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EXPLORATIONS INTO THE NATURE OF INSULIN BINDING TO OXIDIZED DEXTRAN

This thesis was presented in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at Massey University

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Abstract

The results reported in this thesis comprise an investigation into the conjugation of insulin to oxidized dextran, various release studies from the conjugates, and an attempt to interpret the binding nature of the conjugates. A model system involving the sustained release from insulin-dextran conjugates has been employed in this study. For insulin, up to 3 potential sites only (A1-Gly, B1-Phe and B29-Lys) were expected to bind to oxidized dextran. The rate of release and the maintenance of activity of the released protein are vital to such systems. Success in the interpretation of the binding nature of the conjugate will allow us to investigate its relationship to the rate of release. The desired rate of release for the sustained release of protein could then be achieved, once the projected binding could be obtained.

Activation of dextran was achieved by periodate oxidation to give levels of 8%, 16% and 27% oxidized dextran. Insulin was chosen for its relatively 'uncomplicated' structure and few possible sites available for binding with activated dextran. Insulin was bound to the dextran through imine bonds. Complex formation was examined under a wide range of conditions. Initial studies were begun with the determination of a desirable basic molar ratio. A molar ratio of insulin to 8% activated dextran of 10 : 1 arose from this set of experiments. Insulin was bound to 27% activated dextran at pH 7.4, pH 9 and pH 10. In the cases of pH 9 and pH 10, many more lower MW complexes were formed than at pH 7.4. It seemed that the higher the pH of formation, the more crosslinks occurred between an insulin molecule and dextran molecules in the lower MW range. Approximate physiological pHs (pH 7.1-7.8) were used for complex formation in all subsequent experiments.

Release studies were carried out under approximate physiological conditions (pH 7.4, 37°C). Immediate release was observed upon isolation by size exclusion chromatography. The greatest release occurred in the first 24 hours for all three activation levels. The higher the activation level of dextran, the lower the level of release. An equilibrium was established after several days' release and studies at 37°C produced the expected result: greater release relative to ambient.

A number of studies were carried out with complex after sodium cyanoborohydride had been used to reduce the imine bonds.

The first set of experiments on the reduced complexes was enzymatic cleavage studies, which employed trypsin and α-chymotrypsin. The results for trypsin digestion of the reduced insulin-27% oxidized dextran complex indicated partial binding had occurred at B29-Lys, in combination with full binding at B1 and/or A1. Amino acid analysis results of the isolated complex after trypsin digestion indicated about 90% binding occurred at B29-Lys for the complex, which formed at pH 7.1. The results of α-chymotrypsin digestion study were shown questionable due to its incomplete cleavage.

The reduced complexes were analyzed by amino acid analysis. The insulin-27% activated dextran complexes formed at pH 7.4, pH 9 and pH 10 showed similar extents of binding at B1-Phe, indicating B1 might be the prime binding site. There was more binding at B29 and A1 for the pH 9 than at pH 7.4 case. At pH 10 abnormal values arose. The studies for the complexes of insulin with 16% and 27% activated dextran indicated the more highly activated the dextran, the greater the binding at B29 and A1.

Trials with the 2, 4-dinitrophenyl-derivativatization method proved to be a useful way to examine the degree of B1 and B29 binding from the amino acid analysis results of complex. The insulin-16% activated dextran complex formed at pH 7.1 was found to be about 100% binding at B1, 60% at A1 and 50% at B29.

Oxidative and reductive cleavage studies of A and B chains of insulin and the complex were carried out to investigate the level of A1 binding. After chemical cleavage of the three disulfide bonds in insulin and subsequent chromatography, the amino acid analysis results for the treated complexes indicated a significant proportion of A chain had bound to dextran, i.e. at A1. An estimation of 60-70% of A1 binding was achieved for this study.

This exploratory study has shown that varied complex formation conditions such as the level of activation of dextran, pH, and temperature could alter the extent of binding between insulin and dextran molecules. Amino acid analysis of the reduced complex was a useful method to interpret the binding.

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A1-Gly	α-NH_2 terminal group glycine of A chain of insulin
AAA	amino acid analysis
Ab	antibody
B1-Phe	α-NH_2 terminal group phenylalanine of B chain of insulin
B29-Lys	ε-lysine of the 29^{th} residue from the N-termini of B chain of insulin
CD4	cell surface glycoprotein receptor for HIV
CE	capillary electrophoresis
DOR	double oxidized residues
ELISA	enzyme-linked immunosorbent assay
EPC	especially for small proteins
FDA	food and drug administration
FPLC	fast protein liquid chromatography
GP120	glycoprotein-120
HPLC	high performance liquid chromatography
Met-hGH	recombinant methionyl human growth hormone
MW	molecular weight
MWCO	molecular weight cut off
$NaBH_4$	sodium borohydride
$NaBH_3CN$	sodium cyanoborohydride
PBu_3	tributylphosphine
PEG	polyethylene glycol
PHPMA	N-2-hydropropyl methacrylamide
rhGH	recombinant human growth hormone
rIGF-1	recombinant human insulin-like growth factor-1
RIA	radioimmunoassay
RPLC	reverse phase liquid chromatography
rtPA	recombinant human tissue plasminogen activator
SEC	size exclusion chromatography
TFA	trifluoroacetic acid
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
Tris	tris-(hydroxymethyl-)aminomethane

Abbreviations used for amino acids:

Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Cysteine	Cys
Glutamic acid	Glu
Glutamine	Gln
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tyrosine	Tyr
Tryptophan	Trp
Valine	Val

Asx	asparagine and asparatic acid
Glx	glutamine and glutamic acid

CHAPTER ONE
INTRODUCTION

1.1 Drug Delivery

Conventional forms of drug administration generally rely on pills, eye drops, ointments and intravenous solutions (Langer, 1990). Newer and complex drugs such as proteins are becoming available through the recent advances in the application of recombinant DNA technology (Oliyai and Stella, 1993). The delivery of these drugs is often more complicated than that of the conventional drugs. Generally, the larger the molecule the greater the problems that will need to be overcome (Davis, 1992). Rapid clearance mechanisms in vivo of proteins as drugs must be taken into account. In order to maintain the effective drug concentration in the blood, multiple and/or high dosing is often needed. Furthermore, as proteins do not pass through the gut wall and can be degraded by proteases in the gut, they readily lose their medicinal activity in the process. Therefore, it is difficult to deliver them through the conventional oral or transdermal routes. Based on these features, proteins are typically administered by frequent injection into the blood stream (Gianturco, 1992).

A number of new drug delivery approaches have been developed recently. These approaches include drug modification by chemical means, drug entrapment in small vesicles that are injected into the blood, and drug entrapment within pumps or polymeric materials that are imbedded in desired bodily compartments, such as beneath the skin and in the abdomen (Langer, 1990).

Chemical modification of drugs is designed to selectively alter such properties as biodistribution, pharmacokinetics, solubility, or antigenicity, and reduce toxicity as well. One example is drugs that are modified to cross a normally impermeable barrier, such as the blood brain barrier. Several approaches have been developed. Drugs have also been bound to soluble macromolecules such as proteins, polysaccharides, or synthetic polymers via degradable linkages. One example is the antitumor agent, doxorubicin linked to N-(2-hydroxypropyl) methacrylamide copolymers which showed radically changed pharmacokinetics, resulting in reduced toxicity. The half-life of the drug in the bloodstream and the drug levels in the tumor were increased while the

concentrations in the periphery dropped (Kopecek and Duncan, 1987). Polymers, such as polyethylene glycol (PEG), could be linked to drugs to either prolong their lifetime or alter their immunogenicity (Hershfield et al, 1991).

In an attempt to lengthen the lifetime of drugs in terms of reducing multiple or high dosing, a number of approaches have been investigated for the sustained release of drugs in vivo (Battersby et al, 1996). One example is to either administer a soluble protein-carrier conjugate, or covalently modify the protein to decrease the breakdown and/or clearance rates. The attachment of polyethylene glycol, glycosylation and acylation are examples of the covalent modifications (Oliyai and Stella, 1993).

1.2 Controlled Release Systems

Controlled release systems deliver a drug at a desired rate for a definite time period. One problem of conventional drug administration is the fluctuation of drug blood concentration. Since each drug has a therapeutic range above which it is toxic and below which it is ineffective, oscillating drug levels may lead to alternating periods of ineffectiveness and toxicity. In contrast, the biggest advantage of controlled release systems is to maintain the drug in the desired therapeutic range by a single administration. From the viewpoint of patients, convenience and compliance are dramatically improved. Another strength of controlled release systems is the increased local concentration of drugs while lowering the systemic drug level. Such targeted controlled release systems are generally desirable, when efficacy cannot be obtained by systemic delivery either because of adverse reaction at sites other than the target or because insufficient drug reaches the target at maximum achievable doses. Furthermore, the stability of drugs, such as proteins which are biologically sensitive molecules, can be increased by protecting from rapid degradation (Langer, 1990).

In the controlled release systems, release rates are controlled by the design of the system and are nearly independent of environmental condition, such as pH. These systems can also be applied to release drugs for long time periods (days to years). The optimal control can be achieved if the drug is put in a polymeric material or pump. Pumps have advantages of very accurate release rate, when compared with polymeric systems, though they are more costly and need surgery for implantation (Langer, 1990).

The drug-release mechanism can be physical or chemical in nature. Diffusion is always involved (Leong and Langer, 1987). In the physically controlled release systems, release is driven by the concentration gradient, in other words, it is by diffusion. It can also be driven by osmotic pressure or matrix swelling. Non-biodegradable matrix and membrane devices belong to this group catalogue. The two common chemically controlled systems are A. biodegradable matrices in which the drug is dispersed and B. polymer-drug conjugates in which the drug molecules are bound to the side chains of the polymer. Release is mainly controlled by the hydrolytic or enzymatic cleavage of the relevant chemical bonds, though diffusion of the reactants and the liberated drug molecules may still be rate limiting.

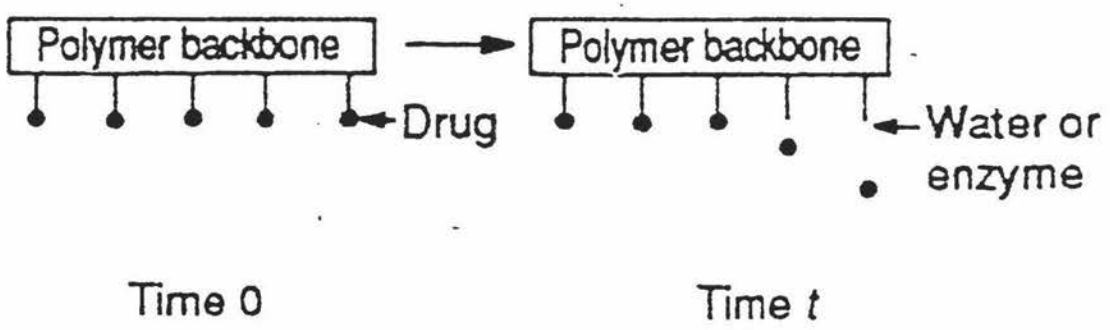
Controlled release of a macromolecule from a biocompatible polymer was first reported in 1970. Cyclazocine, an analgesic was released from a poly(lactic acid) implant (Dang & Saltzman, 1994). Polymer systems are now being applied to animal studies to release many proteins, including insulin, growth factors, and angiogenesis inhibitors (Brown et al, 1986). The first USA Food and Drug Administration (FDA) approved system for controlled release of a peptide, Lupron was introduced for the treatment of prostate cancer (Langer, 1990). A month-long effect from a single injection of microencapsulated human hormone via sustained release was reported in 1996 (Johnson & Cleland, 1996).

Microspheres containing proteins have been designed to provide a continuous release of protein overtime by using the technique of microencapsulation in biodegradable microspheres. Three phases are involved in the release of protein: An initial burst, diffusion controlled release, and erosion controlled release (Cleland, 1995, 1996). In order to maintain a continuous release, the initial burst phase must be minimized, the protein loading within the microspheres should be maximized. Stable formulations of recombinant human growth hormone (rhGH) and interferon-γ for microencapsulation have been achieved by Cleland and Jones (1996). Undoubtedly, encapsulation has been one of the main drug delivery systems studied for the last fifteen years (Langer et al, 1996), which provides an option for slow release of drugs.

1.3 Polymer-Drug Conjugates

Over the last three decades, intensive efforts have been made to design systems capable of delivering the drug more efficiently to the target site. Since one of the main problems in chemotherapy is the limited selectivity of most common drugs, a variety of approaches have been made: low molecular weight prodrugs, macromolecular carriers including immunoconjugates (Baldwin et al. 1990), natural polymers (Sezaki, et al. 1989) and synthetic polymers (Putnam and Kopecek, 1995). Most of these are the combinations of the drug with a polymer. A polymer acts as carrier wherein the drug is dispersed or dissolved, or to which it is covalently linked.

Water-soluble polymers to which a drug is covalently linked have been investigated widely. The carrier can be a nonbiodegradable or a biodegradable polymer. The drug can be linked either directly or via a spacer group onto the polymer backbone. A proper selection of spacer offers the possibility for controlling the site and the rate of release of the active drug from the conjugate by hydrolytic or enzymatic cleavage (Soyez et al. 1996).



Scheme 1.3.1 Drug release mechanism (Langer, 1990)

Several criteria should be taken into account when selecting a polymer as carrier:

- chemical composition: suitable functional groups necessary to permit covalent linkage to drug or target moiety.
- water solubility.
- biodegradable: degradable and/or excretable from the body.
- biocompatibility: non-toxic, non-immunogenic.
- availability: reproducibly manufactured and conveniently administrated to patients.

A number of carriers have been examined, such as vinylpolymers (e.g. N-2-hydroxypropyl methacrylamide, PHPMA); polysaccharides (e.g. dextran); synthetic poly(-amino acids) (e.g. poly(L-amino acids)); proteins (e.g. serum albumin); poly(ethylene glycol) (PEG).

Dextran has a number of attributes that are required of a good drug carrier and it has few side-effect (Larsen 1989). Since the clinical use of dextran as plasma expander and its claimed biodegradability has lasted for four decades, these side effects (anaphylactoid reactions) have been well studied and minimized. Dextran is a generic name for a large class of α-D-glucans that have high water solubility, a well-defined repetitive chemical structure, and are available commercially in a range of molecular weights from 2 kilodaltons to 1 megadalton. They are one of a number of macromolecules that are devoid of structure transport. The overall carbohydrate structure is linear with 95% of the linkages being α-1,6 and the remainder being α-1,3. The α-1,3 linkage has branch points with 85% of the branches being 1 to 2 residues in length. The remaining 15% of the side chains are of multiple residue length. The hydroxyl groups on the glucose sub-units that make up dextran are accessible for direct drug fixation or they may be further derivatized for drug immobilization (Larsen, 1989). Dextrans above 55 Kda that are not cleared via kidneys, are partially depolymerized by dextranases in the liver and spleen, and then excreted (Molteni, 1979; Larsen, 1989). The attachment of ligands may increase or decrease the rate of depolymerization. Rare dextran induced anaphylactoid reactions can be circumvented by preinjection of the monovalent hapten dextran preparation, promiten. However, covalent modification of pendant groups has been shown to reduce the susceptibility of normally biodegradable polymers to enzymatic attack. It has been demonstrated that modification of dextran reduces to the rate of enzymatic hydrolysis by dextranases (Vercauteren, et al. 1992).

Dextran has also be employed as a link between drugs and antibody (Ab) carriers that are used to direct the drug to the site of action (Larsen, 1989; Berstein, et at, 1978; Hurwitz, 1983). At high drug concentrations the drug-dextran-Ab carrier conjugates have been shown to be more efficient than the free drug in vivo experiments.

1.4 Modification of Proteins

As requirements for high quality protein increase, it has become more and more necessary to improve the properties of food proteins to meet the world's protein need. Demands for therapeutic proteins as drugs are so urgent that more and more protein drugs are being developed and have already lessened many patients' suffering. Chemical and enzymatic treatments are described as two main approaches. For many years a motivation factor in the study of the chemical modification of proteins was the desire to determine quantitatively the amounts of proteins or their component amino acids. Commercial application of the chemical modification of proteins has a long history related to the pharmaceutical, dyeing, and clothing industries. An early application in the pharmaceutical industry was the use of formaldehyde to modify bacteria toxins and viruses, similar procedures are still important commercially. The purpose of this treatment is to kill, inactivate, or so change the virus or toxin as to render it incapable of eliciting its toxic or pathological response, while retaining its ability to elicit an immunogenic response when injected into an animal. The bacterial toxins, when so modified, are known as toxids (Feeney and Whitaker, 1977).

The investigation of relationships between biological proteins and specific residues is currently one of the most active applications of the chemical modification of proteins. An essential group in a protein is any group that is required for the protein's function. An essential group could be one of several active-site groups or the remote groups, which would be essential to maintaining the protein's correct conformation. Another objective is to introduce Special Purpose Groups (Reporter Groups), which may serve as labels for tracing the protein through some physical or biological series of events or as labels to monitor changes in the conformation of the protein. For example, a small molecule attached to a protein for the purpose of eliciting antibodies to the small molecule when the molecule-protein conjugate is injected into an animal is called a hapten. When antibodies are made for the hapten, many different types of experiments can be done, either with the free hapten and the antibody, or with the hapten attached to the protein and the antibody (Feeney and Whitaker, 1977).

Modifications have been used extensively to change physical properties of proteins, although they are used more frequently to modify specific active-center residues for changing biochemical or chemical functions. Changing physical properties can, of

course, result in changes in the biochemical functions as well. Modifications, which alter the charges on amino acid side chains, usually effect profound changes in the properties. The most obvious change is one in the isoelectric point of a protein, but the changes in charge can also affect the conformation of the protein and thus its overall functional activity.

Reversible modification can be used to form derivatives with chemical reagents that can easily be removed later. Such reagents can be useful to temporarily block the reaction of active or reactive groups with other reagents (Means and Feeney, 1971).

Chemical modification of proteins is an important everyday tool of basic protein chemistry. Its role in applied protein chemistry appears to be increasing and branching into different areas such as food, nutrition, and pharmaceuticals. Modifications also occur in natural deterioration. Generally these modifications are with the most reactive side chains and are predominantly oxidations, reductions, nucleophilic and electrophilic substitutions. Deteriorations include peptide bond scissions, racemizations, β-eliminations, and formation of products by the reaction of proteins with added chemicals. Proteins are modified intentionally for structure-function relationship studies or for development of new and improved products. Although appearing quite varied, the techniques used in pharmacological, food and feed, or other industrial areas differ more operationally than from major differences in the levels of chemical sophistication that are used.

Enzymes catalyze two types of modifications of proteins, hydrolytic and non-hydrolytic. The modifications, occurring in vivo during or after translation of the protein, are highly specific and may affect markedly the biological, chemical and physical properties of the protein. Specific proteolysis can be a control process, allowing the biological activity of a number of proteins to be expressed. Although a number of enzymatic modifications are poorly investigated or understood, enzyme-catalyzed reactions, which take place under mild conditions, may be useful when chemical methods either are not feasible or may present potential health hazards due to possible toxicant formations (Feeney and Whitaker, 1982).

1.5 Periodate Oxidation of Dextran

Dextran T-40 was chosen as the model carrier for this study. Actually, Dextran T-40 covers a range of molecular weights, but averages 40,000 Da, and most of them are in the range between 30 K and 50 K. The hydroxyl groups on the glucose could be oxidized to give aldehydes, which form imine linkages with the amino groups on proteins. Several agents have been used for the activation of dextran, including periodate, azide, cyanogen halides, organic cyanates and epoxyhalopropyl. When the vicinal diols of dextran are reacted with periodic acid, dialdehyde products are formed (Perlin, 1980). The periodate oxidation of dextran is a competitive, consecutive reaction, with the added complication that the intermediates formed after the first oxidative attack may exist either in a reactive, acyclic form or as an unreactive cyclic hemiacetal. The complete reaction scheme is shown in Fig 1.5.1 (Aalmo and Painter, 1981; Yu and Bishop, 1967). The first step involves the formation of single oxidized intermediate (2 possible forms and their unreactive hemiacetals). A single active double oxidized species and formic acid are formed as final products from a second oxidation step.

Periodate oxidation of Dextran T-40 has been examined in both buffered and unbuffered conditions (Puchulu-Campanella 1991-1993). It has been confirmed that under buffered conditions, there is a more rapid decay of single oxidized intermediates due to the instability of hemiacetals (k_4, k_4', Fig.1.5.1), and there is greater formation of the double oxidized residues in Dextran T-40. It is more likely that the double oxidized residues are the more desirable form for reaction with nucleophilic agents, and the hemiacetal dead-end reduces the accessibility of aldehyde for the reaction with the nucleophiles. Imine bonds can be formed when aldehydes react with the amino groups in proteins. More precisely, the oxidized dextran can react with ε-lysine and the N-terminal amino groups in proteins to form imine complex.

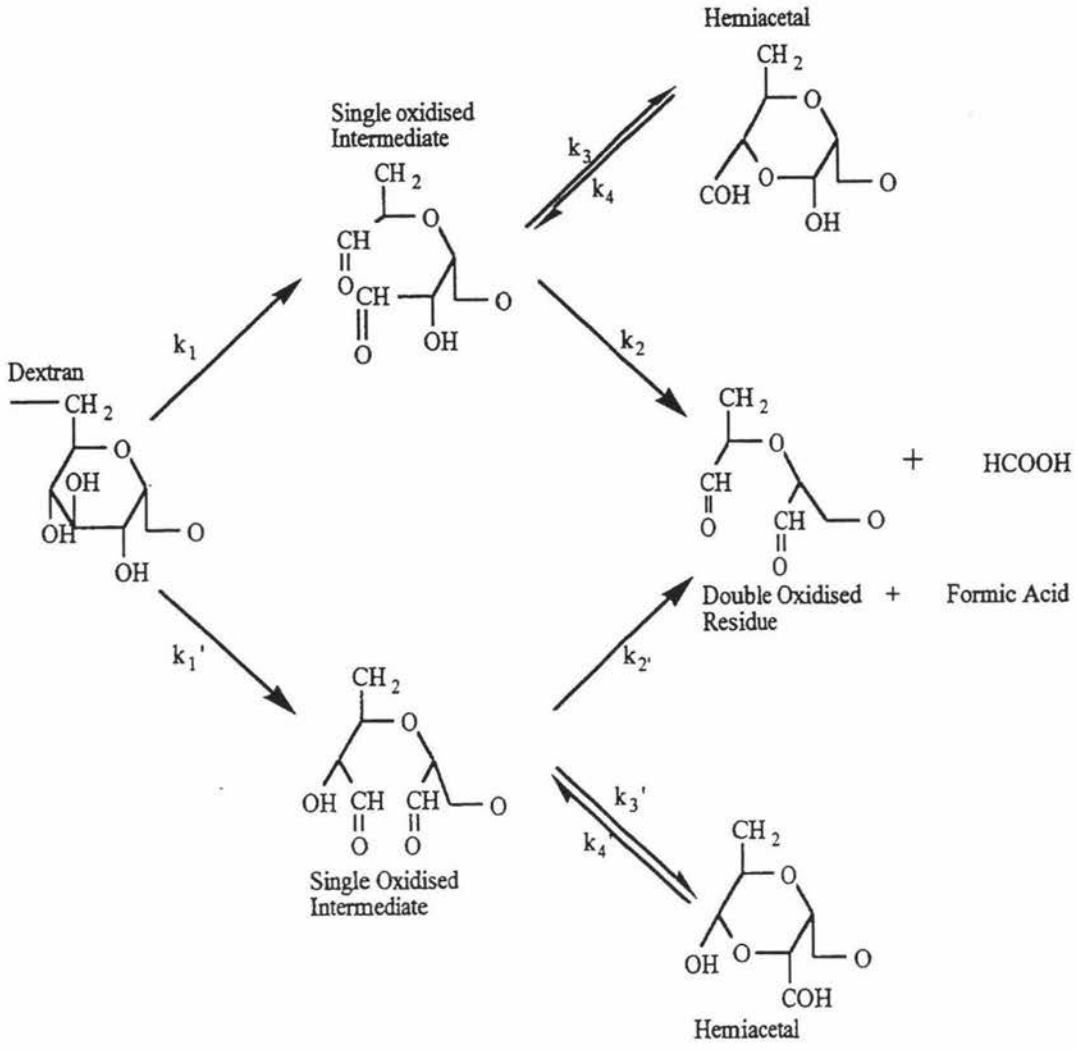


Figure 1.5.1 Periodate Oxidation of Dextran (Battersby et al 1996)

The level of activated or oxidized dextran molecules can be expressed as a percentage of glucose units that have been oxidized by partial sodium periodate oxidation of dextran (Puchulu-Campanella 1991-1993). The assumption is made that 2 moles of oxidant are consumed per glucose residue. This calculation is performed by taking aliquots of oxidized dextran and titrating with iodine following the addition of arsenite.

$$H_2AsO_3^- + IO_4^- \rightarrow H_2AsO_4^- + IO_3^-$$

$$H_2AsO_3^- + I_2 + H_2O \rightarrow H_2AsO_4^- + 2I^- + 2H^+$$

The arsenite reacts with the unreacted periodate and the excess of arsenite is then titrated with iodine. Moles of periodate consumed are determined from the difference

between the amount of arsenite added and the amount of iodine required to take the titration to the end point. 1 mg of dextran is equivalent to 6.25 μmole glucose. Therefore the amount of glucose present can be calculated. As it takes 2 moles of periodate to oxidize 1 mole of glucose, the percentage of activation is equal to half the moles periodate used over the moles of dextran present times 100.

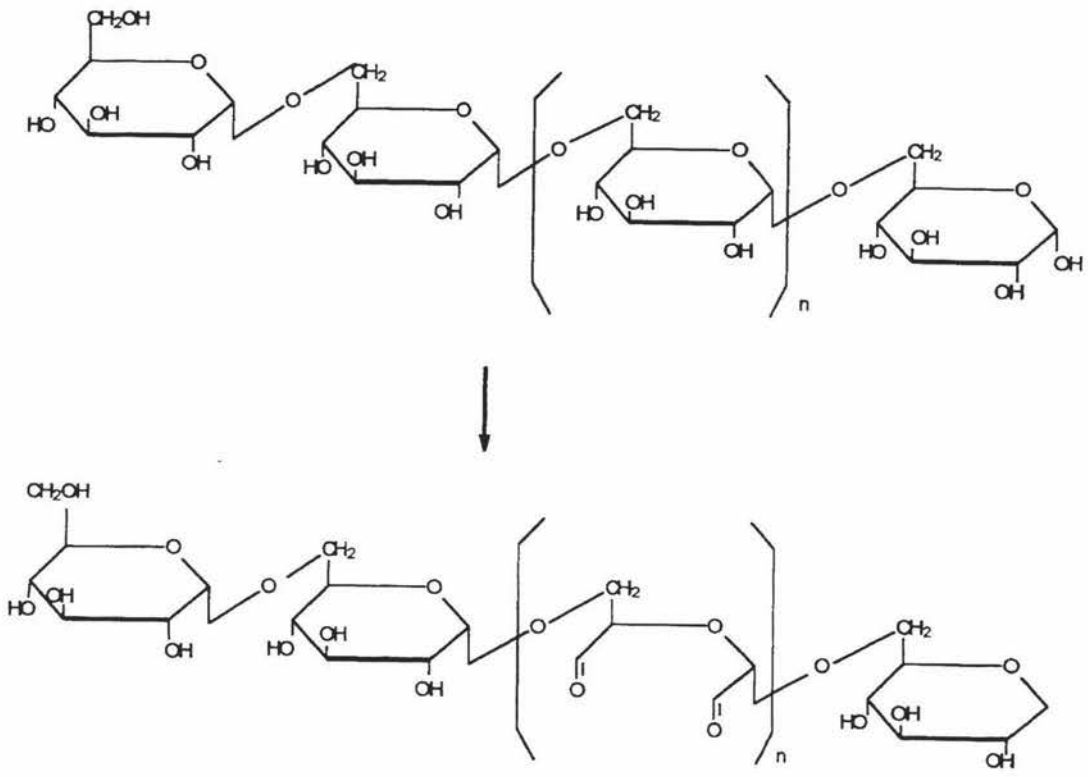


Figure 1.5.2 Overall reaction individual glucose molecule periodate oxidation

Activation of dextran (Fig. 1.5.2) can be expressed as a percentage of double oxidized residue (%DOR), which it is equal to:

$$Ax^2 + Bx + C$$

Where x is the percentage oxidation and A, B and C are constants (8.336×10^{-3}, 7.648×10^{-2} and 2.63 respectively). The equation was derived from the curve obtained from the comparison of two groups of experimental data (Fig. 1.5.3) (Battersby et al. 1996).

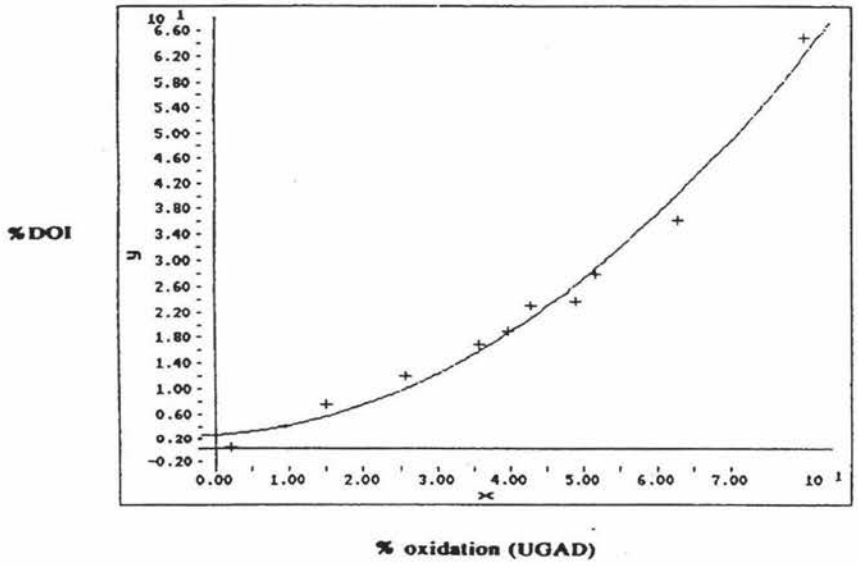


Figure 1.5.3 Relationship between degree of oxidation and % double oxidized residues.

1.6 Imine Formation

The dialdehyde of activated dextran mentioned above may react with amino groups in different ways. Two of these possibilities, an imine or an oxazepine (Kobayashi, et al. 1990), were investigated with reference to the dialdehyde of cyclodextrin, an α-1,4 linked cyclic glucan. While the oxazepine linkage is stable, it caused irreversible inactivation between oxidized dextran and phosphorylase kinase. Reaction conditions dictate to a certain degree the type of product formed. Imine formation is favored at higher temperatures and pH (Kobayashi and Funane, 1993).

In the presence of sodium borohydride or sodium cyanoborohydride, the imine product (or labile Schiff's base), forms a stable, non-labile, single-bond linkage upon reduction due to the reduction of the carbon-nitrogen double bonds. This has been used for immobilization of several proteins on to periodate oxidized dextran, such as daunomycin, hemoglobin-dextran conjugates, kallikrein, bradykinin and several enzyme inhibitors (Battersby et al, 1996). Modification by reduction of imine conjugates was seen as a way to avoid the unstable conjugate. There is now evidence that imine conjugates are more stable than once was thought, and hence the reduction may not be necessary to obtain a useful product. This was evident in the case of trypsin (Kobayashi and Takatsu, 1994) which after conjugating with dextran was stable enough without reduction to be isolated by size exclusion chromatography (SEC).

$$RCHO + H_2N\text{-Protein} \leftrightarrow RCH{=}N\text{-Protein}$$
$$\downarrow$$
$$RCH_2\text{-}NH\text{-Protein}$$

Several criteria should be considered upon the binding of a protein to dextran: the MW range of dextran, the type of activation and the amount of protein desired in the resulting complex (Molteni, 1979). It is also feasible to dictate coupling via the protein (Mitra et al, 1993). For example, bilirubin oxidase had amino groups (spacer groups) introduced to allow and enhance its binding to PEG. With purine nucleoside phosphorylase, PNP, additional binding sites for PEG were required. This was achieved by site-directed mutagenesis of some of the arginine residues to lysine, giving rise to additional binding. As a result, the mutant enzyme maintained its catalytic activity and the resulting conjugate was less susceptible to immune responses.

