ISOELECTRIC FOCUSING OF INSULIN, ^{125}I-INSULIN AND THE ^{125}I-INSULIN-ANTIBODY COMPLEX

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Received September 28th, 1978

Isoelectric focusing on polyacrylamide gel columns of three native crystalline commercial preparations of insulin and ^{125}I-labelled insulin was carried out. All the compounds studied contained three components of different isoelectric points. The largest fraction, having pI 5·60 ± 0·05, was common to all preparations. The other two fractions were situated in the acid region of pH between pI 4·5 and 5·2. The presence of these fractions is explained by the contamination of crystalline insulins by proinsulin and by the formation of des-amido derivatives during the dissolving and storage of insulin samples, and, in case of labelled insulin, also by the presence of heavily iodinated insulin and contaminating components. The isoelectric focusing of the complex ^{125}I-insulin-antibody showed a peak of radioactivity having pI 6·15 ± 0·05.

The purity of labelled compounds can be improved using isoelectric focusing in polyacrylamide gel columns[1-3]. The application of this method for purifying insulin on a microscale is an easy matter. Nevertheless, there are few data available concerning the pI of insulin and its labelled products and most of them are controversial[4,5]. Such data for the insulin-antibody complex are lacking altogether. The aim of this work is to compare the pI of various commercial insulins, and to determine the pI of ^{125}I-labelled insulin, and of the insulin-antibody complex.

EXPERIMENTAL

Materials

Crystalline insulins were purchased from the following firms: British Drug House, England (bovine insulin, 23.4 U/mg), NOVO, Denmark (10times crystallized porcine insulin, 27 U/mg), and Hoechst, German Federal Republic (porcine insulin, 27 U/mg). Guinea-pig anti-insulin serum (anti-porcine NOVO insulin) was obtained in the laboratory in Sofia, Na^{125}I was purchased from Isotop, Hungary, Coomassie brilliant blue from Serva, German Federal Republic, Charcoal Norit A and Dextran T 150 from Pharmacia Fine Chemicals, Sweden,

human serum albumin from NIEM, Bulgaria, and ampholines from LKB-Produkter AB, Sweden. All other chemicals were of Analytical-R grade.

Methods

Insulin labelling: The chloramine T method[6] was used. The specific activity of the labelled product was 150—200 μCi/μg.

Polyacrylamide gel electrophoresis: The gels were prepared according to Reisfeld and coworkers[7], the concentrations being 7·5% T and 2·5% C. Polyacrylamide gel electrophoresis was used for the purification and separation of ^{125}I-labelled insulin from free iodide and degradation products and was performed before each experiment of isoelectric focusing. The peak gel slices containing the maximum of labelled insulin (about 100000—300000 cpm) were put on the top of the gel for isoelectric focusing without elution, or incubated with the antibody and then focused.

Isoelectric focusing in polyacrylamide gel: The method of Doerr and Chrambach[8] was used with minor modifications. The concentrations of the gel components were as follows: 7% T and 4% C in 25% sucrose, and 1% ampholine (pH range 5—8 and 7—9). $K_2S_3O_7$ was used as catalyst of the polymerisation in the final amount 0·07%. The gel size was 85 × 5 mm. The upper reservoir contained 1% ethanolamine and the lower one 0·2% sulfuric acid. Isoelectric focusing was run for 18 h at 4°C and 150 V, the starting current being 2 mA per gel. After this time the gels were taken out and cut into 2 mm slices, the radioactivity and pH of which were measured in a well-type scintillation γ-counter VAM-120 "Vakutronic", GDR, and on a Radiometer Copenhagen pH-meter, Denmark, respectively. The pH of the gel slices was measured after 2 h of extraction with preboiled 0·05M solution of NaCl at 24°C or after 24 h at 4°C. Staining and destaining of proteins was performed according to Vesterberg[9].

Affinity chromatography of insulin antibody described in refs [10] and [11] was used for the isolation of insulin antibodies from guinea-pig anti-insulin serum.

Affinity of ^{125}I-insulin to insulin antibody. Fractions of ^{125}I-insulin after polyacrylamide gel electrophoresis and isoelectric focusing were tested according to Herbert and coworkers[12] with minor modifications.

Incubation of samples before isoelectric focusing: Anti-insulin serum, normal guinea-pig serum or purified immunoglobulins were incubated with ^{125}I-insulin in 0·05M phosphate buffer, pH 7·5, for 24 h at 4°C. The labelled insulin was purified by polyacrylamide gel electrophoresis as mentioned above. The sample volume was 0·05 ml (having 50—400 μg of protein) and was laid on the top of the gel for isoelectric focusing in 25% sucrose solution.

