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Research Article

Chemical Stability of Insulin. 1. Hydrolytic Degradation During Storage of Pharmaceutical Preparations

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Hydrolysis of insulin has been studied during storage of various preparations at different temperatures. Insulin deteriorates rapidly in acid solutions due to extensive deamidation at residue Asn^{A21}. In neutral formulations deamidation takes place at residue Asn^{B3} at a substantially reduced rate under formation of a mixture of isoAsp and Asp derivatives. The rate of hydrolysis at B3 is independent of the strength of the preparation, and in most cases the species of insulin, but varies with storage temperature and formulation. Total transformation at B3 is considerably reduced when insulin is in the crystalline as compared to the amorphous or soluble state, indicating that formation of the rate-limiting cyclic imide decreases when the flexibility of the tertiary structure is reduced. Neutral solutions containing phenol showed reduced deamidation probably because of a stabilizing effect of phenol on the tertiary structure (α-helix formation) around the deamidating residue, resulting in a reduced probability for formation of the intermediate imide. The ratio of isoAsp/Asp derivative was independent of time and temperature, suggesting a pathway involving only intermediate imide formation, without any direct side-chain hydrolysis. However, increasing formation of Asp relative to isoAsp derivative was observed with decreasing flexibility of the insulin three-dimensional structure in the formulation. In certain crystalline suspensions a cleavage of the peptide bond A8–A9 was observed. Formation of this split product is species dependent: bovine > porcine > human insulin. The hydrolytic cleavage of the peptide backbone takes place only in preparations containing rhombohedral crystals in addition to free zinc ions.

KEY WORDS: insulin; chemical stability; deamidation; hydrolysis; autocatalysis; chain cleavage.

INTRODUCTION

Insulin has been in therapeutic use for 70 years but its stability in pharmaceutical formulations has so far been studied systematically only in terms of biological potency (1,2). Little information has appeared in the literature on the chemical transformation of insulin during storage of insulin preparations, and most studies have dealt with the hydrolysis in acid medium into insulin desamido products (3,4). Decomposition of insulin during storage of bovine insulin in solid state has been studied by Fisher and Porter (5). Today virtually all insulin preparations are neutral solutions or suspensions in which deamidation occurs at a much slower rate (6,7).

Insulin has been formulated into many different pharmaceutical preparations varying with respect to type (timing characteristics), which differ as to exact formulation and composition (for review see Ref. 8). However, very little has been reported about the influence of formulation and composition on the chemical stability of insulin during storage of the preparations.

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Until the late 1960s crystalline insulin was considered essentially pure insulin, but the introduction of new analytical methods made it possible to detect the presence of significant amounts of protein impurities by disc electrophoresis and gel filtration. The purity of recrystallized insulin as revealed by these methods was only 80–90% (8), rendering exact assessment of chemical stability extremely difficult. Therefore, a prerequisite for the present studies has been the introduction of monocomponent (MC) insulins (9) which are, by chromatographic methods, purified to the extent that impurities are virtually undetectable by the above methods.

The purpose of our studies was to investigate the degradation or transformation of insulin during storage of pharmaceutical preparations, using disc electrophoresis, size exclusion chromatography (SEC), and HPLC.

We report here on the quantitative aspects of the hydrolytic deterioration of insulin during storage of different preparations whereas the chemical transformation into higher molecular weight products is communicated in a subsequent paper (10).

Proteins are known to be degraded nonenzymatically by various chemical reactions (11). The most prevalent of these is deamidation, a reaction in which the side-chain amide group in glutaminyl or asparaginyl residues is hydrolyzed to form a free carboxylic acid (12,13). Insulin contains six such residues, Gln^{A5}, Gln^{A15}, Asn^{A18}, Asn^{A21}, Asn^{B3}, and Gln^{B4}

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(Fig. 1A), of which the three asparagine residues (Figs. 1A and B) are likely to be the most labile sites (12), in particular the C-terminal residue A21 upon acid treatment (3,4).

The preparations studied are listed in Table I together with their naming in different major pharmacopoeias and the abbreviations used in the text. The compositions of these preparations appear in Table II. Preliminary accounts have been published in abstract form (14,15) and in excerpt (8).

MATERIALS AND METHODS

Chemicals

Insulins used for the pharmaceutical preparations were monocomponent (MC) insulins of different species (porcine, bovine, and human) with the following characteristics: potency, 28 IU/mg (dry weight), corresponding to 1.68×10^8 IU/mol; Zn^{2+} content, 0.4%; and insulin, >99% by RP-HPLC. The content of impurities was as follows: (a) desamidoinsulins, nondetectable (<0.2%) by disc electrophoresis; and (b) di- and polymerization products, <0.2% as detected by size exclusion chromatography.

Distilled water was used for the preparation of electrophoretic buffers and chromatographic eluents. All other chemicals used were either official (Ph. Eur. or BP) or analytical grade. Porcine proinsulin was obtained from Ole Hallund, Novo Research Institute.

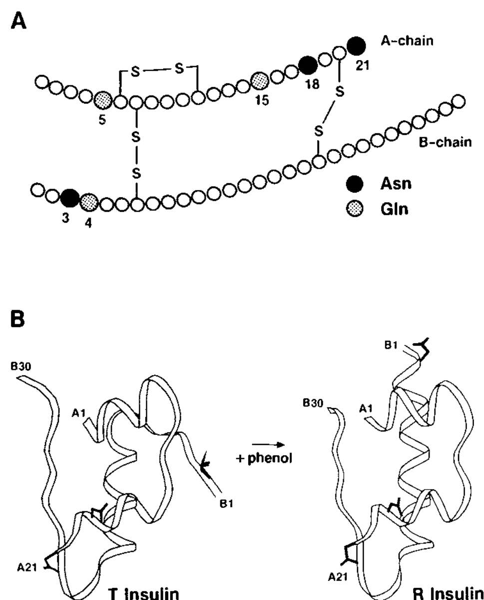

Fig. 1. Structures of insulin. (A) Primary structure of insulin shown schematically, with indications of the amino acid residues prone to deamidation. (B) Ribbon drawings of the tertiary structure (backbone) of the insulin monomer in its so-called 2 Zn structure (T Insulin) and in the phenol-induced "6 Zn" structure (R insulin) showing the most labile amide side chains (Asn residues). The view is approximately perpendicular to the crystallographic two-fold axis. It appears that the most dramatic change in the T → R structure transformation is the formation of an α-helix at the N terminal end of the B chain. This change also involves a large movement of the N terminal residues.

