INSULIN STABILITY AND AGGREGATION IN AGITATED AQUEOUS SOLUTIONS

by

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ABSTRACT

Stability of protein-based pharmaceuticals is compromised by a variety of physical and chemical degradation pathways which substantially lower formulation efficacy. Biological activity can be lost as a result of chemical decomposition (cleavage of peptide bonds and such destructions of amino acid residues as oxidation, deamidation and ß-elimination), as well as physical (conformational) instability--loss of native structure caused by the disruption of non-covalent interactions (e.g. aggregation and adsorption).

The instability and aggregation of insulin solutions present serious problems for the traditional injection therapy of diabetes mellitus, and for the design of alternative methods of drug delivery and administration: aggregation of insulin preparations causes loss of potency and the inert aggregates obstruct tubing and catheters. The mechanism and kinetics of insulin aggregation have not been fully elucidated; yet, a better understanding of the degradation process is necessary for the design of rational stabilization strategies which specifically target the destabilizing pathways.

The goals of this investigation were to gain a fundamental understanding of insulin aggregation and to improve insulin stability in aqueous environments. Aggregation rates were studied by varying some of the basic destabilizing parameters: agitation rates, interfacial interactions, insulin concentration, Zn^{2+} content, solvent, and temperature. The reaction time course was followed by monitoring the concentration of non-aggregated species using UV absorbance at 280 nm. Quasielastic light scattering analysis was

used to determine the particle size distribution and to detect the formation of high molecular weight intermediates.

The combination of experimental observations and mathematical modeling led to the formulation of a reaction scheme describing insulin aggregation in agitated aqueous solutions containing solid hydrophobic surfaces. Initial destabilization was attributed to monomer denaturation at hydrophobic surfaces, followed by the formation of stable intermediate species which facilitated subsequent aggregation. The proposed mechanism identified possible pathways of destabilization, predicted the qualitative shape of kinetic profiles, and explained increased stability at higher insulin concentrations.

Stabilization strategies (based on the developed model for insulin aggregation) were proposed and verified. The mechanism of stabilization in the presence of an air-water interface was elucidated. Sugar-based nonionic surfactants (which excluded insulin from the hydrophobic interface) increased solution stability from 6 hours to more than 6 weeks for bovine Zn-insulin; Na-human insulin's stability was similarly enhanced from 2 hours to 6 weeks.

The model's ability to predict trends in insulin aggregation behavior suggested that it was an adequate representation of the aggregation processes, while the success of the stabilizing strategy indicated that the model identified one of the most important pathways of destabilization. These results illustrate how a fundamental understanding of physical degradation can be used to improve the stability of protein formulations.

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CHAPTER 1.

INTRODUCTION

1.1 Stability Issues for Protein-based Pharmaceuticals.

Recent advances in biotechnology and genetic engineering have made possible the production of many important proteins. The successful implementation of these products, however, requires careful handling and processing conditions which do not interfere with the stability and biological activity of the proteins. Protein-based pharmaceuticals are subject to many chemical and physical degradation pathways: chemical decomposition leads to loss of native structure through cleavage of peptide bonds and the destruction of amino-acid residues, while physical instability refers to conformational changes resulting from disruption of non-covalent interactions (Manning et al., 1989). In most cases, only the native conformation is biologically active, so it is important to preserve both the chemical integrity and the three-dimensional structure or folding of the protein.

While the mechanisms of many chemical degradation processes have been identified (e.g. proteolysis, oxidation, deamidation and ß-elimination) and ways to improve chemical stability have been proposed (Manning et al., 1989; Banga and Chien, 1988), physical instability (particularly protein aggregation) continues to present serious problems during production, storage and administration of protein-based drugs. Protein denaturation or unfolding is usually accompanied by the exposure of hydrophobic groups which are

normally buried in the interior of the molecule; the resulting conformations are unstable and seek to minimize their surface energy by interacting with other unfolded molecules in order to shield the hydrophobic moieties from the aqueous environment (Joly, 1965). In effect, aggregation locks in the denatured structure, making it nearly impossible to recover the protein's biological activity.

A fundamental understanding of the degradation mechanisms is necessary to protect the protein's biological functions: when the causes and pathways of instability are identified, rational stabilization strategies can be designed. While every protein will present the investigators with problems and concerns unique to its structure and function, some trends in physical stability will be common to many systems, so the understanding gained with a model protein (or at least the experimental approach used to study the mechanism of degradation) can contribute to improving formulation stability of other biomolecules.

1.2 Insulin: a Model Protein for Studying Aggregation.

Insulin regulates the transport of glucose from the bloodstream into cells where glucose is metabolized (Orci et al., 1988). The inability to produce adequate amounts of this hormone and the associated glucose intolerance and accumulation in the blood are the characteristic symptoms of diabetes mellitus (Orci et al., 1988). Since its discovery in the early 1920s (Banting and Best, 1922), insulin has become one of the most important and commonly used therapeutic proteins.

Insulin is a globular hydrophobic protein made up of two polypeptide chains (A and B) held together by 3 disulfide bonds (Figure 1.1). The hormone contains 51 amino acid residues and weighs ~5800 daltons (Blundell et al., 1972). The insulin precursor, proinsulin, is synthesized in the pancreatic beta cells, and subsequently converted to insulin by the enzymatic cleavage of the C peptide (Derewenda et al., 1989). Because of its medical importance, insulin is one of the most thoroughly studied molecules; it is the first protein whose chemical sequence was determined (Ryle et al., 1955) and it is the first to be commercially produced by recombinant DNA techniques (Johnson, 1983). Extensive studies of its 3-dimensional structure by X-ray crystallography have been undertaken (Blundell et al., 1972), and the amino-acid sequence of the insulin receptor has been recently determined (Derewenda et al., 1989). Insulin's small size, which makes it a particularly attractive protein for sequencing studies, puts it at the detection limits of many analytical methods used in solution studies. The low solubility of Zn-insulin at physiological pH and its tendency to adsorb to many different materials, further increase the difficulties involved in experimental work (Helmerhorst and Stokes, 1986; Jeffrey, 1986; Cuatrecasas and Hollenberg, 1975). Despite these problems, insulin's conformation in solution has recently been investigated using advanced NMR (Weiss et al., 1989; Ramesh and Bradbury, 1987), circular dichroism (Pocker and Biswas, 1980; Sato et al., 1983) and SEM techniques (Salemnik et al., 1989).

The biologically active form of the hormone (which circulates <u>in vivo</u> and binds to the receptor) is monomeric, but insulin readily

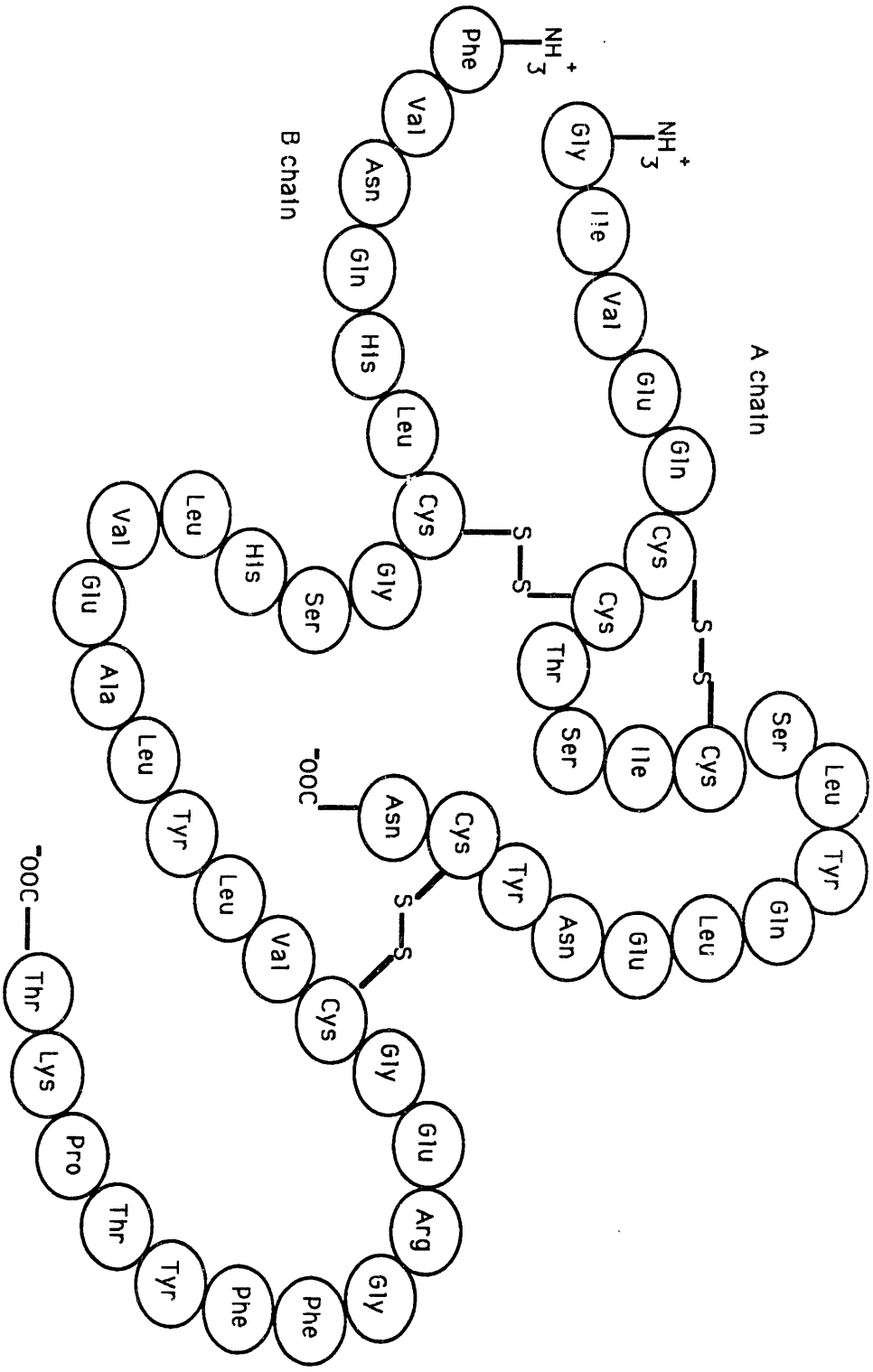

Figure 1.1: A schematic representation of the insulin molecule.

associates into dimers, and in the presence of zinc, three dimers assemble into a hexamer (Figure 1.2). The hydrophobic region of the insulin monomer involved in dimer and hexamer formation is the same portion of the molecule which binds to the receptor, and is responsible for the regulatory function of the hormone (Helmerhorst and Stokes, 1986). The monomer's self-association into dimers and hexamers leads to the burial of hydrophobic moieties, resulting in stable structures with largely polar surfaces. The hexamers crystalize readily; this conformation, which is used for storage in the pancreatic beta cells, has a very low solubility (Derewenda et al., 1989; Brange, 1987). Despite the wealth of information available in the literature about insulin's structure and function, the mechanism of physical degradation has not been fully elucidated.

1.3 Diabetes Mellitus: Current Therapies and Problems

Diabetes mellitus is an auto-immune disease which affects millions of people in the U.S. and world-wide; in this country, diabetes is the leading cause of blindness and one of the leading causes of death. Left untreated, the disease is fatal: the patients cannot use carbohydrates to fulfill their energy requirements, and must rely instead on fat and muscle metabolism; this leads to elevated blood levels of organic acids which cause ketoacidosis and diabetic comma (Brown, 1983).

The two most commonly encountered disorders are insulin dependent (Type I) and non-insulin dependent (Type II) diabetes (Pyke, 1989). The causes behind the onset of diabetes remain unknown, although there is convincing evidence that the destruction

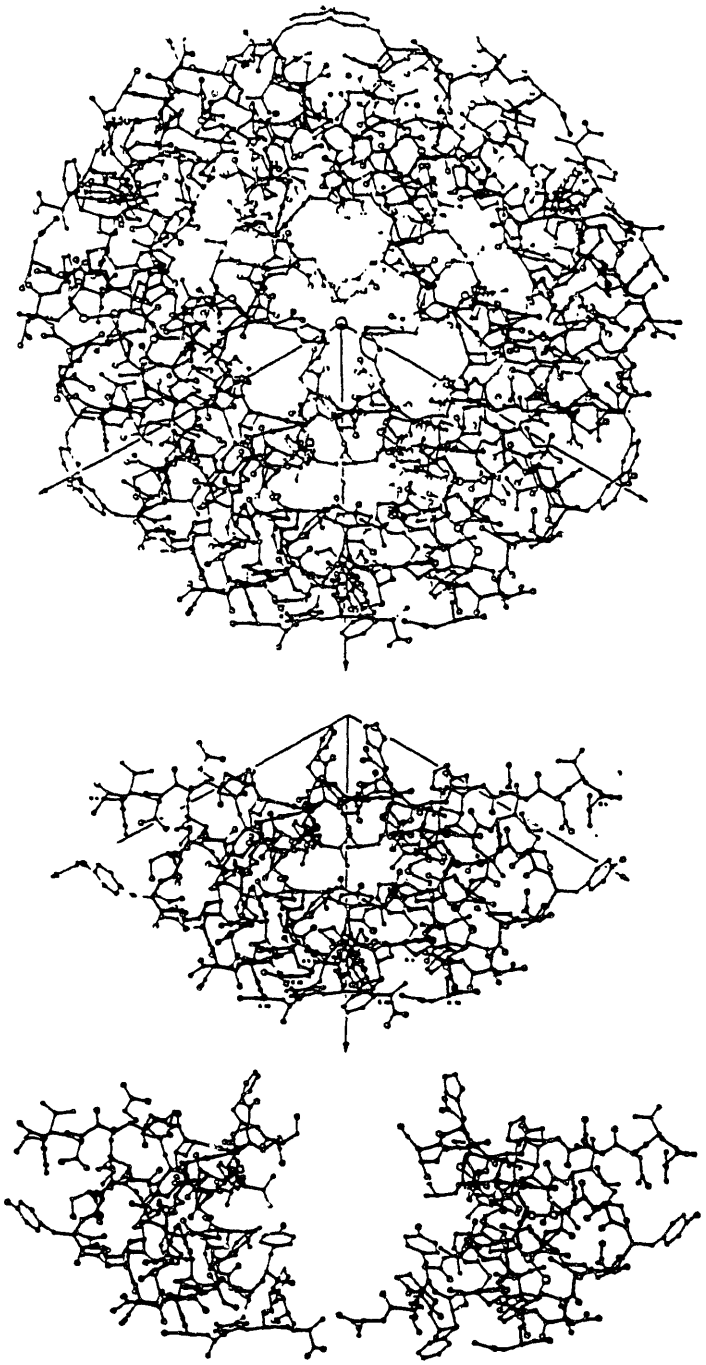

Figure 1.2: The assembly of insulin hexamer from monomers and dimers (from Blundell et al., 1972; reproduced with permission of Academic Press, Inc.).

of insulin secreting beta cells is mediated by the immune system (Pyke, 1989). Ultimately, addressing the problem of autoimmunity my lead to a different way to treat and even cure diabetes, but that area of research is new and relatively unexplored, so for the time being attention must be given to optimizing the currently available treatment.

1.3.1 Traditional Injection Therapy.

For insulin dependent patients (Type I diabetes), the classical method of achieving glycemic control is via subcutaneous or intramuscular injections of concentrated insulin preparations (Brange, 1987). The preferred regimen is based on twice daily injections -- before breakfast and at bedtime. Mixtures of short, intermediate and long acting (slowly dissolving) insulins are used to counteract the increased blood sugar content at meal times and to maintain a basal insulin concentration between meals. Unfortunately, the insulin profile generated is far from physiological (Figure 1.3): the slow rise to peak concentrations causes post-prandial hyperglycemia, and the slow return to baseline concentrations may result in hypoglycemia between meals (Home et al., 1989). Other problems are caused by variable insulin adsorption and dispersion rates, variable dissolution rates for the long acting insulin-protamine complexes, disruption of subcutaneous tissue and night-time hypoglycemia. The differences in patient behavior, metabolism, eating habits and physical exertion, further complicate the administration of insulin preparations (Home et al., 1989; Brange et al., 1990).

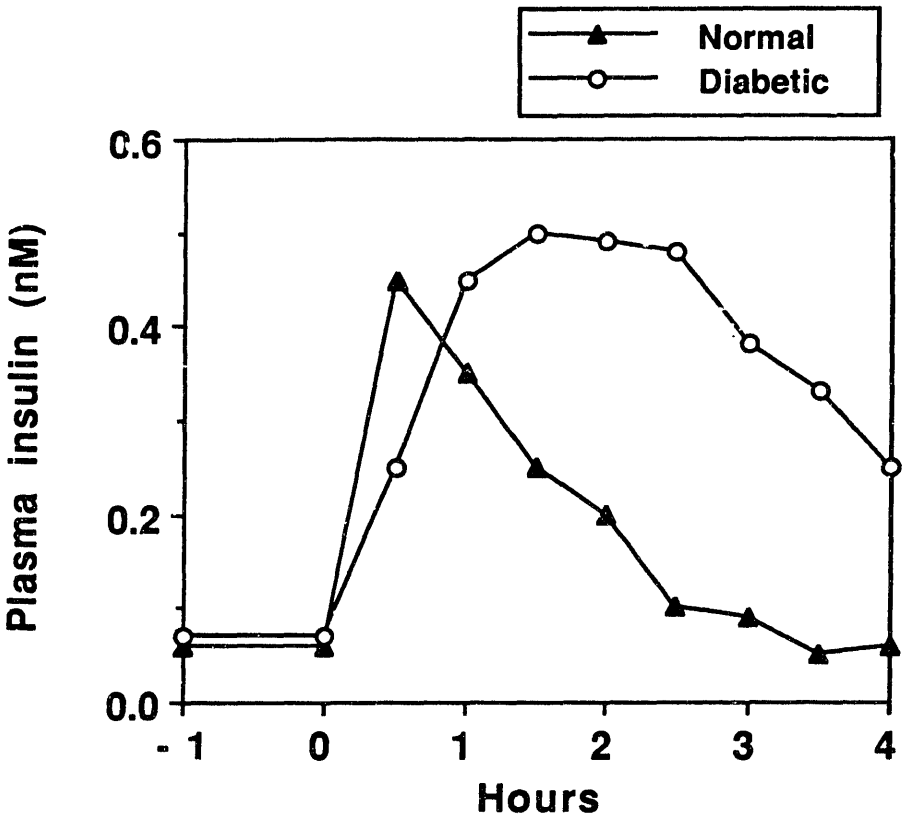

Figure 1.3: Typical profiles of normal and diabetic plasma insulin levels after a meal at time 0 (adapted from Brange et al., 1990).

1.3.2 Alternative Treatments of Diabetes

Despite the problems associated with traditional injection therapy, subcutaneous administration remains the most efficient way to deliver insulin solutions to the patient. Enzymatic degradation prevents oral insulin administration, as most of the drug is metabolized prior to reaching the bloodstream. Chemically modified gell-encapsulated insulins are currently being investigated (Sato et al., 1984); these systems are "based on the competitive binding of

glucose and derivatized carbohydrate-coupled insulin to Concanavalin A; the glycosylated insulin is displaced from Con A by glucose in response to, and proportional to," blood glucose levels (Jeong et al., 1984).

Slow transdermal diffusion prevents topical administration of insulin, making nasal, rectal, buccal and sublingual delivery routes at best 50% as effective as intramuscular injections (Aungst et al., 1987), although some aerosol dosages have been proposed (Lee and Sciarra, 1976). Recently, iontophoresis has been used to facilitate transdermal transport of insulin (Siddiqui, 1987), but with limited success (90 minutes required to deliver 1/2 required dose) and only on animal models. Transplantation of neonatal rabbit pancreatic tissue (Gates and Lazarus, 1977) and insulin encapsulation in erythrocytes (Bird et al., 1983) have also been attempted, but proved not to be clinically useful.

During the past decade, advances have been made in the development of artificial pancreas and pancreatic transplantation, and much research has been devoted to the development of self-regulating artificial infusion and controlled release systems (Home et al., 1989). Unfortunately, the initial enthusiasm about the implementation of these novel treatments has subsided due to the high cost and technical difficulties surrounding the transplantation procedures (Pyke, 1989), while the self-monitoring infusion pumps are proving to be cumbersome, difficult to control, and subject to problems related to insulin aggregation (Home et al., 1989); Lougheed et al., 1980; James et al., 1981; Lougheed et al., 1983). Pancreatic transplantation remains a rare and drastic treatment with

a success rate of 40-50% (Home et al., 1989), and the majority of insulin infusion pumps (both subcutaneous and intravenous) are limited to clinical use. Furthermore, insulin infusion pumps have not shown significant improvements over the more common insulin injection therapy (Leichter et al., 1985).

An alternative method of insulin administration is controlled release of the hormone from polymer matrices. The development of such devices is still in experimental stages because the problems of insulin stability within the polymer and regulation of the release rates have not been resolved (Creque et al., 1980; Brown et al., 1986). The only new device which is gaining widespread use is the pen injector, which combines the insulin vial and syringe with a dose measuring device (Home et al., 1989). The pen injector may allow for greater meal-time flexibility, but it has yet to undergo conclusive clinical trials; further design modifications may be necessary to accommodate the resuspension of complexed insulin preparations. Although the portable pen injector offers the patient greater convenience and flexibility, it does not differ fundamentally from the traditional injection therapy, and will be subject to some of the same problems.

1.3.3 Changes in Pharmaceutical Formulations.

Despite numerous efforts to improve diabetes therapy, the regulation of blood glucose levels has changed very little since the original introduction of insulin injections. While the methods of insulin administration have remained more or less the same, the quality of insulin preparations has increased dramatically because of

improvements in isolation and purification techniques (Brange, 1987).

Acidic preparations of insulin were required to solubilize the impure form of the hormone available initially; neutral formulations (which were more stable and better tolerated by the patients) were made possible only in the 1960s (Brange, 1987). In addition to these rapid-acting insulins, formulations designed to prolong insulin effects were introduced in the 1930s (Hagedorn et al., 1936). The protracted action was achieved by complexing insulin with protamine or other basic peptides which depressed insulin solubility at neutral pH; similarly long-acting Zn-insulin suspensions were developed at NOVO labs in the 1950s (Brange, 1987). Mixtures of short and intermediate acting formulations could be used to counteract the glucose challenge at meal times and to maintain a basal insulin level between meals.

Traditionally, the hormone was isolated from bovine and porcine sources; animal derived protein formulations proved to be compatible with the majority of insulin-dependent diabetics, although some patients were allergic to such preparations. In recent years, concern about insulin availability led to the development of recombinant human insulin which assured unlimited supply and improved compatibility (reduced antigenicity) (Johnson, 1983).

1.4 Goals and Motivation

To improve the treatment of diabetes, novel methods of insulin administration are needed. New therapies capable of providing a more physiological response to blood glucose levels could improve

the diabetics' quality of life and reduce the risk of microvascular renal and retinal complications (Home et al., 1989). The design and implementation of all drug delivery systems must take into consideration the delicate nature of the hormone, and steps must be taken to improve stability of insulin formulations. While some causes for physical, chemical and biological instability of insulin have been identified (Lougheed et al., 1980; Grau, 1985; Brange et al., 1987) and some stabilizing agents have been partially successful (see chapter 2), the pathways and kinetics of insulin aggregation have not been unequivocally determined. Rather than continue an empirical investigation of destabilizing/stability enhancing additives, this project addressed the fundamental nature of insulin aggregation. The ultimate goal of this study was the design of rational stabilization strategies based on a basic understanding of insulin behavior in solutions and a clear picture of the aggregation mechanism.

1.5 Overview of Thesis

In this study, a rigorous kinetic analysis was used to investigate the mechanism of insulin aggregation. Time-dependent concentration profiles were obtained by monitoring the levels of non-aggregated insulin remaining in solution (UV absorbance at 280 nm), and changes in particle size distribution were determined using quasielastic light scattering (QELS). The experimental set-up allowed for a systematic study of the parameters affecting insulin behavior in aqueous solutions. Possible reaction mechanisms were formulated based on the overall kinetic pattern and information about insulin's

physical and chemical properties available in the literature. To determine the validity of proposed reaction schemes, mathematical models of possible reaction pathways were constructed, and results of computer simulations were compared with empirical evidence.

The proposed mechanism included insulin's self-association into dimers and hexamers, the interactions with hydrophobic surfaces and the formation of stable intermediate species. The model successfully predicted trends in insulin aggregation as well as the effects of some external perturbations. It identified monomer destabilization at hydrophobic surfaces as one of the main pathways leading to aggregation. Stabilizing additives which prevent unfavorable conformational changes by excluding insulin from the hydrophobic interface, were shown to dramatically increase insulin solution stability. The mathematical model presented here served two purposes: it clarified the aggregation mechanism, and suggested rational stabilization strategies. Hopefully, a better understanding of insulin aggregation can eventually contribute to improvements in diabetes therapy.

CHAPTER 2.

BACKGROUND

2.1 Insulin Structure and Conformation

Most proteins' biological functions depend on their native conformations. Insulin's ability to recognize and bind to its receptor, thus signalling the need for glucose metabolism, is closely linked to its folded, three-dimensional structure. The role of native conformation in this regulatory mechanism has long been recognized, and much effort has been devoted to elucidating not only the amino-acid sequence, but also the spacial orientation of the residues making up this protein.

2.1.1 Crystal Structure

Insulin's ability to crystallize in the presence of Zn^{2+} has been known since the early 1930s; during the 1960s, several groups have investigated insulin's crystalline structure by X-ray diffraction (Blundell et al., 1972), revealing the three-dimensional orientation of the amino-acid sequence. Crystalline systems of hexamers and dimers have been elucidated, as have the monomeric crystals of beef and sheep DPI (despentapeptide) insulins (native insulin monomers self-associate to dimers and hexamers at concentrations required for crystallization) (Derewenda et al., 1989b). Since the Zn-insulin hexamer crystals are the naturally occuring species used for insulin storage in the pancreas, the following discussion will be restricted to their structure.

The shape of insulin hexamer crystalls depends on the concentration of Zn^{2+}, Ca^{2+} and phenol. In the presence of Zn^{2+}, insulin hexamers form nearly-symmetric (2 Zn^{2+}) and assymetric (4 Zn^{2+}). rhombohedral crystals; hexamers containing 2 Zn^{2+} and a phenol molecule form symmetric monoclinic crystals (Derewenda et al., 1989a). Although crystal geometry varies with insulin species, pH and zinc content, hexamerization produces "an almost spherical, very stable structure with a largely polar surface, and very suitable for crystal formation. In all likelihood it has evolved in response to the advantages that crystal deposits represent in storage" (Derewenda et al., 1989b) (Figure 2.1). Recent studies have examined insulin-metal ion interactions, binding affinities and rates (Coffman and Dunn, 1988). The contributions of Zn^{2+} and Ca^{2+} to insulin hexamer assembly, stability and storage have also been investigaed (Hill et al., 1991).

For years, protein chemists have hypothesized that a protein's conformation in aqueous solution was the same as its crystalline structure. Thanks to the advances in two-dimensional NMR technology, that assumption can now be tested: insulin's conformation in solution has recently been determined, and was found to be nearly identical to that in the crystalline state (Weiss et al., 1989; Kline and Justice, 1990). The investigations of insulin's solution stability can now safely draw upon the wealth of structural information available from X-ray diffraction studies.

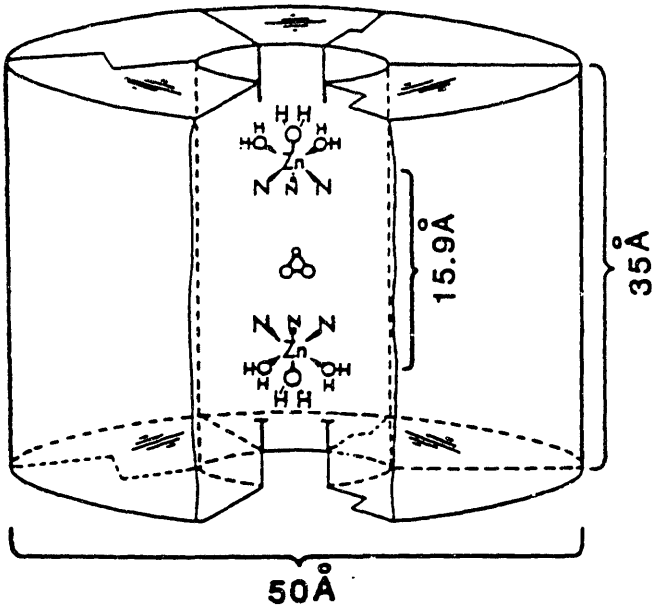

Figure 2.1: Structure of the Zn-insulin hexamer. Jagged lines between monomers on the top and bottom of the hexamer indicate a dimer-dimer interface. Straight lines between monomers on the top and bottom of the hexamer indicate a monomer-monomer interface within a dimer. The "hash marks" indicate the top of monomers. Zn^{2+} ions are shown on the 3-fold axis in the positions they occupy in two-zinc insulin. Each of the zinc ions is coordinated by three His (B10) side chains and by three water molecules (adapted from Hill et al., 1991; used by permission of the American Chemical Society).

2.1.2 Insulin's Self-Association in Aqueous Solutions

Insulin's ability to form dimers and hexamers has long been recognized, and its equilibrium self-association pattern has been the subject of numerous studies. Insulin's association state has been investigated by sedimentation equilibrium studies as early as 1953 (Fredericq, 1953). Equilibrium ultracentrifugation remained the instrument of choice for researchers examining insulin's self-association pattern; from the observed diffusion coefficients, molecular weights, degrees of association and various thermodynamic parameters ($\Delta G°$, $\Delta H°$ and $\Delta S°$) could be determined. The accuracy of early investigations may have been compromized by impurities (such as proinsulin), but the general trends in association behavior are in good agreement with more recent studies.

Jeffrey and Coates examined bovine insulin's self-association at pH 2 and various ionic strengths and temperatures (Jeffrey and Coates, 1966a&b), finding increased ionic strength to favor dimer and hexamer formation; they also reported the prevalence of the insulin dimer, which they attributed to its high association constant. Self-association of porcine insulin and proinsulin at neutral pH was also investigated, and the results fitted to a model involving monomer, dimer and hexamer formation (Pekar and Frank, 1972). The degree of association was found to be higher at neutral than acidic pH even in the absence of Zn^{2+}, and proinsulin's self-association was reported to be similar to that of insulin (Pekar and Frank, 1972). Bovine Zn-insulin's oligomerization was compared with Zn-free insulin; a higher proportion of high molecular weight species was reported in the presence of 2 g-atoms of Zn^{2+} per insulin hexamer, and glucose was

shown to favor dissociation (Jeffrey, 1974). The self-association pattern of bovine Zn-free insulin at pH 7 and room temperature was further investigated, and equilibrium constants for the formation of dimers, hexamers and higher molecular weight species were determined (Jeffrey et al., 1976). A similar analysis was done for bovine Zn-insulin at pH 7 (25°C, ionic strength 0.2) (Milthorpe et al., 1977), and Zn-free bovine insulin at pH 7.4 (20°C, ionic strength 0.368) (Holladay et al., 1977). The technique of concentration difference spectroscopy was used to determine the equilibrium association constants for bovine and porcine Zn-free insulin and proinsulin (at pH 2 and 7, 25°C) (Strazza et al., 1985). Although the general trends reported in the above study were in good agreement with previous investigations, the constants based on concentration difference spectroscopy deviated from values reported by others. This could probably be explained by the inappropriateness of the assumption that in the absence of Zn^{2+}, insulin would not form hexamers even at neutral pH. The results of equilibrium self-association studies are summarized in Table 2.1.

A mathematical model describing self-association of Zn-free bovine insulin at acidic, neutral and basic conditions was proposed recently (Mark et al., 1987); a revised version incorporated four different patterns of association (Figure 2.2) and effects of non-ideality (Mark and Jeffrey, 1990). Model 1 assumed that one association constant could be used to describe insulin oligomerization, which proceeded by "head-to-tail" addition of monomers and resulted in an equilibrium of odd- and even-numbered species. Model 2 proposed that dimerization was followed by a

Table 2.1: Summary of equilibrium constants for insulin self-association to dimers (K_{dim}) and hexamers (K_{hex}) reported in the literature.

Insulin Species*	pH	Ionic Strength	Temperature (°C)	K_{dim} ($\times 10^{-5}$ M^{-1})	K_{hex} ($\times 10^{-8}$ M^{-2})	Reference
porcine	7.0	0.1	24.5	1.4	4.0	(Pekar and Frank, 1972)
bovine	8.0	0.1	27	2.2	NA	(Goldman and Carpenter, 1974)
bovine	7.0	0.2	25	1.1	2.9	(Jeffrey et al., 1976)
bovine	7.4	0.368	20	0.22	86	(Holladay et al., 1977)
bovine	7.0	0.05	25	7.5	NA	(Poker and Biswas, 1981)
bovine	2.0	0.1	25	1.2	NA	(Strazza et al., 1985)
bovine	7.0	0.01	25	2.0	NA	(Strazza et al., 1985)
porcine	2.0	0.1	25	0.65	NA	(Strazza et al., 1985)
porcine	7.0	0.01	25	0.055	NA	(Strazza et al., 1985)
bovine	2.0	0.1	25	0.46	0.014	(Mark and Jeffrey, 1990
bovine	10.0	0.1	25	0.032	0.045	model 4, ideal treatment)
bovine	7.0	0.1	25	0.85	5.17	(Mark and Jeffrey, 1990
bovine	7.0	0.05	25	0.41	19.5	model 4, ideal treatment)
bovine	7.0	0.1	37	0.568	4.26	(Mark and Jeffrey, 1990)
bovine, Zn	7.0	0.2	25	1.1	1.17×10^5	(Milthorpe et al., 1977)

*Values are for Zn-free insulin solutions, unless specified otherwise.

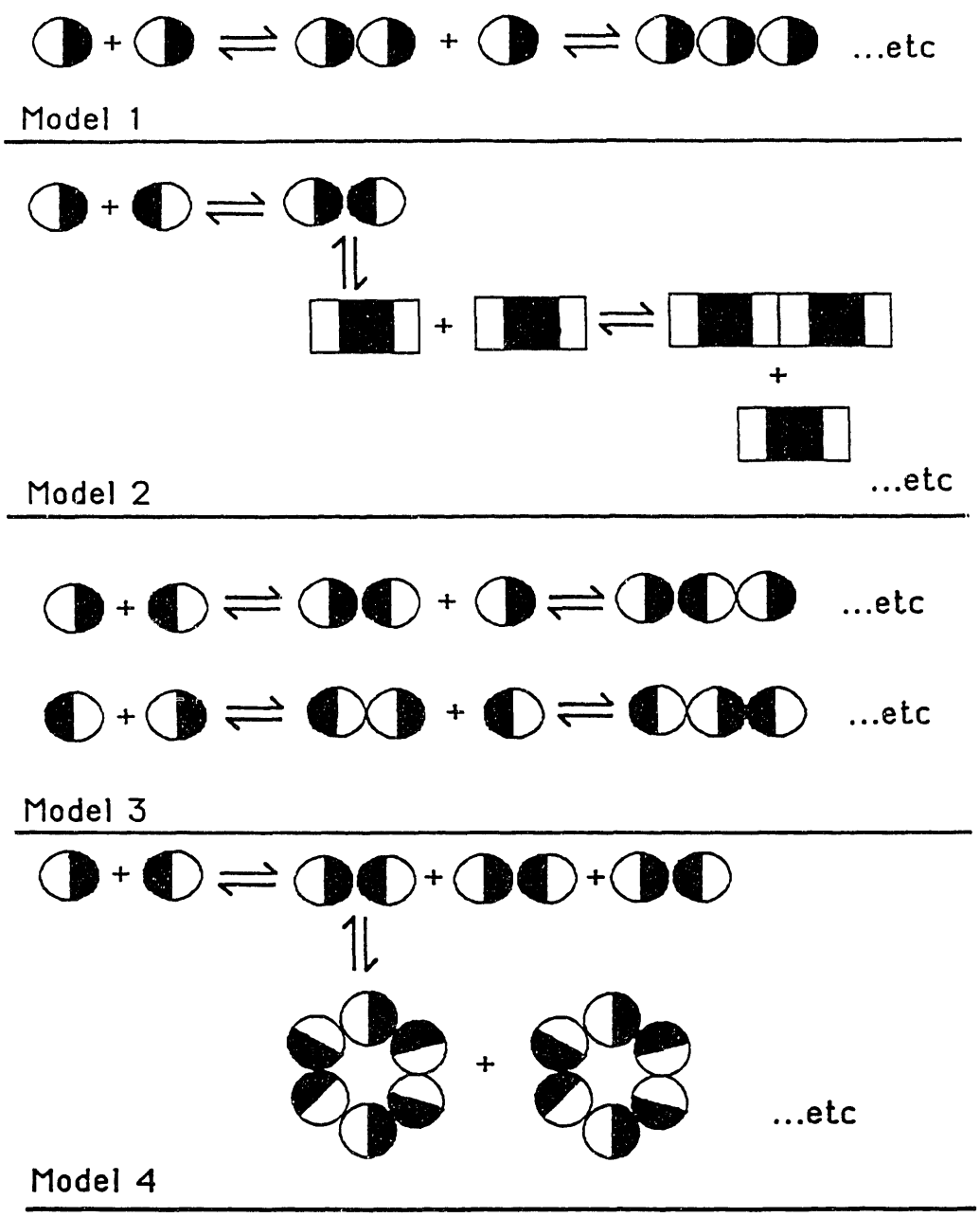

Figure 2.2: Diagrammatic representation of 4 model patterns for insulin self-association used in determining equilibrium association constants (adapted from Mark and Jeffrey, 1990). Each circle represents the insulin monomer; different shades are used to distinguish between the dimer- and hexamer-forming regions of the assymetric molecule. Please see text for more details about these association mechanisms.

conformational change which led to subsequent interactions between dimers only. Model 3 defined two association constants: one for "head-to-head" and one for "tail-to-tail" monomer addition; based on interactions known to occur during hexamer formation in the crystal, this scheme allowed for the existence of odd- and even-numbered insulin oligomers in solution. In model 4 (the mechanism used by Pekar and Frank), dimerization was governed by one association constant, while the constant for hexamer assembly from three dimers was assumed to be so high as to render intermediate species (tetramers) virtually nonexistant. The authors commented on the usefulness of various models and concluded that while no preferred (uniquely correct) pattern can be established at this time, "it is better to use one of the models...than none at all" (Mark and Jeffrey, 1990).

2.1.3 Structure of Insulin Aggregates

A certain amount of confusion exists in the literature about the term "aggregate", which in the case of insulin has been used to describe both the products of equilibrium self-association (dimers, hexamers, etc.), and the high molecular weight, biologically inactive species. This investigation reserves the term "aggregation" for the non-covalent association between partially denatured insulin molecules, which leads to the formation of large, inactive agglomerates. Dissolution of these aggregates requires extreme conditions (pH>10, 6 M GdHCl or Urea), and recovery of biological activity is questionable. In contrast, the oligomeric species formed by equilibrium self-association, readily dissociate to the monomeric state and retain full biological activity upon dilution at physiological

conditions.

