Aggregation of Insulin, Containing Surfactants, in Contact with Different Materials

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SUMMARY

The aggregation of insulin and of insulin protected with surfactants was studied by shaking at 37°C in glass, in polypropylene and polystyrene vials, and in CPI and Auto-Syringe insulin syringes and infusion sets. Surfactants such as Pluronic 17R8 and 25R5 hastened the aggregation, whereas Pluronic F68 was effective in preventing it. Furthermore, there was no change in the immunoreactivity of insulin containing Pluronic F68 even after 8 days of shaking. Unprotected insulin aggregated in all the vials.

There appears to be little problem with the commercial syringes tested, but the infusion sets could cause aggregation of insulin if used over an extended period of time. Although Pluronic F68 prevented insulin aggregation in situ, it extracted more impurities from the contacting plastics. Further development in materials and design of insulin sets and insulin containers appears necessary. DIABETES 1985; 34:420–24.

Portable insulin infusion pumps are increasingly being used for the treatment of patients with insulin-dependent (type I) diabetes. These pumps are designed for continuous subcutaneous infusion of insulin (CSII) through indwelling catheters. One of the problems associated with CSII is the tendency of insulin to aggregate in the reservoir and, more so, in the fine delivery catheter. Insulin is known to polymerize[1,2] and form aggregates.[3–7] Macroscopic aggregates may precipitate and impede or even stop the delivery of insulin. Furthermore, the aggregated insulin may not have the required pharmacologic properties and may lead to abnormal immune response.[8]

Sulfated insulin, which is recommended for patients who have developed antibodies to regular pork and beef insulins, does not appear to form insoluble aggregates at physiologic pH.[9] This insulin is not the material of choice; rather, the tendency is to treat patients with insulin that is closer to human insulin. Therefore, the sulfated insulin is not used in infusion pumps on a regular basis.

It has been suggested that the addition of surfactant could prevent the aggregation of insulin and that the surfaces contacted by the insulin may affect its tendency to aggregate.[10] In the present investigation, several surfactants were assessed as stabilizing additives to prevent aggregation. In addition, vials of different materials, together with insulin syringes and infusion sets (catheters) were assessed for aggregation of various insulin preparations. From these studies, practical solutions to forestall insulin aggregation are proposed.

MATERIALS AND METHODS

Insulin-Toronto, 100 U/cm^3 (Connaught Laboratories, Willowdale, Ontario), was selected for this work. This is a mixed solution of beef and pork insulins at neutral pH containing *m*-cresol as preservative.

Three different container systems, glass vials (Wheaton), polypropylene test tubes (Fisher Scientific), and polystyrene vials (Scientific Products) were used. Syringes and infusion sets from CPI (Hooksett, New Hampshire) were also tested for their ability to induce aggregation of insulin. Surfactants used were Pluronics F68, 17R8, and 25R5 (compliments of BASF Canada, Montreal).

Some of the Pluronics have undergone extensive toxicologic studies that indicate that they are relatively nontoxic.[11] Thus, Pluronic F68 has been used as plasma substitute in animal experiments,[12] and it was thought that Pluronics could be used to prevent the aggregation of insulin. Of course, additional data would be required to show that it is safe to use in insulin formulations.

The chosen concentrations of these Pluronics were such that for every insulin molecule, there were two surfactant molecules in the preparation. For example, for a 10-ml vial

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Received for publication 23 February 1984 and in revised form 15 October 1984.

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TABLE 1
Aggregation of insulin and insulin surfactants in glass vials

Time (days)	Surfactant	Turbidity (OD at 600 nm)	Agitation*
2	25R5	Precipitated	Yes
2	25R5	Precipitated	Yes
2	25R5	0.143	Not
8	17R8	Precipitated	Yes
8	17R8	Precipitated	Yes
8	17R8	0.000	Not
17	F68	0.005	Yes
17	F68	0.017	Yes
17	F68	0.000	Not
17	None	1.722	Yes
17	None	2.008	Yes
17	None	0.002	Not

*Agitation using eccentric shaker at 37°C.
†Control samples.

of insulin, 58.4 mg of Pluronic F68 dissolved in 0.5 ml of normal saline was added. This solution is referred to as insulin-surfactant solution. Control insulin was prepared by adding 0.5 ml of normal saline to 10 ml of insulin solution.

