Immobilization of Urokinase on Agarose Matrices

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Abstract □ Immobilization of urokinase, a plasminogen activator, was carried out to determine the effect of spacer length used on the immobilized enzyme activity. The enzyme was covalently coupled to agarose gel, both directly to the matrix and also *via* interposing different lengths of spacer groups. The specific activity of immobilized urokinase increased as the spacer length (n') increased to a certain length and tended to decrease thereafter. The maximal activity was shown when the value of n' was 7 for the agarose-NH-$(CH_2)_n$-CO-NH-$(CH_2)_2$-CO-NH-urokinase series. The coupling yield of the enzyme activity was from 33 to 68% depending on various forms of immobilized urokinase. The immobilized urokinase was characterized with regard to pH, temperature, storage, and thermal stabilities.

Keyphrases □ Urokinase—immobilization on agarose matrices, effect on spacer length, enzyme activity □ Agarose—immobilization of urokinase on matrices, effect of spacer length, enzyme activity □ Immobilization—urokinase on agarose matrices, effect of spacer length, enzyme activity

Urokinase, a plasminogen activator, has been used for the treatment of thrombosis and/or vascular obstruction. For the purpose of thrombolytic therapy the immobilization of urokinase was attempted (*a*) to increase the *in vivo* half life, (*b*) to obtain local fibrinolytic activity for thromboembolic disorders, and (*c*) to develop antithrombogenic materials (1–6). Previous results suggested the potential clinical application of the immobilized urokinase (2–6).

Urokinase has been immobilized (1, 2) onto agarose matrices using the cyanogen bromide activation technique. Polyamide has been used as a matrix for the immobilization of urokinase (3, 5, 6) and other studies have utilized polyamide, polyester, polyvinyl chloride, polyurethane, and polydimethyl siloxane for this purpose (4, 6). The results demonstrated that the immobilized urokinase activated plasminogen. In a previous study streptokinase, a metabolic product of β-hemolytic *Streptococci* was immobilized for this purpose (7).

Steric considerations in the immobilization of bioactive agents appear to be important. It has been reported that urokinase immobilized onto a polyethyleneimine copolymer *via* a nitrophthalic acid spacer group showed better fibrinolytic activity than urokinase immobilized directly onto polyamide (4). Recently, researchers (8) immobilized heparin on polymers using different spacer group lengths. When immobilized, heparin activity increases rapidly, beginning with 8 carbon diaminoalkane spacer groups. In this work, the immobilization of urokinase onto an agarose surface was carried out by varying the spacer length between the enzyme and substrate.

EXPERIMENTAL

Materials—Crude urokinase was prepared from fresh human urine and human Cohn fraction III[1]. Crude urokinase, adsorbed onto silica gel, was extracted with 30% ethanol followed by dialysis and freeze-drying. Human plasminogen was purified from human Cohn fraction III according to a previous method (9) by using a lysyl-agarose affinity column. Human plasmin was prepared by eluting plasminogen into the column containing immobilized urokinase. One hundred milliliters of plasminogen solution, consisting of 0.5 CTA[2] units/ml of 0.1 *M* tromethamine hydrochloride buffer (pH 7.5), was passed through the column at a flow rate of 0.3 ml/min. The eluted plasmin was used for the standard curve calibration of the urokinase assay. Bovine serum albumin, α-casein, α-aminobutyric acid, ε-aminocaproic acid, and ω-aminocaprylic acid[3] were used as received. Glycine, *N*-hydroxysuccinimide, β-alanine[4], 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (I), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[5] (II) were used as received. Agarose beads (III)[6] were used as the substrate for urokinase immobilization. (All other reagents were commercially available reagent grades.)

