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RADIOIODINATION OF HUMAN GROWTH HORMONE WITH CHARACTERIZATION AND MINIMIZATION OF THE COMMONLY DEFINED "DAMAGED PRODUCTS"

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Summary

The radioiodination and chromatographic purification of human growth hormone (hGH) has been studied in order to better define and control the so-called "preparation damage", which is often a cause of interferences, loss in specific activity and sensitivity, misclassification errors in radioligand assays, and a source of misinterpretation when the tracer is used in receptors or in vivo studies.

A series of labelings and false labelings, with and without protein carrier in the buffer used for Sephadex purification, indicate that the "preparation damage" peak is made up of two components: aggregated ^{125}I-hGH and BSA-carried radioactivity. The former can be minimized by the use of recently extracted non-lyophilized hGH, and the latter by enzymatic labeling. Both components can be better resolved, and thus eliminated, when Sephadex G-100 is employed rather than G-75.

Introduction

The difficulties encountered in obtaining reproducible labeling reactions with well-controlled specific activity in the product, principally with regard to the nature and extent of the alterations occurring and the influences of the reagents and carriers used during the preparation process, are widely known [1].

In referring to this problem, the vague term "damaged antigen" is often used; depending on the particular case and the authors, this "damaged antigen" is attributed to cleaved labeled antigen carried by serum albumin [2—11], to a type of aggregate [10—17], or to immunologically inactive, though uncleaved antigen bound or not to the protein carrier [5,6,8,18—20].

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Previous work on labeled hGH, in which we attempted to identify the various electrophoretic components of this so-called "damaged antigen" [21], suggested the existence of a relationship between albumin used as protein carrier and the presence of such "undesirable components", as we prefer to refer to these forms. Using a modified electrophoretic technique developed in this laboratory [22], we have now closely examined this relationship and these components, with the objective of minimizing or, possibly, eliminating them.

Material and methods

Immunoassay grade human growth hormone (NIH-GH-HS2160 E) prepared by Dr. A.E. Wilhemi, was a gift of the National Pituitary Agency (NIH-NIAMDD) Bethesda, MD, U.S.A., as were also the guinea pig antiserum against hGH, which was furnished at a dilution of 1 : 2000, and human luteinizing hormone (hLH-LER-960) for radioiodination. The second hGH preparation used was prepared in this laboratory at the Instituto de Energia Atômica (hGH-IEA Somatormon, Central fraction of peak III, prep. 270777, 110478 and 200979) according to the method of Roos et al. [23], with some modifications [24].

$Na^{125}I$, free of carriers and reductors at a specific activity of about 500 mCi/ml (New England Nuclear, Boston, MA, U.S.A.) was used in quantities of about 1 mCi per μg hormone, dissolved in 5 μl phosphate buffer 0.05 mol/l, pH 7.4, in the labeling experiments, which were performed according to the method of F.C. Greenwood et al. [20], using 50 μg of Chloramine T and 200 μg of metabilsulfite.

Crystalline lactoperoxidase (Calbiochem Co., Los Angeles, CA, U.S.A.) was used for the enzymatic labeling according to the method described by Thorell and Johansson [25].

The purification of the labeled hormone was performed on a Sephadex G-75 or G-100 (Pharmacia, Uppsala, Sweden) 2 X 45 cm column, at a flow rate of 12 ml/h (fraction volume 2—2.5 ml) in 0.025 mol/l Veronal buffer, pH 8.6, with or without 1% BSA, according to the method of Cerasi et al. [9]. The void volume was determined with Blue Dextran and the elution volume of BSA with BSA-bound bromophenol blue.

