Diabetologia 19, 1–9 (1980)

Diabetologia

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Review Articles

Insulin Aggregation in Artificial Delivery Systems

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Miniature "open-loop" devices have been used successfully to deliver insulin intravenously and maintain normoglycaemia for periods of up to 2½ years in diabetic dogs [1, 2]. However, a tendency of insulin to aggregate at both 25 and 37 °C has led to serious problems in delivery and has necessitated frequent replacement or flushing of the insulin reservoir-catheter system. The heterogeneous mixture produced by insulin aggregation in portable and implantable delivery systems does not allow delivery of the hormone within the fine limits of tolerance that are required to maintain euglycaemia. The premature end of attempted extended infusion is thus often caused by the obstruction of internal pump parts and/or the delivery catheters [3]. Insulin has an inherent tendency to polymerize [4] and form large molecular weight aggregates [5] that do not equilibrate rapidly with the rest of the solution. Insulin aggregation in artificial devices is thus the major impediment to their further technological development and clinical application. With subcutaneous infusion devices [6, 7], these problems have not been reported, possibly due to the frequent changing of the insulin containing portion of the devices. Only upon the complete resolution of the aggregation problem can the new intravenous therapeutic methods be properly assessed as to their effect on the aetiology of diabetic complications.

This paper reviews the relevant published literature and attempts to consolidate the physical, chemical and thermodynamic factors that lead to insulin aggregation. Such knowledge may expedite the development of insulin formulations and the selection of materials suitable for the uncomplicated long-term administration of the hormone.

Relevant Literature

The aggregational behaviour of pork and beef insulins in both acid and neutral formulations has been discussed [4, 5, 8–21]. In this paper, unless otherwise stated, it will be assumed that 'insulin' refers to these four insulin formulations only. The terms self-association, polymerization and aggregation have been used interchangeably in the literature. We assume that all authors in fact refer to the same phenomenon but in the sections relevant to our work we shall use the term "aggregates" to describe those particles (visible at 400 X magnification) which have been responsible for the occlusion of our insulin infusion devices.

The stoichiometry of the self-association or aggregation of insulin was described by Steiner as early as 1952 [20]. These early studies at pH 2.06–3.07 (19–40 °C) proposed a model which involved dimers, tetramers and hexamers in reversible equilibrium. Since then, many models have been proposed to describe the self-association of insulin in solution at both acid and neutral pH. The early studies used sedimentation velocity measurements to determine the species in suspension. More recent work has included sedimentation equilibrium and circular dichroism techniques. Most early models were qualitatively similar to each other involving monomer-dimer-hexamer-polymer formation [9, 11, 21] although disagreement existed regarding the size of the monomer [11]. Grant et al. [13] reported that the hexamer was the sole component present at zinc concentrations higher than two g-atoms/hexamer (pH 7.0). Models which incorporated the finite existence of the tetramer were also proposed but work with zinc-free insulin at neutral pH seemed to indicate monomer-dimer association followed by dimer-hexamer association.

In 1977, Holladay et al. [22] reported association constants for these reactions ($K_2 = 2.2 \times 10^4$ 1/mol, $K_6 = 8.6 \times 10^9$ l^2/mol^2) which were in fairly close agreement with those reported earlier by Pekar and Frank [18] ($K_2 = 1.4 \times 10^5$ l/mol, $K_6 = 4 \times 10^8$ l^2/mol^2). In 1976 Jeffrey et al. [4] using a broader range of zinc-free insulin concentrations fitted their results to a model in which monomer-dimer association (K_2