Urokinase Immobilization—*Direct Coupling of Urokinase on the Matrix*—Compound III was activated with cyanogen bromide according to a previously described procedure (11). A 10-ml slurry of washed III, consisting of equal volumes of the gel and water, was added to 10 ml of 2 *M* sodium carbonate and gently mixed. One gram of cyanogen bromide in 0.5 ml of acetonitrile was then added, and the slurry was stirred for 2 min. The cyanogen bromide-activated beads were then collected by filtration on a glass filter and washed successively with 100 ml each of cold 0.1 *M* sodium bicarbonate and 0.1 *M* sodium borate buffer.

To 10 ml of an activated slurry of III was added 50 mg of urokinase (1500 CTA units) in 20 ml 0.1 *M* sodium borate buffer (pH 7.5) containing 0.05 *M* HCl and 50 m*M* $CaCl_2$, which was then gently swirled overnight at 4°. The beads were collected on a coarse glass filter and washed with 300 ml each of cold water, 0.5 *M* NaCl, 2 *M* urea, and 0.1 *M* sodium borate buffer.

Coupling of Diaminoalkanes to Cyanogen Bromide-Activated III—Ten milliliters of activated III, prepared as previously described, was suspended in 10 ml of cold 0.1 *M* sodium bicarbonate, pH 9.0, and mixed with 10 ml of the various 1 *M* α,ω-diaminoalkane solutions which had been previously adjusted to pH 9.0 with 6 *N* HCl. The coupling was carried out overnight at 4° by stirring with a synchronized stirrer. After the reaction was completed, III was again sequentially washed with cold water, 0.1 *M* $NaHCO_3$, 0.05 *M* NaOH, water, 0.1 *M* CH_3COOH, and water.

Coupling of Urokinase to ω-Aminoalkyl-III with I and II—Ten milliliters of each of the above aminoalkyl-III beads was suspended in 10 ml of 0.1 *M* borate buffer solution, pH 7.5, containing 40 mg of I or II and 50 mg of urokinase (1500 CTA units). The reaction was continued overnight at 4°. Compound III was filtered and thoroughly washed with cold water, 0.5 *M* NaCl, and 2 *M* urea. The final immobilized urokinase was stored in 0.9% NaCl solution at 4° until use.

Coupling of Aminoalkyl Carboxylic Acids to Cyanogen Bromide-Activated III—Aminoalkyl carboxylic acid-coupled III was prepared according to the procedure for diaminoalkane coupling described above. Ten milliliters of the various 1 *M* aminoalkyl carboxylic acids was added to 10 ml of cyanogen bromide-activated III. The other procedures were similar to those described for diaminoalkane coupling.

Coupling of Urokinase to Carboxylic Alkyl-III with N-hydroxysuccinimide—The ω-carboxylic alkyl-III beads were activated with *N*-hydroxysuccinimide according to previous procedures (12, 13).

Ten milliliters of each washed carboxylic alkyl-III, which was prepared as previously described, was washed with anhydrous dioxane extensively to obtain anhydrous conditions. Compound III was suspended in dioxane to make a total volume of 30 ml. The *N*-hydroxysuccinimide, 346 mg, was

[1] Korea Green Cross Corp., Seoul, Korea.

[2] As defined by the Committee on Thrombolytic Agents from Ref. 10.
[3] Sigma Chemical Co., St. Louis, MO 63178.
[4] Aldrich Chemical Co., Inc., Milwaukee, WI 53233.
[5] Pierce Chemical Co., Rockford, IL 61105.
[6] Sepharose 4B, Pharmacia Fine Chemicals, Piscataway, NJ 08854.

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Table I—Comparative Results of Various Immobilized Urokinases on III

