The Labelling of Proteins to High Specific Radioactivities by Conjugation to a 125I-Containing Acylating Agent

APPLICATION TO THE RADIOIMMUNOASSAY

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1. A new method is described for labelling proteins to high specific radioactivities with 125I. The protein is treated with a 125I-labelled acylating agent, iodinated 3-(4-hydroxy-phenyl)propionic acid N-hydroxysuccinimide ester, which reacts with free amino groups in the protein molecule to attach the 125I-labelled groups by amide bonds. 2. Three protein hormones have been labelled by this method, human growth hormone, human thyroid-stimulating hormone and human luteinizing hormone. Specific radioactivities of up to 170, 120 and 55 μCi/μg respectively have been obtained for these hormones. 3. The immunoreactivity of these labelled hormones has been investigated by using a radio-immunoassay system specific for each hormone. These preparations have also been compared with and found to be equal or superior to labelled hormones prepared by chemical substitution of 125I into tyrosine residues of the proteins by using the chloramine-T-oxidation procedure. 4. With some antisera the immunoreactivity of the antigen was diminished by the introduction of a single I atom into the tyrosyl groups, whereas antigen containing a single 125I-labelled 3-(4-hydroxyphenyl)propionamide group showed the same immunoreactivity as the unmodified antigen.

Proteins that have been labelled to high specific radioactivities with 125I are currently employed in a variety of biochemical studies, for example in the investigation of hormone metabolism (Ansorge et al., 1971) and hormone/target tissue interactions (Cuatrecasas, 1971) and in studies on immunoglobulin metabolism (Motas & Ghetie, 1969). The most widespread use of 125I-labelled proteins, however, is as high-specific-radioactivity antigens used as tracers in the radioimmunoassay. The chloramine-T procedure (Hunter & Greenwood, 1962; Greenwood et al., 1963) was developed for this purpose and is an efficient method for the direct substitution of 125I into the tyrosyl residues of proteins. During the past decade this method has been almost universally used to label the ever-increasing number of proteins and polypeptides for which radioimmunoassays have been reported (Yalow & Berson, 1968; Kirkham & Hunter, 1971; Greenwood, 1971). Two specific disadvantages are associated with this method. First, this approach may be unsuitable for peptides that lack tyrosine, for example secretin (Mutt et al., 1970) and porcine parathyroid hormone (Woodhead et al., 1971). Secondly, some proteins, for example parathyroid hormone and calcitonin, are regularly altered during iodination (Buckle & Potts, 1970; Buckle, 1971), whereas others are subject to intermittent alterations. These changes may result in loss in the affinity of the labelled antigen for antibody, causing

a decrease in the sensitivity of the assay system. However, iodination damage can be so severe as to render the preparation of labelled antigen unsuitable for use. There is some evidence that damage of this kind is caused by noxious impurities present in the solution of 125I obtained from the manufacturers (Hunter, 1971), although exposure of some proteins to the oxidizing agent (chloramine-T) and reducing agent (sodium metabisulphite) used in this method may also disrupt their structural integrity.

The present paper describes a method of labelling proteins with 125I in which the protein itself is not directly exposed to the 125I solution or to the reagents used in the iodination reaction. This method has been used to label protein hormones, and the effect of the labelling procedure on the reaction of the hormones with specific antisera has been investigated. The new procedure involves reacting the protein under mild conditions with the N-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid that has previously been labelled with 125I and separated from the products of the iodination reaction by solvent extraction. Hydroxysuccinimide esters react with free amino groups of proteins or peptides to form amides (Anderson et al., 1964). This method has the additional advantage that the 125I label is introduced into a group other than the tyrosine residues involved in simple iodination, so that an alternative form of chemical modification to that generally employed now

becomes available. A preliminary report of this work has already been published (Bolton & Hunter, 1972).

Materials

General

Carrier-free ^{125}I, at a concentration of 100–240mCi/ml, was obtained as preparation IMS30 from The Radiochemical Centre, Amersham, Bucks., U.K.

