PREPARATION AND PURIFICATION OF

[HOMOARGININE^{12}]-GLUCAGON

by

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PREFACE

This work was supported in part by a grant from the National Institutes of Health (NOI-AM-1-2125) and the United States Public Health Service (AM 15504). All optically active amino acids are the L variety. The symbols for the amino acid residues follow the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature.

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ABSTRACT

[Homoarginine^{12}]-glucagon was prepared by reacting native bovine glucagon with o-methylisourea at pH 10.5. Several purifications using SP-Sephadex ion exchange chromatography were attempted, however after purification each [homoarginine^{12}]-glucagon sample still apparently contained a small percentage of native glucagon. DEAE and QAE anion exchange resins were also employed, but were unable to eliminate the apparent 3 to 4% contamination by native glucagon. A sample of [homoarginine^{12}]-glucagon was reacted with p-nitrophenylacetate at pH 11.1 in order to convert any unreacted native glucagon into the ε-acetyl-derivative. SP-Sephadex ion exchange chromatography was then used for purification, but the sample of [homoarginine^{12}]-glucagon still contained a small percentage of native glucagon. [Des-histidine^{1}]-[homoarginine^{12}]-glucagon was prepared by reacting [homoarginine^{12}]-glucagon with Edman's reagent. Subsequent purification however by SP-Sephadex ion exchange chromatography was incomplete since the sample of [des-histidine^{1}]-[homoarginine^{12}]-glucagon still contained a small percentage of both lysyl and histidyl residues.

CHAPTER 1

INTRODUCTION

Glucagon is a single chain polypeptide hormone consisting of 29 amino acid residues having a molecular weight of 3485 (see Figure 1). In 1921, Banting and Best injected newly prepared pancreatic extracts into a diabetic dog, however the hoped for hypoglycemic effect was preceded by a short-lived but reproducible hyperglycemia. They attributed this phenomenon to epinephrine release, though others attributed it to an insulin contaminant. Murlin and collaborators (1923) suggested that the contaminant might be due to a second compound. Postulating a physiological role, they called it "glucagon" or "mobilizer of sugar." Additional evidence for the glycogenolytic action of glucagon was obtained by several investigators, among them Burger and Brandt (1935), who made the first attempts to purify the material. Glucagon's primary sequence was established by Bromer, Sinn and Behrens (1957) and is identical in many species, including man, pig, rat, cow, and camel, though it does differ slightly in some avian species. Circulating glucagon is a mixture of pancreatic glucagon, the single chain 29 amino acid polypeptide, which is secreted by the α_2 cells of the islet of Langerhorns in the pancreas, and two partially identified substances which constitute "gut glucagon," which appears to be similar to pancreatic glucagon by biologic and immunometric methods and is secreted by the mucosa of

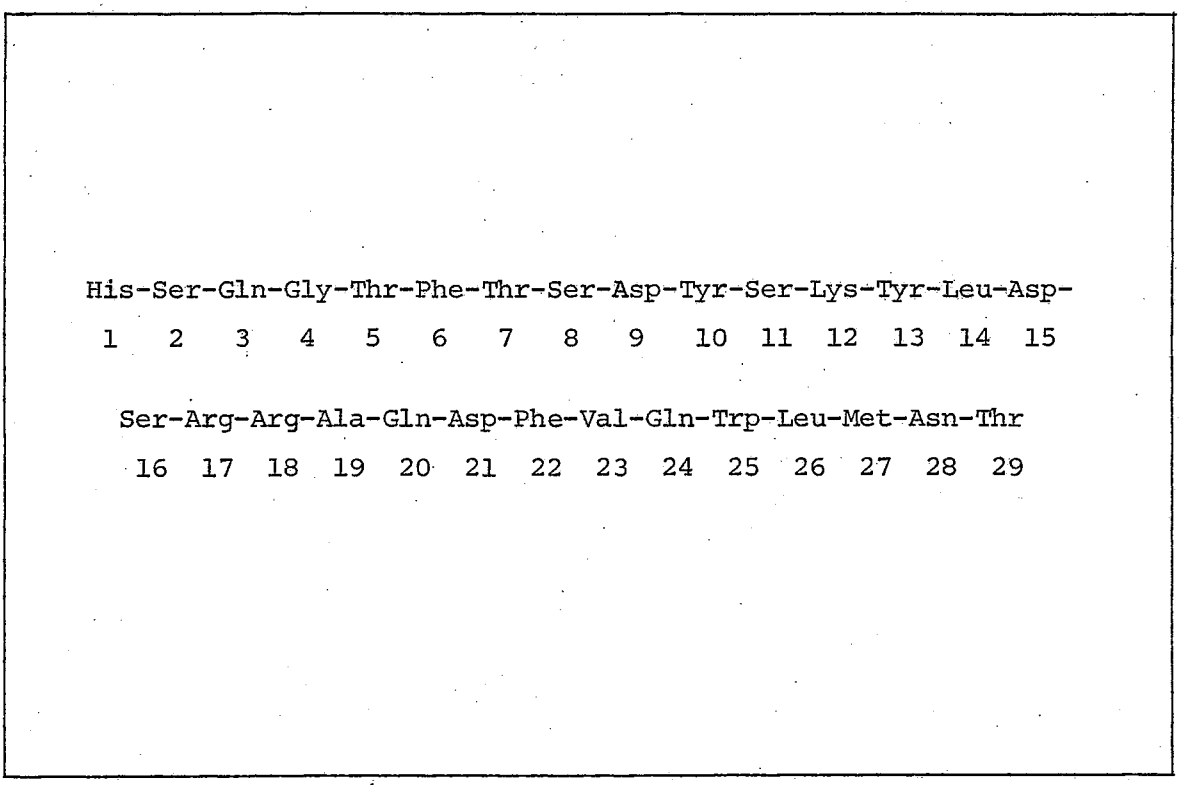

Figure 1. The Primary Structure of Glucagon

the gastrointestinal tract, these cells being ultrastructurally indistinguishable from the pancreatic alpha cells (Sasaki, Rubalcava, et al. 1975). There is some evidence that glucagon may be secreted as a higher molecular weight precursor prohormone, since high molecular weight polypeptides containing the structure of glucagon have been found (Hew and Yip 1976). Most work has been done with pancreatic glucagon and the studies reported in this thesis make use of bovine pancreatic glucagon. Unless otherwise designated, glucagon and pancreatic glucagon are synonymous.

Glucagon serves as a hormone of fuel mobilization, giving energy-yielding substrates to satisfy tissue needs in the fasting state, extended starvation, and physical exertion. Released into the portal blood, glucagon reaches the liver, where its biologic action is glycogenolysis or the breakdown of glycogen to glucose. By binding to specific receptors on the cell membrane, glycogenolysis is stimulated and gylcogen synthesis is inhibited. This process appears to be mediated by activation of adenyl cyclase which acts, in the presents of Mg^{+2}, to convert adenosine triphosphate to cyclic 3' of 5'-adenosine monophosphate (cAMP). According to Sutherland, Rall, and Menon (1962), cAMP acts as an endocellular "second messenger," conveying the stimulus delivered by glucagon on the cell surface to the effector enzymes in the cytoplasm. Here the primary action of cAMP 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 yield glucose. One molecule of glucagon can potentially stimulate the production of one and one-half million molecules of cAMP and the release of about 3 million molecules of glucose from hepatic glycogen (Foa 1973). As a lipolytic hormone, glucagon's action on adipose tissue is to initiate gluconeogenesis or the production of glucose from the breakdown of fatty acids. The mechanism by which glucagon stimulates lipolysis follows the same cascade pattern as described for glycogenolysis except that near the end a proteinkinase activates a glyceride lipase which results in the release of glycerol and free fatty acids, with a corresponding increase in esterification (or re-esterification). The free fatty acids are converted to acetyl-coenzyme 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 (Foa et al. 1973). Other effects of glucagon include depression of appetite, augmentation of renal excretion of water and of most ions producing osmotic diuresis, 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. In the heart, glucagon activates adenyl cylase and stimulates glycogenolysis (Foa et al. 1973). As a result the strength and rate of cardiac contraction is stimulated and cardiac output is increased. Glucagon dilates the

coronaries and the systemic arterioles, which decreases vascular resistance and increases bloodflow.

Glucagon has been found to play a major role in diabetes mellitus (Unger et al. 1970). It has generally been thought that diabetic hyperglycemia was due to a decrease in the amount of insulin in the blood stream, but it has been shown that whereas insulin normally is deficient in diabetes, glucagon usually is present in excess and that this excess is a cause, not an effect, of the disease. Bihormonal control of glucoregulation, through a push-pull system between insulin and glucagon is therefore necessary so that a varying turnover of glucose in the extracellular space can be achieved, without allowing that space to exceed relatively narrow limits of glucose concentration. The hormonal antagonists are thought to be directed by a glucose sensor, which allows the islets to respond in a corrdinated fashion by maintaining an equality of glucose influx and efflux at all times. The islet of Langerhorns contain the necessary anatomical equipment (see Figure 2) which could allow the hormone secreting cells, through surface contact, to achieve their bihormonal oscillations. In severe diabetes, the outer layer of B cells 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. In severe cases, however,

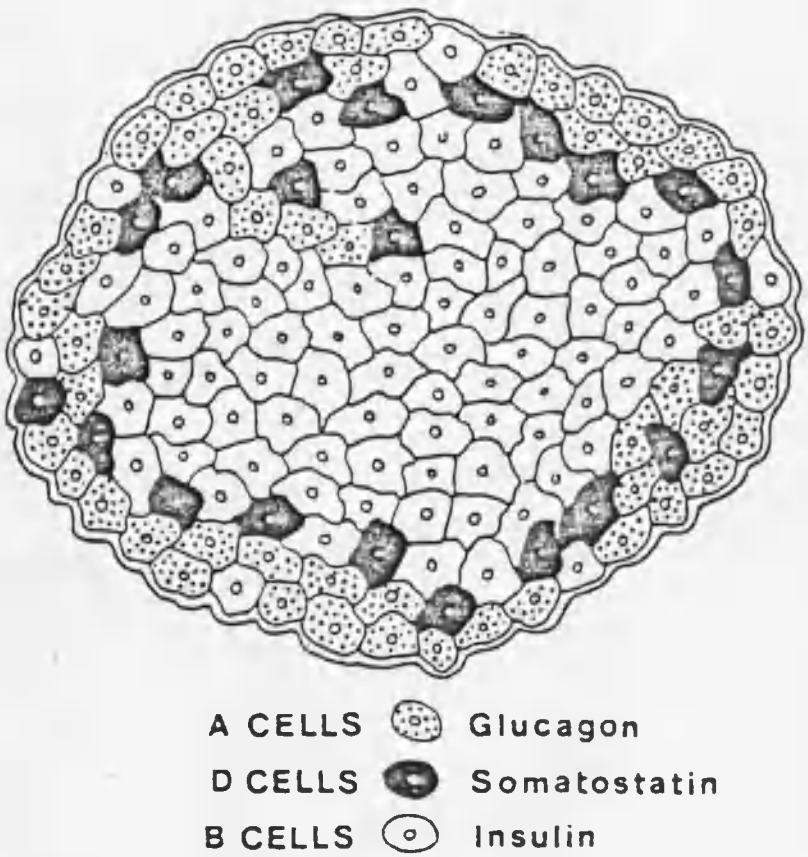

Figure 2. Schematic Representation of an Islet of Langerhans Showing Distribution of Glucagon, Somatostatin, and Insulin-containing Cells. -- (Unger and Orci 1977)

the elevated glucagon levels can lead to an increase of ketone bodies, or ketoacidosis, and other diabetic complications (Alberti, Iverson, and Christensen 1974). This leads in turn to the lowering of the blood pH and if left untreated can lead to the inducement of a coma. Severe ketoacidosis fails to suppress the diabetic alpha cells' secretion (Asson et al. 1969), but rather appears to stimulate glucagon release (Unger et al. 1970). Somatostatin, the D cells, suppresses both insulin and glucagon release (Koerker, Ruch, and Chideckel 1974), which results in the restoration of the blood surgar concentration to normal limits and the alleviation of certain symptoms of diabetes.