To study insulin aggregation, about 2 ml of control insulin or insulin-surfactant solution was placed in one of the containers and shaken in a water bath (Precision Scientific, Model 50) at 37°C and at 100 oscillations/min with a stroke length of 35 mm. The course of aggregation was followed turbidimetrically by measuring the increase in optical density (OD) at 600 nm using a Beckman Spectrophotometer, Model 25. Eventually, almost all insulin preparations that did not contain a surfactant precipitated, and solutions became clear.

Radioimmunoassay. To find the immunoreactive insulin (IRI) in insulin samples, radioimmunoassay was performed using Immo-Phase Insulin Radioimmunoassay kits (Corning Glass Works, Medfield, Massachusetts). This system uses an antibody that is chemically immobilized on porous glass particles, and separation of bound from free antigen is accomplished by centrifugation.

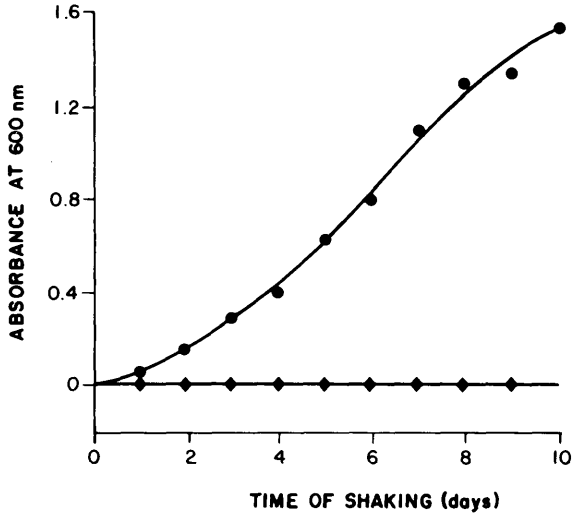

FIGURE 1. Aggregation of insulin in glass vials. Change in absorbance with time of shaking for (●) unprotected insulin and for (◆) Pluronic F68–containing insulin or control insulin.

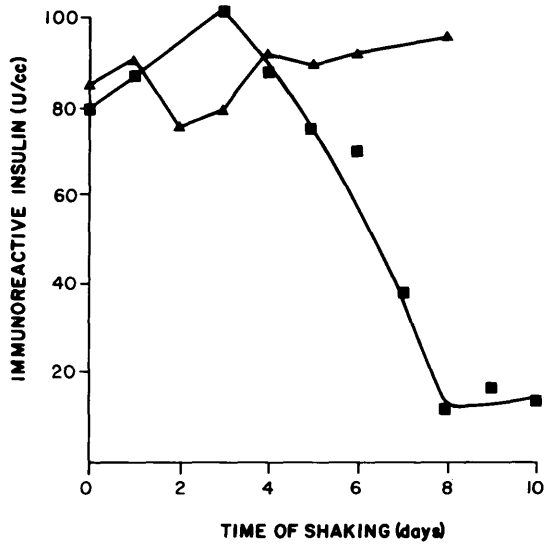

FIGURE 2. Aggregation of insulin in glass vials: changes in immunoreactivity of supernatant insulin with time of shaking. (■) Immunoreactivity of unprotected insulin, (▲) immunoreactivity of insulin containing Pluronic F68.

In samples in which aggregation had occurred, the solution was clarified by centrifugation and the clear supernatant liquid was used to determine the IRI. Precipitated insulin was not used because it is not used for the treatment of diabetic subjects and, in fact, it may be harmful to the host.[13] All sample dilutions were done using 0.1 M phosphate buffer at pH 7.4 and contained 5 g/L of bovine serum albumin (Sigma Chemicals, St. Louis, Missouri). Only plasticware was used for handling insulin samples. Gamma counting was done using an LKB Commpu Gamma system (Fisher Scientific).

