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The Preparation of ^{131}I-Labelled Human Growth Hormone of High Specific Radioactivity

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The radio-immunoelectrophoretic technique for the assay of insulin in human sera developed by Yalow & Berson (1960) has been applied to glucagon (Unger *et al.* 1959) and to growth hormone (Hunter & Greenwood, 1962*b*). The ability of ^{131}I-labelled hormones to compete with unlabelled hormones for antibody to the unlabelled hormone has also been utilized in assays based on the precipitation of antigen–antibody complex for glucagon (Grodsky, Hayashida, Peng & Geschwind, 1961) and for human growth hormone (Utiger, Parker & Daughaday, 1962). Immunoelectrophoretic procedures require the routine preparation of small amounts of ^{131}I-labelled hormones of very high specific radioactivity. The method used successfully for insulin by Yalow & Berson (1960) requires the handling of 30–80 mc of [^{131}I]iodide in volatile solvents. Samols & Williams (1961) have used 10–20 mc of [^{131}I]iodine monochloride (McFarlane, 1958) for the labelling of insulin but no experimental details are available. Human growth hormone has been iodinated by Utiger *et al.* (1962) by using modifications of the method of Yalow & Berson (1960) and Staub, Springs & Elrick (1957). They report specific radioactivities of up to 5 μc/μg., with 0·25–2·0 atoms of total iodine/molecule, by using 10–20 mc of carrier-free sodium [^{131}I]iodide and 0·2–1·0 mg. of human growth hormone. This level of specific radioactivity, though adequate for methods based on precipitation, is inadequate for the immunoelectrophoretic technique.

The following method has been developed to allow the rapid preparation of 1·5–2·5 μg. of ^{131}I-labelled human growth hormone with specific radioactivities of 250–590 μc/μg. (60–75% efficiency) by using 2–4 mc of carrier-free sodium [^{131}I]iodide and 5 μg. of human growth hormone. Chloramine-T is used as the oxidizing agent. The ^{131}I-labelled hormone is calculated to contain up to 1·2 atoms of iodine/molecule.

In these preparations there are no discrete immunologically inactive components. With specific radioactivities of up to 300 μc/μg. the preparations are quantitatively identical with the unlabelled hormone as measured by an immunoelectrophoretic method (Hunter & Greenwood, 1962*b*). At 500 and 750 μc/μg. there is a progressive loss of affinity for the rabbit anti-(human growth hormone) serum.

A preliminary report of the method has been published (Hunter & Greenwood, 1962*a*).

MATERIALS

Buffers. All buffers were sodium salts.

Human growth hormone. Human growth hormone (Raben, 1959) is referred to as growth hormone. Solutions for iodination were made by adding growth hormone (5 mg.)

to water (1 ml.), with stirring of the suspension, followed by the addition of 1 N-NaOH (0·1 ml.). The clear solution was diluted to 200 μg./ml. with 0·05 M-phosphate buffer, pH 7·5, and samples were stored at $-20°$ for up to 2 months. Initially a gift of human growth hormone from the Clinical Endocrinology Committee of the Medical Research Council was used. Batches of growth hormone were then prepared by the method of Raben (1959) by using acetone-dried pituitaries. These were kindly collected by Dr J. Clark, New Sussex Hospital, Brighton. Both of these preparations were subsequently shown to contain an immunologically inert component which was removed by gel-filtration on Sephadex G-200 in 0·05 M-borate–0·05 M-KCl buffer, pH 8·6. Preliminary experiments with growth hormone batches 13 and 14 kindly supplied by Dr M. S. Raben have not shown the presence of this component.

[^{131}I]*Iodide.* Sodium [^{131}I]iodide (IBS-3) was obtained from The Radiochemical Centre, Amersham, and is the distillate from the target material collected in 0·02–0·04 N-NaOH. The material is specified as being between pH 8 and 11, and is thiosulphate-free and carrier-free, with an isotope abundance of not less than 90%.

Albumin. Crystalline bovine plasma albumin was a commercial sample (Armour). Human serum albumin (AP 3) was a gift from the Blood Products Laboratory, The Lister Institute of Preventive Medicine.

Sephadex G-50. This was obtained from Pharmacia, Uppsala, Sweden.

METHOD

Preparation of ^{131}I-labelled growth hormone. Reaction is carried out in the rubber-capped vial (10 ml.) in which the [^{131}I]iodide sample is packaged. Care is taken that the vial is kept upright in transport by requesting delivery in a 1 in. lead pot. This ensures that the small volume (0·05 ml.) of [^{131}I]iodide solution is not spread over the surface of the vial and its cap.

The radioactivity is counted by using a Panax scintillation counter (USC-B) with a well-type sodium iodide crystal with the geometry modified such that 2 mc in 0·05 ml. gives approx. 400 counts/sec. The lid of the lead castle is removed and the sample, in a 1 in. lead pot, placed 30 cm. above the aperture. The rubber cap is removed after the preparation is completed, and counted separately, with the same geometry.

To the sample is added 0·025 ml. of 0·5 M-phosphate buffer, pH 7·5. Immediately thereafter growth hormone (5 μg.) followed by fresh chloramine-T (100 μg.), each in 0·025 ml. of 0·05 M-phosphate buffer, pH 7·5, are added. After each addition, made by injection through the rubber cap with an Agla micrometer syringe. the contents of the vial are briefly mixed. Immediately after mixing the chloramine-T, sodium metabisulphite is added [0·1 ml. of a solution (2·4 mg./ml.) in 0·05 M-phosphate buffer, pH 7·5]. This addition prevents any subsequent iodination of the Sephadex-gel bed by converting [^{131}I]iodine into [^{131}I]-iodide. This residual iodide is diluted with carrier KI [0·2 ml. of a solution (10 mg./ml.) in the 0·05 M-phosphate buffer]. The reaction mixture is transferred to a Sephadex column followed by a single wash with further KI [0·4 ml. of a solution (10 mg./ml.) in the 0·05 M-phosphate buffer]. The reaction vial and pipette are then counted.