1.7 Complex Formation of Proteins with Dextran

It has been concluded that the rate of formation of the complex is proportional to the level of oxidation of dextran (Fig.1.7.1), while the rate of release of the protein from dextran conjugate is inversely proportional (Puchulu-Campanella, 1991-1993). Since the level of activation of dextran is controllable (Battersby et al. 1996), a balanced intermediate needs to be sought. The specific degree of oxidation should be chosen so that a desired rate of release from the complex is obtained. Three levels of activated dextran (7%, 21% and 56%) were chosen for a recent investigation (Fisher, 1997).

The circulatory lifetime of dextran could be increased due to the conjugation of a protein to dextran (Marshall, 1978; Mitra, et al. 1993). Clearance of the protein via renal filtration is prevented when the molecular weight of a complex containing it is above 70 Kda. Once conjugated there is reduced likelihood of the protein being recognized by proteases, inhibitors and pro-existing antibodies. Conformational stability of the tertiary structure of the protein has been suggested to result from the multipoint binding that occurs with dextran conjugates (Mitra, et al. 1993). Proteins are also protected by the steric hindrance of the complex structure. If the steric hindrance proves to be a drawback, spacer arms can be introduced (Larsen, 1989). This requires

careful selection of the spacer arm and spacer-drug bond. It should therefore be possible to choose a spacer-drug link that will be cleaved at the target cells by enzymes secreted locally. Spacer arms can also be used when direct binding between the protein and the polymers is impossible.

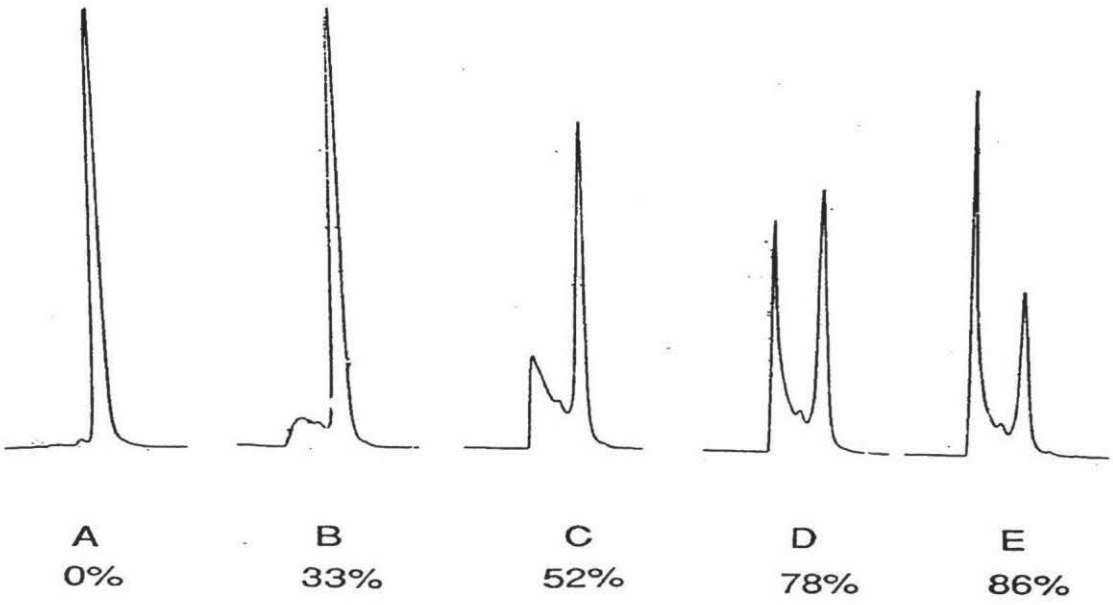
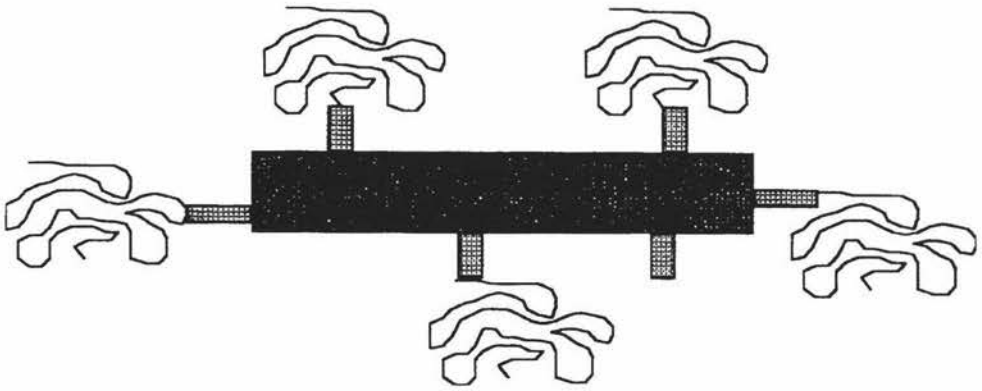
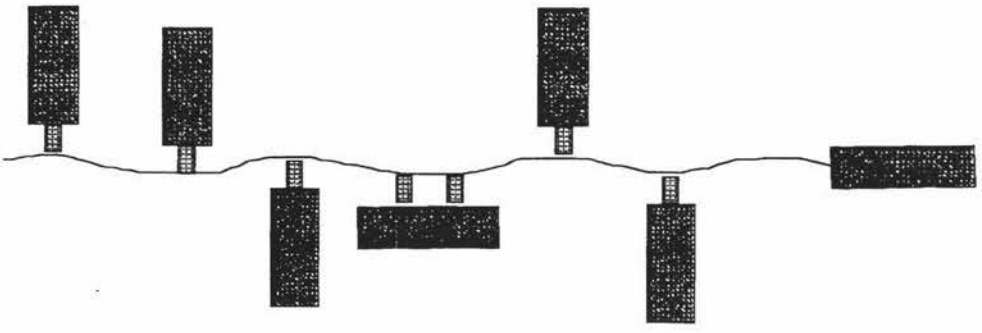


Figure 1.7.1 Extent of complex formation over increasing dextran activation levels for a 24hr period (Puchulu-Campanella 1991-1993).
met-hGH was incubated with increasing levels of activated dextran for 24hrs.
Aliquots were taken for each level of activation for SEC.

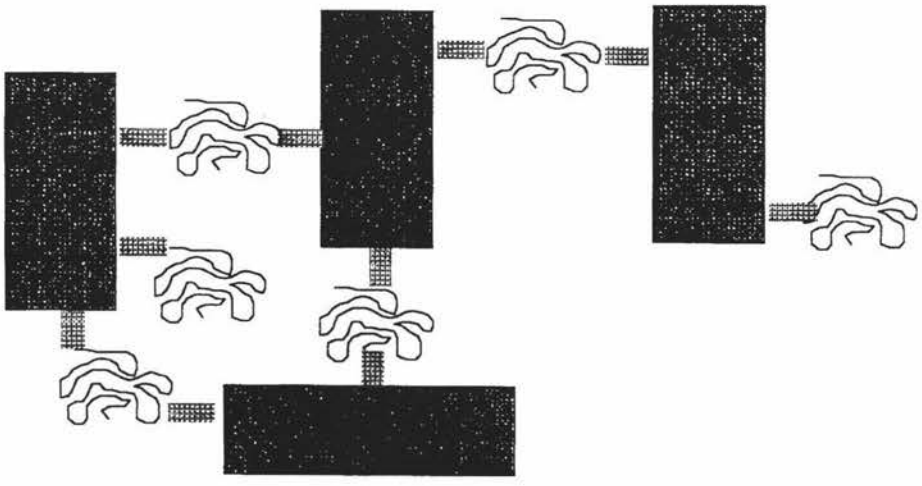
The exact final structure of protein-dextran complexes is not known. It is possible that there is one dextran molecule binding to several protein molecules or vice versa (Fig.1.7.2 (a) and (b)), but it is probably more likely that the monosaccharide molecules within the dextran bind to several different protein molecules which in turn bind to several different dextran molecules, resulting in intermolecular crosslinking (Fig.1.7.2 (c)). The situation however may vary depending on the size of the protein, the number of lysine residues present (Fisher, 1997), or the ratio of activated dextran to protein used.



a)



b)



c)

Figure 1.7.2 Possible structure of Protein-Dextran Complex (Fisher, 1997)

a) Several dextran molecules bound to one protein molecule
b) Several protein molecules bound to one dextran molecule
c) Random crosslinking between dextran and protein molecules

1. 8 Sustained Release of Proteins from Dextran

Genentech Inc., a South San Francisco based company, has preliminarily investigated a method for sustained release of proteins. A soluble, reversible protein-dextran conjugate was chosen for the study of the potential for prolonging the biological half-life of a protein (Hancock and Battersby, 1991). Unmodified recombinant human growth hormone (rhGH) was released from the rhGH-activated dextran conjugate in vitro. This was determined by N-terminal sequencing and tryptic mapping (Battersby et al. 1996). The results in vivo found that the dextran-GH conjugate showed a larger initial weight gain in rats between days 1 and 2. At the same time control experiments indicated that the effect of injected rhGH, on weight gain, is transient in the same hypophysectomized rats. It was shown that the rhGH-dextran conjugate slowly released growth hormone and maintained a higher level of growth hormone in the blood over a 2-day period.

Although this initial release of rhGH only covered a two-day period, it suggests the possible extension of this technique by either varying the activation level of the dextran or by using different range MW of dextran polymers, or both. The complex itself was formed readily and was stable at 4°C at least ten days (Puchulu-Campanella 1991-1993). It could be stored for longer periods at -70°C without loss of activity. Protein release was studied by comparing reduced (with sodium cyanoborohydride) and non-reduced complexes. It was shown that release required a non-reduced bond, presumed to be an imine. Overall it was found that the adduct formed between the rhGH and oxidized dextran was biologically active, and the presence of rhGH in rats was shown to be prolonged. When rhGH is released in the bloodstream, formation of non-specific complexes between dextran and other proteins was not found to be a problem in the limited study carried out (Battersby et al. 1996).

Several other proteins have been studied for their ability to form complexes with the activated dextran (Puchulu-Campanella 1991-1993). These included rIGF-1 (MW 7,655Da), CD4 (MW 40,962Da), rtPA (recombinant tissue plasminogen activator, MW 59,042Da), and GP120 (glycoprotein-120 MW 120,000Da). All were found to have the ability to bind to oxidized dextran. From the kinetics of complex formation, it was found that rIGF-1 (the smallest) was the fastest, while GP120 (the largest) was the slowest at complex formation. CD4, a cell surface glycoprotein receptor for HIV, is twice the molecular weight of Met-hGH. Kinetic studies showed complex formation to

be slower for CD4 which was thought to result, at least in part, form steric hindrance caused by the carbohydrate attached to the protein. IGF-1, recombinant human growth factor-1, has the fastest kinetics for complex formation. Release studies for IGF-1 showed a drop in the complex concentration, but no signs of the free protein. Size exclusion chromatography (SEC) showed that upon release, the protein had been hydrolyzed into smaller fragments, which had longer retention times than IGF-1. GP120 was the largest protein studied (MW 120,000) and it was also shown to form a complex with the dextran. Thus the formation of imine adducts between proteins and organic polymers appeared to be a general phenomenon.

Enzyme-dextran conjugates were investigated for gaining further quantitative knowledge of models of reversible association-dissociation between the macromolecular components (Fisher 1997). The enzymes studied range form 14 Kda up to ~250 Kda, while different activities of enzymes was chosen to gain an initial impression of the generality of this approach. These included Lysozyme (14.3 Kda), Trypsin (23 Kda), Amylase (48 Kda), Alcohol Dehydrogenase (150 Kda) and Catalase (225 Kda). All were found to have the ability to bind to 7 & 21% oxidized dextran, as with the previous studies the speed of complex formation was shown to be greater at the 21% level of activation than the 7% activation of dextran in all cases studied. At 56% activation of dextran it was shown that the complex was unable to release the conjugated protein (lysozyme). Investigations have shown that the level of activation chosen affects the extent of binding and therefore the functions of the resultant complex.

Lysozyme, the smallest protein studied, while binding to lower levels of activated dextran, forms a complex relatively easily. A lysozyme-like species released showed the normal lytic activity. However, upon conjugation lysozyme exhibited only minimal activity. Similarly, released trypsin-like species and unreacted trypsin showed similar tryptic maps for a synthetic peptide designed to show distinctive fragments. While complexed to dextran trypsin showed no sign of activity. Conjugation to dextran did not effect the activity of α-amylase i.e.: it was active. Over time the release of an α-amylase-like species from the complex was observed. Alcohol dehydrogenase and catalase are both high MW proteins. Subsequent experiments showed that upon release the proteins appeared to dissociate, most probably into their subunits. The ADH-complex exhibited enzymatic activity.

1.9 Investigations into the Binding Sites of Insulin-Activated Dextran Complex and Subsequent Release

The complex study reported in this thesis was aimed at studying insulin-activated dextran complex formation and release, and finding a reliable method to interpret the binding nature of protein-dextran conjugates. The next target of our research was to investigate the conditions for the formation of the complex and see whether they could alter the tendency of the binding. The release study should allow the examination of the relationship between the rate of release and the binding nature of the protein-dextran conjugates. The desired binding might lead to the right rate of release for controlled release of proteins.

Insulin was chosen for its low MW, relatively "uncomplicated" structure and stability. Relatively few possible binding sites (A1, B1 α-NH_2 terminal groups and B29 ε-lysine amino residues) with activated dextran was one of the main reasons insulin was chosen for this research study. Successfully solving the "simpler" insulin problems may provide a useful method for exploring more complex protein structures.

The study reported in this these has concentrated on the possibility and propensities of all potential binding sites. Activation of dextran was performed by periodate oxidation. Three activation levels were selected to examine the subsequent release rates of insulin-activated dextran complexes. Formations of complexes at a range of conditions, including pH, molar ratio and shaking, were examined to choose 'ideal' conditions. The resulting complexes were isolated and analyzed for release of insulin. The released insulin was characterized and compared to the original insulin used.

The complexes formed were selectively reduced by sodium cyanoborohydride to obtain non-release complexes, due to the selective reduction of the imine linkages between insulin and activated dextran. The reduced complexes were subjected to the following binding studies. Trypsin digestion was initially applied to determine the extent of the ε-lysine binding. Amino acid analysis (AAA) studies were further employed for the comprehensive understanding of those three binding sites. Further study by chymotrypsin digestion attempted to solve the A1-Glycine binding. Oxidative cleavage by performic acid and reductive cleavage by tributylphosphine were finally used to determine the A1-Gly binding.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents and Equipment

Dextran T-40 was from Pharmacia, Uppsala, Sweden; Trypsin type XIII, (TPCK treated), α-Chymotrypsin type II, Porcine Insulin and Trizma base (RG) were from Sigma Chemical Co., St Louis, MO, USA; Sodium acetate and potassium iodide were from Scientific Supplies Ltd., Auckland, New Zealand; Sodium periodate, sodium azide, acetone, ammonium acetate were from Ajax Chemicals Ltd., Auburn, NSW, Australia; Sodium cyanoborohydride and 4-Vinylpyridine were from Aldrich Chemical Company, Inc., Steinheim, Germany; Formic acid 98/100% was from May & Baker Ltd., Dagenham, England; Ammonia hydroxide was from J.T. Baker Chemical Co., Phillipsburg, NJ, USA; Calcium chloride was from Merck; Convol arsenite, Convol iodine, hydrogen peroxide (30% w/v), hydrochloric acid, HPLC grade, acetonitrile, HPLC grade, 2,4-dinitrofluorobezene(DNFB), tri-n-butyl-phosphine, isopropanol, HPLC grade and ethanol, HPLC grade were from BDH Chemicals Ltd., Poole, England; Sodium hydrogen carbonate (RG) was from Paul Industrial Ltd., Kelston, Auckland; Triethylamine and sodium di-hydrogen phosphate were from Riedel-de Haen AG., Germany; All other reagents were analytical grade wherever possible. Water was MilliQ grade.

Centricon-10 tubes (used for concentration of macromolecular solutions by ultrafiltration through low-adsorption, hydrophilic membranes) MWCO 10,000 were from Amicon®, New Zealand Medical and Scientific Ltd. FPLC and SMART columns (SEC: Superdex 75, HR 10/30 or HR 3.2/30 and RPLC: PepRPC HR 5/5) were from Pharmacia Biotech, Uppsala, Sweden. HPLC columns, Vydac Protein C4 and Econosphere C18 5μm 250mm x 4.6mm, were from Alltech, Auckland, New Zealand. FPLC and SMART systems were from Pharmacia, Uppsala, Sweden. HPLC system was Waters Associate comprising of a model 660 solvent programmer, 2 model 510 pumps, a model 441 of absorbance detector and a Rheodyne injection system. Centrifugation was carried out using a Clandon Model T52. Spectrophotometer was from Hewlett Packard, a model 8452A Diode Array Spectrophotometer, HP 89531A MS-DOS-UV/VIS operating software. Capillary Electrophoresis (CE) was from

Applied Biosystems, Model 270A Analytical Capillary Electrophoresis System. Ultrasonic cleaner was from Cole-Parmer, Model 8891 sonicator. Diaflo ultrafilters, MWCO 3,000 (YM3) membrane in an ultrafiltration device, was from Amicon Division, W. O. Grace & Co., MA, USA.

2.2 Periodate Oxidation

Dextran T-40 (400 mg) was dissolved in 0.2M sodium acetate buffer (50 ml), pH 4.5 and added to 0.1M sodium periodate in 0.2M acetate buffer (50 ml). A blank solution was also prepared by using acetate buffer (50 ml) instead of the dextran solution. Both blank and dextran solutions were kept in the dark with constant stirring at 4°C. Aliquots were taken at time intervals of 0, 15, 30, 45 and 60 minutes for iodometric titration. The time intervals were varied to obtain a range of choosing desired % activations.) Oxidations were halted by the addition of 2 x w/w ethylene glycol. Oxidized dextran was then purified by ultrafiltration using a Diaflo ultrafilter with a 3,000 MW cut off. Purified oxidized dextran was then freeze dried for storage. Level of oxidation was determined by iodometric titration.

2.3 Iodometric Titration

Saturated sodium bicarbonate solution (10 ml), reported to be 6.9 g/100 ml at 0°C and 16.4 g/ml at 60°C (CRC, Handbook of Chemistry and Physics), was added promptly to an aliquot of oxidized dextran (5 ml) or blank solution followed by the addition of sodium arsenite (8 ml) and 20% KI solution (1 ml, 2g in 10 ml saturated bicarbonate solution). This solution was left in the dark for 15 minutes at room temperature. Starch (0.4g) was added and the solution was then titrated against 0.0484M iodine. The end point of the titration was taken at the volume of iodine required to give the solution a faint blue purple color, stable for 5-10 seconds with stirring.

2.4 Complex Formation

Insulin (6 mg) was dissolved in 50/100 mM sodium phosphate buffer, 0.02% sodium azide, pH 7.1/7.4/7.8 (2 ml), and then added to activated dextran (4 mg) in phosphate buffer (2 ml). The molar ratio of insulin over dextran was 10:1. At the beginning of mixing, [insulin] is 1.5 mg/ml and [dextran] is 1 mg/ml. The mixture was shaken gently for 30 seconds, then left in the dark without stirring at room temperature. The progress of the complex formation was monitored with runs of FPLC/SMART system using a

Superdex 75 column. Flow rate was 0.4 ml/min and the chart speed was 0.4 cm/min on FPLC. Before loading, all samples were filtered using 0.2μm Millipore membrane in a Swinny filter. Running buffer was 0.05/0.1M sodium phosphate, pH 7.1/7.4/7.8. Maximum 300μl of complex formation incubation sample was injected per run. All runs on the FPLC were monitored at 214 nm.

2.5 Complex Release

Insulin-dextran complexes were buffer exchanged into 0.05M sodium phosphate, 0.02% sodium azide, pH 7.1/7.4/7.8, on the FPLC using a Superdex 75 column and then incubated in the dark at room temperature at 37°C with shaking. Aliquots were taken and applied on FPLC using a Superdex 75 column at 0.4 ml/min or on the SMART (20-50 μl) using a Superdex 75 (PC 3.2/30) column at 40 μl/min. Released protein species were isolated for further analysis. In some cases, several runs were made on the FPLC, and the peaks were pooled and concentrated to obtain enough material for experiments to follow the release of insulin from the complex. All runs on the FPLC and SMART system were monitored at 214 nm.

2.6 Complex Reduction Studies

Insulin-dextran incubations were set up for several days to allow complex formation. Sodium cyanoborohydride, $NaBH_3CN$, was added to give two fold concentration excess (w/w, $NaBH_3CN$/insulin). Upon addition of sodium cyanoborohydride the incubations were kept in the dark with shaking at room temperature. Incubations were then analyzed for complex formation using SEC (Superdex 75 column) and the peaks were collected. These samples were then studied for any signs of release by pooling and concentrating the desired fractions and subjecting them to further SEC (SMART and FPLC, Superdex 75 column). All runs on the FPLC and SMART were monitored at 214 nm.

2.7 Trypsin Digest and Peptide Mapping

Insulin (1 mg) was dissolved in trypsin digest buffer (1 ml), 100 mM sodium acetate, 10 mM Tris base, 1 mM $CaCl_2$, pH 8.3. Insulin was then digested by addition of 0.02M TPCK-treated trypsin (1:100 trypsin : protein w/w) dissolved in 1 mM HCl (0.5 ml) and incubated at 37°C. After 2 hours a further addition of trypsin (0.5 ml) was added to

give a final ratio of 1:50 (trypsin : insulin, w/w). After 4 hours the incubation was halted by the addition of formic acid to lower the pH to 2-3.

Trypsin mapping was then established with an FPLC using PepRPC HR5/5 column. Buffer A was 0% acetonitrile, 0.1% formic acid and buffer B was 60% acetonitrile, 0.1% formic acid. All samples were filtered through a 0.2 μm Millipore membrane in a Swinny filter before loading onto the column. A linear gradient was run from 0% to 100% B over 30 minutes. Concentration was held at 100% B for 10 minutes and a short gradient of five minutes was run back to 0% B, and re-equilibrated with 0% B for 25 minutes. Flow rate was 0.5 ml/min.

Trypsin digestion and mapping of the isolated reduced insulin-dextran complex were followed the same procedures as above. The dextran-insulin fragment complex was isolated by SEC (Superdex 75) and subjected to acid hydrolysis and AAA.

2.8 Chymotrypsin Digest and Peptide Mapping

Porcine insulin (4.3 mg) was dissolved in digest buffer (0.86 ml of 100 mM sodium acetate, 10 mM Tris base and 1 mM $CaCl_2$, pH 8.3). Insulin was then digested by addition of 17.2 μl α-chymotrypsin (2.5 mg/ml in 1 mM HCl) and incubated at 37°C. After 2 hours a further addition of chymotrypsin (17.2 μl) was added to give a final ratio of chymotrypsin to insulin of 1 : 50 (w/w). After 4 hours the incubation was halted by the addition of formic acid to lower the pH to 2-3.

Chymotryptic mapping was then followed on the SMART system using the μRPC C2/C18, PC3.2/3 column. Buffer A was 5% acetonitrile, 0.1% TFA and buffer B was 90% acetonitrile, 0.1% TFA. All samples were filtered through a 0.2 μm Millipore membrane in a Swinny filter before loading onto the column.

After an isocratic hold at 0% B for three minutes, a linear gradient was run from 0% to 76% B over 24 minutes followed by a 2 minutes gradient to 100% B. Concentration was held at 100% B for 2 minutes and a short gradient of five minutes was run to return to 0% B, and re-equilibrated with 0% B for 7 minutes. Flow rate was 100 μl/min. All runs were monitored at 214 nm and 280 nm.

Chymotrypsin digestion and mapping of the isolated reduced insulin-dextran complex were followed the same procedures as above.

2.9 Preparation of DNP-Insulin

0.1g crystalline insulin and 0.1g $NaHCO_3$ were dissolved in 1 ml water and 2 ml ethanol. 0.1 ml DNFB was then added, and the mixture mechanically shaken for 2 hours. The DNP-insulin, which had precipitated as an insoluble yellow powder, was centrifuged and the precipitate washed with water, ethanol and ether, and air-dried. DNP-insulin prepared above was then sent for amino acid analysis.

Preparation of DNP-insulin-dextran followed similar procedures as above. 1.2 ml of reduced insulin-dextran complex (~0.9 mg insulin) was placed in 2 Centricon-10 tubes, topped up with MilliQ water and centrifuged. Once the retentate was reduced to a small volume, it was diluted with MilliQ water, and centrifuged again twice until a limited amount of sample was left.

Then 1 mg $NaHCO_3$, double the volume of the liquid sample of ethanol and 1 µl DNFB was added to the liquid sample. The solution was shaken for a day until a precipitate appeared. The mixture was then placed in ordinary centrifuge tubes, centrifuged, washed with MilliQ water, ethanol and ether, dried in the air. The yellow precipitate was subjected to acid hydrolysis for 8 hours and AAA.

2.10 Oxidative Cleavage of Disulfide Bonds of Insulin

25 mg of insulin was dissolved in 0.9 ml formic acid and 0.1 ml 30% (w/w) hydrogen peroxide was added. The mixture was then allowed to stand for 15 minutes at room temperature. Water (1 ml) was added and the mixture evaporated in vacuo to a small amount. The oxidized insulin was then precipitated by a large amount of acetone, centrifuged, washed with acetone until free of formic acid and dried in air.

Freshly oxidized insulin (25 mg) was dissolved in 1.2 ml 0.1M NH_3, and 0.1M acetic acid was added to bring the pH of the solution to 6.5. The precipitate was centrifuged and used in the preparation of fraction B. The precipitate was washed with 10 ml 0.01M acetic acid, dissolved in 5 ml 0.1M HCl and 40 ml absolute ethanol added. This brought about the separation of a precipitate, which was centrifuged down and washed well with 80% ethanol. The combined supernatant solution and washings were taken almost to dryness in vacuo and a further precipitate was isolated by adding a large volume of acetone. This residue was chain B.

The solution was brought to pH 4.5 with 0.1M acetic acid and, after removal of the precipitate, was taken almost to dryness in vacuo. It was then transferred to a centrifuge tube with a minimum volume (about 3 ml) of water, and an equal volume of 50% (w/v) ammonium acetate, which had been brought to pH 5.5 with glacial acetic acid, was added. This brought about the separation of a small precipitate, which was discarded. The water and ammonium acetate solution was then removed by leaving the solution in a high vacuum over H_2SO_4 and NaOH until it reached constant weight. The residue was a white powder, chain A.

Capillary Electrophoresis Method

Fresh oxidized insulin was dissolved in 0.02M ethanolamine buffer, pH 7.8 (titrated by H_3PO_4, filtered with C_{18}-SEP-PAK filter). The buffer for CE was the same buffer as above, and the buffer was degassed by ultrasound before being used. 20 KV was applied for each CE run at 30°C. The rate of the chart recorder was 0.1 cm/min. Each running time ranged from 30 minutes to 60 minutes.

Reverse Phase Liquid Chromatography

Peptide mapping was followed by FPLC by using PepRPC HR5/5 column. Buffer A was 0% acetonitrile, 0.1% formic acid, and buffer B was 100% acetonitrile, 0.1% formic acid. All samples were filtered through a 0.2 μm Millipore membrane in a Swinny filter before loading onto the column.

A linear gradient was run form 10% to 60% B over 30 minutes. A short gradient of 5 minutes was run to return to 10% B, and re-equilibrated with 10% B for 25 minutes. Flow rate was 0.4 ml/min.

Isolated reduced insulin-dextran complex (containing about 0.5 mg insulin) was dissolved in 60 μl formic acid and 5 μl 30% (w/w) hydrogen peroxide was added. The mixture was then allowed to stand for 15 minutes at room temperature. Water (0.1 ml) was added and the mixture evaporated in vacuo to a small amount. The oxidized insulin-dextran complex was then precipitated by a large amount of acetone, centrifuged, washed with acetone until free of formic acid and dried in air.

The oxidized insulin-dextran complex was isolated by using SEC (Superdex 75) and concentrated by using Centricon-10 tubes, then subjected to hydrolysis and AAA. The peptide mapping was followed the same procedures as that of oxidized insulin.

2.11 Reductive Cleavage of Disulfide Bonds of Insulin

Porcine insulin (5.2 mg) was dissolved in 1M triethylamine-acetic acid, pH 10 (500 µl). After evacuating and flushing with nitrogen, 500 µl of PBu_3 was added as an aliquot of a 2% solution of PBu_3 in isopropanol. 5 µl 4-vinyl-pyridine was added together with the phosphine. After 5 hours, the solution was reduced by rotary evaporation (bath temperature 37°C) and resuspending in 500 µl of isopropanol. The evaporation, resuspension and evaporation were repeated. The procedure cleaved the disulfides and modifies the resultant -SH groups.

The products were dissolved by adding 50% acetic acid solution. Peptide mapping was followed by FPLC by using PepRPC HR5/5 column. Buffer A was 0% acetonitrile, 0.1% formic acid, and buffer B was 60% acetonitrile, 0.1% formic acid. All samples were filtered through a 0.2 µm Millipore membrane in a Swinny filter before loading onto the column.

After an isocratic hold at 15% B for 15 minutes, a linear gradient was run form 15% to 50% B over 30 minutes following by a 10 minutes gradient to 100% B. A short gradient of 5 minutes was run to return to 15% B, and re-equilibrated with 15% B for 20 minutes. Flow rate was 0.3 ml/min. All runs were monitored at 214 nm.

Isolated reduced insulin-16% activated dextran complex (containing about 0.2 mg insulin) was dissolved in 80µl, 1M triethylamine-acetic acid, pH 10. After evacuating and flushing with nitrogen, 20µl of PBu_3 was added as an aliquot (2% in isopropanol). 10 µl 4-vinyl-pyridine (2% in isopropanol) was added together with the phosphine. After 5 hours, the solution was reduced by rotary evaporation (bath temperature 37°C) and resuspending in 300 µl of isopropanol. The evaporation, resuspension and evaporation were repeated.

Peptide mapping of treated insulin-dextran complex was followed the same procedures as that of insulin. The insoluble precipitate was centrifuged and washed with 50% acetic acid and 0.1% formic acid several times, and then subjected to acid hydrolysis and AAA.

2.12 Mass Spectrum Sample Preparation

Samples (peptides) containing salts other than formates must be desalted for mass spectrum. RP-FPLC system is used to obtain suitable samples. Buffer A was 0% acetonitrile, 0.1% formic acid and buffer B was 100% acetonitrile, 0.1% formic acid. All samples were filtered through a 0.2 μm Millipore membrane in a Swinny filter before loading onto the column.

After an isocratic hold at 0% B for five minutes, a linear gradient was run from 0% to 100% B over 30 minutes. Concentration was held at 100% B for 10 minutes and a short gradient of five minutes was run to return to 0% B. Flow rate was 0.3~0.5 ml/min. The isolated samples were analyzed by Ms Jo Mudford and Dr. Gill Norris, Biochemistry Section, Institute of Biomolecular Sciences, Massey University.

