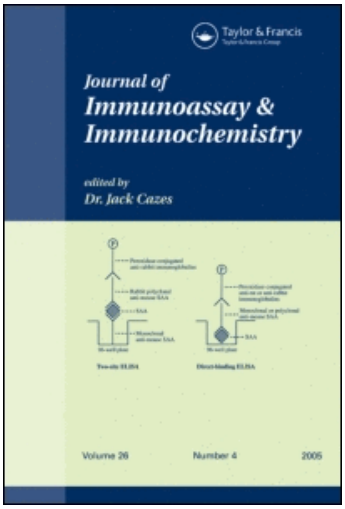

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Mohammed-all, S. A. A. , Salacinski, P. R. and Landon, J.(1981) 'Effect of Temperature on the Radioiodination of Human Growth Hormone', Journal of Immunoassay and Immunochemistry, 2: 3, 175 — 186
To link to this Article: DOI: 10.1080/15321818108056976
URL: http://dx.doi.org/10.1080/15321818108056976

JOURNAL OF IMMUNOASSAY, 2(3&4), 175-186 (1981)

EFFECT OF TEMPERATURE ON THE RADIOIODINATION OF HUMAN GROWTH HORMONE

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ABSTRACT

Studies have been undertaken to assess the effect of altering the temperature at which human growth hormone is radioiodinated on the incorporation of ^{125}Iodine and the immunoreactivity and stability of the labelled hormone. Employing highly purified monomeric hormone it proved possible, by the iodogen procedure, to prepare a labelled product of high specific activity irrespective of temperature. However, in radioiodinations performed at ambient temperature (20 to 25°) significant amounts of the labelled hormone were in an aggregated form which was less immunoreactive than the ^{125}Iodine-labelled monomeric hormone. Such aggregation was largely prevented by radioiodinating at low temperature (0 to 4°) and even the large monomeric peak was more immunoreactive (about 95% bound in antibody excess) than the monomeric peak from iodinations performed at room temperature (maximum binding 87%, or less).

INTRODUCTION

All radioimmunoassays (RIA) employ antigen labelled with a radionuclide as tracer and a successful RIA depends on the quality of the tracer [1]. Thus, use of a labelled antigen of different immunoreactivity than that of the unlabelled antigen in the samples and standards generally results in loss of sensitivity [2] and impaired precision [3]. ^{125}Iodine is, in general, the radionuclide of choice and human growth hormone (hGH) was used for the development of the highly successful chloramine T radioiodination procedure [4,5]. Several alternative radioiodination procedures

have since been introduced including an electrolytic technique [6], use of the enzyme, lactoperoxidase [7], the iodogen method [8] and indirect labelling [9].

An ideal radioiodination procedure would ensure maximum incorporation of the radionuclide and, thereby, a labelled product of high specific activity; the immunoreactivity of the labelled antigen would be unimpaired; and it would be stable throughout several half-lives of the isotope. While there have been few problems in obtaining ^{125}I-hGH (and other proteins and glycoproteins) of high specific activity, radioiodination is often associated with some loss of immunoreactivity and the useful life of the product is usually limited to a few weeks.

The precise causes of the loss of immunoreactivity associated with labelling remain uncertain [10]. Some proteins are so unstable that they may become seriously damaged in the dilute, protein-free solution essential for labelling. They may sustain damage by contact with noxious substances present in a commercially-available radioactive iodide preparation, or with the oxidising agent or due to gamma irradiation during the radioiodination or during the subsequent reduction step. In addition, the incorporation of iodine atoms may alter the immunochemical properties of a protein [11]. Such damage must be distinguished from the production of labelled product with impaired immunoreactivity as a result of the protein being impure, aggregated or damaged in some other way prior to radioiodination. It must also be distinguished from that occuring during storage, upon which the stability of the labelled product depends.

This repord describes a simple procedure for the production of undamaged, aggregate free ^{125}I-hGH using the iodogen method, with especial reference to the importance of the temperature at which the radioiodinations were performed.

MATERIALS

1, 3, 4, 6-Tetrachloro-3α, 6α-diphenyl glycol uril (iodogen) was from Pierce and Warriner, Chester, Cheshire, U.K.; carrier-free Na^{125}I

in NaOH (100 mCi/ml) from Amersham International, Amersham, Bucks, U.K.; iodination vials (polypropylene tubes, No 690) from Sarstaedt, Leicester, U.K.; Sephadex G-100 from Pharmacia, Hounslow, Middlesex, U.K.; chromatography columns from Whatman, Maidstone, Kent, U.K.; bovine serum albumin (BSA) from Sigma, Poole, Dorset, U.K.; and polyethylene glycol (PEG) 6000 and other Analer grade chemicals from BDH Chemicals, Poole, Dorset, U.K.