N-Succinimidyl 3-(4-hydroxyphenyl)propionate was prepared for us by the method of Anderson et al. (1964) by Mr. U. Ruegg, Institut für Molekular-biologie ünd Biophysik, Eidgenössische Technische Hochschule, Zurich, Switzerland (see the Appendix; Rudinger & Ruegg, 1973).

Thin-layer chromatography was carried out on 0.25mm-thick Merck silica-gel F254 pre-coated plates (E. Merck A.-G., Darmstadt, Germany).

Whatman CF-11 cellulose powder was used for cellulose adsorption.

Buffers

Sodium salts were used to prepare buffers.

Hormones

Human growth hormone was prepared by Dr. Anne Stockell Hartree by using the method of Wilhelmi (1961), and was distributed as preparation 69/46 by the Medical Research Council Division of Biological Standards, National Institute for Medical Research, Hampstead Laboratories, Holly Hill, London NW3 6RB, U.K.

Human thyroid-stimulating hormone was preparation DE-32-3 from Dr. Anne Stockell Hartree, Department of Biochemistry, University of Cambridge, U.K.

Human luteinizing hormone was fraction IRC-50, also prepared by Dr. Hartree and distributed in microcapillary ampoules designated 71/53 by the Medical Research Council Division of Biological Standards.

Bovine insulin (6 times recrystallized) was obtained from Boots Pure Drug Co., Nottingham, Notts., U.K.

Antisera

Antisera to human growth hormone (HGH-R6b and HGH-R2) and to human luteinizing hormone (LH GP 7253) were raised in this laboratory; that to human thyroid-stimulating hormone was a guinea-pig anti-human thyroid-stimulating hormone serum raised in this laboratory. Antiserum to insulin

(insulin-neutralizing/precipitating serum; lot K1571) was obtained from Burroughs Wellcome, Beckenham, Kent, U.K.

Methods and Results

Iodination of the hydroxyphenylpropionic acid ester

The reaction was carried out at room temperature (about 20°C). Crystalline N-succinimidyl 3-(4-hydroxyphenyl)propionate (0.2–0.25μg) was treated with 2–5mCi (10–20μl) of Na^{125}I and 50μg of chloramine-T in 10μl of 0.25M-phosphate buffer, pH7.5, as oxidizing agent. The reaction was immediately terminated by the addition of 120μg of sodium metabisulphite in 10μl of 0.05M-phosphate buffer, pH7.5, after which 200μg of carrier KI in 10μl of this buffer was added. Under these conditions and by using a reaction ratio of 1.5–3.0 atoms of I/molecule of ester a substitution of 0.8–1.8 atoms of I/molecule of ester (30–75% yield) was obtained. The iodinated product was extracted into redistilled benzene (two 0.25ml portions) and recovered by evaporation of the solvent under vacuum, taking precautions to trap any volatile radioactive material. The addition of 5μl of dimethylformamide before the benzene was necessary for quantitative extraction of the ester into the solvent. Because of the instability of the hydroxysuccinimide ester under the conditions of the iodination reaction, the procedure must be carried out as rapidly as possible. With experience, the time taken from the addition of the Na^{125}I to the solvent extraction of the product can be decreased to less than 20s. The reaction volume at this stage is largely determined by the concentration of ^{125}I obtained from the manufacturers. If this is less than about 100mCi/ml, the yield of the iodination reaction is diminished.

Identification of the labelled products of the iodination reaction

A series of iodinations of the N-succinimidyl 3-(4-hydroxyphenyl)propionate was carried out by using K^{127}I trace-labelled with Na^{125}I, reaction ratios of between 0.1 and 10.0 atoms of I/molecule of ester being used. The products of the iodination reactions were separated by t.l.c. on silica gel by using ethyl acetate–toluene (1:1, v/v), and the distribution of radioactivity on the plates was determined. By using a low ratio of I to ester (0.1 g-atom/mol), two radioactive spots were obtained on t.l.c., one remaining at the origin (corresponding to free I) and the second with R_F 0.5. By using an excess of I over ester (10 g-atoms/mol), a maximum of 2 atoms of I became incorporated/molecule of ester, presumably at positions 3 and 5. T.l.c. of the reaction mixture revealed two radioactive spots, one at the origin and the second

with R_F 0.7. It was concluded that mono- and di-iodo-3-(4-hydroxyphenyl)propionic acid hydroxy-succinimide ester were formed in the iodination reaction and that these products had R_F values 0.5 and 0.7 respectively in the t.l.c. system used. The hydrolysis product of the iodinated succinimide ester, labelled 3-(4-hydroxyphenyl)propionic acid, had an R_F value 0.2 in this system. Any of this material formed during the iodination reaction should not be extracted with the labelled ester at the pH used.