Results

The influence of bovine serum albumin on the purification process was first studied by performing two labelings in the complete absence of hGH, the data being presented in Fig. 1. One was "purified" using Veronal buffer without BSA and the other using the same buffer containing the usual 1% BSA. As can be seen in the first case (Fig. 1A), the elution profile is virtually identical to that obtained upon passing $Na^{125}I$ alone under the same conditions. The only difference is a small amount of radioactivity immediately following the void volume, probably carried by Blue Dextran. In the presence of albumin (Fig. 1B) a considerable peak of "damaged material", accounting for about 20% of the recovered radioactivity, can be seen in the BSA elution volume. It should be emphasized that in this case nothing was present during the labeling that might

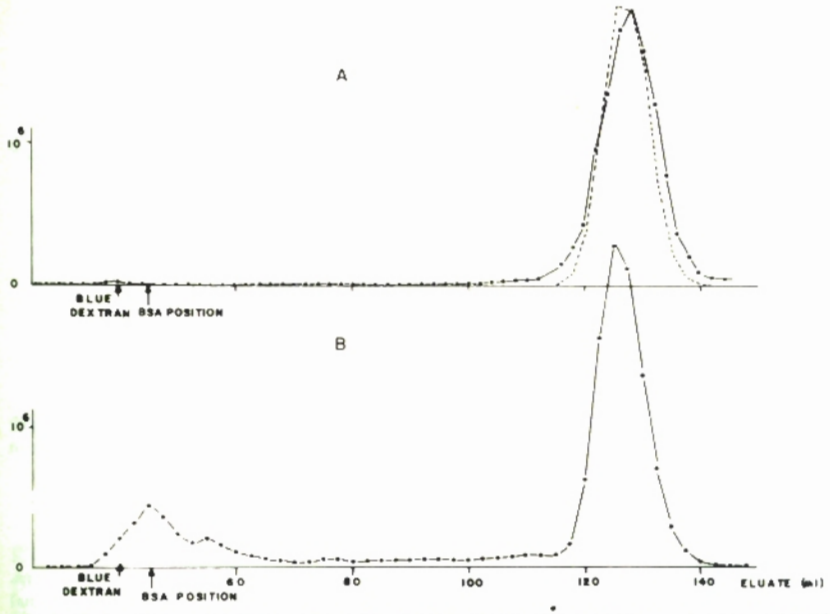

Fig. 1. Gel chromatography on Sephadex G-75 of two false labelings. Column size 2 × 45 cm, flow rate 12 ml/h, fraction volume 2—2.5 ml. Elution buffer: Veronal 0.025 mol/l, pH 8.6 (A) with no BSA present in the buffer. Dashed line corresponds to a direct run of untreated $Na^{125}I$ on the same column but in a separate experiment. (B) With 1% BSA in the buffer. The void volume is indicated by Blue Dextran and the BSA elution volume by bromophenol blue.

somehow be damaged. The total recovered radioactivity, around 70% in the absence of BSA, went up to 90% in the presence of this protein.

The same type of experiment was carried out introducing 5 μg of hGH, (IEA), as is usually done in our labelings. It is evident (Fig. 2A) that the complete lack of protein carrier led to the formation of a species eluting immediately after the void volume and therefore less retarded than albumin, the elution volume of which on the same column was determined in a separate run.

One of the less satisfactory, but still useful, labelings of hGH (IEA-270777), under normal conditions, using a solution of the hormone prepared several weeks before and kept frozen at —20°C, produced the known chromatographic pattern in Fig. 2B. This pattern was also obtained many times using equivalent solutions of hGH (NIH). It can be seen that the position and form of the "damaged zone" (PI) are those of the first two bands in Figs. 1B and 2A. The overlapping of these two components was further confirmed by analyzing the two tubes, which were collected at the Blue Dextran and BSA peak positions of this regular hGH labeling on PAGE. Their different composition is clearly evident in the electrophoretograms of Fig. 3, and supports our assignment of the components to an aggregate of ^{125}I-hGH (Fig. 3A) and of BSA-carried ^{125}I (Fig. 3B). In addition, the two electrophoretograms of Fig. 3 are practically identical to those obtained from the first elution peaks in the chromatography carried out without BSA in the buffer (Fig. 4), and in the chromatography of the false labeling carried out with BSA (Fig. 5), respectively. Moreover, in both cases, BSA-carried ^{125}I exhibited essentially no binding in the presence of