Hormone-Receptor Interactions

A receptor is an element of a target cell that specifically recognizes and binds the hormone and, as a consequence of this recognition, can induce other changes or a series of changes which ultimately result in the biological response. This proposal is made in analogy with classical enzyme-substrate systems in which substrate binding and catalysis are separate and discrete but sequential processes which 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, as, for example, adenosine triphosphate being converted into cAMP. The generation of this stimulus from 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). Several models have been proposed in an

attempt to explain the interaction process of a hormone with its receptor (Rudinger, Pliska and Krejci 1971; Burgen, Roberts and Feeney 1975; Cuatrecasas et al. 1975; Hammes and Rodbell 1976; Haën 1974; Salomon et al. 1975). In order to bring the hormones in contact with the receptor, two general models are usually considered. The "lock and key" model allows only those molecules with the correct conformation and correct orientation to form a complex. In this single step binding, all the favorable interactions between hormone and receptor are formed simultaneously. Such a model would indicate a decreasing association constant as the size of the molecule increased. An alternative model likens to a zipper. Here an initial "nucleation" complex can be formed by the interaction of a single segment of the hormone with its specific subsite. This would be followed by changes in the receptor to accommodate the binding of the rest of the hormone or it may reduce the number of possible conformations and preferentially "freeze" the active conformation of the hormone. The association time for this model would be comparably greater than that for the "lock and key" model. Once the hormone has made contact, it may participate in the molecular event of stimulus generation. This "receptor occupation" theory of the participation model regards the complex as active. In binding, the complex may acquire specificty 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. Another

consideration of the participation model allows for the formation of an intermediate transition state after the hormone has coupled to the receptor. In this three step model, one would observe no increase in enzyme activity in the intermediate transition state, but would observe a time lag begore isomerization to a higher activity state occurs. 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 binding 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 have been valid in a particular case, no one model or combination of models has gained widespread acceptance.

Two adenylate cyclase systems, the rat liver membranes and the fat cell ghosts, have been used most extensively to study the action of glucagon, in vitro. The rat liver plasma membranes are stimulated by the hormone glucagon, and also by 5'-guanylyl-imidodiphosphate, fluoride, and epinephrine (Birnbaumer 1973). Secretin, vasopressin, thyroid-stimulating hormone, para-thyroid 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'guanylyl-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. In contrast, the fat cell ghost is stimulated by many different hormones including glucagon, secretin, epinephrine, adrenocorticotropin, leutenizing hormone, vasoactive intestinal polypeptide, and insulin. Also the ligands guanidinetriphosphate 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. Since the liver membrane is not stimulated by as many different hormones as the fat cell ghost and has a reliable binding assay, it is easier to study glucagon derivatives using this system.

Structure-Function Relationships

An understanding of the nature of glucagon receptor binding depends on a proper description of the conformation of the hormone in particular environments. At physiological concentrations, approximately 10^{-10} molar in the blood stream, glucagon exists as a single strand of amino acids and in dilute aqueous solution, the monomeric glucagon does

not have a stable globular structure, but is a flexible chain existing in an equilibrium of possible conformations containing less than 15% of a helical structure (Panijpan and Gratzer 1974). Variation in conformation occur as a result of changes in hormone concentration, in temperature, in concentration of denaturants present, or in pH. As the concentration of glucagon in solution increases, the molecule associates into trimers (Blundell et al 1975) and possibly into higher oligomers (Swann and Hammes 1969) when crystallized. Optical rotatory dispersion (Blanchard and King 1966) and circular dichroism (Panijpan and Gratzer 1974) indicate 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 solution the α-helical structure occurs (Swann and Hammes 1969). In the pH range of 5.8-7.5, glucagon assumes the α-helical structure. In 6M guanidine hydrochloride, the helical conformation is destroyed (Panijpan and Gratzer 1974). However the α-helical conformation 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), which increase the hydrophobic environment of the molecule. X-ray analysis at 3.0 Å resolution has shown that the molecule has a well defined α-helical region between residues 6-27 and large volumes of low density corresponding to regions of solvent, resulting in PHE 6, TYR 16, TYR 13, and LEU 14 forming one mainly hydrophobic

patch and ALA 19, PHE 22, VAL 23, TRP 25, LEU 26 and MET 27 another (Sasaki, Dockerill, et al. 1975). Residues 1-5 are not constrained by intermolecular interactions and appear, even in crystals, to be flexible. The trimeric arrangement of the molecule appears to be due to heterologous contacts between two groups of hydrophobic residues to form hydrophobic regions, the intermolecular contacts being located between tryptophan and tyrosine. Since the glucagon receptor complex is destabilized at lower temperatures or by the presence of urea (Rodbell, Birnbaumer et al. 1971), it is suggested that the complex is formed through hydrophobic interactions. The receptor bound glucagon may be stabilized by two hydrophobic regions on the receptor. Fragments which are unable to form helics (1-21, 1-23, 20-29, 22-29) in these systems only weakly compete with glucagon at the receptor and have weak or no glucagon activity (Contaxis and Epand 1974). 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 et al. 1975). Using their rules for predicting protein confromations, Chou and Fasman (1975) suggest that in dilute form, glucagon has an α-helix region in residues 19-27, but with aggregation this region is converted into a β-sheet. It was proposed that on interacting with the receptor, the β-helix conformation is induced.

In addition to physical studies on the conformation of the hormone, knowledge of the regions or functional groups on the hormone which are involved in binding only and which are required for the biochemical

stimulus also is necessary for a complete understanding of glucagon-receptor interactions. Modifications of glucagon fall into two broad categories: changes in the peptide chain length and changes in the side chains and terminal functional groups, which are brought about by enzymatic or chemical means. A list of various glucagon derivatives that have been made and studied on rat liver membranes is presented in Table 1. A list of glucagon derivatives acting through lipolysis, as well as glyconeogenesis, is given by Wright (1976). Most of the derivatives have not been highly purified.

Glucagon has been synthesized twice (Wünsch et al. 1967, Protein Synthesis Group 1975) but both syntheses proved laborious and no derivatives have been reported to have been made outside of synthetic intermediates. The native hormone is rather used as a starting material for modifications.

One inherent problem in this study is that a steric modification of the hormone molecule may take place due to the modification of a functional group. Since any change in biological activity or association rates may then be due not to a change of the charge of the functional group, but rather due to steric or conformational perturbations, care must be taken in comparing hormone-receptor interactions.

The N-terminal of glucagon has a more flexible conformation than most of the rest of the molecule. It is important in eliciting a biological response as well as in providing stabilization of hormone-receptor complex. The terminal histidyl residue has been shown to play an important role in the expression of hormone action in addition to imparting

Table 1. 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]	N.T.	Lande, Gorman and Bitensky (1972)
N^{ε}-Boc-glucagon	Electrophoresis	12%, N.T.	N.T.	Ditto
N^{α}-Carbamyl-glucagon	None	10%, F.A.[c]	N.T.	Epand, Epand and Grey (1973)
N^{α}, N^{ε}-Acetyl-glucagon	None	10%, F.A.	N.T.	Ditto
{12}-Homoarginine-glucagon	None	10%, F.A.	N.T.	Ditto
Imidazole-ethoxyformyl-N^{α}, N^{ε}-acetyl-glucagon	None	None	N.T.	Ditto
N^{α}-Acetyl-glucagon	Ion Exchange	10%, F.A.	12%	Desbuquois (1975b)
N^{α}, N^{ε}-Acetyl-glucagon	Ion Exchange	1%, F.A.	1%	Ditto
N^{α}, N^{ε}, Tyr-acetyl glucagon	Ion Exchange	0.1%, F.A.	0.1%	Ditto
N^{α}-Trinitrophenyl-glucagon	Non	10%, N.T.	N.T.	Epand and Wheeler (1975)
N^{α}-Trinitrophenyl-{12}-homoarginine-glucagon	None	15%, N.T.	N.T.	Ditto

Table 1, (Continued). Activity of Glucagon Derivatives Acting on Liver Membranes.

Peptide	Purification	Activity[a]	Binding	Reference
N^{α}-Carbamyl N^{ϵ}-trinitrophenyl-glucagon	None	23%, N.T.	N.T.	Epand and Wheeler (1975)
N^{α}, N^{ϵ}-Trinitrophenyl-glucagon	Ion Exchange	0.02%, N.T.	N.T.	Ditto
Tyrosine				
Iodo-glucagon	Ion Exchange	100%, F.A.	100%	Rodbell, Birnbaumer and associates (1971)
Iodo-glucagon	Gel Filtration	5-10 x	N.T.	Bromer, Boucher and Patterson (1973)
Iodo-glucagon	Ion Exchange	5 x	2 x	Desbuquois (1975a)
Mononitro-glucagon	Ion Exchange	77%, F.A.	N.T.	Patterson and Bromer (1973)
Dinitro-glucagon	Ion Exchange	22%, F.A.	N.T.	Ditto
Monoamino-glucagon	Ion Exchange	40%, F.A.	N.T.	Ditto
Diamino-glucagon	Ion Exchange	14%, F.A.	N.T.	Ditto

Table 1, (Continued). Activity of Glucagon Derivatives Acting on Liver Membranes.

Peptide	Purification	Activity[a]	Binding	Reference
Carboxyl groups				
Glucagon tetramethylester	Gel Filtration	None	N.T.	Epand and Epand (1972)
Glucagon tetraglycineamine	Gel Filtration	None	N.T.	Ditto
Monotaurine-glucagon	Gel Filtration	15%, F.A.	N.T.	Wheeler, Epand and Barrett (1974)
Tetrataurine-glucagon	Gel Filtration	0.1%, N.T.	N.T.	Ditto
Ethylenediamine-glucagon	Gel Filtration	None	N.T.	Ditto
Desamido-glucagon	Ion Exchange	60%, N.T.	N.T.	Bromer et al. (1972)
Fragments				
$Glucagon_{2-27}$	Electrophoresis	None	10%	Rodbell, Birnbaumer and associates (1971)
$Glucagon_{2-27}$	Electrophoresis	None	N.T.	Lande, Gorman and Bitensky (1972)
$Glucagon_{2-27}$	Ion Exchange	2%, P.A.[d]	7%	Lin et al. (1975)

Table 1 (Continued). Activity of Glucagon Derivatives Acting on Liver Membranes.