T.l.c. of benzene extracts of two 'hot' iodination reactions (average substitution of about 0.8 atoms of ^{125}I/molecule of ester) revealed the presence of a major radioactive spot at R_F 0.5 and a minor spot at R_F 0.7, together accounting for 95% of the total radioactivity recovered from the plate. Sufficient material has not been available for detailed characterization of the iodinated compounds.

Labelling the protein by conjugation with the hydroxy-succinimide ester of 3-(4-hydroxyphenyl)propionic acid

Protein (5 μg) in 10 μl of 0.1 M-borate buffer, pH 8.5, was added to the dried iodinated ester and the reaction mixture agitated for 15 min at 0°C. Thus 3–4 mol of labelled ester was reacted/mol of protein. Labelled ester hydrolyses in aqueous media, the half-life of this material being about 9 min in 0.1 M-borate buffer, pH 8.5.

For human growth hormone, 5 μg of protein reacted with 0.2 μg of iodinated ester (3.2 mol of ester/mol of protein) has resulted in the incorporation of 13–53% of the radioactivity (mean 32%) into the conjugate in 14 preparations yielding specific radioactivities of 22–170 μCi/μg. This reaction is markedly concentration-dependent, the yield being decreased at human growth hormone concentrations below 5 μg/10 μl. To obviate subsequent conjugation to carrier proteins, unchanged ester was reacted with 0.5 ml of 0.2 M-glycine in 0.1 M-borate buffer, pH 8.5, for 5 min at 0°C.

The ^{125}I-labelled protein can be separated from the other labelled products of the conjugation reaction, glycine conjugate and 3-(4-hydroxyphenyl)propionic acid, by gel filtration. For human growth hormone, this was accomplished by using Sephadex G-50 (fine grade). To minimize the loss of labelled protein by adsorption, the Sephadex was equilibrated and eluted with 0.05 M-phosphate buffer, pH 7.5, containing 0.25% (w/v) gelatin. The low-molecular-weight labelled products bind to serum albumin and other serum proteins; buffers containing these proteins should therefore be avoided until the separation stage has been completed. Recovery of ^{125}I is generally about 90% of the column load. Both the ^{125}I-labelled glycine conjugate and labelled free 3-(4-hydroxyphenyl)propionic acid can be recovered quantitatively from Sephadex G-50 gel-filtration columns. It follows

therefore that of the labelled compounds present only ^{125}I-labelled protein is subject to losses by adsorption and the proportion of labelled protein eluted can consequently be calculated.

Labelling by the conjugation technique has been used to make 14 preparations of labelled human growth hormone, six preparations of labelled human thyroid-stimulating hormone and four preparations of labelled human luteinizing hormone. With human thyroid-stimulating hormone specific radioactivities of 44–120 μCi/μg have been obtained. Labelling of human luteinizing hormone by the conjugation method has resulted in specific radioactivities of 20–55 μCi/μg. This hormone is more susceptible to
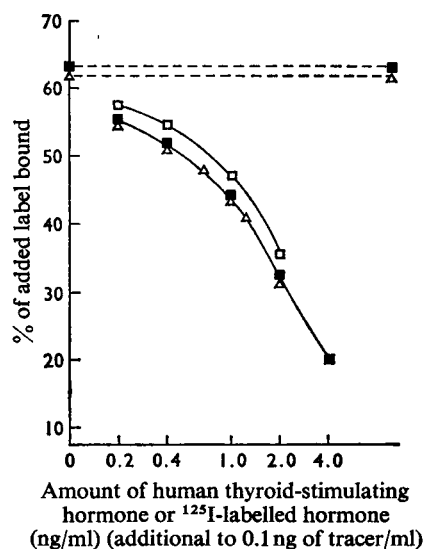