Manufacture

Preparation of the different formulations was performed according to the various pharmacopoeias (Table I). The types of preparations studied together with their content of auxiliary substances are listed in Table 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. Within a few days after filling into 10-ml vials samples were stored protected from light in different-temperature environments thermostatically controlled within ±1°C.

Insulin preparations from other manufacturers were purchased from drug stores and showed at least 1-year residual shelf lives.

Isolation and Storage of Samples

At appropriate intervals, 1-ml homogeneous samples were withdrawn from the vials and pH measured. Samples containing insulin in solution were adjusted to pH 6, zinc acetate (pH 6) was added to 0.01 *M*, and the samples were allowed to stand at 4°C overnight for complete precipitation of insulin and derivatives. Samples containing insulin solely in suspension were isolated by centrifugation, as were materials precipitated from solutions. Supernatants from suspensions as well as from precipitates were randomly controlled for derivatives appearing in solution (testing with ninhydrin). The amount was always below 0.2%. The isolated material was stored, without drying, at below −18°C until analysis.

Quantification

The studies were initiated in the early 1970s and concluded in the late 1980s, therefore the analytical techniques used have changed during the studies along with the development of modern high-performance liquid chromatographic (HPLC) methods. Initially a combination of polyacrylamide disc electrophoresis (PAGE) and densitometric scanning was used for separation and quantification. During the last 10 years HPLC has also been used.

Disc electrophoresis was performed as described earlier (9), with the following modifications: 2.5 ml of small-pore gel solution and 0.2 ml of spacer gel solution were used per column (140 × 5 mm); 10–200 μg of protein was applied (with double-constriction micropipettes) in 50 μl of spacer buffer containing 8 *M* urea and 0.02% (w/v) porcine proinsulin as an internal standard. The samples were run in two to four concentrations to ensure that the amounts of insulin and its derivatives were within the linear part of the standard curves (2–15 μg; see Densitometry). Electrophoresis was continued at 3 mA per column until 10 min after the tracker dye (bromphenol blue) reached the bottom of the gels. Staining was performed in 1% amido black in 7% acetic acid for 20 hr. The gels were destained, protected from light, by diffusion in 3% acetic acid for 5 days, with change of destainer three times per day. The gels with different dilutions of the same sample were destained in the same container. The stained gels were kept at room temperature in the dark until scanning, which was performed within 14 days after staining.

Table I. Types of Insulin Preparations

Classification after timing of action	Name of Preparation	Abbreviation used in text	Pharmacopoeia name		
			USP	BP	Ph. Eur.
Rapid-acting	Acid regular	Regular A	Insulin injection	Acid insulin injection	Insulini solutio iniectabilis
	Neutral regular	Regular N		Insulin injection	
Short-acting	Insulin zinc suspension, amorphous	IZS amorph.	Prompt insulin zinc suspension	Insulin zinc suspension (amorphous)	Insulini zinci amorphi suspensio iniectabilis
Intermediate-acting	Insulin zinc suspension, mixed	IZS mixed	Insulin zinc suspension	Insulin zinc suspension	Insulini zinci suspensio iniectabilis mixta
	Isophane insulin (NPH)[a]	NPH	Isophane insulin suspension	Isophane insulin injection	Insulini isophani protaminati suspensio iniectabilis
Long-acting	Insulin zinc suspension, crystalline	IZS cryst.	Extended insulin zinc suspension	Insulin zinc suspension (crystalline)	Insulini zinci cristallisati suspensio iniectabilis
	Protamine zinc insulin	PZI	Protamine zinc insulin suspension		Insulini zinci protaminati suspensio iniectabilis
Biphasic-acting	Biphasic insulin	Biphasic		Biphasic insulin injection	

[a] Neutral protamine Hagedorn.

To ensure sufficient separation of the protein bands for scanning purposes, not more than 200 μg of sample could be loaded on the disc gel. The smallest amount which could be clearly distinguished from baseline readings (see densitometry) was about 2 μg of protein, meaning that the detection limit with respect to quantitative estimation was about 1%. On the other hand, by loading larger amounts visual comparison of the test gel and standard gels allowed for a semiquantitative detection limit of about 0.2%.

Densitometry. The absorbance of the stained bands was measured on a Joyce-Loebl chromoscan (Mk II double-beam) recording densitometer (Team Valley, England) with automatic electronic integration of densitometric readings and with high-resolution transmission attachment to magnify the image of the sample. The balancing wedge ranged from 0 to 2 optical density units, the nominal wavelength of the filter at maximum transmission was 595 nm; the scanning slit was set to approx. 3×0.2 mm, and the scan expansion on the original record relative to the gel was 9×. The gels were placed submerged in 3% acetic acid in a quartz cuvette with a fixed distance between slit and gel. Integrator counts were corrected for baseline counts recorded cathodically to the insulin band.

The absorbance of the stained bands on the disc gels was shown to be linear at least up to 15 μg of applied insulin and of the individual desamido insulins including isoAsp B3. On a weight basis, insulin and the desamido insulins gave identical standard curves. The densitometric readings were shown to decrease by approx. 30% during storage of the stained gel for 4 weeks. Therefore scanning of gels with different dilutions of the same sample was performed the same day and always within 1–2 weeks after destaining. Reproducibility of the results obtained after scanning of the same set of gels (same sample, different dilutions) at 1-week intervals was about ±10% (coefficient of variation, 0.11; $n = 14$), with more than 5% of derivative formed (corresponding to 10–20 μg in the band). With <10 μg in the band the variation was doubled (CV = 0.22, $n = 19$).

The relative content of the individual derivatives was calculated as the ratio of the densitometric reading of the derivative band (sample gel) to that of the insulin band (diluted sample gel, corrected for dilution factor) plus the derivative bands with adjustment for difference in internal standard readings.

