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# Prevention of Insulin Self-Association and Surface Adsorption

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Received November 30, 1982, from the *Department of Pharmaceutics, University of Utah, Salt Lake City, UT 84112.* Accepted for publication April 14, 1982.

**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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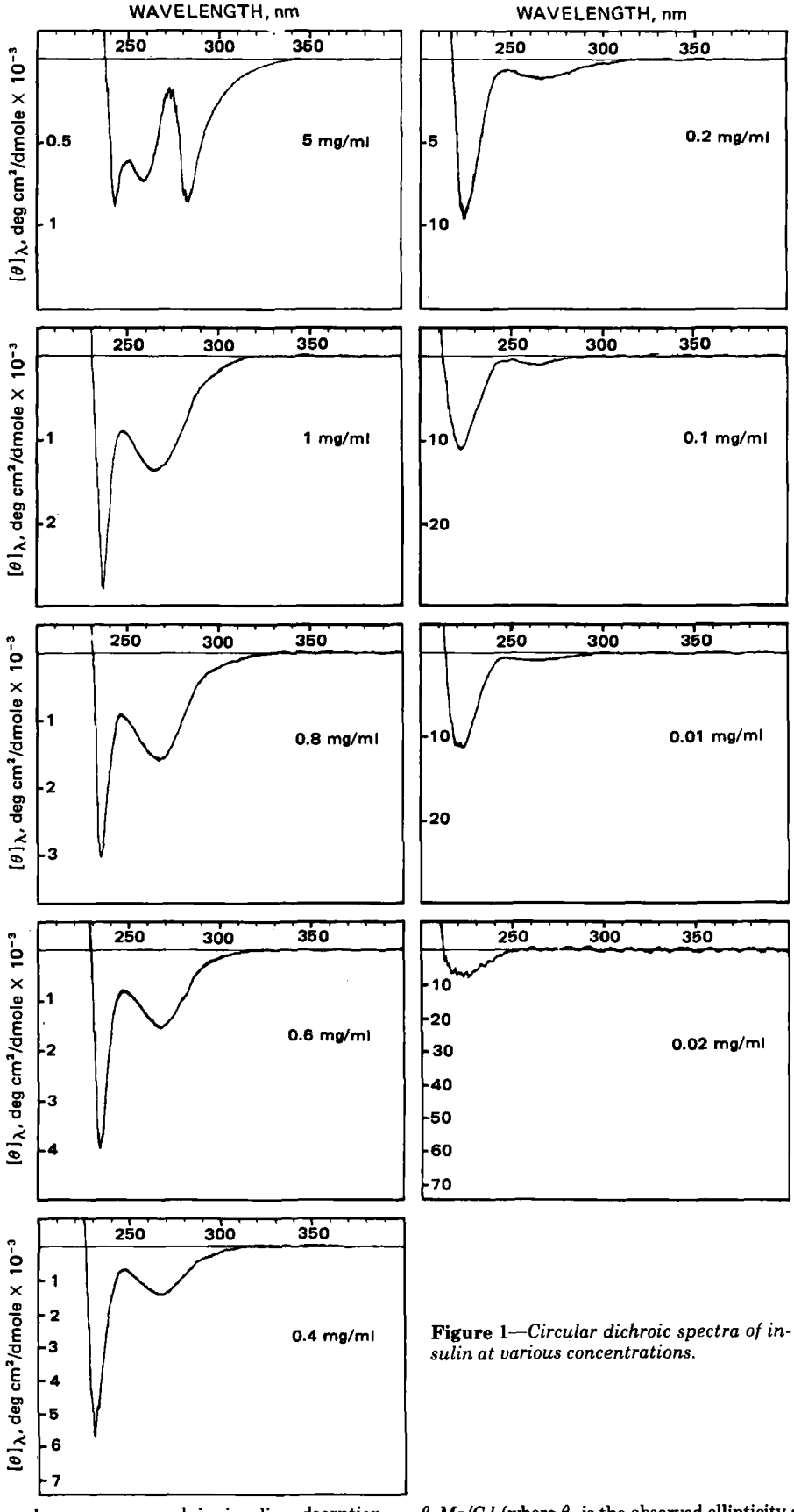



**Figure 1**—*Circular dichroic spectra of insulin at various concentrations.*

into sheets. The above polymers were used in insulin adsorption studies.