Peptide	Purification	Activity[a]	Binding	Reference
$Glucagon_{1-21}$	None	None	None	Rodbell, Birnbaumer and associates (1971)
$Glucagon_{1-21}$	Partition Chromatography	0.5%, F.A.	N.T.	Wright (1976)
$Glucagon_{1-23}$	Synthetic	None	N.T.	Spiegel and Bitensky (1969)
$Glucagon_{1-23}$	Synthetic	0.01%, F.A.	N.T.	Epand and Grey (1973)
$Glucagon_{20-29}$	Synthetic	None	None	Rodbell, Birnbaumer and associates (1971)
$Glucagon_{22-29}$	Synthetic	None	None	Ditto
Glucagon trypic fragments	None	None	N.T.	Spiegel and Bitensky (1969)
CNBr-glucagon	Electrophoresis	18%, F.A.	N.T.	Ditto
CNBr-glucagon	Gel Filtration	10%, F.A.	N.T.	Epand and Grey (1973)

Table 1 (Continued). Activity of Glucagon Derivatives Acting on Liver Membranes.

Peptide	Purification	Activity[a]	Binding	Reference
CNBr-glucagon	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 η-butylamide	Partition Chromatography	2%, F.A.	2%	Hruby et al. (1976)

a. as compared with native glucagon at half maximal activity and at high concentrations

b. not tested

c. full agonist

d. partial agonist

some contribution to the binding of the hormone at specific binding sites in hepatic plasma membranes (Lin et al. 1975). Epand, Epand, and Grey (1973) have suggested that the imidazole group is the essential portion of the terminus, since blocking of the α-NH_2 group or the sole ε-NH_2 group of lysine did not result in complete loss of activation by the respective derivatives. From titration data, Epand, Epand and Grey (1973) noticed the imidazole ring did not appear to have an abnormal pK for an amino terminal histidine, indicating that this particular group did not have any specific interactions with other parts of the glucagon molecule, but rather was free for other intermolecular interactions. Removal of the histidyl group produces the derivative des-histidine-glucagon or DH-glucagon, which has allowed a closer look at the function of the N-terminal amino acid. DH-glucagon has been shown to be a partial, weak agonist since at maximal stimulating concentration it is able to induce only 70% of the maximal stimulation of 10^{-8}M native glucagon at a saturated concentration of 10^{-6}M. The derivative displayed a 15-fold decrease in affinity but a 50-fold decrease in biological activity relative to that of native glucagon (Lin et al. 1975). Iodoglucagon, which has the same biological activity as the unlabeled hormone at neutral pH (Rodbell, Michiel et al 1971), was found to dissociate from liver plasma membranes in the presence of DH-glucagon, giving evidence that DH-glucagon displaces native glucagon from its receptor (Birnbaumer and Pohl 1973). However, at the same concentrations iodoglucagon was displaced more by the native hormone than by DH-glucagon, suggesting

that the hormone derivative has a different affinity for the receptor. Similar results have been found with DH-glucagon acting on insulin-secreting tumors of the Syrian (golden) hamster (Goldfine, Roth and Birnbaumer 1972). In these β cell particles DH-glucagon had no effect on adenyl cyclase activity, inhibited the binding of ^{125}I-glucagon, and was about one-third as potent as native glucagon in displacing the labeled hormone from the receptor. Also when DH-glucagon was added to native glucagon, adenylate cyclase activation was blocked. The affinity of native glucagon and DH-glucagon for binding sites in hepatic plasma membranes treated by Bacillus cereus phospholipase C (an enzyme which hydrolyzes acidic phospholipids) was the same. DH-glucagon was found with equal affinity to either untreated or phospholipase C treaded membranes (Rubalcava and Rodbell 1973), while glucagon binding was decreased only when treated with phospholipase C. Guanine nucleotides did not alter the rate of dissociation rate of glucagon when the native hormone was bound to untreated membranes. In aqueous solution, lysolecithin forms micelles which have an apolar interior and a charged exterior resembling the lipid structure in cellular membranes. Interaction of DH-glucagon with lysolecithin was found to have the same affinity as the native hormone with lysolecithin, whereas glucagon 1-21 and glucagon 22-29 showed no effect (Schneider and Edelhoch 1972). In all of these studies, DH-glucagon was prepared by removing the N-terminal histidyl group by a one-step Edman degradation. However glucagon has a lysine residue and since the difference between the pK_a

values of the alpha and epsilon amino groups is too small to restrict the phenylisothiocynate reaction to only the N-terminal alpha amino group, the N^{ε}-phenylthiocarbamyl derivative probably constitutes a contaminant in significant amounts. This side product was not analyzed for in most of the DH-glucagon prepared and the amino acid analysis for lysine was low indicating that this somewhat acid stable derivative may be present even after final purification by isoelectric focusing (Rodbell, Birnbaumer et al. 1971). In another preparation of DH-glucagon (Lande, Gorman and Bitensky 1972), glucagon was reacted with t-butylazidformate in order to selectively protect the epsilon amino group on lysine, thereby preventing formation of the N^{ε}-phenylthiocarbamyl derivative. However when this N^{ε}-boc derivative was reacted with phenylisothiocyanate, a pure product was not obtained even though disc gel electrophoresis was used in purification. The resulting DH-glucagon still had 6% histidine, indicating contamination from native glucagon and other possible side products. Because the reactions do not go to completion, competition from side products such as N^{α}-boc-glucagon, [des-histidine^{1}]-N^{ε}phenylthiocarbamyl-glucagon, N^{α}, N^{ε}-boc-glucagon, N^{ε}-phenylthiocarbamyl-glucagon, or N^{α}-boc, N^{ε}-phenylthiocarbamyl-glucagon, can be expected and the only recourse is to separate the desired starting material from whatever else is present. Since the role of histidine and/or the imidazole group could play such a significant part in understanding hormonal interactions, it would be advantageous to have a scheme for preparing and purifying significant amounts of DH-glucagon and use the derivative as an intermediate for further

semi-synthetic work. In particular, by replacing the histidyl residue with different imidazole derivatives would allow a closer look and a better understanding of the ring's interaction with the glucagon receptor.

The object of this investigation is to prepare highly pure [des-histidine^{1}]-glucagon so that the histidyl residue can be replaced by derivatives of the imidazole ring, and provide a better understanding of hormone-receptor interactions. Such a preparation requires the limiting of the Edman degradation specifically to the N-terminal alpha amino group. Thus provisions must be made to selectively block the amino group on lysine. Several reversible and non-reversible blocking agents have been developed (Hirs 1967) which are claimed to be limited to the epsilon amino groups. One of these procedures is for converting the lysine residue into a homoarginine residue by guanidylation (Hirs 1967). Guanidylation has the advantages that it is apparently highly specific for epsilon amino groups, it gives high reaction yields, and it maintains a basic moiety at the lysine residue, but a moiety which is not nucleophilic under most conditions. A previous investigation (Epand, Epand and Grey 1973) indicated that glucagon could be guanidylated and the derivative retained about 10% of its natural activity. However, more recent studies (Hruby and Lin, personal communication) indicate the derivative may have virtually the same activity as native glucagon, but the compound was not well characterized. This investigation involves the conversion of the lysine^{12} residue of glucagon into a homoarginine^{12} residue and the subsequent purification of the hormone.

The [homoarginine^{12}]-glucagon is then modified by Edman degradation and purified. In an effort to completely purify the [homoarginine^{12}]-glucagon, three ion exchange resins have been investigated and variations in solvent concentrations have been tested on each attempted purification in an effort to obtain those conditions that will give a complete and quantitative separation of native glucagon from [homoarginine^{12}]-glucagon.

CHAPTER 2

EXPERIMENTAL SECTION

In all synthetic work native bovine glucagon was used. After an ion exchange or gel filtration, the peptide content of the various fractions was determined by the ultraviolet absorbance at 280 mm. All gel filtrations were eluted with 30% acetic acid on either a (2.3 x 107 cm) Sephadex G-25 column (#7) or a (2.3 x 67 cm) Sephadex G-15 column (#12). All samples for amino acid analysis were hydrolyzed for 22 hours in 6N HCl at 110° in a vacuum (Spackman et al. 1958), and the analyses were done on a Beckman 120 Amino Acid Analyzer. No corrections were made for hydrolytic destruction of susceptible amino acids. All temperatures are in degrees centigrade (°C).

Preparation of [Homoarginine^{12}]-Glucagon

The procedure used was according to the method described in Methods of Enzymology (Hirs 1967) adapted by Hruby and Lin (personal communication). Native glucagon (52.7 mg) was dissolved in 5 ml of a 25% aqueous solution of N, N-dimethylformamide. The solution was heated over a steam bath and the pH was adjusted to 10.5, using a dilute solution of NaOH. The solution was cooled to 4° in the refrigerator for one hour and then the pH was adjusted back to 10.5 using a dilute NaOH solution. o-Methylisourea hydrogen sulfate (0.86 g, 5 mmole) was dissolved

in another 5 ml of a 25% aqueous solution of N, N-dimethylformamide. The solution adjusted to a pH of 10.5 with dilute NaOH and then cooled to 4° in the refrigerator. The o-methylisourea solution was added gently to the glucagon solution and the mixture allowed to sit in the cold room (4°). The solution was not agitated in any manner so as to prevent any salts from precipitating out, though, in this case, precipitation of transparent needles was observed along the sides of the reaction flask. After five days the reaction was warmed to room temperature and 3 ml of lgacial acetic acid was added to the solution, bringing the pH to 3 and the sample was gel filtered on a Sephadex G-15 column (#12) using 30% acetic acid as the eluent solvent. The peptide fractions were pooled and lyophilized to give a white powder.

Separation and Purification of [Homoarginine^{12}]-Glucagon

The peptide sample was dissolved in 30 ml of 15% acetic acid and applied to a 10 x 2.3 cm column of SP-Sephadex C-25 resin. The cation exchange column, which makes use of sulfopropyl binding groups, was previously washed and equilibrated at 25° with several column volumes of an aqueous buffer at pH3 containing 10% acetic acid, 20 millimolar sodium acetate, and one molar urea. An increasing linear chloride gradient of volume 800 ml was used for elution. The first 400 ml of buffer contained 0.1 molar (2.32 g) sodium chloride and the second 400 ml contained 0.3 molar (6.96 g) sodium chloride. After the 800 ml had passed through the column, another 400 ml of 0.3 molar sodium chloride was added to bring off any lingering glucagon (see Figure 3). The three