2.13 Amino Acid Analysis Preparation

Samples were prepared for amino analysis by the addition of 6M HCl and 0.1% phenol (1 ml per mg sample or 200μl). The sample was then hydrolyzed under vacuum at 110°C for 24 hours. Finally the sample was dried down in the dessicator. Amino acid analysis, AAA, was performed by Ms Debbie Frumau, Biochemistry Section, Institute of Biomolecular Sciences, Massey University, Mr. Brian Treleor, Grasslands Research Center, AgResearch, Palmerston North, or Dr. Nick Greenhill, Wellington School of Medicine, Wellington.

CHAPTER THREE
COMPLEX FORMATION AND RELEASE STUDIES

3.1 Introduction

Insulin is a polypeptide hormone produced in the pancreas of all vertebrates. It is primarily a metabolic hormone and plays a vital role in the regulation of energy metabolism in higher animals. The biological effects of insulin embrace most of the vital functions of living organisms. It is essential for development and growth and its absence accelerates aging, and even causes death. Basically insulin acts as a messenger which notifies tissues to store foodstuffs. Insulin stimulates not only anabolic processes leading to storage of carbohydrate in the form of glycogen, but also storage of fats in the form of tricyclglycerol in adipose tissue and incorporation of amino acids into proteins. The major sites of action are the liver, adipose tissue and muscles. Failing to secrete enough insulin to the bloodstream leads to the disease `Diabetes`, from which millions of people are suffering. Animal insulin mainly extracted from the pancreas of pigs and beef or recombinant human insulin must be supplied to diabetics to satisfy the need of their bodies and produce normal function bodily. As a protein whose lifetime is only 5-16 minutes, insulin needs to be injected frequently.

Insulin is a small protein with a MW of about 6,000. Insulins (human, porcine and bovine) are composed of two polypeptide chains, an A chain of 21 amino acids and a B chain of 30 amino acids. Most mammalian insulins have similar sequences and also consist of 3 disulfide bonds. One intramolecular disulfide bond is between amino acids A6 and A11 in the A chain and the other two intermolecular disulfide bonds are A7 and B7 and A20 and B19. These later two hold the 2 peptide chains together. This dual chain structure at the positions of the inter/intra molecular S-S bridges has remained relatively conserved throughout evolution. The function of insulin may be lost if the 3 dimensional structure is upset. Some sequences are invariant. For example, the position of the 3 disulfide bridges, the N and C terminal regions of the A chain and the hydrophobic residues in the C terminal region of the B chain where the active site is, are the same for human, porcine and bovine insulins.

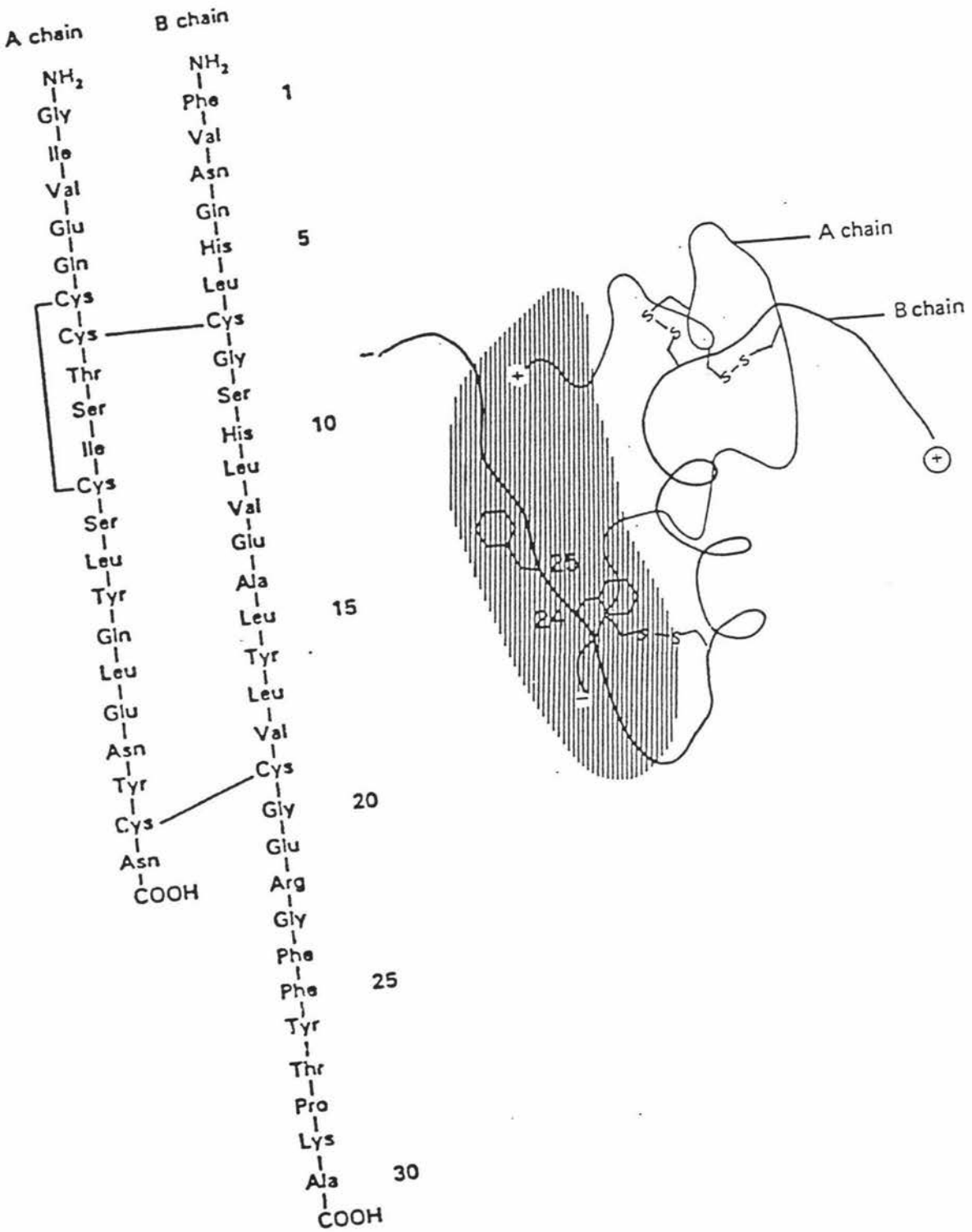


Figure 3.1.1 The Primary Structure and 3-D Structure of Monomeric Porcine Insulin

The shaded area illustrates the portion of insulin that is thought to be most important in conferring biological activity to the hormone. The Phe residues B24 and B25 are the sites of mutations that affect insulin bioactivity. The N termini of the insulin A and B chains are indicated by +, whereas the C termini are indicated by -. (Tager, 1984)

Insulin can exist as a monomer (Fig.3.1.1), or by the aggregation of 2 monomers to form a dimer. Three dimers can aggregate in the presence of 2 Zn ions to form a hexamer (MW 36,000). The degree of aggregation is a function of various factors including insulin concentration and the presence of zinc. In the presence of zinc and acid pH, most insulins form crystals composed of hexamers. However, insulin circulates essentially in the monomeric form because physiological concentrations of the hormone (0.05-1.0 nM) are much lower than the dissociation constant for dimer to monomer equilibrium which is ~1 μM at neutral pH. Thus the monomer is the physiologically active moiety.

Insulin was the first protein for which a chemical structure and a precise molecular weight were determined (Sanger et al. 1951). From partial synthesis to advanced biotechnology in commercial manufacturing of recombinant human insulin, from modification of a single amino acid to attempts at insulin pills, gradual progress has been made towards the most convenient administration for patients (Starr, 1992).

Modification of insulin has been underway since the 1960's. Cuatrecasas (1969) synthesized polymeric insulin derivatives in which insulin is covalently attached to the larger polymers of Sepharose through the α-amino group of N-terminal residue of the B chain, or through the ε-amino group of the lysyl residue. Suzuki et al. (1972) studied the properties and biological activity of an insulin-dextran complex in which dextran have been activated with cyanogen bromide. A modification of insulin by periodate-activated dextran was reported in 1986 (Li et al.). They did not determine the oxidation extent of dextran and no release or reduction studies on the complex were carried out. They attempted to discover which amino group bound to dextran by directly running the isolated complex on AAA, however, glycine was used to block the aldehyde groups in dextran molecules. It was therefore difficult to tell the actual composition of glycine due to insulin. Some characteristics, distribution and metabolic rates of the modified insulin were studied and compared with those of native insulin. The modified insulin showed increased blood sugar lowering activity and increased stability to heat and resistance to proteolysis. It showed that the dextran-insulin conjugate not only had higher capacity of lowering blood sugar than the native insulin, but also lengthened the half-life of the insulin in blood.

Following the discovery of insulin in 1921, the development and progress of tests for the activity assay of insulin have been made, because of lack of knowledge of its chemical structure at the early days, biological assays were developed to assess the potency of insulin based on the measurement of the response in animals, mainly rabbits and mice (Banting and Best, 1922). In order to control the potency of insulin in pharmaceutical preparations, different approaches have been employed. A sensitive, precise and specific radioimmunoassay (RIA) for the determination of insulin at physiological concentrations was devised (Yalow and Berson, 1960). Some disadvantages of this assay were the requirement of expensive equipment, the instability and biological hazard of isotopic reagents (Tandhanand-Banchuin et al. 1993). In the radioreceptor assay, insulin binding is detected by using a competition between the unknown or reference insulin and insulin labeled with I^{125}. The amount of radioactivity bound to cells is the measured parameter and as in the radioimmunoassay, the higher the insulin concentration in the test material, the lower the binding of I^{125} (Trethewey, 1989). Other alternative methods have been introduced, including enzyme-linked immunosorbent assay (ELISA) (Dean et al. 1989) and haemagglutination (Wolk and Kieselstein, 1984). The nature of these methods made them being more applicable, because there was no need for expensive equipment and/or no radiation hazard. The reagents used have relatively long shelf-lives and are less expensive.

The advent of HPLC columns with great resolving power has enabled further development in this field. Fisher and Smith (1986) produced a precise chromatographic assay of insulin. This enabled species to be identified and the proteins in the formulations to be separated and quantified. HPLC has proved to be a very satisfactory method. The 1993 edition of the British Pharmacopoeia has introduced an HPLC method for assaying insulin, which replaces animal testing.

3.2 Results and Discussion

3.2.1 Periodate Oxidation of Dextran

Previous studies (Battersby et al., 1996) have indicated that level of oxidation of dextran is inversely proportional to the rate of release of the complexes. The first step of this research was to examine the reproducibility in the production of a chosen level of

oxidation of dextran. Acetate buffer was normally prepared at least one day ahead of reactions. Once those periodate and dextran solutions were cooled to 4°C with constant stirring, the periodate solution was added to the dextran solution. Half the molar ratio of periodate to those of previous studies (Battersby et al., 1996 and Fisher, 1997) was used to slow down the rate of oxidation, since low oxidation of dextran (8-30%) was employed in this study. Results for one study are shown in Table 3.2.1.1 and Figure 3.2.1.1. A typical calculation for % activation is shown below.

Table 3.2.1.1 Typical results for activation of dextran

Time (minutes)	Blank (ml)	Dextran (ml)	% Activated Dextran
5	6.30	6.60	6
15	6.25	7.05	16
30	6.30	7.54	25
45	6.28	7.90	32
60	6.26	8.20	39

Typical calculation:

1 mg, 25 nmol of Dextran T-40 (MW 40,000) is equivalent to 6.25μmol of glucose (MW 160). In 5 ml aliquot samples have 20 mg of dextran (400mg in 100 ml).

mmoles of glucose $= 20 \times 6.25 \times 10^{-6}$ mole

$= 0.25$ mmole

mmoles of IO_4^- = mmoles of arsenite - mmoles of iodine

Δmmoles of IO_4^- = Δmmoles of iodine

Differences in mmoles of IO_4^- consumed between the dextran and the blank aliquots:

= [iodine] x Difference in volume required for titration (ΔV)

$= 0.0484 \times \Delta V$ mmole

It takes 2 moles of periodate to oxidize 1 mole of glucose

$$\% \text{ activation} = \frac{0.5 \times \text{mmoles of } IO_4^- \text{ consumed}}{\text{total mmoles of glucose}} \times 100$$

$$= \frac{0.5 \times 0.0484 \times \Delta V}{0.125} \times 100$$

$$= 19.36 \times \Delta V$$

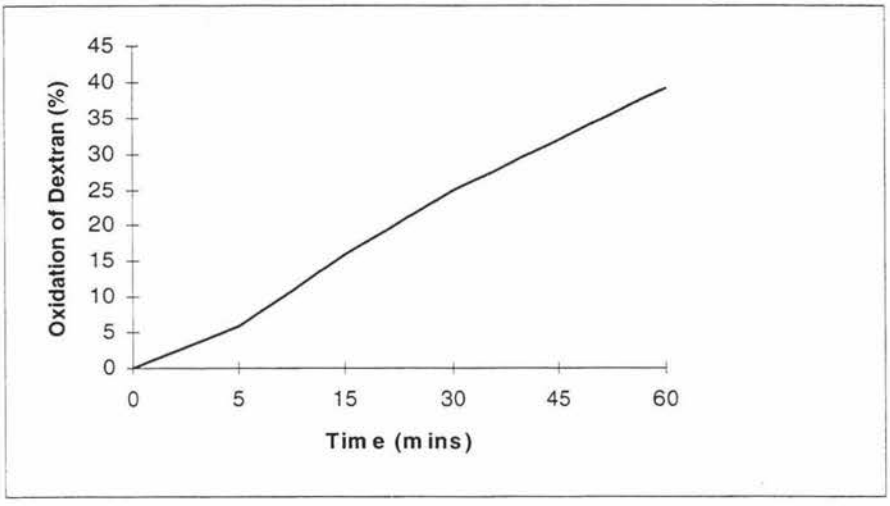


Figure 3.2.1.1 Periodate oxidation of dextran

It was found that the results for a particular level of periodate oxidation of dextran within the 0-40% range were similar under the same conditions. However, higher temperature and faster stirring may result in an increase rate of oxidation of dextran. It was also thought necessary that the periodate solution be prepared fresh daily, since some precipitates occurred after the first few hours.

Ultrafiltration of the oxidant was carried out using a Diaflo ultrafilter. Precautions should be taken to make sure there are no leaks through the membrane or around the rubber seal. Several flushing with water were needed to remove all the inorganic salts. The examination of the conductivity of the waste washes was used to determine if the solution still had inorganic salts.

3.2.2 Initial Studies of Insulin

Porcine insulin was used for this study. An absorbance scan experiment was carried out to choose the best absorbance wavelength for this study. A sample of insulin in sodium phosphate solution pH 7.4, was scanned from 190 to 820 nm (Fig. 3.2.2.1).

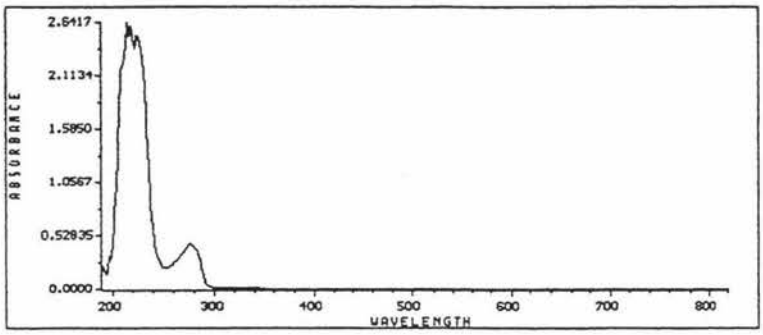


Figure 3.2.2.1 Absorbance wavelength scan of porcine insulin

The diagram indicated insulin had much higher absorbance at 214 nm than that at 280 nm. Porcine insulin has only 3 tyrosines, 3 phenylalanines and 1 tryptophan. Tryptophan is the only major absorbing group at 280 nm. At 214 nm absorbance results from peptide bonds and carboxylic acid groups. Oxidized dextran was also examined for absorbance. It showed no obvious absorbance. Therefore, 214 nm was chosen as the major wavelength for this study.

RP-HPLC and CE were used to examine the quality of insulin. In HPLC, a C-4 column was used, with buffer A (H_2O : CH_3CN, 9:1) and buffer B (H_2O : CH_3CN, 2 : 3) which contained 0.05% formic acid and 1% NaCl (Fig. 3.2.2.2).

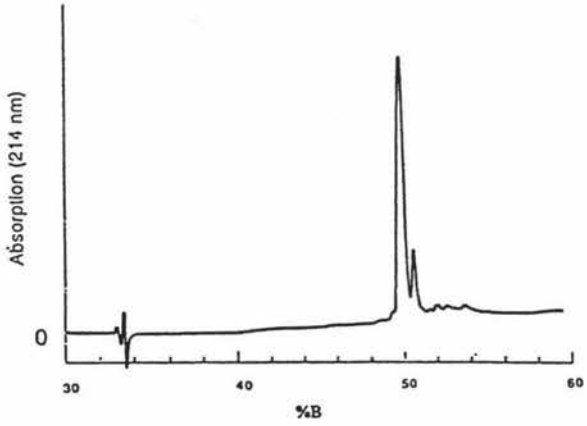


Figure 3.2.2.2 Reverse-phase analytical chromatogram of porcine insulin
Buffer A: 0.05% HCOOH, 1% NaCl, 10% CH_3CN; Buffer B: 0.05% HCOOH, 1% NaCl, 60% CH_3CN Initial concentration at 30% B, a linear gradient was run from 30% to 60% over 30 minutes. Flow rate was 1ml/min.

The small peak (51% B) after the principal peak was desamidoinsulin. The tiny noisy absorbance (52-54% B) might be the impurities of the sample.

CE was also applied to examine the insulin sample. Buffer pH 9.2 was used under the high volts of 20 KV at 30°C. Each run was over 30 minutes (Fig.3.2.2.3).

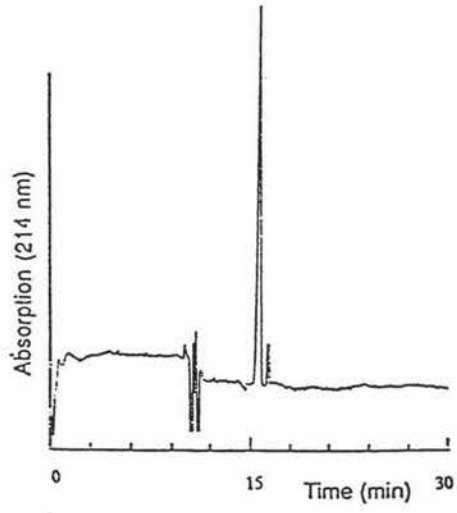


Figure 3.2.2.3 Analytical capillary electrophoretogram of porcine insulin
Buffer: pH 9.2. 20 KV was applied at 30°C. Each run was 30 minutes.

3.2.3 Complex Formation

3.2.3.1 Determination of Initial Molar Ratio

Initial studies were started with the activation level of oxidized dextran at 8%. Three molar ratios of insulin to 8% activated dextran were chosen to find out the proper initial ratio (5 : 1, 10 : 1 and 15 : 1). This was necessary to give a reasonable excess of insulin to activated dextran, and hence resulted in a better separation. The initial incubation buffer chosen for complex formation was 0.1M sodium phosphate, pH 7.8. FPLC (Superdex 75) chromatograms (Fig. 3.2.3.1.1, Fig. 3.2.3.1.2 and Fig. 3.2.3.1.3) showed that loss of the insulin peak during the complex formation was observed in accordance with the formation of a broader peak at higher MW. The shape of the growing peak was similar to that might be expected from the MW distribution of Dextran T-40 (Fig. 3.2.3.2.2).

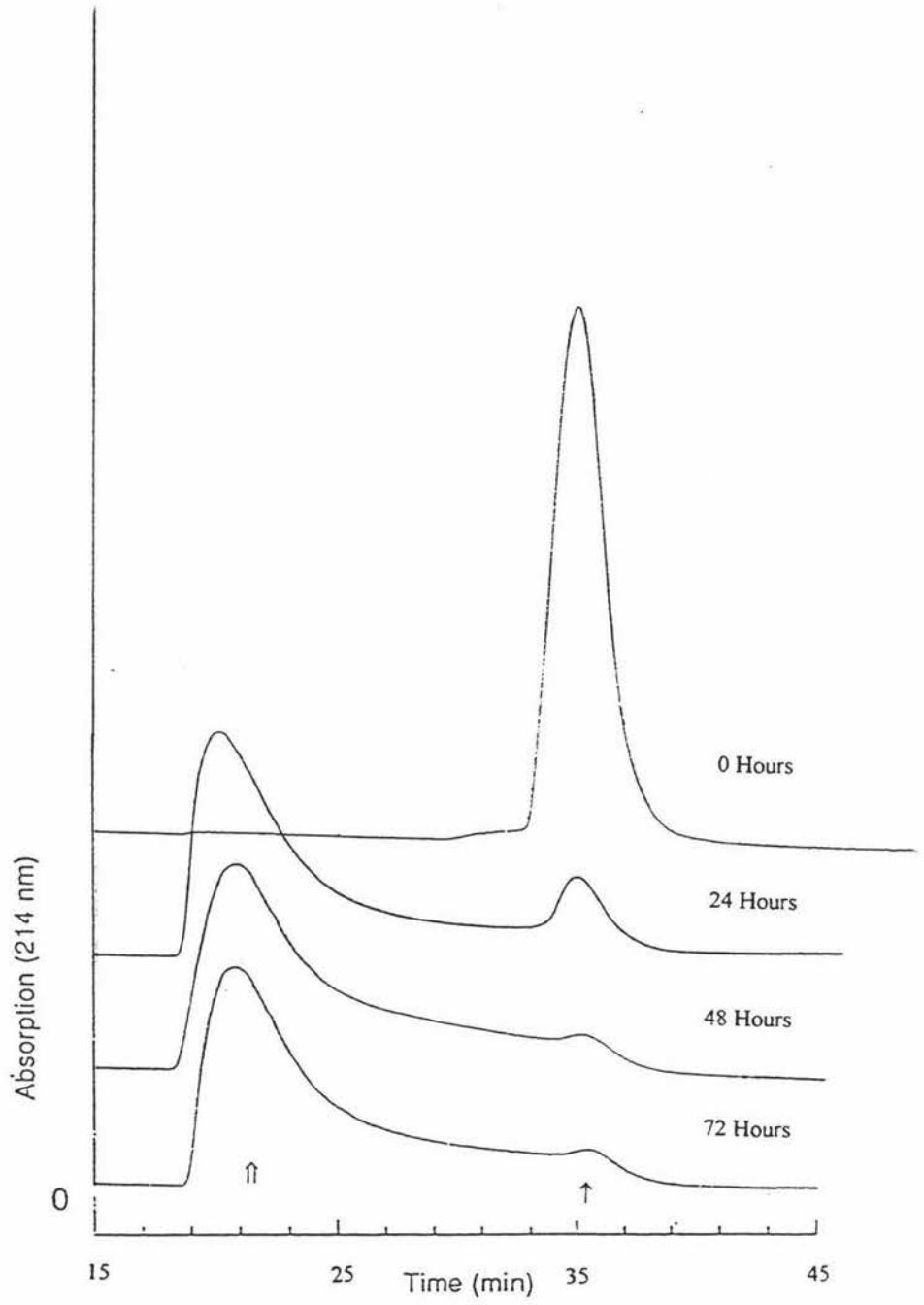


Figure 3.2.3.1.1 **Complex (⇑) formation over time for insulin (↑) and 8% activated dextran with a molar ratio of 5 : 1**

Insulin incubated with 8% activated dextran with a molar ratio of 5 : 1 was subject to SEC (Superdex 75, FPLC) at 0hrs, 24hrs, 48hrs and 72hrs in 0.1M sodium phosphate, pH 7.4.

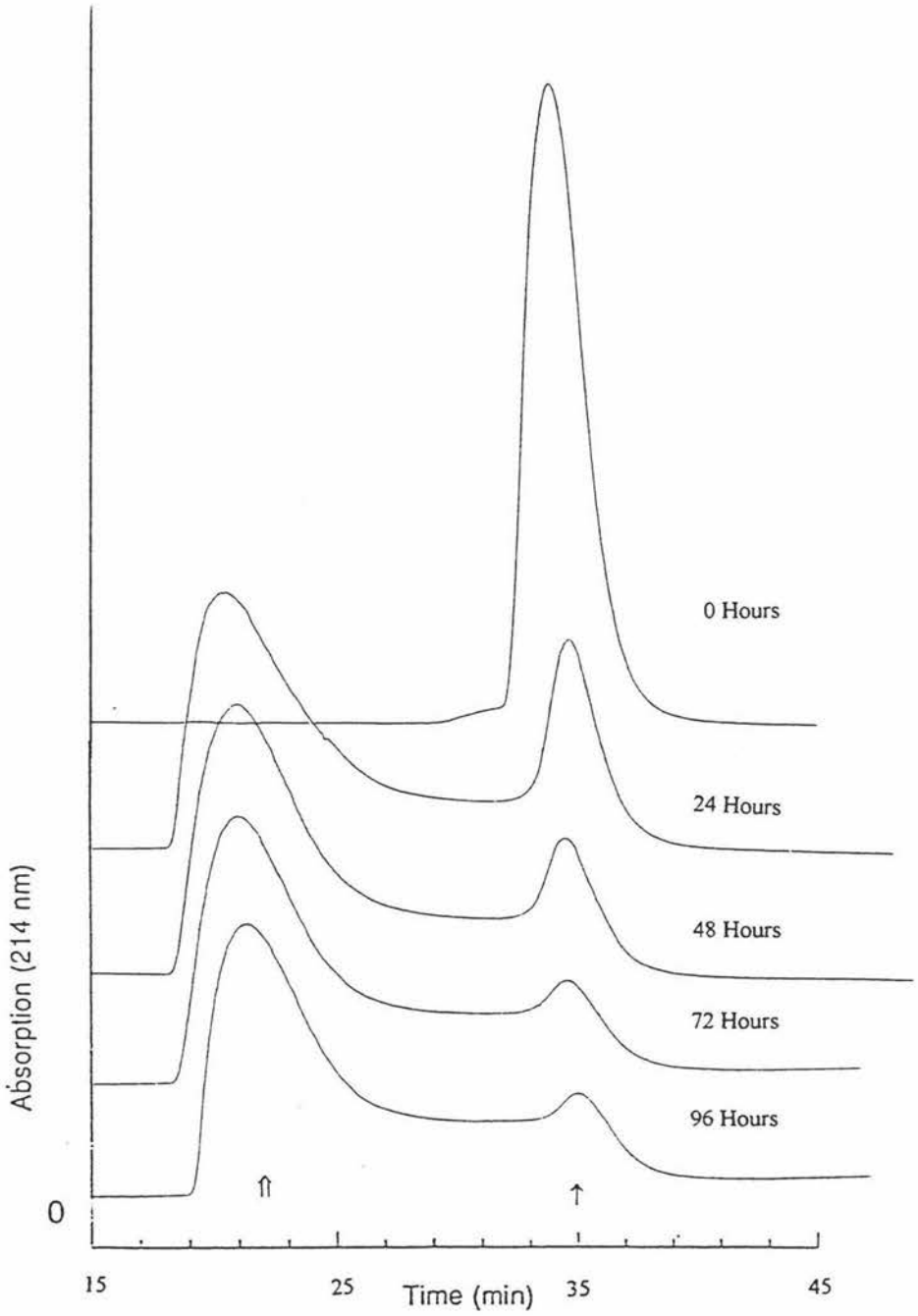


Figure 3.2.3.1.2 **Complex (⇑) formation over time for insulin (↑) and 8% activated dextran with a molar ratio of 10 : 1**

Insulin incubated with 8% activated dextran with a molar ratio of 10 : 1 was subject to SEC (Superdex 75, FPLC) at 0hrs, 24hrs, 48hrs, 72hrs and 96hrs in 0.1M sodium phosphate, pH 7.4.

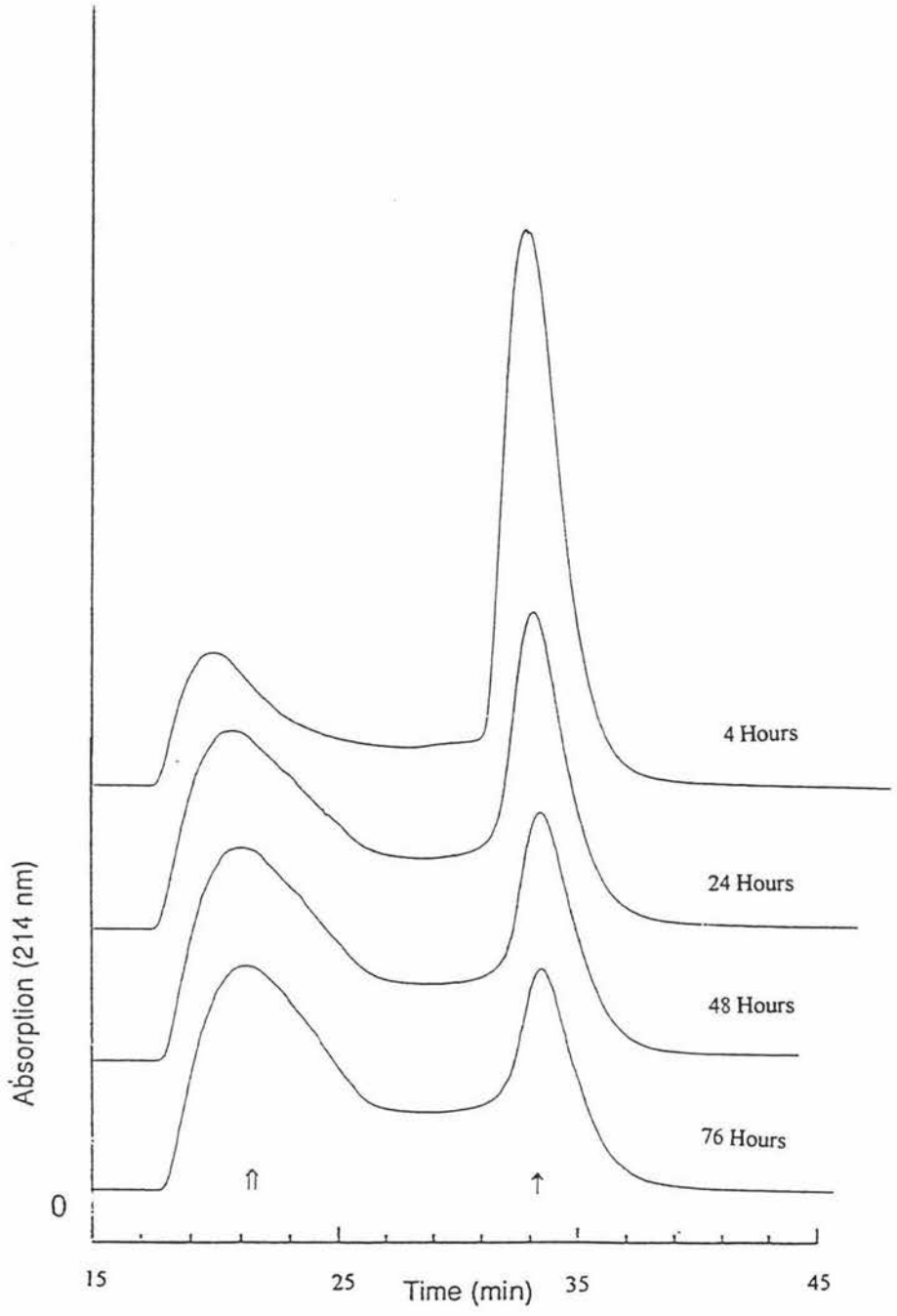


Figure 3.2.3.1.3 **Complex (⇑) formation over time for insulin (↑) and 8% activated dextran with a molar ratio of 15 : 1**

Insulin incubated with 8% activated dextran with a molar ratio of 15 : 1 was subject to SEC (Superdex 75, FPLC) at 4hrs, 24hrs, 48hrs and 76hrs in 0.1M sodium phosphate, pH 7.4.

