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# Stability of bovine insulin

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A stability experiment on purified crystalline bovine insulin showed that it deteriorates at normal refrigeration temperature (5 °C) and that storage at −20 °C is necessary to ensure stability. Purified insulin degrades by two mechanisms: deamidation and polymerization. Immunochemical potency determinations alone are insufficient to determine the stability of insulin as they may be little affected by the chemical changes which occur on storage. The activation energy for the polymerization of insulin was found to be 66 kJ $mol^{-1}$ and for deamidation 45 kJ $mol^{-1}$. It is recommended that crystalline bovine insulin be stored at −20 °C.

Since the current trend is towards using highly purified insulins for the treatment of diabetes mellitus (see e.g. Alberti & Nattrass 1978) it is essential to determine the extent to which the insulin remains free from degradation products after the expensive and time-consuming processes of purification.

Most previous work on the stability of insulin has been concerned primarily with the changes in biological potency on storage of insulin formulations, with no investigation of the chemistry giving rise to these changes (Krough & Hemmingsen 1928; Sahyun et al 1937, 1939; Lens 1947; Stephenson & Romans 1960; Storvick & Henry 1968; Pingel & Volund 1972; Storring et al 1975).

Schlichtkrull et al (1975) have shown that in injections, insulin degrades by two mechanisms: deamidation and polymerization. Neither the deamidated nor the polymerized insulin showed any significant immunogenicity in rabbits. Chance (1972) and Schlichtkrull et al (1975) (citing Sorensen, personal communication) stated that deamidated insulin showed almost the same potency as insulin itself; however, no potency determinations were reported on the polymerized insulin. Rates of deamidation and polymerization were not measured although it was noted by Schlichtkrull et al (1975) that higher temperature accelerated both reactions.

This report describes experiments carried out to determine the stability of solid insulin, as monitored by changes in immunochemical potency and in amounts of high molecular weight material (HMW) and desamido insulin (DAI) present. Rate constants and activation energies for the conversion of insulin to HMW and DAI are determined, and recommendations made for the storage of insulin.

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## MATERIALS AND METHODS

The insulin used was purified bovine insulin (Wellcome Foundation, Dartford, Batch Number CD 345/5/XST). 100 mg samples of the insulin were sealed into clear, soft glass vials and stored at 60, 50, 37, 25, 5 and −20°C in the dark. Vials were removed after 3, 6 and 12 months for analysis.

### *Gel filtration chromatography*

The method was an adaption of that described by Rolando & Torroba (1972). Approximately 3 mg of sample was loaded onto a column (0·9 × 40 cm) containing Sephadex G50 Superfine (Pharmacia Fine Chemicals) in 1M acetic acid, and eluted with 1M acetic acid at a flow rate of 8 ml $h^{-1}$. The effluent from the column was monitored at 276 nm with a Cecil CE212 Variable Wavelength Ultraviolet Monitor, fitted with a CE213 AutoRange Unit (Cecil Instruments Ltd) and the output recorded on a Bryans Southern Chart Recorder, model 28000. A typical chromatogram is shown in Fig. 1.

The amount of HMW material present was calculated from the percentage of the total response given by the area under the peaks eluting faster than insulin.

### *Polyacrylamide gel electrophoresis*

The method was based on the original work of Davis (1964). The running gel was prepared from acrylamide (7·5 g), ethylene diacrylate (200 μl) and tetramethylethylene diamine (TEMED, 50 μl) in tris-(hydroxymethyl)-methylamine hydrochloride (Tris-HCl) buffer (50 ml, 0·38 M, pH 8·9), polymerization being initiated by the addition of ammonium persulphate (50 mg). 1·1 ml of this mixture was placed in each running tube and allowed to polymerize. The spacer gel was prepared from Cyanogum 41 (British