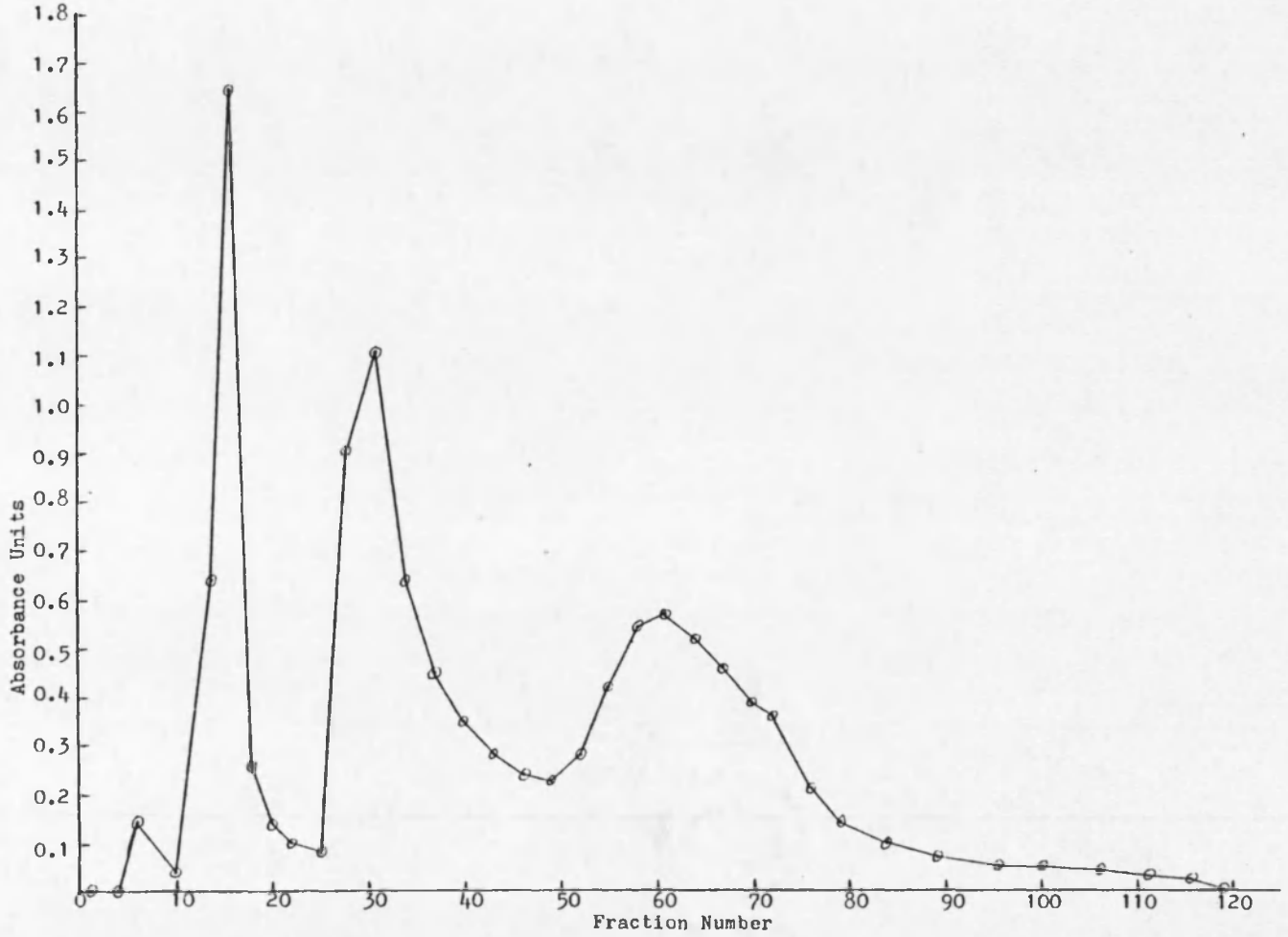

Figure 3. SP-Sephadex Ion Exchange Chromatography Using a 0.1 to 0.3 M Sodium Gradient: Optical Density versus Fraction Number.

peaks were pooled separately, the salts removed by gel filtration on a Sephadex G-15 (#12) column and the resulting peptide fractions were isolated by lyophilization. Amino acid analysis indicated the first peak to be composed entirely of salts. The third peak gave the following molar ratios: lysine 0.02 (0), ornithine 0.33 (0), histidine).69 (1), arginine 2.08 (2), homoarginine 1.09 (1), aspartic acid 4.55 (4), threonine 2.78 (3), serine 3.43 (4), gluatmic acid 4.09 (3), glycine 1.19 (1), alanine 1.00 (1), valine 1.00 (1), methionine 0.86 (1), leucine 1.98 (2), tyrosine 1.98 (2), phenylalanine 2.14 (2). A white powder, 23 mg, was obtained. The middle peak, 7 mg, gave the following ratios after amino acid analysis: lysine 0.92 (1), histidine 0.68 (1), arginine 1.81 (2), homoarginine 0.15 (0).

Large Scale Synthesis of [Homoarginine^{12}]-Glucagon

Crystalline bovine glucagon (315.0 mg) was dissolved in 30 ml of a 25% aqueous solution of N, N-dimethylformamide. A 10% NaOH solution was added dropwise, along with heating, until all the glucagon had dissolved. The pH was then increased to 10.5. The solution was allowed to cool to room temperature and the pH was adjusted to 10.5 using the 10% NaOH solution. o-Methylisourea hydrogen sulfate (5.16 g, 30 mmole) was dissolved in 30 ml of a 25% aqueous solution of N, N-dimethylformamide and raised to a pH of 10.5 by the dropwise addition of a 10% NaOH solution. Both solutions were cooled to 4° in the cold room and then the isourea solution was poured gently into the flask containing the glucagon solution. The reaction flask was not agitated

in any manner and no precipitation was observed. After twelve days in the cold room, 18 ml of glacial acetic acid was added to the solution and the pH was lowered to 3. Since the total volume was 92 ml, the solution was divided into thirds and each third was gel filtered on a Sephadex G-25 column (#7). Following this, the tubes containing the peptide fractions from each respective run were pooled and lyophilized and a white powder was recovered in each case. Each sample was then purified on an ion exchange column (see parts A, B, and C).

Part A

The peptide sample (98 mg) was dissolved in 20 ml of a 10% aqueous acetic acid solution. Gentle heating was required to dissolve the sample. The solution was applied to an 18 x 25 cm SP-Sephadex cation exchange (C-25) column and followed by three 15 ml washes of a 10% aqueous acetic acid solution. The column had at first been washed and equilibrated with 500 ml of the buffer used in the previous ion exchange chromatography. An increasing linear sodium gradient was set up with 1000 ml of the buffer solution; 500 ml contained 0.1 molar NaCl (2.9 g) and the second 500 ml contained 0.3 molar NaCl (8.7 g). The flow rate was about 0.63 ml per minute. After 115 fractions of 10 ml each had been collected, not all the glucagon had eluted off the column, so a new gradient was used with 400 ml of 0.3 molar NaCl (6.96 g) buffer solution and in another 400 ml of buffer 0.6 molar NaCl (13.92 g). An additional 80 tubes were collected (see Figure 4). Two fractions were made, one from tube numbers 80 to 120 and the other from tube numbers

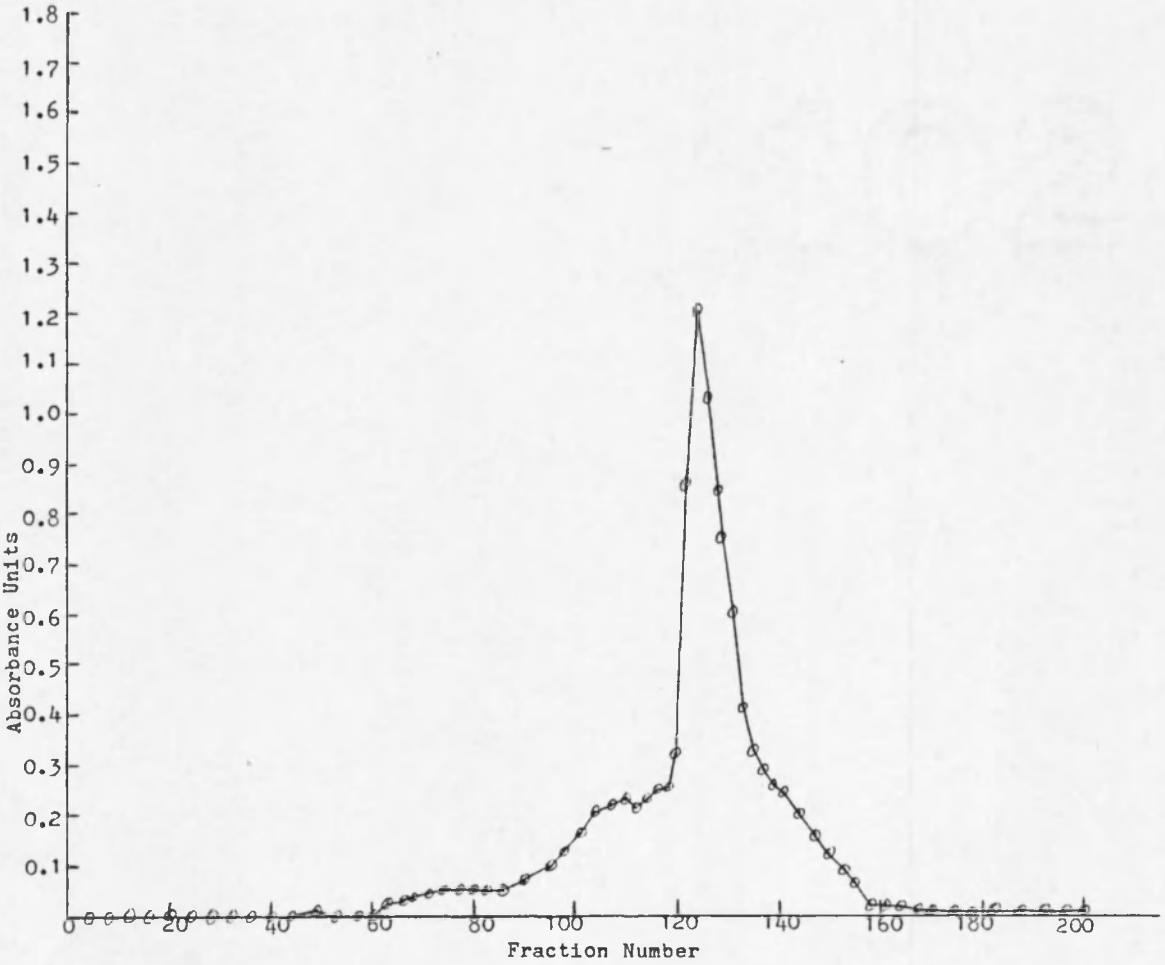

Figure 4. Large Scale SP-Sephadex Ion Exchange Chromatography Using a 0.1 to 0.3 M Sodium Gradient: Optical Density versus Fraction Number.

121 to 153. This first fraction was pooled separately and its salts were removed by ultrafiltration on an Amicon UM-2 membrane filter. The final white lyophilized powder weighed 49.0 mg. Amino acid analysis gave the following molar ratios: lysine 0.04 (0), histidine 0.76 (1), arginine 1.98 (2), homoarginine 1.00 (1), aspartic acid 4.23 (4), threonine 2.93 (3), serine 3.91 (4), glutamine 3.11 (3), glycine 1.04 (1), alanine 1.03 (1), valine 0.98 (1), methionine 0.95 (1), leucine 2.07 (2), tyrosine 2.14 (2), phenylalanine 2.10 (2).

Part B

The lyophilized sample (97 mg) was dissolved in 30 ml of a 20% aqueous acetic acid solution and then applied to the column used in Part A. The column had been previously washed with 800 ml of water and then with 400 ml of the buffer used in the preceding section. Three washes of nine ml each using warm buffer followed. An increasing linear sodium gradient was established using 500 ml of an 0.2 molar NaCl (5.80 g) solution of the buffer and a second 500 ml containing an 0.4 molar NaCl (14.5 g) solution of the buffer. The flow rate was maintained at 0.62 ml/minute and 120 fractions of 10 ml each were collected. Since no clear separation was seen (see Figure 5), fractions 40-103 were recombined. The solution was passed through an Amicon UM-2 membrane filter, the peptide lyophilized to 72 mg, and saved for subsequent purification.

