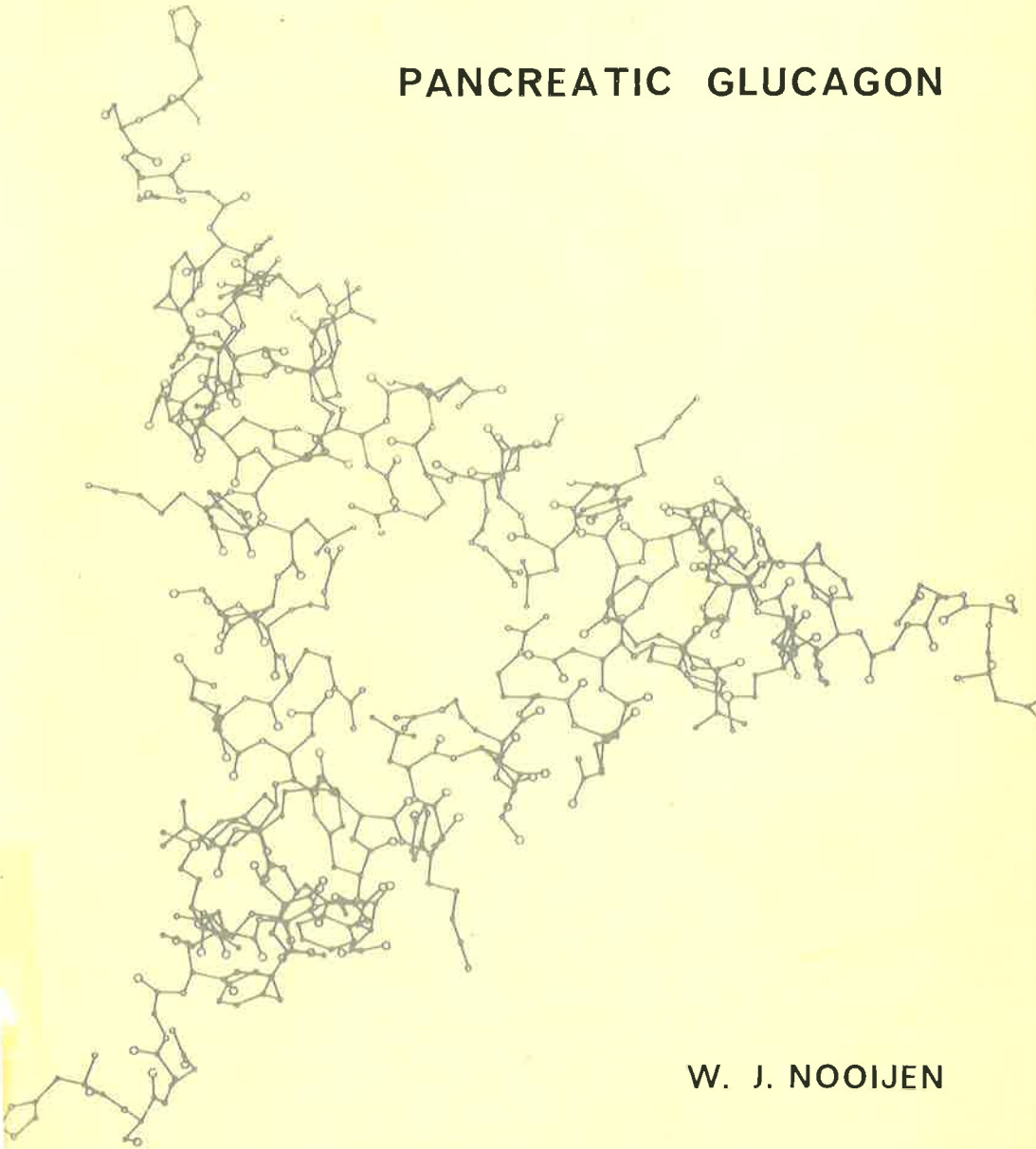
RADIOIMMUNOASSAY

OF

PANCREATIC GLUCAGON



W. J. NOOIJEN

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RADIOIMMUNOASSAY OF PANCREATIC GLUCAGON

S T E L L I N G E N

behorende bij de dissertatie van

W.J. Nooijen

I

De resultaten van Budzynski en Marder, verkregen met het abnormale fibrinogeen Paris I, kunnen even goed verklaard worden met een verschil in affiniteit van de γ-keten voor Ca-ionen als met de door hun geponeerde verschillen in cross-linking.

A. Budzynski and V. Marder: J. Lab. Clin. Med. 88(5): 817, 1976.

II

De interpretatie, die Lawrie en Kemp geven aan de resultaten van hun electroforese-experimenten met fibrinogeen, is niet de meest aannemelijke.

N.S. Lawrie and G. Kemp: Biochim. Biophys. Acta 577: 415, 1979.

III

De validiteit van de bepaling van de ratio apolipoproteïne E_2/apolipoproteïne E_3 volgens de methode van Utermann et al., is discutabel.

G. Utermann, G. Albrecht and A. Steinmetz: Clinical Genetics 14: 351, 1978.

IV

De bepaling van de ratio HDL_2/HDL_3, zoals beschreven door Terebus-Kekish et al., is zeer twijfelachtig.

O. Terebus-Kekish, M. Barclay and C. Chester Stock: Clin. Chim. Acta 88: 9, 1978.

V

Hoewel Ozaki et al. voor de vorming van α-gefunctionaliseerde aminozuurderivaten met α-acetoxy aminozuurderivaten een eliminatie-additie mechanisme postuleren, lijkt een Sn^2 mechanisme waarschijnlijker.

Y. Ozaki, T. Iwasaki, H. Horikawa, M. Miyoshi and K. Matsumoto: J. Org. Chem. 44: 391, 1979.

VI

De door Snatzke en Snatzke opgestelde "planar rule" ter bepaling van de absolute configuratie van sulfoxides met behulp van circulair dichroïsme is een te ver gaande extrapolatie van de experimenteel, door Mislow, gevonden regel.

G. Snatzke en F. Snatzke: Fundamental aspect and recent development in optical rotatory dispersion and circular dichroism. Editors: F. Cardelly and P. Salvadory. Publisher: Heyden & Son Ltd., 1973, pp 186.

VII

Het carnitine tekort in dwarsgestreept spierweefsel bij patiënten met intermitterende hemodialyse moet eerder op het nierlijden dan op de dialyse teruggevoerd worden.

T. Böhmer, H. Bergrem, K. Eiklid: The Lancet 126, 1978 I.

VIII

Bij een tijdschrift dat een grote tijdsmarge heeft tussen het moment van aannemen en van in druk brengen van manuscripten, zouden deze kort voor publicatie nogmaals op hun inhoud bekeken moeten worden.

W.A. Andes: Thromb. Haemostas. 39: 437, 1978.

IX

Zolang een patiënt die ernstig ziek is alleen toegesproken wordt door juristen, planologen en agogen, is het overleven een kwestie van de goden. Nu ons ecosysteem ziek is en vele belangrijke beslissingen door juristen van de kroon genomen worden, wordt de kans van het overleven kleiner. Door biologen en ecologen in de Raad van State op te nemen krijgen ecologische overwegingen een kans en is het misschien mogelijk belangrijke natuurgebieden te behouden.

X

Antisera af konijnen, zijn vaak bij de konijnen af.

14 november 1979

RADIOIMMUNOASSAY OF PANCREATIC GLUCAGON

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A.A.H. KASSENAAR, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 14 NOVEMBER 1979 TE KLOKKE 14.15 UUR

DOOR

WILHELMUS JACOBUS NOOIJEN

GEBOREN TE DELFT IN 1947

1979
ELVE/LABOR VINCIT LEIDEN

PROMOTOR: PROF. DR. A.A.H. KASSENAAR
CO-REFERENTEN: DR. IR. L.W. HESSEL
DR. W.H.L. HACKENG

Dit proefschrift werd bewerkt in het Gaubius Instituut van de Gezondheidsorganisatie TNO te Leiden.

Manuscript: Mevr. C. Horsting-Been
Tekeningen: De heer J.J. Nieboer

to my wife Carla
my son Jasper
and my parents

C O N T E N T S

GENERAL INTRODUCTION

Of the numerous in vitro methods available for estimating the concentrations of antigen or antibody in biological fluids no single method of measurement has been adopted and applied so rapidly as the radioimmunoassay (RIA) procedure. Among the first to employ RIA procedures were Berson et al. (1956) and Farr (1958). Since these early studies there has been an increasing use of RIA for the detection of antibodies and a variety of antigens.

RIA has also found increasing use in the fields of immunology, enzymology, microbiology, oncology and pharmacy. During the past decade, observations based on RIA which have demonstrated that many, if not all, peptide hormones are found in more than one form in plasma have certainly introduced complications in the interpretation of hormonal concentrations as measured by RIA. These forms may or may not have biologic activity and may represent either precursors or metabolic products of the well-known, well-characterized, biologically active peptide hormone. The measurement of glucagon concentrations in plasma is complicated by the heterogeneity of glucagon-like immunoreactivity (GLI; Table below) in plasma, which entails special difficulties in the interpretation of radioimmunoassay data. Furthermore, it is complicated by the presence of glucagon-degrading enzymes and the presence of various serum proteins and substances which interfere nonspecifically in the assay.

In this thesis some of the problems and concepts are presented which are related to the development of a radioimmunoassay of pancreatic glucagon. We have introduced a specific derivatization of glucagon for raising specific anti-glucagon antisera and special procedures for diminishing the non-specific effect. These methods might be applied to other radioimmunoassay systems.

The nomenclature and the abbreviations to be found in this thesis, and their definitions, are tabulated below.

Terminology, abbreviations and definitions

Name or abbreviation	Synonyms	Definition
specific anti-glucagon antiserum		Serum which contains antibodies specifically directed against the C-terminal sequence of glucagon (residue 19-29)
non-specific anti-glucagon antiserum		Serum containing antibodies directed against the N-terminal sequence (residue 1-12) and/or mid-portion (residue 13-18) of glucagon
glucagon	pancreatic glucagon	29-Amino acid peptide, originally extracted from the pancreas, MW 3,485 with the following sequence H-His-Ser-Gln--Gly-Thr-Phe-Thr-Ser-Asp-Tyr--Ser-Lys-Tyr-Leu-Asp-Ser-Arg--Arg-Ala-Gln-Asp-Phe-Val-Gln--Trp-Leu-Met-Asn-Thr-OH
GLI	glucagon-like immunoreactivity	Any peptide, regardless of origin, which is bound by non-specific antibodies raised against glucagon. Glucagon is included in this group.
PTG	pancreatic-type glucagon	Peptides, including glucagon, which will bind to specific antibodies raised against glucagon. The PTG's behave immunologically like glucagon. They are, except for glucagon, of extrapancreatic origin and indistinguishable from glucagon by varying methods of physicochemical characterization.
GTG	gut-type glucagon, gut-glucagon, gut GLI	Peptides, which do not bind to the specific glucagon antisera, but only to non-specific antisera. They are from gastrointestinal origin. The GTG's behave immunologically like GLI-1.

Name or abbreviation	Synonymes	Definition
IRG	immuno-reactive glucagon	Peptides which will bind to specific glucagon antisera. The various molecular weight components of IRG are: big plasma glucagon (MW > 20,000), proglucagon (MW ± 9,000), glucagon and small glucagon (MW ± 2,000).
GLI-1	glicentine, gut GLI-1	A highly purified porcine GTG component, MW 11,625. The amino acid composition is known.

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CHAPTER 1

INTRODUCTION

1.1 THE IMPORTANCE OF PANCREATIC GLUCAGON

Pancreatic glucagon is a 29-amino acid straight chain peptide (Bromer et al., 1957). It is secreted by the A-cells in the pancreatic islets (Baum et al., 1962). Together with insulin, secreted by the B-cells in the pancreatic islets, it plays a central role in the blood glucose homeostasis (Alford et al., 1974). The insulin:glucagon ratio is an important determinant of the anabolic-catabolic setting of the organism (Unger, 1971).

Pancreatic glucagon secretion is stimulated by many factors such as amino acids, especially alanine (Muller et al., 1971), the gut hormones vasoactive intestinal polypeptide (VIP), gastric inhibiting polypeptide (GIP) and gastrin (Bloom et al., 1976). Its secretion is inhibited by glucose (Unger et al., 1962), fatty acids and ketone bodies (Gerich, 1976), somatostatin (Koerker et al., 1974) and secretin.

The major target organ of circulating pancreatic glucagon in mammals is the liver, where it stimulates glycogenolysis (Sutherland et al., 1951) and gluconeogenesis (Felig et al., 1976a); both effects are mediated by cyclic AMP. Pancreatic glucagon also facilitates the extraction of amino acids from the blood, especially alanine, by the liver (Mallette et al., 1969).

Pancreatic glucagon increases the ketogenic capacity of the liver through activation of the hepatic acylcarnitine transferase enzyme system (McGarry et al., 1976). A low level of hepatic glycogen and a high level of carnitine are required (McGarry et al., 1975). Pancreatic glucagon inhibits the secretion of very low density lipoprotein (VLDL) by the liver (Heimberg et al. 1969).

Under appropriate conditions, physiologic levels of pancreatic glucagon can stimulate lipolysis in adipose tissue (Bjorntorp et al., 1969). The influence of pancreatic glucagon on proteolysis in muscle under physiologic conditions is doubtful.

After the discovery of somatostatin (Koerker et al., 1974), a tetradecapeptide secreted by the D-cells in the pancreatic islets (Orci et al., 1975) the relative importance of pancreatic glucagon could be established (Alberti et al., 1973; Koerker et al., 1974).

Although the importance of pancreatic glucagon may have been overstressed in the past, its role as one of the catabolic hormones in short-term regulation is now well established (Felig et al., 1976b; Bombay et al., 1977).

1.2 ASSAYS FOR PANCREATIC GLUCAGON

A. Bioassay

Pancreatic glucagon can be determined by measuring its biological activity. The major advantage of a bioassay is that it distinguishes between the active hormone and the inactive precursors or degradation products.

Several bioassays for glucagon have been developed, based on the ability of the hormone to provoke hyperglycemia in vivo or to stimulate glucose production, phosphorylase activity or adenylate cyclase activity in vitro (for a review, see Luyckx and Lefebvre, 1970). Most studies in vitro have been done with liver cells. There are a few exceptions, e.g. with isolated fat cells from chicken adipose tissue (Langslow et al., 1970).

The major disadvantages of a bioassay are that it is not specific, because other substances which are not glucagon-like

in chemical structure may possess strong glucagon-like bioactivity (e.g. the cathecholamines), it is very insensitive, it is not very accurate, it is expensive and it requires considerable technical experience.

B. Competitive protein binding assays

1. Radioreceptorassay

It is assumed that the first step in the action of pancreatic glucagon is binding to a specific cell plasma membrane receptor site. It is possible to use the displacement of labeled glucagon as an assay for pancreatic glucagon.
The radioreceptorassay has been proved to be sensitive and simple to perform.
A disadvantage is its lack of specificity for pancreatic glucagon. Gut glucagons and pancreatic glucagon share a common binding site (Bataille et al., 1973).

2. Immunoassays

a. Enzyme-immunoassay

The enzyme-immunoassay is a method in which the binding of enzyme-labeled antigen or antibody to its immune reactant is measured by its reaction with a substrate.
The enzyme-immunoassay technique is further identical to the radioimmunoassay technique described below. The enzyme-immunoassay is simple and fast and the labeled compound is very stable. However, the sensitivity is too low to measure the small quantities of pancreatic glucagon present in plasma.

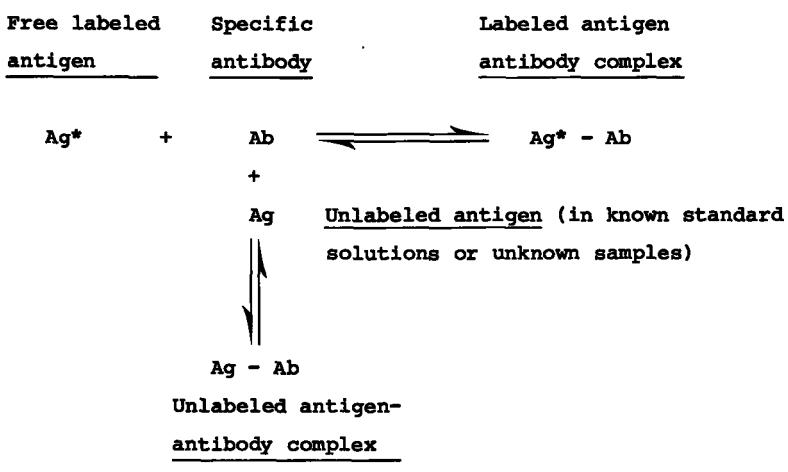
b. Radioimmunoassay

The major advantages of radioimmunoassay are the capacity to label most antigens with a radioactive isotope usually without unduly altering them; the possibility to

measure samples with a high sensitivity and accuracy and a low détection limit (dependent on the antiserum) and to count these samples with automatic equipment.

Disadvantages are the rather short half life of iodine--125 which is the most suitable radioactive isotope, the radiation damage which may affect the immunochemical activity of the labeled compound, the restrictions of its use to a highly specialized laboratory and the non--specificity with respect to inactive precursors or degradation products of pancreatic glucagon.

The basis of the radioimmunoassay method is the competitive inhibition by unlabeled hormone of binding of labeled hormone to its specific antibody (Yalow and Berson, 1959).



As the concentration of unlabeled antigen (Ag) is increased, the formation of labeled antigen-antibody

complexes are diminished because of the formation of more unlabeled antigen antibody complexes.
The concentration of antigen in an unknown sample is calculated from the degree of binding of labeled antigen in the sample, by comparison with that in standard solutions, containing known amounts of added antigen.

1.3 *PROBLEMS IN ESTIMATING PANCREATIC GLUCAGON IN PLASMA BY RADIO-IMMUNOASSAY*

During the past twenty years lower and lower "normal" values of pancreatic glucagon in plasma have been reported i.e. from 300-400 pg/ml (Unger et al., 1959) to 40-50 pg/ml (Alford et al., 1977). This downward tendency is probably due to a decreasing influence of cross-reacting proteins or interference factors, and this is due to an increased specificity of the used antiserum. Indeed there are several factors which may completely invalidate the radioimmunological determination of pancreatic glucagon in plasma and other biological fluids. These factors make the development of a radio immunoassay of sufficient sensitivity and specificity for pancreatic glucagon in plasma considerably more difficult than e.g. for insulin.
There are nine problems to be considered:

1. Sensitivity
   Fasting levels are probably in the order of 20-40 pg/ml. This requires an assay of very high sensitivity.
2. Low immunogenicity of glucagon
   Pancreatic glucagon is a poor immunogen by itself. Since its immunogenicity has to be increased by modification of the molecule there is an increased risk of cross-reaction with protein in plasma.
3. Specificity
   It was shown for the first time by Unger et al. (1961) that the human stomach, duodenum and small bowel possess glucagon-like

immunoreactivity (GLI). The presence of gut glucagon-like immunoreactivity in blood was suggested by Samols (1966) and confirmed by Unger et al. (1968). There are also other sources of GLI materials in humans, i.e. in the submaxillary gland (Lawrence et al., 1976). It is evident that an anti-pancreatic glucagon antiserum should not cross-react with gut GLI, neither with other GLI materials except for pancreatic glucagon itself.

4. Glassware adsorption of pancreatic glucagon
   The adsorption of pancreatic glucagon in buffer systems to glass is extremely strong. The solution of this problem by adding protein might result in partial proteolysis (Hazzard et al., 1968).
5. Radiation damage
   The short half life and the high energy of iodine-125 cause radiation damage, which may affect the immunochemical activity of the labeled hormone.
6. ^{125}I-glucagon damage in incubation medium
   During an incubation of ^{125}I-glucagon in plasma some proteolytic degradation occurs and this varies from plasma to plasma. This degradation of ^{125}I-glucagon in plasma is not prevented entirely by the addition of aprotinin or benzamidine.
7. Interference factors in plasma
   Even with the most specific antisera available some plasma effects may persist, i.e. from gamma-globulins (Unger et al., 1968; von Schenck, 1977).
8. Degradation of pancreatic glucagon and other proteins in blood
   As it is possible that part of the interference originates from the degradation of proteins by proteolytic enzymes present in blood, special care must be given to blood sampling and storage.
9. Errors in separating "free" from "bound" hormone activity
   Achievement of a satisfactory and reproducible separation between "free" and "bound" hormones depends on a careful proportioning of the concentration of the individual molecules in the reaction mixture.

Thus, it is clear that in order to determine only pancreatic glucagon in plasma and biological fluids, the assay should be corrected for any remaining interference factor or known sources of error.

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CHAPTER 2

PRODUCTION AND SCREENING OF ANTI-GLUCAGON ANTISERA

2.1 METHODS FOR PRODUCING ANTIBODIES

2.1.1 Production of antibodies in animals whose glucagon is different from man

Human, porcine, bovine rabbit, rat and camel glucagon for instance have identical sequences. The sequence of chicken and turkey glucagon is different from man. Markussen et al. (1972) described the sequence of turkey glucagon.
Turkey glucagon has the same number of residues as human glucagon, but the asparagine residue, located at position 28 in human glucagon, is replaced by a serine residue in turkey glucagon.
This difference in primary structure changes its immunological properties considerably. Turkey glucagon reacts strongly with a non-specific anti-glucagon antiserum (antibodies directed against the N-terminal part of glucagon), but reacts weakly with a pancreas specific anti-glucagon antiserum (antibodies directed against the C-terminal part of glucagon).
Therefore human or bovine glucagon might be immunogenic in the turkey.

2.1.2 Coupling methods

Pancreatic glucagon like most other small peptides is, by itself, a poor immunogen. The problem of generating antisera against pancreatic glucagon may be overcome by the use of larger molecules covalently coupled to pancreatic glucagon or by forming glucagon-polymers (Table 1).
In general, antisera raised against glucagon conjugates have a higher titre than antisera raised to glucagon itself. However,

all these approaches do not guarantee antibody specificity, i.e. the ability of the antibody to distinguish between pancreatic glucagon and glucagon-like immunoreactive materials. Almost all antisera to pancreatic glucagon cross-react with gastrointestinal extracts.

Table 1 Treatments of glucagon for the production of anti-glucagon antisera

1. Glucagon emulsified in adjuvant	Unger et al., 1959 Schopman et al., 1967
2. Glucagon-bovine serum albumin	Assan et al., 1965 Tager, 1976 Holst et al., 1974
3. Glucagon-polyvinylpyrrolidone	Assan et al., 1965
4. Alum-precipitated mixture of glucagon and egg albumin	Probst and Colwell, 1966 Heding, 1969
5. Glucagon-homopolymer	Heding, 1969 McEvoy et al., 1977
6. Glucagon-hemocyanin	Grey et al., 1970
7. Glucagon-poly-L-lysine	Cuatrecasas and Illiano, 1971
8. Glucagon-keyhole limpet hemocyanin	McEvoy et al., 1977
9. Glucagon-poly-γ-D-glutamic acid	
10. Glucagon-thyroglobulin	Sperling et al., 1974 Schenck, 1977

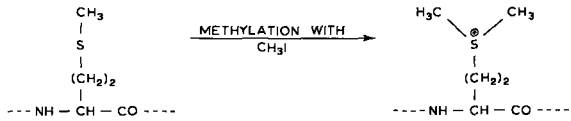
2.1.3 Methods utilizing derivatives of pancreatic glucagon

As antisera specific for pancreatic glucagon should be directed against the C-terminal part of the molecule (Assan and Slusher, 1972), we reasoned that the preparation of 27-S-methylglucagon might give us a tool for developing a specific glucagon assay.

We considered the possibility that the introduction of a bulky methyl group together with a change of the S-atom would make the C-terminal part of the molecule foreign to the immunological recognition system and enhance the triggering of antibody production; moreover, these antibodies would be raised against the C-terminal rather than against the unchanged N-terminal region of the molecule. We expected that glucagon in serum samples would have to be converted to the methyl-derivative in order to obtain sufficient sensitivity for a radioimmunoassay. As the oxidation of methionine to methionine-sulfoxide is described as easy (Shechter et al., 1975), we also tried to modify glucagon to glucagon-methionine-sulfoxide.

A. Methylation of the methionine residue in glucagon

Methylation changes the methionine residue from an uncharged hydrophobic residue into a hydrophilic sulfonium salt.



Preparation and purification of 27-S-methylglucagon

Methylation was performed with magnetic stirring at 22°C within a dark box. Glucagon was dissolved in 0.5 M phosphate (1 mg per ml), then 2 M urea was added. The pH of the solution was adjusted to 3.5 with 1 N phosphoric acid.
To this solution was added CH_3I (about 0.1 M). The biphasic reaction mixture was stirred for 24 hours.