The absorbance was monitored at 214 nm, a wavelength that does not detect non-conjugated dextran. The Superdex 75 column MW ranges from 3,000 to 70,000, was initially chosen on the assumption that it would have the capacity for separating the insulin-dextran complex and free insulin. In the mixture with a molar ratio of 5 : 1, within 48 hours of complex formation, the peak characteristic of free insulin had diminished with only a trace left. It indicated that the activated dextran might still have a strong tendency to couple insulin, in other words, a number of free aldehyde groups might still remain. In the mixtures with molar ratios of insulin to dextran of 10 : 1 and 15 : 1, the status of saturation had been shown on the chromatograms. That is to say, an equilibrium had been established between the insulin-dextran complex and free insulin. In the case with a molar ratio of 15 : 1, a significant proportion of insulin molecules remained in the solution. This increased the difficulty of separation between the complex and free insulin. Therefore, in the following experiments, the molar ratio of insulin to dextran of 10 : 1 was used as a basic ratio.

3.2.3.2 Differences in Various pHs

Varying experimental conditions might result in different binding between insulin and activated dextran. In this set of experiments, 27% activated dextran was used. It was understood that higher activation of dextran lead to more binding between proteins and activated dextran from previous studies (Fisher, 1997 and Puchulu-Campanella, 1991-1993). Higher concentration of reactants was used. Porcine insulin (4.5 mg/ml) and 27% activated dextran (3 mg/ml) were prepared separately. Equal volumes of both solutions were mixed together, resulting in final concentrations of insulin (2.25 mg/ml) and dextran (1.5 mg/ml) in the mixture in a molar ratio of insulin to dextran of 10 : 1. The incubation buffer was 0.05M sodium phosphate, 0.02% sodium azide, pH 7.4, pH 9, and pH 10. The SEC column was Superdex 75. The sensitivity of the UV detector was 2.0. Fig.3.2.3.2.1 shows the chromatograms for complex formation under different pHs at 48 hours.

It was obvious from the shapes of the curves produced from the complexes formed at different pHs that a change occurred above physiological pH. Under the formation conditions of pH 9 and pH 10, the shapes of complexes were similar. It appeared that the complexes formed at pH 7.4 had a greater percentage of higher MW material than

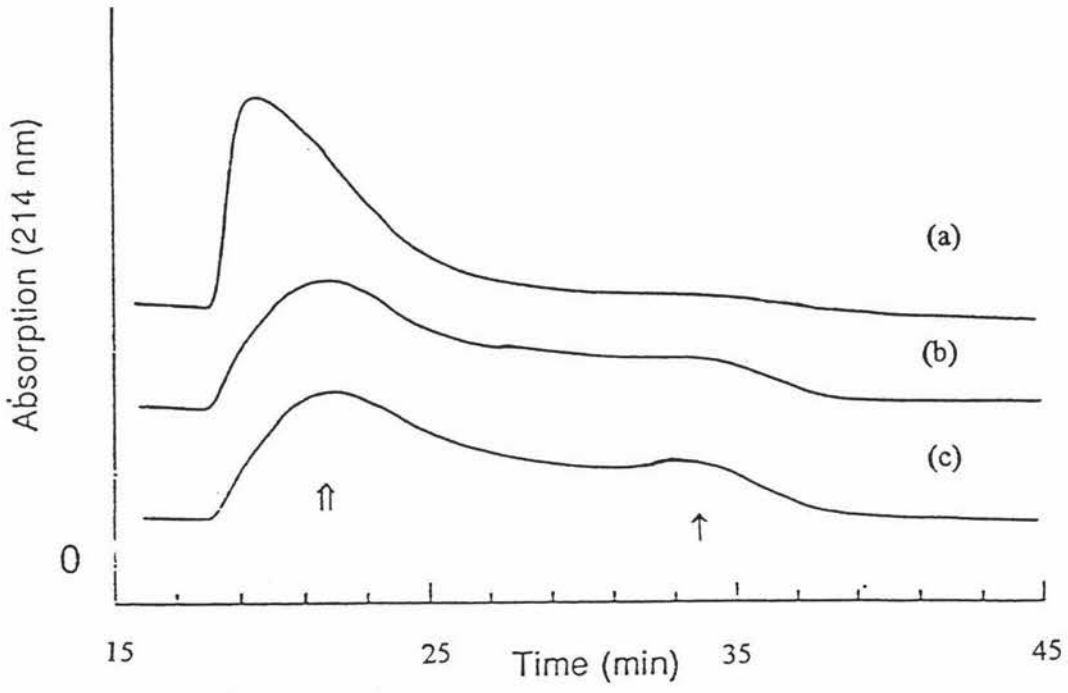


Figure 3.2.3.2.1 **Complex (⇑) formation for insulin (↑) and 27% activated dextran at (a) pH 7.4, (b) pH 9, and (c) pH 10 at 48 hrs.**
Insulin incubated with 27% activated dextran at pH 7.4, pH9 and pH 10 after 48hrs was subjected to SEC (Superdex 75) in 0.05M sodium phosphate, pH7.4.

those at pH 9 and pH 10. The complexes formed at pH 9 and pH 10 were shown to have wider and flatter range of MW distribution. It also seemed that at the higher pHs of formation, there was an indication that more free insulin was in solution. It could be deduced that at the higher pH of complex formation, there was more binding between one activated dextran and one insulin molecule. In other words, more aldehyde groups on a single dextran were occupied by a single insulin molecule, resulting in more free insulin left in the solution. Furthermore, it might be postulated that more cross-links were present at higher pH, since more lower MW complexes were formed at higher pH. In comparison with the shapes of MW distribution between dextran and insulin-dextran conjugate which formed at pH 7.4 (Fig. 3.2.3.2.1a), it could be pointed out that it was very similar to Fig.3.2.3.2.2. It was more likely that the pH 7.4 case had less cross-links. In other words, an insulin molecule might be more likely to bind to a dextran molecule at pH 7.4 and less binding sites of an insulin molecule would be involved, thus one dextran would contain many insulin molecules (Fig. 1.7.2b).

It seemed at higher pHs, the rates of imine bond formation were faster for the lower MW molecules (Fig.3.2.3.2.1, 25-30 minutes). It was thought that this resulted in cross-links leading to a spread of MWs toward the lower end of the MW range on the SEC trace. This was thought to lead to an increase in the degree of limited cross-linking. Extensive cross- linking would lead to a shift to the high MW end of the SEC chart. For the case of higher MW molecules, ample aldehyde groups in one dextran molecule were capable of catching almost all possible bond-forming amino groups of a single insulin molecule, resulting in hardly any intermolecular cross-links. In contrast, one low MW dextran molecule might not have enough aldehyde groups to satisfy all amine groups in one insulin molecule at high pHs, resulting in more cross-links than had occurred at the higher pHs.

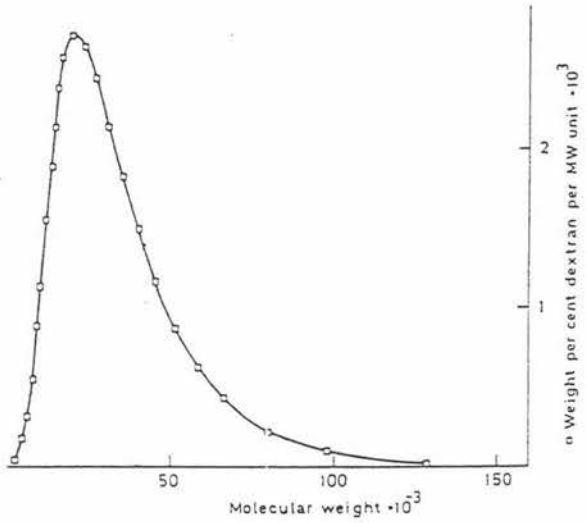


Figure 3.2.3.2.2 **Molecular weight distribution by SEC of Dextran T-40**
(Courtesy of Pharmacia)

Additional studies were carried out to make sure that the pH of running buffer did not affect the MW distribution of complex while running through the column. A 0.05M sodium phosphate, 0.15M NaCl, pH 10 buffer was run at Superdex column, i.e. the same conditions as the injection conditions discussed above. It was found that the chromatograms obtained were the same as those at pH 7.4 running buffer. In other words, the nature of the initial insulin-dextran complex formation conditions dictates the shapes on the chromatograms, i.e. the running buffer (pH 7.4 in all cases) did not change the shape of all 3 traces in Fig. 3.2.3.2.1.

Based on the deduction above, physiological conditions pH 7.1~7.8 were chosen for all future complex formation studies. Not only was easier separation obtained, simpler binding was also desired for interpretation and release studies.

Sodium phosphate was selected as the incubation buffer and running buffer for SEC as well, since it has no absorbance occurred at 214 nm and 280 nm. The wavelength at 214 nm is essential for a small protein, like insulin. Other buffers, which absorb at 214 nm, could not be considered here. However, phosphate is a perfect nutrient for bacteria, so sodium azide should be used to prevent bacterial growth. Bacteria decompose the target protein as well as produce other proteins. However because azide absorbs at 214 nm, phosphate buffers containing no azide was prepared every two days as a running buffer for SEC. Incubation buffers did contain azide, which would then elute (in SEC) in the salt peak.

3.2.3.3 Other Conditions for Complex Formation studies

Previous studies indicated that complex formation with shaking might result in loss of protein via adsorption to the vial. In particular this would be important consideration for very expensive and rare proteins as well as dose related pharmaceutical preparations. It was also thought worth examining for insulin to compare conditions of both shaking and non-shaking. 0.1M Phosphate, pH 7.8 was prepared for incubation for complex formation and running buffer for SEC. 2 mg 8% activated dextran was dissolved in 3 ml buffer, while 5.1 ml insulin in 4 ml. Two solutions were mixed together with brief gently shaking, then one half was shaken, the other was kept still. The shaking was carried out on a shaker. All were wrapped with tin foil for avoiding light. Then aliquots were taken for SEC progress examination (Fig.3.2.3.3.1).

The irregular shape of the complex formed with shaking at 24 hrs indicated higher formation rate occurred at some particular MW distribution. However, at 48 hours the peak shape returned to normal. It could be pointed out that the complex formation with shaking was a bit faster than that without shaking. However, the differences were not striking. It seems obvious that molecules should collide with each other more readily, resulting in greater complex formation with shaking. It also probably happened that some unexpected or abnormal binding might happen. Based on these results and

considerations, complex formation without shaking was selected as one of conditions for all following experiments.

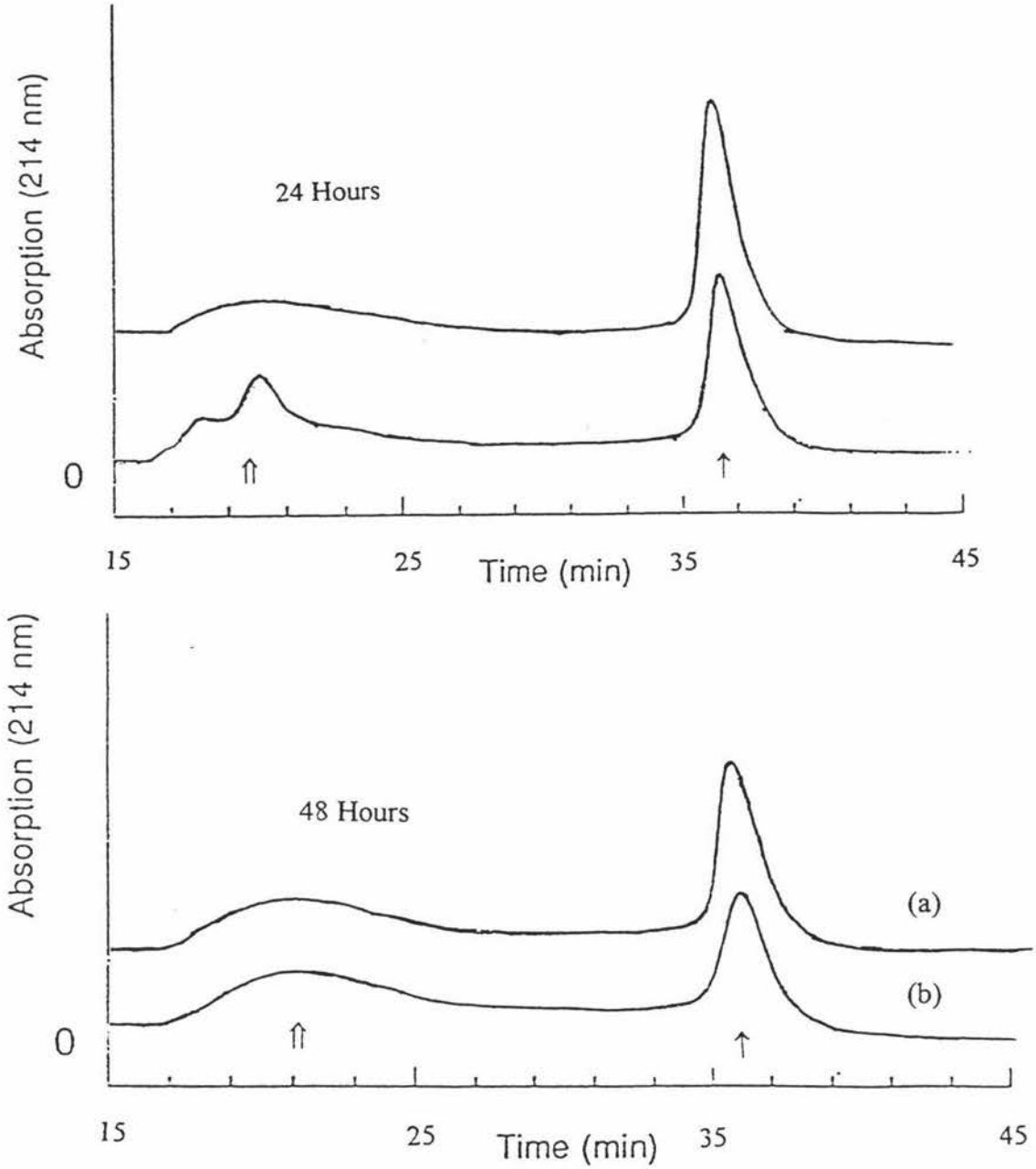


Figure 3.2.3.3.1 **Complex (⇑) formation over time for insulin (↑) and 8% activated dextran (a) without shaking and (b) with shaking.**
SEC (Superdex 75, FPLC) at 24 hrs, 48 hrs in 0.1M sodium phosphate, pH 7.8, sample injected: 50µl per run.

3.2.4 Complex Release Studies

The rate of release was of primary concern. It could be deduced from the studies of complex formation that an equilibrium was established between the free insulin and insulin-dextran complex. In order to examine this deduction, complexes isolated from

SEC were analyzed by SEC (Superdex 75) again immediately without any treatment (Fig.3.2.4.1). The complex formed at pH 7.8 with a molar ratio of insulin to 8% activated dextran of 15 : 1 at Fig.3.2.3.1.3 was isolated. The chromatogram showed that insulin-like species (same retention time as insulin for SEC) was released immediately at 0.1M phosphate buffer, pH 7.8 at room temperature. At this level of activation, insulin could be released readily, where an equilibrium was more likely to exist readily and be established quickly.

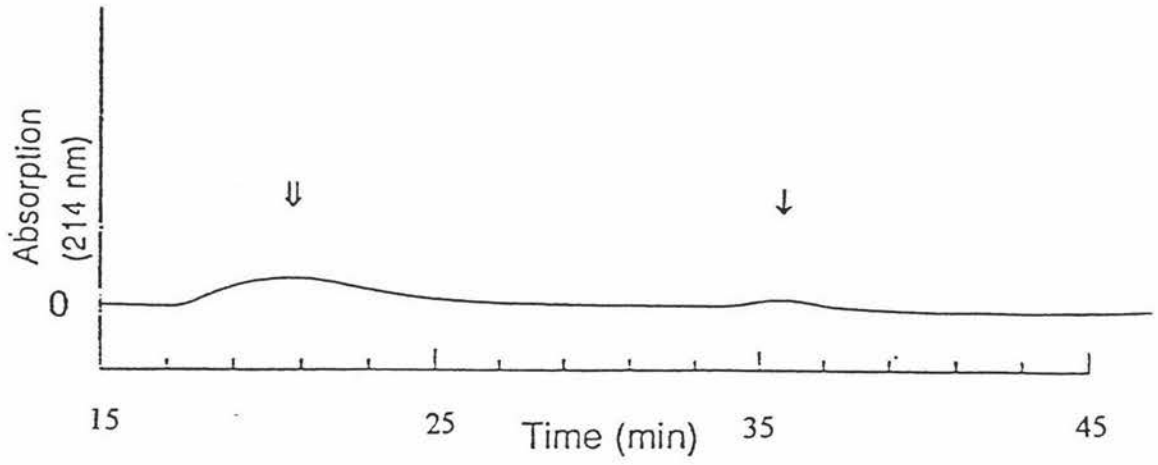


Figure 3.2.4.1 Release of insulin-like species (↓) from complex (⇓)
Insulin-8% activated dextran complex isolated from the FPLC was subjected to SEC immediately in a running buffer of 0.1M phosphate, pH 7.4.

Previous studies (Fisher, 1997) demonstrated that high activation of dextran binds protein too tightly to release protein readily at room temperature. More than 30% activation of dextran appeared to lead readily to irreversible binding (Puchulu-Campanella, 1991-1993), or especially for small proteins (Fisher, 1997). It was also understood that Dextran T-40 could be excreted in 2 days from the body, and about 70% had left the body via the kidney after first 24 hours (Arturson and Wallenius, 1964). A reasonable rate of complex release should be taken into account. So 8% activated dextran-insulin complex was initially chosen for the release studies.

To follow insulin release, complexes isolated from SEC (Superdex 75) were concentrated and then kept at 37oC after azide was added with shaking. 0.1M phosphate buffer, pH 7.8 was used as incubation buffer for the initial release study. The complexes formed with a molar ratio of insulin to dextran of 15 : 1 at pH 7.8 (Fig.3.2.3.1.3) were isolated and concentrated with Centricon-10 tubes, then aliquots were analyzed by SEC (Superdex 75, SMART System) over a set period of time. In

each case, a decrease in the size of the peak due to the complex was associated with an increase in a lower MW species with the same retention time as that of the original insulin (Fig.3.2.4.2 and Table 3.2.4.1).

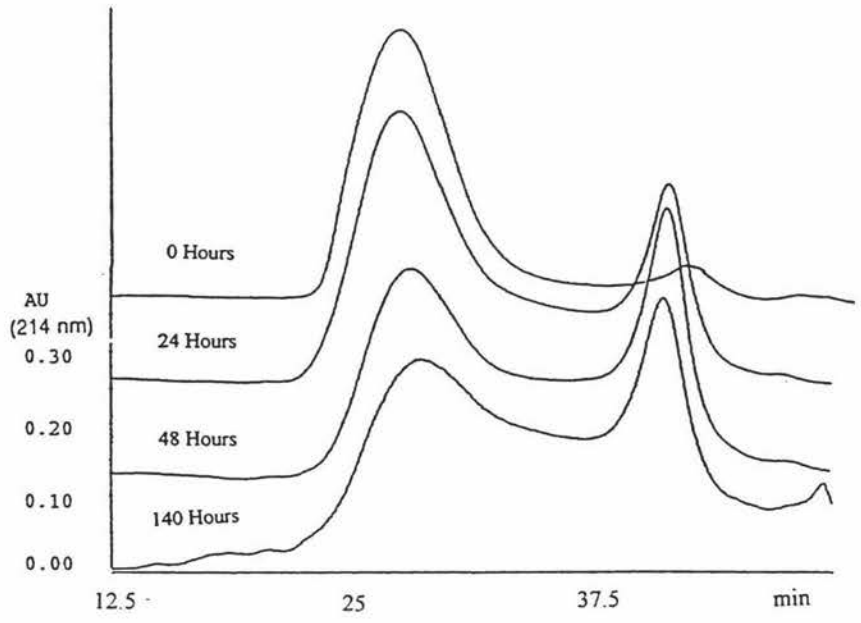


Figure 3.2.4.2 Release of insulin-like species from complex (insulin-8% activated dextran) over time.

Insulin-dextran complex isolated from the FPLC was subjected to SEC (Superdex 75, using the SMARTsystem) at 0hr, 24hrs, 48hrs and 140hrs in 0.1M phosphate, pH 7.8. Flow rate: 40 µl/min. Y axis was true only for the lowest trace.

During the first 24 hours a large decrease of the complex peak indicated that release readily took place from the insulin-8% activated dextran complex. The chromatogram at 140 hours showed that traces of higher MW species than that of the complex formed ambient and some low MW ones, which had lower MWs than insulin appeared in the solution. It might indicate insulin decomposed into small fragments, which were free in the solution and cross-linked complexes as well.

Table 3.2.4.1 Release study of insulin-8% activated dextran complex by SMART system (Superdex 75)

Time (hours)	Complex peak area	Released 'insulin' peak area	Ratio: Complex : 'Insulin'
0	65.8	5.9	11
24	46.3	17.9	2.5
48	31.7	22.2	1.4
140	28.8	23.7	1.2

'Insulin' – Insulin-like species

16% and 27% activated dextran-insulin have also been employed for release studies in order to get an understanding of the release properties of insulin-dextran conjugates over a range of % activated dextran. Complexes formed in 0.05M phosphate, pH 7.1, 5 days before were isolated by SEC (Superdex 75 column), while running buffer was 0.05M phosphate, pH 7.4. The isolated complexes were concentrated with Centricon-10 tubes, after a proper amount of azide was added, then put in 37°C water bath with shaking in the dark. Aliquots were subjected to the SMART system for release studies over a set period of time (16%: Fig.3.2.4.3 and Table 3.2.4.2; 27%: Fig. 3.2.4.4 and Table 3.2.4.3). In each case, a decrease in the size of the peak due to the complex was associated with an increase in a lower MW species with the same retention time as that of the original insulin. The chromatogram of release study for insulin-27% activated dextran complex at 96 hours indicated traces of higher MW species formed upon release.

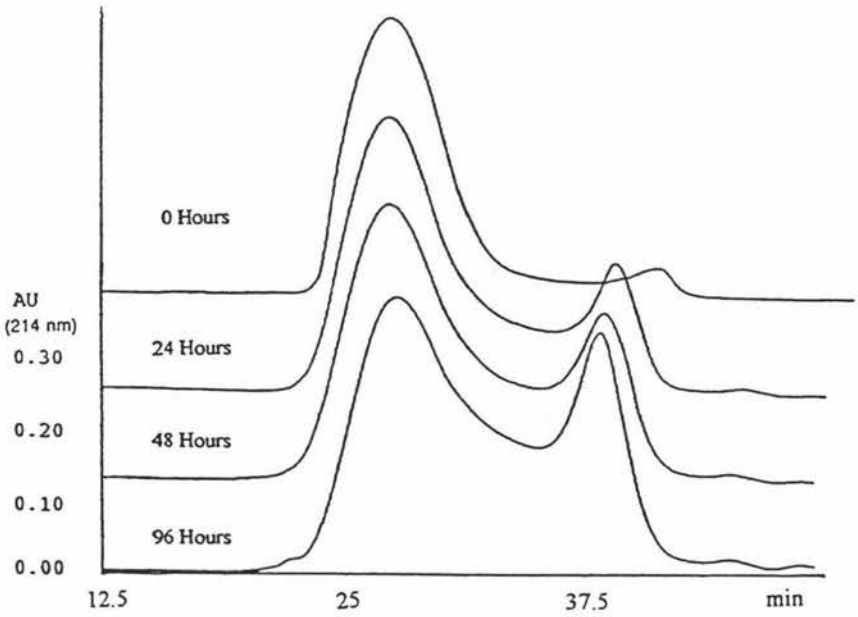


Figure 3.2.4.3 Release of insulin-like species from complex (insulin-16% activated dextran) over time

Insulin-dextran complex isolated from the FPLC was subjected to SEC (Superdex 75, using the SMART system) at 0hr, 24hrs, 48hrs and 96hrs in 0.05M phosphate, pH 7.4. Flow rate: 40 µl/min. Y axis was true only for the lowest trace.

Table 3.2.4.2 Release study of insulin-16% activated dextran complex by SMART system (Superdex 75)

Time (hours)	Complex peak area	Released 'insulin' peak area	Ratio: Complex : 'Insulin'
0	90.8	6.1	15
24	69.3	18.2	3.8
48	73.3	26.6	2.7
96	65.0	30.5	2.1

'Insulin' – Insulin-like species

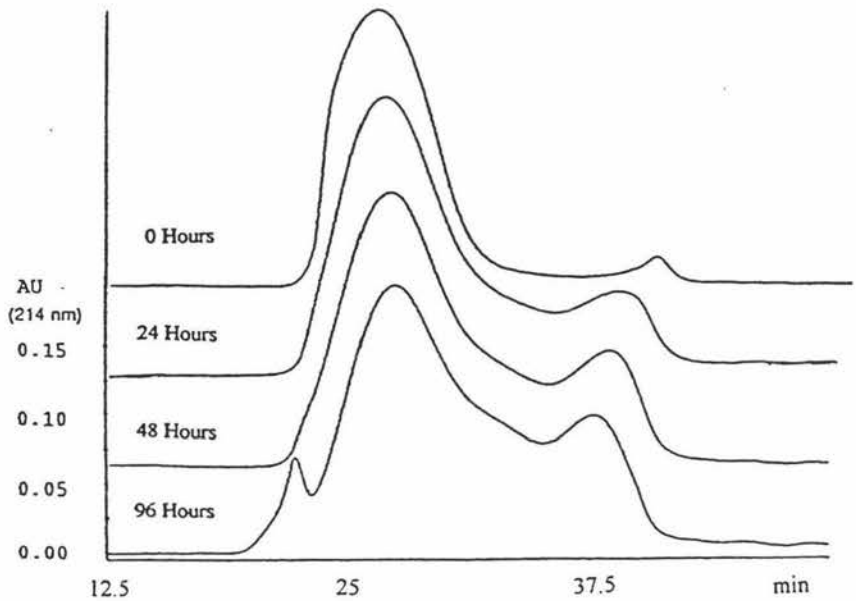


Figure 3.2.4.4 Release of insulin-like species from complex (insulin-27% activated dextran) over time

Insulin-dextran complex isolated from the FPLC was subjected to SEC (Superdex 75, using the SMART system) at 0hr, 24hrs, 48hrs and 96hrs in 0.05M phosphate, pH 7.4. Flow rate: 40 µl/min. Y axis was true only for the lowest trace.

Table 3.2.4.3 Release study of insulin-27% activated dextran complex by SMART system (Superdex 75)

Time (hours)	Complex peak area	Released 'insulin' peak area	Ratio: Complex : 'Insulin'
0	68.3	3.2	21.6
24	62.6	11.8	5.3
48	61.2	16.2	3.8
96	50.6	14.9	3.4

'Insulin' – Insulin-like species

Comparison of these release studies for three levels (8%, 16% and 27%) of activated dextran, showed that the lower the level of activation of dextran, the easier the release took place as expected. The greatest decrease occurred during the first 24 hours. It could be assumed that the increase in temperature to 37°C for release study in comparison to ambient for complex formation, was one of the main driving forces to drive the equilibrium toward the dissociation direction. After a 4 days period, a new equilibrium established.

The insulin-16% activated dextran complex was isolated by SEC (Superdex 75) with a running buffer of 0.05M phosphate, pH 7.4. Without concentrating, the isolated complex was shaken at ambient. Aliquots were taken to SMART system (Superdex 75) for release study over a period of time (Table 3.2.4.4). The results indicated a much slower rate of release occurred at ambient than at 37°C. As the initial concentration of the complex under the conditions of 37°C, the run was much higher than that of the ambient run, the data could not be used to compare ambient to 37°C quantitatively. Qualitatively however it can be seen that temperature played an important role in the rate of release.

Table 3.2.4.4 Release of insulin-16% activated dextran complex by SMART system (Superdex 75) at ambient.

Time (hours)	Complex peak area	Released 'insulin' peak area	Ratio: Complex : 'Insulin'
0	23.9	3.5	6.8
24	22.8	4.5	5.1
48	21.0	8.8	2.4
96	19.9	11.2	1.8

'Insulin' – Insulin-like species

Released species isolated by SEC were adjusted to pH 2-3 with formic acid, and then was run on the FPLC (PepRPC HR 5/5 column). This reverse phase column which is designed to separate peptides and small proteins, is capable of separating insulin molecules or even smaller fragments. Since the salts in the sample could interfere the mass spectral analysis, desalting samples is necessary for the mass spectrometer, hence the use of the volatile formic acid buffer. Samples isolated by SEC might contain impurities because SEC peaks normally cover a much wider band than those obtained

by RP-HPLC systems. The sample isolated by PepRPC column (Fig. 3.2.4.5) was submitted to Biochemistry Section for mass spectral analysis.

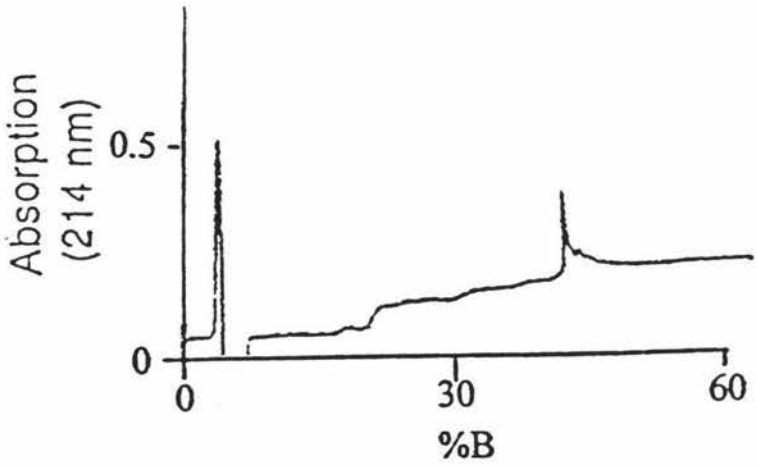


Figure 3.2.4.5 Reverse phase chromatography of the isolated released insulin-like species for mass spectrum after SEC isolation.
Buffer A: 0.1% HCOOH, 100% H_2O; Buffer B: 0.1% HCOOH, 100% CH_3CN. A linear gradient was run from 0 to 60% B over 30 minutes.

Initial trials were done in an attempt to separate the released species from the complex formation solution directly by using the PepRPC column. However, the results were not encouraging, because the peak of released species and that of complex overlapped, while the peak of the complex covered a wider range of area (Fig.3.2.4.6). Therefore, one has to isolate the released peak by SEC then do RPLC.

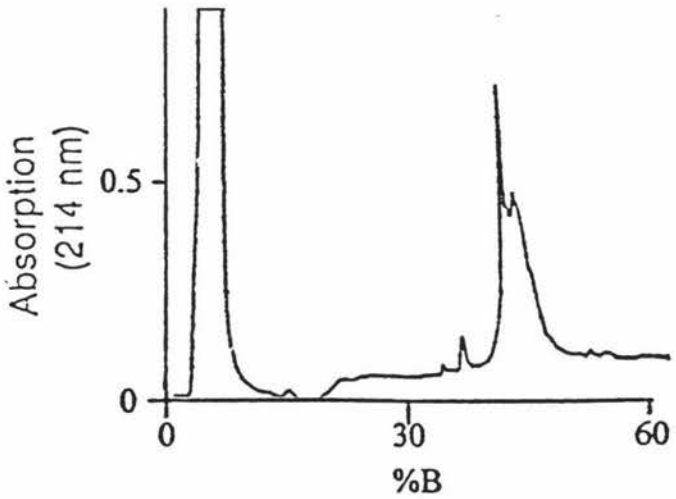


Figure 3.2.4.6 Reverse phase chromatography of released insulin-like species from release mixture
Buffer A: 0.1% HCOOH, 100% H_2O; Buffer B: 0.1% HCOOH, 100% CH_3CN. A linear gradient was run from 0 to 60% B over 30 minutes.