**Circular Dichroic (CD) Studies**—All CD spectra were obtained with a CD spectrophotometer[7] at 25°. Mean residue ellipticities, $[\theta]_\lambda = \theta_\lambda Mo/C \cdot l$ (where $\theta_\lambda$ is the observed ellipticity at wavelength $\lambda$, $Mo$ is the mean residue molecular weight for insulin (112 g/residue), $C$ is the insulin concentration in grams per milliliter, and $l$ is the pathlength in centimeters) were calculated for the various wavelengths and insulin concentrations.

**Effects of Shear on Insulin Self-Association and Adsorption**—To

[7] JASCO model 40A, Japan Spectroscopy Co., Tokyo, Japan.

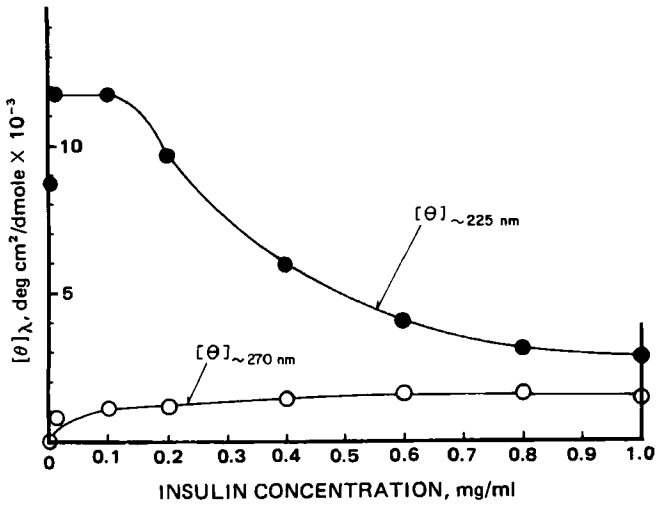
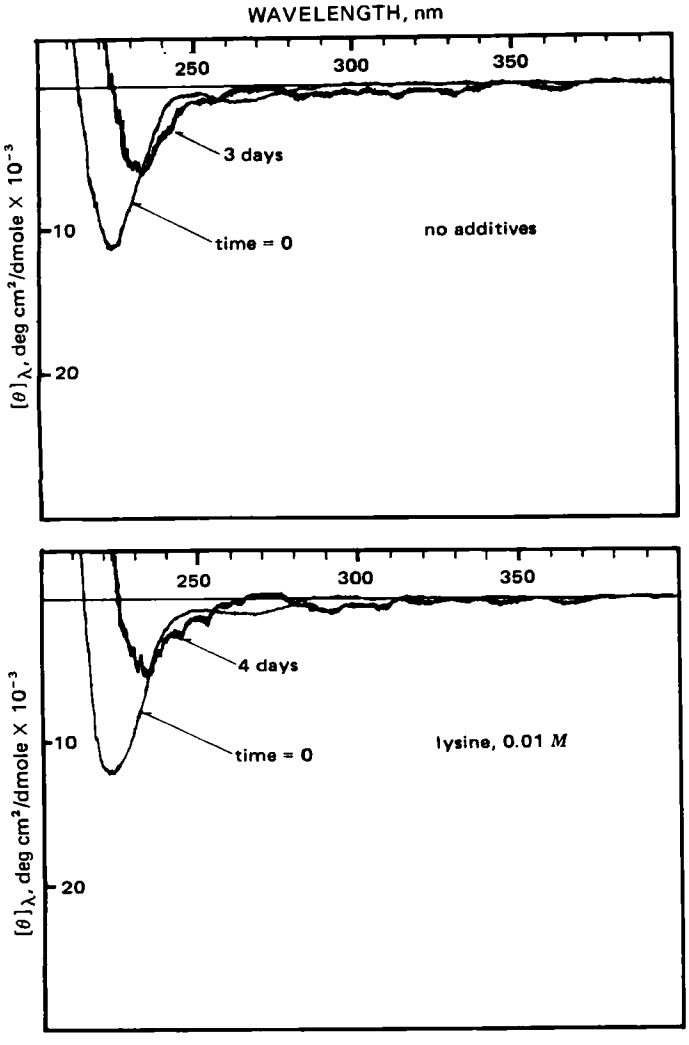