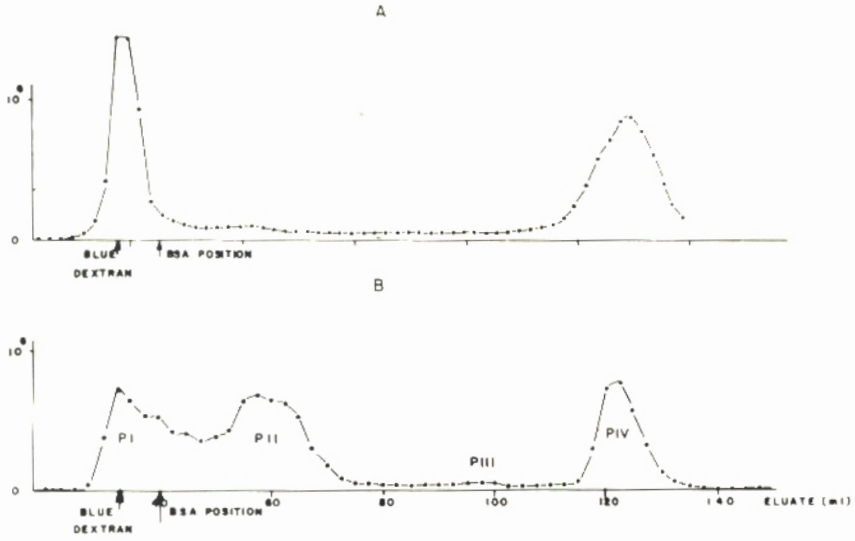

Fig. 2. Chromatogram on Sephadex G-75 of ^{125}I-hGH (IEA), after labeling under the usual conditions. Column specifications and elution conditions as before. (A) No BSA present in the buffer; (B) veronal buffer with 1% BSA. Void volume and BSA elution volume have been determined as in the previous experiment. PI, "preparation damage"; PII, monomeric ^{125}I-hGH; PIII, unidentified non-immunoreactive peak; PIV, free ^{125}I.

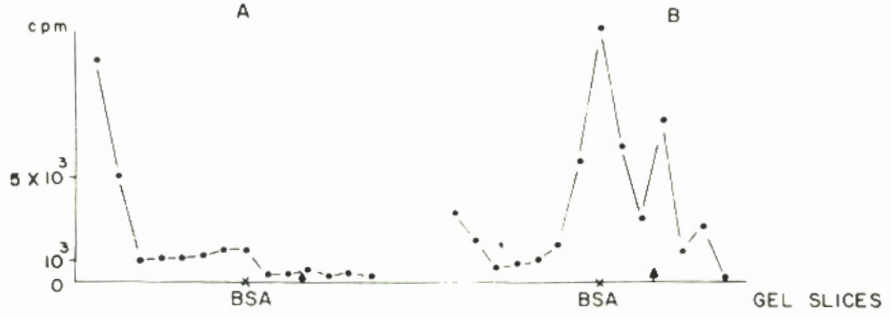

Fig. 3. Polyacrylamide gel electrophoresis of two fractions obtained upon gel filtration of labeled hGH (IEA) in the presence of 1% BSA and shown in Fig. 1B. (A) Fraction corresponding to the Blue Dextran peak position; (B) fraction corresponding to BSA elution volume.

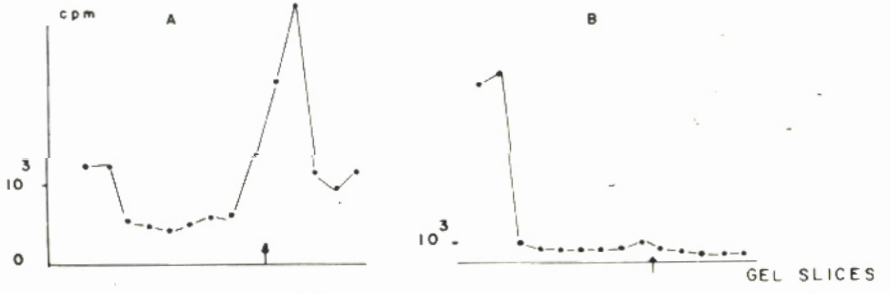

Fig. 4. Gel electrophoresis of ^{125}I-hGH, stored and purified in the absence of BSA. (A) Unpurified labeling mixture; (B) fraction corresponding to the Blue Dextran peak position.