High-performance liquid chromatography. High performance liquid chromatography (HPLC) was used to identify and quantitate beef and pork insulins. Equipment used was a Beckman Dual Pump HPLC system fitted with an automated sample injection accessory. The column chosen was a 25 cm × 0.25 in. Zorbax TMS (DuPont) thermostated at 45°C. All solvents were of HPLC grade. The procedure was essentially that described by Chance et al.[14] and is summarized as follows. The flow rate of the mixed solvent was 1 ml/min and the UV detector was set at 210 nm. Injection volume was 5 μl. Solvent A was a mixture of 25% acetonitrile and 75% 0.1 M aqueous phosphate buffer, pH 2.5. Solvent B was acetronitrile. All the solvents were filtered and degassed before use. The samples were eluted isocratically with solvent A for the first 7 min, then pump B was started to provide a linear gradient. At the end of an additional 10-min period, solvent B constituted 10% of the total eluant.

RESULTS

The aggregative behavior of different insulin formulations in glass vials is shown in Table 1. Samples containing Pluronic 25R5 precipitated after about 2 days. Similarly, samples to which Pluronic 17R8 had been added precipitated after about 8 days. Control samples, which were not agitated but were otherwise treated under identical conditions, did not precipitate. The insulin preparations without additives be-

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TABLE 2
Effect of container material on the aggregation of insulin

No. of days of shaking	Polystyrene		Polypropylene		Glass	
	Without additive	With F-68	Without additive	With F-68	Without additive	With F-68
0	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
11	0.10	0.00	0.08	0.00	0.08	0.00
14	PPT*	0.00	0.47	0.00	PPT*	0.00

OD measurements at 600 nm.
*PPT, precipitation.

came turbid after about 3 days, but macroscopic precipitates were seen only after about 17 days. Insulin samples containing Pluronic F68 remained clear for at least 17 days and did not deposit solids. From these results, it is evident that Pluronic F68 could be used to protect insulin from aggregation.

The kinetics of phenomena taking place in the preparation with Pluronic F68 are shown in Figure 1. Pluronic F68 had been added directly to glass vials containing the insulin. Controls show that the turbidity (OD at 600 nm) of unprotected insulin solutions increased with time, confirming that aggregative processes were taking place. On the other hand, insulin protected by Pluronic F68 remained clear and without any increase in absorbance. Protected and unprotected insulin kept at 37°C, but without agitation, also remained clear, and even after 11 days these preparations showed no increase in OD. Thus, only the unprotected and agitated control insulin aggregated, whereas the other three insulin preparations remained clear.

The pH of protected and unprotected insulin was also measured. Values measured were 6.93 ± 0.09 (mean ± SD, N = 8) for the unprotected formulation and 6.89 ± 0.02 (mean ± SD, N = 8) for the insulin containing Pluronic F68. It is evident that there is not much difference in the pH values of these insulin formulations, and it may be concluded that Pluronic F68 addition does not affect the pH values of insulin formulations.

Immunoreactivity of Pluronic F68–containing insulin. Figure 2 shows the results of IRI measurements taken in the supernatant liquid. For unprotected insulin, IRI decreased with time of agitation as expected, since the aggregated insulin precipitated, resulting in a decreased concentration of active insulin in the supernatant.

Effect of different materials on the aggregation of insulin. Three materials (glass, polypropylene, and polystyrene) were used. Aggregation was followed both with and without the addition of Pluronic F68. Results are shown in Table 2. As there was no increase in the OD of insulin samples containing Pluronic F68, it may be concluded that Pluronic F68 protects insulin in all of the materials tested. Unprotected insulin started to aggregate and turbidity was detected after 11 days of agitation in all of the vials. After 14 days, the insulin had precipitated in glass and polystyrene. In polypropylene test tubes, its OD had increased to 0.47 but there was no settling of the precipitates.

Aggregation of beef and pork insulins. The insulin-Toronto used is a mixture of beef and pork insulins. It was important to know if one or the other of these preferentially aggregates. To achieve this objective, HPLC analyses of the insulin samples were performed before and after partial aggregation. For the partially aggregated insulin, OD was 0.41. Figure 3 (A and B) shows the results for the fresh and partially aggregated insulin. There is hardly any change in the peak position or the peak areas, suggesting that the two insulin species differ little in their aggregation. Figure 3C shows the HPLC analysis of Pluronic F68–containing insulin. Again, it is evident that there is hardly any change in insulin composition by the added surfactant, although peak positions have shifted a bit. This shift is probably due to the eluting action of the surfactant, Pluronic F68.