Sheep anti-hGH serum, donkey anti-sheep immunoglobulin G serum (second antibody) and pooled normal human serum with an hGH content of less than 2 ng/ml, were from ILS, 14-15 Newbury Street, London EC1, U.K. The highly purified hGH used had been prepared in the Department by the method described by Jones and his colleagues [12] and comprised the monomeric peak which had then been freeze-dried in 5 mg amounts.

METHODS

Repurification of hGH

Working at 0 to 4° throughout, 5 mg of hGH was dissolved in 250 µl of phosphate buffer (50 mmol/l, pH 7.4) and loaded onto a column of Sephadex G-100 (1 x 36 cm) previously equilibrated with the same buffer containing 1 g/l BSA and then washed with phosphate buffer alone. The column was eluted with the phosphate buffer at 3 ml/h and 0.5 ml fractions collected. The protein content of the fractions was monitored by measurement of optical density at 280 nm, those corresponding to the homogeneous monomeric hGH peak pooled and the concentration of the hormone determined. This pool was divided into small aliquots and stored frozen at -20°.

Radioiodination of hGH

Iodination vials containing a film of 2 µg of iodogen were prepared by drying 50 µl of a 40 mg/l solution in dichloromethane in each vial. All radioiodinations were performed by the sequential addition of 20 µl of phosphate buffer (500 mmol/l, pH 7.4), 10 µg of the repurified hGH in 10 µl of phosphate buffer (50 mmol/l, pH 7.4) and 10 µl (1 mCi) of $Na^{125}I$

to an iodogen-coated vial, followed by gentle mixing. After 10 min, a further 210 µl of phosphate buffer (50 mmol/l, pH 7.4) was added and the total radioactivity of the iodination mixture determined. The reaction mixture was then loaded onto a Sephadex G-100 column (1 x 36 cm), previously equilibrated with sodium phosphate buffer (50 mmol/l, pH 7.4) containing 1 g/l BSA and 1 g/l sodium azide, and eluted at 3 ml/h with this buffer. Fractions (0.5 ml) were collected and the radioactivity of the ^{125}I-hGH determined.

A total of seven radioiodinations, and subsequent purification of the ^{125}I-hGH, were performed at 0 to 4° in the cold room. On each occasion a second radioiodination and purification was performed at ambient temperature (20 to 25°) using the same batches of reactants. One further radioiodination and purification was carried out at 40°.

Repurification of ^{125}I-hGH

One batch of ^{125}I-hGH containing 0.45 µg in 750 µl, which had been prepared six months previously at 0 to 4°, was repurified in the cold on a Sephadex G-100 column as described above, except that 1 ml fractions were collected - to prevent excessive counting times.

Assessment of the Immunoreactivity of ^{125}I-hGH

Sodium phosphate buffer (50 mmol/l, pH 7.4) containing BSA (5 g/l) and sodium azide (1 g/l) was used throughout.

The immunoreactivity of the ^{125}I-hGH in each fraction from a column was assessed by the amount bound in antiserum excess. Human serum (50 µl), 100 µl of a 1:100 dilution of each fraction and 100 µl of a 1:10,000 dilution of sheep anti-hGH serum were incubated at ambient temperature overnight. The antibody bound ^{125}I-hGH was then precipitated by addition of 100 µl of a 1:40 dilution of donkey second antibody followed, 2 h later, by 1 ml of 5.4% PEG in sodium phosphate buffer (50 mmol/l, pH 7.4) and centrifugation. The supernate was aspirated to waste and the radioactivity in the precipitate counted. Non-specific binding of each fraction

was also determined in the same way except that the antiserum was replaced by 100 µl of buffer.

Aliquots from the pools of each peak of ^{125}I-hGH were assessed by means of antiserum dilution and standard curves. The antiserum dilution curves were constructed by incubating 100 µl of tracer (1 ng) with 50 µl of human serum and 100 µl of doubling dilutions of the antiserum in assay buffer. After overnight incubation, the antibody bound and free fraction were separated as above. The standards comprised highly purified hGH in normal human serum and 50 µl of each was preincubated with 100 µl of a dilution of antiserum which would bind about 50% of the labelled hormone in the absence of unlabelled hGH. After 60 min, 100 µl of tracer (1 ng) was added and, after further incubation, separation achieved as above.