HPLC. A two-step HPLC method in which the 6000 MW fraction from a separation on a size exclusion chromatography (HP-SEC) column in the second step is analyzed on a reverse-phase HPLC (RP-HPLC), as described earlier (16), was used to relate the peaks in the chromatogram to the bands on the disc gel. It appeared that the insulin di- and polymerization products removed in the first chromatographic step did not elute in the second RP-HPLC step at the

Table II. Composition of Insulin Preparations

Type of preparation[a]	Physical state of insulin[b]	pH	Auxiliary substance(s): Preservative	Isotonic agent	Other additives
Regular A1	D	3	Phenol, 0.2%	Glycerol, 1.6%	
Regular A2	D	3–4	Methylparaben, 0.1%	Glucose, 5%	
Regular N1	D	7.4	Methylparaben, 0.1%	NaCl, 0.7%	Na-acetate
Regular N2	D	7.4	Phenol, 0.2%, or *m*-cresol 0.3%	Glycerol, 1.6%	None or Na-phosphate
Insulin zinc suspension, amorphous (IZS amorph.)	A	7.4	Methylparaben, 0.1%	NaCl, 0.7%	Zn^{2+}, Na-acetate
Insulin zinc suspension, mixed (IZS mix.)	3 parts A 7 parts C	7.4	Methylparaben, 0.1%	NaCl, 0.7%	Zn^{2+}, Na-acetate
Insulin zinc suspension, crystalline (IZS, Cryst.)	C	7.4	Methylparaben, 0.1%	NaCl, 0.7%	Zn^{2+}, Na-acetate
Isophane insulin (NPH)[c]	C	7.3	Phenol and *m*-cresol, total 0.3%	Glycerol, 1.6%	Protamine sulfate, Na-phosphate
Protamine zinc insulin (PZI)	A	7.3	Methylparaben, 0.1%	Glycerol, 1.6%	Protamine sulfate, Na-acetate
Biphasic insulin	1 part D (P) 3 parts C (B)	7.1	Methylparaben, 0.1%	NaCl, 0.7%	Na-acetate

[a] The abbreviations used in the text are shown in parentheses.
[b] A, amorphous; B, bovine; C, crystalline; D, dissolved; P, porcine.
[c] NPH, neutral protamine Hagedorn.

same positions as the products formed by hydrolysis, and therefore the first step has been omitted in later analyses. The RP-HPLC step was performed on a Nucleosil C_{18} column (4 × 200 mm). Reservoir A contained ammonium sulfate, 0.1 *M*, pH 2.3; reservoir B, acetonitrile. Elution was isocratical at 11 min, with 26% B and gradient (60 min) to 32% B; flow rate, 0.8 ml/min; and monitoring, by absorbance at 214 nm. Figure 2 illustrates the relation between the bands on the disc gel and the peaks in the HPLC chromatogram. It appears that one of the hydrolysis products (the Asp^{B3} product) is not separated from the insulin peak. However, as the ratio between the $isoAsp^{B3}$ and the Asp^{B3} products for every type of preparation has been shown within experimental error to have a fixed value for a given formulation, the value for the content of $isoAsp^{B3}$ can be used to calculate the total amount formed of the B3 transformation products.

Data Analysis

The experimental data were analyzed by polynomial regression analysis including a linear and a quadratic term: $D = a \times t + b \times t^2$ (D = total fraction of hydrolyzed insulin, t = time in months). This model was chosen since it describes the initial linearly increasing formation of hydrolysis product, which later on may proceed at a faster or slower rate as a consequence of pH changes in the preparations during storage. Thus the linear coefficient (a) will be an accurate estimate of the initial rate even if data at later times, when the rate of hydrolysis may have changed, are included in the regression analysis. In some cases the simple linear model appeared to be the most qualified to fit the experimental data, and in a few cases (split product formation) neither of these models could be used.

Temperature coefficient Q_{10} was calculated on the basis of the expression: $\log Q_{10} = 10 \times (\log k_2 - \log k_1)/(t_2 - t_1)$.

RESULTS

Three major degradation products are formed during storage of insulin solutions as revealed by disc electrophoresis, all appearing anodically to the insulin band on the gel (Fig. 3). The two bands appearing in neutral solutions of porcine (or human insulin) are different from the major band resulting from storage in acid. In neutral solutions of bovine insulin, only one band appears on the disc gel. During prolonged storage of acid solutions progressive hydrolysis and formation of desamido insulins with two or more deamidated side chains also occur (3).

It has been demonstrated (14,15) that the product in the first band anodic to insulin (R_f insulin, 1.12) is identical to monodesamido-(A21)-insulin (3). Bands 2 and 3 (R_f 1.20 and 1.26, respectively) are both deamidated in position B3, but while the upper band contains normal desamido-(B3)-insulin, the product in the lower band is isoAsp-(B3)-insulin. The single band formed during storage of bovine neutral solutions contains a mixture of the two B3 derivatives. The two B3 deamidation products can also be observed in most of the insulin suspensions after prolonged storage. In certain crystalline suspensions an additional third band (peak) arises (Fig. 2). This band (R_f, 1.17 on the disc gel) contains a hydrolysis product in which the peptide linkage between amino acid residue A8 and residue A9 has been cleaved.

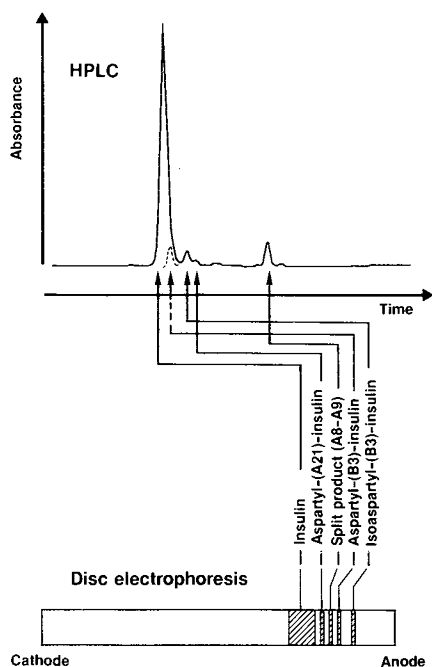

Fig. 2. Relationship between peaks in the RP-HPLC chromatogram and bands in the disc electrophoresis pattern after analysis of a pharmaceutical insulin suspension (IZS, mixed; porcine insulin) stored for 3 years at 25°C. It appears that the aspartyl-(B3)-insulin is clearly separated from insulin using disc electrophoresis, whereas the two compounds elute at approximately the same position by HPLC.

Effect of Insulin Concentration, Species, and Formulation

The rate of formation of the hydrolysis products did not vary significantly with the strength (40–400 IU/ml) for any of the preparations as exemplified in Figs. 4–6, nor could any statistical difference be observed between species of insulin (porcine, human, or bovine) with respect to formation of deamidation products (data not shown) except for preparations containing insulin zinc suspension (IZS)-type crystals. Accordingly, data on deamidation have subsequently been pooled irrespective of the strength and, in most cases, also the species of insulin. The IZS (mixed) and the IZS crystalline formulations, however, showed significant differences between species. The rate of split product formation was

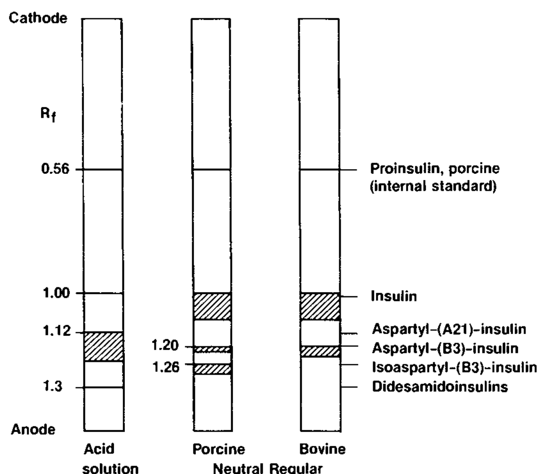

Fig. 3. Disc electrophoresis pattern of different insulin solutions (see Materials and Methods) after storage for approx. 6 months at 25°C.