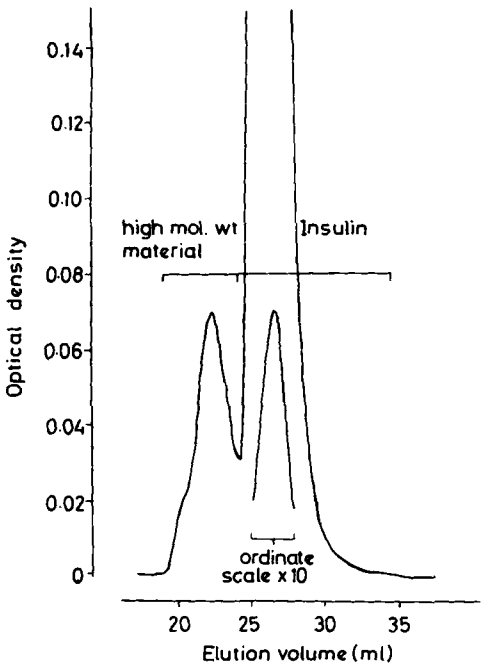



FIG. 1. Typical gel filtration chromatogram of a partially polymerized insulin sample.

Drug Houses, 1·9 g), ethylene diacrylate (115 $\mu$l) and TEMED (50 $\mu$l) in Tris-HCl buffer (50 ml, 0·062 M, pH 6·7), polymerization being initiated by the addition of ammonium persulphate (50 mg). 0·2 ml of this spacer gel mixture was placed on top of each running gel and allowed to polymerize. 1 mg of sample was dissolved in 20% sucrose solution (1 ml) containing hydrochloric acid (0·01 M) and $6 \times 10^{-4}$% bromophenol blue. 50 $\mu$l of the solution was loaded onto a gel. The running buffer (pH 8·3) for the anode and cathode baths was prepared from Tris (1·21 g litre$^{-1}$) and glycine (5·8 g litre$^{-1}$). With the anode at the bottom, a current of 1 mA per tube was passed for the first 15 min and then increased to 2 mA per tube until electrophoresis was complete, when the bromophenol blue marker bands reached the bottoms of the running gels. Gels were then removed from the tubes and each gel placed in 12·5% aqueous trichloroacetic acid solution (10 ml) for at least 1 h. 0·25% aqueous Coomassie Brilliant Blue G250 solution (0·5 ml) was added to each tube, mixed and allowed to stand overnight. Destaining was carried out in 5% acetic acid.

The apparatus used was supplied by Shandon Scientific. A typical gel pattern shows a narrow band of arginyl insulin (AI) separated by about $6 \times$ AI band widths from a wide band of insulin ($\simeq 3 \times$ AI bandwidths), itself separated by $\simeq 2 \times$ AI bandwidths from closely associated mono, di and tridesamido insulin each $\simeq$ 1·5AI bandwidths. The amounts of DAI were estimated by comparison with standard loadings of insulin run on the same occasion.

*Immunochemical potency*

This was determined by radioimmunoassay using a modification of the method of Hales & Randle (1963).

*Analysis of kinetic data*

This was carried out by standard methods (e.g. Laidler 1963), assuming first order kinetics, not unreasonable for the decomposition of a solid. A plot of ln (a-x) against t gave a line of slope $-k$, where a is the initial concentration, x is the extent of reaction at time t, and k is the rate constant. A plot of log k against 1/T then gave a line of slope $-E_A/2{\cdot}303R$, where T is the absolute temperature, $E_A$ is the activation energy and R is the gas constant. In all cases the slopes were calculated from a least squares fit of the data, using the methods of Draper & Smith (1966).

*Amino acid analysis*

This was by the method of the British Pharmacopoeia (1973) using 2 mg of sample heated at 110°C for 24, 48 and 72 h. Cysteic acid was measured after performic acid oxidation using the methods of Hirs (1967) followed by hydrolysis.

## RESULTS AND DISCUSSION

Table 1 shows the results of measurements of the content of HMW material at the times and temperatures stated. Similar data are shown in Table 2 for the content of desamido insulin. The results of the potency determinations are shown in Table 3.