RESULTS

Repurification of hGH

Column chromatography of the freeze-dried hGH revealed a peak in fractions 20 to 25, comprising about 10% of the total, thought to represent aggregates. A second, in fractions 27 to 30, comprised about 18% and probably represented dimers of the hormone. About 72% eluted in the third monomeric peak. Aliquots of the latter, kept frozen at -20° over a 13 month period, eluted as a single monomeric peak when rechromatographed.

Radioiodination of hGH

Figure 1a illustrates the profile obtained when the reaction mixture from a radioiodination performed at 0 to 4° was chromatographed and Figure 1b that from a radioiodination performed on the same day at 20 to 25°. The results of all 14 radioiodinations are summarised in Table 1.

The percentage incorporation of ^{125}Iodine and, therefore, the specific activity of the ^{125}I-hGH was similar irrespective of temperature. Following radioiodination at low temperature, nearly 90% of the labelled hormone was in the monomeric peak, about 95% of which was bound in

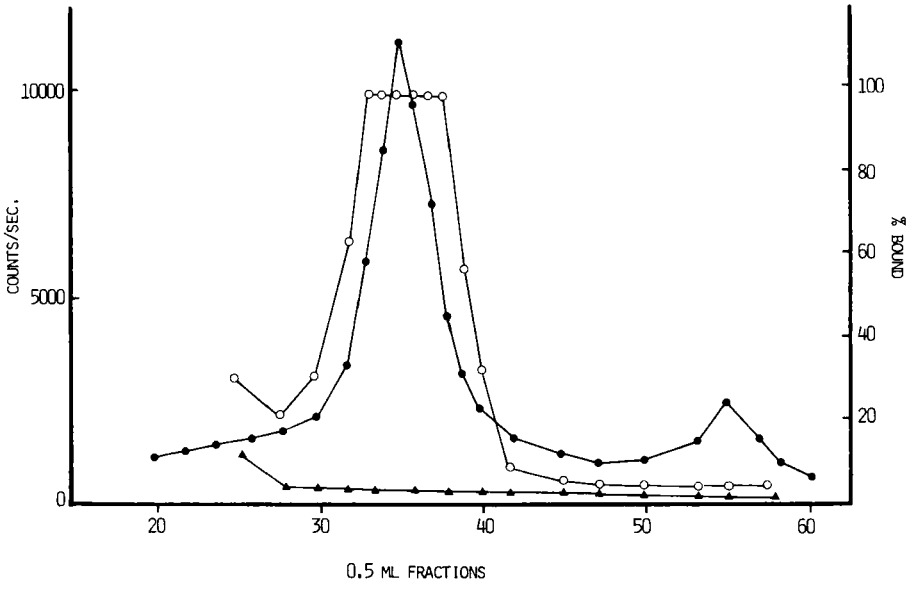

Figure 1 A. Sephadex G-100 column chromatography of ^{125}I-hGH prepared at 0 to 4°, showing the radioactivity of the fractions (●——●), and maximum binding (o——o) and non-specific binding (▲——▲) of the labelled hormone in the various fractions.

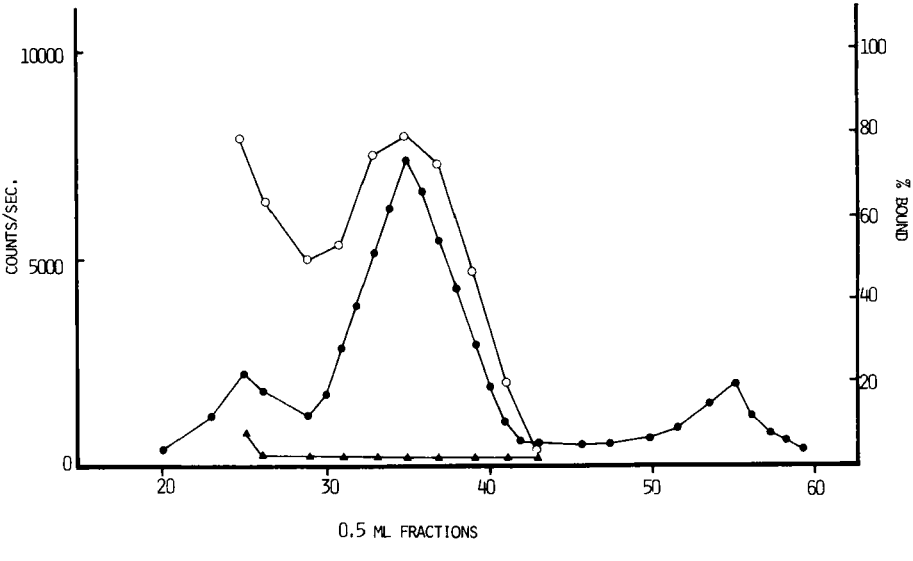

Figure 1 B. As above other than that the ^{125}I-hGH was prepared at 20 to 25°.