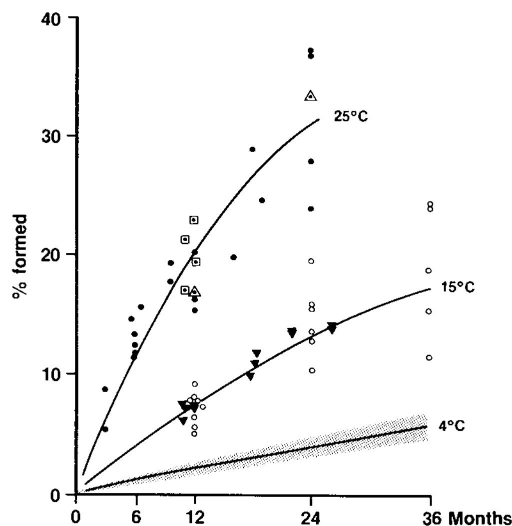

Fig. 4. Time courses of formation of total B3 hydrolysis products in regular N1 insulin solutions (porcine or human insulin formulated with methylparaben + NaCl) at 4, 15, and 25°C. The curves represent the best fit of the data by polynomial regression analysis (see Materials and Methods). 4°C: Mean ± SE, n = 20. 15°C: Data obtained by disc electrophoresis + scanning (○); data on isoAsp^{B3} formation (HPLC) transformed into total B3 transformation using the isoAsp/Asp ratios (Table III) (▼). 25°C: Data on 40 IU/ml (●); 80 IU/ml (□); 400 IU/ml (△).

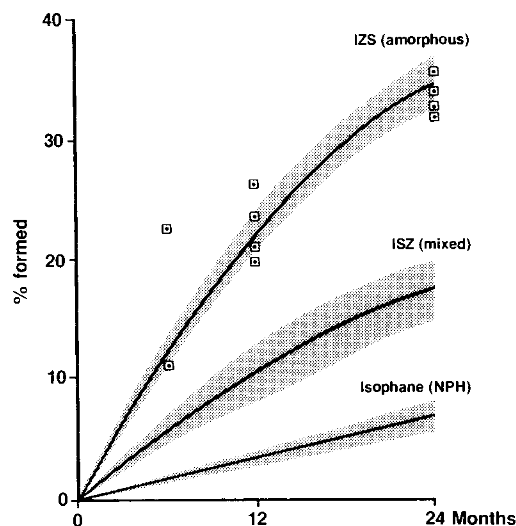

Fig. 5. Time courses of formation of hydrolysis products (mean, with 95% confidence interval hatched) during storage at 25°C of different types of pharmaceutical insulin suspensions (all formulated with porcine insulin). The curves represent the best fit of the data by polynomial regression analysis (see Materials and Methods). IZS (amorphous): 40 IU/ml (n = 23) + 10 results (□) on four batches with 80 or 100 IU/ml. IZS (mixed): 40 IU/ml (n = 15); data include split product (A8–A9) formed in the crystalline phase as well as the B3 deamidation products, which are formed mainly in the amorphous phase of the suspension. Isophane (NPH): 40 IU/ml (n = 17).

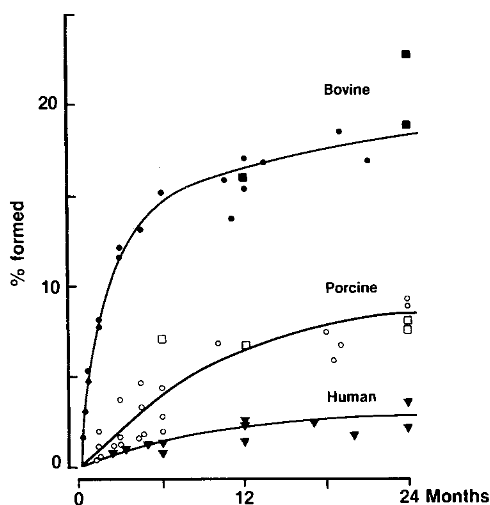

Fig. 6. Time courses of formation of the split product (A8–A9) during storage at 25°C of insulin zinc suspension, crystalline prepared from different species of insulin. Bovine: 40 IU/ml (●); 80 or 100 IU/ml (■). Porcine: 40 IU/ml (○); 80 or 100 IU/ml (□). Human: 40 IU/ml (▼).

much lower in the porcine and, especially, the human versions compared to the bovine versions of these types of preparations. To the contrary, the rate of deamidation was highest in human and negligible in bovine IZS crystalline preparations.

The composition of the acid as well as the neutral solutions had a profound influence on the rate of hydrolytic degradation of the insulin (Table III), and significant variation could also be observed between different formulations of the neutral suspensions (Table IV, Figs. 5 and 6).

Time Courses and Rate Data

Examples of the time courses of total hydrolytic degradation are given in Figs. 4 and 5. The courses with respect to formation of A8–A9 split product during storage of the long-acting crystalline Insulin Zinc Suspensions (IZS cryst.) formulated with different species of insulin are shown in Fig. 6. The effect of temperature on such time courses of cleavage in the bovine version of this type of preparation is illustrated in Fig. 7. Large variations are observed with respect not only to the initial rate of hydrolytic degradation but also to how the degradation develops with time. The bovine IZS cryst. (Fig. 6), for example, initially degrades at a high rate, but after 12 months the rate slows down and levels off at around 20%. In contrast, the IZS amorphous (amorph.) type (Fig. 5) initially degrades at a lower rate but does not exhibit the same large reduction in the rate of formation with time.