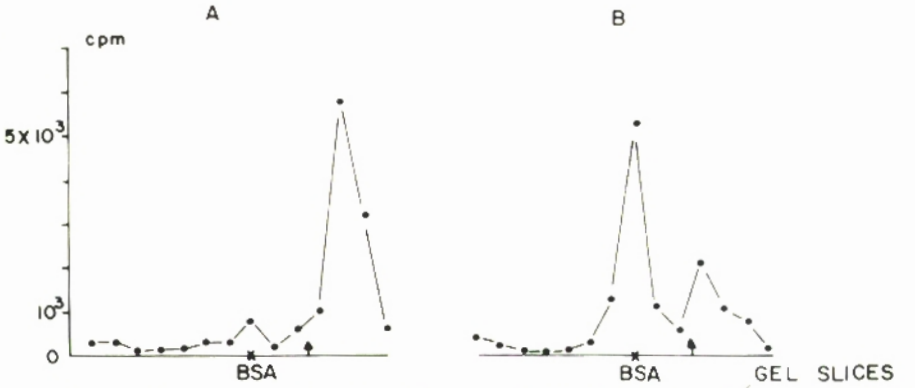

Fig. 5. Electrophoretograms of the false labeling, purified in the buffer with 1% BSA. (A) Unpurified mixture; (B) fraction corresponding to BSA elution volume.

guinea pig antiserum against hGH, which is always very active in binding purified ^{125}I-hGH, and this confirms experiments already published by this same laboratory [21].

In the light of these findings, a new purification process to better resolve the damaged zone components was standardized employing Sephadex G-100 on the same column (2 X 45 cm). A recent (less than 1 week old) hGH extract IEA-140878), freshly dissolved on the day of labeling, in Cerasi et al. [9] buffer, was used. Under these conditions the two components eluting first from Sephadex (PIa and PIb) are clearly resolved and occur immediately after the void volume and in the BSA elution volume, respectively. Fig. 6 shows this chromatogram, together with PAGE analysis of the various fractions. PIa is clearly smaller in this experiment (relative to Fig. 2B) and was practically eliminated in a second experiment. The latter was performed under exactly the same

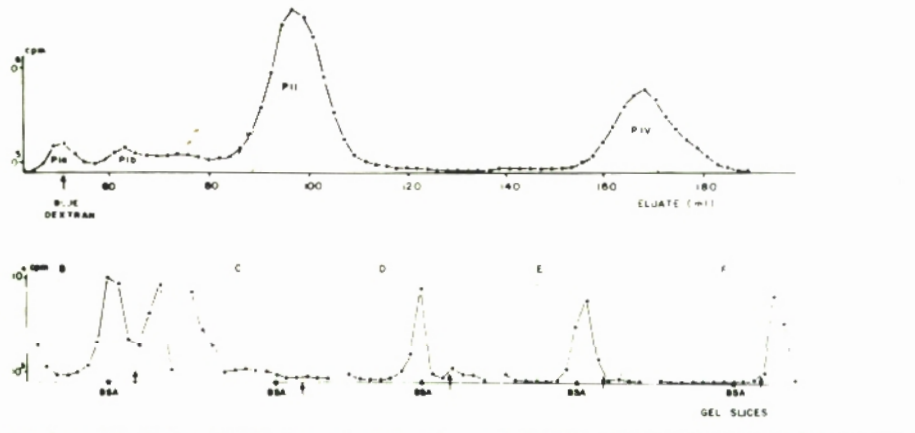

Fig. 6. (A) Sephadex G-100 chromatogram of conventionally labeled hGH (IEA). Column specifications and elution conditions as usual. PIa and PIb, undesirable components; PII, monomeric ^{125}I-hGH; PIV, free ^{125}I. (B) PAGE analysis of the unpurified labeling mixture. (C), (D), (E) and (F), PAGE analysis of, respectively, PIa, PIb, PII and PIV.