TABLE 1

Comparison of Seven Radioiodinations of hGH Performed at 0 to 4° and seven at 20 to 25°

Parameter	Radioiodination at 0 to 4°	Radioiodination at 20 to 25°C
Percent incorporation of ^{125}Iodine	83.5 (81 to 85)*	82.3 (80 to 85)
Specific activity (μCi/μg)	83.5 (81 to 85)	82.3 (80 to 85)
Percentage in monomeric form	87.7 (84 to 91)	68.8 (62 to 75)
Maximum binding (%)	95 (93 to 97)	84 (82 to 87)
Non-specific binding (%)	3.2 (2.5 to 3.8)	4.6 (4.0 to 5.5)

* Mean (range)

antibody excess while non-specific binding was less than 4%. However, following radioiodination at 20 to 25°, an additional peak due to aggregated labelled hormone was apparent, less than 75% was in the monomeric form and maximum binding of the ^{125}I-hGH in the latter peak was less while non-specific binding was greater. The final peak of radioactivity eluted from the columns was not immunoreactive and ran in the same position as unreacted ^{125}Iodine.

Column chromatography of the reaction mixture following the single radioiodination performed at 40° showed that more than 50% of the ^{125}I-hGH was in the aggregated form. Repurifying an aliquot of the monomeric labelled hormone, which had been prepared six months previously at 0 to 4°, showed three peaks of which the first was aggregate, the second monomer and the third degraded fragments of ^{125}I-hGH (Figure 2).

Assessment of the Immunoreactivity of ^{125}I-hGH

The immunoreactivity of the labelled hormone from the monomeric peaks was considerably greater than that of the ^{125}I-hGH from the peaks

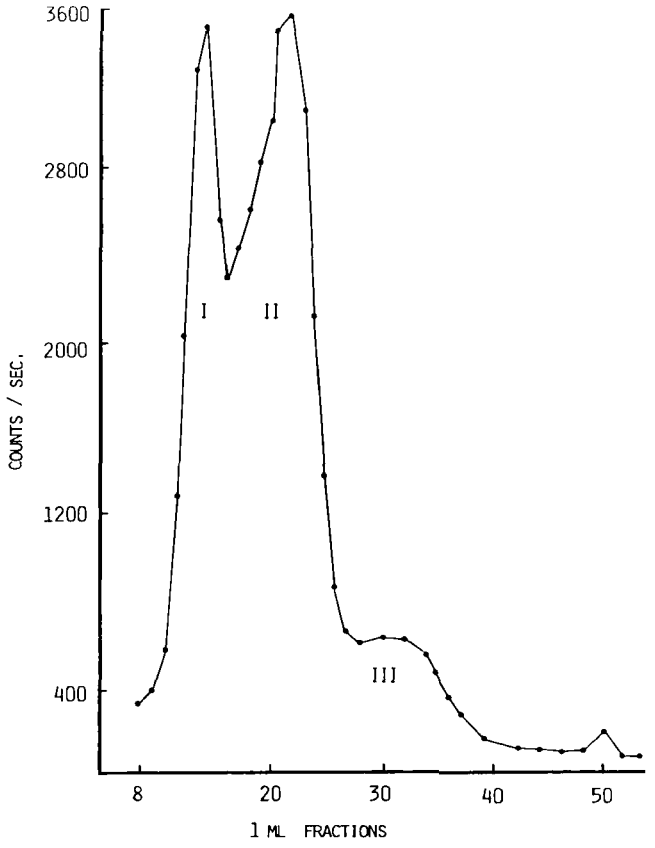

Figure 2. Sephadex G-100 column chromatography of ^{125}I-hGH prepared six months previously at 0 to 4° and stored deep-frozen. There are three peaks of radioactivity of which I represents aggregated labelled hormone, II is the monomeric peak and III is degraded ^{125}I-hGH.

of aggregated material. This was evidenced by antiserum dilution curves in which binding was always greater and the slope of the curve steeper with the monomeric form. Of especial note, the standard curve obtained using ^{125}I-hGH from the monomeric peak of radioiodinations performed at 0 to 4° was always more sensitive than with that from the monomeric peak of those carried out at the higher temperature (Figure 3). Nonetheless, both were suitable for assay purposes.