The chemistry of aggregate (or fibril) formation was investigated by Waugh in the 1950s, who induced destabilization by repeatedly heating and freezing acidic solutions of insulin (Waugh et al., 1953). Under these conditions, aggregation was found to be reversible, leading to the conclusion that the fibrils were formed by hydrophobic interactions between slightly altered conformations of native insulin molecules (Waugh, 1955). The reaction was reported to be autocatalytic: small concentrations of insulin fibrils added to previously unperturbed solutions, nucleated aggregation, progressively depleting the concentration of soluble protein (Waugh et al., 1953). Electron microscopy and X-ray diffractions studies indicated that "the fibrils are composed of polypeptide chains which are in the ß conformation and which extend transverse to the fibril axis (i.e., cross-ß structure)" (Burke and Rougvie, 1972). The role of hydrophobic interactions was emphasized once again: "extensive interactions involving nonpolar residues on the surface of the fibrils serve to protect the interior hydrogen bonding from disruptive effects of polar solvents" (Burke and Rougvie, 1972).

Although insulin aggregation in pharmaceutical preparations is observed under much less stringent conditions (neutral pH, mild agitation and only slightly elevated temperatures), and leads to precipitation rather than gel-like structures, the basic elements involved are independent of the macrostructure (Brange et al., 1987). Increased concentrations of insulin monomer have been shown to accelerate the fibrillation process, indicating that aggregation involves partially denatured monomeric species (Brange et al., 1987).

In the fibrills, the antiparallel ß-pleated sheet structure involved in dimer formation, is replaced by a parallel arrangement, which increases the hydrophobic surface area and provides additional sites for hydrophobic interactions (Brange et al., 1987).

The information about structural properties of both native and aggregated insulin species provides invaluable insights for the formulation of a physically reasonable mechanism of destabilization.

2.2 Previous Investigations of Insulin Stability

Stability and efficacy of insulin formulations is a serious concern for diabetic patients, their doctors, pharmaceutical companies and students of protein chemistry. It comes as no surprize that the stability of this medically significant protein has been the subject of numerous investigations.

2.2.1 Stability of Insulin Crystals

In the absence of water, insulin is remarkably stable. Crystalline preparations of insulin (5-6 wt% water content) stored at -20°C for over 20 years retain >99% of their original activity (Storring et al., 1975; Fisher and Porter, 1981). Loss of potency is accelerated by elevated temperatures, yet even after being stored at 37°C for 14 years, the preparations retained ~70% of original activity (Storring et al., 1975).

2.2.2 Stability of Insulin Solutions (Static Storage)

In aqueous solutions, insulin stability decreases drastically (compared to dry storage of insulin crystals): the population of

native insulin is reduced by deamidation and aggregation (formation of high molecular weight polymers which do not redissolve at neutral pH) (Grau, 1985). While desamido insulin retains biological activity, the aggregates are largely responsible for the loss of potency of insulin preparations in a variety of delivery environments (Lougheed et al., 1983; Grau, 1985). Earlier studies have shown that neutral regular insulin solutions are more stable than acidic preparations: upon storage at 5°C, neutral solutions were stable for over 4.5 years, while acidic solutions were stable for only 2 years (Jackson et al., 1972). Elevated storage temperatures accelerated insulin degradation, deamidation being more pronounced in the acidic preparations. No concentration dependence was observed, and biological potency was shown to be a function of time and temperature only (Pingel and Vølund, 1972). Despite the claim that neutral commercial preparations are stable for more than 4 years, "frosting" or "hazing" of refrigerated insulin solutions accompanied by a marked loss of biological potency has been recently reported by diabetic patients (Benson et al., 1988). Thus, insulin aggregation remains a problem even under the most favorable conditions: static storage at low temperatures.

The gain more insights into protein solution behavior, denaturation of insulin and proinsulin induced by guanidine hydrochloride was investigated using near- and far-ultraviolet (UV) circular dichroism (CD) (Brems et al., 1990). Denaturation was found to be a two state transition, with a Gibbs free energy of unfolding of 4.5±0.5 kcal/mol.

2.2.3 Solution Stability in Artificial Infusion Systems

Insulin aggregation presents serious problems for prolonged artificial infusion systems. In a portable or implanted insulin pump, highly concentrated insulin solutions are exposed to elevated temeratures (25-37°C), continuous motion, and a large surface contact area in small diameter tubing and catheters. Untreated insulin solutions remain stable for 1-3 days, and must be replaced to prevent aggregation and occlusion of delivery passages. Inconsistent aggregation patterns make it difficult to maintain a stable basal glucose level in diabetic patients relying on this method of drug administration.

The work of Pickup and co-workers stimulated more than a decade of attemps to perfect portable continuous infusion systems which promised to provide improved glycemic control: a dual rate pump could maintain a basal insulin concentration between meals and provide an increased infusion dose before and during meals to counteract elevated glucose levels (Pickup et al., 1978). Early studies indicated that insulin dependent diabetics using such devices exhibited near-normal metabolism resulting from stricter regulation of blood glucose concentrations (Pickup et al., 1979; Nathan, 1982). The initial enthusiam began to subside as technical problems with the pumps became apparent: long-term glycemic control was compromized the instability and aggregation of insulin formuations (Irsigler and Kritz, 1979). Many researchers attempted to identify the causes of instability and to delay the onset of aggregation in order to make the artificial infusion systems more feasible.

Lougheed and co-workers reviewed the effects of purity, metal

ions, temperature, motion and other physical, chemical and thermodynamic factors influencing insulin aggregation (Lougheed et al., 1980). Destabilization was attributed primarily to elevated temperature, agitation, and the presence of impurities/man-made materials in contact with insulin solutions. The studies that followed observed insulin aggregation under conditions mimicking the artificial infusion environment: solutions containing a variety of additives and pump materials were agitated at 37-50°C and stability was measured by the presence or absence of visible micron-sized agglomerates. Many potential stabilizing reagents were tested: autologous serum (Albisser et al., 1980), bicarbonate ion (Lougheed et al., 1981), lysine, aspartic and glutamic acid (Bringer et al., 1981; Quinn and Andrade, 1981), EDTA (James et al., 1981) and glycerol (Blackshear et al., 1983) slightly prolonged the lifetime of insulin solutions, but were not adequate for long-term use.

Another comprehensive study examined the effects of physiological and synthetic surfactants on insulin aggregation, and identified some pump materials (i.e. silicone rubber) which significantly accelerated the degradation reaction (Lougheed et al., 1983). Some of the surfactants (SDS, Brij 35, Tween and Triton X) were shown to maintain solution stability for up to 150 days, but were required at concentrations which were unphysiological. The same investigation suggested that insulin's interactions with solid-liquid and liquid-air interfaces possibly contributed to aggregation by inducing partial conformational changes in the protein. Increased stability at higher concentrations was also reported, but no adequate explanation was provided for this unusual behavior (Lougheed et al.,

1983). A similar concentration dependence was observed and increased stability in the presence of small amounts of Ca^{2+} was found at NOVO labs (Brange and Havelund, 1983).

A correlation between the nature of contact material and aggregation rates was also observed: hydrophilic materials (e.g. cellulose butyrate) were shown to be much more compatible with insulin solutions, while hydrophobic ones (polypropylene and polyvinylchloride) increased insulin's propensity to aggregate (Feingold et al., 1984; Chawla et al., 1985). Solution stability was found to depend not only on the polymer used, but on the sterilization method used for the material: gamma irradiated polymers were shown to be particularly deleterious to insulin solutions (Melberg et al., 1988). Others reported that insulin was more stable in polyethylene than glass reservoirs, and that chemical and physical transformations occured in passage through the pumping mechanism tubing and catheters (Selam et al., 1987).

The influence of physical parameters on insulin aggregation did not escape the researchers' scrutiny: low flow-rates in infusion pumps were shown to reduce solution stability (Brennan et al., 1985). Aggregation was also attributed to high shear-rates in tubing and needles (Hutchison, 1985) and the volume of air in contact with protein solutions (headspace in agitated insulin vials) (Hansen et al., 1987).

2.2.4 Insulin Stability in Controlled Release Polymer Matrices

Many of the aggregation inducing parameters were absent in polymeric controlled release devices. High concentrations of insulin were loaded into the matrix, preventing dissolution within the device (Brown et al., 1986). Interfaces with air were absent, and agitation effects could be eliminated, since the protein remained in its dry form. However, the devices were exposed to elevated temperatures (37°C), and as the dissolved insulin diffused out of the matrix, it came in contact with hydrophobic polymer surfaces. An additional complication was the moisture content of the device, which probaby increased after implantation. One or a combination of these factors led to insulin aggregation, and prevented the release of 37-40% of the incorporated protein (Brown et al., 1986).

The nature of insulin aggregates inside the polymer marix remains unclear. If covalent interactions are involved, then the destabilization could be similar to moisture induced aggregation of BSA (which was shown to result from intermolecular S--S bond formation via the thiol-disulfide interchange reaction (Liu et al., 1991)). The addition of inorganic salts, lyophilization from acidic aqueous solutions, and co-lyophilization with water soluble polymers minimized BSA's solid-state aggregation (Liu et al., 1991); similar approaches to reduce insulin's moisture induced aggregation are currently being investigated in Dr. Langer and Dr. Klibanov's research groups.

2.2.4 Insulin Adsorption

Poteins' interactions with solid surfaces have often been found to cause losses in biological activity (Mizutani, 1979). Addition of excess amount of BSA (which preferentially adsorbs to glass and other container materials) is usually suggested to decrease protein binding and subsequent unfavorable reactions (e.g., denaturation and aggregation).

Insulin was shown to bind to glass, especially at low (10^{-10} M) concentrations (Cuatrecasas and Hollenberg, 1975). Various plastics (polythene, siliconized glss, nylon, teflon, cellulose nitrate and parafin wax) were found to bind >50% of insulin in solutions containing physiological concentrations (10^{-9} to 10^{-11} M) of the protein; adsorption was attributed to non-covalent interactions between insulin's hydrophobic groups and the hydrophobic plastic surfaces (Cecil and Robinson, 1975). Insulin's conformational change prior to adsorption was thought to be responsible for the slow binding rate, and coating the containers with cetyl alcohol was found to significantly reduce adsorption at these low concentrations (Cecil and Robinson, 1975).

Insulin's susceptibility to denaturation and loss of activity upon binding to glass was found to be greatly reduced at higher concentrations (0.17 mM); in fact, unlike horse-radish peroxidase, alkaline phosphatase and catalase, which lost 52, 86 and 98% of activity, respectively, after 3 months of adsorption to porous glass at 4°C, insulin's activity declined by less than 11% (Mizutani, 1979). Adsorption to various polymeric materials used in artificial infusion devices was shown to be reduced by urea (1-3 mg/ml) (Sato et al.,

1983), and insulin was found to bind preferentially to hydrophobic surfaces (siliconized glass and polyvinylchloride), once again suggesting the possibility of hydrophobic interactions between insulin's moieties and container materials (Asakawa, 1984).

A comprehensive study of insulin adsorption isotherms on Teflon, silastic, glass, polyacrylamide, and polystyrene in phosphate (pH 7.4) and acetate (pH 3.5) buffers and at 23 and 37°C, provided a rigorous quantitative relationship between insulin concentration and surface binding (Sefton and Antonacci, 1984).

Increased adsorption was observed in monomeric solutions of insulin (Jeffrey, 1986), while Zn-insulin binding to hydrophillic metal surfaces was attributed to deposition of hexamers, rather than the more hydrophobic monomers and dimers (Arnebrant and Nylander, 1988). Insulin adsorption and interactions between layers bound to hydrophobic surfaces were recently examined using a surface force apparatus (Claesson et al., 1988). And yet, the relationship between adsorption and aggregation remains speculative.

2.3 Proposed Mechanisms of Insulin Aggregation

Despite the large number of studies conducted to examine insulin stability, little is known about the kinetics of aggregation. Several pathways have been suggested based on the qualitative observations and stabilizing trends: Lougheed and co-workers proposed a competitive inhibition mechanism which addressed the phenomenon of increased stability at higher concentrations (Lougheed et al., 1983), while Thurow and Geisen speculated on the role of interfacial interactions on insulin aggregation (Thurow and

Geisen, 1984).

The competitve inhibition mechanism assumed that aggregate formation began with partial unfolding of insulin monomers; through non-covalent interaction, these destabilized specied formed high molecular weight polymers. Since this process involved partially unfolded molecules, the concentration of unaltered monomers was expected to competitively inhibit the interactions between the denatured species (Lougheed et al., 1983). Increased stability of insulin solutions at higher concentrations was attributed the the higher concentration of native species. However, this mechanism neglected the fact that although a smaller proportion of insulin molecules remained monomeric as concentrations increased, the absolute number monomers increased with total concentration. If aggregation was initiated by partial denaturation of insulin monomers, the driving force for the reaction would still increase with concentration, even if relative amount of susceptible molecules decreased.

The second mechanism addressed the problem of insulin denaturation at interfaces. The dimer was assumed to preferentially adsorb and partially denature at hydrophobic surfaces, where high molecular weight aggregates formed in the adsorbed layer (Thurow and Geisen, 1984). Two stabilizing strategies implied by this model were 1) elimination of hydrophobic surfaces in contact with insulin solutions, and 2) stabilizing reagents favoring hexamer formation, which would reduce the dimer population. While this kinetic scheme pointed out important destabilizing interactions, it did not provide an adequate description of insulin aggregation behavior. Ample

evidence suggested that insulin aggregation was initiated by the monomer and not the dimeric species. This mechanism also failed to explain increased solution stability at higher insulin concentrations. Both reaction schemes attempted to provide qualitative explanations for experimenatlly observed trends, but neither successfully described the dynamics of insulin aggregation.

2.4 Current Stabilization Strategies

Once the causes of insulin aggregation became apparent, researchers attempted to eliminate, or at least modify, the destabilizing interactions.

2.4.1 Design of Stability Enhancing Additives

Despite the large number of investigations addressing the problem of insulin stability in artiticial infusion devices, the mechanism of degradation remained speculative. Scientists at Hoechst proposed that aggregation was initiated by insulin's adsorption to and denaturation at hydrophobic surfaces; their stabilizing reagent Genapol PF-10 (a polypropylene glycol/polyehtylene glycol block co-polymer) was aimed at preventing insulin adsorption to solid surfaces (Thurow and Geisen, 1984). Increased solution stability was attributed to the fact that Genapol contained both hydrophobic and hydrophilic portions. The hydrophobic regions would bind to glass, while the hydrophilic groups extended into the solution, effectively reducing the hydrophobic surface area exposed to insulin solutions. In-vitro and in-vivo studies with implantable infusion devices containing Genapol

PF-10 stabilized insulin solutions were quite successful, with an average of 90% of insulin retaining activity for up to 5 months (Grau and Saudek, 1987). Pluronic F68 (a similar polypropylene glycol/polyehtylene glycol block co-polymer) was also reported to prevent insulin aggregation in samples agitated for 8 days (Chawla et al., 1985).

The recognition that the insulin hexamer had a very stable protein conformation, promted attempts to design additives which would favor hexamer formation. Solution stability was shown to be increased by the addition of Zn^{2+} (Brange et al., 1986), and phenol was reported to stabilize hexamer's helical structure (Derewenda et al., 1989a). Others tried to design reagents or ligands aimed specifically at hexamer stabilization (Manallack et al., 1985), (McGraw et al., 1990). The roles of Ca^{2+} and Zn^{2+} in hexamer assembly and stability were also investigated recently (Palmieri et al., 1987), (Hill et al., 1991). However, it is important to remember that while hexamer stabilization may reduce the risk of aggregation, it may adversely affect insulin absorption, since dissociation to the monomeric state is necessary prior to entrance into the bloodstream (Brange et al., 1990). Rather than improving the quality of insulin preparations, increased hexamer stability may dely the response to elevated blood glucose levels, making normoglycemia even more difficult to achieve.

2.4.2 Chemical Modifications of Insulin Structure

Another approach to increasing insulin stability was through chemical modification of the protein. Since aggregation was thought

to occur via hydrophobic interactions of partially denatured molecules, various modifications/mutations could be introduced to reduce hydrophobicity of exposed surfaces. Sulfated insulin (SI), used to treat diabetics with crystalline zinc-insulin intolerance, does not self-associate at neutral pH due to the electrostatic repulsion imparted by the SO_4^{2-} groups. SI solutions used in in-vivo studies with portable infusion pumps, remained stable for 140 days and provided adequate glycemic control in pancreatomized dogs (Nomura et al., 1983). Despite the fact that SI was shown to be resistant to aggregation in artificial delivery environments, it did not become a substitute for zinc insulin; concerns about its antigenicity and receptor binding (as well as the cost associated with the sulfation-sulfonation procedure) probably imposed limitations on its widespread use (Pongor et al., 1983).

Other structural modifications were attempted to prevent insulin self-association and aggregation. Most changes were accompanied by reduced biological potency, even if they succeeded in improving stability (Carpenter et al., 1980). Efforts to engineer monomeric insulins (Bi et al., 1984; Brange et al, 1988) for better absorption profiles met with some success: some of the modified insulins exhibited equal or higher biological potency (Schwartz et al., 1989), but physical stability was reduced (Brange et al., 1990). The changes which prevented insulin monomer's association into dimers and hexamers actually increased its propensity to aggregate.

2.5 Conclusions

The medical significance of insulin prompted numerous researchers to examine its physical, chemical and biological properties, making it one of the most thoroughly investigated and characterized proteins to date. Studies of insulin paved the way for exploring the biochemistry of other proteins and enzymes. It seemed that every aspect of insulin structure and function had undergone compelete and exhaustive analysis, and yet, insulin's tendency to aggregate had not been fully explained. This work aimed to combine the information obtained by previous investigators with an original kinetic analysis in order to elucidate the pathways of insulin's instability and aggregation.

CHAPTER 3.

EXPERIMENTAL APPROACH

3.1 Introduction

To study the fundamental nature of insulin aggregation, it was necessary to have:

a) control of the aggregation process (the ability to achieve rapid, reproducible aggregation, and to vary parameters responsible for destabilization; and

b) quantifiable analytical methods (the ability to monitor aggregation kinetics, determine the nature of association, and identify native and intermediate species involved in aggregate formation).

While insulin aggregation rates could be controlled by changing such physical parameters as temperature, agitation rates, and hydrophobicity of surfaces in contact with insulin solutions, analysis of protein behavior presented a more challenging problem. The desired analytical technique had to detect changes in native conformation, and be able to identify and quantify the species involved in the aggregation reaction, all without interfering with the protein. The methods best suited for kinetic studies had to be rapid as well as quantitative and non-invasive. To determine which analytical tools were most appropriate for this work, a literature review was done to examine the advantages and drawbacks of various techniques.

3.1.1 Analytical Methods Used in Previous Studies

In the investigations reviewed in Chapter 2, the presence of aggregates and native insulin species was monitored by a variety of experimental techniques:

1) visual inspection for turbidity {physical stability} (Lougheed et al., 1980; Lougheed et al., 1983)

2) absorbance at 540 and 600 nm {physical stability} (Lougheed et al., 1983)

3) ultracentrifugation {sedimentation experiments to determine insulin's self-association state} (Jeffrey, 1974; Pekar and Frank, 1972)

4) circular dichroism (CD) {changes in helical content representative of conformational changes} (Pocker and Biswas, 1980; Sato et al., 1983; Truskey et al., 1987)

5) reverse-phase High Performance Liquid Chromatorgraphy (HPLC) {chemical stability}, (Chance et al., 1981; Grau, 1985; Arakawa et al., 1989; Adams and Haines-Nutt, 1986; Fisher and Smith, 1986)

6) size-exclusion HPLC {chemical stability} (Schrader and Pfeiffer, 1985a&b; Brange et al., 1990)

7) gel electrophoresis {chemical stability, aggregate size} (Lougheed et al., 1980; Brown, 1983)

8) differential gel adsorption {self-association} (Helmerhorst and Stokes, 1986)

9) concentration difference spectroscopy {self-association} (Strazza et al., 1985)

10) scanning electron microscopy (SEM) {aggregate size} (Sato et al., 1983; Lougheed et al., 1983)

11) radioimmunoassays (RIA) {biological activity} (Lougheed et al., 1983; Musial et al., 1986)

12) in-vivo blood glucose levels {biological activity} (Jackson et al., 1972; Albisser et al., 1980; Benson et al., 1988; Maislos et al., 1988)

13) ^{1}H nuclear magnetic resonance (NMR) spectroscopy {metal binding and equilibrium self-association} (Ramesh and Bradbury, 1986; Ramesh and Bradbury, 1987; Palmieri et al., 1987; Weiss et al., 1989; Kline and Justice, 1990)

14) quasielastic light scattering (QELS), or dynamic light scattering, or photon correlation spectroscopy {diffusion coefficient and hydrodynamic radius determination} (Martindale et al., 1982; Bohidar and Geissler, 1984; Bohidar, 1989; Dathe et al., 1990)

15) surface force apparatus of Israelachvili {protein-surface and protein-protein interactions in adsorbed layers} (Claesson et al., 1988)

16) radiolebelling with ^{125}I and ^{65}Zn {adsorption studies} (Sefton and Antonacci, 1984; Dathe et al., 1990)

The choice of analytical methods depended on the specific aims of a particular project. As with other biological molecules, care had to be taken to prevent denaturation and unfolding during sample preparation and analysis. No single analytical technique could provide a complete picture of insulin behavior; ideally, the results of

several complementary analyses were used the describe the experimental system.

3.2 Experimental Approach and Choice of Analytical Tools

Only a few of the above mentioned techniques satisfied the requirements of the proposed kinetic analysis. Optical density measurements and circular dichroism were found lack specificity; reverse-phase HPLC, differential gel adsorption and gel electrophoresis interfered with insulin's native conformation and self-association equilibrium; ultracentrifugation and NMR spectroscopy required long equilibration times; concentration difference spectroscopy would be ill applied to systems containing more than two association states; the small size of native insulin species and the extremely low concentrations of soluble intermediates, prevented the use of SEM techniques; and RIA techniques, which were extremely sensitive at low concentrations, did not have significant advantages over simpler and quicker methods, such as size-exclusion HPLC or ultraviolet spectrophotometry.

To construct time-dependent concentration profiles of aggregating insulin solutions and to identify the species involved in the reaction, this work relied on size exclusion HPLC and UV absorbance measurements at 280 nm (which determined the concentrations of non-aggregated insulin), and on QELS (which analyzed particle size distributions and detected the formation of reaction intermediates). The latter two methods were particularly attractive because of their rapidity and non-invasiveness, while the

HPLC analysis provided important information about insulin's chemical stability.

The experimental approach was to study the effects of aggregation inducing parameters on the overall kinetic pattern, and to infer the reaction mechanism. The legitimacy of proposed schemes was tested by constructing mathematical models of possible reaction pathways, and comparing theoretical predictions with empirical evidence. This was an iterative process: the insights gained about the physical nature of insulin aggregation were used to refine the mathematical model, until computer simulations were in reasonable agreement with experimental trends.

3.3 Materials and Methods

3.3.1 Physical Set-up

The experimental set-up was similar to systems used to study shakeproofness of insulin preparations, as well as the stability of insulin solutions in artificial infusion devices (Figure 3.1). The incubating orbital shaker allowed variation and control of temperature and agitation rates, while air or Teflon spheres in glass vials containing insulin solutions mimicked the destabilizing interactions with hydrophobic interfaces present in many drug delivery systems. (In a portable pump, air could be eliminated, but insulin solutions would still come in contact with solid hydrophobic surfaces.)

Aggregation rates were easily varied by changing insulin concentrations, the size and nature of materials contacting insulin

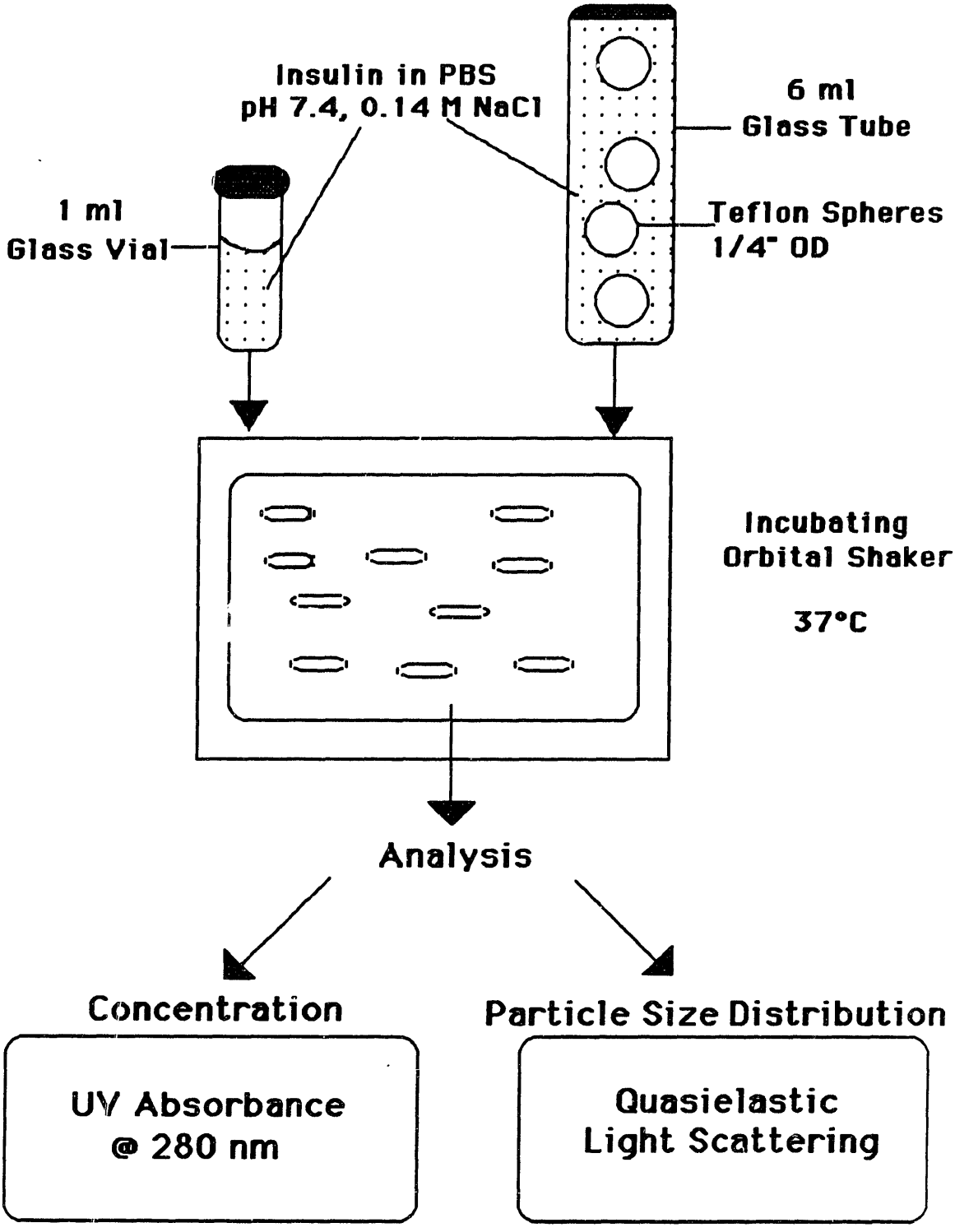

Figure 3.1: A schematic representation of the experimental system used to study insulin aggregation in aqueous solutions.

solutions, temperature, agitation rates, and chemical composition of the solvent. Control of the air-water interface proved to be more difficult; measurement and characterization of the air-water interface were hindered by bubble formation and foaming. Consequently, the air-water interface was replaced with a Teflon-water interface in order to facilitate characterization, quantification and modeling of surface interactions. Like air, Teflon was an inert and hydrophobic surface, thus protein-surface interactions at the Teflon-water interface were expected to be very similar to those at the air-water interface present in vials used in this study (and in the diabetes injection therapy).

3.3.2 Insulin Solution Preparation

Bovine Zn-insulin (Sigma I5500, lots 38F-0827 {25.6 IU/mg}, 128F-0523 {24.4 IU/mg} and 79F-0092 {25.7 IU/mg}, Zn^{2+} <0.5% (w/w)) was used without further purification. Phosphate buffered physiological saline (PBS, pH 7.4, 0.14 M NaCl/0.1% NaN_3 preservative) was filtered through 0.45 μm Millipore HV filter (Millipore Co., Bedford, MA) and degassed. Stock solutions were prepared by adding Zn-insulin to PBS; the resulting cloudy mixture was sealed with Parafilm and placed in an incubating shaker (Lab-Line Orbital Shaker), where it was gently agitated (70 rpm) for 3 hours at 37°C. Under these conditions, Zn-insulin dissolved completely at concentrations up to 0.6 mg/ml. Stock solutions were sterilized by filtration with 0.22 μm ultra low binding cellulose acetate bottle filters (Costar, Cambridge, MA). All glassware used in sample preparation and storage was rinsed with 0.01N HCl and MilliQ

water, followed by drying at 100°C. Zn-insulin concentrations were determined by measuring UV absorbance at 280 nm (ε = 5.53 mM^{-1} cm^{-1}).

3.3.3 Concentration Dependence Studies: Air-Water Interface

Borosilicate glass HPLC vials (1.1 ml, Sun Vial with polyethylene cap, Sun Brokers, Wilmington, NC) were acid rinsed and dried as described above. Each vial was filled with 0.75 ml of insulin solution of appropriate concentration (0.6, 0.3, or 0.1 mg/ml), capped, and sealed with Parafilm to prevent leakage. The samples (each contributing a time point) were taped horizontally to the platform of the incubating shaker and agitated at 250 rpm and 37°C. (The importance of shaking speed was examined by agitating 0.6 mg/ml samples at 120 rpm.) Every 20 minutes, one vial was removed and the extent of aggregation was determined by size-exclusion isochratic HPLC analysis (Bio-Sil SEC-125 column from Bio-Rad Laboratories; mobile phase consisting of 10% acetonitrile and 90% aqueous solution containing 0.02 M NaH_2PO_4 and 0.05 M Na_2SO_4, pH 6.8; flow rate of 1.2 ml/min, detection at 280 and 217 nm). Insulin concentration was determined by integrating the peak area on the chromatogram. The decrease in concentration was due to aggregation, whose manifestation was a change in the physical appearance of the insulin solution.

3.3.4 Concentration Dependence Studies: Teflon-Water Interface

Five Teflon spheres (Polysciences, Inc., #17650), 0.64 cm in diameter, were placed in each borosilicate glass disposable culture-tube (12x75 mm, Pyrex) to provide the hydrophobic surface. Insulin solution (approximately 5 ml) of appropriate concentration (0.6, 0.4 or 0.2 mg/ml) was added to each tube so that there was no headspace left, and the samples were sealed with Parafilm to completely eliminate the presence of air (so that the only hydrophobic interactions would occur on the Teflon surface). The Parafilm closures were secured with time-labeling tape to prevent the seals from melting in the incubator. The samples were then taped horizontally to the platform of the incubating shaker and agitated at 80 rpm and 37°C. Each sample contributed a time point, and thus the number of culture tubes depended on the intended duration of the experiment. Every 30 minutes one sample was removed from the shaker to measure the progress of aggregation. The contents of the culture-tube were filtered through sterile 0.8 μm Millex-PF filters (Millipore) to remove the fully aggregated, micron-size Zn-insulin particles. The concentration of Zn-insulin remaining in solution was subsequently determined by absorbance at 280 nm, and the size distribution of insulin species was determined using QELS. For each set of conditions, the experiments were repeated in triplicate.

3.3.5 Effects of Agitation and Increased Teflon Surface

To examine the effects of agitation rates and Teflon surface area on insulin aggregation rates, the number of Teflon spheres was increased from 5 to 10, and samples were agitated at 80 and 160 rpm. Sample preparation and analysis was as described in section 3.3.4.

3.3.6 Adsorption Studies

To determine the extent of insulin adsorption to glass and Teflon, several samples (initial insulin concentration of 0.6 mg/ml) containing 10 Teflon spheres were agitated at 160 rpm and 37°C overnight. The fully aggregated samples were removed from the shaker, and 1.5 ml aliquots from each test tube were transferred to Eppendorf vials and centrifuged at 14,000xg (Eppendorf micro-centrifuge, Brinkmann Instruments) for 10 minutes. Insulin concentrations in the supernatants were determined using absorbance at 280 nm. The precipitated pellets were resuspended in 1.5 ml of 8 M urea and incubated at 37°C for 6 hours. The glass vials and Teflon spheres were rinsed twice with distilled water; the spheres were transferred to glass vials containing 2 ml of 8 M urea and placed in the incubating shaker. One ml of 8 M urea was added to each of the original culture tubes which were subsequently capped and placed horizontally in the incubating shaker. Following the incubation period of 6 hours, insulin concentrations were determined using absorbance at 280 nm (ε =6.0 $mM^{-1}cm^{-1}$ for Zn-insulin in 8 M urea), and size-exclusion HPLC (as described in section 3.3.3).

3.3.7 Sodium Insulin

Bovine sodium insulin (Calbiochem, lot # 084991, 29.5 USP U/mg) was used without further purification. Na-insulin solutions were prepared as described in section 3.3.2, and concentrations were monitored at 280 nm (ε=5.17 mM^{-1} cm^{-1}). Kinetic studies were performed at 37°C and 80 rpm, with 10 Teflon spheres providing the hydrophobic surface.

3.3.8 Surface Interaction Studies: Solid-Water Interface

Siliconized glass beads were prepared by immersing glass beads in siliconizing agent SurfaSil™ (Pierce # 42800), followed by drying at 100°C for 1 hour. Teflon or polypropylene spheres (Polysciences, 0.64 cm diameter, 10 spheres per sample), or borosilicate glass beads (unmodified or siliconized) (Kimble #13500, 0.6 cm diameter, 11 spheres per sample) were added to each borosilicate glass disposable culture tube (Pyrex, 12x75 mm). All materials were cleaned by rinsing with 0.01N HCl, followed by repeated rinsing with MilliQ water and air-drying. Insulin solution (approximately 4.4 ml) was added to each tube until there was no headspace left (thus eliminating the hydrophobic interactions at the air-water interface). Samples were sealed with Parafilm and secured with time-labelling tape to prevent the seals from melting and rupturing during the experiment. Samples were taped horizontally to the platform of the incubating shaker and agitated at 160 rpm and 37°C. Each sample contributed a time point; sampling intervals varied with the material used: samples with Teflon were analyzed

every 30 minutes; those with polypropylene and siliconized glass -- every 4 hours for the first 20 hours and every 1 hour thereafter; samples containing unmodified glass beads were examined once a day. When a sample was removed from the shaker to determine the progress of aggregation, its contents were filtered with sterile 0.8 μm Millex-PF filters (Millipore) to remove the fully aggregated, micron-size Zn-insulin particles which interfered with both quasielastic light scattering (QELS) and ultraviolet absorption spectroscopy (UV) analyses.

3.3.9 Zn-insulin in Organic Solvents

Zn-insulin was lyophilized from a 1 mg/ml solution (prepared by dissolving Zn-insulin in MilliQ water (pH 3) and then adjusting the pH to 7.3). All organic solvents were of analytical grade: DMSO (Fisher), ethylene glycol (Fisher), and formamide (Fluka). Lyophilized insulin readily dissolved in the neat organic solvents to desired concentrations. Zn-insulin concentration was determined by UV absorbance at 280 nm (ε = 8.74, 6.86 and 7.07 mM^{-1} cm^{-1} for DMSO, ethylene glycol and formamide, respectively). QELS analysis could not be performed due to the small difference between the refractive indices of these organic solvents (n=1.48, 1.43 and 1.45 for DMSO, ethylene glycol and formamide, respectively) and insulin's index of refraction (n=1.59); (for aqueous solutions n=1.33). Sample preparation and experimental conditions were as in air-water interfacial studies.

3.3.10 Quasielastic Light Scattering

Light scattering measurements were made using a Lexel argon-ion laser (model 95-2), with a Brookhaven apparatus consisting of a goniometer (Model BI-200SM), and a 136-channel digital correlator and signal processor (Model BI-2030AT), which incorporated an IBM-AT™ compatible computer for measurement control, data accumulation, and particle-size distribution analysis. All measurements were made with a laser wavelength λ=488 nm at a scattering angle of 90°. The latter was particularly useful for observing Rayleigh scatterers (i.e., particles much smaller than the wavelength λ). Non-aggregated insulin molecules behaved as Rayleigh scatterers, since the hexamer's 5 nm diameter was small compared to λ. The presence of peaks at diameters >50 nm was verified at 135°, where scattering contributed by dust and rotational diffusion was minimized (Brookhaven, 1986; Tanford, 1961).

For each sample, light-scattering measurements were accumulated during 1 minute intervals in order to reduce random noise in the signal and to ensure a stable baseline. The experimentally determined autocorrelation function $g(\tau)$ was used to obtain the size-distribution function $G(\gamma)$ using inverse Laplace transform algorithms Non-Negatively Constrained Least Squares (NNLS) and CONTIN (of Provencher) (Brookhaven, 1986; Stock and Ray, 1985).

3.3.11 Computer Simulations

Mathematical modelling of aggregation kinetics was accomplished by simultaneously solving a set of differential equations describing each step in the reaction scheme. Due to the number and complexity of the combined equations, the dynamics of insulin aggregation were simulated using a Stella II software package (High Performance Systems, Hanover, NH), with integration by a Runge-Kutta fourth-order method (Richmond et al., 1990). Details of model simulation parameters are provided in Chapter 5.

CHAPTER 4.

RESULTS OF KINETIC STUDIES

4.1 Control Studies

Insulin aggregation rates are affected by a variety of physical and chemical factors; small changes in temperature, solvent composition, pH, agitation rates, and contact surface areas, can significantly affect the overall pattern of destabilization (see Chapter 2). To elucidate the nature of the aggregation reaction, it was necessary to control aggregation inducing parameters in order to obtain reproducible kinetic profiles. Care was taken to prepare buffers, solutions, and samples under identical conditions; temperature and agitation rates were closely monitored throughout each study, and to ensure the reliability of experimental results, most studies were repeated in triplicate.

4.1.1 Reproducibility

Different lots of bovine Zn-insulin were used in this work, and therefore it was necessary to determine how lot to lot variations affected the pattern of aggregation. Small differences in Zn^{2+} content and specific activities appeared to have little or no effect on insulin aggregation. As can be seen in Figure 4.1, solutions prepared from lots 38F-0827 and 128F-0583 (specific activity 25.6 and 24.4 IU/mg, respectively) exhibited nearly identical aggregation profiles when agitated in the presence of an air-water interface. Similarly, the

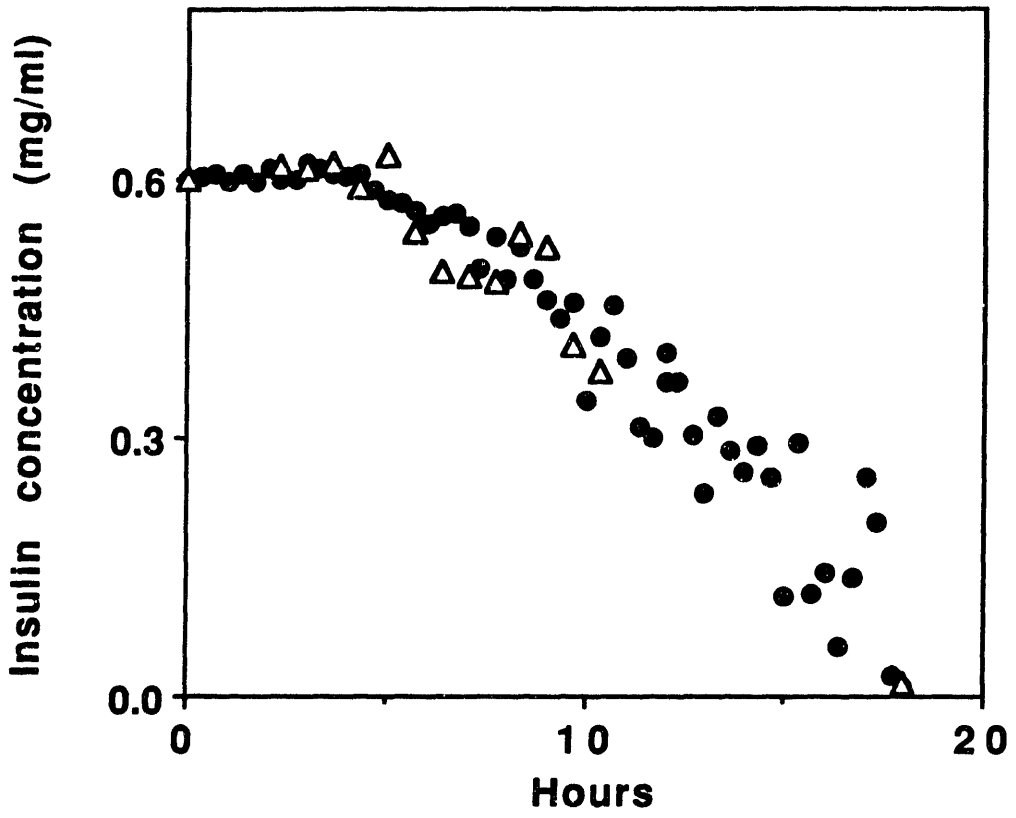

Figure 4.1: Reproducibility of insulin aggregation rates in the presence of an air-water interface. Solutions prepared from lots 38F-0827 (●) and 128F-0523 (Δ) were compared; samples were agitated at 37°C and 250 rpm in 1 ml glass vials.