Rate data are shown in Tables III and IV together with the ratios in which $isoAsp^{B3}$ and Asp^{B3} products are formed. The rate of deamidation in neutral solution is reduced by 1 order of magnitude as compared to deamidation in acid solutions, and the rate is lower in regular N2, formulated with glycerol and phenol/cresol, than in regular N1, with NaCl and methylparaben. The initial rate of hydrolysis in neutral medium varies at 4°C from 0.3% per year in the IZS cryst. (formulated with porcine insulin) to 2.3% per year in the IZS amorph. type of formulation. At 25°C the lowest rate of formation of total hydrolysis products is seen in the NPH type (0.3% deamidation per month) and the highest rate in the IZS cryst. type formulated with bovine insulin (6% per month of mainly split product). Rate data for formation of the individual hydrolysis products in the IZS cryst. preparations are shown in Table V.

The two B3 transformation products are formed at a fixed ratio independent of temperature and time, which is in agreement with observations on succinimide formation and subsequent hydrolysis of asparaginyl residues in model peptides (13,17). The ratio is, however, influenced by composition and formulation. Thus, while the formation of the $isoAsp^{B3}$ derivative dominates in neutral solution (Table III), the Asp derivative is the main product of deamidation in the semisolid state in the neutral suspensions (Table IV).

Formation of the A8–A9 split product was observed only in the IZS types of formulations containing crystals, i.e., IZS, cryst. and mixed. Its rate of formation varies at 4°C from below the detection limit (approx. 0.05% per year) in the human insulin version to 0.6% per year in the bovine formulation. At 25°C the rates for the human, porcine, and

Table III. Formation of Insulin Deamidation Products in Pharmaceutical Insulin Solutions[a]

Insulin formulation	Temperature (°C) 4	15	25	37	45	Ratio isoAsp/Asp derivative
Regular A1	1.4					
	(0.1, n = 4)					
Regular A2	Approx. 4[b]					
Regular N1	0.18	0.65	2.10	15.3	31.0	1.9
	(0.02, n = 20)	(0.05, n = 34)	(0.16, n = 27)	(0.4, n = 10)	(0.3, n = 11)	
	b = −0.07[c]	b = −0.43	b = −3.3	b = −250	b = −760	
Regular N2	0.11	0.32	1.06	5.1	14.4	1.4
	(0.02, n = 5)	(0.02, n = 7)	(0.18, n = 22)	(0.2, n = 9)	(0.2, n = 15)	
			b = −0.04	b = −44	b = −530	

[a] The data are the apparent rate constants $k_{obs.} \times 10^2$/month (±SE).
[b] Based on data from Ref. 8.
[c] b values ($\times 10^4$)—the coefficients to the quadratic term—are given when the polynomial equation showed the best fit to the experimental data (see Materials and Methods). In all other cases the best fits are linear.

Table IV. Formation of Insulin Hydrolysis Products (Total Amounts) in Intermediate- and Long-Acting Insulin Suspensions

Type of preparation	Storage temperature (°C)[a] 4	15	25	Ratio isoAsp/Asp
Insulin Zinc Suspensions:				
Amorphous	0.19 (0.02, n = 28)	0.77 (0.06, n = 15) b = −1.0[b]	2.23 (0.14, n = 33) b = −3.3	0.6
Crystalline				
Porcine	0.027 (0.006, n = 13)	0.30 (0.01, n = 10) b = −0.09	1.1[c] (0.1, n = 5)	0.5
Human	0.03 (0.01, n = 12)	0.12 (0.01, n = 8) b = −0.1	1.4[c] (0.1, n = 5)	0.6
Bovine	0.10 (0.02, n = 14) b = −0.02	0.58 (0.05, n = 10) b = −0.8	5.9[d] (0.2, n = 6)	
Mixed (amorph./cryst.)				
Porcine or Human	0.10 (0.01, n = 6)	0.32 (0.03, n = 4)	1.0 (0.2, n = 15) b = −1.2	0.6
Porcine/Bovine	0.12 (0.05, n = 11)		2.0 (0.2, n = 13) b = −5	0.6
NPH	0.07 (0.02, n = 7)		0.15 (0.04, n = 13)	0.6
PZI	0.09 (0.02, n = 3)		1.7 (0.2, n = 5) b = −4	
Biphasic	0.05 (0.02, n = 8)		0.54 (0.07, n = 17) b = −0.3	

[a] The figures are the apparent rate constants $k_{obs.} \times 10^2$/month (±SE).
[a] The b-values ($\times 10^4$) indicate the best fit to the expression: Fraction formed = $kT + bT^2$.
[c] Linear over 1.5–6 months.
[d] Linear over 0.3–1.5 months, for time course of formation of the main hydrolysis product (A8-A9 split product) (see Fig. 6 and Table V).

bovine formulations are 0.4, 1.0, and 5.9% per month, respectively. The split product formation seems to depend not only on the type of crystals but also on the composition of the suspension. In biphasic insulin with the same rhombohedral crystals as in bovine IZS cryst., split product formation is virtually undetectable. These two types of preparations differ with respect to pH (7.1 versus 7.4, respectively), the content of porcine insulin in the solution (biphasic insulin only), and their zinc-ion content. The crystalline part of biphasic insulin contains only the amount of zinc ions (4 Zn/hexamer) necessary for crystallization, and essentially all Zn^{2+} is structurally bound in the crystals. In contrast, the IZS preparations contain a large surplus of zinc ions, of which only approximately 50% are bound to the crystals. As illustrated in Fig. 8 the presence of free zinc ions rather than pH seems to be crucial for the formation of the split product.

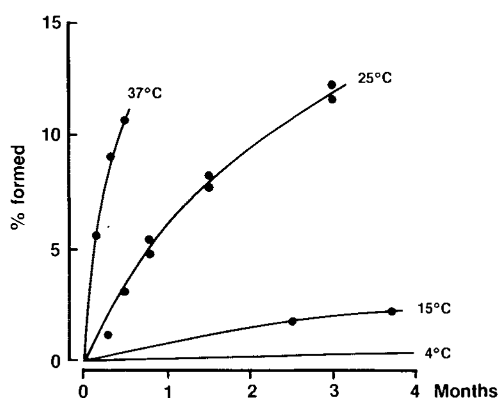

Fig. 7. Time courses of formation of the split product (A8–A9) during storage of bovine insulin zinc suspension, crystalline at different temperatures. The course at 4°C, based on results (n = 6) from 2 to 10 years of storage, corresponds to the formation of approx. 0.05% per month.

