Chemical Stability of Insulin. 2. Formation of Higher Molecular Weight Transformation Products During Storage of Pharmaceutical Preparations

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Formation of covalent, higher molecular weight transformation (HMWT) products during storage of insulin preparations at 4–45°C was studied by size exclusion chromatography. The main products are covalent insulin dimers (CID), but in protamine-containing preparations the concurrent formation of covalent insulin–protamine (CIP) products takes place. At temperatures ≥25°C parallel or consecutive formation of covalent oligo- and polymers can also be observed. Rate of HMWT is only slightly influenced by species of insulin but varies with composition and formulation, and for isophane (NPH) preparations, also with the strength of preparation. Temperature has a pronounced effect on CID, CIP, and, especially, covalent oligo- and polymer formation. The CIDs are apparently formed between molecules within the hexameric unit common for all types of preparations and rate of formation is generally faster in glycerol-containing preparations. Compared with insulin hydrolysis reactions (see the preceding paper), HMWT is one order of magnitude slower, except for NPH preparations.

KEY WORDS: insulin; insulin preparation; chemical stability; covalent dimerization; polymerization; covalent insulin protamine.

INTRODUCTION

In the preceding paper (1) the chemical degradation of insulin in pharmaceutical formulations was studied with respect to formation of hydrolytic degradation products. However, insulin in preparations has also been found to be chemically transformed by formation of insulin di- and polymerization products (2–5).

Steiner and co-workers were the first to show the presence of a (''nonconvertible'') insulin dimer in insulin crystals (6) and suggested that it was an artifact associated with commercial production (7). Similar covalent dimers have been isolated by Helbig (8) as a heterogeneous mixture of different insulin derivatives, in some of which the N-terminal amino groups from one insulin molecule had reacted with the A21 amido group from another molecule. Partial dimerization of insulin at pH 7 as a result of disulfide exchange was reported by Csorba *et al.* (9). The potential sites for formation of such insulin transamidation (intermolecular aminolysis) and disulfide exchange products are illustrated in Fig. 1.

We report in this communication the formation of covalent, higher molecular weight transformation (HMWT) products during storage of insulin preparations of different types and formulations, as revealed by the appearance of peaks eluting in front of the insulin peak when analyzed by size exclusion chromatography (SEC). Preliminary accounts have been published in abstract form (3,4) and in excerpt (5). For detailed information about the different types of insulin preparations studied, reference is made to the preceding paper (1).

MATERIALS AND METHODS

Materials

Insulins and chemicals used and the preparation of the different formulations were described in the preceding article, in which also a survey of the different preparations and their content of auxiliary substances were included (Ref. 1, Tables I and II). The majority of batches was from the production line of Novo Industri A/S, but batches produced on a smaller scale in the laboratory were also studied. The insulin preparations including samples from other manufacturers were handled as described earlier (1).

Methods

Isolation and Storage of Samples. Dissolved insulin was isolated by zinc precipitation and subsequent centrifugation as described earlier (1). Due to the requirements of low ion strength mentioned below, the precipitate was washed with 20 μl of 1 m*M* zinc acetate/mg of insulin precipitate. Insulin in suspensions was isolated by centrifugation and washed as described above. Samples were stored, without drying, in the deep freeze (−20°C) until analysis.

Analytical Procedures. Samples were analyzed for content of HMWT products either by conventional size exclusion chromatography (CSEC) or by high-performance size exclusion chromatography (HPSEC). CSEC was performed on columns (25 × 400 mm) of Bio-Gel P 30, 100–200 mesh (Bio-Rad Laboratories) equilibrated for at least 20 hr with 1 *M* acetic acid in order to ensure the lowest possible baseline readings. Separation efficiency of each column packing was checked by running a standard impure insulin sample (once-crystallized porcine insulin) and columns were repacked after every 10 runs. Insulin samples (10–100 mg of insulin depending on the expected content of HMWT products) were dissolved in 2 *M* acetic acid and applied on the column in a total volume not exceeding 1.5 ml. It was found necessary to keep the ion strength of the applied sample as low as possible in order to avoid artificial splitting of the insulin peak into two peaks. In cases of voluminous insulin samples (amorphously precipitated insulin) from preparations made isotonic with NaCl, the salt content in the supernatant was reduced by washing with 1 m*M* zinc acetate and centrifugation. Elution with 1 *M* acetic acid was performed at ambient temperature at a rate of 3 cm/hr and 1.5-ml fractions were