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aggregation patterns of insulin samples prepared from lots 128F-0583 and 79F-0092 (specific activity 24.4 and 25.7 IU/mg, 0.40 and 0.46 wt% Zn^{2+}, respectively, Zn^{2+} content determined by Galbraith Laboratories) and agitated in the presence of Teflon, were within experimental error (Figure 4.2). The reproducibility of kinetic profiles indicated good control over experimental conditions, and

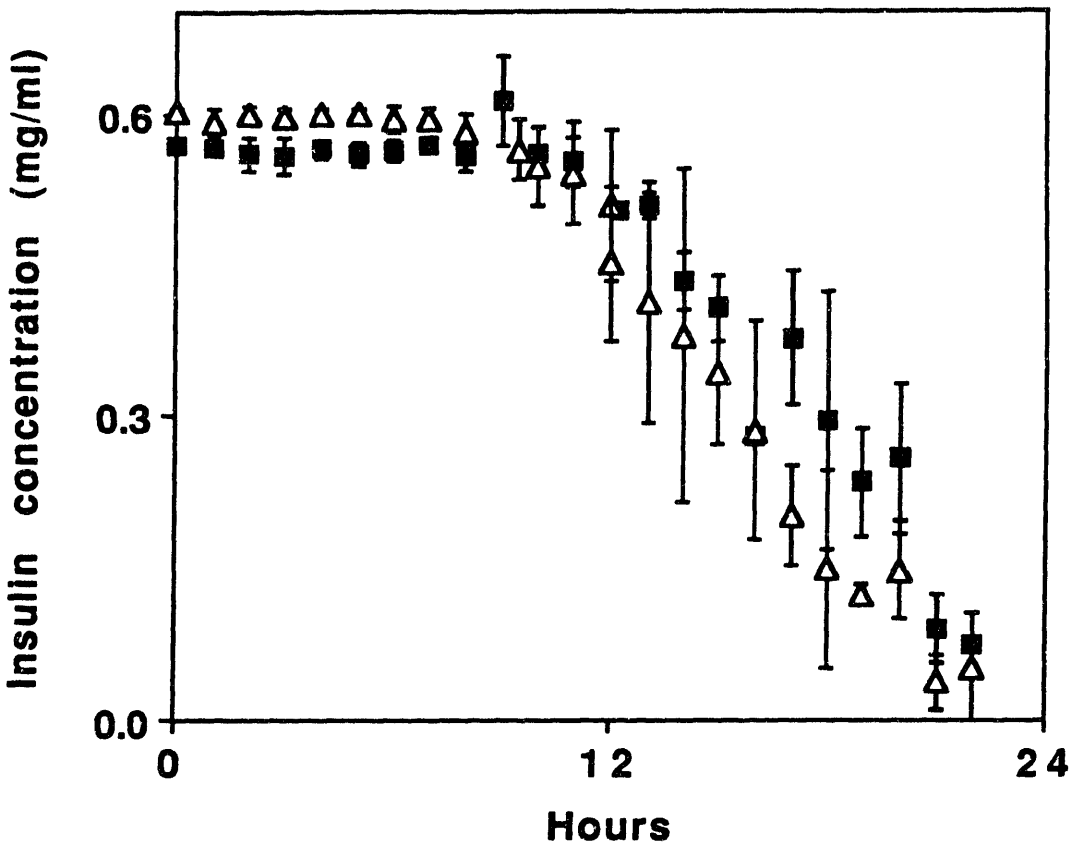

Figure 4.2: Reproducibility of insulin aggregation rates in the presence of a Teflon-water interface. Solutions prepared from lots 79F-0092 (■) and 128F-0523 (Δ) were compared. Samples containing 10 Teflon spheres were agitated at 37°C and 80 rpm. Each curve was averaged over 2 experiments, with error bars representing standard deviation from the mean.

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suggested that the observed trends were not lot-specific, but representative of general insulin aggregation behavior.

4.1.2 Stationary Samples

Previous studies suggested that agitation significantly contributed to the destabilization of insulin solutions. In the absence of shaking, no aggregation was observed in samples containing an air-water (Figure 4.3) or a Teflon-water interface (Figure 4.4 A), even though the solutions were exposed to elevated temperature (37°C). QELS analysis performed on stationary samples with Teflon detected only one peak representing native insulin molecules (monomers, dimers and hexamers), indicating that no destabilizing interactions were taking place in the absence of agitation (Figure 4.4B).

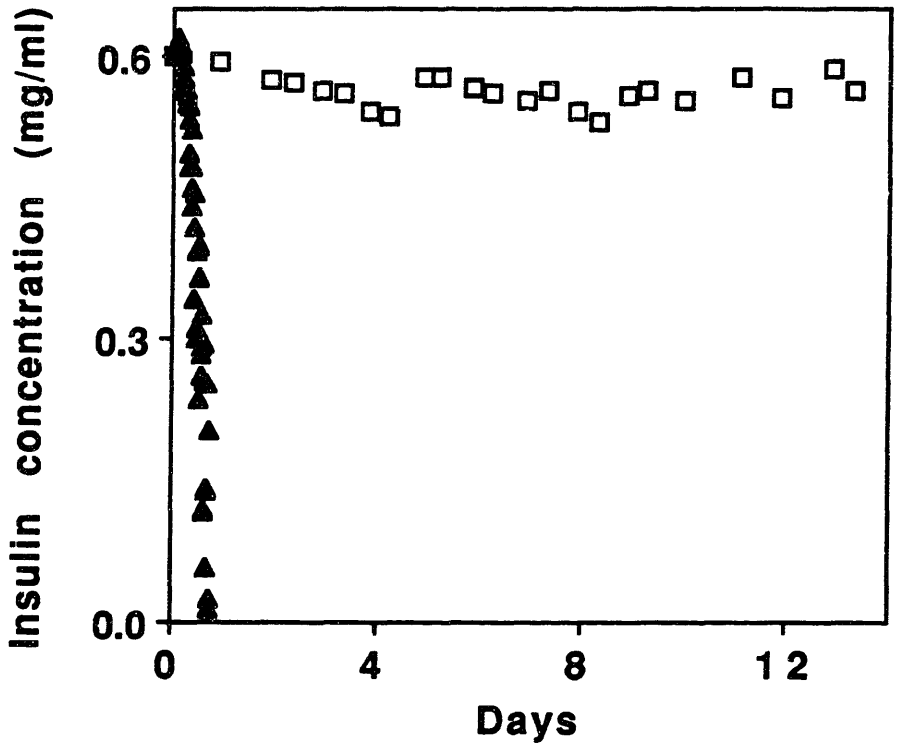

Figure 4.3: Effects of agitation on Zn-insulin aggregation in the presence of an air-water interface. Both stationary samples (□) and samples agitated at 250 rpm (▲) were kept at 37°C.

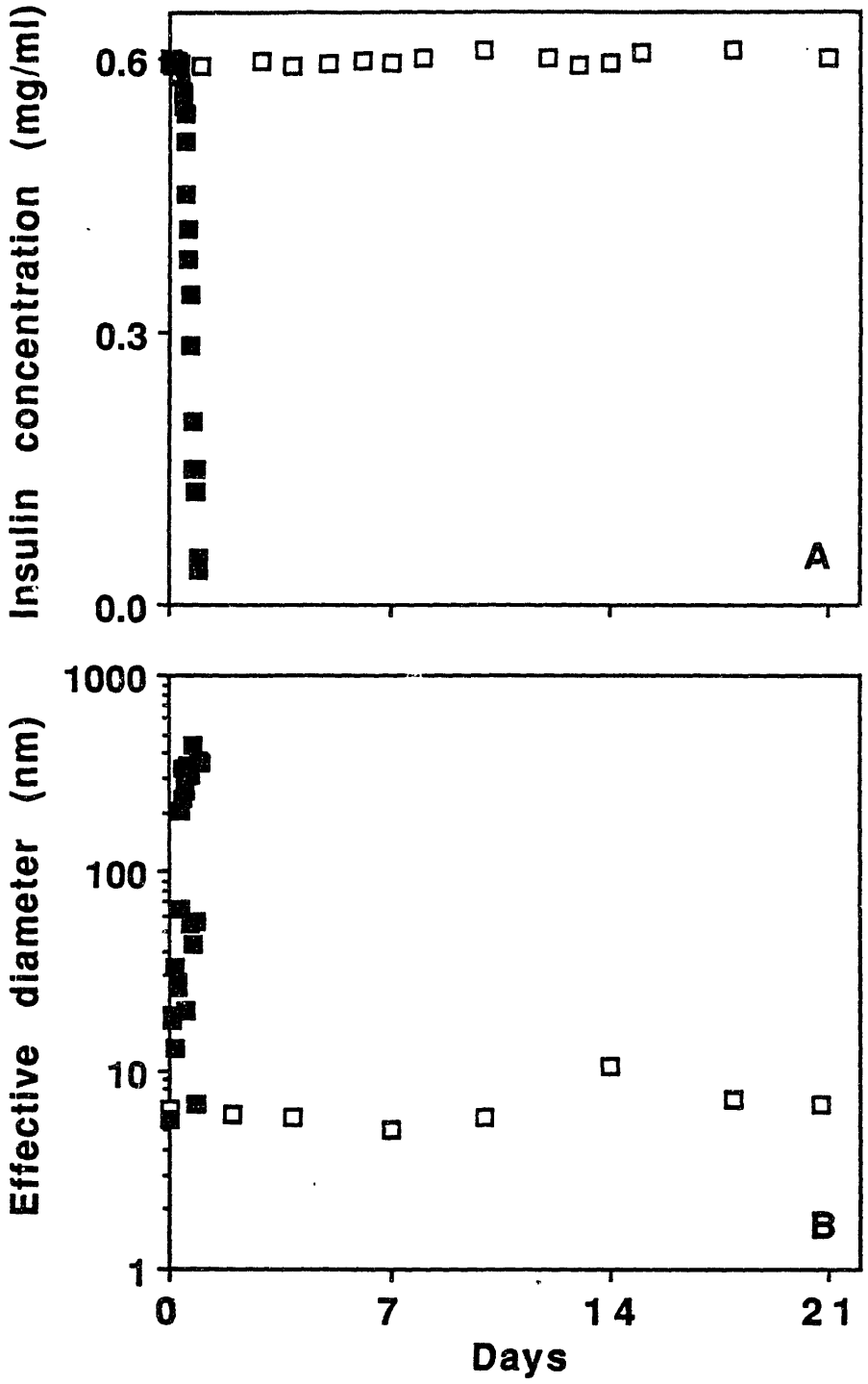

Figure 4.4: Effects of agitation on Zn-insulin aggregation in the presence of Teflon. Both stationary samples (□) and samples agitated at 80 rpm (■) contained 10 Teflon spheres and were kept at 37°C. (A) Concentration profiles of Zn-insulin aggregation; (B) results of QELS analysis.

4.1.3 Comparison of HPLC and UV Analyses

In the initial kinetic studies with an air-water interface, a small volume of insulin solution was used per sample (0.75 ml), and the concentration of non-aggregated protein was determined using the HPLC analysis, which required only 25 μl per injection. Although aggregated solutions were injected without prior filtration, the fully aggregated, micron-size species never reached the column, as they could not pass through the 2 μm frit at the inlet. The large aggregates were not broken down during the analysis: supernatants of centrifuged samples gave results identical to aggregated samples which were injected directly onto the column. More than 97% of injected insulin was recovered, making HPLC analysis a quantitative and reproducible technique for measuring protein concentrations.

It was hoped that in addition to measuring insulin concentrations, the HPLC analysis would detect the formation of high molecular weight intermediate species (less than 1 micron in size), and provide information about changes in insulin's self-association equilibrium. At protein concentrations used in these studies, almost 90% of insulin molecules were expected to be in the hexameric form (Milthorpe et al., 1977). QELS measurements indicated that insulin's effective diameter in stock solutions was 5 nm (Figure 4.5), which confirmed the hexamer's presence and predominance. Yet, despite the mild conditions used with the HPLC analysis, dilution effects and column-packing interactions disrupted the formation of dimers and hexamers, and only one peak -- that of monomeric insulin -- was observed throughout the experiment. No intermediate species were

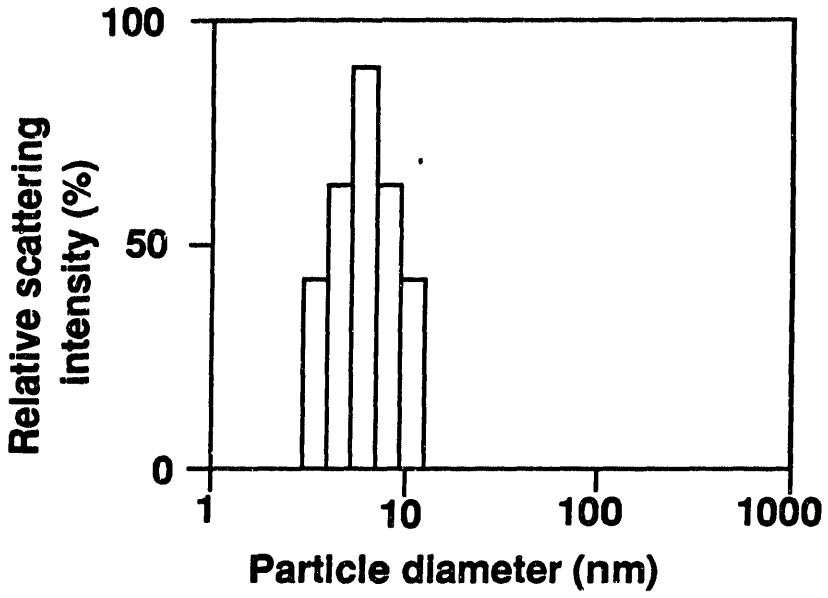

Figure 4.5: Results of QELS analysis on non-aggregated (freshly prepared stock) Zn-insulin solutions. Relative scattering intensity (% of total) is plotted versus the diameter of the particle responsible for the scattering. The size distribution histogram was generated using the program CONTIN.

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observed, most likely because they were present in concentrations below the detection limits of the HPLC analysis. Thus, the only information provided by size-exclusion chromatography was the concentration of non-aggregated insulin remaining in solution.

The HPLC analysis proved to be a quantitative and reproducible method for measuring insulin concentrations; at the same time, it was slow (requiring ~20 minutes for analysis of each sample), labor intensive (mobile-phase preparation, column cleaning and equilibration, common switch-overs between aqueous and

organic conditions required by other users of the system), and less than reliable (mechanical problems with pumps, UV detector and automated injector). For these reasons, an alternative technique was evaluated.

The rapidity and non-invasiveness of UV spectrophotometry made it an attractive alternative to HPLC analysis. The main difference between the two techniques was that the HPLC analysis measured the UV absorbance of a monodisperse solution of insulin monomers, while UV spectrophotometry measured the absorbance of a polydisperse solution. For unperturbed insulin solutions, both techniques appeared to work well, generating linear calibration curves (goodness of fit parameter R^2 = 0.984 and 1.000 for HPLC and UV spectrophotometry, respectively). However, absorbance measurements in aggregating solutions were complicated by the formation of intermediate species (the micron-size aggregates were filtered out), whose presence was expected to interfere with the analysis. To determine the extent of such interference, UV absorbance measurements at 280 nm were used to monitor the concentrations of aggregating insulin solutions, and the results compared with HPLC analysis performed on the same samples (Figure 4.6). Fortunately, the concentration of intermediates was so small as to make the contribution to total absorbance measurements negligible, making UV spectroscopy an excellent replacement for the HPLC analysis.

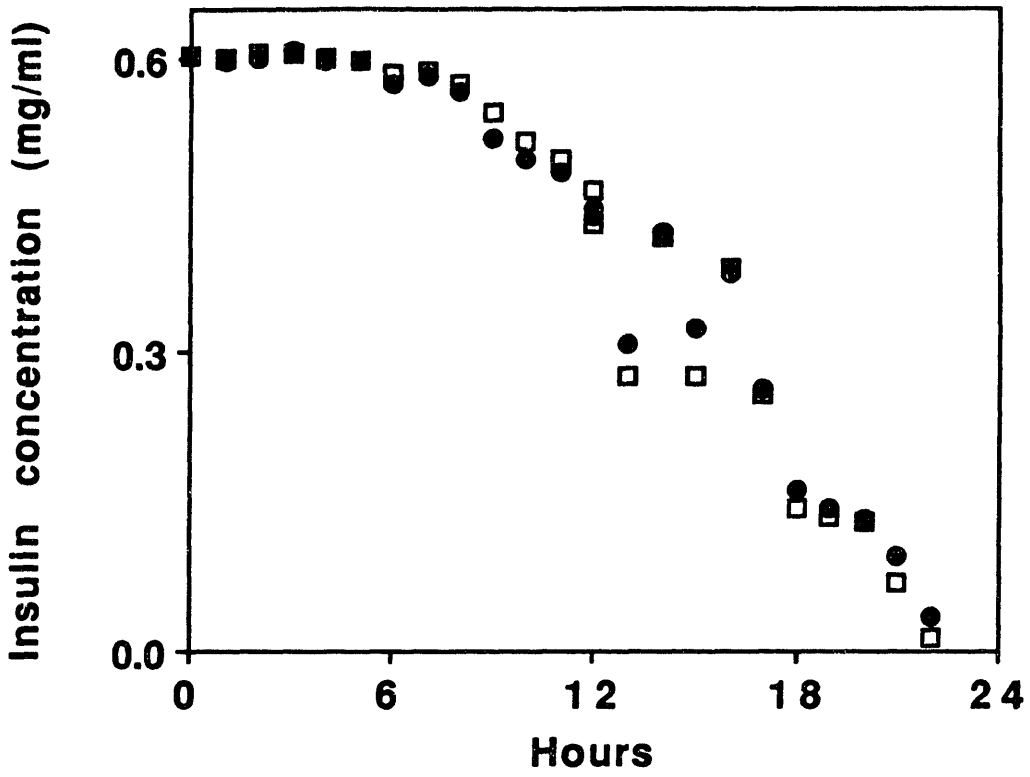

Figure 4.6: Comparison of UV (●) and HPLC (□) analyses used to determine the concentration of non-aggregated Zn-insulin in samples agitated at 80 rpm and 37°C, in the presence of 10 Teflon spheres.

4.1.4 Adsorption to Glass and Teflon

Insulin's interactions with solid surfaces were expected to play an important role in the aggregation reaction. Since insulin is known to bind to glass and other materials (Cuatrecasas and Hollenberg, 1975; Sefton and Antonacci, 1984), it was of interest to determine how much protein was lost due to aggregation, and how much adsorbed unto container surfaces. The results of adsorption studies (described in section 3.3.6) indicated that most of the protein ended up in the aggregated material. On average, 4.4% of initially dissolved

insulin adsorbed to 10 Teflon spheres, less than 0.1% adsorbed onto glass walls of the sample container, and the remaining 95.5% were found in the precipitated pellet (Table 4.1).

Table 4.1: Extent of adsorption to glass and Teflon in aggregated insulin samples (initial concentration 0.6 mg/ml). Results of UV measurements @ 280 nm on insulin aggregates redissolved in urea¶.

Sample Number	Aggregated Material*	Adsorbed to Teflon*†	Adsorbed to glass*†	Total Absorbance‡
1	0.786	0.034	0.002	0.822
2	0.775	0.030	0.002	0.807
3	0.775	0.045	0.003	0.823
4	0.760	0.032	0.002	0.794
5	0.782	0.037	0.003	0.822
6	0.761	0.040	0.002	0.803
Average	0.773	0.036	0.002	0.812

¶ The concentration of non-aggregated insulin remaining in solution was negligible (less than 1 wt%).

* Absorbance units (±.003). Results are reported in absorbance units rather than weight percent because of the difference in absorbance of native and aggregated insulin in urea.

† Corrected for dilution effects (i.e., 2/5 and 1/5 of measured absorbance for Teflon and glass, respectively).

‡ Absorbance of 0.6 mg/ml native insulin in 8 M urea was 0.609.

4.1.5 Nature of Aggregate Interactions

To determine the nature of protein-protein interactions involved in aggregate formation, an HPLC analysis was done on aggregates redissolved in 8 M urea. Only one peak was observed, indicating the presence of only one species, which was equivalent in size to the insulin monomer. The chromatogram was indistinguishable from that of freshly prepared insulin solution in PBS. This indicated that no fragmentation of insulin occurred during aggregation and that the aggregates were held together through non-covalent (probably hydrophobic) interactions, as was suggested previously (Lougheed et al., 1983; Brange et al., 1987). It is interesting to note, however, that when the concentration of redissolved insulin was determined by UV spectrophotometry, the absorbance measurements were approximately 33% higher than the absorbance of native insulin in 8 M urea (Table 4.1). This suggested that a conformational change took place during aggregation, leading to the formation of a partially denatured or scrambled structure when insulin aggregates dissociated in a denaturing reagent. The increased absorbance could probably be attributed to the exposure of tyrosine groups, which were shielded from the solvent in the native structure.

4.1.6 Comparison of Filter Performance

The QELS analysis was extremely sensitive to dust content in insulin samples, making it essential to remove the fully aggregated species prior to light scattering measurements. These micron-sized agglomerates interfered with the UV analysis as well, and filtration

was necessary for accurate determination of insulin concentrations. Filtration through polyhydroxypropylacrylate grafted polyvinylidene difluoride (poly-HPA-PVDF, Millex GV) and polyester-backed cellulosic (Millex PF) membranes was expected to have little or no effect on insulin's conformation and self-association (Truskey et al., 1987), but it was important to determine how much protein was retained by the filters.

For QELS analysis, the removal of large agglomerates (greater than 800 nm) was required, so 0.800 μm Millex-PF filters were used. They removed the fully aggregated particles, leaving the intermediate species unperturbed and available for light scattering analysis. When the concentration of intermediate species was high, UV absorbance measurements gave anomalously high readings, making it necessary to remove the intermediates with 0.200 μm Millex-GV filters (this happened very rarely). Very little material was lost: on average, the 0.200 μm and 0.800 μm filters retained 2.7 and 1.3 wt% of dissolved protein, respectively. The amount of adsorbed insulin could be reduced even more by pre-wetting the filters with fresh (non-aggregated) insulin solutions.

4.2 Air-Water Interface

The initial studies with an air-water interface concentrated on identifying general patterns in the aggregation reaction. These experiments were done before the light-scattering equipment was available, so only time-dependent concentration profiles were obtained. The studies provided important qualitative information about the role of various parameters affecting insulin aggregation

rates; they were also used to evaluate the reliability of the experimental system, and to identify problems which interfered with adequate characterization of destabilization pathways.

4.2.1 Concentration Dependence Studies

The first series of experiments focused on the effects of insulin concentration on overall aggregation rates. It soon became apparent that at higher insulin concentrations, more time was required for aggregation. Subsequent studies were done to obtain detailed kinetic profiles; the results are shown in Figure 4.8. At each concentration, a characteristic curve shape was observed: initially, there was a period of stability (a flat portion during which no aggregation was detected); it was followed by a sloping portion, which indicated insulin depletion resulting from aggregation. Comparison of solution half-lives ($t_{1/2}$ defined as the time when insulin concentration were reduced to 50% of the original concentration) revealed greater stability (longer half-lives) at higher concentrations. For initial concentrations of 0.6, 0.3 and 0.1 mg/ml, the $t_{1/2}$ values were 12, 6 and 3 hours, respectively (Figure 4.8).

This positive correlation between stability and concentration was somewhat anomalous. In general, protein aggregation processes appear to be kinetically controlled, and rates of aggregation tend to be proportional to protein concentrations (Zettlmeissl et al., 1979). For example, thermally induced aggregation has been reported to increase with protein concentration (Klibanov, 1983), and dilution

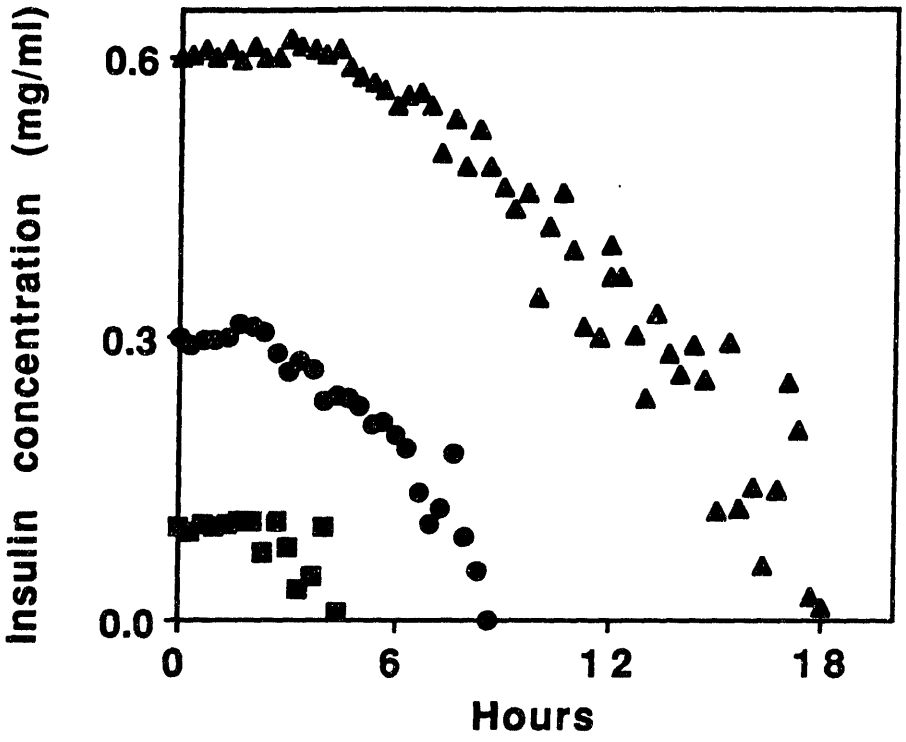

Figure 4.7: Concentration dependence of Zn-insulin aggregation rates in the presence of an air-water interface. Samples with initial concentrations of 0.6 mg/ml (▲), 0.3 mg/ml (●), and 0.1 mg/ml (■) were agitated at 250 rpm and 37°C.

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was shown to enhance stability. The opposite was observed in Zn-insulin solutions, where the propensity to aggregate was lower at higher protein concentrations. Similar results have been reported previously (Lougheed et al., 1983; Brange and Havelund, 1983); yet, no adequate explanation has been proposed for this anomalous behavior. A successful kinetic model of insulin aggregation would

have to explain and predict both the concentration dependence and the characteristic curve shape.

4.2.2 Role of Agitation

The next set of experiments examined the effects of agitation rates on Zn-insulin aggregation (Figure 4.8). In the absence of agitation, no aggregation was observed for more than 2 weeks, while rapid agitation (250 rpm) led to complete aggregation in only 18

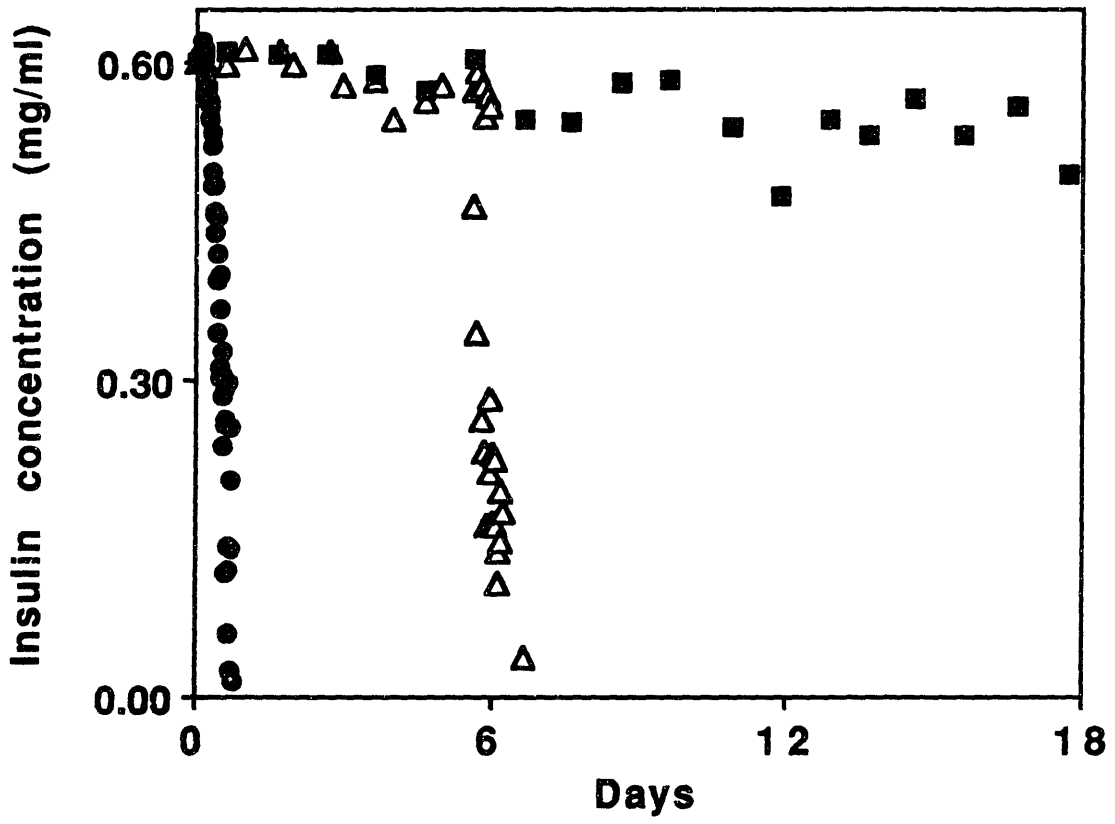

Figure 4.8: Effects of agitation rate on Zn-insulin aggregation. Samples (initial concentration of 0.6 mg/ml, 37°C) were shaken at 250 rpm (●), 120 rpm (Δ) and 0 rpm (■) in the presence of an air-water interface.

hours. When the agitation rate was reduced to 120 rpm, the induction period (flat portion of the curve) increased from 4 hours to almost 6 days, and the solutions aggregated fully in 6.5 days. However, a direct correlation between increases in agitation and aggregation rates could not be drawn, since the air-water interface also increased with agitation (because of foaming and bubble formation). Thus, while the results of these experiments pointed to the importance of agitation, the difficulties associated with decoupling the contributions of improved mixing and a larger air-water interface prevented the formulation of a quantitative relationship between agitation and aggregation. To further investigate the role of agitation, it was necessary to vary agitation rates without changing the size of the hydrophobic interface.

4.2.3 Role of Air-Water Interface

The importance of both mixing rates and air-water interfaces became evident once again when insulin samples with and without headspace were agitated at 250 rpm (Figure 4.9). Samples with an air-water interface aggregated in less than a day, while those without any headspace remained stable for over a week. In the samples lacking headspace, mixing (as well as the air-water interface) was eliminated, and mass transfer inside the vials was limited by diffusion. As in the no agitation case described above, it was difficult to distinguish between the effects of mixing and hydrophobic interfaces on insulin destabilization, although it became clear that both parameters were necessary for rapid and reproducible aggregation.

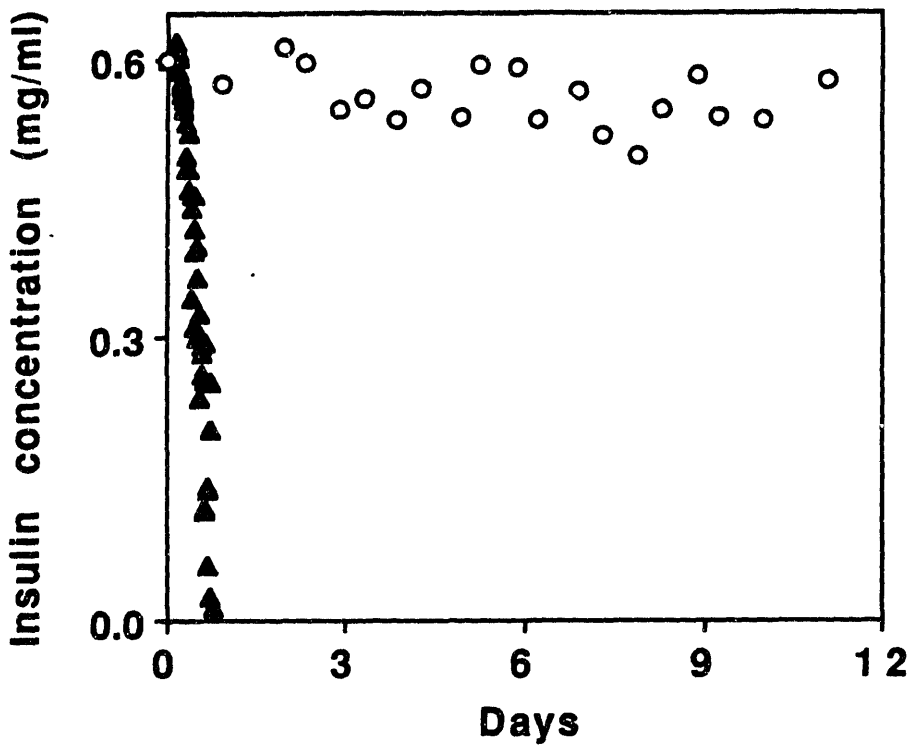

Figure 4.9: Effects of air-water interface on Zn-insulin aggregation rates. Samples (initial concentration of 0.6 mg/ml) with (▲) and without (O) an air-water interface were agitated at 250 rpm and 37°C.

4.3 Teflon-Water Interface

The experiments with an air-water interface provided valuable insights about the concentration dependence of insulin aggregation. However, it was very difficult to characterize and quantify the air-water interface, and nearly impossible to independently evaluate the contributions of the hydrophobic interface and mixing rates to the overall pattern of aggregation. Replacement of headspace with

Teflon spheres provided the necessary control of agitation rates and hydrophobic surface areas, and allowed for a quantitative analysis of surface interactions and mass transfer. In addition to monitoring concentration changes, light scattering measurements were done to determine the particle size distributions in the destabilized samples. The combination of these two analytical methods provided a more complete picture of the aggregation reaction.

4.3.1 Concentration Dependence

Insulin aggregation in the presence of Teflon spheres was very similar to the behavior observed with an air-water interface. As insulin concentrations increased, so did solution stability, with $t_{1/2}$ values increasing from 16 to 30 hours for initial concentrations of 0.2 and 0.6 mg/ml, respectively (Figure 4.10).

The characteristic curve shape -- an induction period followed by a sloping section -- was also observed, and was in good qualitative agreement with the air-water interface results. This suggested that the use of Teflon spheres did no introduce any artificial effects, and that the experimental set-up indeed mimicked the destabilizing conditions present in many drug-delivery environments.

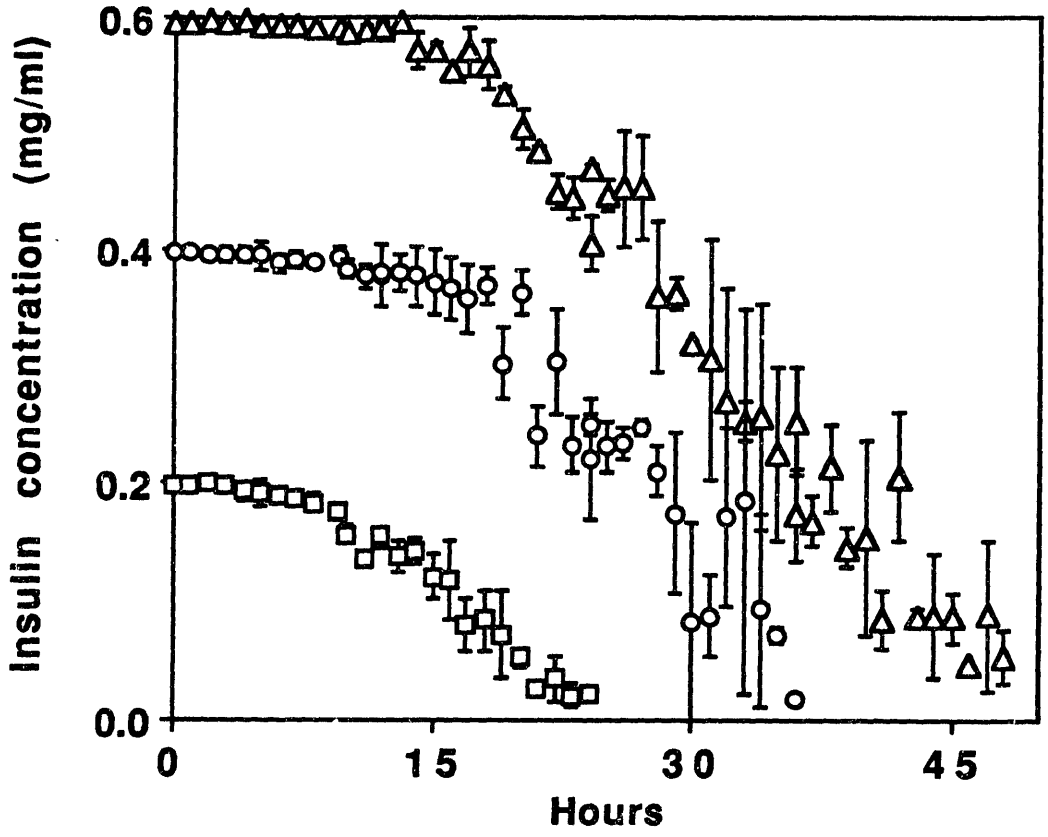

Figure 4.10: Concentration dependence of Zn-insulin aggregation rates in the presence of a Teflon-water interface. Samples containing 5 Teflon spheres were agitated at 80 rpm and 37°C. Initial concentrations were 0.6 mg/ml (Δ), 0.4 mg/ml (O), and 0.2 mg/ml (□). Each curve is the average of 3 experiments, with error bars representing standard deviation from the mean.