**Figure 2**—*Mean residual ellipticities at ~225 and ~270 nm* versus *insulin concentration.*



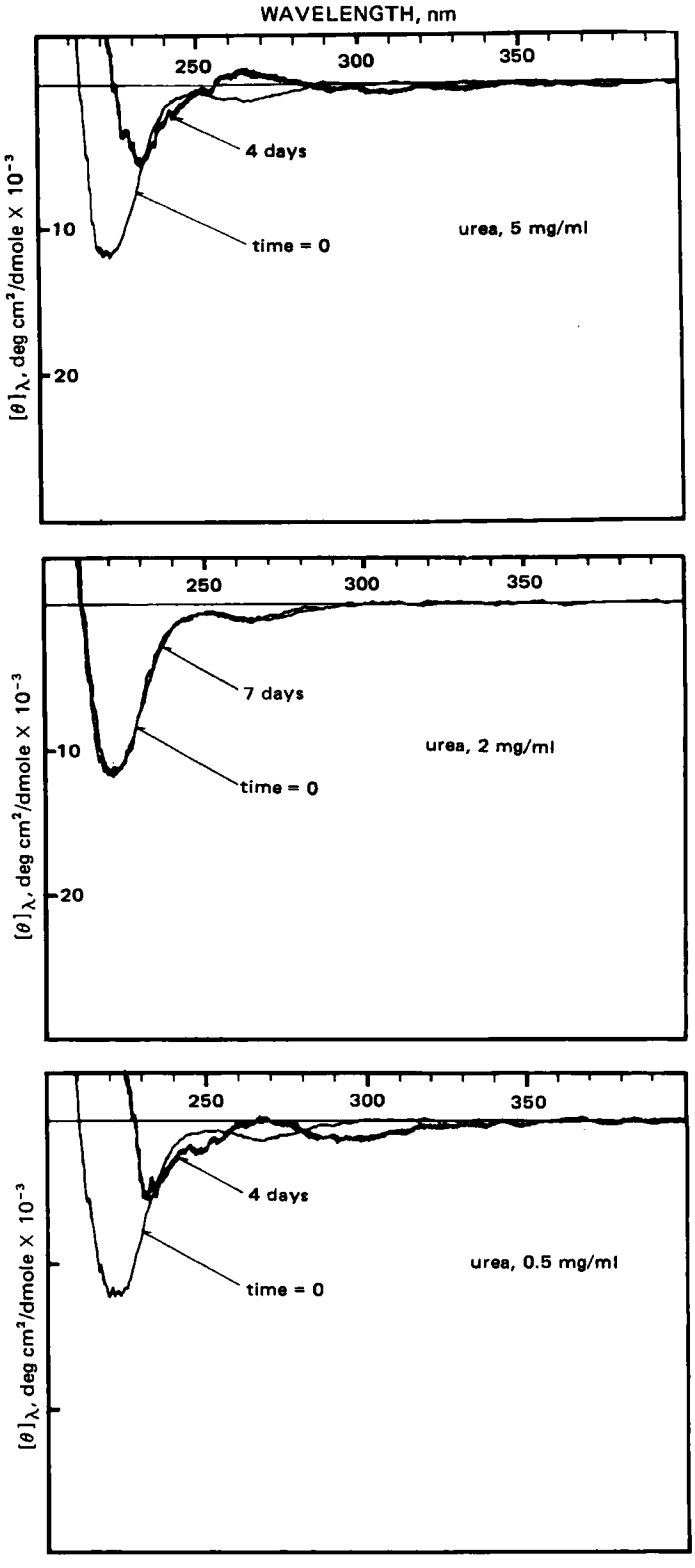




**Figure 3**—*Effects of additives on changes of the circular dichroic spectra of insulin subjected to high shear rates. All insulin concentrations were set at 0.1 mg/ml.*

evaluate the effects of shear on insulin macromolecular aggregation, 100-ml portions of various concentrations of insulin were stirred at 1550 rpm with a mechanical stirrer. This stirring velocity was selected because it represents an extreme case for shears that could be developed with infusion devices, and because these conditions will be utilized in current insulin–polymer diffusion experiments for the development of the self-regulating insulin delivery system previously described. Periodic samples were withdrawn and visibly evaluated for macroaggregation. Approximately 1-cm$^2$ disks of poly(hydroxyethyl methacrylate), cellulose, poly(dimethylsiloxane), and poly(urethane ether) were placed in the above insulin solutions and withdrawn after 7 days. These polymer samples were vacuum dried, gold coated, and a scanning electron microscopy[8] of the resultant polymer surfaces was conducted.