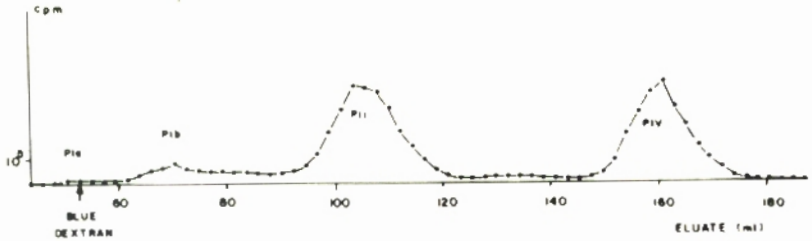

Fig. 7. Sephadex G-100 chromatogram of recently extracted hGH, labeled directly without intermediate lyophilization. Column specifications and elution conditions as usual.

conditions, except that the hGH to be labeled (IEA-200979) was obtained directly by Sephadex G-100 purification of pituitary extracts using the method of Roos et al. [23], followed by dialysis against 0.05 mol/l phosphate buffer, pH 7.5 (Fig. 7), without intermediate lyophilization.

A preliminary labeling experiment was also carried out to determine the influence of the type of hormone being labeled. Using a glycoprotein (hLH) in the usual procedure, with and without BSA, the formation of undesirable components was found to be qualitatively similar to that of hGH. However, only about 30% of the hormone aggregated in the absence of BSA, and there is some evidence which needs further study, that other modifications might have occurred in the ^{125}I-hLH molecule (Fig. 8).

To check the possible effects of Chloramine T or the particular labeling technique, parallel labelings and purifications were carried out using the lactoperoxidase method [25] and recently extracted, freshly dissolved hGH. Fig. 9

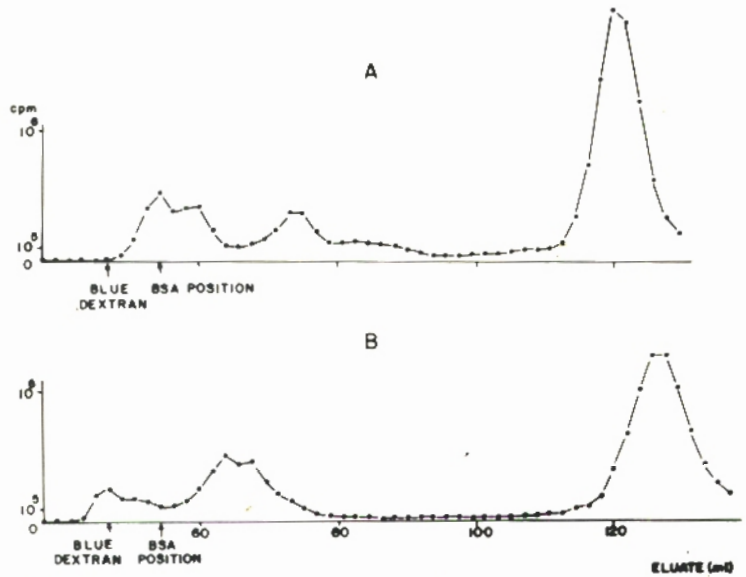

Fig. 8. Sephadex G-75 chromatogram of ^{125}I-hLH labeled and purified under the usual conditions. (A) Regular Veronal buffer with 1% BSA; (B) no BSA present in the buffer.

