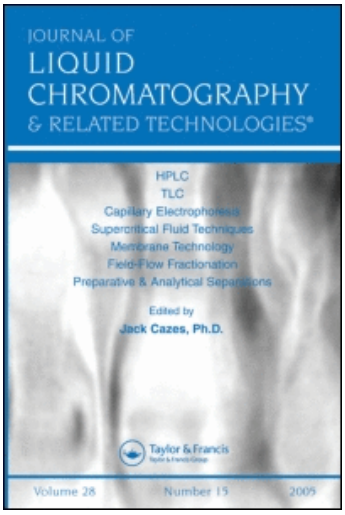

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The Influence of Motion and Temperature upon the Aggregational Behavior of Soluble Insulin Formulations Investigated by High Performance Liquid Chromatography

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THE INFLUENCE OF MOTION AND TEMPERATURE UPON THE AGGREGATIONAL BEHAVIOR OF SOLUBLE INSULIN FORMULATIONS INVESTIGATED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY:

We examined insulin formulations of soluble pork and human insulin with High Performance Liquid Chromatography (HPLC), using reversed phase and gel-filtration chromatography (column temperature:ambient temperature and 37°C). We were unable to find any impurities or aggregates of higher molecular weight, nor detect differences in the composition of the investigated insulins even ten months after production. The effects of temperature, salt concentration and dilution upon gel-filtration were also studied. After shaking at 37°C during 72 h, under exactly controlled conditions, some insu-

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lins showed a milky suspension. However, the immediately performed gel-filtration surprisingly showed in no case aggregates of higher molecular weight, but a reduction of the quantity of insulin. At ambient temperature the shaken insulin formulations remained clear. Here again no associations were detectable. To elucidate the nature of the sedimentation further investigations are necessary. But these investigations are not possible with HPLC.

INTRODUCTION:

It has been suggested that the aggregational behaviour of insulin might complicate the treatment of insulin dependent patients. This tendency to self-association of insulin is well documented in the literature and is influenced by many factors such as motion, temperature, impurity, pH, refrigerating temperatures and buffering systems (4). However, the extent of the formation of dimeres, tetrameres and aggregates of a higher molecular weight has not been elucidated yet. In this study we investigated with High Performance Liquid Chromatography (HPLC) (reversed phase chromatography and gel-filtration) the aggregational behaviour of pork and human soluble insulin formulations under variations of motion and temperature.

MATERIAL AND METHODS:

Apparatus:

The apparatus consisted of a programmer series 200 (Kontron, Munich, Germany), a switch valve LMV 870 (Kontron), two LC 414 pumps (Kontron), a high temperature oven (Knauer), an Uvikon photometer 720 LC (Kontron) and a Shimadzu integrator C-R1B (Kontron).

Insulins:

We examined Lilly Human insulin R (Eli Lilly GmbH, Gießen, Germany), Velasulin R Nordisk (Gentofte, Denmark), Hoechst Human insulin 31H (0-40) R (Hoechst AG, Frankfurt, Germany), Actrapid R Human insulin (Novo, Denmark) and Actrapid MC R insulin (Novo). Between the experiments the insulin preparations were stored at 4°C. We received from the company of Hoechst an insulin formulation which was stored during three years at 37°C. This preparation contained aggregates and smaller parts of the insulin molecule (personal communication Dr.Grau, Hoechst).

We appreciate the fact that the companies of Eli Lilly and Nordisk have been so kind as to give us the date of production of the tested insulin. All these preparations were soluble neutral insulins, 40 E/ml.

Methods:

Concerning the reversed phase chromatography, the procedure was carried out under the following conditions:
Ambient temperature, 20 µl loop injector, a spher C18 10 µl Chrompack column, flow rate 1.25 ml/min, isocratic 40 % acetonitrile at 215 nm. In a phosphate buffer we dissolved 0,1 M NaCl as well as a small bottle of ion-pair reagent PIC A (Waters, Eschborn, Germany; unknown concentration of Tetrabutylammoniumphosphate) to each liter.
We repeated the experiments several times; the retentions differed 0,1 min at most from each other; to compare the peaks we tested buffer without insulin.
Concerning the HPLC gel-filtration we worked with a TSK 125 column (Bio-Rad, Munich, Germany). Our experiments were performed at ambient temperature as well as at 37°C, flow rate 0,75 ml/min, wavelength 215 nm and measurements of control

at 280 nm; the recovery was 98-100 %, photometrically determined.