	Spacer length, n'	Activity, CTA units/ml, wet gel	Specific Activity, CTA units/mg, bound urokinase
Direct-coupled urokinase (III-urokinase)	0	56	14.4
III—NH—$(CH_2)_n$—C(=O)—NH-urokinase			
$n = 2$	3	49	33.6
$n = 3$	4	52	37.0
$n = 5$	6	112	45.0
$n = 7$	8	89	31.6
III—NH—$(CH_2)_n$—C(=O)—NH—$(CH_2)_2$—C(=O)—NH-urokinase			
$n = 2$	6	78	56.2
$n = 3$	7	102	64.0
$n = 5$	9	93	42.4
$n = 7$	11	84	41.4

added to the gel suspension to obtain 0.1 *M* concentration, an equal molar quantity of dicyclohexylcarbodiimide was then added and the suspension was stirred gently for 70 min at room temperature. Activated III was washed with dioxane, followed by washing with methanol and dioxane. After drying the gels briefly in air, slightly moist cakes of the activated gels were subjected to coupling. Each gel was added rapidly to 10 ml of 0.1 *M* sodium borate buffer, pH 7.5, which contained 50 mg of urokinase. After stirring for 8 hr at 4°, 750 mg of glycine was added to react any remaining *N*-hydroxysuccinimide-activated spacer groups. After 2 hr, the gels were sequentially washed with cold deionized water, 2 *M* urea, and 0.5 *M* NaCl.

Coupling of β-Alanine to Carboxylic Alkyl-III—β-Alanine-coupling reaction was conducted for 8 hr as described in the previously mentioned experiment. β-Alanine (890 mg) was mixed with 10 ml of each *N*-hydroxysuccinimide-activated alkyl-III.

Coupling of Urokinase to β-Alanine-Linked III—Ten milliliters of each β-alanine-linked III was activated with *N*-hydroxysuccinimide as previously described. Urokinase (10 mg) was coupled to 2 ml of each β-alanine-linked III.

Determination of Urokinase Bound to III—To determine the amount of the immobilized urokinase on III[6] the amino acid content was determined by an auto amino acid analyzer after the immobilized urokinase, on the various gel matrices, was hydrolyzed with 6 *N* HCl (13). Soluble urokinase was also subjected to amino acid determination for reference purposes.

One milliliter of packed gel volume of the various immobilized urokinase gels was filtered on a glass filter and washed thoroughly with distilled water, followed by amino acid hydrolysis with 2 ml of 6 *N* HCl in a sealed tube at 110° for 24 hr. After hydrolysis, the hydrogen chloride was neutralized with 0.1 *M* sodium citrate, and the amino acid solutions were concentrated by rotoevaporatization. The amounts of amino acids were determined with a fully automated amino acid analyzer[7]. With a comparison to soluble urokinase, the amount of urokinase bound to the various gels was calculated based on quantities of alanine, lysine, and arginine.

Procedures for Enzyme Activity Assays—The activities of urokinase, plasminogen, and plasmin were determined by the caseinolytic assay method (10). Thirty CTA units of urokinase was used in the experiment. The activity of the immobilized urokinase was also determined by the same methods, constantly stirring the immobilized enzyme with the incubation mixture using a water-immersed magnetic stirrer. Protein concentrations were determined according to a previous method (14) using bovine serum albumin as a standard. The medium in which the immobilized enzyme was stored was assayed and showed no urokinase activity due to possible leakage.

RESULTS

Immobilization of Urokinase—To minimize steric hinderance for improved plasminogen accessibility to the coupled urokinase, various immobilized systems were prepared interposing different spacer group

[7] Beckman Model 118-BL, Mountain View, CA 94043.