Separation of ^{131}I-labelled growth hormone from the reaction mixture is carried out by gel-filtration with a column (10 cm. × 1 cm.; 1 g.) of Sephadex G-50 (Fig. 1). Equilibration of the gel and elution from it is carried out with 0·07 M-barbitone buffer, pH 8·6. Before the Sephadex column is used crystalline bovine plasma albumin (20 mg. in 1 ml. of pH 8·6 buffer) is passed through it, followed by a 20 ml. wash with the same buffer. This 'presaturation' of the Sephadex with albumin allows a subsequent recovery of 70% of 3–4 μg. of ^{131}I-labelled hormone from the Sephadex.

The elution pattern, determined by counting 1 ml. eluates, is reproducible and allows for a routine collection of a non-radioactive discard fraction (1·5 ml.), a protein peak (4·0 ml.) and a salt peak (three 5 ml. fractions) into calibrated counting vials. The protein peak is collected into a vial containing bovine plasma albumin [1·0 ml. of a solution (50 mg./ml.) in 0·07 M-barbitone buffer, pH 8·6]. After the counting of each fraction the amount of ^{131}I-labelled hormone eluted is calculated and a sample diluted as required for the assay with 0·07 M-barbitone buffer, pH 8·6, containing bovine plasma albumin (0·5 mg./ml.).

The time taken from the mixing of the hormone and [^{131}I]iodide to the loading of the column is about 4 min. Within a further 15 min. the ^{131}I-labelled hormone is eluted, freed of unchanged [^{131}I]iodide and other low-molecular-weight additions. The counting schedule of a typical preparation is shown in Table 1. In control experiments in which 0·025 ml. of buffer is substituted for the

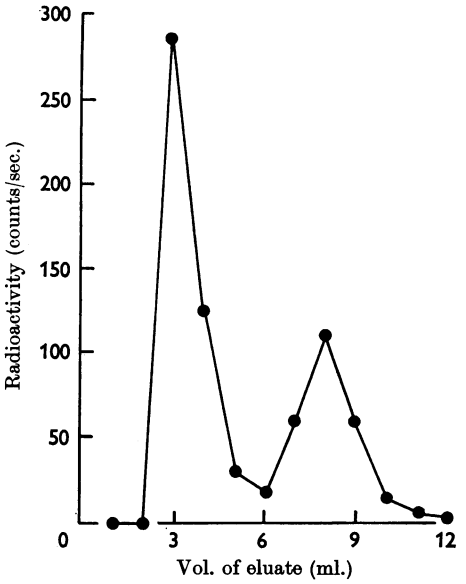

Fig. 1. Separation of ^{131}I-labelled growth hormone from [^{131}I]iodide on Sephadex G-50. The separation was carried out by gel-filtration of an iodination reaction mixture containing ^{131}I-labelled growth hormone (3·4 μg.) of specific radioactivity 590 μc/μg., sodium metabisulphite (240 μg.), chloramine-T (100 μg.), and KI (2 mg.) and approx. 1 mc of [^{131}I]iodide in a volume of 0·4 ml. The column (1 g.) of Sephadex G-50 was equilibrated with 0·07 M-barbitone buffer, pH 8·6, and presaturated with crystalline bovine plasma albumin (20 mg.). Unretarded iodinated protein was eluted first, followed by a smaller [^{131}I]iodide peak.

growth hormone solution, 96% of the total [^{131}I]iodide is recovered in the salt fractions. The calculation of the yield, specific radioactivity and distribution of ^{131}I-labelled protein through the procedure is based on this quantitative recovery of unchanged [^{131}I]iodide.

In the preparation shown in Table 1, the total radioactivity of the salt peak (106 counts/sec.) represents all of the unchanged [^{131}I]iodide, since [^{131}I]iodide is recovered quantitatively in the salt peak in the absence of added protein. The remaining radioactivity (405 − 106 = 299 counts/sec.) represents ^{131}I transferred to 5 μg. of growth hormone. The specific radioactivity is thus 2 × 299/405 mc/5 μg. (i.e. 295 μc/μg.): a percentage transfer of 73·8% of the initial [^{131}I]iodide.

The total ^{131}I-labelled growth hormone radioactivity of 299 counts/sec. (5 μg.) is made up of 87 counts/sec. (1·45 μg.) adsorbed to the vial, 51 counts/sec. (0·85 μg.) adsorbed to the pipette tip, 105 counts/sec. (1·75 μg.) eluted from the Sephadex and 56 counts/sec. (0·93 μg.) adsorbed to the Sephadex. The percentage recovery of ^{131}I-labelled hormone added to the Sephadex is 65·2%; the overall yield of hormone is 35·1%.

Table 1. *Counting schedule in the preparation of ^{131}I-labelled growth hormone*

Details are given in the text. All samples were counted for 100 sec. and corrected for dead-time and background.

		Radioactivity (counts/sec.)
Sodium [^{131}I]iodide (2·0 mc) in vial		486
Rubber cap		2
[^{131}I]Iodine in reaction mixture (484 counts/sec.)		405*
Reaction vial after KI wash	87 counts/sec.*	
Pipette tip after wash	51 counts/sec.	
Column load (calc.)	267 counts/sec.	

