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Studies on Solid-Phase Peptide Synthesis: The Synthesis of Glucagon

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STUDIES ON SOLID-PHASE PEPTIDE SYNTHESIS:

THE SYNTHESIS OF GLUCAGON

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TABLE OF CONTENTS

ABSTRACT

The feasibility of the stepwise-solid phase approach was
studied in the synthesis of the peptide hormone glucagon. The
choice of this molecule was based upon: (1) the unusual chemical
structure; (2) biological significance.

The main features of the strategy employed are: (1) the use
of Biphenylisopropyloxycarbonyl group (Bpoc) for N^α-amino protection;
(2) the use of $\underline{t}$-butyl based protecting groups for side chains of
the trifunctional amino acids; and (3) the use of $\underline{p}$-alkoxybenzyl-
alcohol-resin as a solid support. At each step of the synthesis
the N^α-Bpoc moiety was removed by treatment with 0.5% trifluoracetic
acid in dichloromethane. Deprotection of most of the side-chain
protecting groups as well as cleavage of the peptide from the
resin at the end of the synthesis was accomplished through treatment
of the protected peptide resin with 50% TFA in dichloromethane in
the presence of carbonium ion scavengers.

The crude synthetic glucagon was purified by gel filtration
and ion exchange chromatography. The pure synthetic glucagon was
isolated in 5.4% overall yield based upon the amount of the first
amino acid anchored to the resin. The similarity between synthetic
and natural glucagon was demonstrated through the use of amino acid
analyses, ultraviolet absorption and fluorescence spectrometry,
polyacrylamide gel electrophoreses, gel filtration and ion exchange
chromatograms. Additionally, the synthetic material was isolated
as crystalline rhombic dodecahedra. Injection of rabbits with three
different dosages of synthetic glucagon did cause a rise in the
blood sugar levels. Within the limits of the bioassay, the maximal
hypeglycemic effect was indistinguishable from that caused by
native glucagon itself.

Note to the reader:

The references are listed after each chapter and are not complete. For the final draft of the manuscript the references will be completed, arranged in the correct order, and listed as a last chapter of the manuscript.

I. RATIONALE FOR SOLID-PHASE SYNTHESIS OF GLUCAGON

Solid-phase peptide synthesis has become a well-established method for the preparation of biologically active peptides and proteins. The feasibility of this method was first demonstrated by the synthesis of a simple tetrapeptide, leucylalanylglycyl-valine. Within only a few years, solid-phase progressed towards the synthesis of chemically more complex peptides such as brady-kinin, oxytocin, and angiotensins. The total synthesis of an enzyme with the activity of ribonuclease A demonstrated for the first time the possibility of assembling amino acids by laboratory techniques into structurally complex molecules posessing specific biological function.

Most solid-phase syntheses are dependent upon the differing acid sensitivity of two categories of protecting groups: the moder-ately labile N^α-amino protecting group such as Boc, as well as the relatively stable side-chain protecting groups and C-terminal anchoring bonds based on benzyl derivatives. The structures of a great number of peptides and proteins are sensitive to the strong acid conditions of liquid HF employed in the removal of the pro-tected peptide chains from the solid support. In addition, strong acid conditions employed in the deprotection steps can give rise to various side reactions which can lead to the formation of peptide byproducts. All of these factors can contribute to the failure of a given synthesis.

The purpose of this work was to explore the use of mild acidic conditions in the stepwise solid-phase synthesis of a pep-tide of moderate size. The peptide hormone glucagon was chosen because of its unusual chemical structure as well as its biological significance. Almost all of the naturally occurring amino acids are present in its sequence with the exception of isoleucine, proline, čysteine and glutamic acid. Twenty-three out of the twenty-nine residues are trifunctional amino acids requiring protection of their side chains. Tryptophan and methionine are

two residues which are sensitive to oxidation and alkylation under acidic conditions and are present at positions 25 and 27, the C-terminal portion of the molecule. Furthermore, there are three aspartic residues in the sequence which may undergo acid-catalyzed rearrangement resulting in β-aspartyl bonds in the final peptide chain. This reaction is sequence dependent and is especially likely at Asp-Ser sequences such as at residues 15 and 16.

Thus far, the successful synthesis of glucagon by the stepwise solid-phase method has not been reported.

Glucagon has been synthesized before in two other labora-tories. The first synthesis, by Wünsch, made use of classical fragment methods, and the second, recently reported by the Protein Synthesis Group in Shanghai, used a combination of classical and solid-phase fragment methods. Peptide chemists consider the Wünsch synthesis to be an outstanding achievement representing a standard of excellence against which other syntheses must be compared. This was a collaborative effort of many people over a long period of time. The solid-phase fragment condensation method employed by the Chinese group is only slightly easier to perform since the fragments must still be prepared in solution.

Generally, peptide synthesis has three goals: 1) to show that a natural product can be synthesized and thereby unambiguosly establish the proposed structure; 2) to furnish the natural product in sufficient quantities to replace the natural source of supply; 3) to enable studies regarding the mode of action of the peptide.

The Wünsch synthesis clearly accomplished the first goal, but even after ten years it was not followed by the further syn-theses of glucagon analogs, either from his or any other labora-tory.

The function of glucagon is that of an essential regulator of carbohydrate, fatty acid and amino acid metabolism in mammals. It promotes the breakdown of glycogen and fat into smaller energy-

yielding components, as well as the use of amino acids for gluconeo-
genesis at the expense of protein synthesis. The bihormonal control
by glucagon and insulin is responsible for maintaining extracellular
concentrations of glucose within a narrow range and is thus required
for the prevention of hypoglycemia. There is compelling evidence
that glucagon plays as important role as insulin in the pathogenesis
of diabetes mellitus.

Very little experimental evidence is available regarding the
molecular basis of glucagon action at its target tissue, the liver.
Chemical modifications are limited to partial chemical degradation
at either end of the molecule, to specific cleavages at selected
sites within the peptide chain, and to derivatization of certain
functional groups of the molecule. In general, the separation of
byproducts produced by a chemical modification of the unmodified
peptide is quite difficult. Interpretation of the experimental
data may, therefore, lead to incorrect conclusions.

If we could synthesize pure, crystalline glucagon by the
stepwise solid-phase method, a quick and simple route for the
synthesis of a number of glucagon analogs would then be available
for the first time. These analogs would be designed to probe the
relationship between the conformation and biological activity of
this important hormone.

In this thesis, I report the synthesis of pure and crystalline
glucagon by the stepwise solid-phase method.

II. GLUCAGON: A REVIEW

Kimball and Murlin[1] were the first to report, in 1923, the presence
of a glucose elevating substance in the pancreas, which they named "glu-
cagon". Although their pancreatic extracts could raise the blood sugar
level of depancreatized dogs by 200%, glucagon was long regarded as an
unimportant hormone, or even simply as an artifact with no hormonal action
of its own. During the past twenty years, however, glucagon has been firmly
established as a true hormone with very important functions.

1. Origin and Biosynthesis

Little is known about the biosynthesis of glucagon. A precursor
of glucagon with a molecular weight of 9,000 has been isolated from man[2]
as well as anglerfish[3]. A 37-amino acid fragment of proglucagon con-
taining the 29-amino acid sequence of bovine glucagon was identified
by Tager and Steiner[4]. The remaining eight residues of the fragment were
located at the C-terminus. In anglerfish, proglucagon is a 78-amino
acid single chain polypeptide from which glucagon is liberated by tryptic
digestion[3].

Fractionation of dog plasma has resulted in the demonstration of
four components which are believed to be related to glucagon on the basis
of immunological cross-reactivity.[4] These include: 1) an immunoreactive
fraction of the correct size, presumed to be true glucagon; 2) an immuno-
reactive substance with a molecular weight less than that of glucagon
presumed to be a derivative of glucagon; 3) an immunoreactive fraction
with a molecular weight in the range of 9,000, presumed to be proglucagon;
and 4) a globulin-sized immunoreactive glucagon with undetermined bio-
logical activity, which has been referred to as interfering factor[5] or
big plasma glucagon[4].

In the pancreas, glucagon is produced[6] in the A-cells of the
islets of Langerhans; that organ also contains insulin-producing B-
cells and somatostatin-producing D-cells. However, morphological evi-
dence shows that the pancreas is not the only source of biologically and
immunologically-active glucagon.[7] A crucial observation in support of

this evidence was dicovery of residual glucagonemia in insulin-deprived dogs following pancreatectomy.[8] Secretory granules closely resembling pancreatic A-cells were identified in the upper gastrointestinal tract of dogs. The physiological significance of extrapancreatic glucagon remains to be determined.

2. Glucoregulatory Functions

Of all the metabolic fuels, glucose is the one that is most nearly maintained at essentially constant concentration in the bloodstream. In order to keep the concentration variation within narrow limits, the rates of both influx and efflux of glucose must be similar. This is accomplished through the coupled insulin-glucagon system, where glucagon regulates endogenous glucose influx into the extracellular space, and insulin regulates glucose efflux from the extracellular space. Control is brought about by the A-cell-B-cell unit of the pancreas, which changes the secretory mixture of insulin and glucagon, and in turn the glucose flux required in meeting fuel needs.

This concept of gluco-regulation[9] is summarized in Fig. 1. During exercise, efflux of glucose into muscle tissue is increased. Hypoglycemia is prevented through a rise in glucagon production, thus maintaining adequate glucose delivery to the central nervous system. Intake of carbohydrates results in increased exogenous glucose efflux which in turn causes a drop in glucagon production. Under normal conditions, insulin prevents hyperglucemia by promoting incorporation of glucose in glycogen and fat. Malfunction of the bihormonal coupled system will result in disorders of nutrient metabolism, such as is observed in diabetes mellitus.

3. The Role of Glucagon in Diabetes

Until recently, diabetes was thought to be primarily an insulin deficiency disease. This belief began in 1889 with the experiments of von Mering and Minkowski[10], and was placed on firmer ground by the historic achievements of Banting and Best[11] in 1922. There is now considerable evidence that glucagon is also involved in diabetes and that the
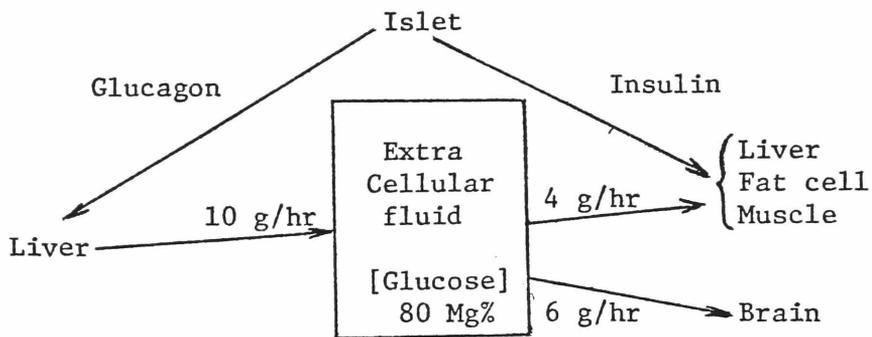
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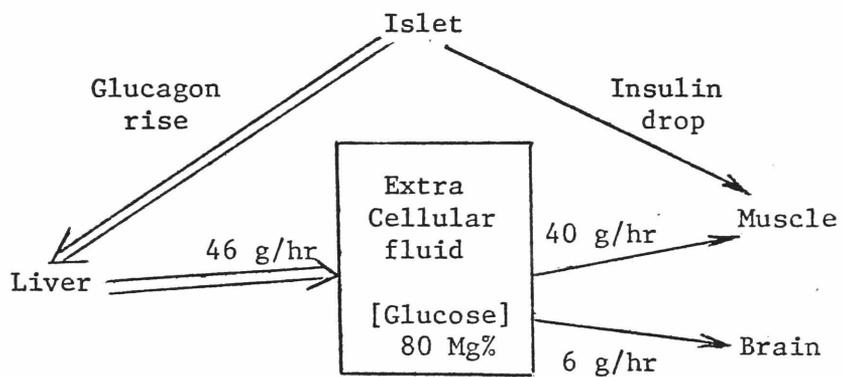
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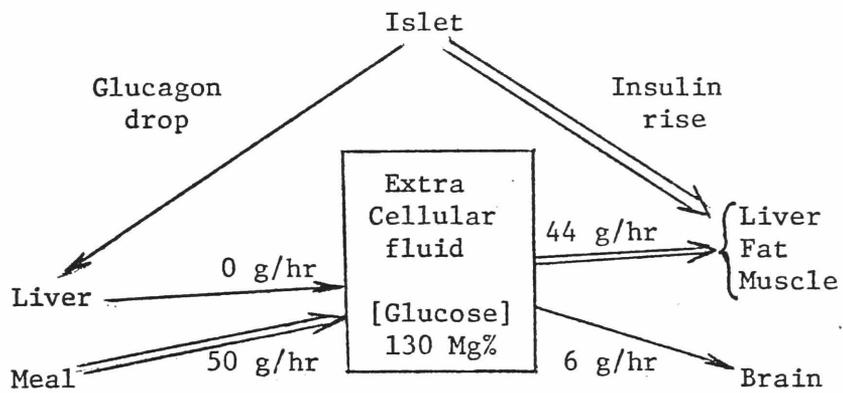
III Carbohydrate

Glucoregulatory function of pancreatic islet cells

unihormonal deficiency hypothesis should be replaced with a bihormonal hypothesis[12]. This hypothesis states that in diabetes, the underutilization of glucose is a consequence of lack of insulin, while overproduction of glucose is a consequence of excess of glucagon. The main evidence for this hypothesis is that hyperglucagonemia is observed in every known form of diabetes. This hypothesis was further supported with the discovery of high circulating levels of immunoreactive glucagon in insulin-deprived depancreatized dogs[8]. An important development in diabetes research was the discovery[13] that the hormone somatostatin blocks both glucagon and insulin secretion, thus suppressing hyperglycemia. When, however, glucagon levels are restored to normal by infusion of exogenous glucagon, a rise in glucose levels is observed, which cannot be prevented by addition of insulin. From these observations, it was concluded that glucagon is more important than insulin in the control of hepatic glucose production. In the diabetic state, somatostatin-induced glucagon suppression reduces hyperglycemia in spite of the presence of exogenous insulin. In summary, therefore, the principal glucoregulatory consequence of the diabetic state may be a reduced rate of glucose removal manifested by hyperglycemia due to a relative lack of insulin. The major effect of a relative glucagon excess may be an inappropriately high level of endogenous glucose production, in relation to the prevailing hyperglycemia.

4. The Molecular Basis of Glucagon Action

After glucagon is released by the A-cells of the pancreatic islets of Langerhans into the circulation it reaches the liver by way of the portal vein. Here is is bound to its specific receptor on the cell membrane and activates the adenylate cyclase system of the cell. To understand the action of glucagon we must first examine the mechanism of its binding and the relationship of the process to that of adenylate cyclase activation, and finally the metabolic consequences of an increase in cellular cAMP levels.

a. Binding of glucagon to the liver cell membrane and activation of adenylate cyclase

Adenylate cyclase of the liver cell is located in the membrane and has not yet been solubilized in an active form. The cyclase is composed of two subunits; the regulatory (receptor) component, and the stimulatory (catalytic) component[14]. The reaction between glucagon and the regulatory component of cell-free liver membranes is highly specific for glucagon and the reaction is rapidly reversible. The reaction in the membrane also leads to activation of the catalytic subunit(coupling) which then catalyses the conversion of ATP to cAMP. The concentration range of glucagon is the same for binding as for activation of the cyclase[15]. The coupling might be due to the regulator acting as an enzyme itself, which modifies the catalytic subunit in a manner similar to the activation of phosphorylase b kinase by protein kinase. Alternatively, the coupling may be via a physical change that activates the catalytic subunit in a manner similar to the activation of protein kinase by cAMP (which causes dissociation of the regulatory subunit and leaves the catalytic subunit active).

The relative position of the two components in the membrane has not been established. However, the regulatory unit appears to be a phosphoprotein and is accessible to the external environment. The catalytic unit is not accessible to trypsin in intact cells and may be situated near the cytoplasmic face of the plasma membrane[16]. There seem to be two reactions between glucagon and the receptor; a weak, reversible binding which does not inactivate the hormone, and an in-activation reaction. The binding reaction is unique for liver, since fat cells require 10 times higher concentrations of glucagon, and it is specific for glucagon. It is not altered by secretin, insulin or ACTH. The evidence shows that the binding is a hydrophobic interaction. Phospholipase A and digitonin selectively diminish the binding and action of glucagon by removal of the lipid from the membrane and the inactivated membrane can be significantly reactivated by addition of membrane lipids or phosphatidylserine[17]. Furthermore, binding is decreased at lower temperature and in low concentrations of urea, which are characteristics of hydrophobic interactions. Unless GTP is present, the binding of glucagon to membranes is slow (15 min) and incomplete,

and dissociation is also slow[18]. The reversibility in the binding
process induced by purine nucleotides means that the regulatory compo-
nent must exist in at least two states of activity. The effect is not
due to phosphorylation by GTP and the site of binding of GTP is not
at the binding site of glucagon[19]. It serves as an allosteric
effector to change the physical relationship between the regulatory
and catalytic omponents.

The rapdily reversible state of the adenylate cyclase system was
demonstrated by the following experiment[14]. Liver membranes were
incubated with high glucagon, but no ATP. The mixture was then diluted
and [^{32}P]ATP added. The rate of cAMP formation corresponded to the
diluted concentration of glucagon, showing that there was a rapid
dissociation of bound glucagon. The rapidly reversible equilibrium
between glucagon and receptor requires the continuous presence of
glucagon in the system. The inactivation process is responsible in
part for the control of the adenylate cyclase system. The product of
inactivation is similar to glucagon chromatographically and therefore
is not a result of enzymatic cleavage into small fragments. The
inactivator may be the regulatory component itself.

Rodbell has pointed out that, "although the structure of the
receptor is unknown, it should be possible to gain insights into
intermolecular forces responsible for binding and action of glucagon
by knowing the structure-function relationships in the hormone"[14].

b. Metabolic effects of adenylate cylcase activation

The net effect of adenylate cyclase activation and cyclic 3',5',-
adenosine phosphate production is to convert the liver from an organ
of glucose storage to one of glucose production. This is accomplised
by decreasing the uptake of glucose by liver cells through the sup-
pression of glucokinase, the inhibition of glycogen synthesis, the
stimulation of gluconeogenesis, and the stimulation of glycogenolysis.
The pathways of these processes are outlined in Fig. 2, redrawn after
a review[20] by Foa.

Figure 2. Action of Glucagon on the Liver Cell.

The primary action of cAMP is to activate protein kinases that then catalyze the phosphorylation of several enzymes and other proteins. Thus, phosphorylase kinase is phosphorylated and thereby activated and goes on to phosphorylate glycogen phosphorylase and glycogen synthetase. These reactions activate the phosphorylase and inactivate the synthetase and result in stimulation of glycogen breakdown and inhibition of glycogen synthesis. This sequential effect of one enzyme on another magnifies the original signal and enhances the effectiveness of the cascade system. Thus, one molecule of glucagon stimulates the production of 10^6 molecules of cAMP and the eventual release of 2 or 3×10^6 molecules of glucose. When the hormonal signal ceases, the enzymes are rapidly deactivated by specific phosphatases and cAMP is destroyed by phosphodiesterase.

Gluconeogenesis from fatty acids and amino acids is also stimulated by cAMP. This second messenger mediates the synthesis and activation of several enzymes that catalyze the urea cycle, the deamination of amino acids and the activation of enzymes, such as pyruvate kinase and phosphoenolpyruvate carboxykinase, which lead to phosphoenolpyruvate and eventual synthesis of glucose. cAMP activation of lipase also leads to gluconeogenesis via free fatty acids and acetyl CoA. Furthermore, glucagon activation of liver adenylate cyclase is reflected in the phosphorylation of histones and ribosomal proteins. DNA is derepressed, genes are activated, transcription and translation are enhanced and protein synthesis is promoted.

The pancreatic B-cell is also a target for glucagon, where release of insulin is promptly stimulated. The mechanism of this action is not known and it is not clear whether glucagon analogs would be effective inhibitors of insulin release. Glucagon has a host of other reported effects that cannot be discussed here.

The reactions described show how glucagon functions as one of the essential regulators of carbohydrate, fatty acid and amino acid levels in the blood. The overall, primary role of glucagon is that of a mobilizer of nutrients in time of metabolic need.

5. <u>Conformation of Glucagon</u>

The conformation of glucagon in solution depends on several factors, including solvent, pH, concentration and time, and it is not the same as found in the crystal. All studies indicate that there is flexibility in the molecule and a delicate balance between different conformational states. The important question of what conformation is assumed when the hormone binds to its specific receptor in the cell membrane is not resolved and is a subject of much interest and debate.

The primary structure of glucagon as determined by Bromer[21] is shown in Fig. 3.

```
 1   2   3   4   5   6   7   8   9   10  11  12  13  14
His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-
 15  16  17  18  19  20  21  22  23  24  25  26  27  28  29
Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr
```

<u>Fig. 3</u> The amino acid sequence of procine glucagon

a. <u>Conformation in solution</u>

Optical rotary dispersion curves of glucagon in dilute solutions (less than 1 mg/ml) at acid, neutral and alkaline pH show that the glucagon molecule possess about 10% of α-helix, which corresponds to one turn of a helix[22]. The shapes of the curves at these different pHs are almost identical, indicating that under these conditions the conformation of the molecule does not depend on the pH of the solution. Addition of 6M guanidine hydrochloride and different concentrations of urea[23] changes the shape of the curve to the one which is characteristic of a random coil. If the dilute solution is in 2-chloroethanol, the molecule becomes about 90% helical.

A ten-fold increase of the concentration of glucagon at pH 10.2 increases the helicity of the molecule to 40-50%[24,25]. The same dependence of the extent of helical conformation upon glucagon concentration is observed in 2-chloroethanol[24]. The lower limit of the concentration-induced conformation is reached at 0.5 mg/ml. Below

this concentration little further change in curcular dichroism is observed[26]. Ellipticity, though, never reaches the values of a fully random coil, as in the case of glucagon in 6M guanidine hydrochloride. The simplest way to interpret the above observation is by assuming the confomational equilibrium between α-helix and random coil. The results with chloroethanol, however, indicated that besides the helix-coil equilibrium some further interactions between glucagon molecules occur and this stabilizes a predominantly α-helical conformation. Blanchard and King[27] from their results on the concentration difference spectra of glucagon, and model manipulation have suggested that at higher concentrations glucagon aggregates in the form of helical trimers. Upon dilution, the molecules dissociate to a non-helical form. On the other hand Swann and Hammes[28] interpreted their difference spectral data, as well as the qualitative results from sedimentation equilibrium and gel filtration to fit a monomer-dimer-hexamer equilibrium. On a qualitative level, though, the results presented in those two studies show that a major conformational change occurs upon self-association of glucagon. By contrast a succinylated derivative of glucagon which contains two extra carbonyl groups arising from acylation of α-amino group and ε-amino group of the peptide does not show concentration dependence of conformation[29]. This means that there are precise steric requirements for the association reaction.

At acid pH, moderately concentrated glucagon solutions on standing at room termperature become viscous and form gels. Upon further standing a precipitate forms. Electron microscopy shows that this precipitate consists mainly of long well-developed fibrils[22]. ORD of films prepared by slow drying of acidic solutions show the presence of β-structure. By IR spectroscopy the structure was further characterized as an antiparallel β-conformation. Gel formation is characterized by changes in the absorption spectrum involving Tyr and Trp residues[24] and the difference is time-dependent. The kinetics of gel-formation may be followed by measuring the changes in viscosity[30]. Viscosity-time curves show the presence of a lag phase which can be abolished by addition of a small seed of preformed glucagon gel into a fresh

glucagon solution. Therefore, the kinetics of initiation and propagation reaction differ. The gel formation is concentration dependent and at concentrations below 1.5 mg/ml the reaction is slow. Ionic strength and temperature increase the rate of formation of fibrils [maximum rate of polymerization is at 30°C]. Aggregation is completely inhibited by 5% dioxane, and acetylated and carbamylated glucagon molecules aggregate without a lag period whereas esterfied glucagon shows no trace of aggregation even after a prolonged standing at 30°C. Thus, the formation of β-structure involves a considerable degree of specificity.

The CD spectra of glucagon also showed a time-dependent transition of α-helical to β-sheet structure[31]. The results are summarized in Table I.

It seems that the most stable conformation of glucagon in solution, is one containing at least 50% β-structure, and this is independent of concentration and pH. These results are in agreement with theoretical predictions[32].

b. <u>Conformation in the crystalline state</u>

The structure of glucagon in the crystalline state has recently been determined by x-ray analysis[33]. It is unlike any protein structure that has been studied before and may provide valuable clues about the way in which the hormone and receptor bind. It was observed that, not only is the glucagon molecule flexible in solution, but it is also flexible in the crystal.

In the crystal the individual glucagon molecules have a distorted α-helical structure, with the N-terminal region somewhat extended, and they are associated in a complex arrangement as trimers, with a cubic symmetry. The hydrophobic parts are packed against each other and the polar side chains are free to interact with the solvent, but there is no globular structure, as found in proteins. From CD spectra a similar structure is found in solution under conditions of aggregation, where glucagon can also form trimers. Glucagon may even be stored in the granules of the A-cell as trimers of similar structure and stability.

Table I. Time-dependent transitions in glucagon solutions

Glucagon Concentration (mg/ml)	Solvent/pH	Distribution of Conformers Time (days)			
		1	5	12	16
1	0.01N HCl	39%β 51%C			82%β 18%α
1.68	0.01N Phosphate pH 9.2	28%α 17%β 55%C	18%α 27%β 55%C	0%α 51%β 49%C	
3	2 Cl-ethanol	59%α 24%β 17%C			
9.14	0.01N HCl	46%α 5%β 49%C		3%α 54%β 43%C	
2.52	93% Propylene glycol	36%α 29%β 34%C	31%α 30%β 34%C	6%α 52%β 42%C	

However, when glucagon is released into the blood, it circulates in such low concentrations (10^{-11}M) that the trimers must dissociate into monomers. Binding to the receptor has been shown to depend on hydrophobic interactions[34] and glucagon has been shown to assume a helical conformation in the presence of hydrophobic surfaces[35,36] or in apolar solvents[22,23]. From these considerations it has been argued that the structure of glucagon when bound to the membrane receptor resembles the structure found in the trimers of the crystal[33].

The x-ray analysis was performed on crystals grown from 0.3% solutions in phosphate buffer at pH 9.2 and soaked in acetate buffer at pH 5.6 to 7.5. Three heavy-atom derivatives were used: Ag^+, $PtCl_4^=$, $Pt(NO_2)_4^=$. They gave similar, but not identical diffraction patterns. Electron density maps show helical regions corresponding to 16 residues of α-helix located between residues 10 and 25, with approximate two-fold symmetry. This helix is extended at each end by 4 residues of less regular right handed helix. The chain between residues 1 and 5 is flexible. The glucagon molecules associate through contacts between side chains of Trp^{25}, Leu^{26}, and Phe^{22} of one molecule and Tyr^{10}, Tyr^{13} and of Phe^6 of the second. This leads to a triangular structure with the central region around a three-fold axis containing solvent and side chains of Arg and Asp. Association around a second three-fold axis leads to contacts between equivalent amino acids Phe^{22}, Val^{23}, Leu^{26}. This hydrophobic region is extended by close packing of side chains of Phe^6 and Tyr^{10} of other molecules, so that the first region of nonpolar contacts is continuous with the second. The result is to bury the hydrophobic regions, and the driving force of the association is this burying of the hydrophobic residues. In the crystalline structure region 1-4 does not form a well-defined helical arrangement and can change its conformation easily.

c. <u>The conformation of glucagon when bound to its membrane receptor</u>

The biological action of glucagon must depend on the conformation which it assumes when bound to its receptor. Despite extensive experimental evidence, it is still difficult to say if it is the helical

structure stabilized by hydrophobic contacts as suggested by the x-ray data, or the thermodynamically more stable β-sheet conformation which the molecule assumes upon standing in solution, as detected by spectral methods. The contribution of both hydrophobic interactions and hydrogen bonding in the β-sheet should contribute a greater free energy of binding than would the α-helical hydrophobic interaction alone.