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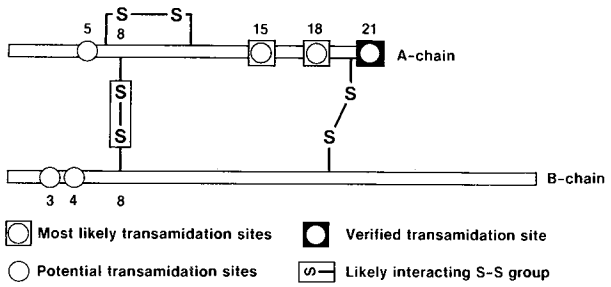

Fig. 1. A schematic illustration of potential sites involved in covalent di- and polymerization of insulin.

collected. Insulin and HMWT products were detected as the area under the curve by measuring absorbance at 276 nm. As protamine has no absorbance at this wavelength, the measured amount of CIP is not a weight fraction but accounts only for the fraction of chemically transformed insulin. Using the procedure described the detection limit for HMWT products was found to be 0.05–0.1% and reproducibility was approx. ±3% for estimation of CID content above 1% (coefficient of variation = 0.033, n = 7) and ±9% for values below 1% (CV = 0.094, n = 6). For a covalent polymer content of about 2%, the reproducibility was ±6% (CV = 0.056, n = 5).

HPSEC was performed on a Waters I-125 column (7.8 × 300 mm) using an eluent with 2.5 *M* acetic acid, 4 m*M* *l*-arginine, and 4% (v/v) acetonitrile. Elution was performed at ambient temperature at a rate of 1 ml/min and insulin and HMWT products were detected by their absorbance at 280 nm. The reproducibility of this method was found to be similar to that of the CSEC method.

Data Analysis. The experimental data were analyzed by least-squares polynomial regression analysis including a linear and a quadratic term:

$$D = at + bt^2$$

where D is the fraction of transformed insulin as percentage of total and t is time as months. In the cases where coefficient b is not significantly different from zero, using a statistical significance level of 0.05, the quadratic term is excluded. Results are generally quoted as the initial rate constant, i.e., a, and its standard error, supplemented with b when it is significant. Significance testing was performed by standard F tests.

RESULTS

Covalent insulin dimer (CID) products are formed in all types of insulin formulations during storage of the preparations. In the preparations formulated with protamine an extra peak eluting in front of the peak containing the CID products can be observed at all storage temperatures. This peak which has been shown to contain reaction products of insulin linked covalently to protamine (10) is subsequently referred to as covalent insulin protamine (CIP). At higher storage temperatures (≥25°C) additional formation of covalent insulin polymerization products can be observed in all preparations. Figures 2 and 3 illustrate the diversity in the time courses of formation of the HMWT products in different types of pharmaceutical insulin preparations.

