SYNTHESIS OF A POSSIBLE GLUCAGON INHIBITOR BY SEMISYNTHESIS OF A GLUCAGON ANALOGUE OF [HARG^{12}]-GLUCAGON AND [DES-HIS^{1}][HARG^{12}]-GLUCAGON

by

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STATEMENT BY AUTHOR

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PREFACE

This work was supported in part by a grant from the United States Public Health Service AM 15504. All optically active amino acids are the L variety unless otherwise indicated. The symbols for the amino acids residues follow the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature.

The author sincerely thanks Dr. Marvin D. Bregman for all the amino acids analyses, and for his helpful discussion and assistance in this project. She thanks Dr. Venkatappa Viswanatha for the purification techniques of BOC-amino acids and Mr. Tom K. Sawyer for his assistance in acid hydrolysis. She thanks the professors who served in her research committee, Dr. Robert B. Bates and Dr. Cornelius Steelink. The author thanks Mr. Oluchukwu E. Ekechukwu for his encouragement and friendship.

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ABSTRACT

The goal of this study was to prepare an antagonist to glucagon, a peptide responsible for the control of glucose levels in the blood. This work described two synthetic strategies in this preparation, which involved N-terminal modifications of [HArg^{12}]-glucagon and its purified Edman degradation products, [DH^{1}, HArg^{12}]-glucagon, to prepare analogues of glucagon. The peptide syntheses were achieved by two types of azide couplings, the conventional acid azide coupling and the diphenylphosphoryl azide (DPPA) coupling, with the latter being extensively investigated.

Phe-[HArg^{12}]-glucagon and [Phe^{1}, HArg^{12}]-glucagon were prepared using the conventional azide method. No side reaction seemed to occur. Purification by precipitation (0.001 N HCl) did not significantly improve the quality of the peptide synthesized, but lowered the yield greatly.

DPPA coupling was investigated for the semisynthesis of the following glucagon analogues: Gly-[HArg^{12}]-glucagon, Ala-[HArg^{12}]-glucagon, [Gly^{1}, HArg^{12}]-glucagon, Phe-[HArg^{12}]-glucagon, and Val-[HArg^{12}]-glucagon. The reactions were done at 4°C or lower for 10-12 hr. For bulky amino acids such as Phe, longer reaction times (61 hr) were required. At a higher temperature (room temperature), side reaction was observed, which gave apparent high lysine content in the amino acid analysis. This side reaction could be suppressed at a lower temperature (-10°C).

Purifications were investigated using gel filtration chromatography, precipitation (0.001 N HCl), and DEAE anion exchange chromatography in a 0.01 M Tris-buffer containing 7 M urea at pH 7.7.

CHAPTER 1

INTRODUCTION

Glucagon is a single-chain polypeptide comprising 29 amino acids residues and having a molecular weight of 3485. It crystallizes readily without metals in the pH range 5 to 8, appearing as rhombic dodecahedra that conform to the cubic system. Crystalline metal-glucagon complexes (e.g., zinc-glucagon) are easily prepared and are known to prolong biological action. The molar absorptivity of glucago in dilute aqueous acid (pH 2, at λ_{max} = 278 nm) is 8310 $M^{-1}cm^{-1}$, with E = 2.38 at 1 mg/ml, 1 cm (Gratzer, Bailey, and Beaver, 1967). The isoelectric point of glucagon in dilute aqueous solutions lies near pH 7, and its solubility in the physiological pH range is quite low. Other solubility and stability characteristics of glucagon are given in Table 1.

The discovery of glucagon (for reviews, Lefebre and Unger, 1972; Metabolism, 1976) began in 1921, when Banting and Best injected pancreatic extracts in depancreatized dogs, and noticed that a mild but reproducible hyperglycemia preceded the hoped-for hypoglycemic effect. They thought that this (hyperglycemia) might be due to epinephrine liberation. The phenomenon, noted also by other workers (Collip, 1923), was correctly attributed to a glucogenic substance by Murlin and his coworkers (1923) who called this substance "glucagon" or mobilizer of glucose. Several investigators, among them Bürger and Brandt (1935) who made the first attempts to purify the material, provided additional

Table 1. Some solubility and stability properties of glucagon -- From Bromer (1972).

Condition	Value
Solubility (aqueous, 25°C)	
pH 3.5 to 8.5	50 μg/ml or less
pH 2 to 3 and 9 to 11	> 10 mg/ml
Solubility (nonaqueous)	
90% formic acid	
90% chloroethanol	≥ 5 mg/ml
70% dimethylformamide	
Stability (0.2 to 1 mg/ml, aqueous)	
pH 2.5 to 3	100% for 3 months, 4°C
pH 9 to 10	100% for 4 hours, 25°C
	50% after 4 months, 4°C

evidence for the glycogenolytic action of glucagon. Its primary sequence was established by Bromer et al. (1956) and Bromer, Sinn, and Behrens (1957) (see Figure 1) and is identical in many species, including man, pig, rat, cow, and camel, while differing slightly in some avian species (pigeon, chicken, duck, etc.). There are two forms of glucagon: the pancreatic glucagon, which is secreted by the $alpha_2$ cells of the islets of Langerhans in the pancreas; and the gut glucagon which appears to be similar to pancreatic glucagon by biologic and immunometric methods, and is formed in specific cells of the digestive tract which are ultrastructurally indistinguishable from the pancreatic $alpha_2$ cells (Sasaki, Rubalcava, et al., 1975). Some evidence indicated that glucagon may be secreted as a higher molecular precursor since high molecular weight polypeptides containing the structure of glucagon have been found (Hew and Yip, 1976). The isolation and crystallization of porcine pancreatic glucagon (Staub, Sinn, and Behrens, 1953, 1955) and its consequent availability made possible extensive biological and chemical studies of the hormone. Since 1953, little attention has been given to improve methods of isolation, perhaps because of the discouragingly low content of glucagon (10 ng/g tissue) in the pancreas of most mammals. Most work has been done with pancreatic glucagon, including the studies made in this thesis. Unless otherwise designated, glucagon and pancreatic glucagon are synonymous.

Pancreatic glucagon release is stimulated by hypoglycemia and suppressed by rising blood glucose concentrations (Foá, Galansino, and Pozza, 1957). These results have been confirmed by radioimmunoassay *in vivo* and *in vitro* using isolated pancreatic islets (Foá, 1971). The

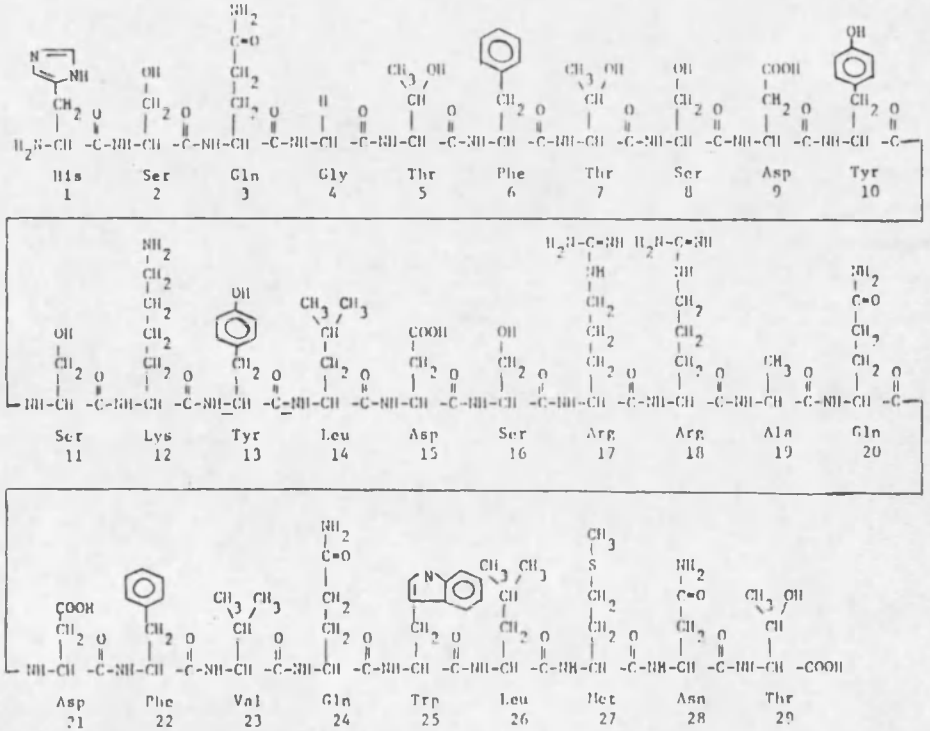


Figure 1. The primary structure of glucagon.

secretion of pancreatic glucagon is stimulated also by amino acids and inhibited by fatty acids. Since glucagon increases glucose production and lipolysis and decreases the levels of circulating amino acids, these actions help create the conditions necessary for the feedback control of its own secretion.

During fasting, extended starvation, and physical exertion, glucagon serves as a fuel mobilization hormone producing energy-relea releasing substrates to satisfy tissue needs. Glucagon is secreted by the $alpha_2$ cells into the portal blood and is transported to the liver as its first and main target, where its biological action is glycogenolysis or the breakdown of glycogen to glucose. The binding of glucagon to specific receptors on hepatic plasma membrane stimulates glycogenolysis, and inhibits glycogen synthesis and activates the enzyme adenylate cyclase which converts adenosine triphosphate to cyclic 3'-5'-adenosine monophosphate (c-AMP), in the presence of Mg^{++}. Sutherland, Rall, and Menon (1962) showed that c-AMP acts as an endocellular "second messenger" (see Figure 2), conveying the stimulus delivered by glucagon on the cell surface to the effector enzymes in the cytoplasm. Here the primary action of c-AMP is to activate several protein kinases that may in turn catalyze the phosphorylation of specific enzymes such as phosphorylase, glycogen synthetase, and lipase. The net result is the inhibition of the enzyme glycogen synthetase and the breakdown of glycogen to give glucose. Foá (1973) has calculated that one molecule of glucagon can stimulate the production of one and one-half million molecules of c-AMP and the release of about 3 million molecules of glucose from the hepatic glycogen.

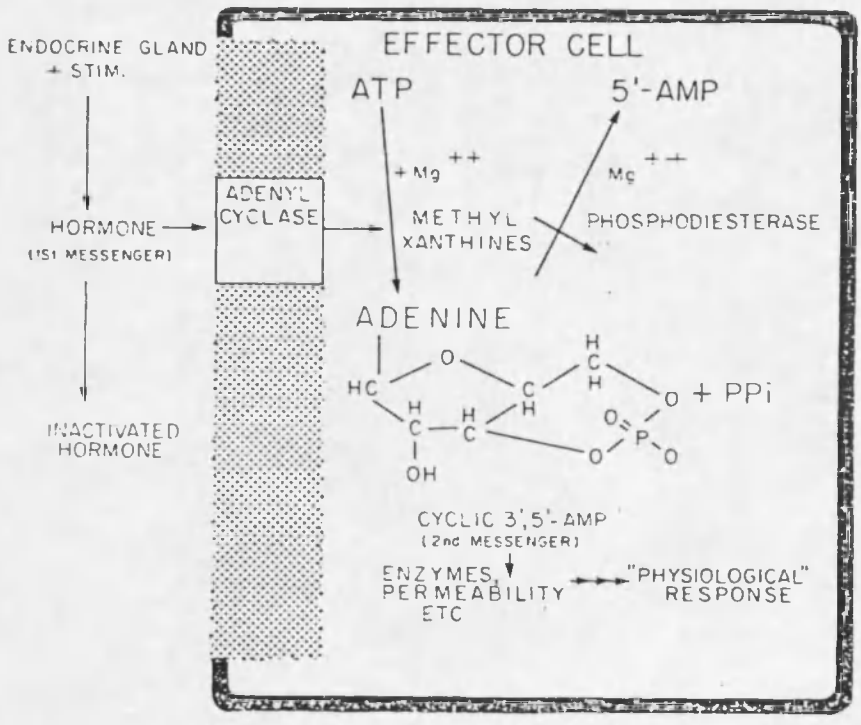


Figure 2. Second messenger concept involving c-AMP -- From Sutherland, Robison, and Butcher (1968); Sutherland and Robison (1966).

Glucagon in adipose tissue acts to initiate gluconeogenesis or the degradation of fatty acids. The stimulation of lipolysis by glucagon is accompanied by a marked stimulation of glucose uptake (Vaughan, 1960; Lee, Ellis, and Bromer, 1960; Hagen, 1961) and oxidation (Foresch et al., 1960; Lee et al., 1960; Wörner and Weinges, 1961). The mechanism by which glucagon stimulates lipolysis (see Figure 3) is similar to that described for glycogenolysis. The only difference is, toward the end, the glyceride lipase is activated by a protein kinase resulting in the liberation of glycerol and free fatty acids, with a corresponding increase in esterification (or re-esterification). The free fatty acids are then converted to co-enzyme A, a precursor of glucose.

Other enzyme activities stimulated by glucagon are those that catalyze the urea cycle, and the deamination of amino acids, with a resulting increase in excretion of nitrogen products (Foá et al., 1973). Other biological effects of glucagon include depression of appetite; augmentation of renal excretion of water, and most ions producing osmotic diuresis; stimulation of the release of potassium from the liver; lowering the concentration of serum phosphate; stimulation of the release of growth hormone from the anterior pituitary and of catecholamines from the adrenal medulla; increase in the release of insulin, epinephrine, calcitonin, and growth hormone; and inhibition of gastrointestinal movements and secretion. Glucagon also exhibits chronotropic and inotropic effects on myocardium (Lucchesi and Stutz, 1970). stimulates the strength and rate of cardiac contraction (Regan et al., 1964; Whitehouse and James, 1966), and increases cardiac output (Farah

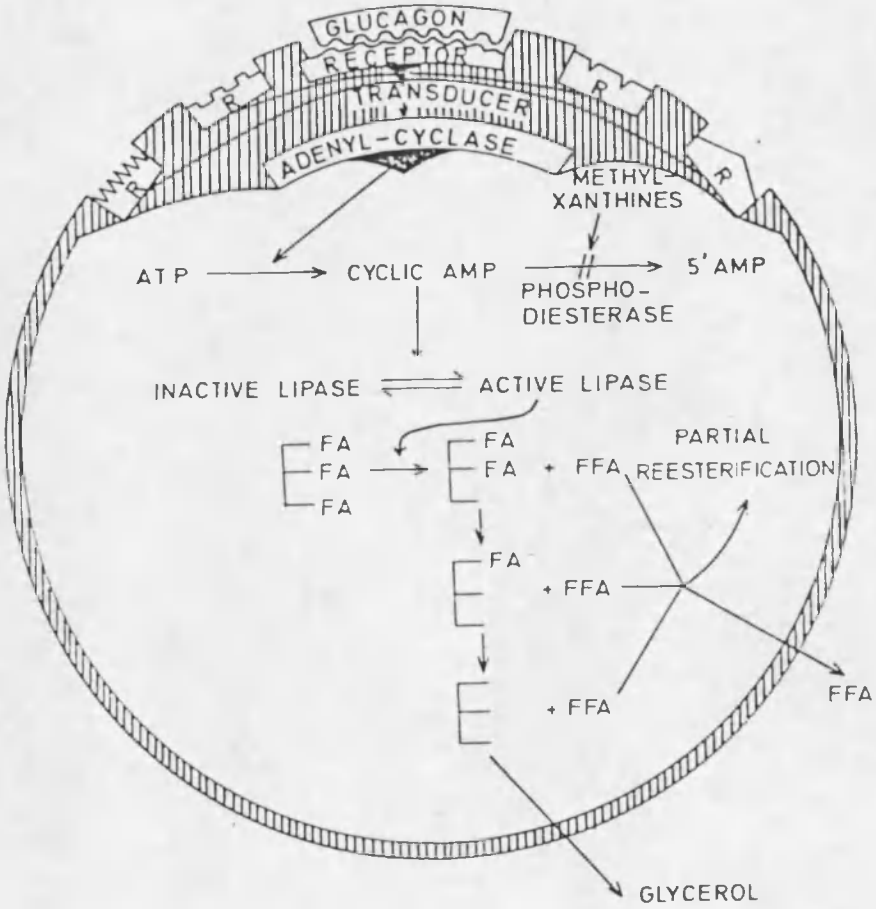


Figure 3. Mode of action of glucagon on the fat cell according to Rodbell's theory -- The number of specific receptors indicated in the figure is conventional (Rodbell, 1970; Rodbell, Birnbaumer, and Pohl, 1970; Birnbarmer and Rodbell, 1969).

and Tuttle, 1960). It dilates coronaries and the system arterioles, which decreases vascular resistance and increases bloodflow. Several recognized endocrinopathies and metabolic disorders related to glucagon are shown in Table 2.

In 1970, Unger and coworkers illustrated the close relationship between glucagon and diabetes mellitus (Unger and Orci, 1977; Raskin and Unger, 1978; Unger, 1978; Lefebvre and Luycks, 1979). There are two major forms of the disease: (1) juvenile-onset diabetes which is characterized by a complete lack of the production of the peptide hormone insuline by the beta cells in the pancrease; (2) adult-onset diabetes in which insulin levels are often normal or above normal, but elevated blood glucose levels (hyperglycemia) and other symptoms of the disease (glucosuria, ketosis, etc.) generally are observed. In virtually all cases, diabetics tend to be hyperglycemic and there is increased evidence that this is not due only to impairment of insulin but the presence of an excess amount of glucagon in the blood (hyperglucagonemia) (Unger and Orci, 1975; Unger, Madison, and Muller, 1972; Unger, 1976; Gerich, Tsalikian, et al., 1975; Koerker, Ruch, et al., 1974; Gerich, Lorenzi, Hane, et al., 1975; Gerich, Lorenzi, Karam, et al. 1975; Chideckel et al., 1975; Raskin and Unger, 1976; Soman and Felig, 1978; Bhathena et al., 1978). A bihormonal control of glucoregulation, through a push-pull system between insulin and glucagon is, therefore, necessary in control of glucose levels in the blood. The hormonal antagonists are believed to be directed by a glucose sensor, which allows the islets to respond in a coordinated fashion by maintaining an equality of glucose influx and efflux at all times.