0012-186X/80/0019/0001/$01.80

$= 1.1 \times 10^5$ l/mol) was followed by indefinite self-association of the dimer to form higher polymers (K $= 1.7 \times 10^4$ l/mol). Blundell et al. [8] and Thurow [23] agreed with Jeffrey that the dimer was the fundamental unit for further association. Blundell et al. proposed that dimer formation involved relatively weak intermolecular Van der Waal's forces as well as hydrogen bonding in the monomer interface region (pleated sheet region) which is common to both insulin and proinsulin. Recently, Jeffrey [5], employing similar sedimentation equilibrium techniques (pH 2 and 7) reported: (1) that zinc insulin species of higher molecular weight than the hexamer were present and could exist in solution for sufficient time to conduct equilibrium sedimentation experiments; (2) that these aggregates were not in rapid equilibrium with the monomer-dimer-hexamer system; (3) that as reported by others [11, 18] the aggregation process was concentration-dependent with disassociation to the monomer occurring at very low concentrations and the formation of dimers, tetramers, hexamers and higher polymers proceeding as the insulin concentration was progressively raised; (4) that the aggregation behaviour of zinc-free insulin (pH 7.0) as reported by Pekar and Frank [18] was qualitatively similar to that of zinc insulin (pH 7.0); (5) that glucose (7.76 g/100 ml) favoured dissociation at both pH 2 and pH 7 but that large molecular weight aggregates still made a significant contribution to the sedimentation coefficient at pH 7. Some of the cited authors, notably Jeffrey [5], have reported variable aggregation behaviour in samples from the same lot and attribute this to the retention of varying amounts of large molecular weight aggregates in solution.

Wu [24] has criticized Pekar and Frank [18] and Jeffrey [5] in that they failed to consider the effect of storage time on aggregation behaviour in solution. Thus, it must be noted that the quoted experiments were conducted with protocols that would not detect any relationship between stability and increased aggregation. In sedimentation velocity experiments Wu [24] has also noted that insulins from the same batch showed an inconsistent propensity for aggregation. In addition he reported that on at least two occasions when a solution of 125 U highly purified insulin/ml at pH 7 (in 0.1 mol/l Tris buffer, 0.1 mol/l NaCl, 0.001 mol/l, EDTA) was refrigerated for a period of a few days a precipitate formed in the glass vials. The precipitate did not redissolve in fresh diluent. The author added that the large molecular weight aggregates may be 'other forms' of "denatured" insulin molecules present in the stock solution.

Contrary to the above reports describing insulin aggregation by simple thermodynamic equilibria it has been proposed that the aggregation of insulin is a complex phenomenon. In support of this the literature abounds with articles that cite the following as variables which influence aggregation behaviour: insulin purity, nature and concentration of metal ions, processing methods, refrigeration temperatures, elevated temperatures (above 31 °C), ionic strength, elevated salt concentrations during processing, motion and denaturation. In the remainder of this review we shall summarize some of these variables which reportedly contribute to the process of insulin aggregation.

Purity

Jackson et al. [25] suggested that the purity of insulin solutions at neutral pH was a prerequisite for solubility. In addition, other authors have proposed that impurities (such as proinsulin) may have been responsible for or enhanced aggregation [5, 24]. Regular (soluble) insulin preparations usually contain varying low levels of contaminants [25, 26] e. g.: desamido insulin, ethyl esters of insulin, proinsulin and other partially processed forms of the hormone [27]. From 1972–1975 advances in purification technology considerably reduced the level of these contaminants in special preparations. "Single Component" (SC) insulin (Eli Lilly) and "Monocomponent" (MC) insulin (Novo) which were purified by both molecular sieve and ion exchange chromatography contained 99% pure insulin and less than 1 ppm of such contaminants as proinsulin or glucagon [29]. Unfortunately the insulin molecule is not a stable entity. Although biological potency is not substantially reduced under the appropriate storage conditions (losses of less than 1% during shelf life at 2–8 °C), insulin transformation products are formed [29]. The products are mainly desamido insulins and insulin polymers, primarily dimers.

The claims of purity for MC insulin have been challenged by some investigators. Yue and Turtle [30] from their chromatographic analysis claimed that 5% of the protein in MC insulin was covalently linked dimer and that its proinsulin content was 0.049%. Schlichtkrull [31] refuted the analysis of the above authors attributing their results to the presence of preservative in their insulin samples.

In view of these controversial data one cannot draw any definitive conclusions except that all insulin solutions regardless of purity demonstrate a propensity towards aggregation. Until a truly homogeneous solution of insulin is obtained the precise relationship between purity and aggregation cannot be determined.