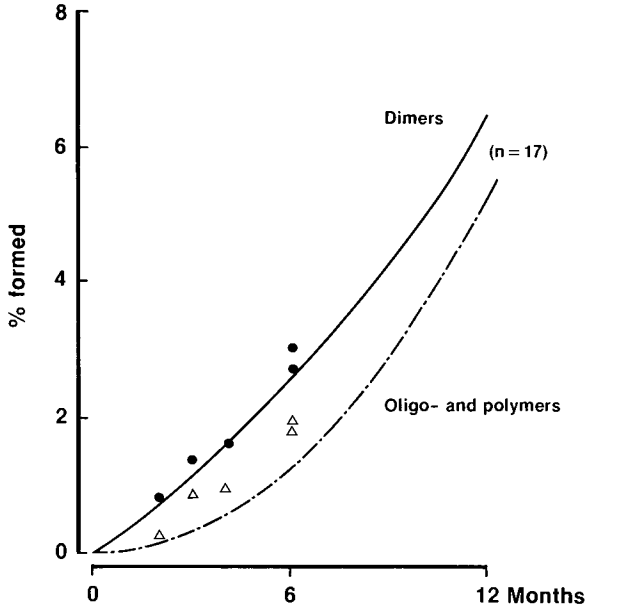

Fig. 2. Time courses of formation of dimers (——) and oligo- and polymers (– · – · –) during storage of regular N1 porcine insulin at 37°C. The lines represent the best fits of data (n = 17) on preparations of U 40 strength. Additional data on formation of covalent dimers (●) and oligo- and polymers (△) in preparations of U 100 strength reveal a tendency to increased formation of covalent oligo- and polymers with increasing strength of insulin.

Rate Data

Rate data for the different types of preparation with respect to the total formation of HMWT products are shown in Tables I–III. Except for the protamine-containing prepa-

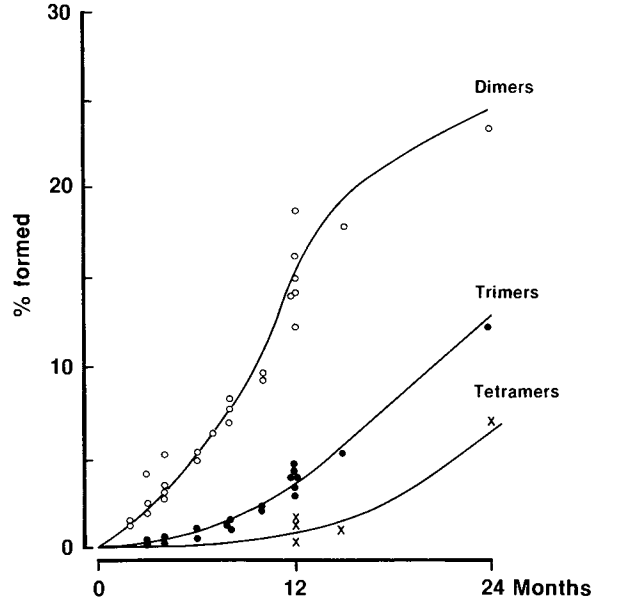

Fig. 3. Time courses of formation of insulin oligomers during storage of insulin zinc suspension, crystalline (bovine or porcine), at 37°C.

Table I. Total HMWT Product Formation in Protamine-Containing Preparations[a]

Preparation	Species[b]	Strength	Temperature (°C) 4	15	25	37	45
NPH	Bovine	U 100	3.1 ± 0.7 (n = 8)	6.6 ± 1.1 (n = 8)	22.1 ± 1.4 (n = 14)	116 ± 33 (n = 5)	
			n.s.	$P < 0.05$	$P < 0.001$	$P < 0.05$	
	P and H	U 100	4.0 ± 0.5	9.7 ± 0.9	27.8 ± 1.0	160 ± 5	462 ± 27 $b = -92$[c]
			(n = 23)	(n = 17)	(n = 34)	(n = 20)	(n = 9)
			n.s.	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$
	P and H	U 40	5.0 ± 0.4	18.9 ± 1.6	53.7 ± 3.0 $b = -0.6$	271 ± 17 $b = -19$	773 ± 48 $b = -178$
			(n = 31)	(n = 24)	(n = 46)	(n = 31)	(n = 6)
			n.s.	$P < 0.01$	$P < 0.001$		
	P	U 80	3.8 ± 0.8 (n = 10)	14.0 ± 1.3 (n = 11)	27.0 ± 2.7 (n = 10)		
PZI	P	U 40	6.2 ± 0.8	16.3 ± 0.5	80 ± 9	1180 ± 80 $b = -98$	
			(n = 11)	(n = 7)	(n = 8)	(n = 4)	