4.3.2 Effects of Surface Area and Agitation Rates

In the presence of Teflon spheres, it became possible to vary the size of the hydrophobic surface without changing the agitation rates. Figure 4.11 illustrates the effects of an increased hydrophobic

interface on insulin aggregation. When the number of Teflon spheres was doubled from 5 to 10 per sample, the flat portion of the profile (the induction period) became shorter, and the slope of the curve became steeper. Similar results were obtained when the agitation rate was doubled (Figure 4.12). These observations pointed to the importance of insulin interactions with the hydrophobic interface and to the role of mass transfer and/or shear denaturation in the aggregation process.

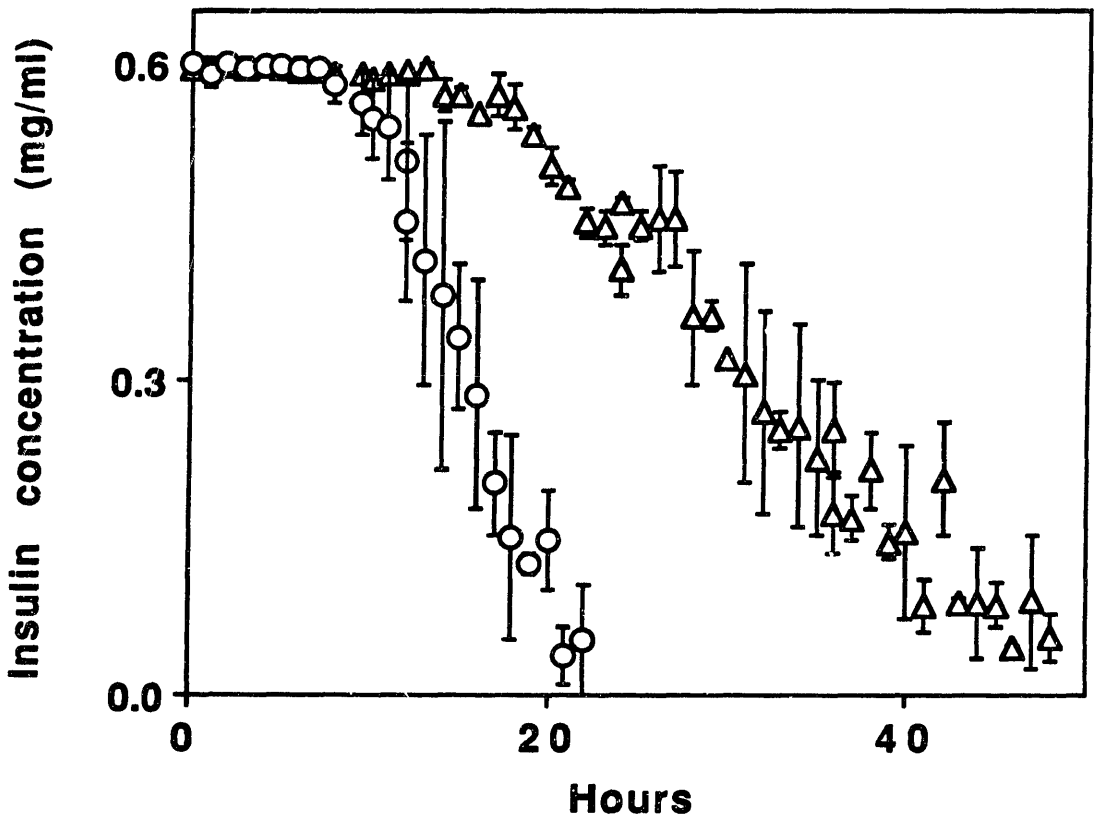

Figure 4.11: Effects of Teflon surface area on Zn-insulin aggregation rates. Samples (initial concentration of 0.6 mg/ml) were agitated at 80 rpm and 37°C, with 5 (Δ) and 10 (O) Teflon spheres. Each curve is the average of 3 independent experiments, with error bars representing standard deviation from the mean.

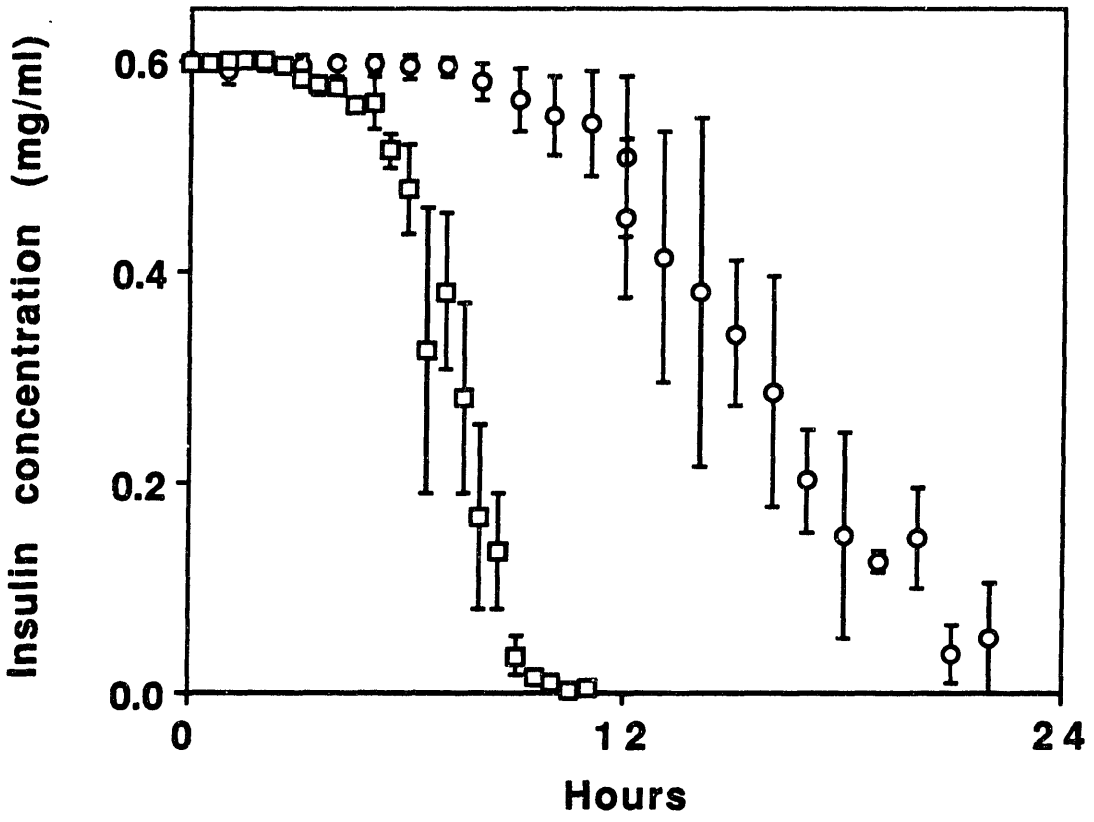

Figure 4.12: Effects of agitation rate on Zn-insulin aggregation. Samples (initial concentration of 0.6 mg/ml) containing 10 Teflon spheres were agitated at 80 (O) and 160 (□) rpm. Each curve is the average of 3 independent experiments, with error bars representing standard deviation from the mean.

4.3.3 Results of QELS Analysis

The overall shape of the curves suggested that aggregation was analogous to a phase transition, such as the formation of ice, protein precipitation, or crystallization. In each case, the first step is nucleation -- formation of stable intermediate species which facilitate the growth of crystals or precipitates (Kam et al, 1978). QELS analysis revealed the presence of such stable intermediates in aggregating insulin solutions. Typical experimental results are presented in Figure 4.13.

Initially, only native insulin molecules were present in solution, as reflected by the peak at 5 nm (Figure 4.13 A). Due to the small size of the insulin monomer and the small differences in the hydrodynamic diameters of the monomers, dimers, and hexamers, it was difficult to distinguish among these species.

(The scattering intensity is proportional to the particle diameter raised to the sixth power. At Zn-insulin concentrations used in these experiments, the monomer made up only 4% by weight of particles in solution. Therefore, the monomer's scattering intensity was only 0.1% of hexamer's scattering, while the dimer was responsible for approximately 0.3% of the signal -- contributions too small for meaningful resolution of particle sizes (see appendix A for details). The algorithm CONTIN used to generate the depicted histograms sought continuous distributions rather than discrete spikes; while this reduced the problem of spurious peaks, it also smoothed out the particle size distribution (Brookhaven, 1986), making it nearly impossible to separate out the populations involved in Zn-insulin's dynamic self-association equilibrium.)

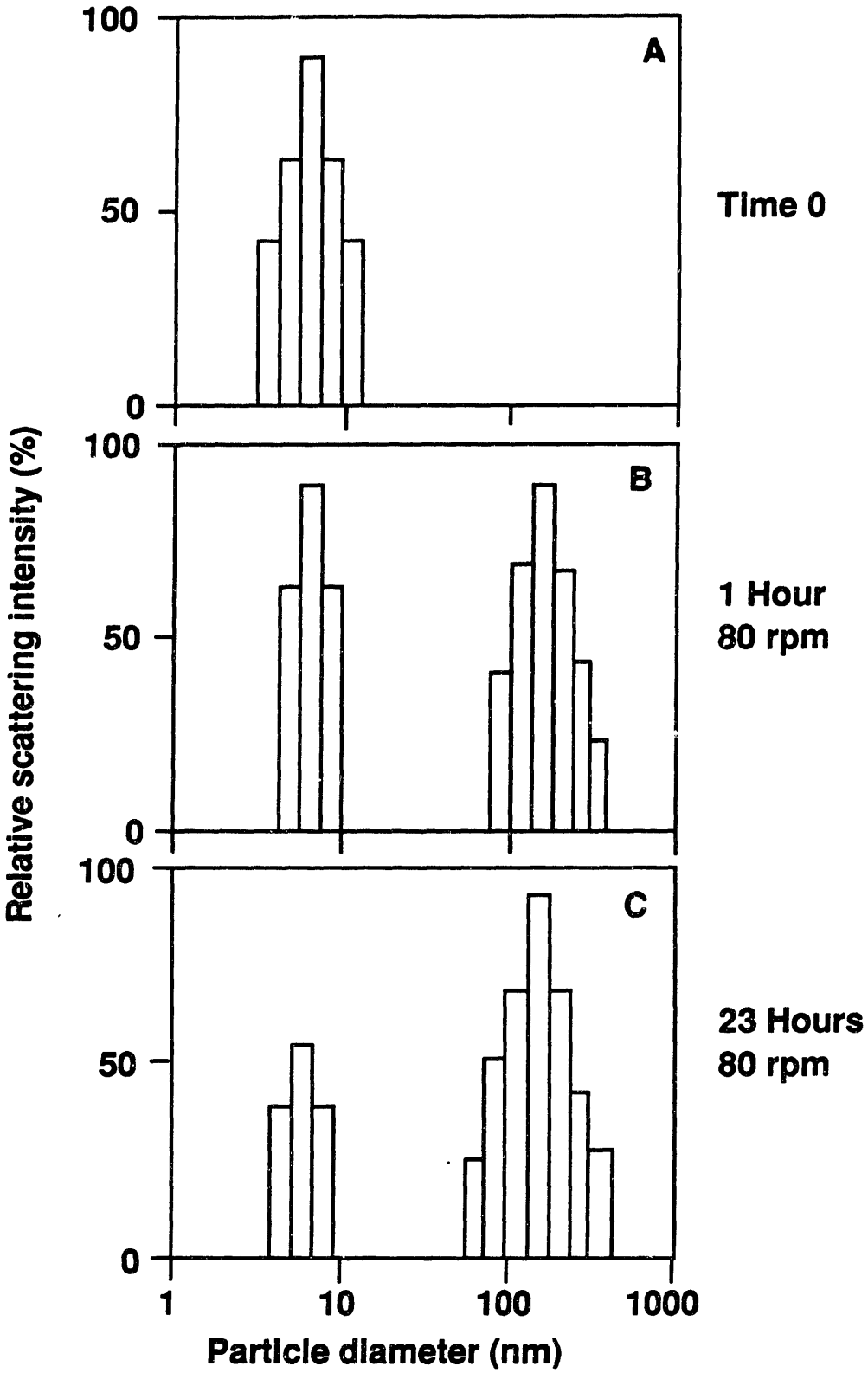

Figure 4.13: Representative results of QELS analysis of insulin solutions (0.6 mg/ml) agitated in the presence of 10 Teflon spheres.

Just in an hour of agitation in the presence of Teflon, a second peak appeared (Figure 4.13 B), corresponding to particles of approximately 150 nm in diameter, which made up less than 0.01% (w/w) of total protein in solution. This bi-modal distribution was observed throughout the experiment: even in an almost fully aggregated solution (following 23 hours of agitation), a native peak at approximately 5 nm and an intermediate peak at 170±20 nm were present (Figure 4.13 C). Thus, there were three distinct types of species in solutions of aggregating insulin: the native molecules (monomers, dimers, hexamers), represented by the peak at 5 nm; the fully aggregated micron-size particles (larger than 0.8 μm), filtered out prior to analysis; and the stable intermediates represented by the peak at 170±20 nm. It is important to note that the intermediate species appeared before aggregation could be detected by other means (such as changes in concentration or visible turbidity). This suggested that the initial stages of aggregation involved relatively few destabilized molecules, and that the intermediate species facilitated subsequent aggregation.

It is necessary to emphasize that QELS is a low resolution technique. For monodisperse samples, it can be used to determine particle sizes with ±3% accuracy, but for polydisperse solutions, the certainty in the measured diffusion coefficients is much lower (Brookhaven, 1986). The analysis of polydisperse samples had to assume a particular form (bi-modal, tri-modal, etc.), but choosing an appropriate form using the best statistical fit is not recommended, because function $g(\tau)$ is an ill-conditioned equation (Brookhaven, 1986). Furthermore, the analysis assumed that all particles were

spherical. While this assumption held up well for the insulin hexamer (a globular protein), there was no evidence about the shape of the intermediate species. It is possible that the intermediate's shape would have been better described as a rod or a random coil. This uncertainty also reduced the accuracy of particle size measurements. Given these complications, the intermediate's diameter could not be determined with better than ±10% accuracy, and the weight distribution of insulin species could be only roughly estimated. Despite these limitations, the insights provided by QELS analysis were instrumental in the formulation of the kinetic scheme.

4.3.4 Na-insulin Aggregation

In the absence of Zn^{2+}, insulin's self-association was disrupted; fewer dimers assembled into hexamers, and a larger proportion of molecules remained monomeric. To examine the effects of increased monomer concentrations on insulin aggregation rates, the destabilization of bovine Na-insulin was examined.

Figure 4.14 illustrates that solutions of Na-insulin aggregated faster than equimolar solutions of Zn-insulin; the induction period was reduced from 8 to less than 1 hour, and the slope of the curved portion became steeper. Lower hexamer content in Na-insulin solutions was evident from QELS analysis (Figure 4.15 A): the peak representing native insulin species shifted from 5 to 4 nm, indicating the predominance of monomers and dimers over hexamers. While hexamer formation was reduced, it was not eliminated entirely. Because of the strong correlation between particle size and scattering intensity, the presence of even a minute amount of hexamers would

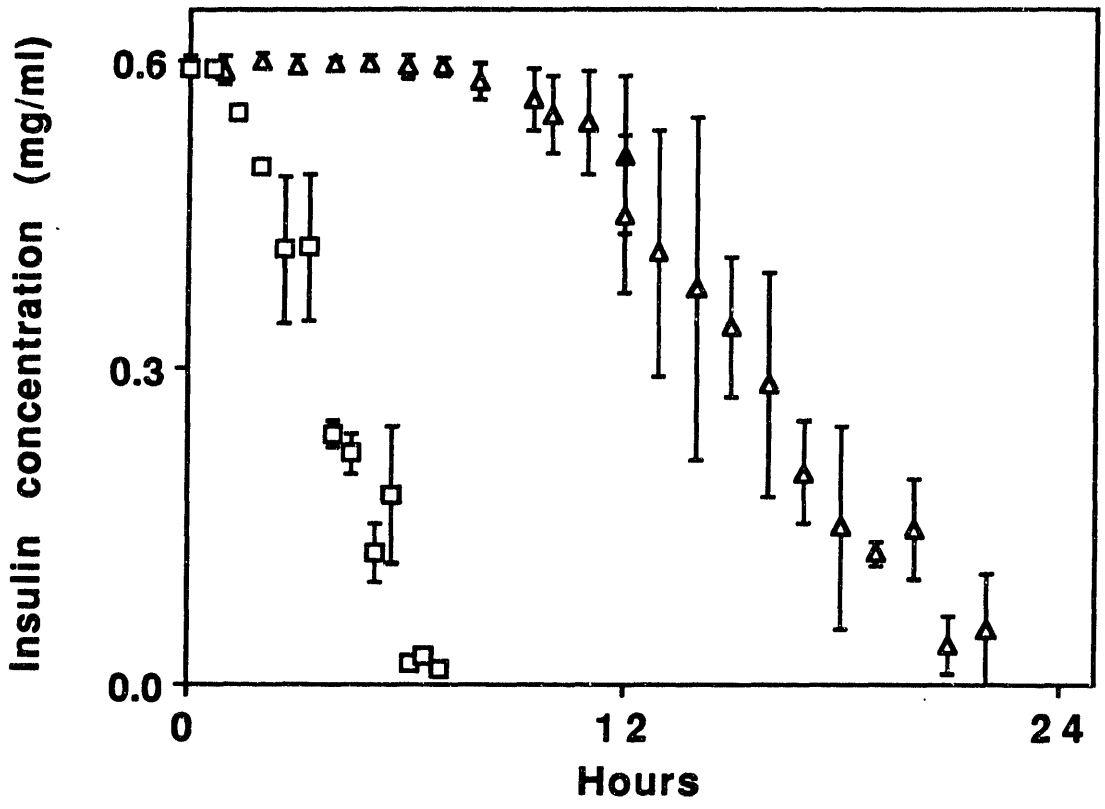

Figure 4.14: Concentration profiles for Na- (□) and Zn- (Δ) insulin solutions, aggregating in the presence of 10 Teflon spheres at 80 rpm and 37°C. Results were averaged over three experiments, with error bars representing standard deviation from the mean.

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significantly affect the QELS results. This was why only a small change was observed between the effective diameters of scattering species in Zn- and Na-insulin solutions. In addition to the native peak at 4 nm, stable intermediate species (150±20 nm) were detected in aggregating Na-insulin solutions (Figure 4.15 B); this was consistent with insulin aggregation behavior observed in Zn-insulin

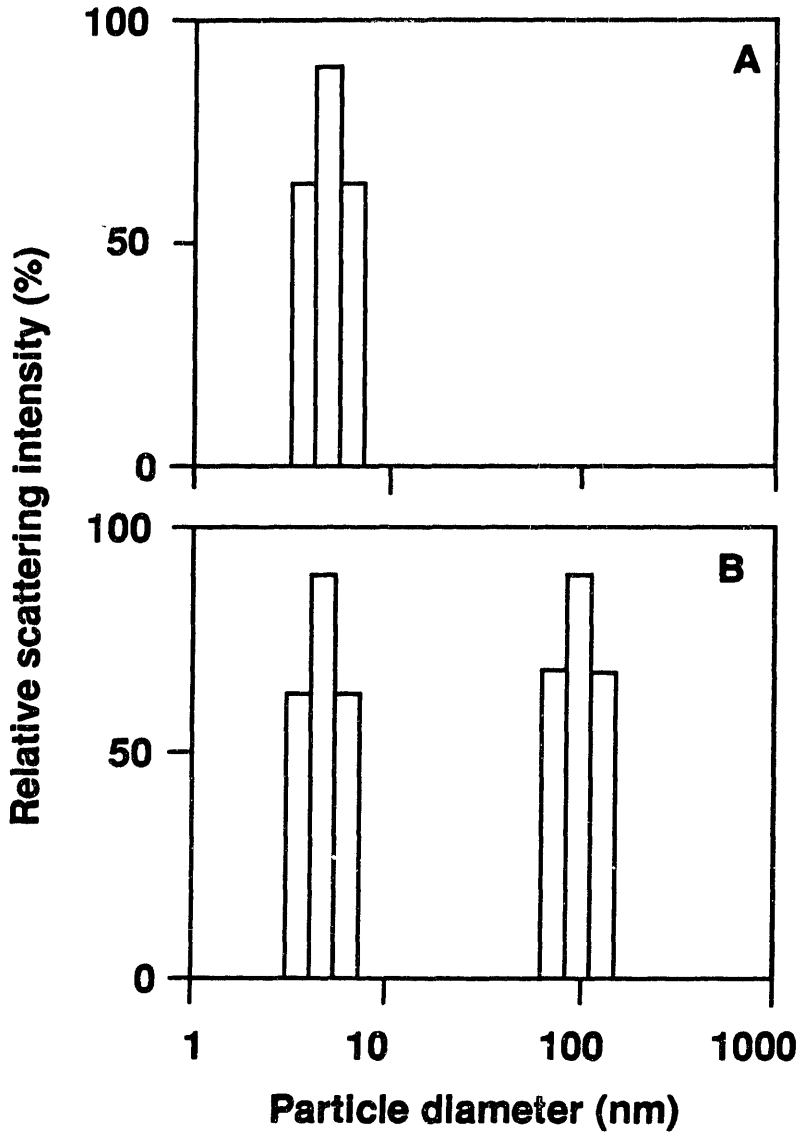

Figure 4.15: Results of QELS analysis on Na-insulin solutions. Samples (initial concentration 0.6 mg/ml) were analyzed (A) at time 0, and (B) after 2.4 hours of agitation at 80 rpm and 37°C in the presence of 10 Teflon spheres. Relative scattering intensities (% of total) are plotted versus the diameter of the particle responsible for the scattering. These size distribution histograms were generated using the program CONTIN.

solutions. The observed decreases in solution stability of Zn-free insulin were in good agreement with reports in the literature which suggested that aggregation was initiated by insulin monomers (Brange et al., 1987).

4.3.5 Temperature Dependence

The detrimental effects of elevated temperature on protein stability are well known (Ahern and Klibanov, 1988). It was of interest to determine how temperature changes influenced insulin aggregation rates. As can be seen in Figure 4.16, solution stability declined dramatically as temperature increased from 4 to 47°C. No aggregation was observed and no intermediate species were detected in insulin solutions agitated at 4°C even after one week. Insulin solutions aggregated at the other examined temperatures; the half-life values were 3, 6 and 72 hours at 47, 37 and 27°C, respectively. QELS analysis detected stable intermediate species (170±20 nm) in all destabilized solutions.

Temperature changes probably affected insulin's self-association equilibrium and the rates of many steps involved in the aggregation reaction, making it difficult to attribute the accelerated destabilization to any one set of interactions. Rather, it suggested that as the temperature was raised, the combination of reduced self-association, increased monomer denaturation and faster diffusion, led to more rapid aggregation.

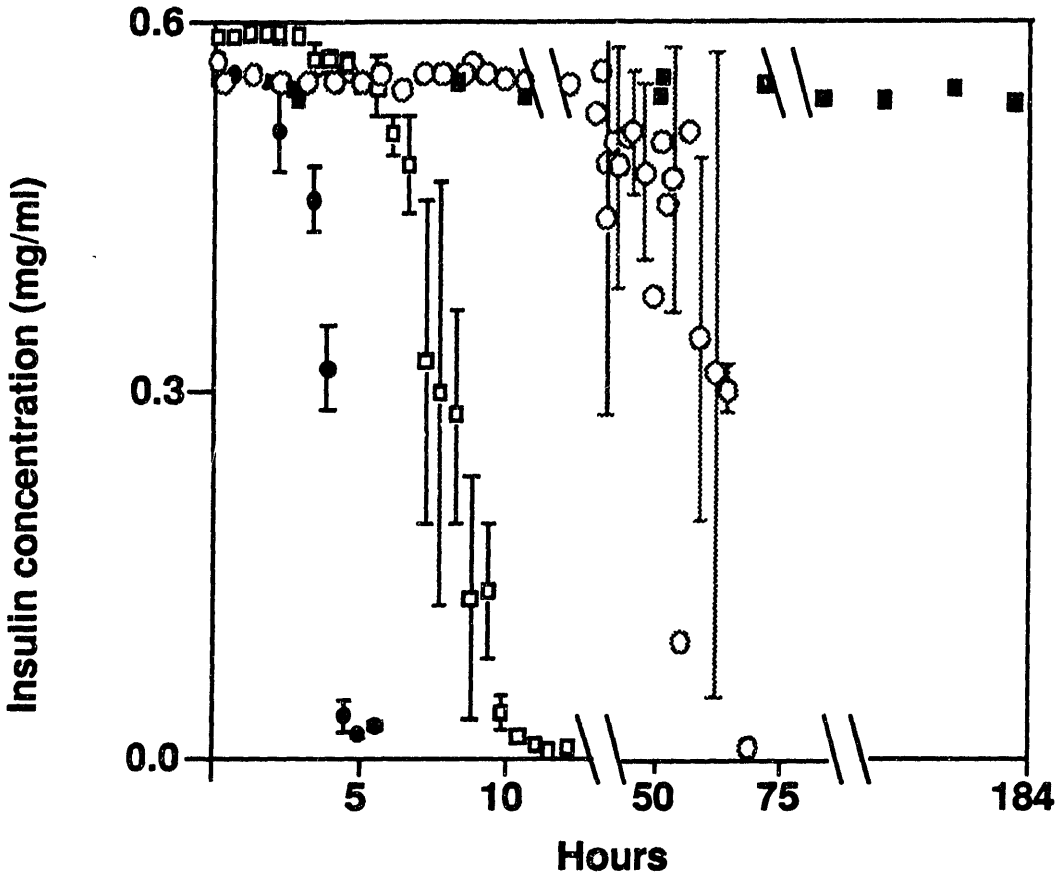

Figure 4.16: Temperature dependence of Zn-insulin aggregation rates (samples agitated at 160 rpm in the presence of 10 Teflon spheres): (●) 47°C, (❑) 37°C, (○) 27°C and (■) 4°C.

4.4 Other Hydrophobic Interfaces

Insulin aggregation in the presence of air-water and Teflon-water interfaces suggested that initial destabilization was mediated by insulin's interactions with hydrophobic surfaces. It was hypothesized that the monomer's reversible adsorption to Teflon resulted in conformational changes (exposure to solutions of internal

hydrophobic moieties) conducive to aggregation; the association of destabilized monomers removed exposed hydrophobic groups from the aqueous environment before the native conformation could be restored. To verify that destabilization was not specific to air or Teflon, but was a result of interactions between any hydrophobic surface and insulin, aggregation in the presence of polypropylene, siliconized glass, and unmodified glass beads was examined.

4.4.1 Polypropylene

Aggregation was indeed observed in solutions containing hydrophobic materials. Solutions agitated in the presence of polypropylene spheres aggregated completely in 3 days (Figure 4.17). The characteristic biphasic shape was observed, although the induction period increased to 16 hours (compared to 4 hours for samples with Teflon spheres), and the slope of the curved section became less steep. Stable intermediate species were also detected in the presence of polypropylene, and the results were in excellent qualitative agreement with insulin aggregation in the presence of Teflon (Figure 4.18). The relatively slow aggregation with polypropylene spheres was attributed to the fact that polypropylene's low density (ρ=0.9 g/ml) reduced the amount of agitation. While Teflon spheres (ρ= 2.16 g/ml) moved up and down the sample vials as the shaker platform rotated, polypropylene spheres floated at the top of the samples and hardly moved at all. Slower fluid motion in insulin samples with polypropylene reduced the extent of mixing, which meant that fewer interfacial interactions occurred in a given time period. As no destabilization was observed

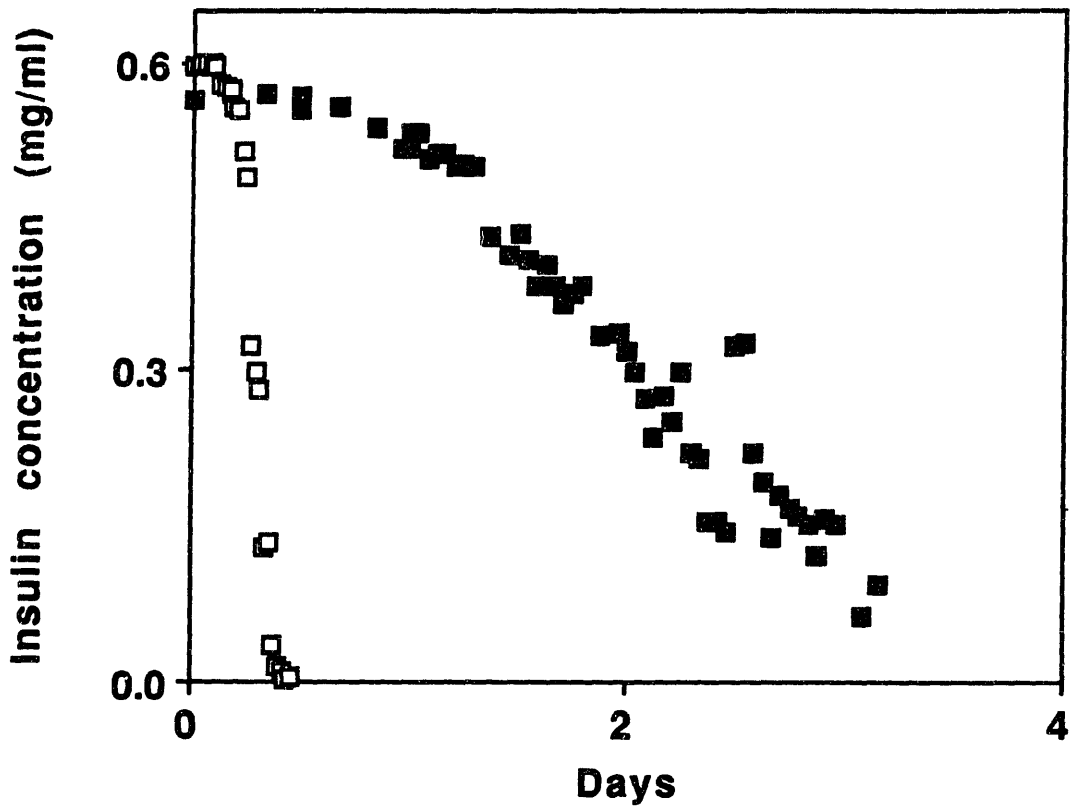

Figure 4.17: Effect of surface hydrophobicity on Zn-insulin aggregation. Samples were agitated at 160 rpm and 37°C in the presence of 10 Teflon (□) and 10 polypropylene (■) spheres.

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in stationary samples (see 4.1.2), it was expected that reduced agitation would lead to slower aggregation. Consequently, even though the surface hydrophobicity of polypropylene was similar to that of Teflon (critical surface tension of 34 and 22 dyne/cm for polypropylene and Teflon, respectively, (Shafrin, 1975)), reduced mass transfer and/or shear denaturation at the polypropylene surface led to slower destabilization and aggregation (see 5.3.5).

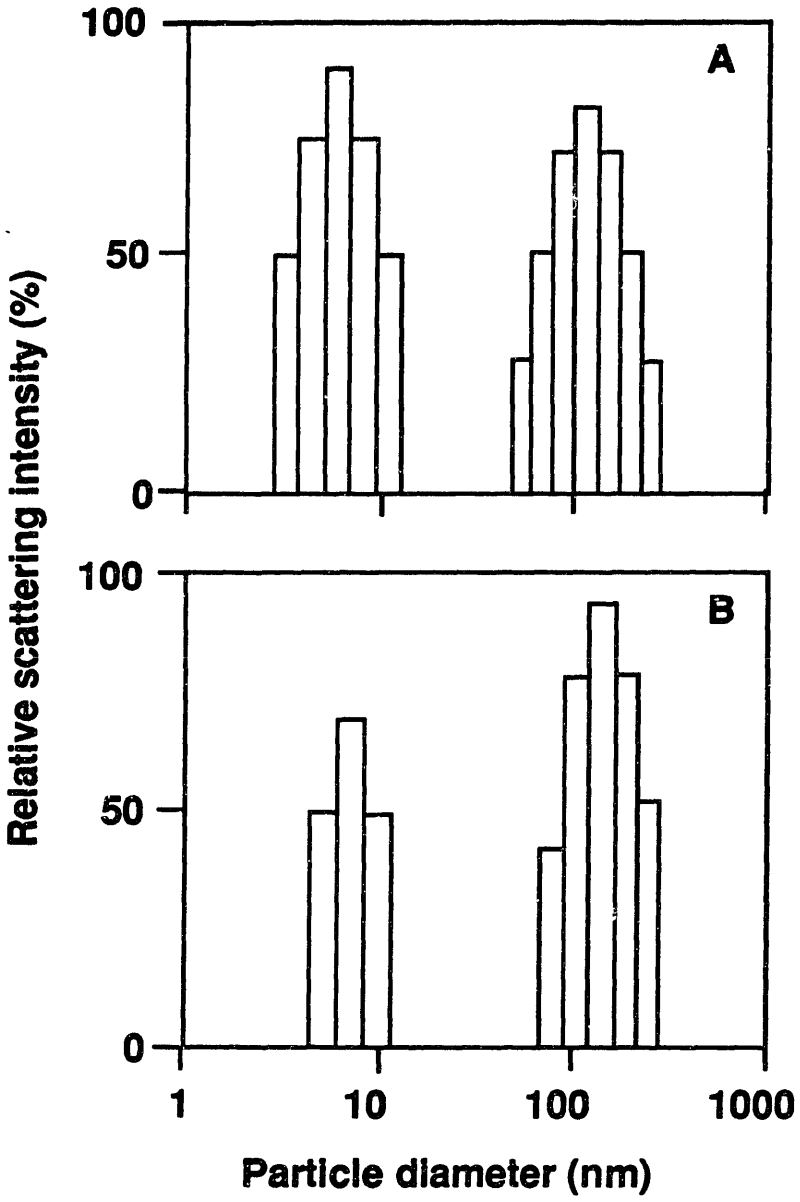

Figure 4.18: QELS analysis of Zn-insulin aggregation upon shaking. relative scattering intensities (% of total) are plotted versus the diameter of the particle responsible for the scattering. These size distribution histograms were generated using the program CONTIN. Results shown are for $t_{1/2}$ of Zn-insulin solutions agitated at 160 rpm and 37°C, in contact with (A) 10 Teflon spheres (5 hours) and (B) 10 polypropylene spheres (42 hours).

4.4.2 Siliconized Glass

In the presence of siliconized glass beads, the induction period was increased to 12 hours, and insulin solutions aggregated fully in less than 2 days (Figure 4.19); formation of stable intermediate species was also detected by QELS (Figure 4.20 A). No aggregation was observed for more than a week in solutions containing relatively hydrophilic, unmodified glass beads (Figure 4.19). As can be seen in Figure 4.20 B, a small amount of the intermediate species was also detected in samples with unmodified glass. This was probably due to the fact that the surface of glass beads was not perfectly hydrophilic, and that shear effects could have contributed to the formation of intermediate species.

In the case of siliconized glass beads, the mixing rates (and thus mass transfer and shear effects) were similar to those observed with unmodified glass beads, while the surface was much more hydrophobic. It was difficult to estimate the extent and reproducibility of surface modifications; the large standard deviation from the mean in the aggregation profile suggested that hydrophobic functional groups were not uniformly distributed on the bead surface. Although uncertainty about the actual aggregation profile prevented mathematical modeling of insulin's interactions with siliconized glass, the most important observation was that insulin aggregated fully within 2 days in the presence of this modified hydrophobic surface, while no aggregation was observed in the presence of the much more hydrophilic glass beads.

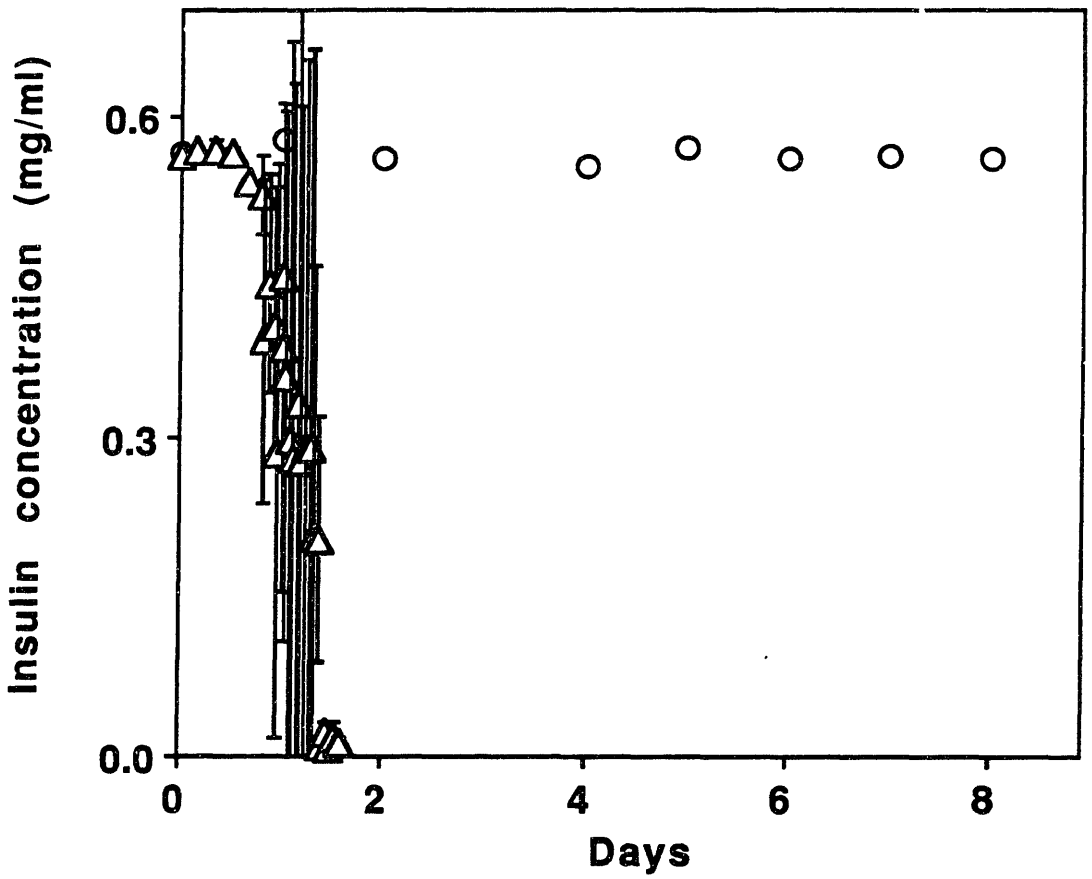

Figure 4.19: Effect of surface hydrophobicity on Zn-insulin aggregation. Samples were agitated at 160 rpm and 37°C in the presence of hydrophobic siliconized glass (Δ) and hydrophilic unmodified glass spheres (O). Results were averaged over 2 experiments, with error bars representing standard deviation from the mean.