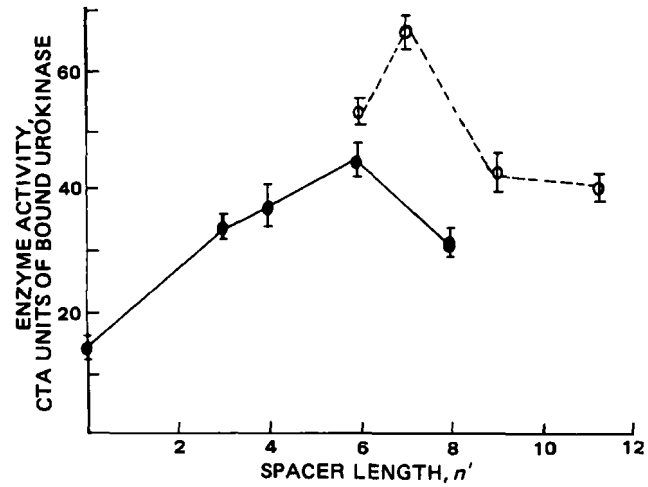

Figure 1—*Effect of spacer length on the activity of the immobilized urokinase. The length of spacer is represented by n' corresponding to: [—$(CH_2)_n$—C(=O)—] for III—NH—$(CH_2)_n$—C(=O) NH-urokinase series and [—$(CH_2)_n$—C(=O)—NH—$(CH_2)_2$—C(=O)—] for III—NH—$(CH_2)_n$—C(=O)—NH—$(CH_2)_2$—C(=O)—NH—urokinase series, respectively; (●—●) III—NH—$(CH_2)_n$—C(=O)—NH—urokinase series; (○ . . . ○) III—NH—$(CH_2)_n$—C(=O)—NH—$(CH_2)_2$—NH-urokinase series.*

lengths between III and the immobilized urokinase, as described. The results are summarized in Table I.

In the III—NH—$(CH_2)_n$—C(=O)—NH-urokinase series, the activity of immobilized urokinase increased as the length of spacer increased up to $n = 5$ ($n' = 6$). However, the highest activity was observed when $n = 3$ ($n' = 7$) in the III—NH—$(CH_2)_n$—C(=O)—NH—$(CH_2)_2$—C(=O)—NH-urokinase series. Above $n = 3$ ($n' = 7$) the activity decreased.

In immobilized urokinase activity experiments where it was immobilized onto diaminoalkane-derivatized III through carboxylic groups on the enzyme, it was found that the immobilized urokinase retained no activity. The loss in activity was found to be due to urokinase cross-linking by the carbodiimides during the immobilization step, resulting in the complete loss of enzyme activity.

The directly coupled urokinase ($n' = 0$) showed higher immobilization yields than those for III—NH—$(CH_2)_n$—C(=O)—NH-urokinase, when $n' = 3$ or 4. However, the enzymatic activity was much lower for the directly coupled urokinase relative to spacer arm mediated immobilized urokinase.

Figure 1 illustrates the effects of increasing spacer length (n') between the matrix and enzyme on the activities of the immobilized urokinase. The activity per milligram of bound enzyme was calculated from the data obtained by amino acid analysis of the immobilized urokinase. The enzymatic activity increased as spacer lengths extended to a certain length and tended to decrease thereafter. The maximum enzymatic activity was observed when $n' = 7$ for the III—NH—$(CH_2)_n$—C(=O)—NH—$(CH_2)_2$—C(=O)—NH-urokinase series.

Properties of the Immobilized Urokinase—Properties of the immobilized urokinase in terms of activity *versus* pH and temperature and the thermal stability were measured for III-urokinase and III—$(CH_2)_5$—C(=O)—NH-urokinase ($n' = 6$). Figure 2 illustrates the pH effects on the activity of urokinase in solution and the immobilized urokinase. The optimum pH of the soluble urokinase and III-urokinase was between 7.5 and 8.0, while III—$(CH_2)_5$—C(=O)—NH-urokinase was most active over a pH range from 7.5 to 9.0.

The effect of temperature on the activity of the immobilized urokinase was compared with that of the solution. The result indicated that the optimal temperature was 40° for both enzymes (Fig. 3).

Figure 4 shows the retention of the activity of immobilized urokinase in 0.9% NaCl solution at 4°. Both urokinase solution and III—NH—$(CH_2)_5$—C(=O)—NH-urokinase are stable in these storage conditions for 10 days without loss of activity, while III-urokinase (direct coupled urokinase) lost almost 40% of its original activity within 10 days. The urokinase immobilized with aminoalkane carboxylic acid spacer groups maintained well over 90% activity even after 60 days.