[a] Figures are the apparent first-order rate constants ($\times 10^4$ month^{-1}) ± SE.
[b] P, porcine; H, human.
[c] b values ($\times 10^4$), the coefficient to the quadratic term, are given when the polynomial expression gives the best fit of the time course.

rations, the CID fraction is the only kind of HMWT product formed at the lower storage temperatures (4–15°C). Data for the parallel formation of CID and CIP products during storage of NPH at several temperatures are listed in Table IV. At the higher storage temperatures (25–45°C) additional formation of products with a MW higher than the CID occurs in some of the preparations (Table V).

Effect of Concentration and Species of Insulin

For most preparations there is no major influence of the insulin concentration on the formation of CID. In contrast, increasing the concentration of insulin in neutral solution tends to increase the rate of formation of covalent oligo- and polymers at the higher storage temperatures (Fig. 2 and Table VI). The NPH formulation is the only type of preparation in which the strength of insulin has a highly significant effect on the formation of HMWT products including CID as well as CIP (Table I and IV).

There is no overall tendency that one particular species of insulin is more stable than another. Thus bovine insulin is the most stable species of insulin in the NPH formulation but exhibits the fastest rate of transformation when used in the regular N1 formulation. Generally human and porcine insulin exhibit the same rate of HMWT product formation, and only in the preparations containing rhombohedral crystals [insulin zinc suspensions (IZS), crystalline and mixed] is porcine insulin significantly more stable than human insulin.

Effect of Composition and Formulation

In neutral solution formation of HMWT products is 40–160% higher in regular N2 (with phenol + glycerol) compared to regular N1 (with methylparaben + NaCl) (Table III). In the protamine-containing preparations, neutral protamine Hagedorn (NPH; crystalline suspension) and protamine zinc insulin (PZI; amorphous suspension), formation of HMWT products is comparable at the lower temperatures, but at 25 and 37°C the PZI formulation is becoming increasingly more unstable than the crystalline NPH preparation (Table I). The same tendency to inferior stability of amorphously precipitated insulin at the higher storage temperatures, when compared with insulin in crystalline suspension, can be observed for the IZS type of preparation (Table II).

Manufacturer

Comparison of human insulin preparations from four manufacturers revealed that the rate of formation of HMWT products in neutral solutions can vary up to 100% between different manufacturers, whereas only small differences are seen within NPH preparations of the same strength (Fig. 4).

DISCUSSION

The present study demonstrates the following.

(1) Intermolecular chemical reactions occur in all pharmaceutical insulin formulations but at rates which are generally much slower than those observed for hydrolytic reactions (1).

(2) CID are the main products formed in most preparations, but in protamine-containing preparations the concurrent formation of covalent insulin–protamine reaction products takes place at all storage temperatures.

(3) At storage temperatures ≥25°C parallel or consecutive reactions are seen in most types of preparations, resulting in the formation of covalent insulin oligo- and polymers.

Table II. Total HMWT Product Formation in Insulin Zinc Suspensions (IZS Types of Preparations)[a]