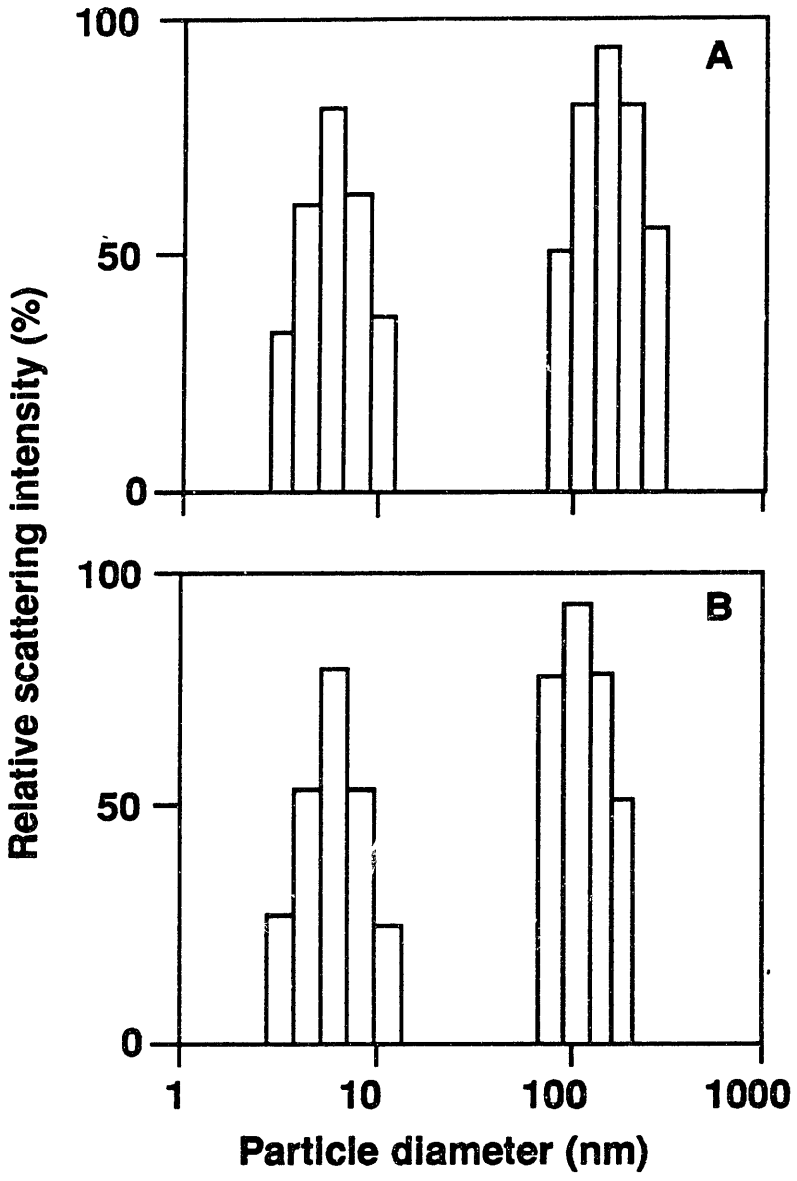

Figure 4.20: QELS analysis of Zn-insulin aggregation upon shaking. Relative scattering intensities (% of total) are plotted versus the diameter of the particle responsible for the scattering. These size distribution histograms were generated using the program CONTIN. Results shown are for $t_{1/2}$ of Zn-insulin solutions agitated at 160 rpm and 37°C, in contact with (A) 11 siliconized glass spheres (24 hours) and (B) 11 unmodified glass spheres (6 days).

4.5 Insulin Aggregation in Organic Solvents

Insulin aggregation was assumed to be analogous to other protein systems, in which destabilization was initiated by denaturation, or partial conformational changes in native structure:

> Since configurational changes occur in protein denaturation (with frequent liberation of functional groups previously buried in the interior of the molecule), it is to be expected that the interaction energy between the molecules of any protein varies when this protein undergoes denaturation. As a consequence of such a variation of interaction energy, the state of dispersion of the molecules in solution may change. For instance, association or polymerization, and more generally aggregation or even coagulation may occur. (Joly, 1965).

This generally accepted mechanism which identified hydrophobic interactions as the driving force for protein aggregation in aqueous solutions (Volkin and Klibanov, 1990), appeared to be consistent with the formation of non-covalent insulin aggregates. Furthermore, the experiments with solid-water and air-water interfaces suggested that insulin's interactions with hydrophobic surfaces produced partially denatured species (Figure 4.21), which minimized their surface energy by interacting with each other in order to shield exposed hydrophobic groups from unfavorable contacts with the aqueous environment.

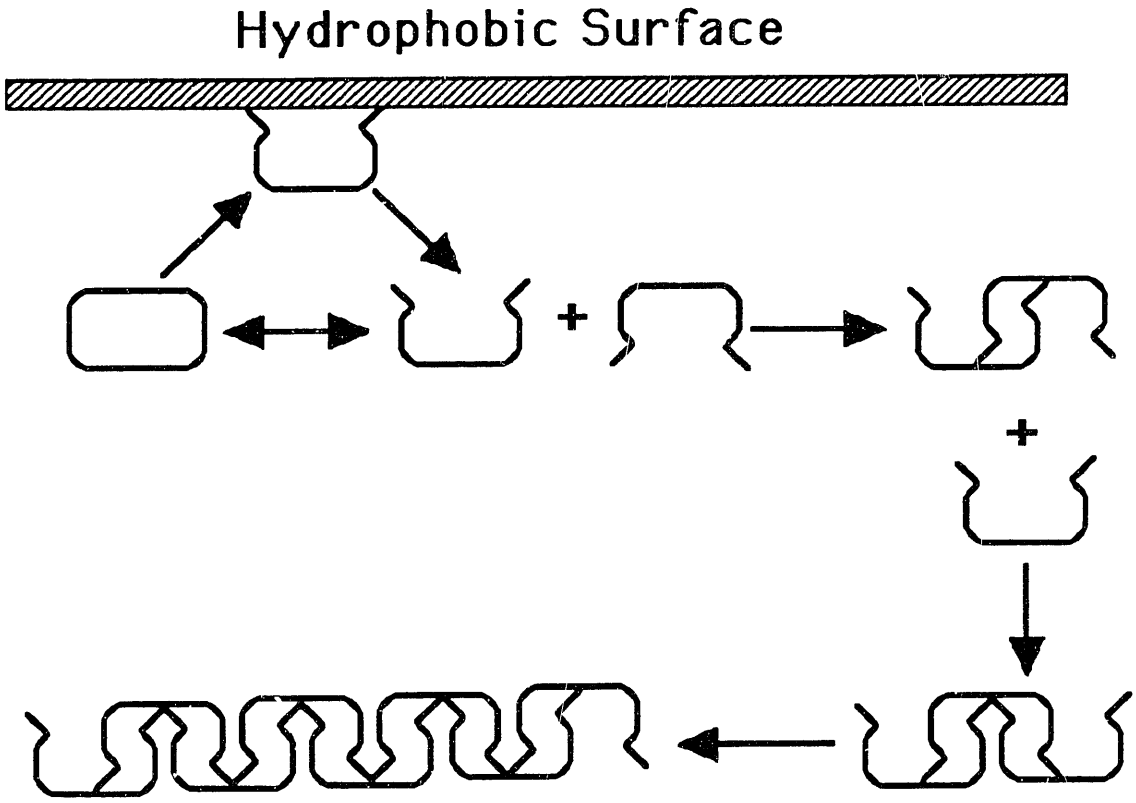

Figure 4.21: Schematic illustration of protein aggregation initiated by conformational changes at hydrophobic surfaces.

Species: native  , denatured  , aggregated  .

In an organic solvent, insulin's native conformation would be lost, but exposure of hydrophobic moieties would no longer be a thermodynamic liability. Consequently, conformational changes at an air-liquid interface should not lead to aggregation, since there would be no incentive to minimize contacts between exposed hydrophobic groups and the solvent. To verify this reasoning, samples of insulin in various protein-dissolving (Chang and Klibanov, 1992) organic solvents (formamide, DMSO and ethylene glycol) were subjected to the same physical perturbations which led to rapid aggregation in aqueous solutions. As seen in Figure 4.22, no aggregation was observed during the 7-day experiment. This result was revealing from a mechanistic standpoint, since it lent additional support to the proposed model of insulin destabilization and aggregation (see chapter 5). Together with the fact that insulin aggregates were held together by non-covalent interactions (which could be disrupted by overnight incubation in 8 M urea, as discussed in section 4.1.5), insulin's stability in organic solvents supported the idea that in aqueous environments, aggregation was initiated by exposure of, and interactions between, hydrophobic groups.

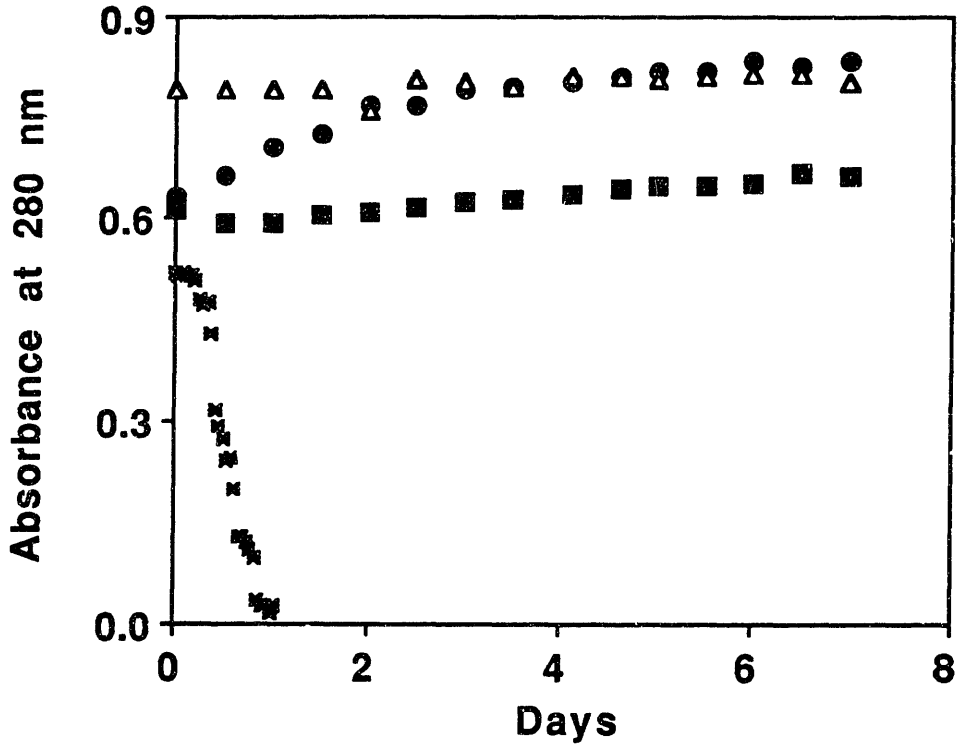

Figure 4.22: Aggregation of Zn-insulin solutions agitated at 100 rpm and 37°C in the presence of an air-liquid interface. Insulin in PBS (*), ethylene glycol (■), formamide (●), and DMSO (Δ). Insulin's absorbance at 280 nm was determined by subtracting the absorbance of the solvent (0.010, 0.042, 0.096 and 0.260 for PBS, ethylene glycol, formamide and DMSO, respectively) from the total absorbance. The increased absorbance in formamide was probably due to insulin's slow, time-dependent conformational transition in this organic solvent.

CHAPTER 5.

MODEL FORMULATION AND RESULTS OF COMPUTER SIMULATIONS

5.1 Formulation of Aggregation Scheme

The mechanism of insulin destabilization and aggregation in aqueous solutions remains obscure. It has been suggested that the process is mediated by adsorption at hydrophobic interfaces (air-water and water-pump materials) (Thurow and Geisen, 1984), and that the initial step is the formation of small intermediate aggregates which serve as precursors to large precipitates (Dathe et al., 1990). These elements alone, however, fail to describe the insulin aggregation behavior: a successful kinetic scheme has to predict both the bi-phasic nature of this reaction's time-course and the increased insulin stability at higher concentrations (which in the past has been attributed only to the concentration dependent self-association equilibrium).

To identify the insulin species most susceptible to destabilization, conformational stability of different association states was considered. In solutions, insulin monomers are in equilibrium with the more stable dimers and hexamers (Jeffrey, 1974). The insulin hexamer has a compact globular structure with a largely polar surface (Blundell et al., 1972); it has been shown that increasing hexamer concentration by addition of phenol (Derewenda

et al., 1989) or Zn^{2+} (Brange et al., 1986) enhanced solution stability. The hexamer's conformational stability makes it an unlikely candidate for denaturation at a hydrophobic surface. Partial unfolding of the insulin dimer at hydrophobic surfaces can be discounted for similar reasons: one of the monomer's two hydrophobic regions is involved in dimer formation (Blundell et al., 1972), and these intermolecular interactions greatly stabilize its tertiary structure. Thus, of the three insulin species present in solution, the monomer is the most likely to undergo conformational changes when it interacts with hydrophobic surfaces. This is consistent with the increased aggregation rates observed in Na-insulin solutions, in which higher monomer concentrations were present (see 4.3.4). In solutions where insulin's self-association had been eliminated altogether (Zn-free insulin in 0.1 M TRIS and 60% ethanol, pH 7), aggregation rates increased with increasing insulin concentrations (Brange et al., 1987), implying that it was the monomer which initiated the formation of insoluble agglomerates.

Insulin denaturation at hydrophobic interfaces (solid-water and air-water) has been reported (Feingold et al., 1984; Hansen et al., 1987), and the extent of insulin adsorption to glass and Teflon has been determined (Sefton and Antonacci, 1984), making possible a semi-quantitative characterization of insulin's interactions with these surfaces. Due to the difficulties associated with measuring and controlling the air-water interface, mathematical modeling was restricted to insulin aggregation in the presence of solid surfaces. The results of kinetic studies done with Teflon spheres were particularly well suited for computer simulations, as it was possible

to estimate the sizes of hydrophobic surface areas, extent of insulin adsorption, and changes in mass transfer resulting from variations in agitation rates. A model explaining insulin destabilization at solid hydrophobic surfaces could then be used to elucidate insulin aggregation in the presence of air-water interfaces, as the two phenomena were qualitatively similar.

Based on the overall kinetics of insulin aggregation, the formation of stable intermediate species, and the importance of interfacial interactions, a mechanism of insulin destabilization and aggregation in the presence of solid hydrophobic surfaces was formulated. The proposed reaction pathways are depicted schematically in Figure 5.1. As no aggregation was observed in the absence of hydrophobic interfaces (and in the presence of hydrophilic glass beads), insulin's interactions with glass were assumed to have no detrimental effects.

The unfavorable conformational changes leading to aggregation were assumed to be mediated by insulin's interactions with hydrophobic surfaces. It was hypothesized that dimers and hexamers reversibly adsorbed to Teflon without undergoing significant conformational changes. However, the less stable monomeric species became partially unfolded upon adsorption to Teflon. The unfolded species either snapped back to the native conformation (the most likely event), or combined with other unfolded species, initiating nucleation (Kam et al, 1978) -- the formation of intermediate aggregates of approximately 170 nm in diameter. The smaller intermediates (U_2, U_3, etc.) were short-lived transients: they continuously combined and fell apart, and were able

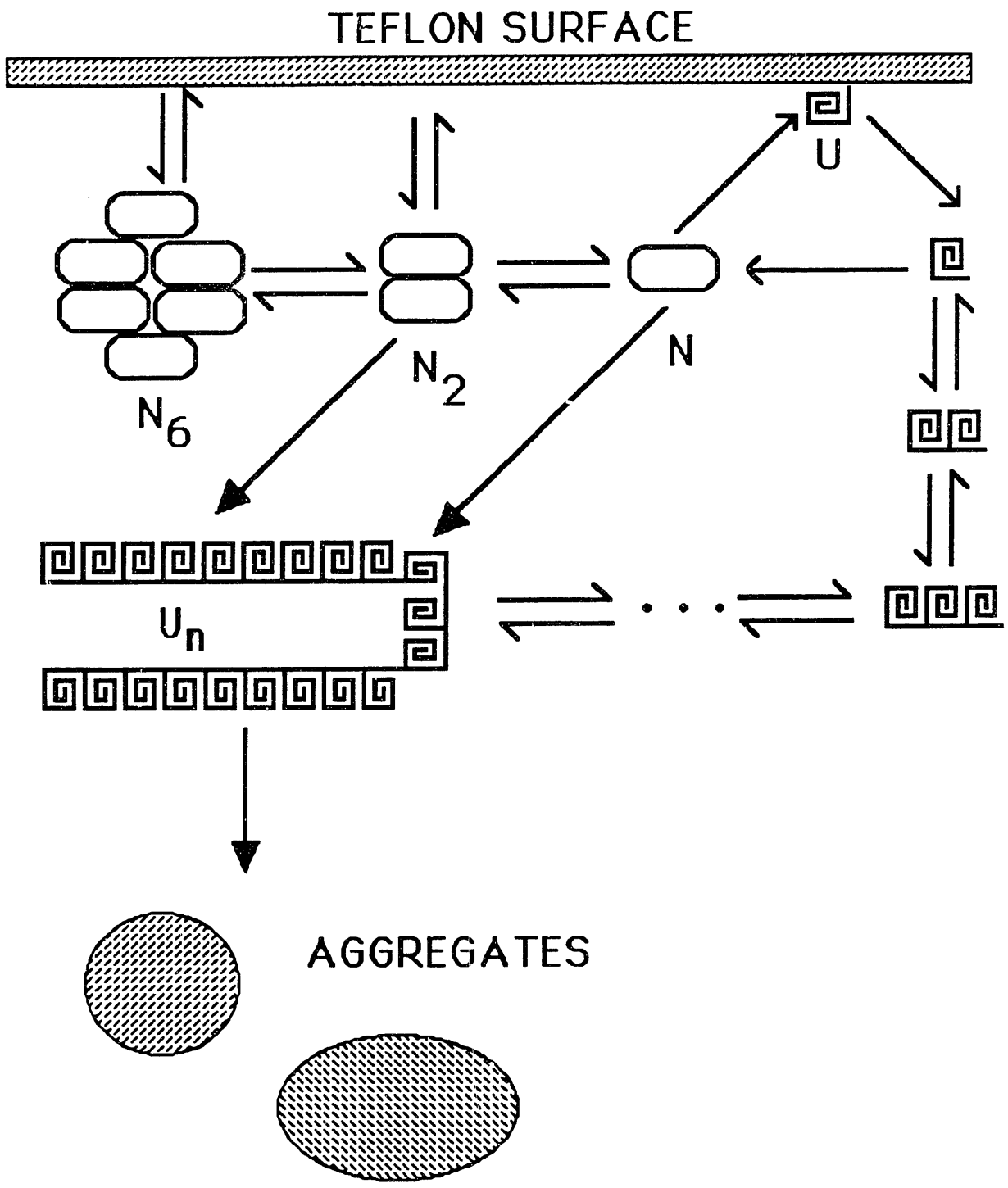

Figure 5.1: Schematic representation of the proposed mechanism of insulin aggregation in the presence of a Teflon-water interface. Species: native ⬭, unfolded 🌀, aggregates ●.

to interact only with other unfolded species. Once a critical size was reached, the intermediates had enough surface area for stability, and could start trapping the native molecules. The slow formation of the 170±20 nm stable intermediates explained the lag time: no significant aggregation was observed until a population of nucleating species was established (Sluzky et al., 1991).

The increased stability at higher concentrations (when a larger proportion of insulin was oligomeric) was attributed to the occupation of the Teflon surface by dimers and hexamers, which reduced the surface available to unfold insulin monomers. As insulin concentration increased, so did the monomer concentration. In the absence of dimer and hexamer adsorption to Teflon, higher insulin concentrations would lead to more rapid aggregation, as the rate of denaturation (which initiated the process of destablilization) depended on the concentration of both monomers and interfacial sites (Table 5.1). In order to reduce the driving force for aggregation at higher concentrations, the number of interfacial sites causing monomer denaturation had to be reduced. One way to accomplish this, given a fixed surface area, was to allow the more stable species to occupy interfacial sites without undergoing conformational changes.

Table 5.1: Simulation of monomer denaturation rate (proportional to free monomer [N] and interfacial site [I]) with and without dimer [N_2] and hexamer [N_6] adsorption to Teflon.

Zn-insulin, mg/ml	Monomer [N], molecules per ml ($\times 10^{15}$)	Teflon surface [I] after adsorption,* sites per ml ($\times 10^{12}$)	[I]x[N] without adsorption, sites•molecules per ml^2 ($\times 10^{31}$)	[I]x[N] with adsorption†, sites•molecules per ml^2 ($\times 10^{27}$)
0.6	2.47	3.1	3.2	7.7
0.2	2.03	4.7	2.6	9.4

* Concentration of interfacial sites before protein adsorption [I_0] was 6.3×10^{12} sites per ml (based on 5 Teflon spheres per sample, i.e., initial surface area [A_0] = 1.3×10^{16} Å^2/ml).

† Interfacial site concentration [I] after adsorption was computed as:
([A_0](Å^2/ml) - (750 Å^2/dim)x(N_2ads) - (2250 Å^2/hex)x(N_6ads))/(2000 Å^2/site)),
where $N_2ads = K_{2ads} \times [N_2] \times [I_0]$ and $N_6ads = 0.4 \times K_{2ads} \times [N_6] \times [I_0]$.

5.2 Mathematical Formulation of Reaction Kinetics

The interactions in the proposed aggregation model were as follows:

$$6N \overset{K_{dim}}{<=======>} 3N_2 \overset{K_{hex}}{<=======>} N_6 \qquad \{1\}$$

$$N_2 + I \overset{K_{2ads}}{<=======>} N_2I \qquad \{2\}$$

$$N_6 + I \overset{K_{6ads}}{<=======>} N_6I \qquad \{3\}$$

$$N + I \overset{k_{unf}}{======>} U + I \qquad \{4a\}$$

$$U \overset{k_{ref}}{======>} N \qquad \{4b\}$$

$$U + U \underset{k_{U\text{-}diss}}{\overset{k_{U\text{-}form}}{<=======>}} U_2 \qquad \{5\}$$

$$U_2 + U <======> U_3$$
$$\text{"} \quad \text{"} \quad \text{"} \quad \text{"}$$
$$U_{n-1} + U <=======> U_n \text{ etc.}$$

$$U_n + (N \text{ or } N_2) \overset{k_{agg}}{======>} \text{Aggregates} \qquad \{6\}$$

Notation:

N = native insulin monomer (concentration, M)

N_2 = native insulin dimer (concentration, M)

N_6 = native insulin hexamer (concentration, M)

U = partially denatured insulin monomer (concentration, M)

U_2 = transient intermediate species (concentration, M)

U_n = stable intermediate species (~170±20 nm) (concentration, M)

I = interfacial sites (concentration, sites/ml)

N_2I = dimer adsorbed on hydrophobic surface (concentration, M)

N_6I = hexamer adsorbed on hydrophobic surface (concentration, M)

Step 1 represented insulin's self-association equilibrium, while steps 2 and 3 were used to describe the adsorption of dimers and hexamers to Teflon. Step 4 in the reaction scheme represented monomer denaturation induced by contacts with the hydrophobic surface; this was assumed to be a pseudo-equilibrium process (since intramolecular changed occurred on a much shorter time-scale than bimolecular interactions (Creighton, 1984)), in which $K_{denat}=k_{unf}/k_{ref}$. Step 5 (the formation of stable intermediate species) was modelled as a stepwise polymerization with equal probability of subsequent addition. To simplify the computations without changing the qualitative features of the kinetic scheme, the number of steps needed to reach a critical size was limited to 9 (with n=10). As a result, the numbers used in the simulations were pseudo-rate constants.

In step 6, the stable intermediates reacted with native insulin monomers and dimers, to form large, insoluble agglomerates. The hexamer did not participate in step 6, since interactions between stable intermediates and native species were assumed to be hydrophobic. While insulin monomers and dimers had large hydrophobic areas which could easily interact with the intermediates, the hexamer's hydrophilic surface (Blundell et al. 1972) made such associations much less likely (J. Brange, personal communication).

Only qualitative predictions could be expected from this model since the actual kinetic rate constants (for formation of aggregates and intermediates) could not be determined independently; yet, the computer simulations proved to be very helpful in elucidating the nature of aggregation and stabilization mechanisms. Most of the parameters used in the model were estimated from the literature; the results are summarized in Table 5.2, and details about how these parameters were determined are provided in sections 5.2.1-5.2.4.

Table 5.2: Thermodynamic and kinetic parameters used to model insulin aggregation in the presence of solid hydrophobic surfaces.

Parameter	Value	Reference
K_{dim}	6.9×10^4 M^{-1}*	(Mark and Jeffrey, 1990)
K_{hex} (Zn-free)	3.2×10^8 M^{-2}*	(Mark and Jeffrey, 1990)
K_{hex} (Zn-insulin)	1.3×10^{13} M^{-2}*	(Milthorpe et al., 1977)
K_{2ads}	$(1.2\text{-}2.5) \times 10^{-16}$ ml/site†	(Sefton et al, 1984)
K_{6ads}	$(4.8\text{-}9.9) \times 10^{-17}$ ml/site†	(Sefton et al, 1984)
K_{denat} (Teflon, 80 rpm)	2.0×10^{-16} ml/site§	(Brems et al., 1990)
K_{denat} (polypropylene)	5.5×10^{-17} ml/site	see text
$k_{U\text{-}form}$	6.0×10^6 M^{-1} min^{-1}	see text
$k_{U\text{-}diss}$	3.0×10^5 min^{-1}	see text
k_{agg}	3.0×10^5 M^{-1} min^{-1}	see text

* Extrapolated to our experimental conditions (37°C, I = 0.14)

† Calculated assuming $K_{6ads}=0.4 \times K_{2ads}$. Adsorption coefficients increased as insulin concentration decreased from 0.10 to 0.017 mM.

§ K_{denat} was estimated to be one order of magnitude greater than the value reported for bulk solutions; it was then scaled by the average number of hydrophobic interfacial sites. $K_{denat} = 4 \times 10^{-16}$ ml/site at 160 rpm

5.2.1 Parameters for Self-Association Equilibrium

Insulin's oligomerization has been investigated by numerous researchers (see 2.1.2), who have determined the equilibrium constants for dimer and hexamer formation at various temperatures, pHs, and ionic strengths. The majority of these studies focused on Zn-free insulin solutions, and the equilibrium constants were calculated based on a variety of self-association patterns (see Figure 2.2). The dimerization and hexamerization parameters in our model were extrapolated from the results of (Mark and Jeffrey, 1990), (Jeffrey et al., 1976) and (Milthorpe et al., 1977), as Table 5.3 illustrates. The values reported for the "Indefinite Isodesmic of Hexamers" model for Zn-free insulin self-association at pH 7, I 0.05 and 0.1, and 25°C and 37°C, (Mark and Jeffrey, 1990) (model 4 in Figure 2.2) were compared with the numbers reported by (Jeffrey et al., 1976) for Zn-free insulin at pH 7.0, I 0.2, and 25°C. The equilibrium constant for hexamer formation in the presence of Zn was based on values determined by (Milthorpe et al., 1977), while dimer formation was assumed to remain unchanged in Zn- and Zn-free insulin solutions.

5.2.2 Adsorption Coefficients

The coefficients for dimer and hexamer adsorption to Teflon were calculated from the adsorption isotherms at 37°C reported by (Sefton and Antonacci, 1984). A total Teflon surface area of 1.19 cm^2 was used in Sefton's studies, and results were given in terms of insulin units (U) adsorbed per 1 cm^2 of Teflon at specified bulk concentrations of Zn-free insulin in a phosphate buffer. The dimer

Table 5.3: Determination of insulin self-association constants (based on literature values).

Parameter	Temperature (°C)	Value at I=0.05*	Value at I=0.10*	Value at I=0.20*	Extrapolated to I=0.14
K_{dim}	25	4.1×10^4 M^{-1}	8.53×10^4 M^{-1}	1.1×10^5 M^{-1}	9.8×10^4 M^{-1}
K_{dim}	37		5.68×10^4 M^{-1}		6.9×10^4 M^{-1}
$K_{hex\ (Zn\text{-}free)}$	25	1.95×10^9 M^{-2}	5.17×10^8 M^{-2}	2.9×10^8 M^{-2}	4.0×10^8 M^{-2}
$K_{hex\ (Zn\text{-}free)}$	37		4.26×10^8 M^{-2}		3.2×10^8 M^{-2}
$K_{hex\ (Zn)}$	25			1.17×10^{13} M^{-2}	
$K_{hex\ (Zn)}$	37				1.3×10^{13} M^{-2}†

* See text for references

† At 25°C and I=0.20, $K_{hex\ (Zn)}=(4 \times 10^4) \times K_{hex\ (Zn\text{-}free)}$; so at 37°C, the hexamerization constant was calculated as $K_{hex\ (Zn)}=(4 \times 10^4) \times K_{hex\ (Zn\text{-}free,\ 37°C,\ I=0.14)}$

was assumed to be responsible for adsorption, as it was the predominant species in Zn-free solutions. In this work, it was assumed that the hexamer also adsorbed to Teflon, although not as readily as the more hydrophobic dimer. Hexamer's interactions with Teflon probably resulted in dissociation into dimers, which then adsorbed to the surface. To reflect the lower affinity of the hexamer for the hydrophobic surface, its adsorption coefficient was subsequently assumed to be smaller than that of the dimer.

The surface area occupied by adsorbed dimers was reported to be 750 $Å^2$; the hexamer's surface area was similarly calculated to be 2250 $Å^2$. Thus, in Sefton's experiments there were $1.59x10^{13}$ dimer ($[I_0]_{dim}$) or $5.29x10^{12}$ hexamer ($[I_0]_{hex}$) sites per ml (based on available Teflon surface area of $1.19x10^{16}$ $Å^2$/ml). The average specific activity of insulin (25 U/mg) was used to calculate the number of units per individual dimer and hexamer molecule as follows:

$$\frac{12{,}000 \text{ mg dim}}{1 \text{ mMol}} \times \frac{1 \text{ mMol}}{6 \times 10^{20} \text{ dim}} \times \frac{25 \text{ U}}{1 \text{ mg}} = 5x10^{-16}\text{U/dim} \qquad \{5.1\}$$

$$\frac{36{,}000 \text{ mg hex}}{1 \text{ mMol}} \times \frac{1 \text{ mMol}}{6 \times 10^{20} \text{ hex}} \times \frac{25 \text{ U}}{1 \text{ mg}} = 1.5x10^{-15}\text{U/hex} \qquad \{5.2\}$$

The concentration of insulin adsorbed on Teflon ($[N]_{Tef}$, U/ml) could then be expressed in terms of bulk concentrations (molecules/ml) of dimers $[N_2]$ and hexamers $[N_6]$:

$$[N]_{Tef}=K_{2ads}x[N_2]x[I_0]_{dim}x[U/dim]+K_{6ads}x[N_6]x[I_0]_{hex}x[U/hex] \quad \{5.3\}$$

Equation 5.3 could be simplified by calculating

$$[I_0]_{dim}x[U/dim]=[I_0]_{hex}x[U/hex]=7.9x10^{-3}\ (\text{U site/ml molecule}) \quad \{5.4\}$$

and expressing K_{6ads} as a function of K_{2ads}:

$$K_{6ads}=Y \times K_{2ads} \quad \{5.5\}$$

The adsorption coefficient for the insulin dimer could then be calculated from

$$K_{2ads} = \frac{[N]_{Tef}\,(U/ml)}{7.9x10^{-3}\left(\frac{U\ site}{ml\ molec}\right)} x \frac{1}{([N_2]+Yx[N_6])(molec/ml)} \quad \{5.6\}$$

The results presented in Table 5.4 were calculated using equation 5.6; hexamer adsorption was varied by setting Y to 0, 1 and 0.4 to represent the cases of no hexamer adsorption, equal dimer and hexamer affinity for the surface, and higher dimer adsorption,

Table 5.4: Concentration dependence of dimer adsorption coefficients in Zn-free insulin solutions.

Bulk Concentration (mg/ml)	Surface Concentration (U/ml) $x10^{-3}$	K_{2ads} (ml/site) x 10^{16}		
		Y=0*	Y=1†	Y=0.4§
0.6	16	1.3	1.1	1.2
0.4	13	1.5	1.3	1.4
0.2	8.7	1.9	1.9	1.9
0.1	5.0	2.5	2.5	2.5

* K_{2ads} calculated assuming no hexamer adsorption.

† K_{2ads} calculated assuming $K_{2ads} = K_{6ads}$.

§ K_{2ads} calculated assuming $K_{6ads} = 0.4xK_{2ads}$.

respectively. It became evident that K_{2ads} remained virtually unchanged in all three cases, indicating that hexamer adsorption had little or no effect on the stability of Zn-free insulin solutions. This was not surprising, as in the absence of Zn^{2+}, hexamers made up a very small fraction of molecules in solution. The situation would change dramatically in Zn-insulin solutions, where hexameric species predominated; the extent of hexamer adsorption significantly affected the predictions of solution stability, and the observed concentration dependence could not be modelled successfully in the absence of hexamer occupation of destabilizing interfacial sites (see

Appendix B). Consequently, adsorption constants calculated assuming $K_{6ads} = 0.4xK_{2ads}$ were used in computer simulations of insulin aggregation in the presence of Teflon.

5.2.3 Monomer Denaturation at Hydrophobic Surfaces

To describe conformational changes resulting from insulin monomer's interactions with hydrophobic surfaces, the equilibrium constant for unfolding and refolding the absence of destabilizing interactions was estimated from the literature. Insulin's Gibbs free energy of unfolding at room temperature (23°C) was reported to be 4.5±0.5 kcal/mol (Brems et al., 1990). The equilibrium constant of unfolding (K_{unf}) could be calculated from the thermodynamic relationship

$$K_{unf} = \exp(-\Delta G_{unf}/RT) \qquad \text{(Modell and Reid, 1983)} \qquad \{5.7\}$$

Using equation 5.7, the equilibrium constant for unfolding was determined to be $2x10^{-4}$.

To include the destabilizing effects of insulin's interactions with hydrophobic surfaces, $K_{unf'}$ (the equilibrium constant for unfolding in the presence of Teflon) was estimated to be one order of magnitude greater than K_{unf} in bulk solutions. K_{denat} was then calculated by scaling $K_{unf'}$ ($2x10^{-3}$) by the average number of hydrophobic interfacial sites (order 10^{13} sites/ml) present in samples used in kinetic studies with solid hydrophobic surfaces. This order of magnitude estimate for K_{denat} ($2x10^{-16}$ ml/site) was used to

describe monomer unfolding resulting from interactions with Teflon spheres at 37°C and 80 rpm.

K_{denat} contained information about both mass transfer to the hydrophobic surface and the rate of unfolding at that solid-water interface. In subsequent computer simulations, the value of K_{denat} was varied to reflect changes in agitation rates, shear-denaturation and surface hydrophobicity.

5.2.4 Interfacial Sites Available for Monomer Denaturation

The concentration of destabilizing interfacial sites was determined in the following way. Total available surface area A_0 was calculated as (number of spheres)x(sphere's surface area), e.g. $5x(\pi xD^2)$ for five spheres of diameter D. A_0 was then divided by total sample volume, 5.1 and 4.4 ml for samples containing 5 and 10 Teflon spheres (0.635 cm OD), respectively. The concentration of interfacial sites was determined by dividing initial surface area ($Å^2$/ml) by the size of an individual site, which was assumed to be 2000 $Å^2$ (based on hexamer's cross-sectional area (Blundell et al, 1972), since the hexamer was the predominant insulin species in solution at these concentrations).

Thus, in samples containing 5 and 10 Teflon spheres, there were $6.3x10^{12}$ and $1.4x10^{13}$ sites/ml, respectively, at the beginning of each experiment. Adsorbed dimers and hexamers were assumed to occupy 750 and 2250 $Å^2$ (based on dimer's cross-sectional area (Sefton and Antonacci, 1984)) of the Teflon surface, respectively, so the concentration of destabilizing interfacial sites was calculated by

dividing the unoccupied surface area ($Å^2$/ml) by 2000 $Å^2$/site. As the concentrations of dimers and hexamers decreased, more interfacial sites were freed up, leading to a greater number of destabilizing interactions between insulin monomers and the hydrophobic surface.

5.2.5 Rate Constants for the Formation of Intermediates and Aggregated Species

Rate constants for steps 5 and 6 (Table 5.2) were empirically determined based on one data set: Zn-insulin with 10 Teflon spheres at 80 RPM and 37°C, assuming $K_{6ads}=0.4xK_{2ads}$). These parameters had to be internally consistent, with $k_{U\text{-}form}$ being greater than $k_{U\text{-}diss}$ (to allow for the formation of stable intermediate species) and $k_{U\text{-}form}$ being greater than k_{agg} (to prevent transient intermediates from reacting with native species). Once determined, these pseudo-rate constants remained unchanged in all subsequent simulations, and only those parameters which could be measured or estimated independently (such as insulin concentrations, hydrophobic surface areas, Zn^{2+} content, and agitation rates) were varied. These external perturbations should have had no effect on the rates of intermediate formation and aggregation (since those intermolecular interactions were diffusion controlled), and hence the constants determined for one set of conditions should have been valid throughout. The good agreement between experimental results and computer simulations indicated that this assumption was reasonable and that our model was an adequate representation of insulin aggregation in aqueous solutions.

5.3 Results of Computer Simulations

5.3.1 Comparison of Na- and Zn-insulin Aggregation

It came as no surprize that results of computer simulations for Zn-insulin aggregation (10 Teflon spheres, 37°C and 80 rpm) were in good agreement with experimental observations (Figure 5.2). The kinetic parameters were determined based on this set of conditions, with rates of intermediate and aggregate formation chosen to simulate the relative durations of the induction and aggregation periods of insulin destabilization.

The same set of parameters (with the exception of K_{hex}) was used to simulate Na-insulin aggregation (Figure 5.2). The model predicted that in the absence of Zn^{2+}, higher monomer concentrations led to faster destabilization and aggregation. As the equilibrium constant for hexamer formation decreased, the induction period became shorter and the slope of the curve became steeper. Computer simulations of Na-insulin aggregation were in good qualitative and quantitative agreement with the experimentally determined kinetic profile.

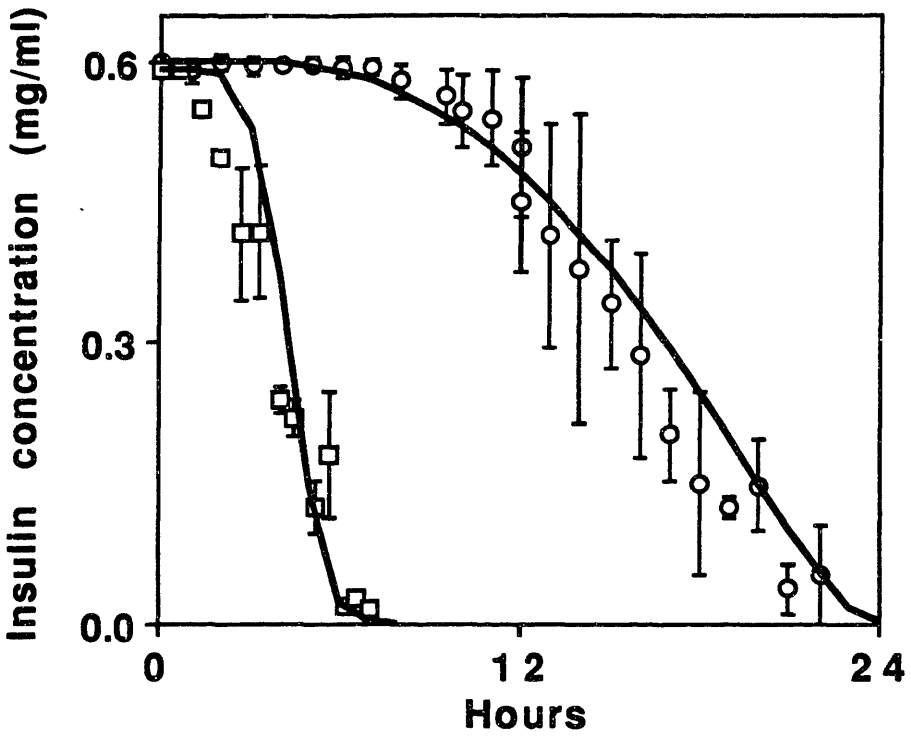

Figure 5.2: Mathematical modelling of aggregation in Na-(□) and Zn-(○) insulin solutions agitated at 37°C and 80 rpm, in the presence of 10 Teflon spheres. Results of computer simulations are depicted by solid lines.