6. Natural and Chemical Modification of Glucagon

There is a high degree of conservation of the primary structure of glucagon among species and very few useful analogs are available from natural sources[37]. The unit evolutionary period for glucagon has been estimated to be 43; as compared with 4 for growth hormone and 400 for the very highly conserved histone H4[38]. Thus human, bovine and porcine glucagons have identical sequences and the camel, rabbit and rat hormones are also thought to be identical with the others. Duck glucagon differs only by an exchange of threonine for serine at position 16 and of serine for asparagine at 28. Turkey and chicken are probably equivalent and differ from the mammalian glucagons by exchange of a serine for asparagine at residue 28. Shark glucagon differs in only three positions. More extensive changes have been found for the anglerfish which has differences at eight amino acid residues even though the total number remains 29. Guinea pig glucagon may be an exception. This hormone as isolated has a molecular weight of 4,000 to 5,000 and a composition corresponding to 40 residues. It has a much higher pI due to additional arginine and lysine residues. However, like the other mammalian glucagons, it contains tryptophan and an N-terminal histidine. Although other species variations will probably be found, this does not appear to be a fruitful way in which to obtain the structural variants required for structure-function studies or to obtain analogs that can function as inhibitors of glucagon.

Chemical modifications of glucagon have been achieved by partial degradation at either end of the molecule, by specific cleavage at selected sites within the peptide chain or by derivatization of

Table II. Activity of Modified Glucagon

Compound	Receptor binding activity	Adenyl cyclase activity	In vivo activity	Ref.
1. Glucagon	100	100	100	
2. $[^{125}I]$Glucagon	30-300*	90-300*		39
3. Mononitro-Glucagon			100	45
4. Dinitro-Glucagon			100	45
5. N^α-Trinitrophenyl-Glucagon				41
6. N^α,N^ϵ-Bisiodoacetyl-Glucagon	0	0		43
7. Desamido-Glucagon			<100	44
8. [Des-His^1]Glucagon	2	2	2.4	40
9. [Des-His^1,Ser^2]Glucagon	0			46
10. [Des-Asn^{28},Thr^{29}][Hse lactone^{27}]-Glucagon	2-3	2-3		43
11. CNBr-Glucagon-NHNH$_2$	3	3		43
12. CNBr-Glucagon-NHC$_4$H$_9$	3-4	3		43
13. CNBr-Glucagon-NH(CH$_2$)$_6$NH-Biotin		0.1-0.2		43
14. N^α,N^ϵ-Bis-Boc-CNBr-Glucagon	0	0		43
15. [Des-Met^{27},Asn^{28},Thr^{29}]Glucagon	0.5		1.4	42
16. [Des 26-29]Glucagon	0			46
17. Secretin	0			14

* Activity depends on the pH of the assay medium.

certain functional groups of the molecule. These products have been extremely valuable in studies designed to define the role of various regions or individual residues of the structure in determining the conformation of the molecule, in binding to receptors or to antibodies, and in the activity of the hormone. Of course, the changes that can conveniently be made are relatively limited and predetermined by the positions of labile groups in the natural structure. Table II summarizes the structure and activity of some of the glucagon derivatives that have been made in these ways.

The main findings are:

(1) Iodination of tyrosine residues gives an analog with an increased affinity for binding to the receptor and subsequent activation at adenylate cyclase. That shows that tyrosine residues play an important role in the binding and action of glucagon. The effect is dependent on the pH of the incubation medium. From this it was concluded that the ionization of the phenoxy groups disrupts the structure of the hormone, possibly through the loss of hydrogen bonding[39].

(2) [Des-His[1]] Glucagon retains about 10% of the affinity for the receptor and 2% of the adenylate cyclase activity of native glucagon and at saturation can induce 70% of the maximum stimulation of the hormone[40]. Therefore, the histidyl residue plays a very important role in the binding and action of glucagon but does not appear to be absolutely obligatory. The potential use of this derivative as an inhibitor of glucagon action has been pointed out[47].

(3) N^α-Trinitrophenyl-glucagon is exceptional because it contains 100-fold greater binding affinity compared with its activity in the adenylate cyclase assay[41]. In addition N^{im}-His(acrylonitrile)-glucagon, a weak agonist, was also shown to compete with glucagon to lower its activity. It seems likely that appropriate synthetic modifications or substitutions of His[1] could lead to improved inhibitors.

(4) There is a parallel loss of both binding and biological activity when the C-terminal residues Asn[28] and Thr[29] are removed, suggesting

that they are involved only in the recognition process and are not involved in activation of adenylate cyclase[40]. The cyanogen bromide product, of course, contains a modified C-terminal methionine residue and this homoserine lactone may be in part responsible for the observed effects and interpretation. However, the derivative missing Met[27], Asn[28] and Thr[29] that was isolated from a commercial preparation of glucagon[42] has a normal C-terminal carboxyl group and also showed low binding and low in vivo activity. [Des-Met[27],Asn[28], Thr[29]] Glucagon could not be crystallized but did form fibrils, and the CD spectra were different from glucagon. It was concluded[42] that some or all three of the C-terminal residues are necessary for the hormone to assume a helical conformation. Further removal of Leu[26] by N-bromosuccinimide treatment of glucagon gave an inactive product[48].

(5) By reaction of [Des-Asn[28], Thr[29]] [homoserine lactone[27]] Glucagon with amines it was possible to prepare a series of amides[43]. These derivatives, with the C-terminal carboxyl blocked, were approximately equal in cyclase and binding activity with the parent derivation if the amine component was small. When the amine was bulky the activity was greatly reduced, suggesting that steric and lipophilic properties are important factors in determining these activities.

(6) Blocking the two primary amino groups of glucagon with iodoacetyl groups or of cyanogen bromide-cleaved glucagon with Boc groups gave inactive, non-binding derivatives, suggesting that steric or charge differences at these sites prevent receptor binding[43]. In view of the data on the Tnp derivative, it seems likely that the change at lysine was probably responsible for the effect.

(7) Loss of the amide group from one or more of the three Gln residues only slightly reduces activity[44].

The overall conclusion from studies of modified glucagon is that the C-terminal part is primarily concerned with binding to the receptor, while the N-terminal and internal portions of the molecule are important both to binding and to activation of adenylate cyclase. The entire molecule appears to be necessary for full binding.

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III. PEPTIDE SYNTHESIS

1. Introductory Remarks

The field of peptide synthesis was founded by Emil Fischer and Theodor Curtius at the beginning of this century. In 1901, Fischer and Forneau[1] prepared glycylglycine by partial hydrolysis of 2,5-diketopiperazine. In the following year, Curtius[2] described a completely different approach involving treatment of N-benzoyl-amino acid hydrazides with nitrous acid to give azides which were then reacted with amino acids, peptides or their esters; this azide coupling method is still used today in special instances.

Fischer's work clearly anticipated the strategic requirements for the synthsis of non-trivial free peptides (greater than two residues); foremost among them was the development of the easily removable N^{α}-amino protecting group. Although specific chemical problems prevented a general solution along these lines, the power of Fischer's technique is shown by his impressive synthesis[3] of the octadecapeptide, Leu-(Gly)$_3$-Leu-(Gly)$_3$-Leu-(Gly)$_9$ in 1907. The next methodological breakthrough in the field did not occur until the work of Bergmann and Zervas[4], who in 1932, introduced the first successful N^{α}-amino protecting group, the carbobenzoxy functionality. Use of this group allowed the synthesis of such naturally occurring small peptides as carnosine and glutathione, incorporating for the first time, trifunctional amino acid residues. The synthesis of larger peptides with complex amino acid sequences was not possible until two decades later with the introduction of the mixed anhydride coupling method as well as a number of selectively removable protecting groups.

The synthesis of the pituitary hormone oxytocin[5] by V. du Vigneaud and his associates in 1953 marked the beginning of stage in peptide chemistry. About the same time, it was realized that peptides and proteins play a major role in metabolic processes. The amino acid sequences of a great number of peptides and proteins were deduced along with those regions responsible for biological activity of the

molecule. With these discoveries, a new target was set for peptide chemists who now proceeded in two directions: 1) towards the total synthesis of naturally occurring peptides in an effort to prove their amino acid sequences, and 2) towards the synthesis of analogs of these peptides which could be used to probe the mode of action of their parent molecules.

In an effort to achieve the aforementioned goals, three basic steps are employed: (1) coupling, the racemization free creation of a peptide bond; (2) linkage of amino acids in the correct order (specificity), and (3) purification of the desired peptide or peptide intermediates from the starting materials.

Coupling of the carboxyl group of one amino acid to the amino group of another amino acid is generally achieved through activation of the carboxyl component. The peptide can then be built through: (1) stepwise elongation of the peptide chain starting with the N-terminal amino acid; (2) stepwise elongation of the peptide chain starting with the C-terminal amino acid; or (3) condensation of small peptide fragments of proper sequence.

Specificity in peptide synthesis is achieved through the blocking and subsequent deblocking of functionally reactive sites which have been selectively protected.

During coupling and deprotection of peptide chains, a number of side reactions might result. Purification, therefore, requires not only separation of the desired peptide from starting materials, but removal of accumulated by-products. In the synthesis of large peptides, the use of fragment intermediates is often required. However, purification may be difficult due to insolubility of these fragments when fully protected.

Classical peptide synthesis as described above, including isolation of the desired peptide, can be a tedious and laborious process. It was soon apparent that if peptide chemistry were to become a useful tool in biomedical research, a radically new approach was

necessary. In 1959, R.B. Merrifield introduced for the first time, the concept of stepwise solid-phase synthesis[6-12]. Use of this method greatly alleviated many of the problems previously encountered in peptide synthesis.

2. Solid-Phase Peptide Synthesis

Solid-phase peptide synthesis involves the use of an insoluble solid support to which the carboxyl end of an amino acid can be attached. Additional residues are then sequentially attached to the amino end of the growing peptide chain. The advantage of this method is that problems arising from isolation of intermediates during the synthesis and their separation from excess reagents and undesirable products are avoided. This is possible through simple filtration of the reaction mixture: unwanted materials are washed away off while the growing peptide chain remains covalently bound to the insoluble support.

The basic steps outlining the procedure of a simple solid phase synthesis are shown in Figure 4. Beads of polystyrene cross-linked with divinylbenzene to make them insoluble in organic and aqueous solvents are derivatized by reaction with chloromethyl ethyl ether. The cesium salt of the N^α-protected amino acid is then reacted with the chloromethyl group to form a benzyl ester link to the polystyrene-resin. Following this, the N^α-protecting group is removed by treatment with acid in order to liberate the protonated amino group, which is neutralized by treatment with a tertiary amine. The free amino group becomes a nucleophile which can then attack the carbonyl of the new activated amino acid derivative. Deprotection, neutralization, and coupling are repeated until the total amino acid sequence is assembled. The peptide chain is cleaved from the resin and most of the protecting groups removed at this stage.

Steps 1,2,6, and 7 occur only once during a single synthesis and need not be quantitative or rapid. Steps 3,4, and 5 are repetitive and must be quantitative, rapid, and free of side reactions. A major advantage of the solid phase method is the possibility for automation of the repetitive steps (deprotection, neutralization, coupling, and

ClCH$_2$OCH$_3$ + ⬡—◯ R

ZnCl$_2$ ↓   1. Functionalization

Boc-NHCHR^{1}COO$^-$Cs$^+$ + ClCH$_2$—⬡—◯ R

↓   2. Anchoring

Boc-NHCHR^{1}COOCH$_2$—⬡—◯ R

Acid ↓   3. Deprotection
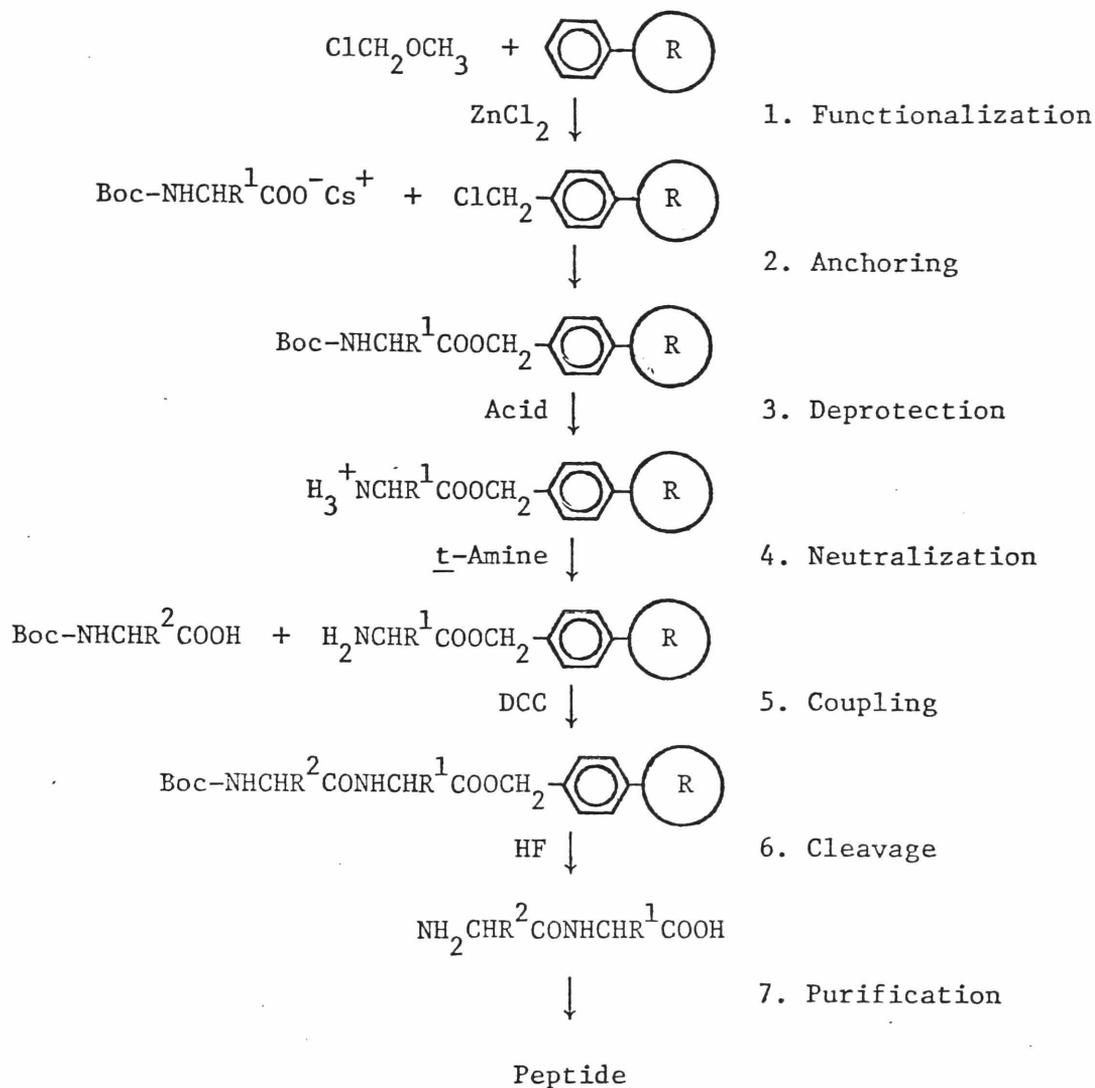
H$_3$$^+$NCHR^{1}COOCH$_2$—⬡—◯ R

$\underline{t}$-Amine ↓   4. Neutralization

Boc-NHCHR^{2}COOH + H$_2$NCHR^{1}COOCH$_2$—⬡—◯ R

DCC ↓   5. Coupling

Boc-NHCHR^{2}CONHCHR^{1}COOCH$_2$—⬡—◯ R

HF ↓   6. Cleavage

NH$_2$CHR^{2}CONHCHR^{1}COOH

↓   7. Purification

Peptide

Figure 4.   A typical scheme for solid phase synthesis.

all intervening washes). Following the construction of the first instrument[11,12] used to carry out all steps of a synthesis under programmed control, several other instruments have been made and are commercially available.

The method of stepwise solid-phase synthesis can also be applied in the assembly of peptide chains in the N to C direction[13,14]. After the N^{α}-amino group of the first amino acid is anchored to the resin, the peptide chain is extended through condensation of the carboxyl group of the first amino acid with the amino group of the second amino acid. One drawback of this method lies with the possibility of low coupling yields. Since quantitative coupling depends upon the activation and total reaction of the carboxyl end of the peptide chain with the incoming amino acid, and since the activated peptide cannot be used in an excess, coupling reactions may not proceed to completion. Additionally, any side reaction of the activated carboxyl group will result in incorporation of the undesired by-product into the growing chains. Finally, activation of the carboxyl component might lead to racemization.

Alternatively, solid-phase peptide synthesis may be accomplished through anchoring of the side chain[15-17] of a trifunctional amino acid to the solid support. The peptide chain may then be extended in two directions: by addition of an activated amino acid derivative at the amino terminus or by activation of the carboxyl end of the peptide and extension at the carboxyl end.

Solid-phase synthesis is also useful in the preparation of peptide fragments[10]. Fragments may be cleaved from the resin, purified in solution, and then coupled in solution or reattached to the support. Fragments must be made in protected form in order to be further coupled to the growing peptide chain without interference by side chains of the trifunctional amino acids. A new set of conditions must necessarily be developed for application of this strategy.

Since the introduction of solid-phase peptide synthesis, efforts have been directed towards improving this method. Specifically, the development of more selectively removable protecting groups[18] as well as the development of new and different solid supports[19-22] to which peptide chains may be attached have been studied.

3. Protecting Groups

In principle, the formation of a peptide bond between two arbitrary amino acid, A_1 and A_2, can occur in at least two ways: the carboxyl group of amino acid A_1 may react with the amino group of amino acid A_2 to form the dipeptide A_1A_2, or the reverse order reaction may occur where the dipeptide A_2A_1 is formed. In order to permit selective formation of the peptide bond corresponding to the desired sequence, only the appropriate amino and carboxyl groups of the respective amino acids should be allowed to participate in the acylation. The remaining amino and carboxyl groups must be protected. Similarly, all other functional groups which may interfere with the peptide-bond forming reaction must be protected.

a. Protection of the N^α-amino group

The protecting groups used for the N^α-amino moiety must satisfy the following criteria: (1) stability during coupling and neutralization reactions; (2) quantitative removal without side reactions; and (3) prevention of racemization in the activated amino acid that is being coupled.

Since the introduction of the benzyloxycarbonyl N^α-amino protecting group by Bergman and Zervas[4] in 1932, a large number of urethane-based protecting groups have been developed for use in peptide synthesis. Those urethane groups most commonly used in solid-phase peptide synthesis are listed in Table 3., along with several other groups. These groups are listed in order of acid-lability.

b. Protection of side chains of amino acids

Those amino acids possessing inert side chains, glycine, alanine, valine, leucine, isoleucine, phenylalanine, as well as the amino acid proline, do not require protection. The side chains of trifunctional amino acids can undergo a series of side reactions during the various steps in the course of the synthesis. In particular, they can serve as initiation sites for peptide chain growth thereby resulting in branched peptides. The choice of side chain protecting groups depends upon the strategy employed for the given synthesis. The side chain protecting

Table 3 . Some N^α-Amino Protecting Groups for Solid-Phase Peptide Synthesis

Structure/Name	Deprotection		Remarks
	Acidolytic	Other	
1. [structure: 2-(p-biphenyl)isopropyloxycarbonyl] 2-(p-biphenyl)isopropyl-oxycarbonyl (Bpoc)	0.5% $CF_3COOH-CH_2Cl_2$; 4:1 $CH_3COOH : H_2O$; 3.4 M $ClCH_2COOH-CH_2Cl_2$		3000-fold acido-lytic selectivity with respect to Boc group. Resistant to bases and cata-lytic hydrogenolysis.
2. [structure: 2-nitrophenylsulfonyl, with S- and NO_2] 2-nitrophenylsulfonyl (Nps)	2 eq. of HCl in ethanol in ether	0.1 M thioacetamide; β-mercaptoethanol	resistant to base; several-fold more acid-labile than Boc group. When HCl cleavage is used the by-product Nps-Cl irreversibly alkylates trypto-phan residue.
3. [structure: 2-(3,5-dimethoxyphenyl)-isopropyloxycarbonyl, with CH_3O groups] 2-(3,5-dimethoxyphenyl)-isopropyloxycarbonyl (Ddz)	5% $CF_3COOH-CH_2Cl_2$	photolysis (280 nm)	1400-fold acidolytic selectivity with respect to Boc.

Table **3** (cont'd). Some N^α-Amino Protecting Groups for Solid-Phase Peptide Synthesis

Structure	Deprotection		Remarks
	Acidolytic	Other	
4. $CH_3-\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{C}}-O-\overset{O}{C}-$ <u>t</u>-butyloxycarbonyl (Boc)	50% $CF_3COOH-CH_2Cl_2$; 4 N HCl-dioxane		resistant to catalytic hydrogenolysis and alkali During deprotection scavengers for reactive carbonium ions added
5. $CH_3-\!\!\bigcirc\!\!-CH_2-O-\overset{O}{C}-$ 4-methoxybenzyloxycarbonyl (4-MeOZ)	40% CF_3COOH-anisole; 0.9 M HCl in 1:10 (v/v) anisole-acetic acid		uv absorbance which permits monitoring of the deprotection
6. $\bigcirc\!\!-CH_2-O-\overset{O}{C}-$ benzyloxycarbonyl (Z)	HBr-CH_3COOH; anhydrous HF +10% anisole, 0°C	catalytic hydrogenolysis	resistant to base

Table 3 (cont'd). Some N^α-Amino Protecting Groups for Solid-Phase Peptide Synthesis

Structure/Name	Deprotection		Remarks
	Acidolytic	Other	
7. ![Fmoc structure] $CH_2-O-C(=O)-$ 9-fluorenyl methyloxycarbonyl (Fmoc)	stable	liquid ammonia morpholine	Resistant to catalytic hydrogenolysis.
8. ![Dts structure] The N is part of the amino acid that is protected dithiasuccinoyl (Dts)	stable	β-mercapto-ethanol +Et$_3$N; NaBH$_4$; Bu$_3$P or ϕ_3P	conditions for removal very mild and specific

groups must be stable under the conditions of N^α-deprotection, and subsequent neutralization, and preferably removable in a single step at the end of the synthesis.

At present, an ideal combination of compatible N^α-amino and side chain protecting groups does not exist. Side-reactions can occur during the removal of the protecting groups giving rise to undesireable peptide mixtures. The successful synthesis of any peptide or protein will accordingly depend upon careful evaluation of the anticipated levels of byproducts.

Those side chain protecting groups most commonly employed in solid phase peptide synthesis are listed in Table 4 along with conditions for their deprotection. Structures and names of protecting groups are listed under the method used in their deprotection. The formation of byproducts which result during their removal is given in a separate column.

A brief discussion of the properties of the aforementioned protecting groups follows. Protection of the sulfhydryl group of cysteine and γ-carboxyl group of glutamic acid is not discussed since neither of these amino acids is present in the sequence of glucagon. Introduction of each of these residues into peptide sequences presents certain unique problems, which can be minimized under appropriate conditions[23,24].

i. Arginine

The very basic guanidino group of arginine is commonly protected with the nitro-, 4-toluenesulfony, or a variety of urethane groups. In some syntheses, protonation of the guanidinium group has provided sufficient protection against undesired side reactions.

The nitro-group can be removed by catalytic hydrogenation, electrolytic reduction reduction with zinc in HCl, but is stable to HBr in HOAc. Catalytic hydrogenation does not always proceed smoothly in those peptides containing methionine and cysteine due to poisoning of the catalyst by sulfur[25]. The nitro-group is quantitatively removed by liquid

Table 4. Some Side-Chain Protecting Groups for Solid-Phase Peptide Synthesis

Amino acid side-chain	Protecting Group/Removal			Remarks
	liquid HF	50% TFA-CH_2Cl_2	Other	
1. $H_2N-\underset{+\ NH_2}{\overset{\parallel}{C}}-NH-(CH_2)_3-$ (arginine)	a. $CH_3-\langle\bigcirc\rangle-SO_2-$ p-toluenesulfonyl (tosyl)	c. bis(adamantyl-oxycarbonyl)		Tosyl and (Adoc)$_2$Arg stable to catalytic hydrogenolysis. Latter derivative undergoes intramolecular cycli-zation side reaction.
	b. NO_2- nitro		catalytic hydrogenolysis	HF-cleavage of NO_2-Arg gives some ornithine peptides.
2. $H_2N-(CH_2)_4-$ (lysine)	a. $\langle\bigcirc\rangle-CH_2-O-\overset{O}{\overset{\parallel}{C}}-$ benzyloxycarbonyl (Z)	c. $CH_3-\underset{CH_3}{\overset{CH_3}{\overset{\mid}{\underset{\mid}{C}}}}-O-\overset{O}{\overset{\parallel}{C}}-$	Z-group removed by catalytic hydrogenolysis	Premature depro-tection of Z-group leads to branched peptides
	b. Cl-phenyl-Cl (2,4-Cl$_2$Z)			

Table 4 (cont'd). Some Side-Chain Protecting Groups for Solid-Phase Peptide Synthesis

Amino acid side chain	Protecting Group/Removal			Remarks
	liquid HF	50% TFA-CH$_2$Cl$_2$	Other	
3. (imidazole ring, CH$_2$– side chain) (histidine)	a. CH$_3$–⟨⟩–SO$_2$– p-toluenesulfonyl (tosyl) b. ⟨⟩–CH$_2$– benzyl (Bzl)	c. adamantyloxy-carbonyl or Boc	d. O$_2$N–⟨⟩–NO$_2$ 2,4-dinitrophenyl (Dnp), removed by thiolysis, but stable to acid	Unprotected or Bzl-His racemizes upon coupling.
4. (indole ring, CH$_2$– side chain, N–H) (tryptophan)			a. HCO (formyl, on indole N), removed pH ≥ 9	Unprotected indole ring very acid-sensitive (alkylation or oxidation).
5. CH$_3$–S–(CH$_2$)$_2$– (methionine)			a. –S– with =O (sulfoxide) removed by thiolysis	Unprotected thio-ether susceptible to alkylation and oxidation.

Table 4 (cont'd). Some Side-Chain Protecting Groups for Solid-Phase Peptide Synthesis

Amino acid side-chain	Protecting Group/Removal			Remarks
	liquid HF	50% TFA-CH$_2$Cl$_2$	Other	
6. HO$_2$C-(CH$_2$)$_n$- (aspartic acid, n=1) (glutamic acid, n=2)	a. ⬡-CH$_2$- (benzyl ester) (OBzl)	b. CH$_3$-C(CH$_3$)(CH$_3$)- (t-butyl ester) (O-tBu)	c. ⬡-C(=O)-CH$_2$- (phenacyl ester) removed by thiolysis (O-Pac)	Acidolytic or basic cleavage of Asp(OBzl), gives rise to mixture of α and β-peptides. Glutamine acid forms which react with anisole or lead to pyrollidiones.
7. HO-CH$_2$- (serine) HO-CH(-CH$_3$) (threonine)	a. ⬡-CH$_2$- (benzyl ether) (Bzl)	b. CH$_3$-C(CH$_3$)(CH$_3$)- (t-butyl ether) (t-Bu)	catalytic hydrogenolysis of Bzl ethers	N→O acyl shift under acid conditions, readily reversed at pH >7.