Effect of Temperature

The temperature dependence of the different hydrolysis reactions is shown in Table VI, in which the coefficient Q_{10} is calculated for different temperature intervals. An increasing effect of temperature on the rate of B3 transformation by raising the temperature by 10°C can be observed, especially for the N2 formulation. The coefficients for the B3 transformation are approximately 3 (4–25°C) for most formulations, whereas the corresponding temperature coefficients for the

Table V. Initial Rate Constants of Formation of Desamido and Split Products in Insulin Zinc Suspensions, Crystalline[a]

Insulin species	Desamido-(B3)-insulin, 25°C	Split-(A8–A9)-product		
		4°C	15°C	25°C
Human	1.0 (0.04, n = 8)			0.4 (0.1, n = 5)
Porcine	0.2 (0.01, n = 5)	0.008 (0.001, n = 10)	0.14 (0.02, n = 8)	1.0 (0.1, n = 10)
Bovine	<0.02	0.05 (0.01, n = 9)	0.52 (0.04, n = 9)	5.9 (0.2, n = 6)

[a] The rate data ($k \times 10^2$/month ± SE) are calculated from the initial linear part of the curves; see Fig. 6.

formation of the split product in porcine and bovine IZS cryst. formulations are approximately three times higher.

Manufacturer

There seem to be no major differences among four manufacturers with respect to the formation of the isoAsp^{B3} hydrolysis product in different preparations or formation of the A8–A9 split product in the IZS mixed type of preparation (Table VII).

Other Degradation Products

When using disc electrophoresis other possible insulin transformation products were below the detection limit (approximately 0.2% by visual inspection), but by using the more sensitive two-step HPLC method (with initial removal of di- and polymerization products), supplemental distinct peaks in the chromatogram were observed to increase with time. However, these additional transformation products with approximately the same molecular weight as insulin are distributed over 10–15 peaks in the chromatogram.

Calculation of the total sum of all these additional insulin transformation products (excluding di- and polymerization products; see the following paper) detectable by RP-HPLC reveals that ≤0.3% (versus 1–2% of the B3 transformation product) is formed per month at 25°C, with a tendency to slightly higher values for regular N2 than for N1.

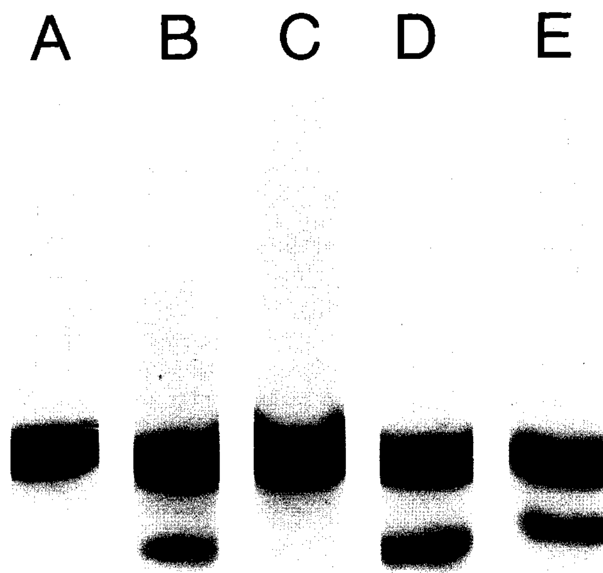

Fig. 8. Gel electrophoresis (performed as disc electrophoresis) of different formulations of bovine insulin zinc suspensions, crystalline (40 IU/ml), after storage of the preparations for 2 months at 37°C. (A) Containing 12 μg Zn^{2+}ml, corresponding to the amount of zinc structurally bound to the insulin (4 zinc atoms/hexamer of insulin), pH 7.0; (B) 83 μg Zn^{2+}/ml, pH 7.0; (C) 12 μg Zn^{2+}/ml, pH 7.5; (D) 83 μg Zn^{2+}/ml, pH 7.5; (E) for comparison, porcine insulin containing 10% monodesamido-(A21)-insulin.

DISCUSSION

This study demonstrates the following.

(1) Deamidation of insulin occurs in neutral solution but the rate of formation is reduced by one order of magnitude compared to the rate of deamidation at residue A21 in acid formulations.

(2) In neutral solutions and suspensions deamidation takes place almost exclusively at residue Asn^{B3} and leads to a mixture of aspartyl and isoaspartyl derivatives.

(3) The rate of hydrolysis is independent of the concentration (strength) and, in most cases, the species of insulin (human, porcine, or bovine) but is strongly influenced by the composition and formulation of the preparations.

(4) The ratio at which the isoAsp^{B3} and Asp^{B3} derivatives are formed is independent of time and temperature but varies with the formulation. In neutral solution the formation of the isoAsp derivative dominates relative to the Asp derivative, whereas the transformation of insulin in suspension leads mainly to formation of the Asp^{B3} derivative.

(5) In certain crystalline insulin suspensions cleavage of the peptide bond A8–A9 can be observed. This hydrolytic cleavage of the A chain takes place only in suspensions containing rhombohedral crystals in addition to a relatively high Zn^{2+} content (>4 Zn/hexamer). Rate of formation of the A8–A9 split product is species dependent (bovine > porcine > human insulin).

Deamidation Reactions

Site and Mechanism

Deamidation of glutaminyl and asparaginyl residues in peptides and proteins has been extensively studied and reviewed by Robinson and Rudd (12), who concluded that the rate of deamidation is dependent upon the primary sequence, secondary and tertiary structure, temperature, pH, ion strength, and special intermolecular interactions. They

Table VI. Effect of Temperature on the Rate of Hydrolysis of Insulin in Neutral Formulations[a]

Preparation	Temperature interval (°C)					
	B3 deamidation					A8–A9 cleavage
	4–15	15–25	4–25	25–37	37–45	4–25
Regular N1	3.2	3.2	3.2	5.2	2.4	
Regular N2	2.6	3.3	2.9	3.7	3.7	
IZS, amorphous			3.3			
IZS, crystalline						
Bovine						9.7
Porcine			3.1			10.0
Isophane (NPH)			1.4			
Biphasic			3.1			

[a] The figures, calculated from data for the individual products in Tables III–V, are the temperature coefficient Q_{10}, i.e., the increase in rate of reaction when the temperature increases 10°C in the temperature interval indicated.

also showed that asparaginyl residues are much more prone to deamidation than glutaminyl residues.

Insulin deamidates rapidly in acid formulations due to hydrolysis at residue Asn^{A21} (3). The particular lability of the A21 amide is due to its position as a C-terminal residue, where deamidation is catalyzed by the terminal uncharged carboxyl group (18,19).