5.3.2 Concentration Dependence in the Presence of Teflon

The kinetic scheme had to explain the observed concentration dependence of insulin aggregation rates. As can be seen from Figure 5.3, the model predicted both the characteristic curve shape (an initial lag time followed by a sloping sections) and increased solution stability at higher insulin concentrations. During the computer

simulations, all kinetic and thermodynamic parameters were kept constant; the only parameter that did change was the number of insulin molecules present in the beginning of each experiment. The close agreement between theoretical predictions and experimental results suggested that the model was a reasonable kinetic representation of Zn-insulin aggregation upon shaking in the presence of Teflon.

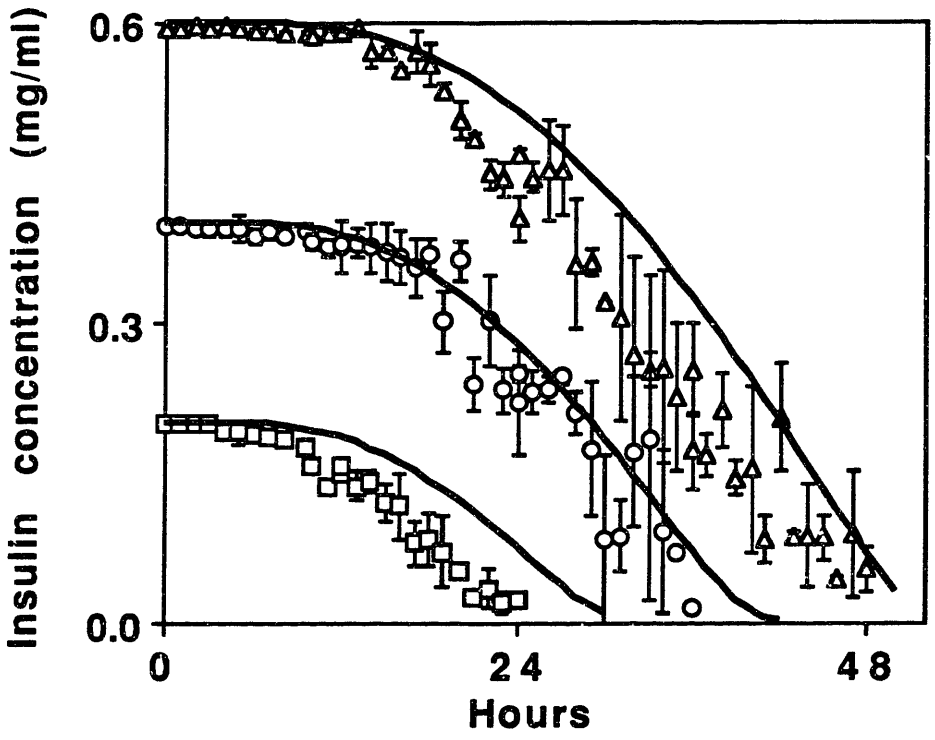

Figure 5.3: Mathematical modelling of concentration dependence of Zn-insulin aggregation. Samples containing 0.6 mg/ml (Δ), 0.4 mg/ml (○) and 0.2 mg/ml (□) were agitated at 37°C and 80 rpm in the presence of 5 Teflon spheres. Results of computer simulations are depicted by solid lines.

5.3.3 Role of Teflon Surface Area

The proposed kinetic scheme was used to predict changes in insulin aggregation rates when Teflon surface area was reduced from 10 to 5 spheres per sample (Figure 5.4). All kinetic and thermodynamic parameters were kept constant during these simulations; the only parameter that changed was the concentration of initially available interfacial sites.

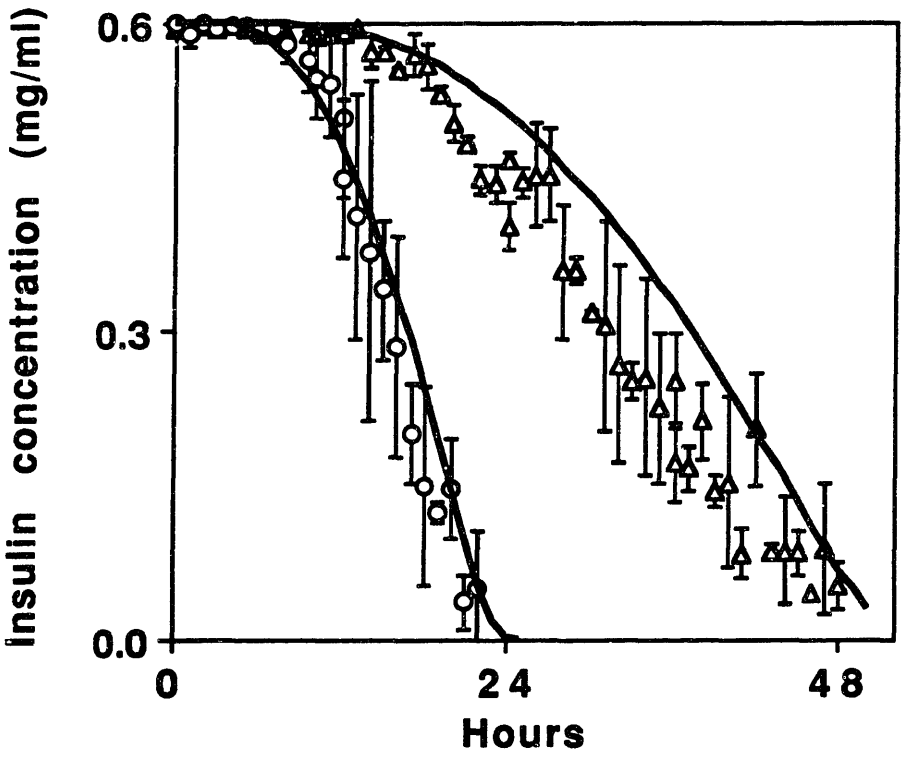

Figure 5.4: Mathematical modelling of Zn-insulin aggregation in the presence of 5 (Δ) and 10 (O) Teflon spheres, at 37°C and 80 rpm. Results of computer simulations are depicted by solid lines.

The model predicted the increased induction period and decreased slope steepness which were observed in insulin samples containing 5 Teflon spheres. Results of computer simulations were in good agreement with the experimentally determined concentration profiles.

5.3.4 Role of Agitation Rate

The effects of increased agitation on Zn-insulin aggregation rates were modelled by changing K_{denat} in proportion to the increases in the mass transfer coefficient. To evaluate the dependence of mass transfer coefficient on rates of agitation, several models were considered. At small mass-transfer rates and constant surface temperature and composition, forced convection around a sphere of diameter D could be described as

$$\frac{k_m D}{c_f \mathcal{D}_{ABf}} = 2.0 + 0.60 \left(\frac{D v_\infty \rho_f}{\mu_f}\right)^{\frac{1}{2}} \left(\frac{\mu}{\rho \mathcal{D}_{AB}}\right)_f^{\frac{1}{3}} \quad \text{(Bird et al., 1960)} \quad (5.8)$$

where

k_m = mass transfer coefficient

c_f = bulk fluid concentration

v_∞ = approach velocity

$\mathcal{D}_{ABf}$ = diffusion of components A and B relative to bulk flow

ρ_f = fluid density

μ_f = fluid viscosity

$\left(\frac{\mu}{\rho \mathcal{D}_{AB}}\right)_f^{\frac{1}{3}}$ = Schmidt number for the fluid

Equation 5.8 indicated that k_m was proportional to $\sqrt{(v_\infty)}$. A similar dependence of the mass transfer coefficient on the square root of fluid velocity was found when k_m was calculated using the Sherwood number (Sh) for mass transfer in a fluidized packed bed:

$$Sh = 1.8\ Re^{0.5} Sc^{0.33} \propto k_m \quad \text{(Froment and Bischoff, 1979)} \quad \{5.9\}$$

where Re and Sc were the Reynolds and Schmidt numbers for the fluid.

Equations 5.8 and 5.9 suggested that when the agitation rate was increased from 80 to 160 rpm, K_{denat} should have increased by a factor of $\sqrt{2}$. It should be noted, however, that the aforementioned analyses described mass transfer under conditions analogous to, but not exactly like the ones used in the kinetic studies; they also neglected the contribution of shear denaturation to monomer destabilization.

Since an exact analytical relationship between mass transfer and agitation rates was not available in the literature, K_{denat} was increased by a factor of 2 when the agitation rate was doubled from 80 to 160 rpm. This change was proportional to the increase in the mass transfer coefficient calculated using equations 5.8 and 5.9; it

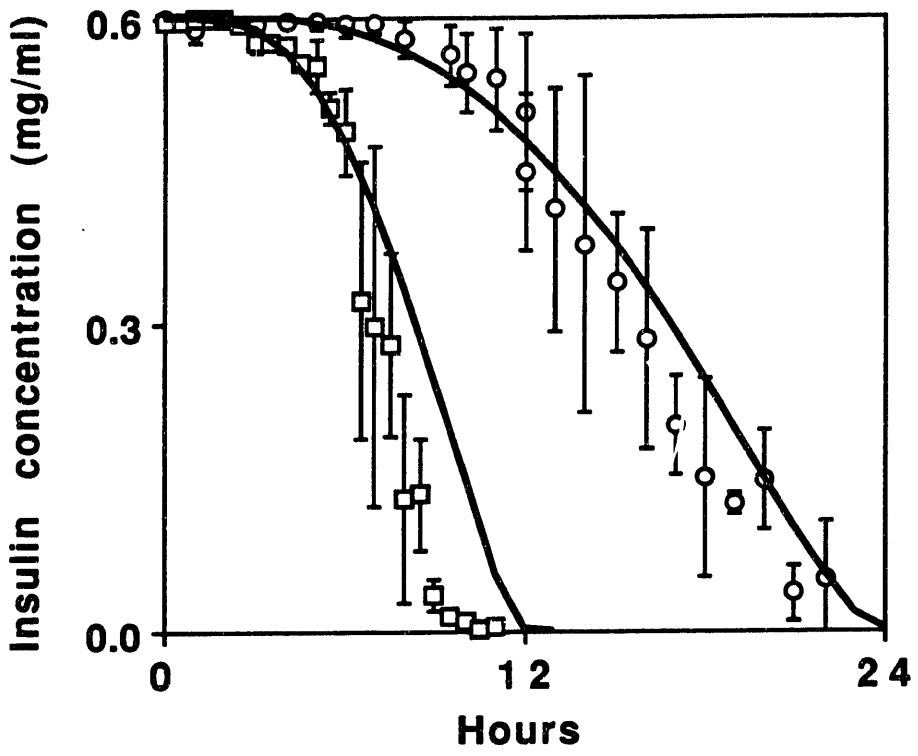

Figure 5.5: Mathematical modelling of Zn-insulin aggregation in solutions agitated at 80 (O) and 160 (□) rpm (in the presence of 10 Teflon spheres, at 37°C). Results of computer simulations are depicted by solid lines.

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also compensated for the possible increase in shear denaturation at higher agitation rates. Figure 5.5 illustrates that insulin aggregation at 160 rpm could be successfully modeled with K_{denat} = $4x10^{-16}$ ml/site. The simulated profile was in good qualitative agreement with the experimentally observed changes in aggregation kinetics. The increase in K_{denat} led to a reduced induction period and a steeper slope, characteristic of faster aggregation at increased agitation rates.

5.3.5 Aggregation in the Presence of Polypropylene

When Teflon spheres were replaced with polypropylene, mass transfer in insulin samples changed drastically. Polypropylene's low density reduced agitation to a minimum; in fact, movement of the spheres was barely noticeable, making it extremely difficult to estimate the rates of mixing in these samples. Insulin's interactions with polypropylene were assumed to be qualitatively similar to those at the Teflon surface, and as no detailed information was available about insulin adsorption on polypropylene, adsorption coefficients were left unchanged.

Mathematical simulation of insulin aggregation in the presence of polypropylene (Figure 5.6) was accomplished by changing only one model parameter, K_{denat}, which was reduced to 5.5×10^{-17} ml/site to reflect the decreases in mass transfer and shear effects. This change in K_{denat} was sufficient to desribe the increased induction period and the reduced slope steepness of the experimentally determined kinetic profile.

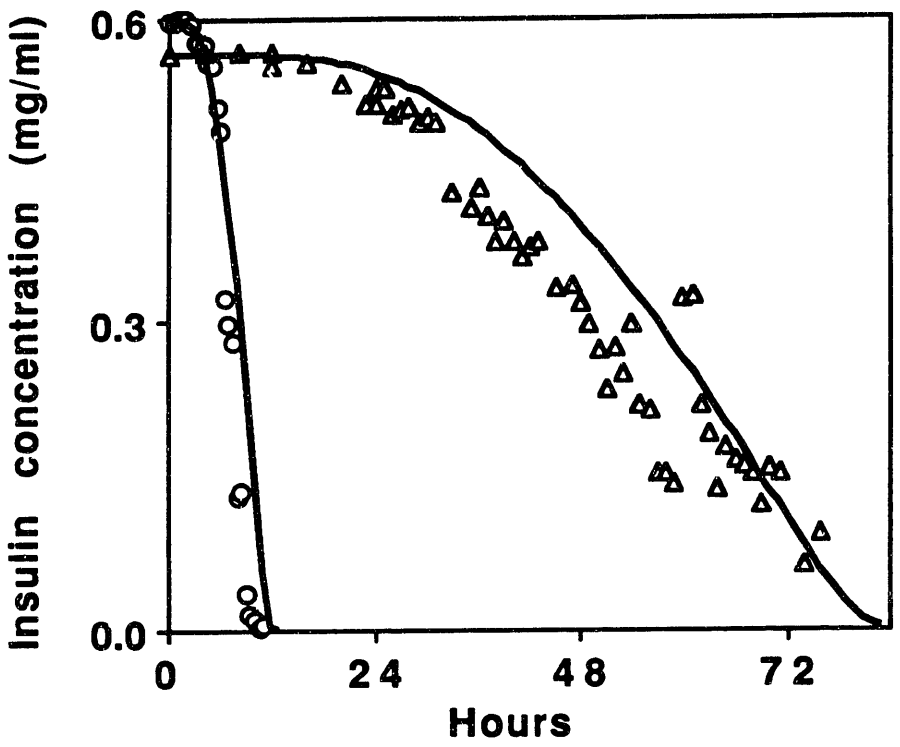


Figure 5.6: Mathematical modelling of Zn-insulin aggregation in the presence of Teflon (O) and polypropylene (Δ). Samples containing 10 spheres were agitated at 37°C and 160 rpm. Results of computer simulations are represented by solid lines.

5.4 Conclusions

The proposed kinetic scheme was successful in predicting and/or simulating insulin aggregation in the presence of solid hydrophobic surfaces. It was a minimal kinetic model, containing the fewest number of steps required to adequately describe system dynamics. The close agreement between empirical observations and theoretical predictions suggested that the model provided a reasonable description of pathways and interactions leading to

instability, and that it could be used to predict the effects of stabilizing reagents on insulin's aggregation behavior. The insights provided by mathematical modelling of reaction kinetics could be used to select stability enhancing conditions and to design additives aimed specifically at blocking unfavorable interactions. Computer simulations also allowed a separate evaluation of the contributions made by different pathways to the overall pattern of instability (Appendix B). This analysis could be used to identify the pathways most sensitive to perturbations, and to propose and test ways to mitigate destabilizing interactions.

CHAPTER 6.

STABILIZATION STUDIES

6.1 Stabilization Strategy

The ultimate goal of this work was to enhance insulin solution stability, and to do so with a clear understanding of the aggregation mechanism. The stabilization strategy was to target the destabilizing pathways and to prevent, or eliminate altogether, the unfavorable interactions leading to insulin aggregation. Since the proposed kinetic model appeared to be in good agreement with experimental observations, stabilization efforts were aimed at minimizing insulin denaturation at hydrophobic interfaces. The model suggested that these interactions initiated the process of aggregation, and that solution stability would be improved if contacts between insulin molecules and hydrophobic surfaces could be eliminated. In fact, experiments with hydrophilic glass beads showed that no aggregation occurred in the absence of solid hydrophobic surfaces (section 4.4.2).

Results of kinetic studies described in chapter 4 indicated that similar pathways were responsible for destabilization in the presence of both solid and gaseous hydrophobic surfaces. Thus, similar stabilization strategies should have succeeded in preventing aggregation induced by both air-water and Teflon-water hydrophobic interfaces. Since air could not be eliminated in insulin vials used in the traditional injection therapy for diabetes mellitus, stabilization studies aimed to reduce insulin aggregation in the

presence of an air-water interface. The addition of surface active reagents, which would occupy interfacial sites and minimize insulin's contacts with a hydrophobic surface, was expected to enhance solution stability.

6.2 Materials and Methods

6.2.1 Control Studies with an Air-Water Interface (Bovine Zn- and Human Na-insulin)

Stock solutions were prepared as described in section 3.3.2, using bovine Zn-insulin (Sigma, lot 79F-0092 {25.7 IU/mg}) and human Na-insulin (Lilly Labs, lot 048 EMO). Samples were prepared by adding 4.0 ml of stock solution to borosilicate glass culture tubes (Pyrex, 12x75 mm), which were capped with polyethylene stopper pugs and agitated at 100 rpm and 37°C. QELS and UV analyses were performed every hour. Sample volume and agitation rate were optimized for maximum reproducibility and rapidity of aggregation.

6.2.2 Stabilizing Reagents

To improve insulin stability, small amounts of surfactants were added to protein solutions. Since insulin was negatively charged at neutral pH, cationic surfactants precipitated it from solution and could not be used. Some anionic detergents (e.g., SDS) had been previously shown to reduce insulin aggregation, but at concentrations too high (1 wt%) to be physiologically compatible (Lougheed et al., 1983). It was therefore of interest to explore the stability enhancing

properties of compounds which had low toxicity and could be used at low concentrations.

The effects of nonionic detergents on insulin solution stability were examined in the presence of an air-water interface at 100 rpm and 37°C. Most of the reagents (Table 6.1) were used at approximately one half of critical micelle concentration (CMC) to examine the interactions with the air-water interface and to prevent micelle formation from interfering with the QELS analysis.

Table 6.1: Nonionic detergents used in preliminary stabilization studies.

Detergent	Source	Lot number	CMC
n-octyl-ß-D-glucopyranoside	Sigma	60H5010	19-25 mM*
n-dodecyl-ß-D-maltoside	Sigma	110H5013	120 µM*
Brij-35	Sigma	10H0353	100 µM†
Tween 20	Sigma	70H0171	59 µM†
Pluronic F68	Calbiochem	018191	NA‡
Pluronic F127	Calbiochem	910104	NA‡

* CMC at neutral pH and I=0.10 (Neugebauer, 1988).

† CMC at neutral pH and I=0.05 (Neugebauer, 1988).

‡ No CMC values were available for these reagents, which were used at concentrations recommended by (Chawla et al., 1985) (2x insulin concentration).

To determine whether the addition of another protein to insulin solutions would reduce aggregation, 30 mg/ml (0.45 mM) of BSA (Sigma) was co-dissolved with 0.6 mg/ml (0.10 mM) insulin in PBS. If BSA preferentially adsorbed at hydrophobic interfaces, it would reduce the number of unfavorable contacts leading to insulin denaturation. This competition for interfacial sites was expected to contribute to solution stability.

6.2.3 Surface Tension Effects

Surface tension of Zn-insulin solutions (0.6 mg/ml in PBS) was varied by adding 1% (w/v) of either acetonitrile (Baker, HPLC grade) or n-propanol (Mallinckrodt, analytical grade). Sample preparation and experimental conditions were the same as in air-water interfacial studies.

6.3 Results of Preliminary Stabilization Studies

In the absence of nonionic detergents, Zn-insulin solutions aggregated in 24 hours (Figure 6.1 A). Both the formation of stable intermediates species (Figure 6.1 B) and the characteristic curve shape were observed, with the induction period lasting approximately 6 hours, and a $t_{1/2}$ value of 12 hours.

The addition of proposed stabilizing reagents had variable effects on overall solution stability. Solutions containing n-octyl-ß-D-glucopyranoside (10 mM), n-dodecyl-ß-D-maltoside (100 µM) and Brij 35 (50 µM) showed no signs of aggregation even after seven days (Figure 6.2), while solutions with Tween 20 (30 µM), Pluronic

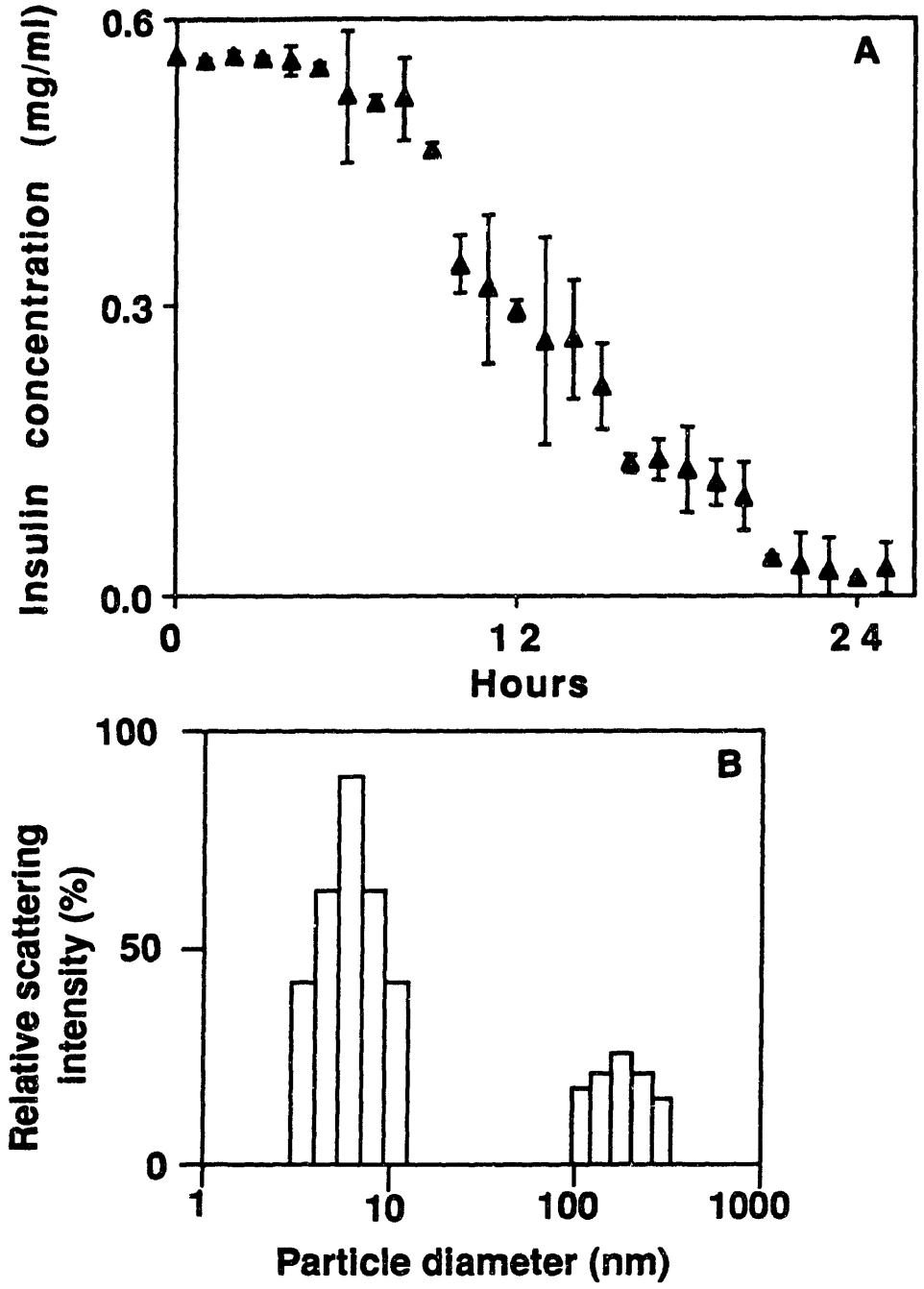


Figure 6.1: Zn-insulin aggregation in the presence of an air-water interface at 37°C and 100 rpm. (A) Time-dependent concentration profile; (B) results of QELS analysis after 2 hours of agitation.

68 (200 μM) and Pluronic 127 (200 μM) aggregated after 72, 50 and 36 hours, respectively (Figure 6.3). The presence of BSA had no effect on insulin solution stability: insulin solutions with and without BSA aggregated in approximately 24 hours (Figure 6.3).

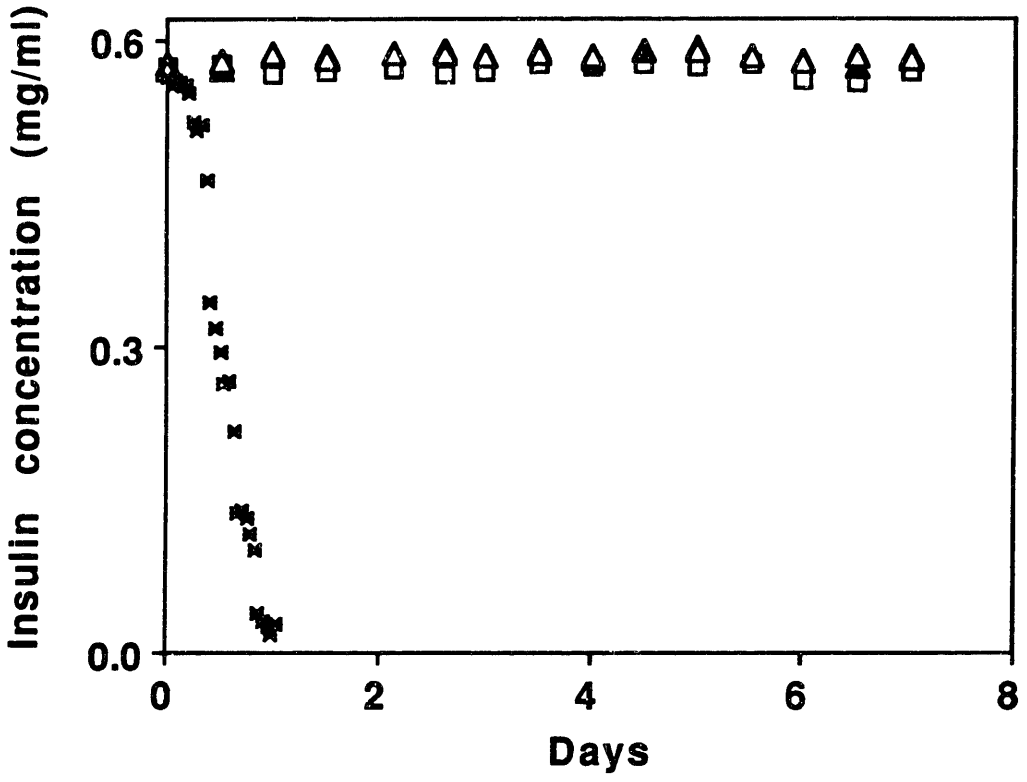


Figure 6.2: Stability of aqueous solutions of Zn-insulin (agitated at 100 rpm and 37°C in the presence of an air-water interface) containing no additives (*), Brij 35 (□), n-octyl-ß-D-glucopyranoside (▲), and n-dodecyl-ß-D-maltoside (Δ). Results of a seven day study.

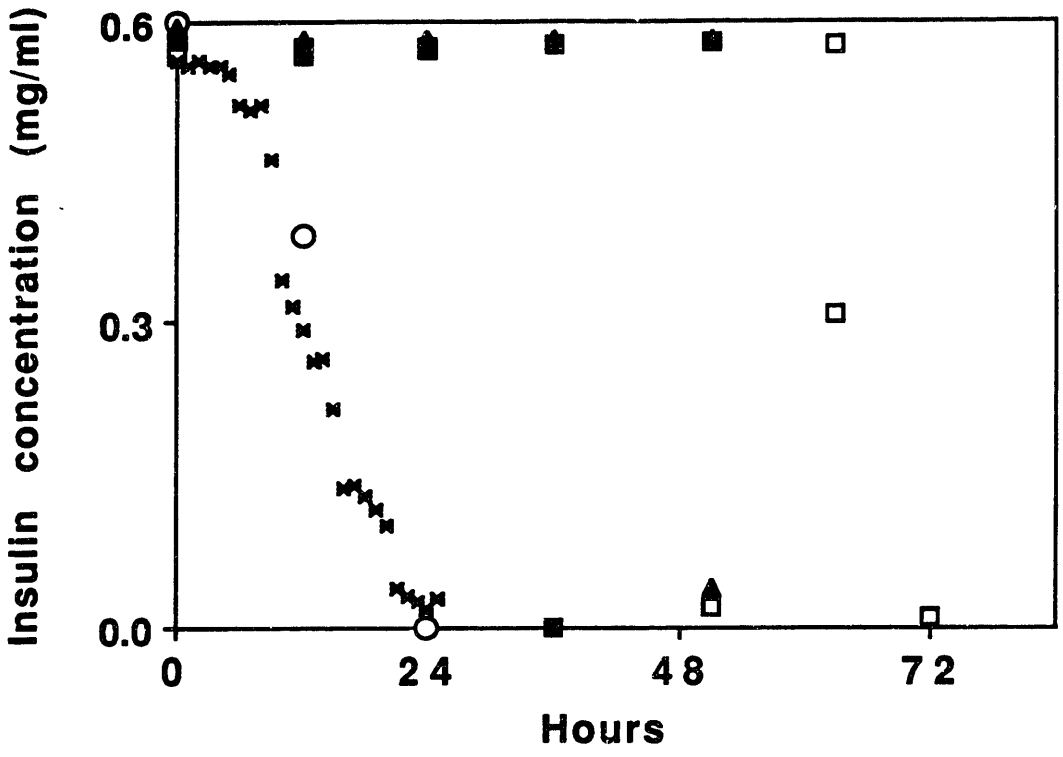


Figure 6.3: Stability of aqueous solutions of Zn-insulin (agitated at 100 rpm and 37°C in the presence of an air-water interface) containing no additives (*), Tween 20 (□), Pluronic F68 (▲), Pluronic F127 (■), and BSA (○). Results of a three day study.

6.4 Mechanism of Stabilization

Nonionic detergents were expected to improve solution stability because of their affinity for the hydrophobic interface, where the hydrophilic head groups faced the aqueous environment, while the hydrophobic tails extended into the gaseous phase (Figure 6.4). As a result, insulin would be excluded from the interface and, consequently, its adsorption induced conformational changes would be minimized.

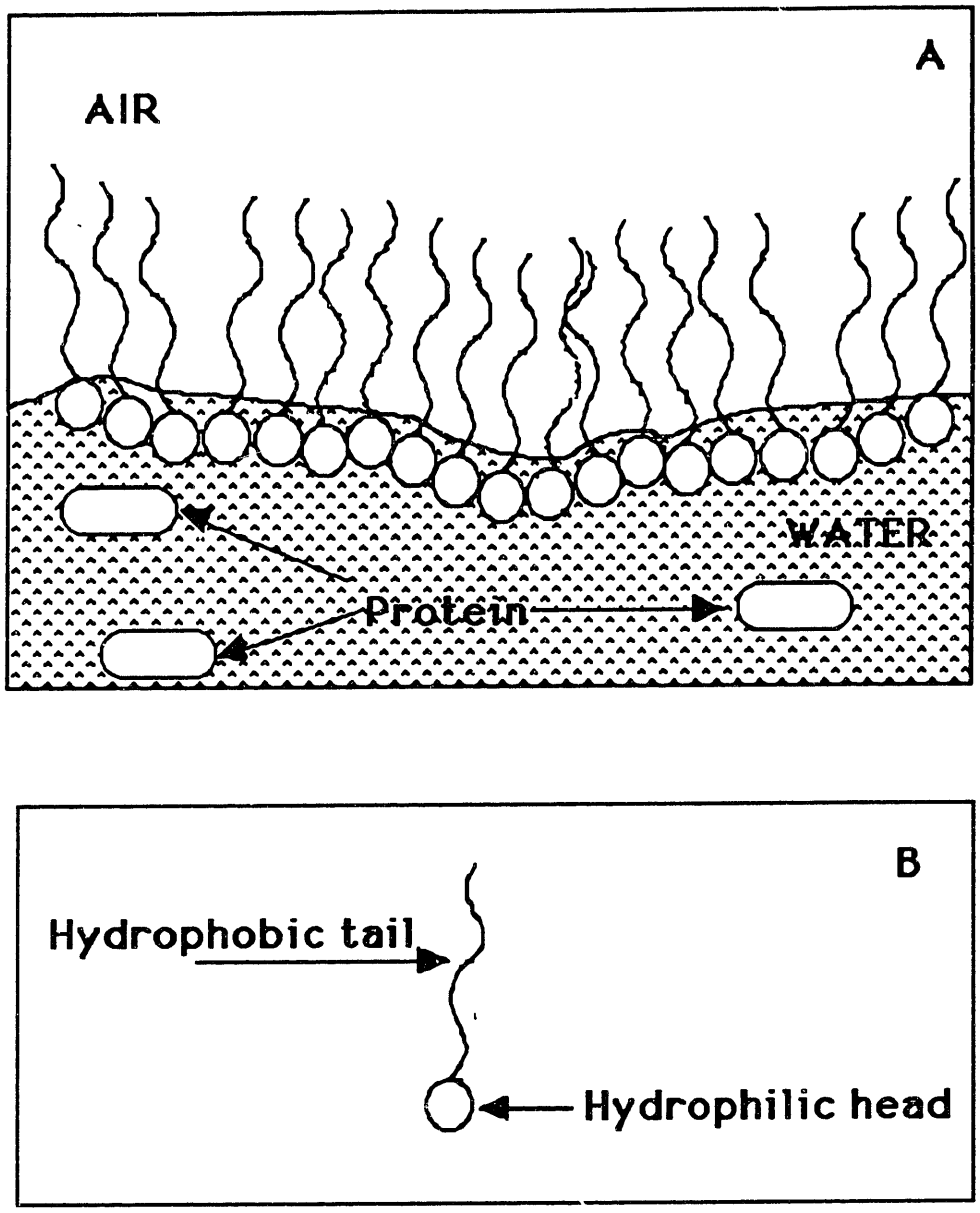


Figure 6.4: (A) Surfactant molecules' orientation at and occupation of the air-water interface; (B) schematic representation of a surfactant molecule.

6.4.1 Good vs Poor Stabilizing Reagents

It became clear from the initial stabilization studies that some of the tested detergents were much more effective in preventing insulin aggregation. To explain the differences in their stability enhancing properties, molecular structures of these reagents were examined (Figures 6.5 and 6.6). The smaller, less sterically hindered surfactant molecules (Figure 6.5) could easily orient themselves at (and fully occupy) the air-water interface, which is probably why they exerted the most dramatic stabilizing effects. In contrast, such bulky nonionic detergents as Tween 20 and Pluronic 127 (Figure 6.6) may not have been closely packed at the interface because of steric factors. This inability to fully cover the hydrophobic surface probably impaired their stability enhancing properties.

BSA's inability to prevent aggregation was not surprising. Insulin was a much more hydrophobic molecule than BSA, and probably had a higher affinity for the air-water interface. BSA's polar surface made interactions with hydrophobic surfaces thermodynamically unfavorable, leaving insulin's contacts with the air-water interface unchanged even at relatively high concentrations of BSA.

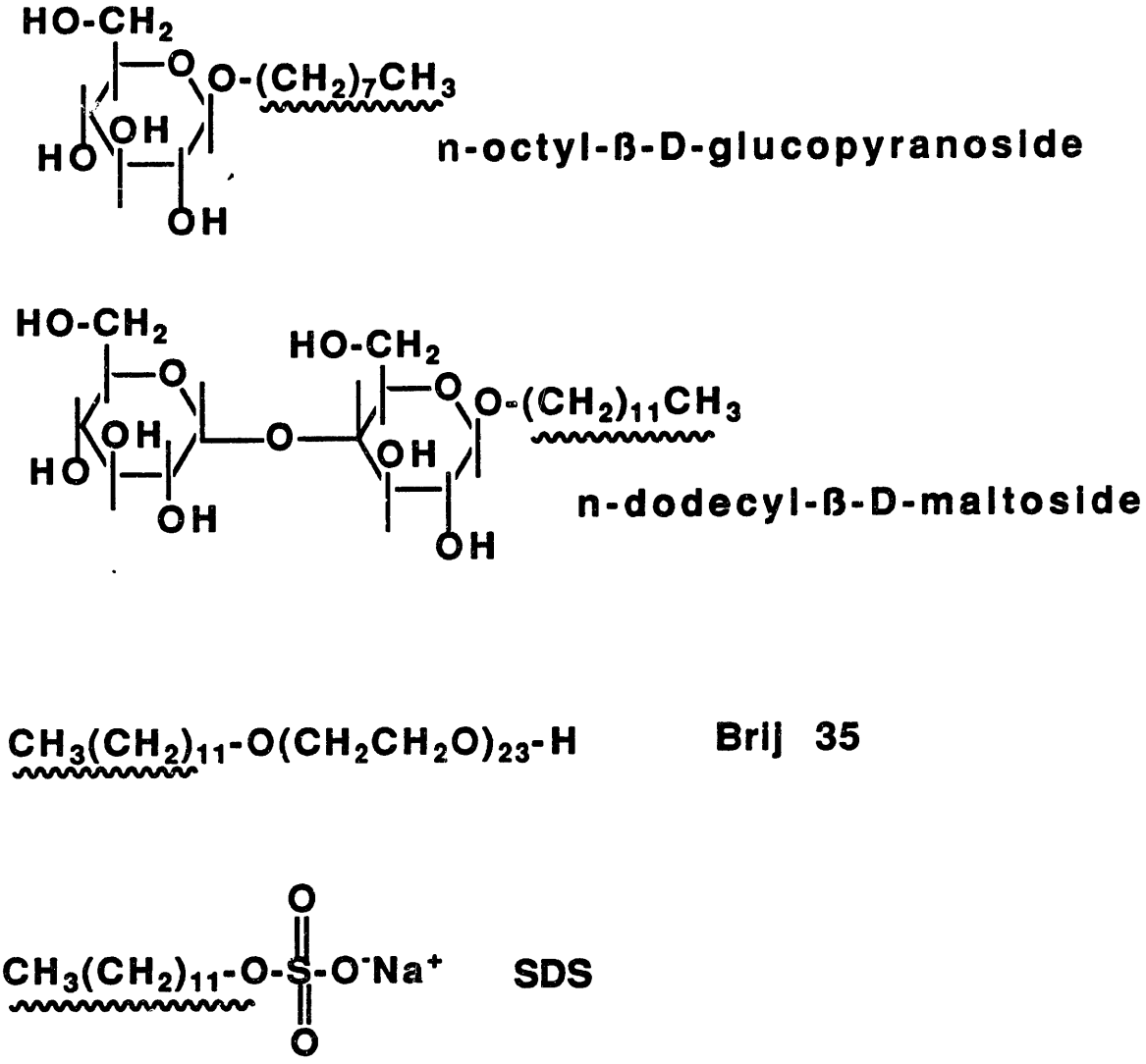
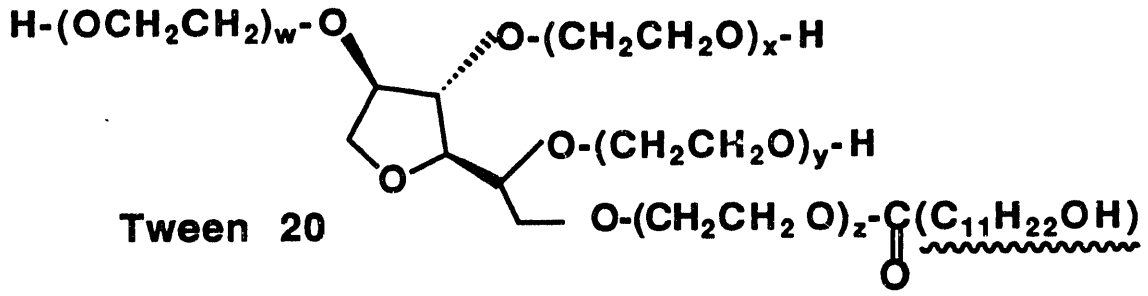
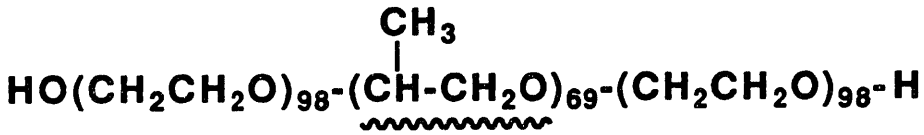


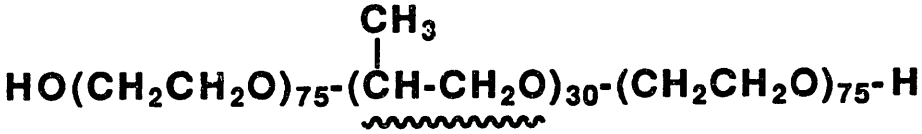
Figure 6.5: Molecular structures of detergents which enhanced insulin solution stability. The hydrophobic portions of the molecules are underlined.