After heat treatment at 70° for 30 min, the urokinase solution lost activity completely, while III—NH—$(CH_2)_5$—C(=O)—NH-urokinase retained 45% of its activity by the same treatment.

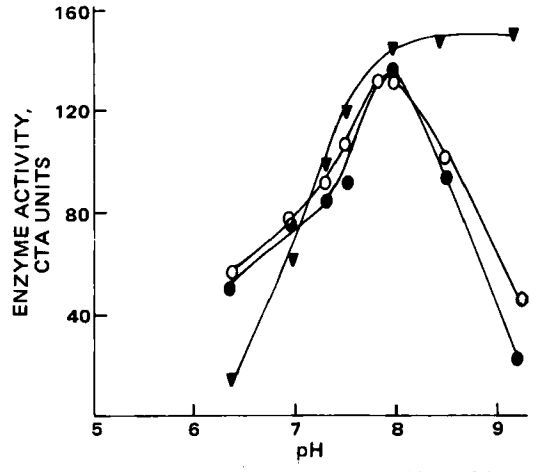

Figure 2—*Effects of pH on the activity of soluble and immobilized urokinase at 40°. Key: (○—○) soluble urokinase; (●—●) direct coupled urokinase (III-urokinase); (▼—▼) III—NH—$(CH_2)_5$—C(=O)—NH-urokinase.*

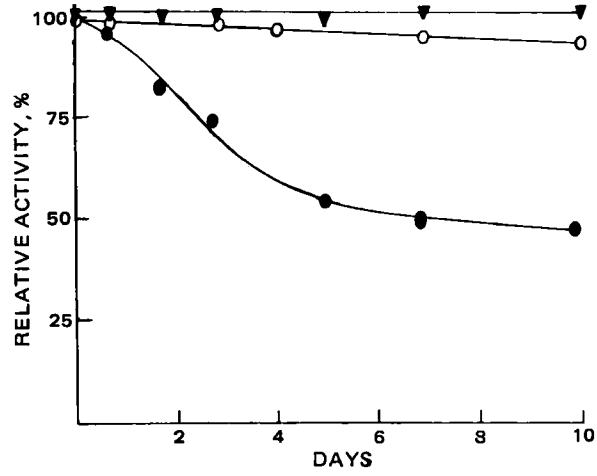

Figure 4—*Storage stability of soluble and immobilized urokinase. Key: (○—○) soluble urokinase; (●—●) direct coupled urokinase (III-urokinase); (▼—▼) III—NH—$(CH_2)_5$—C(=O)—NH-urokinase.*

DISCUSSION

Urokinase has been immobilized onto various matrices, and the results suggest a potential clinical use for thrombolytic therapy (2–6). Among them, polyamide, polyesters, and polyurethane-immobilized urokinase are useful clinically (3–6). Nevertheless, it can be said that an immobilized urokinase may experience steric hindrance from the polymer matrix. This has been postulated previously (4), where the polyamide–spacer–urokinase showed better fibrinolytic activity than the directly coupled polyamide–urokinase.

We first immobilized urokinase onto aminoalkyl-III which was prepared by a previously described method (13, 15). Unfortunately, unlike pronase (13), aminoalkyl-III-urokinase and III—NH—$(CH_2)_n$—NH—C(=O)-urokinase retained <5% of the initial urokinase enzymatic activity. It appears that urokinase was inactivated due to cross-linking by the carbodiimide I or II, which was used as a condensing agent in the immobilization step. We tested the inactivation of urokinase by treating free urokinase in solution with I or II in the presence or absence of aminoalkyl-III. In either case it was shown that urokinase retained no activity, whereas pronase activity remained when treated in a similar way.

Interposing spacer lengths (n') from 0 to 11, we have observed that the enzymatic activity of the immobilized urokinase increased as the spacer length (n') increased to a certain length, and tended to decrease thereafter (Table I, Fig. 1).