Concerning the gel-filtration we used phosphate buffer, pH 7,40 (+/- 0,05), without any admixtures or with the addition of NaCl, 0,1 M. To test the column quality, we regularly separated a mixture of Bio-Rad containing five calibration proteins; our samples of insulin were not diluted or were diluted in the proportion 1:4 with phosphate buffer,pH 7,40, without any admixtures.

To calibrate the column, we separated a mixture composed of thyreoglobulin, IGg, ovalbumin, myoglobin and cyancobalamin.

To test the influence of movement and temperature, we shook two preparations each from the above mentioned insulins in a Kottermann shaker (Hänigsen, Germany) for 72 h at 37°C (+/- 0,5°C; the speed was 75 rotations/minute. At the same time we shook at ambient temperature two formulations from the same charge in a mini-shaker (model Kühner, B.Braun) at 75 and 150 rotations/minute for 72 h.

All the chemicals we worked with were of HPLC-grade (Fluka, Neu-Ulm, Germany). We used water which was distilled after ion-exchange procedure. Before injection, the samples and the buffer were all filtered by a filter RC 55 (0,45 µm) from Schleicher & Schüll (Dassel, Germany). To avoid unspecific absorption of the filters, we compared the chromatographic experiments of unfiltered and filtered samples. The solvents were degassed by helium.

We collected samples of about 1 ml at the retention time of insulin during the separations before and after shaking. Then we tested the radioimmunological activity with an insulin kit from Sorin Biomedica (Saluggia, Italy).

Secondary to our experiments we inoculated parts of the insulin sediment on culture plates to look for bacterial growth.

Concerning the Shimadzu integrator, we performed all the experiments at a recorder attenuation 2^3 and 2^5 mV/full scale.

RESULTS:

At first, we separated the insulin preparations without any motion or changes of the temperature. No differences in the composition of the soluble insulins were seen; each of the insulin formulations composed of two main peaks: the insulin and the added phenol derivative. The tested insulin formulations had an excellent grade of purity (figure 1 and 3).

With the method of HPLC gel-filtration no aggregate of a high molecular weight like dimere or tetramere was detected, even up till ten months after the production of the insulin formulation. There were no differences between the insulin formulations separated at ambient temperature as well as at 37°C.

We emphasize that the determinations of the molecular weight of insulin were in the limits of 5000 and 7000 Daltons (figure 2). To identify the peaks, we tested pure cristalline insulin which we received from Eli Lilly Company (Gießen, Germany).

The addition of 0,1 M NaCl was necessary for obtaining good results with gel-filtration. With 0,05 M NaCl, insulin shifted to lower retentions, and the lower molecular weights of the calibration curve shifted to greater retentions; furtheron the results became less reproducible. With 0,1 M NaCl, the retentions of the substances used for calibration were reproducible within the range of seconds.