		Radioactivity (counts/sec.)	
	Discard fraction (1·5 ml.)	0	
	Protein peak (4·0 ml.)	105	
Represents quantitative recovery of all unchanged [^{131}I]iodide	Salt peak (5 ml.)	87	106
	Salt peak (5 ml.)	14	
	Salt peak (5 ml.)	5	
	Retained on column	56	

Counts associated with protein: total counts reacted − unchanged [^{131}I]iodide recovered in the salt peak = (405 − 106) = 299 counts/sec.

Distribution of radioactivity of reaction mixture (405 counts/sec.)

	% of total	% of protein	μg. of protein equivalent
Vial	21·5	29·1	1·45
Pipette	12·6	17·1	0·85
Protein	25·9	35·1	1·75
Salt	26·2		
Column (by difference)	13·8	18·7	0·93
Total	100·0	100·0	4·98

* Corrected for volume.

The ^{131}I-labelled hormone, of specific radioactivity 295 μc/μg., contains 0·53 atom of ^{131}I/molecule of growth hormone (mol.wt. 29000; Squire & Pedersen, 1961). Since the isotope abundance of ^{131}I in IBS-3 is not less than 90%, the ^{131}I-labelled hormone is then calculated to contain 0·53–0·59 atom of total iodine/molecule.

The calculations of specific radioactivity etc. assume that the specific radioactivities of the ^{131}I-labelled growth hormone adsorbed to the reaction vial and to the Sephadex are the same as that eluted from the column. It has not been possible to verify this assumption experimentally.

The slight trailing of protein into the salt peak of a Sephadex column (Fig. 2) was determined experimentally by using unlabelled hormone (4 μg.) together with a trace of salt-free ^{131}I-labelled growth hormone. It was then possible to calculate that the observed trailing at this level could be corrected for by increasing the calculated percentage efficiencies of transfer of ^{131}I and specific radioactivities, as shown above, by 5%. It was considered that a correction factor of this order could be disregarded.

RESULTS

Adsorption of radioactivity to Sephadex G-50 columns

The separation of 4 μg. of ^{131}I-labelled protein from approx. 0·005 μg. of unchanged [^{131}I]iodide presents problems of adsorption either by dialysis

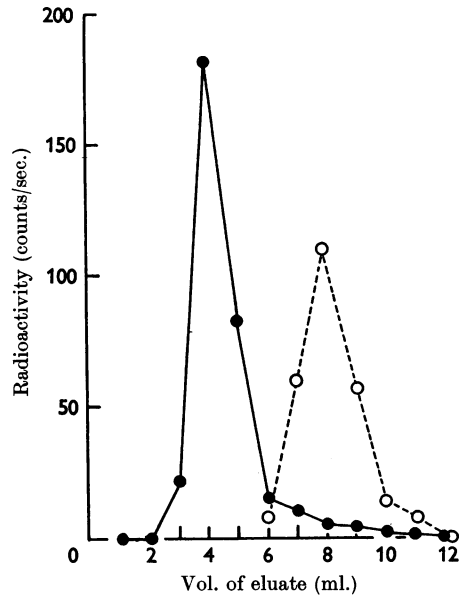

Fig. 2. Determination of trailing of ^{131}I-labelled growth hormone on Sephadex G-50. ●, Gel-filtration of ^{131}I-labelled growth hormone (trace) with growth hormone (4 μg.) was carried out. Equilibration and elution were with 0·07 M-barbitone buffer, pH 8·6; the column (1 g.) was presaturated with crystalline bovine plasma albumin (20 mg.). ○, Elution pattern of [^{131}I]iodide and KI (2 mg.) in 0·4 ml. volume run separately on the same column.

or column chromatography. The gel-filtration method has the advantage of rapidity and was therefore investigated as the method of choice.

A mixture of sodium [^{131}I]iodide and sodium [^{127}I]iodide (total iodide 0·2 μg.) was recovered quantitatively (94 %) in the salt peak of a Sephadex column by using barbitone buffer, pH 8·6, for equilibration and elution. A similar mixture with chloramine-T added as oxidant was subsequently recovered in the salt peak in only 62 % yield. No activity was found in the protein peak and the residual activity was not eluted with 0·1 N-sodium hydroxide. It was judged that the Sephadex was capable of undergoing iodination since the addition of sodium metabisulphite to an iodination mixture before the column step gave quantitative recovery of the label (94 %) in the salt peak. Under these conditions chloramine-T is not available for the oxidation of sodium [^{131}I]iodide and the iodination of the Sephadex is prevented.

By using these conditions for the quantitative recovery of unchanged [^{131}I]iodide, the adsorption of ^{131}I-labelled growth hormone to Sephadex could be demonstrated. Growth hormone (20 μg.) was iodinated with sodium [^{131}I]iodide (2 mC) by using chloramine-T (100 μg.) as oxidant followed by sodium metabisulphite (120 mg.). Of the radioactive mixture loaded on to the column, 14·4 % of the radioactivity was recovered in the protein peak and 26·6 % in the salt peak. The latter represents all of the non-protein-bound ^{131}I. Of the ^{131}I bound to protein (73·4 % calc.) only 14·4 % was recovered in the protein peak, an 80 % loss at this level of added protein. The following methods, designed to saturate the active binding sites on the Sephadex, were investigated.

Presaturation of the binding sites with unlabelled growth hormone. A preliminary chromatogram was carried out in which unlabelled growth hormone (1 mg.) was put through the Sephadex column and followed by extensive washes with eluting buffer. Growth hormone (20 μg.) was allowed to react with sodium [^{131}I]iodide (2 mC) with the use of chloramine-T followed by the addition of sodium metabisulphite. The recovery of ^{131}I-labelled growth hormone (approx. 80 μC/μg.) from the Sephadex column was increased from 20 % to 57 %. In further experiments the amount of ^{131}I-labelled hormone eluted from the column was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). These showed that, of the unlabelled growth hormone adsorbed by the preliminary treatment, some 10 μg. was subsequently eluted from the Sephadex with the ^{131}I-labelled hormone. This rendered estimates of specific radioactivity based on the recovery of radioactivity invalid.