Table 4 (cont'd). Some Side-Chain Protecting Groups for Solid-Phase Peptide Synthesis

Amino acid side chain	Protecting Group/Removal			Remarks
	liquid HF	50% TFA-CH$_2$Cl$_2$	Other	
8. HO–⟨phenyl⟩–CH$_2$– (tyrosine)	a. ⟨phenyl⟩–CH$_2$– benzyl (Bzl)	d. CH$_3$–C(CH$_3$)(CH$_3$)– t-butyl ether (t-Bu)		During acidolytic cleavage, 3-alkyl tyrosine by-products formed (most with a, least with c, none with d).
	b. Cl,Cl-⟨phenyl⟩–CH$_2$– 2,6-Cl$_2$-Bzl			
	c. ⟨cyclohexyl ring⟩ cyclohexyl (cHex)			

Table 4 (cont'd). Some Side-Chain Protecting Groups for Solid-Phase Peptide Synthesis

Amino acid side chain	Protecting Group/Removal			Remarks
	liquid HF	50% TFA-CH₂Cl₂	Other	

$$9. \quad H_2N-CO-(CH_2)_n-$$

(asparagine, n=1)
(glutamine, n=2)

a. CH_3O—⬡—$\overset{|}{CH}$—⬡—OCH_3

4,4'-dimethoxybenzhydryl

(Mbh)

Remarks: Asn(Mbh) and Gln(Mbh) couple slowly. The stability to acids is intermediate, so they are not particularly useful for either a Bzl or tBu-based protection strategy.

When coupled unprotected with DCC, nitrile derivatives are formed; prevented by addition of 1-hydroxybenzotriazole.

10. $HS-CH_2-$

(cysteine)

a. CH_3—⬡—CH_2-

4-methylbenzyl
(4-MeBzl)

b. $\emptyset_3C-$

trityl
(Trt)

c. $CH_3-\overset{O}{\overset{\|}{C}}-NH-CH_2$

Acetamidomethyl
(Acm)
Removed by 0.05 M mecuric acetate in 30% aq. acidic and stable to HF

HF under standard conditions (0°C; 30 min) in the presence of a sca-
venger such as anisole. In some instances, ornithine-containing peptides
are produced[27] using liquid HF , although the reaction appears to be
sequence dependent.

The 4-toluenesulfonyl group has been frequently used in recent
years in the protection of the guanidino group. It is cleaved with
liquid HF. Formation of ornithine-containing peptides has not been
detected.

The guanidino group has also been protected with several urethane-
type protecting groups such as: N^{ω}-benzyloxycarbonyl, N^{ω}-butyloxycarbonyl,
or N^{ω}-adamentyloxycarbonyl. These are removed under acidic conditions.

ii. Lysine

The basic ε-amino group of lysine can be protected with the
same set of groups used for the α-amino group of amino acids. However,
the ε-amino protecting group must be stable under the acidic conditions
employed in the repeated deprotection of the α-amino group during stepwise
synthesis. Premature N^{ε}-deprotection will provide a new initiation site
for the growth of peptide chains. As a result, a new mixture of branched
peptides of varying chain lengths will occur[28] and separation may become
very difficult.

N^{ε}-Benzyloxycarbonyl protection has been used in combination with
N^{α}-Boc protection. The benzyloxycarbonyl group is removed with HBr in
TFA or liquid HF under the same conditions employed in the cleavage of
peptides from the solid support. However, cleavage with 50% TFA/CH_2Cl_2
employed for N^{α}-Boc deprotection slowly removes it. This limits the feasi-
bility of this combination for use in a long stepwise synthesis.

Introduction of electron-withdrawing halogen substituents, such
as 2-chloro-,3-chloro-, 2,4-dichloro, 2,6-dichloro, and 3,4-dichloro-
to the aromatic ring of the benzyloxycarbonyl group, increases
stability towards TFA 60 to 1,000 times[29], and therefore these derivatives
can be usefully employed in combination with the Boc group for N^{α}-amino
protection. The chlorinated benzyloxycarbonyl derivatives are all

deprotected with liquid HF in 1 hr at 0°C with the exception of 2,6-dichloro-benzyloxycarbonyl which requires treatment for 1 hr at 20°C.

The N^ε-amino group can also be successfully protected with the tert-butyloxycarbonyl group if a very acid-labile group such as the bi-phenylisopropyloxycarbonyl (Bpoc) group for N^α-amino protection. The mild acidic conditions required for removal of the N^α-Bpoc group should not cause a significant amount of branched peptide chains.

iii. Histidine

Histidine has been incorporated into several peptides and even a protein synthesized by the solid phase method without protection of the imidazole ring[30]. A great deal of experimental evidence has shown[31,32], however, that Boc-Histidine racemizes (5-25%) during dicy-clohexylcarbodiimide coupling. Racemization occurs by intramolecular abstraction of the α-proton adjacent to the activated carboxyl group by the basic nitrogen of the imidazole ring:

Protection of the imidazole nitrogen with strong-electron with-drawing groups should minimize this reaction, and the N^{im}-Tos and N^{im}-DNP are best used for this purpose. The N^{im}-4-toluenesulfonyl group[33] is removed by liquid HF. The N^{im}- 2,4-dinitrophenyl group can be removed by thiolysis[34] with 2-mercaptoethanol. mercaptoacetic acid, dithiothreitol or thiophenol. A 20-fold excess of thiophenol cleaves quantitatively[36] in 1 hr. The course of thiolysis can be followed spectrophotometrically. The stability of 2,4-dinitrophenylhistidine to acids allows selective deprotection of the imidazole ring before or after cleavage of the peptide from the resin. This selectivity can be very useful in studing the role played by histidine residues in the expression of biological activity of peptides and proteins.

The imidazole ring of histidine has also been protected with the benzyl[37], benzyloxycarbonyl[38], adamantyloxycarbonyl[39], and t-butyloxy-carbonyl groups[40].

iv. Tryptophan

During solid-phase peptide synthesis, tryptophan residues have been incorporated into peptide chains without protection of the indole ring. In order to prevent air oxidation under acidic conditions of the indole ring, a nitrogen atmosphere within the reaction vessel is maintained. Alternatively, addition of indole or 2-mercaptoethanol to the N-deprotecting reagent will give the same effect.

A t-butyl-alkylation of the indole nitrogen during acidic removal of protecting groups has been observed[41]. Levels of alkylation byproducts are high (up to 50%) in liquid HF.

Recently, N^i-formyltryptophan has been successfully applied in the synthesis of several trytophan-containing peptides. The N^i-formyl group is removed under basic conditions.

v. Methionine

During solid-phase synthesis, the thioether group of methionine is frequently left unprotected.

Unprotected methionine residues can undergo two major side reactions under acidic conditions (employed in the N^α-deprotection or in the removal of peptide chains from the resin): 1. Oxidation to the sulfoxide or sulfone derivatives. Upon treatment with thiol, the methionine-sulfoxide derivatives reverse back to methionine. Formation of the sulfone is irreversible. 2. S-alkylation with carbonium radicals. The addition of scavengers such as 2-mercaptoethanol or methionine to the reagent mixture should minimize formation of the byproduct. In order to prevent alkylation, methionine has been protected as the sulfoxide[42]. However, the reduction with 2-mercaptoethanol is not always quantitative.

vi. Aspartic Acid

Protection of the β-carboxyl group of aspartic acid causes several problems. In principle, introduction of the aspartyl residue

as the aspartylben^{zyl} ^{ester} should be straightforward. It has, however, been observed that removal of the benzyl group under acidic or basic conditions in classical solution[43] or solid-phase syntheses[44], results in a mixture of α- and β-aspartyl peptides. This effect is sequence dependent and is especially pronounced at the Asp-Ser and Asp-Gly sequences. The reaction proceeds through a cyclic aspartimide derivative most probably formed through protonation of the β-carboxyl oxygen followed by nucleophilic attack of the amide nitrogen and elimination of the benzyl group[45]. Treatment with base leads to the α- and β-aspartyl peptides (Figure 5.).

Peptides containing the free β-carboxyl group do not rearrange when treated with liquid HF. This finding supports the conclusion that steric and electronic effects determine the extent of aspartimide formation.

The formation of α- and β-aspartyl peptides is greatly reduced when aspartic acid is introduced into the peptide as the β-phenacyl ester[46]. The protecting group is selectively removed by thiolysis prior to HF treatment of the protected peptide-resin. Studies with model peptides have shown β-aspartyl peptides to be formed in less than 2.4%[47].

The β-t-butyl protected aspartyl peptides do not produce β-aspartyl byproducts. This protection scheme can be applied only in combination with acid labile N^{α}-amino-protecting groups such as Bpoc or Nps.

vii. Serine and Threonine

When unprotected, serine and threonine undergo O-acylation during the coupling step. The hydroxyl group is usually protected as an ether with the benzyl or t-butyl group. The O-benzyl group is stable to 50% TFA in dichloromethane and is used in combination with N^{α}-Boc-protection. Removal of the O-benzyl group is by HBr in TFA, liquid HF, or catalytic hydrogenolysis. The O-t-butyl group is cleaved by 50% TFA/CH$_2$Cl$_2$ and is used when the α-amino group is protected with the acid labile Bpoc or Nps group.

Reversible migration of the acyl moiety from the amino to hydroxyl group of serine has been observed[48]. Under acidic conditions

Figure 5 . Reaction mechanism of α- to β- rearrangement of aspartyl peptides.

the following takes place slowly:

$$\underset{\text{N-Acyl derivative}}{\overset{\overset{\displaystyle HO-CH_2}{\underset{\displaystyle \underset{O}{\overset{\|}{R-C}}-NH-CH-R^1}{|}}}{}} \quad \underset{OH^-}{\overset{H^+}{\rightleftarrows}} \quad \underset{\text{2-Hydroxy-}\atop\text{1,3-oxazolidine}}{\overset{\overset{HO}{\underset{R}{>}}C\overset{O-CH_2}{\underset{NH-CH-R^2}{<}}}{}} \quad \underset{OH^-}{\overset{H^+}{\rightleftarrows}} \quad \underset{\text{O-Acyl derivative}}{\overset{\overset{\displaystyle \overset{O}{\overset{\|}{R-C}}-O-CH_2}{\underset{\displaystyle \overset{+}{N}H_3-CH-R^2}{|}}}{}}$$

Studies on model peptides have shown that treatment with liquid HF at room temperature gives 8% of the O-acyl derivative in 24 hrs. Under standard conditions employed in solid phase peptide synthesis, removal of protecting groups with liquid HF (30 to 61 min at 0°C) should not result in this rearrangement. In any event, when the pH of the solution is adjusted to near or above 7, the reverse O to N shift is very fast[49].

viii. Tyrosine

The phenolic hydroxyl group of tyrosine has also been pro-
tected with the O-benzyl moiety. The latter is partially removed in 50%
TFA/CH_2Cl_2 and as a result is satisfactory only in the stepwise synthesis
of small peptides. During acidolysis with HBr in TFA or liquid HF, rear-
rangement to 3-benzyltyrosine is observed. This rearrangement can be
avoided by cleavage of the resin-bound protected peptide under non-
acidic conditions and removal of the benzyl protecting group by catalytic
hydrogenolysis. The stability of O-benzyltyrosine towards acidolysis is
increased through introduction of electron-withdrawing halogen sub-
stituents at the O-benzyl moiety. O-(3-bromobenzyl)tyrosine[50] and
O-1(2,6-dichlorobenzyl)tyrosine[51] show increased stability towards acido-
lysis by factors of 50 and 5,000-fold, respectively. The use of O-(2,6-
dichlorobenzyl)tyrosine also reduced the formation of 3-alkyl-tyrosine
byproducts to 5%. This level of tyrosine byproducts is still unacceptable
in the stepwise synthesis of a long peptide containing several tyrosine
residues.

Limitations of use of O-benzyl based tyrosine protecting groups are largely overcome if tyrosine is introduced into the peptide chain as O-(cyclohexyl)tyrosine[52]. In this case, there is satisfactory stability towards 50% TFA/CH$_2$Cl$_2$ and quantitative removal with liquid HF in 30 min is possible. Furthermore, formation of the cyclohexyl-tyrosyl rearrangement product is suppressed to levels of less than 0.3%.

Tyrosine-O-t-butyl ether has frequently been used in combination with N$^\alpha$-Bpoc protection. The t-butyl group is cleaved by 50% TFA/CH$_2$Cl$_2$. Use of this group is not possible in a prolonged stepwise synthesis which is based on differential acid stability of α-amino and side-chain protecting groups.

ix. Asparagine and Glutamine

When the side chains of asparagine and glutamine are not protected, coupling with dicyclohexylcarbodiimide causes intramolecular dehydration of the amides to nitriles[53]. This side reaction has been observed in both classical solution and solid-phase syntheses. Other dehydrating agents such as acyl-or acylsulfonyl chlorides, as well as phosphorus pentachloride also cause the nitrile formation. In contrast, formation of a mixed anhydride with isovalareyl chloride does not lead[54] to the nitrile product. The dehydration reaction occurs during the activation step and not after the asparagine or glutamine have been incorporated into the peptide chain. Two reaction pathways have been proposed[55,56] and are shown in Figure 6 .

If the dehydration reaction goes through Mechanism A, the amide oxygen of the carbobenzoxyasparagine will appear in the carboxyl group of of β-cyanoalanine derivative, and the oxygen of Z-Asn. If the β-cyanoalanine derivative is formed through Mechanism B, then the oxygen atoms of the carboxyl group will be derived from the carboxyl group of Z-Asn. The amide oxygen of Z-Asn then will be incorporated into dicyclohexylurea. Therefore, in principle, labeling the carboxyl group with oxygen-18 will differentiate between the two mechanisms. From the results of such labeling studies, it was qualitatively concluded that the dehydration reaction proceeds mainly via Mechanism A[57,58]. However,

Figure 6 . Proposed literature mechanisms and expected labeling patterns for dehydration of Z-asparagine upon activation with dicyclohexylcarbodiimide.

the experiments with carbobenzoxyasparagine labeled with 0-18 in the car-
boxyl group do not exclude Mecahism B. Furthermore, the appearance of
oxygen-18 labeled dicyclohexylurea is not unambiguous proof of Mechanism A.

One of the major reactions[59] which carboxylic acids undergo with
dicyclohexylcarbodiimide is formation of the symmetrical anhydride of the
carboxylic acid. Therefore, some incorporation of the label in the by-
product dicyclohexylurea is possible from any carboxylic acid labeled with
oxygen-18 as shown below:

The dehydration product may them be formed through an alternative
mechanism as suggested by Kashelikar and Ressler[57] (Figure 7).

If the symmetrical anhydride intermediate (IIIa) is formed from
the reaction of intermediate (III) with a second mole of Z-asparagine,
then (IIIa) can decompose through Z-asparagineisoimide (V) to Z-β-
cyanoalanine (VI). The oxygen-18 label will be incorporated in both
dicyclohexylurea and Z-β-cyanoalanine. The experimental design in which
the carboxyl group of Z-asparagine is labeled with oxygen-18 cannot
distinguish between Mechanism A and Mechanism A'. Labeling at the amide
oxygen and determining the amount of O^{18} incorporated in dicyclohexylurea
should provide a better way to distinguish among the different mechanisms
involved in the dehydration reaction. However, this experiment was not
performed.

Hemmasi and Bayer[59] have proposed that, by mixing the protected

Figure 7. An alternative mechanism and expected labeling patterns for dehydration of Z-asparagine upon activation with dicyclohexylcarbodiimide.

asparagine and glutamine derivatives with dicyclohexylcarbodiimide in the molar ratio 2:1, all the carbodiimide will be consumed in the formation of the amino acid anyhydride. Thus, formation of the nitrile derivative was expected to be avoided, based on the assumption that only Mechanism A (not Mechanism B or A') describes the reaction pathway. These workers analyzed for the presence of the nitrile derivative after the final product was treated with anhydrous HF. Absence of the nitrile compound (<5%) was confirmed by ion exchange chromatography, IR spectral analysis, mass spectrometry and enzymatic digestion of the peptides. It is quite probably that the conditions which were applied for coupling of Boc-asparagine and Boc-glutamine did lead to formation of the nitrile compound. The presence of a strong acid such as HF could have caused complete or partial rearrangement of the dehydration product back to the Boc-asparagine and Boc-glutamine peptides by the following mechanism:

$$
\begin{array}{ccccc}
N & & NH & & \\
\|\| & & \| & & \\
C & \xrightarrow{\text{HF}} & C\text{-F} & \xrightarrow{\text{H}_2\text{O}} & \\
| & \rightleftharpoons & | & \xleftarrow[-\text{HF}]{} & \\
CH_2 & & CH_2 & & \\
| & & | & & \\
NH_2\text{-CH-COOH} & & NH_2\text{-CH-COOH} & &
\end{array}
$$

β-cyanoalanine

$$
\begin{array}{ccc}
NH & & NH_2 \\
\| & & | \\
C\text{-OH} & \longleftrightarrow & C=O \\
| & & | \\
CH_2 & & CH_2 \\
| & & | \\
NH\text{-CH-COOH} & & NH_2\text{-CH-COOH}
\end{array}
$$

asparagine

Hagenmaier and Frank[60] have also introduced glutamine into the peptide chain as a symmetrical anhydride by premixing the Boc deriva-

tive with dicyclohexylcarbodiimide in the molar ratio 2:1. The insoluble dicyclohexylurea was removed by filtration prior to the coupling step. In the final product the nitrile was present in less than 5%.

Coupling of Bpoc asparagine and Bpoc glutamine as p-nitrophenyl esters[61] completely circumvents the problem of the nitrile formation. However, the reaction proceeds very slowly in the solid-phase and often is not complete even after prolonged reaction times.

Dehydration is completely prevented in solution synthesis when 1-hydroxybenzotriazole is added with dicyclohexylurea to the N^α-protected asparagine or glutamine[62]. This method has also been applied for coupling of asparagine and glutamine in solid-phase synthesis[63,64]. However, no experimental data are available regarding the completeness of suppression of nitrile formation under conditions of solid-phase synthesis.

An alternate way to avoid the rearrangement is to protect the amido function of asparagine and glutamine with the Mbh group (4,4-dimethoxybenzyhydryl)[65]. The products are free of C≡N, but coupling yields are not very high due to the steric effect.

c. Solid phase anchoring of the C^α-carboxyl group

In the most widely used variations of solid-phase peptide synthesis, chain growth proceeds stepwise in the C to N direction. First, the C^α-carboxyl group of the terminal amino acid must be anchored to the support. Some linkages that are employed are listed in Table 5 .

4. Coupling Reagents

In forming a peptide bond between two amino acids, two steps are involved: (1) addition of a coupling reagent to an amino acid derivative to form a reactive intermediate, followed by (2) reaction of this intermediate with the amino component of a second amino acid. In most coupling reactions of solid-phase peptide synthesis, the reagent of choice is dicyclohexylcarbodiimide (DCC). The reaction scheme[66] for coupling is shown in Figure 8 . The first step involves addition of the N^α-protected amino acid to the coupling reagent to form the

Table 5. Common Linkages of C-terminal Amino Acid for Solid-Phase Peptide Synthesis

Structure/Name	Cleavage	Product
1. $R-\overset{O}{\underset{\|}{C}}-O-CH_2-$⟨ring⟩$-R$ p-alkyl benzyl	HF, 0°, 30 min NH_3 NH_2NH_2 R^1OH	$R-\overset{O}{\underset{\|}{C}}-OH$ $R-\overset{O}{\underset{\|}{C}}-NH_2$ $R-\overset{O}{\underset{\|}{C}}-NHNH_2$ $R-\overset{O}{\underset{\|}{C}}-OR^1$
2. $R-\overset{O}{\underset{\|}{C}}-O-CH_2-$⟨ring⟩$-CONHCH_2-$⟨ring⟩$-R$ p-acetamido benzyl ester	HF, 0°, 30 min (more acid-stable than number 1)	$R-\overset{O}{\underset{\|}{C}}-OH$
3. $R-\overset{O}{\underset{\|}{C}}-O-CH_2-$⟨ring⟩$-OCH_2-$⟨ring⟩$-R$ p-alkoxybenzyl ester	50% TFA–CH$_2$CH$_2$, 25°, 30 min	$R-\overset{O}{\underset{\|}{C}}-OH$
4. $R-\overset{O}{\underset{\|}{C}}-O-\underset{CH_3}{CH}-\overset{O}{\underset{\|}{C}}-$⟨ring⟩$-R$ α-methylphenacyl ester	NaSØ, DMF hν NH_2NH_2	$R-\overset{O}{\underset{\|}{C}}-OH$ $R-\overset{O}{\underset{\|}{C}}-OH$ $R-\overset{O}{\underset{\|}{C}}-NHNH_2$

	Structure/Name	Cleavage	Product
5.	$$R\overset{O}{\underset{\parallel}{C}}-O-NHNHCO_2\overset{CH_3}{\underset{CH_3}{C}}-CH_2CH_2$$ —〈R〉 alkoxycarbonyl hydrazide	50% TFA–CH_2CH_2 25°, 30 min	$R\overset{O}{\underset{\parallel}{C}}-NHNH_2$
6.	$$R\overset{O}{\underset{\parallel}{C}}-NH-CH-〈 〉$$ —〈R〉 benzhydrylamide	HF, 0°, 60 min	$R\overset{O}{\underset{\parallel}{C}}-NH_2$

50
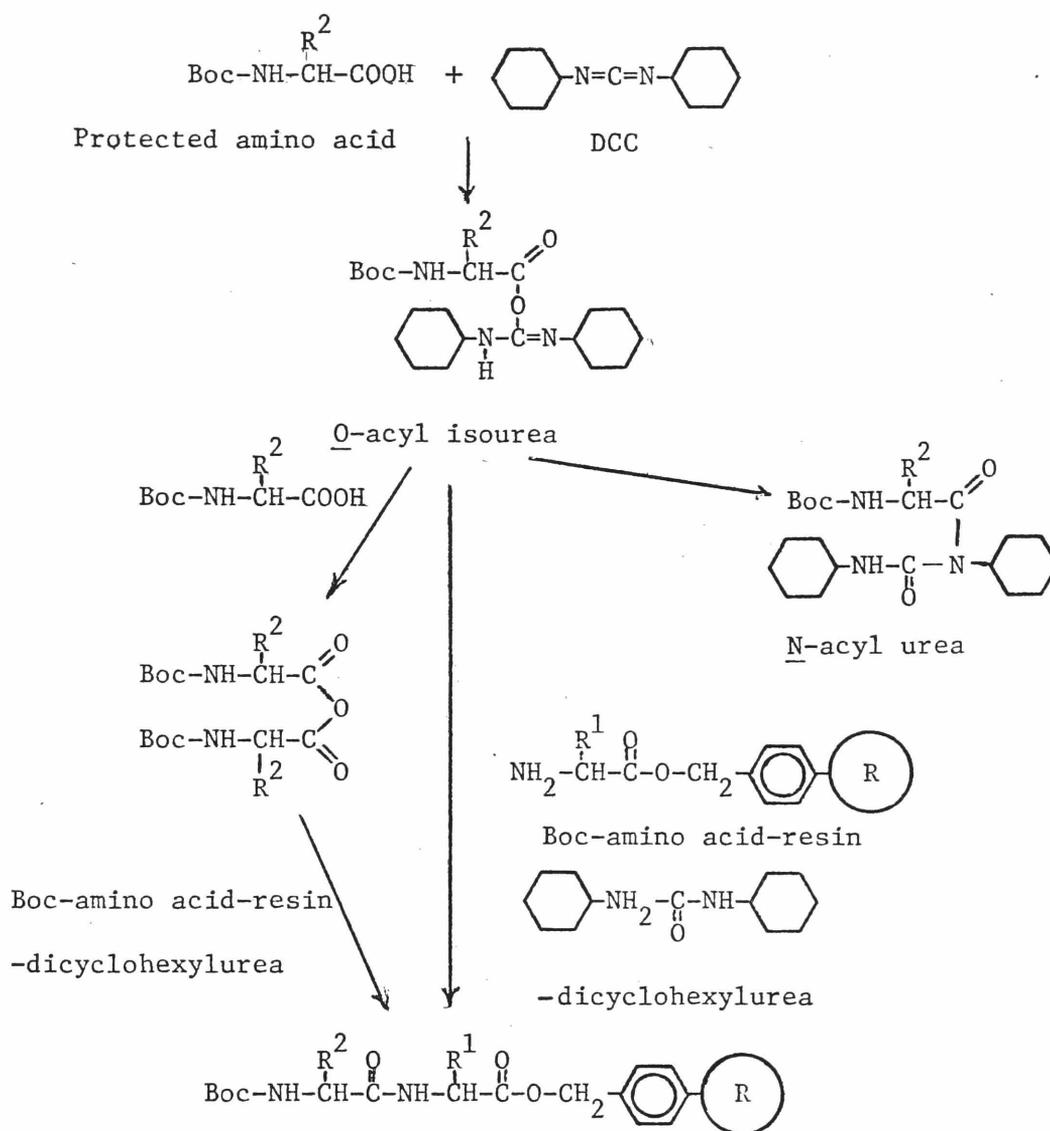
Figure 8 . Activation and coupling with dicyclohexylcarbodiimide.

O-acyl isourea which may then react in three different ways.

The O-acyl isourea may react with the amino group of the resin-bound amino acid or peptide to form a peptide bond. The byproduct of the reaction, N,N'-dicyclohexylurea is removed from the resin-bound peptide by extensive washing. The coupling reaction is solvent dependent and is most efficient in such strong resin swelling solvents as DMF or dichloromethane. In order to achieve a quantitative coupling, molar excesses of the added protected amino acid and dicyclohexylcarbodiimide are used.

The O-acyl isourea may react in part with another molecule of the amino acid derivative before it reacts with the resin-bound amino component. As a result, a symmetrical anhydride of the protected amino acid is formed. If the amino acid derivative and dicyclohexylcarbodiimide are mixed in solution prior to addition of the resin-bound amino group, the symmetrical anhydride would act as the acylating species.

Finally, the O-acyl isourea may undergo O to N acyl migration resulting in the formation of the N-acyl urea, which does not acylate amine nucleophiles. In this case, the coupling yields will be lowered. To overcome this problem, an excess of coupling reagent is used.

Amino acids have been coupled in solid-phase synthesis[67] as p-nitrophenyl esters. Their coupling rates are lower relative to coupling with dicyclohexylcarbodiimide and often are not quantitative. Consequently, this method of coupling is not frequently used.

Activation of amino acids with dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole[68] has also been employed. This aspect of coupling has been discussed in detail in section V d .

An alternate coupling technique recently developed and applied in solid-phase synthesis is the oxidation-reduction method[69]. During formation of the peptide bond, triphenylphosphine is oxidized and dipyridyl disulfide is reduced and an equivalent of water is eliminated. This coupling reaction proceeds at 10% of the rate of coupling mediated by dicyclohexylcarbodiimide.