Tween 20



Pluronic F-127



Pluronic F-68

Figure 6.6: Molecular structures of detergents which failed to improve insulin solution stability. The hydrophobic portions of the molecules are underlined.

6.4.2 Role of Surface Tension

It was suggested previously that insulin stabilization in the presence of surfactants was partly due to reduced surface tension (Lougheed et al, 1983), which minimized shear denaturation effects at the air-water interface. However, the addition of 1 wt% of n-propanol (which reduced surface tension (Weast, 1978) in the absence of a surface active reagent) did not increase solution stability; quite the contrary, it resulted in faster aggregation (Figure 6.7). At the same time, the addition of 1 wt% of acetonitrile had practically no effect on either surface tension (Weast, 1978) or aggregation (Figure 6.7). In the presence of n-propanol, lowered surface tension increased the air-water interface available to destabilize insulin monomers, thus leading to more rapid aggregation.

It should be noted that both n-propanol and acetonitrile had denaturing properties (Musial et al., 1986); however, in the unlikely event that the small amounts (1%) added actually contributed to monomer denaturation in solution (rather than at the air-water interface), similar destabilizing effects should have been observed with both reagents. Since insulin aggregation was not affected by the addition of acetonitrile, denaturation in bulk solution could be dismissed, and increased aggregation with n-propanol attributed to decreased surface tension.

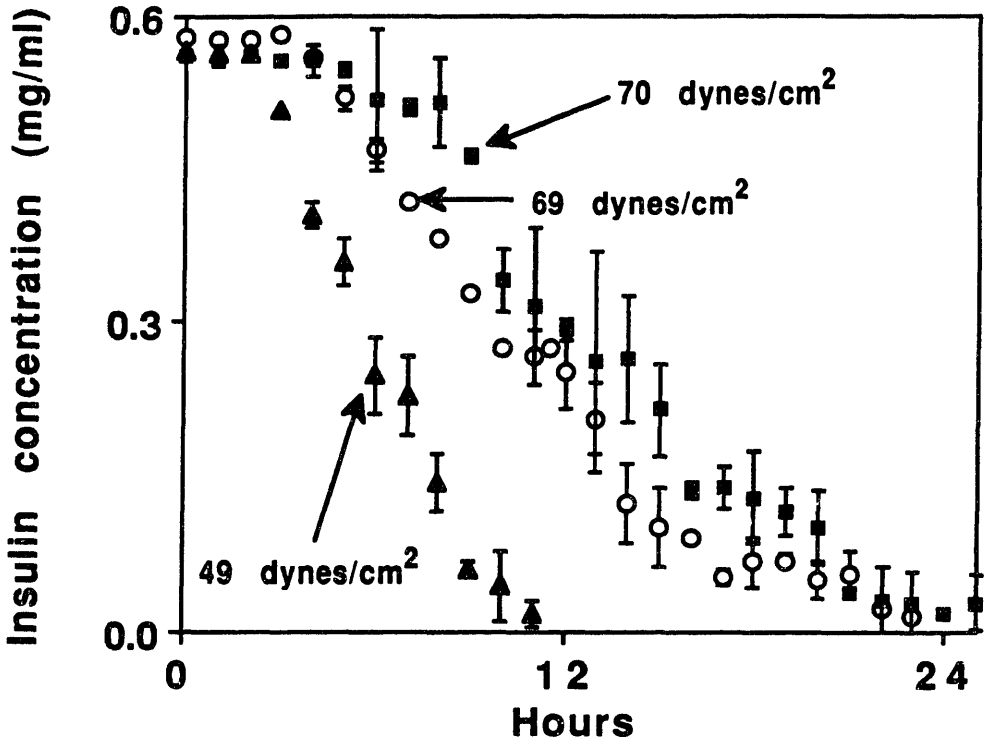


Figure 6.7: Effects of surface tension (values depicted in the figure) on insulin aggregation rates (air-water interface, 100 rpm, 37°C): no additives (■), 1 wt% n-propanol (▲), 1 wt% acetonitrile (○).

6.5 Long Term Stabilization with Bovine Zn-Insulin

The results of preliminary stabilization studies indicated that Brij 35 and the sugar-based detergents n-octyl-ß-D-glucopyranoside and n-dodecyl-ß-D-maltoside significantly reduced insulin's propensity to aggregate even in the presence of elevated temperature, agitation and an air-water interface. To further explore the stability enhancing properties of these three surfactants, a long term study was undertaken. Samples were prepared as described in section 6.2; UV and HPLC analyses were performed every two days during the first four weeks, and every seven days thereafter.

As can be seen in Figure 6.8, no aggregation was observed in samples containing n-octyl-ß-D-glucopyranoside or n-dodecyl-ß-D-maltoside, and no intermediate species were detected in them. In the presence of Brij 35, the onset of aggregation was delayed, with the induction period lasting over 10 days. After 12 days of agitation, large intermediate species were detected and insulin concentrations began to decrease; these insulin samples aggregated completely within 2 weeks. The difference in the stability enhancing properties of these surfactants could probably be attributed to their structural differences. Brij 35 (by far the largest and bulkiest molecule of the three) was probably less effective in excluding insulin from the hydrophobic surface, allowing a few protein molecules to interact with the air-water interface. The slow destabilization of these molecules probably led to the subsequently observed aggregation.

The stabilizing effects of sugar-based nonionic detergents are particularly encouraging. Since elevated temperatures contribute to insulin destabilization, one can expect that the stabilization observed

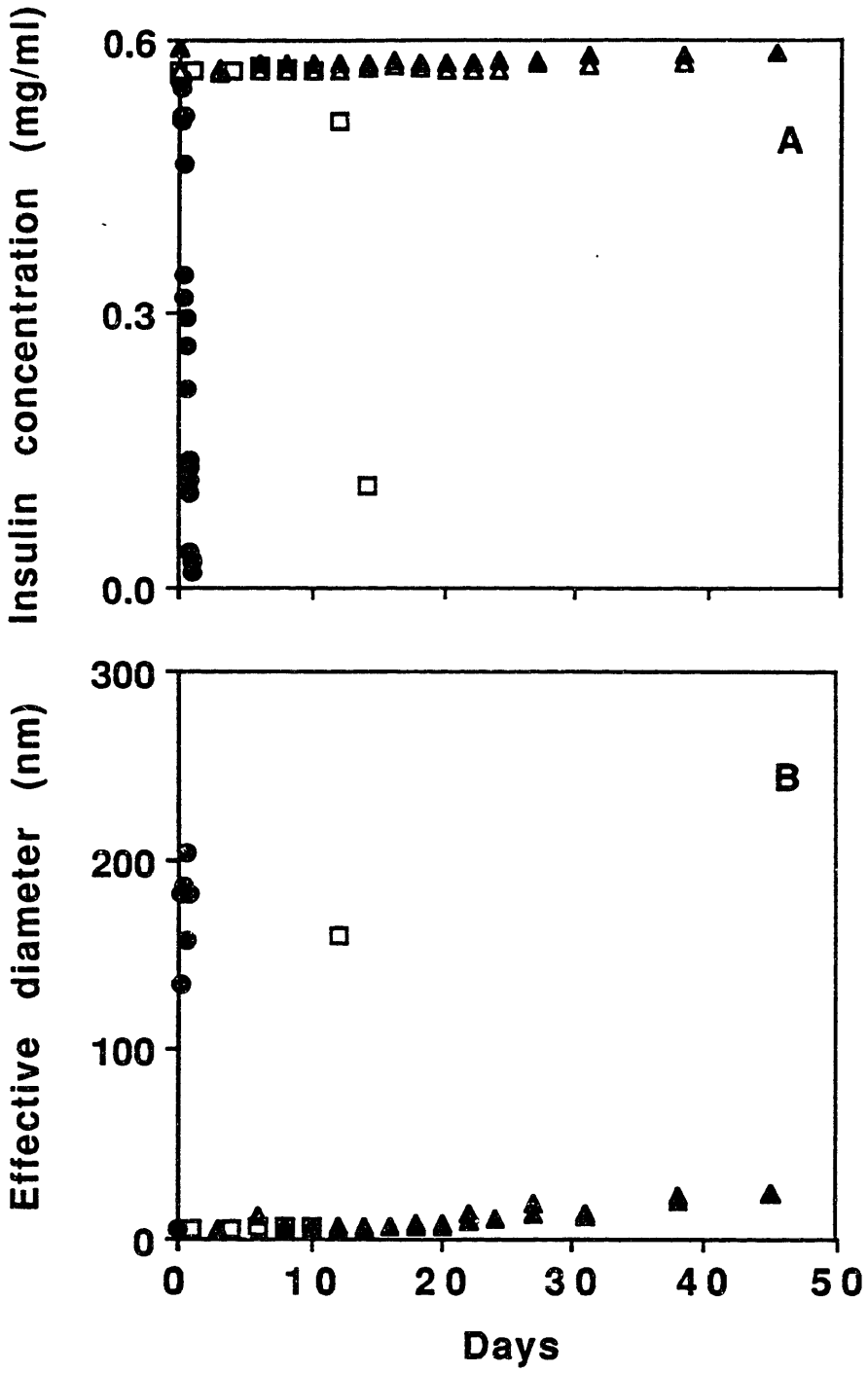


Figure 6.8: Long term stability of aqueous solutions of bovine Zn-insulin (agitated at 100 rpm and 37°C in the presence of an air-water interface) containing no additives (●), Brij 35 (□), n-octyl-ß-D-glucopyranoside (▲), and n-dodecyl-ß-D-maltoside (Δ). (A) Time-dependent concentration profiles; (B) results of QELS analysis.

with these reagents at 37°C could be enhanced still further at room temperature.

6.6 Long Term Stabilization with Human Na-Insulin

In the absence of stabilizing reagents, solutions of bovine Zn-insulin aggregated within a day (Figure 6.1). Human insulin (which is more hydrophilic and hexamerizes less readily than its bovine and porcine counterparts (Brange et al., 1990)) had been shown to be slightly more prone to aggregation and destabilization. The reduced stability was attributed to higher monomer concentrations; at the same time, insulin dissociation to monomeric and dimeric species was desirable, since it facilitated adsorption into the bloodstream and provided better glycemic control (Brange et al., 1990). Since the development of genetic engineering techniques, insulin preparations have been formulated using recombinant human insulin, making the stability of this species an immediate concern for diabetes therapies.

To examine the stability of this recombinant protein, solutions of human Na-insulin were tested with and without stabilizing reagents. Samples containing no additives aggregated in approximately 7 hours (Figure 6.9 A). The induction period was reduced to less than one hour, and $t_{1/2}$ of approximately 4 hours was observed. This behavior was very similar to the aggregation of bovine Na-insulin in the presence of Teflon (section 4.3.4). The reduced concentration of hexamers was evident from the QELS analysis (Figure 6.9 B): here too the native peak shifted from 5 to 4 nm, indicating the predominance of dimeric and monomeric species

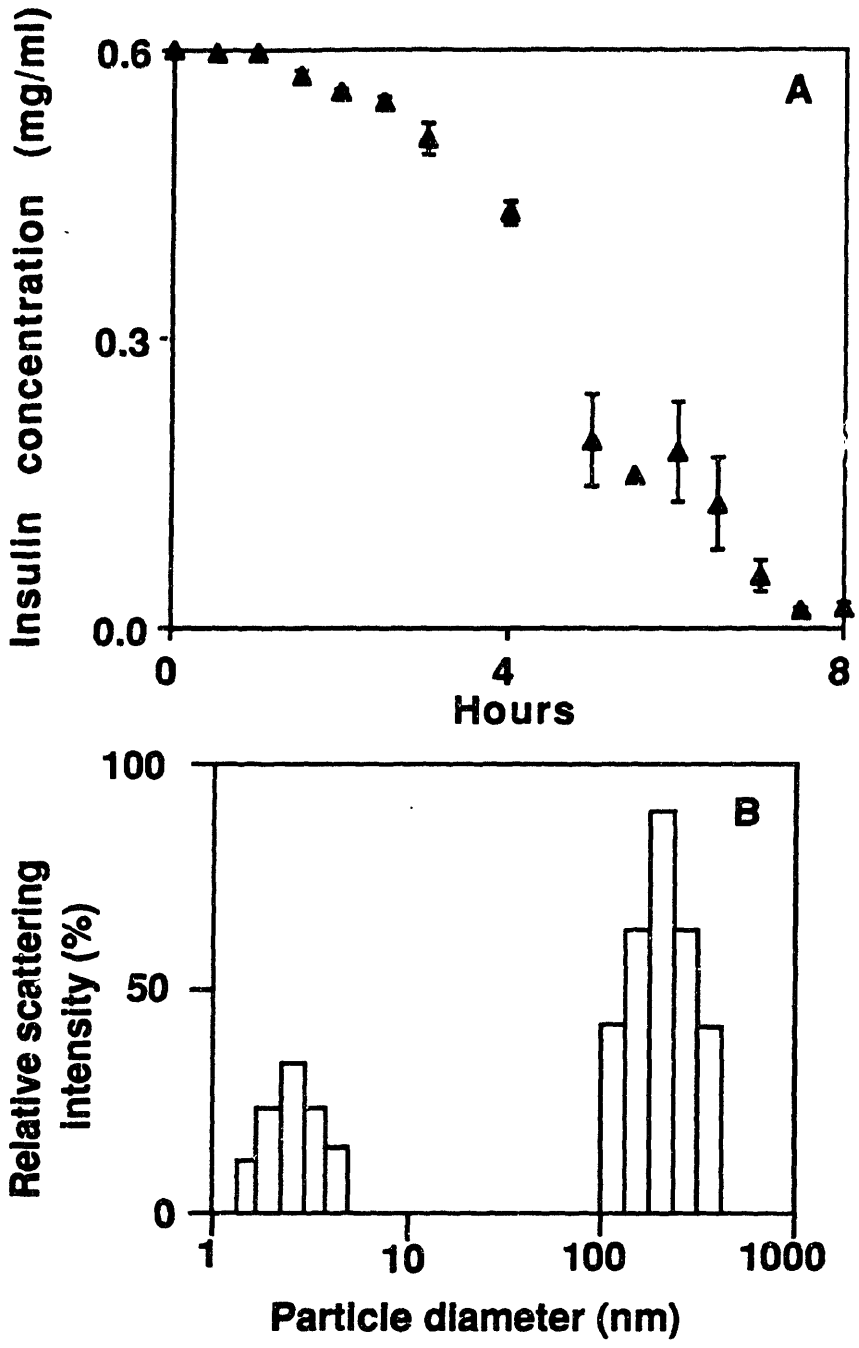


Figure 6.9: Human Na-insulin aggregation in the presence of an air-water interface at 37°C and 100 rpm. (A) Time-dependent concentration profile; (B) results of QELS analysis after 2 hours of agitation.

(see 4.3.4 and Appendix A for a more detailed discussion of QELS results). QELS also detected stable intermediate species (indicated by the peak at approximately 200 nm) in solutions of aggregating human Na-insulin.

To reduce aggregation, n-octyl-ß-D-glucopyranoside and n-dodecyl-ß-D-maltoside (which demonstrated the best stability enhancing properties) were added to solutions of human Na-insulin. During this long term study, UV and QELS analyses were performed on duplicate samples twice a week. As can be seen from Figure 6.10, samples containing 100 µM n-dodecyl-ß-D-maltoside remained stable for almost two weeks, at which point the majority of these samples aggregated. In the presence of 10 mM n-octyl-ß-D-glucopyranoside, no aggregation was observed during the 6 weeks of the experiment.

Although these nonionic detergents significantly improved the stability of both human and bovine insulin, there were some notable differences between the stabilization of Na- and Zn-insulin solutions. Both n-octyl-ß-D-glucopyranoside and n-dodecyl-ß-D-maltoside were equally effective in preventing insulin aggregation in solutions containing Zn^{2+}. However, in the absence of Zn^{2+}, n-dodecyl-ß-D-maltoside failed after two weeks, while n-octyl-ß-D-glucopyranoside continued to protect insulin stability for six weeks. The molecular structures of the two surfactants were not significantly different (the maltoside's hydrophobic tail was slightly longer and its hydrophilic head group slightly larger than the respective portions of the glucoside molecule), and should not have caused large differences in their interactions with the air-water interface. The success of both

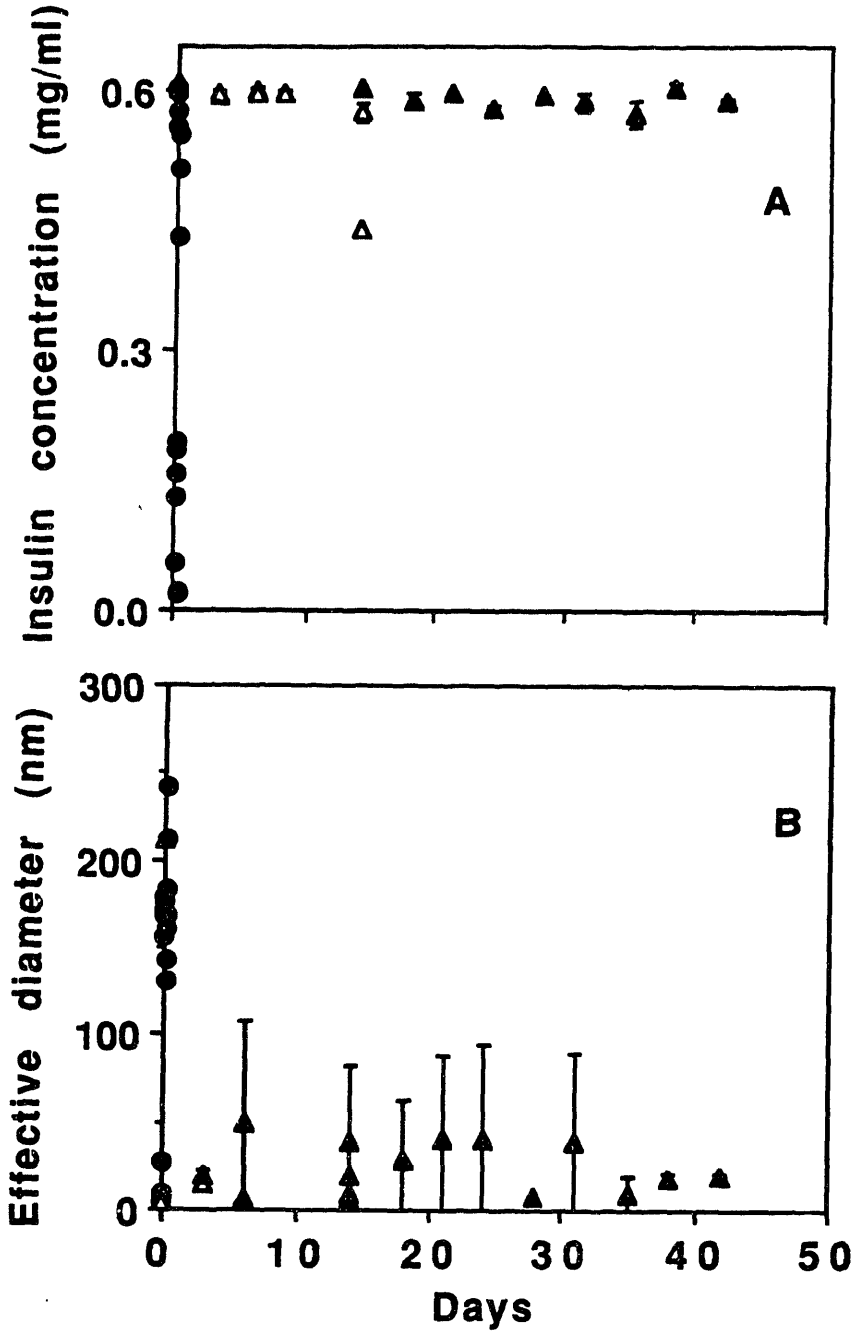


Figure 6.10: Long term stability of aqueous solutions of human Na-insulin (agitated at 100 rpm and 37°C in the presence of an air-water interface) containing no additives (●), n-octyl-ß-D-glucopyranoside (▲), and n-dodecyl-ß-D-maltoside (Δ). (A) Time-dependent concentration profiles; (B) results of QELS analysis. Results were the averages of two measurements, with error bars representing standard deviation from the mean.

reagents in Zn-insulin solutions also supported the conclusion that something other than structural differences compromised the effectiveness of n-dodecyl-ß-D-maltoside in Na-insulin solutions.

In the absence of Zn^{2+}, the concentration of insulin monomers became much higher. As a result, the ratio of monomers to surfactant molecules was approximately 1 to 1 in solutions containing n-dodecyl-ß-D-maltoside, while in the presence of n-octyl-ß-D-glucopyranoside the ratio of surfactant to protein was closer to 100 to 1. In the presence of maltoside, the monomer had to compete for interfacial sites with just one surfactant molecule, and not 100, as was the case in solutions stabilized with glucopyranoside. This probably made monomer interactions with and denaturation at the air-water interface much more likely in solutions containing n-dodecyl-ß-D-maltoside.

Although insulin solutions stabilized with n-octyl-ß-D-glucopyranoside did not aggregate during the six weeks of the experiment, QELS results indicated that some destabilizing interactions did occur. A small amount of stable intermediate species (approximately 120 nm in diameter) was observed in some samples (Figure 6.11). As the formation of this intermediate did not result in aggregation, it was concluded that it reached neither the critical size nor the concentration necessary to initiate interactions with native insulin molecules. It is also possible that this species was formed as a result of interactions between protein and surfactant molecules (i.e., micelle formation).

These minor side effects, however, should not detract from the most important observation that in the presence of n-octyl-ß-D-

glucopyranoside, insulin concentrations remained virtually unchanged during the 42 days of the experiment.

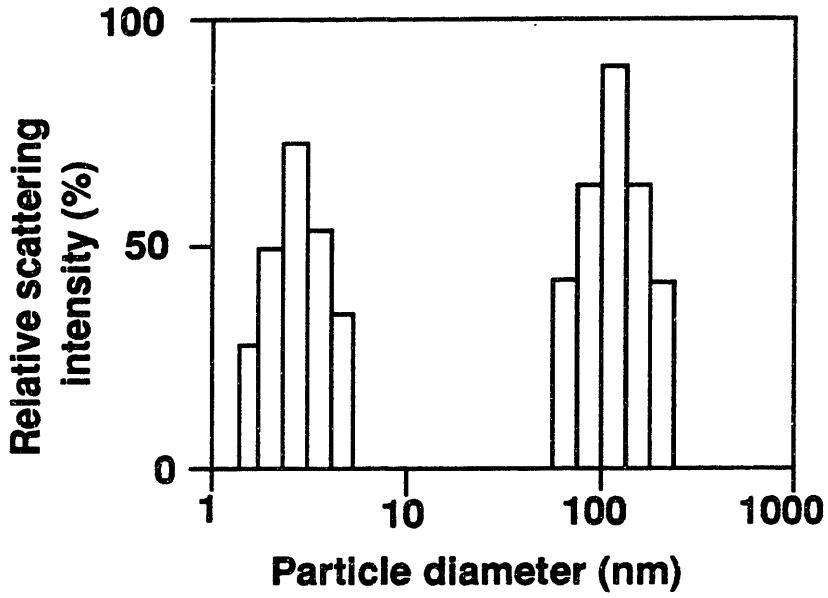


Figure 6.11: QELS analysis of human Na-insulin solutions stabilized with n-octyl-ß-D-glucopyranoside (after 14 days of agitation at 100 rpm and 37°C, in the presence of an air-water interface). This size distribution histogram was generated using the program CONTIN.

6.7 Safety of Stabilizing Reagents

The nonionic surfactants which proved to be particularly successful in preventing insulin aggregation in agitated aqueous solutions, have not been approved by the FDA (although Brij 35 was approved by the regulatory agency of the UK (Pharmaceutical Society of Great Britain, 1986)). Unfortunately, Tween 20 and Pluronic 68 -- which according to US pharmacopeia (U.S. Pharmacopoeial Convention, 1979) are safe for use in some drug formulations -- did not have a satisfactory stabilizing effect on insulin solutions. This brought into question the safety of n-octyl-ß-D-glucopyranoside and n-dodecyl-ß-D-maltoside.

One reason why these two surfactants have not yet received FDA approval is that both are quite new, and have not been used in drug formulations, finding their primary application in biological research (where they are used to solubilize membrane proteins (Gould et al., 1981; Muccio and DeLucas, 1985)). However, if one compares the structures of these molecules to those of reagents which are considered to be safe by the FDA (U.S. Pharmacopoeial Convention, 1979), many similarities can be found. For example, SDS (an anionic detergent, approved for use in some drug formulations) has a hydrophobic portion identical to that of n-octyl-ß-D-glucopyranoside, and has a hydrophilic headgroup (SO_4^-) which appears to be even less innocuous than the glucose moiety in the proposed nonionic detergent. Similarly, the hydrophobic tail of n-dodecyl-ß-D-maltoside is slightly longer than that of SDS (by 4 carbon groups), and its hydrophilic headgroup is the maltose moiety, which would not be expected to cause toxic side effects.

Furthermore, the concentrations of nonionic surfactants shown to be sufficient to prevent insulin aggregation were very small (100 μM - 10 mM, compared to 35 mM for SDS (Lougheed et al., 1983)), thereby reducing the likelihood of toxicity. This also suggested that FDA approved reagents such as SDS could be used at much lower concentrations (than previously tested) to stabilize insulin solutions.

Nevertheless, extremely strict standards will be applied to additives used in insulin formulations, since these pharmaceuticals are used to treat a chronic illness over a patient's lifetime. If the proposed nonionic detergents do not meet such stringent requirements, other (safer) reagents must be found. The following criteria should be used in selecting stabilizing additives for insulin formulations: a) they must exhibit high affinity for hydrophobic interfaces, b) their structure must be compact, allowing them to easily orient at and fully occupy the hydrophobic surface, c) they must have no denaturing properties, and should not interfere with insulin's self-association, and d) they must be nontoxic, and be required at low concentrations. Reagents which satisfy these conditions should prevent insulin aggregation in aqueous solutions exposed to hydrophobic surfaces, as they are targeted at minimizing monomer denaturation resulting from interfacial contacts.

CHAPTER 7.

CONCLUSIONS

Stability of protein based pharmaceuticals is essential for their production, storage, and delivery. The specific formulation of these therapeutic reagents must anticipate and prevent all unfavorable interactions which may lead to loss of biological activity. There are no uniform guidelines for improving protein stability; the diversity of protein sizes and conformations presents new challenges and problems specific to the individual molecule. In order to reduce the amount of guesswork going into the formulation of protein-based drugs, rational stabilization strategies are needed to address the specific problems of a particular protein system. All degradation mechanisms must be explored, so that stability enhancing additives can be chosen based on their ability to block unfavorable interactions.

This investigation was aimed at gaining a fundamental understanding of insulin solution stability and aggregation. The effects of agitation rates, air-water and Teflon-water interfacial interactions, insulin concentration, solvent composition, surface tension and Zn^{2+} content were examined. Detailed kinetic studies were performed to determine how these parameters affected the overall pattern of aggregation. Time dependent concentration profiles of insulin aggregation were generated, and quasielastic light scattering measurements were used to monitor changes in particle

size distribution. Based on the experimentally observed trends and a comprehensive literature review, a mechanism of insulin aggregation in aqueous solutions in contact with solid hydrophobic surfaces was formulated, and mathematical modelling was used to simulate reaction kinetics.

The proposed reaction scheme incorporated insulin's self-association equilibrium, and identified monomer denaturation at hydrophobic interfaces as the step which initiated the process of destabilization. The model was used to explain both the anomalous concentration dependence and the initial lag time observed in aggregating insulin solutions. It correctly predicted trends in insulin destabilization, and appeared to be valid in the presence of both solid and gaseous hydrophobic surfaces. Computer simulations helped to identify the pathways leading to insulin destabilization and aggregation, and to propose rational stabilization strategies, which aimed specifically at minimizing or eliminating unfavorable interactions.

To prevent insulin destabilization induced by contacts with hydrophobic surfaces, several stabilizing reagents were evaluated. These nonionic surfactants successfully inhibited monomer denaturation at the air-water interface, and prevented insulin aggregation for up to 6 weeks. The stabilization mechanism was consistent with the destabilizing role attributed to the hydrophobic surface: only those surfactants able to readily orient themselves at the interface and thus exclude insulin from surface interaction, were effective in preventing aggregation. It was also shown that occupation of interfacial sites, rather than reduction in surface

tension, enhanced insulin solution stability. It is hoped that better understanding of the aggregation mechanism and greater formulation stability may lead to improvements in diabetes therapy, particularly the design of new devices for prolonged insulin administration

This work illustrates an engineering approach to improving protein stability. Rather than rely on empirical stabilization strategies, this study identified the mechanism of destabilization, and chose stability enhancing additives which specifically targeted the unfavorable interactions causing monomer denaturation. Improved stability was achieved in a controlled and rational manner, bringing more science to the current "art" of drug formulation.

7.1 Suggestions for Future Work

The current model of insulin aggregation in aqueous solutions in the presence of solid hydrophobic surfaces allowed one to make qualitative predictions about trends in aggregation behavior. The model was particularly successful in describing changes resulting from variations in insulin concentration and in the size of the Teflon surface area. However, the experimental system used in this study did not allow for a separate evaluation of the effects of surface hydrophobicity and mass transfer on the overall aggregation behavior. It would be of interest to decouple these two parameters.

To investigate the role of mass transfer (in the presence of a fixed hydrophobic surface area), another sample geometry could be chosen, one with well characterized fluid mechanics (i.e., where an

analytical expression exists to relate the mass transfer coefficient to fluid velocity). For example, agitation could be provided by a disk spinning in an enclosed container. Mixing rates could then be varied by changing the speed of rotation, and the mass transfer coefficient could be determined analytically.

This system could also be used to study the effects of surface hydrophobicity. The disk's surface could be modified with different concentrations of hydrophobic groups, and the degree of hydrophobicity determined by measuring the contact angle of water with the surface. Using a fixed agitation rate, insulin aggregation could be examined in the presence of a range of hydrophobic surfaces. A better understanding of insulin interactions with polymer surfaces could also be used to improve insulin stability in controlled release devices. The same type of analysis could be used to study other proteins as well, since conformational changes induced by surface interactions and/or shear denaturation are important issues for many other protein systems.

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APPENDIX A

PRINCIPLES OF QUASIELASTIC LIGHT SCATTERING ANALYSIS

This section is intended to be a brief overview of QELS (or photon correlation spectroscopy); more indepth discussions of light scattering theory and interpretation of QELS results can be found in the references sited herein.

A.1 Theoretical Basis for Particle Size Determination

Quasielastic light scattering is a non-invasive technique (Weiner, 1984) which allows one to measure the size of particles smaller than 1 micron. A typical experimental set-up used for making light scattering measurements is depicted in Figure A.1: a laser beam shines on a static sample; the light scattered at an angle θ (90° in this study) is collected by the photomultiplier, and the output is analyzed by an autocorrelator. This analysis assumes that incident and scattered light are of the same wavelength.

If the particles in the sample are much smaller than the laser wavelength λ, they re-radiate like small dipoles (Weiner, 1984). Since the dissolved particles undergo diffusion (Brownian motion), their relative positions are constantly changing, causing instantaneous fluctuations in the signal detected by the photomultiplier. These fluctuations are detected as noise in the scattered radiation (Figure A.2). The autocorrelator analyzes scattered data by comparing the photomultiplier output (scattering

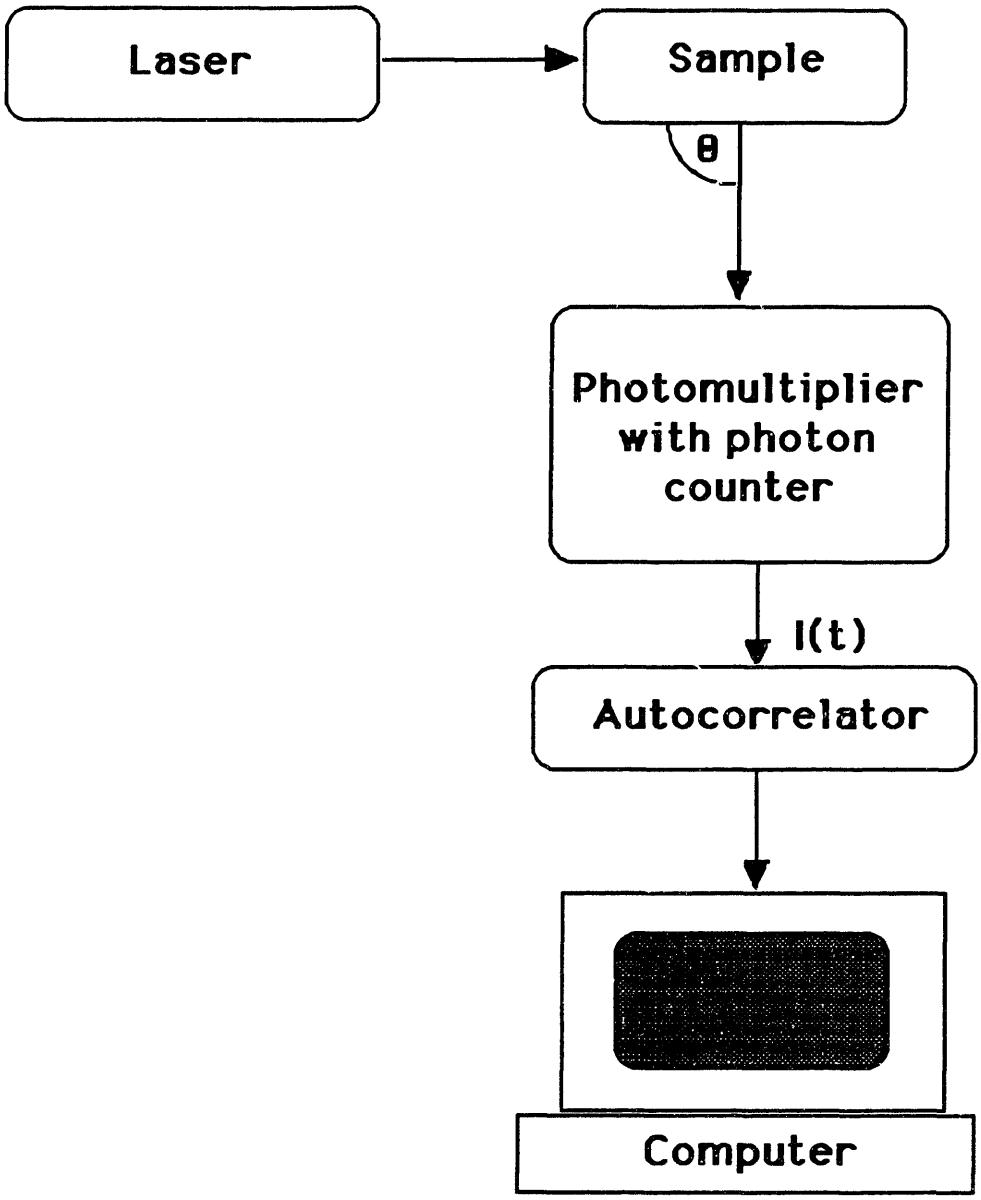

Figure A.1: Schematic representation of the experimental set up used to make light scattering measurements.

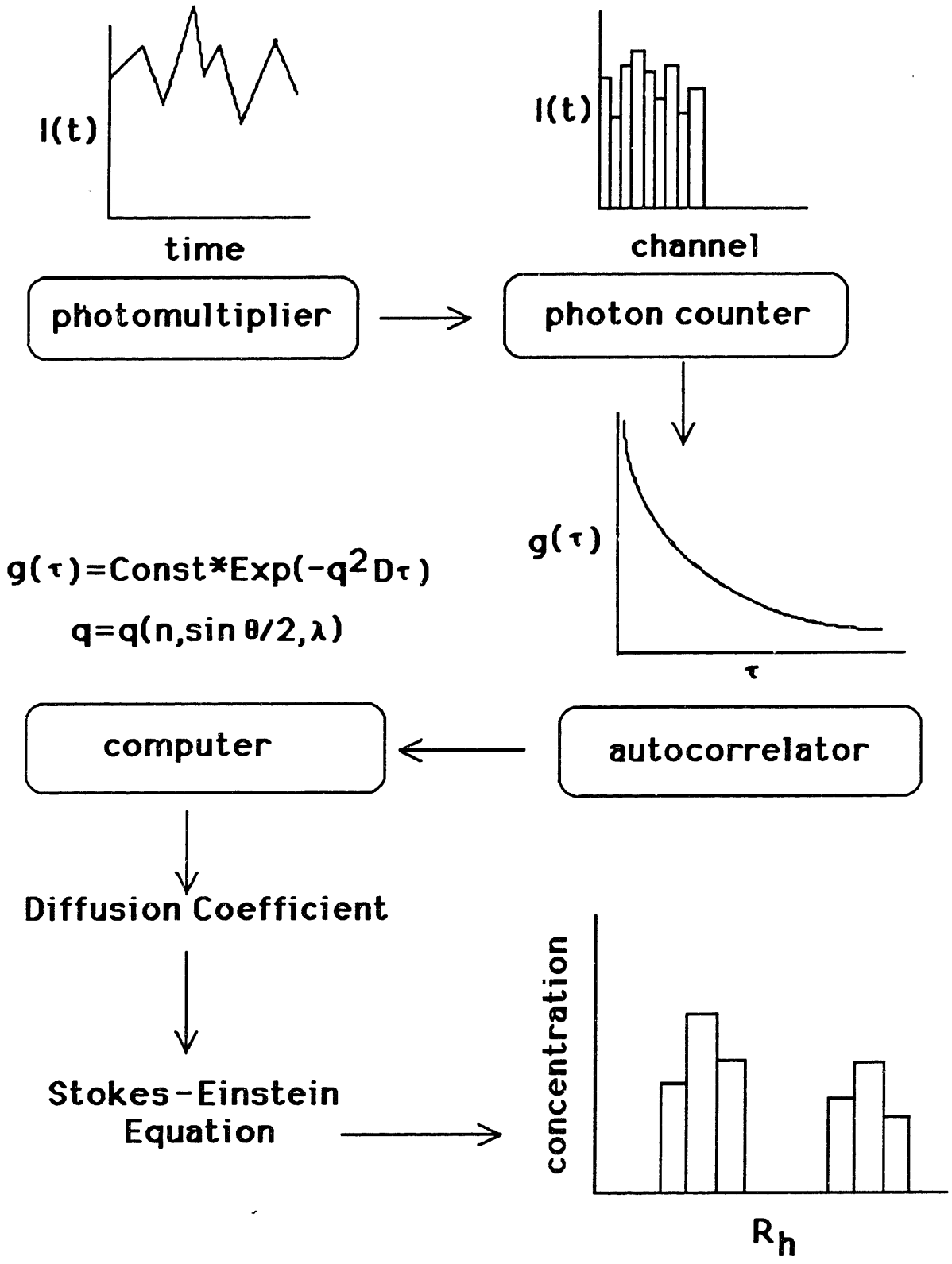

Figure A.2: Schematic representation of QELS analysis.

intensity I) at some time t with the output at time $(t + \tau)$:

> If the outputs are similar at the two times, they are said to be **correlated.** If the motion of the scattering particle is rapid compared to τ, then there will be no average correlation between the two output levels. If the motion is slow compared to τ, there will be, on average, a correlation between the output levels at time t and $(t + \tau)$ [which is why the choice of $\Delta\tau$ (sample time) is extremely important for making meaningful QELS measurements]. This correlation is defined by a function $g(\tau)$. It is common for the correlation function $g(\tau)$ to decrease exponentially with increasing τ. The time constant of the decay is related to the product of the scattering vector **q** and the diffusion coefficient **D**. Thus,
>
> $$g(\tau) \approx e^{-\mathbf{D}q^2} \qquad \{A1\}$$
>
> A plot of $\ln\{g(\tau)\}$ versus τ then gives a straight line with slope $-\mathbf{D}q^2$ (Cantor and Schimmel, 1980).