Metal Ions

It has been reported that excess amounts of divalent metal ions, notably Zn^{2+}, Cu^{2+}, Fe^{2+}, will cause insulin at neutral pH's to form large molecular weight aggregates (mol. wt. = 10^5 to 2×10^5) [9, 32]. This seems reasonable as the addition of Zn under appropriate conditions results in crystallization of the hormone. However, Wu [24], reported that aggregation occurred during experimentation with zinc-free insulin in the presence of a chelating agent such as EDTA. It was, however, the above author's contention that the removal of zinc was necessary to reduce the tendency towards self-association. In addition, Massey and Smyth [33] reported that zinc-free insulin revealed aggregates during gel filtration but to a lesser extent than zinc insulin. Sulphated insulin which is zinc-free tends to exist as a monomer [34], but this may be due to additional charges on the molecule introduced by the sulphate groups.

Temperature

The stability of insulin solutions at temperatures between −20 and +37 °C is well documented [35]. One notable study [36] has defined stability as $P(t) = P_o e^{-kt}$ where t = time, P_o = biological activity at t = 0, and $k = e^{BT}$, where T = absolute temperature and B is a proportionality constant. Instability of insulin solutions at 25 and 37 °C has been noted [19, 23]. Whether denaturation of the hormone at elevated temperatures caused aggregation of the hormone is not clear from the literature. It has been observed that the decline with time in immunological activity is less than the decline in biological activity [35] which might perhaps be explained on the basis of aggregation or partial denaturation.

Processing

The presence of high molecular weight aggregates in freshly prepared insulin solutions stored at 4 °C has been reported [27]. At the time the above authors reported that the chemical nature of these cross-linkages had not been elucidated but evidently these aggregates were formed during the extraction and isolation of insulin under acid conditions and were present at low levels in all solutions unless further methods for their removal were implemented [26, 28]. With respect to salting out of the hormone during processing it has been postulated that hypertonic salt concentrations may alter the conformation of the crystalline protein [19].

Motion

The effect of movement upon the hormone is not well documented. Irsigler and Kritz [3] reported that the precipitation of insulin in infusion devices appeared to be an unavoidable consequence of continuous motion of the hormone and that aggregation or precipitation occurred irrespective of the materials used in the pumping system. Thurow [23, 38] reported that factors which accelerated denaturation also increased aggregation and he cited agitation as an important variable.

Other Variables

Among other variables noted in the literature are: (1) that high ionic strength favours association [19]; (2) that association or aggregation affects only the average mobility of the protein with conformation remaining unchanged [19]; (3) that the usual low storage temperature of 4 °C favours association [24].

Purity, homogeneity, temperature, metal ions, processing methods, ionic strength, and motion are all variables implicated in the aggregation of insulin. The relative importance of the above variables has not yet been established. Presented below are the results of our own investigations.

Biomaterials and Construction

One of the more disputed points is whether insulin adsorption to the pump and/or catheter surfaces is fundamental to the insulin plugging problem. Although several experiments conducted over the last year do not entirely answer this question they do provide definite insights into the behaviour of insulin and its compatability with delivery systems.

Insulin solutions appear to polymerize preferentially in solution rather than forming complex coatings on the surfaces of pumping systems. Polymerization may, however, be a surface process and thus influenced by materials employed within the system. The materials tested included polytetrafluoroethylene (PTFE), titanium, silicone rubber, glass, polyvinylchloride (PVC) and cellulose acetate in the form of 0.22 micron pore filters.

Two implanted pumps (Infusaid®, Metal Bellows Corp., Sharon, Mass.) were explanted after 6 to 9 weeks of intravenous insulin delivery. The pumps were disassembled and all parts concerned were viewed with scanning electron microscopy (SEM) to determine the extent of insulin aggregation on the surfaces. Both pumps were found to be occluded

within the fine 61 μm diameter PTFE flow restricting capillary 1–2 cm down stream from the pump. The plugs, analysed by Eli Lilly and Co., contained substantial amounts of immunoreactive insulin. However all other sections of the teflon capillary were free of deposition (SEM at 1000 X) with no difference between these and control sections. It is noteworthy to add that the capillaries in the pump were approximately 2.7–3.0 m in length and that if adhesion to the teflon surface were indeed a significant precipitating factor then at least some form of distributed deposition should have been visualized. The cellulose acetate filter discs also showed little or no deposition as compared to controls. Any tendency towards aggregation on the surface of this material would be expected to occlude the 0.22 micron pores. The titanium surfaces of the pump reservoir were discoloured but this was found to be copper deposition due to a welding procedure employed during manufacture.