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IV. STRATEGY FOR THE STEPWISE SOLID-PHASE SYNTHESIS OF GLUCAGON

The strategy usually employed in stepwise solid-phase synthesis is based upon protection of the N^{α}-amino group by the Boc-moiety, and protection of the side chains of amino acids by the benzyl group and its derivatives[1,2]. Anchoring of the peptide as an ester to the resin is also based upon the benzyl functionality. In the synthesis of certain peptides, application of this strategy may not, however, be feasible. In the case of glucagon, a series of side reactions may be anticipated if this strategy is followed. These side reactions are due to the inherent amino acid sequence of glucagon and may be separated into two categories: (a) those occurring during deprotection of the Boc-groups with 50% TFA/CH_2Cl_2 and (b) those occurring during removal of the protecting groups from the side chains and cleavage of the peptide from the resin with liquid HF. These may be further summarized as follows:

Side-reactions occurring during each deprotection step with 50% TFA in dichloromethane.

(1) Alkylation and oxidation of the unprotected thioether group of methionine and the indole ring of tryptophan. Both methionine and tryptophan are incorporated into the peptide chain of glucagon at an early stage of the synthesis. Alkylation of the thioether and N-indole functions with the t-butyl group during repeated cycles of Boc-deprotection with 50% TFA/CH_2Cl_2 is well-documented[3]. In the case of the thioether, alkylation may be prevented through introduction of the methionine residue as the sulfoxide derivative[4]. However, under the acidic conditions employed for the deprotection of the Boc-group, further oxidation of the sulfoxide to the sulfone may occur. This scheme is additionally complicated since reduction of methionine sulfoxide back to methionine may not always proceed smoothly. In the case of tryptophan, alkylation as well as oxidation may be prevented through protection of the indole-moiety with the N^i-formyl group[5]. The formyl group is easily removed at alkaline pH (≥ 9). Under these condtions, however, some peptide bonds such as $Asn^{28}-Thr^{29}$

may be unstable[6]. Therefore, if the tryptophan residue were to be introduced into the peptide as the N^i-formyl derivative, a detailed study of the Asn^{28}-Thr^{29} lability would have to be undertaken. In the event that the methionine and tryptophan residues are introduced into the peptide unprotected, the side reactions of alkylation and oxidation may be minimized through the addition of scavengers[7].

(2) Cyclization of glutamine residues situated at the N-terminal position to pyroglutamyl derivatives. An amino terminal glutamine residue can be converted to a pyroglutamyl (pyrrolidone carboxylic acid) residue through intramolecular acylation of its free N^α-amino group with its own carboxamide group as follows:

α-Glutamyl   Pyroglutamyl
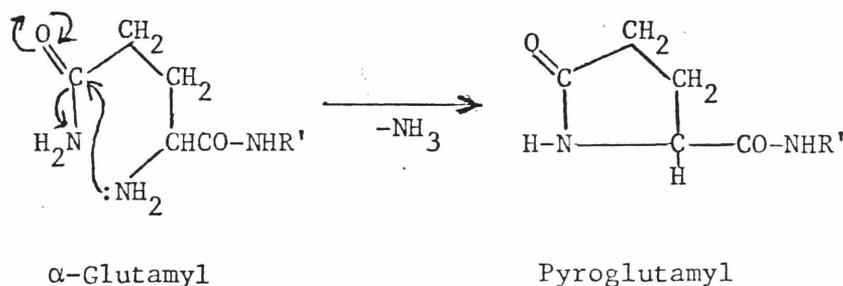
Systematic studies[8] of this reaction show it to occur in 11% yield with 50% TFA/CH_2Cl_2, and in 1% yield in 1 N HCl in acetic acid. This reaction was not detectable in 4 N HCl in dioxane. Therefore, use of the latter reagent has been recommended. In general, the procedure for the preparation of 4 N HCl in peroxide-free dioxane is tedious and time-consuming. Once prepared, this reagent must be used immediately due to rapid formation of peroxides in the acid/dioxane solution.

Side-reactions occurring during treatment of the protected peptide-resin with liquid HF.

(1) During treatment of the peptide with liquid HF, alkylation of the thioether and the indole ring of tryptophan with carbonium ions is more likely to occur than in the previously discussed case of TFA treatment. Again, scavengers are commonly added in an effort to

minimize this problem.

(2) Formation of 3-alkyl-tyrosine by-products[9]. Rearrangement of the O-(2,6-dichlorobenzyl)-tyrosine to the 3-(2,6-dichlorobenzyl)-derivative occurs in 5% yield upon treatment in liquid HF. Since there are two tyrosine residues in the amino acid sequence of glucagon, the total yield of byproducts present in the final peptide will be roughly 10%.

(3) Rearrangement of aspartyl residues to aspartimides leading, upon ring opening, to a mixture of α- and β-aspartyl peptides[10]. When the carboxyl group of aspartic acid is protected as the benzyl ester, treatment by liquid HF leads to protonation of the β-carboxyl oxygen followed by rearrangement to the cyclic-aspartimide. Nucleophilic attack at either carbonyl of the aspartimide will give a mixture of α- and β-aspartyl peptides. In general, resistance to cyclization is correlated to the size of the residue following the aspartic acid itself. Whereas Asp-Ser and Asp-Gly sequences are very sensitive to cyclization, Asp-Val sequences are not. In the amino acid sequence of glucagon, cyclization may occur at three different positions: $Asp^{21}-Phe^{22}$, $Asp^{15}-Ser^{16}$, and Asp^9-Tyr^{10}. In the past, two glucagon fragments have been synthesized by the stepwise solid-phase method[11]. The tetrapeptide containing the $Asp^{21}-Phe^{22}$ sequence resulted in 80% of the β-aspartyl peptide. The second decapeptide fragment containing the $Asp^{15}-Ser^{16}$ and Asp^9-Tyr^{10} sequences resulted in 40% of the β-aspartyl peptide and 30% of the cyclic aspartimide product.

In summary, application of this strategy would most likely result in a high level of byproducts; separation of these from the desired synthetic peptide might not be feasible. Clearly, consideration of an alternative route was necessary for the successful synthesis of glucagon.

The alternative strategy to be employed requires the use of milder acidic conditions in the deprotection and cleavage steps. Additionally, it was desirable to choose for this strategy a series

of acid-labile protecting groups which could be easily removed, preferably in one step, at the end of the synthesis.

In the amino acid sequence of glucagon, the three aspartic acid residues, one lysine residue, as well as the nine hydroxyl-containing residues, may be protected at their side chains with the same type of protecting group: t-butyl esters, t-butyloxy-carbonyl urethanes, and t-butyl ethers. Therefore, a more acid-labile moiety must be selected for the N^{α}-amino protecting group. The protecting group which meets this requirement is the biphenyl-isopropyloxycarbonyl (Bpoc) group[12].

In order to remove the majority of the protecting groups as well as to cleave the peptide from the resin in a single step, the p-alkoxybenzylalcohol resin of Wang[13] was chosen as the solid support for the assembly of the peptide chain. In this resin, the anchoring bond is a p-alkoxybenzyl ester linkage. The additional presence of the p-methoxyl-moiety of the Wang resin renders the

benzyl ester bond more acid-labile than in the commonly employed Merrifield resin. Using the Wang resin, treatment with 50% TFA/ CH_2Cl_2 for 60 min. cleaves the peptide chain from the support quanti-tatively and also results in removal of all t-butyl-based protecting groups. Only trace amounts of peptides are cleaved during the treat-ment with 0.5% TFA/CH_2Cl_2 for 20 min. These are the conditions used to cleave the Bpoc group.

The three main features of the proposed strategy are: 1) For N^{α}-amino protection, use of the Bpoc group which can be removed with 0.5% TFA/CH_2Cl_2; 2) For most of the trifunctional amino acids, use of t-butyl-based protecting groups which can be removed by 50% TFA/ CH_2Cl_2; and 3) For the solid support, use of the alkoxybenzylalcohol resin from which the peptide can be cleaved by 50% TFA/CH_2Cl_2.

The detailed protection scheme for the stepwise solid-phase synthesis of glucagon is shown in Figure 9.

(1) Hydroxyl groups of serine, threonine, and tyrosine are

DNP Bu^t Bu^t Bu^t Bu^t OBu^t Bu^t
 | | | | | | |
Boc-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr
  1   2   3   4   5   6   7   8   9   10

                                            11 Ser-Bu^t

OBu^t NO$_2$ NO$_2$ Bu^t OBu^t Bu^t Boc
 | | | | | | |
Asp-Gln-Ala-Arg-Arg-Ser-Asp-Leu-Tyr-Lys
 21 20 19 18 17 16 15 14 13 12

Phe 22

                              Bu^t
                               |
Val-Gln-Trp-Leu-Met-Asn-Thr-OCH$_2$-⬡-OCH$_2$-⬡-Ⓡ
 23 24 25 26 27 28 29

Figure 9.   Protection scheme for the stepwise solid–phase
            synthesis of glucagon.

protected as t-butyl ethers, and the carboxyl group of aspartic acid is protected as t-butyl ester. It has been shown in the past[14], that protection of aspartic acid as the tbutyl ester prevents formation of β-aspartyl peptides in the final peptide product. Furthermore, intramolecular rearrangement of O-t-butyl-tyrosine derivatives does not occur[15].

(2) The ε-amino group of lysine is protected as the t-butyloxy-carbonyl (Boc) group. The ratio of stability[12] of the Boc to Bpoc group is 3,000:1. Therefore, premature deprotection of the lysine side chain resulting in branched peptides was not expected.

(3) The thioether function of methionine and the indole ring of tryptophan need not be protected due to the mild acid conditons employed. Protection of tryptophan from undesired side-reactions during both the deprotection and cleavage steps is through the addition of 0.1% indole (w/v) into corresponding deprotection reagents.

(4) The guanidino groups of the two arginine residues are protected with the nitro-function. It is stable to TFA but can be removed by two methods following cleavage of the peptide from the resin: liquid HF and catalytic hydrogenolysis. Treatment of the nitro-peptide with liquid HF should not cause formation of the β-aspartyl peptide, since it has been previously established that aspartic acid with a free carboxyl group does not undergo rearrangement to the aspartimide[16]. We showed that treatment with liquid HF does not cause any structural damage to native glucagon. Catalytic hydrogenolysis of the nitro-groups may be complicated by the presence of methionine in the amino acid sequence of glucagon since the sulfur groups of this residue may poison the catalyst. However, choice of a suitable catalyst should allow quantitative removal of the group.

(5) The imidazole ring of histidine is protected with the dinitro-phenyl-function which may be removed selectively by thiolysis[17] prior to deprotection and cleavage of the peptide from the resin. Since histidine is present as the last amino acid introduced into the chain, its N^{α}-amino group is protected as Boc and may be removed by treatment

of the peptide resin with 50% TFA/CH$_2$Cl$_2$.

(6) In order to determine optimal coupling conditions for the introduction of Bpoc-asparagine and Bpoc-glutamine into the peptide chain, we examined in detail the recommended literature procedures of Hemmasi and Bayer[18] and König and Geiger[19] on the model dipeptides Asn-Gly and β-Ala(CN)-Gly. On the basis of our findings (see Section V.d.), we decided to introduce Bpoc-asparagine and Bpoc-glutamine with dicyclohexylcarbodiimide in the presence of hydroxybenzotriazole. In the model system, no nitrile formation ($\leq$ 0.1%) was detected under these condtions.

(7) Two radioactively labeled amino acids are introduced into the peptide at two stages of the synthesis: Bpoc-[^{14}C]-Leu at position 26, and Bpoc-[^{3}H]-Phe at position 6. Incorporation of the label should allow monitoring of the loss of peptide chains from the solid support as well as monitoring of the purification of the synthetic material. The choice of the positions for the label was based upon the selection of amino acids from both ends of the glucagon sequence that do not require side chain protection. The ratio of [^{3}H] to [^{14}C] label should provide an indication of the homogeneity of the purified peptide.

In order to ensure maximal incorporation of labeled amino acids into the peptide chain, two methods were considered.

(a) Radioactive amino acids could be coupled to the growing peptide chain in a slight molar excess during the first two couplings. In the second coupling, an excess of unlabeled amino acid is used to drive the reaction to completion. This method of introduction cannot predict beforehand the exact amount of specific activity of the peptide chain to be synthesized since the first coupling of the radioactive amino acids is not quantitative. Determination of the amount of label incorporated into the peptide chain would be calculated in the following way: a sample of protected peptide resin is hydrolyzed and the amount of amino acid determined by amino acid analysis. The degree of incorporation of radioactivity in the sample is then determined and correlated to the amount of amino acid present.

(b) Protected labeled amino acids could be coupled in an adequate molar excess, which would ensure maximal incorporation of the label with the same pre-defined specific activity. In this instance, however, the majority of labeled amino acid would be wasted. Due to the high cost of radioactively labeled amino acids, use of this method is not practical.

Incorporation of radioactively labeled amino acids into the peptide is according to Method (a). Based upon previous experimentation using this method[20], 1.5 eq. of radioactive amino acids were introduced during the first coupling, followed by 4 eq. of unlabeled amino acids in the second coupling.

(8) The synthesis is performed using an automotated Beckman Model 990 Synthesizer. The program for a single cycle is given in Figures 23 and 24. The reaction vessel is maintained under a nitrogen atmosphere to provide additional protection from oxidation of the unprotected methionine and tryptophan residues.

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V. RESULTS

1. Model Studies on Incorporation of Asparagine in Solid Phase Peptide Synthesis

a. Experimental rationale

The degree to which the formation of nitrile is suppressed during the introduction of Bpoc-asparagine into the peptide chain (using different coupling conditions) in solid-phase synthesis was determined by chromatographic analysis. Using a cation exchange resin, asparagine-glycine and β-cyanoalanyl-glycine can be separated. At pH 2.80 Asn-Gly has an absorbance maximum elution time at 255 min and β-Ala(CN)-Gly has an absorbance maximum at elution time 280 min. When the sample is overloaded, the level of detection of nitrile is 1 part per 1,000.

In this system, under the same conditions, the elution maximum for β-cyanoalanine is at 52 min, and for asparagine at 95 min. As a control experiment for the above studies we decided first to check if the nitrile compounds could rearrange to the corresponding carboxamide derivatives when treated with HF and TFA.

b. The influence of strong acids on the conversion of β-cyanoalanine to asparagine

(i) We initially observed that β-cyanoalanine was partially converted (21%) to asparagine upon treatment with anhydrous HF under standard conditions at 0°C for 30 min. More extensive treatment with HF at 0°C for 300 min caused a complete conversion to asparagine.

In contrast, only trace amounts of asparagine (<1%) were detected after treating β-cyanoalanine with 50% TFA/CH_2Cl_2 at room temperature for 45 min; after 300 min, β-cyanoalanine was partially converted (8%) to asparagine.

(ii) The influence of strong acids was also evaluated using Boc-β-cyanoalanylglycine-t-butyl ester. The chromatographic profile of the reaction mixture obtained after treatment with anhydrous HF at 0°C for 30 min showed a complete disappearance of the elution maximum of

β-Ala(CN)-Gly at 280 min. Instead, the major component was eluted at 255 min, which is the elution maximum of the asparaginylglycine dipeptide. The rough estimate of the amount of Asn-Gly present in the sample gave a value of 63%. Together with Asn-Gly, two additional components were eluted: one at 185 min and one at 467 min. This experimental design permitted only analytical runs. As a result of this, the two additional products could not be isolated and characterized.

In contrast, the chromatogram obtained after the β-cyanoalanylglycine-t-butyl ester had been treated with 50% TFA/CH_2Cl_2 at room temperature for 30 min showed that the major component is β-Ala(CN)-Gly. Asn-Gly was present in the amount of approximately 2.7%. No additional components were detected.

The above results clearly show that β-cyanoalanine alone or incorporated into a dipeptide undergoes reaarangement to asparagine after treatment with anhydrous HF. These results are qualitative and do not provide information regarding the reaction mechanism of this rearrangement.

c. Coupling of Bpoc-asparagine as preformed symmetrical anhydride

Bpoc-asparagine and dicyclohexylcarbodiimide were mixed in the molar ratio of 2:1 in the cold for 15 min. The insoluble dicyclohexylurea was removed by filtration and the solution containing the symmetrical anhydride added to the deprotected glycine-p-alkoxybenzyl resin. After 20 min coupling, the peptide-resin was filtered, washed and dried and separated into two parts: (i) the dipeptide was cleaved from the resin using anhydrous HF at 0°C for 30 min; (ii) the dipeptide was cleaved from the resin using 50% TFA/CH_2Cl_2 at room temperature for 45 min.

(i) A chromatogram of the dipeptide cleaved from the resin using anhydrous HF showed no presence of β-Ala(CN)-Gly. The major component found was Asn-Gly with an elution maximum at 255 min. Additional peaks were eluted at 185 min in 4% as determined by integration of areas, 200 min (0.4% amounts), and 218 min (0.4%). The chromatographic run was not followed for a long period of time to detect the compound which eluted at 467 min (see above). The presence of a

trace amount of glycine in the chromatogram indicated that the coupling yield was slightly less than quantitative. Due to the incomplete coupling as well as the presence of additional components in the sample, the amount of Asn-Gly formed could not be estimated.

(ii) A chromatogram of the dipeptide cleaved from the resin using 50% TFA/CH$_2$Cl$_2$ showed the presence of both Asn-Gly and β-Ala(CN)-Gly, in a ratio of 2.4:1, by area. Additional components were found to eluted at 185 min (4%), 200 min (0.4%), and 227 min (0.2%), as in the previous run.

The above experimental data indicate that introduction of Bpoc-asparagine into the peptide chain with dicyclohexylcarbodiimide by the preformed symmetrical anhydride method gives the desired product as well as the nitrile derivative of the product. Upon treatment with anhydrous HF, the nitrile derivative is partially converted to the desired asparaginyl-glycine peptide, but also to other unidentified products. (These products do not necessarily originate from the nitrile compound.)

The assumption that dehydration of asparagine compounds with dicyclo-hexylcarbodiimide proceeds primarily via mechanism A (see Fig. 6) is not consistent with our data. The experiments described above are not de-signed to and cannot distinguish among the possible reaction mechanisms of nitrile formation. Although our experimental evidence is limited to the case of the dipeptide, it is reasonable to conclude that the method for coupling asparagine derivatives by preforming symmetrical anhydride does give rise to undesired peptide products. Separation of these unde-sired peptides from the correct peptide chain may become increasingly difficult and would depend on the length and amino acid composition of a given peptide.

d. Coupling of Bpoc-asparagine with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole

No quantitative experimental data were available regarding the degree to which nitrile formation is suppressed when asparagine and glutamine derivatives are incorporated into the peptide chain with

dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in solid phase
synthesis.

It has been suggested that coupling of protected amino acids with
dicyclohexylcarbodiimide and 1-hydroxybenzotriazole proceeds through
an hydroxybenzotriazole ester intermediate. Two different orders of
addition of reactants were explored:

(i) 1-Hydroxybenzotriazole and dicyclohexylcarbodiimide
were mixed in the molar ratio of 1:1 in CH_2Cl_2 for 10 min and then
added to the deprotected glycine resing at 0°C. After 10 min, Bpoc-
AsnOH (1 equivalent) was added to the reaction mixture. The coupling time
was 40 min at 0°C and 70 min at room temperature.

(ii) In a second experiment, the 1-hydroxybenzotriazole ester
of Bpoc-asparagine was formed by reaction of DCC, 1-hydroxybenzotriazole
and Bpoc-asparagine for 10 min at 0°C, followed by addition to the depro-
tected glycine-resin. The coupling time with the ester was 40 min at
0°C and 70 min at room temperature.

In both experiments the dipeptide was cleaved from the resin by
treatment with 50% TFA/CH_2Cl_2 for 40 min. Chromatograms of the two
asparaginyl-glycine dipeptides showed no presence of β-cyanoalanyl-
glycine ($\leq$ 0.1%). The major component was Asn-Gly dipeptide. When
the Asn-Gly dipeptide was synthesized by method (ii), an extra peak
was found to elute at 216 min.

In conclusion we have confirmed that dicyclohexylcarbodiimide and
1-hydroxybenzotriazole are the reagents of choice in the coupling of
Bpoc-asparagine and presumably for Bpoc-glutamine to the peptide chain.

e. Stability of the Bpoc group to 1-hydroxybenzotriazole

1-Hydroxybenzotriazole is a moderately strong acid (pKa = 4.32) and
may cause partial deprotection of the acid-labile Bpoc moiety from the
peptide during coupling. The stability of the Bpoc group was determined
as follows: Bpoc-asparagine and 1-hydroxybenzotriazole were added
together at concentration of (i) 0.03 mM and (ii) 0.2 mM to the pro-

tected Bpoc-glycine resin. After 10 min, dicyclohexylcarbodiimide was added and stirred over a period of 90 min. The peptide was then treated with 50% TFA/CH_2Cl_2 for 40 min and the sample was analyzed according to the chromatographic system described in the previous sections. If Bpoc-Gly-resin were deprotected under these conditions, then the presence of the asparaginyl-glycine dipeptide would be detected. If Bpoc-asparagine were deprotected, two additional components would be found: asparaginylasparagine dipeptide and asparaginylasparaginylglycine tripeptide. If the Bpoc-group would be entirely stable to 1-hydroxybenzotriazole, only glycine would be found. The amount of asparaginyl-glycine dipeptide in the sample found was respectively, (i) 0.2% and (ii) 1.3% by comparison to glycine. No additional peaks were detected.

It was concluded that dicyclohexylcarbodiimide and 1-hydroxybenzotriazole are satisfactory reagents in the coupling of Bpoc-asaparagine and Bpoc-glutamine to the peptide chain. It was concluded that Bpoc-group showed a satisfactory stability during the coupling reaction with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole.

f. Summary

The results obtained from different methods of coupling Bpoc-asparagine to the peptide chain are summarized in Table 6:

Coupling Method	Products[a]	
	Asn-Gly	β-Ala-(CN)-Gly
1. Dicyclohexylcarbodiimide[b]	48%	52%
2. Bpoc-AsnOH : DCC (2:1)	71%	29%
3. DCC + 1-hydroxybenzotriazole	99.9%	<0.1%

[a] Lines 1,2 are determined by area; line 3 by quantitation of Asn-Gly and correction for color factor.

[b] Performed in this work as a control for line 2; confirms literature results.

2. Assembly of the Amino Acid Sequence of Glucagon

Bpoc-Thr(t-Bu)-alkoxybenzyl-resin (5.1 g, 1.1 mmol) was placed in the reaction vessel of a Beckman 990 Automatic Synthesizer.

The chemical steps for a cycle consisted of deprotection with 0.5% TFA/CH_2Cl_2 (3 x 1 min prewashes + 20 min), neutralization of the peptide chain with 5% diisopropylethylamine/CH_2Cl_2 (2 x 5 min) and coupling (120 min, performed twice). After incorporation of tryptophan at position 25, 0.1% indole (w/v) was added to the deprotection reagent to protect against oxidation of the indole ring. For the first coupling, four equivalents of Bpoc-amino acid and dicyclohexylcarbodiimide were used. The second coupling was with two equivalents of the reagents. Radioactive Bpoc-[^{14}C]Leu at position 26 was introduced as 1.5 eq. (1 eq. of Bpoc-Leu diluted with 0.5 eq. of Bpoc-[^{14}C]Leu) in the first coupling step, and as 4 eq. of unlabeled Bpoc-Leu in the second). Radioactive Bpoc-[^{3}H]Phe at position 6 was coupled as roughly one equivalent (1 eq. of Bpoc-Phe and 10^{-3} eq. Bpoc-[^{3}H]Phe) in the first coupling step, and as 4 eq. unlabeled in the second. DCC, the activating agent was used in equimolar excess during Bpoc-Leu and Bpoc-Phe couplings. All amino acids were added in a volume of 20 ml; the final concentration was 0.24 M for the first coupling and 0.12 M for the second coupling.

Bpoc-Asn and Bpoc-Gln were coupled as hydroxybenzotriazole esters. 1-Hydroxybenzotriazole and dicyclohexylcarbodiimide in the ratio of 1:1 were premixed for 10 min at 0°C. The mixture was added manually to the peptide-resin folllowed immediately by addition of Bpoc-Asn and Bpoc-Gln.

After each coupling, an aliquot of peptide-resin was treated with flourescamine to test for the presence of free amino groups[1]. Unfortunately, the presence of the N^{α}-Bpoc group gave a slight background which obscured the accuracy of determination at low levels. As a

standard, the flourescamine test was performed on resin-samples obtained following each deprotection. The absence of free amino groups was judged through comparison of the fluorescence of these two samples. Since no difference in background flourescence was ever observed between the samples removed after the first and second couplings, it was assumed that the coupling steps had gone to the maximum extent.

For the first six cycles of the synthesis, including the introduction of Bpoc-Val at position 23, benzoylation was performed in the following manner: following completion of the second coupling, the protected peptide-resin was treated with benzoyl chloride (10eq) and pyridine (20 eq) for 15 min. The excess of reagents was based upon the amount of the first amino acid attached to the resin. The benzoylation was performed because of the possible formation of a diketopiperizine at the dipeptide stage[2], which will be lost from the resin. Thus, there is a possibility for formation of new hydroxyl sites which could initiate growth of new peptide chains by reaction with DCC-activated amino acids. One might suppose that the couplings of the first few amino acids may not be quantitative due to proximity to the polymeric backbone of the solid support. Once the peptide chain is sufficiently removed from the resin, incomplete couplings were not anticipated.

Following incorporation of the radioactive label at position 26 (the 4th residue in the synthetic chain), loss of peptide chains from the resin during the deprotection step could be followed. Filtrates from the 0.5% TFA/CH_2Cl_2 prewash step, the deprotection step, as well as the CH_2Cl_2 washes following deprotection, were combined and diluted to a total volume of 500 ml. 1 ml-aliquots were counted with 1.5%-7% error. The results are summarized in Table 7. The percentage of loss is calculated from the amount of ^{14}C counts. For calculations of the amount of radioactivity incorporated into the peptide chains, see page 122. The differences of the percentage loss per step were not significant due to low amount of counts present in the sample. The average value was 0.2% per step. The total loss

Table 7 . Summary of the results on the loss of peptide chains
from the solid support during the deprotection step.

Amino acid in the sequence that was deprotected	14-C counts[a] [cpm/1 ml]	14-C counts [total cpm in the sample]	% loss[b]
Gln 24	54.4	27,200	0.20
Val 23	27.2	13,600	0.10
Gln 20	60.5	30,250	0.22
Ala 19	75.4	37,700	0.27
Arg 18	69.9	34,950	0.25
Arg 17	69.9	34,950	0.25
Leu 14	101.7	50,850	0.37
Lys 12	44.4	22,200	0.16
Ser 11	61.7	30,850	0.22
Asp 9	47.3	23,650	0.17
Thr 7	49.3	24,650	0.18
Gln 3	27.1	13,550	0.10
His 1	31.9	15,950	0.12

[a] The values are corrected for blank.

[b] % of loss was calculated as follows:

$$\frac{14[-C]\text{cpm in the sample}}{14[-C]\text{cpm incorporated into peptide chains on 5.1 g resin}} \times 100$$

of chains during the synthesis was calculated to be 5.6% according
to the formula: The % of peptide chains retained on the resin equals

$$(100 - x)^n \ (\%) \ = \ (100 - nx) \ (1\%) \quad \text{for small } x$$

$$x = \text{amount of loss per step (in 1\%)}$$
$$n = \text{number of steps}$$

After incorporation of phenylalanine at positions 22 and 6,
aliquots of protected peptide-resin were hydrolyzed and analyzed
for their amino acid content. The results are given in Table 8 .
Following incorporation of histidine-1 into the peptide chain, resin
hydrolysis was performed. The results of the amino acid analysis
are listed in the last column of the same table. The values repre-
sent averages of the two determinations.

