Minimizing the Aggregation of Neutral Insulin Solutions

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Received November 16, 1981, from the *Department of Bioengineering, University of Utah, Salt Lake City, UT 84112.* Accepted for publication October 21, 1982.

Abstract □ Various solution additives affect the solubility and macroaggregation of insulin in buffered aqueous solutions at physiological pH. The solubility of insulin may be improved with the addition of small amounts of aspartic acid, glutamic acid, EDTA (ethylenediaminetetraacetic acid), lysine, Tris buffer, or bicarbonate buffer. In addition, the propensity of dissolved insulin to reaggregate and precipitate may be inhibited by such additives. Buffered physiological (pH 7.4) saline solutions containing 0.001–0.003 *M* lysine in the presence of 0.005 *M* EDTA or 0.01 *M* lysine in the absence of EDTA improve insulin solubility and are effective in minimizing aggregation. Solutions thus prepared may be suitable for application in intravenous insulin infusion devices and may be useful commercial insulin preparations.

Keyphrases □ Insulin—minimizing aggregation, neutral solutions, lysine, solubility □ Aggregation—minimization, solubility of neutral insulin solutions, lysine solubility □ Lysine—minimizing aggregation of neutral insulin solutions, solubility □ Solubility—minimizing aggregation of neutral insulin solutions, lysine

The tendency of insulin solutions to form macroaggregates is an obstacle in the development of long-term insulin delivery systems (1–5). The macroaggregation of the insulin molecule often limits prolonged infusion to a few days unless the device is regularly flushed during the test period. This problem, as well as a desire to characterize the adsorption of insulin, have led us to search for a physiological solvent or additive that will stabilize insulin solutions. Insulin solubility and prolonged prevention of macroaggregation has been achieved by addition of various agents to dilute insulin solutions (4–8).

EXPERIMENTAL

The Tris buffer contained 0.1 *M* NaCl, 0.005 *M* EDTA (ethylenediaminetetraacetic acid) (Tris-HCl 14.04 g/liter; Tris, 1.34 g/liter)[1]. The phosphate-buffered saline solution was prepared using 1.36 g of Na_2HPO_4, 0.22 g of KH_2PO_4, 0.005 *M* EDTA, and 8.5 g of NaCl/liter (0.01 *M* phosphate and 0.145 *M* NaCl). The pH of both solutions was adjusted to 7.2–7.4, as needed, by addition of 0.1 *M* HCl or 0.1 *M* NaOH. Bicarbonate buffer was prepared using 1.428 g of $NaHCO_3$ and 8.070 g of NaCl diluted to 1 liter. A mixture of 5% CO_2 and compressed air was bubbled through the solution to adjust the pH to 7.4. Amino acids and other additives were added to the buffered solutions in varying concentrations as desired.

Crystalline insulin[2] at a potency of 25.2 U/mg was used in an attempt to regulate solution additives. Many other studies have used commercially available insulin preparations which usually contain additives that influence solubility and aggregation.

Solutions of 1 ml were sealed with paraffin film in 16-ml glass tubes (16 mm × 100 mm) and continuously agitated in a shaking water bath at 100–200 cycles/min and 37°. Solution turbidity was evaluated twice daily. The degree of aggregation of the solution was assessed visually on a five-plus scale: (+) meant clear, no observable particles, and (+++++) meant large aggregates or cloudy. Initially instrumental turbidity measurements were used to assess the degree of aggregation, but because of the macroscopic nature of the aggregate, this method did not accurately reflect the amount of aggregation. "First day" results indicate apparent solubility of insulin after 2–4 hr. The "5–6 day" results indicate degree of aggregation present at that time.

RESULTS

Additives tested were aspartic acid, EDTA, glutamic acid, bicarbonate buffer, ethanol, glycerol, leucine, lysine, and Tris buffer. When increased solubility or prolonged prevention of aggregation was observed, an attempt was made to determine the minimum amount of the additive required to produce the observed result. This was done by serially diluting the additive in the buffered solution while other buffer conditions were held constant. Results are given in Table I.