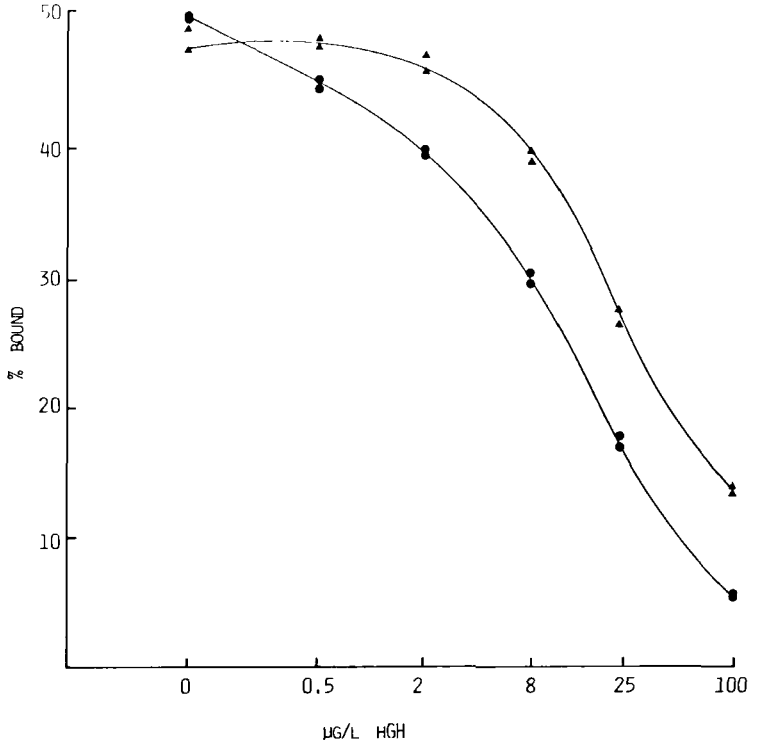

Figure 3. Standard curves for hGH prepared using 1 ng of either ^{125}I-hGH prepared at 0 to 4° (●——●) or at 20 to 25° (▲——▲).

Following storage for more than about five weeks in the case of monomeric ^{125}I-hGH prepared at 20 to 25° and over about eight weeks for that prepared at the lower temperature, the tracers were no longer suitable for assay purposes. Their immunoreactivity, as determined by maximum binding in antibody excess, continued to decline at a similar rate. However, even after six months, the monomeric peak obtained by repurification proved a suitable tracer for assay purposes apart, of course, from the unacceptably long counting time required.

DISCUSSION

^{125}I-hGH may be unsuitable for use in RIA because the initial hormone preparation was impure or damaged or as a result of the radioiodination

procedure or because of subsequent damage occuring during storage. This study illustrates the need for careful assessment prior to use and, if necessary, a repurification step. Thus highly purified, monomeric hGH had been prepared which, kept frozen, was stable for more than a year. However, its lyophilisation had resulted in significant aggregation, which necessitated column chromatography.

The main points to emerge from this study concerning radioiodination damage of hGH are that this appears to reflect aggregation (rather than fragmentation) and, as suggested many years ago by Hughes [13], is not necessarily due to the radioiodination. Thus, using the relatively gentle iodogen technique [8, 14], the temperature at which the reaction was performed played a major role in determining whether or not aggregation and loss of immunoreactivity occurred. It is also apparent that more subtle damage of the labelled hormone was produced than that shown by aggregation. Thus the standard curves obtained using ^{125}I-hGH from the monomeric peak of iodinations performed at 0 to 4° were more sensitive than those using labelled hormone from the monomeric peak following radioiodinations at ambient temperature.

Damage becoming apparent during storage of monomeric ^{125}I-hGH differs from that found immediately after radioiodination in that there was a peak of radioactivity representing fragments of the hormone as well as an aggregate peak, noted previously by Sönksen and West [15]. The rate at which storage damage occurred was not significantly influenced by the temperature at which the radioiodination had been performed and was a consequence of iodination - since unlabelled hGH is stable when stored in the same way, at -20°, and the damage is unlikely to be due to the bovine serum present in the buffer [16]. Such damage differs from that occurring during radioiodination and recent studies indicate the need for including a free radical scavenger, such as methanol, to minimise storage damage [17].

It remains to be seen whether performing radioiodinations at 0 to 4° may be of benefit in respect of other proteins.

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