Aggregation in syringes and infusion sets. Having established that Pluronic F68 is effective in preventing aggregation, it was tested in Auto-Syringe syringes and infusion sets. Results are shown in Table 3. It is evident that insulin in syringes and infusion sets that were not shaken showed no increase in absorbance. Also, insulin shaken in syringes did not show marked increase in absorbance. For the infusion set, the absorbance of unprotected insulin increased to

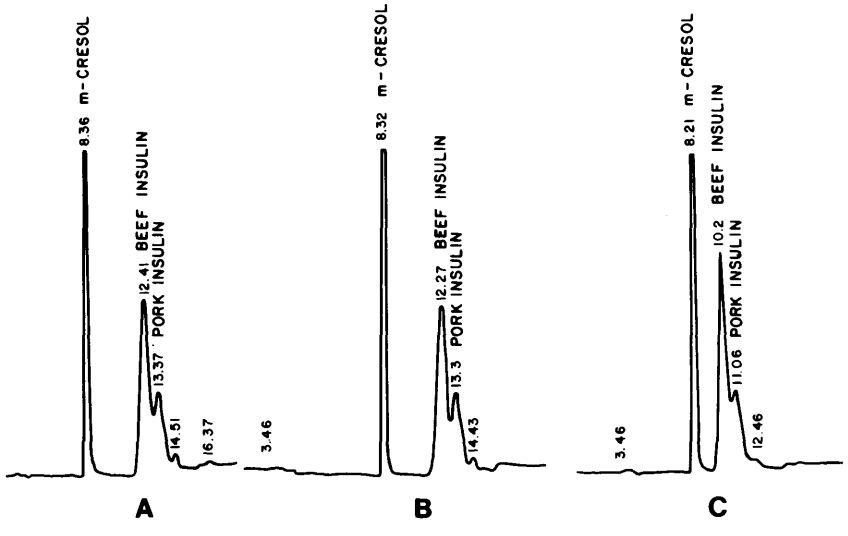

FIGURE 3. HPLC analysis of insulin-Toronto. Peaks are as indicated. (A) Fresh insulin, (B) insulin that had undergone partial aggregation, and (C) insulin to which Pluronic F68 had been added.

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TABLE 3
Aggregation of insulin and Pluronic F68–containing insulin in Auto-Syringe syringes and infusion sets (catheters)

No. of days at 37°C	Syringe with			Infusion set with		
	Control insulin (no shaking)	Insulin	Insulin with F-68	Control insulin (no shaking)	Insulin	Insulin with F-68
0	0.00	0.00	0.00	0.00	0.00	0.00
17	0.00	0.006	0.001	0.00	0.020	0.040
20	0.00	0.008	0.007	0.00	0.020	0.067
24	0.00	0.013	0.059	0.00	0.054	0.845

OD measurements at 600 nm.
"Control" were syringes or infusion sets filled with insulin at 37°C but not shaken.

0.054, whereas that for the Pluronic F68–containing insulin increased to 0.845.

Two sets of syringes and infusion sets (CPI and Auto-Syringe) used by diabetic subjects were tested for their tendency to induce aggregation in insulin formulations. No surfactant was added; this was to simulate conditions appropriate to treatment of diabetes. Results are shown in Table 4. There did not appear to be much problem with the syringes, but insulin in infusion sets began to turn turbid. Thus, after 5 days, the OD of insulin in infusion sets was 0.014 compared with 0.007 for the syringes.

DISCUSSION

The results in Table 1 show that insulin samples containing Pluronic F68 remained clear for at least 17 days and no solids were deposited. From these results, it is evident that Pluronic F68 could be used to protect insulin from aggregation even when it is agitated. Figure 1 confirms these results by showing that there is no increase in absorbance at 600 nm of insulin containing Pluronic F68.