Methylation of glucagon was followed by a titrimetric method and by a chromatographic method.
In the titrimetric method, portions of the reaction mixture

were pipetted into a 10-fold or greater excess of 50% acetic acid. After a few drops of erythrosin (a fluorescent indicator) had been added, iodide was titrated with 0.01 M $AgNO_3$. A stock solution of $AgNO_3$ was stored in the dark and titrated daily against freshly made solutions of KI. The reaction can be followed by determining the rate of appearance of iodide ion. The methylation of glucagon could also be determined by chromatography, because methylation of a methionine residue increases the net positive charge of the protein and accelerates its elution from an anionic exchange resin or retards its elution from a cationic exchange resin. The reaction mixture was fractionated on a DEAE-Sephadex A 25 anionic exchange resin (350 x 10 mm) at 22°C.

The rate of methylation of glucagon was constant between pH 2.0 and pH 4.5. At higher, including physiological, pH values the rate was much slower, probably because of the low solubility of glucagon in this pH-range.

Administration of 2 M urea to the reaction mixture prevents precipitation of glucagon during the reaction but it has no effect on the reaction rate between pH 2.0 and pH 4.5. The methylation of methionine is pH-independent, while that of histidine and lysine is pH-dependent. Because the rate is dependent on the concentration of the unprotonated nucleophile to inhibit any reaction of these residues, the reaction was carried out at pH 3.5 and in the presence of 0.05 M phosphate (Crestfield et al., 1963).

Since protons are neither consumed nor liberated in the reaction of CH_3I with thioether sulfur, no buffer is needed to keep the pH constant.

The reaction of the methionine residue of glucagon with CH_3I in 0.05 M phosphate (pH 3.5) and 2 M urea at 22°C is shown in Fig. 1.

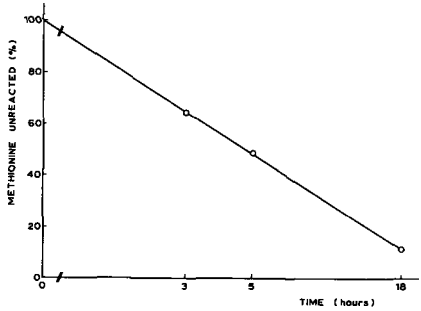


Fig. 1.
Methylation of the methionine residue of glucagon with CH_3I as a function of time.

At the end of the reaction the mixture, containing up to 10 mg protein, was placed on a BioGel P-2 column (16 x 100 mm) and eluted with 0.07 M NH_4HCO_3 (pH 7.5). The column was monitored at 280 nm; 1.5 ml fractions which contained protein were pooled and lyophilized. The desalted 27-S-methylglucagon was then purified on a DEAE-Sephadex A 25 column (10 x 350 mm) and also monitored at 280 nm. The column was eluted with a linear gradient from 0.01 M NH_4HCO_3 (pH 7.5) to 1.0 M NH_4HCO_3 (pH 7.5) (Fig. 2).

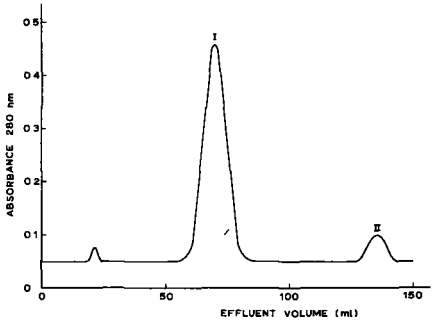


Fig. 2.
Elution profile for the purification of 27-S-methylglucagon (I) on a DEAE-Sephadex A 25 column from unreacted glucagon (II).

Characterization of 27-S-methylglucagon

Because it might be possible that the 27-S-methylglucagon fractions from the DEAE-Sephadex A 25 column contain small quantities of glucagon with a methylated histidine or methylated lysine or of an association product between native and 27-S-methylglucagon, we checked the absolute purity of 27-S--methylglucagon as follows:

a) Polyacrylamide gel electrophoresis

Electrophoresis was conducted in 20% polyacrylamide gels at pH 8.3 using the method given by Ornstein and Davies (1964).

Samples were prepared by dissolving them at 1 mg/ml in 8 M urea with ^{125}I-glucagon (15,000 cpm) as reference. A maximum of 100 μl was put onto each gel (6 x 75 mm). The loading buffer contained 200 mM glycine and 50 mM Tris (hydroxymethyl) methylamine at pH 8.3.

Gels were fixed and stained in 0.25% Coomassie Brilliant Blue, 45% methanol, 9% acetic acid and destained in 25% methanol and 12.5% acetic acid. The running time was 2.5 hours at 2 mA per gel.

For the determination of radioactivity, gels were sliced into 3 mm sections and these sections were counted with a gamma scintillation counter (Packard, model 5110) (Fig. 3).

b) Amino acid analysis

Amino acid analysis was performed by the method of Spackman et al. (1958) on a Technicon NCL, equipped with a 650 mm column of C2 resin (Technicon).

S-methylmethionine cannot be recovered quantitatively after HCl hydrolysis. Lavine et al. (1954) demonstrated that HCl hydrolysis causes a 50% conversion of S-methylmethionine sulfonium bromide to methionine in 5 hours.

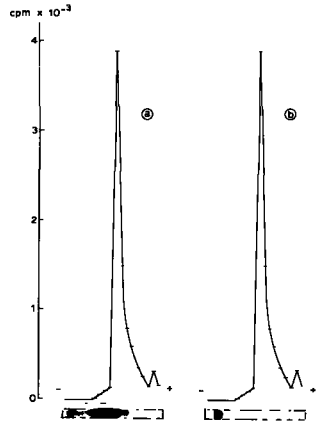


Fig. 3.
Patterns of native glucagon with ^{125}I-glucagon (a) and of 27-S-methylglucagon with ^{125}I-glucagon (b) after polyacrylamide gel electrophoresis. Staining patterns with Coomassie Brilliant Blue (below) and distribution of radioactivity (top).

Link and Stark (1968) also reported a conversion to homoserine (lactone) from S-methylmethionine sulfonium after acid hydrolysis.

Unmodified and modified methionine in glucagon preparations were determined as follows: the methylated protein was subjected to performic acid oxidation. In this way methionine residues are converted to methionine sulfone, while the S-methylmethionine sulfonium remains intact (Hirs et al., 1953).

The oxidation product was hydrolysed in 6 N HCl at 110°C for 22 hours. Methionine sulfone represents the methionine content of the original sample, while methionine, S-methyl methionine and possible homoserine (lactone) represent its S-methylmethionine sulfonium content.

Native glucagon was hydrolysed directly in 6 N HCl at 110°C for 22 hours. Table 2 presents the amino acid composition of native glucagon and purified 27-S-methylglucagon.

No homoserine (lactone) or methionine sulfone could be detected in the 27-S-methylglucagon hydrolysate. Detec-

Table 2. Amino acid composition of native glucagon and 27-S-methylglucagon

Amino acid	Number of amino acid residues per molecule			
	Native glucagon		27-S-methylglucagon	
	theoretical	experimental	theoretical	experimental
Aspartic acid	4.0	3.9	4.0	3.8
Threonine	3.0	3.0	3.0	3.0
Serine	4.0	4.1	4.0	3.9
Glutamic acid	3.0	2.8	3.0	2.6
Glycine	1.0	1.2	1.0	1.1
Alanine	1.0	1.2	1.0	1.0
Valine	1.0	1.0	1.0	1.0
Methionine	1.0	0.9	-	0.4
Leucine	2.0	2.2	2.0	2.1
Tyrosine	2.0	2.1	2.0	1.8
Phenylalanine	2.0	2.0	2.0	2.0
Lysine	1.0	1.0	1.0	1.0
Histidine	1.0	1.1	1.0	0.9
Arginine	2.0	1.9	2.0	1.8
S-methylmethionine	-	-	1.0	0.7
Homoserine (lactone)	-	-	-	-
Tryptophan	1.0	n.d.*	1.0	n.d.*
Methionine sulfone	-	-	-	-

*n.d.: not determined.

tion limit was about 2%. The overall yield of 27-S-methylglucagon after its purification was more than 75%.

c) Biological activity

Isolated parenchymal cells were obtained from the liver of normally fed rats, essentially according to the procedure of Seglen (1976). The cells were finally suspended in Krebs Henseleit HEPES (pH 7.49 to approximately 8 mg dry weight/ml.

Incubation tubes (polystyrene) contained 0.5 mg cell suspension, 0.1 ml glucagon (or 27-S-methylglucagon) dilution (in Krebs Henseleit HEPES, containing 1 mg/ml bacitracine) and 0.4 ml Krebs Henseleit HEPES and were filled with O_2 before capping.

Incubation was carried out in a Dubnoff shaker at 37°C for 30 minutes and stopped by addition of 0.5 ml 1.2 M $HClO_4$. After centrifugation, glucose was determined in 50 μl samples supernatant with a coupled glucose oxidase/peroxidase method (GOD-Perid kit from Behringer) according to Bergmeyer and Bernt (1970).

The dose-response characteristics of native glucagon and 27-S-methylglucagon are shown in Fig. 4. Glucose production during incubation was calculated by correcting for the glucose content in the extracts of cells, which are mixed with $HClO_4$ at t = 0.

For each experiment, glucose production in the individual incubations was expressed as a percent of the glucose production in the incubations with 10^{-6} M native glucagon. The data in Fig. 4 represent mean value (±SD) of these percent values for three separate experiments. Fig. 4 shows that the maximum level of stimulation with native glucagon is about 2.5 times the basal glucose production. The S-methyl derivative seems to be able to reach this level and the lowest concentration that causes a clear effect is 10^{-8} M for the S-methyl deriva-

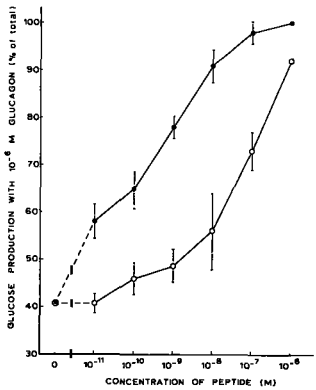


Fig. 4.
Effect of native glucagon (●) and 27-S-methylglucagon (O) on glucose production by isolated hepatocytes of fed rats (n=3; mean ± SD).

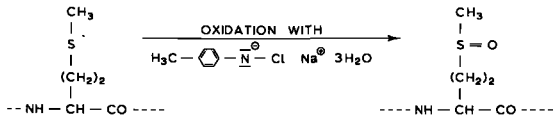
tive. Glucagon already stimulates glucose production significantly at 10^{-11} M. Although it cannot be ruled out entirely that the observed stimulation is due to traces of native glucagon in the 27-S-methylglucagon sample, this would seem extremely unlikely.

Firstly, glucagon is very well separated on a DEAE-Sephadex A-25 column (Fig. 2) and secondly, further purification of 27-S-methylglucagon did not influence the biological activity. It might be concluded from these results that a modification in the C-terminal part probably reduces the affinity of the glucagon molecule to its receptor but does not interfere with its maximum glucose mobilizing activity.

From the above data, we may conclude that methylation of glucagon with CH_3I, under the proper conditions, is a convenient and specific method for modifying glucagon at the methionine residue.

B. Oxidation of the methionine residue in glucagon

Oxidation with chloramine-T changes the methionine residue to a methionine-sulfoxide residue.



Preparation and purification of glucagon-methionine-sulfoxide

We oxidized glucagon according to the method of Shechter et al. (1975) under alkaline conditions. To a stirred solution of glucagon (2 mg glucagon in 1 ml Tris-HCl buffer, pH 8.5) an excess (2.0 equivalent) of chloramine-T was added from a solution of chloramine-T (approximately 10 μmoles/ml aqua dest).

After 30 minutes glucagon was purified by elution from a Sephadex G-25 column (20 x 250 mm) with 0.07 M NH_4HCO_3 (pH 7.5). The eluate was monitored at 280 nm, 1.5 ml fractions which contained protein were pooled and lyophilized.

Characterization of glucagon-methionine-sulfoxide

a. Amino acid analysis

Amino acid analysis was performed by the method of Spackman et al. (1958), already described with the methylation of glucagon. The completeness of the conversion to the sulfoxide was tested as follows: about 2 mg of the modified glucagon was treated with 0.3 ml 70% formic acid with 5 mg CNBr for 24 hours. Under these conditions the methionine residues are quantitatively converted to homoserine and its lactone while the methionine-sulfoxide

residues remain intact. The sample was evaporated and hydrolysed in 6 N HCl at 110°C for 22 hours in the presence of about 2 mg dithioerythritol. Under these acid hydrolysis conditions, methionine sulfoxide is converted back to methionine and the amount of methionine obtained on amino acid analysis is actually the amount of methionine sulfoxide.

A blank experiment with the non-modified protein was run under the same conditions.

A second amino analysis method was performed to determine methionine, methionine-sulfoxide and tryptophan directly. For this purpose, samples were hydrolyzed in 3.75 N sodium hydroxide at 110°C for 22 hours.

However, alkaline hydrolysis was found to have the following shortcomings:

1st Methionine-sulfoxide could not be recovered quantitatively. Losses of 10-15% during alkaline hydrolysis were seen from a conversion of methionine-sulfoxide to methionine.

2nd Tryptophan, which is stable to alkaline hydrolysis, could not be determined, since it was found to have the same retention volume as ornithine (a product which is formed from arginine during alkaline hydrolysis).

3rd After alkaline hydrolysis, the solution has to be acidified to pH 1.5 with a few drops of concentrated hydrochloric acid before amino acid analysis. This resulted in an incomplete separation of aspartic acid, threonine and serine.

Table 3 presents the amino acid composition of native glucagon and glucagon-methionine-sulfoxide, both treated with formic acid and CNBr.

In conclusion, we have demonstrated that with the blank experiment the CNBr cleavage yields are between 80-90%;

Table 3. Amino acid composition of native glucagon and glucagon-methionine-sulfoxide

Amino acid	Number of amino acid residues per molecule			
	Native glucagon		Glucagon-methionine-sulfoxide	
	theoretical	experimental	theoretical	experimental
Aspartic acid	4.0	3.9	4.0	3.6
Threonine	3.0	3.0	3.0	3.2
Serine	4.0	4.0	4.0	3.7
Glutamic acid	3.0	2.9	3.0	2.9
Glycine	1.0	1.1	1.0	1.0
Alanine	1.0	1.1	1.0	1.1
Valine	1.0	0.9	1.0	0.9
Methionine	-	0.1	1.0	0.9
Methionine-sulfoxide	-	-	-	0.1
Homoserine (lactone)	1.0	0.8	-	0.1*
Leucine	2.0	1.9	2.0	1.9
Tyrosine	2.0	2.1	2.0	2.0
Phenylalanine	2.0	2.1	2.0	2.0
Lysine	1.0	1.0	1.0	1.1
Histidine	1.0	1.0	1.0	1.1
Arginine	2.0	1.9	2.0	1.9
Tryptophan	1.0	n.d.**	1.0	n.d.**

* = value was rounded upwards
**n.d. = not determined

that with the oxidized protein, the conversion of methionine-sulfoxide to methionine is more than 90% and that the content of methionine-sulfoxide in the original sample of the oxidized protein is at least 90%.

b. Spectrophotometric measurements

Besides methionine, tryptophan is very sensitive to oxidation. The extent of oxidation of tryptophan, was ascertained spectrophotometrically.

We performed these measurements with a Pye Unicam SP6-500 spectrophotometer. Quartz cells of 10 mm light path were used. The extinction was measured at 290 nm. The following results were obtained.

Sample	Concentration (μM)	E_{290}	ε (litre/moles.cm)
Native glucagon	597	0.949	1.59×10^3
Oxidized glucagon	306	0.502	1.64×10^3

From these data it is evident that under alkaline conditions chloramine-T does not modify tryptophan.

c. Biological activity

The same method and calculations have been applied as described for the glucose production with 27-S-methylglucagon.

In Fig. 5 the dose-response characteristics of native glucagon and glucagon-methionine-sulfoxide are shown.

The maximum level of stimulation is about 2.5 times the basal glucose production and is equal for glucagon and the sulfoxide. The lowest concentration that causes a clear effect on glucose production is 10^{-9} M for the sulfoxide derivative.

The same conclusion can be drawn with respect to the biological activity for glucagon-methionine-sulfoxide as for 27-S-methylglucagon.

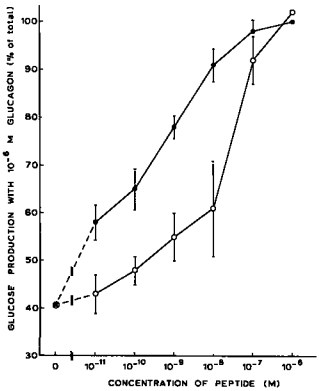


Fig. 5. Effect of native glucagon (●) and glucagon-methionine-sulfoxide (○) on glucose production by isolated hepatocytes of fed rats (n = 3; mean ± S.D.)

Essentially the same results were obtained when glucagon--methionine-sulfoxide was prepared by either an equivalent amount or a 10-fold excess of oxidizing agent indicating that the stimulation was not due to a residual contamination with unmodified glucagon.

2.2 IMMUNIZATION OF MAMMALS

2.2.1 Choice of animal

It is well-known that immunogenicity not only depends on the characteristics of the injected compound but also on the qualities of the animal species.

Species used for immunization are usually rabbits and guinea--pigs. The advantages of rabbits are that 30 to 40 ml blood can be taken every month, not by cardic puncture, which is very risky, but from an ear vein and that they have a strong responsiveness to an injected compound. Albinos are especially good anti-

body producers for unknown reasons (Heinemann and Fedulin, 1970).

From several rabbit strains, we finally selected the New Zealand white rabbit, which is an albino. This strain proved to be the best antibody producer against glucagon derivatives. We used them for the production of antibodies between the age of 4 months and 24 months.

Consideration of the aspects mentioned in chapter 2.1, led us to immunize 4 turkeys with native porcine glucagon and 19 rabbits with glucagon derivatives. The results are presented in Table 4, chapter 2.3.2.

2.2.2 Immunization procedures

Schedule:

1. A suspension was made of native porcine glucagon (or its derivatives) in phosphate-buffered saline (PBS; pH 7.3; 1 mg/dl).
2. An emulsion was made of 1 ml PBS containing glucagon (or its derivatives) and 1 ml Freund's complete adjuvant for each animal, by joining one syringe containing the water-phase and another syringe containing adjuvant with a connector, passing the content of one syringe to the other and the other way round, at least 20 times.
3. Primary injections were given at intramuscular and/or subcutaneous sites. In each site 0.5 ml PBS-adjuvant emulsion was injected.
4. Booster injections were given with 1 ml PBS containing 0.5 mg glucagon (or its derivatives) and 1 ml Freund's incomplete adjuvant, every 4 weeks.
5. Ten days after each booster injection, 30-40 ml of blood were taken from an ear vein.

2.3 ANTI-GLUCAGON ANTISERA

2.3.1 Immunohistochemical characterization

Human pancreas tissues were collected at the time of autopsy and fixed immediately in 10% phosphate-buffered neutral formaldehyde. After fixation, the pancreas was dehydrated and embedded in paraffin and studied immunohistochemically (Fig. 6). Immunohistochemical studies were carried out by the indirect method of Nakane and Pierce (1967).

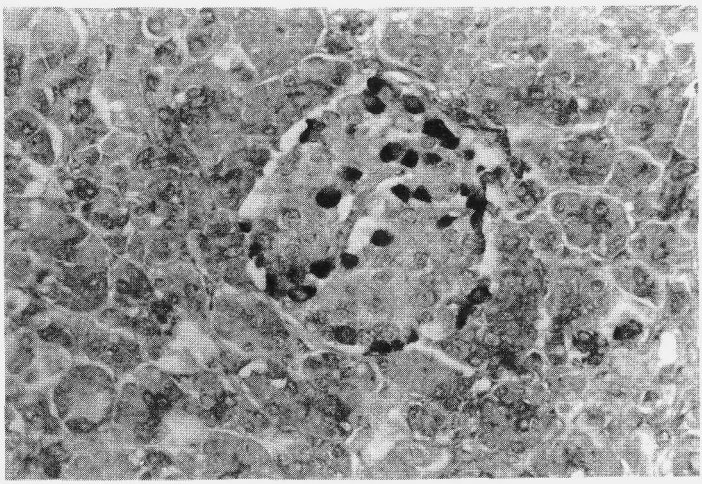


Fig. 6.
A pancreatic islet, treated with antiserum raised against 27-S-methylglucagon with stained pancreatic A-cells.

Horse-radish peroxidase (Sigma Chemical Company) was conjugated to goat anti-rabbit gammaglobulin (Nordic) by the method of Avrameas and Ternynck (1971). Four micron paraffin sections of human pancreas tissue were first treated with rabbit anti-glucagon antiserum K 1711 for 30 minutes, washed in phosphate-buffered saline (pH 7.4) and incubated with the peroxidase-labeled goat anti-rabbit gammaglobulins for 30 minutes.

Peroxidase was revealed by 0.05% 3-3'-diaminobenzidine tetrahydrochloride (DAB, Aldrich) and 0.001% hydrogen peroxide by the method of Graham and Karnovsky (1966). Specificity controls on the immunohistochemical preparation included non-immune rabbit anti-porcine ACTH antiserum.

Human tissues of the gastrointestinal tract were examined also with the immunoperoxidase technique, using anti-glucagon antiserum K 1711, which had been shown to be specific for pancreatic glucagon (see specificity, chapter 2.3.2). No immunohistochemical reactive cells were present in anthrum, duodenum, and jejunum.

It is difficult to evaluate the histological and morphological evidence that there is one cell type in the gastrointestinal tract which stores gut GLI's, since the protein(s) is (are) still unknown and is (are) only related to it(s) ability to prevent the binding of radio-iodinated glucagon to anti-glucagon antisera.

For instance, acid-ethanol extracts of stomach, duodenum, fundus, jejunum, colon and ileum made by Samols (1966) and Schopman (1967) have shown cross-reactivity with anti-glucagon antisera, but these cross-reactions varied considerably amongst each other.

A-cells have been identified electron microscopically in the human fundus (Orci, 1976) and in the human duodenum (Saragawa et al., 1974). On the other hand we have seen two immunoreactive cells in one section of human terminal ileum, revealed with the immunoperoxidase method and with anti-glucagon antiserum K 1711 (Fig. 7).

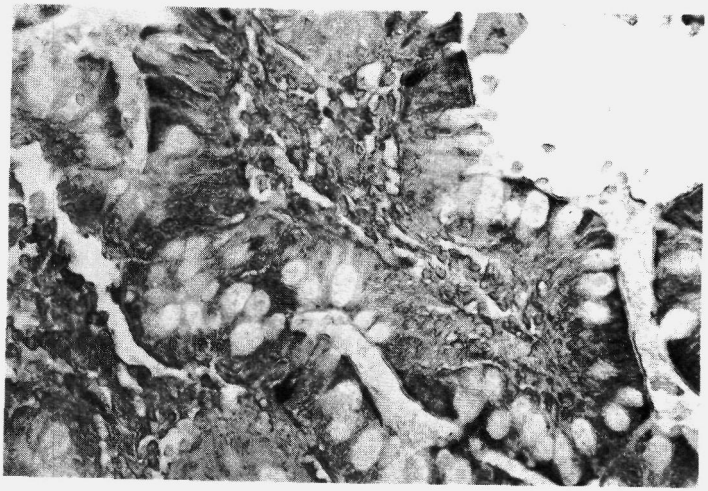


Fig. 7.
Two cells immunoreactive with anti-glucagon antiserum K 1711 located between the epithelium and the lamina propria, next to the goblet cells of an intestinal villus. Morphologically these areas cannot be identified as A-cells with certainty.