The scattering vector **q** is a function of the solvent's index of refraction n, the scattering angle θ and the wavelength λ of the incident light:

$$\mathbf{q} = \frac{4\pi n}{\lambda} \sin\frac{\theta}{2} \qquad \{A2\}$$

which is a constant for a given set of experimental conditions. The translational diffusion coefficient **D** can then be determined from

equations A1 and A2. The Stokes-Einstein equation relates the hydrodynamic radius R_h to the diffusion coefficient as follows:

$$D_T = \frac{kT}{6\pi\eta R_h} \qquad \{A3\}$$

where k = Boltzman constant

T = absolute temperature

η = solvent viscosity

R_h = radius of a spherical particle

For monodisperse solutions of spherical particles, this analysis provides a rapid and accurate (±3% (Brookhaven, 1986)) method for particle size determination. For non-spherical particles, it calculates an R_h which is defined as the product of the radius of a sphere with the equivalent volume (R_e) and a form factor $f_o \geq 1$ (which becomes very large when the shape of the particle deviates significantly from a sphere) (Degiorgio, 1980). Thus, the accuracy of the analysis decreases as the uncertainty about the particle shape increases. For polydisperse solutions, QELS can also be used to determine approximate particle size distributions (see A.3 Signal Processing).

Note that this technique is different from classical light scattering, which averages out the fluctuations in the signal and uses time averaged intensity $\langle I_s \rangle$ to determine molecular weight and radius of gyration (from angle dependent intensity measurements). Classical light scattering is particularly useful for monodisperse solutions, since information about sample polydispersity is lost during signal averaging. When combined with an appropriate

separation technique (such as GPC or HPLC), classical light scattering can be used to determine molecular weight distributions by calculating weight averaged molecular weights for the individual peaks.

A.2 Scattering from a Single Particle

For spherical particles, scattering intensity can be represented by (Weiner, 1984)

$$\langle I \rangle_i \propto N_i M_i^2 P_i(\theta) \qquad \{A4\}$$

where N = number of particles

M = particle mass

$P(\theta)$ = general form factor at the scattering angle θ

It follows that scattering intensity is proportional to particle diameter **d** raised to the sixth power, which is why high intensity laser sources are required to measure submicron-size particles. This dependance of scattering intensity on $\mathbf{d}^6$ also explains why this analysis is extremely sensitive to dust content in the sample: the signal contributed by these large particles can be so strong as to completely overshadow the scattering from the small particles being examined. The scattering also depends upon the differential refractive index (dn/dc) of the particle with the surrounding medium and concentration (i.e., a large dn/dc facilitates the detection of a particle in a given solvent, while if the ratio is not sufficiently big, it becomes impossible to distinguish between the signal emanating from solvent and solute molecules).

Let's consider the case of a polydisperse solution of Zn-insulin monomers, dimers and hexamers. The total scattering intensity is

$$I_{total} = I_{mon} + I_{dim} + I_{hex} \qquad \{A5\}$$

where $I_{mon} \propto N_{mon} x (d_{mon})^6$

$I_{dim} \propto N_{dim} x (d_{dim})^6$

$I_{hex} \propto N_{hex} x (d_{hex})^6$

At 0.6 mg/ml, there were 2.5×10^{15} monomers, 6.4×10^{14} dimers and 9.4×10^{15} hexamers per ml of insulin solution, with diameters of 2, 3 and 5 nm, respectively (Blundell et al., 1972). Thus, the relative scattering of monomer to hexamer molecules could be calculated as

$$\frac{I_{mon}}{I_{hex}} \cong \frac{2.5 \times 10^{15} x (2)^6}{9.4 \times 10^{15} x (5)^6} \cong 0.001 \qquad \{A6\}$$

and the relative scattering of dimeric to hexameric particles as

$$\frac{I_{dim}}{I_{hex}} \cong \frac{6.4 \times 10^{14} x (3)^6}{9.4 \times 10^{15} x (5)^6} \cong 0.003 \qquad \{A7\}$$

This clearly illustrates that the signal from solutions of Zn-insulin was dominated by the scattering caused by the hexameric species, as was evident from the peak centered at 5 nm (Figure 4.5).

The miniscule contributions to total scattering intensity by the dimers and monomers (0.3 and 0.1%, respectively) explain why it was impossible to distinguish among these species in solutions of Zn-insulin.

A.3 Signal Processing

The time-dependent intensity fluctuations detected by the photomultiplier can be represented by a second-order intensity autocorrelation function (Yarmush et al., 1988)

$$G^{(2)}(\tau) = \langle I(t)I(t+\tau)\rangle = \lim_{t\to\infty} \frac{1}{T}\int_0^T I(t)I(t+\tau)dt \qquad \{A8\}$$

where T is the duration of the experiment, and $G^{(2)}(\tau)$ is the time average intensity evaluated at various values of τ. In practice, the integral in equation A8 is approximated by a sum of discrete values (i.e. the number of photons n_i counted by the photomultiplier during a time interval $\Delta\tau$) (Yarmush et al., 1988). A8 can then be represented by (Stock and Ray, 1985)

$$G^{(2)}(j\Delta\tau) = \lim_{N\to\infty} \frac{1}{N}\sum_{i=1}^{N} n_i n_{i+j} = \langle n_i, n_{i+j}\rangle \qquad \{A9\}$$

where $\Delta\tau$ is the sample time, N the total number of samples, and $\tau=j\Delta\tau$ the autocorrelation delay time, with j taking integer values from 1 to m (m being the number of correlator channels). A normalized second

order intensity autocorrelation function is obtained by dividing $G^{(2)}(\tau)$ by the baseline value

$$g^{(2)}(\tau)=\frac{G^{(2)}(\tau)}{G^{(2)}(\infty)}=\frac{\langle n_i, n_{i+j}\rangle}{\langle n\rangle^2} \qquad \{A10\}$$

In this study, $G^{(2)}(\tau)$ was determined using the first 135 channels of the autocorrelator, while the baseline $G^{(2)}(\infty)$ was measured by the last 6 of 8 channels delayed by 1024 sample periods (Brookhaven, 1986).

For a dilute solution of independently scattering macromolecules, the electric field of the scattering light is a Gaussian random process (Yarmush et al., 1988), for which the Siegert relation

$$g^{(2)}(\tau) = 1 + \beta| g^{(1)}(\tau) |^2 \qquad \{A11\}$$

may be derived; (β is an experimental variable ($0<\beta<1$) limited by the signal-to-noise ratio of the measurement system) (Stock and Ray, 1985).

A.3.1 Polydispersity Analysis

If the Siegert relation applies, the electric field autocorrelation function can be represented as a sum or a distribution of single exponentials:

$$g^{(1)}(\tau)=\int_0^\infty G(\gamma)\exp(-\gamma\tau)d\gamma \qquad \{A12\}$$

The distribution $G(\gamma)d\gamma$ is the fraction of the total integrated intensity scattered by particles which obey $\gamma = \mathbf{D}\,q^2$ within the interval $d\gamma$, where $\mathbf{D}$ is the diffusion coefficient and q is the scattering vector defined in [equation A2]. For spherical particles, the radius is related to diffusion coefficient through the Stokes-Einstein formula [equation A3].

To find a size distribution from [QELS] data, one must transform the measured intensity autocorrelation function into an electric field autocorrelation function using equations [A10 and A11]. Then the task of each of the data interpretation methods is to invert equation [A12] to obtain $G(\gamma)$ from electric field autocorrelation data $g^{(1)}(\tau)$.

Unfortunately, the inversion of the integral equation [A12] is in general an ill-posed problem. There are, in principle, an infinite number of solutions $G(\gamma)$ which will satisfy equation [A12] to within experimental error present in $g^{(1)}(\tau)$. Hence, it is necessary to put some reasonable constraints on $G(\gamma)$ in order to find a unique solution. Two strategies are useful: (i) incorporation of a priori information [i.e., non-negativity of $G(\gamma)$], and (ii) parsimony, which requires that the simplest of all possible solutions be chosen. (Stock and Ray, 1985).

The polydispersity analysis can resolve between two populations of particles only if their ratio of sizes exceeds 1.5 (Brookhaven, 1986). The determination of size distribution is further complicated by the presence of non-Rayleigh scatterers (particles larger than $\lambda/10$), when back-scattering and other interference effects must also be considered (Degiorgio, 1980). These effects can be taken into account by evaluating the θ-dependent form factor $P(\theta)$

(see equation A4) using the relationship derived by Gustave Mie in 1908. The complete treatment, which includes the distortion of the electric field of the incident radiation produced by the difference between the refractive index of the particles and the solvent, is exceedingly complicated (Tanford, 1961).

In this work, particle size distributions were determined using Non-Negatively Constrained Least Squares (NNLS) and Constrained Regularization (CONTIN, of Provencher) algorithms. For a detailed discussion and comparison of the two methods, please see (Stock and Ray, 1985). In general, there was good agreement between the results of these inversion schemes. However, since CONTIN used parsimony in choosing a solution, it often smoothed out the particle size distribution (i.e., instead of reporting peaks at 5 and 30 nm, only one broad peak (4-40 nm) centered at 15 nm was detected). The NNLS method was particularly useful for multimodal distributions with narrow, widely spaced peaks, which was the case in this study (i.e. native peaks at 5 nm and intermediate peaks at 170±20 nm). Nevertheless, the uncertainty about the shape of the intermediate species prevented a quantitative determination of its size and weight distribution.

A.4 Concluding Remarks

It is important to emphasize that the accuracy of the inversion algorithms depends on the quality of the measured autocorrelation function. To ensure the reliability and reproducibility of QELS measurements, great care was taken to prepare dust-free samples, to control sample temperature, to increase the signal-to-noise ratio, and

to choose appropriate duration and sample times. For polydisperse samples, multiple sample times were used (ranging from 0.5 to 40 μsec), so that the autocorrelation function contained sufficient information about both small (5 nm) and large (~200 nm) particles present in solution.

QELS analysis can be a powerful tool for studying physical properties of macromolecules. However, it is important to remember the limitations of this technique and to refrain from overinterpreting the results of light scattering measurements (particularly when a polydispersity analysis is involved). It is advisable to measure particle sizes using other techniques (i.e., SEM, HPLC, etc.) to confirm QELS results. In this study, the sizes of native insulin species were known from the literature, and QELS measurements were found to be in good agreement with the reported values. However, the intermediate species were present in such low concentrations (less than 0.1wt%) that detection by other methods was impossible. The formation of particles larger that the 5 nm hexameric species was evident not only from the size-distribution analysis, but also from the shape of the autocorrelation function, the increased scattering intensity, and the need for larger sample times. The fact that the intermediates were detected in all aggregating insulin solutions also indicated that the peak at ~170 nm was not an artifact of the inversion algorithm, but represented a real, physical entity.

APPENDIX B

SENSITIVITY ANALYSIS

It was of interest to determine model sensitivity to changes in the values of kinetic and thermodynamic parameters. To identify the reaction steps most affected by perturbations, computer simulations were performed using various magnitudes of a particular rate or equilibrium constant. This analysis provided additional insights into the nature of the aggregation process, because it allowed a separate evaluation of the contributions of different pathways to the overall pattern of instability.

None of the simulated perturbations changed the shape of the kinetic profile, i.e., the initial period of stability, followed by a slightly sigmoidal curve. However, the duration of the induction period, the steepness of the slope, and the relative durations of the two phases of aggregation did change.

B.1 Model Sensitivity to Fitted Kinetic Parameters

The kinetic parameters $k_{U\text{-}form}$ and k_{agg} were determined based on the results obtained with aqueous Zn-insulin solutions agitated at 37°C and 80 rpm, in the presence of 10 Teflon spheres. Since these empirically fitted parameters differed only by an order of magnitude, it was impossible to significantly change one without compromising the internal consistency of the proposed aggregation mechanism. However, as long as these constants remained within the specified order of magnitude range, it was possible to test model

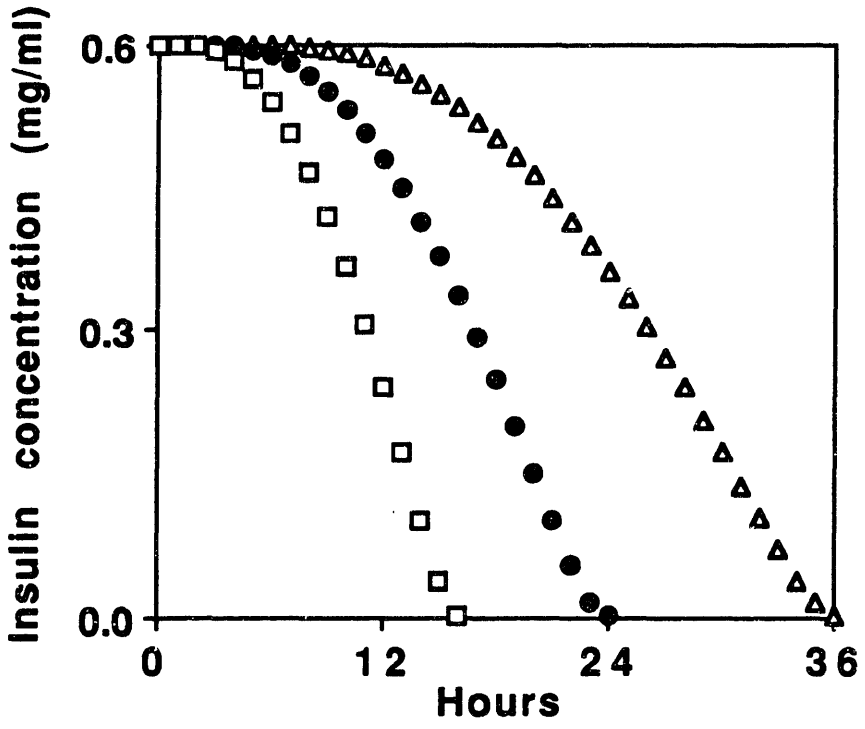

Figure B.1: Model sensitivity to changes in the rate constant of intermediate formation ($k_{U\text{-}form}$). Computer simulations were done using: the model value (●), 1/2 of the model value (Δ), and twice the value used in the model (□).

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sensitivity to such variations. Changes in $k_{U\text{-}form}$ affected the duration of the induction period and the slope of the curve (Figure B.1). This reflected the fact that the aggregation rate was affected both by the rate of intermediate formation and the concentration of the intermediate species.

These effects would have been less pronounced if the size of the intermediate species had been on the order of 100 (rather than

10) molecules. The value of the pseudo-rate constant used in the model was significantly lower than the actual value (which could not be estimated because of the uncertainty about the size and shape of the intermediate species). Thus, it took the same amount of time to reach the critical size U_{10} in the model as it did to reach ~U_{100} in the actual reaction. The sensitivity analysis illustrated that underestimating the value of $k_{U\text{-}form}$ affected the overall kinetic pattern to a greater extent than overestimating this rate constant.

Variations in the rate of aggregate formation (k_{agg}) had very little effect on the kinetic profile (Figure B.2). Changes in the duration of the induction period were barely noticeable, but the steepness of the slope changed slightly, while the overall pattern of aggregation remained the same. Since the steepness of the slope represented the rate of aggregation following the formation of the intermediate species, it was not surprising that changes in k_{agg} affected this portion of the kinetic profile.

Because of the uncertainty about the actual values of the kinetic constants, the model was used to make only qualitative predictions about Zn-insulin aggregation. This sensitivity analysis confirmed that the overall pattern of the reaction was independent of the values of the fitted rate constants, and that mathematical modelling could be used to identify trends in insulin aggregation.

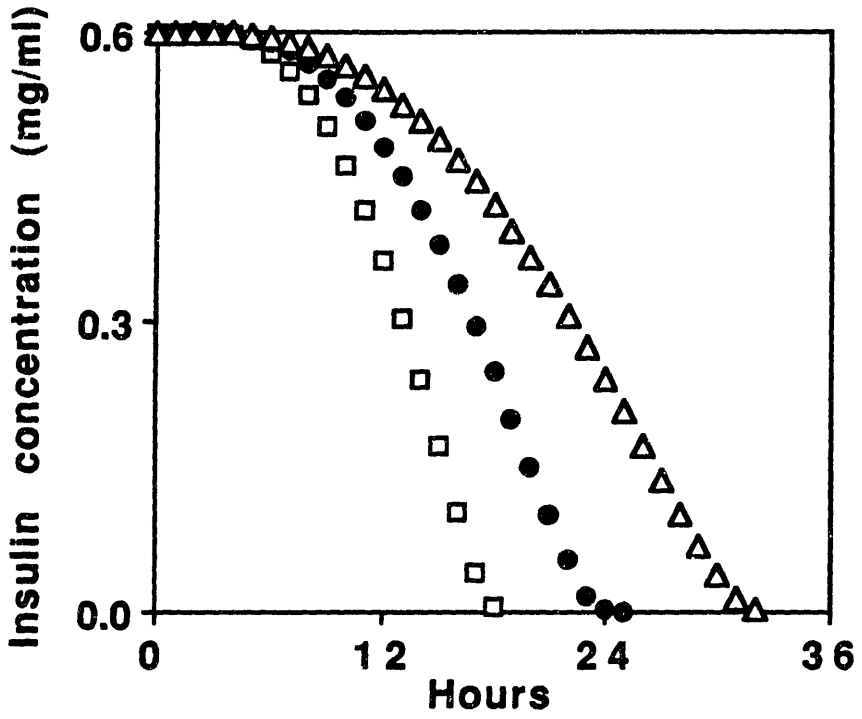

Figure B.2: Model sensitivity to changes in the rate of aggregate formation (k_{agg}). Computer simulations were done using: the model value (●), 1/2 of the model value (Δ), and twice the value used in the model (□).

B.2 Model Sensitivity to Changes in Equilibrium Constants for Self-Association

The contributions of dimer and hexamer formation to the stability of aqueous insulin solutions were examined by varying the equilibrium constants K_{dim} and K_{hex}. These thermodynamic parameters were based on values reported in the literature, and were extrapolated to experimental conditions used in this study. The

sensitivity analysis indicated how the uncertainty about the actual magnitude of these parameters affected the pattern of aggregation. Results of computer simulations using various values of the dimerization constant are presented in Figure B.3. Changes of plus or minus 10% in K_{dim} used in the proposed model had very little effect on the kinetic profiles: the induction period remained virtually unchanged, while the slope was only slightly affected, becoming less steep when the dimerization constant was increased by 10%. These

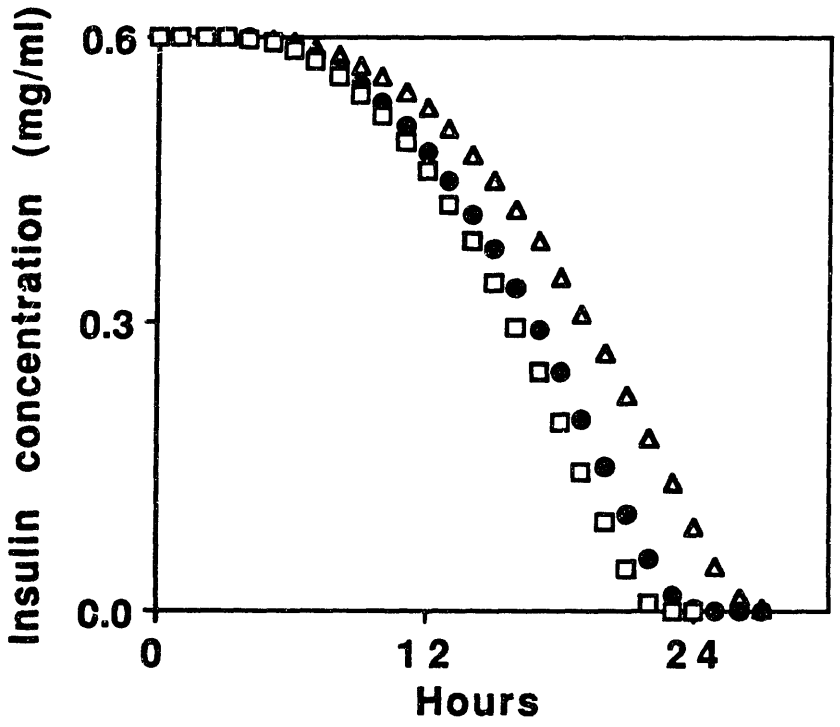

Figure B.3: Model sensitivity to changes in the equilibrium constant of dimer formation (K_{dim}). Computer simulations were done using: the model value (●), the model value +10% (Δ), and the model value -10%(□).

results indicated that the extent of dimerization directly affected insulin stability, since this was the main route for decreasing monomer concentration.

Results of computer simulations using various values of the hexamerization constant are presented in Figure B.4. When this thermodynamic parameter was increased or decreased by 50% of the original value used in the model, the shape of the kinetic profile remained unchanged, the duration of the induction period was not affected, and only the slope of the curved portion varied slightly. Smaller values of K_{hex} resulted in a slightly steeper slope, since a decreased concentration of hexamers coincided with increased populations of dimers and monomers -- which were directly involved in aggregate formation (step 6 in the reaction scheme, Chapter 5). Similarly, larger values of K_{hex} reduced monomer and dimer concentrations, thereby slowing down the rate of aggregation. However, these effects were very small, and changes in the kinetic profile were hardly noticeable as long as K_{hex} remained within the specified order of magnitude range.

Simulations of Na- and Zn-insulin (Figure 5.2) indicated that when K_{hex} was changed by several orders of magnitude, both the induction period and slope steepness were significantly affected. This suggested that hexamerization played an important role in increasing insulin solution stability, both by reducing dimer and monomer concentrations, and by decreasing the number of destabilizing (unoccupied) interfacial sites. Although the extent of hexamer formation directly affected the rate of monomer

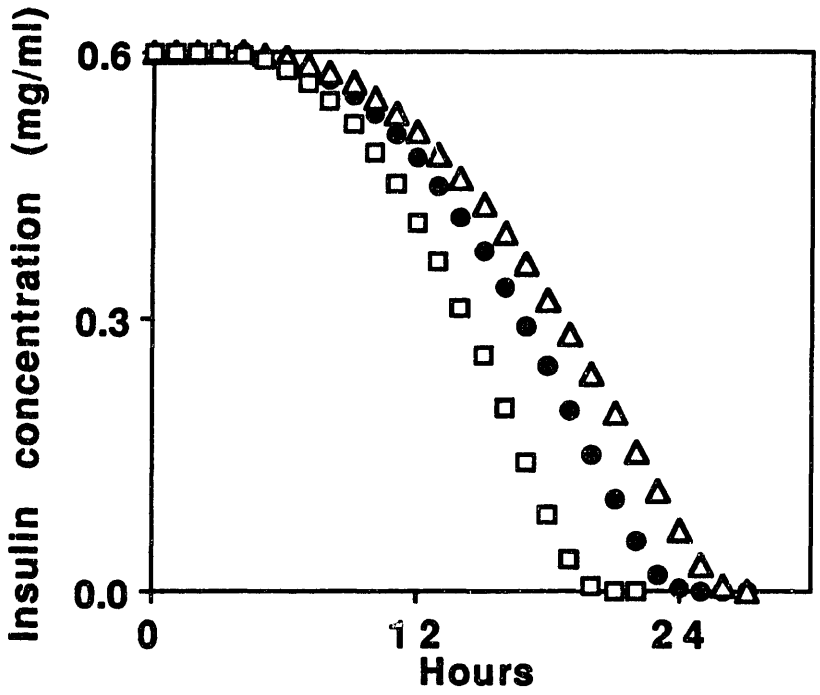

Figure B.4: Model sensitivity to changes in the equilibrium constant of hexamer formation(K_{hex}). Computer simulations were done using: the model value (●), the model value +50% (Δ), and the model value -50%(□).

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denaturation and subsequent aggregation, for the purpose of predicting trends in insulin aggregation behavior, it was sufficient to know K_{hex} with ±50% accuracy.

B.3 Model Sensitivity to Changes in the Equilibrium Constant of Denaturation

The agitation dependence studies and experiments with polypropylene (Chapter 4) pointed to the importance of K_{denat}, which contained information about mass transport to the solid-liquid interface, shear effects, and destabilizing surface interactions. The sensitivity analysis confirmed these conclusions; while variations in K_{denat} did not change the shape of the kinetic profile, both the duration of the induction period and the steepness of the slope were affected (Figure B.5).

When K_{denat} was doubled, the total reaction time similarly increased from 24 to 48 hours (the induction period became longer and the slope less steep). A 50% reduction in the value of this parameter led to a decreased induction period and a steeper slope, while the total reaction time was shortened to 12 hours. These were by far the most dramatic changes in the simulated kinetic profiles, suggesting that monomer denaturation at the hydrophobic interface was the rate limiting step in the aggregation reaction. This analysis also identified this reaction step as the one most sensitive to perturbations, and therefore the best candidate for stabilization strategies. Stabilizing reagents aimed at reducing the value of K_{denat} (either by reducing mass transfer and shear effects, or by modifying the nature of interfacial interactions) would have the most profound effect on overall solution stability. Based on this sensitivity analysis, nonionic surfactants (which prevented monomer denaturation by occupying the destabilizing interfacial sites and minimizing the number of unfavorable contacts between insulin and the

hydrophobic surface) were chosen to prevent insulin aggregation in agitated aqueous solutions containing an air-water interface.

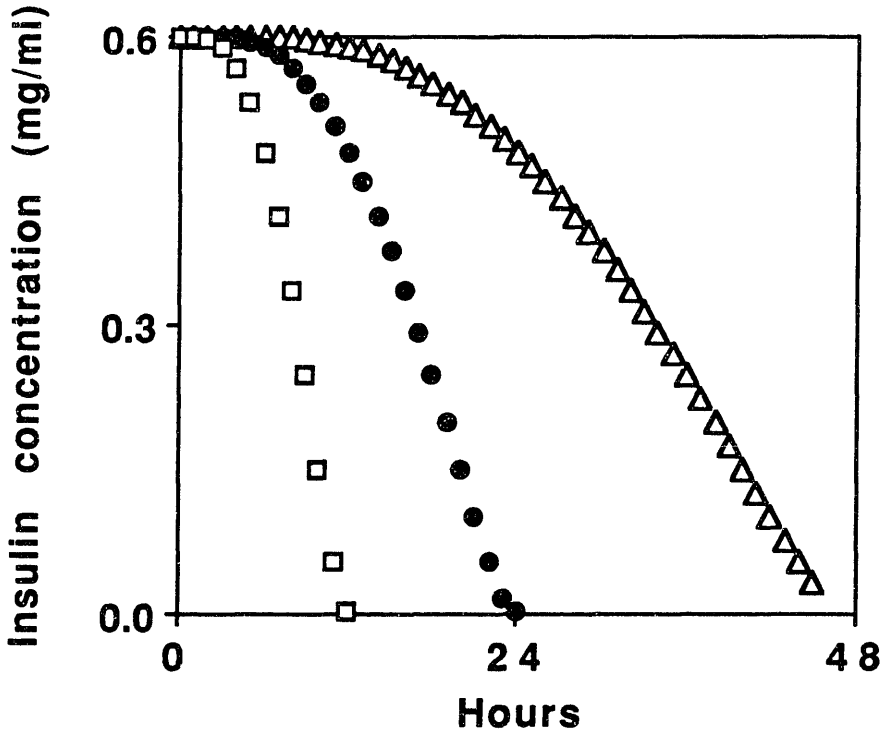

Figure B.5: Model sensitivity to changes in the equilibrium constant of denaturation (K_{denat}). Computer simulations were done using: the model value (●), 1/2 of the model value (Δ), and twice the value used in the model (□).

B.4 Model Sensitivity to Changes in the Size of Intermediate Species

In the proposed kinetic scheme, it was assumed that once the intermediate species reached the critical size U_{10}, they had enough surface area for stability and could interact with native monomers and dimers (Chapter 5). Results of QELS analysis indicated that the critical size was reached when some 100 unfolded monomers combined to form a stable intermediate species (this rough estimate of the intermedite's size is based on the 2 nm diameter of the insulin monomer (Blundell et al, 1972)). However, because of the uncertainty about the shape of the intermediate species and the low resolution of light scattering measurements, the actual number of steps required to reach the critical size could only be estimated. For modelling purposes, the exact size of U_n was not important, since the characteristic shape of the kinetic profile could be simulated using a any number (>1) of association steps to represent the delayed onset of aggregation.

To illustrate this point, the number of association steps needed to reach a critical size was varied from 4 to 14 (i.e., U_n=U_5 and U_{15}, respectively). As can be seen from Figure B.6, these changes had very little effect on the overall kinetic profile: the induction period decreased by approximately one hour when U_5 was used instead of U_{10} to represent the critical size; likewise, when 14 association steps were required to reach U_n, the lag time increased by approximately one hour. The slope of the curve remained unchanged during all three simulations. This confirmed that the general shape of the kinetic profile was independent of the number of association steps,

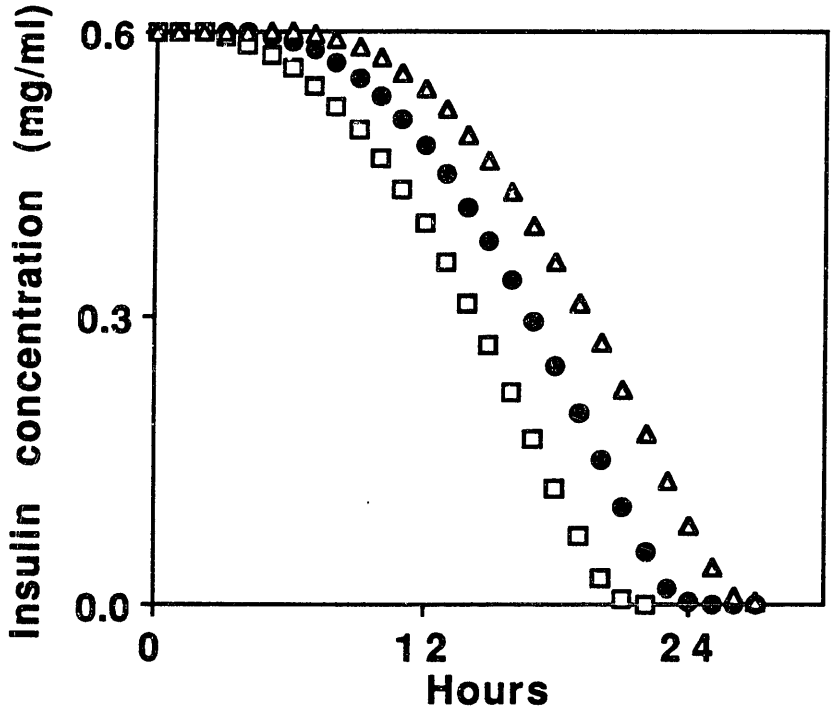

Figure B.6: Model sensitivity to changes in the critical size of the intermediate species. Computer simulations were done using U5 (□), U10 (●) and U15 (Δ) to represent the critical size.

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and that the assumption of a smaller critical size did not compromise the model's ability to make qualitative predictions about insulin aggregation.

However, better quantitative predictions could probably be made if the size of the intermediate species used in the model was closer to the size observed using QELS analysis. For example, a better agreement between computer simulations and experimental results of concentration dependence was obtained when the kinetic

parameters were determined based on 14 rather than 9 association steps (Figure B.7). So while this simplifying assumption did not hinder the model's ability to predict trends in insulin aggregation behavior, quantitative modelling of the reaction scheme was limited by the uncertainty about the size and shape of the intermediate species.

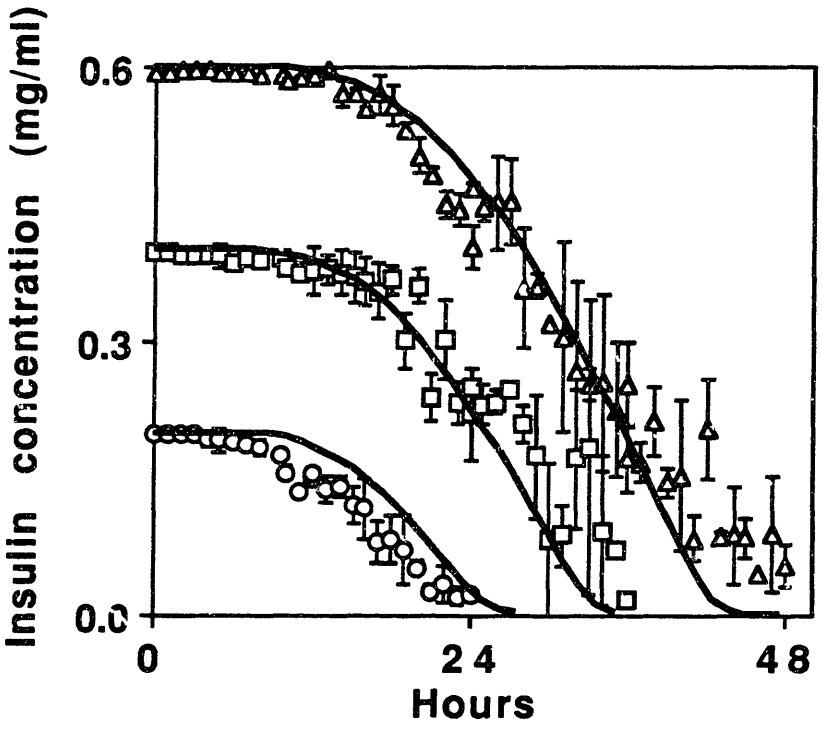

Figure B.7: Computer simulations of concentration dependence of Zn-insulin aggregation (37°C, 80 rpm, 5 Teflon spheres) using U_{15} as the critical size of the intermediate species. Results of computer simulations are represented by solid lines. See Chapter 5 for simulations using U_{10} as the critical size.

B.5 Model Sensitivity to Insulin Adsorption at Hydrophobic Surfaces.

The concentration dependence of insulin aggregation was attributed to dimer and hexamer adsorption to and occupation of hydrophobic interfacial sites. It was shown in Table 5.1 that in the absence of adsorption, increased insulin concentrations would lead to more rapid aggregation, since the driving force for monomer denaturation would be elevated in proportion to the increase in monomer concentrations. To further illustrate this point, the concentration dependence of insulin aggregation was simulated assuming no hexamer adsorption to Teflon (Figure B.8). The results confirmed that in the absence of hexamer adsorption, higher insulin concentrations would result in faster aggregation. If only hexamer adsorption was eliminated from the model, solution half-lives increased proportionally with total protein concentrations ($t_{1/2}$= 19, 18, and 16 hours for initial concentrations of 0.6, 0.4 and 0.2 mg/ml), but mathematical simulations were in poor agreement with experimental results, and underpredicted solution stability at higher concentrations. The good agreement between the experimental results and computer simulations at 0.2 mg/ml suggested that the adsorption coefficient used in the model overpredicted insulin adsorption at lower concentrations.

This analysis underscored the destabilizing effects of monomer interactions with hydrophobic surfaces, and supported the hypothesis that greater stability at higher concentrations was due to dimer and hexamer interactions with interfacial sites. It also indicated that dimer adsorption alone was not sufficient to explain

increased solution stability, and that hexamer formation not only reduced the number of unstable monomeric species, but also contributed to excluding monomers from unfavorable interactions with the hydrophobic surface.

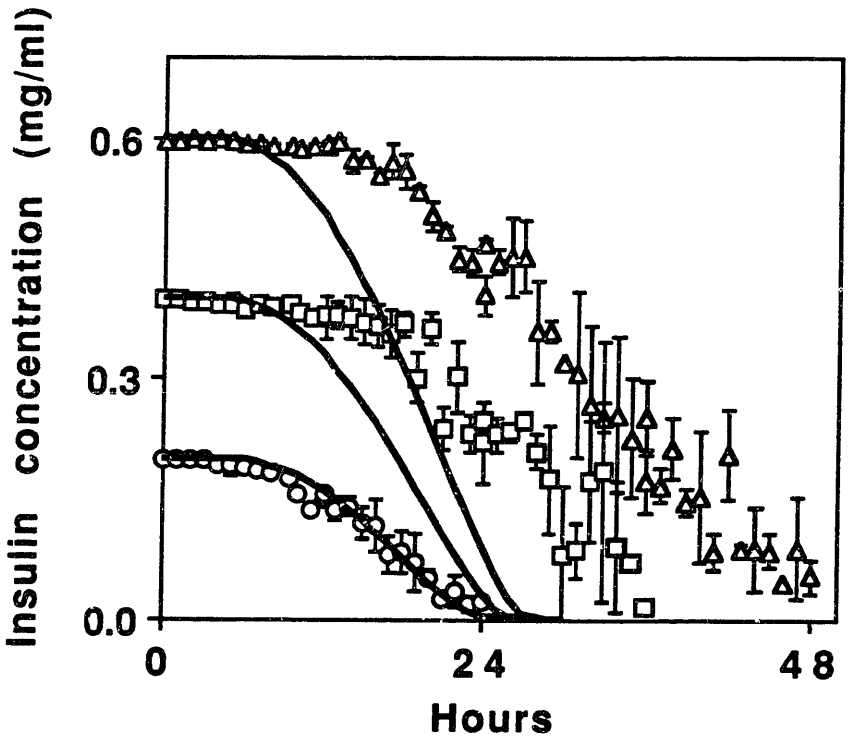

Figure B.8: Effects of hexamer adsorption to Teflon on the stability of Zn-insulin solutions. Comparison of experimental results (symbols) and computer simulations (solid lines) of the concentration dependence of Zn-insulin aggregation assuming no hexamer adsorption. See Figure 5.3 for simulation of Zn-insulin concentration dependence in the presence of dimer and hexamer adsorption to Teflon.