Analysis of the data in Tables 1 and 2 gives the values for the rate constants and activation energies shown in Table 4. In the Arrhenius plots there is some departure from linearity, as shown in Fig. 2,

Table 1. Percentages of high molecular weight material in insulin after storage.

| Storage (months) | Temperature (°C) | | | | | |
|---|---|---|---|---|---|---|
| | 60 | 50 | 37 | 25 | 5 | −20 |
| 0 | 0·20 | 0·20 | 0·20 | 0·20 | 0·20 | 0·20 |
| 3 | 16·12 | 8·16 | 1·65 | 0·56 | 0·19 | 0·18 |
| 6 | 24·63 | 12·14 | 4·18 | 1·09 | 0·31 | 0·24 |
| 12 | — | 16·97 | 6·29 | 4·98 | 0·41 | 0·22 |

Table 2. Percentages of desamido insulin in insulin after storage.

| Storage (months) | Temperature (°C) 60 | 50 | 37 | 25 | 5 | −20 |
|---|---|---|---|---|---|---|
| 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 3 | 13 | 8 | 7 | 5 | 4 | 4 |
| 6 | 15 | 11 | 7 | 5 | 5 | 4 |
| 12 | — | 14 | 7 | 5 | 5 | 4 |

Table 3. Insulin-like immunoactivity (units $mg^{-1}$) of insulin after storage. Results are expressed as: potency (fiducial limits, $P = 0.95$).

| Storage (months) | Temperature (°C) 60 | 50 | 37 | 25 | 5 | −20 |
|---|---|---|---|---|---|---|
| 0 | ← | | 28·0 (25·9–30·2) | | | → |
| 3 | 26·8 (24·9–28·9) | 30·3 (28·2–32·5) | 31·0 (28·8–33·2) | 29·8 (27·5–32·4) | 31·4 (29·3–33·7) | 29·4 (22·6–38·0) |
| 6 | 24·9 (23·3–26·6) | 28·5 (26·7–30·4) | 28·7 (26·9–30·6) | 30·0 (28·1–31·9) | 32·4 (30·5–34·3) | 30·1 (28·1–32·3) |
| 12 | | 25·5 (23·6–27·6) | 28·7 (26·7–30·9) | 25·3 (24·0–26·6) | 28·0 (26·0–30·2) | 27·7 (26·2–29·4) |

Table 4. Rate constants and activation energies (with 95% confidence intervals) for the generation of high molecular weight (HMW) material and desamido insulin (DAI).

| Temperature °C | K | Rate constants, k ($months^{-1}$) HMW generation | DAI generation |
|---|---|---|---|
| 60 | 333 | $4.68 (\pm 0.22) \times 10^{-2}$ | $2.03 (\pm 0.28) \times 10^{-2}$ |
| 50 | 323 | $1.46 (\pm 0.03) \times 10^{-2}$ | $8.93 (\pm 0.08) \times 10^{-3}$ |
| 37 | 310 | $5.37 (\pm 0.02) \times 10^{-3}$ | $2.12 (\pm 0.10) \times 10^{-3}$ |
| 25 | 298 | $4.19 (\pm 0.04) \times 10^{-3}$ | $6.98 (\pm 0.11) \times 10^{-4}$ |
| 5 | 278 | $1.94 (\pm 0.001) \times 10^{-4}$ | $9.97 (\pm 0.08) \times 10^{-4}$ |
| −20 | 253 | $2.67 (\pm 0.003) \times 10^{-5}$ | — |
| Activation energy ($kJ\ mol^{-1}$) | | 65·52 (±2·76) | 44·83 (±16·47) |

this being more noticeable in the case of desamido insulin formation with its less precise method of determination.

From the Arrhenius plots in Fig. 2 it can be seen that below approximately 10 °C the main mechanism of insulin breakdown is deamidation, but above this temperature polymerization predominates.

Deamidation occurs due to progressive loss of $-NH_2$ groups from the glutamine residues at positions 5 and 15 and the asparagine residues at positions 18 and 21 in the A-chain of insulin and from the asparagine residue at position 3 and the glutamine residue at position 4 in the B-chain of insulin (Schlichtkrull et al 1975).

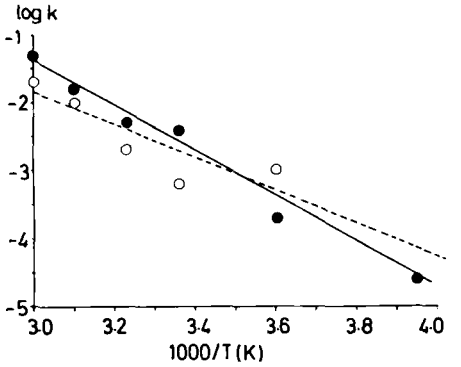



FIG. 2. Arrhenius plots for HMW generation (●——●) and DAI generation (○- - -○).