Fig. 1. *Assessment of immunoreactivity of* [125]*I-labelled human thyroid-stimulating hormone in the radio-immunoassay system*

Hormone (either all labelled or unlabelled + tracer) was incubated with antiserum for 3 days at 4°C before separation of antibody-bound from free hormone by the double-antibody method. Radioimmunoassay standard curves with: ■, unlabelled human thyroid-stimulating hormone + 0.1 ng of ^{125}I-labelled hormone tracer/ml; △, ^{125}I-labelled human thyroid-stimulating hormone prepared by the conjugation method (specific radioactivity 45 μCi/μg, about 0.8 mol of labelled residue/mol of hormone); □, ^{125}I-labelled human thyroid-stimulating hormone prepared by the chloramine-T method (specific radioactivity 48 μCi/μg, about 0.6 g-atom of I/mol of hormone). ■---■, Binding of 0.1 ng of tracer labelled by chloramine-T method/ml; △---△, binding of 0.1 ng of tracer labelled by conjugation method/ml.
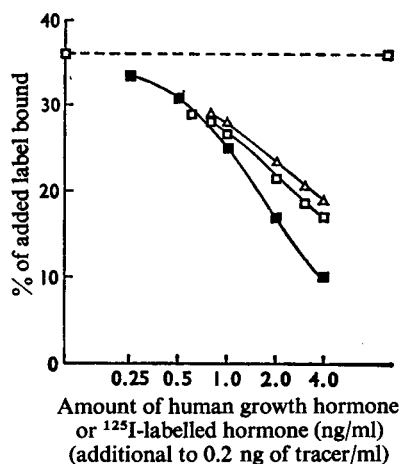
Fig. 2. *Assessment of the immunoreactivity of* ^{125}I-*labelled human growth hormone in the radioimmunoassay system with antiserum HGH-R6b at a final dilution of* 1:654000

Experimental procedure is as described for Fig. 1. ■, Unlabelled human growth hormone+0.2ng of ^{125}I-labelled human growth hormone tracer/ml; △, ^{125}I-labelled human growth hormone labelled by the conjugation method (specific radioactivity 140μCi/μg, about 1.5mol of labelled residue/mol of hormone); □, ^{125}I-labelled human growth hormone labelled by the chloramine-T method (specific radioactivity 135μCi/μg, about 1.3g-atoms of I/mol of hormone). □---□, Binding of 0.2ng of tracer/ml.

iodination damage during conventional iodination with chloramine-T than are human growth hormone and human thyroid-stimulating hormone. After iodination by the conventional method, ^{125}I-labelled human luteinizing hormone requires further purification by adsorption to cellulose powder (Hunter, 1969). ^{125}I-labelled human luteinizing hormone prepared by the conjugation method and purified by cellulose adsorption on a 75mg-cellulose column was usable as a tracer in a human luteinizing hormone assay at a time when tracers iodinated by the chloramine-T method showed excessive damage.

Testing the immunoreactivity of the proteins labelled by the conjugation method

The immunoreactivity of ^{125}I-labelled hormones prepared by the method of conjugation-labelling was investigated by using the radioimmunoassay system and compared with that of hormones labelled by the chloramine-T method. In all such experiments, pairs of tracers labelled on the same day from the same ^{125}I solution by these two methods were com-
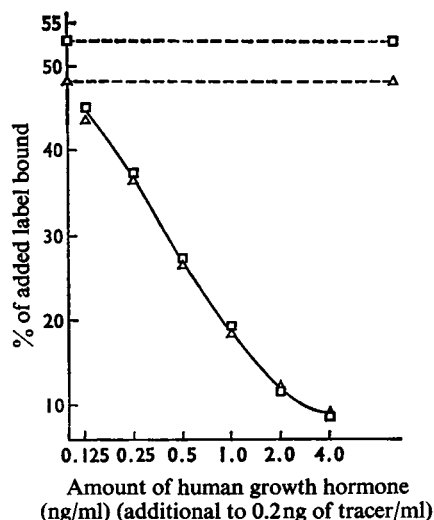