2.3.2 Radiochemical characterization

Antisera were screened for titre, affinity and specificity with the following routine procedures:

a) titre

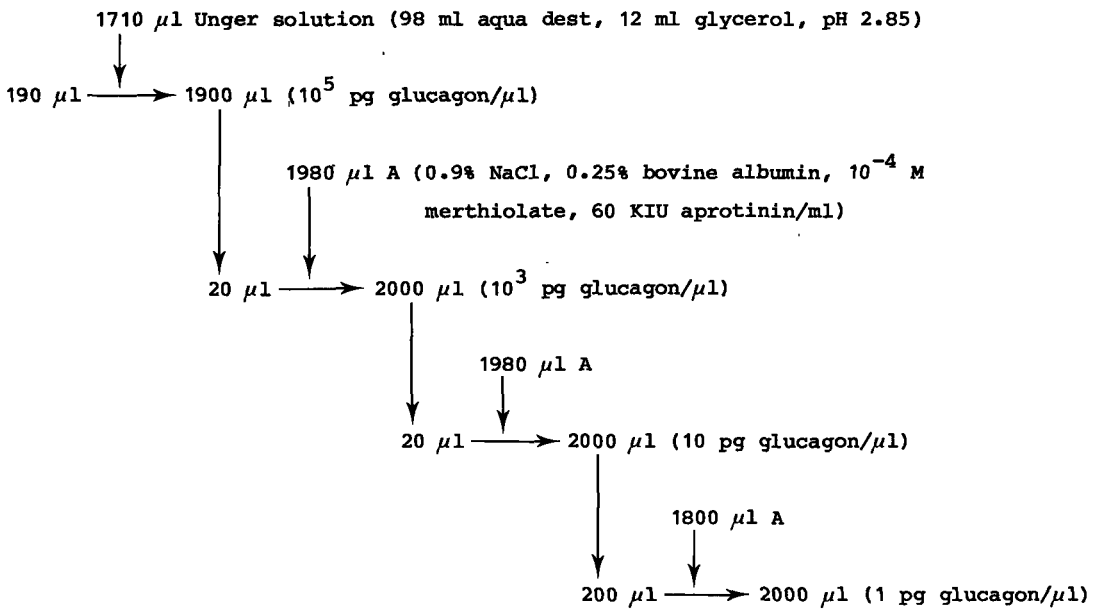
In duplicate, 350 μl of borate buffer (0.1 M; pH 8.6; 0.25% bovine albumin; 10^{-4} M merthiolate), 50 μl of ^{125}I-glucagon (40-60 pg glucagon), 50 μl aprotinin (500 KIU) and a dilution of antiserum (final dilution of antibody from 1:10 to 1:20,000) in 0.9% NaCl, 0.01% bovine albumin and 10^{-4} M merthiolate, were incubated for two days at 0-4°C. A control on the "damaged" fraction (that part of ^{125}I-glucagon which is damaged and not binding to the antiserum) was performed with 400 μl of borate buffer, 50 μl of ^{125}I-glucagon and 50 μl aprotinin without addition of antiserum.

After the incubation period 200 μl bovine serum was added to each incubation vial. The separation of free and antibody--bound glucagon was accomplished with dextran-coated charcoal. The dextran-coated charcoal was prepared in borate buffer (0.1 M; pH 8.6; 10^{-4} M merthiolate), 300 mg dextran and 2 g charcoal were mixed in 200 ml of borate buffer. Each incubation vial was shaken after addition of 1 ml dextran-coated charcoal suspension. The contact time before centrifugation was 8 minutes at 4°C. The centrifugation time was 4 minutes at 2000 x g. The supernatant was decanted and the residue was counted with a gamma scintillation counter (Packard, model 5102).

The fraction of antibody-bound radioactivity was calculated from the formula (B-TxD):(T-TxD), B being the antibody-bound radioactivity, T the total amount of radioactivity added to the vial, and D the fraction of radioactivity measured as bound in the absence of antibody (non-specifically bound). The titre is defined in this thesis as the final dilution of antiserum which bound 50% of the maximum ^{125}I-glucagon bound by excess antibody.

b) affinity

Pure synthetic porcine glucagon (Eli Lilly) was freshly dissolved to a concentration of 1 mg/ml in 10^{-3} N NaOH. A dilution of glucagon was made according to the following scheme:


1710 µl Unger solution (98 ml aqua dest, 12 ml glycerol, pH 2.85)
190 µl
1900 µl (10^5 pg glucagon/µl)
1980 µl A (0.9% NaCl, 0.25% bovine albumin, 10^{-4} M merthiolate, 60 KIU aprotinin/ml)
20 µl
2000 µl (10^3 pg glucagon/µl)
1980 µl A
20 µl
2000 µl (10 pg glucagon/µl)
1800 µl A
200 µl
2000 µl (1 pg glucagon/µl)

In duplicate 0 μl, 10 μl, 20 μl and 40 μl of a standard solution with 1 pg glucagon/μl and 8 μl, 16 μl, 32 μl and 64 μl of a standard solution with 10 pg glucagon/μl was added to 50 μl diluted antiserum (1:10,000), 50 μl aprotinin (500 KIU) and enough borate buffer (0.1 M, pH 8.6, 0.25% bovine albumin, 10^{-4} M mertiolate) to make the final incubation volume 0.5 ml.

The mixtures were preincubated for three days at 0-4°C. The fraction bound ^{125}I-glucagon was measured two days after the addition of 50 μl radioactive glucagon (20-40 pg/reaction mixture). The separation of free and antibody-bound glucagon was accomplished with dextran-coated charcoal.

In Table 4 we used the criteria suggested by Heding (1972) for the description of the affinities (Fig. 8a,b,c).

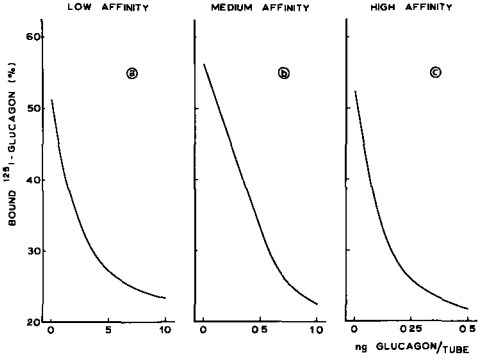


Fig. 8 (a,b,c)
Standard curves leveling off at 10 (a), 1 (b) and 0.5 (c) ng glucagon/tube are characteristics of antisera with respectively low, medium and high affinities.

c) specificity

The specificity of an antiserum and its detection limit also depend on the selectivity and the affinity of the antibody.

Table 4 Results of immunizition

Antigen	Animals	Number	Methods of injection	Number died during immunization period	Number of cases with antibodies	titre-range	affinity	non-specificity*
Glucagon in Freund's adjuvant	turkey	4	intra-muscular	-	-	-	-	
27-S-methyl-glucagon in Freund's adjuvant	turkey	1	intra-muscular	-	-	-	-	
	female Wener Alaska rabbits	3	intra-muscular and sub-cutaneous	-	1	80	low	n.d.
	female New Zea-land white rabbits	3	subcutan-eous	1	-	-	-	

Table 4 Results of immunizition (continued)

Antigen	Animals	Number	Methods of injection	Number died during immunization period	Number of cases with antibodies	titre-range	affinity	non-specificity*
	female New Zealand white rabbits	12	intra-muscular	2	9	500-10,000	2 high 2 medium 5 low	< 1 and > 30 < 1 and > 50 n.d.
glucagon-methionine-sulfoxide in Freund's adjuvant	female New Zealand white rabbits	1	intra-muscular	-	1	1000	medium	n.d.

n.d.: not determined;
*non-specificity is expressed as percentage of bound labeled glucagon displaced in the presence of 500 pg gut GLI-1/ml.

Thus, antisera with a low affinity for porcine glucagon were not tested for specificity on gut GLI. The specificities were evaluated by displacement of ^{125}I-glucagon with highly purified gut GLI-1 (Novo Research). The same procedure was followed as with the estimation of the affinities. Instead of porcine glucagon, gut GLI-1 was taken for the standard solution.

From the antisera tested, one antiserum, K 1711, had a sufficient affinity and specificity for undertaking a radioimmunoassay for pancreatic glucagon in human plasma (Fig. 9).

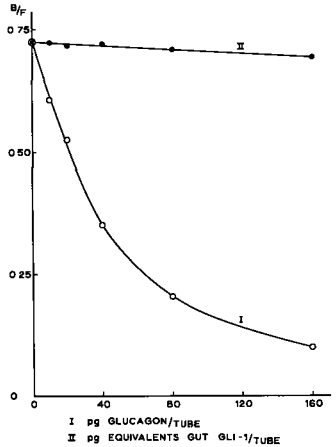


Fig. 9.
Glucagon standard curve obtained with anti-glucagon antiserum K 1711 and apparent glucagon content of gut GLI-1.

2.4 DISTRIBUTION OF ANTIBODY-BINDING AFFINITY

Each antiserum against a protein antigen usually comprises a collection of heterogeneous populations of antibodies against different determinants distributed all over the protein. Thus, whereas antibodies produced by two rabbits against 27-S-methylglucagon are bound by the C-terminal portion of glucagon, two antisera obtained against the same modification of glucagon are bound by the N-terminal portion, since only antisera bound by the C-terminal undecapeptide (residue 19-29 of glucagon) are specific for pancreatic

glucagon (Table 4). This suggests that different individuals of the same strain of animal recognize different portions of the same antigen. By comparing the antibody-binding affinity of glucagon and 27-S-methylglucagon (Scatchard plots, Fig. 12 and Fig. 13), we can obtain information on the location of determinants that interact with antibodies induced against 27-S-methylglucagon. A reduction in affinity is normally associated with a chemical modification of the protein. However, this decrease in reactivity is also related to the degree of unfolding of the polypeptide. Glucagon in diluted aquaeous solutions from pH 1 to 10 (< 1 mg/ml) has a predominantly random coil conformation with at most 15% α-helix (Blanchard and King, 1966; Srere and Brooks, 1969). Thus, a reduction in affinity could be explained by having either an antigenic determinant in the neighbourhood of a methionine residue or an antigenic determinant containing a methionine residue. The antibody-binding affinity was determined by measuring the capacities of glucagon or 27-S-methylglucagon to inhibit the binding of respectively ^{125}I-glucagon and ^{125}I-(27-S-methylglucagon) by antibodies generated against 27-S-methylglucagon.
For any further investigations we chose antiserum K 1711 (generated against 27-S-methylglucagon) because it has a high affinity for glucagon and it also proved to be specific.

Methods and calculations

Antibodies were directly incubated with both labeled and unlabeled hormones. The incubation time was three to four days. The specific activity of ^{125}I-glucagon and ^{125}I-(27-S-methylglucagon) was determined by self-displacement curves, where the immunoreactivity of respectively ^{125}I-glucagon and ^{125}I-(27-S-methylglucagon) was found to be equal to that of unlabeled glucagon and unlabeled 27-S-methylglucagon.
Binding data were plotted as B/F v.s. B (where B and F are the concentration of bound and free antigen, respectively, at equili-

Table 5 Binding data for glucagon and 27-S-methylglucagon to antiserum K 1711

Glucagon (pg)	^{125}I-glucagon (pg)	B/F	B (pg)	27-S-methyl-glucagon (pg)	^{125}I-(27-S--methyl-glucagon (pg)	B/F	B (pg)
0	50	1.02	25.25	0	50	0.56	18.00
10	50	1.02	29.70	10	50	0.51	20.40
25	50	0.84	34.35	25	50	0.46	23.45
50	50	0.71	41.70	50	50	0.43	30.00
100	50	0.59	67.95	100	50	0.42	44.78
200	50	0.39	121.20	250	50	0.40	86.20
500	50	0.19	173.80	500	50	0.37	149.50

Table 6 Antibody-binding capacity and equilibrium constants of anti-glucagon antiserum K 1711 for native glucagon and 27-S-methylglucagon

Antigen	Antibody population	Antibody-binding capacity (moles/litre)	Equilibrium constant (litre/moles)
Glucagon	high affinity	4.50×10^{-11}	3.4×10^{10}
	low affinity	1.26×10^{-10}	6.8×10^{9}
27-S--methyl-glucagon	high affinity	3.08×10^{-11}	2.64×10^{10}
	low affinity	4.69×10^{-10}	9.60×10^{8}

brium) and the curve extrapolated to its interception with the Y axis and the X axis. Extrapolation of the binding curve to infinite free antigen concentration will indicate the total amount of antibody binding sites present.

Theoretical approach of a binding curve

The antibody concentration is [Ab]
The free antigen concentration is [Ag] = [F]
The concentration of bound antigen is [AgAb] = [B]

$$[Ag] + [Ab] \underset{K_2}{\overset{K_1}{\rightleftharpoons}} [AgAb]$$

The equilibrium constant K is:

$$K = \frac{K_1}{K_2} = \frac{[AgAb]}{[Ag]\,[Ab]}$$

$[Ab_t]$ = total antibody concentration = [AgAb] + [Ab]

$$K = \frac{[AgAb]}{[Ag]\,[Ab_t - AgAb]} \quad \text{or} \quad K = \frac{B}{F[Ab_t - B]}$$

$$B/F = K[Ab_t - B]$$

Results

In Table 5, the data for the binding curves is given.
The binding data were plotted on B/F v.s. B for glucagon (Fig. 10) and 27-S-methylglucagon (Fig. 11).

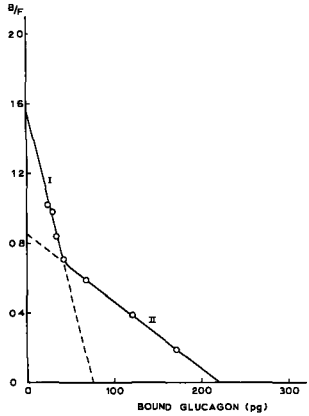


Fig. 10.
Scatchard-plot from a glucagon standard line with anti-glucagon antiserum K 1711. It shows a high affinity antibody population (I) and a medium affinity antibody population (II).

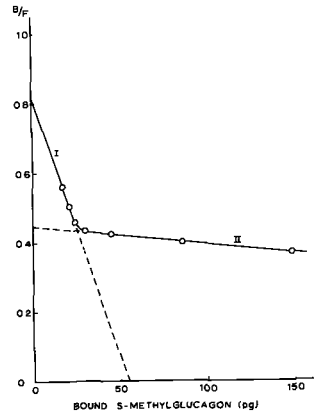


Fig. 11.
Scatchard-plot from a 27-S-methylglucagon standard line with anti-glucagon antiserum K 1711. It shows a high affinity antibody population (I) and a low affinity antibody population (II).

By extrapolation we found the antibody-binding capacity and the equilibrium constant of antiserum K 1711, for glucagon and 27-S--methylglucagon (Table 6).

It should be noted that in the presence of high affinity antibodies the shape of the curve is such that there could be an underestimation of the total antibody-binding capacity and that in Fig. 11 we made a long extrapolation to the X-axis which results also in an imprecise value.

Conclusions

Firstly, antiserum K 1711 contains two populations of antibodies, one with a high affinity, the other with a low affinity. Secondly, only the low affinity population is directed against a determinant

which contains an unchanged methionine residue, because it is sensitive to a modification of methionine. Thirdly, because antiserum K 1711 is specific for glucagon, we can distinguish between two determinants, both located on the C-terminal undecapeptide (residue 19-29) of glucagon. One determinant (low binding capacity for A.S. K 1711) is located on the C-terminal position of the undecapeptide, the other (high binding capacity for A.S. K 1711) on the N-terminal position of the undecapeptide (residue 19-29) of glucagon.

It is surprising however, that the low affinity antibodies have a higher affinity for glucagon than for 27-S-methylglucagon. An explanation might be that antibody production was preceded by a demethylation, so that in fact the antibodies were produced against unchanged glucagon. The presence of an extra electrical charge induces an immune response and affects the immunogenicity drastically, but this charge is apparently not responsible for the recognition of antigenic determinants.

2.5 *AFFINITY CHROMATOGRAPHY OF ANTIBODIES RAISED AGAINST GLUCAGON DERIVATIES*

Ligands coupled to Sepharose or other immobile material can be used to purify specific molecules such as antibodies. This method was introduced by Campbell et al. (1951).

Sepharose-insulin (Cuatrecasas, 1969) has been used to isolate insulin-specific antibodies from whole antiserum. It also provides a method for the fractionation of individual antisera, which are known to contain populations of antibodies heterogeneous with respect to their antigen-binding properties.

Although isolation of the highest affinity antibodies would increase the sensitivity of an immunoassay, this method as far as we know, has been used only by Miles and Hales (1968) and Weintraub and Kadesky (1971). This method may also be of value in increasing

the specificity of the immunoassay for glucagon.

Specific anti-glucagon antibodies may be obtained on affinity columns prepared by covalent linkage of the C-terminal undecapeptide of glucagon, prepared by tryptic digestion of glucagon, to Sepharose gel.

The two populations of antibodies of antiglucagon antiserum K1711 were separated by isolating the gammaglobulins from antiserum by means of a Sepharose-protein A column, followed by affinity chromatography on a C-terminal undecapeptide-Sepharose 4B column.

Theoretically we may expect the following peptides after tryptic digestion of glucagon:

a) residue 1 to 12: His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-
-Lys

b) residue 13 to 17: Tyr-Leu-Asp-Ser-Arg

c) residue 18 : Arg

d) residue 19 to 29: Ala-Glu-Asp-Phe-Val-Glu-Trp-Leu-Met-Asn-Thr

However, Bromer et al. (1971) did not find residue 18 separated from residue 19-29.

Methods

A. Digestion of glucagon

Glucagon (2 mg/ml diluted NaOH, pH 10.0) was digested for 24 hours with 2% by weight of trypsin at pH 7.8 and 37°C. The pH was adjusted to 7.8 with HCl and the enzyme was added immediately. The hormone solution became clear within 5 minutes. The pH was kept at 7.8 during the reaction time.

Purification of the enzymatic digests

The peptides were purified on a CH-Sepharose C-25 column (10 x 280 mm). The column was monitored at 280 nm. The column was equilibrated with 0.1 M phosphate buffer (pH 5.0) and eluted

with 0.05 M phosphate buffer (pH 5.0). Two ml protein containing fractions were pooled and lyophilized. The separation of the enzymatic digest on this column is shown in Fig. 12.

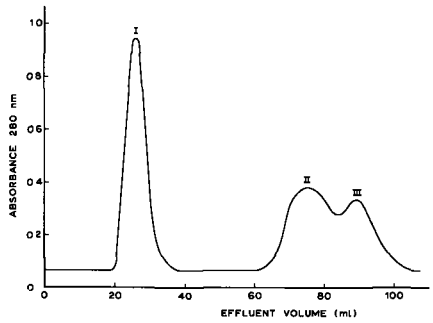


Fig. 12.
Elution profile, on a CH-Sepharose C-25 column, of glucagon after tryptic digestion. Peak I represents the undecapeptide (residue 19-29), peak II and III represent the dodecapeptide (residue 1-12) and the pentapeptide (residue 13-17).

Characterization of the enzymatic digests

a. When glucagon was digested in the presence of traces ^{125}I-glucagon, radioactivity appeared mainly in peak II and III (± 95%), suggesting a correspondence of these peaks with the dodecapeptide (residue 1-12; His-Ser-Gln-Gly-Thr--Phe-Thr-Ser-Asp-Tyr-Ser-Lys) and the pentapeptide (residue 13-17; Tyr-Leu-Asp-Ser-Arg). No radioactivity was found in peak I.

b. Spectrophotometric measurements

The extinction was measured at 290 nm with a Pye Unicam SP6-500 spectrophotometer. Only peak I contained tryptophan.

c. Amino acid analysis

The amino acid composition of tryptic digest from bovine glucagon was investigated only for peak I from Fig. 12 (Table 7).

The analysis was performed on a Technicon NC1, equipped with a column of C2 resin (Technicon). Peak I was pooled, lyophilized and hydrolyzed in 6 N HCl at 110°C for 22 hours.

Table 7 Amino acid composition of the undecapeptide (residue 19-29) of glucagon

Amino acid	Number of amino acid residues of the C-terminal undecapeptide of glucagon	
	Theoretical	Experimental
Aspartic acid	2.0	1.90
Threonine	1.0	0.99
Serine	-	0.13
Glutamic acid	2.0	2.16
Glycine	-	0.08
Alanine	1.0	1.10
Valine	1.0	1.05
Methionine	1.0	0.91
Leucine	1.0	0.93
Tryosine	-	0.04
Phenylalanine	1.0	1.03
Lysine	-	0.04
Histidine	-	0.03
Arginine	-	0.03
Tryptophan	1.0	not determined

From these data, it is concluded that the C-terminal undecapeptide of glucagon was pure enough (± 95%) to use for affinity chromatography (and that it did not contain an arginine residue).

B. Covalent linkage of the purified C-terminal undecapeptide of glucagon to activated CH-Sepharose 4B (Pharmacia)

Activated CH-Sepharose 4B is a N-hydroxysuccinimide ester derivative of CH-Sepharose 4B. The N-hydroxysuccinimide leaves the activated CH-Sepharose 4B when the ester is attacked by the unprotonated form of a primary amine and a stable amide linkage is formed. No coupling reagents are required.

The activated CH-Sepharose 4B (0.5 g freeze-dried powder) was suspended in 10^{-3} M HCl and washed on a glass filter (B3) with 100 ml 10^{-3} M HCl. The C-terminal undecapeptide

of glucagon was dissolved in the coupling buffer (0.1 M $NaHCO_3$; 0.5 M NaCl; pH 8.0) and mixed with the activated Sepharose gel at 4°C. 0.3 μmol C-terminal undecapeptide was coupled to 750 μl swollen Sepharose gel during 30 minutes at pH 8.0 and 4°C by an end-over-end mixing. Excess ligand was removed by washing with coupling buffer. Remaining active groups were blocked with 1 M ethanolamine (pH 9.0).
The gel was washed on a glass filter (P_3) with three cycles of alternating pH, using acetate buffer (0.1 M; 1 M NaCl; pH 4.0) and Tris buffer (0.1 M; 1 M NaCl; pH 8.0). The yield after coupling was estimated to be 70-80% by optical density measurement at 280 nm of uncoupled proteins. The gel was stored at 4°C. Before the antiserum K 1711 was applied to the C-terminal undecapeptide-Sepharose conjugates, the antibodies were isolated on a protein A--Sepharose CL-4B column.

C. Isolation of IgG's on a protein A-Sepharose CL-4B column
Protein A (a single polypeptide chain of MW 42,000) was coupled covalently to Sepharose CL-4B (Pharmacia), using CNBr. It interacts specifically with the Fc fragment of gammaglobulins. One gram freeze-dried protein A-Sepharose CL-4B powder was swollen and equilibrated in phosphate buffer (0.1 M; pH 7.0). Two ml antiserum K 1711 (diluted to 10 ml with phosphate buffer) was applied to a protein A-Sepharose CL-4b column (10 x 50 mm). The column was then irrigated with the following solvents:
a 0.1 M phosphate buffer (pH 7.0)
b 1 M acetic acid.
Fig. 13 illustrates the elution pattern of antiserum K 1711 on a column of protein A coupled to Sepharose CL-4B at 4°C. The flow rate was 30 ml/hour.

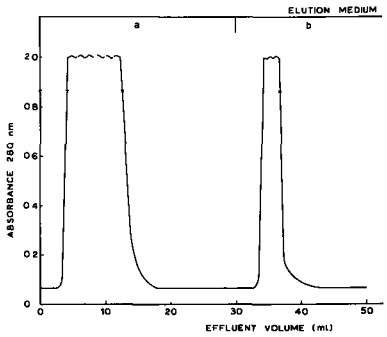


Fig. 13.
Elution profile of gammaglobulins on a protein A-Sepharose Cl-4B column.
The first peak represents non-retarded serum proteins and the second peak represents the gammaglobulins of anti-glucagon antiserum K 1711.

The material in the first peak consisted of serum proteins which were not retained on the column. Gammaglobulins of type IgG3 were not retained on the column. The second peak was pooled and dialyzed against saline and lyophilized.

D. Antibodies coupled to immobilized antigens

The isolated IgG's were dissolved in phosphate buffer (0.04 M; 0.15 M NaCl; pH 7.4) and applied to the C-terminal undecapeptide-Sepharose 4B column, which was equilibrated with the same phosphate buffer.

The IgG's were allowed to pass the column by an end-over--end mixing at 23°C for one hour to ensure maximum adsorption of the IgG's.

E. Elution of coupled antibodies

The following solvents were used to irrigate the affinity column at 4°C (Fig. 14).

a phosphate buffer (0.04 M; 0.15 M NaCl; pH 7.4). In order to recover all unretained antibodies.

b aqua dest

c 0.15 M NaCl adjusted to pH 11 with NH_3

d 0.1 M glycine-HCl buffer (pH 2.8). Weak or moderately strong interactions betwen antigen-antibody are characterized with desorption at low pH

e 2.5 M KI (neutral solution).

Iodide is a chaotropic ion. It causes a breakdown of ordered structure of water, leading to a suppression of hydrophobic bonding, which is usually involved in an antigen-antibody interaction.

f 6 M guanidine hydrochloride

Guanidine hydrochloride breaks hydrogen bonds, which are also considered to contribute towards an antigen-antibody interaction.