Having established that Pluronic F68 could protect insulin against aggregation, it is important to know if the insulin is still immunoreactive. Figure 2 shows that, for the Pluronic F68–containing insulin, the IRI remained near its nominal value of 100 U/cm^3. This compares well with pure insulin kept at 37°C for 3 mo in a quiescent state; it loses only 3% of its biologic activity and does not aggregate.[15] It appears that the loss of IRI in such unprotected insulin is due principally to polymerization phenomena. Therefore, from the results in Figure 2, it may be concluded that the immunoreactivity of insulin can apparently be protected by the addition of Pluronic F68.

Pluronic F68 is a short-chain, block co-polymer consisting of a mildly hydrophobic polyoxypropylene center block flanked at both ends with slightly more hydrophilic polyoxyethylene segments; it has a number-average molecular weight of 8350. For Pluronic 17R8 and 25R5, the structure is reversed, consisting of a polyoxyethylene center block with polyoxypropylene segments at both ends. It appears that surfactants having the hydrophilic groups at the end(s) are more effective agents in preventing insulin aggregation. To this end, it may be noted that the special surface-active polymer developed by Hoechst AG, Frankfurt, for preventing the aggregation of insulin has a structure[10] in which central hydrophobic units have hydrophilic end groups, a structure very similar to Pluronic F68.

It has been shown that different materials adsorb different amounts of insulin.[16,17] Also, the aggregation is affected by materials coming in contact with it.[3,6,7] Our investigation was directed to materials that could prevent or at least delay aggregation and that might be used in portable insulin infusion pumps. From the results in Table 2, it is evident that the three materials (glass, polypropylene, and polystyrene) exhibit an effect on the aggregation, but none could be effective in preventing it.

It may be mentioned here that shaking in vials is an extreme condition for introducing aggregation. Proteins are known to undergo conformational changes at the air-liquid interface.[18] With shaking, the air-liquid and the liquid-solid interfaces increase, leading to greater conformational changes and denaturation. It has been suggested that the denatured insulin forms a nucleus for aggregation.[4] Shaking would enhance the denaturation and increase the chances for aggregation. These conditions are not present to the same degree when a diabetic subject is using CSII therapy.

We were concerned whether beef or pork insulins aggregated at different rates. HPLC results shown in Figure 3 indicate that both insulins aggregate equally.

TABLE 4
OD measurements of insulin in contact with Auto-Syringe and CPI syringes and infusion sets

No. of days	Auto-Syringe				CPI			
	Syringe		Infusion set		Syringe		Infusion set	
	Control	Sample	Control	Sample	Control	Sample	Control	Sample
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.000	0.007	0.005	0.014	0.00	0.011	0.005	0.040
11	0.001	0.007	0.001	0.145	0.001	0.007	0.067	0.085

"Controls" were syringe or infusion sets similar to test samples but were not shaken. Temperature of experiments was 37°C.

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Aggregation in syringes and infusion sets was carried out to simulate actual use conditions. Our results with glass, polypropylene, and polystyrene vials had shown that Pluronic F68 is an effective stabilizing agent and prevents aggregation. Therefore, the high absorbance value of 0.845 (shown in Table 3 for the Pluronic F68–containing insulin in contact with the infusion set) suggests that this formulation extracts some material from the poly(vinyl chloride) infusion sets. In fact, the color of the insulin-surfactant formulation had turned brownish compared with white-colored aggregates in all other cases.

Our test results (Table 4) with two sets of commercially available syringes and infusion sets show that, with normal use of about 4 days per syringe and infusion set, such devices should work well. Problems could develop if these products are used over extended periods, especially for the infusion sets. Improvement in the materials of construction of the infusion sets could eliminate or at least reduce the problem of aggregation of insulin, even when these are used for longer periods than recommended by the manufacturer.

Pluronic F68 surfactant prevents motion-induced aggregation of insulin up to 17 days. It does not significantly reduce the immunoreactivity or change pH of the insulin. As an insulin-surfactant formulation it may, however, extract some material from poly(vinyl chloride) infusion sets with apparent increase in OD of the solution. Under normal use by the patient, aggregation of insulin does not appear to be a significant problem in the commercially available syringes and infusion sets tested. Should these be used over extended periods, aggregation could develop in these systems; this is especially true for the infusion sets. Our results suggest that aggregation of insulin can further be delayed by improvement in the materials used for fabricating infusion sets.

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