Fig. 3. *Comparison of* ^{125}I-*labelled human growth hormone prepared by the conjugation method and chloramine-T method as tracers in human growth hormone-radioimmunoassay-standard curves with antiserum HGH-R6b at a final dilution of* 1:654000

The experiment was performed by using delayed addition of tracer (see the text). Antibody-bound was separated from free hormone by a double-antibody system. △, Standard curve with unlabelled human growth hormone standard+0.2ng of tracer labelled by the conjugation method/ml; □, standard curve with unlabelled human growth hormone standard+0.2ng of tracer labelled by the chloramine-T method/ml. □---□, Binding of 0.2ng of tracer labelled by the chloramine-T method/ml; △---△, binding of 0.2ng of tracer labelled by the conjugation method/ml. The tracers used in these experiments were the same preparations as were used in Figs. 2 and 4.

pared. Antibody-bound and free hormone were separated by means of a double-antibody system (Morgan & Lazarow, 1962), the percentage of labelled hormone bound to antibody being measured.

A direct comparison of the immunoreactivity of labelled and unlabelled proteins was carried out by the method suggested by Hunter (1971), in which radioimmunoassay standard curves with increasing amounts of unlabelled hormone and a constant amount of labelled tracer hormone are compared with standard curves containing corresponding increasing amounts of labelled hormone alone. If these two curves are superimposible, the labelled and unlabelled hormones are immunologically indistinguishable in this system.
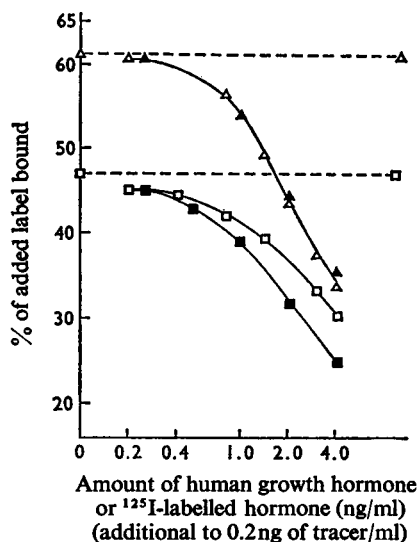
Fig. 4. *Assessment of the immunoreactivity of ^{125}I-labelled human growth hormone in the radioimmunoassay system with antiserum HGH-R2 at a final dilution of* 1:512000

Experimental procedure is as described in Fig. 1. Radioimmunoassay standard curves with: ▲, unlabelled human growth hormone+0.2ng of ^{125}I-labelled hormone tracer prepared by the conjugation method/ml; △, ^{125}I-labelled human growth hormone labelled by the conjugation method; ■, unlabelled human growth hormone+0.2ng of ^{125}I-labelled hormone tracer prepared by the chloramine-T method/ml; □, ^{125}I-labelled human growth hormone labelled by the chloramine-T method. △---△, Binding of 0.2ng of tracer prepared by the conjugation method/ml; □---□, binding of 0.2ng of tracer prepared by the chloramine-T method/ml. The tracers used in these experiments were the same preparations as were used in experiments shown in Figs. 2 and 3.

Fig. 5. *Comparison of ^{125}I-labelled human growth hormone prepared by the conjugation method and chloramine-T method as tracers in human growth hormone radioimmunoassay standard curves with antiserum HGH-R2 at a final dilution of* 1:520000

Experimental details are as described in Fig. 3. △, Standard curve with unlabelled human growth hormone standard+0.2ng of tracer labelled by the conjugation method/ml (specific radioactivity 78 μCi/μg, about 0.7mol of labelled residue/mol of hormone); □, standard curve with unlabelled human growth hormone standard+0.2ng of hormone tracer labelled by chloramine-T method/ml (specific radioactivity 75 μCi/μg, about 0.75g-atom of I/mol of hormone). △---△, Binding of 0.2ng of tracer labelled by the conjugation method/ml; □---□, binding of 0.2ng of tracer labelled by the chloramine-T method/ml.