Table 2. Possible disease states associated with glucagon deficiency and glucagon excess -- From Lawrence (1972).

Glucagon deficiency	Glucagon excess
Hypopituitarism	Alpha cell tumors
Hypertriglyceridemias	Acromegaly
Idiopathic hypoglycemia	Pheochromocytoma
Reactive hypoglycemia	Diabetes mellitus
	Hyperparathyroidism
Chronic pancreatitis	Polyglandular syndrome
	Obesity
	Fatty liver
	Hypocalcemia
	Hypomagnesemia
	Starvation ketosis
	Pancreatitis

In severe diabetes, the outer layer of B cells (see Figure 4) surrounding the A cells has been disrupted and no B cells are detected (Unger and Orci, 1977). The A cells, in contact with each other, appear to lose their glucose-sensing capacity. The A cells hypersecrete, and in most juvenile type diabetes, aggressive therapy with insulin fails to restore blood glucose levels to normal. In adult-onset type diabetes, there is evidence of partial preservation of the glucose-sensing ability. The accelerated catabolism of lipids and fats, due to elevated glucagon level, leads to increased production of ketone bodies and other diabetic complications (Alberti, Iverson, and Christensen, 1974). This can lead to ketoacidosis which lowers the blood pH, resulting in a coma and even death if not treated. Severe ketoacidosis fails to suppress the secretion of diabetic alpha cells (Asson et al., 1969), but rather appears to stimulate glucagon release (Unger et al., 1970). Somatostatin (see Figure 5) and its analogs (Brown, River, and Vale, 1977; Meyers et al., 1977; Sarantakis et al., 1978; Märki et al., 1979) suppress both insulin and glucagon release (Koerker et al., 1974; Gerich, 1978; Dobbs and Unger, 1979) to restore blood sugar concentration to normal and alleviate certain other symptoms of diabetes. Recently, diazoxide is reported to inhibit the secretion of glucagon as well as insulin in response to certain secretagogues independent of any changes in prevailing levels of glucose (Urdanivia, Pek, and Santiago, 1979).

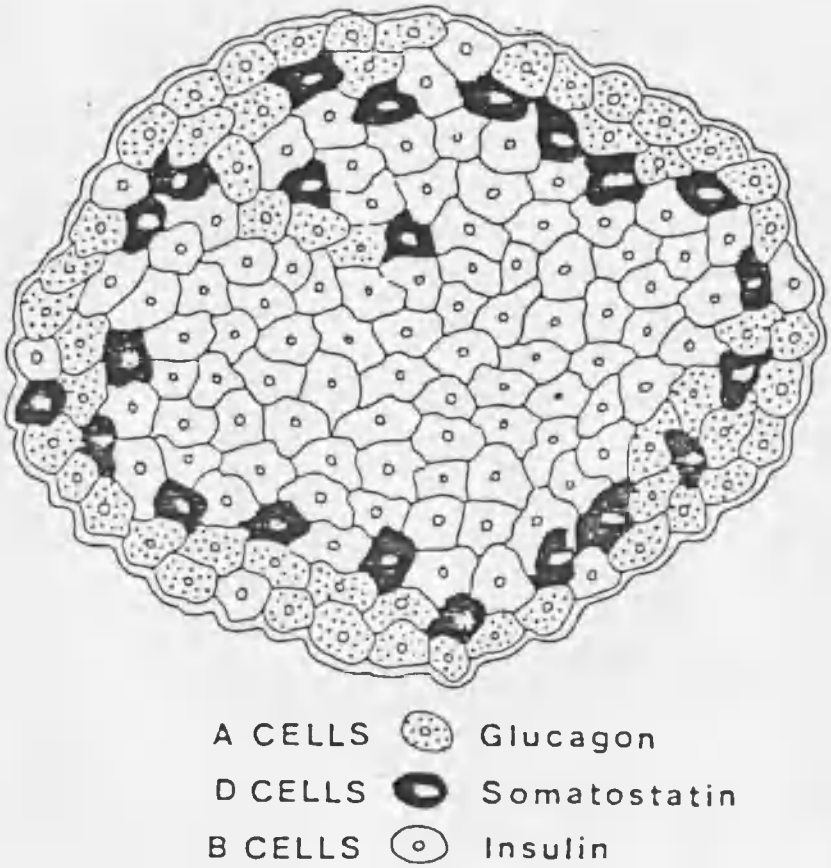


Figure 4. Schematic representation of an Islet of Langerhans showing distribution of glucagon, somatostatin, and insulin-containing cells -- From Unger and Orci (1977).

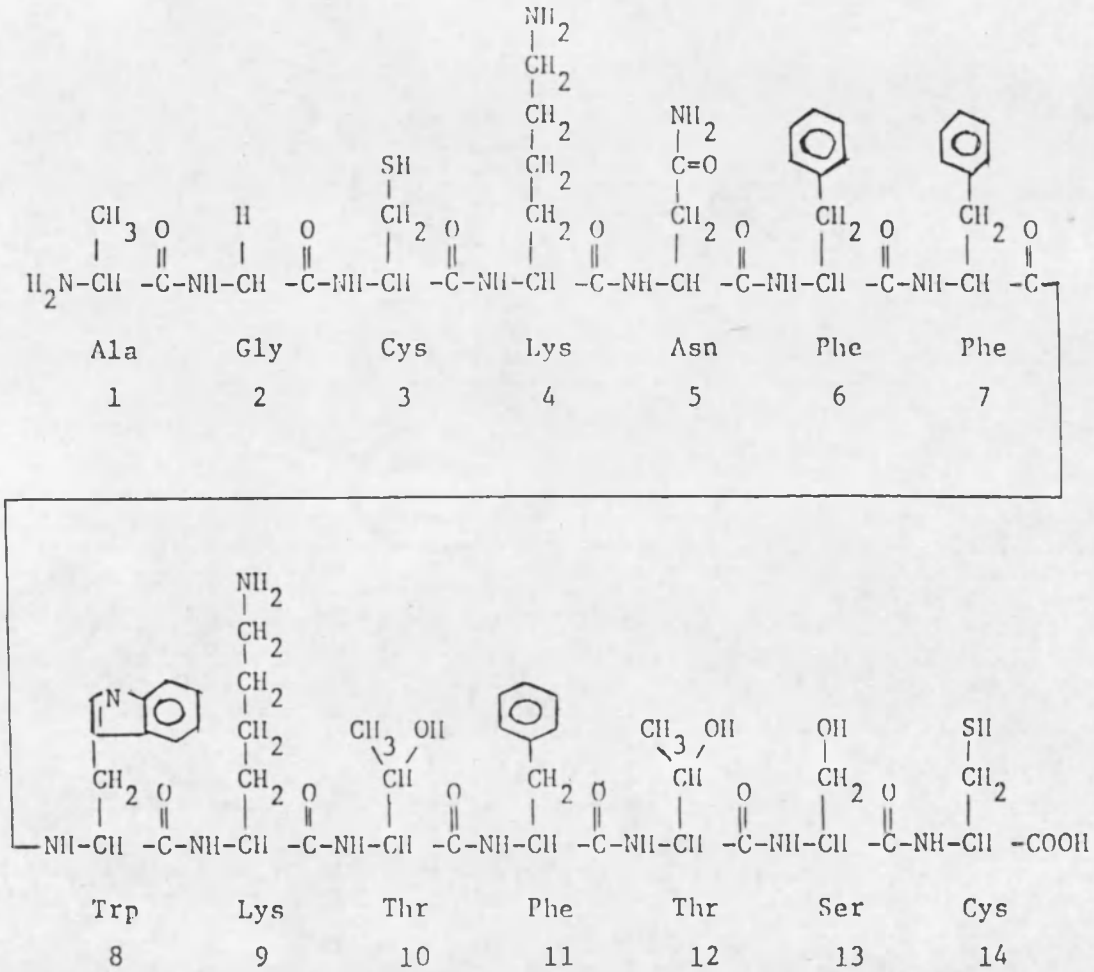


Figure 5. The primary structure of somatostatin.

Hormone-Receptor Interactions

Adenylate cyclase is the primary enzyme system (for review, Sutherland et al., 1962; Rodbell, 1972; Sutherland, 1972) by which a number of peptide hormones and biogenic amines alter the metabolism, structure, and function of their target cells (Robison, Butcher, and Sutherland, 1968). This system selectively receives, translates, and amplifies the "message" contained within the structure of the hormone to give the new message, adenosine cyclic 3'-5'-phosphate (c-AMP), formed by the catalytic hydrolysis of ATP. The molecular basis for specificity and action of the hormones affecting the system lies, therefore, within the processes leading to the production of c-AMP. Since adenylate cyclase systems retain their sensitivity to hormones in cell-free preparations, it should ultimately be possible to ascertain the physiochemical basis for hormone action; i.e., the nature of hormone receptors.

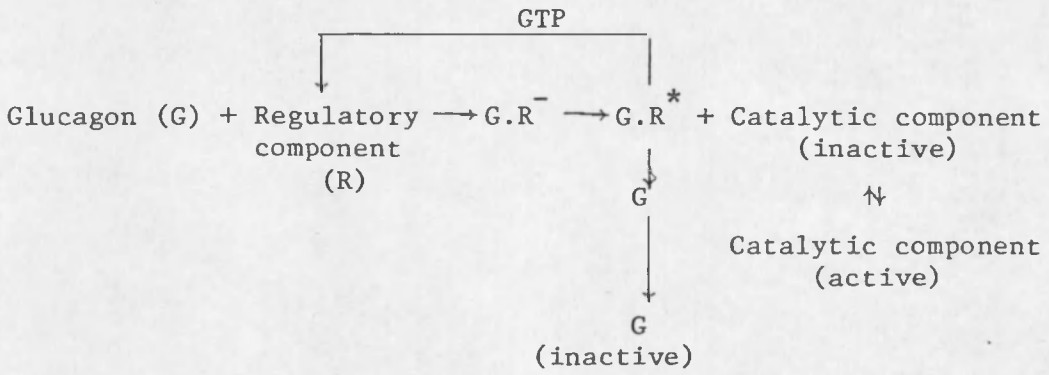
The first event in the cascade of reactions leading to hormone response is the interaction of hormone with its recognition site termed the "receptor." A receptor is an element of the target cell that specifically recognizes and binds the hormone and initiates other changes which ultimately result in the biological response. This may be considered as analogous to the classical enzyme-substrate systems in which substrate binding and catalysis are separate, but sequential processes can be studied independently (Sutherland et al., 1962). Once the hormone-receptor complex is formed, a stimulus can be generated to elicit a biological response (in this case the formation of c-AMP from adenosine triphosphate). The generation of the stimulus from the

hormone-receptor to the catalytic unit is not well understood, although this so-called coupling process appears to be dependent on phospholipids in the cell membrane (Pohl et al., 1971).

Attempts have been made to explain the interaction process of a hormone with its receptor (Rudinger, Pliska, and Krejci, 1971; Bürgen, Roberts, and Feeney, 1975; Cuatrecasas et al., 1975; Hammes and Rodbell, 1976; Häen, 1974; Salmon et al., 1975). Two general models are usually considered for hormones in contact with receptors. The "lock and key" model allows only those molecules with the correct conformation and orientation to form a complex. In this single step binding, all the faborable interactions between hormone and receptor are formed simultaneously. In the zipper model, a portion of the hormone is necessary for initial binding, followed by changes in the receptor to accommodate the binding of the rest of the hormone. This initial binding may reduce the number of possible conformations and preferentially "freeze" the active conformation of the hormone. The association time for this model generally would be greater than that for the "lock and key" model. Once the hormone-receptor complex is formed, it may participate in the event of stimulus generation. In binding, the complex may acquire specificity and affinity for the catalytic enzyme, indicating that the receptor-hormone-enzyme are not one unit, but rather separate multiple subunits, drawn together as one by the hormone. This is termed the mobile receptor theory and allows for the combining of separate structures due to the fluidity of cell membrane structures. In this three-step model, one would observe no increase in enzyme activity in the intermediate transition state, but would observe a time lag before

isomerization to a higher activity state occurred. However, the receptor complex may also fall into an inactive state, once it is formed, as suggested by the "rate theory." In the allosteric model, the bonding of the hormone induces a conformational change in the receptor, which then generates a stimulus. However, the hormone does not directly participate in the activation. In this respect antagonists, which act as competitive inhibitors, provide insight into the process of stimulus generation, since they have retained the structural elements required for binding to the receptor, but have lost a structural feature necessary for the initiation of the stimulus (Meraldi, Hruby, and Brewster, 1977). Since each of the theories has been valid in a particular case, no one model or combination of models has been fully accepted.

The two most extensively used adenylate cyclase systems for the studies of glucagon action, in vitro, are the rate liver membranes and the fat cell ghosts. Glucagon does not activate adenylate cyclase unless either GTP (or GDP), at concentrations as low as 5 nM, or ATP (or ADP), at concentrations greater than 50 μM, is present (Rodbell, 1972). The nucleotides, through an allosteric type of action, decrease the affinity of the regulatory component for glucagon and change the binding of glucagon from a slow, nearly irreversible process to one that exhibits both rapid binding and dissociation of the hormone. These findings are compatible with the reaction scheme:



Adenylate cyclase and its response to glucagon

in which GTP (or GDP, ATP, ADP) changes the state of the hormone-regulatory complex from one that is stable ($G.R^{-}$) and inactive to one that is unstable ($G.R^{*}$), but which is capable of allowing excitation of the catalytic component to its active state. The release of glucagon from the active state results in generation of the inactive, but high affinity state of the regulatory component. Although the released glucagon should react again with the regulatory component, an inactivating process in the plasma membranes, specific for glucagon, but otherwise uncharacterized, apparently modifies glucagon such that the hormone no longer binds and activates the enzyme. According to this scheme, therefore, the activity of the enzyme is dependent upon the continuous process of glucagon and the nucleotides.

The rat liver plasma membranes are stimulated by the hormone glucagon, and also by 5'-guanyl-imidodiphosphate (Iyengar, Swartz, and Birnbaumer, 1979), fluoride, and epinephrine (Birnbaumer, 1973). Secretin, vasopressin, thyroid-stimulating hormone, parathyroid hormone, and growth hormone were all found to be ineffective. Glucagon and

epinephrine both interact with a receptor on the outer surface of the plasma membrane, while fluoride and 5'-guanyl-imidodiphosphate appear to act directly on the catalytic unit exposed on the inner surface of the membrane. Half maximal stimulation of enzymatic activity is obtained with as little as 4×10^{-9} M glucagon. The reaction of hormone with receptor is reversible and the proportion of enzyme in the stimulated state depends on the concentration of free hormone in the medium. Recently, evidence has shown that the coupling of glucagon receptor to adenylate cyclase is regulated by GDP (Iyengar and Birnbaumer, 1979; Kimura and Nagata, 1979).

In contrast, the fat cell ghost is stimulated by many different hormones including glucagon, secretin, epinephrine, adrenocorticotropin, leuteinizing hormone, vasoactive intestinal polypeptide, and insulin. Also the ligands guanidine-triphosphate and fluoride act in the same manner on the fat cell ghosts as they do on the liver plasma membranes. Each hormone has a separate receptor site, but all the receptors are linked up in some as yet unknown manner to a single adenylate cyclase system since the addition of two or more hormones at maximal stimulating concentrations do not produce the additive effect if each were taken singularly. The liver membrane is the preferred system to study the action of glucagon and its derivatives, since this system is not stimulated by as many different hormones as the fat cell ghost and has a reliable binding assay.