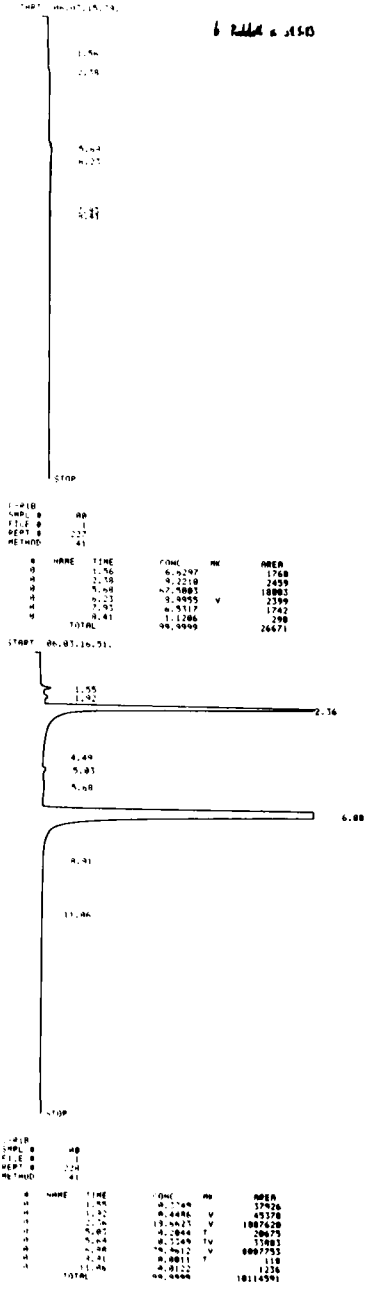

Fig.1: Typical reversed phase chromatogram of an insulin formulation. Flow 1,25 ml/min, spher C18 10 µm Chrompack column; ambient temperature, isocratic 40% acetonitrile, phosphate buffer 7,40 and 0,1 M NaCl; 215 nm. The chromatogram on the left side has no content of insulin. Att 2^5 mV/full scale.

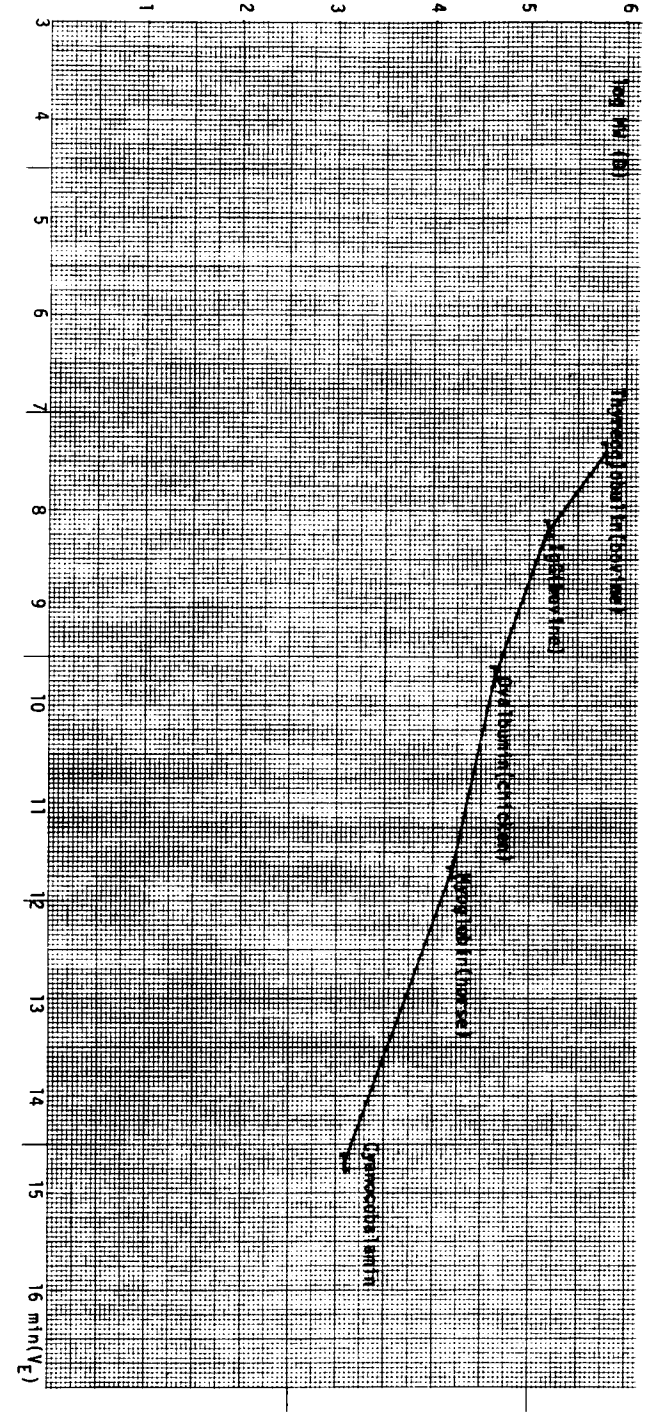

Fig.2: Calibration curve. TSK 125 column of Bio-Rad. Flow 0,75 ml/min, 215 nm, column temperature 37°C, phosphate buffer pH 7,40 and 0,1 M NaCl. Att.2^5 mV/full scale.