Preparation	Species[b]	Strength	Temperature (°C) 4	15	25	37	45
IZS, amorph.	P	U 40, 80	0.28 ± 0.06 (n = 12)	1.2 ± 0.1 (n = 15)	7.9 ± 0.3 (n = 45)	154 ± 6 (n = 29)	
IZS, cryst.	B	U 40, 80				107 ± 15 b = 3.1[c] (n = 7)	
				2.0 ± 0.1	6.8 ± 0.9 b = 0.2	↑	366 ± 18
				(n = 18)	(n = 43)	P < 0.05 ↓	(n = 9)
			0.27 ± 0.04 (n = 19)				
	P	U 40, 80			↑ P < 0.001 ↓	52 ± 19 b = 8.5 (n = 21)	
						P < 0.01	
	H	U 40, 100		No data	11.3 ± 4.1 b = 0.2 (n = 8)	141 ± 38 b = 8.1 (n = 6)	
IZS, mix.	B/P, P, H	U 40, 80, 100	0.66 ± 0.08 (n = 74)				
	H	U 40, 100		2.2 ± 0.1	10.3 ± 0.9 b = 0.17	134 ± 24 b = 3.7	517 ± 87 b = 12
				(n = 8)	(n = 23)	(n = 15)	(n = 11)
				P < 0.02	P < 0.001	P < 0.001	P < 0.002
	P	U 40, 80, 100		1.5 ± 0.3	6.2 ± 2.1 b = 0.15	88 ± 12 b = 2.0	365 ± 92 b = 15
				(n = 5)	(n = 7)	(n = 9)	(n = 15)
					P < 0.001		n.s.
	B/P	U 40, 80, 100			12.7 ± 1.5 b = 0.13 (n = 10)		449 ± 15 (n = 8)

[a] Figures are the apparent rate constants ($\times 10^4$ month^{-1}) ± SE.
[b] B, bovine; P, porcine; H, human; B/P, mixture of bovine crystals and porcine amorphous phase.
[c] b values ($\times 10^4$), the coefficient to the quadratic term, are given when the polynomial expression gives the best fit of the time course.

(4) The rate of formation of HMWT products varies with the composition and formulation of the preparations and, in some cases, with the strength and species of insulin. Differences with respect to HMWT product formation can be observed within the same type of preparation from various manufacturers.

CID Formation

Covalent dimers are the main products formed in all preparations except for the protamine-containing preparations (NPH and PZI). In the neutral solutions the insulin molecules are associated mainly into noncovalent, Zn^{2+}-containing hexamers (5,11). The observation that the rate of formation of CID in the neutral solutions is independent of the insulin concentration strongly indicates that the intermolecular chemical reaction occurs mainly within the hexameric units, and not to any significant extent between the hexamers in the solution. As the crystalline and amorphous suspensions share with the solutions the hexamer as the common unit, CID is again most likely to arise mainly from intermolecular reactions within the hexamer in these preparations. This theory is supported by the fact that CID formation is of the same order of magnitude in neutral solution and in suspensions when these preparations contain similar auxiliary substances (regular N1 versus IZS types).

It is normally assumed that chemical decomposition of drug suspensions takes place solely in the part of the drug in solution (12). In the IZS types of formulations the amount of insulin in solution is extremely small (<0.1 IU/ml) but the CID formation, nevertheless, occurs to the same extent as in the neutral solutions containing 100 IU/ml, indicating that the transformation reactions in these suspensions occur mainly within the solid crystalline phase. This is conceivable because the molecules in an insulin crystal have some conformational flexibility of their side chains and backbone for movements within the crystal lattice (13).

CID formation is generally fastest in preparations containing glycerol and phenol/cresol (regular N2 and NPH). Glycerol is well recognized to be able to stabilize proteins

Table III. Total HMWT Product Formation in Insulin Preparations Containing Dissolved Insulin[a]

Preparation	Species[b]	Strength	Temperature (°C) 4	15	25	37	45
Regular A2	P, B	U 40	9.3 ± 1.1 (n = 4)		102 ± 3 (n = 3)		
Regular N1	B	U 40, 80			15 ± 1 (n = 4)	104 ± 11 b = 1.7[c] (n = 7)	
					$P < 0.001$	$P < 0.001$	
	P, H	U 40–200	0.65 ± 0.06 (n = 33)	1.95 ± 0.13 (n = 39)	8.1 ± 0.2 (n = 67)	54 ± 6 b = 3.6 (n = 32)	186 ± 14 b = 4.5 (n = 33)
N1 vs N2 (P, H) at all temperatures: $P < 0.01$							
Regular N2	P, H	U 40–500	1.64 ± 0.13 (n = 37)	3.5 ± 0.3 (n = 17)	11.2 ± 0.4 (n = 32)	140 ± 17 b = −12 (n = 25)	456 ± 56 b = −53 (n = 16)
Biphasic	P/B	U 40	0.95 ± 0.13 (n = 17)	3.5 ± 0.2 (n = 14)	11.9 ± 3.1 b = 0.36 (n = 23)	106 ± 20 b = 4.6 (n = 5)	