Structure-Function Relationships

The first step in the activation of adenylate cyclase by glucagon is the recognition of a membrane receptor (Robison et al., 1968; Hammes and Rodbell, 1976; Sonne and Gilemann, 1977). An understanding of the nature of the glucagon-receptor interactions depends critically on a proper description of the conformation of the hormone when bound to the receptor. Preliminary studies show that crystalline glucagon is a rhombic dodecahedron (see Figure 6) containing 12 molecules packed with cubic symmetry (King, 1965). At physiological concentration, about 10^{-8}-10^{-11}M, glucagon exists as a monomer, and in dilute aqueous solution, monomeric glucagon does not have a stable globular structure but exists in equilibrium as a flexible chain containing litt1 little (15%) helical structure (Edelhoch and Lippoldt, 1969; Gratzer et al., 1968; Panijpan and Gratzer, 1974; Blanchard and King, 1966). As the concentration of glucagon increases, the structure becomes more ordered on self association (Panijpan and Gratzer, 1974; Gratzer and Beaven, 1969) into trimers (Sasaki, Dockerill et al., 1975; Johnson, Hruby, and Rupley, 1979) and possibly into higher oligomers (Swann and Hammes, 1969) when crystallized. Optical rotatory dispersion (Blanchard and King, 1966), circular dichroism (Panijpan and Gratzer, 1974), magnetic resonance, and Zimm-Bragg analysis (Deranleau et al., 1978) indicated that an increase in helical conformation accompanies molecular association. The conformation of the associated glucagon is pH dependent. At low acid pH, glucagon appears to form antiparallel β-conformations (Beaven, Gratzer, and Davies, 1969), while in alkaline

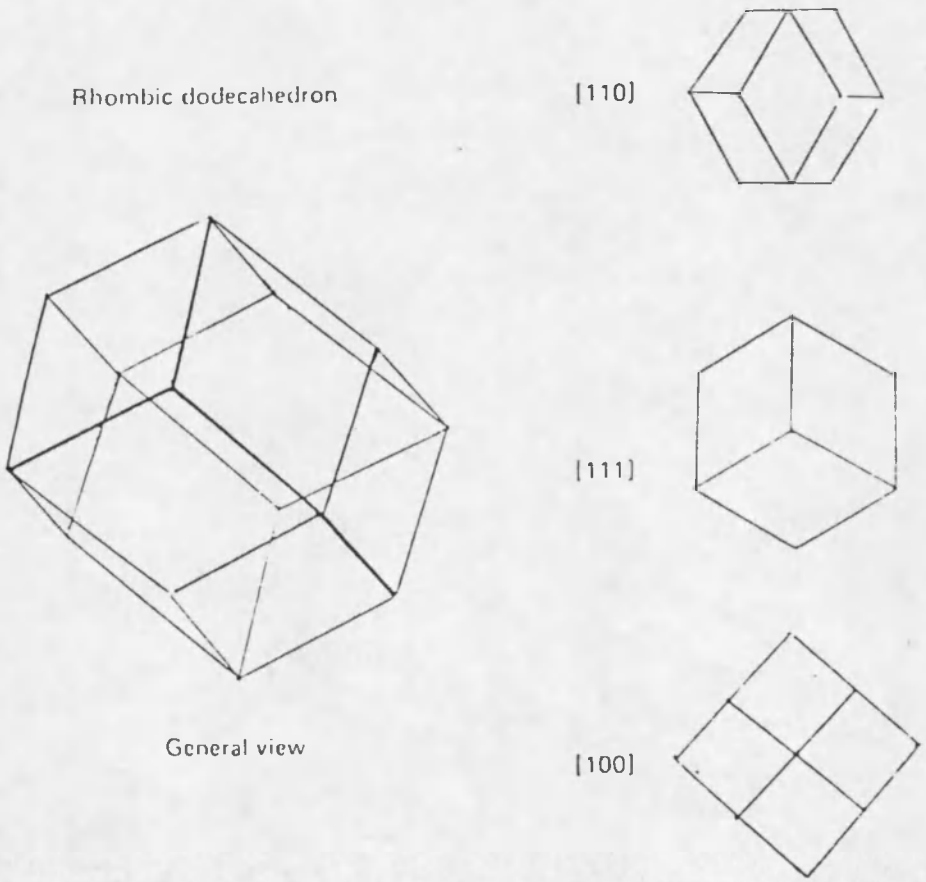
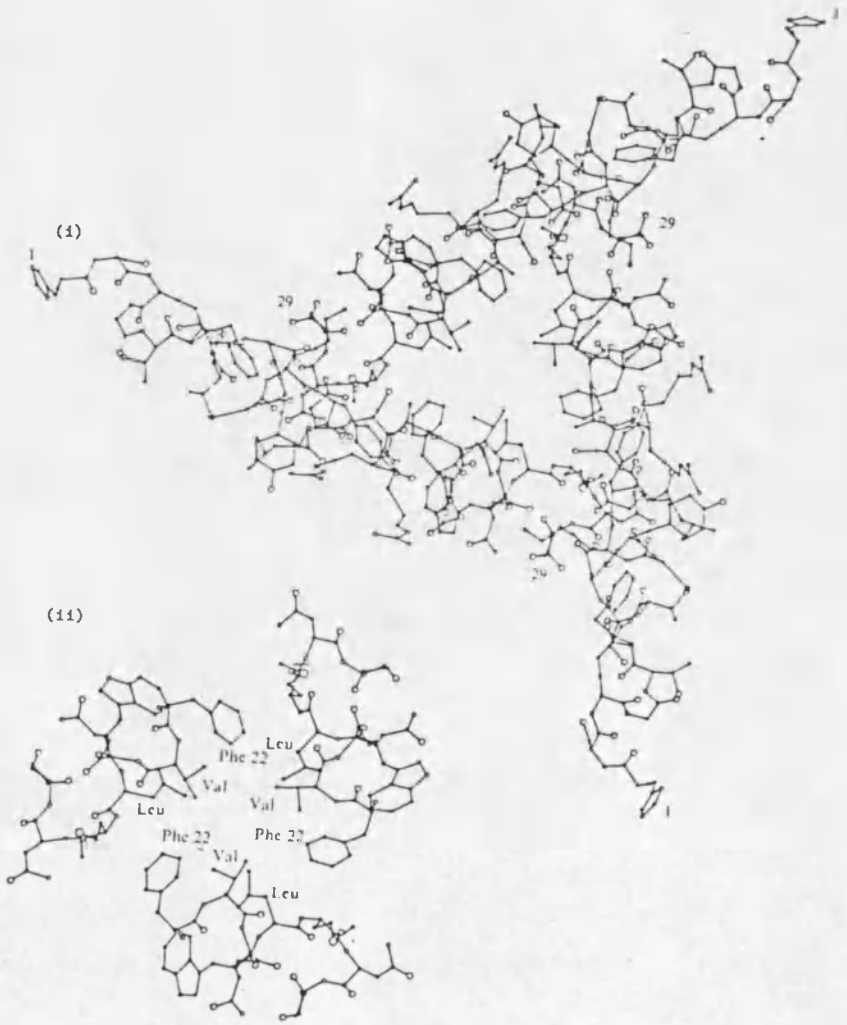


Figure 6. Schematic representation of the rhombic dodecahedron characteristic of cubic glucagon crystals -- From Blundell (1979).

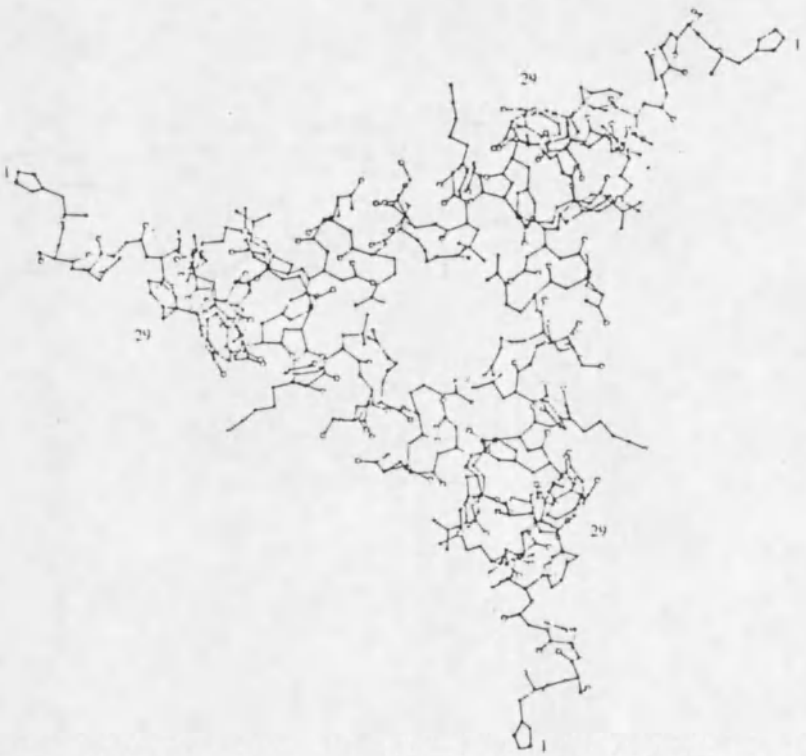
solution, the α-helical structure exists (Swann and Hammes, 1969). In the pH range 5.8 to 7.5, glucagon assumes the α-helical structure. In 6M guanidine hydrochloride, the helical structure is destroyed (Panijpan and Gratzer, 1974). However, the α-helical can be regenerated or stabilized in the presence of detergents (Bornet and Edelhoch, 1971), glycols (Contaxis and Epand, 1974), or lipid micelles (Schneider and Edelhoch, 1972).

An X-ray study (Sasaki, Dockerill et al., 1975) indicates an α-helical conformation between residues 6 and 27 resulting in the formation of one mainly hydrophobic region Phe 6, Tyr 10, Tyr 13, and Leu 14, and another by Ala 19, Phe 22, Val 23, Trp 25, Leu 26, and Met 27. Parts of these hydrophobic regions interact to form two kinds of trimers in the crystals (see Figure 7). The center of the trimer is mainly hydrophilic, involving charged residues such as Asp 15, Arg 17, Arg 18, and Asp 21. It has secondary structure in the helical conformers and quaternary structure. It is the hydrophobic quaternary structure interactions that stabilize the helix which is found only at the concentrations that favor self-association. Self-association of the hormone to trimers and higher oligomers is accompanied by the formation of α-helix (Blundell, 1979). It is probable that a helical conformer is also stabilized when glucagon binds its membrane receptor (see Figure 8). The same suggestion was made earlier (Rodbell et al., 1971), since the glucagon receptor complex is destablized at lower temperatures or by the presence of urea. However, residues 1-5 are not constrained by intermolecular interactions, and appear to be flexible. Fragments 1-21, 1-23, 20-29, and 22-29, which are unable to form



(a) (i) resulting from contacts around the threefold axis involving Phe 22, Val 23, and Leu 26, which are shown enlarged in (ii)

Figure 7. A view of a glucagon trimer.



(b) Resulting from heterologous contacts between hydrophobic residues. The region around the threefold axis of symmetry in the center comprises hydrophilic residues including arginines and aspartates.

Figure 7.--Continued

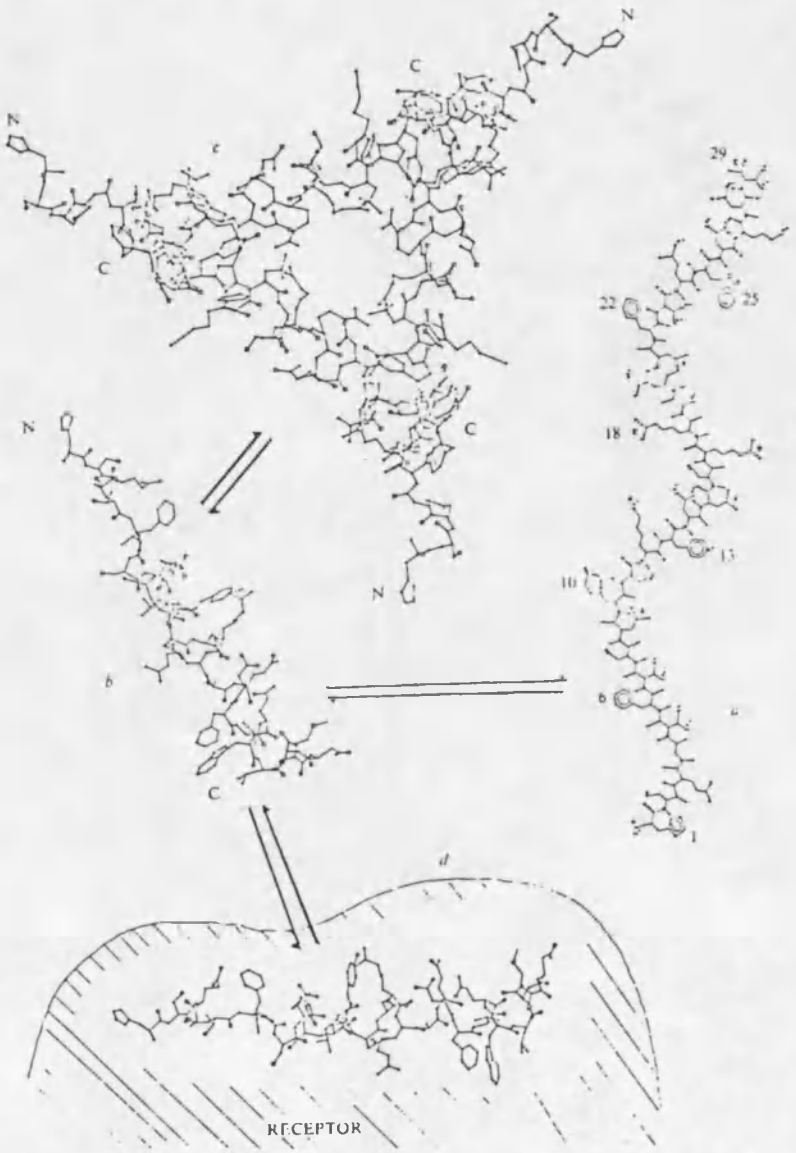


Figure 8. A schematic diagram of the equilibrium between conformers of glucagon -- (a) Hypothetical random coil in equilibrium with a helical form (b). The helical form is stabilized either as trimers (c) or by association with a receptor (d) by hydrophobic interactions.

helices in these systems, only weakly compete with glucagon at the receptor and have weak or no glucagon activity (Contaxis and Epand, 1974; but see Wright, Hruby, and Rodbell, 1978). The N-terminal may initially remain flexible, but subsequent binding becomes important in eliciting the biological response and providing a further stabilization of the hormone receptor complex (Hruby et al., 1976; Lin, Wright et al., 1975). A predicted conformation of glucagon in dilute solution contains an α-helix region residing in residues 19-27, but on aggregation this region is coverted into a β-sheet (Chou and Fasman, 1975) which is induced by interacting with the receptor.

In order to have a complete understanding of hormone-receptor interactions, modifications of the hormone are made to provide the knowledge of the regions or functional groups on the hormone which are involved in binding only and those which are required for activity. These modifications (peptide chain length, side chains, and terminal functional groups) are brought about either by enzymatic or chemical means. Table 3 (Wright, 1976) presents a list of various glucagon derivatives that have been made and studied on rat liver membrane. A list of glucagon derivatives acting through lipolysis, as well as glyconeogenesis, is given by Asson and Slusher (1972).

Examination of the structure of glucagon suggested it would be difficult to specifically modify the C-terminal end. In addition to the C-terminal carboxyl group, glucagon contains three other carboxyl groups, and it is unlikely that specificity could be obtained for the terminal carboxyl group, though recently Wheeler and associates (1974) have obtained some evidence that under certain conditions one of the

Table 3. Activity of glucagon derivatives acting on liver membranes.

Peptide	Purification	Activity[a]	Binding	Reference
α and ε amino groups				
N^{α}-Boc-glucagon	Electrophoresis	35%	N.T.[b]	Lande, Gorman, and Bitensky (1972)
N^{ε}-Boc glucagon	Electrophoresis	12%	N.T.	Lande et al. (1972)
N^{α}-Carbamyl-glucagon	None	10%, F.A.[c]	N.T.	Epand, Epand, and Grey (1973)
		6%	33%	Epand et al. (1979)
N^{α},N^{ε}-Acetyl-glucagon	None	10%, F.A.	N.T.	Epand et al. (1973)
	Ion Exchange	1%, F.A.	1%	Desbuquois (1975b)
		0.15%	3,5%	Epand et al. (1979)
[12]-Homoarginine-glucagon	None	10%, F.A.	N.T.	Epand et al. (1973)
	Ion Exchange	7-fold less	N.T.	Ross et al. (1979)
		17%	38%	Epand et al. (1979)
Imidazole-ethoxyformyl-N^{α}, N^{ε}-acetyl-glucagon	None	None	N.T.	Epand et al. (1973)
N^{α}-Acetyl-glucagon	Ion Exchange	10%, F.A.	12%	Desbuquois (1975b)
N^{α}, N^{ε}, Tyr-acetyl-glucagon	Ion Exchange	0.1%, F.A.	0.1%	Desbuquois (1975b)
		10%	N.T.	Epand and Wheeler (1975)

Table 3.--Continued Activity of glucagon derivatives acting on liver membranes.

Peptide	Purification	Activity[a]	Binding	Reference
N^{α}-Trinitrophenyl-glucagon	Ion Exchange	0.02%	N.T.	Epand and Wheeler (1975)
	Ion Exchange	0.16%	7%	Epand et al. (1979)
	Ion Exchange	None	7%	Cote and Epand (1979)
N^{α}-Trinitrophenyl-[12]-Homoarginine-glucagon	None	15%	N.T.	Epand and Wheeler (1975)
N^{α}-Carbamyl N^{ε}-trinitrophenyl-glucagon	None	23%	N.T.	Epand and Wheeler (1975)
N^{ε}-4-azido-2-nitrophenyl-glucagon	Ion Exchange	None	100%	Bregman and Levy (1977)
	Ion Exchange	None	100%	Bregman, Cheng, and Levy (1978)
N^{ε}-t-butyloxycarbonyl-glucagon	Electrophoresis	10-15%	N.T.	Lande et al. (1972)
N^{α}-Trifluoroacetyl-glucagon	Electrophoresis	N.T.[b]	N.T.	Lande et al. (1972)
Tyrosine				
Iodo-glucagon	Ion Exchange	100%, F.A.	100%	Rodbell et al. (1971)
	Gel Filtration	5-10x	N.T.	Bromer, Boucher, and Patterson (1973)

Table 3.--Continued Activity of glucagon derivatives acting on liver membranes.

Peptide	Purification	Activity[a]	Binding	Reference
	Ion Exchange	5x	2x	Desbuquois (1975a)
	Ion Exchange	3x	3x	Lin, Nicosia, and Rodbell (1976)
Mono-nitro-glucagon	Ion Exchange	77%, F.A.	N.T.	Patterson and Bromer (1973)
Dinitro-glucagon	Ion Exchange	22%, F.A.	N.T.	Patterson and Bromer (1973)
Mono-amino-glucagon	Ion Exchange	40%, F.A.	N.T.	Patterson and Bromer (1973)
Diamino-glucagon	Ion Exchange	14%, F.A.	N.T.	Patterson and Bromer (1973)
Carboxyl groups				
Glucagon tetramethylester	Gel Filtration	None	N.T.	Epand and Epand (1972)
$Asp^{9,15,21}$-trimethyl-ester-glucagon-methylester		0.01%	0.02%	Epand et al. (1979)
$Asp^{9,15,21}$-triglycinamide-glucagonyl-[glycinamide]		0.004%	0.05%	Epand et al. (1979)
Glucagon tetraglycineamine	Gel Filtration	None	N.T.	Epand and Epand (1972)
Monotaurine-glucagon	Gel Filtration	15%, F.A.	N.T.	Wheeler, Epand, and Barrett (1974)
		2.3%	2.5%	Epand et al. (1979)

Table 3.--Continued Activity of glucagon derivatives acting on liver membranes.

