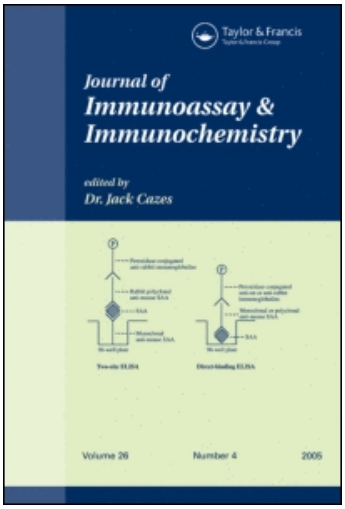

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INFLUENCE OF CHLORAMINE T IODINATION ON THE BIOLOGICAL AND IMMUNOLOGICAL ACTIVITY OR THE MOLECULAR RADIUS OF THE HUMAN GROWTH HORMONE MOLECULE

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ABSTRACT

Potential alterations of the somatotropic activity of human growth hormone (hGH) resulting from Chloramine T labelling reaction, iodination up to 2.7 atoms/molecule and indirect radiation effects, have been studied. Three 2x2 factorial assays, performed in hypophysectomized rats, failed to reveal any significant difference ($P > 0.05$) in true growth promoting activity between hGH and (127-I)hGH, even after storing the latter with 125-I. Similar results were obtained applying a sensitive and precise gel filtration technique for Stokes Radius determination and radioimmunoassay.

The Chloramine T labelling reaction is still the most widely used technique for protein radioiodination (1,2). At present there is no clear evidence as to whether the original (3),still commonly employed (4), high concentration of oxidizing reagent is deleterious to the antigen being labelled (5).

Many authors have studied the effects of iodination on various proteins and hormones, reaching a variety of conclusions (6-10). While there seems to be general agreement with respect to retention of immunoactivity of moderately iodinated human and bovine growth hormone (11-13), possible alterations in its somatotropic activity have been much more difficult to exclude due to the limited sensitivity of bioassays. Hughes et al. (12) studied alterations due to lactoperoxidase iodination using a sensitive bioassay (14), related to lactogenic rather than somatotropic activity, and found loss of bioactivity. In a simi-

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lar study using bovine growth hormone, Mattera et al. (13) found full retention of growth-promoting activity after introduction of ^{127}I. Their product was, however, labelled using the milder Chloramine T technique of Roth (15) at an iodination degree of 1 atom/molecule. Goodman et al. (16) used two "in vitro" bioassays to show that, at moderate levels of iodination, hGH retained full potency with respect to stimulation of glucose oxidation and lipolysis.

The present study, which takes advantage of a sensitive 2x2 factorial bioassay (17), was mainly designed to investigate changes in the growth-promoting activity of hGH due to relatively drastic Chloramine T iodination (up to ~2 atoms/molecule) and indirect radiation effects. Alterations in immunological activity and in molecular radius were also studied.

Our study thus bears directly on the basic assumption made when a tracer is employed, i.e., that the biological, immunological and physico-chemical properties of the iodinated ligand do not differ significantly from those of the native hormone. In the case of a molecule like (^{125}I)hGH, this assumption is relevant to its valid use in radioligand assays and in its application in "in vivo" studies of the mechanism of action and metabolism of this hormone.

METHODS

hGH preparations: The 2nd. and 3rd. standard-IPEN of hGH, prepared and calibrated in this laboratory as previously described (18), and hGH-IPEN lot 24, were used for the labelling and control.

Iodination: 0.25, 1.6 or 3.2 mg of hGH were iodinated using (with minor modifications) the original Chloramine T labelling technique of Greenwood et al.(3), maintaining reagent concentrations and conditions identical to those used in our routine labelling of 5 µg hGH with 125-I. This was done by multiplying by 50, 320 or 640-fold the mass and the volumes of the following reagents, added in the order listed: 40 µl 0.5 M phosphate buffer, pH 7.4; 0.136 µg NaI in 4 µl 0.1 M NaOH; 5 µg hGH in 10 µl 0.05 M phosphate buffer, pH 7.4; 50 µg Chloramine T in 10 µl of the same buffer. After waiting 30 seconds, 200 µg of sodium metabisulfite, dissolved in 20 µl 0.05 M phosphate buffer, pH 7.4, were added. Continous magnetic stirring was maintained throughout. In the macroiodinations, instead of using 0.91×10^{-3} µg-atoms of 125-I (~2 mCi), a 50, 320 or 640-fold amount of 127-I was added, together with about 45, 300 or 600 µCi of 125-I, in order to permit calculation of labelling yields and average iodination degree, as well as to reproduce indirect radiation effects. Thus the re-

agent concentrations at the moment of the oxidizing reaction were: hGH= 3.7×10^{-3} mM; I_2= 7.4×10^{-3} mM; Chloramine T= 2.9 mM.