[8] Cambridge Stereo Scan Mark IIA, Cambridge, England.

**Table I—The Effect of Additives on Insulin Aggregation Time[a]**

| Additive | Concentration of Additive | Time for Aggregation |
|---|---|---|
| No Additive | — | 2–3 days |
| Lysine | 0.01 *M* | 3–4 days |
| Lysine | 0.002 *M* | 3–4 days |
| + I[b] | 0.005 *M* | |
| I[b] | 0.005 *M* | 2–3 days |
| Urea | 0.5 mg/ml | 3–4 days |
| Urea | 1.0 mg/ml | 7 days |
| Urea | 2.0 mg/ml | 7 days |
| Urea | 3.0 mg/ml | 7 days |
| Urea | 5.0 mg/ml | 3–4 days |

[a] Insulin concentration set at 0.1 mg/ml in all cases. [b] EDTA (I).

**Effects of Additives on Insulin Self-Association and Adsorption Under High Shear**—The effects of various concentrations of lysine[1], urea[1], and disodium edetate (I)[1] on insulin circular dichroism, macromolecular aggregation, and adsorption on polymer surfaces were evaluated under high shear conditions as described.

## RESULTS AND DISCUSSION

The CD spectra of insulin at various concentrations is presented in Fig. 1, where mean residue ellipticity, $[\theta]_\lambda$ in deg cm$^2$/*d*mol × $10^{-3}$, is plotted *versus* wavelength. At an insulin concentration of 5 mg/ml, negative maxima were observed at 245, 262, and 283 nm, the trough at 262 nm having been assigned to phenylalanine at B24 and/or B25 residues, while the trough at 283 nm was assigned to tyrosine at B26 (4).

Two negative maxima at ~245 and 270 nm were observed between 1.0 and 0.4 mg/ml. The trough at ~270 nm was assigned to tyrosine and phenylalanine aromatic residues in the B23–28 region of the antiparallel $\beta$-structure formed between insulin monomers in insulin aggregated states. Attenuation of this band has been associated with disaggregation of insulin (5), while strengthening of the band is associated with conditions that enhance insulin aggregation (6). The optical activity of the aromatic residues contributing to this band is, therefore, dependent upon the state of insulin self-association. As insulin concentration decreased, the strength of this band progressively decreased indicating reduced insulin self-association. At 0.2 mg/ml the 270-nm trough was greatly diminished and a negative maxima at ~225 nm was the dominant band. This trough was the predominant feature of the insulin CD spectra for 0.2, 0.1, and 0.01 mg/ml insulin concentration. The appearance of this trough at ~225 nm was assigned to the antiparallel $\beta$-structure of insulin dimers (7). This band was the predominant feature of the insulin dimer. Furthermore, this peak was not attenuated upon dilution from 0.2 to 0.01 mg/ml, suggesting that insulin dimers are the prominent species in that concentration range. At an insulin concentration of 0.02 mg/ml, mean residue ellipticity at 225 nm decreased and the trough was broadened, demonstrating negative ellipticity at ~210 nm. Negative ellipticity at ~210 nm has long been associated with an $\alpha$-helical structure. The attenuation of negative ellipticity at 225 nm combined with the appearance of negative ellipticity at 210 nm suggests that insulin dimers are less prevalent at this concentration.