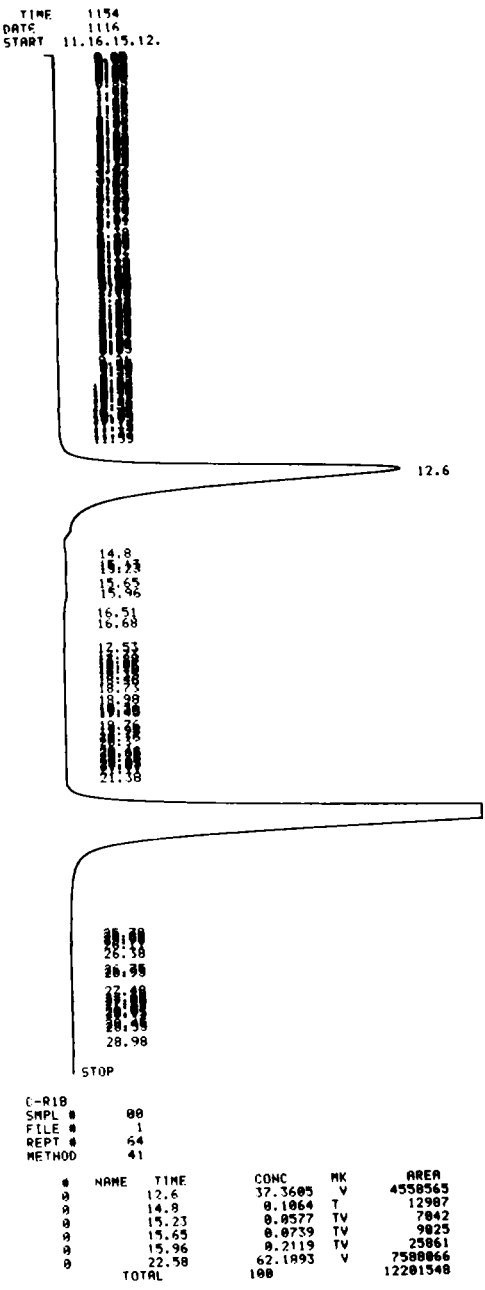

Fig.3: Typical chromatogram of gel-filtration. Column temperature 37°C, TSK 125 column of Bio-Rad. Flow 0,75 ml/min, phosphate buffer pH 7,40 and 0,1 M NaCl; 215 nm. You see at the retention time 12,6 the insulin peak, at 22,58 the phenol peak. Att.2^5 mV/full scale.