The two silastic catheters leading from the plugged pumps were also cut transversely into 1 cm lengths and viewed with light and scanning electron microscopy. Both catheters contained white aggregates of insulin along their entire length. The viscous white aggregates within the sections dissolved after standing for 5–10 min at room temperature and the catheter sections all became patent. This phenomenon will be discussed in a later section under the heading of CO_2 diffusion and pH. The sections of silastic catheter were rinsed lightly in distilled water and viewed (SEM at 1000 X magnification) with no significant difference being noted between the above surfaces and control sections.

Further experiments to determine the effect of luminal restrictions on insulin plug formation were conducted. To this end ten 20 cm long PTFE capillaries were cut at both ends using a method [39] that ensures an undistorted entrance or exit to flow. In addition, 10 similar control capillaries were cut as above at the upstream end only. The downstream ends however were cut with a sharp razor blade. This method caused distortion and barbing of the lumen. Regular pork insulin (10 U/ml, pH 7.5) was pumped from a 1 l glass vessel (at 25 °C) through the capillaries (at 37 °C) and into sterile glass vials. No decrease in flow rate was observed in 9 out of 10 well cut capillaries after 90 days. The one occluded capillary was sectioned for microscopic examination and the occlusion was found to be particulate matter. Flow in all of the poorly cut PTFE tubes ceased within 20 days. The plugs in these were analyzed by Eli Lilly and Co. and viewed under the SEM. Formed aggregates were partially amorphous and partially crystalline insulin. All of the above plugs were located at the down stream poorly cut exit with insulin aggregates being clearly held within the PTFE barbs protruding into the exit region of the capillaries.

In addition, experience over the last year with our portable insulin delivery systems [1] has revealed that the majority of occlusions occur or collect at tubing junctions where the lumen narrows. The insulin reservoirs (Silicone rubber, PVC and titanium) for both the implantable and portable systems have been inspected regularly and insulin aggregates have been found in all reservoirs that had not been flushed or changed every 3–4 days.

We conclude that flow path restrictions and/or irregularities are contributing variables in the plugging of insulin delivery systems, and that the self-associating behaviour of insulin formulations most probably allows aggregates generated upstream to accumulate in these regions. The degree to which surface interactions, temperature and motion affect the above phenomena has only been partially addressed but will be discussed in a subsequent section.

Metal Ions and Aggregation

As stated previously it has been suggested that the removal of zinc and other metal ions from insulin solutions may decrease self-association. To address this problem three vials (5 U insulin/ml, 0.154 mol/l NaCl, pH 7.5) containing clean etched copper and controls without copper were subjected to gentle rocking at 38 °C. Visible precipitation occurred in those vials containing copper within 4 h. Visible aggregation appeared in the controls only after 3–4 days.

Two occlusions within the teflon capillaries of the implanted pumps were attributed to the above mechanism. Unlike the white insulin precipitates that we have repeatedly found in our insulin delivery systems, these plugs were a transluscent brown. Analysis using atomic absorption techniques showed the plugs to contain 1.6×10^4 ppm Cu and 2.3×10^4 ppm Zn while the reservoir solution contained only 1.4 ppm Cu, 2.0 ppm Zn. Subsequent radioimmunoassay substantiated the presence of insulin. The divalent copper ions were evidently highly concentrated within the plug material. Investigation revealed that copper contamination had occurred during manufacturing and that electrolysis (emf of 0.14 volts between solution and reservoir) was responsible for driving the copper into solution. Implantable pumps (Infusaid™) employing a titanium insulin reservoir and a stainless steel capillary produced similar occlusions which contained substantial amounts of Fe. The same electrolytic process can be attributed to these plug formations.

The presence of certain metal ions is without question a contributing factor in insulin aggregation.

With the employment of more insulin-compatable metallic pump parts (ie. titanium) in combination with zinc-free insulin formations the catalysing effect of metal ions upon aggregation can be eliminated.

Thermodynamic Factors

To test the thermodynamic effects of temperature, motion and certain diluents an in vitro experiment was performed in which neutral pork insulin (5 U/ml) contained in 50 ml capacity sterile glass vials was gently rocked (Pharmacia Roto-Tak Rotor) at 37 and 25 °C for 30 days. Control vials for each of the solutions tested were placed at 37 and 25 °C but not subjected to movement. The solutions were tested in triplicate.

