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# Effect of contaminating proteolytic activity upon insulin and insulin-protamine complex

CHRISTINE P. J. CAYGILL* AND CHRISTINE M. AYLING†

*Division of Hormones, National Institute for Biological Standards and Control, Hampstead, London NW3 6RB U.K.*

The proteolytic contamination in batches of various insulin preparations was studied using chromogenic synthetic peptide substrates, synthetic ester substrates, and a test similar to the European Pharmacopoeia test for 'Limit of Proteolytic Activity'. In order to study the latter test, insulin-protamine complexes were prepared using salmine sulphate and samples of crystalline insulin, some of which contained peptidase activity. All insulin-protamine complexes prepared from insulin crystals in which peptidase activity had been detected using a synthetic chromogenic substrate were degraded on storage at 37 °C, whereas those prepared from insulin crystals in which no peptidase activity could be detected showed no significant loss in weight. Chromatography on Sephadex G50 revealed degradation of insulin in solutions containing traces ($\approx$0·5 p kat mg$^{-1}$ preparation) of peptidase activity and stored for 2 weeks at 37 °C at pH 7·4, but not of such insulin stored at pH 2·5 at 37 °C for up to 6 weeks. Since insulin preparations formulated at neutral pH are so susceptible to molecular degradation by traces of peptidase contamination, it is suggested that the test using a chromogenic peptide substrate could be applied to bulk crystals before vialing of insulin preparations and could also replace the current test specified in the monograph on Isophane Insulin of the European Pharmacopoeia.

The British Pharmacopoeia and the European Pharmacopoeia (Eur. P) specify a test for a permissible limit of proteolytic activity in Isophane Insulin, formulated at neutral pH. This test involves measurement of the loss of weight of a sample of insulin-protamine complex incubated at 37 °C for 30 days. The recent introduction of synthetic chromogenic peptide substrates, originally developed for use in the blood coagulation field, offers a basis for a rapid and sensitive alternative test for traces of peptidase activity (Svendsen et al 1972; Amundsen et al 1973; Seghatchian & Miller-Andersson 1976; Caygill 1977).

Substrate S-2160 (AB Kabi) (Benzoyl-Phe-Val-Arg-*p*-nitroanilide HCl) was synthesized as a thrombin substrate but is relatively non-specific for most serine peptidases (Svendsen et al 1972) and has been found useful in the detection of peptidase contamination of albumin preparations (Caygill 1977). This paper describes its use in the measurement of peptidase activity in samples of insulin used to make preparations for clinical use, and the degradation of insulin in solution by such proteases.

## MATERIALS AND METHODS

### *Materials*

Samples of bovine insulin crystals used in the manufacture of clinical insulin preparations were provided by Weddel Pharmaceuticals Limited, St. Albans, Herts, and Burroughs Wellcome Laboratories, Dartford, Kent.

Chromogenic peptide substrate S-2160 (Benzoyl-Phe-Val-Arg-p-nitroanilide HCl) was obtained from AB Bofors, Nobel Division and Kabi Diagnostics, Stockholm, Sweden; synthetic esterase substrate BAEE (*N*-Benzoyl-L-arginine ethyl ester HCl) from the Sigma Chemical Company; Sephadex G50 from Pharmacia (G.B.) Ltd.; salmon protamine sulphate (shown to be free of peptidase activity) and Folin and Ciocalteau reagent from British Drug Houses Ltd., and acrylamide and methylenebisacrylamide from the Eastman Chemical Company. All other chemicals were of Analar grade.

*Insulin-protamine complexes.* Twenty three preparations of insulin-protamine complex were made as follows. Samples of crystalline insulin (as used for manufacture) dissolved in 0·01M HCl (250 U ml$^{-1}$) were added to an aqueous solution of peptidase-free salmine sulphate (0·6 mg per 100 units of insulin) at room temperature (20 °C) and the pH adjusted to 7·4 by the addition of 1M sodium phosphate. The suspension, which formed immediately, was diluted with distilled water to a final concentration of approximately 20 units insulin per ml. Between 50 and 85% of the insulin was incorporated in the complex. Sodium azide (1:10 000) was added as preservative.

† Correspondence.
* Present address: Bacterial Metabolism Research Laboratory, Public Health Lab. Service, Colindale Ave., London NW9 5HT, U.K.

*Methods*

*Non-specific proteolytic activity.* This was determined by a modification of the Eur. P. method. A suspension of each insulin-protamine complex (24 ml) was divided into 3 equal portions. Portions were taken immediately and after 18 and 30 days incubation at 37 °C, centrifuged (1000 *g*, 5 min, 5 °C) and the sediment collected, washed with absolute ethanol and dried at 37 °C to constant weight. The weights were recorded; the Eur. P. limit permits a difference of up to 10% in weight.