Peptide	Purification	Activity[a]	Binding	Reference
Tetrataurine-glucagon	Gel Filtration	0.1%	N.T.	Wheeler et al. (1974)
Ethylenediamine-glucagon	Gel Filtration	None	N.T.	Wheeler et al. (1974)
Desamido-glucagon	Ion Exchange	60%	N.T.	Bromer et al. (1972)
Amino (terminal) groups				
[Des-His^1]-glucagon	Electrophoresis	6%	N.T.	Lande et al. (1972)
	Ion Exchange	2%, P.A.[d]	15-fold less	Lin et al. (1975)
[Des-His^1][N^{ε}-phenylthio-carbamoyl]-glucagon	Ion Exchange	None, antagonist	1/18	Bregman and Hruby (1979)
Fragments				
$Glucagon_{1-6}$	Synthetic	75%, P.A.	0.001%	Wright and Rodbell (1979)
$Glucagon_{1-21}$	None	None	None	Rodbell et al. (1971)
	Partition Chromatography	0.5%, F.A.	N.T.	Wright (1976)
	Partition Chromatography	0.5%, F.A.	0.1%	Wright et al. (1978)

Table 3.--Continued Activity of glucagon derivatives acting on liver membranes.

Peptide	Purification	Activity[a]	Binding	Reference
$Glucagon_{1-23}$	Synthetic	None	N.T.	Spiegel and Bitensky (1969)
$Glucagon_{1-23}$	Synthetic	0.01%, F.A.	N.T.	Epand and Grey (1973)
$Glucagon_{2-27}$	Electrophoresis	None	10%	Rodbell et al. (1971)
	Electrophoresis	None	N.T.	Lande et al. (1972)
	Ion Exchange	2%, P.A.	7%	Lin et al. (1975)
$Glucagon_{20-29}$	Synthetic	None	None	Rodbell et al. (1971)
$Glucagon_{22-29}$	Synthetic	None	None	Wright et al. (1978)
Glucagon tryptic fragments	None	None	N.T.	Spiegel and Bitensky (1969)
CNBr-glucagon	Electrophoresis	18%, F.A.	N.T.	Spiegel and Bitensky (1969)
	Gel Filtration	10%, F.A.	N.T.	Epand and Grey (1973)
	Partition Chromatography	2%, F.A.	2%	Lin et al. (1975)
CNBr-glucagon hydrazide	Partition Chromatography	2%, F.A.	2%	Hruby et al. (1976)
CNBr-glucagon, n-butylamide	Partition Chromatography	2%, F.A.	2%	Hruby et al. (1976)

Table 3.--Continued Activity of glucagon derivatives acting on liver membranes.

Peptide	Purification	Activity[a]	Binding	Reference
S-Methyl-glucagon	Ion Exchange	None	N.T.	Rothgeb et al. (1977)

[a]As compared with native glucagon at half maximal activity and at high concentrations.

[b]Not tested.

[c]Full agonist.

[d]Partial agonist.

carboxyl groups (probably the terminal carboxyl) is more reactive. The presence of a lone methionine residue (Met 27) near the C-terminal of the glucagon suggested that treatment of the peptide with cyanogen bromide would give a slightly abbreviated glucagon analogue, namely cyanogen-bromide glucagon (CNBr-glucagon) which has a new functional group (a γ-lactone). CNBr-Glucagon is synthesized and is found to be a full agonist, that is, at appropriate concentration, it could stimulate adenylate cyclase to the same maximal activity as native glucagon (Lin et al., 1975). The affinity of CNBr-glucagon for specific glucagon binding sites, determined by measuring the ability of CNBr-glucagon to displace [^{125}I]-glucagon bound to the hepatic plasma membranes, was about 40-50 fold less than that of native glucagon. These results indicate that the major role of the missing or modified residue of CNBr-glucagon appears to be primarily in the recognition process at the hormone receptor and not in activation of adenylate cyclase. Again, glucagon$_{1-21}$ (Wright et al., 1978) is a full agonist and has an adenylate cyclase activity 1/200 that of glucagon. These findings provide evidence that the C-terminal moiety of glucagon is primarily concerned with recognition of the receptor and not activation of adenylate cyclase.

The effects of changes at the N-terminal residue and the amino groups of glucagon are more complex. Previous preparation of [des-His^{1}]-glucagon (DH-glucagon, His 1 residue removed) by the Edman degradation procedure (Rodbell et al., 1971; Lande et al., 1972) gave different results. Highly purified DH-glucagon was synthesized (Lin et al., 1975) and shown to be a partial agonist (Hruby et al., 1976).

It possesses about 60% of the maximal activity, 2% of the potency, but about 10% of the binding activity of glucagon. This illustrated that the histidyl residue or the amino terminal and perhaps the central portion of the molecule in glucagon plays an important role in binding to the receptor as well as activation of adenylate cyclase.

The chemical modification of the hydrophobic amino acids tryptophan (Epand and Cote, 1976; Demolious and Epand, 1979) and tyrosine (Lin et al., 1976; Patterson and Bromer, 1973) results in derivatives which exhibit equal or even enhanced potency compared with the native glucagon. When the hydrophobic amino acids tyrosine (Lin et al., 1976) or methionine (Rothgeb et al., 1977) become modified in such a manner that they carry a charge and thus become polar they they can no longer interact with the hydrophobic sites on the membrane and greatly decreased activity is observed. In agreement with the idea that the peptide can interact with biological membranes in the form of an amphipathic helix, Epand and Wheeler (1975) have observed that making these residues more hydrophobic by neutralizing the charge enhances the activity of the peptide. In general, modification of the polar amino acids led to a reduction in activity even in the case of [homoarginine^{12}]-glucagon (Epand et al., 1973; Ross et al., 1979; Morin, 1977) and glucagon-[taurine] (Wheeler et al., 1974) where only one group was modified and the change at neutral pH was retained in the analogue. The results suggest that these polar groups have a functional role in addition to receptor binding and that their modification can result in derivatives which behave as glucagon antagonists. In fact, another derivative, [des-His^{1}] [N^{ε}-phenylthiocarbamoyl]-glucagon,

modified at polar groups, has been shown to act as a glucagon antagonist in adenylate cyclase assays (Bregman and Hruby, 1979) and the photosensitive derivative, N^{ε}-4-azido-2-nitrophenyl-glucagon, can bind to glucagon receptor sites with an affinity apparently equal to that of the native hormone but is incapable of stimulating adenylate cyclase activity (Bregman and Levy, 1977; Cote and Epand, 1979). Though N^{α}-trinitrophenyl-glucagon inhibits the elevation of cyclic AMP levels caused by glucagon, it nevertheless stimulates glycogenolysis (Cote and Epand, 1979).

In partial sequence modification, $glucagon_{1-21}$ (Rodbell et al., 1971; Wright, 1976) is a full agonist (Wright et al., 1978). It stimulates hepatic adenylate cyclase activity to the same extent as native glucagon, but with 0.1% of the potency. $Glucagon_{1-21}$ also displays 0.1% of the binding affinity of native glucagon to glucagon receptor in hepatic membranes. $Glucagon_{22-29}$ alone or in combination with $glucagon_{1-21}$ did not activate adenyl cyclase or displace [^{125}I]-glucagon from its receptor (Wright et al., 1978). Recently, a much shorter sequence, $glucagon_{1-6}$, has been shown to be capable of activating adenylate cyclase (Wright and Rodbell, 1979). This result suggests that virtually all the groups required for glucagon function are contained in the N-terminal region. However, the potency of this fragment was 0.001% that of the native hormone and about 1% that of $glucagon_{1-21}$. It must be further considered that this derivative may not be operating solely by a glucagon-specific mechanism.

The objective of this investigation is to prepare a possible glucagon inhibitor. The approach in obtaining a hormone antagonist to

glucagon by N-terminal modifications involves using [homoarginine^{12}]-glucagon or one of its purified Edman degradation products, particularly [des-His^{1}] [homoarginine^{12}]-glucagon, to prepare analogues of glucagon. Such a preparation requires the limitation of the Edman degradation specifically to the N-terminal α-amino group. Thus provisions must be made to selectively block the amino group on lysine. Guanidylation (Hirs, 1967), converting the lysine residue into a homoarginine residue, has the advantages that it is apparently highly specific for epsilon amino groups, it gives high reaction yields, and maintains a basic moiety at the lysine, but the moiety is not nucleophilic under most conditions. The histidine is then removed by one step Edman degradation to give [des-His^{1}-homoarginine^{12}]-glucagon. Using [homoarginine^{12}]-glucagon or [des-His^{1}-homoarginine^{12}]-glucagon and azide coupling with various azide compounds, it will be possible to study structure-function of glucagon with regard to the histidine residue in a number of ways. The most recent peptide coupling reaction, diphenylphosphoryl azide (DPPA) coupling, is being extensively investigated in this study for preparing glucagon analogues.

The acid azide method has the chief advantage over the other conventional methods of peptide synthesis of no racemization (Smart, Yang, and Williams, 1960). Among the drawbacks of the method are the various and often awkward solubility properties of hydrazides and the multiplicity of reaction paths open to acid azides, with the consequent possibility of side reactions. The Curtius rearrangement (see Figure 9) has been encountered relatively infrequently under the conditions of peptide synthesis by the azide route (Hinman, Carson, and Christensen,

Curtius Rearrangement

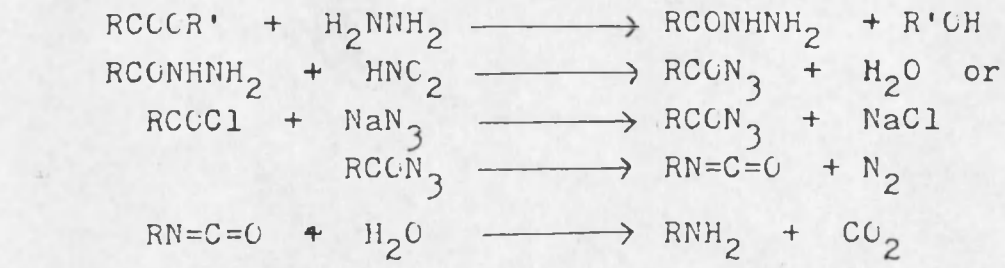


Figure 9. Curtius rearrangement.

1950; Hooper et al., 1956; Stoll and Petrzilka, 1952; Fruton, 1942; Harris and Fruton, 1951; Jost, Rudinger, and Sorm, 1963; Jost et al., 1964; Zaoral et al., 1965). On the other hand, the reaction which gives rise to the amide corresponding to the starting hydrazides has been shown to be difficult in many cases.

The formation of amide from the azide has been suggested (Cook and Elvidge, 1949) to occur by a reaction such as (1) (see Figure 10), or that it is formed from an intermediate, presumably a nitrosohydrazide (A) (Cook and Elvidge, 1949; Thompson and Wolrom, 1946) or its tautomeric form (B) by elimination of nitrous oxide (2a), whereas the azide is formed from the same intermediate.[1] However, the second alternative is favored with circumstantial evidence.

The azide coupling reaction designed to prepare the glucagon analogues is shown in Figure 11a. Rudinger (Honzl and Rudinger, 1961) reported that at high acidities and low temperature in homogeneous solution, the Curtius rearrangement is largely suppressed. He introduced the use of butyl nitrite (BuONO), in the presence of HCl, for the preparation of azides from hydrazides in fairly good yield (Dutta et al., 1978). The starting amino acid hydrazide is synthesized from the corresponding BOC-amino acid and hydrazine by dicyclohexylcarbodiimide-hydroxybenzotriazole (DCC-HOBT) coupling (Wang et al., 1978).

Diphenylphosphoryl azide (Yamada, Yokoyama, and Shioiri, 1974; Lal et al., 1977) is introduced as a new convenient reagent for a

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1. Though, in principle, two nitrosohydrazides might exist, with the nitroso group on the α and β nitrogen, respectively, the disposition of bonds in the products of both reactions requires precursors with the arrangement C-N-N-N.

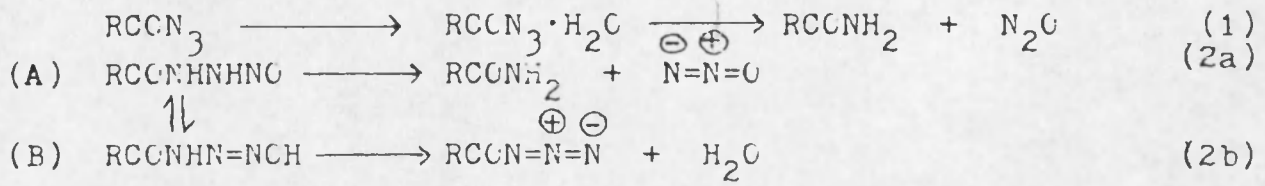


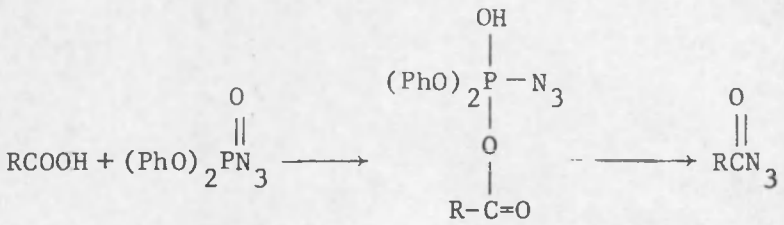
Figure 10. Possible by-products and intermediates in the azide reaction.

modified Curtius reaction and for peptide synthesis (Shioiri, Minomiya, and Yamada, 1972). DPPA allows direct conversion of carboxylic acids to urethanes (1) and amides (2).

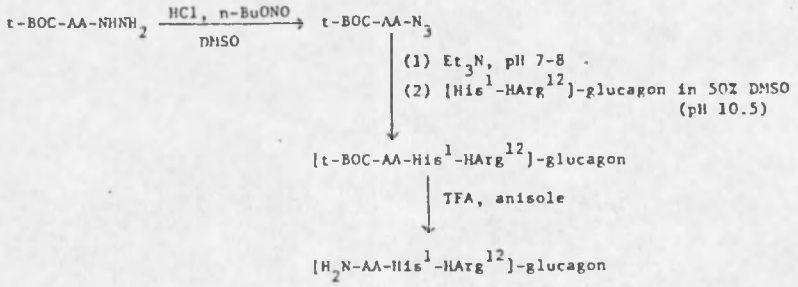
$$\text{RCOOH} + \text{N}_3\text{PO(OPh)}_2 \xrightarrow{\text{R'OH, Et}_3\text{N}} \text{RNHCOOR'} \quad (1)$$

$$\text{RCOOH} + \text{N}_3\text{PO(OPh)}_2 \xrightarrow{\text{R''NH}_2\text{, Et}_3\text{N}} \text{RCONHR''} \quad (2)$$

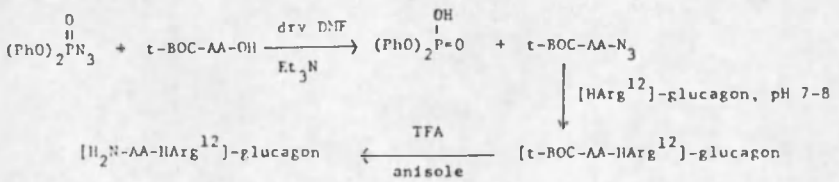
The reaction proceeds under more or less neutral and non-oxidizing conditions, and neither strong alkali as in the Hofmann reaction nor strong acids as in the Schmidt reaction is required. The key intermediate is apparently the carboxylic acid azide which is produced by the transfer of the azide group from DPPA to the carboxylic acid.



This leads to a convenient method for azide coupling in peptide synthesis, in which the hydrazide intermediate is eliminated. DPPA provides the coupling in high yield of acylamino acids or peptides with amino acid or peptide esters in the presence of base in a single operation and with partial (Rosowsky and Yu, 1978) or no racemization. Figure 11b illustrates the DPPA coupling reaction designed for the preparation of the glucagon analogues in this study.



(a) azide coupling



(b) DPPA coupling

Figure 11. Schematic diagram for the preparation of glucagon analogues.

CHAPTER 2

EXPERIMENTAL SECTION

In most synthetic work, crystalline glucagon purchased from Elanco Products Company was not purified. The peptide fractions were determined by ultraviolet absorbance (Gilford 240 Spectrophotometer) at 280 nm or 289 nm after gel filtration or ion exchange chromatography. All samples for amino acid analysis (AAA) were hydrolyzed for 22 hr in 6 N HCl at 110°C in a vacuum ampule (Spackman, Stein, and Moore, 1958) and the amino acid contents (Table 4) were quantitatively determined by a Beckman 120C Amino Acid Analyzer. No corrections were made for hydrolytic destruction of susceptible amino acids. All temperatures are in degrees centigrade (°C). An International Refrigerated Centrifuge Model B-20 or a Beckman Microfuge B was used in all cases for centrifugation. The following chromatograhy methods were employed:

Column #	Type	Solvents	Dimensions
Gel filtration:			
7	Sephadex G-15	30% HOAc	2.2 cm x 90 cm
6	Sephadex G-15	30% HOAc	2.2 cm x 90 cm
A	Sephadex G-10	33% Pyr/H_2O	2 cm x 36 cm
B	Sephadex G-15	33% Pyr/H_2O	2 cm x 36 cm
C	Sephadex G-10	30% HOAc	2 cm x 34 cm
Ion exchange:			
D	SP Sephadex C-25	20 mmole NaOAc · $3H_2O$, 1 M urea in 10% HOAc, pH 3	2 cm x 19.5 cm
E	DEAE Sephadex A-25	0.01 M Tris-buffer, 7 M urea, pH 7.7	1.7 cm x 12 cm

Table 4. Amino acids analyses of glucagon derivatives prepared.