Addition of diluent protein after iodination. Previous experiences with isotopically labelled human serum albumin suggested that the capacity of Sephadex to bind protein was non-specific. Crystalline bovine plasma albumin (2–5 mg.) was added after sodium metabisulphite in an attempt to decrease the amount of ^{131}I-labelled growth hormone adsorbed. The recovery of 4 μg. of ^{131}I-labelled hormone was increased by this method to 66 % as calculated from radioactivity measurements.

A control experiment without added growth hormone gave an unexpected 5 % of the radioactivity in the protein peak, suggesting some iodination of the albumin despite the addition of sodium metabisulphite.

^{131}I-labelled growth hormone was prepared and bovine plasma albumin added after sodium metabisulphite. The protein peak obtained by subsequent gel-filtration was subjected to electrophoresis in horse serum on cellulose acetate (Kohn, 1958) in 0·035 M-barbitone buffer, pH 8·6. A trail of radioactivity was obtained from the albumin region to a peak of radioactivity in the growth hormone region, and in a single experiment a small but significant peak of radioactivity was found associated with albumin. The results suggested that the bovine plasma albumin added as carrier after sodium metabisulphite, although unlabelled, had an adsorptive capacity for sodium [^{131}I]iodide. The addition of up to 1 mg. of carrier potassium iodide before the bovine albumin was added decreased but did not eliminate the adsorption of sodium [^{131}I]iodide to the albumin.

Presaturation of the Sephadex with bovine plasma albumin. Bovine albumin (20 mg.) in 0·07 M-barbitone buffer, pH 8·6, was passed through a column (1 g.) of Sephadex G-50 and the column was then washed with a further 20 ml. of the equilibrating buffer. This column was used to separate ^{131}I-labelled growth hormone from unchanged sodium [^{131}I]iodide, chloramine-T, sodium metabisulphite and carrier potassium iodide, without the addition of diluent protein. In a series of six experiments the mean recovery of approx. 4 μg. of ^{131}I-labelled growth hormone was 78 %. This method obviates the dilution of ^{131}I-labelled hormone with unlabelled hormone, and the adsorption of sodium [^{131}I]iodide to albumin and was adopted as the routine procedure.

Adsorption of ^{131}I-labelled growth hormone to glass

The recovery of unchanged sodium [^{131}I]iodide from the reaction vial and the column was 94 % (see above). The radioactivity remaining in the vial in the routine preparation of ^{131}I-labelled growth hormone was assumed for the purposes of calculation to be a mixture of free [^{131}I]iodide and protein-bound ^{131}I. However, the percentage left in the

vial increased in direct proportion to the calculated specific radioactivity, suggesting a specific adsorption of the ^{131}I-labelled growth hormone to the glass vial. This was confirmed experimentally in a series of six experiments by adding unlabelled growth hormone (5 μg. in 0·025 ml.) together with a trace of ^{131}I-labelled growth hormone to a reaction vial containing 0·375 ml. of 0·05 M-phosphate buffer, pH 7·5. After mixing, the contents of the vial were discarded and the vial was washed with 0·4 ml. of potassium iodide solution (10 mg./ml.). The radioactivity still retained in the vial was equivalent to 23 % of the initial, and two further washes only decreased this to 22 and 21 % respectively. The insertion of a single wash of the vial and pipette with 0·4 ml. of potassium iodide solution (10 mg./ml.) in control experiments without added protein gave a 94 % recovery of the sodium [^{131}I]iodide in the salt peak. This suggested that any ^{131}I retained in a vial after such a wash was bound to protein. Adsorption of the eluted protein to the collection vial was eliminated by eluting into 1·0 ml. of a solution of bovine serum albumin (50 mg./ml.) in 0·07 M-barbitone buffer, pH 8·6.

Optimum conditions for iodination

Concentration of reactants. It was early decided to limit the amount of radioactivity handled to 2–4 mc as acceptable levels for routine laboratory work. The absolute loss of some 1·3 μg. of ^{131}I-labelled hormone in the reaction vial precluded the lowering of the amount of growth hormone used to below 2·5 μg. Maximum specific radioactivities were obtained with 5 μg. of growth hormone. A decrease in the amount of the protein resulted in a decreased yield with essentially the same specific radioactivities, and the amount of hormone was fixed at 5 μg.

A preliminary series of trace-labelling experiments was carried out by allowing 10 μg. of growth hormone to react with 0·04 μg. of [^{127}I]iodide together with a negligible amount of [^{131}I]iodide. The reaction volume was varied in six steps from 0·03 to 1·5 ml. As would be expected the percentage of ^{127}I transferred to protein by the reaction decreased from 89 to 4 % with the decrease in concentration of the reactants.

The preparations of high radioactivity were not investigated systematically but showed the expected increase in percentage transfer of ^{131}I with decreasing volume.

Table 2 shows the increase in percentage transfer of ^{131}I when 2 mc of [^{131}I]iodide was allowed to react with 5 μg. of growth hormone in 0·3–0·1 ml. volumes. A further decrease to approx. 0·05 ml., obtained by heating to evaporate the [^{131}I]iodide sample before use, gave no further increase in efficiency.