[a] Figures are the apparent first-order rate constants ($\times 10^4$ month^{-1}) ± SE.
[b] B, bovine; P, porcine; H, human; P/B, mixture of porcine dissolved and bovine crystalline insulin.
[c] b values ($\times 10^4$), the coefficient to the quadratic term, are given when the polynomial expression gives the best fit of the time course.

physically (14,15) and phenol has been demonstrated to enhance the stability of the insulin hexamer (16). It might be expected that such increased structural stability would result in diminished conformational flexibility and, consequently, decreased chemical reactivity as actually seen in the hydrolytic decomposition of insulin in the presence of phenol (1). However, glycerol at a high concentration has been shown to cause substantially increased formation of covalent insulin di- and polymers (17). Glycerolaldehyde, a potential impurity in glycerol formed by oxidation of the glycerol during

Table IV. Formation of Insulin Covalent Dimer and Covalent Insulin Protamine (CIP) Products in NPH Preparations[a]

Species[b]	Strength	Product	Temperature (°C) 4	15	25	37	45
P, H	U 40	Dimer	1.86 ± 0.17	8.3 ± 0.7	20.8 ± 0.8	127 ± 8 b = −12[c]	353 ± 24 b = −88
		CIP	2.54 ± 0.13	9.0 ± 0.6	23.5 ± 0.9	144 ± 10 b = −7	420 ± 51 b = −90
			(n = 27)	(n = 18)	(n = 38)	(n = 31)	(n = 6)
P	U 80	Dimer	1.60 ± 0.38	6.9 ± 0.4	12.9 ± 1.4		
		CIP	1.73 ± 0.31	6.8 ± 0.6	13.7 ± 1.6		
			(n = 9)	(n = 6)	(n = 9)		
P + H, U 40 vs U 100 (dimer and CIP):			n.s.	$P < 0.001$			
P, H	U 100	Dimer	1.53 ± 0.22	4.3 ± 0.5	14.3 ± 1.0	69 ± 3	205 ± 23 b = −35
		CIP	2.4 ± 0.3	5.5 ± 0.5	18.1 ± 1.4	90 ± 3	257 ± 11 b = −56
			(n = 21)	(n = 15)	(n = 29)	(n = 20)	(n = 9)
P + H vs B (U 100)		CIP: n.s. at all temperatures Dimer:	n.s.	$P < 0.02$	$P < 0.001$	$P < 0.005$	
B	U 100	Dimer	1.2 ± 0.3	2.5 ± 0.3	7.4 ± 0.8	41 ± 11	
		CIP	2.0 ± 0.4	4.9 ± 0.7	14 ± 1	75 ± 21	
			(n = 7)	(n = 7)	(n = 14)	(n = 5)	

[a] Figures are the apparent first-order rate constants ($\times 10^4$ month^{-1}) ± SE.
[b] P, porcine; H, human; B, bovine.
[c] b values ($\times 10^4$), the coefficient to the quadratic term, are given when the polynomial expression gives the best fit of the time course.