RESULTS AND DISCUSSION

Isoelectric Focusing of Unlabelled Insulin

Three crystalline commercial insulins (see Experimental) were studied. The stock solutions were prepared several days before the experiments in $3·3 . 10^{-3}$ M-HCl. Isoelectric focusing revealed heterogeneity of all the three preparations, three components being found. Common to all preparations was the most visible component having pI 5·6. The other two components were situated between pI 4·5 and 5·2

with slight variations between different insulins. Even the 10times crystallized preparation contained these components. The first component may correspond to insulin and the other two to proinsulin and des-amido-insulins, as proposed earlier[13-15]. A medium of $3\cdot3 \cdot 10^{-3}$M-HCl prevents the formation of aggregated material from insulin molecules and by repeated isoelectric focusing of every single component the formation of a complex between insulin and ampholine was excluded. Polyacrylamide gel electrophoresis of these three commercial insulins shows only one sharp band without additional fractions, which indicates the same electromobility of insulin and the contaminating components under the conditions chosen.

Isoelectric Focusing of ^{125}I-Insulin

The experiments were carried out with the insulin preparation purchased from NOVO. Before isoelectric focusing, the labelled insulin was purified on polyacrylamide gel electrophoresis. It is evident from Fig. 1, that after polyacrylamide gel electrophoresis the labelled insulin shows only one peak if the gel is cut into 2 mm slices, but the peak is not sharp, especially in its descending shoulder. If the gel is cut into 1 mm slices, a second peak appears in the descending shoulder[16]. Different results (Fig. 2*a*,2*b*) were found if a gel slice with the maximum radioactivity (Fig. 1, part *A*) or a gel slice from the descending shoulder (Fig. 1, part *B*) were focused. The positions of the peaks of radioactivity and the percentage of the total radioactivity in them are summarized in the Table. All three components show the same immunoreactivity (about 95%) with guinea-pig anti-insulin serum. This fact is not surprising considering that guinea-pigs were immunized with the same preparation of insulin as was used for

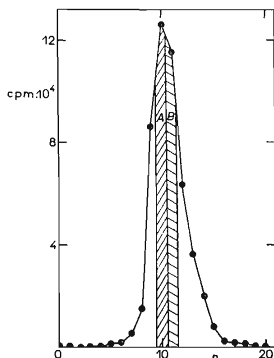

FIG. 1

Distribution of Radioactivity in the Gel after Polyacrylamide Gel Electrophoresis of ^{125}I--Insulin

The gels were cut into 2 mm slices, *n* number of slices; *A* indicates the slice of the gel with the maximum of radioactivity and *B* indicates the gel slice from the descending shoulder. For details see Experimental.

TABLE I

Isoelectric Points of the Components of the Native Crystalline NOVO Insulin and the Iodinated NOVO Insulin Purified by Polyacrylamide Gel Electrophoresis

In the case of labelled insulin the percentage of total radioactivity in the peaks is given in parentheses.

Sample	Fraction number		
	1	2	3
Native insulin	5·60 ± 0·05	5·20 ± 0·10	4·5 ± 0·10
^{125}I-insulin A[a]	5·60 ± 0·05 (70%)	4·80 ± 0·05 (30%)	—
^{125}I-insulin *B*[a]	5·60 ± 0·05 (35%)	4·80 ± 0·05 (30%)	4·55 ± 0·05 (35%)

[a] See Fig. 1.

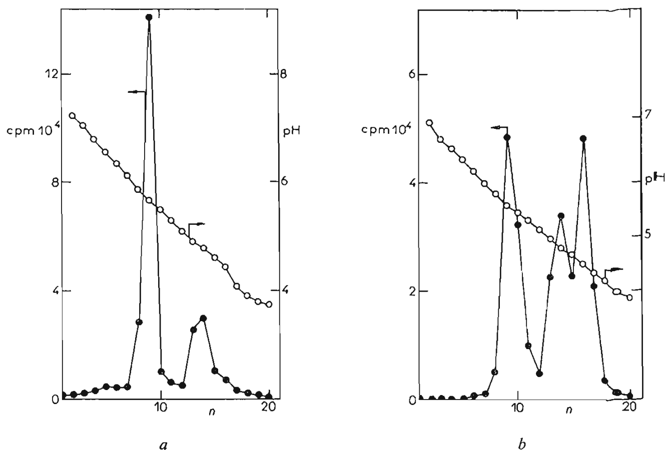

FIG. 2

Distribution of Radioactivity in the Gel after Isoelectric Focusing of ^{125}I-Insulin

a Isoelectric focusing of labelled insulin from the gel slice with the maximum of radioactivity after polyacrylamide gel electrophoresis (see *A* in Fig. 1); *b* isoelectric focusing of labelled insulin from the gel slice from the descending shoulder after polyacrylamide gel electrophoresis (see *B* in Fig. 1). *n* number of slices.

labelling and that, as mentioned above, the contaminating components, proinsulin and des-amido-insulin (they are labelled together with insulin) show a high degree of cross-reactivity with the anti-insulin serum.