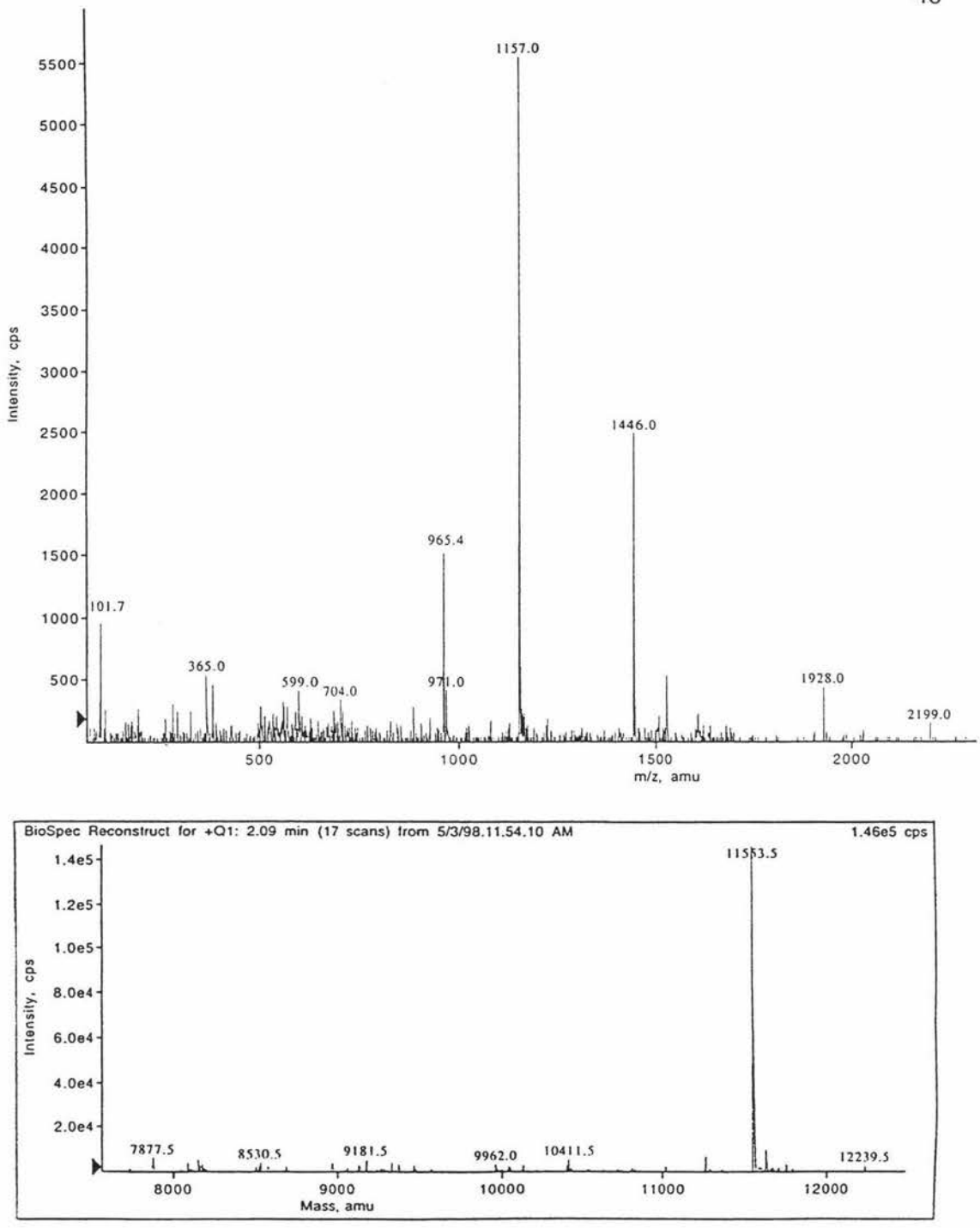


Figure 3.2.4.7 Mass spectrum for release insulin

The result of mass spectrum confirmed that the released insulin-like species, MW 11553.5 (as a dimer), had the same MW as that of original porcine insulin (Fig.3.2.4.7). It also indicated that most released insulin was at least stable at pH 7.4 at 37°C for 4 days. Li et al. (1986) pointed out that the modified insulin (insulin-dextran conjugate) showed increased blood sugar lowing activity in vivo. In other words, released insulin retained activity.

Activity assays have been examined for several proteins (Fisher, 1997; Battersby et al., 1996). Similar activities between free proteins and the released protein-like species from complexes have been observed. In general, these reversible conjugates formed under mild conditions between activated dextran and proteins do not seem to destroy the activities. From the limited studies done to date, the released species seem to be unmodified.

Bioactivity assays were not available for this study. However, previous studies have indicated the bioactivity of insulin dextran conjugate in vivo (Li et al. 1986). The result of mass spectrum confirmed that the released insulin-like species had the same MW as original insulin and the behaviors in HPLC were very similar as well. It was most likely that released insulin remained active.

3.2.5 Complex Reduction Studies

Sodium cyanoborohydride was used as a reducing agent instead of sodium borohydride. It is a more selective reducing agent, which is able to entirely reduce imine compounds but not aldehyde and ketone groups (Borch et al. 1971). The reductive amination of oxidized pyranoside was taken between 24 and 72 hrs to reach completion by using sodium cyanoborohydride (Osuga et al. 1989). Therefore, similar reactive conditions were used in this study.

Once an equilibrium has been reached, a solution of complex formation mixture (containing free insulin and complex) and sodium cyanoborohydride (2 x w/w) were mixed along with ample sodium azide. A control experiment without addition of cyanoborohydride was set up at the same time (Fig. 3.2.5.1).

The chromatograms showed after reduction, the increase of the complex peak was associated with the decrease of the free insulin one. The control experiment showed little change, in comparison of that of 3 days before. This suggested, after the carbon-nitrogen double bonds (imine bonds) were reduced to carbon-nitrogen single bonds by cyanoborohydride, the existing equilibrium between free insulin/dextran and complex was altered irreversibly in favor of complex formation (Scheme 3.2.5.1).

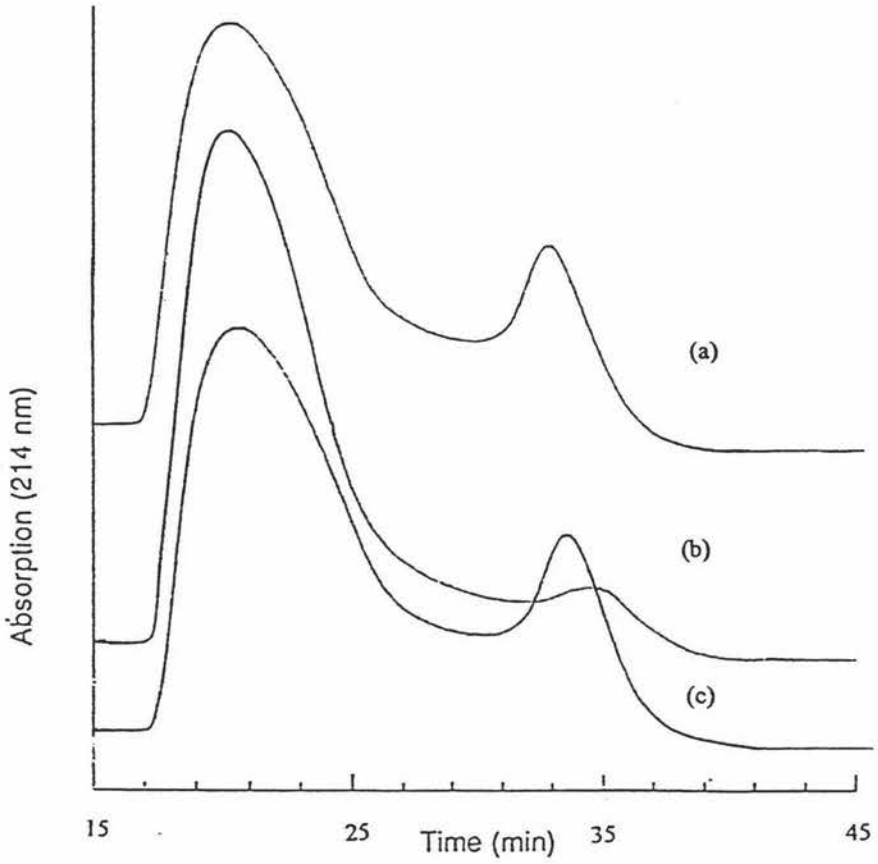


Figure 3.2.5.1 SEC chromatograms of reduced complex and control experiment
(a) Complex mixture before reduction. (b) complex mixture after reduction by $NaBH_3CN$. (c) control experiment without adding $NaBH_3CN$. Samples were subjected to SEC (Superdex 75, using SMART system) with a 0.05M phosphate, pH 7.4 running buffer.

$$RCH_2\text{-}NH\text{-insulin} \xleftarrow{NaBH_3CN} RCH{=}N\text{-insulin} \leftrightarrow RCHO + \text{free insulin}$$

Scheme 3.2.5.1 Cyanoborohydride reduction of insulin-dextran complex

Initial trials for the reduction of imine bonds used the complexes isolated by SEC (Superdex 75). As insulin was released immediately from an isolated complex (Fig. 3.2.4.1), it seemed that the separation of complex from free insulin was not necessary. It also helped to reduce the procedures of isolation and concentration. In other words, I tried to reduce the interruption and losses to the minimum in dealing with the complexes. Of course, differences of binding under the complex formation conditions

and under reduction conditions ($NaBH_3CN$) may have existed. However, to find a reliable method to interpret the nature of binding was our primary concern. Therefore, the simpler method where $NaBH_3CN$ was directly added to the mixture being studied was chosen.

After reduction, the complex was isolated by SEC, following the same treatment for the release study. After being concentrated, the isolated complex was subjected to release study under the conditions of pH 7.4 at 37°C for 24 hours, aliquots were analyzed by SEC (SMART, Superdex 75). The SEC chromatograms were shown in Fig. 3.2.5.2. The released species from the reduced complex had an even longer retention time than insulin (mostly dimers). In other words, they probably were insulin monomers (low concentration) or insulin fragments. Azide was eluted at 55-60 minutes.

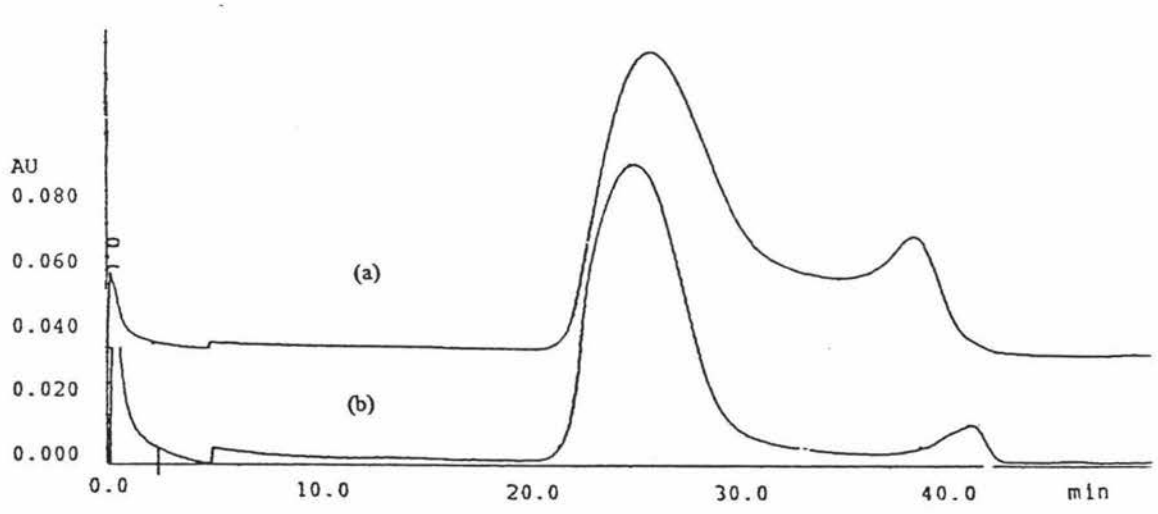


Figure 3.2.5.2 Comparison of release from (a) non-reduced complex and (b) reduced complex.

Insulin-16% activated dextran complex formed at 0.05M phosphate, pH 7.1. Complexes were isolated by SEC (Superdex 75, FPLC) with 0.05M phosphate, pH7.4. Flow rate: 0.4 ml/min. Y axis was true only for the low trace.

Oauga et al. (1989) suggested that if the reduction of the imine was slow, some protein could release from the non-reduced imine linkage. The results showed little release from the reduced complex in comparison with the non-reduced complex. Reduction dragged equilibrium one way. The presence of reducing agents contributed to little insulin release. In other words, which were attributed to the binding between insulin

and activated dextran, were reduced by selective reducing agent cyanoborohydride (imine bonds but no aldehyde and ketone groups). This indicated that imine bonds were involved in the binding.

The dialdehyde of dextran (single and double oxidized intermediates) may react with amino groups in proteins in different ways. An imine and an oxazepine may be the products for the reaction of amino groups with the dialdehyde of cyclodextrin (an α-1,4 linked cyclic glucan, Kobayashi et al. 1990). The oxazepine linkage between oxidized dextran and phosphorylase kinase caused irreversible inactivation (King and Carlson, 1981). Therefore, stable linkage can be obtained by forming an oxazepine. On the other hand, an imine product is thought to be labile, reversible linkage, which is contrast to the irreversible one above. The type and proportion of the dominant structure depends at least some extent on the reaction conditions. It was shown that high release rate was obtained for the release of insulin-dextran complex (Table 3.2.4.1). It indicated that the dominant linkage should not be similar to that of the oxazepine case because reversible reaction between insulin and activated dextran clearly demonstrating the dominant linkage should be imine bonds. Furthermore, the aldehyde groups must be adjacent in the structure if the oxazepine linkage is formed, like those in the single oxidized intermediate. However, the formation of formic acid which can be titrated with sodium hydroxide after the periodate oxidation of dextran indicated the double oxidized intermediates were dominant under these oxidation conditions.

It happened that the reduced insulin-dextran complex release some insulin-like species. One reason was probably due to not enough reducing agents present. Because sodium cyanoborohydride is very hygroscopic, once the jar was opened to the air, the solid-like substance readily becomes a liquid-like viscous one. However, even more cyanoborohydride was added, more reduction time was given, little release still occurred. The second possible reason, was that insulin which existed as a dimer formed complex with activated dextran. In other words, not every insulin molecule formed imine bonds with activated dextran in the complex. It is known that insulin exists as the dimer in mild acid and as the monomer in 30% acetic acid. The dissociation constant for dimer to monomer is ~1 μM (0.006 mg/ml) at neutral pH. Insulin concentration for the complex formation ranged from 1 to 2.5 mg/ml. So they were much higher than the dissociation constant for insulin dimer. Only trace insulin-like species were released

from the reduced complex over 24 hours under approximately physiological conditions (Fig. 3.2.5.3).

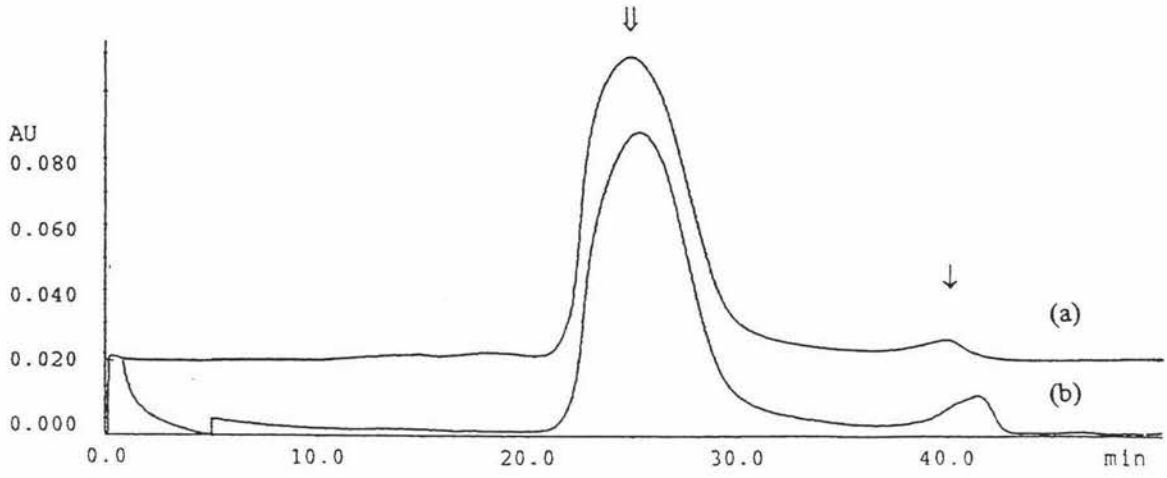


Figure 3.2.5.3 Insulin (↓) release study from reduced complex (⇓) by SMART system (Superdex 75).
Insulin-16% activated dextran complex formed at 0.05M phosphate, pH 7.1. Complexes were isolated by SEC (Superdex 75, FPLC) with 0.05M phosphate, pH7.4. Aliquots were subjected to SEC (SMART, Superdex 75) at 0hr, 24hrs. Y axis was true only for the low trace.

Two insulin monomers form a dimer in a specific conformation (Fig. 3.2.5.4). Only those bound insulin molecules, which had no or little conformational change, would most likely form dimers with unbound insulin monomers. It could be expected that these dimers contribute only very low proportions in the insulin-dextran complex. The low level of release from the reduced complex confirmed this deduction. Due to only little insulin release from the reduced complex, it unlikely interfered with the results of the following binding studies.

Once the reduced insulin-dextran complex showed almost no release under approximately physiological conditions, the reduced complexes were isolated by SEC (Superdex 75) and concentrated with Centricon-10 tubes to minimal amounts. Then they were isolated by SEC (Superdex 75) and concentrated with Centricon-10 tubes to minimal amounts. Then they were subjected to the following experiments, to determine the binding nature of the insulin-activated dextran conjugates.

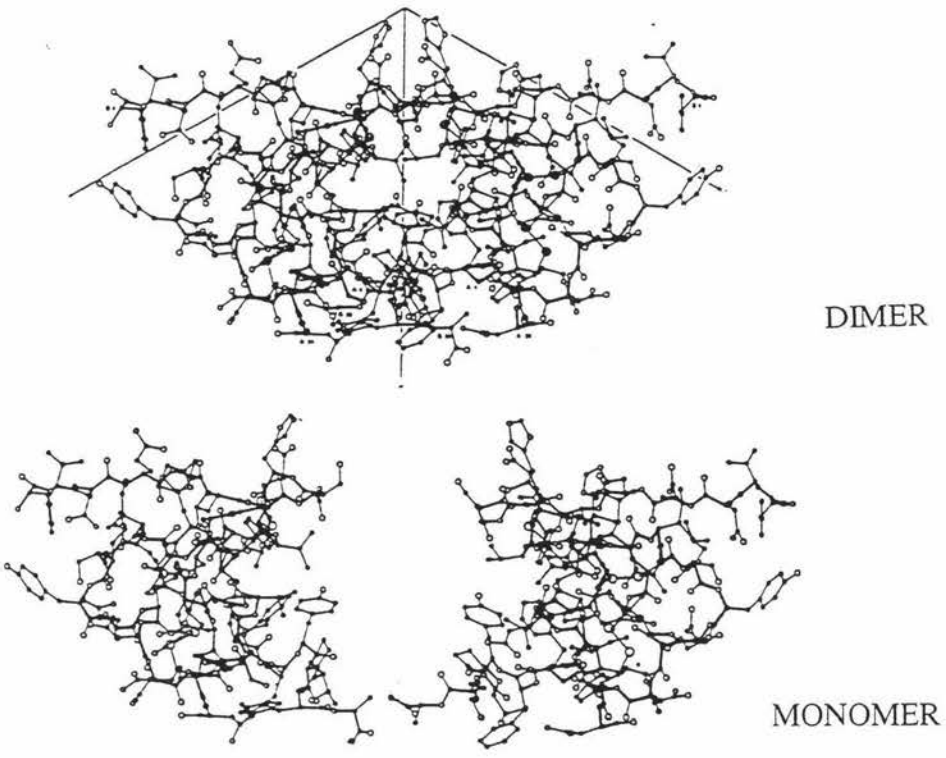


Figure 3.2.5.4 Insulin monomer, dimer: three-dimensional atomic structure (Blundell et al 1977).

CHAPTER FOUR
ENZYMATIC CLEAVAGE STUDIES WITH TRYPSIN AND α-CHYMOTRYPSIN

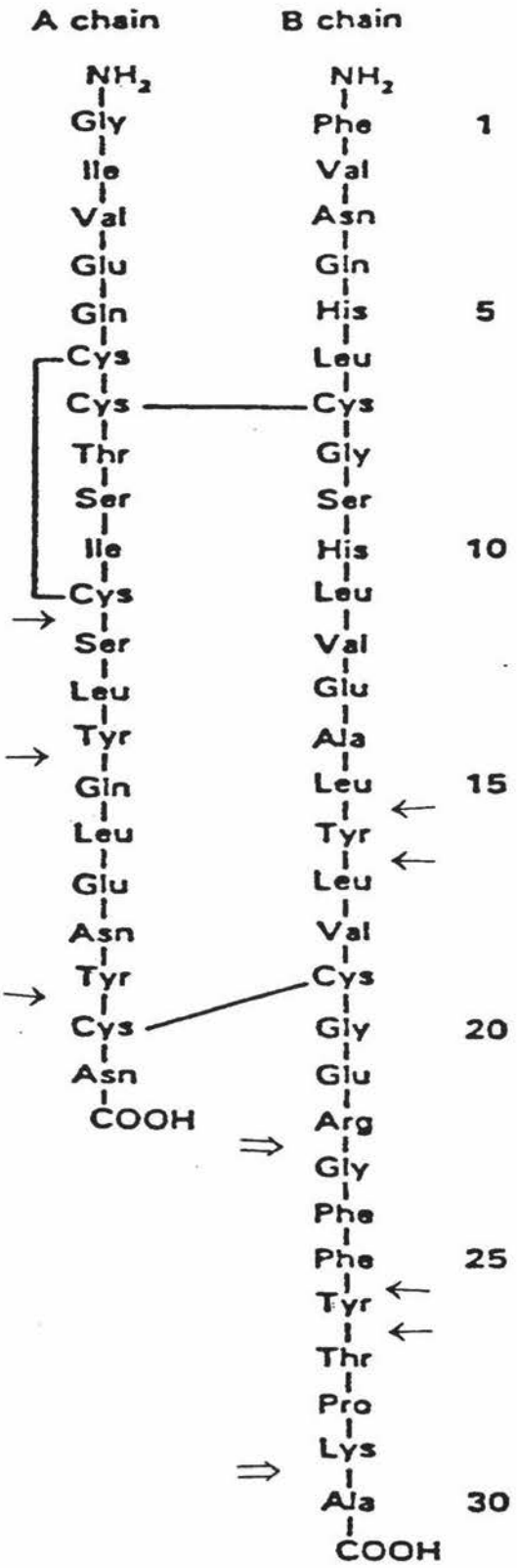
4.1 Introduction

Trypsin and α-chymotrypsin, the major proteolytic enzymes secreted by the pancreas into the intestinal lumen, are both known to cleave various bonds within the insulin molecule. Trypsin only cleaves insulin at only two sites: on the carboxyl side of residue B29-Lys and B22-Arg (Young and Carpenter 1961; Bromer and Chance 1967). Previous studies indicated that B29-Lys was one of the binding sites (Li, et al. 1986).

If the cleavage of insulin by trypsin is complete, one large fragment (desoctapeptide-insulin), one small fragment (heptapeptide) and one amino acid (Ala for porcine insulin) are expected.

The selection of chymotrypsin favors those hydrophobic residues, with cleavage on the carboxyl side of tryptophan, tyrosine, phenylalanine, and leucine (Wilcox, 1970 and Blow, 1971). However, previous studies (Smyth, 1967 and Kaspar, 1970) demonstrated that many other peptide bonds might be hydrolyzed, such as methionine, asparagine, glutamine, histidine, threonine and lysine, but usually at a lower cleavage rate. The pattern of chymotrypic cleavage cannot be accurately anticipated from the amino acid composition of the polypeptide substrate. In some cases, chymotrypsin can be employed for some very selective cleavages under mild digestion conditions, with either the cleavage of the most labile bonds or local structural constraints (Hill, 1965 and Kaspar, 1975).

α-chymotrypsin can cleave at least seven bonds in insulin (Sanger and Tuppy, 1951; Singer and Thompson, 1953; Ginsburg and Schachman, 1960). The carboxyl sides of Phe, Tyr, Leu and 1 Cys throughout the A and B chains of insulin were expected to be scattered to form several main peptide fragments (Scheme 4.1.1).



Scheme 4.1.1 The cleavage sites of insulin by trypsin (⇑) and α-chymotrypsin (↑)

4.2 Results and Discussion

Trypsin digestion and peptide mapping of insulin and insulin-dextran complex were performed under the conditions described by Battersby et al. (1995).

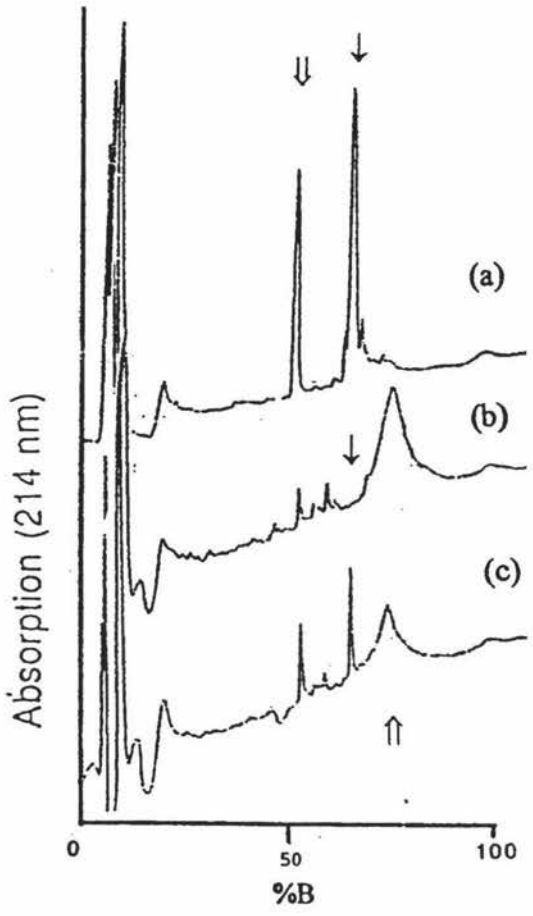


Figure 4.2.1 Peptide mapping of trypsin digestion of insulin and reduced complex
(a) Trypsin digestion of porcine insulin. (b) Trypsin digestion of the isolated reduced insulin-27% activated dextran complex. (c) Mixed sample of insulin digest and reduced complex digest. The first main peak (⇓) and the second main peak (↓) were trapped. The peptides were separated on a PepRPC column using an acetonitrile gradient with a flaw rate of 0.5 ml/min. Details were given in the method section.

2 main peaks from the insulin trypsin digest (Fig.4.2.1a) were isolated and subjected to mass spectral analysis (Fig.4.2.2 and Fig.4.2.3). It was confirmed that the first peak (⇓) was the small fragment (heptapeptide MW 860) peak, and the larger peak (↓) was the main fragment peak (desoctapeptide-insulin MW 4865). The free amino acid 'alanine' was believed to be eluted with the salt peak at the void volume of the chromatograms.