Amount of chloramine-T. The theoretical amount of chloramine-T required for the oxidation of the sodium [^{131}I]iodide and sodium [^{127}I]iodide in a sample of 2 mc can be calculated to be 0·04 μg. Preliminary iodination experiments were carried out by substituting amounts of [^{127}I]iodide for the IBS-3 sample together with a negligible amount of [^{131}I]iodide and by varying the amounts of chloramine-T (up to 5000 equiv.). Only approximate percentage transfers were obtained since at this stage the adsorption of protein to the reaction vial was not appreciated. It was apparent, however, that the amount of chloramine-T required for optimum iodination was for all practical purposes independent of the iodide concentration.

For preparations of high radioactivity obtained by using 2–4 mc of [^{131}I]iodide and 5 μg. of growth hormone in a total reaction volume of 0·1 ml., calculated equivalence data were discarded and the

Table 2. *Effect of reaction volume on the specific radioactivity of the ^{131}I-labelled growth hormone*

Experimental details are given in the text. Specific radioactivities are given as arithmetical means with the numbers of experiments in parentheses.

Growth hormone (μg.)	^{131}I (mc)	Reaction volume (ml.)	Specific radioactivity (μc/μg.)	Percentage of ^{131}I utilized
5	2	0·30	113 (1)	30
5	2	0·15	212 (2)	53
5	2	0·10	257 (3)	64
5	2	0·05*	259 (3)	64
10	2	0·30	157 (3)	79
5	2·8	0·10	416 (2)	74
5	3·0	0·10	405 (1)	67
5	4	0·15*	440 (1)	55
5	4	0·10	555 (4)	70
10	4	0·30	188 (5)	47
10	4	0·30*	261 (1)	65

* [^{131}I]Iodide sample heated in the vial for 1 hr. at 100° in a water bath to decrease the volume: approximate final volumes are recorded.

amount of chloramine-T was determined empirically. With chloramine-T (5 μg.) no iodination took place, but transfer of ^{131}I with up to 76 % efficiency was obtained with amounts ranging from 35 to 450 μg. In the routine preparations 100 μg. of chloramine-T was chosen.

Exposure of human growth hormone to an oxidizing agent might be expected to cause some damage to the hormone. The degree of immunological integrity of the final product of the reaction is dependent on the specific radioactivity, as shown below. Judged immunologically, the short exposure to chloramine-T does not modify the hormone.

Iodate content of the isotope sample. The radioactivity of the IBS-3 sample is derived solely from its ^{131}I content, but no specifications were available from The Radiochemical Centre at the time this work was carried out for the chemical form of this iodine. The sample is mainly sodium [^{131}I]-iodide but various amounts of sodium [^{131}I]iodate may be present. The latter is not made available for iodination by using chloramine-T and this may limit the specific radioactivity obtained. The present material has a specification of 95 % of sodium [^{131}I]iodide.

That the iodate content is not the only variable limiting factor is shown by the results in Table 3. The percentage yields and specific radioactivities calculated from radioactivity data are summarized for 2 mc and 4 mc samples of IBS-3. The [^{131}I]-iodate contents of nine batches of IBS-3 were determined by electrophoresis of samples on cellulose acetate strips for 30 min. under standard conditions. The mean value obtained was 11·7 ± 6·5 % (S.D.).

By assuming 100 % utilization of the ^{131}I the theoretical specific radioactivity obtainable by using a 2 mc sample of IBS-3 and 5 μg. of growth hormone is 400 μc/μg. A 10 % content of [^{131}I]-iodate would decrease this to 360 μc/μg. if [^{131}I]-iodide utilization were maximal. In practice the maximum [^{131}I]iodide utilization was 79 %.

Results obtained with the method

Table 3 shows that in a series of 50 experiments there is a significance difference in the percentages of the radioactivity utilized when 2 and 4 mc samples of IBS-3 were used (t = 2·91; P < 0·01, 48 degrees of freedom). With one 4 mc batch of IBS-3, no iodinated protein was obtained but degraded material was eluted with unchanged non-protein-bound ^{131}I on the column and in a trail thereafter.

Prior treatment of several 4 mc samples of IBS-3 by heating did not produce a consistent improvement in yield. In some cases failure was thought to be caused by excessive alkalinity of the IBS-3 solution (pH > 10). It was not practical to measure the pH of the IBS-3 sample. The buffering capacity of the reaction mixture was therefore increased by the addition of 0·025 ml. of 0·5 M-phosphate buffer, pH 7·5, before the addition of growth hormone and chloramine-T. Thereafter no discrete degradation of the hormone was observed but no consistent improvement in yields was obtained. Electrophoresis of a sample of the salt peak from the Sephadex column separates in 30 min. any degraded protein from [^{131}I]iodate and [^{131}I]-iodide.

The use of 2 mc samples of IBS-3 (thiosulphate-free and carrier-free, in small volumes) imposes a limit of 400 μc/μg. on the specific radioactivity possible. This is compensated for by the reproducibility obtained. Results presented below show that there is a decrease in immunological reactivity with increasing specific radioactivity, and for this reason no further attempts were made to obtain consistent yields with 4 mc samples.

Effect of iodination on growth hormone

Modification of the immunological properties of growth hormone by labelling with ^{131}I could be caused by chemical alteration due to the substitution of iodine, radiation damage and chemical damage by the reagents used in the reaction.

Table 3. *Summary of preparations of ^{131}I-labelled growth hormone*

Experimental details are given in the text. The percentage of [^{131}I]iodide utilized was calculated from the observed percentage of radioactivity utilized after correction for the percentage [^{131}I]iodate content of the IBS-3 sample where this was determined by electrophoresis. The results are given as arithmetical means ± S.D., with the numbers of experiments in parentheses.