The resin hydrolysis of the protected (22-29) glucagon showed
that a major loss (50%) of growing peptide chains had occurred after
incorporation of the glutamine residue at position 24. In general,
resin hydrolysis of larger protected peptide fragments do not yield
quantitative results and cannot be used for accurate monitoring of
the synthesis.

The amount of peptide chains at the end of the synthesis was
determined from the value of valine to be 0.061 mmoles per g resin.
(The weight of the protected peptide-resin is corrected for the
weight of the protected peptide anchored to the resin).

The weight of the peptide on the resin was calculated as
follows:

$$\frac{\text{mmol/g Pep-Res}}{1 \ g_{\text{Pep-Res}} - \left(\text{mmol}_{\text{Pep.}} \ x \ \frac{\text{M.W.}}{1000} \frac{\text{Pep. [gm]}}{\text{mmol}} \right)}$$

Incorporation of the radioactive label into the peptide chain
was calculated in the following way: an aliquot (500 μl) from the
hydrolysate solution was counted and the radioactivity (^{14}C or ^{3}H)
in the final volume was correlated with the amount of Leu or Phe
in the sample as determined by amino acid analysis. The yields are

Table 8 . Amino Acid Analysis of the Protected Peptide-Resins[a]

Amino Acid	Protected (22-29) glucagon		Protected (6-29) glucagon		Protected complete (1-29) glucagon	
	Expected	Found[b]	Expected	Found[b]	Expected	Found[b]
Asp+Asn	1	1.7	4	3.82	4	4.2
Thr[c]	1	1.7	3	2.51	3	3.3
Ser[c]			–	–	4	2.1
Glu(Gln)	1	0.99	2	1.82	3	2.9
Gly					1	1.00
Ala			1	0.82	1	0.14
Val	1	1.00	1	1.00	1	1.00
Met[d]	1	1.2	1	–	1	–
Leu	1	1.6	2	2.30	2	2.3
Tyr[c]			2	1.64	2	1.4
Phe	1	0.97	2	2.5	2	2.00

[a] The values for Lys and Arg are not given, because during the acid hydrolysis nitroarginine is partially converted to ornithine. Ornithine and lysine elute at the same position on the column on the Beckman 121 analyzer. N^{α}-Boc, N^{im}-Dnp-His is not hydrolyzed under the conditions employed for resin-hydrolysis. Trp is completely destroyed during resin hydrolysis.

[b] The values are averaged taking Val as 1.00.

[c] The values for Ser, Thr and Tyr are low due to partial destruction during the acid hydrolysis, and are not corrected.

[d] The values of Met following incorporation of Bpoc-Arg(NO_2)OH are not accurate, because during the resin hydrolysis, Arg(NO_2)OH is partially stable and elutes at the same position on the column of the Beckman 121 analyzer.

as follows: 21% for Bpoc-[^{14}C]Leu and 22% for Bpoc-[^{3}H]Phe.

3. Work-up and Purification of Synthetic Glucagon

a. Thiolytic cleavage of the N^{im}-dinitrophenyl group. The dinitrophenyl group of His(Dnp) was deprotected by thiolysis with thiophenol while the protected peptide was anchored to the resin.

The protected peptide-resin (3.2 g, 0.20 mmol of peptide chains) was placed in a manual shaker. 0.2 M Thiophenol in DMF (19.5 ml, 3.9 mmol, 19 eq) was added to the resin and the mixture was shaken for 60 min.

The filtrate was separated from the resin and diluted to a total volume of 2000 ml. Removal of the dinitrophenyl-group was determined spectrophotometrically by measuring the absorption at λ_{max} = 337 nm of the byproduct 2,4-dinitrophenyl-phenylsulfide The amount of deprotected dinitrophenyl-group corresponds to the amount of peptide chains present on the resin since the thiolysis reaction is quantitative[3].

The absorption of 1-ml aliquots of sample was 0.747 OD units. The extinction coefficient[3] of 2,4-dinitrophenyl-phenylsulfide is 10,250 l mol^{-1} cm^{-1}. The total amount of 2,4-dinitrophenyl-phenyl-sulfide was therefore calculated as 0.146 mmoles. This value corresponds to 0.046 mmoles of peptide chains per 1 g of protected peptide-resin. After correction for the weight of the protected peptide, a value of 0.055 mmoles of peptide chains per 1 g of resin was obtained. This value is in reasonably good aggreement with the one obtained from the amino acid analysis of the resin hydrolysate of the protected peptide-resin (0.061 mmol/g).

In order to determine the presence of residual His(Dnp), the elution time of His(Dnp) was determined on a cation exchange chromato-graphic system. At pH 5.26, a standard of dinitrophenylhistidine gave a peak at an elution time of 140 min. This peak was well-separated from other amino acids. An aliquot of the protected peptide-resin was hydrolyzed and the hydrolyzate applied to the column.

No His(Dnp) ($\leq$ 0.1%) was detected in the sample, although if there had been any present, it would have survived the conditions of acid hydrolysis.

b. <u>Cleavage of the peptide from the resin and simultaneous</u> <u>deprotection of all t-butyl groups.</u> Treatment of the protected peptide alkoxybenzyl-resin with TFA cleaves peptide chains from the solid support and removes all protecting groups with the exception of the nitro-moiety from the guanidino groups of the two arginine residues.

In a pilot experiment, an aliquot of protected peptide-resin (20 mg) was treated with 50% TFA/CH$_2$Cl$_2$ for 45 min. at room temperature. The solution was separated from the resin by filtration. The resin was washed with 50% aq. acetic acid (3 x 15 ml, 1 min). The cleavage yield was calculated from the residual peptide chains left on the resin as determined by resin hydrolysis followed by amino acid analysis, and found to be 32%.

In a control experiment, an aliquot of protected peptide-resin (11 mg) was treated with anhydrous HF/anisole for 30 min. at 0°C. The resin was washed with anhydrous ether (3 x 15 ml) to extract anisole and 50% aq. acetic acid (3 x 5 ml). The cleavage yield was calculated in the same manner as above, and found to be 59%.

In order to determine the optimal conditions for cleavage, of the peptide from the alkoxybenzyl-resin, a series of conditions were tested on 10-20-mg aliquots of protected peptide-resin. The results are summarized in Table 7 . Cleavage yields were calculated from the residual peptide chains left on the resin as determined by resin hydrolysis followed by amino acid analysis.

As seen from Table 9 , the best cleavage yield was achieved when the protected peptide was treated for 120 min. with the mixture of TFA/anisole/CH$_2$Cl$_2$ (1:1:1). However, this yield was unsatisfactory and not in complete agreement with the experimental data reported by

Wang[4]. It is quite possible that the cleavage yield was much higher
since extraction of all the peptide chains from the resin may have
been incomplete. Several additional washes were, therefore, inserted
following separation of the filtrate from the resin. These were per-
formed by alternating washes with 50% aq. acetic acid (15 ml, 1 min)
and DMF (15 ml, 1 min) repeated three times. The aqueous acidic
washes were inserted to ensure complete solubility of all components
present in the reaction mixture. Treatment of the alkoxybenzyl-
resin with aqueous solutions caused considerable shrinkage. Each
aqueous acidic wash was followed by washing with DMF to ensure good
extraction of peptide chains from the resin through reswelling. The
cleavage yield was 70%.

Table   . Summary of Cleavage Yield Results in the Removal of
         Peptide from the Resin.

Solvent	Reaction Time	Cleavage Yield
TFA/CH$_2$Cl$_2$ (1:1)	45 min	32%
TFA/CH$_2$Cl$_2$ (1:1)	120 min	35%
TFA/CH$_2$Cl$_2$ (1:3)	120 min	40%
TFA/anisole (1:1)	120 min	41%
TFA/anisole/CH$_2$Cl$_2$ (1:1:1)	120 min	49%
TFA/anisole/CH$_2$Cl$_2$ (1:1:1) (with additional washes to extract peptide)[a]	120 min	70%

[a]see Text for experimental details

    Protected peptide-resin (3.2 g, 0.20 mmol of peptide chains),
previously treated with thiophenol, was placed in a manual shaker.

A mixture of TFA/anisole/CH$_2$Cl$_2$ (1:1:1) (20 ml) was added to the resin and mixed for 120 min. at room temperature. The deprotection mixture also contained 0.1% indole (w/v) to protect tryptophan and methionine against alkylation and oxidation. The filtrate was separated from the resin and then washed as described above. The main solution and the washes were combined. TFA, anisole and DMF were evaporated under vacuum. The concentrated aqueous phase was lyophilized to yield 150 mg of crude peptide.

c. <u>Gel filtration chromatography of the synthetic dinitro-glucagon</u>. The preliminary step in the purification of the synthetic material involved separation of peptide chains of the correct size from shorter terminated peptides, as well as other compounds such as indole present in the mixture obtained following cleavage from the resin.

A Sephadex G-25 (fine) coulumn (2.5 cm x 90 cm) was equilibrated with 1% acetic acid. The lyophilized material (65 mg) was dissolved in 20 ml of 50% aqueous acetic acid to ensure solubility of all the components present. A 10-μl aliquot was counted and the amount of radioactivity present in the sample was found to be 4.3 x 10^6 cpm. The sample was applied to the column and eluted with 1% acetic acid with a flow rate of 17 ml/hr. A total of 320 fractions (896 ml) were collected. The elution pattern was followed by counting 100-μl aliquots obtained from every other fraction. Three peaks were separated. The first peak eluted at the void volume of the column (154 ml); the second eluted at the same volume as natural glucagon (210 ml); and the third eluted as a late peak (322 ml) containing a mixture of short peptides, amino acids and other small compounds. The distribution of counts was found to be in the ratio of 18%;25%;53%. The remaining 4% was due to small extraneous peaks. Recovery of the (^{14}C + ^{3}H) radioactivity from the column was quantitative.

All peaks, the first (fractions 48-60), the second (fractions 62-90) and the third (fractions 107-131) were pooled and lyophilized. The total dry weight of material recovered was 27.0 mg (42%), 30.7 mg

(47%), and 4.8 mg (7.4%) respectively. The calculation of the yield
was based upon the dryweight of material applied to the column. The
specific activity of the material recovered from the late peak was
much higher than in peaks 1 and 2. These results suggest that the
radioactivity absorbed in peak 3 represents incorporation into
short terminated peptide chains. Further characterization of this
synthetic material was not attempted.

d. <u>Removal of the Nitro-protecting group by liquid HF</u>. An ali-
quot of the synthetic dinitroglucagon recovered from peak 2 (11.3 mg)
was added to the Diaflon reaction vessel of the HF cleavage appara-
tus. Anisole (0.5 ml, final 10% v/v) was added as a scavenger for
NO_2-ions followed by the addition of indole (10 mg, final 0.2% w/v)
as a scavenger agent for tryptophan. Liquid HF (5 ml) was distilled
into the reaction vessel which had been previously cooled to -78°C.
The temperature was raised to 0°C and maintained at this temperature
for 30 min. The HF was evaporated by water aspiration and the final
traces of the acid and indole removed by vacuum with an oil pump.
The peptide was dissolved in 30 ml of 1% acetic acid. Residual
anisole and its derivatives were extracted from the sample using
15 ml of ether. The aqueous phase was lyophilized. Recovery of
the sample was quantitative.

As a control, performed before this synthetic strategy was under-
taken, the same procedure was carried out using natural glucagon.
No damage to the peptide chain was noted as evidenced by ion-exchange
chromatography.

e. <u>Ion-exchange chromatography of synthetic glucagon</u>. Further
purification of the synthetic glucagon was achieved through cation
exchange chromatography. A DEAE-cellulose column (2.6 cm x 30 cm)
was poured in 0.01 M Tris-7 Urea-10^{-3} M EDTA buffer, pH 8.5, and was
equilibrated using the same buffer. Following HF treatment, the
lyophilized sample (6.5 mg) was dissolved in 7 ml of buffer, and a
20-μl aliquot was counted for radioactivity. The amount of radio-
activity present in the sample was calculated to be 1.49 x 10^5 cpm.

The sample was applied to the column and a linear gradient of
0-0.4 N NaCl was started using a flow rate of 20 ml/hr. A total
of 121 fractions (360 ml) were collected. The eluent was monitored
by radioactivity of sample aliquots obtained from every other frac-
tion. The chromatogram is shown in Figure 10.

The first peak (peak I) was well-separated from two later
peaks. This peak eluted at an ionic strength of 0.10 N NaCl solu-
tion and had a radioactivity maximum at an elution volume of 96 ml.
The material from peak I was recovered by lophilization, and accounted
for 57% of the radioactivity applied to the column.

The chromatographic run of the synthetic glucagon was followed
immediately on the same column by a run of the native glucagon,
which was commercially available. The eluent was monitored by UV
absorption at 280 nm. In addition to the major component eluting
at 93 ml (0.1 N NaCl), an extra component was found to elute at
221 ml (0.25 N NaCl) in the ratio of 3.4:1 as determined by areas.
The additional component probably represented deamidated glucagon
as has been reported by Bromer et al[5].

f. Desalting of peak I from ion-exchange chromatography. The
lyophilized sample from peak I of the DEAE-cellulose column was
desalted on a Sephadex G-25 column (2.5 cm x 95 cm) in 1% acetic
acid with a flow rate of 15 ml/hr. The elution was followed by count-
ing radioactivity of 100- μl aliquots of sample obtained from every
other fraction.

The peak which eluted at 193 ml was lyophilized. Recovery
of the material was quantitative.

g. Rechromatography of synthetic glucagon by ion-exchange. The
homogeneity of the synthetic material was checked by reapplication
of 2 mg of sample to the DEAE-cellulose column. Conditions were the
same as those employed in Section e. The eluate was monitored by
radioactivity counting in 500- μl aliquots. A single symmetrical
peak was found at 102 ml.
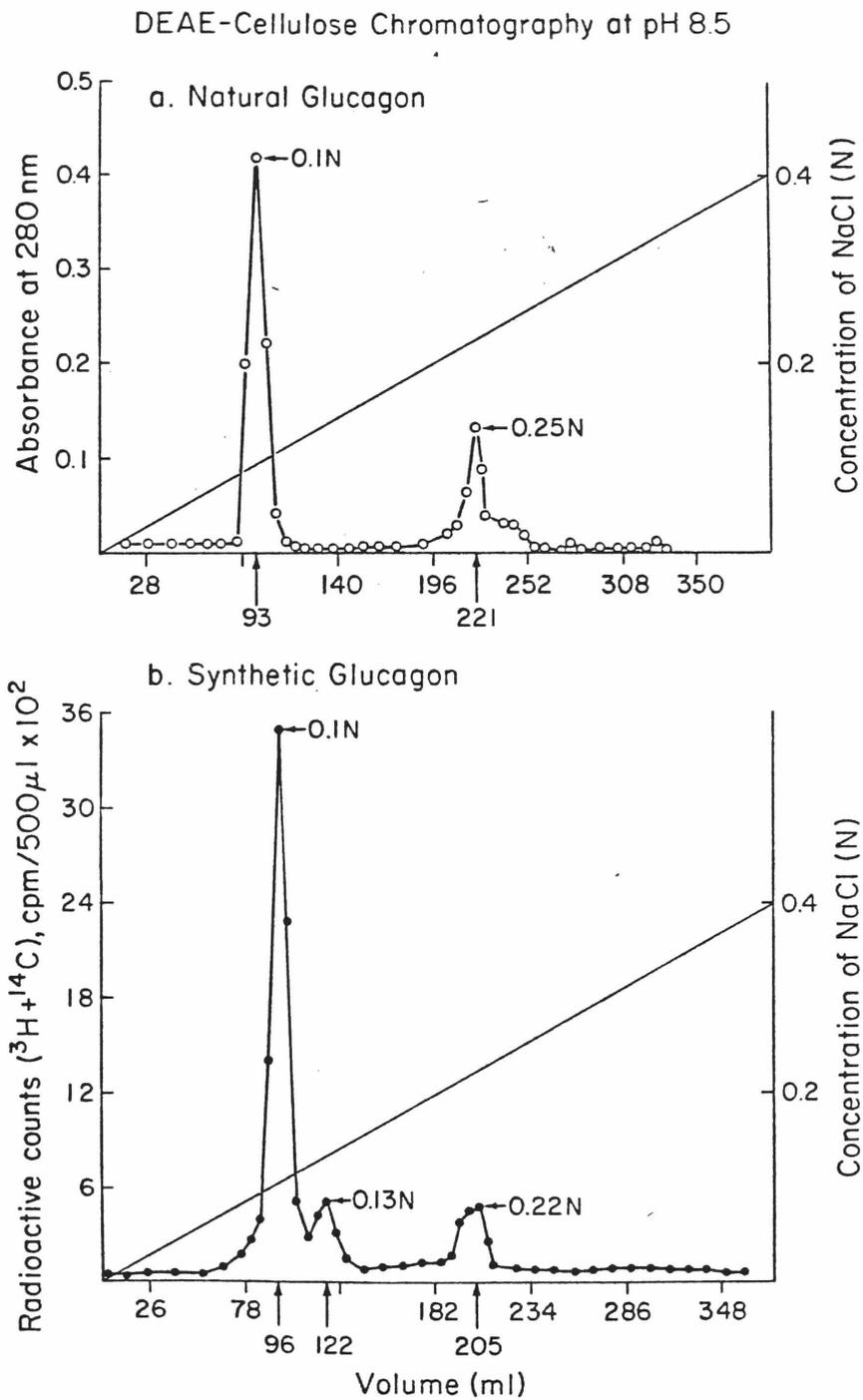
Figure 10. Ion-exchange chromatography of natural and synthetic glucagon.

h. Summary of the cleavage and purification scheme. The
cleavage and purification steps of synthetic glucagon are
summarized in Figure 11.

4. Characterization of Synthetic Glucagon.

All further characterization of synthetic glucagon was per-
formed on the material recovered from peak I of DEAE-cellulose
chromatography and desalted on a Sephadex G-25 column.

a. Amino acid composition. Samples of synthetic (0.9 μmol,
1.05 μmol) and natural (0.8 μmol, 1.2 μmol) were hydrolyzed in
acid and the composition determined by amino acid analysis. The
results are given in Table 10. As seen from Table , the amino
acid composition was in agreement with the one expected from the
sequence of glucagon.

Aliquots from the hydrolyzate of the synthetic glucagon were
counted. The radioactivity determined in the sample was: 42.5 cpm/
nmole of peptide for the ^{14}C-label and 125 cpm/nmole of peptide
for the ^{3}H-label. (The values are corrected for background). Spe-
cific activity was calculated to be 28 μCi/mmole for the ^{14}C-label
and 142 μCi/mmole for the ^{3}H-label.

b. Spectrophotometric characterization.

i. Ultraviolet absorption spectra. This is not a very sensi-
tive tool in the determination of the homogeneity of peptides. How-
ever, any modification of the two tyrosine residues and/or the indole
ring of the tryptophan, should result in alteration of the absorp-
tion maximum of the peptide.

Samples of synthetic and natural glucagon (0.3 mg) were dissolved
in 1% aqueous acetic acid (1 ml) and scanned between 240 nm and 400 nm.
The curves were similar in shape to one another. The absorption maxi-
mum for the synthetic material was at $\lambda = 276$ nm and for the natural
glucagon at $\lambda = 278$ nm. The values of these two figures are within the
sensitivity of the method employed. The curves are shown in Figure 12.

Protected Peptidyl-Resin

1. Thiophenol

2. TFA/anisole/CH_2Cl_2 (1:1:1)

Crude mixture of peptides

Sephadex G-25

Higher mw peptides   Dinitroglucagon   Lower mw peptides

HF

Crude Synthetic Glucagon

DEAE-Cellulose, pH 8.5

Sephadex G-25

Purified Synthetic Glucagon
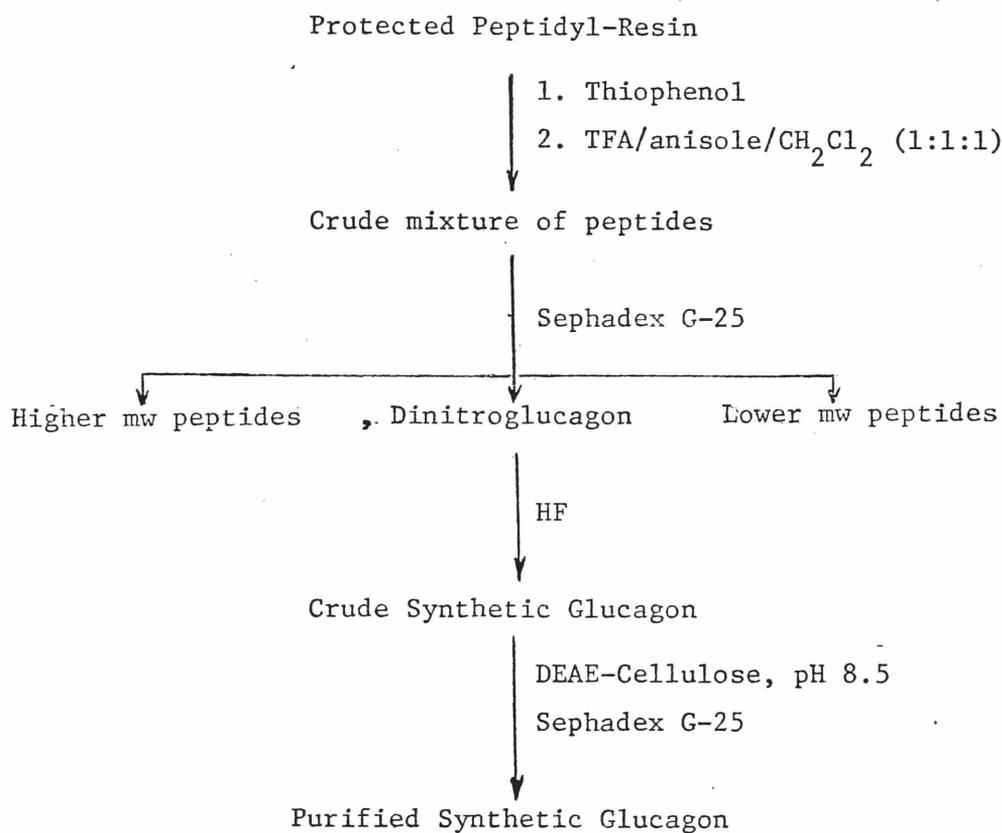
Figure 11 .   Summary of the steps in the cleavage and purification
of synthetic glucagon.

Table 10. Amino Acid Composition of Synthetic and Natural Glucagon

Mole Ratio

Amino Acid	Expected	Found Synthetic	Natural
Trp [a]	1	0.98	1.05
Lys	1	0.97	0.95
His	1	0.95	0.95
Arg	2	2.05	1.91
Asp+Asn	4	4.10	4.20
Thr	3	2.96	3.03
Ser [b]	4	3.75	3.90
Glu(Gln)	3	3.02	3.03
Gly	1	1.05	1.07
Ala [c]	1	1.00	1.00
Val	1	0.95	0.97
Met	1	0.98	1.07
Leu	2	2.01	2.05
Tyr	2	1.95	2.00
Phe	2	1.95	1.98

[a] The value for tryptophan was determined by alkaline hydrolysis with 4 N NaOH.

[b] Values for serine are corrected 15% for partial destruction during the acid hydrolysis.

[c] The value of alanine taken as 1.00.

Absorbance Spectra

a. Natural Glucagon

b. Synthetic Glucagon
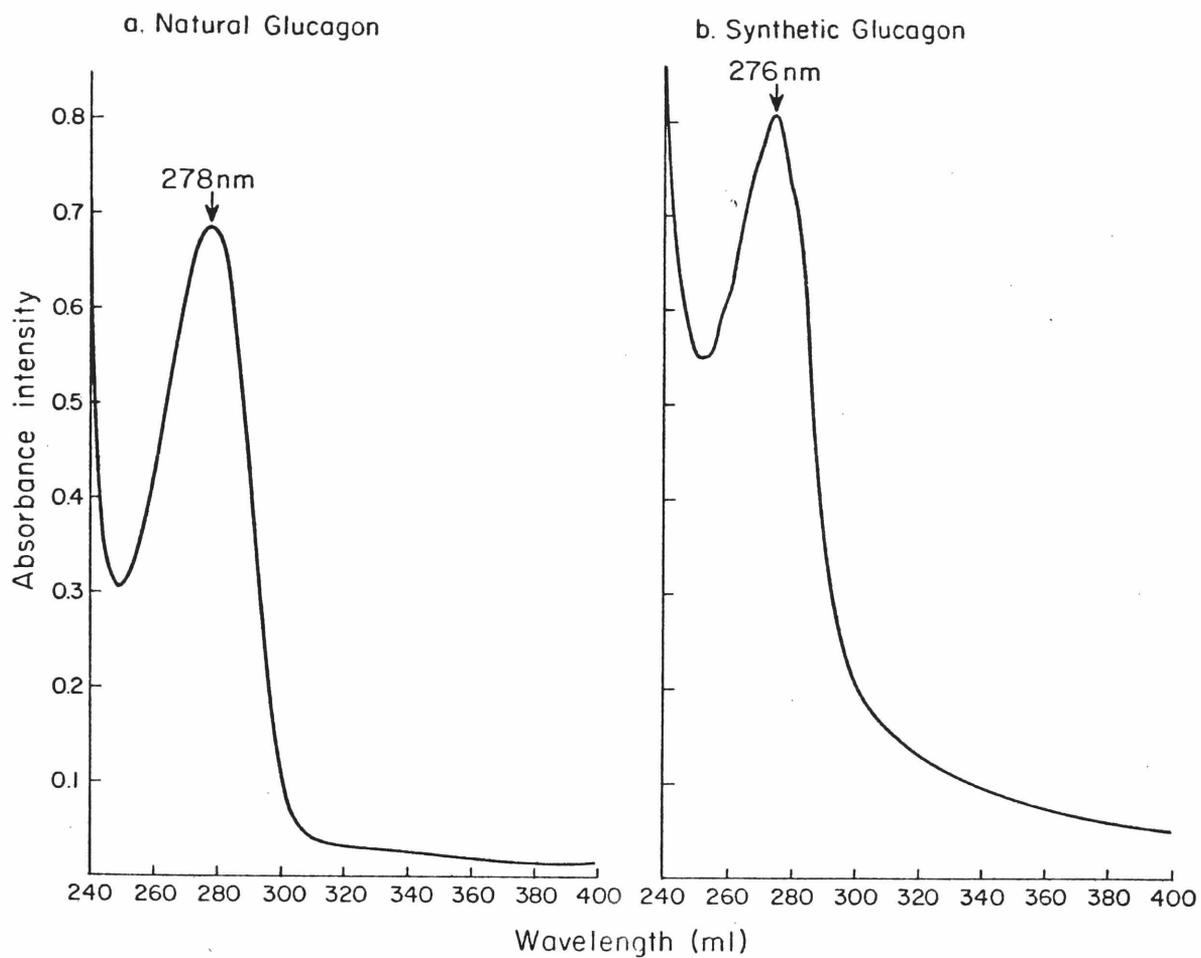
Figure 12. Ultraviolet absorption spectra of natural and synthetic glucagon.

(ii) <u>Fluorescence spectra</u>. Alkylation of the indole ring of tryptophan during deprotection of the peptide was one of the anticipated side-reactions. 1% Indole (v/v) was added to the deprotection mixture, TFA/CH$_2$Cl$_2$/anisole (1:1:1), as a trap for $\underline{t}$-butyl ions. Any change in the fluorescence of the resulting tryptophan residues would reflect the degree to which alkylation of the indole ring occurs.