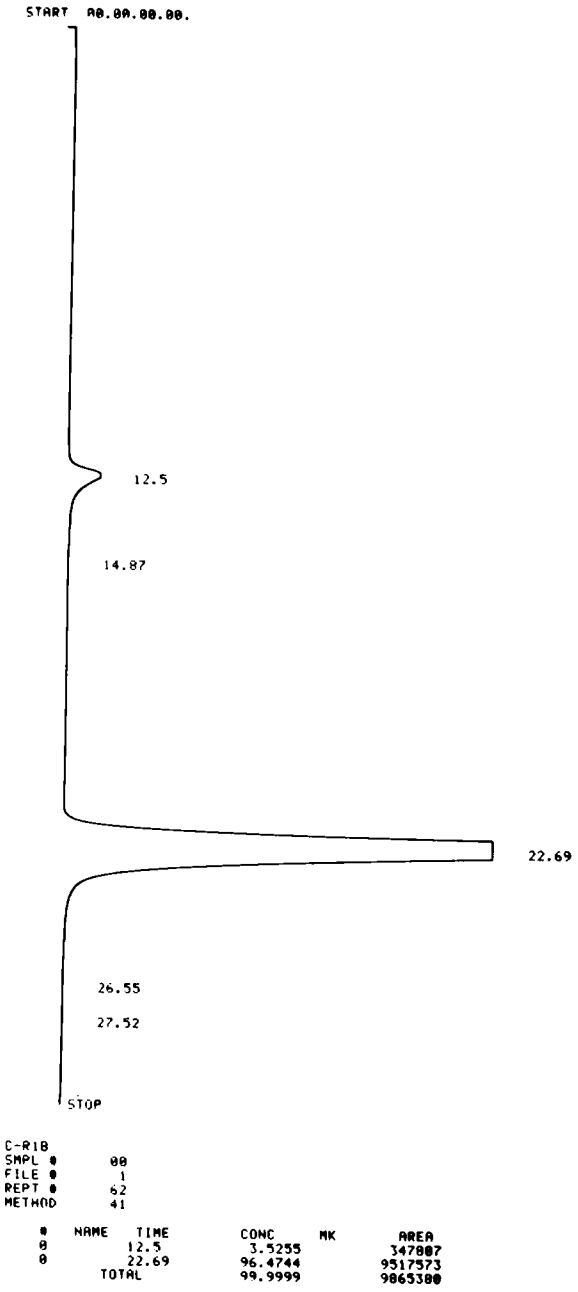

Fig.4: Gel-filtration of the milky suspension after shaking at 37°C during 72 h. The insulin peak is reduced. No aggregates of higher molecular weight are detectable. TSK 125 column of Bio-Rad, 215 nm, Flow 0,75 ml/min, phosphate buffer pH 7,40 and 0,1 M Na Cl. Att.2^5 mV/full scale.

Investigations performed at ambient temperature showed the same molecular weight range for insulin as investigations performed at 37°C.

The dilution of insulin, in proportion 1:4, did not influence the distribution of the molecular weight, only the retentions became greater.

After shaking at 37°C for 72 h, the Hoechst Human Insulin and Actrapid MC showed a milky suspension. Under the same conditions the Human insulin of Lilly remained a clear solution. After shaking at ambient temperature and at two different velocities, each of the three formulations remained clear. The immediately performed gel-filtration surprisingly showed in no case higher molecular aggregates but the formulations showing milky suspensions contained insulin in an obviously reduced quantity (figure 4). Further investigations are necessary to elucidate the nature of the sediment, but this is not possible with HPLC.

Because of filtration before injection we determined in a controlling test that the used filters did not retain substances up to a molecular weight of 600 000 Daltons.

The material, inoculated on culture plates, showed no bacterial growth. The sample of insulin received during separations before and after shaking showed radioimmunological activity.

Furthermore, it is possible for the insulin, stored for three years at 37°C, to indicate separations of insulin aggregates, e.g. dimers and larger molecules (figure 5).

DISCUSSION:

The method of reversed phase chromatography of soluble insulin is well documented in the literature (4,5,8,13,14),