When investigated by this method human thyroid-stimulating hormone labelled on four different occasions by the conjugation technique and by the chloramine-T method showed little or no loss of immunoreactivity compared with the unlabelled hormone (Fig. 1). In the radioimmunoassay system for human growth hormone at present in use in this laboratory, utilizing antiserum HGH-R6b, human growth hormone labelled by the conjugation-labelling and by the chloramine-T methods generally showed similar small losses of immunoreactivity when compared with unlabelled human growth hormone (Fig. 2).
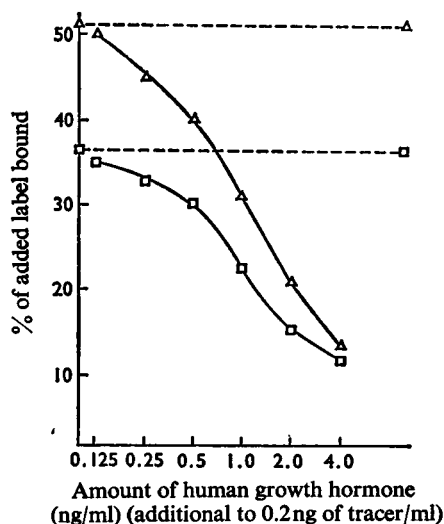
A comparison of the behaviour of hormone preparations as tracers in radioimmunoassays was also carried out. A typical assay standard curve from such a comparison with a human growth hormone radioimmunoassay system is shown in Fig. 3. In this experiment unlabelled human growth hormone standards were incubated with antiserum HGH-R6b for 3 days at 4°C before the addition of the labelled tracer, the incubation then being continued for a further 3 days before separation of antibody-bound from free hormone. This delayed addition of tracer is widely used because it improves the detection limit (sensitivity) of the assay (Hales & Randle, 1963; Rodbard *et al.*, 1971). From these results it can be seen that
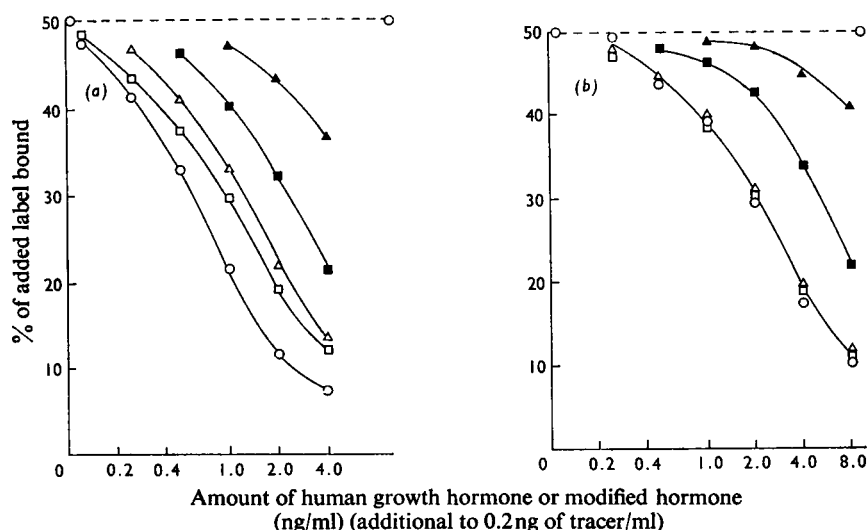
Fig. 6. *Effect of increasing the modification of human growth hormone with iodinated 3-(4-hydroxyphenyl)-propionamide groups on the reactivity of the hormone in radioimmunoassay systems with two different antisera*

Radioimmunoassays were carried out by using delayed addition of tracer (tracer prepared by chloramine-T method). Antibody-bound was separated from free hormone by a double-antibody method. (*a*) Antiserum HGH-R6b; (*b*) antiserum HGH-R2. Human growth hormone: ○, unmodified; modified with an average of: □, 1.2 residues/molecule; △, 2.5 residues/molecule; ■, 7.3 residues/molecule; ▲, 10.0 residues/molecule. ○---○, Binding of 0.2 ng of tracer/ml.

tracers labelled by the two methods produce similar standard curves in this assay system.