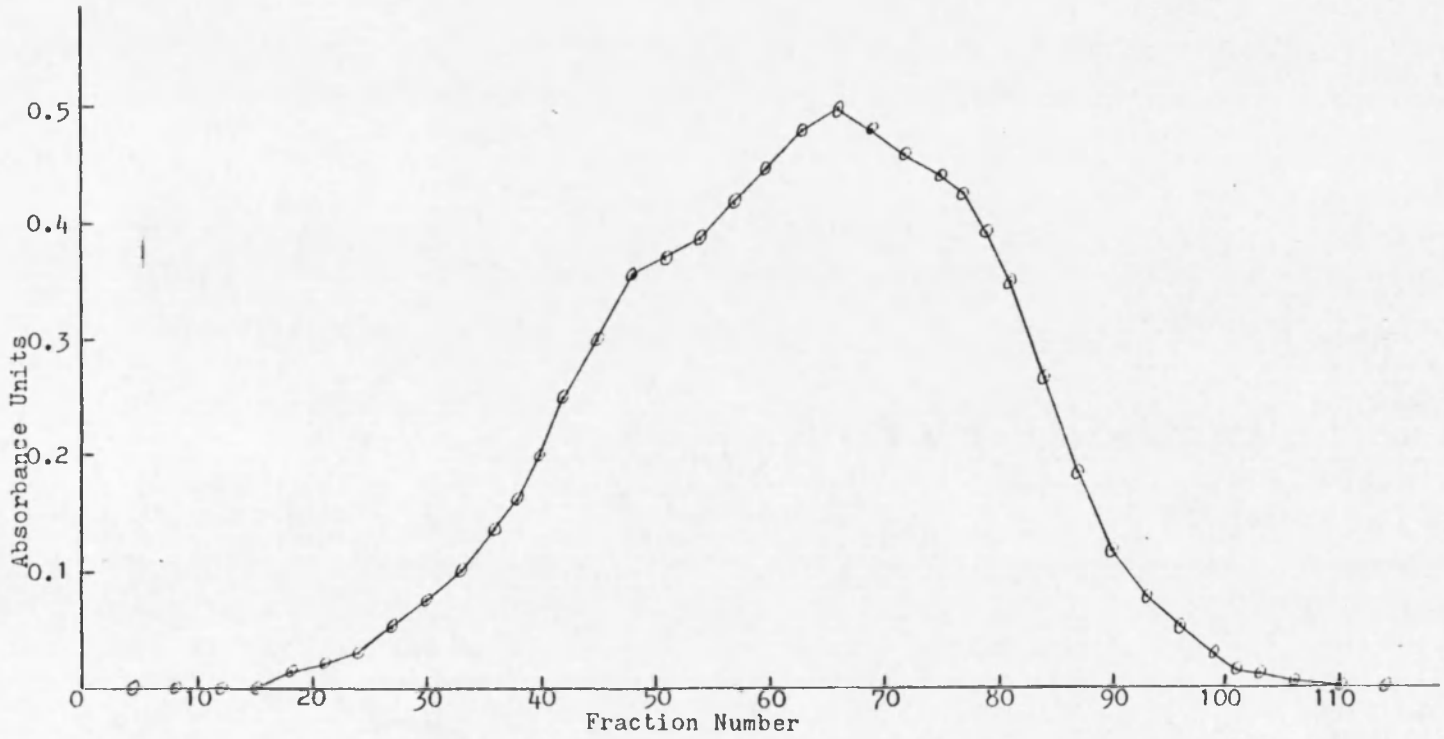

Figure 5. Large Scale SP-Sephadex Ion Exchange Chromatography Using a 0.2 to 0.4 M Sodium Gradient: Optical Density versus Fraction Number.

Part C

The final portion (95 mg) was dissolved in 25 ml of a warm 20% aqueous solution of acetic acid. The solution and three subsequent washings of 11 ml of the warm buffer were applied to the column used in the preceding two purifications. The buffer was also the same as that used previously. The column had been first washed with 600 ml of water and then by 400 ml of the buffer. A 600 ml reservoir containing 0.1 molar NaCl (3.48 g) in the buffer was connected to the column along with a 400 ml reservoir containing a 0.4 molar NaCl (9.28 g) buffer solution. First 200 ml of the 0.1 molar NaCl solution was passed through the column. An increasing linear sodium gradient then followed using 400 ml of each salt solution. After 105 fractions of about 10 ml each had been collected at a rate of about 1 ml/minute, another 400 ml of an 0.6 molar NaCl (13.9 g) buffer solution was eluted through the column, removing any residual peptide (see Figure 6). The fractions from tubes 105-125 were pooled and their salts removed by ultrafiltration on an Amicon UM-2 membrane filter. The final product, 29 mg, was obtained by lyophilization. Amino acid analysis gave the following molar ratios: ornithine 0.25 (0), lysine 0.03 (0), histidine 0.76 (1), arginine 2.00 (2), homoarginine 0.95 (1).

In the three purification procedures a yellow layer was observed which would sometimes move slowly down the column as the gradient was increased. The resin in the column would expand to 19 cm when water was eluted through it and shrink to about 10 cm as a result of the sodium gradient.

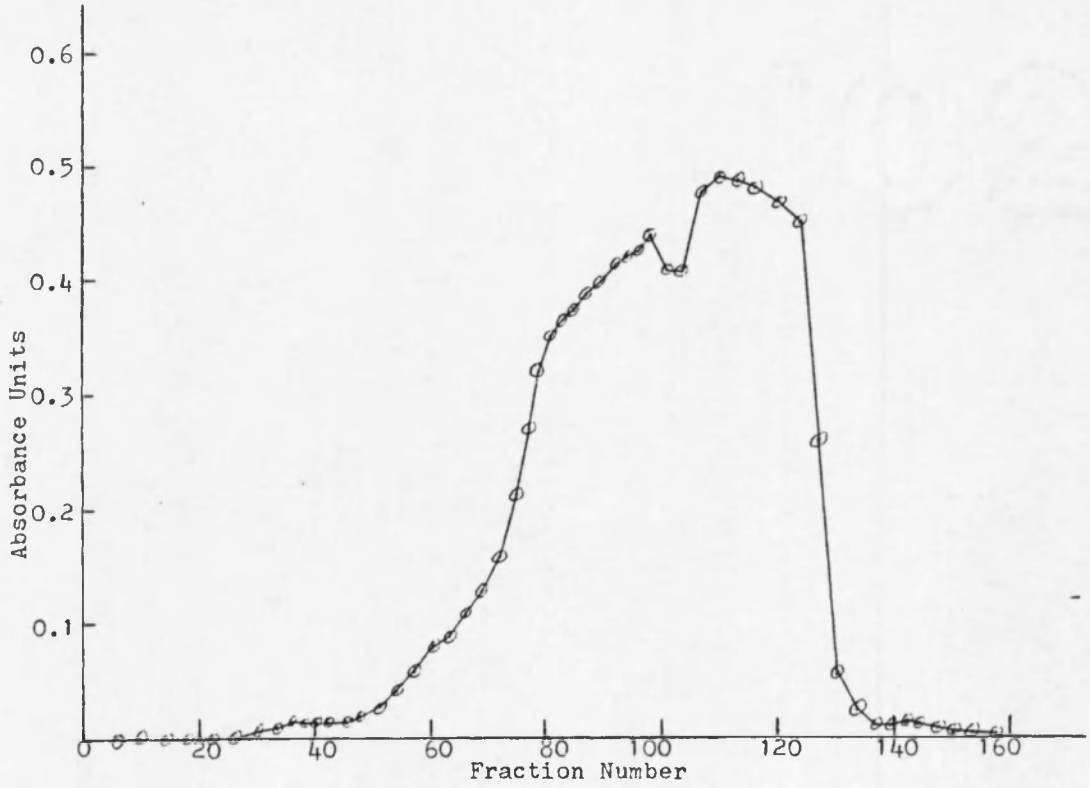

Figure 6. Large Scale SP-Sephadex Ion Exchange Chromatography Using a 0.1 to 0.5 M Sodium Gradient: Optical Density versus Fraction Number.

Use of a Larger Ion Exchange Column for Purification

A larger column (45 x 2.5 cm) of SP-Sephadex (C-25) cation exchange resin was washed and equilibrated with 800 ml of the buffer used previously. The lyophilized powder (72 mg) from Part B of the previous purification was dissolved in 30 ml of a warm 15% aqueous acetic acid solution and applied to the column followed by three 10-ml rinses of 10 ml of buffer. An 800 ml increasing linear sodium gradient was eluted through the column using 400 ml of a 0.1 molar NaCl (2.32 g) and 400 ml of a 0.3 molar NaCl (6.96 g) buffer solution. After 91 fractions of about 17 ml each had been collected at a rate of 0.7 ml/minute, no absorbance was seen for the peptide and a second increasing linear sodium gradient was started, using 300 ml of a 0.2 molar NaCl (3.48 g) buffer solution and 300 ml of a 0.4 molar NaCl (6.96 g) buffer solution. The same flow rate was maintained and after 37 more fractions, no peptide was indicated. Another 600 ml gradient was started using a 0.3 molar NaCl (5.2 g) buffer solution and a 0.6 molar (10.4 g) NaCl buffer solution. This was followed by a 1000 ml sodium gradient from a 0.6 molar NaCl (17.4 g) to a 1.0 molar (29.0 g) buffer solution. Finally 1 liter of a 1.0 molar (58.0 g) NaCl buffer solution was eluted through the column, but no peptide was indicated at any time.

Synthesis of p-Nitrophenyl Acetate

A Schotten-Baumen reaction was run to prepare the title compound using 14 g (.101 moles) of p-nitrophenol in 500 ml of an 0.3 N NaOH (6g, 0.15 moles) solution. The phenol turns yellow and does not

completely dissolve. Acetic anhydride (12.5 ml, 11.55 g, 0.13 moles) was then added with vigorous stirring of the exothermic reaction for one hour. The solution was cooled in an ice bath and the yellow precipitate was filtered out. The crystals were redissolved in hot 95% ethanol, precipitated in an ice bath, and refiltered. The product was then recrystallized from hot water, filtered, and dried. The melting point was 77° - 78° (Lit. mp, 77° CRC Handbook, 1968). About 15 g (82%) of off-white crystals were obtained. The n.m.r. confirmed the product.