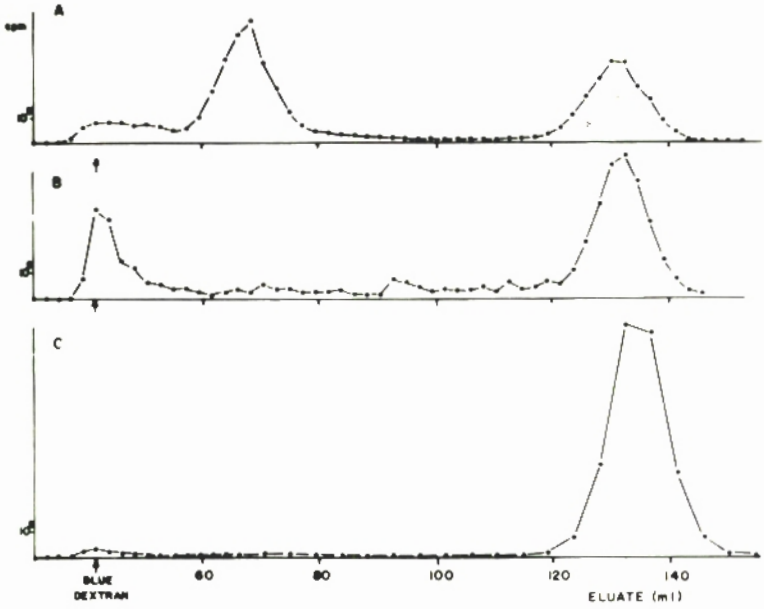

Fig. 9. Purification on Sephadex G-75 under the same conditions as in Fig. 1 of ^{125}I-hGH labeled with enzymatic method of Thorell and Johansson [25]. (A) Regular labeling with 1% BSA in the elution buffer; (B) same labeling without BSA in the buffer; (C) false labeling: all components except hGH are present.

presents the results of two enzymatic labelings with BSA (A) and without BSA (B) in the purification buffer, and of a false enzymatic labeling with protein carrier added during purification (C). In this case, the ^{125}I-hGH obtained was also cleanly resolved, though it exhibited a somewhat lower specific activity resulting from the labeling method. BSA still served to prevent aggregation, but, in contrast to the Chloramine T technique, it did not carry any radioactivity.

Discussion

Any "damage" occurring during the radioiodination of a protein hormone, as is suggested by many authors, is not supported by the results presented here. The first peak eluting from Sephadex appears to be derived principally from the overlapping of a peak of aggregate with a certain amount of radioactivity carried by BSA. The fundamental role of this protein carrier seems to be to prevent aggregate formation during purification and storage of the labeled product. The possibility that Blue Dextran might be acting as an extremely efficient ^{125}I-hGH carrier in absence of BSA has been eliminated by a chromatography carried out in complete absence of this marker, which showed a pattern exactly identical to that presented in Fig. 2A. BSA also seems to generally affect the total recovery of radioactivity.

Our results are consistent with the findings published by Bieler et al. [26], with regard to better hGH recovery in the presence of serum albumin (human

in their case); nonetheless, in their experiment aggregation seems to be favoured by increased concentration of albumin, in contrast to our results.

A role of hSA in stabilizing the monomeric form of ^{131}I-hGH in vitro, upon incubation at 37°C, has also been present in work of Beitins et al. [27]; the absence of BSA could also explain some of the results obtained by Schwartz et al. [15] in aggregation of ^{125}I-hGH monomers during storage at −20°C.

The use of Sephadex G-100 instead of G-75 results in a much better resolution of PIa, PIb and PII (Fig. 6) and permits one to obtain a monomeric form of ^{125}I-hGH of higher purity. This opens the way to a more precise calculation of its specific activity (unpublished data). The result shown in Fig. 7 and its comparison with the previous purifications seems to indicate that this aggregate exists prior to the labeling reaction, and that it can be eliminated by using freshly extracted hGH, avoiding long storage in solution, freezing and thawing and lyophilizations.

The data in Fig. 9C suggest that the presence of PIb might somehow be dependent upon the particular labeling technique, or more specifically, upon an imperfect or still uncontrolled Chloramine T-metabilsulfite ox-redox system [20,28]. On the other hand, PIa formation favoured principally by the absence of BSA seems to depend on the type and conditions of the cold hormone preparation being independent from the labeling technique used. Thus the enzymatic labeling with lactoperoxidase does not, by itself, eliminate the problem of "preparation damage", especially thinking in terms of aggregates. Our preliminary results with human luteinizing hormone (hLH), which is not routinely labeled here, might reflect a different tendency to aggregate, possibly related to the type of protein.

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