				(A) Azide Couplings					(B) DPPA Couplings											
Amino acids	Glucagon calculated	[HArg^{12}]-glucagon	[DH^{1},HArg^{12}]-glucagon	Phe-[HArg^{12}]-glucagon (SL23bls)	Phe-[HArg^{12}]-glucagon, 2nd attempt (SL45a1s)		[Phe^{1},HArg^{12}]-glucagon (SL48a2s)		Gly-[HArg^{12}]-glucagon (SL38a1s)	[Gly^{1},HArg^{12}]-glucagon (SL38b1s)	Gly-[HArg^{12}]-glucagon, 2nd attempt (SL42a2s)		Gly-[HArg^{12}]-glucagon, 3rd attempt (SL50b1s)		Ala-[HArg^{12}]-glucagon (SL40b1s)	Ala-[HArg^{12}]-glucagon, 2nd attempt (SL45a2s)		Ala-[HArg^{12}]-glucagon, 3rd attempt (SL48a2s)	Phe-[HArg^{12}]-glucagon, 1st attempt (SL41a1s)	Phe-[HArg^{12}]-glucagon, 2nd attempt (SL49a2s)	Edman Degradation of Ala-[HArg^{12}]-glucagon (SL42b1s)
Lys	1.00(1)	0.037(0)	0.059(0)	0.042(0)	0.012(0)[a]	0.04(0)[b]	0.033(0)[a]	0.03(0)[b]	0.73(0)	0.79(0)	0.04(0)[a]	0.055(0)[b]	0.113(0)[a]	0.037(0)[b]	0.038(0)[d]	0.308(1)	0.109(0)[a]	0.06(0)[b]	0.11(0)[b]	0.304(0)	0.24 (0)[b]	0.231(0)
His	1.00(1)	0.878(1)	0.092(0)	0.495(1)	0.33 (1)	0.43(1)	0.052(0)	0.03(0)	1.12(1)	0.11(0)	0.72(1)	0.753(1)	0.865(1)	0.86 (1)	0.761(1)	0.743(1)	0.638(1)	0.67(1)	0.66(1)	0.660(1)	0.706(1)	0.679(1)
Arg	2.00(2)	1.922(2)	2.068(2)	2.00 (2)	2.21 (2)	2.40(2)	2.02 (2)	2.06(2)	0.78(1)	2.14(2)	2.01(2)	2.01 (2)	1.956(2)	1.96 (2)	1.94 (2)	2.01 (2)	1.99 (2)	2.05(2)	2.05(2)	2.06 (2)	2.07 (2)	2.00 (2)
HArg	0.00(0)	0.907(1)	0.933(1)	0.960(1)	0.787(1)	0.60(1)	0.974(1)	0.93(1)	--[c]	0.86(1)	0.99(1)	0.988(1)	1.04 (1)	1.03 (1)	1.05 (1)	0.962(1)	1.00 (1)	0.96(1)	0.96(1)	0.94 (1)	0.925(1)	0.864(1)
Asp	4.00(4)	3.97 (4)	4.16 (4)	4.18 (4)	4.36 (4)	4.25(4)	4.31 (4)	4.34(4)	--	4.33(4)	--[c]	3.87 (4)	4.19 (4)	4.09 (4)	4.33 (4)	3.73 (4)	4.08 (4)	4.51(4)	4.13(4)	4.23 (4)	4.20 (4)	4.51 (4)
Thr	3.00(3)	2.83 (3)	2.75 (3)	2.90 (3)	2.80 (3)	2.90(3)	2.86 (3)	3.11(3)	--	2.18(3)	--	--[c]	2.80 (3)	2.74 (3)	2.89 (3)	2.71 (3)	2.76 (3)	2.84(3)	2.75(3)	2.56 (3)	2.30 (3)	2.85 (3)
Ser	4.00(4)	3.83 (4)	3.52 (4)	3.64 (4)	3.70 (4)	3.63(4)	3.46 (4)	3.58(4)	--	3.62(4)	--	--	3.86 (4)	3.73 (4)	3.80 (4)	3.96 (4)	3.86 (4)	4.00(4)	3.82(4)	3.91 (4)	3.88 (4)	3.44 (4)
Glu	3.00(3)	2.94 (3)	3.16 (3)	2.88 (3)	2.98 (3)	2.91(3)	2.99 (3)	3.00(3)	--	2.94(3)	--	--	2.97 (3)	3.16 (3)	3.06 (3)	3.09 (3)	3.03 (3)	2.66(3)	3.18(3)	3.00 (3)	3.03 (3)	3.13 (3)
Gly	1.00(1)	1.04 (1)	1.14 (1)	1.16 (1)	2.46 (1)	1.17(1)	1.08 (1)	1.01(1)	2.13(2)	2.77(2)	Multiple	3.12 (2)	7.02 (2)	3.46 (2)	2.63 (2)	1.09 (1)	1.20 (1)	1.29(1)	1.23(1)	1.19 (1)	1.10 (1)	1.25 (1)
Ala	1.00(1)	1.00 (1)	1.09 (1)	1.06 (1)	1.10 (1)	1.10(1)	1.29 (1)	1.11(1)	--[c]	1.14(1)	--[c]	1.28 (1)	1.11 (1)	1.45 (1)	1.03 (1)	2.35 (2)	6.80 (2)	2.50(2)	2.97(2)	1.18 (1)	1.12 (1)	0.849(1)
Val	1.00(1)	0.94 (1)	1.12 (1)	1.26 (1)	1.06 (1)	1.03(1)	1.04 (1)	1.07(1)	--	0.85(1)	--	1.06 (1)	1.05 (1)	1.06 (1)	0.95 (1)	0.961(1)	0.92 (1)	0.56(1)	1.23(1)	0.82 (1)	1.05 (1)	1.005(1)
Met	1.00(1)	0.79 (1)	0.797(1)	1.189(1)	0.625(1)	0.92(1)	0.898(1)	0.92(1)	--	0.37(1)	--	0.715(1)	0.92 (1)	0.87 (1)	0.87 (1)	0.00 (1)	0.89 (1)	0.14(1)	0.81(1)	0.685(1)	0.775(1)	0.846(1)
Leu	2.00(2)	2.11 (2)	2.11 (2)	1.94 (2)	2.05 (2)	2.02(2)	2.05 (2)	2.08(2)	--	1.96(2)	--	2.02 (2)	2.11 (2)	2.00 (2)	1.92 (2)	2.33 (2)	1.97 (2)	1.87(2)	2.03(2)	1.98 (2)	2.02 (2)	2.03 (2)
Tyr	2.00(2)	2.14 (2)	1.97 (2)	1.98 (2)	1.94 (2)	1.99(2)	1.86 (2)	1.70(2)	1.95(2)	1.92(2)	--	1.88 (2)	1.97 (2)	1.92 (2)	1.96 (2)	2.00 (2)	2.05 (2)	2.00(2)	2.66(2)	2.09 (2)	2.19 (2)	2.025(2)
Phe	2.00(2)	2.16 (2)	1.94 (2)	2.72 (3)	3.18 (3)	2.68(3)	2.44 (3)	2.21(3)	2.05(2)	1.93(2)	--	1.88 (2)	1.93 (2)	1.89 (2)	2.05 (2)	2.12 (2)	2.11 (2)	1.80(2)	1.97(2)	2.29 (2)	3.27 (3)	1.76 (2)

Glucagon and all the derivatives contain 1 residue of tryptophan which is lost on acid hydrolysis.

[a] Before purification.

[b] After purification.

[c] Unable to determine due to poor chromatogram.

[d] After second gel filtration.

Purification of Glucagon

A procedure by Bromer et al. (1972) with some modifications was used to purify the commercial glucagon. Glucagon (132 mg) in 6 ml 0.01 M Tris-buffer (pH 7.7, 4°) which contained 7 M urea, was divided into two equal aliquots. The first aliquot was applied to a DEAE ion exchange column (E) previously equilibrated with the Tris-buffer. The sample was eluted with 90 ml Tris-buffer and then 75 ml of a second Tris-buffer which contained 0.5 M NaCl in addition to the first buffer. Similarly, the second aliquot sample was applied to the same column. Fractions #4-25 (Figure 12a) and #3-20 (Figure 12b) were pooled and lyophilized into white solids which were dissolved in 70 ml 30% HOAc. The solution was divided into two equal aliquots and applied to column #6 and #7. The column was eluted with 300 ml 30% HOAc. Fractions #21-35 (Figure 13a) and #19-30 (Figure 13b) were lyophilized to give 117 mg of a white fluffy solid. No AAA was done.

Preparation of [Homoarginine^{12}]-Glucagon ([HArg^{12}]-Glucagon)

The procedure was modified slightly from the guanidination method established earlier by Kimmel (1967). O-Methylisourea hydrogen sulfate (1.989 g) dissolved in 11.7 ml water and 3.51 g barium hydroxide was added slowly to precipitate the sulfate. The insoluble sulfate was filtered through two sheets of Whatman filter paper. The filtrate was centrifuged for 15 min at 6000 rpm with an International Refrigerated Centrifuge. The clear supernatant was carefully removed with a Pasteur pipette, the volume was adjusted to 18 ml and the pH to 11 with 1 N

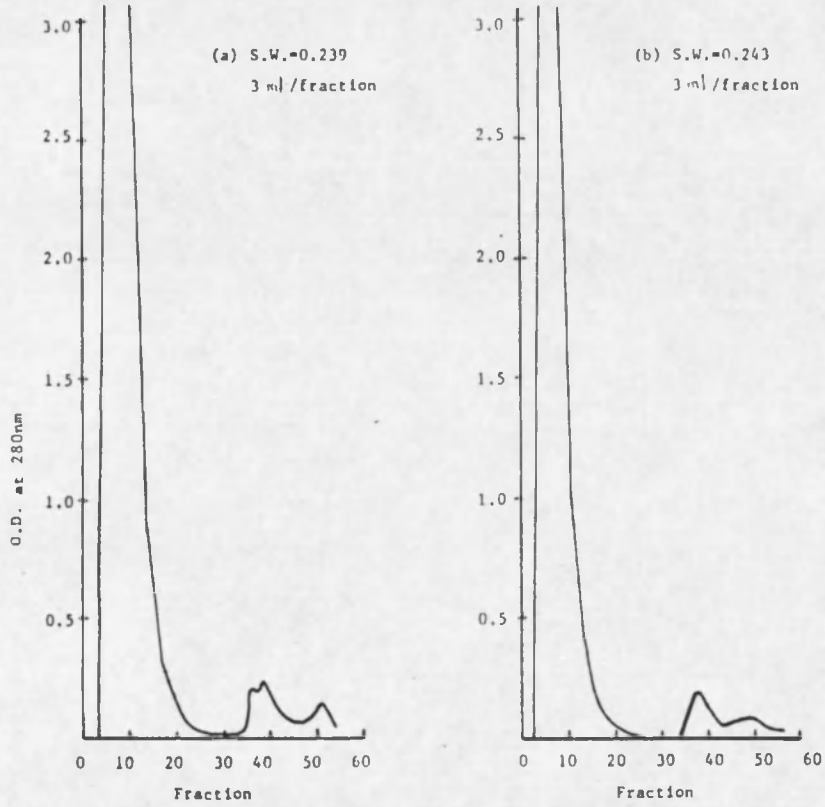


Figure 12. DEAE ion exchange chromatography of glucagon.

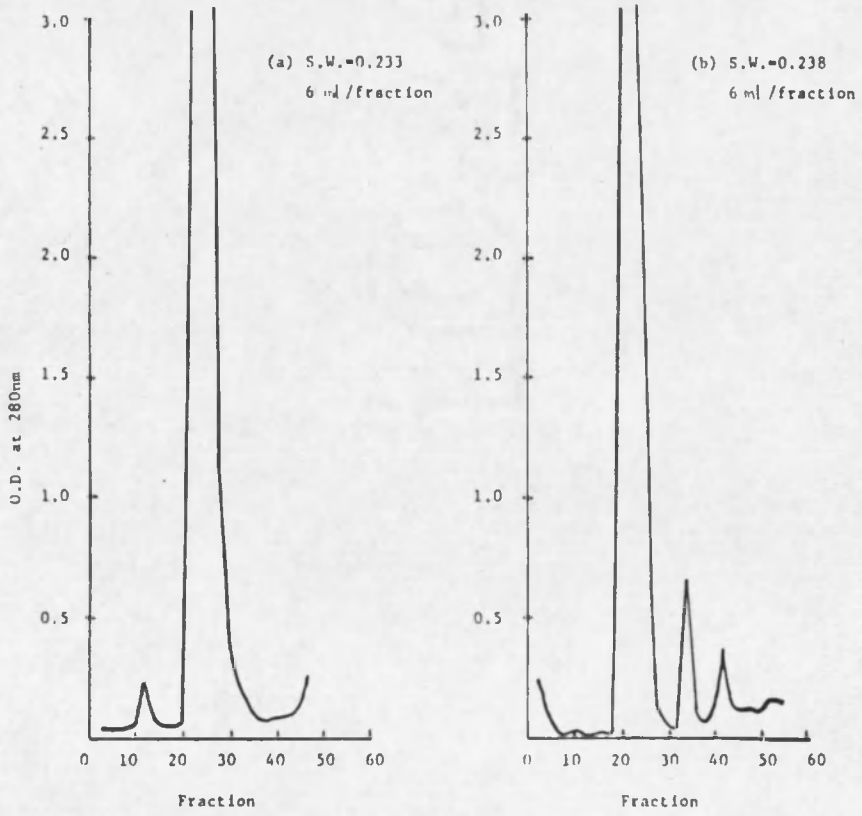


Figure 13. 30% HOAc Sephadex G-15 chromatography of glucagon.

NaOH. This solution was then cooled to 0° in an ice bath and added to a cold solution of 117 mg glucagon in 5.85 ml water (pH 11) with a final pH of 11.05. The reaction mixture was left aside in a cold room (4°) for 14 hr. Then 11.7 ml glacial HOAc was added to stop the reaction. The whole reaction mixture was desalted on column #6 eluted with 340 ml 30% HOAc. Fractions were pooled and lyophilized to give 100.73 mg of white powder (Table 4).

Preparation of [Des-His^{1}][HArg^{12}]-Glucagon ([DH^{1}, HArg^{12}]-Glucagon)

To achieve the removal of histidine from [HArg^{12}]-glucagon, an Edman degradation procedure (Lande et al., 1972; Peterson et al., 1972) with some modifications (Bregman and Hruby, 1979) was followed closely. [HArg^{12}]-Glucagon (20 mg) was dissolved in 2 ml Pyr/H_2O/Et_3N &70/30/0.1, pH 10.4) in a 10 ml pear-shaped round bottom (r.b.) flask and the solution was adjusted to pH 10.27 with 10 µl Et_3N and 10 µl 10% HOAc. Nitrogen gas was bubbled through into the vessel for 2 min and 100 µl phenyl isothiocyanate (PITC) was added under nitrogen. The reaction flask was sealed and incubated in a water bath for 2 hr, at 40° in the dark. The reaction mixture was then extracted 5x with benzene (or until the benzene layer was clear). The contents were transferred to a 50 ml r.b. flask with two rinsings of 33% Pyr/H_2O and was quickly frozen in a dry ice/acetone bath. The solvent was evaporated to dryness in vacuo. Into the r.b. flask was added 15 mg dithioerythritol (DTE) and 5 ml TFA under nitrogen. The reaction mixture was incubated in a water bath for 15-20 min at 50° and evaporated to

dryness in vacuo. The white solid mass was dissolved in 8 ml 30% NOAc and 100 µl Pyr, and the sample was desalted on column #6 eluted with 440 ml 30% HOAc. Fractions #21-20 (Figure 14) were lyophilized into 13.3 mg white solid (Table 4).

Purification of [DH^{1}, HArg^{12}]-Glucagon

The attempt to purify [DH^{1}, HArg^{12}]-glucagon was carried out by ion exchange chromatography with a continuous salt gradient of 0.1 M NaCl to 0.35 M NaCl. An SP Sephadex C-25 column (D) was washed with 100 ml 0.1 M NaCl buffer. The buffer contained 20 mmole NaOAc · $3H_2O$ and 1 M urea in 10% HOAc, pH 3. [DH^{1}, HArg^{12}]-Glucagon (36 mg) in 8 ml buffer was applied to the column and the gradient began with equal volumes (250 ml) of 0.1 M NaCl-buffer and 0.35 M NaCl-buffer. Finally, the column was eluted with 175 ml 0.5 M NaCl-buffer. Fractions #33-66 (Figure 15) were collected, lyophilized, taken into 10 ml 30% HOAc, applied to column #7, and eluted with 480 ml 30% HOAc. Fractions #19-35 (Figure 16) were lyophilized to give 30.7 mg white material. AAA still showed about 3% histidine both before and after purification.