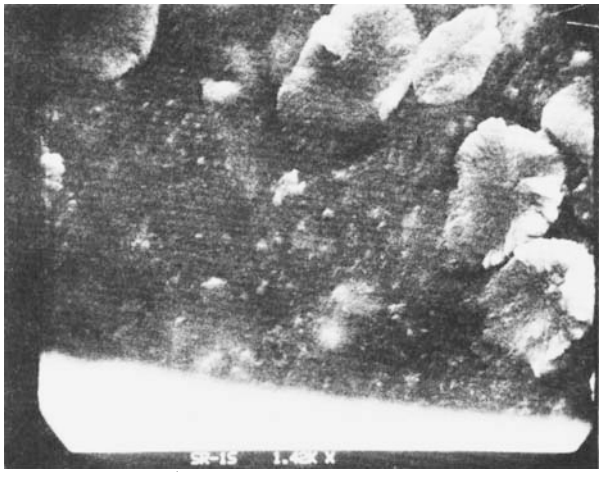


**Figure 4**—*Insulin adsorbed onto silicone rubber under high shear, 1600 ×.*

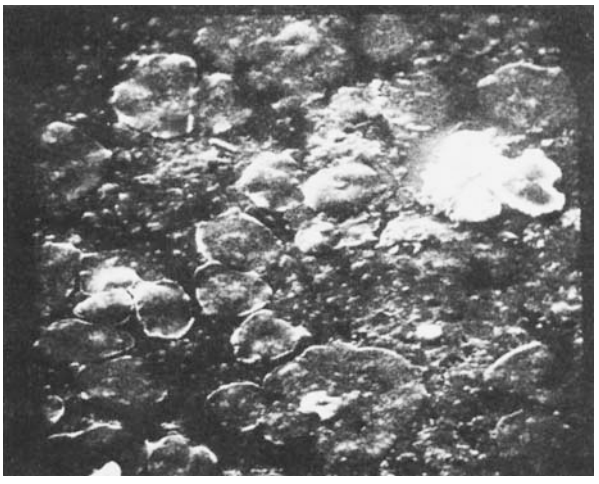


**Figure 5**—*Insulin adsorbed onto the segmental poly(urethane ether) copolymer under high shear, 600 ×.*

Results from the data reported here and studies by others (4, 7) suggest that the ~225- and 270-nm bands provide an indication of insulin self-association. When mean residue ellipticity is plotted *versus* insulin concentration, Figure 2, it is evident that the ~225-nm band is the more sensitive indicator of insulin self-association, insulin self-association into dimers, tetramers, hexamers, *etc.*, in turn having previously been correlated by others with insulin concentration (4, 8).

Using insulin CD as the indicator of insulin self-association, the effects of various additives on insulin self-association under high shear rate conditions were evaluated. These results are presented in Fig. 3, where the lighter tracing represents the CD spectra at time 0 and the darker tracing represents the CD spectra for the same insulin solution at the indicated time. The insulin concentration was set at 0.1 mg/ml for all spectra presented. At a urea concentration of 2.0 mg/ml, no changes in the 225-nm peak were observed even after 7 days under high shear rate conditions. Changes in the CD spectra were observed for higher and lower concentrations of urea at ~4 days providing spectra similar to aggregated insulin without additives. Interestingly, the small trough at 270-nm disappears under high shear rates indicating a loss in optical activity for the B23–28 aromatic residues.

Table I summarizes the effects of additives on shear-induced insulin macromolecular aggregation, all data obtained at an insulin concentration of 0.1 mg/ml where insulin dimers were the prevalent species. Macromolecular aggregation was observed without additives within 2–3 days. The same aggregation time was observed for 0.005 *M* EDTA (I). The addition of 0.01 *M* lysine or a combination of 0.01 *M* lysine and 0.005 *M* I prolonged macromolecular aggregation of insulin by ~2 days. The addition of 0.5 mg/ml of urea produced aggregation times similar to the lysine–I combination; however, aggregation times were >7 days

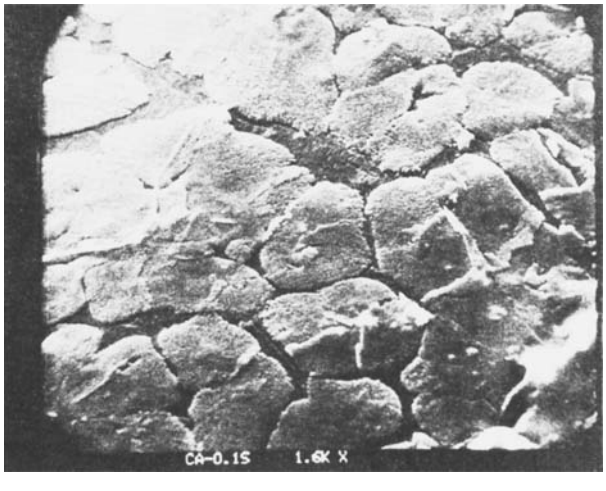



**Figure 6**—*Insulin adsorbed onto cellulose under high shear, 1600 ×.*

**Figure 7**—*Insulin adsorbed onto the segmental poly(urethane ether) copolymer under high shear with 2.0 mg/ml urea, 2100 ×.*