The results of the experiment are presented in Table 1. Noteworthy conclusions are: (1) After 30 days only small amounts of aggregates were observed in those vials that were stationary and at 25 °C. (2) All vials at 37 °C (no motion) demonstrated moderate to severe aggregation (granular particles). Birefringence indicated that these aggregates were partially crystalline. One exception to the above was sulphated insulin. Only minor aggregation was witnessed under 400 X mag. (3) All vials subjected to gentle rocking contained moderate to extremely severe granular and strand-like formations excepting the sulphated insulin which demonstrated only a minor tendency towards aggregation. The strand-like or fibrous formations were observed only in those vials subjected to continuous motion. (4) The combination of elevated temperature (37°) and gentle motion resulted in a higher degree of aggregation than was observed in those vials subjected only to elevated temperature or continuous motion alone. (5) Those solutions containing 0.154 mol/l saline demonstrated the most severe propensity towards aggregation. (6) Extremely small quantities of microscopic granular like aggregates were present in 3 of the 12 vials of regular pork insulin (100 U/ml) before the initiation of the experiment. These aggregates varied from 2.5–25 μm in diameter. Samples from a random selection of vials from a number of manufacturers also showed varying but minute quantities of crystalline and amorphous aggregates. The small quantities would have negligible impact on normal usage of the formulation but may well promote occlusion under the specific conditions encountered during slow pumping through narrow extended capillaries. During this experiment no attempt was made to maintain pH levels by the addition of buffers. However, in all cases the pH remained between 6.65 and 7.5 over the 30 day period.

As stated, the instability of insulin solutions at room temperature has been noted and the propensity for insulin to precipitate when in continuous motion has also been acknowledged by Irsigler and Kritz [3] and Thurow [23].

The successful delivery of insulin described earlier through well cut PTFE capillaries is undoubtedly due to the fact that the insulin delivery vial was maintained at 25 °C and was kept stationary. Thus aggregates large enough to occlude the lumen were not formed.

A further observation on the effect of motion was made during an experiment in which two vials of Insulin Toronto (Connaught Laboratories Ltd.) were checked for signs of gross aggregation. One vial (100 U/ml) was shaken rapidly (low speed – Eberbach Reciprocal Shaker) and became opaque with precipitate after less than 4 days of this motion. A second vial which has been rocked gently (Pharmacia Roto-Tak Rotor) for over 4 months does not yet contain precipitates detectable by eye. Both experiments were carried out at 38 °C.

Experiments were also carried out to test the effect of vigorous shaking (38 °C) in glass containers. Connaught Neutral Pork, specially purified pork insulin and Actrapid (Novo MC Pork) in 3–5 replicates and at a concentration of 5 U/ml were used in neutral 0.154 mol/l NaCl solutions (pH 7.3–pH 7.5). The formulations were examined by the unaided eye and by light microscopy for signs of aggregation. Gross aggregation occurred in all vials within 3–8 days.

Increased purification of the insulin may enhance the stability of the formulations, but in the above experiments increased purification of insulin did not delay the onset of aggregation.

Many other solutions were screened in the preliminary experiments described above and the results, listed in Table 2, show the time at which solutions demonstrated severe aggregation.

The preliminary results reported above indicate that although some additives, namely benzyl alcohol and SDS, do stabilize the formulations to a limited extent, further experiments are required to produce insulin solutions suitable for protracted infusion. New formulations are currently being tested as above and also by a method developed by Thurow [23], described below.

Thurow used a wheel on which vials were rotated at 60 rpm at a distance of 20 cm from the axis in order to test the stability of insulin solutions. The effect of additives on solutions of zinc-free bovine insulin 400 U/ml in Tris-HCl (0.05 mol/l; pH 7.5) and containing 1.8 g/100 ml glycerol was studied. The times, reported by Thurow at Aachen, in which denaturation of insulin to form a turbid gel occurred, are listed in Table 3. The experiments were conducted at 37 °C.