Azide Coupling

Preparation of t-BOC-Phe-Hydrazide (t-BOC-Phe-$NHNH_2$)

The protected phenylalanine hydrazide was synthesized from the corresponding t-BOC-acid and hydrazine (NH_2NH_2) by dicyclohexylcarbodiimidehydroxybenzotriazole (DCC-HOBT) coupling, using a modified procedure of Wang et al. (1978). t-BOC-Phe (1.615 g, 6.6 mmole) was dissolved in 20 ml DMF and stirred with 200 µl (6.6 mmole) anhydrous

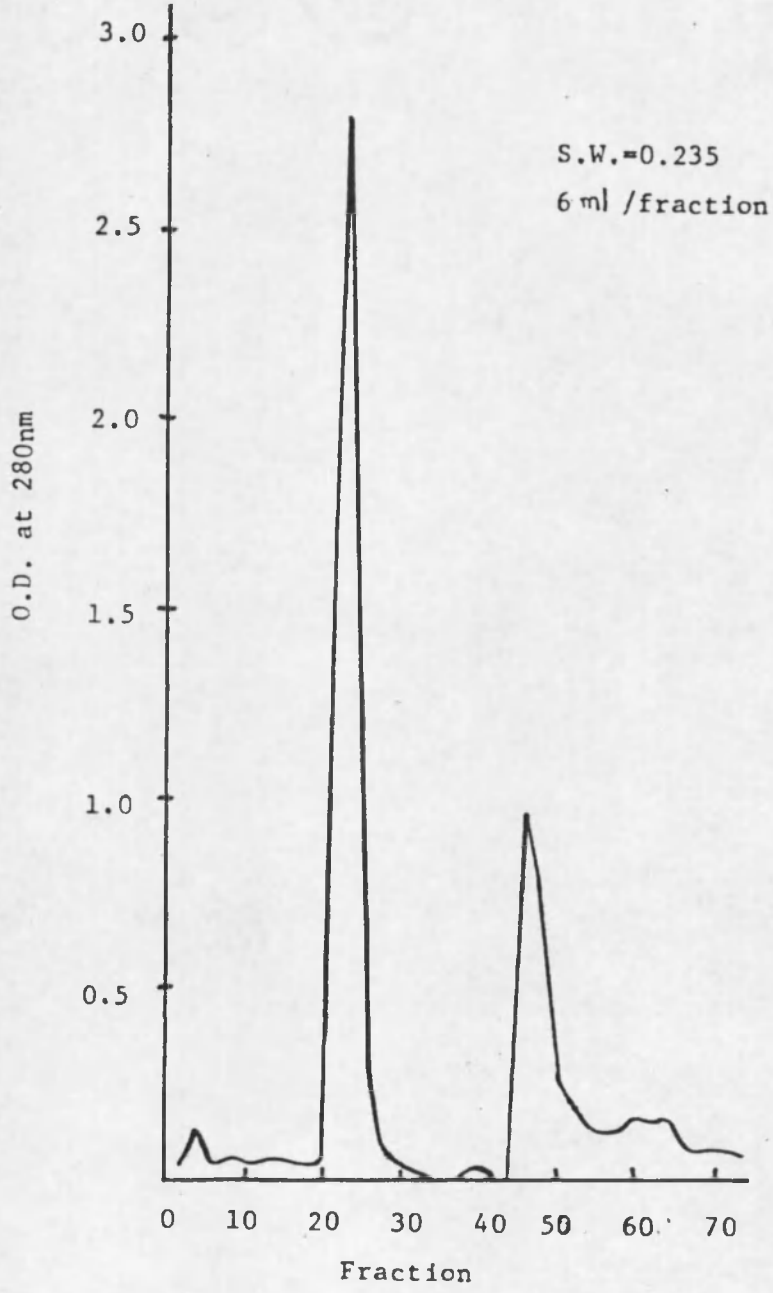


Figure 14. 30% HOAc Sephadex G-15 chromatography of [DH^{1},$HArg^{12}$]-glucagon.

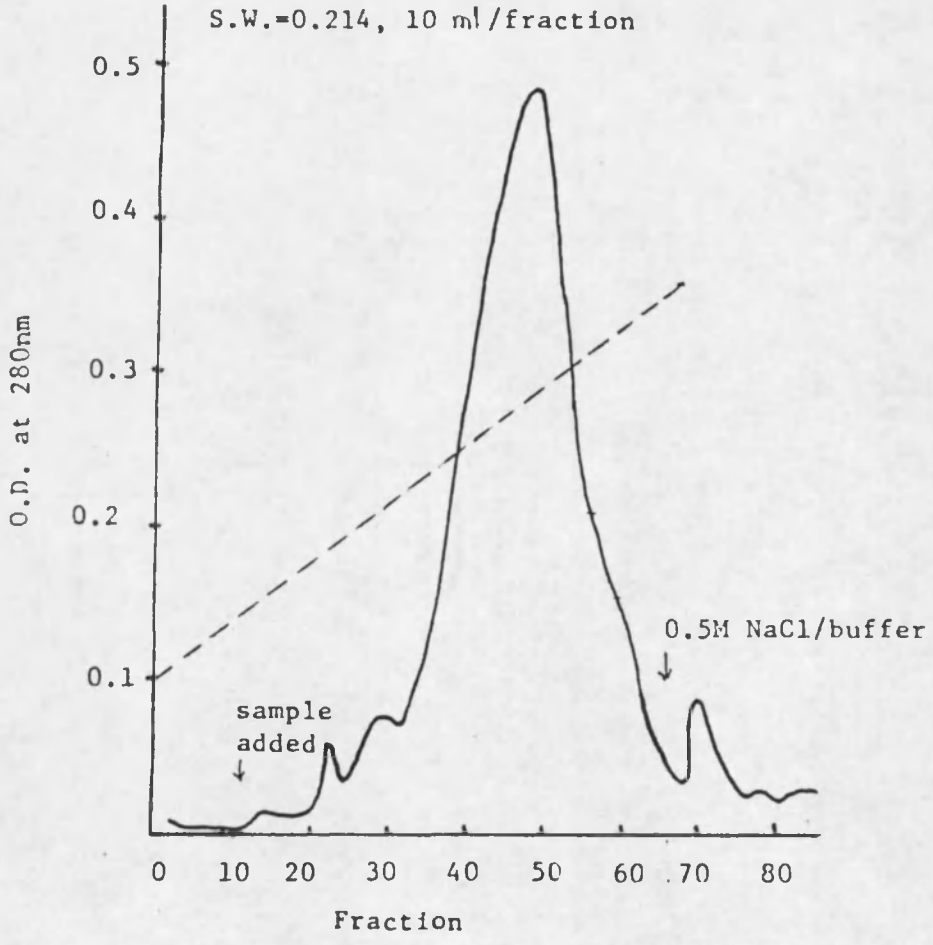


Figure 15. SP Sephadex C-25 ion exchange chromatography of [DH^{1},$HArg^{12}$]-glucagon.

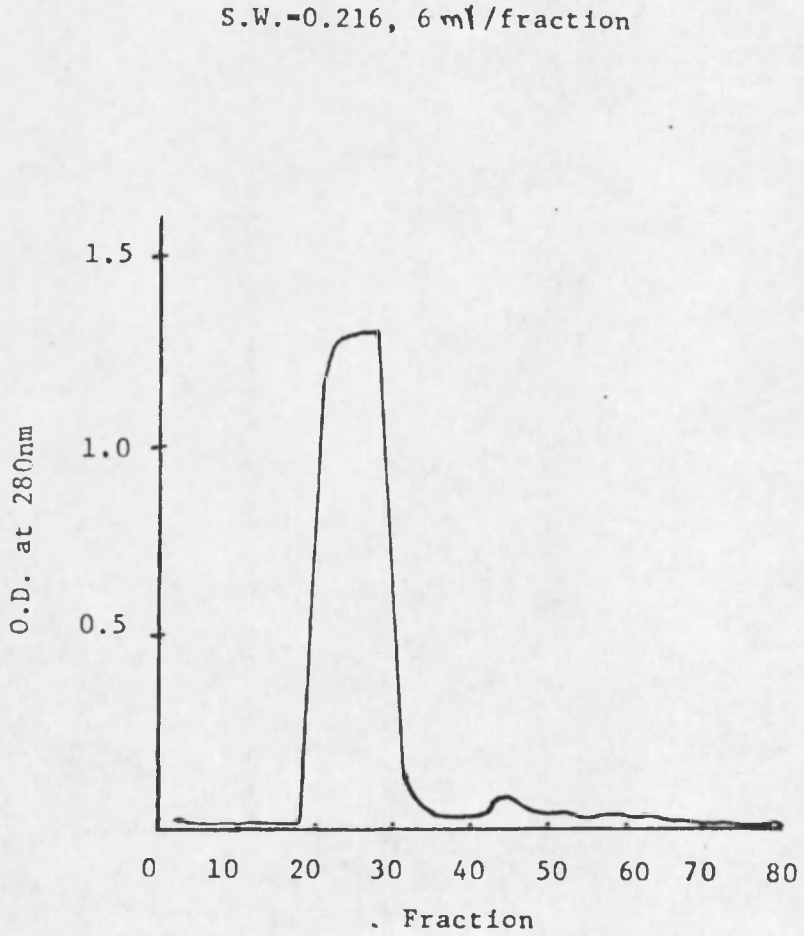


Figure 16. 30% HOAc Sephadex G-15 chromatography of purified [DH^{1},$HArg^{12}$]-glucagon.

NH_2NH_2, 1.834 g (6 mmole) HOBT, and 1.36 g (6.6 mmole) DCC for 24 hr at room temperature. The insoluble byproduct, dicyclohexylurea (DCHU), was filtered off and the filtrate evaporated to a light yellow paste. The residue was taken into 120 ml EtOAc with steam, washed 3x with 50 ml H_2O, dried over Na_2SO_4 (2x), and evaporated. The crude product was recrystallized from THF and petroleum ether (pet. ether, 2x), and then from EtOAc and pet. ether; 111 mg of the white product was obtained, mp = 120.5-122°; R_f = 0.77 (n-BuOH/HOAc/H_2O = 4:1:5).

Preparation of Phe-[HArg^{12}]-Glucagon
by Azide Coupling

Organic nitrite (n-BuONO), as introduced by Honzl and Rudinger (1961) was used in all the azide coupling reactions. t-BOC-Phe-$NHNH_2$ (6.4 mg, 20 μmole, 10xs) was dissolved in 2 ml DMSO, and 2 ml 2 N HCl was added slowly. The solution was then cooled to 0° in an ice bath. n-BuONO (115 μl, 1.2 mmole) was introduced and the reaction mixture was stirred for 1/2 hr at 0°, neutralized to pH 8-9 with 1300 μl Et_3N, and then added to a solution of 7 mg (2 μmole) [HArg^{12}]-glucagon in 2 ml DMSO containing 50 μl Et_3N, pH 8. The resultant solution was stirred in a cold room (4°) for 24 hr and desalted on column A. The column was eluted with 60 ml 33% Pyr/H_2O and the major fraction was collected and lyophilized. The t-BOC was removed by treating the white solid obtained with 10 mg DTE, 2 ml anisole, and 4.5 ml TFA at 0° for 45 min with continuous stirring. The solution was then evaporated to dryness in vacuo. The residue was taken into 8 ml 30% HOAc, desalted on column #7, and eluted with 360 ml 30% HOAc. Fractions were collected and lyophilized to give 4 mg white fluffy material (Table 4, SL23b1s).

Modified Procedure for Preparation of
BOC-Phe-[HArg^{12}]-Glucagon
(Second Attempt)

t-BOC-Phe-$NHNH_2$ (6.4 mg, 20 μmole, 10x) was dissolved in 2 ml DMSO and 2 ml of 2 N HCl was added slowly. The solution was then cooled to 0° in an ice bath, 115 μl (1.2 mmole) n-BuONO was introduced, and the reaction mixture was stirred for 1/2 hr at 0°, neutralized to pH 8 with 400 μl Et_3N, then added to a solution of 7 mg (2 μmole) [HArg^{12}]-glucagon in 2 ml 50% DMSO/H_2O (pH 11). Et_3N was added to maintain the pH at 7-8. The solution was stirred at 4° for 2 hr in the dark. The same azide was generated twice as before at 2-hr intervals, and added to the reaction mixture with continuous stirring at 4° for 2 hr. The final reaction solution was yellow, 15 ml, pH 8. Finally, the solution was stirred for additional 41 hr in the cold and desalted on column B and eluted with 112 ml 33% Pyr/H_2O. Fractions #9-14 (Figure 17) were lyophilized to give 8 mg yellow powder (Table 4, SL45a2s[a]).

Purification of Phe-[HArg^{12}]-Glucagon
(Second Attempt)

BOC-Phe-[HArg^{12}]-Glucagon (8 mg) was placed in a 1.5 ml plastic vial to which 1 ml 0.001 N HCl (pH 3.1) was added and the vial was centrifuged for 3-4 min with a Beckman Microfuge-B. The supernatant was removed with a pipette and the same process was repeated 4x. The pellet formed in the bottom of the vial was taken into 3-4 ml 33% Pyr/H_2O and the solution was lyophilized to a slight cream colored powder. The t-BOC group was removed as previously described and the sample was

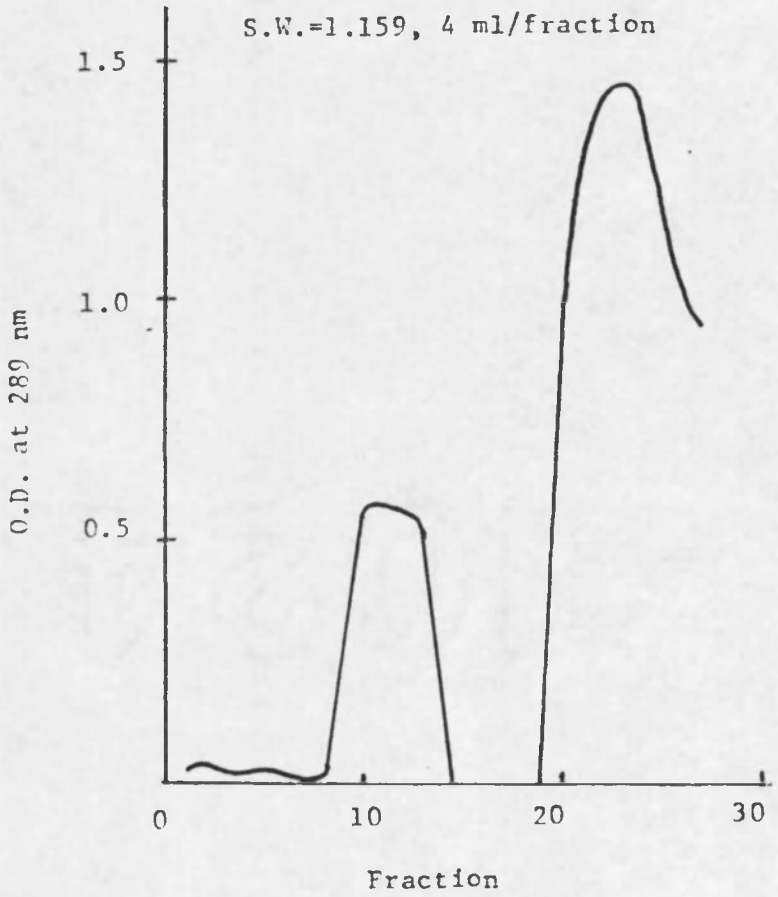


Figure 17. 33% Pyr/H_2O Sephadex G-15 chromatography of purified BOC-Phe-[HArg^{12}]-glucagon via azide coupling.

desalted on column #6. The major fractions were collected and lyophilized to give 5.05 mg of pale yellow powder (Table 4, SL45a2s^{b}).

Preparation and Purification of
[Phe^{1}, HArg^{12}]-Glucagon

The same modified procedure was used to synthesize [Phe^{1}, HArg^{12}]-glucagon. The final product was light yellow; overall yield was 1.77 mg (Table 4, SL48a2s).

DPPA Coupling

Preparation of t-BOC-Gly

The BOC- amino acids was prepared using a new reagent, t-butyloxycarbonyloxyimino-2-phenylacetonitrile (Itoh, Hagaiwara, and Kamiya, 1975). To a solution of glycine (0.75 g, 10 mM) and Et_3N (3.1 ml, 22.5 mM) in 50% aq. dioxane (18 ml) was added BOC-reagent (2.71 g, 11 mM) with stirring at room temperature for 3 hr. Water (15 ml) was added and the mixture was extracted 4x with ETOAc (20 ml wa h). The aqueous phase was neutralized with solid citric acid to pH 3, saturated with NaCl, extracted with EtOAc, and dried by passage through anhydrous Na_2SO_4 on a filter funnel into a 250 ml r.b. flask. The solvent was evaporated to a light yellow oil in vacuo. The residue was dissolved in a small amount of EtOAc with steam and made turbid with petroleum ether and kept cold overnight. Filtered precipitate: white needles, mp = 85-86° (Lit. mp = 86-88°, Nagasawa et al., 1973); yield 0.772 g; R_f = 0.807 (n-BuOH/HOAc/H_2O = 4:1:5); ninhydrin test still positive.

Purification of t-BOC-Amino Acids

The t-BOC amino acids were purified by either one of the methods described as follows:

1. Application of $CuSO_4 \cdot 5H_2O$ column. Crude or impure BOC-amino acids (2 g) were dissolved in a minimum amount of CH_2Cl_2 (re-distilled), applied to a finely-ground $CuSO_4 \cdot 5H_2O$ column (2 cm x 16.5 cm), eluted with 100 ml CH_2Cl_2, evaporated to dryness or viscous liquid, and recrystallized from EtOAc or ether and pet. ether. This method was used to purify BOC-Gly (EtOAc and pet. ether), mp = 86.5-87°, R_f = 0.83 (n-BuOH/HOAc/H_2O = 4:1:5); ninhydrin test negative.
2. Extraction with dilute HCl. Impure BOC-amino acid (10 g) in EtOAc (100 ml) was shaken with dil. HCl (4%, saturated with NaCl, cooled to 0° in an ice bath, 3x, 30 ml). A few pieces of ice were added before each shaking. The EtOAc solution was then washed with cold water saturated with NaCl (2x, 30 ml), then cold water alone (2c, 30 ml), dried (Na_2SO_4) and solvent removed in vacuo. The following BOC-amino acids were purified by the above method and crystallized from the solvents indicated: BOV-Val (ether and pet. ether), mp = 76-78°; ninhydring test negative; BOC-Ala (ether and pet. ether), mp = 80-81°; ninhydrin test negative; BOC-Phe (ether and pet. ether); mp = 85-88°; ninhydrin test negative.