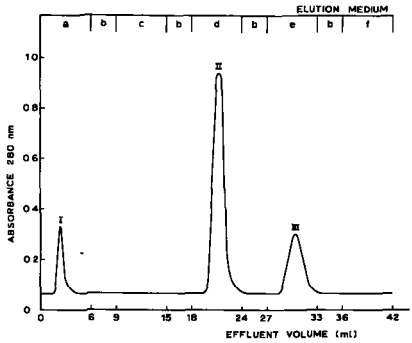


Fig. 14.
Elution profile for the separation of the IgG populations bound to the C-terminal undecapeptide-Sepharose 4B column. Peak I represents non-retarded gammaglobulins, peak II represents gammaglobulins with a medium affinity and peak III with a high affinity.

Antibodies in effluent fractions were detected by incubation with ^{125}I-glucagon under conditions of radioimmunoassay as described previously (Table 8).

Table 8 Antibody-binding capacity and equilibrium constants of the IgG populations of anti-glucagon antiserum K 1711 separated by means of a C-terminal undecapeptide-Sepharose 4B column

Effluent fraction	Antibody-binding capacity (moles/litre)	Equilibrium constant (litre/moles)
I	5.10^{-12}	5.10^{9}
II	1.10^{-10}	5.10^{9}
III	1.10^{-11}	1.10^{10}

From these results, it was concluded that at least two antibody populations (peak II and III) can be distinguished in antiserum K 1711 directed against the C-terminal undecapeptide (residue 19-29) of glucagon, as mentioned previously, and that it is possible to separate these two populations from each other.

Apparently there is a considerable loss of antibodies and a decrease in affinity of the high affinity antibody population, presumably caused by rather extensive exposure to the chaotropic ion, iodide.

A control experiment showed that from a non-specific anti-glucagon antiserum only a small portion of antibodies (< 5% of total antibody concentration) had affinities on the C-terminal undecapeptide-Sepharose 4B column.

With an improvement of the elution procedure, it should be possible to isolate antibodies specific for glucagon when present in a non-specific antiserum and so obtain an improvement of sensitivity and specificity for a radioimmunoassay.

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CHAPTER 3

RADIOIMMUNOASSAY OF PANCREATIC GLUCAGON

3.1 TRACER

3.1.1 Techniques for radioiodination of peptide hormones

The labeling of peptide hormones with radioactive iodine has become increasingly important in recent years in connection with the development of radioimmunoassays.

An important requirement for a successful radioimmunoassay for peptide hormones is the production of a radioiodinated hormone, which is immunologically identical to the unlabeled hormone.

The process of iodination may induce chemical changes in the hormone, which are reflected in a decreased affinity for antibodies or a decreased biological activity.

The labeling of peptide hormones has to be carried out under relatively mild conditions. Ordinarily, the mono- and diiodination of tyrosyl residues are the principal modifications involved in the incorporation of iodine; to a lesser extent iodohystidyl residues are formed. The oxidizing activity of iodine or of other oxidizing agents, used in the procedure, may cause a modification of tryptophan, methionine or cyst(e)ine. The presence of these modified components in the peptide hormone can seriously decrease the affinity for antibodies.

To establish the best method for preparing radioactive iodoglucagon four methods were investigated.

a The most widely used method of iodination is based on the chloramine-T oxidation of iodide to iodine. Although this method is rapid and easy to perform, exposure of the hormone to a strong oxidizing agent may damage the protein.

Rae and Schimmer (1974) suggested that the observed loss of hormonal activity of ACTH during the iodination reaction with

chloramine-T was due to an oxidation rather than an iodination. Slagg et al. (1970) proved by amino acid analysis that such an oxidation reaction occurred during iodination of gastrin with chloramine-T, which was responsible for a loss of biological activity.

b Of the more commonly used procedures for iodination of peptide hormones, the iodine monochloride (ICl) method seems to cause less damage and denaturation than the classical chloramine-T method. However, Sammon et al. (1973) reported that iodination with iodine monochloride led to a complete loss of biological activity of PTH. Rosa et al. (1967) showed detectable damage of albumin when this was radioiodinated with iodine monochloride.

c Electrochemical iodination proved to be a more suitable method to iodinate albumin and PTH (Rosa et al., 1967; Sammon et al., 1973). The electrochemical approach has many advantages. It is not hampered by a difficult separation of oxidant from protein such as is found in the chloramine-T procedure; the oxidation reaction can be monitored continuously and the reaction conditions can be altered during the procedure.

d Enzymatic iodination of glucagon, insulin, the B-chain of insulin and gastrin with lactoperoxidase showed significantly better antibody binding of the labeled hormone than with chloramine-T (Holohan et al., 1973).

3.1.2 Iodination of glucagon (experimental)

The present study examines the influence of various iodination methods on the immunoreactivity of glucagon. The following methods were examined:

(a) iodine monochloride method
(b) chloramine-T method
(c) electrolytic method
(d) enzymatic method.

ad (a) Iodine monochloride method

Iodine monochloride was prepared by the method of McFarlane (1963), by dissolving 150 mg of sodium iodide and 99 mg of sodium iodate in 2.5 ml of aqua dest and adding 2.4 ml of concentrated hydrochloric acid.

Two ml carbon tetrachloride and 0.1 M sodium iodide were added until a pink colour appeared in the organic solvent. The aquaeous phase was diluted with aqua dest up to 10 ml. One ml of this stock solution was partially neutralized by adding 2 ml 1 N sodium hydroxide and stabilized with 48 ml 1 M sodium chloride.

The method of iodination was based on McFarlane's iodine monochloride method with small modifications. Urea was purified on a mixed resin Amberlite MB-1.

Glucagon was dissolved (1 mg/ml) in 0.5 M glycine-NaOH buffer (pH 8.5, containing 8 M urea). Ten μl of this hormone solution was mixed with 2-10 μl of a diluted ICl solution (42 μg/ml of iodine as monochloride), 20 μl of 1 M glycine-NaOH buffer (pH 8.5, containing 2 M NaCl) and 10-50 μl carrier-free iodine-125 solution (100 mCi/-ml). The solution was allowed to stand for 5 minutes at 4°C. The reaction was stopped with 100 μl sodium metabisulphite (2.5 mg $Na_2S_2O_5$/5 ml 0.05 M phosphate buffer, containing 0.9% NaCl and 10^{-4} M merthiolate).

Iodoglucagon was purified by gel filtration on a Sephadex G-15 column (10x180 mm). The iodination vial was rinsed twice with 100 μl potassium iodide (50 μg KI/5 ml 0.1 M borate buffer, containing 0.9% NaCl, 0.25% bovine albumin and 10^{-4} M merthiolate, pH 8.6) in order to replace any adsorbed radioiodide. The column was eluted with 0.1 M borate buffer (0.25% BSA, 60 KIU aprotinin/-ml, 10^{-4} M merthiolate, pH 8.6), 0.5 ml fractions were collected and counted with a gamma scintillation counter. The specific activity was determined with self-dis-

placement curves.

ad (b) Chloramine-T method

Radioiodination with chloramine-T was carried out according to the method of Hunter and Greenwood (1962). Iodination was performed at pH 7.4. Five to 10 μl of a glucagon solution (1 mg/ml phosphate buffer, 0.2 M disodium hydrogen phosphate - sodium dihydrogen phosphate, pH 7.4) was mixed with 10 μl carrier-free iodine-125 solution (100 mCi/ml). The reaction was initiated by addition of 25 μl chloramine-T solution (4 mg/ml phosphate buffer, pH 7.4) and terminated after 10-45 seconds by addition of 100 μl sodium metabisulphite (0.5 g/l). Purification and determination of specific activity of iodoglucagon were carried out according to the method described with the radioiodination of glucagon with iodine monochloride.

ad (c) Electrolytic method

Electrolytic iodination may be carried out either at constant current (Pennisi and Rosa, 1969), or at constant potential (Sammon et al., 1973).

We chose the constant potential approach because it produces less oxidative damage. This method favours iodination of the most susceptible amino acid residue tyrosine.

A diagram of the electrolytic iodination cell is shown in Fig. 1.

The anode compartment was a platinum crucible, into which the hormone solution was iodinated in a total volume of 0.5 ml. The cathode compartment was a coiled platinum wire, enclosed in a glass tube and closed at the bottom by a dialysis membrane.

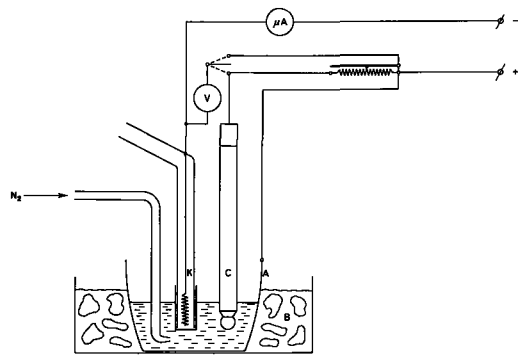


Fig. 1.
Diagram of the electrolytic iodination.
B = ice bath; A = platinum crucible (anode);
K = platinum kathode with membrane;
C = calomel reference electrode

Iodination procedure:

1. The anode compartment was filled with 0.1 ml hormone solution (10 to 100 μg of glucagon into 100 μl of 0.06 M phosphate buffer; pH 7.4 containing 0.1 M NaCl as supporting electrolyte), 0.2 ml phosphate buffer, pH 7.4, and 0.2 ml KI solution (equimolar with hormone solution, i.e. 2.9×10^{-9} to 2.9×10^{-8} mole of KI) labeled with 0.5 mCi iodine-125.
2. The cathode compartment was filled with 0.5 ml phosphate buffer, pH 7.4.
3. A constant electrode potential of 800, 700, 600 or 500 mV was adjusted between the anode and the calomel electrode.
4. The electrolysis was continued until the current dropped to zero.
5. After the reaction was completed, iodoglucagon was purified according to the method described with the ICl method.

ad (d) Enzymatic method

Iodination was performed at:

pH 5.0 : 0.05 M sodium acetate-acetic acid

pH 7.5 : 0.05 M sodium phosphate buffer

pH 9.0 : 0.1 M sodium borate buffer

pH 10.0 : 0.1 M glycine-NaOH buffer

Reaction mixture consisted of:

Ten, 5 or 2.5 μl hormone solution (freshly made: 1 mg/ml 0.001 N NaOH), 15 μl, 10 μl, 5 μl, H_2O_2 (8.5 μl H_2O_2 30% by weight/100 ml aqua dest) or 5 μl H_2O_2 (4.5 μl H_2O_2 30% by weight/100 ml aqua dest), 30 μl, 20 μl, 15 μl, 10 μl or 5 μl lactoperoxidase (50 μg lactoperoxidase/ml buffer), 10, 5 or 2.5μl carrier-free iodine-125 solution (100 mCi/ml).

The reaction was carried out at room temperature in small glass vials. The reaction time was 1, 2, 5, 10, 15 or 20 minutes. The reaction was terminated by the addition of 100 μl of sodium metabisulphite (0.5 g/l).

Iodoglucagon was purified by gel filtration on a Sephadex G-15 column and the specific activity was determined with self-displacement curves. Experimental details are as given with the ICl-method.

Results

ad (a) The preparations of iodoglucagon obtained with the four radioiodination methods, were adjusted to equal specific activity. Iodination of glucagon with chloramine-T results in derivatives that bind less to specific anti-glucagon andibodies than derivatives prepared by the iodine monochloride method (Fig. 3), but bound to a similar extent to non-specific anti-glucagon antibodies (Fig. 2). This might indicate an alteration of the C-terminal portion of glucagon, by exposure to chloramine-T, presumably an oxidation of methionine residue to

methionine sulfoxide, as was postulated by Shima (1975) and/or an oxidation of tryptophan residue.

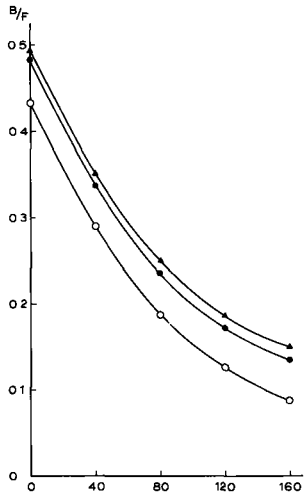


Fig. 2.
Comparison of the immunoreactivity of ^{125}I-glucagon preparations towards a non-specific glucagon antiserum (antiserum dilution 1:2,000).
Displacement curves were obtained using glucagon preparations treated with chloramine-T, exposure time 10 sec (▲) and 30 sec (●) and with ICl, exposure time 5 min (○). Experimental details of the displacement curves are as given in chapter 2.3.2.

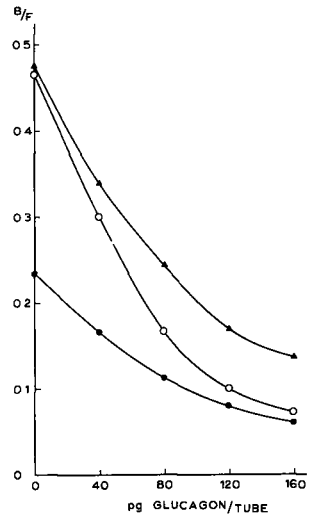


Fig. 3.
Comparison of the immunoreactivity of ^{125}I-glucagon preparations towards a specific glucagon antiserum (antiserum dilution 1:10,000).
Details are as given in Fig. 2.

Presence of a reducing agent like thiolglycollic acid (10^{-3} M) in the reaction mixture had indeed a beneficial effect (Figs. 4 and 5).

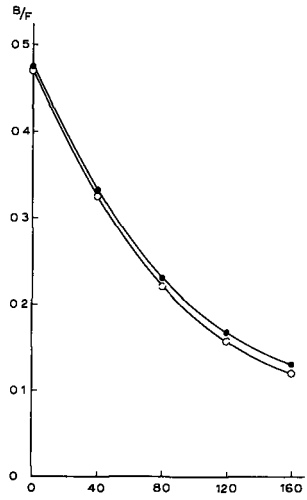


Fig. 4.
Effect of the presence of thiolglycollic acid on the immunoreactivity of iodinated glucagon towards a non-specific glucagon antiserum (final dilution 1:2,000). Displacement curves were obtained using glucagon preparations treated with chloramine-T (exposure time 45 sec) in the presence (O) or absence (●) of thioglycollic acid (10^{-3} M).

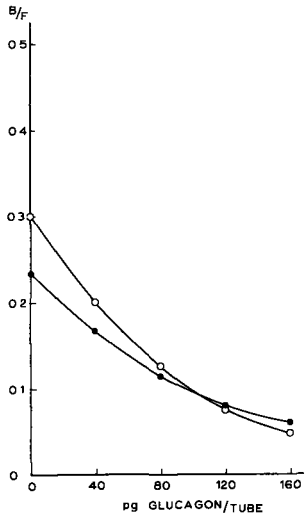


Fig. 5.
Effect of the presence of thioglycollic acid on the immunoreactivity of iodinated glucagon towards a specific glucagon antiserum (final dilution 1:10,000). Details are as given in Fig. 4.

To confirm that this decreased immunoreactivity towards specific anti-glucagon antibodies is due to an oxidation reaction, we exposed glucagon to chloramin-T in a similar way as described with the radioiodination method, but in the absence of iodide.

Amino acid analysis performed after treatment with CNBr, as described with the characterization of oxidized glucagon, of a preparation of glucagon exposed to chloramine-T for 45 seconds demonstrated that more than 70% of the methionine residues were oxidized. Apparently, the other amino acid residues (tryptophan excluded) in the glucagon molecule remained unaffected, except for the histidine residue of which only 80% was recovered. Tryptophan measured with a Pye Unicam SP6-500 spectrophotometer showed modifications for up to 70%. Glucagon preparations treated with chloramine-T for 45 seconds (in the absence of iodide) showed a decreased ability to replace iodinated glucagon from specific anti-glucagon antibodies (Fig. 6).

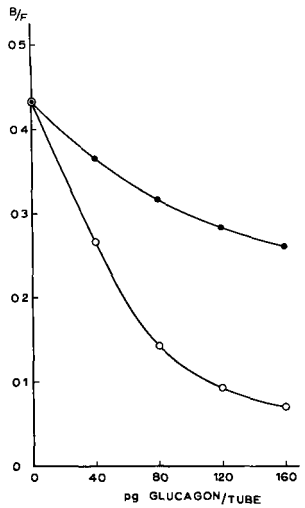


Fig. 6.
Comparison of the immunoreactivity of glucagon preparations treated without chloramine-T (O) or with chloramine-T (●) for 60 sec, in the absence of iodide, towards a specific glucagon antiserum (final dilution 1:10,000).
^{125}I-glucagon preparations obtained with the ICl method were used as tracers.

From the above results we concluded that the methionine residue is essential for binding of the glucagon molecule to specific anti-glucagon antibodies as seen before (chapter two). Thus, we have to be sure that the oxidative damage to the iodoglucagon preparation during the

iodination procedure is minimal in order to obtain a maximum immunoreactivity of the iodoglucagon molecule and a maximum sensitivity. Obviously, the chloramine-T method is not the best choice for radiolabeling of glucagon.

ad (b) In the iodination of glucagon by iodine monochloride the iodinating species (^{125}ICl) has to be prepared in situ by reacting "cold" ICl (^{127}ICl) with radioactive NaI ($Na^{125}I$) : $^{127}ICl + {}^{125}I \rightleftharpoons {}^{125}ICl + {}^{127}I$.

Some excess of ^{127}ICl is necessasy for an efficient conversion of ^{125}I to ^{125}ICl.

The addition of more "cold" ICl will result in the incorporation of more than one iodine atom per glucagon molecule, which is undesirable (Fig. 11). If less "cold" ICl is added the resulting tracer will be a mixture of ^{125}I-labeled glucagon with a low specific activity and with still a considerable amount of ^{127}I-labeled glucagon (Fig. 7). We found that even under optimum conditions the efficiency of radioactive labeling was less than 35%.

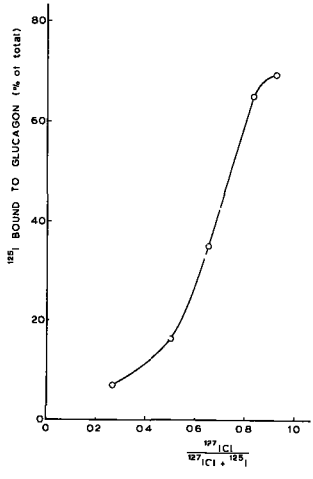


Fig. 7. Influence of the amount of ICl on the incorporation of ^{125}I into iodinated glucagon.

ad (c) The electrolytic iodination method was studied, firstly because no other oxidizing agents are present and secondly because the rate of formation of iodine can be controlled. This method allows us to keep the amount of iodine into glucagon molecule under control. Using the above method, a number of iodinations of glucagon were carried out at different values of electrode potential. The yield of the iodinated glucagon was between 20-40%. However, none of the iodinated products showed an immunoreactivity towards either specific or non-specific anti-glucagon antiserum. Moreover, the biological activity of these products, determined according to the method described in chapter 2.1.3 was nil. Paper electrophoresis revealed degradation products derived from glucagon, which contained more than 50% of the bound iodine. Gel chromatography (Sephadex G-25) revealed the presence of products with a molecular weight greater than 3500 daltons.

Fig. 8 also shows the results obtained with an iodination mixture after electrolysis at an electrode potential of 800 mV separated on a Sephadex G-10 column (11 x 300 mm). The elution profile showed a protein peak (I) and an iodide peak (III) as well as an unknown one (II). Peak I is asymmetric and presumably contains split products of glucagon. To indicate if peak II could be due to a formation of small amounts of iodate (Sammon et al. 1973) approximately 2 mg KIO_3 was added to the iodination mixture after electrolysis and before any separation on the Sephadex G-10 column. The column was eluted with borate buffer (0.1 M, 0.25% BSA, 10^{-4} M merthiolate, 60 KIU aprotinin/ml, pH 8.6).

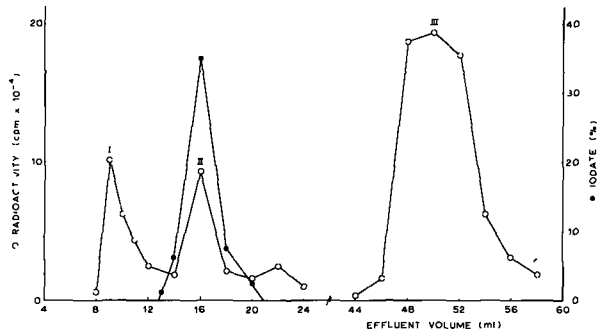


Fig. 8.
Separation of glucagon (+ split products) (peak I), iodate (peak II) and iodide (peak III) on a Sephadex G-10. The separation was carried out by gel filtration of an iodination mixture obtained after electrolytic iodination containing ^{125}I--glucagon (+ split products), KI, 0.5 mCi of iodine-125, sodium metabisulphite and approximately 2 mg of iodate.

Iodate was measured as follows:

To each column fraction was added: 5 μl NaI solution (10 mg NaI/ml), 20 ml aqua dest, 20 ml 1 N HCl and 5 ml starch solution (1 g starch/100 ml), thiosulphate (140 mg $Na_2S_2O_3 \cdot 5\ H_2O$/200 ml) until colour disappears. From these data we concluded that peak II was iodate and might cause this pronounced damaging effect on the glucagon molecule. Addition of reducing agents, thioglycollic acid, glutathion and sulphite during electrolysis improved the immunoreactivity of the iodinated products only slightly.

ad (d) Experiments with lactoperoxidase to iodinate glucagon over the pH range 5.0-10.0 showed that maximal incorporation of iodine and maximal binding of ^{125}I-glucagon to excess antiserum K 1711 occurred at pH 5.0 (Fig. 9). Unless indicated otherwise, the following conditions

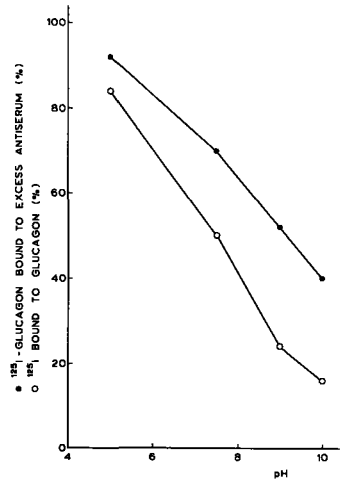


Fig. 9.
Influence of pH on the yield of ^{125}I-labeled glucagon measured as percentage radioactivity incorporated (O) and on the immunoreactivity of ^{125}I-labeled glucagon measured as percentage radioactivity bound to excess glucagon antiserum (●).

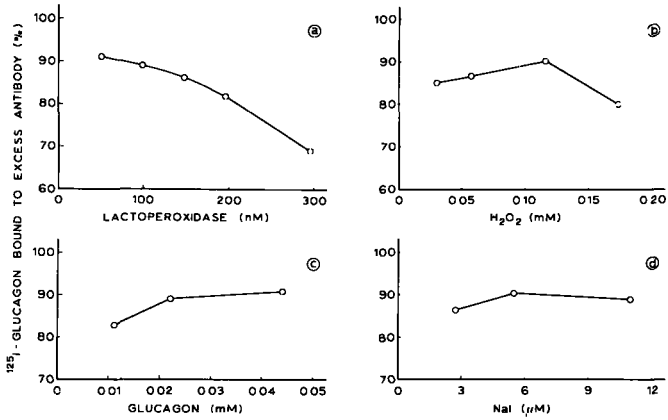


Fig. 10a,b,c,d.
Immunoreactivity of ^{125}I-labeled glucagon at varying concentrations of lactoperoxidase (a), H_2O_2 (b), glucagon (c) and iodide (d), measured as percentage radioactivity bound to excess antiserum.

were employed for assay of the lactoperoxidase-catalyzed iodination of glucagon at pH 5.0. The final concentrations were 2.2×10^{-5} M glucagon, 1.1×10^{-5} M $Na^{125}I$, 1.1×10^{-4} M H_2O_2 and 4.9×10^{-8} M lactoperoxidase in 0.05 M acetate buffer (pH 5.0).

The immunoreactivity of iodinated glucagon towards excess antiserum K 1711 (final dilution 1:40) was investigated as a function of lactoperoxidase concentration, H_2O_2 concentration, glucagon concentration and sodium iodide concentration. The results are given in Fig. 10a,b,c,d and summarized in Table 1.

Table 1 Effect of variation in lactoperoxidase concentration, H_2O_2 concentration, NaI concentration and glucagon concentration on the immunoreactivity of ^{125}I-labeled glucagon towards a specific glucagon antiserum.