Purification and storage of I-hGH: Part of the sample was purified on Sephadex G-100 to allow precise calculation of the labelling yield and iodination degree and for Stokes Radius determination. The remainder was dialyzed immediately after the labelling. The labelled hormone was frozen and stored for about 10 days at -20ºC before initiating the injections or the radioimmunoassays.

Bioassay: The body weight gain test was performed in hypophysectomized rats in 5 and 10-day assays, using doses of 10 and 20 µg/rat·day, 10 rats per group, with statistical treatment as described previously (17). Potencies of I-hGH were calculated in terms of the original, dialyzed preparation used for the labelling.

Radioimmunoassay: An immunoassay technique as previously described (18), used overnight incubation at 4ºC and PEG 6000 as separating agent. Relative potencies were calculated from the median effective doses (ED_{50}).

Stokes Radius determination: The gel filtration technique described by Martenson (19) was used for molecular radius determination. Values of the frictional Stokes Radius (R_e) were calculated from literature values (19-21) of the diffusion coefficient ($D_{20,w}$) by the equation $R_e = kT/6\pi\eta D_{20,w}$ where k is the Boltzmann constant (1.36231×10^{-16} erg/degree), T the absolute temperature (293.16) and η the viscosity of the medium (0.010 poise).

Stokes Radii (indicated in parentheses) for the standard proteins and hGH were calculated from the following $D_{20,w}$ values: cytochrome C (16.5 Å) 13×10^{-7} $cm^2 s^{-1}$; myoglobin (19.0 Å) 11.3×10^{-7} cm^2/s; soybean trypsin inhibitor (22.6 Å) 9.4×10^{-7} cm^2/s; ovalbumin (28.1 Å) 7.63×10^{-7} cm^2/s; bovine serum albumin monomer (34.8 Å) 6.15×10^{-7} cm^2/s; hGH (23.9 Å) 8.88×10^{-7} cm^2/s.

The calibration curve, $K_d^{1/3} = \alpha - \beta R_e$, was obtained in 0.05 M phosphate buffer, pH 7.4, from triplicate runs of 2-4 mg of each protein standard (Fig.1) dissolved in 1-1.5 ml of buffer, together with 1 mg of dextran blue to indicate the void volume (V_o) and about 10^7 cpm of 125-I in carrier 127-I for the total volume (V_t) determination. The distribution coefficient (K_d) was calculated as $K_d = (V_e - V_o)/(V_t - V_o)$ where V_e is the elution volume of the protein under study.

RESULTS

Fig.2 presents a typical Sephadex G-100 chromatogram of I-hGH purification and Stokes Radius determination, together with the protein profile of the cold preparation used for the labelling and run simultaneously on the same column. Considering the 67.3 % labelling yield, the average iodination degree was, in