Preparation of Gly-[HArg^{12}]-Glucagon, Ala-[HArg^{12}]-Glucagon, Val-[HArg^{12}]-Glucagon, Phe-[HArg^{12}]-Glucagon

The new reagent, diphenylphosphoryl azide (DPPA), introduced by Shioiri et al.,(1972) and the procedure described by Rosowsky and Yu (1978) were used in this coupling reaction. However, the procedure was modified using a large excess of t-BOC-amino acids (5x DPPA, 100x hormone) and imidazole as buffer. A solution of 11.66 mg (200/3 μmole) t-BOC-Gly in 1 ml anhydrous DMF (redistilled), 2.87 μl (40 μmole, 1 eq.) Et_3N were stirred in an ice bath for 2 hr. Then 13.62 mg (200 μmole, 5 eq.) imidazole was added, followed by 10 ml DMF, 7 mg (2 μmole) [HArg^{12}]-glucagon and 5 μl Et_3N to pH 7. The reaction mixture was kept at 4° for 3-4 hr with continuous stirring in the dark. The azide was regenerated twice as before, at 2-hr intervals and added to the reaction mixture with continuous stirring at 4° for 3-4 hr each time. Finally, the mixture was stirred for an additional 37 hr at room temperature, desalted on column B eluting with 80 ml 33% Pyr/H_2O. Fractions #7-13 and #17-27 (Figure 18) were collected. The first fraction was identified as the hormone peak while the second fraction was the salt peak. The t-BOC group was removed from the first fraction as usual (10 mg DTE, 2 ml anisole, 5 ml TFA, 50 min at 0°). The content was evaporated to dryness, taken into 10 ml 30% HOAc with steam, and finally desalted on column #7, eluting with 360 ml 30% HOAc. Fractions were lyophilized to give 6.4 mg white solid. AAA showed Gly = 4.5(2). No short column data were recorded. The sample was then purified by gel filtration on column #7. Fractions were collected and

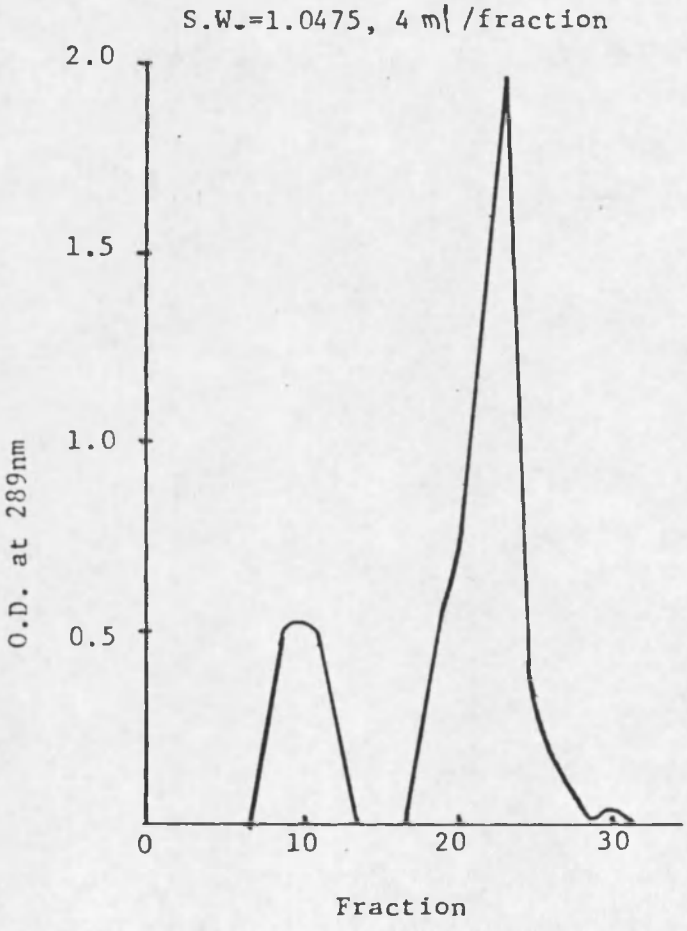


Figure 18. 33% Pyr/H_2O Sephadex G-15 chromatography of BOC-Gly-[HArg^{12}]-glucagon via DPPA coupling.

lyophilized and 1 mg off-white material was obtained; AAA showed Gly = 2.2(2). Again no short column data were taken.

Similarly, 6.5 mg Ala-[HArg^{12}-glucagon was synthesized using the same method (Table 4, SL40b1s). The attempt to synthesize Val-[HArg^{12}]-glucagon and Phe-[HArg^{12}]-glucagon as before failed. AAA showed no coupling (see Table 4, Phe-[HArg^{12}]-glucagon, first attempt, SL41als) even when conditions were changed to more alkaline medium (pH 9) and longer reaction times of up to 45 hr at room temperature were used.

Large Scale Preparation of
Gly-[HArg^{12}]-Glucagon

A solution of 17.5 mg (100 μmole, 1/3 eq.) BOC-Gly in 1 ml anhydrous DMF, 4.31 μl (20 μmole, 1/3 eq.) DPPA, and 8.37 μl (60 μmole, 1 eq.) Et_3N was stirred in an ice bath for 2 hr in the dark. To this solution was added 20.43 mg (300 μmole, 5 eq.) imidazole, 10 ml DMF, 10.5 mg [HArg^{12}]-glucagon (2.5 μmole). The pH was adjusted to 7 with 150 μl Et_3N, and the reaction mixture was stirred at 4° for 3-4 hr (dark). The azide was prepared twice more, at 2-hr intervals and added to reaction mixture with continuous stirring at 4° for 3-4 hr. Finally, the mixture was stirred for 36 hr at room temperature and desalted on column B. Fractions were lyophilized to a white solid which was treated with TFA to remove the BOC group as previously described. The sample was taken into 10 ml 30% HOAc and applied to column #7. Fractions were collected and lyophilized to give 7 mg white powder (Table 4, SL38als).

Large Scale Preparation of
[Gly^{1}, HArg^{12}]-Glucagon

The preparation of [Gly^{i}, HArg^{12}]-glucagon was carried out on the same scale as before. A white powder (5.84 mg) was obtained (Table 4, SL38b1s).

Modified Procedure for Preparation of
Gly-[HArg^{12}]-Glucagon

A solution of 11.6 mg (66.66 μmole, 1/3 eq.) BOC-Gly in 1 ml anhydrous DMF, 2.87 μl (13.33 μmole, 1/3 eq.) DPPA, and 5.58 μl (40 μmole, 1 eq.) Et_3N was stirred in an ice bath for 2 hr in the dark. To this solution was added a solution of 10 ml DMF, 7 mg (2 μmole) [HArg^{12}]-glucagon, and 50 μl Et_3N to pH 7. The reaction mixture was stirred for 3-4 hr at 4°. The same azide was generated twice as before at 2 hr intervals, and added to the reaction mixture with continuous stirring and pH 7-8 was maintained with Et_3N at all times. The final clear solution was then desalted on column B. Fractions were collected and lyophilized to give 8.42 mg of white powder (Table 4, SL42a2s^{a}). The sample was placed in a plastic microvial and 1 ml 0.001 N HCl (pH 3.1) was added, then centrifuged with a Beckman Microfuge B for 4 min. This process was repeated 4x and the supernatant removed carefully with a pipette. The pellet was taken into 1 ml 33% Pyr/H_2O, centrifuged for 3-4 min (3x), lyophilized to a white solid, deprotected as usual, and finally desalted on column #6, eluting with 240 ml 30% HOAc. Fractions were lyophilized to give 1.77 mg (Table 4, SL42a2s^{b}).

Similarly, the same scale preparation of Ala-[HArg^{12}]-glucagon was attempted twice to give 0.8 mg and 3.19 mg overall yield,

respectivesly (Table 4, SL46a2s and SL48b2s). Phe-[HArg^{12}]-glucagon was attempted once more with this modified procedure at a longer reaction time of 61 hr at 4°; 2.27 mg overall yield was obtained (Table 4, SL49a2s).

Modified Procedure for a Large Scale Preparation of Gly-[HArg^{12}]-Glucagon

With the same modified procedure described above, Gly-[HArg^{12}]-glucagon was prepared on a larger scale using 105 mg (600 μmole) BOC-Gly, 25.86 μl (120 μmole) DPPA, 33.5 μl (360 μmole) Et_3N, 21 mg (6 μmole) [HArg^{12}]-glucagon in 30 ml anhydrous DMF at pH 7.2-7.6, -10° for 24 hr. A white fluffy material (25.63 mg) was obtained (Table 4, SL50 bls[a]). The attempt to purify BOC-Gly-[HArg^{12}]-glucagon was carried out by anion exchange chromatography with a discontinuous salt gradient of 0.15 M NaCl, 0.3 M NaCl, and 0.5 M NaCl. A solution of 11 mg BOC-Gly-[HArg^{12}]-glucagon in 3 ml 0.01 M Tris-buffer containing 7 M urea (pH 7.7, 4°) was applied to DEAE anion exchange column previously equilibrated with the Tris-buffer. The sample was eluted with 150 ml Tris-buffer. A discontinuous salt gradient began with 150 ml 0.15 M NaCl-buffer, 100 ml 0.3 M NaCl-buffer, and finally 100 ml 0.5 M NaCl-buffer. Fractions #3-12 (Figure 19) were collected, lyophilized, and then desalted on column #7. White powder was obtained after lyophilization and the protecting BOC-group was removed as previously described. A white powder (3.21 mg) (Table 4, SL50bls[b]) was obtained after gel filtration on column C. A second batch of BOC-Gly-[HArg^{12}]-glucagon (14 mg) was purified in the same manner; 3.32 mg white material was obtained. Both purified materials were combined and gel filtered on

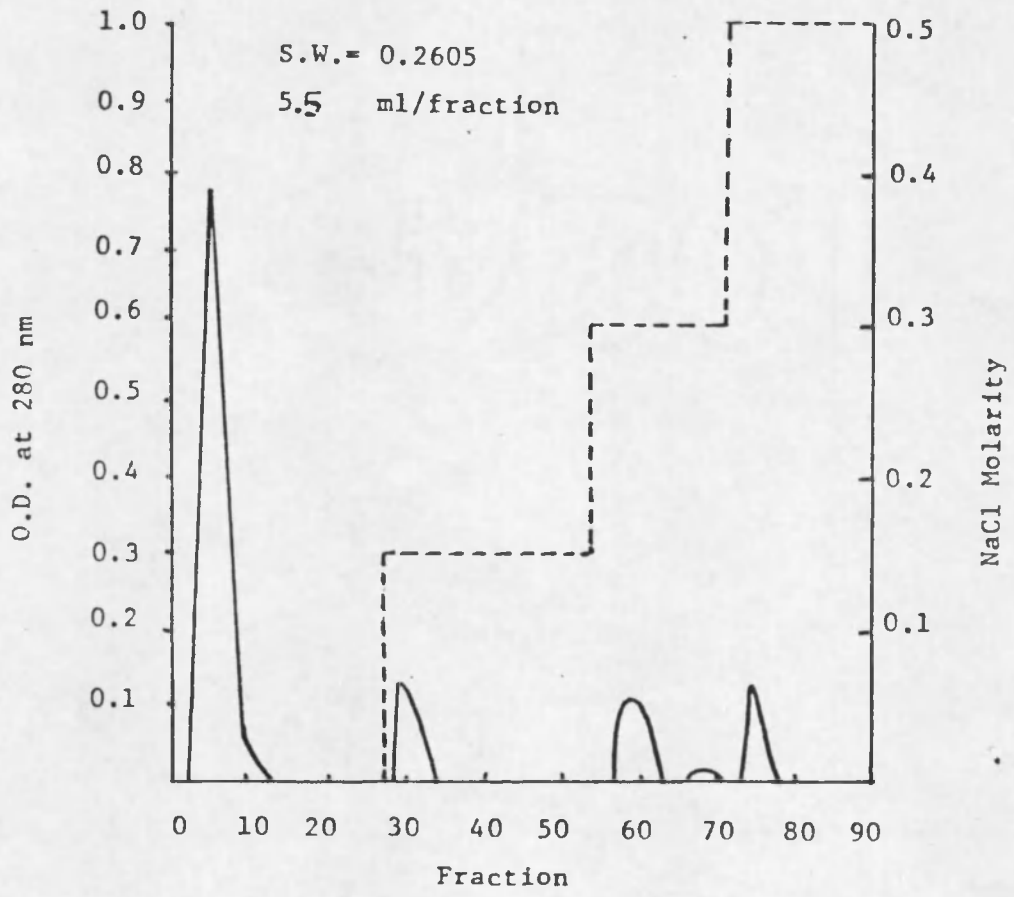


Figure 19. DEAE anion exchange chromatography of BOC-Gly-[HArg^{12}]-glucagon obtained via DPPA coupling.

column C once more. Approximately 3 mg white powder was obtained (Table 4, SL50b1s^{d}).

Edman Degradation of Ala-[HArg^{12}]-Glucagon

Two milligrams Ala-[HArg^{12}]-glucagon was dissolved in 200 µl Pyr/H_2O/Et_3N (70/30/0.1, pH 11) and adjusted to pH 10.45 with Et_3N and 10% HOAc. Nitrogen was bubbled into the reaction vessel for 2 min and 10 µl PITC was added under nitrogen. The reaction mixture was incubated in a water bath for 2 hr at 40°, extracted 4x with benzene and evaporated to dryness. DTE (1.5 mg) and 0.5 ml TFA were added under nitrogen, incubated for 15-20 min at 50° in a water bath, and finally desalted on column C, eluting with 150 ml 30% HOAc, and lyophilized (Table 4, SL42b1s).

CHAPTER 3

DISCUSSION

Guanidylation of glucagon gave the side-chain modified product, [HArg^{12}]-glucagon, in high yield (86%) and purity. Though the reaction is specific for ε-amino groups, on prolonged reaction time (greater than 15 hr), substitution on the α-amino group begins to occur. This was evident by the histidine content from AAA which can be as low as His = 0.71(1). An attempt to purify the hormone further by DEAE Sephadex A-25 anion exchange resin did not seem to provide any improvement. The one step Edman degradation of [HArg^{12}]-glucagon gave a fairly good yield (66.5%) of high purity (97%, with 3% histidine contamination) hormone analogue, [DH^{1}, HArg^{12}]-glucagon. Purification by SP Sephadex C-25 cation exchange resin did not work, but instead, lowered the yield tremendously. However, this modified hormone was pure enough to use as starting material for further coupling reactions.

The low solubility of glucagon and its analogues created the first problem in the attempts at coupling reactions. Being soluble only at two extreme pH's (pH 2-3 and pH 10-11) and having pH sensitive or reactive groups in the molecules, it was necessary to carry out the coupling reactions in excess solvent at neutral pH. By doing so, the concentration of the hormone was greatly reduced and prolonged reaction times were required. The common solvents used in azide coupling are DMF and THF. DMSO was chosen as the solvent for the acid azide

reaction, since glucagon and its analogues are insoluble in a small amount of DMF (2 ml) but fairly soluble in DMSO. However, dry DMSO freezes below o° (azide coupling reactions require low temperature) and is difficult to lyophilize. Fortunately, gel filtration on a 33% Pyr/H_2O Sephadex G-10 or G-15 column enables separation of DMSO and most of the undesirable salt byproducts, prior to lyophilization. On the other hand, anhydrous DMF was used in the DPPA couplings as described in the literature (Shioiri et al., 1972; Yamada et al., 1974; Lal et al., 1977; Rosowski and Yu, 1978), but much excess solvent was used due to the low solubility of glucagon and its analogues in DMF. However, the final peptide product from the DPPA coupling reaction is soluble in the reaction mixture as the reaction goes along. Thus DMSO showed preference over DMF as solvent, it was also used in some DPPA coupling reactions with no further problems.

Table 5 gives a summary of the two coupling reactions investigated, with emphasis on DPPA couplings. The azide intermediate generated, but not isolated, during both coupling reactions is expected to be short lived. The success of the coupling depends solely on the generation of this azide intermediate at proper pH. Using several additions of the azide at two-hr intervals, the reaction could be forced to completion. In some cases, apparently greater than 100% coupling had occurred. This can be explained in several ways. First, a large excess amoung of BOC-amino acid was used, some excess BOC-amino acid remaining even after gel filtration. Second, the BOC-amino acid may not remain fully protected, leaving a reactive amino group which can take part in the reaction. This, however, should be a very minor

Table 5. Summary of azide coupling and DPPA coupling reactions.

Derivative	Conditions pH 7-8	Overall yield (from a 7 mg sample)	Purification	AAA of major residues: Before purification	AAA of major residues: After purification
(A) Azide couplings:					
(1) Phe-[$HArg^{12}$]-glucagon (SL23b1s)	4°,+24 hr	4 mg	None	Lys=0.042 (0) His=0.495 (1) Phe=2.72 (3) Arg=2.00 (2) HArg=0.960 (1)	
(2) Phe-[$HArg^{12}$]-glucagon (SL45a2s)	4°,+41 hr	8 mg (before purif.) 5.05 mg (after purif.)	Precipitation 0.001 N HCl	Lys=0.0118(0) His=0.33 (1) Gly=2.46 (2) Phe=3.18 (3) Arg=2.21 (2) HArg=0.787 (1)	Lys=0.04 (0) His=0.43 (1) Gly=1.17 (1) Phe=2.68 (3) Arg=2.40 (2) HArg=0.60 (1)
(3) [Phe^{1},$HArg^{12}$]-glucagon (SL48a2s)	4°,+57 hr	5 mg (before purif.) 1.77 mg (after purif.)	Precipitation 0.001 N HCl	Lys=0.033 (0) His=0.052 (0) Arg=2.02 (2) HArg=0.974 (1) Phe=2.44 (3)	Lys=0.0321(0) His=0.0286(0) Arg=2.065 (2) HArg=0.9356(1) Phe=2.21 (3)
(B) DPPA Couplings:					
(4) Gly-[$HArg^{12}$]-glucagon (SL26a2s)	4°,+37 hr rt	6.4 mg (before purif.) 1.0 mg (after purif.)	Gel filtration	Gly=4.50 (2) No short column data	Gly=2.20 (2)
(5) [Gly^{1},$HArg^{12}$]-glucagon (SL35b1s)	4°,+36 hr rt	4.92 mg (before purif.)	Gel filtration	Gly=6 (2) No short column data	Gly=2.8 (2)
(6) Gly-[$HArg^{12}$]-glucagon (SL38a1s)	4°,+36 hr rt	7 mg (from a 10.5 mg sample)	None	Lys=0.73 (0) His=1.12 (1) Arg=2.22 (2) HArg=0.78 (1) Gly=2.13 (2)	
(7) [Gly^{1},$HArg^{12}$]-glucagon (SL38b1s)	4°,+36 hr rt	5.64 mg (from a 10.5 mg sample)	None	Lys=0.79 (0) His=0.11 (0) Arg=2.14 (2) HArg=0.86 (1) Met=0.37 (1) Gly=2.77 (2)	

Table 5.--Continued Summary of azide coupling and DPPA coupling reactions.