A mechanism for deamidation at neutral pH of asparaginyl residues involving formation of a cyclic succinimide intermediate followed by nucleophilic attack by hydroxide ion was proposed by Bornstein and Balian (20). This process leads to the formation of a mixture of peptides in which the polypeptide backbone is attached via an alpha-carboxyl linkage (Asp) or via a beta-carboxyl linkage (isoAsp). Strong evidence for this pathway as the dominating mechanism for deamidation in peptides and proteins has been obtained in studies of synthetic model peptides (13,17,21,22) and of proteins (11,23–25). The succinimide formation has been shown to be dependent on the charge and size of the side chain on the adjacent carboxyl-site residue (12,13) but recent investigations have emphasized the role of tertiary structure and its influence on the conformational restraints on imide formation (17,19,22,26).

The rate-determining step in the deamidation reaction via the cyclic imide is the formation of the intermediate succinimide, which is a much slower process than the subsequent hydrolysis into Asp and isoAsp peptides (13,24).

Deamidation at Residue Asn^{B3}

The transformation of insulin in neutral medium into two B3 derivatives probably also involves the formation of a cyclic imide intermediate which hydrolyzes under retention of either a normal peptide linkage corresponding to simple deamidation or a beta-carboxyl linkage (isopeptide).

For neutral solutions as well as suspensions the lowest rates of deamidation are seen when the preparations contain phenol (regular N2 and NPH, respectively). This apparent effect of phenol is consistent with the fact that the B1–B8 α-helix induced by phenol (27,28) reduces the flexibility around the B3 residue and thereby the possibilities for imide formation. The two neutral solutions differ not only with respect to rate of deamidation but also in their time courses of degradation. Regular N2, especially at the lower temperatures, exhibits a more linear course than regular N1, in which the rate of degradation at all temperatures decreases with time (negative b values at all temperatures). In the latter preparation hydrolysis of methylparaben into p-hydroxyben-

Table VII. Influence of the Brand of Insulin on Deamidation and Split Product Formation

Brand[a]	Storage temperature (°C)	Formation of isoAsp[B3] derivative in human insulin preparations (% per month)			Formation of split product in IZS, mixed (bovine crystals) (% per week)
		Regular N2	NPH	IZS, cryst.	
A	25	0.77	0.20	0.32	
	37	2.1	0.6	1.3	
	45				2.0
B	25	0.56	0.23		
	37	1.8	1.0		
C	25	0.63	0.12		
	37	2.0	0.6		
D	25	0.62	0.15	0.29	
	37	1.9	0.7	0.9	
	45				2.0

[a] One or two batches of each brand were analyzed by HPLC.

zoic acid leads to a fall in pH during storage (8), whereas a more constant pH applies to regular N2. Therefore the decreasing B3 deamidation with falling pH in the range 6.8 to 7.6 (data not shown) may account for the different time courses in deamidation of the two neutral solutions.

The insulins of different animal species have identical primary structure at the B-chain N terminal, and it is therefore not surprising that they exhibit the same tendency to deamidation. One exception are the preparations in which the parallel occurring and species-dependent cleavage of the A-chain probably causes an overall change in the flexibility of the molecule.

Human insulin was proposed to be more susceptible to chemical transformation, including deamidation, than bovine insulin (29), but this observation is flawed by the fact that the two neutral preparations also varied with respect to formulation and initial purity.

The total hydrolytic transformation at Asn^{B3} occurs to the same extent in neutral medium containing identical auxiliary substances (NaCl and methylparaben) regardless of whether insulin is in the amorphous state (IZS, amorph.) or in solution (regular N1), indicating similar conformational flexibility and propensity to imide formation at B3. In contrast, when the insulin in suspension is in the crystalline rather than the amorphous state, the transformation at B3 is considerably reduced (Table IV). In the amorphous suspension insulin is precipitated with the individual insulin hexamers loosely packed together in an unordered way, probably with zinc ions binding them together, whereas in the rhombohedral crystals (IZS type of preparation) the hexamers are closely packed, with many direct contacts between the individual hexamers (30). These constraints on the tertiary structure reduce the possibilities for the large movement of the B-chain N terminal necessary for the peptide bond nitrogen atom to approach the B3 side-chain carbonyl carbon in order to form the succinimide ring (23). In the monoclinic NPH crystals the hexamers are more loosely packed, but the phenol content of this formulation induces helix formation at B1–B8, which adds to the conformational stability in this region of the molecule. Thus, the structural restraints put on the insulin molecule when in the crystalline form account for the slow rate of deamidation in the crystalline formulations. In comparison, in the formulations with insulin in the amorphous or dissolved form, allowing more conformational flexibility in the molecule, imide formation at B3 is facilitated, leading to a faster rate of deamidation. These results strongly support the theory that tertiary structure is the main determinant for protein deamidation (17,19,22,26).

Ratio of IsoAsp/Asp Derivatives

The ratios of B3 isomerized to B3 normal peptide observed in the neutral solutions are lower than that seen in deamidation of oligopeptides (13,17) but similar to the value seen in deamidation of α-crystalline subunits (24). The reason for such reduced values might be an additional deamidation pathway via direct solvent hydrolysis of the side-chain amide. However, Meinwald *et al.* (21), in studies on deamidation of asparaginyl-glycyl model peptides, were not able to detect any direct side-chain hydrolysis of the primary amide. The independence of the ratios on time and temperature also indicates that all deamidation proceeds via the imide. It would also be difficult to understand why any direct hydrolysis of the amide in B3 in neutral solution would not occur at the other two Asn residues in insulin. Therefore the reduced ratio is more likely explained by the lower accessibility to hydrolysis of the peptide linkage (isoaspartyl formation) as compared to the other hydrolyzable bond in the imide when this is part of a large peptide with more fixed tertiary structure.

The phenol-containing regular N2 gives a lower isoAsp/Asp ratio than regular N1 with methylparaben as preservative. Thus, the helix formation induced by phenol not only reduces the possibilities for imide formation but also apparently decreases the accessibility of the main chain for hydrolysis relative to the side-chain part of the imide.

Whereas formation of the isoAsp derivative dominates in solution, the Asp derivative is the main hydrolytic product when insulin is in suspension. This is probably due to further reduced accessibility of the carbonyl carbon in the peptide linkage for nucleophilic attack by water (or OH^-) when insulin is in the amorphous or crystalline phase rather than in solution. In that respect the amorphous state is not different from the crystalline state of insulin, as similar ratios of isoAsp/Asp can be observed (Table IV).

The trend toward lower isoAsp/Asp ratios with more stabilized structures of the molecule (effect of phenol and crystallization) indicates that tertiary structure is governing not only imide formation but also the subsequent hydrolysis reactions.