Table 1

Insulin type	Concentration (U/ml)	Diluent	Temperature (°C)	Motion	Severity* of aggregation at 30d.	Fraction aggregated at 30d.
Neutral	5	0.0001%	37	Y	XXXX	3/3
pork		EDTA	37	N	XXX	3/3
		Sodium bicarbonate	25	Y	X	2/3
		(0.10 mmol/l)	25	N	X	2/3
Neutral	5	0.154 mol	37	Y	XXXX	3/3
pork		NaCl/l	37	N	XXX	3/3
			25	Y	XX	3/3
			25	N	X	3/3
Neutral	5	Cysteine	37	Y	XXX	3/3
pork		(0.01 mmol/l)	37	N	XX	3/3
			25	Y	XX	3/3
			25	N	X	2/3
Neutral	5	Cysteine	37	Y	XXXX	3/3
pork		(1 mmol/l)	37	N	XX	3/3
			25	Y	XXX	3/3
			25	N	X	2/3
Neutral	5	1 g Benzyl	37	Y	XXXX	3/3
pork		alcohol/100 ml	37	N	XX	3/3
			25	Y	X	3/3
			25	N	X	2/3
Neutral	5	Sodium bicarbonate	37	Y	XXX	3/3
pork		(0.10 mmol/l)	37	N	XX	3/3
			25	Y	XXX	2/3
			25	N	X	3/3
						2/3
Neutral	5	0.0001%	37	Y	XXX	3/3
pork		EDTA	37	N	XX	3/3
			25	Y	XX	3/3
			25	N	X	3/3
Neutral	5	Cysteine (1 mmol/l)	37	Y	XXX	3/3
pork		Sodium bicarbonate	37	N	XXX	3/3
		(0.10 mmol/l)	25	Y	XX	3/3
			25	N	X	3/3
Neutral	5	Cysteine (1 mmol/l)	37	Y	XXX	3/3
pork		0.0001% EDTA	37	N	XXX	3/3
			25	Y	XX	3/3
			25	N	X	2/3
Neutral	5	Cysteine (1 mmol/l)	37	Y	XXXX	3/3
pork		Sodium bicarbonate	37	N	XX	3/3
		(0.10 mmol/l) 0.0001% EDTA	25	Y	XX	3/3
			25	N	X	2/3
Neutral	5	HCL	37	Y	XXX	3/3
pork		(pH3)	37	N	XX	3/3
			25	Y	X	3/3
			25	N	X	3/3
Neutral	100	–	37	Y	XXX	3/3
pork			37	N	XX	2/3
			25	Y	XX	2/3
			25	N	X	2/3
Sulphated	3	Water for	37	Y	XX	3/3
beef		injection	37	N	X	3/3
		USP	25	Y	XX	3/3
			25	N	X	1/3

X = 10, 2–5 μm aggregates/50 μl
XX = 10–20, 10 μm aggregates/50 μl
XXX = 20–100, 10 μm or > aggregates/50 μl
XXXX = 100, 10 μm or > aggregates/50 μl
Y indicates solution in motion
N indicates no motion