The excitation and emission spectra of the synthetic and natural glucagon (0.3 mg/ml in 1% aqueous acetic acid) are shown in Figures 13,14 The excitation maxima of both the synthetic and natural glucagon were at λ = 292 nm. The broad emission spectra had a maxima at λ = 360 nm for the synthetic glucagon and λ = 350 nm for the natural glucagon. Within the precision of the measurements, spectral curves are equivalent.

c. <u>Polyacrylamide gel electrophoresis</u>

Aliquots of synthetic and natural glucagon (10 µg each) were run on a modified Lemli[6] polyacrylamide gel in which sodium dodecylsulfate (SDS) is omitted from the polyacrylamide solution. This systems allows detection of only a slight charge difference and is thus sensitive in testing for homogeneity.

Both synthetic and natural glucagon at 10 µg loading gave idenical sharp bands at 1.8 cm from the origin. At 100 µg loading, natural glucagon showed in addition to the main bands, two small bands which moved faster. At the same loading, synthetic glucagon showd a faint diffuse streak which moved ahead of the main band (Figure 15).

d. <u>Crystallization</u>

Synthetic glucagon (1 mg) was suspended in distilled water (0.4 ml) in a cluture tube. The peptide was solubilized by raising the pH to 10.2 through the addition of 0.1 N NaOH (60 µl). The pH was then lowered to 8.8 with 0.1 N HCl (50 µl) and placed in the cold. Crystals appeared after a period of 24 hours and could be visualized under a microscope at 660 magnification as rhombic dodecahedra. They are shown in Figure 16.

It should be pointed out that this is the first instance of
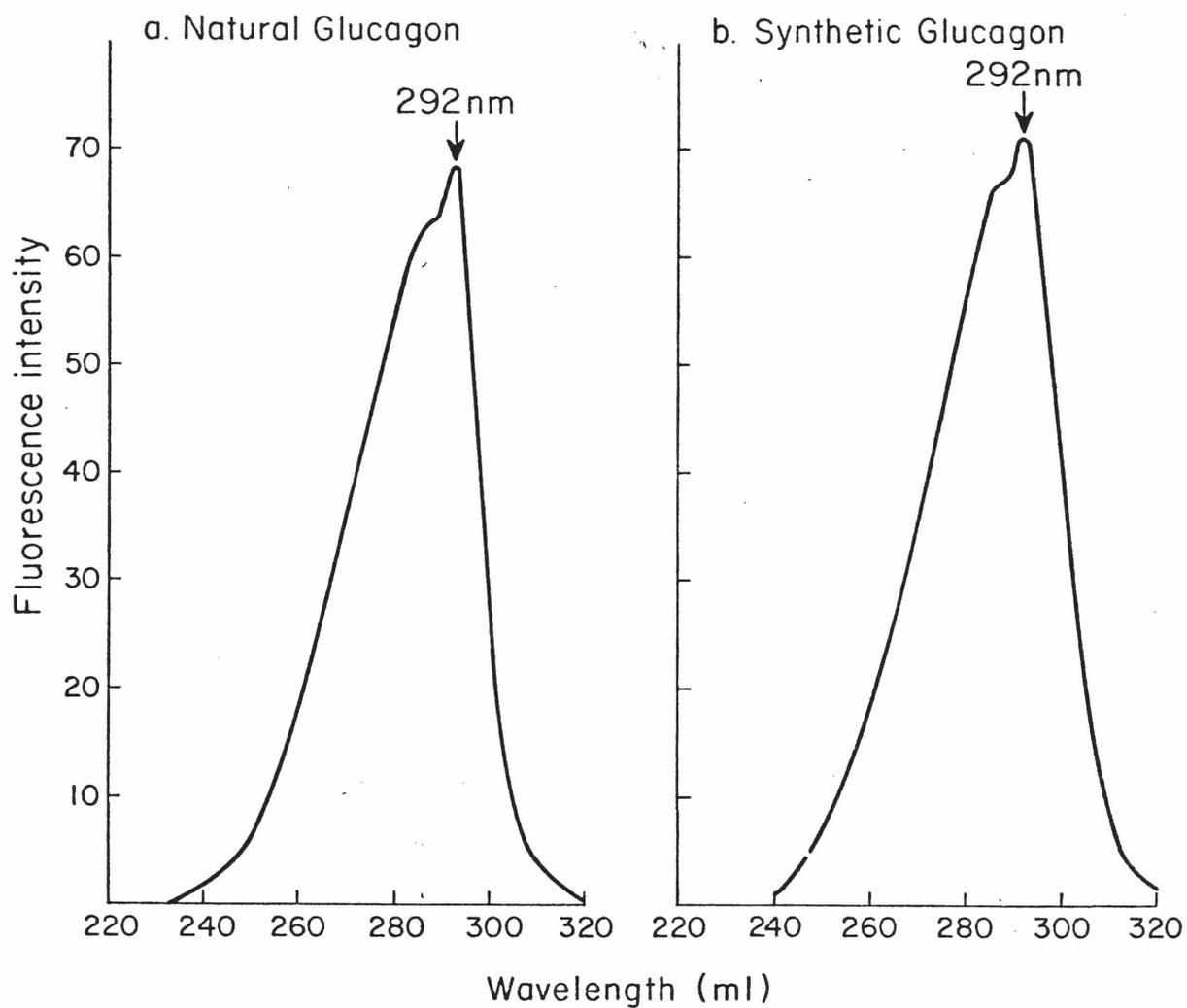
Figure 13. Exitation Spectra of natural and synthetic glucagon.
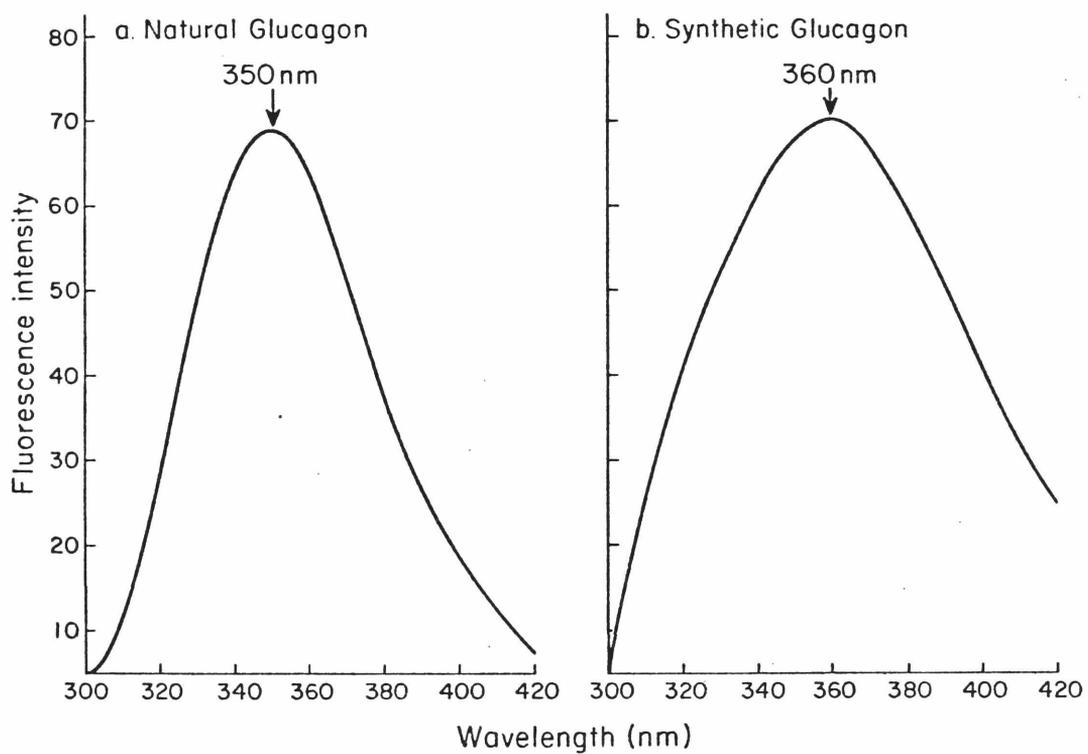
Figure 14. Emission Spectra of natural and synthetic glucagon.

Figure 15 . Polyacrylamide gel electrophoresis of synthetic and
natural glucagon.

    a= 100 μg synthetic glucagon
    b= 100 μg natural glucagon
    c= 10 μg natural glucagon
    d= 10 μg synthetic glucagon

The cathode is at the top of the gel, and the anode is at the
bottom. The separating slab gel employed was 20% polyacrylamide-
0.6% bisacrylamide. The sample was applied to the gel in 0.05 M
Tris·HCl, pH 6.7, and 6 M urea and run for a period of 14 hours
at a current of 10 mA at room temperature. The plates used in the
electrophoretic apparatus were 17 x 19 cm. The gels were stained
with 0.2% Coomasie Blue and destained in 10% aqueous acetic acid.
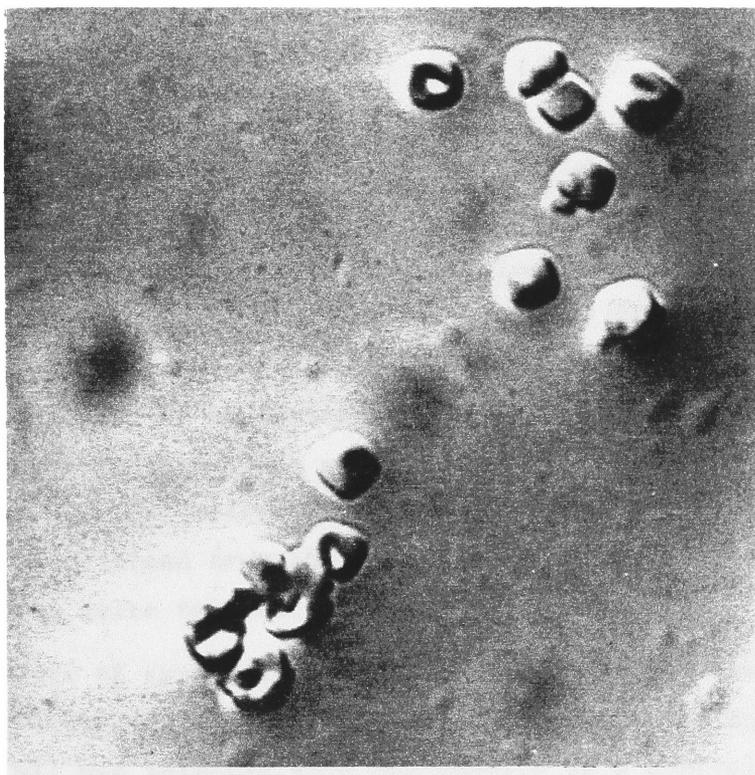
Figure 16. Crystals of synthetic glucagon, at 660 x magnification.

crystallization of a peptide of medium size obtained from the
stepwise solid-phase method. Crystallization is a powerful method
of purification and is considered to be one of the major criteria
of purity. However, taken alone it is not sufficient evidence
because of purity. There are many examples of crystalline
peptides and proteins which remain heterogeneous. In view of
the other results obtained from analytical methods applied in
the characterization of the synthetic glucagon, we believe that
this material is highly pure.

e. In vivo bioassay of hyperglycemic activity of synthetic
and natural glucagon. The hyperglycemic effect of glucagon is
reproducible in all mammalian species, including man[7]. In general,
bioassay of the hyperglycemic effect is not very specific since the
increase of glucose can be caused by a variety of different factors.
However, in the case of the synthetic material, as well as in the
case of glucagon isolated from natural sources, good characteriza-
tion by other chemical criteria is possible prior to the biological
test. Results obtained from the bioassay of synthetic glucagon
are, therefore, quite reliable.

The effect of synthetic and natural glucagon was tested on
rabbits that had been fasted for 18 hrs. In a separate experiment,
saline (1.5 ml) was injected into the rabbits as a control. Between
each test, a week interval was allowed for the animal to recover.
Stock solutions (1 mg/ml) of both the synthetic and natural glucagon
were prepared. The concentration of each solution was determined
by amino acid analysis of an acid hydrolyzate obtained from a 20 μl
aliquot. Blood samples were removed immediately prior to the test,
and following injection, every 3-5 min. for 40 min. The collected
samples were centrifuged in order to separate serum from whole blood.
The amount of glucose in the serum was determined colorimetrically
by the glucose oxidase method. Each determination was usually per-
formed three times (see page 121).

The effect of synthetic glucagon was tested in the following

manner. Three rabbits were injected intraveneously with one of three dosages of synthetic glucagon: 0.6 µg/kg, 2.3 µg/kg, and 3.7 µg/kg of rabbit. Additionally, these three dosages were tested on a single rabbit.

The effect of purified natural glucagon was tested with the same three rabbits using two dosages: 1 µg/kg and 3.6 µg/kg of rabbit.

The increase of blood-sugar content in the serum of the injected rabbits was plotted as a function of time. The data are shown in Figures 17,18. Differences in glucose concentration, as plotted on the abcissa, were due to individual variations between animals. The maximal hyperglycemic effect was achieved within 9-12 min. after the injection and was maintained for an additional period of 10-15 min.

The data obtained on the hyperglycemic effect of a 0.6 µg/kg are listed in Table 11.

Table 11 . Blood sugar rise in rabbits caused by a synthetic glucagon dose of 0.6 µg/kg.

Time (min)	Serum blood sugar (following i.v. injection) (mg%)
0	96
5	99
10	101
17	105
20	103
25	112
35	102

The maximal increase of serum blood sugar is 16 mg%.

Rabbit Blood Sugar Assay
for Glucagon
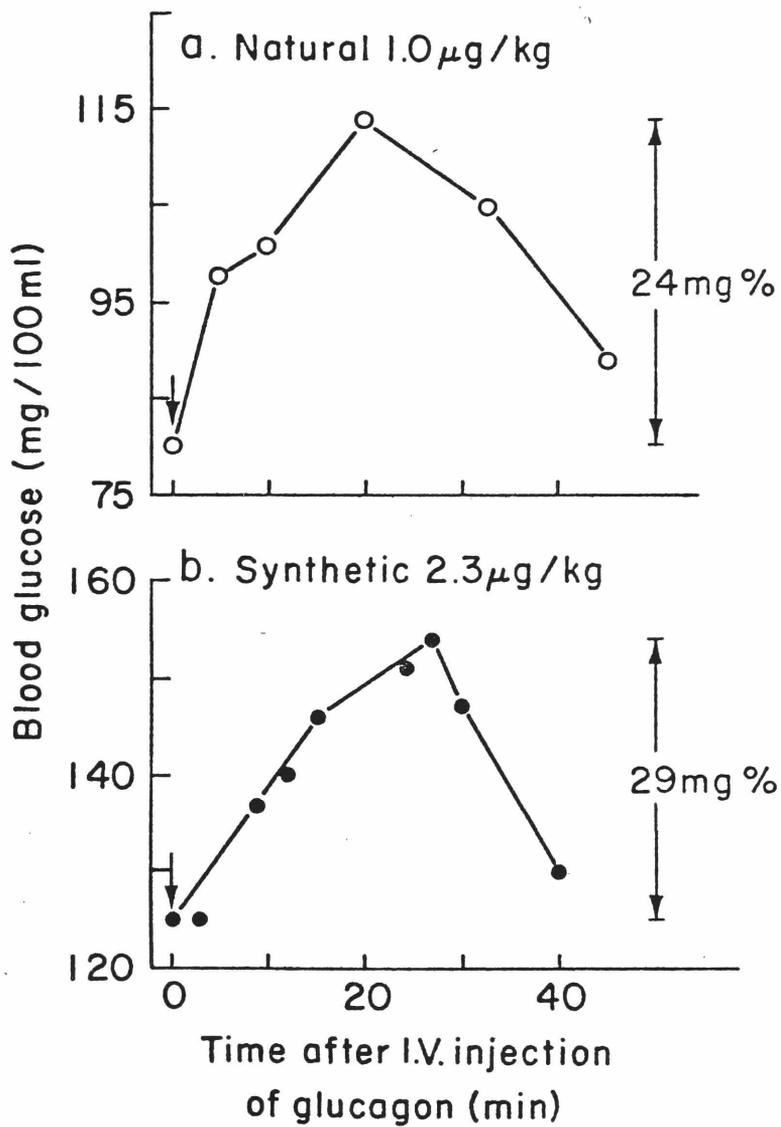
Figure 17. Bioassay for glucagon.

Rabbit Blood Sugar Assay
for Glucagon

a. Natural 3.6 μg/kg

39 mg %

b. Synthetic 3.7 μg/kg

48 mg %

Saline Control

Blood glucose (mg/100ml)

Time after I.V. injection
of glucagon (min)
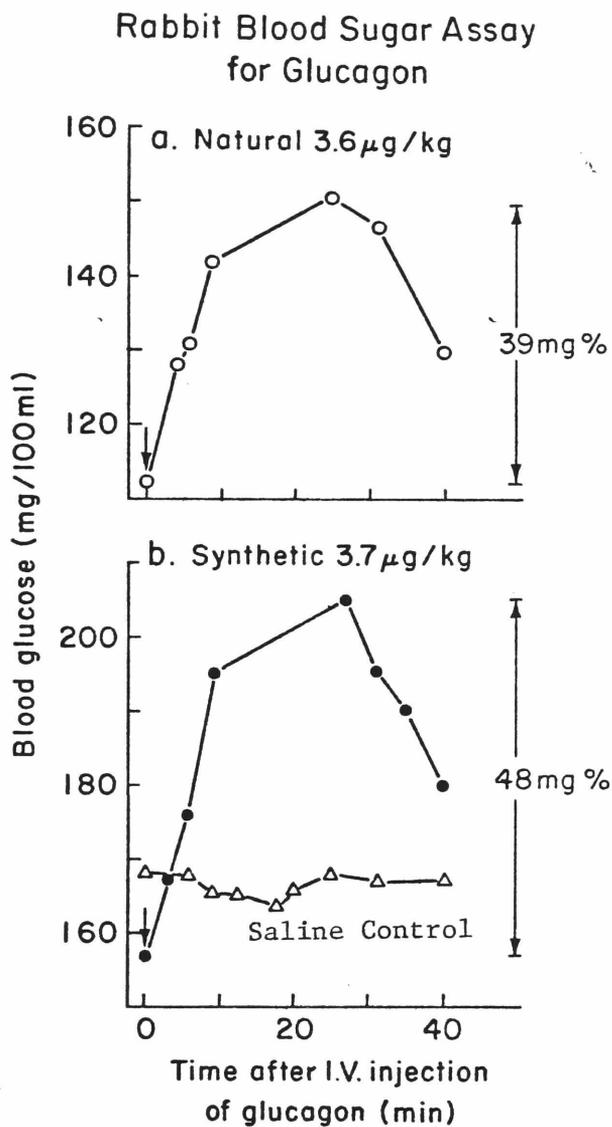
<u>Figure 18</u> .   Bioassay for glucagon, discussed in text.

The dose response curve of synthetic and natural glucagon plotting the maximal rise of serum is shown in Figure 19.

The drawn line was calculated as the best least squares fit to all the experimental points for synthetic glucagon. The linear correlation coefficient is r = 0.93. The points in the curve are usually means of triplicate determinations and the error bars correspond to ± one standard deviation. The agreement between the values for natural and synthetic glucagon was within the accuracy of both the bioassay and subsequent colorimetric determination of glucose content.

5. Preliminary Experiments on the Scope of the Synthetic Strategy.

a. Removal of nitro-groups by catalytic hydrogenolysis. In addition to HF cleavage, deprotection of nitro-groups from the two arginine residues in the synthetic material may be through the use of catalytic hydrogenolysis[8]. Using this method, harsh treatment by liquid HF is avoided. The presence of byproducts in the mixture of synthetic material following HF treatment was identified. Whether these products result from HF treatment, or from side-reactions occurring during the actual synthesis itself, is not known. Therefore, the presence of byproducts following catalytic hydrogenolysis of the synthetic material would be of particular interest.

Thus far, recovery of synthetic glucagon following catalytic hydrogenolysis of the dinitroglucagon compound has not been satisfactory.

Two types of catalysts were employed:
(1) $PdCl_2$ catalyst (5 mg) was activated with formic acid prior to the reaction[9]. An aliquot of synthetic dinitroglucagon (2 mg) was dissolved in 50% aqueous acetic acid (1 ml) and added to the reaction vessel of the hydrogenation apparatus. Reaction time was 24 hours at a hydrogen pressure of 30 psi. The catalyst was removed by filtration and an aliquot of the solution (100 µl) removed and applied to the slab gel electrophoretic system. Sample aliquots (100 µl) of synthetic dinitroglucagon were applied on the same slab gel.
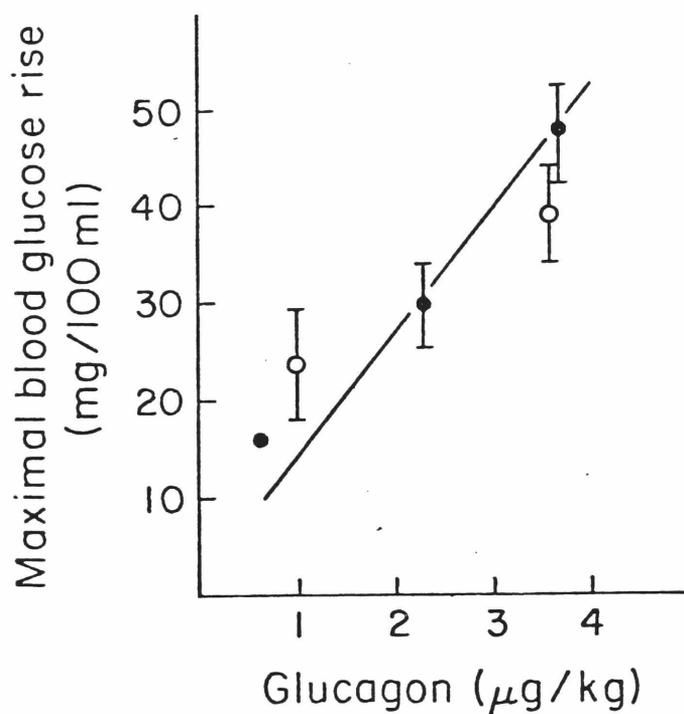
Figure 19. Dose-response curve of glucagon activity.

The drawn line was calculated at the best least squares fit to all the experimental points for synthetic glucagon (represented with full squares). The error bars correspond to ± one standard deviation.

The conditions of the run were the same as previously described
(see page   ). Both synthetic samples showed a single band at
the top of the gel in addition to a diffuse band throughout the
center of the gel (see Figure   ). Therefore, neither of the
nitro-groups were removed.

(2) 5% Pd on BaSO$_4$ (4 mg) was used[10]. An aliquot of synthetic
dinitroglucagon (2 mg) was dissolved in 50% aq. acetic acid (1 ml)
and added to the reaction vessel of the hydrogenolysis apparatus.
The total reaction time was 96 hours, an addtional amount of fresh
ctalyst (4 mg) was added to the reaction vessel. The sample was
separated from the catalyst by filtration and the solution lyophi-
lyzed. The lophilyzed material was dissolved in 0.01 N Tris-7 M
Urea buffer, pH 8.5 (1 ml) and applied to a DEAE-cellulose column
(2.5 cm x 30 cm). The conditions of the run were the same as
described on page   . The elution pattern was followed by
radioactivity counting on sample aliquots (500 µl) obtained from
every other fraction. Three peaks were separated: the first eluted
at the position expected for glucagon at a volume of 102 ml (0.1 N
NaCl); the second eluted at a volume of 187 ml (0.2 N NaCl); and
the third eluted at a volume of 231 ml (0.24 N NaCl). The amount
of material which eluted at 0.1 N NaCl was found to be 10% as com-
pared to the other two peaks and was determined by integration of
their individual areas. The other components were probably partially
reduced mononitro or starting dinitroglucagon.

Both catalysts tried have been successfully used in the past to
remove nitro-protecting groups from the arginine residues in methio-
nine-containing peptides.

b. Identification of high-molecular weight components. Since
glucagon in concentrated solutions aggregates[11], it is possible that
material isolated from the eluant in peak I is actually an aggregated
form of material eluted in peak II. In order to test the above
assumption, an aliquot of synthetic material from peak I (3 mg)
was mixed with natural glucagon (6 mg), dissolved in 13 ml of 50%
acetic acid, and applied to the column. The conditions employed
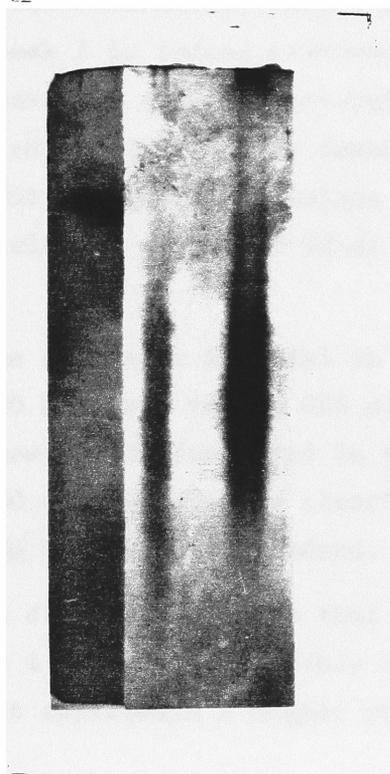
a   b   c

Figure 20 .   Polyacrylamide gel electrophoresis of synthetic
               dinitroglucagon before and after catalytic hydrogenolysis.

               a= purified synthetic glucagon (shown for comparison)
               b= synthetic dinitroglucagon after catalytic hydrogenolysis
               c= synthetic dinitroglucagon before catalytic hydrogenolysis

               For further experimental details, see legend to Figure 15 .

in the run were the same as those used in the previous run (see page 73). A total of 150 fractions (390 ml) were collected. UV absorption at 280 nm was measured at every other fraction. From the same fractions, 100 μl aliquots were counted. The absorption maxima represent material from both natural and synthetic peptide, while the radioactive maxima are from the synthetic peptide only. If peak I is indeed a reversible aggregate of synthetic dinitroglucagon (peak II), then the resulting radioactivity should be distributed between the two peaks. The chromatogram showed a radioactivity maximum at a volume of 52 ml and an absorption maximum at an elution volume of 52 ml and 204 ml as shown in Figure 21.

Aliquots of the synthetic material in peak I (20 μg) and natural glucagon (20 μg) were run on SDS slab gel electrophoresis. Synthetic peak I showed a diffuse band in the area of molecular weights 3500 to 4000 daltons and was clearly separated from the 3000 dalton glucagon band of the standard.