Preparation of [Homoarginine^{12}]-Glucagon

Another batch of [homoarginine^{12}]-glucagon was prepared as described previously. Crystalline bovine glucagon (315.0 mg) and o-methylisourea hydrogen sulfate (5.16 g, 30 mmoles) was used. Solvation of the glucagon was a problem this time do to difficulties in controlling the pH of the solution, and a final volume of 240 ml was required. Both solutions were cooled to 4° and their pH's held to 10.5. Then the isourea solution was slowly poured into the glucagon solution, making sure that the reaction mixture was not agitated. At the end of the first day, transparent crystals formed along the inside of the flask, presumably salt crystals. After 12 days in the cold room at about 4°, the solution was warmed to room temperature and 70 ml of glacial acetic acid was required to bring the pH down to 3. Some of the solid material at the bottom of the flask did not dissolve. The supernatant liquid was decanted from the now white spherical crystals, which when treated with 10 ml of 50% aqueous acetic acid, 2 ml of glacial acetic acid, and

finally 7 ml of water, dissolved. Five separate gel filtrations of approximately 40 ml each were required using a Sephadex G-25 column (#7). The pooled fractions of peptide after gel filtration were lyophilized and 289 mg of a white powder was obtained. Amino acid analysis of the product yielded the following molar ratios: ornithine 0.38 (0), lysine 0.09 (0), histidine 0.83 (1), arginine 2.03 (2), homoarginine 0.89 (1).

Reaction of [Homoarginine^{12}]-Glucagon with o-Methylisourea

In an effort to force the guanidylation reaction to completion, 101 mg of the crude [homoarginine^{12}]-glucagon was reacted again with o-methylisourea hydrogen sulfate (1.62 g, 10 mmoles) using the previously described procedure. When adding the dilute NaOH solution to dissolve the glucagon, the solution turned yellow and remained colored with cooling. The reaction flask remained in the cold room (4°) for five days, after which the now clear solution was warmed to room temperature. The pH was then brought down to 3 with 3 ml of glacial acetic acid. No precipitation was observed at any time. The soluiton was gel filtered on a Sephadex G-25 (#7) and the peptide fractions were pooled and lyophilized. Amino acid analysis of the final white powder (88.8 mg) gave the following molar ratios: ornithine 0.33 (0), lysine 0.09 (0), histidine 0.76 (1), arginine 2.02 (2), homoarginine 0.89 (1).

Reaction of Twice Reacted [Homoarginine^{12}]-Glucagon

While analyzing the sample from the previous reaction, another guanidylation reaction was intiated using 29 mg of the lyophilized glucagon from the immediately preceding preparation. The glucagon was dissolved and reacted with o-methylisourea hydrogen sulfate (0.86 g, 5 mmoles) as previously described. The reaction flask remained in the cold room for seven days, after which it was heated to room temperature and 3 ml of glacial acetic acid was added bringing the pH to about 3. The contents of the flask was gel filtered on a Sephadex G-25 column (#7) and the peptide fractions were pooled and lyophilized. Amino acid analysis of the final white product (24 mg) gave the following molar ratios: ornithine 0.25 (0), lysine 0.10 (0), histidine 0.61 (1), arginine 1.83 (2), homoarginine 0.90 (1).

Preparation of [Homoarginine^{12}]-Glucagon in a Dilute Solution

In dilute solution, the glucagon molecule dissociates into a monomer. It is possible that the α-helix and trimer conformation may successfully hinder guanidylation at some of the lysyl residues. However as the monomer, this situation possibly could be avoided.

Crystalline bovine glucagon (11.6 mg) was reacted with o-methylisourea hydrogen sulfate (0.194 g, 1 mmole) as described previously. However prior to mixing of the two cool solutions, 300 ml of water was added to the glucagon solution. Then dilute NaOH was used to bring the pH back to 10.5 and the mixture was recooled at 4°. After eleven days in the cold room (4°) subsequent to mixing, the contents of

the reaction flask was evaporated, in vacuo to about 30 ml, and 3 ml of glacial acetic acid was added to bring the pH to 3. The glucagon solution was gel filtered on a Sephadex G-25 column (#7). The peptide fractions were pooled and lyophilized and 8.2 mg of a white powder was isolated. Amino acid analysis gave the following molar ratios: lysine 0.53 (0), histidine 0.70 (1), arginine 2.01 (2), homoarginine 0.47 (1).

Preparation of ε-Acetyl-Glucagon

The acetylation procedure for ε-amino groups of Leclerc and Benoiton (1968) was used with slight modifications in order to put an irreversible blocking group on any unreacted lysyl residues. The authors realized a 100% conversion when their particular conditions are employed.

[Homoarginine^{12}]-glucagon (10.5 mg) obtained from the Part A purification (containing about 3-4% lysyl residues) was dissolved in 1 ml of a 25% aqueous solution of N, N-dimethylformamide. This was followed by 300 ml of water. A 10% aqueous solution of NaOH was added dropwise until a pH of 11.1 was reached. The solution was constantly stirred with a magnetic stirrer and the pH was constantly monitored. p-Nitrophenylacetate (9.0 mg, 0.05 mmole) was dissolved in 1 ml of N, N-dimethylformamide and added to the stirring solution. The pH was maintained at 11.1 by an automatic titrator charged with a dilute NaOH solution. After 24 hours of stirring, 0.2 ml of dicyclohexylamine (0.185 g, 1.0 mmoles) was added to react with any excess p-nitrophenylacetate and the solution was stirred for one hour. The

solvents were evaporated, in vacuo, and a precipitate formed. Glacial acetic acid (7.5 ml) was then added to bring the pH to 3 and the solid material dissolved. The solution was evaporated, in vacuo, until about 30 ml were left. This solution was then gel filtered on a Sephadex G-25 column (#7). The peptide fractions were pooled and lyophilized to give 8.5 mg of a white powder.

A 2.2 x 1.4 cm column of SP-Sephadex (C-25) resin was washed and equilibrated with 200 ml of the buffer used in previous purifications. The lyophilized product was dissolved in 20 ml of a warm 20% aqueous acetic acid solution and applied to the column. This was followed by three 15 ml washes with the warm buffer. The buffer solution (100 ml) with 0.1 molar NaCl (0.58 g) was first eluted through the column. An increasing linear sodium gradient then followed using 200 ml of an 0.1 molar (1.16 g) NaCl buffer solution and 200 ml of an 0.3 molar (3.48 g) NaCl buffer solution. After 79 fractions of 6.8 ml each had been collected at a rate of 0.68 ml/minute, 200 ml of a 0.7 molar buffer solution of NaCl (6.96 g) was eluted through the column, but no more peptide was observed (see Figure 7). Fraction numbers 33 to 45 were pooled and reduced, in vacuo, to 30 ml, and then applied to a Sephadex G-25 column (#7). The pooled glucagon fractions were lyophilized to give 0.38 mg of a white powder. Amion acid analysis gave the following molar ratio: lysine 0.10 (0), histidine 0.65 (1), arginine 2.0 (2), homoarginine 0.98 (1).

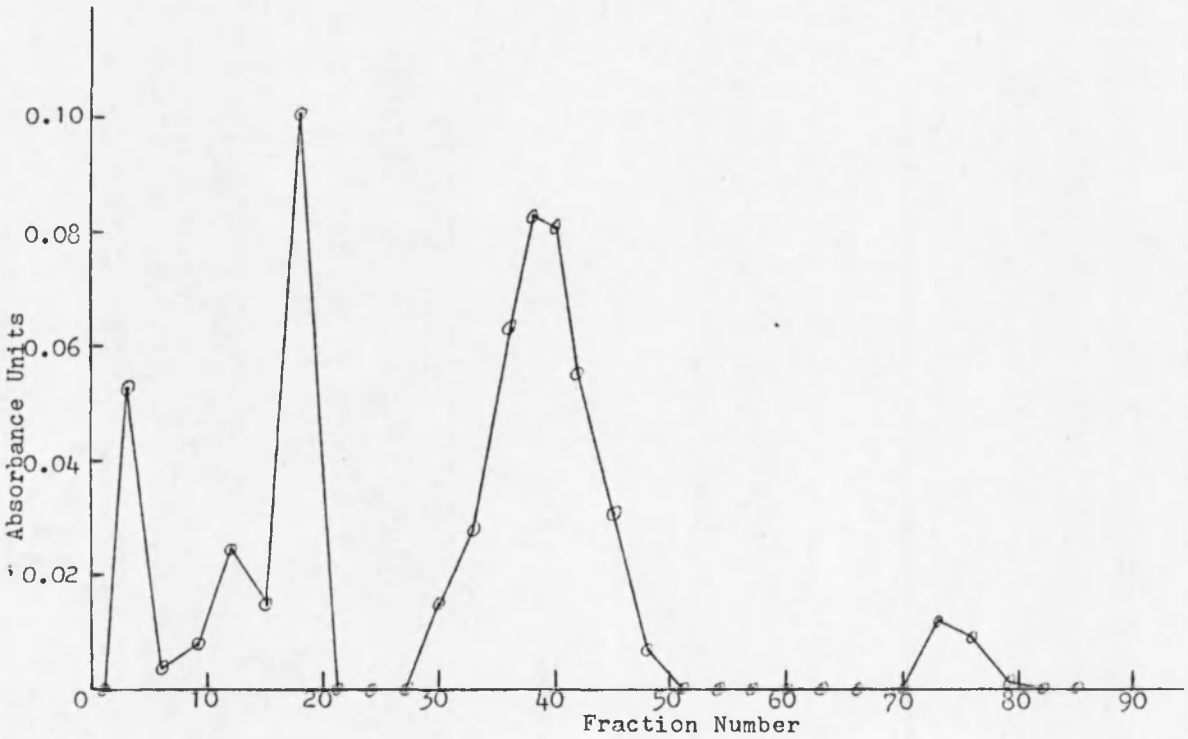

Figure 7. SP-Sephadex Ion Exchange Chromatography of [Homoarginine^{12}]-Glucagon and ε-Acetyl-Glucagon: Optical Density versus Fraction Number.

Purification of [Homoarginine^{12}]-Glucagon Using QAE Resin

The purification procedure of Bromer et al. (1972) with some modifications was used to purify [homoarginine^{12}]-glucagon. Sephadex (A-25) QAE (anionic exchange resin) was suspended in 0.01 molar Trisma-Base (pH 10.5, 4°) which contained 7 molar urea and 0.001 molar disodium ethylenediaminetetraacetate. The column (12 x 1.6 cm) was washed and equilibrated with 400 ml of the Trisma-Base buffer. Crude [homoarginine^{12}]-glucagon (11.7 mg) from Part A was dissolved in 10 ml of the buffer and applied to the column. Fine 8 ml washings with the buffer followed. Then an increasing linear chloride gradient was used for elution. The second 400 ml of the buffer was the same as the first 400 ml except that it contained 0.075 molar NaCl (1.74 g). After 118 fractions of about 7 ml each had been collected at a rate of about 1 ml/minute (see Figure 9), fractions 30 to 55, fractions 56 to 84, and fractions 85 to 118 were pooled. Due to the high concentration of urea, an Amicon ultrafiltration UM-2 membrane filter system was employed. The peptide from fractions 30 to 55 was gel filtered on a Sephadex G-25 column (#7), after membrane filtration, and the peptide fractions were pooled and lyophilized. A white powder, 0.67 mg was obtained. The following molar ratios were given by amino acid analysis: ornithine 0.19 (0), lysine 0.04 (0), histidine 0.83 (1), arginine 1.99 (2), homoarginine 1.00 (1). The peptide from fractions 85 to 118 was lost when the Amicon UM-2 membrane dried out. Another 800 ml of the buffer was eluted through the same column immediately following the initial