*Peptidase activity.* Peptidase activity in the samples of crystals was measured with chromogenic peptide substrate S-2160 as described previously (Caygill 1977). A quantity (0·1 ml) of a solution of the insulin under test (40 U $ml^{-1}$ in 0·01 M HCl) was incubated at 37 °C with 0·2 ml 1 mM substrate in distilled water and 1·5 ml 0·1 M Tris-HCl buffer pH 8·2 for 24 h and 7 days.

*Esterase activity.* This was measured by the National Formulary method using BAEE in solutions containing 10 mg $ml^{-1}$ (approx. 250 U $ml^{-1}$) of the insulin samples.

*Molecular size analysis.* Degradation of insulin in solution was investigated by monitoring changes in molecular weight distribution with a Sephadex G50 column. The column (8 × 190 mm) had a void volume of 3 ml, and a salt volume of 12 ml. Insulin (2–3 mg) was dissolved in 0·01 M HCl (0·6 ml), and an aliquot (0·12 ml) was loaded onto the column and eluted with 0·05 M $NaH_2PO_4/Na_2HPO_4$ buffer pH 7·4. Fractions (1·0 ml) were collected and the protein content estimated by the method of Lowry et al (1951), which was found to be more sensitive for insulin than measurements of absorbance at 280 nm. Of the remainder of the insulin solution, half was adjusted to pH 7·4, and incubated at 37 °C in parallel with the insulin at pH 2·5. Aliquots (0·12 ml) were run on the Sephadex column after 2 and 6 weeks (pH 2·5) and 2 and 4 weeks (pH 7·4). All of the protein loaded was accounted for in the eluate.

*Polyacrylamide gel electrophoresis.* The polyacrylamide gel system used was that of Davis (1964). The gels (7·5%) were prepared in 8M urea and 10 μl of each of the insulin solutions incubated at acid pH (for 6 weeks) and neutral pH (for 4 weeks) were applied. The gel electrophoreses were run in 0·005 M Tris glycine buffer, pH 8·3 at 300 V for 45 min; they were stained in 1% Amido Black for 20 min, and destained overnight in 7% acetic acid.

RESULTS

*Degradation of insulin by contaminating peptidase activity*

None of the preparations of insulin incubated at 37 °C at pH 2·5 for up to 6 weeks showed molecular degradation when examined by chromatography on a Sephadex G50 column. Typical elution profiles are shown in Fig. 1a (Sample 6, lacking peptidase), and Fig. 1b (Sample 23, with peptidase).

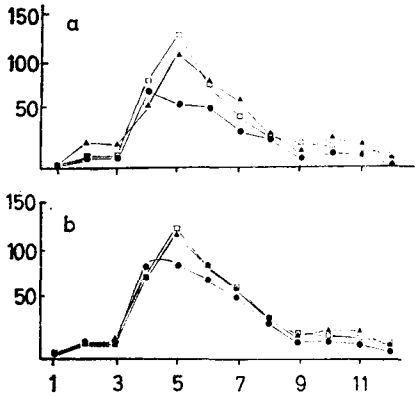


FIG. 1. Elution pattern of insulin solutions on a Sephadex G50 column. Aliquots (0·12 ml) of insulin solutions at pH 2·5 were applied to Sephadex G50 columns before incubation (▲—▲) or after 2 weeks (□——□) or 6 weeks (●——●) storage at 37 °C. Sample 6 (Fig. 1a) was not contaminated with peptidase and sample 23 (Fig. 1b) was contaminated with peptidase.

On the other hand, although no degradation was detected when insulin samples lacking peptidase activity were incubated at 37 °C at pH 7·4 for up to 4 weeks (Fig. 2a), insulin samples contaminated with peptidase activity showed molecular degradation after 2 weeks at 37 °C and pH 7·4. (Fig. 2b).

*Peptidase detection by the 3 methods*

Table 1 shows that the results from the tests on the samples of insulin crystals with the peptidase substrate S-2160, the esterase substrate BAEE and the Eur. P test compare reasonably well in ranking order and there is a significant correlation between all three at the 0·1% level. When proteolytic activity was detected using the Eur.P. method, esterase activity (except in preparation 12) and peptidase activity were also detected. Addition of the protease inhibitor aprotinin (500 units $ml^{-1}$) prevented degradation of the insulin-protamine complex.

*Polyacrylamide gel electrophoresis*

Some of the insulin solutions, pre-incubated for 4 weeks (Samples 1, 6, 18, 19, 22, 23) and 6 weeks

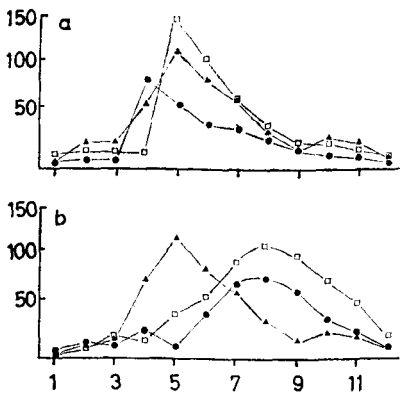


FIG. 2. Elution pattern of insulin solutions on a Sephadex G50 column. Aliquots (0·12 ml) of insulin solutions at pH 7·4 were applied to Sephadex G50 columns before incubation (▲——▲) or after 2 weeks (□——□) or 4 weeks (●——●) storage at 37 °C. Sample 6 (Fig. 2a) is uncontaminated with peptidase and sample 23 (Fig. 2b) is contaminated with peptidase.