Total radioactivity of sample mixed with 5 μg. of growth hormone (mc)	Percentage of total radioactivity utilized	Percentage of [^{131}I]iodide utilized	Specific radioactivity (μc/μg.)
2	63·7 ± 8·6 (10)	—	255 ± 34 (10)
2	57·1 ± 12·8 (8)	66·2 ± 14·1 (8)	228 ± 51 (8)
4	46·5 ± 20·4 (25)	—	372 ± 163 (25)
4	47·9 ± 11·3 (7)	55·6 ± 11·1 (7)	383 ± 90 (7)

The effect of chemically altering the growth hormone by the substitution of iodine has been investigated by preparing [^{127}I]iodinated growth hormone containing 0·94, 9·5 and 19·3 atoms of ^{127}I/molecule of growth hormone (mol.wt. 29000; Squire & Pedersen, 1961). These were obtained by the routine method by using 100 μg. of growth hormone and various amounts of potassium iodide (Fig. 3) together with a negligible amount of sodium [^{131}I]iodide, and were assayed by an immunoelectrophoretic assay (Hunter & Greenwood, 1962*b*). The assay procedure is based on the ability of unlabelled growth hormone to compete with a fixed amount of ^{131}I-labelled growth hormone for the binding sites on the limited and fixed amount of antibody present in the system. This is reflected in a decrease in the percentage of the radioactivity bound to γ-globulin which is then linearly related to the logarithm of the concentration of unlabelled growth hormone. By using this parameter, [^{127}I]iodinated growth hormone is indistinguishable from unlabelled growth hormone when up to 9·5 atoms of ^{127}I are substituted/molecule of growth hormone (Fig. 4). The highest level of substitution (19·3 atoms of ^{127}I/molecule) represents an almost quantitative iodination of the 10 tyrosine residues/molecule of growth hormone present in our sample of growth hormone prepared by the method of Raben (1959) (Dr J. E. Eastoe, Royal College of Surgeons, unpublished work). At this level there is a marked loss of affinity for the antiserum. The residual immunological activity is not due to a small percentage of unlabelled growth hormone in the preparation, since a further experiment showed that it competes over the whole range (70–20 % binding) but with a different slope from that given by the unlabelled growth hormone (Fig. 5).

The degree of chemical substitution achieved in the preparation of ^{131}I-labelled growth hormone is well below the 9·5 atoms/molecule shown above to cause no immunological damage. The highest specific radioactivity obtained (750 μC/μg.) is calculated to contain 1·50 atoms of total iodine/molecule of growth hormone. The electrophoretic pattern of this material on starch-block electrophoresis (Kunkel & Slater, 1952) shows a slight broadening of the peak and a slight increase in mobility towards the anode. This might be expected from the conversion of tyrosine residues into iodinated tyrosine residues.

We have no evidence for radiation damage to growth hormone by the method: no discrete degradation products as noted by Utiger *et al.*

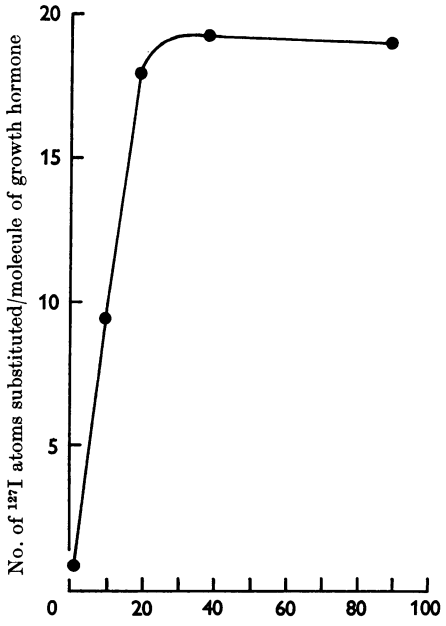

Fig. 3. Iodination of growth hormone with various amounts of ^{127}I. Growth hormone (100 μg.) was allowed to react with KI (0·104–104 μg.) with a trace amount of [^{131}I]iodide by the routine method described in the text, and the number of ^{127}I atoms substituted/molecule of growth hormone was calculated.

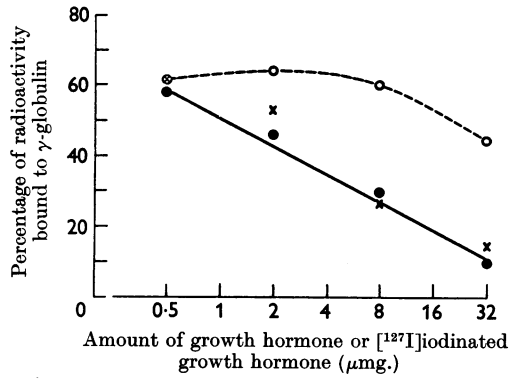

Fig. 4. Comparison of [^{127}I]iodinated growth hormone with unlabelled growth hormone. The abilities of [^{127}I]iodinated growth hormone and growth hormone to compete with ^{131}I-labelled growth hormone for a fixed amount of rabbit antiserum (1:35000 dilution) were compared. Incubations were for 4 days in 0·07 M-barbitone buffer, pH 8·6, containing crystalline bovine plasma albumin (0·5 mg./ml.), followed by the separation of free ^{131}I-labelled growth hormone from that bound to antibody γ-globulin by electrophoresis on cellulose acetate. The [^{127}I]iodinated growth hormone contained: ●, 0·95 atom of ^{127}I/molecule; ×, 9·5 atoms of ^{127}I/molecule; ○, 19·3 atoms of ^{127}I/molecule. The full line represents unlabelled growth hormone (experimental points omitted).