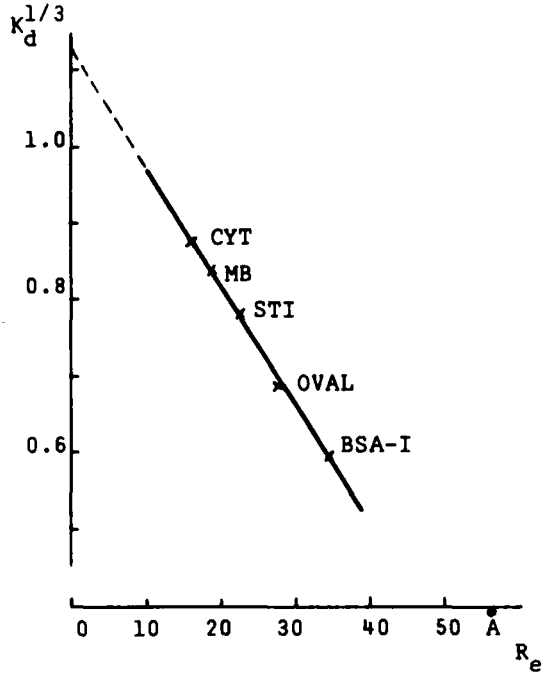

Fig.1 Calibration curve ($K_d^{1/3} = 1.127 - 0.01546\ R_e$ r=-0.9975) obtained with a 1.3x85 cm Sephadex G-100 column, in phosphate buffer 0.05 M, pH 7.4.
Standard proteins: BSA-I, bovine serum albumin monomer; OVAL, ovalbumin; STI, soybean trypsin inhibitor; MB, myoglobin; CYT, cytochrome C.

this case, 2.8 atoms/molecule of hGH. In the experiments reported here, the amount of ^{125}I incorporated into the protein was between 8 and 11 μCi/ml of purified product, which is about the same amount of radioactivity per unit volume as in our regular (^{125}I)hGH preparations. In our regular preparations, however, the hGH is protected by the presence of a large amount of bovine serum albumin carrier (1 mg/ml), while in these macroiodinations the solution contains only hGH (50 μg/ml).

The Stokes Radius determination (five separate runs) for native hGH (3rd. standard-IPEN) provided an experimental value of 22.24±0.30 Å. Based on Student's t test (22), applied to triplicate determinations for ovalbumin (K_d=0.317±0.010) and myoglobin

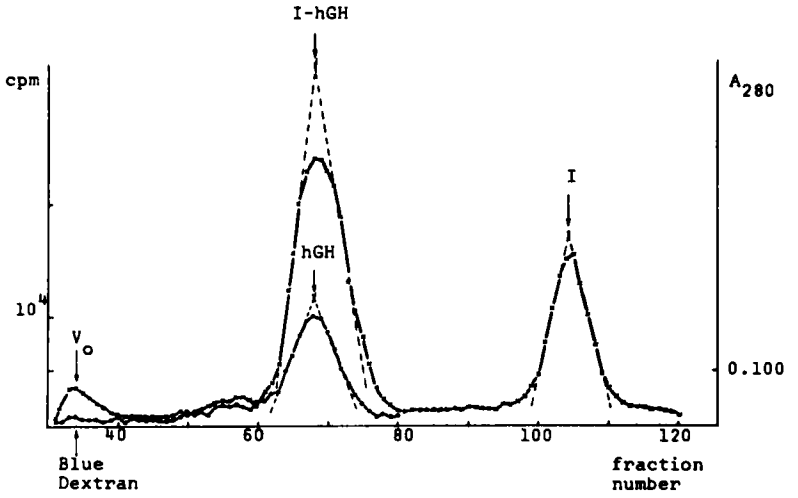

Fig.2 Sephadex G-100 chromatogram of the hGH labelling mixture (127-I-hGH + traces of 125-I-hGH) run together with a sample of the original preparation used for the labelling. Column size 1.3x85 cm, flow rate 6 ml/hr, fraction volume 1.1 ml.
——x—— radioactivity profile
——•—— protein profile
K_d of hGH=0.482 K_d of I-hGH=0.489

(K_d=0.581±0.005), this chromatographic technique was found sensitive enough to detect an increase of more than 2.2% and a decrease of more than 1.2% in molecular radius. Iodinated (^{125}I + ^{127}I) hGH, chromatographed three times on the same Sephadex G-100 column, provided an average R_e= 22.16±0.27 Å, not significantly different from the native hGH value (P> 0.1).

In Table I, we report the relative potencies (95 % fiducial limits) and statistical parameters for the three experiments used for bioactivity determination, with iodination degrees between 1.9 and 2.7 atoms/molecule. Two statistical tests (23), based on the variance ratio "F", indicated no significant difference in potency, as well as no significant departure from parallelism (slope divergence) between native and iodinated hGH.