Ethanol, Glycerol, and Leucine—These three compounds proved to be very unsatisfactory as additives in the concentration range tested (0.001–0.1 *M*). None of the compositions demonstrated delayed onset

Table I—Effect of Additives on Insulin Aggregation

Major Additive	Buffer[a]	pH	EDTA (0.005 *M*)	Insulin Concentration, mg/ml	Effective in Blocking Aggregation?
Lysine (0.0005–0.1 *M*)	PBS	7.4	+	6–10	Yes
Lysine (0.0005–0.1 *M*)	PBS	7.4	–	6	Slight
Lysine (0.001–0.1 *M*)	PBS	9.0	+	6	Yes
Lysine (0.001–0.1 *M*)	PBS	9.0	–	6	Slight
Aspartic Acid (0.00005–0.05 *M*)	PBS	7.4	+	6	No
Aspartic Acid (0.00005–0.05 *M*)	PBS	7.4	–	6	No
Aspartic Acid (0.00005–0.05 *M*)	PBS	3.5	+	3–6	Yes
Aspartic Acid (0.00005–0.05 *M*)	PBS	3.5	–	3–6	Yes
Glutamic Acid (0.00005–0.05 *M*)	PBS	7.4	+	6	No
Glutamic Acid (0.00005–0.05 *M*)	PBS	7.4	–	6	No
Glutamic Acid (0.00005–0.05 *M*)	PBS	3.5	+	3–6	Yes
Glutamic Acid (0.00005–0.05 *M*)	PBS	3.5	–	3–6	Yes
Leucine (0.001–0.1 *M*)	PBS	7.4	+	6	No
Glycerol (0.001–1.0 *M*)	PBS	7.4	+	6	No
Ethanol (0.001–0.1 *M*)	PBS	7.4	+	6	No
Buffer A (0.005–0.1 *M*)[a]	PBS	7.4	+	6	Yes
Buffer A (0.005–0.1 *M*)	PBS	7.4	–	6	No
Buffer A (0.005–0.1 *M*)	Tris	7.4	+	6	Yes
Sodium Bicarbonate	$NaHCO_3$	7.2–7.4	+	0.5	Yes

[a] Key: (PBS) phosphate-buffered saline; (Tris) Tris buffer in 0.1 *M* NaCl.

[1] Chemicals were obtained from Sigma Chemical Co.

[2] Obtained from Calbiochem Behring Corp., La Jolla, Calif.; lot number 003622.

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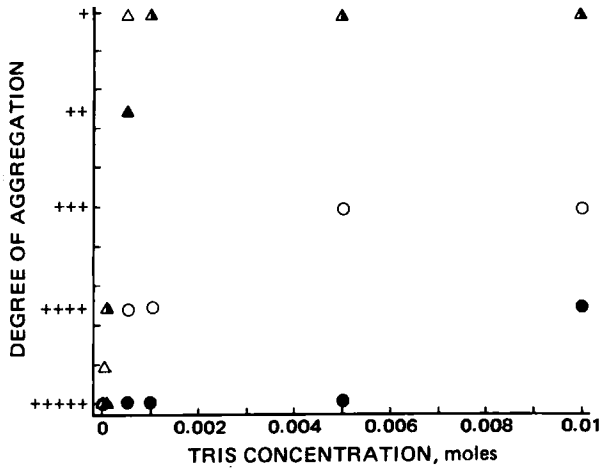

Figure 1—*Comparison of aggregation of insulin in phosphate-buffered saline as a function of Tris concentration. Solution conditions: phosphate-buffered saline, pH 7.2–7.4, temperature 37°, and insulin concentration, 6 mg/ml. Key: Degree of aggregation of solutions with 0.005 M EDTA at 1 (△) and 5 (▲) days; aggregation of solutions without EDTA at 1 (○) and 5 (●) days; (▲) point overlap.*

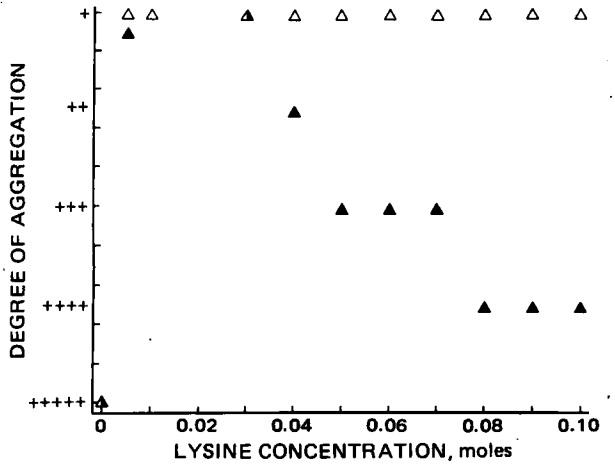

Figure 2—*Concentrations of 0.001–0.01 M lysine in 0.005 M EDTA and phosphate-buffered saline, pH 7.4. Key: degree of aggregation of solutions with 0.005 M EDTA at 1 (△) and 5 (▲) days; (▲) point overlap.*

of aggregation.