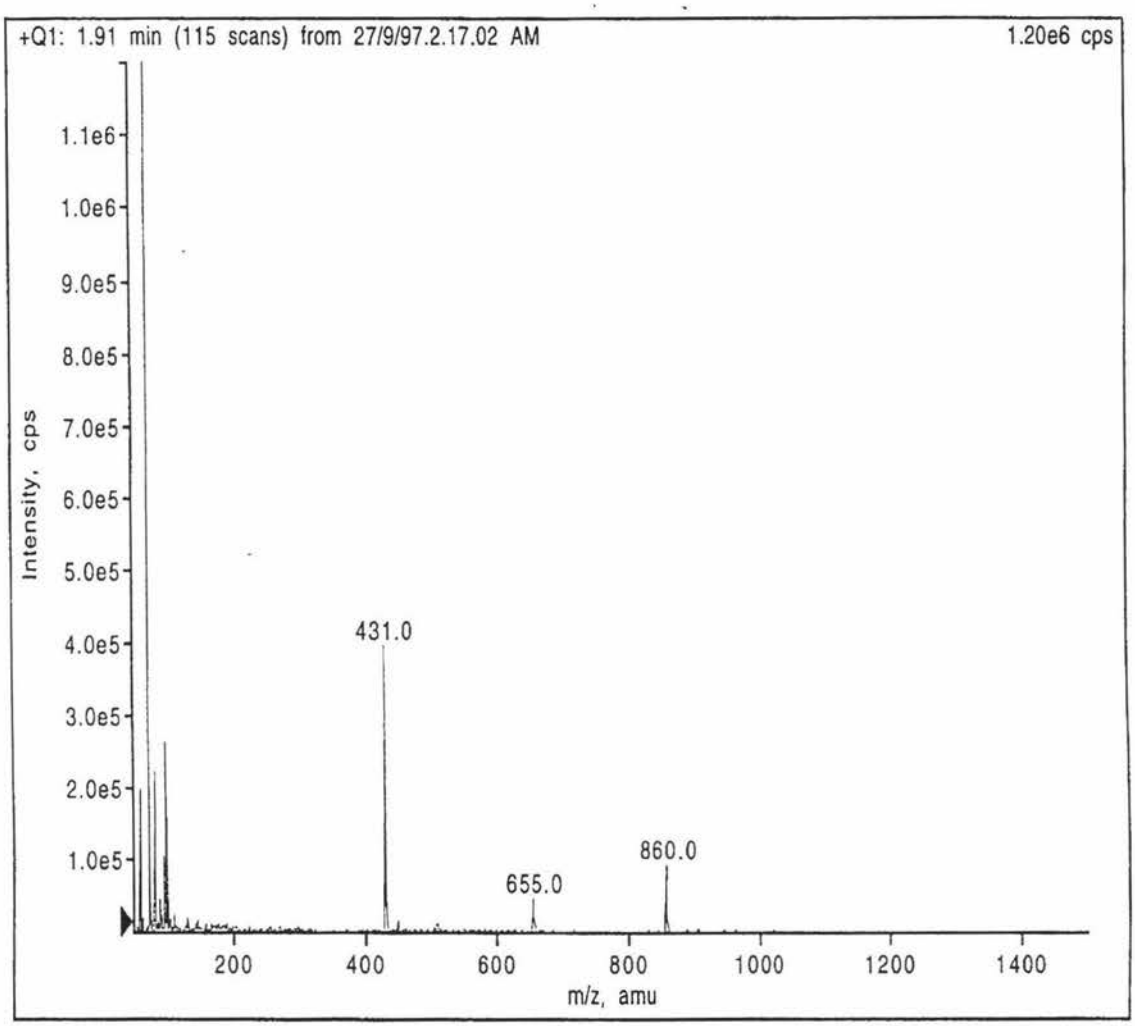
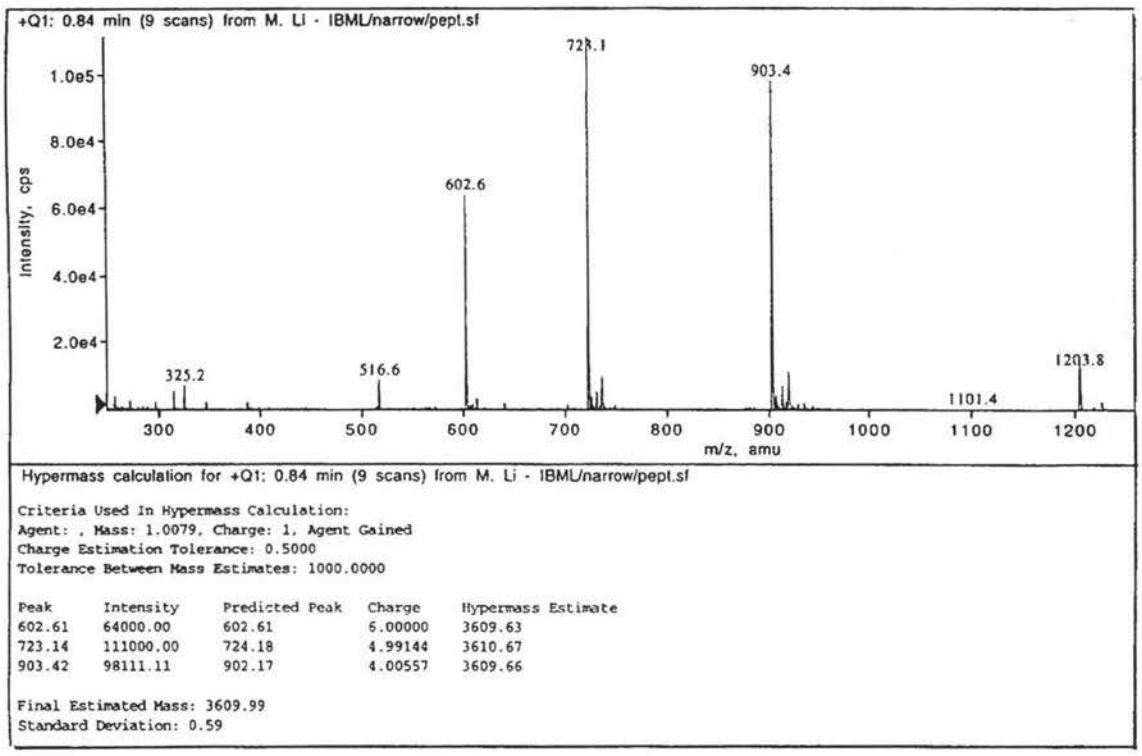


Figure 4.2.2 Mass spectrum for the small fragment of insulin trypsin digestion



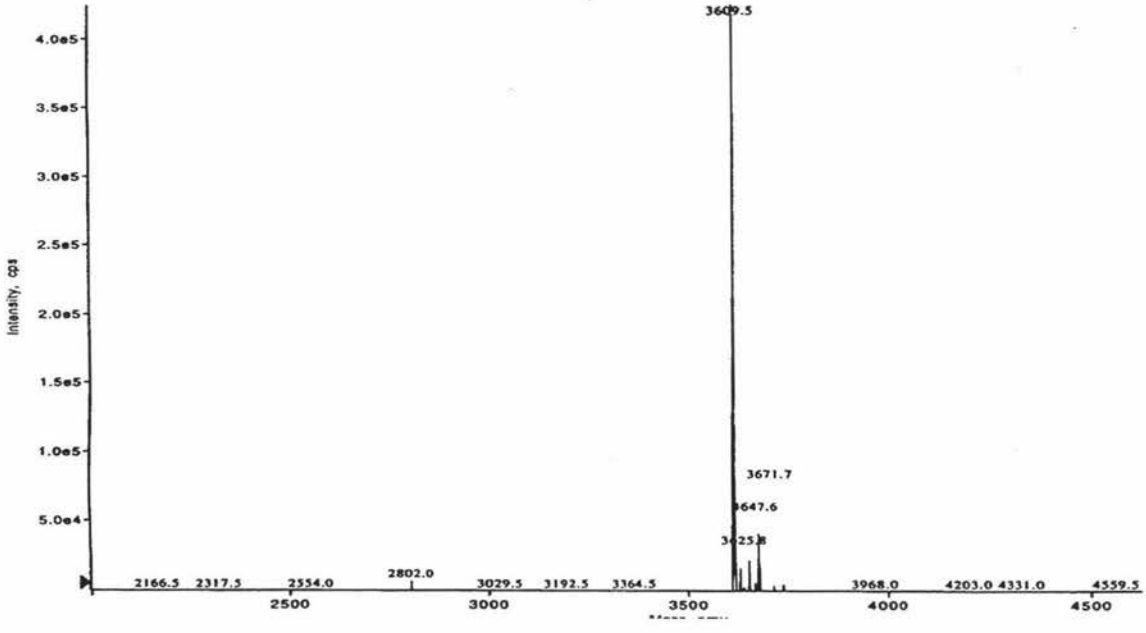


Figure 4.2.3 Mass spectrum for the large fragment of insulin trypsin digestion

The sample for the isolated reduced complex was concentrated with Centricon-10 tubes, washed with MilliQ water twice and then the supernatant reduced to a minimal amount. In order to minimize procedures, which might upset the structure, freeze-drying

technique was not employed here for the treatment of the reduced complexes. A rough calculation was carried out to estimate the concentration of the reduced complex. More than the normal final ratio (1 : 50) of trypsin was added since the trace amount of reduced complex was used. Another concern was that the dextran might act somewhat as a steric inhibitor to prevent any hindered insulin molecules from contacting trypsin molecules.

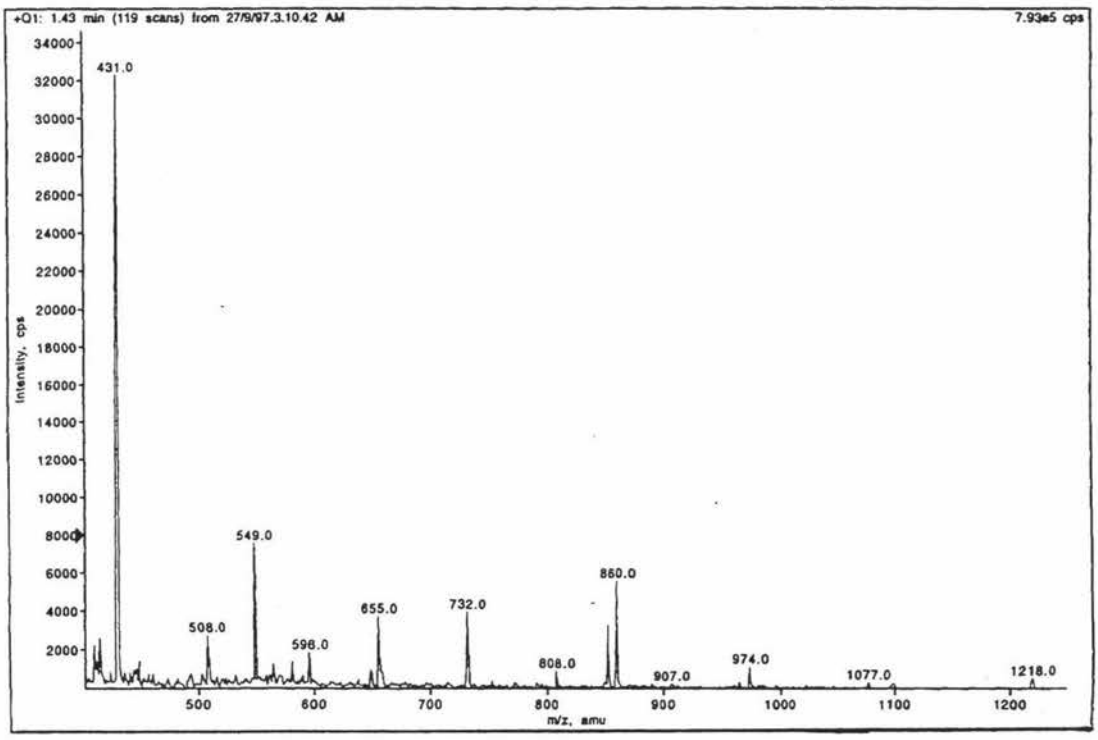


Figure 4.2.4 Mass spectrum for heptapeptide-like peak from reduced complex trypsin digestion

Under the same conditions as the insulin digest, the chromatogram (Fig. 4.2.1b) obtained for the reduced complex clearly showed that the heptapeptide-like peak which appeared at the same retention time as the heptapeptide peak of insulin trypsin digestion was present, but the desoctapeptide-insulin-like peak nearly absent. The heptapeptide-like peak was trapped and subjected to mass spectral analysis. The result confirmed its MW was 860 as well (Fig.4.2.4). When the insulin digest was mixed with the reduced complex digest, and subjected to the PepRPC column as well, the chromatogram indicated that the heptapeptide peaks overlapped with each other, while the desoctapep-

Table 4.2.1 AAA for the complex after trypsin treatment

Residue	Complex I	Complex I corrected	Complex II	Main fragment	Main fragment Theory	'Hepta-peptide	'Hepta-peptide Theory
Asx	0.55	0.28	0.55	0.55	3	-	-
Glx	0.17	0.09	0.17	0.17	7	-	-
Ser	2.67	1.37	2.67	2.67	3	-	-
Arg	1	0.51	1	1	1	-	-
Thr	2.49	1.28	2.49	2.49	2	-	-
Gly	3.3	1.69	3.3	2.3	3	1	1
Ala	2.29	1.18	2.29	1.14	1	1.14	1*
Pro	0.95	0.49	0.95	0	-	0.95	1
Val	3.82	1.96	3.82	3.82	4	-	-
Ile	1.81	0.93	1.81	1.81	2	-	-
Leu	5.4	2.78	5.4	5.4	6	-	-
Phe	1.81	0.93	1.81	0	1*	1.86	2
½ Cys	1.16	0.59	1.16	1.16	6	-	-
Lys	0.49	0	0	0	0	0	0*
His	1.32	0.68	1.32	1.32	2	-	-
Tyr	3.89	2.0	3.9	3	3	0.9	1

Complex I: amino acid composition of the complex after trypsin treatment with respect to arginine. Complex I corrected: amino acids of complex I reduced by 0.49 of a residue after adjusted lysine to zero. Complex II: amino acid composition of Complex I corrected with respect to one arginine again. The Phe* of main fragment and the Lys* of heptapeptide were expected to bind to dextran. The B chain C-terminal Ala* adjacent to the lysine is lost because of trypsin digestion.

tide peak appeared at the same place where it was absent for the reduced complex digest sample. The later broad peak (⇑, 75% B) behind the desoctapeptide fragment was due to the complex (Fig.4.2.1b, c). It can be concluded that the heptapeptide fragment was cleaved but almost no desoctapeptide fragment was released by tryptic digestion of the reduced complex (Fig.4.2.1b). This indicated that one of the potential binding sites, B29-Lys was not 100% bound to activated dextran, and otherwise the heptapeptide would not have shown up. It appears that one or both of the other

potential binding sites A1-Gly and B1-Phe had achieved comprehensive binding. In other words the desoctapeptide peak should be present if A1/B1 binding(s) were absent. The broad peak (⇑, Fig. 4.2.1b) due to the reduced complex after trypsin digest was trapped and subjected to AAA (Table 4.2.1), in order to examine if some heptapeptide fragments were still present in the trypsin-treated reduced complex and determine the percentage of B29-Lys binding.

Relative quantities of each amino acid composition present were determined with respect to arginine (Table 4.2.1). AAA was achieved by RP-HPLC. The amino acid-dextran fragments were thought to be eluted early, since AA-dextran species should be hydrophilic (Scheme 5.1.1). 49% of lysine was present in the complex. It was probably due to incomplete digestion of hindered insulin or heptapeptide fragments adhered to the complex. It was assumed that after correction of 0.49 of lysine in terms of exclusion of 0.49 compositions of insulin molecules, the adjusted values (Table 4.2.1, column 3) reflected the approximately true values of the trypsin-treated complex. The data in column 4 were determined with respect to arginine as well. In other words, lysine was adjusted to zero (column 3, complex I corrected) and other amino acids were reduced by 0.49 of a residue (column 4, complex II). The data were split into 2 columns, which represented the main fragment and heptapeptide fragment, according to the theoretical values. One significant figure was proline, which was only present in heptapeptide fragment, as well as Phe, which should mostly belong to heptapeptide fragment. It could be deduced that about 90% binding occurred at B29-Lys. In summary, the AAA result indicated that a significant proportion of B23-B29 amino acids were still bound to dextran either via B29-Lys or incomplete trypsinolysis had occurred.

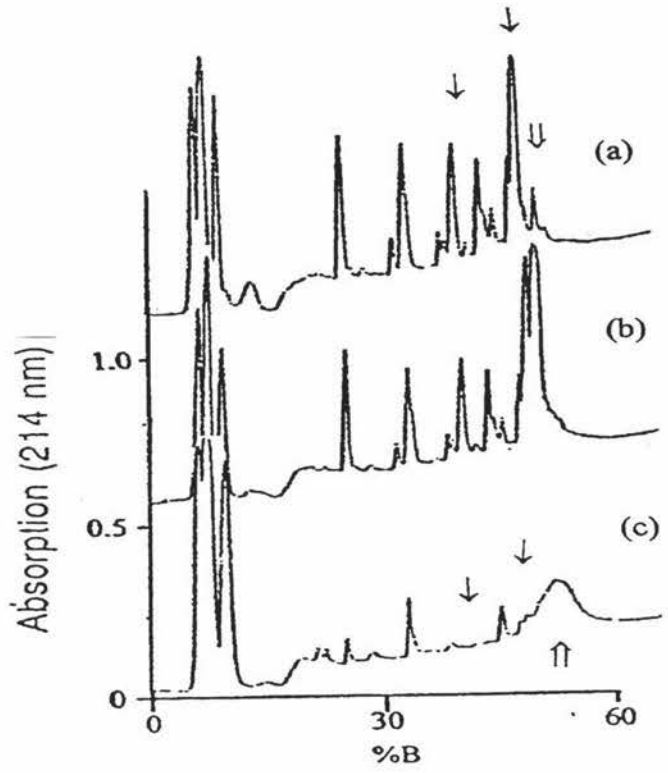


Figure 4.2.5 Peptide mapping of α-chymotrypsin digestion of insulin and reduced complex on FPLC

(a) Chymotrypsin digestion of insulin; (b) Mixed sample of insulin (⇓) + (a); (c) Chymotrypsin digestion of reduced insulin-8% activated dextran complex. Buffer A: 0.1% HCOOH, 100% H_2O; Buffer B: 0.1% HCOOH, 100% CH_3CN. A linear gradient from 0% to 60% B was run over 30 minutes. Flow rate: 0.4 ml/min.

The actual chromatograms (Fig. 4.2.5) obtained from chymotrypsin insulin digestion were more complicated than expected, probably due to the quality of the α-chymotrypsin used. 5 main peaks and several small peaks needed to be identified. The reduced complex was isolated and digested by α-chymotrypsin. 2 main peaks (↓) were nearly absent, while 3 others remained. The broad peak (⇑) after the last main peak was believed to be insulin fragment-dextran complex (Fig. 4.2.5c).

Isolated reduced insulin-dextran complex was also digested by α-chymotrypsin and run on SMART system (Fig. 4.2.6). A control experiment was run to confirm the last twin peak (⇓) was α-chymotrypsin. A larger dose of chymotrypsin (1 : 10) was used in an attempt to digest insulin in order to compare with that of complex (Fig.4.2.6a), since it was believed that overdose of chymotrypsin would be needed to digest the complex due to the relatively tiny amount of insulin present in the complex. In other words, I thought a larger dose

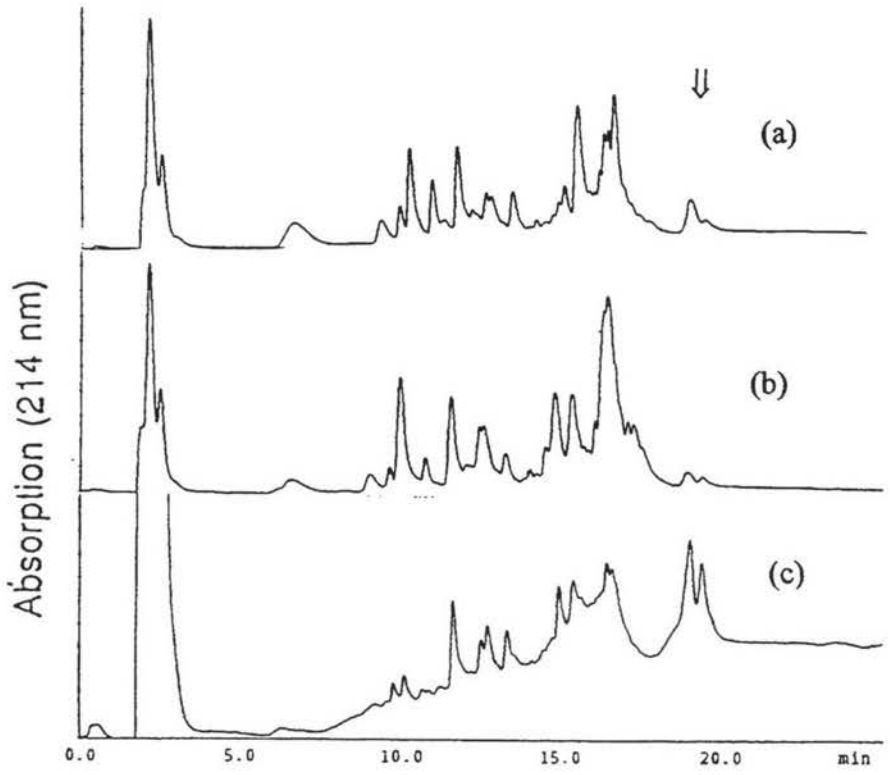


Figure 4.2.6 Peptide mapping of α-chymotrypsin digestion of insulin and reduced complex on SMART system

(a) Insulin digested by overdose chymotrypsin (1/10). (b) Insulin digested by normal dose chymotrypsin (1/50). (c) Insulin-dextran complex digested by chymotrypsin. The details were described in the methods section.

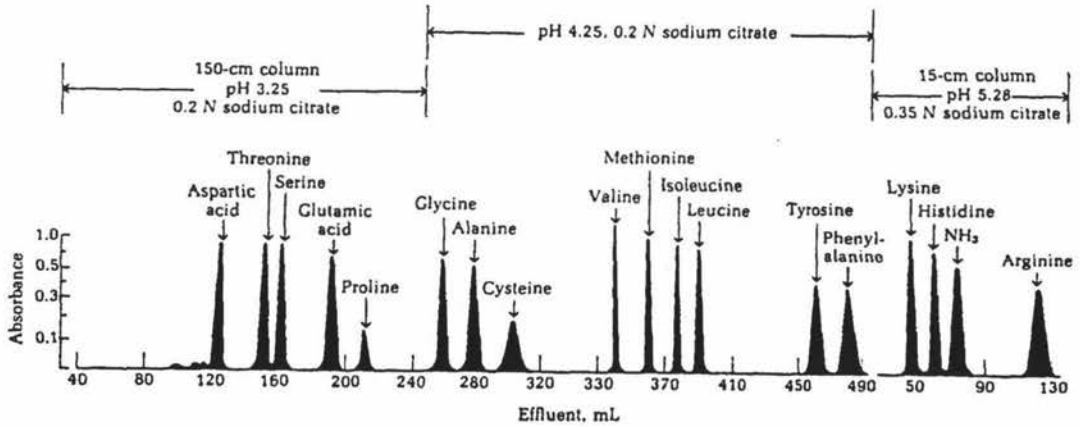
would be advisable as chymotrypsin can autolyze so the dose was raised in order to have a large excess over complexed insulin. Since two potential binding sites A1-Gly and B29-Lys were separately located in 2 peptide fragments, it was expected that after the digestion, the peak area ratio could be used to compare with those of insulin digest to determine the percentage binding at the A1 and B29 sites. However, the incomplete digestion by α-chymotrypsin made it difficult to estimate and interpret the binding nature of the insulin-activated dextran complex. The results between the tryptic digestion and the AAA were in good agreement, therefore, amino acid analysis was chosen to carry out further investigation.

CHAPTER FIVE

ANALYSIS OF INSULIN BINDING TO OXIDISED DEXTRAN BY AMINO ACID ANALYSIS

5.1 Introduction

Amino acid analysis (AAA) based on the principle of ion-exchange chromatography is a widely used technique for separating, identifying and quantifying the amounts of each amino acid in a mixture. The differences in the acid-base behavior of different amino acids, i.e., differences in the sign and magnitude of their net electric charge at a given pH, are the basis for the analysis of amino acids. Additional factors make major contributions to the effectiveness of this procedure. Cation-exchange resins in a chromatographic column at pH 3 can bind the largest positive charge (lysine, arginine, and histidine) most tightly, and the least amount of positive charge (glutamic acid and aspartic acid) least tightly. All the other amino acids will have intermediate amounts of positive charge. The different amino acids will therefore be eluted at different rates, which depend largely on their pKa values but also partly on their adsorption or solubility in the resin particles. The elution is carried out with different buffers of successively higher pH. The area under each peak is proportional to the amount of each

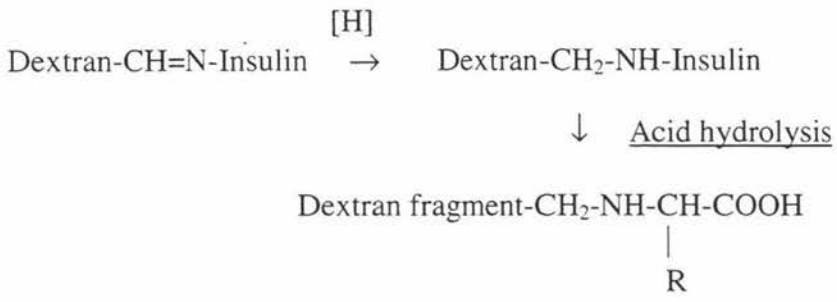


amino acid in the mixture (Fig. 5.1.1).

Figure 5.1.1 Automatically recorded chromatographic analysis of amino acids on a cation-exchange resin. (Lehninger, 1982)

AAA can also be carried out by HPLC. The reverse phase HPLC method was used to analyze a small numbers of samples off campus (Chapter 4 and Chapter 6).

The peptide bonds of peptides or proteins can be hydrolyzed to amino acids by strong acid (6N HCl, 110°C, 24 hours). However, most other chemical bonds cannot be cleaved under the same conditions. Based on these considerations, AAA was applied to the reduced insulin-dextran complexes. Dextran molecules might be chopped down to smaller fragments, but it was thought that the dextran fragments would stick after acid hydrolysis to those single amino acids, which had already bound to dextran molecules with carbon-nitrogen single bonds after reduction. It would be expected that an amino acid-dextran fragment be eluted at a different rate of the unbound amino acid. Because the amino group of the amino acid has thus been modified. One should expect a varied pKa and a different retention time to that of the parent amino acid (Scheme 5.1.1).

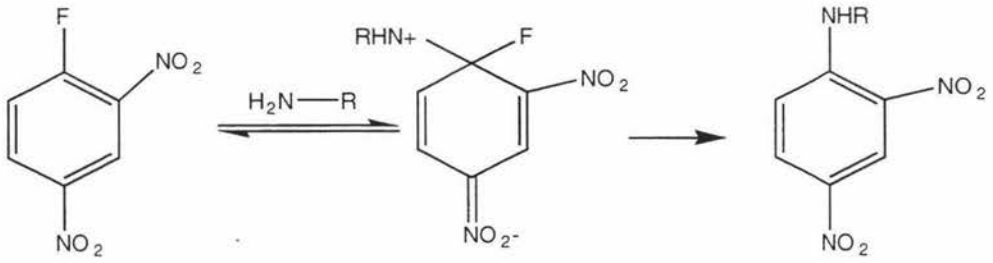


Scheme 5.1.1 Acid hydrolysis of reduced insulin-dextran complex

Therefore, the different value of an amino acid between free insulin and insulin-dextran complexes could be used to estimate the percentage of binding that had occurred to each of the 3 possible NH_2 groups.

In principle, enzymatic hydrolysis is better than acid hydrolysis as a means of obtaining an accurate amino acid analysis, because no covalent change occurs other than peptide bond hydrolysis. Some covalent changes other than peptide bond cleavage may occur in acid hydrolysis, e.g., hydrolysis of N-acyl groups and ω-amides and destruction of the indole side chain of tryptophan. However, it is difficult to completely hydrolyze all peptide bonds by enzyme, due to the resistance of certain peptide bonds and steric constraints (Lehninger, 1982).

2,4-dinitrofluorobenzene (DNFB) was once used to determine the free amino groups of insulin (Sanger, 1945) and the sequence of amino acids which occupied positions near the N-terminal amino acid residues (Sanger, 1949). Although this method has to a large extent been supplanted by the Edman phenyl isothiocyanate degradation procedure (Schroeder, 1967) and by dansylation with 1-dimethyl-aminonaphthalene-5-sulfonyl (Gray, 1967 a, b), dinitrophenylation with DNFB continues to be widely used for purposes of end-group analysis, modification of proteins, and identification of peptide fragments (Bunnett and Hermann, 1970). DNFB reacts readily at room temperature under mild conditions.



Scheme 5.1.2 Formation of DNP-amino acid

After complete hydrolysis, all normal peptide bonds in the peptide/protein chain are hydrolyzed. However, the covalent bond between the 2,4-dinitrophenyl group and the α-amino group of the amino terminal residue is resistant to this treatment. Consequently, the amino-terminal residues will be present in the hydrolysate as its 2,4-dinitrophenyl derivatives. The N-2, 4-dinitrophenyl (DNP) derivatives of the amino acids can be separated from each other and quantitatively estimated. This method has been used to identify the 2 N-terminal residues, the α-Gly and the α-Phe and the ε-Lys of insulin (Sanger, 1945). Based on the different chromatographic behavior of amino acids and their derivatives (DNP-amino acids), different retention time can be expected during the chromatographic run. In other words, the DNP-amino acid would not have the same retention time as that of the normal amino acid. The typical peak of a specific amino acid obtained can only be referred to the unsubstituted amino acids, or non-DNP-derivatives. If the reduced insulin-dextran complex is treated with DNFB, comparison of the AAA results between the DNP-insulin-dextran complex and the original insulin-dextran complex should prove useful. The relative molar difference in a particular

amino acid can thus be used to determine the percentage of the amino acid that is free from binding. It is another way to interpret the binding nature of insulin-dextran complex.

5.2 Results and Discussion

5.2.1 Initial Complex Binding Studies by AAA

Sodium cyanoborohydride was added after 7 days to the porcine insulin-16% activated dextran complex, which formed in 0.05M phosphate buffer, pH 7.4, with the beginning concentration of insulin (1.5 mg/ml), dextran (1 mg/ml), for the reduction of the imine bonds. A control experiment was carried out, with insulin in the same environment as that of the complex only in the absence of dextran. Three days later, the reduced complex and the control insulin were isolated by SEC (Superdex 75) with the running buffer of 0.05M phosphate, pH 7.4. Samples were subjected to acid hydrolysis and AAA (Table 5.2.1).

Table 5.2.1 Amino acid composition with respect to arginine of the complex in comparison to insulin and literature sequence.

Residue	Insulin	Complex	Theory
Asx	3.57	3.18	3
Thr	2.74	2.61	2
Ser	2.92	2.77	3
Glx	7.66	6.93	7
Pro	1.07	1.23	1
Gly	4.17	3.91	4
Ala	2.02	1.85	2
Cys	3.58	2.19	6
Val	2.89	1.95	4
Ile	1.29	0.73	2
Leu	5.19	3.7	6
Tyr	3.39	3.31	4
Phe	3.18	2.09	3
His	1.95	1.62	2
Lys	1.09	0.69	1
Arg	1	1	1

Relative quantities of each amino acid present were determined with respect to arginine (Table 5.2.1). Arginine was chosen as a standard since no other peaks overlapped with it, and it was believed to be less interfered with possible amino acid-dextran fragments, because it is eluted last and separately. Some amino acid values for free insulin did not agree well with the theoretical values. Others were in good agreement, probably due to problems with the Massey University AAA analyzer at the time. A small number of AAA were run off campus toward the end of this study when the Massey IMBS facility closed completely while awaiting parts and then until a steady baseline was achievable.

Since cysteine is partially degraded by the acid hydrolysis, it was not considered during the AAA interpretation of the results. The amino acid composition of the reduced complex was in good agreement with that of the control insulin result. A1-Gly, B1-Phe and B29-Lys were three amino acids of potential binding sites. The biggest variation occurred with Phe. One third of the Phe was lost, while about 0.3 out of one Lys was lost as well and Gly seemed to remain about the same value. It might be speculated that once the imine bonds were reduced, even after the acid hydrolysis, the amino acids, which contributed to the imine bonds would still stick to dextran fragments. Dextran might be degraded to small carbohydrate groups, however, small fragments would still link to those amino acids. The properties of these amino acid-dextran fragment conjugates should be different from those of free amino acids. Therefore, the retention times of them should be different for the runs of AAA as the NH_2 is blocked. In other words, the difference in amino acid compositions between the complex and the free insulin could be used to estimate the actual binding percentage of the potential binding sites. It was also possible that the amino acid-dextran fragment eluted at the same time as other different amino acids. Therefore, some amino acid compositions of the reduced complex may be altered. It can be deduced from the result (Table 5.2.1) that insulin molecules tend to form imine bonds with activated dextran under those conditions predominantly at B1-Phe sites over B29-Lys sites, however, it could not be concluded at this stage if A1-Gly was a binding site as well.

5.2.2 AAA Studies for Complexes Formed at Different pHs

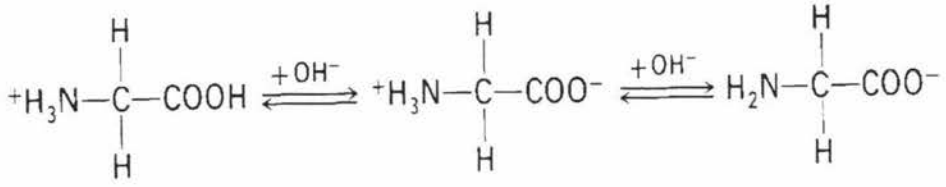
The complex with a molar ratio of 27% activated dextran to porcine insulin of 1 : 10 was formed in 0.05M phosphate pH 7.4, pH 9 and pH 10. The mixture contained 2.25 mg/ml of insulin and 1.5 mg/ml dextran. The reduced complexes were isolated by SEC with 0.05M phosphate, 0.15M NaCl, pH 7.4 running buffer, concentrated with Centricon-10 and then subjected to AAA.