For a series of III—NH—$(CH_2)_n$—NH—C(=O)-urokinase, the highest activity was observed when $n' = 6$ (6.4 Å), while $n' = 7$ (9 Å) for a series of III—NH—$(CH_2)_n$—NH—C(=O)—$(CH_2)_2$—C(=O)—NH-urokinase resulted in maximum activity. Alternatively, direct attachment

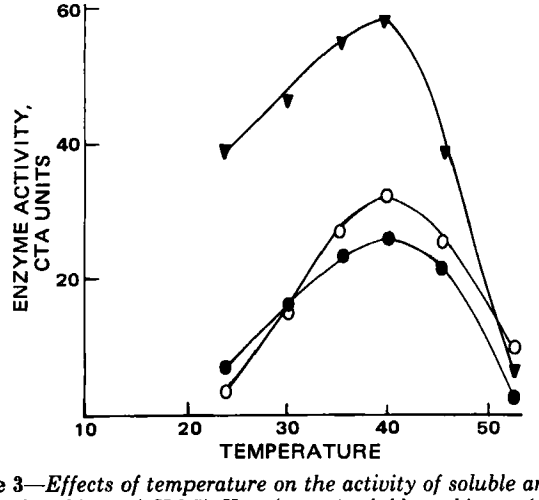

Figure 3—*Effects of temperature on the activity of soluble and immobilized urokinase (pH 8.5). Key: (○—○) soluble urokinase; (●—●) direct coupled urokinase (III-urokinase); (▼—▼) III—NH—$(CH_2)_5$—C(=O)—NH-urokinase.*

of urokinase to the matrix resulted in lower activity for plasminogen as shown in Table I. Differences of maximum enzymatic activity for the above two series of spacer arms are not clear and may be due to the different microenvironment of the hydrophilic polymers. An additional group from β-alanine provides a more hydrophilic environment which may introduce better accessibility of plasminogen to the immobilized urokinase.

As previously suggested (16), the dramatic effects associated with increasing the spacer length may in part be explained by relief of steric restrictions imposed by the matrix and in part by the increased flexibility and mobility of the urokinase as it protrudes further into the solvent phase. Plasminogen could then approach more readily the immobilized urokinase. This flexibility and folding of the spacer could also account for an apparent decrease in urokinase activity observed when the spacer lengths (n') >6 or 7 were interposed between the matrix and urokinase bond. This decreasing trend may be caused by the increased hydrophobicity of the spacer arms and/or rapid folding within immobilized urokinase by cohesive interactions.

The critical role of the matrix in the determination of accessibility of the coupled molecules to interacting macromolecule has been especially emphasized for affinity chromatography (15–17). A previous study (15) reported that a spacer length from 4 to 8 of interposed methylene groups (5–10 Å) elicited a substantial increase in enzyme binding affinities for kinases and pyrimidine nucleotide-dependent dehydrogenases with insoluble derivatives of adenosine triphosphate and nicotinamide adenine dinucleotide.

In conclusion, steric considerations appeared to be important in urokinase immobilization and maximum enzymatic activity was observed when the length of the spacer group was in the region of 6.4–9.0 Å.

The optimum pH with regard to the activity of soluble urokinase and III-urokinase was between 7.5 and 8.5. However, III—NH-$(CH_2)_5$—C(=O)—NH-urokinase showed maximum activity over a pH range from 7.5 to 9.0 (Fig. 2). The unchanged maximum activity for immobilized urokinase over the alkaline pH range may be due to unreacted carboxylic groups from aminoalkyl carboxylic spacers, which could neutralize the alkaline pH at the solvent–immobilized urokinase interface.

After heat treatment at 70° for 30 min, III—NH—$(CH_2)_5$—C(=O)—NH-urokinase retained activity. Activities of the soluble urokinase and III—NH—$(CH_2)_5$—C(=O)—NH-urokinase proved to be stable during storage in 0.9% NaCl at 4°, whereas III-urokinase was unstable under the same conditions (Fig. 4).