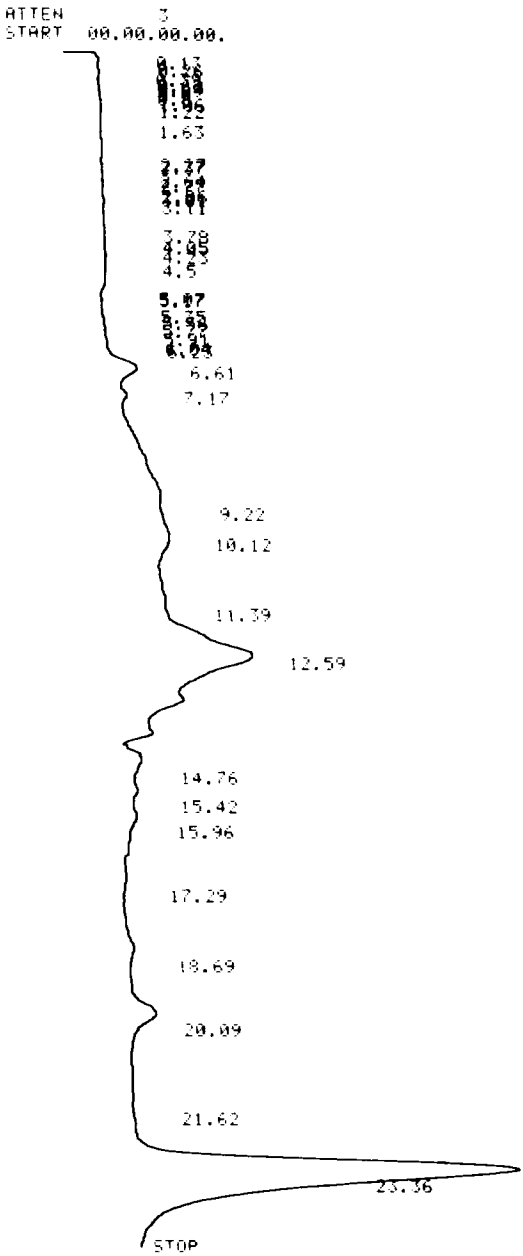

Fig.5: Gel-filtration of insulin which was stored during three years at 37°C. TSK 125 column of Bio-Rad, 215 nm, Flow 0,75 ml/min; phosphate buffer pH 7,40 and 0,1 M NaCl. The retention time of insulin at 12,59 is in good accordance with other figures. Att.2^3 mV/full scale.

but the separations were mainly performed at low pH and using soluble insulins of beef and pork.

The addition of 0,1 M of a salt is necessary to achieve good results. This generally recommended concentration was confirmed by our own results.

We decided to use 0,1 M NaCl because of the self-absorption of Na_2SO_4 at 215 nm. The pressure stability of the molecule, shown by Murphy (17), is very useful for chromatography of insulin. We worked under pressures up to 100 bar, the pH of the buffers was 7,4, receiving reproducible results by the ion-pair reagent PIC A of Waters GmbH (Eschborn, Germany).

We could not find any significant impurities in soluble human insulin or in pork insulin, respectively no proteins originated from Escherichia coli or pancreatic peptides. This result did not change during an observation period of ten months. We obtained the best separations isocratic with 40% acetonitrile, but +/- 3% acetonitrile worsens the chromatographic results, as already shown by Damgaard (5). The accompanying peaks are not significant, as chromatograms without insulin content (only ion-pair reagent and buffer) show similar peaks.

With gel-filtration the molecular weight of proteins cannot be determined exactly, because the retentions of the proteins are influenced by pH, ionic strength, hydrophobic interactions and by ion-exchanges between protein and stationary material (6,16). For example, the elution volume of catalase and ferritin is smaller than expected, but it can be improved by a higher ionic strength of the buffer (6). These facts are responsible for the deviations of the determined molecular weights from the real molecular weight.

The calibration curve is S-shaped because the gel becomes uniformly permeable to molecules which are too large or too small for the exclusion size (9,16). In order to make the curve well readable, we drew the log MW against the elution volume. As we determined the range of the molecular weight between 5000 and 7000 Daltons, we can conclude that the insulin molecule is in the monomere form even after a storage of ten months; under the retentions of the insulin we always detected one single peak, even if we repeated the test at 280 nm.

Aquas gel permeation chromatography of insulin (bovine and porcine insulin, crystallized twice) under denaturating conditions has been already successfully performed (20); the elution time was 10-20 min; our elution times were similar. Twenty-one analogues of ACTH, three analogues of LH-RH and four insulins have been successfully separated on a commercial reversed-phase material with tartrate buffer-acetonitrile systems (pH 3,0)(19). The retention times of the insulins were about five minutes under isocratic conditions; our separations were within the same range.