The high molecular weight material is caused by the polymerization of insulin, since analysis shows it to have amino acids in the same ratio as insulin (see Table 5). This polymerization has been noted also by Fojtik & Kopoldova (1976) during the radiolysis of aqueous solutions of insulin.

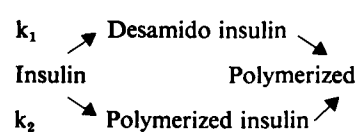
Our model of insulin generating either polymer or desamido insulin on storage is somewhat simplistic and takes no account of the possible deamidation of polymerized insulin nor the polymerization of

Table 5. Comparison of amino acid compositions of the high molecular weight (HMW) material with insulin.

| Amino Acid | Number of residues in bovine insulin (Sanger 1960) | Number of residues found in HMW assuming a total of 51 residues |
|---|---|---|
| Aspartic acid | 3 | 3·16 |
| Threonine[a] | 1 | 1·07 |
| Serine[a] | 3 | 3·31 |
| Glutamic acid | 7 | 7·12 |
| Proline | 1 | 0·95 |
| Glycine | 4 | 4·38 |
| Alanine | 3 | 3·39 |
| Valine[b] | 5 | 4·53 |
| Methionine | 0 | 0 |
| Isoleucine[b] | 1 | 0·99 |
| Leucine | 6 | 5·83 |
| Tyrosine[a] | 4 | 3·50 |
| Phenylalanine | 3 | 2·74 |
| Lysine | 1 | 1·14 |
| Histidine | 2 | 1·79 |
| Arginine | 1 | 0·95 |
| Cysteine[c] | 6 | 6·14 |

[a] Values extrapolated to zero time of hydrolysis.
[b] Values extrapolated to 72 h hydrolysis.
[c] Determined as cysteic acid after oxidation with performic acid.

desamido insulin, hence the system is probably better represented as:




Since our analyses would determine polymerized insulin and polymerized desamido insulin together, the rate constant for the formation of polymerized insulin could be too high i.e. $k_2 + k_1k_4$, and the rate constant for the formation of desamido insulin could be too low, i.e. $k_1$–$k_4$. One would expect these departures to be more apparent at higher temperatures, but since the Arrhenius plots show good linearity at the higher temperatures the simple model seems sufficient.

Of prime importance is the rate at which insulin degrades, by either of the mechanisms, and a simple summation of the data in Tables 1 and 2 gives an indication of the rate at which insulin disappears. This is calculated in Table 6, together with values for $t_{90}$, the time for degradation of 10% of the insulin present.

Table 6. The rate of degradation of insulin by combination of both deamidation and polymerization. Results are expressed as percentages of the initial amounts of insulin remaining. $t_{90}$ is the time for 10% decomposition.

| Time (months) | Temperature (°C) 60 | 50 | 37 | 25 | 5 | −20 |
|---|---|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 3 | 73·99 | 87·52 | 95·36 | 98·58 | 100 | 100 |
| 6 | 63·02 | 79·71 | 92·71 | 98·03 | 98·84 | 99·96 |
| 12 | — | 72·06 | 90·51 | 93·97 | 98·74 | 99·98 |
| $t_{90}$(months) | 1·06 | 2·73 | 11·52 | 21·14 | 88·14 | 419 years |

The data in Table 3 show that the chemical changes are not reflected in the immunochemical potency of the stored samples. Any changes in potency which have occurred are usually less than the fiducial limits of the assay procedure. Although immunochemical potency determinations of insulin do not necessarily correspond with biological (hypoglycaemic) potency determinations, Schlichtkrull et al (1975) have noted a similar lack of change in biological potency in stored insulin samples. Hence potency determinations alone are insufficient to show the degree to which insulin degrades.

It is immediately apparent that insulin as solid drug should be stored at −20 °C, otherwise desamido insulin and polymerized insulin may be formed, even though Schlichtkrull et al (1975) claim that these moieties have no effect on the potency and immunogenicity of the finished product.

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