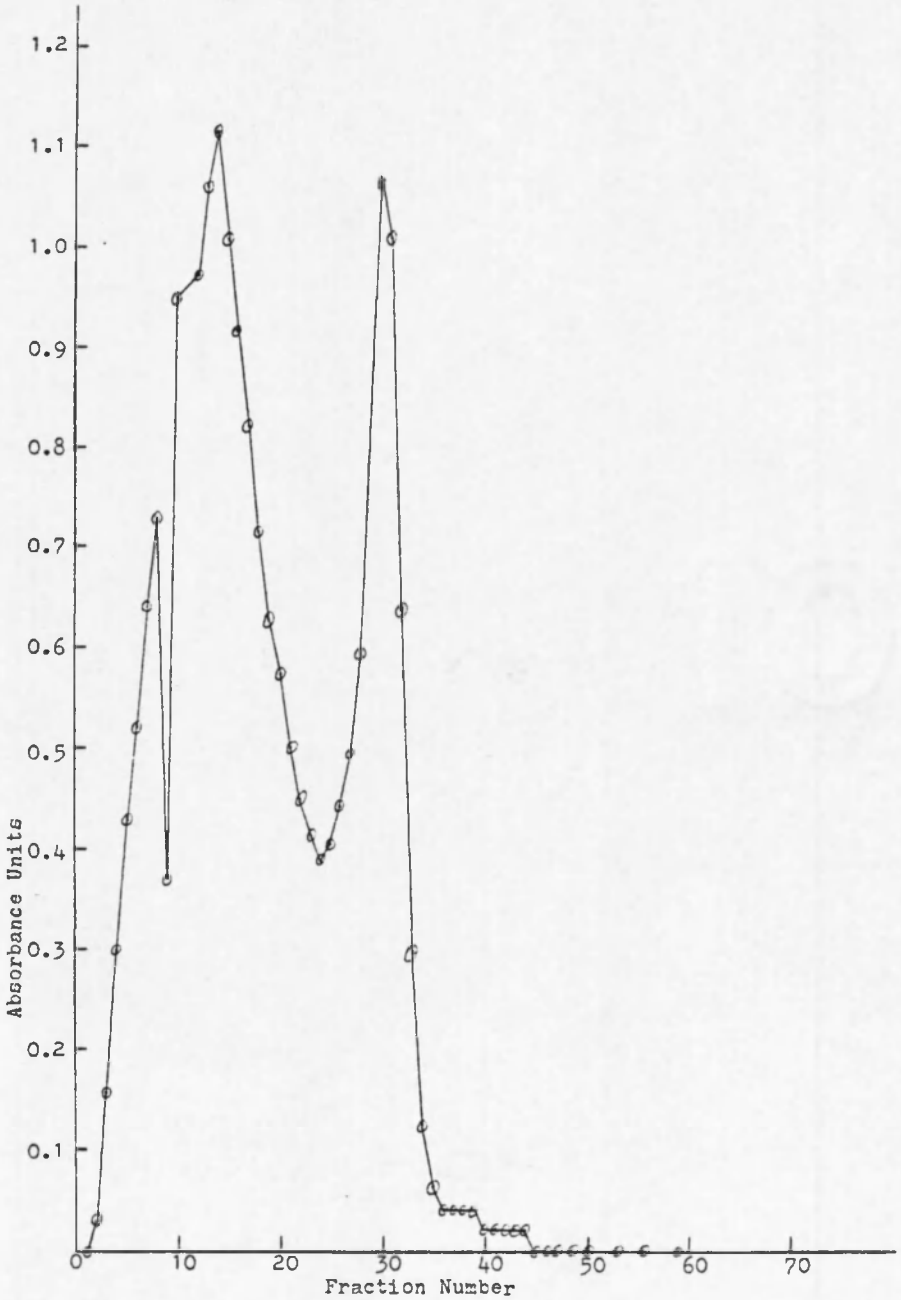

Figure 8. DEAE Sephadex Ion Exchange Chromatography: Optical Density versus Fraction Number.

purification. Two 400 ml portions of the buffer, one with 0.05 molar NaCl (1.16 g) and the second with 0.15 molar NaCl (3.48 g), was used as an increasing linear gradient. After 116 ml fractions had been collected at a rate of about 1 ml/minute, no further evidence of peptide was indicated.

Purification of [Homoarginine^{12}]-Glucagon Using DEAE Resin

The purification procedure of Bromer et al. (1972) with some modifications was used to purify [homoarginine^{12}]-glucagon. Sephadex (A-50) DEAE (anionic exchange) was suspended in 0.01 molar Trisma-Base (pH 9.0, 4°), which contained 7 molar urea and 0.001 molar disodium ethylenediaminetetraacetate. The column (14 x 1.8 cm) was washed with 400 ml of the Trisma-Base buffer. [Homoarginine^{12}]-glucagon (23.6 mg), obtained from the last preparation, was dissolved in 8 ml of the buffer and applied to the column. Six 8 ml washes followed. An 800 ml increasing linear chloride gradient was used for elution, with the second buffer being the same as the first except that the second contained 0.075 molar NaCl (1.67 g). After 140 fractions of about 2 ml each were collected at 4° at a rate of about 0.5 ml/minute (see Figure 8). Fractions 11 to 14 were pooled and gel filtered on a Sephadex G-25 column (#7). The peptide fractions were lyophilized and 2.1 mg of white powder was obtained. Fractions 15 to 25 and 26 to 33 were treated in the same manner giving, respectively, 6 mg and 7 mg. Amino acid analysis of the three samples gave the following molar ratios:

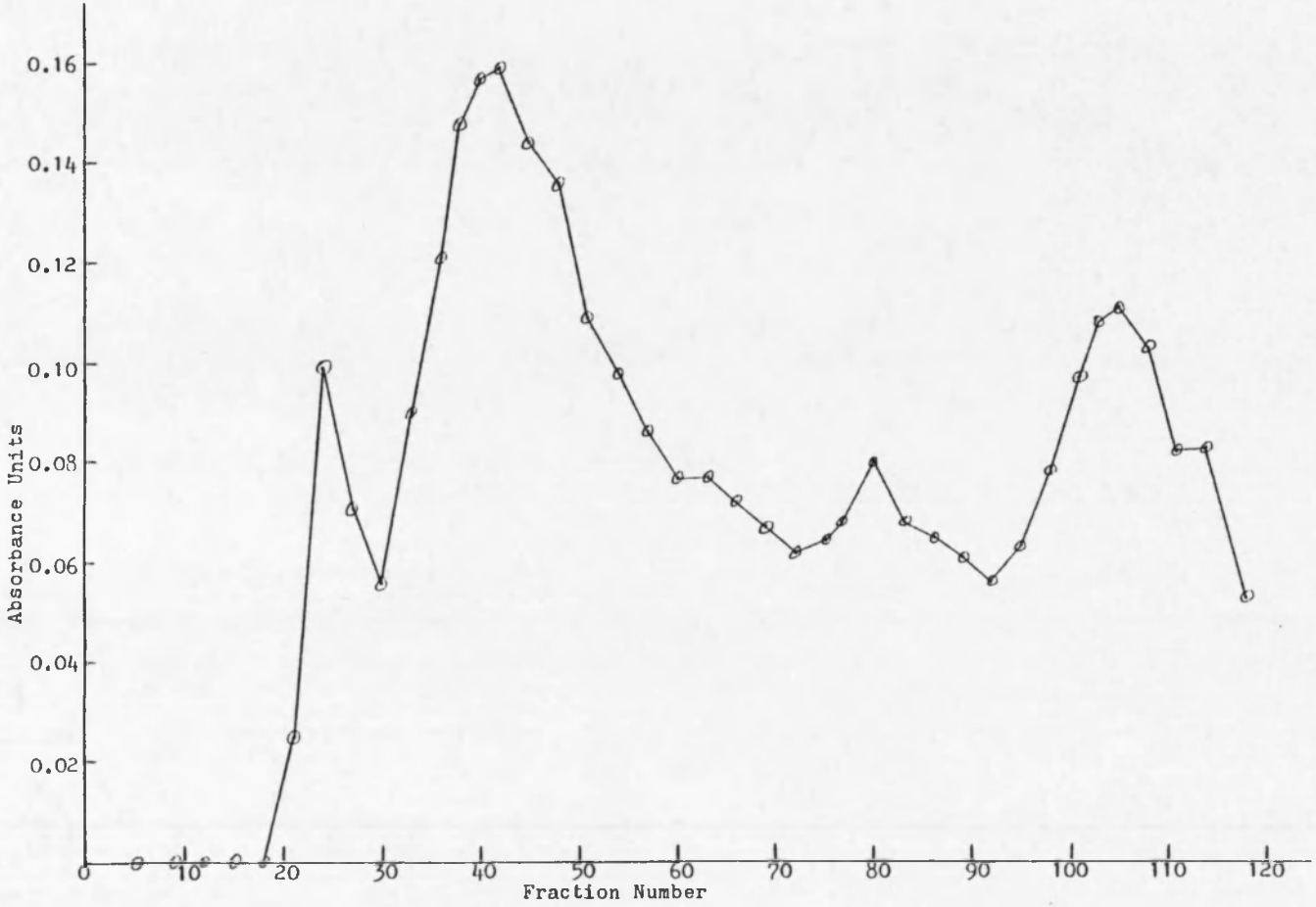

Figure 9. QAE Sephadex Ion Exchange Chromatography: Optical Density versus Fraction Number.

Amino Acid	Fractions 11-14	Fractions 15-25	Fractions 26-33
Ornithine (0)	None	0.22	None
Lysine (0)	.08	.16	.94
Histidine (1)	.49	.75	.34
Arginine (2)	2.17	2.08	1.5
Homoarginine (1)	.91	.91	.56

Preparation of [Des-histidine^{1}]-[Homoarginine^{12}]-Glucagon

The Edman degradation procedure of Peterson et al. (1972) was used with some modiifications to remove histidine from glucagon. [Homoarginine^{12}]-glucagon (10.6 mg), which had undergone the acetylation reaction described earlier, so as to remove any remaining lysyl residues, was dissolved in 0.6 ml of an 0.4 molar solution of triethylamine (distilled over ninhydrin) in 1-propanol-water (3:2 by volume) and brought to pH 9.5 with trifluouroacetic acid. The peptide was dissolved on a vortex mixer. The solution was frozen in a dry ice-acetone bath, evacuated under vacuum, and degassed as the solution warmed. The system was then immediately flushed with nitrogen. While a continuous stream of nitrogen was passed into the vial, 0.03 ml (0.0339 g, 0.25 mmoles) of phenylisocyanate (sequanal grade) was added and rapidly stirred by vortex mixing. The mixture was incubated at 49°-50° for 20 minutes. At this time two layers were seen, with a yellow layer being on top. Benzene (1 ml) sequanal grade) was then added to the vial and, while under a continuous stream of nitrogen gas, mixed well by

vortex mixing. The layers were left to separate for 20 minutes and then the upper benzene layer with a slight yellow color was removed. Nitrogen gas was blown in while keeping the soluiton at 50° for seven minutes and evacuated with a vacuum pump at 50°. The residual oily layer was treated with 0.8 ml of trifluoroacetic acid (1.23 g, 10.8 mmoles) containing 2.5 mg of dithiothreitol (0.016 mmoles) in an atmosphere of nitrogen. The tube was then stoppered and incubated at 50° for seven minutes. The triflouroacetic acid was removed by blowing nitrogen gas until a whitish semi-solid was obtained. The residue was dissolved in 1.5 ml of a 30% aqueous solution and applied to a Sephadex G-15 column (#12). Three washes of 2 ml each using 30% aqueous acetic acid followed. The pooled peptide fractions were lyophilized and amino acid anlaysis gave the following molar ratios: ornithine 0.22 (0), lysine 0.04 (0), histidine 0.10 (1), arginine 2.00 (2), homoarginine 0.99 (1).