Lactoperoxidase ($M \times 10^9$)	H_2O_2 ($M \times 10^5$)	Glucagon ($M \times 10^5$)	$Na^{125}I$ ($M \times 10^6$)	Immunoreactivity (%)
49	11.5	2.2	11.0	90.5
98	11.5	2.2	11.0	89.0
147	11.5	2.2	11.0	86.0
196	11.5	2.2	11.0	82.0
294	11.5	2.2	11.0	66.5
49	3.05	2.2	11.0	85.0
49	5.76	2.2	11.0	86.5
49	11.5	2.2	11.0	90.5
49	17.3	2.2	11.0	80.0
49	11.5	1.1	11.0	83.0
49	11.5	2.2	11.0	90.5
49	11.5	4.4	11.0	91.0
49	11.5	2.2	2.75	86.5
49	11.5	2.2	5.5	90.5
49	11.5	2.2	11.0	90.5

The loss of immunoreactivity of iodinated glucagon towards our specific anti-glucagon antiserum K 1711 was paralleled by the formation of diiodotyrosine-glucagon (Fig. 11).

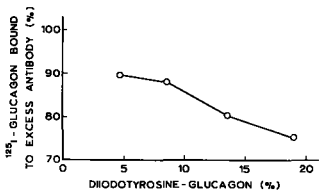


Fig. 11.
Influence of the diiodotyrosine-glucagon content on the immunoreactivity of ^{125}I-labeled glucagon, measured as percentage radioactivity bound to excess glucagon antiserum.

One non-specific antiserum, K 4043, did not show this effect, although another non-specific antiserum, K 1630, did. The determination of the iodine distribution within labeled glucagon is described in chapter 3.1.3 with the characterization of iodinated glucagon.
The influence of the reaction time on immunoreactivity of iodoglucagon is given in Fig. 12.

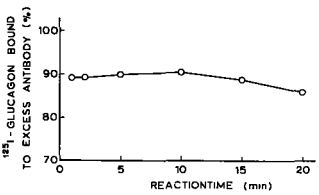


Fig. 12.
Immunoreactivity of ^{125}-labeled glucagon as a function of increasing exposure time to lactoperoxidase, expressed as percentage radioactivity bound to excess glucagon antiserum.

With optimum conditions, the best reaction time proved to be 10 minutes. The yield of iodoglucagon was between 70% and 80%.
The chemical reagents used in the method (lactoperoxidase, sodium metabisulphite and potassium iodide) do not alter the immunological integrity of glucagon. This is

shown by the identical behaviour of non-treated glucagon and glucagon treated with these reagents.
Amino acid analysis after treatment with CNBr on glucagon exposed to lactoperoxidase and H_2O_2 for 10 minutes did not show any oxidation.

3.1.3 Preparation, purification and characterization of iodinated glucagon

Glucagon was radioiodinated according to the enzymatic method as described in chapter 3.1.2.
One mg of porcine glucagon was dissolved in 1.0 ml of 0.001 N NaOH. Five μl of fresh solution was pipetted into a small tube and mixed with 20 μl 0.05 M of sodium acetate-acetic acid buffer, pH 5.0, 20 μl of H_2O_2 (4.5 μl H_2O_2 of 30% by weight/100 ml aqua dest), 10 μl lactoperoxidase (25 μg lactoperoxidase/ml acetate-acetic acid buffer) and 10 μl carrier-free iodine-125 solution (100 mCi/ml 0.001 N NaOH).
After 10 minutes in the dark, the reaction was terminated with 100 μl sodium metabisulphite (2.5 mg $Na_2S_2O_5$/5 ml 0.05 M phosphate buffer, containing 0.9% NaCl and 10^{-4} M merthiolate). The iodination vial was rinsed twice with 100 μl potassium iodide (50 μg/5 ml 0.08 M Tris, 0.02 N HCl and 0.08 M NaCl, 0.25% bovine serum albumin, 60 KIU aprotinin/ml, pH 8.6) in order to replace any adsorbed radioiodide.
Iodinated glucagon was first separated from iodide by gel filtration on a Sephadex G-15 column (10 x 180 mm). The Sephadex column was equilibrated and eluted at a rate of 10 ml/h with 0.08 M Tris, 0.02 N HCl, 0.08 M NaCl, 0.25% bovine serum albumin, 60 KIU aprotinin/ml and 10^{-4} M merthiolate, pH 8.6. Unretarded iodinated glucagon was eluted first, followed by an iodide peak.
An alternative purification of iodinated glucagon is based on adsorption on a cellulose powder column (Whatman; 5 x 40 mm).

Degradation products of glucagon and unreacted iodide were not retarded on the column. Iodinated glucagon was eluted with 12.5% by weight bovine serum albumin (containing 10^{-4} M merthiolate and 60 KIU aprotinin/ml).

Purified in this way, the ^{125}I-glucagon is sufficiently stable for one month if kept frozen, as shown by its immunoreactivity. High specific activity hormone, such as is desirable for radioimmunoassay work, was subject to radiolytic damage on storage. Therefore it is desirable to carry out the final purification of iodinated glucagon immediately before use. The final purification was carried out with about 1.5 μg protein of the fraction corresponding to the single peak of iodinated glucagon recovered from gel filtration or adsorption chromatography on a column (11 x 280 mm) of QAE Sephadex A-25 (Fig. 13) according to K.H. Jørgensen and U.D. Larsen (1972).

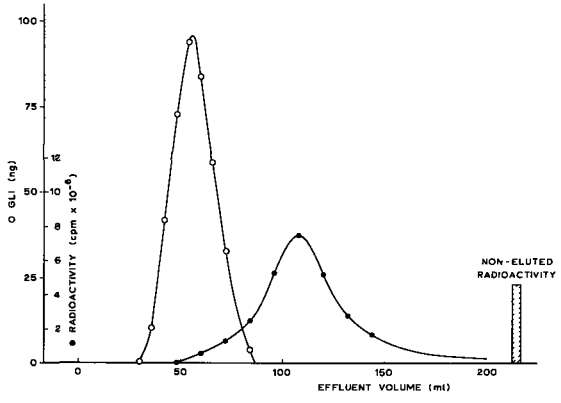


Fig. 13.
QAE-Sephadex A-25 chromatograms of approximately 1.5 μg "hot" glucagon (iodinated glucagon + unreacted native glucagon) from which only the radioactivity peak was drawn (●) and 1.5 μg "cold" glucagon (O). Radioactivity was counted with a gamma scintillation counter and IRG was measured with the radioimmunoassay method as given in chapter 2.3.2.

This column was used to separate iodinated glucagon from unlabeled native glucagon and to separate mono-iodotyrosine-glucagon from other iodoglucagons (DIT-containing material). QAE-Sephadex A-25 was swollen during 24 hours in 0.08 M Tris, 0.02 N HCl, 0.08 M NaCl, 10^{-4} M merthiolate (pH 8.6). This column was equilibrated during 6 hours with the same buffer containing, in addition, 1% bovine serum albumin and 60 KIU aprotinin/ml. The column was eluted with this same buffer at a rate of 15 ml/h at 4°C. The radioactivity of the fractions (2.0 ml) was counted with a gamma scintillation counter. Unlabeled native glucagon (1.5 μg) was passed through the same column under the same conditions. The amount of IRG in each fraction (2.0 ml) was radioimmunologically determined.

The elution pattern of radioactivity from the anion exchange chromatography shows only one peak. This radioactivity peak corresponds to a specific activity of 490 μCi/μg glucagon. A small proportion (5%) of the applied radioactivity remained in the column as indicated by the black bar. The fractions with iodinated glucagon were stored deep frozen. The labeled material and "cold" mono-iodotyrosine and di-iodotyrosine were subjected to thin-layer-chromatography after a treatment with 0.5 mg pronase in 0.5 ml of 0.2 M Tris/HCl buffer (pH 8.0) for 3 days at 37°C to determine the distribution between mono-iodotyrosine and di-iodotyrosine (von Schenck et al., 1976). The chromatogram was developed with n-butanol:acetic acid:water (78:5:17 (v/v)). After locating the spots by radioautography, these were scraped from the plate and counted with a gamma scintillation counter.

The relative amount of di-iodotyrosine was less than 0.2% of the total radioactivity. The iodoaminoacid composition by which a distinction between mono-iodotyrosine-10 and mono-iodotyrosine--13 is obtained, was not determined. Electrophoresis conducted in 20% polyacrylamide gels at pH 8.3 (Ornstein & Davies, 1964) with the labeled material showed one single band migrating more anodally than that given with native glucagon (Fig. 3, chapter 2.1.3).

Discussion

Chemical changes in the glucagon molecule result in a decreased immunoreactivity towards our specific anti-glucagon antiserum K 1711.

The decreased immunoreactivity is due to an increased amount of iodine introduced into the glucagon molecule (di-iodotyrosine formation) and to chemical alteration of the amino acid residues, methionine and tryptophan, in the glucagon molecule during the iodination procedure.

Comparing the results obtained with the four methods of radio-iodination it was concluded that the enzymatic method has several advantages; a high iodination yield and a rather high specific activity can be obtained (400-500 μCi/μg) without any severe damage to the molecule. We have no evidence for radiation damage to glucagon during the reaction time by this method.

3.2 *GUT-GLUCAGON-LIKE IMMUNOREACTIVE MATERIAL (GUT GLI)*

In setting up a reliable glucagon assay, one of the most serious problems is the presence of gut GLI in human plasma (Samols et al., 1966; Faloona & Unger, 1974).

When pancreatic glucagon is used as a standard to quantify GLI material in plasma and the specificity of the particular antiserum is unknown the results are inevitably too high. In order to investigate the specificity of the antiserum several dilutions of human pancreatic extracts and tissue extracts from human gastro-intestinal tract, obtained at autopsy, were assayed to study the parallelism with bovine pancreatic glucagon (Larsson et al., 1975).

Extraction of tissue

Human pancreas tail and human gastro-intestinal tissue stored at -20°C were thawed, cut with scissors, ground with fine sand and

3.5 ml of ethanolic HCl per gram of tissue (ethanol:HCl (37%) : H_2O = 66:1:13). The efficiency of the extraction procedure was followed by monitoring the recovery of a small amount of iodo-glucagon. The material was allowed to stand overnight at 4°C. The mixture was centrifuged and the supernatant was dialyzed in a 20 cm 8/32" Visking tubing against 100 ml polyethyleneglycol (M = 4000; 10% in water) for 4 hours at 4°C to remove the ethanolic HCl. After 4 hours the residue was washed out of the tubing with 2 ml saline (pH 3.3) and centrifuged. The supernatant was diluted with an equal volume of borate buffer (0.1 M; pH 8.6; 0.25% bovine albumin; 10^{-4} M merthiolate). Aliquots of serial dilutions of these tissue extracts and of a solution of bovine pancreatic glucagon were incubated with radio-iodinated glucagon and an antiserum dilution as described in chapter 2.3.2. For valid quantitative results, recovery studies of highly purified iodinated gut GLI on this extraction procedure should be carried out.

Results

Control experiments were performed with two anti-glucagon antisera (K 964 and K 4046) and serial dilutions of crude extracts of human ileum, colon, fundus, pylorus, duodenum and jejunum. Antiserum K 964 is obtained from Novo Research Institute and cross-reacts less than 1% with a crude porcine GLI preparation. Antiserum K 4046 is obtained from Schopman (Schopman et al., 1967) and shows perfect parallelism with a pancreatic glucagon standard curve and with serial dilutions of crude porcine GLI preparations.

Most of these extracts obtained from human intestinal tract contained B/F depressing substance in the radioimmunochemical glucagon assay with antiserum K 1630 and K 4046. No cross-reactivity was seen in the preparation of the oesophagus. Serial dilution curves of pancreatic glucagon obtained from extracts of the

human pancreas tail and dilution curves of the ileum and colon extracts were superimposable (Fig. 14).

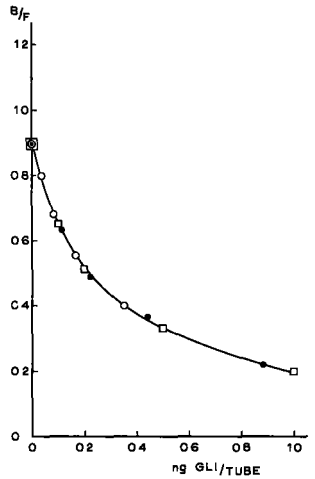


Fig. 14.
Displacement of ^{125}I-glucagon from antiserum K 1630 (final dilution 1:2,000) by: extracts from human pancreas (□), extracts from human ileum (●) and extracts from human colon (○). The GLI content of the extracts was determined with an antiserum which did not discriminate between pancreatic glucagon and gut glucagon.

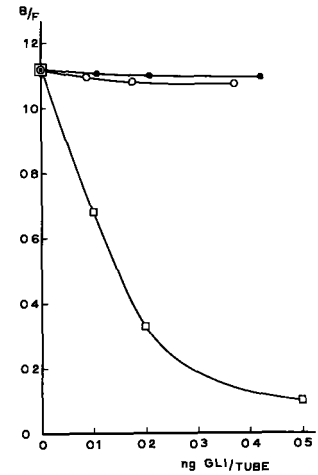


Fig. 15
Displacement curves obtained with antiserum K 1711. Details are as given in Fig. 14.

For the extracts of pancreas, ileum and colon the mean GLI content was calculated from determinations at four concentrations with anti-glucagon antiserum K 4046. Fig. 15 shows the same extracts analyzed with another anti-glucagon antiserum K 1711, which clearly distinguished between pancreatic glucagon and gut GLI. With this antiserum and antiserum K 964 values found for

gut GLI in the colon and ileum extracts varied with varying dilutions of the extract solution (Table 2).
No cross-reacting substance(s) was (were) found in the extracts of fundus, pylorus, duodenum and jejunum with antiserum K 1711 and K 964.

Table 2 Apparent quantity of pancreatic glucagon and gut GLI per gram of tissue, obtained from two different autopsies, for pancreas, ileum and colon, estimated with antisera K 1603, K 1711, K 4046 and K 964.

Anti-glucagon antiserum	Pancreas tail ng glucagon/g wet tissue	Ileum extract ng glucagon/g wet tissue	Colon extract ng glucagon/g wet tissue
K 1630	560	325	55
K 1711	585	n.d.	n.d.
K 4046	614	370	60
K 964	602	n.d.	n.d.
K 1630	580	360	60
K 1711	594	n.d.	n.d.
K 4046	608	400	75
K 964	598	n.d.	n.d.

n.d.= not detectable. No parallelism between standard curve and serial dilutions of extract.

Antisera K 1630 and K 4046 gave identical dilution curves for gut GLI and pancreatic glucagon. Antiserum K 1711 showed a cross-reactivity with a crude gut extract of about 3%, antiserum K 964 was slightly more specific (± 1.5%).
Samples of the same tissues were extracted either in a similar way as described above or with the addition of aprotinin (500 KIU/ml extract) and stored at -20°C for four weeks. Table 3 shows the effect of ageing and aprotinin on the recovery of gut GLI in the extracts as measured with the two pancreatic glucagon specific antisera.

Table 3 Effect of ageing and aprotinin on the cross-reactivity of gut GLI towards specific glucagon antisera

Pancreas glucagon specific antiserum	Ileum extracts cross-reactivity (%)			Colon extracts cross-reactivity (%)		
	freshly prepared	28 days after extraction		freshly prepared	28 days after extraction	
		without aprotinin	with aprotinin		without aprotinin	with aprotinin
K 1711	3.1	6.2	5.5	5.5	13.4	12.8
K 964	1.5	2.5	1.5	3.0	5.0	4.5
K 1711	4.0	8.1	7.5	7.7	15.1	14.6
K 964	2.5	4.4	2.5	2.5	4.0	3.5

From these data, it can be concluded that measurements of gut GLI from tissue extracts were influenced by non-specific interference with binding to both antisera in the colon extract and with binding to antiserum K 1711 in the ileum extract. This is presumably caused by enzymatic degradation. Therefore, direct measurement of crude gut GLI preparations is not possible because substance(s), presumably not gut GLI, interfere(s) with the binding of pancreatic glucagon to antisera K 1711 and K 964. Glucagon antisera can further be characterized by their cross--reactivity against specified components of the gastro-intestinal tract, such as the highly purified peptide GLI-1, isolated from porcine intestinal mucosa (Novo Research Institute). Fig. 9 (chapter 2.3.2) shows the standard curve with antiserum K 1711 and gut GLI-1. Its cross-reactivity against gut GLI-1 proved to be less than 1%.

Antiserum K 1603 gave identical dilution curves for gut GLI-1 and pancreatic glucagon.

The reaction between gastric inhibitory polypeptide (GIP), secretin (two structurally similar peptides) and antiserum K 1711 was less than 0.5%. (GIP was a free gift from H. Yajima, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan).

3.3 PREPARATION AND STABILITY OF PANCREATIC GLUCAGON STANDARDS

Usually both standard and tracer are stabilized and protected against glass surface adsorption by the addition of a second protein in high concentration. It prevents the adsorption of highly diluted antigen to the walls of tubes in which reagents are being stored or incubated. Among the various proteins, the most commonly used is bovine serum albumin. This protection against adsorption is particularly important in radioimmunoassays, where long incubation periods of tracer and standard are used.

Recently, Beverly et al. (1974) reported an enhanced stability of diluted proteins in solution with gelatin which resulted in an

increased sensitivity in competitive binding assays. We could not confirm this result. On the contrary, with gelatin a decreased binding was obtained and a decreased slope as compared with bovine serum albumin (Fig. 16).

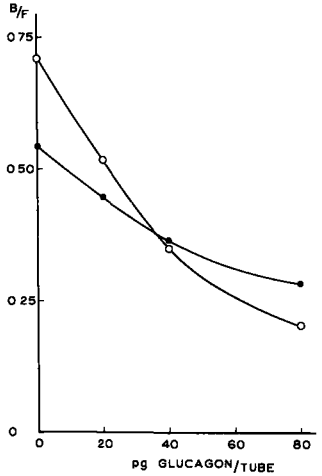


Fig. 16.
Effect of addition of bovine serum albumin (O) or gelatin (●) to the incubation medium. Displacement curves were obtained with ^{125}I-labeled glucagon and antiserum K 1711 (final dilution 1:10,000). Experimental details are as given in chapter 2.3. 2.

These curves were obtained according to the method described in chapter 2.3.2. The competition of glucagon with ^{125}I-glucagon for binding to anti-glucagon antiserum K 1711 was carried out in borate buffer (pH 8.6; 0.1 M 10^{-4} M merthiolate) including 0.25% (w/v) BSA or 0.1% (w/v) gelatin and 250 KIU aprotinin/ml. Interference with antigen-antibody reaction became more pronounced at higher concentrations of gelatin. Fig. 17 illustrates this decrease in initial binding with higher concentrations of gelatin. At very small concentrations of gelatin (0.01%) and bovine serum albumin (0.01%) the adsorption of diluted antigen or antibody to the glass wall caused a remarkable decrease in initial binding.
Much higher albumin concentrations have been used by some workers. Ørskov et al. (1968) used a concentration of 7% (w/v) albumin. Above a concentration of 1% (w/v) albumin we obtained a slight interference which could modify the shape of the standard displacement curve.

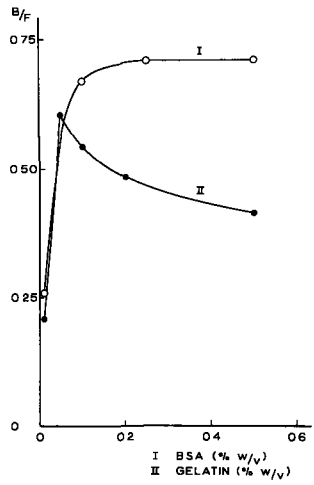


Fig. 17.
Effect of increasing concentrations of bovine serum albumin (O) or gelatin (●) on binding of ^{125}I-labeled glucagon to antiserum K 1711 (final dilution 1:10,000).

At the concentrations of albumin employed, no difference in the standard curve was detected when human albumin instead of bovine serum albumin was used. However, differences exist between the various commercially available bovine serum albumin preparations which we tested. This might be due to an apparent peptidase activity, caused by contaminating enzymes in these albumin preparations which can affect the stability of reagents and possibly the binding of antigen to antibody (Buchanan, 1971). Four commercially available albumin preparations were examined: bovine albumin (Ortho); bovine albumin (fraction I; Armour); bovine albumin (fraction V; Sigma); and bovine albumin (Poviet).

Serial dilutions of unlabeled glucagon were prepared in various albumin solutions and incubated at 37°C for 48 hours. The remaining glucagon was assayed against freshly prepared glucagon standards, according to the method described in chapter 2.3.2. The results are shown in Table 4.

The results in Table 4 suggest that losses of glucagon might be due to proteinase activity. As shown in Table 5, addition of proteinase inhibitors improved the recovery of glucagon, even with the Poviet albumin preparation. The same conditions as described

Table 4 Effect of albumin obtained from various sources, on glucagon recovery

Albumin preparations (0.25%)	Glucagon recovered (%)
Bovine albumin (Ortho)	72
Bovine albumin (fraction V; Sigma)	75
Bovine albumin (fraction V; Armour)	60
Bovine albumin (Poviet)	92

for Table 4 were applied, with the addition of inhibitors.

From Table 5 it can be concluded that soya bean trypsin inhibitor (4 μg/ml) was less efficient in comparison with aprotinin and benzamidine. Aprotinin (250 KIU/ml) and benzamidine (0.05 M) were effective inhibitors. However, benzamidine interfered in the radioimmunoassay of glucagon with antiserum K 1711. The affinity of the antibody for glucagon was significantly decreased even by 0.01 M benzamidine.

Fig. 18 shows the effect of serial dilution of benzamidine in the glucagon radioimmunoassay. Aprotinin shows no effect below the concentration of 1000 KIU/ml.

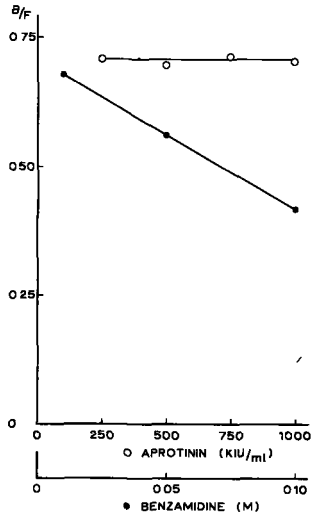


Fig. 18.
Effect of an increasing concentration of aprotinin (O) or benzamidine (●) on binding of ^{125}I-labeled glucagon to antiserum K 1711.

Table 5 Effect of proteinase inhibitors on glucagon recovery from albumin solutions

Albumin preparations (0.25%)	Glucagon recovered (%)		
	Aprotinin	benzamidine	soya bean trypsin inhibitor
Bovine albumin (Ortho)	90	102	86
Bovine albumin (fraction V; Sigma)	96	94	86
Bovine albumin (fraction V; Armour)	92	90	75
Bovine albumin (Poviet)	103	98	95

From the data mentioned, it can be concluded that adsorption of glucagon to glass is completely prevented by the addition of 0.25% bovine serum albumin, that degradation of glucagon by enzyme contamination in the albumin preparation is small with the Poviet preparation as compared with the others and that peptidase activity can be suppressed by the addition of 250 KIU aprotinin/ml buffer solution.

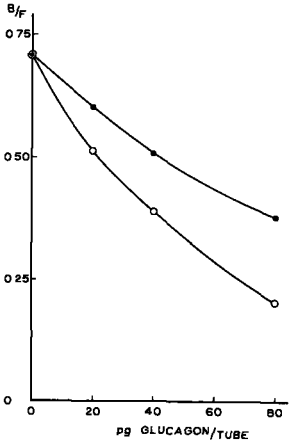


Fig. 19.
Comparison of a freshly made serial dilution of pancreatic glucagon (O) and a glucagon solution after repeated freezing and thawing (●). Displacement curves were obtained with ^{125}I-labeled glucagon and antiserum K 1711 (final dilution 1:10,000). Experimental details are as given in chapter 2.3.2.

Repeated freezing and thawing of a glucagon solution should be avoided because this causes losses in immunoreactive glucagon (Fig. 19). For further experiments we prepared a stock solution of glucagon of 0.1 mg/ml in a glycerol solution (12% v/v) at pH 2.85 and aliquots of 20 μl of this solution were stored at -20°C. Glassware adsorption will not occur at low pH values. When required, one of the aliquots was thawed and used directly for serial dilutions of standard pancreatic glucagon. These serial dilutions were then protected by the addition of bovine serum albumin and aprotinin.

3.4 *SEPARATION OF FREE AND ANTIBODY-BOUND HORMONE*

The conventional procedures of radioimmunoassay have utilized a wide variety of techniques for the separation of free and antibody-bound tracer. The large number of separation techniques (Table 6) seem to indicate that none is completely satisfactory for all purposes.