Table 5.2.2.1 AAA for complexes of 27% activated dextran and porcine insulin formed at pH 7.4, pH 9 and pH 10

Residue	pH 7.4	pH 9	pH 10	Theory
Asx	3.14	2.98	3.45	3
Thr	1.42	1.45	1.68	2
Ser	1.91	1.76	2.03	3
Glx	5.42	>5.28	5.72	7
Pro	2.6	2.23	2.53	1
Gly	3.59	3.55	4.02	4
Ala	2.41	2.23	2.82	2
Cys	0.66	0.85	0.38	6
Val	4.19	>3.2	3.71	4
Ile	1.83	1.63	1.88	2
Leu	6.66	>5.21	5.96	6
Tyr	4.23	3.48	3.59	4
Phe	2.45	2.52	2.4	3
His	1.5	1.5	1.62	2
Lys	0.3	0.35	0.46	1
Arg	1	1	1	1

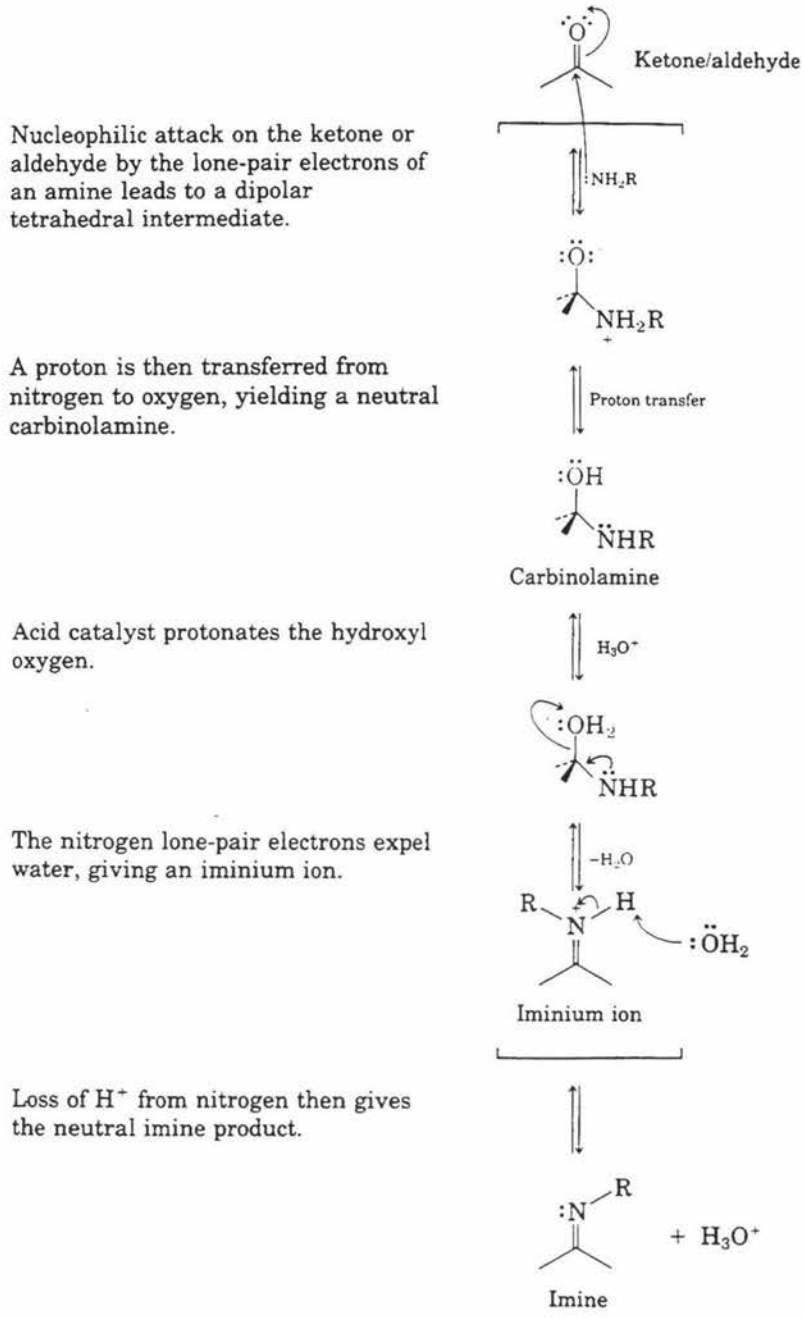
Relative quantities of each amino acid present were determined with respect to arginine (Table 5.2.2.1). The amino acid compositions of all three complexes were not in very good agreement with the theoretical values, perhaps because of the problems with the AA analyzer. Nevertheless attempts were made to extract some indications of what had happened from the data. Most of amino acid compositions of these three complexes were in good agreement with each other. It seemed that the composition of Tyr for the complex formed at pH 7.4 was a bit higher than those of two higher pHs. When the chromatograms were revisited, it was obvious that analyzer fluctuations made it

difficult to achieve a steady baseline. Under these circumstances, the data was then used as a reference in an attempt to compare the relative values and find approximate trends. This might lead to useful interpretation of the binding nature of complexes. B1-Phe, being thought to be the most reactive amino acid, maintained very similar values in terms of similar binding at the three pH levels. In other words, the difference in different pH conditions for complex formation did not affect the binding between B1-Phe and dextran. It could be deduced that B1-Phe was the prime amino acid to bind to the activated dextran. The values of B29-Lys and A1-Gly, were very similar for complexes formed at pH 7.4 and pH 9, however a bit higher recovery of both amino acids was obtained for the complex formed at pH 10. It may be said that lower binding rate for B29-Lys occurred at high pH environment. However, it did not apply to the crosslink rate. As we deduced previously, the higher pH of complex formation environment tended to cause a higher crosslink rate between insulin and activated dextran molecules.



Scheme 5.2.2.1 Acidic and basic nature of an amino acid

If the pKa of an amino acid is lower than the pH environment, the amino acid is more likely to change from unprotonized amine form to protonized amine form (Scheme 5.2.2.1). The protonized amino groups tend to be less nucleophilic than the unprotonized ones, since the long-pair electrons have formed a bond with a proton. The mechanism suggests that the long pair electrons of an amine are needed for the imine formation (Scheme 5.2.2.2).



Scheme 5.2.2.2 **Mechanism of imine formation by reaction of an aldehyde with a primary amine (McMurry, 1996).**

The pKa of α-NH_2 of Phe is 9.1, 9.8 for α-NH_2 of Gly and 10.8 for ε-NH_2 of Lys. In theory, without the consideration of any steric effects and environmental effects, the lower the pH, the higher reactivity the α-Phe is over ε-Lys (Phe > Gly > Lys), or the higher the pH, the higher reactivity the ε-Lys will be. At pH 10, it was expected that more ε-Lys tended to take part in the imine bond formation. This would satisfy the greater crosslink deduction, but not for the data obtained from this set of experiments.

At the same time, the data for A1-Gly could indicate its binding or not as well. In order to solve the puzzle, more experiments were carried out.

8% activated dextran coupled with insulin at pH 7.4 and pH 9, 0.1M phosphate buffer were also reduced, isolated and subjected to AAA.

Table 5.2.2.2 Amino acid composition with respect to arginine of the complex formed at pH 7.4 and pH 9 in comparison to insulin literature sequence

Residue	pH 7.4	pH 9	Theory
Asx	3.34	-*	3
Thr	1.77	4.87*	2
Ser	2.47	4.49*	3
Glx	5.79	7.45*	7
Pro	1.00	2.98*	1
Gly	4.42	3.61	4
Ala	2.05	1.37	2
Cys	0.81	1.96	6
Val	3.67	4.05	4
Ile	1.20	2.25	2
Leu	4.57	5.96	6
Tyr	2.64	3.1	4
Phe	1.95	2.33	3
His	1.47	1.83	2
Lys	0.90	0.65	1
Arg	1	1	1

Relative quantities of each amino acid composition present were determined with respect to arginine (Table 5.2.2.2). The first five results (*) of the reduced complex formed at pH 9 was abnormally high with the absence of Asx. This was apparently due to a machine problem with the first elution buffer (pH 3.25). Other values were in

reasonable ranges. Most of the amino acid compositions of the reduced complex formed at pH7.4 were in good agreement with the theoretical ones. In comparing the values of Lys, Phe and Gly, the higher the values, the less the binding occurred. At the higher pH the more binding occurred at B29-Lys, and A1-Gly, the less at B1-Phe, maybe suggests Gly and Lys bind faster at higher pH. The result of B29-Lys agreed with those of insulin-27% activated dextran complex. However, the large variation was occurred at A1-Gly. It suggested that other approaches were necessary to obtain reliable conclusions.

5.2.3 AAA Studies for Different Extent of Activation of Dextran at pH 7.4

Under the same conditions (pH 7.4 phosphate incubation buffer), 16% and 27% activated dextran reacted with porcine insulin, reduced complexes were isolated and subjected to AAA.

Relative quantities of each amino acid composition present were determined with respect of arginine as well (Table 5.2.3.1). The results showed that about half the amino acid compositions agreed with each other. The biggest variations occurred with Glx, Pro, Val and Leu. The B29-Lys composition for 16% activated dextran complex was twice than that of 27% one. The higher the recovery, the lower the binding involved. In other words, the more highly activated the dextran, the greater the tendency for easy imine formation with B29-Lys. It could be deduced that over 70% binding occurred at B29-Lys. It agreed with the deduction from AAA results (about 90% binding at B29-Lys) from trypsin digestion of insulin-27% activated dextran complex (Table 4.2.1). The composition of Phe for 16% activated dextran complex was abnormally low, it was difficult to make a deduction from these data. The composition of Gly for 16% activated dextran complex was in good agreement with the theoretical value. In other words, no A1-Gly bound to dextran. However, the composition of Gly for 27% activated dextran complex showed decreased value in terms of over 70% A1-Gly binding involved. Due to the fluctuating data, no final judgement could be made at this stage.

Table 5.2.3.1 Amino acid composition with respect to arginine of the complexes (insulin-16% activated dextran and insulin-27% activated dextran) in comparison to insulin literature sequence

Residue	16% activated	27% activated	Theory
Asx	2.84	3.14	3
Thr	1.95	1.86	2
Ser	1.77	2.13	3
Glx	4.76	5.71	7
Pro	0.61	1.46	1
Gly	4.11	3.28	4
Ala	1.91	2.15	2
Cys	0.52	1.01	6
Val	2.64	3.46	4
Ile	1.12	1.33	2
Leu	4.61	5.27	6
Tyr	2.85	3.27	4
Phe	1.19	1.7	3
His	1.23	1.49	2
Lys	0.52	0.27	1
Arg	1	1	1

5.2.4. AAA Study for DNP-Insulin Derivatives

Based on the fluctuating data, believed to be not wholly due to the deficiencies of the AAA at the time, a new DNP-derivative approach was tried in an attempt to solve the problem. The complex was formed at 0.05M phosphate, pH 9 with insulin (1.5 mg/ml) and 8% activated dextran (1 mg/ml). After being reduced for 3 days, reduced complex was isolated by SEC (Superdex 75) with a running buffer of 0.05M phosphate, 0.15M

NaCl, pH 7.4, and then concentrated with Centricon-10 and washed with MilliQ water. The concentrated sample was subjected to acid hydrolysis and AAA.

Table 5.2.4.1 Amino acid composition in respect to arginine of the DNP-insulin-dextran complex in comparison to the complex and literature sequence.

Residue	DNP-	Complex	Theoretical
Asx	2.97	-	3
Thr	1.95	4.87	2
Ser	>1.92	4.49	3
Glx	>5.45	7.45	7
Pro	1.64	2.98	1
Gly	>3.26	3.61	4
Ala	2.16	1.37	2
Cys	3.31	1.96	6
Val	2.61	4.05	4
Ile	1.54	2.25	2
Leu	>4.25	5.96	6
Tyr	0.20	3.1	4
Phe	2.00	2.33	3
His	0.18	1.83	2
Lys	0.09	0.65	1
Arg	1	1	1

The DNP-insulin-dextran complex was prepared as described in the experimental method in Chapter 2. The DNP-insulin can be obtained as powder in 2 hours, however, the DNP-insulin-dextran complex was formed over a much longer time. Once the isolated reduced complex from SEC was reacted with DNFB, the precipitate was hardly obtained since the amount of insulin was too small. The reduced mixture, which was ultrafiltered with a 10K MWCO membrane and washed with buffer several times, was

reacted with DNFB directly. The trace of free insulin can be ignored after the ultrafiltration procedure, since the mixture contained little free insulin after the reduction.

It has been demonstrated that DNP-glycine was relatively unstable to acid hydrolysis (Sanger, 1945). Only 40% DNP-Gly remained after 8 hours boiling with 20% (w/v) HCl, and 5% left after 24 hours acid hydrolysis. Acid hydrolysis for 8 hours was chosen for the DNP-complex, so that if a time period of as long as 8 hours was used, a rather large correction for breakdown must be applied. Due to the overloading of DNP-derivative complex sample, several peaks of residues, Ser, Glx, Gly and Leu were off scale (Table 5.2.4.1), so these values could not be used for the interoperation. Asx, Thr and Ala were in very good agreement with the theoretical values. The big differences occurred with Tyr and His, probably due to the formation of O-DNP-tyrosine and N-(imidazole-DNP)-histidine. Since it was suggested that under the experimental conditions, DNFB reacted with amino, phenolic-hydroxyl, thiol and possible imidazole groups (Sanger, 1945). The recovery of Phe was 2, indicating N-terminal Phe absence. The specific peak for Lys was overlapped by a broader peak, probably due to the presence of DNP-amino acid(s). Since the actual composition of glycine was off scale, it was difficult to consider here, however, it could be predicted being as a similar value as that of complex. The composition of the first five amino acids for the insulin-dextran complex were abnormally higher than those of theoretical values, while the peak of Asx was absent at the chromatogram, indicating a problem occurring with the first eluting buffer run of AA analyzer. The compositions of other amino acids were in agreement with those of theoretical values. The values of Phe and Lys were also in agreement with those of other samples under similar formation conditions.

In summary, this DNP-derivative method could be used to determine the free α-Phe and ε-Lys, however, due to the unstability of DNP-Gly to acid hydrolysis, accurate interpretation of this residue was questionable.

5.2.5. Final AAA Study for Insulin-Dextran Complex

The AAA results obtained from all AAA studies above indicated that B1-Phe in insulin was the predominant binding site, while B29-Lys demonstrated reasonable binding ability as well, especially at higher pH. The fluctuating values for A1-Gly failed to

show the true nature of the binding if any at this site. In order to confirm the conclusions based on the experimental results, the sample was analyzed by a much more sensitive and accurate AA analyzer off campus.

Table 5.2.5.1 Amino acid compositions of insulin-16% activated dextran complex with respect to arginine in comparison to insulin literature sequence

Residue	Complex	Theoretical
Asx	2.99	3
Thr	1.83	2
Ser	2.84	3
Glx	6.81	7
Pro	1.03	1
Gly	3.40	4
Ala	1.96	2
Cys	7.96	6
Val	2.71	4
Ile	1.04	2
Leu	5.93	6
Tyr	3.81	4
Phe	2.03	3
His	1.88	2
Lys	0.51	1
Arg	1	1

Porcine insulin (1.5 mg/ml) was reacted with 16% activated dextran (1 mg/ml) at 0.05M phosphate, 0.02% NaN_3, pH 7.1 buffer. 5 days later, reducing agent, cyanoborohydride was added. The reduced complexes were then isolated by SEC (Superdex 75) 3 days later, and concentrated to a limited amount for AAA.

Relative quantities of each amino acid present were determined with respect to arginine (Table 5.2.5.1). Most of amino acid compositions of the reduced complex were in excellent agreement with those of the theoretical values. The biggest variations occurred with Cys, Val, Ile, Phe, Gly and Lys. The values of Val and Ile were lower while that of Cys was higher than those theoretical values, and the value of Cys should be lower than the theoretical one since it can normally be degraded during the acid hydrolysis carried out before AAA. There may be interference by amino acid-dextran fragments causing the odd values. 2 out of 3 of the Phe recovered indicated that almost all the B1-Phe bound to the activated dextran, while half the Lys recovery demonstrated about half the binding occurred at B29-Lys. These results were in agreement with both that from trypsin digest study and those of AAA studies above. The composition of Gly was a bit lower than the theoretical one (3.4 out of 4), it probably indicated that some binding occurred at A1-Gly site. It has been suggested that the amino group in A1-Gly forms hydrogen bonds with A4-Glu and A5-Gln (Li, et al. 1986), and hence the reactivity of A1-Gly was affected leading to little binding occurring for A1-Gly. However, since Gly was used to block all the unreacted aldehyde groups in activated dextran, the composition of Gly might not reflect the true value for their results. In order to find out the binding nature of A1-Gly, the chemical cleavage of A chain and B chain of insulin was employed next.

CHAPTER SIX
CHEMICAL CLEAVAGE STUDIES OF THE A AND B CHAINS OF INSULIN

6.1. Introduction

Oxidative cleavage of disulfide bonds with performic acid has remained a reliable and often used procedure since it was first introduced in the sequence analysis of bovine insulin (Sanger, 1949; Sanger and Thompson, 1953). First of all, it is a simple, one-step chemical modification. Secondly, the reaction medium (90-95% formic acid) is a strong denaturant. Thirdly, the resultant cystic acid residues are stable during acid hydrolysis as well as under alkaline pH conditions. Finally, high yields of about 90-95% can be obtained when converting disulfide bonds to cystic acid (Hirs, 1967 a, b; Scoffone and Fontano, 1970). However, being a potent oxidant, performic acid produces several other reactions beyond oxidation of sulfur-containing amino acids. The destruction of indole side chain of tryptophan is one of the unavoidable side reactions (Hirs, 1967b). Therefore, it is not suitable for preparation of tryptophan-containing proteins for sequence analysis. Other residues such as Met, Ser, Thr, Tyr may also be modified.

Chemical cleavage of the two chains of insulin whose activated dextran complex has been reduced to form irreversible binding should provide direct information about potential A1 and B1 binding sites. In other words, the question about A1 binding can be answered. Due to its simplicity, this performic acid method was chosen initially for this study. The fact that insulin does not contain tryptophan was of course a bonus in this study.

As indicated above, a significant limitation to the use of performic acid oxidation for the cleavage of disulfide bonds in proteins is the concomitant destruction of tryptophan. This limitation does not apply to procedures which involve reductive cleavage and subsequent blocking of the SH groups (Hirs, 1967c). Reductive cleavage of protein disulfide bonds is nearly universally performed with mercaptans. A linear disulfide is the final product when 2-mercaptoethylamine (Markus et al., 1962) or 2-mercaptoethanol (Crestfield et al. 1963; Hirs, 1967c) is used as reducing agent, and an

intramolecular cyclic disulfide is the product when dithiothreitol or dithioerythritol is the reductant (Cleland, 1964). Following reduction, many alkylating agents (e.g., iodoacetic acid or iodoacetamide) are added in amounts nearly equivalent to the moles of low molecular weight reductant added (Hirs, 1967c).

Iodoacetic acid and its amide have long been employed as S-alkylating agents (Crestfield et al. 1963), usually basing on the desirability of an anionic or a neutral half-cysteine derivative. Exclusion of light, during both alkylation and subsequent removal of excess reagents, is necessary to minimize the side reactions (Hirs, 1967c). Since free iodine, which may react with resides aromatic side chains, can be produced by light-catalyzed oxidation. Alkylation with ethyleneimine (Raffer and Cole, 1963; Hirs, 1967c) produces a cationic derivative, S-(2-aminoethyl) cysteine. However, its elution position between lysine and histidine in the standard amino acid analyzer chromatographic protocol resulted in this method not being considered for this study (Spackman et al. 1958; Cole, 1967). 4-vinylpyridine (Cavins and Friedman, 1968, 1970), an α, β-unsaturated compound has been widely used for sulfhydryl alkylation. The derivative formed, S-(2-pyridylethyl) cysteine, is stable in acid, well resolved on the AAA analyzer, and amenable to spectral quantitation in the intact protein.

Tributylphosphine is a specific and potent agent for the cleavage of disulfides in proteins. First of all, peptides and proteins can be fully reduced with a 5-20% molar excess of tributylphosphine, and no strong denaturing agents such as urea seem to be necessary. Secondly, with large a (e.g., 10-fold) excess of PBu_3 to protein disulfide, no modification other than cysteine could be detected. Furthermore, it is compatible with many alkylating agents, since phosphine and the resulting phosphine oxide are both unreactive toward ethyleneimine, epoxypropane and 4-vinylpyridine. Therefore, reducing agent and alkylating agents can be added together (RÜegg and Rudinger, 1977).

An approach of electrolytic reduction of the disulfide bonds of insulin has been undergone as well (Markus, 1964). Complete reduction was achieved under only slightly alkaline conditions and without the use of reducing or denaturing agents. The subsequent analysis could be carried out during the course of reduction, since there was no reducing agent to remove. However, large amount of highly toxic mercury, which was used as the cathode made it difficult to carry out at this study.

6.2. Results and Discussion

Capillary electrophoresis (CE) was applied to separate the performic acid oxidized insulin. Insulin was initially run to monitor the progress of oxidative reaction. The small peak after the principal one was desamidoinsulin (Fig. 6.2.1a). The diagram (Fig. 6.2.1b) for oxidized insulin clearly showed two main peaks along with several small ones, one main peak along with a small one was a bit ahead of that of insulin, the other main peak accompanied with several small ones had a much longer retention time. It is known that B chain of insulin appeared to be slightly more basic than unchanged insulin, while A chain demonstrated an acidic nature. It was predicted that B chain had similar CE retention time as that of insulin.

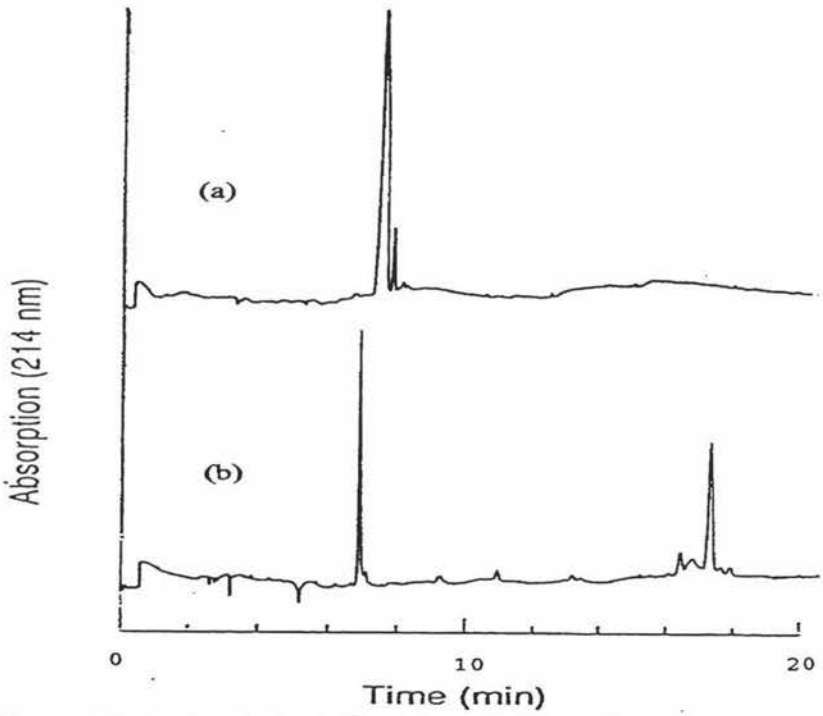


Figure 6.2.1 Analytical Capillary Electrophoretograms of (a) insulin and (b) oxidised insulin by performic acid
CE buffer: 0.02M ethanolamine, pH 7.8. 20 KV was applied for each CE run at 30°C over 30 minutes. The rate of chart recorder was 0.1 cm/min.

The oxidized insulin was fractionated to A chain and B chain by following the method of Sanger (1949). Insulin was dissolved in formic acid, 30% of H_2O_2 was added at ambient temperature. Water was added 15 minutes later, the mixture was then evaporated in vacuo to a small amount. The oxidized insulin was precipitated by acetone. Freshly oxidized insulin was dissolved in ammonia and precipitated with addition of acetic acid to pH 6.5. The supernatant solution (A) was used to prepare the

A chain. The precipitate was dissolved in HCl and ethanol was added. The new precipitate was discarded after centrifugation. The supernatant and washings were taken almost to dryness in vacuo and precipitated by acetone. This was the B chain of insulin.

The above solution (A) was brought to pH 4.5, after removal of the precipitate and was taken to almost dryness in vacuo. The concentrated solution was adjusted to pH 5.5 and the new precipitate discarded. This solution was dried under high vacuum. The residue was the A chain.

Separated A chain and B chain were run on the CE to identify the peaks. The electrophoretogram (Fig. 6.2.2a) was the analytical run of B chain separated by Sanger' method (1949). The reduced complex was isolated and concentrated to a limited amount, ample formic acid and hydrogen peroxide were added. Same procedure was followed as for that of the oxidation of insulin to obtain insulin fractions from the complex. CE diagram (Fig. 6.2.2b) showed a decent peak for chain A-like species (↓) and a small one for chain B-like species. This is in contrast to the decent peak of B chain to a small one of A chain obtained from oxidized insulin. It indicated that the amount of free A chain of insulin from the complex was much more than that of B chain. In other words, the binding of A1-Gly appeared to be much less than that of the combination of B1-Phe and B29-Lys. The peak height ratio of B chain to A chain from oxidized insulin was 2 : 1, in comparison of 0.35 :1 for the oxidized complex. It could be thus deduced that the release of A chain was about 5 times more than that of B chain. The broad peak (⇓) after the A chain (↓) was thought to be the insulin fragment-dextran complex.

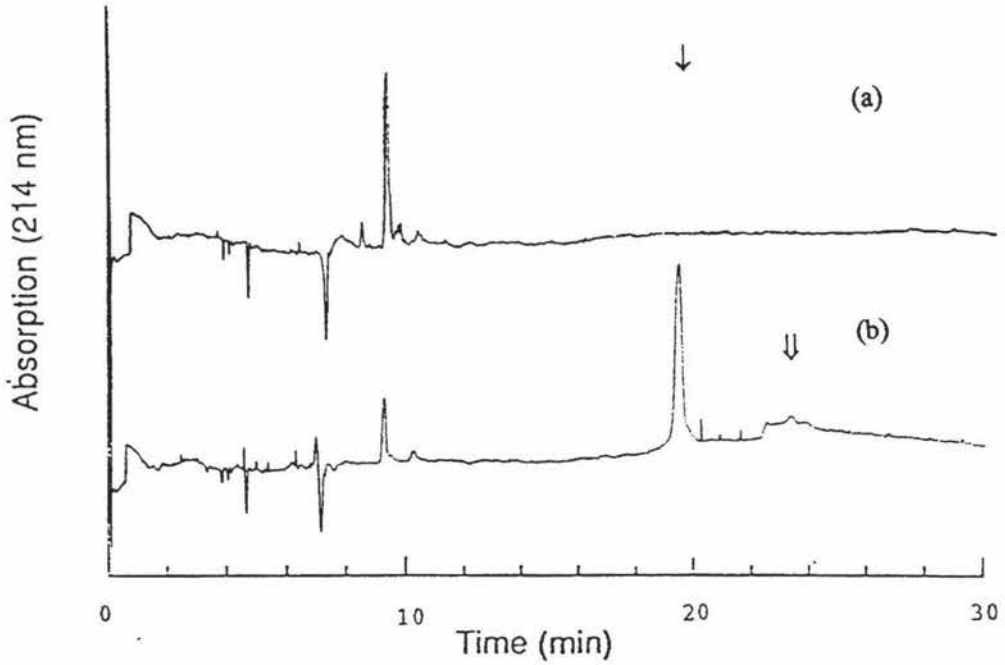


Figure 6.2.2 Analytical capillary electrophoretograms of (a) oxidized B chain and (b) oxidized insulin-dextran complex

CE buffer: 0.02M ethanolamine, pH 7.8. 20 KV was applied for each run at 30°C over 30 minutes. The rate of chart recorder was 0.1 cm/min.

Oxidized complex was run on FPLC (SEC Superdex 75) to examine if performic acid had any effect on dextran. The running buffer was 0.05M phosphate, pH 7.4 (Fig.6.2.3). The chromatogram showed a similar shape of insulin-dextran complex occurred at the oxidized complex. It indicated that performic acid was unlikely to have an effect on both the binding sites and the dextran. Therefore, this performic acid method can be one of the methods to investigate the A1-Gly binding.

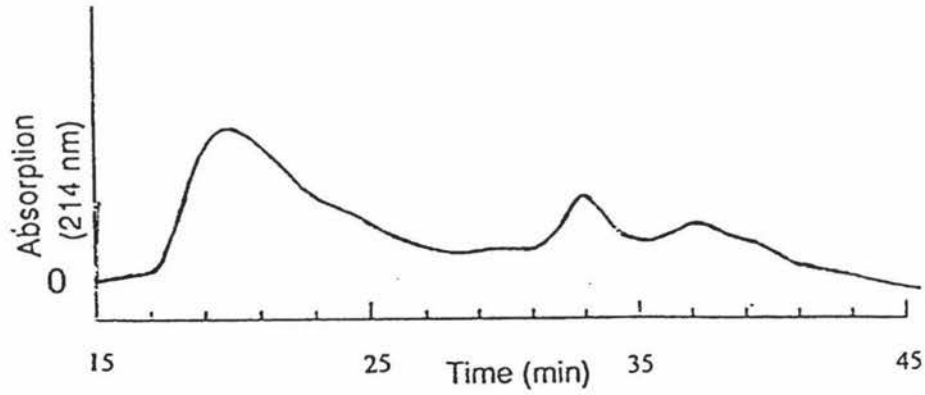


Figure 6.2.3 SEC chromatogram of reduced complex after performic acid treatment

Running buffer: 0.05M sodium phosphate, pH 7.4. Column: Superdex 75 (FPLC). Flow rate: 0.4 ml/min. The rate of chart recorder: 0.4 cm/min. A_{214}=1.0.

The complex after performic acid treatment was isolated by SEC (FPLC, Superdex 75) as well (Fig. 6.2.3). Centricon–10 tubes were used to concentrate and remove the small

molecules. The sample was then freeze-dried and subjected to acid hydrolysis and AAA.

Table 6.2.1 Amino acid composition with respect to alanine of the complex after oxidative cleavage in comparison to insulin A chain and B chain sequences

Residue	Complex	'A chain'	A chain theory	'B chain'	B chain theory
Asx	2.92	1.92	2	1	1
Glx	6.4	3.4	4	3	3
Ser	2.29	1.29	2	1	1
Arg	0.79	0	0	0.79	1
Thr	2.34	1.34	1	1	1
Gly	2.8	0	1	2.8	3
Ala	2	0	0	2	2
Pro	0.8	0	0	0.8	1
Val	3.01	0.01	1	3	3
Ile	1.32	1.32	2	0	0
Leu	3.94	-	2	3.94	4
Phe	1.75	0	0	1.74	3
½ Cys	0.15	-	4	-	2
Lys	0.68	0	0	0.68	1
His	1.17	0	0	1.17	2
Tyr	5.77	3.77	2	2	2

Relative quantities of each amino acid composition present were determined with respect to alanine (Table 6.2.1). AAA in this case was with the RP-HPLC method. There were several strange peaks appeared at the Asx and Glx areas at the beginning of the chromatogram. They were thought to be amino acid-dextran fragments, which should be more hydrophilic than the free amino acids (Scheme 5.1.1). The B chain was used as a standard when compared the percentage of A chain present. The data split to 'B chain' were as close the theoretical values as possible. Ile was only present at the A chain. About 65% recovery of Ile indicated the similar amount of 'A chain' bound to dextran. Asx, Glx and Ser agreed with this deduction. The low value of Gly indicated that bound A1-Gly was eluted at a different time. The significant amount of Ile present indicated the A chain in terms of A1-Gly bound to dextran as well.

Several side products were produced during this performic acid oxidation (Sanger, 1949). If the peak area or height is to be used as a scale to measure the ratio of A chain to B chain, the distribution of the desired products (A chain and B chain), the side products must be taken into account. Reverse Phase Liquid Chromatography (RPLC) was applied for further investigation (Fig. 6.2.4).

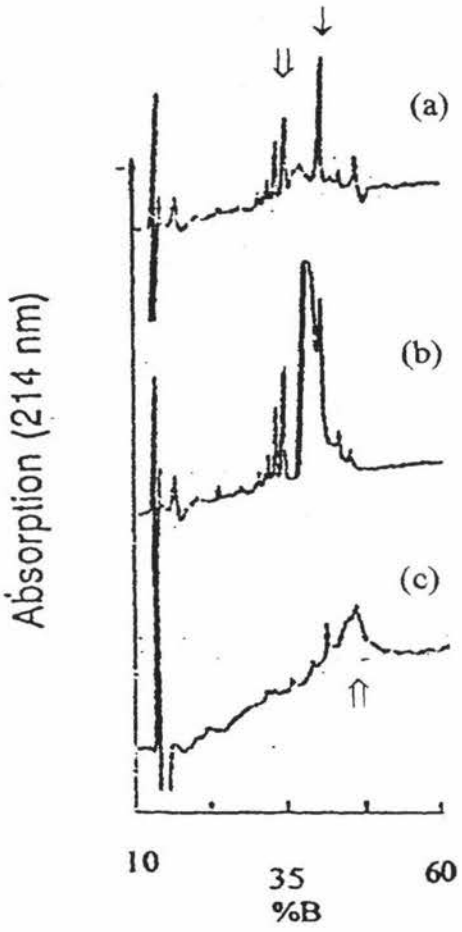


Figure 6.2.4 Peptide mapping of oxidative cleavage of insulin and reduced complex.