The above data clearly indicates that synthetic peptide recovered from peak I is not a reversible aggregate of synthetic dinitroglucagon, but represents a higher molecular weight impurity.
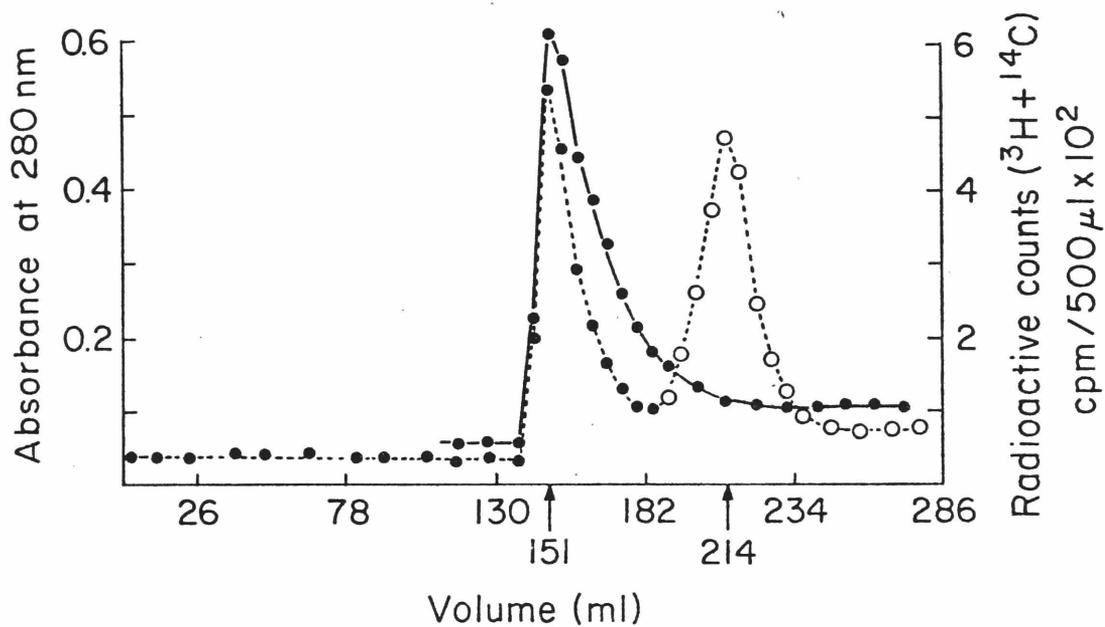
Figure 21. Sephadex B-25 chromatography of the mixture of high molecu-
lar weight synthetic peptide and natural glucagon.

High molecular weight synthetic peptide represents peak I
on Sephadex G-25 (p. 73). For clarity, the solid line is
the measured radioactivity and the dashed line is the absor-
bance. Full circles represent synthetic material and open
circles represent natural glucagon.

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VI. DISCUSSION AND CONCLUSIONS

1. Assembly of the Amino Acid Sequence of Glucagon

a. The p-alkoxybenzyl resin support. The p-alkoxybenzyl alchol
resin has been used in the past in the synthesis of protected peptide
fragments with chain lengths of ten residues or less[1]. The anchor-
ing bond of the peptide to the resin showed satisfactory acid stability
during the synthesis of glucagon. The overall loss of chains following
deprotection of the peptide with 0.5% TFA/CH$_2$Cl$_2$ was 5.6% as determined
by radioactive monitoring of the filtrates. From these data, it appears
that utilization of this resin in the synthesis of a medium sized
peptide containing 29 residues, in combination with deprotection
under mild acidic conditions, is feasible. Furthermore, we predict
that p-alkoxybenzylalcohol resin can be applied as a solid support for
the synthesis of larger peptides with 40 to 50 amino acid residues.

Cleavage of peptides from the alkoxybenzyl resin is facile.
However, the expected cleavage yields of the anchoring ester bond to
the resin may be lowered due to alkylation of the alkoxybenzyl-moiety
of the resin with t-butyl ions during deprotection of the peptide.
Yields may be improved through the addition of a scavenger, such as
anisole, to the deprotection reagent.

Modification of the alkoxybenzyl resin by the aforementioned
alkylation most probably alters its swelling properties. This results
in less efficient extraction of a large number of peptide chains from
the resin following the cleavage step. However, washing with proper
swelling solvents such as dimethylformamide, gives satisfactory
results.

b. Coupling and monitoring. The amount of protected amino acid
and dicyclohexylcarbodiimide used for each coupling step was calcu-
lated from the concentration of the first amino acid attached to the
resin. These values were kept constant throughout the synthesis.
Since the concentration of the growing peptide chains decreased during
the course of the synthesis, the actual molar excess of reagents
increased at every step. Whether this change has any effect on the

extent to which coupling takes place, or whether any side reactions occur, is not known.

The fluorescamine reagent was used to judge the degree of coupling in a qualitative fashion. However, a rather marked background fluorescence prevented detection of low levels of free amino groups.

Incorporation of radioactive label during the first coupling into the peptide chain was low: Bpoc-[^{14}C]Leu, position 26, 22%; and Bpoc-[^{3}H]Phe, position 6, 17%. This was not expected, but the reasons are not known.

Bpoc-[^{3}H]Phe apparently was incorporated into terminated peptide chains. As a result, the precalculated ratio of specific activity of [^{3}H] to [^{14}C] of the peptide was not accurate and could not be used as a criterion of homogeneity of the purified peptide. The purification of the peptide to constant [^{3}H]/[^{14}HC] ratio is still feasible and will be undertaken.

c. Termination side-reactions. In this synthesis of glucagon, results obtained from resin hydrolysis showed that the majority of peptide chains ($\leq$70%) stopped growing due to termination. A major loss ($\gtrsim$50%) of the growing peptide chain occurred following coupling of the glutamine residue at position 24. Since the second coupling of this residue was followed by benzoylation, it was not known if

termination of the peptide was through formation of the pyroglutamyl derivative or a result of incomplete coupling. It is not possible to draw any conclusions about the reasons for the remaining (20%) loss of peptide-chains, because of limitations in the accuracy of the peptide-resin hydrolysis technique.

Termination of the growing peptide chain is a major factor in the decrease of yield of the synthetic peptide.

Clearly, a detailed investigation of the termination side-reaction is necessary.

d. Branching side-reactions. The isolation by gel filtration chromatography of peptides with a higher molecular weight that glucagon suggests premature deprotection of the N^ε-amino group of lysine. This would result in branched peptides which were not originally expected since the ratio of stability[2] of the Boc- to Bpoc-protecting group is over 3,000. Data regarding this aspect of the synthesis are preliminary.

2. Deprotection of Dinitroglucagon

Removal of the nitro-protecting groups from the two arginine residues of the synthetic dinitroglucagon was accomplished using liquid HF. This method was chosen since we had shown that HF does not cause any structural damage to the natural peptide. Recovery of the synthetic peptide following acid treatment was quantitative.

Up to now, catalytic hydrogenolysis of the synthetic dinitroglucagon has not given satisfactory yields of synthetic glucagon. The low yields may be due to poisoning of the catalyst by methionine residues[3].

It is also possible that the dinitroglucagon molecule assumes a conformation in which the two adjacent nitroarginine residues become buried in the interior of the molecule and are accordingly, sterically inaccessible to the catalytic reagent.

Differing methods in the reduction of the nitro-protecting group must further be explored.

3. Purification of Synthetic Glucagon

Purification of synthetic glucagon from other byproducts was monitored by measuring the amounts of radioactivity incorporated into the peptide chain. This method was chosen and found to be more selective than the more conventional method of UV absorption, and is especially useful in distinguishing among the byproducts themselves.

Preliminary purification of the synthetic dinitroglucagon from other small compounds, as well as from higher molecular weight peptides,

was on a Sephadex G-25 chromatography column. After this step, the nitro-protecting groups were removed to give crude synthetic glucagon. In the past, DEAE-cellulose anion exchange resin had been used extensively for the fractionation and purification of both synthetic and natural glucagons[4]. Accordingly, the same method was chosen as a selective way with which to further purify the synthetic material.

Recovery of pure synthetic glucagon from DEAE-cellulose chromatography was high (57%) based on the amount of radioactivity applied to the column.

The ease of purification of the synthetic glucagon reflects to a major degree, the suitability of the synthetic strategy employed. Although formation of peptide byproducts occurred at varying stages of the synthesis, their separation from the correct synthetic peptide was facile.

4. Evidence for the Chemical and Physical Purity of Synthetic Glucagon and for its Similarity to Native Glucagon

The similarity between synthetic and native glucagon was demonstrated through the use of amino acid analyses, ultraviolet absorption and fluorescence spectrometry, polyacrylamide gel eletro-phoreses, gel filtration and ion exchange chromatograms. Additionally, both the synthetic and native materials were isolated as crystalline rhombic dodecahedra.

The aforementioned experimental data show the synthetic peptide to be reasonably homogeneous and to bear a close resemblance to the natural molecule.

At this time, characterization of the synthetic glucagon is not complete. In particular, the absence of low levels of β-aspartyl peptides must be demonstrated.

5. In Vivo Bioassay of Hyperglycemic Activity

The ability to cause hyperglycemia[5] is the ultimate property of a molecule with the glucagon structure. Injection of rabbits with three

different dosages of purified synthetic glucagon did cause a rise in the blood sugar levels. In fact, maximal hyperglycemic effect, within the limits of the bioassay, was indistinguishable from that caused by the native glucagon itself. Therefore, synthetic glucagon acts in a similar fashion to and posesses the properties of a true hormone.

6. Yields.

It is helpful to examine the yields obtained at various steps of this synthesis in order to better understand the improvements which must be made in the future. Since this synthesis of glucagon was exploratory, examination of samples at different points along the way for both analytical and preparative purposes was performed. For this reason, all of the starting material of the protected peptide was not carried though to the final step of purification.

The overall yield has been calculated from yields obtained at various steps of the synthesis as well as the purification. These yields are based upon the amount of the first amino acid attached to the resin, and are summarized in Table 12.

(1) During the assembly of the amino acid sequence of glucagon on the p-alkoxybenzylalcohol resin, 5.4% of the peptide chains were lost through cleavage of the alkoxybenzyl ester linkage of the peptide to the resin.

(2) A major termination occurred following incorporation of the glutamine residue at postion 24. This caused 50% loss of the growing peptide chains.

(3) By the end of the synthesis, an additional 20% of the peptide chains were lost through termination.

(4) Treatment of the protected peptide-resin with TFA/anisole/ CH_2Cl_2 (1:1:1) for 120 min. at 25°C, cleaved 70% of the peptide chains to give a crude mixture of peptides.

(5) Chromatography on Sephadex G-25 gave a dinitroglucagon fraction in 47% yield from the crude cleaved product.

(6) Removal of the nitro groups from dinitroglucagon with liquid HF was quantitative.

Table . Summary of the Yields Obtained During the Synthesis and
Purification of Glucagon.

Stage	Amount of material obtained in each step, calculated from Bpoc-Thr($\underline{t}$-Bu)/ g resin	Yield per Step	Overall Yield
Bpoc-Thr($\underline{t}$-Bu)-resin	210 μmoles	100%	100%
Protected peptidyl-resin	61 μmoles	29.1%	29.1%
Crude mixture of peptides after cleavage from the resin	43 μmoles	70%	20.3%
Crude dinitro-glucagon from Sephadex G-25	20 μmoles	47%	9.5%
Crude Glucagon	20 μmoles	100%	9.5%
Purified Synthetic Glucagon from DEAE-cellulose chromatography	11.4 μmoles	57%	5.4%

(7) Following DEAE-cellulose chromatography, synthetic glucagon was isolated in 57% yield.

As seen in Table 12, the overall yield of synthetic glucagon was 5.4%. The calculation is based upon the amount of C-terminal threonine anchored to the resin.

Considering that this is my first attempted synthesis of glucagon, the final yield of the synthetic glucagon is reasonable. In previous stepwise syntheses of similar chain-length molecules, initial yields were low. For example, in the synthesis of adreno-corticotropin (ACTH) by Yamashiro and Li[6], the purified synthetic material was obtained in 3% yield. In their second synthesis[7], more stable side chain protecting groups were used and the yield was considerably higher (20-30%).

As it was pointed out earlier in the discussion, the major losses occurred during the assembly of the peptide chains. The yield of the purified synthetic glucagon as calculated from the amount of N-terminal histidine was 19%. Clearly, a greater under-standing of those reactions leading to termination of peptide chains during the synthesis, is necessary in order to increase the yield.

7. General Remarks and Conclusions

Since glucagon has been successfully synthesized by the classical fragment method as well as the combined classical and solid-phase frag-ment method, it is useful to examine these apporaches in order to com-pare their relative merits. These syntheses are outlined in Figure 22.

The first synthesis of glucagon was accomplished by Wünsch[8] and made use of classical fragment methods (Figure 22, part A). Four fragments were prepared by solution methods and purified thoroughly before proceeding. They were sequence 1-6, 7-15, 16-21, and 22-29. The fragments were then coupled one at a time starting at the C-terminus to give the fully proteced glucagon (1-29). The success of the synthesis depended upon the use of side chain protecting groups based on t-butyl derivatives, the 2-nitrophenylsulfenyl (Nps) group
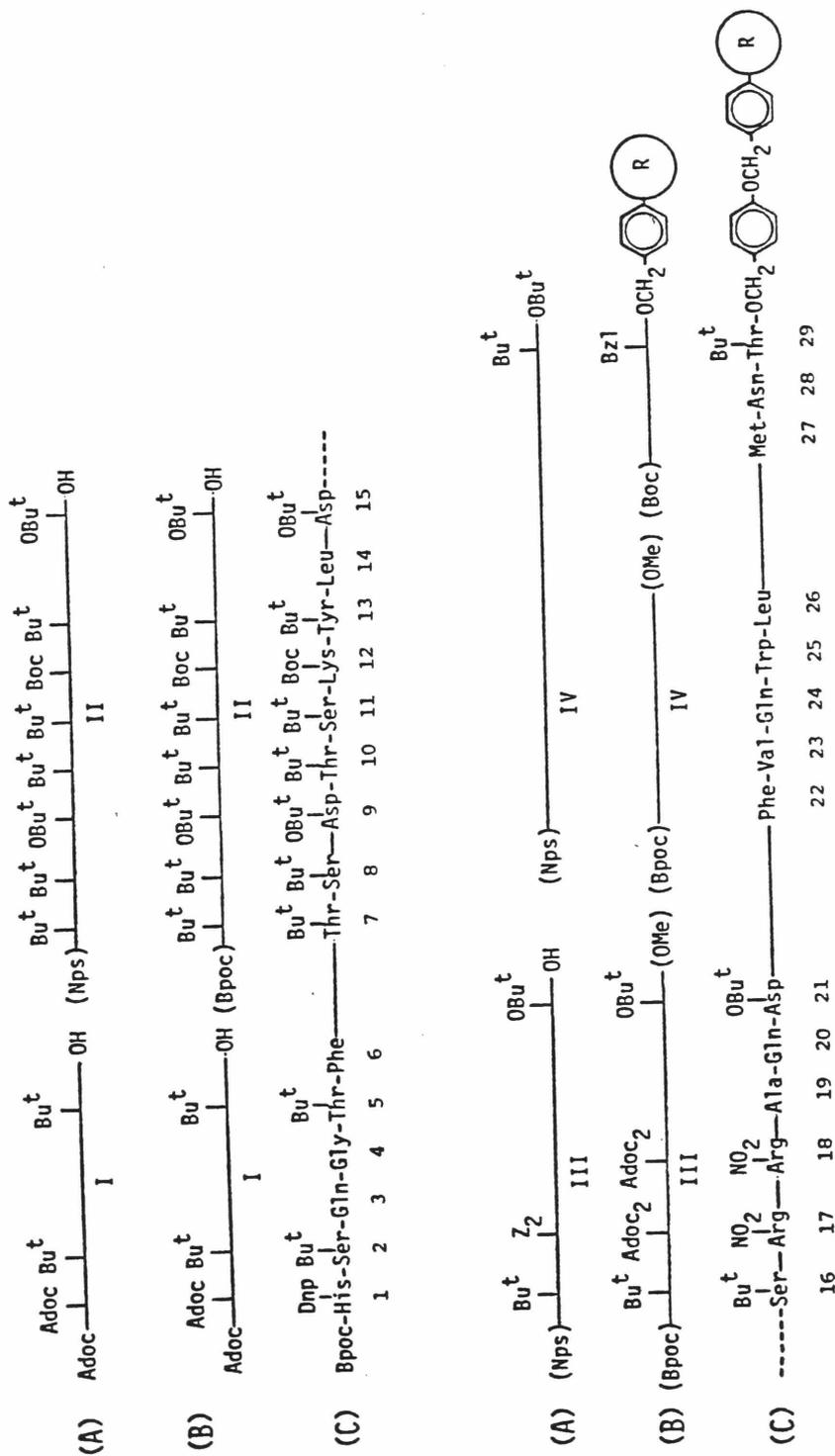
Figure 22. Strategies for the Synthesis of Glucagon.

for reversible N^α-protection, and the coupling of fragments with the new dicyclohexylcarbodiimide-N-hydroxysuccinimide (DCC-HOSu) procedure that was developed in their laboratory. The t-butyl groups were labile to relatively mild acid and made it possible to avoid strong reagents such as HBr, which could lead to serious side-reactions. The Nps group is very acid labile and could be used in combination with t-butyl groups. The DCC-HOSu method allowed peptide coupling without racemization and avoided the azide procedure which would otherwise have been required. The special problem of an Arg-Arg sequence was solved by using only a proton-protection for one guanidine side chain and dicarbobenzoxy-protection for the other. This was necessary to obtain the required solubility of intermediates. In addtion, the rarely used N^α-N^{im}-bisadamantyloxycarbonyl histidine ($Adoc_2$-His) was chosen for the N-terminal residue. The final protected peptide was deprotected with trifluoroacetic acid in the presence of scavengers and treated with a basic ion exchange resin. The crude product showed two main components by gel filtration and several other smaller ones by thin layer chromatography. It was about 50% active. After purification, the synthetic product was crystallized from aqueuous solution at pH 9.3 in the form of rhombic dodecahedra. The product was identical with natural glucagon by thin layer chromatography, amino acid analysis of enzymatic hydrolysates, and UV spectra. The biological activity was equivalent to natural pork glucagon by blood glucose, lipolysis, glycogenolysis, and radioimmune assays. This synthesis constituted a classical proof of structure of glucagon.

The synthesis of glucagon by the Protein Synthesis Group[9] in Shanghai (Figure 22, part B) also depended upon the preparation of four protected fragments. These fragments were synthesized by solution methods according to a scheme varying only slightly from the one employed by Wünsch. Protection of the side chains was with Boc and t-butyl groups. The very acid-labile Bpoc group replaced Nps as the N^α-protecting group. Both arginines as well as histidine were protected with Adoc groups. The molecule was divided into four fragments: 1-6, 7-15, 16-21, and 22-26,

This last fragment was three residues shorter that the fourth frag-
ment of Wünsch. In this instance, these last three residues were
synthesized as a tripeptide by the stepwise solid-phase method.
Thus, Boc-Thr(Bu^t) was esterified to a pellicular polystyrene
resin as a benzyl ester and residues of Asn and Met were then
coupled to give the Boc-tripeptide-resin. The four purified
protected fragments were then coupled one at a time to this tri-
peptide anchored to the solid support. Coupling of the fragments
was with dicyclohexylcarbodiimide-hydroxybenzotriazole. These
couplings were reported to be essentially quantitative. The com-
pleted protected 29-residue peptide-resin was cleaved and fully
deprotected in HF-anisole. Two peaks were obtained by gel filtra-
tion. Further purification of the sample was by DEAE-cellulose
chromatography. The overall yield obtained from the first amino
acyl-resin was about 17%. The final preparation (3.4 mg) was
homogeneous, indistinguishable from natural glucagon by polyacryl-
amide electrophoresis, and gave typical crystals. In the rabbit
blood sugar assay, essentially full activity was obtained.

The third synthesis of glucagon was accomplished by the
stepwise solid-phase method as discussed in this thesis (Figure 22,
part C).

The common feature of the three approaches discussed here
is the side chain protection by t-butyl groups, and N$^\alpha$-amino group
protection by acid-labile groups such as Bpoc or Nps. The purity
and homogeneity of synthetic glucagon obtained from each of these
three methods were comparable. The yields are very difficult to
compare because of a lack of published data on intermediates. None
of the overall yields is high, and they may actually be rather
comparable.

In the syntheses of glucagon, both the classical fragment
method employed by Wünsch, as well as the combined classical and
solid-phase method employed by the Protein Synthesis Group in
Shanghai, proved to be important contributions in the field of

peptide chemistry. However, by relying on these methods, a systematic study of the relationship of structure to biological function will not be practical for a twenty-nine residue peptide. This is due to the time and man-power necessary for thesynthesis and purification of the synthetic product. We believe that automated stepwise solid-phase synthesis is the method which can be used to overcome these limitations.

Criticism of the stepwise solid-phase method has been focused on two major points: (1) laborious steps involved in the purification of a desired synthetic product; and (2) inability to prove homogeneity of biologically active peptides and proteins through isolation of the material in a crystalline form. This synthesis of glucagon has shown that purification was simple and homogeneity could be demonstrated by a variety of discriminating techniques. Difficulties in previous syntheses have often been attributed to inherent problems in the method when they were more likely attributable to inappropriate chemical conditions employed in the synthesis itself. The work presented in this thesis proves that thoughtful planning of a strategy is a necessary prerequisite in the synthesis of a given molecule.

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VII. EXPERIMENTAL

1. Materials and Methods.

a. Solvents and Reagents. The solvents used were purchased from the following sources:

Trifluoroacetic acid - Halocarbon Products Corp., Hackensack, N.J.

Dichloromethane - Eastman; distilled from Na_2CO_3

Isopropanol - Baker Reagent

Diisopropylethylamine - Aldrich; distilled over ninhydrin

Dimethlformamide - M.C. & Bell, Spectroquality Reagent, stored over 4 A molecular sieve

Benzoyl Chloride - Aldrich

Pyridine - Aldrich; distilled over ninhydrin

The synthetic reagents were purchased from the following sources:

Dicyclohexylcarbodiimide - Schartz Mann

1-hydroxybenzotriazole - Aldrich

Fluorescamine - gift from Dr. A. Felix

For column chromatography, the highest grade reagent buffers were used:

Tris·HCl and urea - Schwartz Mann

b. Instrumentation. Amino acid analyses were performed on a Beckman 121 analyzer equipped with a Beckman system AA integrator. Liquid scintillation counting was performed on a Beckman LS 355 model. The efficiency of counting was 70% for ^{14}C-labeling and 40% for ^{3}H-labeling.

Ultraviolet spectra were taken on a Cary 14 Spectrophotometer.

Fluorescence spectra were taken on a Perkin-Elmer Fluorescence Spectrophotometer.

Absorption measurements were taken on a Beckman Spectrophotometer with a Gilford Multi-Sample Absorbance attachment.

Infra-Red spectra were recorded on a Perkin-Elmer 237 spectrophotometer.

Model Studies of Asparaginylglycine and β-cyanoalanylglycine Dipeptides

a. Esterification of Bpoc-GlyOH to alkoxybenzylalcohol resin

Preparation of alkoxybenzylalcohol resin is described on page 115.

Conditions used in the esterification were: 3 eq. Bpoc-GlyOH, 3 eq. DCC, and 0.05 eq. 4-dimethylaminopyridine over the hydroxyl groups on the resin.

Bpoc-GlyOH (1.13 g; 3.6 mmol) in CH_2Cl_2 (10 ml) dicyclohexylcarbodiimide (0.75 g; 3.6 mmol) in CH_2Cl_2 (3.5 ml) and 4-dimethylaminopyridine (3.8 mg; 0.03 mmol) were mixed and added to the alkoxybenzylalcohol resin (2.02g; 1.2 mmol OH sites). After 60 min reaction time at 25°, the resin was filtered and washed with CH_2Cl_2 (3 x 10 ml, 2 min), i-PrOH (2 x 10 ml, 2 min), and CH_2Cl_2 (3 x 10 ml, 2 min). Coupling was repeated one more time for a period of 60 min with the same molar amounts of Bpoc-GlyOH, dicyclohexylcarbodiimide, and 4-dimethylaminopyridine.

The substitution of Bpoc-GlyOH on the resin was determined by hydrolysis on 3-4 mg resin aliquots with a mixture of acetic acid: 12N HCl:phenol (1:2:1, v/v), followed by amino acid analysis[1]. An average of four determinations gave a value of 0.60 mmol Bpoc-Gly per 1 g of resin.

b. Peptide synthesis

The protocol for one synthetic cycle was: 1) prewash with 0.5% TFA/CH_2Cl_2 (v/v), 3 x 10 ml, 1 min); 2) deprotection with 0.5% TFA/CH_2Cl_2 (v/v) for 20 min; 3) neutralization with 5% diisopropylethylamine (v/v), (2 x 10 ml, 4 min); 4) coupling procedures for Bpoc-AsnOH are described in separate sections (see pp. 61-64).

All syntheses were performed using a manual shaker.

c. Peptide analysis

The following procedure is used routinely in our laboratory[2]. In

general, chromatograms were heavily overloaded with the major component
to permit detection of small amounts of peptide byproducts. Peptide
samples were run on a Beckman Model 120 B Amino Acid Analyzer equipped
with an Altex 1.0 ml rotary-valve manual injector. Standard conditions
for all runs were: 58 cm x 0.9 cm column; Beckman AA 15 cation exchange
(sulfonated) resin; buffer flow rate 61 ml/hr; ninhydrin flow rate
32 ml/hr; column temperature 57°C. The buffer (pH 2.80) was prepared
by dilution of Beckman 0.2N sodium citrate (pH 3.49) to four liters fol-
lowed by the addition of 4 ml of 6N HCl.

Elution times: 255 min for Asn-Gly and 280 min for β-Ala(CN)-Gly.

d. Determination of color value of Asn-GlyOH

Asn-Gly was synthesized as described under V.d. The column was
loaded with 3.5 mg (0.19 μmol) in 1 ml H_2O. The area of the peak eluted
at 255 min was 2.6 units.

The concentration of the asparaginylglycine peptides were cal-
culated from the equation:

$$C \text{ (unknown)} = \frac{\text{Area (unknown)}}{\text{Area (standard)}} \quad x \quad C \text{ (standard)}$$

e. The influence of strong acids on a conversion of β-cyanoalanine to asparagine

β-cyanoalanine was purchased from Aldrich. The elution maximum on
the described chromatographic system was 52 min. The main characteristic
of the symmetrical peak was that the channel ratio of 570/440 was
reversed in the ratio 1:5. The β-cyanoalanine used was free (< 0.17)
of ninhydrin-positive impurities as determined by an overloaded run.

The elution maximum of asparagine on the same chromatographic
system was 95 min.

(i) β-cyanoalanine (a: 0.74 mg; 6.5 μmol) and (b: 1.42 mg;
12.4 μmol) were treated with 2.5 ml anhydrous HF at 0°C for 30 min and
300 min, respectively. Following treatment, the sample was dissolved in
15 ml of 50% aq. acetic acid. The acetic acid was evaporated and the

sample was dissolved in 5 ml water. A 100-fold dilution of this initial concentration was required to bring the major peak on-scale.

(ii) β-cyanoalanine (a: 0.51 mg; 4.5 μmol) and (b: 2.36 mg; 20.7 μmol) were treated with 50% TFA/CH$_2$Cl$_2$ at room temperature for 45 min and 300 min, respectively. The solution was evaporated and the sample dissolved in 5 ml water. 1 ml of the sample was applied to the column.

Boc-β-cyanoalanylglycine-t-butylester was provided by Dr. A.R. Mitchell.

(iii) Boc-β-cyanoalanylglycine-t-butylester (6.8 mg; 15 μmol) was treated with 2.5 ml of anhydrous HF at 0°C for 30 min. Proline (0.99 mg; 8.6 μmol) was added to the sample prior to HF treatment as a control for possible loss of the sample during the workup. Following the treatment, the sample was dissolved in 30 ml (3 x 10 ml) 10% aq. acetic acid. Acetic acid was evaporated and the sample dissolved in 5 ml of water for chromatographic analysis of β-Ala(CN)Gly and diluted to 65 ml for analysis of AsnGly. We found: AsnGly: 9.5 μmol (63.5%) β-Ala(CN)Gly < 10 nmoles (<0.1%).