Derivative	Conditions pH 7-8	Overall yield (from a 7 mg sample)	Purification	AAA of major residues: Before purification	AAA of major residues: After purification
(8) Gly-[HArg^{12}]-glucagon (SL42a2s)	4° only 10-12 hr	8.42 mg (before purif.) 1.77 mg (after purif.)	Precipitation 0.001 N HCl	Lys=0.04 (0) His=0.72 (1) Arg=2.01 (2) HArg=0.99 (1) Gly=Multiple>>2	Lys=0.055 (0) His=0.753 (1) Arg=2.01 (2) HArg=0.988 (1) Gly=3.12 (2)
(9) Gly-[HArg^{12}]-glucagon (SL50b1s)	-10°,24 hr	25.63 mg (before purif. from a 23 mg sample) 3.21 mg (after purif., from a 11 mg sample)	DEAE ion exchange	Lys=0.113 (0) His=0.865 (1) Arg=1.956 (2) HArg=1.04 (1) Gly=7.02 (2)	Lys=0.037 (0) His=0.86 (1) Arg=1.97 (2) HArg=1.03 (1) Gly=3.46 (2)
(10) Ala-[HArg^{12}]-glucagon (SL40b1s)	4°,+36 hr	6.5 mg	None	Lys=0.308 (0) His=0.743 (1) Arg=2.038 (2) HArg=0.96 (1) Ala=2.36 (2) Met=0 (1)	
(11) Ala-[HArg^{12}]-glucagon (SL46a2s)	4° only (first attempt) 10-12 hr	11.55 mg (before purif.) 0.8 mg (after purif.)	Precipitation 0.001 N HCl	Lys=0.109 (0) His=0.638 (1) Ala=6.8 (2) Met=0.89 (1) Arg=1.99 (2) HArg=1.00 (1)	Lys=0.06 (0) His=0.667 (1) Ala=2.50 (2) Met=0.143 (1) Arg=2.05 (2) HArg=0.957 (1)
(12) Ala-[HArg^{12}]-glucagon (SL48b2s)	4° only (second attempt) 10-12 hr	8.97 mg (before purif.) 3.19 mg (after purif.)	Precipitation 0.001 N HCl	No AAA	Lys=0.1096(0) His=0.660 (1) Arg=2.05 (2) HArg=0.95 (1) Ala=2.97 (2) Met=0.81 (1)
(13) Val-[HArg^{12}]-glucagon (SL35a1s)	4°,+36 hr	6.4 mg	None	No coupling; no short column data	

Table 5.--Continued Summary of azide coupling and DPPA coupling reactions.

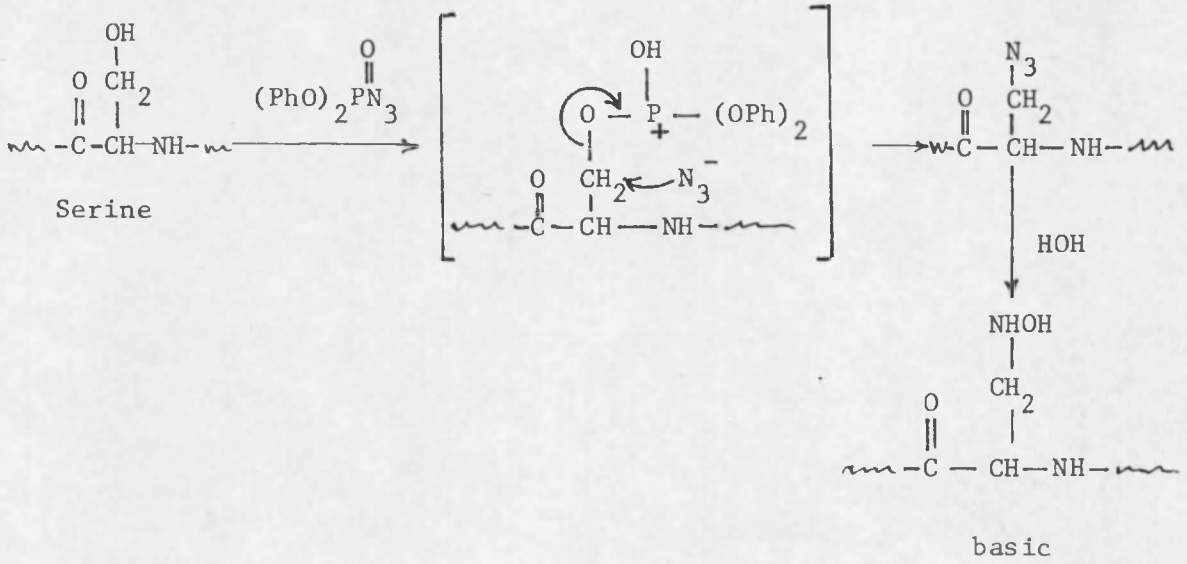
Derivative	Conditions pH 7-8	Overall yield (from a 7 mg sample)	Purification	AAA of major residues: Before purification	AAA of major residues: After purification
(14) Phe-[HArg^{12}]-glucagon (SL41als)	4°,+36-38 hr rt	6 mg	None	Lys=0.304 (0) His=0.661 (1) Arg=2.06 (2) HArg=0.938 (1) Phe=2.29 (3)	
(15) Phe-[HArg^{12}]-glucagon (SL49a2s)	4°,+61 hr	11.78 mg (before purif.) 2.27 mg (after purif.)	Precipitation 0.001 N HCl	No AAA	Lys=0.24 (0) His=0.71 (1) Arg=2.07 (2) HArg=0.93 (1) Phe=3.27 (3)

component. Lastly, it could be that the unprotected imidazole ring of histidine residue in the hormone interferes with the reaction. Partial coupling may occur at this reactive site. The last reasoning seems very unlikely since no such side reaction has been reported in the literature and it is not observed in the conventional azide coupling method.

In attempts to develop the best conditions for the DPPA coupling to the hormone, several interesting findings were observed. When the reaction mixture was brought to room temperature, the apparent lysine content from AAA was observed to rise sharply from Lys = 0.03(0) to Lys > 0.30(0) or Lys > 0.70(0), whereas when the reaction mixture remained at low temperature at all times, no apparent increase in lysine (or ornithine) was observed. This suggested a possible side reaction occurred at a higher temperature (> 4°). Such a finding has never been reported in the literature on DPPA couplings. Ornithine and lysine appear as a peak approximately at the same position on the AAA chromatogram. Since deguanidylation of arginine produces ornithine and of homoarginine produces lysine, both ornithine and lysine could appear as a result of 6 N HCl hydrolysis. Studies (Ormberg, 1979) have shown that acid hydrolysis of homoarginine gives 1% to 1.5% lysine appearing on AAA. Since the calculations for the basic amino acid contents in Table 4 were based on arginine and homoarginine, one can only assume that 3% to 5% lysine (or ornithine) should appear from the degradation of arginine and homoarginine.

Alternatively, the possible side reaction as mentioned may somehow give rise to a basic residue which accidentally appears at the

same place as lysine on the chromatogram. A possible mechanism for this side reaction is proposed.



If this side reaction occurred, the serine residue would be lowered in the AAA. However, the serine contents were observed to be normal in all cases (see Table 4). Hence, the exact origin of the side reaction remains unknown.

Attempts to couple Val and Phe to the hormone by DPPA did not have much success. When conditions were changed to more alkaline (pH 9) and to longer reaction times (greater than 45 hr at room temperature), still no coupling was observed. Finally, full coupling occurred with Phe (greater than 100%, Phe = 3.98(3), after purification), when the reaction was prolonged to 61 hr at 4°, pH 7. Interestingly enough, the lysine content from AAA was up again, Lys = 0.24(0), even after partial purification by 0.001 N NCl precipitation. It appears that there is a competition between the coupling reaction and the side reaction. Two conclusions were reached: (1) lower temperature

is necessary to suppress the side reaction that gives rise to high lysine (or ornithine) content; (b) much longer reaction times are required for bulky amino acids for coupling to the hormone, suggesting some steric hindrance for the reaction.

Purification of the products was carried out prior to the removal of the protecting BOC-group, since this BOC-hormone carries one less positive charge than before. In this case, ion exchange chromatography should be able to bring about separation and purify the product. Due to the insolubility of BOC-hormone in 1 M urea buffer, higher urea concentration, 6 M or 7 M, was necessary for the dissolution to carry out ion exchange chromatography. The attempt to purify the product by DEAE Sephadex A-25 anion exchange resin was carried out with BOC-Gly-[HArg^{12}]-glucagon obtained via DPPA coupling (24 hr, -10°, pH 7.2-7.6). Analyis showed that the anion exchange was effective in reducing the high lysine (or ornithine) content (Lys = 0.113(0), before purification; Lys = 0.037(0), after purification). However, the excess glycine was not completely eliminated. It is also possible that SP Sephadex C-25 cation exchange resin may be useful as another purification alternative.

Gel filtration and precipitation (0.001 N HCl) as purification techniques for both coupling reactions seems to improve matters somewhat. Excess amino acids still remained in the final product as shown from AAA data. When purification by precipitation was done on azide coupling products, the purified product showed lower coupling than before (see Table 5, summary on azide couplings). No reasonable explanation can be offered for this observation at the present moment.

However, combination of the two techniques may be helpful in eliminating all the excess amino acids present in the DPPA coupling products, though the yield will be greatly reduced. Hence, the need for better purification techniques will be important for further investigation.

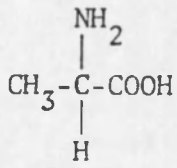
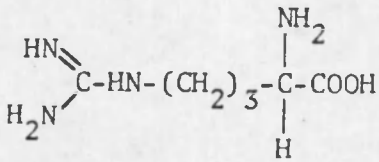
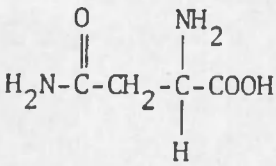
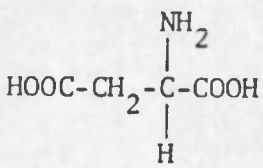
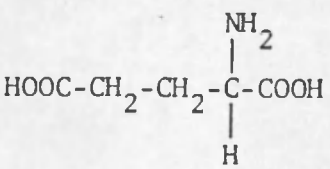
In order to assure that the amino acid is coupled to the first position (N-terminal) of the peptide in the DPPA couplings, an Edman degradation was carried out with a Ala-[HArg^{12}]-glucagon obtained via DPPA coupling. The resultant product showed alanine was reduced to approximately one (see Table 4). This suggests the coupling occurs at the N-terminal, since the degradation is specific for the removal of one amino acid at the first position of the N-terminal of a peptide.

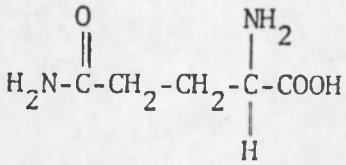
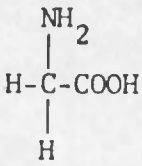
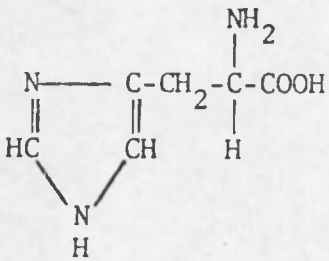
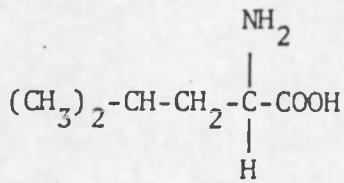
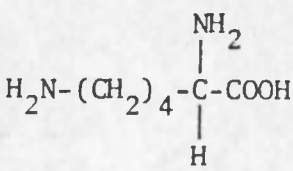
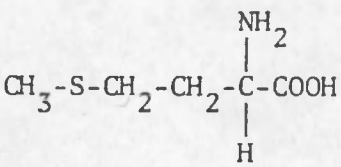
No biological assay has been performed on any of the glucagon derivatives prepared. Adenylate cyclase assays of these derivatives will be made in the near future, when these glucagon analuges are purified further.

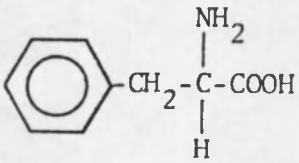
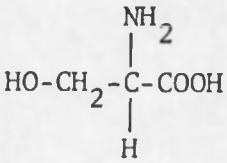
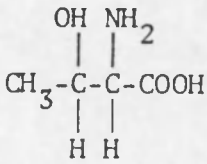
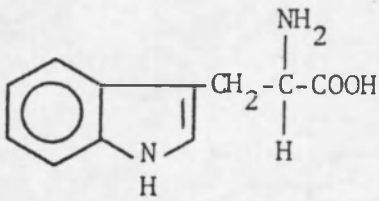
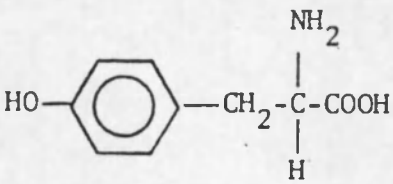
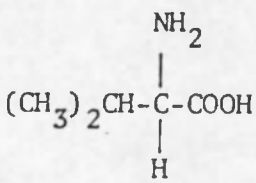
APPENDIX A

LIST OF ABBREVIATIONS

Amino Acids

Name	Formula	Abbreviation
Alanine		Ala
Arginine		Arg
Asparagine		Asn
Aspartic Acid		Asp
Glutamic Acid		Glu

Name	Formula	Abbreviation
Glutamine		Gln
Glycine		Gly
Histidine		His
Leucine		Leu
Lysine		Lys
Methionine		Met

Name	Formula	Abbreviation
Phenylalanine		Phe
Serine		Ser
Threonine		Thr
Tryptophan		Trp
Tyrosine		Tyr
Valine		Val

Protecting Group

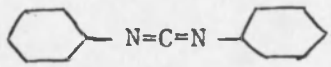
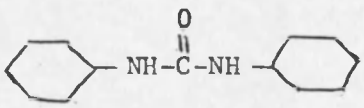
Name	Formula	Abbreviation
t-Butyloxycarbonyl	$(CH_3)_3C\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{—}$	BOC

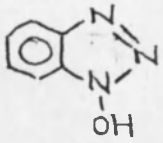
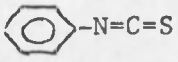
Ion Exchange Resins

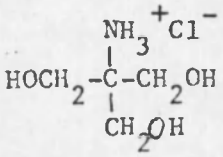
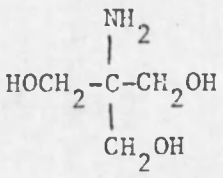
Diethylaminoethyl	$\text{—}C_2H_4\text{-}\overset{+}{N}H(C_2H_5)_2$	DEAE
Sulphopropyl	$\text{—}C_3H_6\text{-}SO_2O^-$	SP

Reagents and Solvents

Acetic acid	CH_3COOH	HOAc
n-Butanol	$CH_3CH_2CH_2CH_2OH$	n-BuOH
n-Butyl nitrite	$CH_3CH_2CH_2CH_2ONO$	n-BuONO
Copper sulfate (hydrate)	$CuSO_4 \cdot 5H_2O$	

Name	Formula	Abbreviation
Dicyclohexylcarbodiimide		DCC
Dicyclohexylurea		DCHU
Dimethylformamide	$(CH_3)_2N-\overset{\overset{O}{\|}}{C}-H$	DMF
Dimethyl sulfoxide	$CH_3\overset{\overset{O}{\|}}{S}CH_3$	DMSO
Diphenylphosphoryl azide	$(\phi O)_2-\overset{\overset{O}{\|}}{P}-N_3$	DPPA
Dithioerythritol	$\begin{array}{l} CH_2-SH \\ \vert \\ CH-OH \\ \vert \\ CH-OH \\ \vert \\ CH_2-SH \end{array}$	DTE
Ethyl acetate	$CH_3\overset{\overset{O}{\|}}{C}OCH_2CH_3$	EtOAc
Hydrochloric acid	HCl	

Name	Formula	Abbreviation
Hydroxybenzotriazole		HOBT
Methylene chloride	CH_2Cl_2	
Phenyl isothiocyanate		PITC
Pyridine		Pyr
Sodium acetate (hydrate)	$CH_3C(=O)O^-Na^+ \cdot 3H_2O$	$NaOAc \cdot 3H_2O$
Sodium chloride	NaCl	
Sodium hydroxide	NaOH	
Sodium sulfate (anhydrous)	Na_2SO_4	
Tetrahydrofuran		THF
Triethylamine	$(CH_3CH_2)_3N$	Et_3N
Trifluroacetic acid	CF_3COOH	TFA

Name	Formula	Abbreviation
Tris(hydroxymethyl)aminomethane hydrochloride		Tris HCl
Tris(Hydroxymethyl)aminomethane		Tris base

Tris-buffer

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