Table 6 Methods of separating bound and free tracer

	Principle	Method/material
a	Differential migration of bound and free tracer	Chromatography Electrophoresis Gel filtration Ultracentrifugation
b	Precipitation of antibody--bound tracer	Ammonium sulphate Sodium hydrogen sulphite Ethanol 2-Propanol Dioxan Polyethylene glycol
c	Adsorption of free tracer	Dextran or albumin-coated charcoal Cellulose Silica or talc Ion exchange
d	Solid-phase antibody	Covalent bonding to insoluble polymer Adsorbed to assay tubes Second antibody

A separation method should meet certain requirements, i.e. the separation material should not disturb the antigen-antibody complex and the separation between antibody-bound and free antigen should be quantitative.

Separation of free and antibody-bound antigen by means of protein

or dextran-coated charcoal in radioimmunoassays has become very popular because of its simplicity. However, it has one undesirable effect: it can disturb the antigen-antibody complex by adsorbing the antigen from this complex. Therefore, optimal conditions have to be worked out separately for each system.
After a dextran-coated charcoal separation method was set up, its validity in comparison with other methods was investigated, i.e. precipitation of the antibody-bound tracer with ethanol and polyethyleneglycol and differential migration of bound and free tracer by chromatography.

Charcoal (and dextran-coated charcoal) method

For each experiment the adsorption of ^{125}I-glucagon by charcoal or dextran-coated charcoal was compared in tubes containing the hormone only or the mixture of hormone and antibody and no added serum, or added normal bovine serum (NBS). The antiserum K 1711 was used in a final dilution of 1:50 (antibody in excess). The charcoal and the dextran-coated charcoal were prepared in borate buffer (0.1 M, pH 8.6, 10^{-4} M merthiolate). The anti-glucagon antiserum K 1711 and ^{125}I-glucagon were incubated for 2 days at 4°C. Afterwards 1 ml of a charcoal suspension (or dextran-coated charcoal suspension) was added, also at 4°C, then the tube was shaken. After centrifugation for 4 minutes at 2000 x g, the supernatant was decanted and the residue was counted with a gamma scintillation counter.
To avoid any disturbance of the antigen-antibody complex, all solutions which had to be added to the incubaton mixture were precooled to 4°C. To saturate the charcoal we employed a dextran-coated charcoal suspension, prepared by mixing 300 mg dextran T-70 and 2 g charcoal in 200 ml borate buffer at 4°C (Luyckx, 1972). For our preliminary studies we used 200 μl normal bovine serum (NBS) added prior to addition of charcoal, and the reaction time with charcoal or dextran-coated charcoal was 10 minutes at 4°C.

charcoal was 10 minutes at 4°C. In Figs. 20 and 21 dose-response curves for charcoal adsorption (coated and uncoated), of free hormone or hormone with excess antibody and with or without the addition of NBS are shown. It seems that at high protein concentration dextran acts as a molecular sieve, excluding the complex, while permitting free hormone adsorption (Herbert et al., 1964). At low protein concentration (Fig. 21), dextran decreases but does not completely eliminate the adsorption of the complex. Addition of NBS is necessary to occupy a number of sites on the charcoal particles and to eliminate adsorption of the complex.

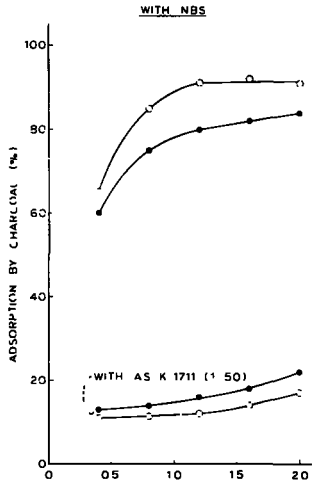


Fig. 20.
Charcoal adsorption of ^{125}I-labeled glucagon and ^{125}I-labeled glucagon bound to antibody as a function of charcoal concentrations. NBS added prior to adsorption. (●) no dextran coating, (○) dextran coating.

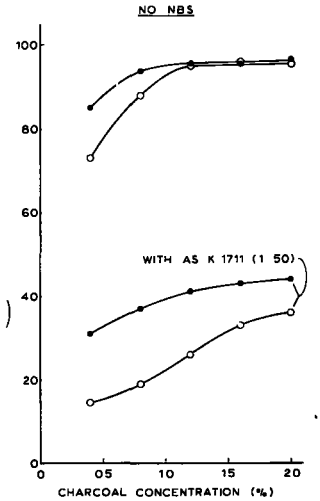


Fig. 21.
Charcoal adsorption of ^{125}I-labeled glucagon and ^{125}I-labeled glucagon bound to antibody as a function of charcoal concentration without addition of NBS. (●) no dextran coating, (○) dextran coating.

After the optimal charcoal concentration (1%) was selected, the binding of free hormone with time after the addition of 1% dextran-coated charcoal was studied (not shown). Eight minutes was sufficient to reach an equilibrium. The dextran-coated charchoal method was compared with the wick chromatography method, the ethanol and polyethyleneglycol method.

Wick chromatography

Wick chromatography was performed according to the method described by Ørskov (1967). After incubation for 48 hours at 4°C of ^{125}I-glucagon and antiserum K 1711 (final dilution 1:50), 100 μl of the reaction mixture was pipetted onto the bottom of round-bottomed test tubes (25 x 100 mm). The longitudinally folded wicks (dry strips of Whatman 3 MC paper, 15 x 120 mm) were placed vertically (Fig. 22). When this solution was completely adsorbed on the wick, 200 μl borate buffer (0.1 M, pH 8.6, 10^{-4} M merthiolate) and another 50 μl borate buffer after adsorption of the former were added. Wicks were then dried, cut into 6 pieces of 20 mm and counted for radioactivity.

Fig. 22.
Principle of wick chromatography.

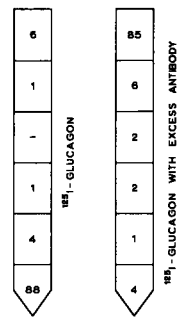


Fig. 23.
Counting results in percentage after wick chromatography. Details are as given in this chapter.

From Fig. 23 we can see that after wick chromatography of free hormone and hormone with excess antibody, the free undamaged labeled hormone remains at the point of application, while the antibody-bound hormone migrates to the top.

Ethanol and polyethyleneglycol 6000 (PEG)

The glucagon antibody complex may be precipitated by ethanol or polyetheleneglycol. A suitable final ethanol concentration was found to be 76% by Edwards et al. (1970) and 80% by Heding (1971), a suitable final polyethyleneglycol 6000 concentration was 12.5% as found by Henquin et al. (1974). After incubation for 48 hours at 4°C of ^{125}I-glucagon and antiserum K 1711 (final dilution 1:10,000), normal bovine serum was added (varying from 0 to 300 μl). Immediately after mixing, either 96% ethanol was added at room temperature (final concentrations 60, 67.5 or 75%) or 25% solution of PEG (final concentration 12.5%) at 4°C.
After 15 minutes the tubes were centrifuged for 20 minutes at 2000 x g. The supernatant was decanted and the tubes left upside down on soft paper to ensure drainage of the supernatant, and the precipitate was counted.
Figs. 24 and 25 illustrate the effect of raising the volume of NBS added to buffer samples. As illustrated in Fig. 24, the final plasma volume necessary for a complete precipitation of the glucagon antibody complex is dependent on the final ethanol concentration. With both ethanol (not shown) and PEG (Fig. 25) a slight increase of precipitated ^{125}I-glucagon was observed in the absence of antibody. Yet the precipitation due to antibodies remained fairly constant, provided a plasma volume of at least 150 μl was present in the assay tubes before PEG addition (final concentration 12.5%) or 200μl before ethanol addition (final concentration 75%).
There was no effect of the time period between addition of ethanol (or PEG) and centrifugation upon ^{125}I-glucagon precipitation.

This indicates the absence of any physico-chemical disturbance in the bound-free hormone equilibrium by ethanol or PEG addition.

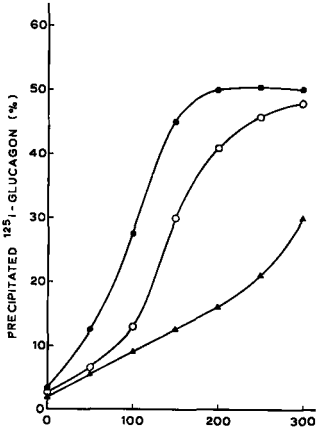


Fig. 24.
Effect of protein concentration upon precipitation of ^{125}I--labeled glucagon bound to antibody of a final ethanol concentration of 60% (▲), 67.5% (O) and 75% (●).

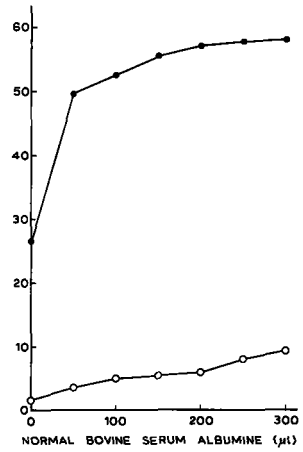


Fig. 25.
Effect of protein concentration upon precipitation of ^{125}I-labeled glucagon (O) and ^{125}I-labeled glucagon bound to antibody (●) of PEG.

There was no effect of the time period between addition of ethanol (or PEG) and centrifugation upon ^{125}I-glucagon precipitation. This indicates the absence of any physico-chemical disturbance in the bound-free hormone equilibrium by ethanol or PEG addition.
Using these carefully selected conditions, identical values were found for precipitated ^{125}I-glucagon in buffer samples with the four separation methods mentioned above. However, there are some restrictions upon the use of these methods. The dextran-coated charcoal method is very sensitive to the protein concentration in tubes which have to be assayed. This amount should be constant in all tubes. Consequently, in tubes containing unknown quantities of serum or plasma spuriously low or even "negative" values may be

observed. It also needs careful control of the time exposure. Wick chromatography is well-suited for single determinations; it is too laborious for routine experiments. However, it meets the requirements for a satisfactory separation of free from bound hormone, without the sources of error, mentioned above and thus it is well-suited as a control of other separation techniques.

With precipitating techniques such as ethanol and polyethyleneglycol, where free radioactive hormone cannot be isolated, it is very difficult to make a correction for the so-called "damage" (the initial radiation damage and the further destruction of ^{125}I-glucagon occurring during incubation). In media which may contain appreciable proteolytic activity, the destruction may be so marked that an assay, which does not correct for this "damage", will result in very high values (false low precipitate count).

The dextran-coated charcoal method was very useful for preparing standard glucagon solution in the same medium as the one which had to be assayed. Damaged hormone will be adsorbed to the proteins in supernatant and free hormone will be adsorbed to the dextran-coated charcoal.

Thus when full standard curves in the various media are set up an accurate determination of the percentage "damage" in each medium is obtained. However, for routine experiments it is too laborious. It was concluded that of all methods tested, the PEG method was to be preferred for routine experiments in our assay system.

3.5 SENSITIVITY, PRECISION AND REPRODUCIBILITY OF THE STANDARD CURVES

After selecting the optimum conditions for producing a standard curve for pancreatic glucagon in buffer samples, standard curves with antiserum K 1711 were obtained as shown in Fig. 26. From these curves the sensitivity and precision was calculated.

Statistical analysis of the results was performed according to Snedecor and Cochran (1967). The results are given in Table 7.

The within assay precision was expressed by the coefficient of

variation (cv) of triplicate determination. The reproducibility or between assay precision (tested by repeated estimation on aliquots of standard glucagon) was calculated as the coefficient of variation of mean of triplicate determinations. Comparison of different standard curves performed during 5 consecutive assays was hampered by the use of different ^{125}I-glucagon batches. However, a high reproducibility of the standard curve was obtained, when expressed as a percentage bound at zero quantity of unlabeled hormone (Fig. 27).

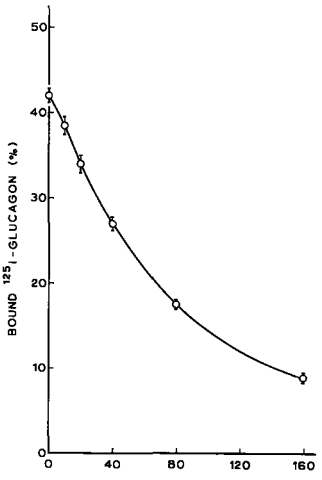


Fig. 26.
Intra-assay variations for glucagon determination. Displacement curve was obtained with antiserum K 1711 as given in chapter 2.3.2. Each point represents the mean ± S (estimate of SD) of tri-triplicate determinations.

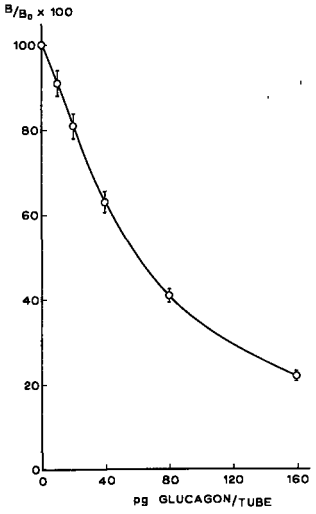


Fig. 27.
Inter-assay variations for glucagon determination. Displacement curve was obtained with antiserum K 1711 as given in chapter 2.3.2. Each point represents the mean ± S (estimate of SD) of 5 consecutive assays.

The sensitivity was taken as 2 standard deviation (SD) from the % bound at a certain quantity of unlabeled hormone. The SD was obtained from 5 consecutive assays. The qualitative sensitivity of

the method or the least amount of glucagon measured which is distinguishable from zero, can be calculated from the formula $\frac{ts}{N}$, where s is the standard deviation, t is derived from student t tables when p is < 0.05 and N is the number of replicates performed on each sample.

Table 7 Estimation of the precision and sensitivity at different points on standard curves for pure glucagon (n = 5)

Amount of glucagon added (pg/tube)	Within assay precision (%)	Between assay precision (%)	Qualitative sensitivity over the range considered (pg/tube)
40	6.1	8.7	6.4
80	3.9	5.2	11.8
160	5.4	6.5	

The qualitative sensitivity enabled us to distinguish in buffer glucagon concentrations of $\geq$ 5.5 pg/tube.

3.6 PLASMA ASSAY SYSTEM

The measurement of pancreatic glucagon concentrations in plasma is complicated by the presence of different forms of glucagon immunoreactivity (3.6.1), proteolytic enzymes (3.6.2), artifacts generated by the assay procedure (3.6.3.1) and various serum proteins or other serum substances which interfere (non)-specifically in some assays (3.6.3.2).

3.6.1 Different forms of glucagon-like immunoreactivity (GLI) and their binding to antiserum K 1711

a True glucagon

A 29-amino acid peptide hormone (MW 3485), originally extrac-

ted from the pancreas. Antiserum K 1711 had high capacity and high affinity for binding of true glucagon.

b GTG's (gut type glucagon)

Peptides derived from the gut which do not bind to the specific anti-glucagon antisera (directed against the C-terminal sequence of glucagon), but only to non-specific antisera which were also raised against glucagon. One gut glucagon, named gut GLI-1, has been isolated in pure form (Sundby et al., 1976). Antiserum K 1711 showed a cross-reactivity with a crude gut extract of less than 3% (chapter 3.2). The GTG's are found in the post duodenal mucosa and their structure and function are as yet unknown. They are secreted by the intestine after oral carbohydrate loads (Samols et al., 1966).

c PTG's (pancreatic type glucagon).

Peptides, which are of extrapancreatic origin and indistinguishable from true glucagon by varying methods of physicochemical characterization. They bind to specific anti-glucagon antisera. Such antisera should not cross-react with GTG's. The PTG's behave immunologically like glucagon. They were found in the plasmas of pancreatectomised patients (Botha et al., 1977; Muller et al., 1974). We have not yet had the opportunity to examine these plasmas with our specific anti-glucagon antiserum K 1711.

The localization(s) of the "extra pancreatic source(s)" in man is (are) not yet clearly known. Lawrence et al. (1976) reported glucagon-like immunoreactivity in human submaxillary salivary glands. Salivary gland glucagon may be one source of circulating glucagon in pancreatectomized patients. The responses to stimulation and suppression are quite different from pancreatic glucagon.

d IRG's (immuno reactive glucagon).

Peptides which circulate in several forms in plasma in normal human subjects and cross-react with specific pancreatic glucagon antisera. They show marked differences to agents which

stimulate or suppress pancreatic glucagon secretion. Nothing is known of the nature or biological importance of the molecules. The various molecular weight components of IRG are:

Big plasma glucagon (or "interference factor") (Weir et al., 1975; Valverde et al., 1974).

By means of gel filtration these authors have reported a high molecular weight immunoreactive glucagon (MW > 20,000) in the plasma of man which binds to a specific anti-glucagon antiserum (Unger 30K). It might be an unrelated protein, containing an immunologic cross-reacting peptide sequence or glucagon bound to an unidentified large molecular weight protein. However, the possibility remained that it might be a pro-hormone. In an attempt to characterize the cross-reactivity of the high molecular weight component with our specific anti-glucagon antiserum K 1711, we have gel filtered blood samples from normal human subjects.

Blood samples were collected in tubes containing aprotinin (500 KIU/ml) and EDTA (1 mg/ml) and centrifuged after standing for one hour at 4°C. Plasma (2.0 ml) was gel-filtered immediately (without any prior freezing) on a Sephadex G-50 column (13 x 300 mm) (Fig. 28).

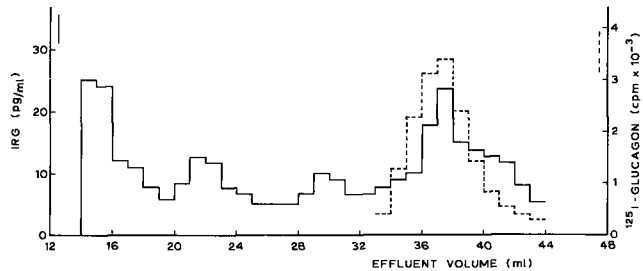


Fig. 28.
Normal plasma IRG pattern on a Sephadex G-50 column. Blood was drawn from a normal human subject after an overnight fast and collected with the addition of aprotinin. The position of the marker radioactive glucagon is indicated.

Elution was carried out at 4°C with 0.1 M NH_4HCO_3 (pH 8.0), containing 0.25% bovine serum albumin (Poviet) and 60 KIU aprotinin/ml.

There is a major IRG component of molecular weight > 20,000 with several smaller components including a 3500 molecular weight species similar to pancreatic glucagon.

The assay method has been described previously (chapter 2.3.2). The amount of the varying fractions can vary considerably from plasma to plasma.

After extraction of plasma with ethanol (1 ml plasma with 1.8 ml 96% ethanol) the IRG component(s) of high molecular weight disappeared.

Pro-glucagon

Kuku et al. (1976) identified an IRG component in the 9000 MW range in patients with chronic renal failure. This component is also present in patients with glucagonomas (Valverde et al., 1976). It is probable that this component is also present in normal plasma in very low concentrations and thus cannot be detected by gel filtration. Limited tryptic digestion of the 9000 MW component resulted in an increase of the 3500 MW component and the appearance of smaller fragments (suggesting the presence of a pro-hormone). Gel filtration of plasma from patients with chronic renal failure in 3 M acetic acid revealed essentially the same 9000 MW component, suggesting that it represents no simple aggregates of 3500 MW glucagon (Jaspan et al., 1976). We have not yet had the opportunity to estimate the immunoreactivity of the 9000 MW component with our antiserum K 1711.

Small glucagon

The 2000 molecular weight fraction is a minor component in normal human plasma (Valverde et al., 1974). We have not been able to detect this component in untreated normal human plasma by gel filtration, probably because of its low concentration (5-10 pg/ml) or its low immunoreactivity with our antiserum K 1711.

Valverde et al. (1974) showed that its concentration may increase in plasma in which proteolytic activity has not been fully inhibited, in which case it would represent an in vitro degradation product. This interpretation would be compatible with our studies (chapter 3.6.2).

3.6.2 Proteolytic enzymes

It has been known for some time that labeled pancreatic glucagon could be degraded in plasma by proteolytic enzymes (Unger et al. 1961) and that this degradation could be reduced, but not eliminated, by the addition of apoprotinin. Therefore, "damage controls" (incubation without antiserum) must be included in each assay for each plasma. However, correction can only be made for a degradation of pancreatic glucagon and not for other IRG (and /or GTG) components which are immunoreactive before and/or after degradation towards a specific anti-glucagon antiserum. When blood was collected from the same person in tubes without the addition of aprotinin and subjected the plasma to gel filtration, after centrifugation and standing for one hour at room temperature, a completely different elution profile was obtained (Fig. 29). Gel filtration was performed in a similar way as described for the gel filtration of plasma with the addition of aprotinin.

A remarkable difference as compared with Fig. 28 is the appearance of IRG components after the ^{125}I-glucagon marker and a

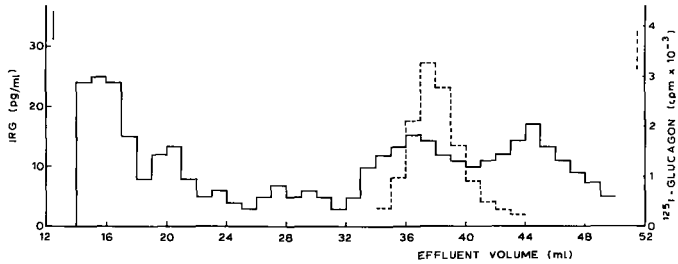


Fig. 29.
Plasma IRG pattern on a Sephadex G-50 column. Blood was drawn from a normal human subject after an overnight fast and collected without the addition of aprotinin. The position of marker radioactive glucagon is indicated.

peak-widening near the glucagon marker.

This increased immunoreactivity might be the result of degradation of IRG (and/or GTG) components of high molecular weight and/or degradation of the 3500 molecular weight species.

The small glucagon components did not show a dilution effect; this suggests an immunoreactivity identical with that of pancreatic glucagon.

Administration of purified gut GLI-1 to plasma without the addition of any proteolytic inhibitor also showed an increased immunoreactivity in a molecular weight zone near the glucagon marker. In this case there was a dilution effect.

To determine whether immunologically active fragments could be produced from glucagon itself, glucagon was digested with trypsin as described in chapter 2.5. The immunoreactivity of the total digests decreased. Only 20% of the initial immunoreactivity remained after incubation with trypsin. From these results it was concluded that an increase of the immunoreactivity in the low molecular weight zone stems mostly from the IRG (and/or GTG) components of high molecular weight.

This degradation by proteolytic enzymes is not completely inhibited by the addition of aprotinin and varies considerably from plasma to plasma, neither is it completely inhibited by freezing the plasma samples. Blood samples collected in tubes with the addition of aprotinin (500 KIU/ml) and stored at -20°C after centrifugation also showed an increase with time of immunoreactivity of the IRG components in the low molecular weight zone, although this increase was less pronounced than shown in Fig. 29.

In order to eliminate this increase of immunologically active fragments from higher molecular weight components, aprotinin enriched plasma was extracted with ethanol immediately after blood collection and centrifugation at 4°C. This extraction removes most of the plasma proteins including the proteolytic enzymes. After extraction of plasma with ethanol no increase of immunoreactivity in the low molecular weight zone was found with increasing time. However, it cannot be excluded that some of the immunoreactivity measured in the glucagon assay stems from proteolytic degradation during blood collection and centrifugation.

3.6.3 (Non-)specific interference factors

When plasma was assayed from subjects who might be expected to have little or no glucagon in the circulation, significant responses were frequently seen. We examined the nature of these responses and tried to find ways of minimizing these non-specific responses.

3.6.3.1 Non-specific interference factors related to the plasma assay procedure

a Anticoagulants

Certain anticoagulants may interfere with the immunochemical reaction as well as with the separation of antibody-

-bound and free hormone (Henderson, 1970). Since heparin is a strongly charged polyanion it might be expected to exert a salt effect and thereby inhibit the antigen-antibody reaction.

An inhibition of the antigen-antibody reaction was observed by more than 50% at a concentration of 25 U heparin/ml. No interference was found with EDTA.

b Incubation temperature

Temperature has a pronounced effect on the equilibrium constant for the reaction of antigen with antibody. Higher B/F ratios are generally observed when mixtures are equilibrated at 4°C, rather than at room temperature (Berson and Yalow, 1959). A dissociation of antigen-antibody complex of more than 10% was observed when mixtures were incubated at 4°C and the antibody-bound and free hormone were separated after standing for one hour at room temperature. Thus, precautions had to be taken to perform all the incubations and separations at 4°C. When labeled glucagon had to be added after a preincubation of antiserum with standard or unknown, we ensured that times for standing at room temperature of all the incubations were very short or at least equal.

c "Incubation damage" of hormone

Damage to labeled glucagon and endogeneous glucagon may differ for each incubation mixture since plasmas vary in their tendency to damage peptide hormones. However, since plasmas were extracted with ethanol after blood sampling, it was not necessary to set up control mixtures for each plasma.