Electrophoretic Mobility of the Deamidation Products

An interesting and peculiar phenomenon in relation to the $isoAsp^{B3}$ insulin derivatives from different species of insulin is their electrophoretic mobilities on the disc gel (Fig. 3). In the electrophoresis buffer (pH 8) the Asp and the isoAsp derivatives both carry a full extra negative charge relative to insulin. Nonetheless, these two derivatives are clearly separated on the disc gel when they are derived from porcine and human insulin, with the isoAsp derivatives moving approx. 30% (relative to insulin) ahead of the other derivative, whereas the two derivatives formed from bovine insulin have the same mobility (that bovine insulin actually is hydrolyzed into the same two derivatives formed at the same ratio has been proven in ion-exchange experiments). As disc gel electrophoresis separates according to charge and also to molecular size, its efficacy in separating the porcine and human derivatives could be explained by the $isoAsp^{B3}$ insulin molecule being slightly smaller in size (more folded tertiary structure). The differences in primary structure (A8 and A10 residues) are located not too far from the B3 residue in the tertiary structure. Therefore, the substitution of A8 Ala in bovine insulin with Thr in human and porcine insulin may, in combination with the conformational changes induced in the main chain (an extra carbon atom) by the isomerization of B3, creates possibilities for hydrogen bonding between the side chain of Thr A8 and the B-chain N-terminal residues. This could result in a more folded and compact molecule in the isoAsp derivatives of human and porcine insulin than in that of bovine insulin.

Hydrolytic Cleavage of the Peptide Bond A8–A9

The cleavage of the peptide bond between residue A8 (Ala in bovine and Thr in porcine and human insulin) and residue A9 (Ser in all three species) is quite unusual, and a similar spontaneous reaction dependent on the presence of higher concentrations of zinc ions has apparently not been described for other nonenzyme proteins. The crucial role played by the availability of free zinc ions (Fig. 8) explains why formation of the split product cannot be detected in the otherwise identical rhombohedral crystals in the biphasic formulation.

The peptide bond involving the amino group of Ser is known to be labile and susceptible to hydrolysis via N–O acyl rearrangement but the intermediate peptidyl shift requires strong acidic conditions. The cleavage is most likely due to an autoproteolytic, metalloproteinase-mediated cleavage by the adjacent hexamer in the stacking of hexamers in the rhombohedral crystal. The key element for enzyme activity, a Glu carboxylate group, is available from the adjacent hexamer, and a zinc ion, supposed to be coordinated to one Glu and two His in the Michaelis complex (31), is available from the medium. This autocatalytic theory is supported by the insulin species variation in the split product formation. Thus bovine insulin with the fastest formation actually has the closest interactions between hexamers in this part of the crystal. The different rate of formation of the split product when comparing bovine insulin with human and porcine insulin can also be accounted for by primary structure differences (A8 Ala and Thr, respectively). The slightly faster formation in porcine relative to human crystals can be explained only by differences in the three-dimensional packing of the hexamers.

The split product formation proceeds initially relatively rapidly, whereafter it slows down (Fig. 6) and eventually stops. As its formation decreased substantially with falling pH (data not shown), this time course is probably caused by the decrease in pH during storage of the IZS preparations (8).

Effect of Temperature

The relatively large temperature effect observed for the B3 deamidation reaction (Table VI), especially around 30°C, most likely reflects the increase in conformational freedom at the deamidating B3 residue with increasing temperature. Because formation of the succinimide ring is rate determining in the transformation reaction (13), the overall reaction is facilitated when higher temperatures increase the possibility for the main-chain and side-chain groups to assume the conformation necessary for succinimide formation. Thus, stereochemical factors also govern the effect of temperature on the deamidation of Asn^{B3}. The much higher temperature effect seen for formation of the split product probably indicates the need for weakening of the conformational interactions within the hexamer before the approach between hexamers can be sufficiently close for the catalytic reaction to take place.

Impact on the Quality of the Preparations

The hydrolytic reactions occurring during storage of insulin preparations are quantitatively predominant and cause transformation of substantially larger amounts of insulin than the parallel occurring di- and polymerization reactions (10). The molecular changes induced by deamidation are relatively small, although the isoAsp formation, in addition to changing the uncharged Asn residue into a charged Asp group, also introduces an extra carbon atom into the peptide backbone. This may cause more extensive structural changes. However, the deamidation products have essentially the same *in vivo* biological potency as the intact molecule (8), and also the immunogenicity in rabbit experiments has been found not to differ from that of the parent insulin (8,9).

The rate-determining step in the deamidation reaction is the formation of the cyclic imide (13,21). Geiger and Clarke (13) found the rate of imide formation in an asparaginyl hexapeptide approximately 40 times slower than the subsequent hydrolysis reaction and calculated the steady-state concentration of the succinimide intermediate to be about 0.2%. Therefore accumulation of the insulin B3 succinimide during storage is likely to be insignificant compared with the end products.

The cleavage of the peptide backbone of the A chain between position A8 and position A9 represents a much more dramatic change and probably induces major alterations in the three-dimensional structure of the molecule. The cleavage is close to two disulfide bridges (A6–A11 and A7–B7), one of which connects the two separated A-chain peptides (A1–A8 and A9–A21) in the split product. Together these disulfide bridges will probably be able to keep the molecule in its folded state and maintain most of the overall tertiary structure, but the much larger retention on the HPLC column (Fig. 2) indicates exposure of some of the hydrophobic core to the surface of the molecule. In accordance, the biological *in vivo* potency of the split product is only about 2% of that of the parent hormone (15). The relatively low biological stability of the preparations containing rhombohedral crystals in combination with surplus zinc ions (IZS cryst. and IZS mixed), especially at higher storage temperatures (2,8), can essentially be accounted for by the split product formation. When stored as recommended (temperature interval, 2–8°C), the fall in potency during shelf life would, however, be less than 5% for the bovine IZS cryst. preparation. Assuming the same temperature coefficient Q_{10} for human as for porcine split product formation, the fall in potency in the human IZS cryst. preparation due to split product formation is calculated to be about 1% after 2 years at 15°C.

Animal insulins vary mainly in primary structure within the A-chain loop (A8 to A10) and these mutations are known to have a great impact on the immunogenicity of the insulin (32). Therefore it could be expected that the molecular change induced by the cleavage of the backbone in the split product would also lead to increased immunogenicity. However, the porcine split product showed the same low immunogenicity in rabbit experiments as the intact parent molecule (15). Insulin preparations formulated with the same species of insulin are generally seen to induce more antibody formation when formulated as a protracted rather than a rapid-acting preparation (33,34). It is, therefore, still an open question whether this enhanced immunogenicity is due pri-

marily to the prolonged stay of insulin in subcutis or to the content of insulin transformation products such as the split product and covalent insulin dimers (35).

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