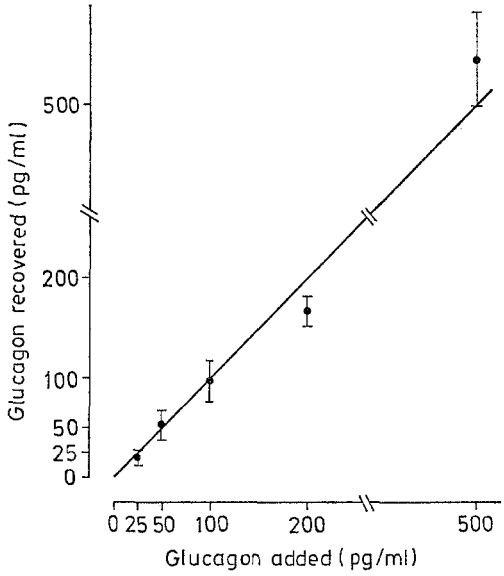

Fig. 3. Recovery of unlabelled glucagon added to plasma. Each point represents the mean ± s (estimate of S.D.) of 7 determinations

For the two assays involved in the study of reproducibility, the mean ± s of the 24 determinations were: 156.5 ± 38.7 pg/ml (range 110 to 280) and 154.4 ± 41.0 pg/ml (range 95 to 220), respectively.

When considering, for each plasma, the glucagon concentrations measured by the two assays as members of a pair, the general mean ± s was 155.4 ± 14.8 pg/ml (for details in calculations, see "Statistical Analysis"). The C.V. of 9.5% observed was of the same importance as that expressing the precision in a single assay, for the same range of values (Table 4).

The reproducibility of our method can hardly be compared with others since few reports were made on that particular aspect of glucagon immunoassay, validity. However, Leclercq-Meyer *et al.* [20] reported a C.V. of 22.7% for a single human plasma (211 ± 48 pg/ml) assayed in 17 different runs and Heding [16] mentioned a C.V. of 8.9% for a gut extract (5.4 ± 0.47 ng/ml) analyzed in 25 assays.

Glucagon concentrations (means ± s) of the 9 plasmas thawed only once were 143.9 ± 24.9 pg/ml and 146.1 ± 48.4 pg/ml in the two assays. For the 15 plasmas thawed a second time after refreezing they were 164.0 ± 44.1 pg/ml and 159.3 ± 36.8 pg/ml. These values were not statistically different. Thus thawing and refreezing the samples once did not seem to cause loss of glucagon immunoreactivity in plasma collected on Trasylol®. A similar observation was made by Leclercq-Meyer *et al.* [20] with samples taken or not on Trasylol®, which were thawed 4 times.

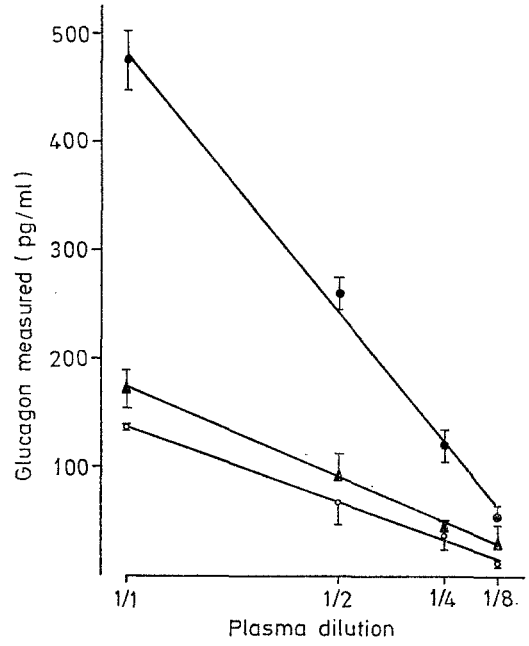

Fig. 4. Effect of dilution on apparent glucagon concentration in plasma. Each point represents the mean ± s (estimate of S.D.) of triplicate determinations

7. Recovery of Glucagon Added to Plasma

Fig. 3 shows the satisfactory recovery of exogenous glucagon added to different plasmas. The mean recoveries were 80, 104, 99, 86 and 109% respectively, for additions of 25, 50, 100, 200 and 500 pg glucagon/ml. Only the 14% underestimation of the 200 pg addition was significant ($p < 0.005$). Our results are comparable with recoveries reported by Hazzard *et al.* [15] for glucagon additions ranging from 100 to 2500 pg/250 µl and by Leclercq-Meyer *et al.* [20] for additions ranging from 250 to 5000 pg glucagon/ml.

8. Effect of Plasma Dilution upon Apparent Glucagon Concentration

Fig. 4 illustrates that the experimental values fit well ($p < 0.01$) with the least square regression lines of measured glucagon concentrations on expected concentrations. From these results, it would seem that plasma constituents, and particularly gamma-globulins, do not interfere with glucagon measurement in our assay system. This is in contrast with previous reports

[4, 37] suggesting that gamma-globulins are responsible for spuriously high plasma glucagon values measured in diluted samples, as compared with undiluted plasma. This interference could be overcome by extraction of glucagon with ethanol [16] or acetone [22].

A recent report by Weir *et al.* [39] suggested the existence of a factor, present in variable amounts in plasma from different individuals, which interferes with the binding of ^{125}I-glucagon to Unger's antiserum 30K. As shown in Fig. 4, the linear relationship observed between dilution of three plasma samples (two fasting samples and one taken during an arginine infusion) and glucagon concentration indicated that, if such factor exists, it does not seem to affect the binding of labelled glucagon to antiserum K814.

In conclusion, the use of PEG to separate free and antibody-bound hormone led to the development of a sensitive and reproducible glucagon immunoassay. Its advantages over customary techniques make it easy and reliable for routine assay of large numbers of samples.

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