Tris Buffer—Phosphate-buffered saline solutions were prepared with and without 0.005 *M* EDTA, at various Tris concentrations (0.001–0.1 *M*). Figure 1 summarizes the aggregation of insulin as a function of Tris concentration in the presence and absence of EDTA, demonstrating that both additives are important in delaying the onset of insulin aggregation.

Lysine—Phosphate-buffered saline solutions containing lysine showed rapid dissolution of insulin, with the dissolution time decreasing as the pH was raised to 8.5–9.0. Solutions containing lysine at pH 7.2–7.4 maintained a clear, unaggregated appearance for 5–6 days. Higher lysine concentrations (0.1–0.01 *M* in 0.005 *M* EDTA) tended to aggregate more than those solutions containing lower lysine concentrations (0.01–0.001 *M*). Lysine (0.001 *M* in 0.005 *M* EDTA) is effective in minimizing aggregation (Fig. 2). However, when 0.005 *M* EDTA was eliminated from the phosphate-buffered saline solution, 0.01 *M* lysine was required to significantly minimize aggregation. Solutions of 0.01 *M* lysine and 0.005 *M* EDTA maintained at 4° without agitation remained not aggregated for periods up to 3 weeks.

Aspartic and Glutamic Acid—Earlier studies in other laboratories (5) showed that glutamic and aspartic acids were important in delaying the onset of aggregate formation. Our studies confirm the results of Bringer *et al.* (5) wherein aggregation was prevented for 6–7 days; however, serial dilution resulted in a decrease in the aggregation time. Aspartic acid proved to be more successful than glutamic acid at blocking insulin aggregation (Table I). It is important to note that due to the acidic nature of these amino acids, the pH of these solutions was 3.5 rather than 7.4. If the solutions were adjusted to pH 7.4, the aggregation was lost. This observation was also noted by Bringer *et al.* (5).

Bicarbonate—Two-milliliter solutions of sodium bicarbonate saturated with insulin were titrated to pH 6.3 with 0.1 *M* HCl, resulting in insulin precipitation. If solutions were back-titrated to pH 7.4 with 0.1 *M* NaOH the insulin remained undissolved. However, if a 5% CO_2–compressed air mixture was bubbled through the solution until pH 7.4 was reached, the insulin redissolved. A similar observation was noted by Lougheed *et al.*, where dissolution times were monitored as a function of bicarbonate concentration (8).

DISCUSSION

These results support the findings of previous researchers that agitation, additives, temperature, pH, and insulin concentration influence the solubility and macroaggregation of insulin. Recent work by Sato *et al.*, demonstrates that urea is effective in minimizing aggregation (9).

Several mechanisms for the prevention of aggregation have been proposed including the possibility of a serum substance (4) that prevents aggregation [newly published data from this group suggests that the bicarbonate concentration is the major factor in mediating insulin solubility (8)]. The chelation effect of the carboxyl groups of amino acids for zinc is believed to block aggregate formation by resulting in a more soluble form of insulin. This data is somewhat supported by the improvement of solubility and prolongation of aggregation time observed in solutions containing EDTA. As a chelating agent, EDTA may compete with insulin for zinc and, therefore, slow aggregate formation (10). Another possible mechanism for minimizing aggregation is that amino acid additives, especially lysine, may interact with the insulin molecule by hydrophobic and ionic means, thereby decreasing insulin–insulin interactions and preventing or slowing the formation of aggregates. More definitive work should be done with detailed analysis of the types of interactions and the conformation of the insulin molecule in these solutions.

Buffered physiological saline solutions containing 0.001 *M* lysine and 0.005 *M* EDTA improve insulin solubility and are effective in delaying the onset of macroaggregation. In the absence of EDTA, 0.01 *M* lysine solutions improve initial solubility and minimize the degree of aggregation. One advantage of the lysine additive is that the solutions are maintained at pH 7.4. A second advantage is that lysine is a common amino acid and is therefore not a synthetic additive.

Results summarized in this study emphasize the importance of additives in improving the solubility and stability of insulin solutions. It should be remembered that the type of insulin and additives used in various insulin preparations influence the properties discussed above, so a comparison of these results with other studies must be done with caution. The only way to accurately assess the contribution of each additive as to its solubility and aggregate-blocking properties is in a study such as this which minimizes the contributions of other solution variables or insulin additives.

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ACKNOWLEDGMENTS

Funded by the Kroc Foundation. We thank G. Iwamoto for helpful suggestions in this work.