The aggregational behaviour of insulin is fully discussed elsewhere (15). The methods used until now were sedimentation velocity measurements, sedimentation equilibrium and circular dichroism techniques. Lougheed (15) reported the existence of calculated association constants for the formation of monomeres-dimeres and dimeres-hexameres. It is shown that in zinc-free insulin solutions the formation of dimeres is very quickly followed by the polymerisation of the insulin molecule. Blundell (2) concluded that the dimere was the fundamental unit for further associations. He proposed that binding forces involve weak Van-der-Waals' forces as well as hy-

drogen bonds. Pekar (18) showed that the aggregational behaviour of zinc-free and zinc-containing insulin is similar at pH 7,0.

Wu (21) has criticized that the relationship between stability and storage time has never been studied. Our results include these data. Until now, the precise relationships between purity and aggregation cannot be defined. Regarding the influence of temperature, insulin is said to be stable between -20°C and +37°C.

Livesey (12) measured by radioimmuno assay LH, FSH, TSH, growth hormone, prolactin and insulin. The concentrations of all these hormones were stable during at least eight days, but insulin was not stable during five freeze and thaw cycles.

When gel-filtration was used, we did not find any changes in the aggregational behaviour during an observation period of ten months, e.g. no formation of dimeres or other aggregates. Even insulin stored for ten months showed no higher molecular aggregates after shaking at ambient temperature during 72 h. At 37°C we observed a milky suspension, but it remained a monomere molecule of insulin, although in lower concentrations. Therefore, we conclude that the stable molecule of insulin exists in its monomere form. At 37°C the molecule folded up its structure, which is perhaps thermodynamically possible at this temperature. This procedure might release groups of the molecule ready for polymerisation, probably after formation of dimere. However, this step has not been observed.

In 1980 Fisher and Porter (7) discussed that purified crystalline bovine insulin is degraded by two mechanisms: deamidation and polymerisation. Both are temperature dependent; higher temperature means higher degradation (10). Fisher and

Porter performed the investigations with gel-filtration chromatography and polyacrylamide gel-electrophoresis. Under appropriate solvent conditions the globular protein insulin polymerizes to form submicroscopic fibrils (3). Infrared dichroism and wide-angle X-ray diffraction clearly demonstrate the cross-ß structure of the fibrils.

After a storage of three years at 37°C aggregates of insulin are to be seen (personal communication Dr.Grau, Hoechst). Therefore we were able to show the separation of insulin aggregates. Due to the large number of aggregates which accumulated during the long storage period, the separation is therefore not ideal.

Blackshear et al. (1) showed that in solutions of insulin in 80 % (v/v) glycerol aggregates of insulin accumulated at 37°C during four weeks. This was subjected to polyacrylamide gel electrophoresis under nonreducing conditions.

We conclude from our results that motion at 37°C can produce very large molecular weight aggregates, the polymerisation is the main form of degradation. A storage without motion at 37°C may cause - perhaps in a time dependent step - dimeres of insulin, but parts of smaller molecular weight obviously are detectable. The deamidation (better "depeptidation") and polymerisation take place. We have to emphasize that we cannot determine the cleavage of amino-acids by our method; the determination of the molecular weight is not accurate enough.

Furthermore, motion at ambient temperature produces small effects on the aggregational behaviour of insulin.

According to Fisher et al.(7), the percentage of high molecular weight material in crystalline bovine insulin after a storage of twelve months at 5°C is about 0,41%; the percent-

age after a storage of twelve months at 37°C is 6,29%. Due to the limited sensibility of our UV-detector we cannot exclude the existence of higher molecular weight aggregates in a very low percentage.

As shown by Schlichtkrull et al. crystalline insulin is fractionated by common gel filtration chromatography into the high-molecular-weight (mol.vt.— 15,000) a-component, the b-component and the c-component. The c-peak consists of arginine-insulins, insulin ethyl esters, the insulin and monodesamido-insulin. The b-component comprises proinsulin, intermediates and dimeres; the a-peak has not been identified.(22). Therefore our results only show the c-peak.