(iv) Boc-β-cyanoalanylglycine-t-butylester (1.75 mg; 3.8 μmol) was treated with 2 ml of 50% TFA/CH$_2$Cl$_2$ at room temperature for 30 min. Following treatment, TFA was evaporated and the sample dissolved in 5 ml water for chromatographic analysis of AsnGly and diluted to 50 ml for analysis of β-Ala(CN)Gly. 1 ml of the sample was applied on the column. The amount of Asn-GlyOH present was calculated to be 0.10 μmol or 0.1/3.8 x 100 = 2.7%.

f. <u>Coupling of Bpoc-asparagine as preformed symmetrical anhydride</u>

Bpoc-AsnOH (224.4 mg; 0.52 mmol; 8 eq.) in 1 ml DMF and dicyclo-hexylcarbodiimide (53.6 mg; 0.26 mmol; 4 eq.) in CH$_2$Cl$_2$ (0.4 ml) were mixed at 0°C for 15 min. The insoluble dicyclohexylurea was removed by filtration and the clear solution added to the deprotected Glycine resin. Reaction time was 20 min. The solution was filtered, and the resin was washed with DMF (3 x 10 ml; 2 min), CH$_2$Cl$_2$ (3 x 10 ml; 2 min), i-PrOH (2 x 10 ml; 2 min), and CH$_2$Cl$_2$ (2 x 10 ml; 2 min).

(i) An aliquot of peptide-resin was treated with 2.5 ml anhy-
drous HF for 30 min. Following treatment, 10 ml of 10% aq. acetic acid
were added into the HF vessel and the solution separated from the resin
through filtration on a scintered glass funnel. This was followed
by two additional washes with 10 ml of 10% aq. acetic acid. Acetic
acid was evaporated and the sample dissolved in 50 ml water for chroma-
tographic analysis.

(ii) The remaining peptide resin was treated with 2 ml 50%
TFA/CH_2Cl_2 at room temperature for 45 min. Following treatment, the
peptide solution was filtered. The resin was washed 3 times with 10 ml
aliquots of 10% aq. acetic acid. The washes were combined with the main
peptide solution and the total volume evaporated to dryness. The sample
was dissolved in 50 ml water.

g. Coupling of Bpoc-asparagine with dicyclohexylcarbodiimide and
1-hydroxybenzotriazole

(i) 1-Hydroxybenzotriazole (40.3 mg; 0.26 mmol; 4.4 eq.) in
DMF (0.5 ml) and dicyclohexylcarbodiimide (49.4 mg; 0.24 mmol; 4 eq.) in
CH_2Cl_2 (0.4 ml) were mixed at 0°C and added to the deprotected glycine
resin (101 mg; 0.06 mmol) still at 0°C in the shaker. After 10 min,
Bpoc-AsnOH (104.7 mg; 0.24 mg; 4.0 eq.) in DMF (0.5 ml) was added to the
mixture still at 0°C. The coupling time was 40 min at 0°C and 70 min at
room temperature. The first part of the coupling reaction was at 0°C
in order to prevent the possible reaction of Bpoc-AsnOH with dicyclohexyl-
carbodiimide which can lead to formation of the nitrile derivative. The
peptide was cleaved from the resin with 50% TFA/CH_2Cl_2 for 45 min. TFA
was evaporated and the sample dissolved in 5 ml water. 1 ml was applied
to the column to give a single peak. To determine the elution time
properly, the final volume of the sample was 500 ml. The elution time of
the peak was 255 min.

(ii) 1-Hydroxybenzotriazole (35.8 mg; 0.24 mmol; 4 eq.) in
DMF (5 ml) and Bpoc-AsnOH (100.0 mg; 0.24 mmol; 4 eq.) were mixed at
0°C. After 10 min, the mixture was added to the deprotected glycine
resin (100.2 mg; 0.06 mmol) still at 0°C. Dicyclohexylcarbodiimide

(49.4 mg; 0.24 mmol; 4 eq.) in CH_2Cl_2 (0.2 ml) was added into the shaker. The reaction time was 40 min at 0°C and 70 min at room temperature. The peptide was cleaved from the resin with 50% TFA/CH_2Cl_2 for 45 min at room temperature. The solution was evaporated and the sample dissolved in 5 ml water. 1 ml of sample was applied to the column. Two peaks were eluted: Asn-GlyOH at 255 min and one unidentified peak at 216 min.

h. Stability of the Bpoc-group to 1-hydroxybenzotriazole

(i) 1-Hydroxybenzotriazole (48.5 mg; 0.32 mmol; 5.4 eq.) in DMF (5 ml) and Bpoc-AsnOH (102.3 mg; 0.24 mmol; 4.1 eq.) in DMF (5 ml) were added to Bpoc-glycine resin (100.0 mg; 0.058 mmol). After 10 min, dicyclohexylcarbodiimide (74.1 mg; 0.36 mmol; 6.2 eq.) was added for 90 min. The resin was treated with 50% TFA/CH_2Cl_2 (5 ml) for 45 min. The solution was evaporated and the sample dissolved in 5 ml water. 1 ml of sample was applied to the column. The glycine peak was eluted at 165 min and Asn-Gly at 255 min.

The concentration of 1-hydroxybenzotriazole was 0.031 μM. In the applied sample there were 29.4 µmol (99.8%) of glycine and 0.06 µmol (0.2%) of Asn-Gly.

(ii) 1-Hydroxybenzotriazole (101.4 mg; 0.24 mmol; 9.3 eq.) in DMF (0.5 ml) and Bpoc-Asn (41.8 mg; 0.27 mmol; 10.5 eq.) in DMF (0.5 ml) were added to the Bpoc-glycine resin (44 mg; 0.026 mmol). After 10 min, dicyclohexylcarbodiimide (41.7 mg; 0.20 mmol; 7.8 eq.) in DMF (0.3 ml) was added. Coupling time was 90 min. The resin was treated with 50% TFA/CH_2Cl_2 (5 ml) for 45 min at room temperature. TFA was evaporated and the sample dissolved in 5 ml water. 1 ml of sample was applied to the column. Glycine and Asn-GlyOH eluted at 165 min and 255 min, respectively. In the sample there were 12.5 µmol (98.7%) of glycine and 0.16 µmol (1.3%) of Asn-Gly.

3. <u>Solid-Phase Assembly of the Glucagon Amino Acid Sequence</u>.

a. <u>Amino acid derivatives</u>. Bpoc-amino acids with protected side chains, Bpoc-Asn·DCHA salt, and Bpoc-Gln·DCHA salt were purchased from Chemical Dynamics. Bpoc-amino acid derivatives with unprotected side chains, Bpoc-Trp·DCHA salt and Bpoc-Met·DCHA salt were prepared by Mr. I. Uhrik according to the procedures of Feinberg and Merrifield[3].

<u>t</u>-Boc-im-Dnp-His-L-Histidine was purchased from Chemical Dynamics and recrystallized from isopropanol prior to use. (Recrystallization was in 70% yield).

The purity of the amino acid derivatives were checked prior to use. Melting points were checked on a Thomas Hoover apparatus. Thin-layer chromatography was performed using 250-micron silica gel G glass plates, Analtech, Inc. Spots were developed in the following system: butanol/pyridine/acetic acid/water (30:20:6:24), followed by preheating to 80°C, spraying with ninhydrin (0.15%) in acetone containing 3% pyridine and 2% acetic acid, and reheating. R_f values were as expected, and no impurities were found.

b. <u>Recovery of Bpoc-amino acids from their DCHA or CHA salts[3]</u>.

The required amount of Bpoc-amino acid derivative was dissolved in CH_2Cl_2 (20 ml) and the solution washed with a sodium citrate buffer, 0.5 M citric acid, pH = 3.5 (3 x 10 ml) in a separation funnel. The solution was back-extracted with CH_2Cl_2. The combined CH_2Cl_2 washes were then washed with water (3 x 10 ml) which was further back-washed with CH_2Cl_2. The combined organic washes were dried over $MgSO_4$, the solution filtered and then evaporated to dryness at 20°C.

c. <u>Preparation of radioactive Bpoc-amino acids</u>. Radioactive amino acids, [14C]Leu (specific activity 50.6 mCi/mmol) and [3H]Phe (specific activity 6.7 mCi/mmol) were purchased from New England Nuclear .

Bpoc-protected radioactive amino acids were prepared following the procedures described by Feinberg and Merrifield[3].

i. Preparation of Bpoc-[^{14}C]Leu·CHA. L-Leu (71.4 mg, 0.55 mmol) and [^{14}C]Leu (0.65 mg, 0.005 mmol, 0.25 mCi) were mixed together in 0.1 N HCl (2.5 ml) and the sample brought to dryness. Triton B (N-benzyltrimethylammonium hydroxide in 40% methanol, 1 ml) was added and the sample brought to dryness. The resulting oily residue was evaporated twice more with 1-ml portions of DMF at 50°C under high vacuum. The residue was then dissolved in DMF (1 ml) and treated with Bpoc-O-phenylcarbonate (0.18 g, 0.55 mmol) for 12 hr. at 46°C. The reaction mixture was diluted with water (25 ml) followed by washing with ether (3 x 25 ml). The aqueous phase was cooled on ice and acidified to pH 4 with 1 M citric acid. The white precipitate was extracted into 25 ml of ether and the ether layer washed with 1 M sodium citrate buffer, pH 3.5, (2 x 10 ml) and water (4 x 20 ml). The organic layer was dried over MgSO$_4$ for 5 min. and CHA (5 drops, pH of the solution was 8) added. The solution was allowed to stand in the cold overnight to crystallize. The crystals were filtered, washed with cold ether and dried. The crystals were characterized as follows: m.p. 147–150°C, R$_f$ = 0.7 in butanol/pyridine/acetic acid/water (15:10:3:12). Yield: 30%.

ii. Preparation of Bpoc-[^{3}H]Phe·DCHA salt. L-Phe (0.108 g, 0.65 mmol) and [^{3}H]-L-Phe (0.025 mg, 5 x 10^{-4} mmol) were mixed together in 0.01 N HCl (1 ml) and the sample brought to dryness. Triton B (N-benzyltrimethylammonium hyroxide in 40% methanol, 1 ml) was added and the sample brought to dryness. The resulting oily residue was evaporated twice more with DMF (2 ml) at 50°C under high vacuum. The residue was then dissolved in DMF (1 ml) and treated with Bpoc-phenylcarbonate (0.29 g, 0.87 mmol) for 6 hrs. at 46°C. Dilution of the reaction mixture with water (25 ml) was followed by washing with ether (3 x 25 ml). The aqueous phase was cooled on ice and acidified to pH 3.5 with 1 M citric acid. The ether phase was washed

with sodium citrate buffer, pH 3.5 (2 x 10 ml) and water (4 x 20 ml) followed by drying over $MgSO_4$ for 5 min. DCHA (5 drops, pH of the solution was raised to 8) was added and the solution placed in the cold overnight to crystallize. Crystals were filtered and characterized as follows: m.p. 117-119°C, R_f = 0.7 in methanol/chloroform/acetic acid (10:85:5).

d. <u>Preparation of p-alkoxybenzylalcohol-resin</u>. The Merrifield resin (chloromethylated copolystyrene-1% divinylbenzene, 0.75 meq/g, 200-400 mesh) was used in this work and purchased from Bio-Rad Chemical Company.

Infra-Red spectra were recorded on a Perkin-Elmer spectrophoto-meter with KBr pellets.

The alkoxybenzylalcohol resin was prepared according to the procedure described by Wang[4].

(i) Preparation of methyl p-alkoxybenzoate resin.

Merrifield-resin (20 g, 15 mmoles chloromethyl groups) in dimethylacetamide (225 ml) was allowed to react with methyl-p-benzoate (9.2 g, 60.2 mmoles, 4 equiv.) and sodium methoxide (3.2 g, 59 mmoles, 4 equiv.) at 85° C for 40 hrs. The resin product was collected and washed with DMF, dioxane, CH_2Cl_2 and methanol (350 ml) and dried under vacuum. The weight of the dried resin was 21.9 g.

The IR spectra showed the expected absorption at 1716 cm^{-1} for the ester band and 1245 cm^{-1} for the ether band. Volhard determination for chloride ions was negative. It contained 0.64 mmol/g of methoxy groups as determined by microanalysis.

(ii) Preparation of p-alkoxybenzylalcohol resin.

Methyl-p-alkoxybenzoate resin (21.5 g, 13.76 mmoles OCH_3) was treated with $LiAlH_4$ (2.2 g, 52.7 mmoles) in 400 ml of dry ether under nitrogen atmosphere for 20 hrs at 55° C. The resin was then collected and washed with ethyl acetate, methanol, dichloromethane, to give a gray-colored product. The color was removed by stirring in 2 liters of a 1:1 mixture of 1 $\underline{N}$ H_2SO_4 and dioxane for 45 hr at room temperature. The resin was collected and washed with CH_2Cl_2

and methanol, and dried under vacuum to give 19.5 g of white product. The IR spectrum showed complete disappearance of the ester band at 1716 cm^{-1}. Microanalysis indicated that the resin contained 0.06 mmole OCH_3/g resin.

e. <u>Esterification of Bpoc-Thr(t-Bu) to alkoxybenzylalcohol resin.</u> Bpoc-Thr(<u>t</u>-Bu) (1.2 g; 2.9 mmol; 1 equiv.) in CH_2Cl_2 (10 ml), dicyclohexylcarbodiimide (0.6 g; 2.9 mmol; 1 equiv.) in CH_2Cl_2 (4 ml) and 4-dimethylaminopyridine (1.1 g; 8.8 mmol; 3 equiv.) in CH_2Cl_2 (10 ml) were added to alkoxybenzylalcohol resin (5.1 g, 2.9 mmol of hydroxyl sites) in a beaker. The reaction mixture was stirred for 6 hours. The product was washed with CH_2Cl_2 (20 ml), DMF (20 ml), and methanol (20 ml) to yield colorless Bpoc-Thr(<u>t</u>-Bu)-resin. The amount of threonine esterified to the resin was determined by two methods: (1) resin hydrolysis on 5-10 mg of sample with phenol:12 N HCl:glacial acetic acid (1:2:1) for 24 hr at 110° C, followed by amino acid analysis gave an average value of 0.20 mmol Bpoc-Thr(<u>t</u>-Bu) per 1 g of resin; (2) picric acid determination on 30 mg aliquots of deprotected resin gave an average value of two determinations of 0.23 mmol of free amino groups per 1 g of resin. The average of the two methods of determination is 0.21 mmol per 1 g of resin. The excess of free hydroxyl groups left on the resin (1.8 mmol) following attachment of Bpoc-Thr(<u>t</u>-Bu) to the resin were benzoylated. The conditions were: benzoyl chloride (5.9 ml; 34.4 mmol; 18 equiv.) and pyridine (5 ml; 64 mmol; 34 equiv.) in CH_2Cl_2 (50 ml) were stirred with the amino acid resin for 15 min at 0° C. The product was washed with CH_2Cl_2 (3 x 3 min, 20 ml), isopropanol (3 x 3 min, 20 ml) and CH_2Cl_2 (3 x 3 min; 20 ml) and dried overnight under high vacuum.

f. <u>Automated Peptide Synthesis</u>. The Beckman Model 990 Peptide Synthesizer was used. The program for one cycle is given in Figures 23 and 24.

g. <u>Fluorescamine monitoring</u>. The procedure applied was a slight modification of the one published by Felix and Jimenez[5]. A Pasteur pipette was plugged with glass wool, so as to serve as a funnel. A small aliquot of peptidyl resin was added, and the Pasteur pipette-funnel

Beckman®

PROGRAM SHEET
MODEL 990
PEPTIDE SYNTHESIZER

PROGRAM NUMBER 1   AUTHOR S. Mojsov   DATE July 1976

DESCRIPTION Deprotection

PROGRAM STATEMENTS

	Reservoir Contents	Program Statement	Mix/Transfer
1 (B)	CH$_2$Cl$_2$	Meter TFA, Drain	1x0 min
2 (A)			
3 (A)	CH$_2$Cl$_2$	Mix, Meter TFA	3x1 min
4 (A)	CH$_2$Cl$_2$	Mix TFA, Meter	1x20 min
5 (A)	CH$_2$Cl$_2$	Mix, Meter CH$_2$Cl$_2$	1x1 min
6 (A)	iPrOH	Mix, Meter CH$_2$Cl$_2$	5x1 min
7 (A)	5% DIEA	Mix, Meter iPrOH	2x1 min
8 (A)		Mix, Meter CH$_2$Cl$_2$	5x1 min
9 (B)	TFA	Mix, Meter iPrOH	2x1 min
1 (A)	DCC	Mix, Meter CH$_2$Cl$_2$	5x1 min
2 (A)		Search	

METER LEVELS

A		B	
1	5ml	1	
2	10 ml	2	
3		3	
4	75 ml	4	75 ml

AMINO ACID TRANSFER PERIOD   0.45 MIN.
ADJUSTABLE DRAIN TIME   0.28

MIX TIMES

A	120	MIN.
B	20	MIN.
C	5	MIN.
D	2	MIN.
E	1	MIN.

MIX SPEED   4.5

Figure 23

Beckman®

PROGRAM SHEET
MODEL 990 PEPTIDE SYNTHESIZER

PROGRAM NUMBER __2__ AUTHOR __S. Mojsov__ DATE __July 1976__

DESCRIPTION __Neutralization, Coupling__

PROGRAM STATEMENTS

Line	Statement	
1	Drain	
2	Meter DIEA	
3	Mix, Meter DIEA	2x2 min
4	Mix, Meter CH$_2$Cl$_2$	1x2 min
5	Mix, Meter CH$_2$Cl$_2$	5x1 min
6	Mix, Meter iPrOH	2x1 min
7	Mix, Meter CH$_2$Cl$_2$	5x1 min
8	Bpoc-AA delivery	1x1 min
9	Check AA level, Transfer	1x0 min
10	Mix AA, Meter DCC	1x5 min
11	Mix AA and DCC, Meter CH$_2$Cl$_2$	1x120 min
12	Mix, Meter CH$_2$Cl$_2$	5x1 min
13	Mix, Meter iPrOH	2x1 min
14	Mix, Meter CH$_2$Cl$_2$	5x1 min
15	Mix, Meter iPrOH	2x1 min
16	Mix, Meter CH$_2$Cl$_2$	1x1 min
17	Drain	1x0 min
18	Mix, Meter CH$_2$Cl$_2$	5x1 min
19	Search	

RESERVOIR CONTENTS

SOLVENTS/REAGENTS

1 (B)	CH$_2$Cl$_2$
2 (A)	CH$_2$Cl$_2$
3 (A)	CH$_2$Cl$_2$
4 (A)	CH$_2$Cl$_2$
5 (A)	iPrOH
6 (A)	5% DIEA
7 (A)	
8 (A)	TFA
9 (B)	

CPLRS.

1 (A)	DCC
2 (A)	

METER LEVELS

A

1	5 ml
2	20 ml
3	
4	75 ml

B

1	
2	
3	
4	75 ml

AMINO ACID TRANSFER PERIOD __0.45__

ADJUSTABLE DRAIN TIME __0.28__

MIX TIMES

A	120	MIN.
B	20	MIN.
C	5	MIN.
D	2	MIN.
E	1	MIN.

MIX SPEED __4.5__

Figure 24

was placed in a rubber stopper with a hole, which was inserted into a suction flask. The resin could thus be rapidly washed by vacuum filtration. Prior to the fluorescamine treatment, the resin was washed with CH_2Cl_2 (3 x 2 ml), isopropanol (3 x 2 ml), CH_2Cl_2 (3 x 2 ml), 5% diisopropylethylamine/CH_2Cl_2 (3 x 2 ml), isopropanol (3 x 2 ml), and CH_2Cl_2 (3 x 2 ml). The pipette was removed from the stopper, a drop of the tertiary amine solution was added, and about 0.5 ml of fluorescamine in CH_2Cl_2 (100 mg/ml) were added. After 10 min, the solution was filtered off, and the resin washed with CH_2Cl_2 (3 x 2 ml), isopropanol (3 x 2 ml), and CH_2Cl_2 (3 x 2 ml). The dried pipette with a dried resin was placed under a long wavelength UV lamp, and assessed visually. The derivative of any free amino groups fluoresced brightly.

h. Resin hydrolysis[6]. Peptide resin samples are placed into Pyrex ignition tubes (16 x 125 mm) and the followed reagents added: 1 ml liquified phenol, 2 ml concentrated hydrochloric acid, and 1 ml glacial acetic acid. The tube was sealed and heated in a 110° oven for 24 hr. The tube was opened, and the suspension transferred to a funnel. The tube and resin were washed with 10-30 ml 1 N HCl, and the solution was evaporated to dryness at 50° C. The sample was taken up in 25 ml water and residual phenol or phenol degradation products were extracted with chloroform (3 x 15 ml). The aqueous phase was evaporated to dryness, and the residue was taken up in 0.2 N sodium citrate buffer, pH 2.2. The volume was calculated to give roughly 60 nmoles/ml of the most abundant amino acid.

4. Characterization of Synthetic Glucagon

a. Peptide hydrolysates.

(i) Acid hydrolysis. Peptide samples were dissolved in 6.0 N HCl containing 0.1% liquified phenol (v/v), in Pyrex ignition tubes (16 x 125 mm). The tube was constricted about 6 cm from the top, and the bottom was frozen in a dry ice-acetone bath. The tube was connected to a vacuum pump, and evacuated for 5-10 min. The tube was then sealed at the constriction and placed in an oven for 24 hr at

110° C. The tube was then cooled to room temperature, cut open, and the solution was quantitatively transferred to a round bottom flask. The solution was evaporated to dryness at 50° C, and the residue was dissolved in 0.2 N sodium citrate buffer, pH 2.2.

(ii) Base hydrolysis. Peptide samples were placed in a poly‑propylene liner from a 400 µl microfuge tube. The liner was then placed in a 10 x 75 mm Pyrex acid-washed tube. A mixture of 5 N NaOH and aqueous thioglycol (1:2.5, v/v) (80 µl was added, and the tubes were evacuated and sealed as described above. They were placed in a 110° C oven for 24 hr. After opening the tube, the solution was quantitatively transferred to an acid-washed 10 x 75 mm test-tube. The liner was rinsed with 0.2 N sodium citrate buffer, pH 2.2 (2 x 100 µl), and an addition 150 µl of buffer was added. The pH of the sample was adjusted to 2.2-2.4 with 6 N HCl.

b. Polyacrylamide gel electrophoresis. The system of Laemli was used. Stock solutions: (1) 30% acrylamide, 0.8% bis-acrylamide; (2) separating gel buffer- 2 M Tris·HCl, pH 8.9; (3) stack gel buffer- 0.5 M Tris·HCl, pH 6.7; (4) chamber buffer- 288 g glycine and 6 g Tris·HCl in 1000 ml H$_2$O. Separating gel: 20% acrylamide-0.6% bis-acrylamide. In a final volume of 30 ml (adjusted with water), the following are compiled: 20 ml of acrylamide stock, 6 ml of separating buffer, 25 µl of TEMED, 100 µl of 10% ammonium persulfate. The gel is immediately covered with 1 ml of butanol. Polymerization time is about 50 min. Stacking gel: 4.5% acrylamide-0.1% bis-- acrylamide, which is layered on top of the separating gel. In a final volume of 30 ml (adjusted with water), the following are compiled: 4.5 ml of acrylamide stock, 6 ml of stacking gel buffer, 25 µl of TEMED and 100 µl of 10% ammonium persulfate.

The sample is prepared in 0.05 M Tris·HCl buffer, pH 6.7, and 7 M urea. Gels are run in an electrophoresis apparatus with 17 x 19 cm plates. The current is 10 mA and running time is 11 hr, at room temperature.

c. <u>In vivo bioassay for hyperglycemic activity of synthetic</u>
<u>and natural glucagon</u>.   The glucose concentration in the blood sera was
determined in the following way.   The coupled enzyme system using
glucose oxidase and peroxidase was purchased from Worthington.   It comes
as a prepackaged set under the name of Glucostat reagent.   The method-
ology is described in the Worthington manual.   The semi-micro method was
used which required 0.1 ml of sample.   The only difference from the
described procedure was that a reaction time of the sample with the
Glucostat reagent was 70 min, instead of 10 min.

Before each experiment, a new calibration curve with glucose
standards (50-250 mg per 100 ml) was made.   Glucose concentrations in
the samples were determined from the linear plot of the absorbance at
λ = 400 nm vs. standard glucose concentrations.   The maximal difference
between two separate determinations was 8 mg per 100 ml.

5. Appendix

a. Calculation for the incorporation of radioactivity into the Peptide chain:

(i) Bpoc-[^{14}C]Leu. The ammount of radioactivity present in the sample prior to coupling was 2.25 x 10^8 cpm in 0.54 mmoles. Specific activity of the sample 4.17 x 10^8 cpm/mmole.

For the coupling reaction this 0.54 mmoles were diluted with 12 mmoles of unlabeled Bpoc-Leu. Therefore 2.25 x 10^8 cpm were now in 1.7 mmoles. Specific activity was 1.3 x 10^8 cpm/mmole.

On the resin there was 1.1 mmole of peptide. Maximal incorporation of radioactivity would be 1.3 x 10^8 cpm/per mmole x 1 mmole = 1.46 x 10^8 cpm.

After incorporation of Bpoc-[^{14}C]Leu into peptide chains the following value was obtained from the amino acid analysis of resin hydrolysates: 2.74 x 10^7 cpm/mmole peptide. On the resin there were 1.1 mmole peptide chains or 2.74 x 10^7 cpm/per mmole x 1.1 mmole = 3.014 x 10^7 cpm. The yield of incorporation was $\frac{3.01 \times 10^7}{1.46 \times 10^8}$ x 100 = 20.7%.

(ii) Bpoc-[^{3}H]Phe. The amount of radioactivity present in the sample prior to coupling was 2.8 x 10^6 cpm in 0.003 mmoles.

For the coupling step, this was diluted with 1.1 mmole unlabeled Bpoc-Phe. Therefore 2.8 x 10^6 cpm were present in 1.103 mmoles. Specific activity was 2.6 x 10^6 cpm/mmole. There were 0.5 mmoles of peptide chains on the resin before incorporation of Bpoc-Phe at position 6 (this value was estimated from the results obtained from the amino acid analysis of resin hydrolysates performed on sample after incorporation of Bpoc-Phe at position 6). Therefore 1.103 mmoles represented 2.2 fold molar excess. Maximal incorporation would 2.6 x 10^6 cpm/mmole x 0.496 mmoles = 1.29 x 10^6 cpm.

After incorporation of Bpoc-[^{3}H]Phe into peptide chains the value of 8.9 x 10^5 cpm/mmole was obtained for the amount of

radioactivity that was incorporated. Since there were 0.5 mmoles of peptide on the resin, the total amount of radioactivity in the peptide chains was 8.9×10^5 cpm/mmol $\times$ 0.5 mmol $= 4.4 \times 10^5$ cpm. The yield of incorporation was: $\dfrac{4.4 \times 10^5}{1.29 \times 10^6} \times 100 = 21.5\%$.

b. <u>Calculation for the incorporation of [^{14}C] radioactivity into the peptide chain</u>:

The amount of radioactivity incorporated per mg of protected peptide resin was calculated from the values of amino acid analysis of resin hydrolysates performed on samples after coupling of Bpoc–Leu at position 26. The following value was determined 2714 cpm/mg peptide. On 5.1 g of resin, therefore would be:

$$2714 \text{ cpm/mg} \times \frac{5.1 \text{ g}}{0.001 \text{ mg}} = 1.38 \times 10^7 \text{ cpm/g}$$

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