Fig.3 compares the radioimmunoassay curves and relative potency of hGH exposed to the labelling reagents in the absence of iodine (false labelled) and iodinated to a low (I.D.= 0.8) or

TABLE I. 2X2 FACTORIAL BIOASSAYS OF I-hGH DETERMINED AGAINST THE ORIGINAL hGH PREPARATION

Experiment No.	Average Iodination Degree (atoms/mol.)	Relative Potency of I-hGH	95% Fid. Limits	Index of Precision (λ)	Combined Slope	Preparations Difference$_2$ (F-test) *^{2}	Slope Divergence$_2$ (F-test)*^{2}
1*^{1}	1.9	1.21	n.c.	0.638	4.58	0.17	260×10^{-3}
2	2.7	0.86	0.65-1.10	0.164	12.20	1.60	292×10^{-2}
3	2.6	0.88	0.52-1.31	0.254	12.70	0.50	0.6×10^{-3}

*^{1} 5-day assay

*^{2} the difference is not significant ($P > 0.05$) when $F < 4.11$

n.c. = not calculated

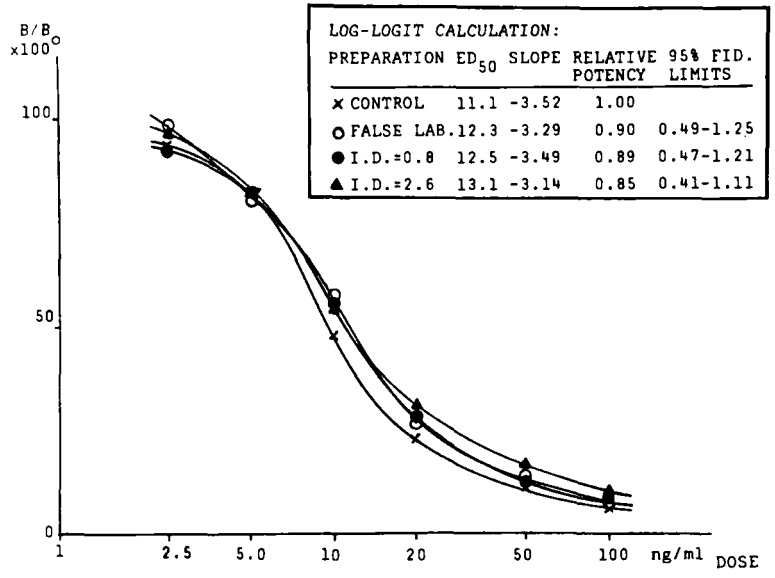

Fig.3 Influence of the labelling reagents, of iodination and of secondary radiation effects on the immunological activity of hGH. Each curve is the result of 3 assays. The index of precision (λ) for the three potency determinations was between 0.100 and 0.105.

high (I.D.= 2.6) degree to those of the original preparation used for the labelling (control).

DISCUSSION

The present work shows that relatively high concentrations of Chloramine T and sodium metabisulfite, high iodination degrees (up to 2.7 atoms/molecule), and indirect radiation effects do not significantly alter the biological and immunological activity of the hGH molecule or its Stokes Radius, as determined by gel filtration.

Our data, based on measurements of the growth-promoting activity of strongly iodinated hGH molecules, suggest a greater retention of the bioactivity than reported by Hughes et al.(12) and are thus in agreement with the results obtained by Mattera et al.

(13) for bovine growth hormone and by Goodman et al.(16) with their "in vitro" bioassays.

The gel filtration technique has proved to be a very useful and sensitive tool for detecting small variations in molecular radius. The experimental value of 22.24 Å is in perfect agreement with the data of Ryan (24) and in fairly good agreement with the theoretical frictional Stokes Radius of 23.9 Å which we calculated from the diffusion coefficient of hGH, especially considering the influence tha pH and ionic strength can have on this determination (19,20).

Since an insignificant fraction of the total hGH was labeled with ^{125}I, the present work provide no information about direct, intramolecular radiation effects, producing a "decay catastrophe" (5), probably due to coulombic explosion (25). Considering that the present tendency is to prepare (^{125}I)hGH with a moderate specific activity (~50 Ci/g; I.D.~0.5 atoms/molecule), where only about 8% of the molecules should be polyiodinated (26), we estimate that, using the tracer within 15 days of the labelling, the percentage of still labelled molecules that had suffered a decay catastrophe (mostly ^{125}I-monoiodo decay products) should be less than 3% of all the growth hormone molecules and less than 9% of all radioiodinated molecules (27). Moreover it has not yet been demonstrated to what extent the immuno- and biological activity of a large protein molecule can be impaired by this decay catastrophe (25). Such studies are currently in progress in our laboratory.

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