for urea concentrations ranging from 1.0 to 3.0 mg/ml. At higher urea concentration, aggregation times were ~4 days.

Scanning microscopy revealed that insulin adsorption onto various polymer surfaces was substantially greater under high shear as compared with static conditions. The morphology of the adsorbed insulin was found also to be dependent on shear conditions. Scanning electron micrographs of insulin adsorbed under high shear onto poly(dimethylsiloxane), poly(urethane ether), and cellulose are presented in Figs. 4–6, respectively. Under high shear, insulin adsorbs onto all of the polymers evaluated as disk-like structures. Without high shear rates, such disk-like insulin adsorbates were not observed over the time frame evaluated. Under high shear, the morphology of the adsorbed insulin appears independent of the polymer nature, disk-like adsorbates were observed on all types of polymer surfaces. However, under static conditions the morphology may be dependent upon the polymer substrate.

Concentrations of urea that prolonged insulin macromolecular aggregation times were found also to inhibit insulin adsorption onto polymer surfaces. Disk-like insulin adsorbates were not observed on the various polymer surfaces with the addition of 2.0 mg/ml urea under high shear, as shown for poly(urethane ether) in Fig. 7 as a representative case.

The self-association of insulin molecules in solution and the adsorption of insulin onto container surfaces pose complications in the administration of insulin. These problems are of particular importance with long-term insulin infusion devices where insulin crystals on the various surfaces of such devices have been observed by several investigators (9, 10). With such systems, insulin can be subjected to shear rates that can greatly effect and potentiate this process.

The addition of urea in a limited concentration range (*i.e.*, 1–3 mg/ml of urea) inhibits both insulin self-association and surface adsorption. It has been suggested that the initial step in insulin self-association is the hydrophobic association of the B23–28 regions on insulin monomers to form insulin dimers which further associate into larger oligomers. Urea, a water structure-breaking solute, was found to greatly inhibit insulin self-association and surface adsorption presumably by decreasing interactions between dimers to prevent further self-association. Higher urea concentrations were found to denature insulin, leading to rapid macromolecular aggregation times. The concentration range of urea that inhibits these processes poses little to no toxicity risks and can stabilize insulin preparations for extended periods, both for conventional administration preparations and for the development of long-term insulin delivery systems.

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# Conformational Study of Two Polymorphs of Spiperone: Possible Consequences on the Interpretation of Pharmacological Activity


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Received December 18, 1981, from the **Laboratoire d'Analyse des Médicaments, Institut de Pharmacie UCL 7340, 1200, Bruxelles, Belgium*, and the ‡*Laboratoire de Chimie physique et de Cristallographie, Université de Louvain, Place Louis Pasteur, 1348, Louvain-la-Neuve, Belgium.* Accepted for publication April 15, 1982.



**Abstract** □ A second polymorph of spiperone, 8-[3-(*p*-fluorobenzoyl)-propyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one, has been isolated and characterized by thermal analysis and IR spectrometry. Its structure was solved by X-ray diffraction analysis. The results are compared with those previously obtained on spiperone, the main difference being in the conformation of the side chain and in the nature of the hydrogen bonding.



**Keyphrases** □ Spiperone—polymorphs, conformational study, possible consequences on interpretation of pharmacological activity □ Pharmacological activity—conformational study of two polymorphs of spiperone, possible consequences of interpretation □ Polymorphs—spiperone, conformational study, possible consequences on interpretation of pharmacological activity


Complete data about the crystal structure and solid-state molecular conformation of different polymorphs of the same drug are rarely available. The main reason lies in the difficulty in obtaining single crystals of good quality from the different polymorphs. Studies of drug polymorphism are generally restricted to determination of IR




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