(1962) have been observed. In excess of antiserum 96% of the ^{131}I-labelled hormone is capable of reacting with the immune γ-globulin of the antiserum to the unlabelled hormone. This suggests that under our conditions growth hormone does not receive a damaging level of radiation. These conditions include the high yield of the reaction, the rapid removal of the unchanged [^{131}I]iodide and the dilution of the ^{131}I-labelled hormone with albumin within 20 min. of its preparation.

The chemical reagents used in the method (chloramine-T, sodium metabisulphite and potassium iodide) do not alter the immunological integrity of growth hormone. This is shown by the identical behaviour of unlabelled growth hormone and growth hormone labelled with up to 9·5 atoms of ^{127}I/molecule with the use of these reagents.

We have evidence that the radiochemical reagent used can cause a subtle change in the immunological reactivity of the growth hormone which is not readily determined. Samples of growth hormone (5 μg.) were allowed to react with 2, 4 and 6 mc samples from an IBS-3 solution. The percentage yields of the reactions and specific radioactivities are shown in Table 4 together with the percentage of the preparations capable of reacting with the immune γ-globulin of the antiserum. There is no difference between these preparations as judged by this parameter. However, these preparations were also assayed by comparison with unlabelled hormone in the immunoelectrophoretic assay. The results are shown in Fig. 6. The higher specific radioactivities (533 and 748 μc/μg.) exhibit a decreased affinity for the antiserum, shown by the decrease in the percentage bound compared with an equal weight of the unlabelled hormone. This decreased affinity is thus shown only when the amount of antibody is limited in a sensitive assay at an antiserum dilution of 1:35000.

It is concluded that preparations with specific radioactivities from 200 to 300 μc/μg. obtained from 2 mc of [^{131}I]iodide and 5 μg. of hormone are indistinguishable from the unlabelled hormone.

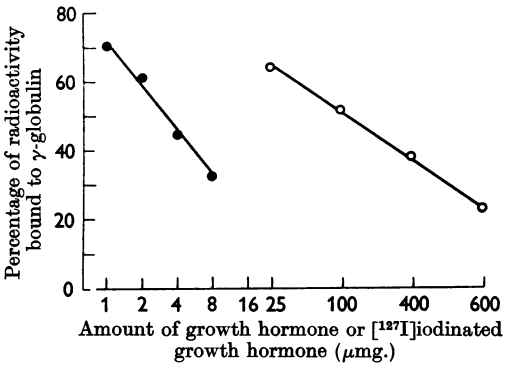

Fig. 5. Comparison of [^{127}I]iodinated growth hormone with unlabelled growth hormone. Experimental details are as given in Fig. 4. ●, Unlabelled growth hormone; ○, [^{127}I]iodinated growth hormone, containing 19·3 atoms of ^{127}I/molecule.

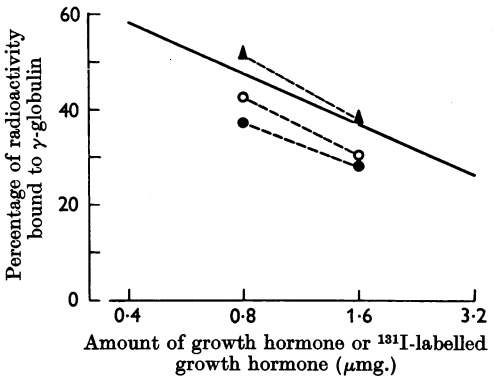

Fig. 6. Comparison of ^{131}I-labelled growth hormone with unlabelled growth hormone. The affinities of ^{131}I-labelled growth hormone of increasing specific radioactivity for a fixed amount of antibody (antiserum dilution 1:35000) were compared. The specific radioactivities of the ^{131}I-labelled growth hormone were: ▲, 283 μc/μg.; ○, 533 μc/μg.; ●, 748 μc/μg. The solid line represents unlabelled growth hormone (experimental points omitted).

Table 4. *Binding of ^{131}I-labelled growth hormone of different specific radioactivities to excess of antiserum*

Growth hormone (5 μg.) was allowed to react with increasing amounts of [^{131}I]iodide by the method described in the text. Then 0·5 μmg. of each preparation of ^{131}I-labelled growth hormone obtained was incubated with antiserum (1:400 dilution) for 4 days at 4°. The percentage of the radioactivity bound by antibody was determined by electrophoresis.

Vol. of [^{131}I]iodide sample used (ml.)	Total radioactivity of sample (mc)	Percentage of ^{131}I utilized	Specific radioactivity (μc/μg.)	Percentage of radioactive protein bound by antiserum
0·025	1·95	72·7	283	85·2
0·05	4·01	72·0	533	85·3
0·073	5·84	64·0	748	87·7

The variable yields (Table 3) and variable affinities (Fig. 6) obtained by using growth hormone of higher specific radioactivity (400–600 μc/μg.) do not allow the routine use of these preparations. The variation appears due to unknown factors in the 4 mc samples of carrier-free [^{131}I]iodide and was not investigated further.

DISCUSSION

There are many methods available for the labelling of proteins with ^{131}I (cf. Taurog & Chaikoff, 1957), and several of these have been applied to hormonally active proteins (Yalow & Berson, 1960; Unger *et al.* 1959; Grodsky *et al.* 1961; Utiger *et al.* 1962; Samols & Williams, 1961).

The present method has several advantages which derive from the use of low amounts of carrier-free [^{131}I]iodide (2 mc) and small quantities of protein (5 μg.) in a high-yield reaction mediated by chloramine-T. No prior treatment of the [^{131}I]-iodide sample is required, the reaction being carried out by injection into the sample vial. After reaction, 70 % of the ^{131}I is combined to protein and the remainder is present in excess of reducing agent. These factors decrease the radiological hazard of manipulating 2 mc amounts of radioactive iodine and no special facilities are therefore required. The rapid removal of the unchanged [^{131}I]iodide by gel-filtration minimizes external radiation of the protein and enables small quantities of protein to be iodinated and recovered in good yield in about 1 hr.