* All observations made at 400 X magnificiation

Table 2

Insulin (5 U/ml)[b]	Diluent	Container	Time to visible aggregation (days)
Sulphated beef	NaCl (0.154 mol/l) pH 6.4	Glass & PVC	13–60[a]
Sulphated beef	NaCl (0.154 mol/l) pH 7.4	Glass & PVC	13–60[a]
Neutral pork	NaCl (0.154 mol/l) with 0.005% SDS	Glass & PVC	4–7
Neutral pork	NaCl (0.154 mol/l) with 0.05% SDS	Glass	35[a]
Sulphated beef	benzyl alcohol 1.0 g/100 ml pH 6.4	Glass & PVC	13–60[a]
Sulphated beef	benzyl alcohol 1.0 g/100 ml pH 7.4	Glass & PVC	13–60[a]
Neutral pork	NaCl (0.154 mol/l) pH 7.4	Glass & PVC	<5
Neutral pork	benzyl alcohol 1.0 g/100 ml pH 7.4	Glass & PVC	60–80[a]
Neutral pork	benzyl alcohol 1.0 g/100 ml, 0.05% SDS	PVC	60–80[a]
Sulphated beef	glycerol 1.27 g/100 ml	Glass	14[a]
Sulphated beef	EDTA (1 mmol/l)	Glass	22[a]
Sulphated beef	polyethylene glycol 1 g/100 ml	Glass	5
Sulphated beef	sodium deoxycholate 1 g/100 ml	Glass	<3
Neutral pork	glycerol 1.27 g/100 ml	Glass	<3
Neutral pork	EDTA (1 mmol/l)	Glass	<3
Neutral pork	polyethylene glycol 1 g/100 ml	Glass	<3
Neutral pork	sodium deoxycholate 1 g/100 ml	Glass	<3
Neutral pork	Tris (10 mmol/l) in Benzyl alcohol 1 g/100 ml	Glass	<5
Neutral pork	Tris (100 mmol/l) with polyethylene glycol 10 g/l	Glass	<5
Neutral pork	histidine (100 mmol/l) with benzyl alcohol 10 g/l	Glass	<5
Neutral pork	histidine (10 mmol/l) with polyethylene glycol 10 g/l	Glass	<5
Neutral pork	histidine (10 mmol/l) with sodium caprylate (10 mmol/l)	Glass	<5
Neutral pork	acetyl tryptophan (10 mmol/l)	Glass	<5
Neutral pork	albumin 50 g/l, sodium caprylate 40 mol/l and acetyl tryptophan 40 mol/l	Glass	<5
Neutral pork	BES (N,N-bis, 2-hydroxy-ethyl-2-aminoethane sulphonic acid) (10 mmol/l)	Glass	<5
Neutral pork	HEPES (N-2-hydroxy-ethylpiperazine-N-2-ethane sulphonic acid) (10 mmol/l)	Glass	<5
Neutral pork	TES (N-tris-(hydroxymethyl) -methyl-2-aminoethane sulphonic acid) (10 mmol/l)	Glass	<5
Neutral pork (2 U/ml)	sodium phosphate (0.02 mol/l)	PVC	8–11
(2 U/ml)	histidine (0.01 mol/l)	PVC	8–11
(2 U/ml)	dimethyl glutarate (0.05 mol/l)	PVC	8–11
(2 U/ml)	glycylglycine (0.01 mol/l)	PVC	8–11
(2 U/ml)	ammonium citrate (0.01 mol/l) in NaCl (0.154 mol/l)	PVC	4
(2 U/ml)	glycine (22.5 g/l) with NaCl (0.154 mol/l)	PVC	4

[a] In these solutions, the time of aggregation was difficult to interpret as small particulate matter was detectable before gross precipitation occurred

[b] Unless otherwise specified

For comparison, it should be noted that in our experiments using a similar rotating apparatus but at lower concentrations (5 U/ml) as required by our pumping systems, neutral pork insulin became cloudy in less than 4 d in 0.154 mol/l NaCl and in less than 20 d in 1 g/100 ml benzyl alcohol. Other formulations are currently being investigated in this manner.

Analysis of Aggregated Material

The precipitated material produced by shaking neutral pork insulin (5 U/ml) in 0.154 mol/l NaCl in both PVC and glass containers was submitted to electrophoretic analysis in an attempt to characterize the type of inter-molecular bonding which causes the for-

Table 3

Solution	Denaturation time (h)
Control (no additives	20
0.196 5,5' dithio-bis-(2-nitrobenzoic acid)	20
0.5% iodoacetic acid	24
0.5% thiosalicylic acid	18
0.196 di-tert-butyl-p-cresol	18
0.2% ascorbic acid	20
0.1% EDTA	19
0.05% nordihydroguajaretic acid	27
0.5% B, B'-thiobis propionic acid	20
In the following 0.1% methyl-4-hydroxybenzoate was also present	
Control (no additives)	20
3.5 M urea	10
3.5 M guanidine	12
7% pyridine	7
1% Triton WR 1339	18
28 μg/ml Zn	5 days

mation of these aggregates. The precipitate was collected by centrifugation, dialyzed to remove salt and lyophilized prior to dissolution in the respective electrophoresis buffers.

Electrophoresis of the samples in urea (8 mol/l) and acetic acid (0.9 mol/l) at pH 3.0 indicated the presence of very high molecular weight material which did not enter the gel, several high molecular weight insulin-derived materials which entered the gel, insulin itself, and some desamido insulins.