The first peak having pI 5·6 most probably corresponds to mono-iodo-insulin, which agrees with the data of Sodoyez and coworkers[4], the other components to heavily iodinated insulins and to labelled proinsulin and des-amido-insulin like compounds. If native insulin and labelled insulin are focused together, the protein band and the peak of radioactivity overlap at pI 5·6. Our results indicate that the difference in pI of native insulin and mono-iodo-insulin is beyond the sensitivity of the method used. Our results differ from those of Lambert and coworkers[3] who stated a lower pI value for native insulin in comparison with mono-iodo-insulin using carrier-free long-lasting isoelectric focusing and a very narrow pH gradient. Repeated isoelectric focusing of each fraction leads to the concentration of the radioactivity at the same pH as in the case of the first focusing. This fact indicates the absence of interaction between insulin and ampholine as in the case of human growth hormone and prolactin[13].

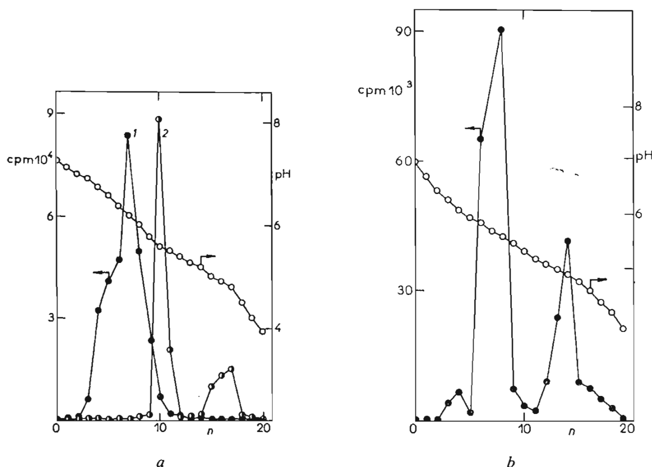

FIG. 3

Distribution of Radioactivity in the Gel after Isoelectric Focusing of the Incubation Mixtures of ^{125}I-Insulin with Guinea-pig Anti-insulin Serum or Guinea-pig Normal Serum or ^{125}I-Insulin alone

a 1 Guinea-pig anti-insulin serum; 2 normal guinea-pig serum, *b* ^{125}I-insulin alone.

Isoelectric Focusing of the ^{125}I-Insulin-Antibody Complex

Labelled insulin purified with polyacrylamide gel electrophoresis as described above was incubated at 4°C for 24 h with: *a*) guinea-pig anti-insulin serum (1 : 1, 1 : 5, and 1 : 100 diluted antiserum having titer 1 : 128 000), *b*) insulin antibodies purified by affinity chromatography, *c*) normal guinea-pig serum, and *d*) isolated bovine immunoglobulins.

In order to check the degree of degradation during incubation, labelled insulin alone was also incubated. The quantity of proteins in the samples amounted to about 400 μg in the case of guinea-pig sera, and 200 μg in the case of purified immunoglobulins. The results are shown in Figs 3*a* and 3*b*. It is evident from the figures that the radioactive complex is situated between pH 6·20 and 5·70 with the maximum at pH 6·15 ± 0·05. An excess of labelled insulin leads to the formation of another maximum of radioactivity at pH 5·70; on the other hand if an excess of antibody is used, the peak at 5·70 will not appear but a precipitate at the beginning of the gel simulates a radioactive peak at pH 8. This fact led us to use polyacrylamide gel electrophoresis after the incubation of an excess of ^{125}I-insulin with the antiserum in order to separate the complex and free labelled insulin according to their different electromobility. The complex is situated near the start and therefore the first slice of the gel was immediately used for isoelectric focusing without elution. However, even after this treatment, the same results were obtained as shown in Figs 3*a* and 3*b*. The extent of the radioactive peak may be explained by the heterogeneity of antibodies. If ^{125}I-insulin is incubated with normal guinea-pig serum or purified bovine immunoglobulins, the peaks of radioactivity are situated at pH 5·60 ± 0·05 and 4·80 ± 0·05 as in the case of isoelectric focusing of ^{125}I-insulin alone.

To conclude, isoelectric focusing in polyacrylamide gel columns under the conditions chosen also without a preceding polyacrylamide gel electrophoresis seems to be a method convenient for the separation of mono-iodo-insulin from the other products of iodination procedure and from contaminating components. It does not, however, manage to separate mono-iodo-insulin from native insulin.

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Translated by L. Servítová.