Table V. Formation of Insulin Covalent Dimer and Covalent Polymerization Products in Insulin Preparations[a]

Preparation	Species[b]	Product	Temperature (°C) 25	37	45
Regular N1	P, H	Dimers	6.4 ± 0.2	54 ± 3	177 ± 12
		Polymers	0.22 ± 0.02	1.7 ± 2	69 ± 6
			(n = 67)	(n = 32)	(n = 33)
IZS, amorph.	P, H	Dimers	5.0 ± 0.3	55 ± 2	97 ± 11
		Polymers	3.2 ± 0.3	92 ± 11	220 ± 16
			(n = 5)	(n = 13)	(n = 4)
IZS, cryst.	B, P	Dimers	8.0 ± 0.8	116 ± 4	
		Trimers	1.1 ± 0.2	29 ± 3	
		Tetramers		8 ± 2	
			(n = 43)	(n = 28)	

[a] Figures are the apparent rate constants ($\times 10^4$ month^{-1}) ± SE.
[b] B, bovine; P, porcine; H, human.

storage, is able to react chemically with amino groups through its carbonyl group under formation of Schiff base adducts. These reaction products are able to undergo Amadori rearrangement (18), which produces a new carbonyl function capable of forming Schiff base linkages with another amino group. Such reactions and subsequent rearrangement can therefore result in covalent cross-linking of proteins (18,19). Thus, in addition to the potential of forming CID by transamidation reactions (10), the CID can also be generated via an initial reaction with glycerol degradation products. The decrease in CID (and CIP product) formation with increasing insulin strength in the NPH preparation clearly indicates involvement of the auxiliary substances, as the concentration of excipient relative to that of insulin decreases with increasing strength of the preparation.

The rate of CID formation seems to increase with time in the preparations containing methylparaben (positive b values), whereas a more constant or declining rate with time applies to the preparations with phenol as preservative. As CID formation at neutral reactions increases with falling pH (unpublished observation), this difference is consistent with the fact that pH changes to lower values during storage of the preparations containing methylparaben as preservative agent (5).

CIP Formation

Although the content of protamine in the NPH crystals corresponds to only 0.15 protamine molecule per insulin monomer, the chemical reaction between protamine and insulin proceeds in the NPH preparation at all temperatures at a rate slightly higher than the rate of CID formation (Table IV). Protamine from salmon (salmine), used in the preparations, does not contain asparaginyl or glutaminyl residues, nor does it include side-chain amine groups (Lys) in the sequence. It is therefore able to participate in transamidation or Schiff base-mediated reactions only with its N-terminal amino group, but because protamine has great conformational flexibility within the crystal lattice (Dodson, personal communication), its N terminal has the capacity to react with insulin by intermolecular aminolysis, resulting in transamidation between the molecules.

Table VI. Effect of Concentration on Formation of HMWT Products in Regular N1[a]

Concentration	Temperature (°C) 25 Dimers	25 Oligo- & polymers	37 Dimers	37 Oligo- & polymers	45 Dimers	45 Oligo- & polymers
U 40	6.6	0.2	52	0.6	139	23
	(n = 53)		(n = 26)		(n = 16)	
U 80	6.3	1.4	48	3.9		
	(n = 8)		(n = 3)			
U 100	1.5	1.3	73	18	171	69
	(n = 4)		(n = 3)		(n = 10)	
U 500					214	78
					(n = 7)	
Conc. effect	n.s.	$P < 0.05$	n.s.	n.s.	n.s.	n.s.

[a] Figures are the apparent rate constants ($\times 10^4$ month^{-1}).

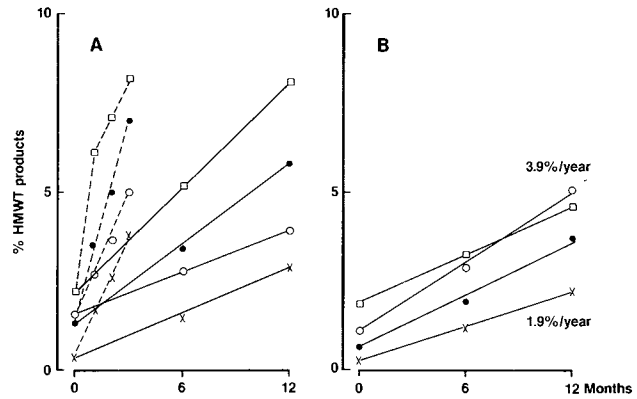

Fig. 4. Formation of HMWT products in human insulin preparations from four manufacturers during storage at 25°C (——) or 37°C (– – – –). Manufacturer 1 (●); 2 (□); 3 (○); 4 (×). (A) NPH insulin: 40 IU/ml (● and □); 100 IU/ml (× and ○). (B) Regular N2: all 100 IU/ml.