For discussion of the biological potency of insulin we refer to the literature (22). After storage of neutral-insulin for 222 months at 4°C the biological potency is reduced by 2%; the biological potency is reduced at the same rate after the storage for 0,4 month at 40°C (both without motion).

We recommend the diabetic patients to store the insulin in a refrigerator and to use it within the period of durability. Also storage temperatures up to 15°C are in order.

We did not confirm, as stated in (15) that storage at 4°C promotes aggregation. Furthermore, we cannot verify the contention (11) that association influences only the mobility of the protein without conformational changes.

Acknowledgment:

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LITERATURE:

1. Blackshear P.J., Rohde T.D., Palmer J.L., Wigness B.D., Rupp W.M. and Buchwald H. — Glycerol prevents insulin precipitation and interruption of flow in an implantable insulin infusion pump Diabetes Care,(1983)6; 387-91

2. Blundell T.L., Dodson G.G., Dodson E.J., Hodgkin D.C. and Vijayan M. — X-ray analysis and the structure of insulin Recent Prog.Horm.Res. 27, (1971) 140

3. Burke J.M. and Rougvie A.M. — Cross-ß protein structures Biochemistry Vol.11 No.13,1972

4. Crawhall I.C. and Posner B. — Separation of bovine and porcine insulin by reversed phase HPLC; J.Clin.Chem.Clin.Biochem. Vol 19, 1981, No.8

5. Damgaard U. and Markussen I. — Analysis of insulins and related compounds by HPLC Horm.Metab.Res.11 (1979),580-81

6. Engelhardt H. — HPLC in protein and peptide chemistry, 1981 by Walter de Gruyter & Co., Berlin, New York

7. Fisher B.V. and Porter B.P. — Stability of bovine insulin J.Pharm.Pharmacol. (1981) 33, 203-206

8. Gazdag M. and Suepesi G. — Separation of large polypeptides by HPLC J.Chrom.218 (1981) 603-612

9. Hancock W. — CRC-Handbook of HPLC of amino acids,peptides and proteins(1981)

10. Hoechst AG — Informationsschrift Januar 1977

11. Irsigler K. and Kritz H. — Long term continuous intravenous insulin therapy with a portable insulin dosage-regulating apparatus; Diabetes (1979) 28 196-203

12. Livesey I.H., Hodgkonson S.C., Roud H.R. and Donald R.A. — Effect of time, temperature and freezing on the stability of immunoreactive LH, FSH, TSH, growth hormone, prolactin and insulin in plasma; Clin.Biochem. 13 (4) (1980) 151-155

13. Lloyd L.F. — Analysis of insulin preparations by HPLC Anal.Proc.March 1982

14. Lloyd L.F. and Calam D.H. — Separation of human insulin and some structural isomeres by HPLC J.Chrom.273 (1982) 511-514

15. Lougheed W.D., Woulfe-Flanagan H. Clement I.R., Albisser A.M. — Insulin aggregation in artificial delivery systems Diabetologica 19,(1980) 1-9

16. Mayer V. Praxis der HPLC, Diesterweg-Salle 1981

17. Murphy R.B. Anomalous stability of insulin at very high pressure Experentia 34/2 (1977) 188

18. Pekar A.H. and Frank B.H. Conformation of proinsulin. A comparison of insulin and proinsulin self-association at neutral pH; Biochemistry 11, (1972) 4013-4016

19. Terabe S., Ryusei Konaka, Ken Inouye Separation of some polypeptide hormones by HPLC J.Chrom.172 (1972) 163-177

20. Welinder B. Gel permeation chromatography of insulin; J.Liqu.Chrom. 3 (9) (1980) 1399-1416

21. Wu Gay-May Purification, stability and physico-chemical behaviour of bovine insulin Dissertation Abstracts Vol 35 (1974) No. 75-7816

22. Schlichtkrull J., Pingel M., Heding L.G., Brange J. and Jørgensen K.H. Insulin preparations with prolonged effect Handbook of Experimental Pharmacology 32/2 Springer Verlag 1975