Non-reducing SDS electrophoresis at pH 8.3 was performed using a modification of the method reported by Laemmli [40]. A certain amount of the sample did not enter the gel and a clear band of high molecular weight material was seen in addition to the insulin band. Layer gel electrophoresis in urea (6 mol/l) at pH 9.0 also indicated the presence of high molecular weight or insoluble components which did not enter the gel.

All of these results point to the irreversible formation of intermolecular covalent linkages. Further analysis should indicate the nature of the groups involved in these covalent reactions.

CO_2 Diffusion and pH

CO_2 diffusion through semi-permeable plastic and rubber components in insulin infusion systems can lead under certain circumstances to significant reduction in pH of neutral insulin solutions. Naturally as the isoelectric point is approached precipitation of the protein occurs. At present, work is being conducted to elucidate buffering systems that are compatible with the hormone. To date all solutions prepared with sodium phosphate buffer have led to precipitation of zinc phosphate from the solution. In lieu of the above, two of our most recent implants in beagles employed insulin solutions containing no buffering system. Flow was maintained in the implants for 6–9 weeks at which time flow terminated due to insulin plugs formed in the 61 μm ID teflon flow restrictor which leads into the 0.4 mm ID silastic delivery catheter. The pumps were implanted subcutaneously and intravenous delivery was through the circumflex iliac vein.

Three days following total occlusion the pumps were explanted and the silastic catheters found to be occluded along their entire length with viscous white flocules. The pH of the viscous material was 5.8 while that of the reservoir was 7.0. As noted previously the catheters quickly became patent after standing on the bench at room temperature. It was suggested that the low flow rates (0–0.2 ml/d) just prior to explantation in combination with the elevated intravascular pCO_2 levels allowed a fall in pH sufficient to initiate precipitation. In vitro experiments verified the above and substantiated that flow rates and catheter wall thickness must be such that the CO_2 diffusion cannot lead to precipitation. Plastic or rubber insulin reservoirs should also be designed with these thoughts in mind. Of course with compatible buffers this restriction would not be necessary.

Conclusions

Abrupt changes in flow path, motion, elevated temperatures, metal ion contamination, impure insulin formulations, CO_2 diffusion, pH drop, dissimilar metal pump components, salt concentration, inappropriate diluents, elevated temperatures, refrigeration temperatures, processing, insulin heterogeneity, and buffering systems have been implicated to a greater or lesser extent in the plugging of insulin delivery devices. Before the rate at which insulin loses its biological activity in delivery systems can be assessed it is obvious that anti-aggregating diluents must be developed and subjected to long-term testing both in vitro and in vivo. Until such stable homogenous formulations are available the knowledge presented in this article will serve to decrease, but not eliminate, the problem of insulin aggregation in delivery systems. Further experiments are in progress and preliminary results [41] provide evidence that the problems cited are not without resolution. In this regard serum apparently contains factor(s) that promote the dissolution of insulin and prevent the formation of peptide aggregates in dilute solutions [41]. Many laboratories are now working to resolve the problem of insulin aggregation in artificial delivery devices.

Acknowledgements. This work was conducted as a research project in the Artificial Pancreas Program at the Hospital for Sick Children and at Connaught Laboratories, Ltd., Toronto, Ontario. It was funded in part by Contract No. NO1-AM-9-2201 from the

National Institutes of Health, Bethesda, Maryland and in part by grants from the Medical Research Council of Canada, and the Metal Bellows Company of Sharon, Mass.

The authors gratefully acknowledge the assistance provided by Dr. L. F. Ellis of Eli Lilly and Co., Indianapolis, Indiana; Mrs. I. Leitis, Mr. B. Wilson and Mr. M. Teitelman of the Dept. of Pathology, Hospital for Sick Children, Toronto; Mr. J. C. Van Loon, Dept. of Geology, University of Toronto, Toronto; Mr. E. Tucker and Mr. F. Prosl of the Metal Bellows Co., Sharon, Mass.; and Dr. A. Bahoric, Hospital for Sick Children, Toronto, Canada. The preparation of this review followed a suggestion to this effect made to one of its authors (AMA) by Dr. J. S. Soeldner of the Joslin Research Laboratories, during the deliberations of the Bioengineering Support Systems Workgroup of the National Conference on Diabetes: Current Status and Future Directions held in Reston, Virginia in October, 1979.

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