The use of carrier-free [^{131}I]iodide of high isotope abundance makes possible high specific radioactivity but with a low degree of chemical substitution, e.g. 0·6 atom of total iodine/molecule of growth hormone for a specific radioactivity of 295 μc/μg.

Bannerjee & Gibson (1962) have used the method described above to prepare ^{131}I-labelled insulin of high specific radioactivity (1000 μc/μg.) from insulin (2·5 μg.). They note that chloramine-T causes damage to the insulin, and the ^{131}I-labelled insulin is purified by dialysis and Sephadex chromatography.

The technique used by Yalow & Berson (1960) for insulin produces discrete degradation products, due apparently to the high amount of [^{131}I]iodide used (30–80 mc). The application of this procedure to human growth hormone by Utiger *et al.* (1962) gave low specific radioactivity (5 μc/μg.), and some degradation of the hormone was reported despite the lower amounts of [^{131}I]iodide used (10–20 mc). Carrier-free [^{131}I]iodide free of reducing agent was not available to these groups. Berson & Yalow (1963) have successfully applied the present method to the preparation of ^{131}I-labelled insulin and other proteins.

In the present method no discrete degradation products have been noted on electrophoresis and, judged by one immunological parameter, growth hormone of high specific radioactivity (up to 700 μc/μg.) was identical with the unlabelled hormone. However, the highest radioactivities obtained by using 4–6 mc gave variable yields due to unknown factors in the samples of [^{131}I]iodide, and by the most sensitive immunological parameter were shown to have a decreased affinity for the antiserum.

With the lower amount of [^{131}I]iodide (2 mc), yielding specific radioactivities of 200–300 μc/μg., the ^{131}I-labelled hormone was indistinguishable from the unlabelled hormone.

The distribution of radioactivity in an experiment, together with the behaviour of protein and iodide on the glass vial and the Sephadex column, allows the specific radioactivity and the amount of hormone recovered to be calculated. It is assumed that none of the [^{131}I]iodide is utilized for non-specific oxidation (cf. Hughes & Straessle, 1950). The theoretical yields of [^{127}I]iodinated growth hormone obtained by using calculated amounts of [^{127}I]iodide is evidence that, under the conditions of the method, namely an excess of oxidizing agent and a step involving sodium metabisulphite, all of the iodine utilized is combined with tyrosine residues.

The rapid separation of protein and salt achieved by gel-filtration is well known, and its application to the method has obvious advantages. The absorptive capacity for protein was shown to be low (approx. 15 μg. of protein/g. of Sephadex) and this was decreased to approx. 1 μg./g. of Sephadex by pretreatment with albumin.

The significant difference between 2 mc and 4 mc samples of carrier-free [^{131}I]iodide both in terms of yields and immunological integrity, despite some attempts at elucidation, remains unexplained.

SUMMARY

1. A simple and rapid method is presented for the preparation of ^{131}I-labelled human growth hormone of high specific radioactivity (240–300 μc/μg.).

2. Low amounts of carrier-free [^{131}I]iodide (2 mc) are allowed to react, without prior treatment, with small quantities of protein (5 μg.) in a high-yield reaction (approx. 70 % transfer of ^{131}I to protein).

3. The degree of chemical substitution is minimized (0·5–1·0 atom of iodine/molecule of protein) by the use of carrier-free [^{131}I]iodide.

4. The ^{131}I-labelled hormone (up to 300 μc/μg.) contains no detectable degradation products and is immunologically identical with the unlabelled hormone.

5. The loss of immunological reactivity at high specific radioactivities or at high levels of chemical substitution with [^{127}I]iodine is demonstrated.

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The Electrophoretic Behaviour of some Trypanosomes

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Evidence has previously been given that certain organic bases show an electrophoretic specificity towards membranes of different cells (Bangham, Glover, Hollingshead & Pethica, 1962). Of particular interest was the effect of the trypanocidal bases Antrycide (quinapyramine), Ethidium (homidium) and Prothidium, and a therapeutically inactive quinapyramine analogue, on the electrophoretic mobility of some neoplastic cells. As trypanosomes are able to circulate freely in the blood during some part of their life-cycle without undergoing phagocytosis, a comparison of their electrophoretic behaviour with that of neoplastic cells may give some indication as to the ability of the latter to metastasize. The present paper describes the effects of these drugs on the electrophoretic mobility of some trypanosomes, and in addition gives quantitative results on the electrophoretic behaviour of these Protozoa, which have previously been examined only qualitatively (see, for example, Broom, Brown & Hoare, 1936; Grodzensky, 1936; Fischer & Fischl, 1933).

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MATERIALS AND METHODS

Trypanosome suspensions. Culture forms of *Trypanosoma rhodesiense* were grown as described by Ryley (1962), and harvested after 5 or 6 days. A rabbit was inoculated intravenously with *T. rhodesiense* from a mouse. Instead of the acute infection seen in rats or mice, a relapsing type of infection resulted which lasted for 3 months. At each relapse, mice were infected from the rabbit, and the variant strains passaged rapidly in mice; one such variant strain has been designated R_2. Blood-stream forms of *T. rhodesiense*, *T. vivax* and *T. equinum* were isolated 3 or 4 days after inoculation from the blood of infected rats by differential centrifuging as described by Ryley (1956), and the blood-stream forms of *T. congolense* and *T. lewisi* were isolated in a similar manner 4–6 days after inoculation.