Covalent Oligo- and Polymer Formation

The covalent trimers and tetramers formed in IZS preparations are most likely products of continued transamidation. In comparison, the formation of covalent polymer in the neutral solutions and in the preparations with amorphously precipitated insulin (IZS, amorph.) seems to occur in parallel with the formation of CID, probably as a result of disulfide interchange (10). Such reactions are catalyzed by thiols, which can arise by initial hydrolytic cleavage of disulfides or via beta-elimination in neutral and alkaline media (20). Intermolecular disulfide exchange requires closeness between disulfide bridges from different insulin molecules. This is not the case within the hexamer or when hexamers pack in the rhombohedral (IZS cryst. preparations) or monoclinic (NPH) crystals. It is therefore not surprising that covalent polymerization due to disulfide reshuffling is undetectable in these types of preparations. However, such reaction becomes possible when the individual hexamers are capable of approaching one another in a random way as in solution or when the insulin is amorphously precipitated. The increasing oligo- and polymer formation with increasing insulin concentration in regular N solutions (Fig. 2 and Table VI) indicates that the reaction takes place between different hexamers in the neutral solution.

Whereas the initial disulfide lysis is a slow process, and therefore rate determining, the subsequent interchange reactions are fast, and as every single interchange leaves a new highly reactive thiolate ion, the initial hydrolysis starts a chain reaction resulting in accelerating polymer formation. Thus, the accumulation of CID and covalent oligomers as a result of disulfide interchange is expected to be low, as also revealed by the low proportion of these products after storage at temperatures at which these processes prevail.

Influence of HMWT Products on the Quality of Preparations

The formation of HMWT products in commercial insulin preparations is generally much slower than the chemical decomposition of the insulin as a result of hydrolytic reactions taking place during storage (1). The impact on the quality and therapeutic usefulness of the preparations might, however, be more serious. Thus, some of the immunological side effects associated with insulin therapy may be due to the presence of covalent aggregates of insulin in the therapeutical preparations (21–23), and specific antibodies against CID have been identified in 30% of insulin-treated diabetic patients (21). Recently cutaneous allergic manifestations resulting from the cell-mediated hypersensitivity response to CID have been reported and the response to stimulation of allergic patients' lymphocytes by commercial insulin preparations was demonstrated to vary with the content of CID in the preparations. A CID level of 2% generated a highly significant response, whereas no significant response was seen when the CID content was between 0.3 and 0.6% (23). Therefore, in order to minimize the frequency of clinical allergic reactions, the content of CID should be kept as low as possible, preferably below 1%. Times to formation of 1% of HMWT products during storage of some of the preparations are shown in Table VII.

The significance of the CIP product in relation to immunological side effects of insulin treatment is unresolved. More than one-third of diabetic subjects treated with NPH have circulating antibodies against protamine (24), and the presence of such antibodies as well as of insulin antibodies might be connected with the content of the CIP products in NPH.

Table VII. Times to Formation of 1% of HMWT Products in Different Preparations (Months)[a]

Preparation	Species	Temperature (°C)			
		4	15	25	37
Regular N1	Human, porcine	154	51	12	1.7
Regular N2	Human, porcine	61	29	9	1.3
NPH (U 100)	Human, porcine	25	10	3.6	0.6
IZS, amorphous	Porcine	357	83	13	0.6
IZS, crystalline	Bovine	370	50	11	0.9
	Human	370	No data	5.2	No data
IZS, mixed	Human	152	45	8.5	0.7

[a] The figures are calculated using the rate data from Tables I–III.

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