Since urokinase immobilized on matrices such as polyamide, polyester, and polyurethane had a proven antithrombotic action *in vivo*, steric problems in urokinase immobilization should be considered to improve immobilized urokinase activity with *in vivo* systems.

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Prevention of Insulin Self-Association and Surface Adsorption

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Abstract □ The self-association of insulin monomers into oligomers and macromolecular aggregates leads to complications in the administration of insulin, both in conventional administration and in the development of long-term insulin delivery systems. These problems are aggravated by the tendency of insulin to adsorb onto the surface of solution containers and infusion devices. Furthermore, with insulin infusion devices, shear rates can be generated which can accelerate the self-association and surface adsorption processes. The effects of urea on shear-induced insulin self-association and surface adsorption were investigated. It was found that the addition of a certain concentration range of urea to insulin solutions greatly reduces both insulin self-association and surface adsorption. Circular dichroic studies established that these concentrations of urea also preserve insulin conformation under high shear rates, where conformations are altered without urea. Higher urea concentrations lead to insulin denaturation and accelerated self-association.

Keyphrases □ Insulin—prevention of self-association and surface adsorption, circular dichroism □ Adsorption, surface—insulin, prevention of self-association □ Self-association—insulin, prevention of surface adsorption

The self-association of insulin molecules into dimers, tetramers, hexamers, and macromolecular aggregates has been studied by numerous groups, and in general, is a multiparameter process dependent upon insulin concentration, pH, solvent composition, ionic strength, and solvent dielectric properties (1–3). This self-association process leads to complications in the administration of insulin for the control of diabetes, both in the conventional administration and in the development of long-term insulin delivery systems. These problems are further complicated by the tendency for insulin to adsorb onto the surfaces of insulin solution containers and infusion devices, perhaps by mechanisms similar to those inducing aggregation.

Investigations have attempted to overcome the self-association and surface adsorption phenomena by the addition of various agents to the insulin preparations. These additives include various organic solvents (1), autologous serum (2), and amino acids (3). This report focuses on the effects of additives on insulin conformation, self-association, and adsorption onto various polymeric surfaces. In addition to the effects on insulin aggregation caused by the additives, the effects of shear stresses on macromolecular aggregation were studied. Depending on the infusion device, substantial shear rates can be developed during insulin infusions which can influence insulin self-association and macromolecular aggregation and limit the effective duration of such devices. These effects must also be considered in the development of insulin delivery systems.

This report studies the effects of additives on the insulin conformation–self-association process under constant shear, solvent pH, and ionic strength. The adsorption of insulin onto various polymers was also studied under the above conditions as a prerequisite to the development of a diffusion controlled, self-regulating insulin delivery system presently under development.

EXPERIMENTAL

Reagents—Bovine zinc–insulin[1] was used without further treatment. This insulin preparation had an activity of 25.5 IU/mg. Gentamicin sulfate[1] was used at a concentration of 25 μg/ml in all insulin aggregation and polymer adsorption studies to prevent bacterial growth. A pH 8.0 phosphate-buffered saline, containing 0.0945 *M* Na_2HPO_4, 0.0055 *M* KH_2PO_4, and 0.015 *M* NaCl, was used as the buffer solution in all studies. Hydroxyethyl methacrylate[2] was polymerized with azobisisobutyronitrile[3]. Poly(dimethylsiloxane)[4] was cured with 0.5% (w/w) stannous octoate. Cellulose sheets were obtained from a hemodialyzer[5] and soxhlet extracted for 24 hr, with double-distilled water. A segmental poly(urethane ether) copolymer[6], was dissolved in dimethylformamide and cast

[1] Sigma Chemical Co., St. Louis, MO 63178.
[2] Polyscience, Inc., Warrington, PA 18976.
[3] Aldrich Chemical Co., Milwaukee, WI 53201.
[4] Silastic 382, Dow Corning Corp., Midland, MI 48640.
[5] Lundia major hemodialyzer, Gambro Inc., Newport News, VA 23605.
[6] Biomer, Ethicon, Somerville, NJ 08876.

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