(a) oxidative cleavage of insulin. (b) mixed sample of (a) + insulin. (c) oxidative cleavage of reduced insulin-16% activated dextran complex. Buffer A: 0.1% HCOOH, 100% H_2O; Buffer B: 0.1% HCOOH, 100% CH_3CN. A linear gradient was run from 10% to 60% B over 30 minutes. Flow rate: 0.4 ml/min.

Comparison of the chromatograms of oxidized insulin and that of the mixed sample of insulin and oxidized insulin, indicated that no insulin was left in the mixture of oxidized insulin. In other words the oxidative cleavage of insulin was complete (Fig.6.2.4a, b). Isolated B chain was run on the FPLC as well (figure no shown here) and it was confirmed that the sharp peak (↓) in Fig.6.2.4(a) which just followed the insulin peak

was B chain (Fig.6.2.4b). In other words, three to four consecutive peaks (⇓) should refer to the A chain and other side products (Fig.6.2.4a). Oxidized complex was run on the FPLC as well (Fig.6.2.4c). It could be expected that the last broad peak (⇑) was derived from insulin-dextran fragment complexes. There is some indication that B chain (↓) and A chain (⇓) fragments were also present.

Tributylphosphine and 4-vinylpyridine were chosen as the reducing and S-alkylating agents, due to the advantages mentioned previously in the introduction section of this chapter. The experimental methods were based on two literature methods (Rüegg and Rudinger, 1977; Aitken and Learmonth, 1996). Ethyleneimine, the S-alkylating agent by Rüegg and Rudinger, is highly toxic. 4-Vinylpyridine was therefor selected instead. After reductive cleavage, the precipitate was found to be only partial soluble in 0.1% formic acid water solution. Therefore, 50% acetic acid was used to dissolve the precipitate before loading on the FPLC (PepRPC column). Chromatograms (Fig. 6.2.5) were shown below.

After reductive cleavage, the insulin fragment-16% activated dextran complex was found nearly insoluble in 50% acetic acid as well, probably alkylated by 4-vinylpyridine. This complex was washed with 50% acetic acid and 0.1% formic acid several times to remove free insulin fragments, then subjected to acid hydrolysis and AAA.

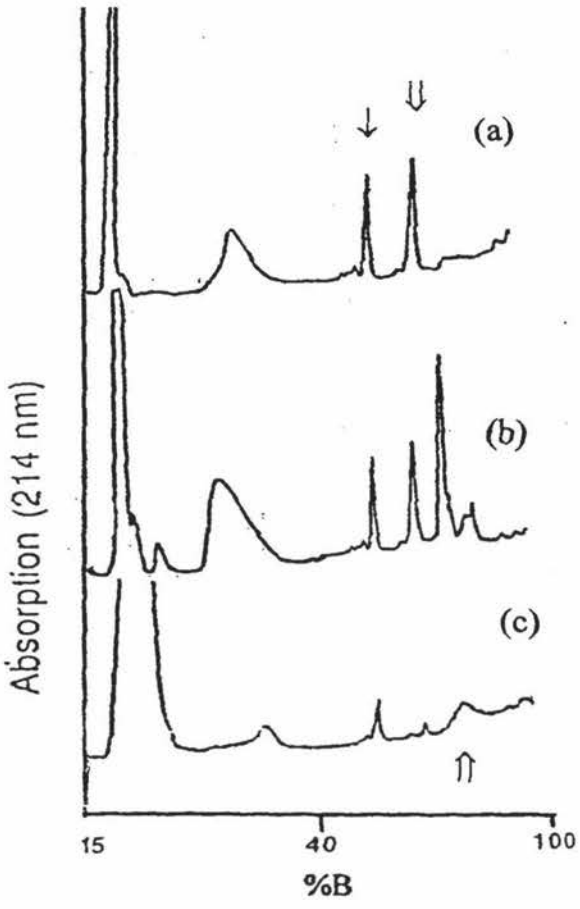


Figure 6.2.5 Peptide mapping of reductive cleavage of insulin and reduced complex

(a) reduction of insulin disulfide bonds. (b) mixed sample of (a) + insulin. (c) disulfide reduction of reduced insulin-16% activated dextran. Buffer A: 0.1% HCOOH, 100% H_2O; Buffer B: 0.1% HCOOH, 60% CH_3CN. A linear gradient was run from 15% to 50% B over 30 minutes following by a 10 minutes gradient to 100% B.

The two peaks (Fig. 6.2.5a) were trapped and subjected to mass spectral analysis. It was confirmed that the first sharp peak (↓) was S-alkylated A chain (MW 2803.5) (Fig. 6.2.6), and the peak (⇓) was S-alkylated B chain (MW 3609.5) (Fig. 6.2.7). When the mixed sample of disulfide reduction insulin (Fig6.2.5a) and insulin was run on FPLC, the insulin peak (Fig. 6.2.5b) had a longer retention time than the split B chain, indicating the cleavage was complete. The reduced insulin-16% activated dextran, which formed in 0.05 M phosphate, at pH 7.1, was cleaved and run on FPLC (Fig. 6.2.5c). The corresponding A and B chains were present on the chromatogram (Fig. 6.2.8 and Fig. 6.2.9). The peak height of A chain was much higher than that of B chain for the cleaved complex, in comparison of the contrast for split insulin. The broad peak

(⇑) after B chain was thought to be the insulin fragment-dextran complex, but in very small amount in comparison of the complex after trypsin digestion. It was thought that 4-vinylpyridine, the S-alkylating agent, was bound to dextran molecules as well, resulting in a more hydrophobic 'family' of compounds which were only slightly soluble in 50% acetic acid (loading solvent for FPLC). Therefore, the insoluble substances were washed with 50% acetic acid and 0.1% formic acid in order to remove the free A and B chains, then subjected to acid hydrolysis and AAA.

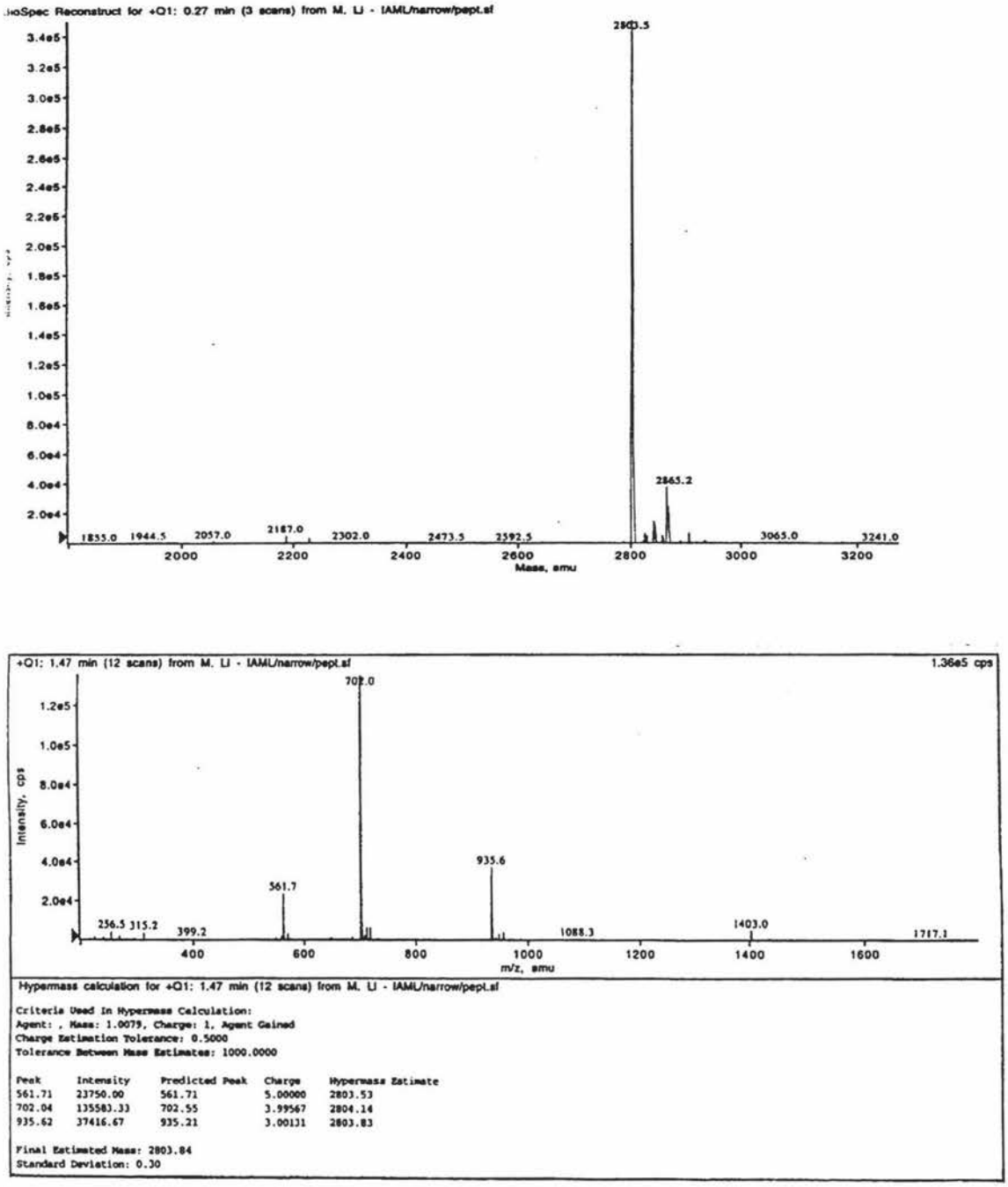
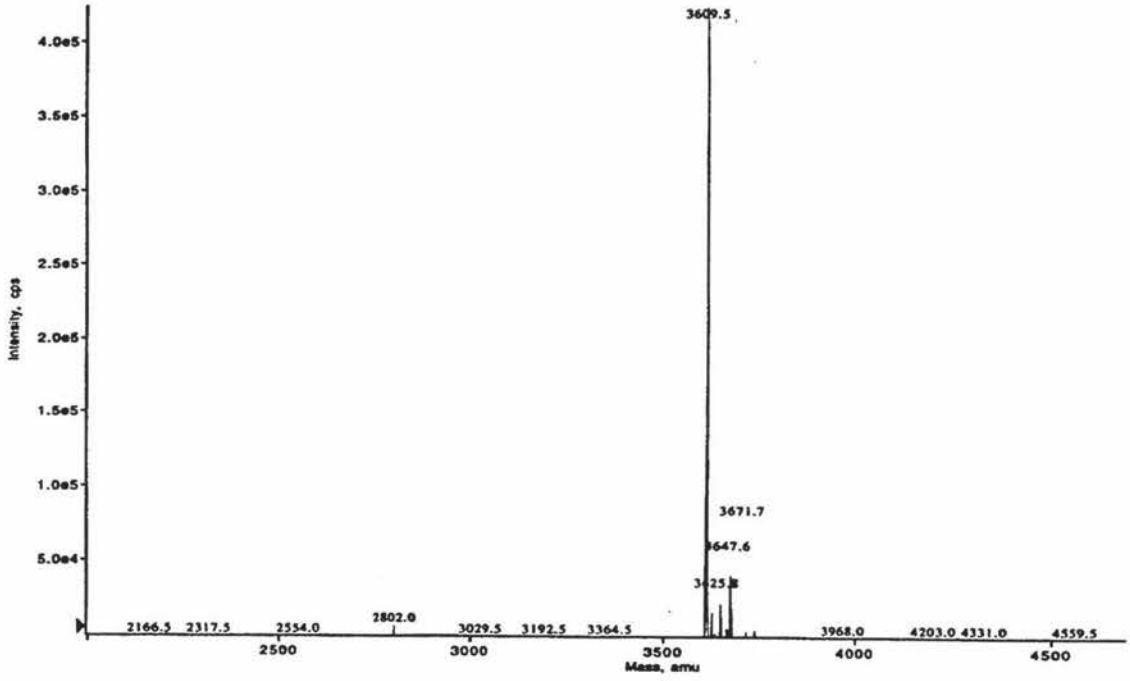
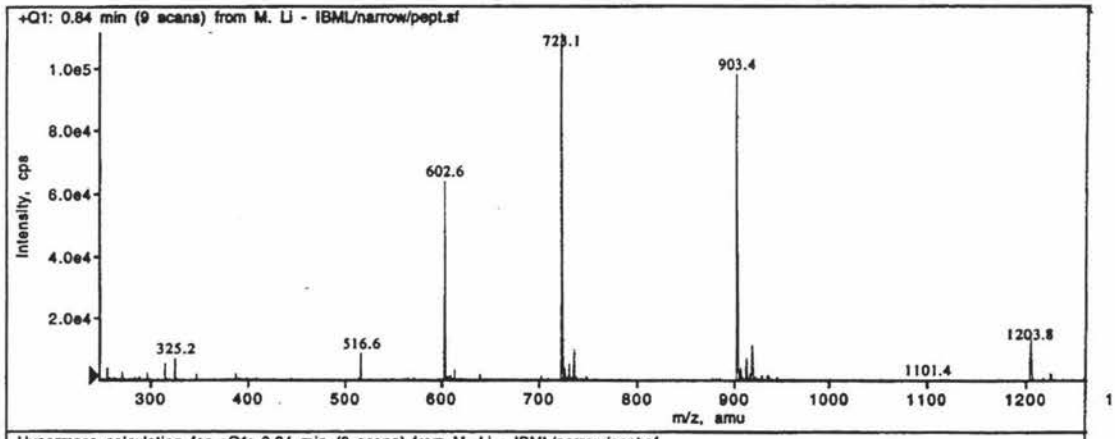


Figure 6.2.6 Mass spectrum for fragment A from reductive cleavage of insulin





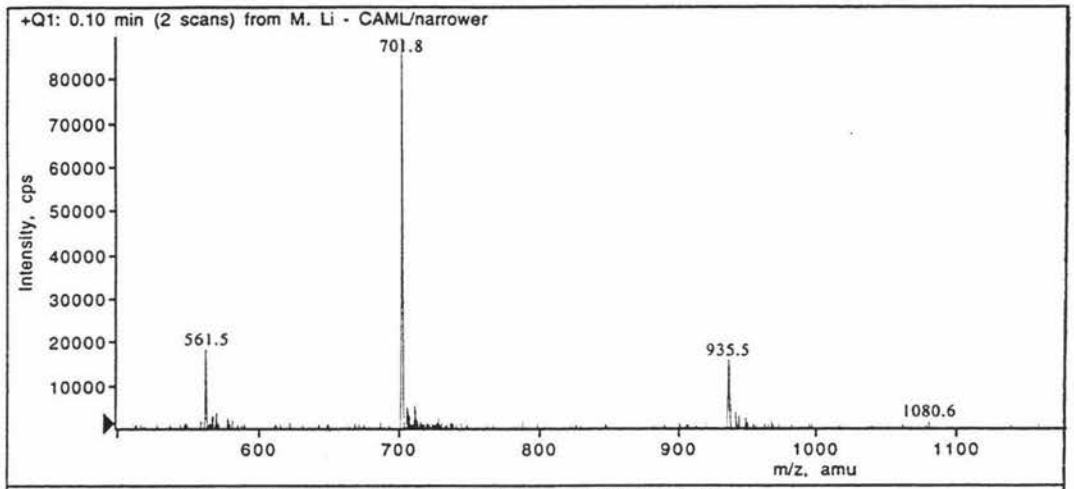
Hypermass calculation for +Q1: 0.84 min (9 scans) from M. Li - IBML/narrow/pept.sf

Criteria Used In Hypermass Calculation:
Agent: , Mass: 1.0079, Charge: 1, Agent Gained
Charge Estimation Tolerance: 0.5000
Tolerance Between Mass Estimates: 1000.0000

Peak	Intensity	Predicted Peak	Charge	Hypermass Estimate
602.61	64000.00	602.61	6.00000	3609.63
723.14	111000.00	724.18	4.99144	3610.67
903.42	98111.11	902.17	4.00557	3609.66

Final Estimated Mass: 3609.99
Standard Deviation: 0.59

Figure 6.2.7 Mass spectrum for fragment B from reductive cleavage of insulin



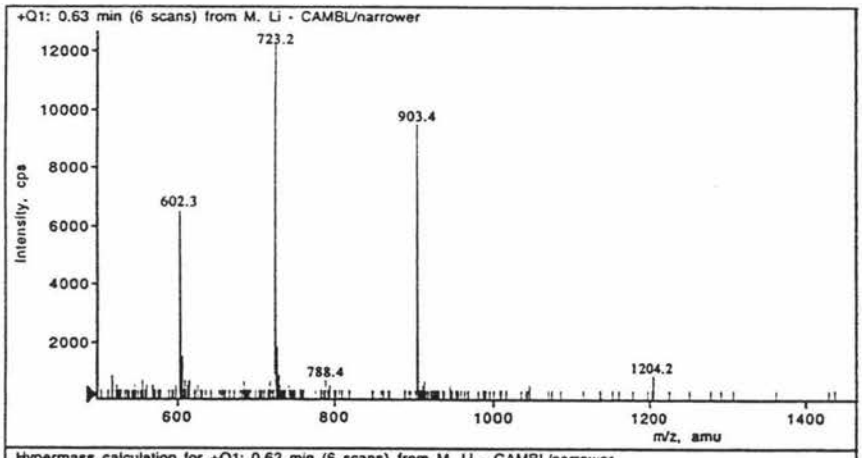
Hypermass calculation for +Q1: 0.10 min (2 scans) from M. Li - CAML/narrower

Criteria Used In Hypermass Calculation:
Agent: , Mass: 1.0079, Charge: 1, Agent Gained
Charge Estimation Tolerance: 0.5000
Tolerance Between Mass Estimates: 1000.0000

Peak	Intensity	Predicted Peak	Charge	Hypermass Estimate
561.52	18500.00	561.52	5.00000	2802.55
701.84	89500.00	702.58	3.99439	2803.34
935.52	16000.00	935.46	2.99912	2803.55
1403.35	1000.00	1404.53	1.99759	2804.67

Final Estimated Mass: 2803.53
Standard Deviation: 0.88

Figure 6.2.8 Mass spectrum for fragment A from reductive cleavage of reduced insulin-dextran complex



Hypermass calculation for +Q1: 0.63 min (6 scans) from M. Li - CAMBL/narrower

Criteria Used In Hypermass Calculation:
Agent: , Mass: 1.0079, Charge: 1, Agent Gained
Charge Estimation Tolerance: 0.5000
Tolerance Between Mass Estimates: 1000.0000

Peak	Intensity	Predicted Peak	Charge	Hypermass Estimate
602.32	6500.00	602.32	6.00000	3607.84
723.24	12666.67	727.03	4.97253	3611.17
903.42	9500.00	901.54	4.00835	3609.66
1204.23	833.33	1204.22	3.00002	3609.66

Final Estimated Mass: 3609.58
Standard Deviation: 1.36

Figure 6.2.9 Mass spectrum for fragment B from reductive cleavage of reduced insulin-dextran complex

Table 6.2.2 Amino acid composition with respect to alanine of the complex after reductive cleavage in comparison to insulin A chain and B chain sequences

Residue	Complex	'A chain'	A chain theory	'B chain'	B chain theory
Asx	3.52	2.52	2	1	1
Glx	7.35	4.35	4	3	3
Ser	2.25	1.25	2	1	1
Arg	0.86	0	0	0.86	1
Thr	1.97	0.97	1	1	1
Gly	3.51	0.51	1	3	3
Ala	2	0	0	2	2
Pro	0.99	0	0	0.99	1
Val	3.32	0.32	1	3	3
Ile	1.45	1.45	2	0	0
Leu	4.67	0.67	2	4	4
Phe	2.12	0	0	2.12	3
½ Cys	0.02	-	4	-	2
Lys	0.53	0	0	0.53	1
His	1.01	0	0	1.01	2
Tyr	2.55	0.55	2	2	2

Relative quantities of each amino acid composition present were determined with respect to alanine (Table 6.2.2). AAA was analyzed by RP-HPLC. There were several strange peaks appeared at the Asx and Glx areas at the beginning of the chromatogram. They were thought to be amino acid-dextran fragments, which should be more hydrophilic than free amino acids (Scheme 5.1.1). The values of Asx and Glx were a bit higher than those theoretical ones, probably due to the interferences near them. It was assumed that the 'B chain' was fully bound in order to make a comparison of the % 'A chain' binding. Therefore, the data split agreed with the 'B chain' first according to the theoretical values of the B chain. Because Ile was only present in the A chain, the 1.45 out of 2 indicated about 70% of A chain present in comparison of whole B chain present. Ser, Thr and Gly had similar values, while some amino acids Val, Leu and Tyr showed a bit lower values, since most of them had been split to satisfy the composition of the 'B chain' first. It also agreed with the AAA result of oxidative cleavage of the reduced complex. In summary, the result indicated about 60-70% binding occurred at A1-Gly.

In conclusion, the results of oxidative and reductive cleavage of the A and B chain of insulin-16% activated dextran complex indicated binding occurred at A1-Gly to a level of 60-70%.

CHAPTER SEVEN
CONCLUSION AND FUTURE WORK

7.1 Conclusion

The results reported in this thesis comprise an investigation into the conjugation of insulin to activated (oxidized) dextran, various release studies from the conjugates, and an attempt to interpret the binding nature of the conjugates. Previous studies (Battersby et al. 1996 and Fisher, 1997) did not study specific binding of any of the protein-activated dextran complexes they studied. A model system involving the sustained release of protein-activated dextran conjugates has been employed in this study. The rate of release and the maintenance of activity of the released protein are vital to the effectiveness of such a sustained release system. The success in the interpretation of the binding nature of the conjugate will lead to the examination of the relationship between the rate of release and the binding nature of the conjugate. If the projected binding could be obtained with certain conditions of complex formation, the desired rate of release for sustained release of protein would be achieved.

Activation of the dextran was obtained by periodate oxidation to give levels of 8%, 16% and 27% activated glucose moieties. The complex formations were examined under a wide range of conditions. Initial studies were begun with the determination of initial molar ratio of insulin to 8% activated dextran, in order to achieve as much binding as possible to activated dextran molecules with reasonable free insulin remaining for better resolution by SEC. A molar ratio of insulin to dextran of 10 : 1 from this set of experiments was used as a basic ratio for all future experiments.

Insulin was bound to 27% activated dextran through imine bonds at pH 7.4, pH 9 and pH 10. The differences in molecular distributions of complexes were examined by SEC. The molecular distribution of the complex formed at pH 7.4 maintained a similarity to that expected from the MW profile of dextran (Fig.3.2.3.2.2). However, those molecular distributions of the complexes formed at pH 9 and pH 10 indicated many more lower MW complexes were formed than at pH 7.4. Correspondingly smaller amounts of higher MW complexes were noted. After excluding the possibility of interference by buffer pH, it seemed most likely that the higher the formation pH, the more cross-links occurred between an insulin molecule and dextran molecules. In other

words, probably more binding sites in each insulin molecule were involved at higher pHs. Complex formation without shaking was chosen after comparison with shaking. Complex formation with shaking resulted in only a small increase in the rate of complex formation, while more cross-links might occur with shaking. Due to the considerations of ease of complex isolation and consistent conditions between the complex formation and complex release, approximate physiological pHs (pH 7.1-7.8) were normally used for complex formation in all subsequent experiments.

An equilibrium was established upon complex formation. Complex release studies were carried out with 8%, 16% and 27% activated dextran-insulin complexes under approximate physiological conditions (pH 7.4, 37°C). Immediate release was observed upon isolation by SEC. The largest release occurred in the first 24 hours for all three activation levels. However, the higher the activation level of dextran, the less the release occurred. An equilibrium was established after several days' release. In comparison to the release study at ambient temperature, which had a slower rate of release, the temperature at 37°C was thought to be one of the main driving forces for greater release.

Reduction of carbon-nitrogen double bonds (imine bonds) was performed by addition of sodium cyanoborohydride instead of $NaBH_4$ because of its specificity. After reduction, almost all the bound insulin showed no release under 37°C, pH 7.4, however, traces of free insulin-like species were still released from the reduced complexes. It could be possible that a chemically bound insulin molecule formed a dimer with an unbound insulin molecule. Other possibilities include a small number of hindered unreduced imine bonds and/or trapped but not bound insulin. The released species had a slightly longer retention time than those of free insulin (probably dimer) in SEC. The trace amounts of insulin present were not thought significant enough to interfere with the following experiments. The reduced insulin-activated dextran complexes were isolated and concentrated for the investigation of the binding nature of the complex.

For insulin, up to three potential sites only (A1-Gly, B1-Phe and B29-Lys) were expected to bind to oxidized dextran. A large part of my thesis was dedicated to seeking clues as to which of these 3 sites and/or to what degree they bound to oxidized dextran.

Enzymatic cleavage was initially employed to interpret some of the binding sites. Trypsin can only split the carboxyl sides of B22-Arg and B29-Lys. Two peptides were obtained after the trypsin digestion of insulin. The trypsin digestion of the reduced complex gave the release of the small fragment (B23-B29) with a nearly absence of the large fragment. It indicated that partial binding had occurred at B29-Lys, while the combination of B1 and/or A1 binding resulted in no release of the large fragment. The AAA result of the isolated complex after trypsin digestion demonstrated the presence of binding at B29-Lys. It was estimated that about 90% binding occurred at B29-Lys for the insulin-27% activated dextran complex, which formed at pH 7.1.

The reduced complexes were analyzed by AAA. Those amino acids at the binding sites were expected to still be bound to dextran fragments after acid hydrolysis. These dextran fragment-amino acids should have different retention time as those unbound amino acids (Scheme 5.1.1). Relative quantities of each amino acid were determined with respect to arginine. Initial studies were begun with reduced insulin-16% activated dextran complex formed at pH 7.4 in comparison of control insulin. 30% binding had occurred at B29-Lys, 100% at B1-Phe and probably no binding at A1-Gly.

The reduced insulin-27% activated dextran complexes formed at pH 7.4, pH 9 and pH 10 were studied by AAA as well. Binding at B1-Phe maintained similar values at the three pHs. It could be deduced that B1 might be the prime binding site for varying as pH did not seem to have an effect on its reactivity. B29-Lys and A1-Gly, had similar values between the complexes formed at pH 7.4 and pH 9, but a bit lower binding at B29-Lys for pH 10 and no binding occurred at A1-Gly. But these results did not agree with the imine formation mechanism, which suggests the long pair electrons of an amine in terms of unprotonized amino groups were needed for imine formation. The higher the pH, the higher reactivity the ε-Lys would be. An estimation of 70% binding at B29-Lys for the insulin-27% activated dextran complex formed at pH 7.4 was in agreement with the result of trypsin digest. Another set of experiments for insulin-8% activated dextran formed at pH 7.4 and pH 9 tended to have reasonable results with agreement with the imine formation mechanism. It indicated that there was more binding at B29-Lys, A1-Gly for the case formed at pH 9 than at pH 7.4. The higher the pH for complex formation, the more binding occurred at B29-Lys and A-Gly.

AAA studies for the complexes of insulin with 16% and 27% activated dextran formed at pH 7.4 indicated that the more highly activated dextran, the greater the tendency for easy imine formation with B29-Lys and A1-Gly.

The insulin-8% activated dextran complex, which formed at pH 9, reacted with DNFB to form DNP-insulin-dextran complex after reduction. The AAA results for control complex and the DNP-derivative indicated this method could be used to determine the amount of free α-Phe and ε-Lys. The different values of Phe and Lys between control complex and its DNP-derivative could be used to examine the binding. It could be deduced that about 70% binding was noted at B1-Phe and 26% binding at B29-Lys for the control complex. This was after consideration of no B1-Phe and 9% B29-Lys obtained for DNP-derived complex. However, due to the unstability of DNP-Gly to acid hydrolysis, the accurate estimation of A1-Gly was thought unreliable.

The reduced insulin-16% activated dextran complex formed at pH 7.1 was subjected to more accurate AAA. Almost all amino acids agreed with the theoretical values except Val, Ile, Cys, Phe, Gly and Lys. The differences in Phe, Gly and Lys indicated that almost all binding occurred at B1-Phe, about 60% at A1-Gly and 50% at B29-Lys.

The oxidative and reductive cleavage studies of A and B chains of insulin and the reduced complex were carried out in an attempt to interpret the A1-Gly binding. After chemical cleavage of the reduced complex, small amount of free split A and B chains were present. The amount of A chain was much higher than the B chain for both cases studied. The treated complexes were isolated and subjected to AAA. The results indicated a significant proportion of A chain bound to dextran, in terms of A1-Gly binding present. An estimation of 60-70% of A1-Gly was achieved for this study.

From these limited studies, insulin-activated dextran complex was seen to be capable of formation and release. The formation under approximate physiological conditions was indicated. The higher the oxidation rate of the dextran, the slower the rate of release from the complex. Trypsin digestion method accompanied by AAA demonstrated the extent of B29-Lys binding (Table 7.1.1). The AAA results of a range of reduced complexes formed at different conditions addressed the possibilities of all three binding sites. B1-Phe was thought to be the prime binding site from the AAA results. The chemical cleavage of A and B chain of insulin allowed us to estimate the extent of A1-

Gly binding. The data (Table 7.1.1) were mainly based on the complexes formed at pH 7.1, some at pH 7.4.

Table 7.1.1 Binding extent of three sites

% Dextran	% at B1-Phe	% at A1-Gly	% at B29-Lys
8%	~100%	n.d.*	10%
16%	~100%	60-70%	50%
27%	~100%	50%	90%

n.d.* = not determined

7.2 Future work

Investigations to date have indicated that the 3 possible sites (A1-Gly, B1-Phe and B29-Lys) of the insulin molecule are all capable of forming imine bonds with activated dextran. AAA results could be used to estimate the extent of the binding involved. Some experimental data was not totally reliable and did not agree well due to problems with the AA analyzer. Thus some AAA experiments need to be examined again to achieve a comprehensive understanding of the binding. Once the reproducible results are obtained, the examination of the relationship between the extent of binding and the experimental conditions could be carried on. This will lead to determine the relationships between the binding and the rate of release. The next step should involve the investigation of the tendency of the binding with a range of complex formation conditions. The desired binding might lead to the designed rate of release for the sustained release of proteins.

In order to make AAA interpretations more reliable, a study of the appropriate AA-dextran fragments produced after acid hydrolysis would be useful.

It is vital for released proteins to remain bioactivity. Bioassays were not available for this study. Although previous studies indicated that most of the proteins studied remained activity after release from the complex, bioassay must be carried out eventually for an insulin study like this one despite the apparent validity of the HPLC method (Fisher and Smith, 1986). This study concentrated on the use of Dextran T-40,

but the 2 days clearance rate from the body should be taken into account. MWs of species above 55 kDa can not be excreted via the kidney. They could only be degraded by a dextranase, so high MW dextrans might be useful to prolong the complex lifetime in the blood stream. Insulin-like growth factor (IGF) I and II have relatively similar structures to proinsulin, the success in solving insulin problems might lead to an investigation of the IGFs.

The problem involving cross-links could be studied further. A single chain peptide without Lys (only one binding site: N-terminal amino group) and with Lys (two binding sites) could be bound to activated dextran. The molecular distribution of the complexes could allow us to examine the differences in the oxidative degrees of dextran over a range of MW. It could also be very useful to find out how many aldehyde groups were present in a certain MW range of dextran molecule.

Applications of protein-activated dextran complex may not be limited to the protein case or the dextran case. Investigations could be extended to small drug molecules and/or different carriers. These preliminary studies into the binding nature of insulin-activated dextran have obtained a rudimentary understanding into protein-dextran complexation.

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