To ensure that the amount of damage to endogeneous glucagon was small during blood sampling, blood was collected and centrifuged at 4°C and protected against proteolytic degradation by the addition of aprotinin (500 KIU/ml) and extracted with ethanol immediately after centrifuging without

any prior freezing and thawing of the plasma. The tracer was now found to be equally stable in serum and diluent as judged by its subsequent binding to antiserum at the dilutions used in the assay.

d Separation methods

We have already mentioned in chapter 3.4 the problems of separating free and antibody-bound hormone as a source of error.

e Use of glucagon-free plasma

Our main problem in attempts to measure the hormone in plasma by the classical radioimmunoassay was that after eliminating the interference factors, mentioned in this chapter, there were still factors remaining which interfered non- specifically in the assay (see chapter 3.6.3.2e). The sensitivity of the assay was not sufficient to permit dilution of the plasma samples to overcome the interference. Moreover, non-specific effects can only be abolished by dilution when the non-specific effect has a steeper response curve than the standard and this was not the case. The regular quantities of plasma (100 μl) added to the assay tubes in many cases contained less immunoreactive glucagon than the detection limit of the assay even under optimal assay conditions. The addition of greater quantities of plasma resulted in an increase in interference in the assay.

Furthermore, plasma samples from different subjects after being made glucagon-free with charcoal did not yield uniformly a zero glucagon level. Our observations indicate that instead of giving the same binding ratio, different glucagon-free plasmas affect antigen-antibody interaction to a considerably different degree as evidenced by the significantly different variations in binding ratio (Figs. 30 and 31).

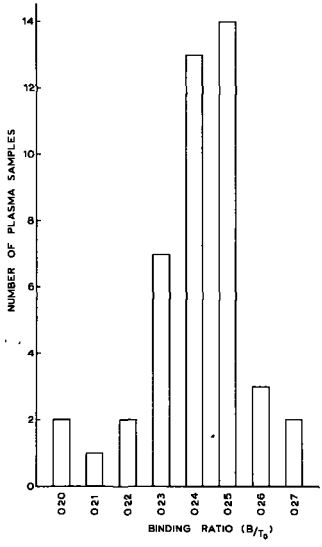


Fig. 30.
Distribution of binding ratios in 44 stripped plasmas. Plasmas were stripped with charcoal and tested with antiserum K 1711 (final dilution 1:10,000).

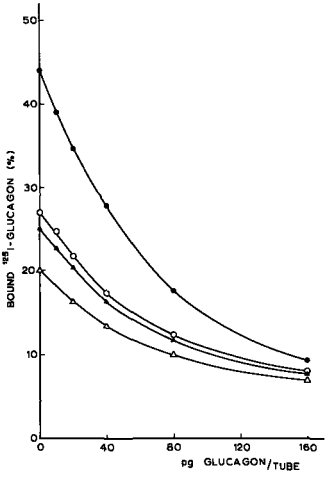


Fig. 31.
Displacement curves set up in buffer (●) and in stripped plasma from three individual patients, A (○), B (▲) and C (△). Plasmas were stripped with charcoal and tested with antiserum K 1711 (final dilution 1:10,000).

As can be seen from Fig. 31 the standard curves obtained from different plasmas were vastly different. The difference in binding was not likely to be caused by different concentrations of proteins or other factors in the plasma, because it was previously demonstrated (chapter 3.4) that separation with polyethyleneglycol was independent of plasma factors over a wide range of concentrations. This was in contrast to the conventional dextran coated charcoal method.

It will be noted that by setting up inhibition curves in 1.0 ml plasma (Fig. 31), these were, when displaced to the right, superimposable on the buffer standard curve. Thus, glucagon can be measured in plasma samples either by setting up full standard curves in each patient's glucagon-free plasma or by subtracting the "apparent" glucagon concentration in the glucagon-free plasma from the "apparent" glucagon concentration in the unknown sample (each assay tube contains 0.5 ml plasma).

Two methods were employed to prepare glucagon-free plasma.

1. Charcoal treatment

   One ml of plasma was treated with 50 mg charcoal and continuously shaken for one hour at 4°C. The tube was then centrifuged at 1500 x g for 20 minutes and the supernatant carefully removed with a Pasteur pipette. If visible charcoal particles remained, the sample was recentrifuged and separated. Immediately after charcoal treatment, plasma was extracted with ethanol and diethylether.

2. Solid-phase coupled antibodies (Alford et al., 1977)

   Firstly, the IgG's from antiserum K 1711 were isolated on a Protein A-Sepharose CL-4B column, described in chapter 2.5. Then the isolated IgG's (80 mg) were coupled covalently to 20 ml (wet volume) cyanogen bromide

activated Sepharose 4B beads (according to Pharmacia, Uppsala, Sweden). Activated CH-Sepharose 4B was freeze--dried in the presence of additives. These additives were removed by washing with about 200 ml 10^{-3} M HCl per gram dry powder. The required amount of freeze-dried powder (1 g gives about 3 ml swelled gel) was suspended in 10^{-3} M HCl. The isolated IgG's were dissolved in coupling buffer ($NaHCO_3$, 0.1 M, containing 0.5 M NaCl, 5 ml per g powder), mixed with the gel and rotated for 18 hours at 4°C. Excess IgG's were removed by washing with coupling buffer and the remaining active groups were blocked with ethanolamine (1 M, pH 9).

Immediately before use, the antibody beads were washed with alternating 0.5 M acetate - 0.5 M NaCl (pH 4) and 0.1 M bicarbonate - 0.14 M NACl (pH 8) on a sintered glass filter. One hundred μl of 1:1, beads:bicarbonate--NaCl (pH 8) buffer were dispensed into 300 μl extracted plasma samples (1 ml plasma was extracted with ethanol and diethylether and after drying under nitrogen dissolved into 300 μl borate buffer, 0.1 M, pH 8.6, including 0.25% bovine albumin, 10^{-4} M merthiolate and 60 KIU aprotinin/ml), mixed for 16 hours at 4°C and separated by centrifugation (see chapter 3.6.4g).

The supernatant was assayed by radioimmunoassay after centrifugation.

Antibody activity was less than 5% in the washings of the solid matrix after each coupling reaction.

Time course at 4°C of binding of ^{125}I-glucagon to the antibody Sepharose complex is shown in Fig. 32. The variation in recovery in 10 plasmas was small (5%). Table 8 demonstrates that the recovery of ^{125}I-glucagon was independent of the plasma concentration of native glucagon over a broad range of concentrations.

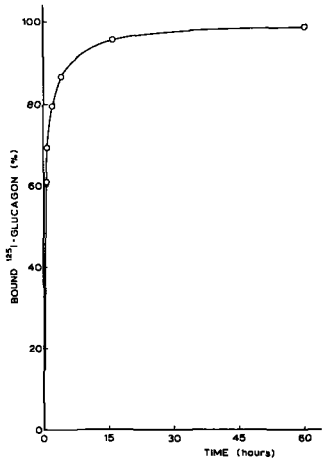


Fig. 32.
Time course of binding of ^{125}I-glucagon at 4°C to 100 µl wet volume of Sepharose antibody complex in plasma.

Table 8 Extraction of ^{125}I-glucagon from plasma in the presence of native glucagon

Glucagon recovered (in % of added dose) in:	Amount of glucagon added (pg/ml)				
	0	100	200	500	1000
supernatant	25.7±0.8	26.1±0.7	25.0±0.4	24.4±0.8	25.2±0.6
extract	73.2±1.8	72.8±1.4	74.0±1.3	73.8±1.3	72.5±1.0

The figures express the percentage of added radioactivity found in plasma supernatant and extract (mean ± SD of four extractions) after an incubation time of 3 hours with an excess of the antibody Sepharose complex (100 µl).

In 20 plasmas the results obtained with the charcoal method were compared with the solid-phase coupled antibodies method. Our results were comparable with those of Alford et al. (1977). Charcoal-treated plasma appears to

be "peptide-free plasma"; this results in some plasmas having less (non-)specific interference. This indicates that when glucagon in plasma was measured by subtracting the "apparent" glucagon concentration in the glucagon--free plasma from the "apparent" glucagon concentration in the unknown sample, higher glucagon values were found for plasmas made glucagon-free with the charcoal method than with the solid-phase coupled antibodies method.

3.6.3.2 (Non)-specific interference factors in plasma

a pH

No significant pH dependency of the dissociation of the antigen-antibody complex was observed in the range 7-8.5. Nevertheless, we think it is desirable to maintain a constant pH for both standards and unknowns.

b Ionic environment

The effect of electrolytes on the association and dissociation of antigen-antibody complexes have been studied with several analytical methods. High concentrations of certain salts, particularly NaCl, may inhibit some hormone-antibody reactions. We assessed the effect of sodium chloride (50 to 1000 mmol x L^{-1}) in buffer samples. The concentration of sodium chloride was found to be of importance only at concentrations exceeding 500 mmol/l.

c Concentration of glucose, urea and albumin

The interference with the binding of labeled glucagon to antiserum K 1711 of buffer samples with increasing amounts of glucose (5 to 20 mmol x L^{-1}) and urea (5 to 50 mmol x L^{-1}) was also assessed. No interference was found in the range examined. As seen in chapter 3.3, increasing the albumin concentration in the incubation mixture above 0.1% did not influence the antibody reaction and in all further experiments at least 0.1% albumin has been used.

d Lipids

Lipids disturb the procedure by giving an unmanageable residue on drying down the plasma after an ethanol extraction, especially when the plasma is assayed in a 1:1 dilution. Extraction with diethylether, after an ethanol extraction of plasma and evaporation of the ethanol under nitrogen, suppressed by 40-70% the interference with the binding of ^{125}I-glucagon to antiserum K 1711.

Fig. 33 shows the effect of extraction with diethylether on assaying 1.0 ml plasma.

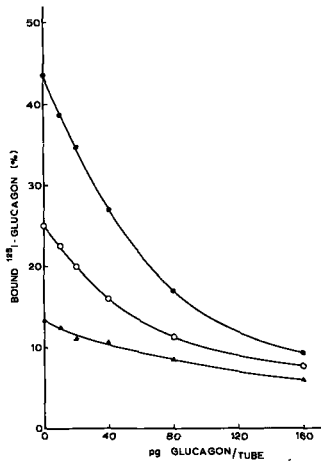


Fig. 33.
Comparison of displacement curves in buffer (●), and in plasmas extracted with ethanol only (▲) or with ethanol and diethylether (○). Displacement curves were obtained with antiserum K 1711 (final dilution 1:10,000). Experimental details are as given in chapter 2.3.2.

Glucagon, added to lipid-free plasma, obtained after ultracentrifugation on a density of KBr of 1.25 was 100% recoverable after a diethylether extraction, measured with the RIA procedure.

Glucagon showed no oxidation products, when glucagon in buffer was treated with peroxide-free diethylether dried down under nitrogen and analysed with an amino acid analysator, as described in chapter 2.1.3.

e Plasma proteins

Finally, there is the non-specific GLI-effect of plasma proteins of high molecular weight, especially the gamma--globulins (Unger and Eisentraut, 1968). The effect can be eliminated by removing these proteins with the already mentioned ethanol extraction method (Heding, 1971). The recovery of labeled glucagon added to plasma and treated with 65% ethanol (1 ml plasma + 1.8 ml 96% ethanol) was more than 95%.

After taking all the precautions mentioned above there were still (non)-specific interference factors in plasma. We investigated whether this phenomenon was introduced during the reaction between (by interference with) antigen and antibody.

In the first experiment the time allowed for the first incubation, i.e. of standards and unknowns with antibody before the addition of tracer was varied between 24 and 72 hours (Table 9).

Table 9 Effect of the duration of the first incubation on measurements of glucagon-like immunoreactive material in ethanol extracted plasma, made glucagon--free with solid-phase coupled antibodies (chapter 3.6.3.1e)

Duration of incubation (hours)	Bound (B;%)	Slope (logit/log)	Apparent content of glucagon (pg/ml)
24 + 48	35.2	- 0.585	85
48 + 48	36.0	- 0.725	62
72 + 48	35.6	- 0.882	55

B_o: tubes containing tracer and antibody in buffer but no standard

As expected, the value of B_o was constant with varying times of the first incubation. From Table 9 it can further be seen that the glucagon-like immunoreactive material seems to decrease, as the sensitivity of the assay improves.

The second experiment in which the second incubation, i.e. between the addition of tracer and the PEG separation stage was varied with time, was then investigated (Table 10).

Table 10 Effect of the duration of the second incubation on measurements of glucagon-like immunoreactive material in ethanol extracted plasma, made glucagon-free with solid-phase coupled antibodies (chapter 3.6.3.1e)

Duration of incubation (hours)	Bound (B;%)	Slope (logit/log)	Apparent content of glucagon (pg/ml)
72 + 24	25.4	- 0.810	52
72 + 48	35.0	- 0.850	55
72 + 72	42.3	- 0.820	60

B_o: tubes containing tracer and antibody in buffer but no standard

As expected the percentage of tracer bound increased with increasing times of the second incubation. However, there was a relatively small difference for the second incubation in the value of glucagon-like immunoreactive material in glucagon-free plasma.

From this experiment it might be concluded that there was no non-specific interaction between tracer and plasma. A probable explanation for the (non-)specific effect is that plasma contains components, presumably small proteins or peptides, which contain structures which resemble the antigenic determinants of glucagon and compete with glucagon

for the antiserum. In the following section we describe attempts to extract these components from plasma.

3.6.4 Methods of extraction of (non)-specific interference factors from plasma

Because our glucagon-RIA was influenced by factors in plasma that interfered (non-)specifically in the assay, it was tried both to eliminate these factors and to extract glucagon from plasma with the following techniques:

a Precipitation technique

A commonly used method is to pour plasma into alcohol or acetone since most of the smaller peptides present in small amounts are soluble in the supernatant, while most of the proteins are precipitated. Centrifugation removes the precipitate and the organic solvent can be removed by drying down. It is important to make sure that the organic solvents are free of peroxides because glucagon is easily oxidized. Another problem is that fat is carried through with the peptide hormone and this gives unmanageable residues on drying down. An extraction of the watery phase with diethylether before drying down under nitrogen is the best solution. An extraction of plasma with acetone is not possible, because glucagon is co-precipitated.

The overall recovery of this precipitation technique was more than 80%. However, as seen in chapter 3.6.3 there still remain factors in the ethanol extracted plasma that interfere non- specifically in the assay.

b Dialysis

Since many peptide hormones are small enough to pass through Visking cellophane (MW of retention about 10,000), use hase been made of dialysis to separate the peptide hormone, glucagon, from proteins. However, the recovery of ^{125}I-glucagon

added to plasma was very low, probably because of adsorption on cellophane.

c Ultrafiltration

Ultrafiltration is a process of selective molecular separation. In our ultrafiltration equipment (thin-channel system), the plasma is passed across the membrane through shallow channels by pumping pressure. Two diaflo membranes with selective pore size were used, PM 10 with a nominal MW cut-off 10,000 and XM 50 with a nominal MW cut-off 50,000. In both cases the recovery of ^{125}I-glucagon added to plasma was very low (30%), probably because of the difficulty in ultrafiltrating solutions with a high protein content, like plasma.

d Gel filtration

The use of molecular sieves like Sephadex has been described in chapter 3.6.1. Chromatography of plasma glucagon immunoreactivity is very useful in excluding glucagon-like immunoreactivity of high molecular weight. It is, however, too laborious for routine experiments. It is of diagnostic value in patients with suspected hyperglucagonemia, because the high molecular weight GLI's can be excluded as the cause of the hyperglucagonemia.

e Electrophoresis

Electrophoresis may be another possibility of removing interference factors from plasma based on a possible difference in mobility. Plasma was obtained after blood was collected in tubes containing aprotinin (500 KIU/ml) and EDTA (1 mg/ml) and centrifuged at 4°C. The supernatant was dried down after centrifugation and then dissolved in 200 μl distilled water. Disc electrophoresis was performed by the method described in chapter 2.1.3 with slight modifications according to Botha et al. (1977). It was carried out with 10% polyacrylamide gels at 4°C in Tris-glycine buffer (pH 8.3). The gels containing

the patient's extracts were cut into sections of 3.5 mm thickness. Each section was extracted with 0.1 N NH_4OH at 4°C for 16 hours. The extracts were lyophilized. After lyophilization the extracts were reconstituted in 350 μl assay buffer (0.1 M borate buffer, pH 8.6) for a glucagon assay with antiserum K 1711.

Glucagon and ^{125}I-glucagon were electrophorized simultaneously with the plasma extract (Fig. 34).

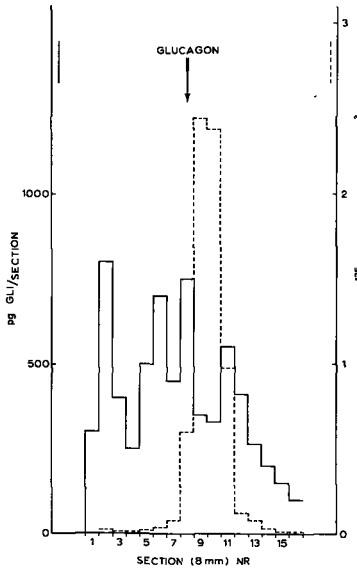


Fig. 34.
Elution pattern of GLI in plasma compared with pancreatic glucagon and ^{125}I-glucagon after polyacrylamide gel electrophoresis. This pattern was obtained after cutting the gel in 8 mm pieces. Each piece was extracted with 0.1 N NH_4OH. GLI-content in extracts was determined with a radioimmunoassay as given in chapter 2.3.2. The positions of marker radioactive glucagon and pancreatic glucagon are indicated.

On polyacrylamide gel electrophoresis of GLI in plasma from a normal man, four fractions were found: two which migrated more towards the cathode than pancreatic glucagon, one which behaved like true glucagon and one which migrated more towards the anode than pancreatic glucagon. Findings on Sephadex G-50 chromatography (chapter 3.6.1) were similar.

We have no explanation for the observed high levels of GLI found after polyacrylamide gel electrophoresis. Therefore, it was concluded that this method is useful in discriminating different forms of GLI, but is not very suitable for a pancreatic glucagon assay in plasma.

f Non-specific adsorption

Since glucagon circulates in plasma in low concentration in comparison with the high concentrations of proteins, the adsorbents must be quite selective. The following adsorbents were tried: charcoal, quso and silica. The recovery of ^{125}I-glucagon added to plasma was low, except for silica (> 70%). This was caused by desorption loss on the various surfaces. All these surfaces have a high affinity for a wide variety of peptides and proteins, as noted from values found for glucagon in the extracts. These varied with varying dilution, indicating factors in the extracts which interfere non-specifically in the glucagon assay.

g Specific immuno-adsorption

The use of antibodies coupled to a solid phase is a reliable and universally applicable method for the separation of peptides from plasma.

A number of different solid phases have been described including Sephadex, cellulose and agarose. In addition, several techniques have been described for antibody coupling, including diazotization, the use of isothiocyanates and cyanogen halides.

In chapter 3.6.3.1 the use of IgG's coupled to cyanogen bromide activated Sepharose 4B beads for extracting glucagon from plasma was described.

By recovering glucagon previously bound to the antibody-Sepharose bead complex it might be possible to have a procedure to concentrate glucagon and at the same time to remove any non-specific interference in the assay, caused by plasma factors. Glucagon was removed from the antibody-Sepharose beads

using 1 ml of an acid mixture (pH 2.0) : HCl/H_2O/aprotinin (60 KIU/ml)/bovine albumin (0.1%). Using this acid mixture, more than 95% of ^{125}I-glucagon was removed within 30 minutes (Fig. 35).

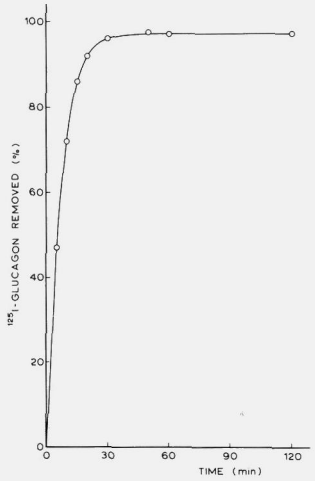


Fig. 35.
Time course of removal of ^{125}I-glucagon from the glucagon-antibody-Sepharose complex by an acid mixture of HCl/H_2O/aprotinin (60 KIU/ml)/BSA (0.1%) (pH 2.0). Percent removed was expressed as cpm in the acid mixture supernatant as compared to total counts on the beads prior to exposure to the acid mixture.

The physical separation of antibody-Sepharose beads and free glucagon was accomplished by centrifugation-filtration using an assembly of plastic test tubes (Fig. 36) (Davies et al., 1977).

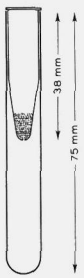


Fig. 36.
Construction of the assembly of test tubes for separation of free glucagon and antibody-Sepharose beads. The outer collection tube is a 6.0 ml polystyrene tube and the inner tube is a 1. 5 ml polypropylene tube with a hole through the bottom of the tube and packed with a small plug of cotton wool at the bottom of the tube.

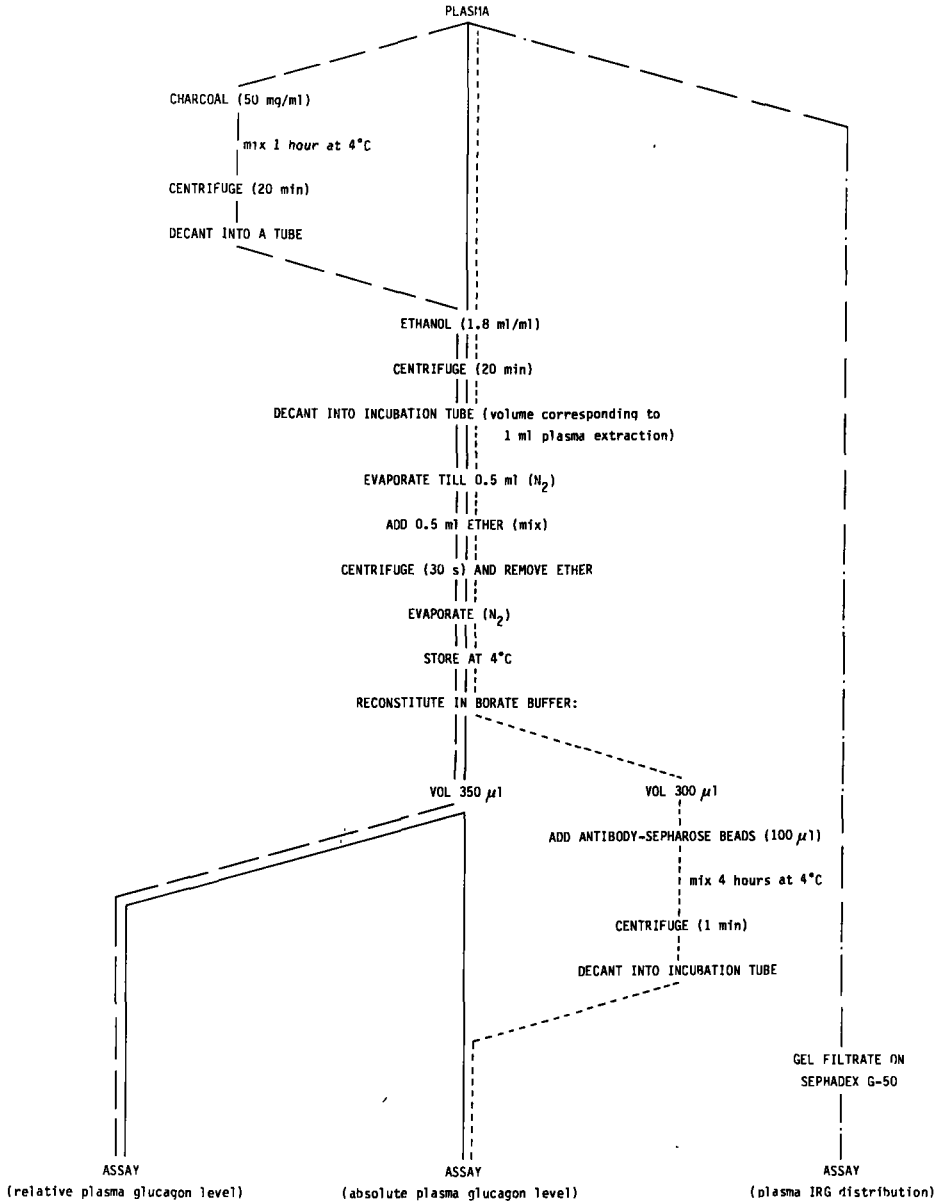
Before the removal of glucagon from the antibody-Sepharose beads, the beads were put into the inner tube and washed three times with aqua dest and centrifuged (1 min, 100 x g). The recovery of glucagon bound to beads was accomplished with 1 ml of the acid mixture, after standing for 30 min at 22°C and centrifuging (1 min, 100 x g). The supernatant was evaporated after neutralization with NaOH and reconstituted in 350 μl borate buffer (0.1 M, pH 8.6). However, recovered glucagon was not 100% immunoreactive. The percentage recovered immunoreactive glucagon varied from 50-80% of the original added immunoreactive glucagon. Three procedures were finally adopted for plasma extraction. These are shown on the diagram on page 113.