(Samples 1, 4, 5, 16, 18, 23) respectively, were analysed by electrophoresis on polyacrylamide gels. Those that had been incubated at acid pH showed the same two protein bands, of insulin and of monodesamido insulin, whether peptidase was present or not. Those devoid of peptidase that had been incubated at neutral pH also showed similar bands. However, in samples contaminated with peptidase incubated at neutral pH, no protein bands were visible, suggesting that degradation had taken place to small fragments which do not stain.

Table 1. Peptidase and esterase activity of bulk crystalline insulin samples and Eur.P. proteolytic activity of Insulin-protamine complexes prepared from these. For details see text.

| Insulin prep. | Peptidase act. p. kat mg$^{-1}$ prep. S-2160 | Esterase act. p. kat mg$^{-1}$ prep. BAEE | Eur. P. Proteolytic act. test % loss of wt* of complex after 30 days |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 |
| 3 | 0 | — | 0 |
| 4 | 0 | — | 0 |
| 5 | 0 | — | 3·0 |
| 6 | 0 | 0 | 6·0 |
| 7 | 0·13 | 0·10 | 22·0 |
| 8 | 0·14 | 0 | 0 |
| 9 | 0·15 | 0·11 | 17·0 |
| 10 | 0·15 | 0·13 | 18·3 |
| 11 | 0·20 | 0·50 | 51·9 |
| 12 | 0·22 | 0 | 100 |
| 13 | 0·24 | 0·17 | 27·6 |
| 14 | 0·26 | 0·33 | 21·5 |
| 15 | 0·28 | 0·31 | 55·6 |
| 16 | 0·33 | 0·24 | 43·0 |
| 17 | 0·52 | 0·30 | 45·6 |
| 18 | 0·53 | 0·81 | 68·0 |
| 19 | 0·57 | 0·66 | 70·0 |
| 20 | 0·61 | 1·14 | 39·0 |
| 21 | 0·61 | 0·74 | 61·0 |
| 22 | 0·69 | 1·52 | 60·0 |
| 23 | 0·87 | 1·98 | 100 |

There is a significant correlation between the three sets of measurements at the 0·1% level.
* Eur.P. limit ±10% weight difference.

## DISCUSSION

The proteolytic enzymes most likely to be found in extracted insulin are those from the pancreas; trypsin, chymotrypsin and carboxypeptidase(s) and perhaps thrombin. These all have pH optima around pH 8·0, and are inactive at the pH of Soluble Insulin Injection preparations (pH 3·0–3·5).

We had previously examined, using S-2160 as substrate, almost 100 batches of various clinical insulin preparations, including Soluble Insulin (34), Isophane Insulin (27), Protamine Zinc Insulin (10) and highly purified insulins (25). Traces of peptidase activity of the same order of magnitude as that found in the insulin crystals used in their formulation, were found also in samples of Soluble Insulin and the highly purified insulins. The trace amounts of peptidase in Soluble Injection (pH 3·0–3·5), one of the most frequently used insulin preparations, would not degrade the insulin at that pH.

Many insulin preparations, however, are formulated at neutral pH, including highly purified and monocomponent preparations which are being increasingly used in the treatment of diabetes. We have shown that trace amounts of peptidase activity cause degradation of insulin preparations stored at 37 °C at pH 7·4; the extent of such degradation during the permitted 2 year shelf-life at 2–10 °C could cause a clinically unacceptable alteration of biological activity.

Of 17 preparations in which peptidase activity was detected with the peptide substrate S-2160, 2 failed to show esterase activity using BAEE. In preparation 12, proteolytic activity was detected using the Eur.P. test. This could occur if carboxypeptidase activity is present alone as this would have no action on an ester substrate, but would degrade protamine.

Providing that these results can be confirmed by other laboratories, it could be possible to use synthetic chromogenic substrates for the screening of bulk insulin crystals for clinical use. Chromogenic peptide substrates have potential advantages of greater sensitivity and speed compared with the pharmacopoeial method currently applied to Isophane Insulin, and could replace it. The Eur. P. test requires 40 ml of Insulin preparation, takes 30 days and is tedious to perform; the method using the chromogenic peptide requires only 0·1 ml sample, takes 1–7 days and is simple to carry out. Further study of the effect of glycerol, phenol, *p*-hydroxy-

benzoate and other additives used in these preparations is necessary, but our original investigations with insulin preparations for clinical use indicated that these have no effect. A limit of contamination detected by the current Eur. P. test would be approximately 0·10 p katals per mg insulin.

The results reported in this paper indicate the value of this alternative simple test to limit peptidase contamination of insulin preparations formulated at neutral pH.

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