Purification of [Des-histidine^{1}]-[Homoarginine^{12}]-Glucagon

The purification procedure used was the same as that used for the purification of [homoarginine^{12}]-glucagon. SP-sephadex (C-25) cation exchange resin was suspended in a buffer at pH 3 containing 10% aqueous acetic acid, 20 millimolar sodium acetate, and one molar urea. The 7.7 x 1.1 cm column was washed and equilibrated wtih 600 ml of the buffer. [Des-histidine^{1}]-[homoarginine^{12}]-glucagon (8.8 mg) was dissolved in 16 ml of a 15% aqueous acetic acid solution and applied to the column. This was followed by two washes with the 15% acetic acid solution and then by two 3 ml washes of the buffer solution. A reservoir

containing 380 ml of 0.1 molar NaCl (2.20 g) buffer solution began eluting through the column. After 130 ml had passed through the column, an increasing linear sodium chloride gradient was set up, using the 0.1 molar NaCl buffer solution and 250 ml of an 0.3 molar NaCl (4.35 g) buffer solution. After 119 fractions of 4 ml each had been collected at a rate of about 0.75 ml/minute (see Figure 10), fractions 16 to 30, 50 to 87, 110 to 119 were pooled. The first fraction was evaporated down, in vacuo, to about 30 ml and gel filtered on a Sephadex G-25 column (#7). No ultraviolet absorbance to suggest the peptide was observed. The next peak was treated similarly and the peptide fractions were pooled and lyophilized, giving a white powder (3.48 mg). Amino acid analysis of this sample gave the following molar ratios: lysine 0.04 (0), histidine 0.03 (0), arginine 2.13 (2), homoarginine 0.96 (1), aspartic acid 4.14 (4), threonine 2.76 (3), serine 3.59 (4), glutamine 3.94 (3), glycine 0.96 (1), alanine 1.04 (1), valine 1.00 (1), methionine 0.72 (1), leucine 2.03 (2), tyrosine 2.07 (2), phenylalanine 2.10 (2).

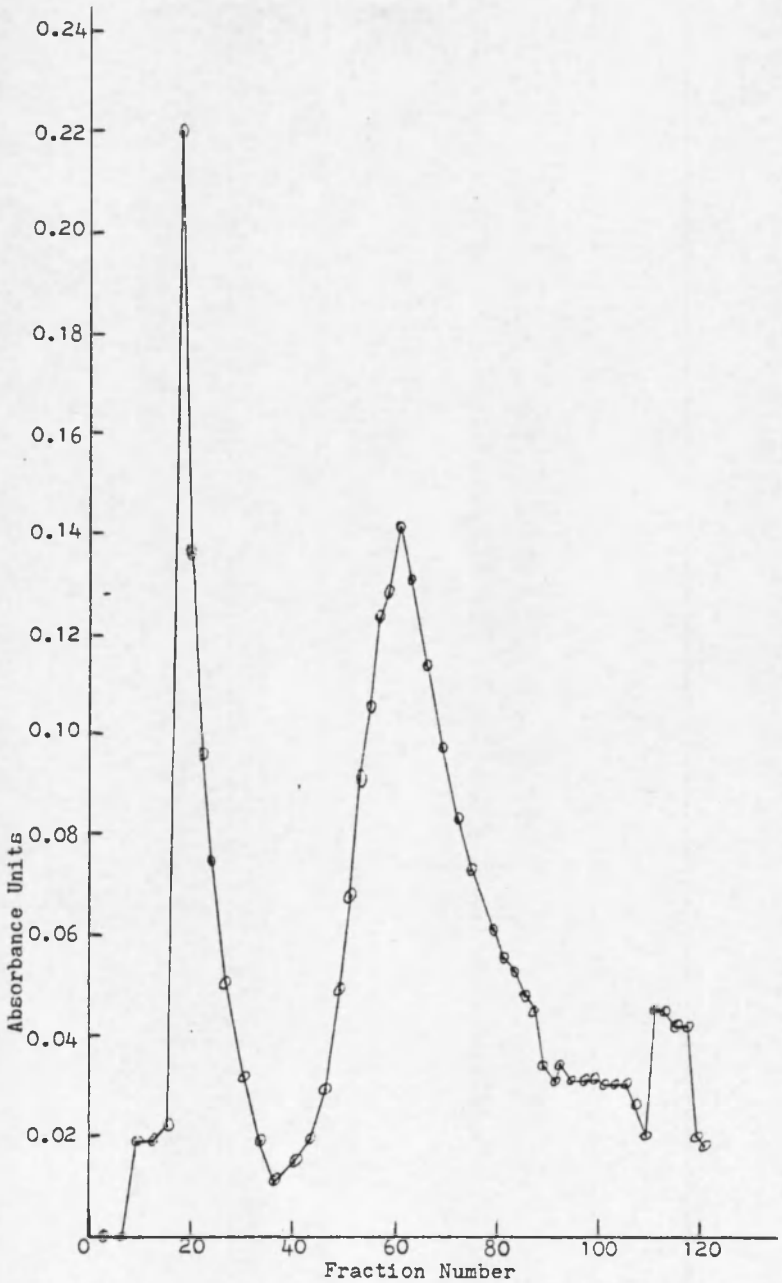

Figure 10. SP-Sephadex Ion Exchange Chromatography of [des-Histidine^{1}]-[Homoarginine^{12}]-Glucagon.

CHAPTER 3

DISCUSSION

The persistance of an apparent lysine contaminate in the preparation and purification of semisynthetic [homoarginine^{12}]-glucagon has retarded further progress towards synthesizing [des-histidine^{1}]-[homoarginine^{12}]-glucagon. The initial reaction of native bovine glucagon with o-methylisourea is a fairly simple reaction and its only limitation is the amount of time required in allowing the reaction to go to completion. Conversion to the homoarginyl residue was about 90% for the initial reaction, and subsequent guanidylation attempts appeared ineffective after the initial reaction. Suitable quantities of [homoarginine^{12}]-glucagon, containing this irreversible blocking group on the lysyl residue was produced to permit further synthetic work. The ε-acetylation with p-nitrophenylacetate reaction is a simple and fairly quick reaction. Leclerc and Benoiton (1968) obtained 100% conversion with lysine, but no work with glucagon has been reported. Selective ε-acetylation would appear to be an easy method in which to convert any unreacted lysyl residues to the amide and thereby remove a positive charge from that group, leaving a two charge difference between [homoarginine^{12}]-glucagon and ε-acetyl-glucagon, and this should allow separation on the basis of charge differences. Ion exchange purification, however, did not separate out any detectable material and gave a

chromatogram very similar to the starting material. The isolated [homoarginine^{12}]-glucagon sample still had an apparent lysine contamination. Further investigation into the ε-acetylation reaction is required to determine the extent of its conversion before conclusions are made. ε-Acetyl-glucagon itself may prove to be a good precursor for Edman degradation, assuming 100% conversion is possible and once its biological activity as compared to native glucagon has been determined.

Ion exchange chromatography appeared to be a good method for [homoarginine^{12}]-glucagon purification. Using SP-Sephadex (C-25 cation exchange seemed to work, but there always appeared to be a 2 to 4% contamination of native glucagon based on lysine content after peptide hydrolysis. In all the purifications run, there was never a complete separation, but some overlapping of the peaks remained. A significant amount of glucagon seemed to be absorbed on the column and lost. It was later found (Lin, personal communication) that even after prodigious column washing, some glucagon would remain, possibly to be eluted later during a subsequent purification. This problem was not encountered with the anionic exchange resins and most of the peptide was recovered after purification. However these resins required the use of a saturated urea solution, which necessitated the removal of urea following ion exchange chromatography. Though not much work has been done with anionic exchange chromatography, there appears to be advantages to going this route. [Homoarginine^{12}]-glucagon elutes before native glucagon or other impurities. Also by doing the purification at a low

temperature and in the presence of urea, a problem that may be causing the lysine contamination may be overcome. Urea and low temperatures have been shown to cause dissociation of the hormone from its receptor (Rodbell, Birnbaumer et al. 1971) and 7M guanidine hydrochloride causes glucagon to close its α-helix conformation and become monomeric in solution. At high concentrations glucagon associates into trimers. It is reasonable to assume that before being put on the column or even while in contact with the ion exchange resin, glucagon may associate into trimers which contain a random mixture of [homoarginine^{12}]-glucagon and unreacted native glucagon. However, if urea acts similarly to guanidine hydrochloride, it would be expected to help dissociate the trimers into monomers. However this approach did not seem to have any effect on the resulting lysine contamination. In each case some overlapping of the peaks were seen, indicating an incomplete separation. The anionic exchange columns appear to offer the best opportunity for obtaining a complete separation of [homoarginine^{12}]-glucagon from its impurities, if the proper conditions can be achieved. Compared to cation exchange, a more complete separation was seen and better peptide recovery was realized. Interference with the trimeric association of glucagon by using urea may prove important also.

Another consideration is the method for identifying the contamination by lysyl residues since acid hydrolysis is a nonspecific reaction. On several amino acid analyses on ornithine peak greater than (2-8 times) the lysine peak was seen. Since deguanidylation of arginine produces ornithine and of homoarginie produces lysine, the relative

amounts of ornithine residues could be indicative of the amount of lysyl residues appearing as a result of 6N HCl hydrolysis. Therefore the [homoarginine^{12}]-glucagon actually may have been purified. If this is the case, then enzymatic hydrolysis may be preferred over acid hydrolysis for amino acid determination and for the detection of any unreacted glucagon in the [homoarginine^{12}]-glucagon preparation.

Conversion of our purified semisynthetic [homoarginine^{12}]-glucagon to [des-histidine^{1}]-[homoarginine^{12}]-glucagon was done according to the standard Edman degradation procedure (Peterson et al. 1972). The conversion was incomplete since about 10% histidine was still present. The attempt to purify [des-histidine^{1}]-[homoarginine^{12}]-glucagon using SP-Sephadex cation exchange chromatography gave a sample that contained 3 to 4% histidyl residues. This contamination by one or more histidine containing impurities suggests the need for further purification.

[Homoarginine^{12}]-glucagon has been shown to be almost as active as native glucagon (90-100%) in gluconeogenesis studies on rat liver plasma membrane. [Des-histidine^{1}]-glucagon's activity is still being studied.

APPENDIX A

FORMULA ABBREVIATIONS

Ion Exchange Resin	Formula
DEAE	$-C_2H_4-\overset{\overset{C_2H_5}{\vert}}{\underset{\underset{C_2H_5}{\vert}}{N^+H}}$
QAE	$-C_2H_4-\overset{\overset{C_2H_5}{/}}{\underset{\underset{CH_2-CHOH-CH_3}{\vert}}{N^+}}-C_2H_5$
SP	$-C_2H_4-SO_2O^-$

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