3.7 SENSITIVITY, PRECISION AND ACCURACY OF THE ASSAY APPLIED TO PLASMA

Glucagon was measured in 1 ml of an unknown sample of plasma (extracted with ethanol and diethylether) by subtracting the "apparent" glucagon concentration in the glucagon-free plasma from the "total" glucagon concentration in the unknown sample. Glucagon--free plasma was obtained with the antibody-Sepharose bead technique. Fig. 37 shows a standard curve set up in a patient's glucagon-free plasma. Fig. 38 shows the same standard curve after a logit-log transformation. The logit transform function is defined as follows:

$$\text{logit } (B/B_o) = \log \frac{B/B_o}{1 - B/B_o}$$

where B is the bound to total ratio of each sample and B_o is the same ratio obtained in the absence of added glucagon. The logit transform function of B/B_o plotted against the logarithm of the concentration of glucagon will give a straight-line standard curve.



Selected procedures for plasma extraction

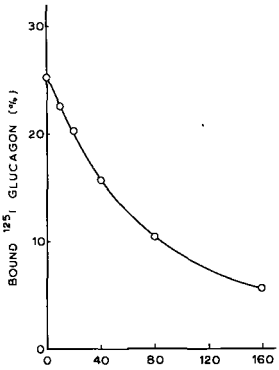


Fig. 37.
Displacement curve set up in a patient's glucagon-free plasma. Stripped plasma was obtained with the antibody-Sepharose bead technique. Plasma was treated with ethanol and diethylether prior to stripping. Experimental details are as given in chapter 2.3.2.

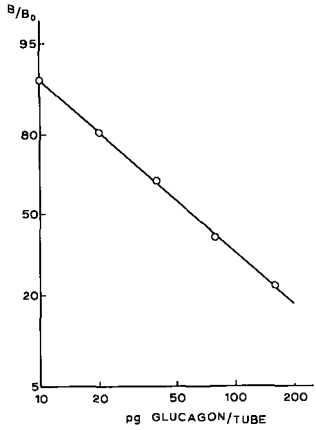


Fig. 38.
Displacement curve set up in a patient's glucagon-free plasma after a logit-log transformation. Details are as given in Fig. 37.

Precision

The within and between assay precision is shown in Table 11. It is expressed by the coefficient of variation (cv) of mean of triplicate determinations.

Table 11 Within and between assay precision of glucagon measurements in plasma

Amount of glucagon added (pg/ml)	Within assay precision (%)	Between assay precision (%)
40	16.0	19.8
80	10.8	16.2
160	9.2	18.6

Sensitivity

The qualitative sensitivity of the method or the least amount of glucagon measured which is distinguishable from zero can be calculated from the formula $\frac{ts}{N}$ where s is the standard deviation, t is derived from Student t tables when p is < 0.05 and N is the number of replicates performed in each sample.

Accuracy

The recovery of known amounts of glucagon added to plasma before extraction with ethanol was determined by radioimmunoassay (Fig. 39). Several dilutions of plasma with a high concentration of exogenous glucagon were assayed. Hormone-free plasma was used for dilution.

The qualitative sensitivity enabled us to distinguish a glucagon concentration in plasma of 12 pg/ml. Fig. 39 shows the recovery of exogenous glucagon added to plasma. The mean recoveries were 62, 70, 72 and 80% respectively for addition of 25, 100, 250 and 500 pg glucagon/ml.

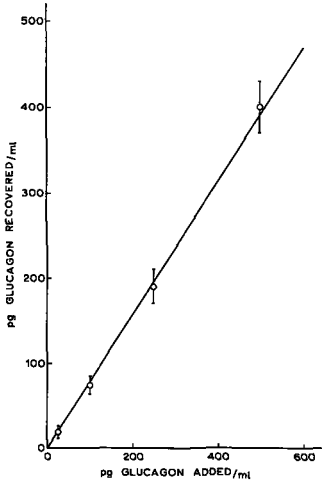


Fig. 39.
Recovery of unlabeled glucagon added to plasma. Each point represents the mean ± S (estimate of SD) of four determinations. Plasma was extracted with ethanol and diethylether.

3.8 CONCLUSIONS

Glucagon in plasma can be assayed by means of antiserum K 1711 with a precision of ± 20%. Necessary precautions are:

- plasma has to be obtained after blood is collected in tubes chilled on ice and containing 500 KIU aprotinin/ml and EDTA (1 mg/ml) and has to be centrifuged at 4°C
- ethanol (1.8 ml/ml plasma) has to be added directly to each plasma sample after centrifugation. Ethanol has to be prechilled on ice
- diethylether, used after the ethanol extraction, should be free of peroxides. It has to be stored in the dark
- drying down of the ethanol and diethylether extract(s) has to be carried out under nitrogen
- plasma extracts have to be stored at 4°C
- glucagon has to be weighed out from a crystalline preparation before iodination
- the carrier-free iodine-125 solution should be centrifuged for a few minutes before use
- the iodination reaction with lactoperoxidase and H_2O_2 has to proceed in the dark
- ^{125}I-glucagon has to be stored at -20°C with the addition of 0.25% (w/v) poviet albumin and 60 KIU aprotinin/ml
- the final purification of ^{125}I-glucagon on a column of QAE--Sephadex A-25 has to be carried out immediately before use. The column has to be eluted at 4°C with a buffer containing 60 KIU aprotinin/ml and 1% BSA (w/v)
- the damage of ^{125}I-glucagon may not exceed 6-7%
- serial dilutions of standard pancreatic glucagon have to be made freshly before use; protected against glass wall adsorption by the addition of 0.25% (w/v) poviet albumin and protected against proteolysis by the addition of 60 KIU aprotinin/ml

- when plasma glucagon is assayed after a gel filtration of plasma on a Sephadex G-50, the column has to be eluted at 4°C with a buffer containing 60 KIU aprotinin/ml
- when plasma glucagon is assayed after a norit extraction, the extraction has to be carried out at 4°C for a maximum of one hour and before the ethanol extraction. After centrifugation the supernatant has to be free of norit particles
- when plasma glucagon is assayed with blanks of each individual plasma, made glucagon-free by affinity chromatography through antiglucagon-Sepharose beads, these beads may not contain any remaining active groups. This has to be checked with the removal of ^{125}I-glucagon from the beads by acid. Excess IgG's should be removed by extensive washing. Incubation of plasma extracts with antiglucagon-Sepharose beads has to be carried out at 4°C. The removal of glucagon from plasma extracts has to be checked each time with a new preparation of antiglucagon--Sepharose beads
- incubation with antiserum and glucagon (standard or plasma) has to be done at 4°C. Incubation buffer should contain at least 250 KIU aprotinin/ml
- when labeled glucagon has to be added after a preincubation of antiserum with standard or unknown, the time for standing at room temperature should be equal for all the incubations
- 150 μl normal bovine serum should be added to the assay tubes after the incubation time and before the separation of "free" and "bound" with PEG. The separation has to be carried out at 4°C

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CHAPTER 4

PLASMA GLUCAGON LEVELS IN SELECTED GROUPS OF PERSONS

4.1 REFERENCE GROUPS

4.1.1 "Normal" fasting plasma glucagon levels

Fig. 1 shows the plasma glucagon levels in ninety fasting, normal subjects (age 20-50 yr). The distribution is slightly skewed to the right with a mean value of 50.3 ± 110 pg/ml. We have found no difference in plasma glucagon level between males and females. Fasting levels of pancreatic glucagon in this assay were much lower than those reported from most other laboratories (Müller et al., 1970; Heding and Rasmussen, 1972; Gerich et al., 1973; Henquin et al., 1974), but were similar to the values obtained by Alford et al. (1977).

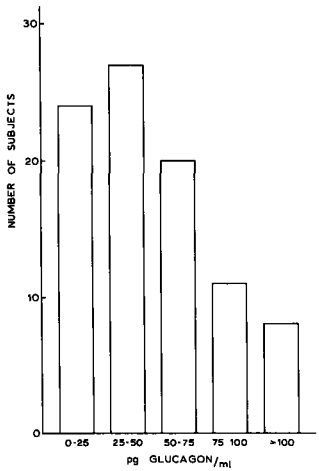

Fig. 1.
Fasting plasma glucagon values in normal subjects; data obtained with antiserum K 1711.

4.1.2 Glucose tolerance tests in normal persons

Oral 50 g glucose tolerance tests were performed on twelve healthy volunteers after an overnight fast. Blood samples were taken immediately before and one hour after glucose administration and collected in tubes chilled on ice.

Glucose and immunoreactive insulin were measured at the Academic Hospital, Leiden. Glucose was analyzed in whole blood by the glucose oxidase reaction and immunoreactive insulin by a RIA. The relation between glucose suppression and insulin stimulation following oral glucose administration is presented in Table 1. It can be seen that insulin is increased and glucagon depressed in most cases, but there is no apparent relation between the changes in both hormones.

Table 1 Suppression of glucagon and stimulation of insulin following oral glucose administration to healthy subjects (age 20-50 yr)

Subject number	Oral GTT					
	Glucagon pg/ml		Insulin μU/ml		Glucose mmol/l	
	Basal	1 h after glucose administr.	Basal	1 h after glucose administr.	Basal	1 h after glucose administr.
1	88	72	7	40	5.7	6.2
2	62	< 10	12	79	4.9	7.6
3	< 10	< 10	10	142	4.6	5.6
4	78	32	7	44	4.7	4.3
5	195	25	9	9	4.2	3.1
6	38	10	8	21	5.0	2.9
7	120	28	7	41	4.6	3.9
8	60	60	< 1	20	4.2	4.4
9	160	30	8	110	4.7	6.5
10	84	36	5	35	5.1	5.1
11	62	10	4	28	3.3	5.6
12	35	30	7	62	3.9	8.6

4.1.3 Arginine tolerance tests in normal persons

Arginine tolerance tests were performed after an overnight fast. An intravenous cannula was inserted in the subject's antecubital vein. Each experiment began with a 30 minutes baseline period during which physiologic saline was infused. Blood was sampled at the end of this period at time t = 0. Thirty grams of L-arginine monochloride dissolved in 300 ml water was then infused over a period of 30 minutes. Blood samples were drawn from the contra-lateral arm at t = 5, 10, 20, 30, 50, 70 and 90 minutes, and collected in tubes chilled on ice. Glucose and immunoreactive insulin were measured and analyzed as described in chapter 4.1.2. Fig. 2 shows that infusion of arginine caused a biphasic release of immunoreactive glucagon characterized in two out of three cases by a sharp first phase and a low sustained second phase.

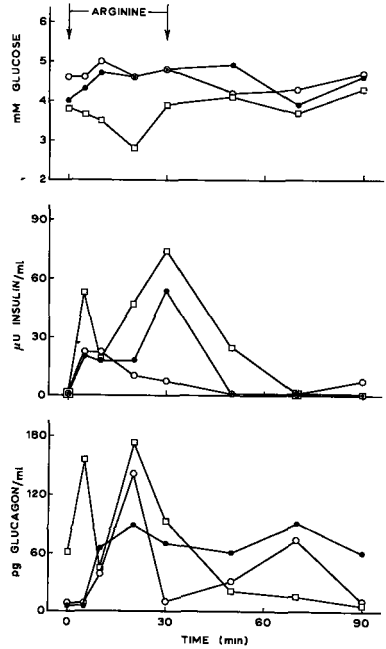

Fig. 2.
Effect of intravenous administration of arginine (30 g/30 min) on venous glucose, insulin and glucagon concentrations in three fasting subjects, (●; ♂, 21 yr), (○; ♂, 26 yr) and (□; ♀, 20 yr).

4.2 PATIENTS WITH HYPERLIPOPROTEINEMIA

4.2.1 Family D

Hyperlipoproteinemia type IIA is inherited as an incompletely dominant trait in which low density lipoprotein (LDL) levels are greatly increased. There is an accelerated rate of coronary artery disease and atherosclerosis in general.

Hyperlipoproteinemia type III (broad beta disease) is an autosomal recessive disease in which the intermediate density (IDL) is often markedly increased.

Advanced atherosclerosis of peripheral and coronary arteries is common.

Family D was studied in our institute (Gevers Leuven, to be published) because traits for HLP type IIA and III seemed to coincide. It has been proposed that the splanchnic hormones and particularly glucagon and insulin play a role in the cholesterol regulation process but the data are scarce and contradictory (Davignon, 1972; Beyers et al., 1975). The glucagon levels of family D, measured in our laboratory, are given in Table 2. It seems that at fasting glucagon levels in affected members are in the normal range.

Table 2 Fasting glucagon levels in some persons with type II and type III hyperlipoproteinemia

Lipid family phenotype	Glucagon plasma level, pg/ml, (number of persons)			
	Spouses		Members of affected	
	Males	Females	Males	Females
normal	10-29(n=3)	<10(n=2)	10-56(n=3)	36(n=1)
HLP type IIA	-	-	-	10-53(n=6)
HLP type IIA + III	-	-	10-32(n=2)	-
HLP type IV	-	-	-	<10(n=1)

4.2.2 Glucagon levels in a type V hyperlipoproteinemic patient

Glucagon levels in a 41 yr old man (110 kg, 172 cms) with hyperlipoproteinemia type V, eruptive xanthoma, hyperglycemia and glucosuria were studied.

Before and after plasmapheresis (carried out in an attempt to reduce insulin resistance) glucagon reponses following insulin adminstration were similar (Fig. 3). Insulin was given intravenously every hour in increasing doses: 11-22-33 units. Glucagon level and blood sugars dropped after the highest dose of insulin only. On plasmapheresis, triglyceride levels dropped from 9000 mg/dl to 3000 mg/dl. Fasting glucagon level, somewhat high before plasmapheresis, was normalized one day after plasmapheresis. Fig. 4 shows the glucagon response following 50 g oral glucose two days after plasmapheresis. There was a striking and unexplained biphasic response of glucagon. The fasting glucagon level was again very high.

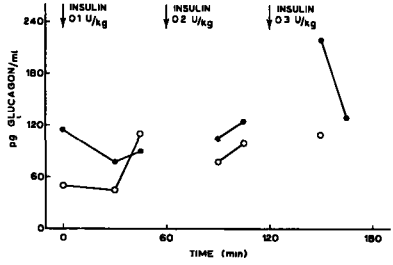

Fig. 3.
Effect of intravenous administration of increasing insulin doses one day before (●) and one day after (O) plasmapheresis on glucagon values in a patient with HLP type V.

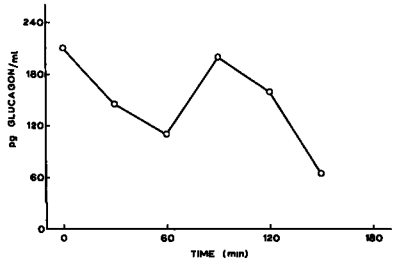

Fig. 4.
Effect of oral glucose administration (50 g) on glucagon values in a patient with HLP type V two days after plasmapheresis.

As can be seen from Table 3 hormone levels fluctuate very rapidly and its seems that glucagon levels in general run parallel to insulin levels.

Table 3 Fasting plasma glucagon, insulin and glucose levels of a type V hyperlipoproteinemic patient

Days after plasmapheresis	glucagon (pg/ml)	insulin (μU/ml)	glucose (mmol/l)	$\frac{\text{insulin}}{\text{glucagon}}\left(\frac{\text{mol}}{\text{mol}}\right)$
13	150	n.d.*	13.0	-
21	65	36	10.2	7.8
24	< 10	12	6.2	28.8
26	52	30	10.0	13.8
28	150	77	10.3	12.4
31	72	32	9.3	10.7

* not determined

Glucose and immunoreactive insulin were measured and analyzed as described in chapter 4.1.2.

4.2.3 Glucagon levels in a homozygous type II hypercholesterolemic child

An 11 yr old girl with the homozygous form of hyperlipoproteinemia type IIA and normal glucose tolerance was treated with diazoxide in an attempt to reduce low density lipoprotein levels (Gevers Leuven, to be published). Hydrochlorothiazide was given as a diuretic. Results of oral glucose tolerance tests (50 g) before and after treatment with diazoxide are given in Table 4. Glucose and immunoreactive insulin were measured and analyzed as described in chapter 4.1.2. The fasting glucagon level seems somewhat high (cf Fig. 1).

Table 4 Effect of diazoxide on plasma glucagon, glucose and insulin levels during an oral GTT in a patient with homozygous type II hyperlipoproteinemia

Treatment	Oral GTT					
	Glucagon pg/ml		Insulin μU/ml		Glucose mmol/l	
	Basal	1 h after glucose administr.	Basal	1 h after glucose administr.	Basal	1 h after glucose administr.
-	150	10	8	44	4.8	6.1
diazoxide	14	13	4	5	13.4	20.8

Apparently, not only insulin but also glucagon section is inhibited by diazoxide. This is in accordance with in vitro data of Urdanivia et al. (1979). LDL levels were not reduced. This lack of effect may be due to suppression of glucagon levels, as glucagon is known to be one of the inhibitors of cholesterol synthesis in isolated hepatocytes.

4.2.4 Glucagon levels in other patients with hyperlipoproteinemia

Hyperlipoproteinemia type IIB is a disease with greatly increased low density lipoprotein and cholesterol levels and moderately increased very low density lipoprotein levels.
Hyperlipoproteinemia type IV is a disease with increased very low density lipoprotein levels and nearly normal cholesterol levels.
Glucose and immunoreactive insulin were measured and analyzed as described in chapter 4.1.2.
Table 5 shows the results of oral GTT's. The glucagon suppression during the GTT in patients with hyperlipoproteinemia type IIB was small.

Table 5 Effect of oral glucose administration on glucagon, insulin and glucose levels with patients suffering from hyperlipoproteinemia

Hyperlipoproteinemia phenotype	Oral GTT					
	Glucagon pg/ml		Insulin μU/ml		Glucose mmol/l	
	Basal	1 h after glucose administr.	Basal	1 h after glucose administr.	Basal	1 h after glucose administr.
IIB	90	87	50.0	50.0	n.d.*	n.d.*
IIB	40	36	14.0	167.0	4.4	7.6
IIB	106	81	2	180.0	4.4	7.4
IV	74	18	4.0	72.0	5.9	8.4
IV**	200	330	3.0	3.0	13.9	19.1

*not determined
**with diabetes mellitus.

4.2.5 Conclusions

1. Glucagon levels are clearly abnormal in some persons with hyperlipoproteinemia, especially those with elevated triglyceride levels.
2. Fasting levels of glucagon in the normal range do not imply normal responses to glucose intake.
3. The glucagon assay developed in this thesis is suitable for further investigations into the relation between hyperlipoproteinemia and glucagon levels (changesis).
4. In further investigations the time course of glucagon responses should be taken into account.

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SUMMARY

Chapter one gives an overall view of the significance of pancreatic glucagon, of several methods to measure plasma glucagon and of the problems to be considered in the development of a specific glucagon radioimmunoassay.

Chapter two deals with the problem of generating specific glucagon antisera with high affinicy. Antisera to pancreatic glucagon were produced against glucagon derivatives, i.e. 27-S-methylglucagon and glucagon-methionine sulfoxide. These derivatives were also screened on their bioactivity. Furthermore, it describes the screening of these antisera and a method to obtain a more specific and more sensitive glucagon radioimmunoassay by means of affinity chromatography of isolated antibodies.

Chapter three is concerned with the problem of the preparation of ^{125}I-labeled glucagon and its purification, the stability of standard glucagon and its degradation caused by albumin preparations added for its protection, the separation of "free" and antibody-bound glucagon. It deals with the difficulties to characterize antibodies to pancreatic glucagon because the terminology and reality of glucagon and glucagon--like substances are very confused. There is a tendency of rapid enzymatic degradation of native endogenous glucagon and radio-labeled glucagon in plasma. There is (non)-specific interference caused by factors present in plasma or caused by the assay system. The significance of the heterogeneity of plasma glucagon-like immunoreactivity is discussed. Finally, suggestions are made for the extraction of glucagon-like substances from plasma, including glucagon. In order to circumvent the problem of interference a technique has been developed whereby the glucagon content of each plasma is measured against the same plasma, made free of glucagon by affinity chromatography.

In chapter four preliminary applications of this technique are shown. The plasma glucagon response to arginine stimulation and the suppression after oral glucose loading were determined in adults. The role of glucagon in some patients with hyperlipoproteinemia was also investigated.

SAMENVATTING

Het eerste hoofdstuk geeft een overzicht van het belang van pancreas glucagon, van verschillende methoden om plasma glucagon te meten en van de problemen die in overweging genomen moeten worden bij het opzetten van een specifieke glucagon radioimmunoassay.

Het tweede hoofdstuk behandelt het probleem om specifieke glucagon antisera, welke een hoge affiniteit bezitten, op te wekken. Pancreas glucagon antisera werden tegen de volgende glucagon derivaten opgewekt, 27-S-methylglucagon en glucagonmethionine sulfoxide. Deze derivaten werden ook op hun biologische activiteit bekeken. Verder beschrijft het het uittesten van deze antisera en een methode om een specifiekere en gevoeligere glucagon radioimmunoassay te krijgen door middel van affiniteitschromatografie van geïsoleerde antilichamen.

Het derde hoofdstuk houdt zich bezig met het probleem van het maken van ^{125}I-glucagon en zijn zuivering, de stabiliteit van standaard glucagon en zijn afbraak, veroorzaakt door albumine preparaten, die voor zijn bescherming worden toegevoegd, en de scheiding van vrij en aan antilichamen gebonden glucagon. Het behandelt de moeilijkheden om antilichamen tegen pancreas glucagon te karakterizeren omdat de terminologie en het al of niet zijn van glucagon en aan glucagon gelijkende substanties erg verwarrend zijn. In plasma bestaat er verder een sterke neiging tot snelle enzymatische afbraak van natief endogeen glucagon. Er bestaat niet specifieke interferentie, veroorzaakt door plasma factoren of veroorzaakt door de bepalingsmethode. De betekenis van de heterogeneïteit van in plasma aan glucagon gelijkende immunoreactiviteit wordt besproken. Tenslotte worden er enkele methoden bekeken om glucagon en aan glucagon gelijkende substanties uit plasma te extraheren. Teneinde om het probleem van interferentie te kunnen heen gaan, werd een techniek ontwikkeld waarin het glucagongehalte van elk plasma gemeten wordt tegen hetzelfde plasma, hetwelk glucagon-vrij gemaakt wordt door middel van affiniteitschromatografie.

In het vierde hoofdstuk worden inleidende toepassingen met deze techniek getoond. De plasma glucagon stimulering door arginine en de onderdrukking door glucose werden bepaald bij volwassenen. De rol van glucagon in enkele patienten met hyperlipoproteinemia werd ook bekeken.

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CURRICULUM VITAE

Na het behalen van het diploma HBS-B in 1967 aan het St. Stanislaus College te Delft, werd in hetzelfde jaar begonnen met de chemiestudie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen (S_2) werd afgelegd in februari 1972. De studie voor het doktoraalexamen werd voortgezet onder leiding van de hoogleraren Prof.Dr. E. Havinga en Prof.Dr. J.H. van Boom met als hoofdvak organische chemie. Het bijvak klinische chemie stond onder leiding van Prof.Dr. A.A.H. Kassenaar en werd begeleid door Dr. C. Hardeveld, de derde richting was fysisch-chemische scheidingsmethoden. Het doktoraalexamen werd in februari 1975 afgelegd. Daarna werd een aanstelling als wetenschappelijk medewerker verkregen bij het Gaubius Instituut (directeur Dr. P. Brakman) van de Gezondheidsorganisatie TNO en begonnen met het in dit proefschrift beschreven onderzoek.