When comparing tracers iodinated by these two methods, it should be remembered that the antisera used above have been selected for their reactivity with antigens iodinated by the chloramine-T method, i.e. the antisera will tend to be indifferent to the presence of I substituted into the tyrosine(s) of the antigen. We have therefore investigated the reactivity of human growth hormone labelled by the conjugation method with other antisera to human growth hormone in our possession. One such antiserum, HGH-R2, reacted identically with unlabelled human growth hormone and that labelled by the conjugation method. However, with this antiserum, human growth hormone iodinated by the chloramine-T method showed a loss of immunoreactivity (Fig. 4). In the human growth hormone assay with antiserum HGH-R2 standard curves with tracer labelled by the conjugation method consistently showed higher binding of tracer in the absence of unlabelled hormone, steeper slopes and greater working range than standard curves with human growth hormone tracers labelled by the chloramine-T method (Fig. 5). However, when used as tracers in the assay

system with antiserum HGH-R6B, the same paired samples of tracers resulted in identical standard curves. The detection limit of the assay system with antiserum HGH-R2 was significantly improved when tracers labelled by the conjugation method were used; the concentration of unlabelled standard hormone giving a 10% fall in binding of tracer decreased from 0.83 ± 0.08 ng/ml (with tracer labelled by the chloramine-T method) to 0.49 ± 0.03 ng/ml (means of determinations on six pairs of labelled preparations $\pm$ s.d.).

In a further investigation of the immunoreactivity of hormones labelled by the conjugation method, insulin and human growth hormone were treated with high molar ratios of iodinated 3-(4-hydroxyphenyl)propionic acid hydroxysuccinimide ester (^{127}I + trace amount of ^{125}I). Preparations of these hormones with different degrees of modification were assayed by radioimmunoassay with tracers prepared by the chloramine-T procedure and their activities thus compared with untreated standard hormone. For insulin, up to an average of 3 mol of the ester/mol of insulin could be substituted. Even the most highly modified of these altered insulins retained full immunological activity when assayed. Up to an

1973

Table 1. Comparison of human growth hormone radioimmunoassay results obtained by using tracer labelled by the conjugation and chloramine-T methods

For details see the text. Zero standards are the percentage of labelled tracer binding to antiserum in the absence of unlabelled hormone. Slope of the curve is the decrease in % of binding of tracer for a twofold increase in concentration of unlabelled hormone taken from the steepest part of the curve. Sensitivity is the concentration of unlabelled hormone (ng/ml) causing a 10% decrease in the % of binding of the tracer.

Tracer prepared by the conjugation method

Date of assay	Age of tracer (days) on addition to assay	Zero standards	Slope of curve	Sensitivity
6. 6.72	1	39.4	7.4	0.180
18. 6.72	1	49.4	10.5	0.110
29. 6.72	5	39.9	8.4	0.125
30. 6.72	6	39.5	10.0	0.210
4. 7.72	3	46.5	8.4	0.180
27. 7.72	3	50.3	9.3	0.120
15. 9.72	1	54.4	10.3	0.125
12.10.72	8	38.8	7.0	0.205
7.11.72	1	58.8	11.6	0.270
8.12.72	6	47.1	8.8	0.155
Means ± S.D.	3.5	46.4 ± 7.0	9.2 ± 1.4	0.168 ± 0.051

Tracer prepared by the chloramine-T method

Date of assay	Age of tracer (days) on addition to assay	Zero standards	Slope of curve	Sensitivity
25. 2.72	7	41.8	8.0	0.330
2. 3.72	3	36.9	7.5	0.270
14. 3.72	Added on day of preparation	42.1	8.0	0.185
22. 3.72	8	41.3	7.2	0.245
21. 3.72	1	52.9	9.0	0.140
5. 4.72	3	32.6	6.4	0.400
20. 4.72	Added on day of preparation	27.7	5.6	0.205
27. 4.72	2	40.5	9.2	0.180
12. 5.72	1	31.6	6.4	0.250
31. 5.72	1	41.3	7.2	0.245
	2.6	38.9 ± 7.1	7.5 ± 1.1	0.245 ± 0.076

average of 10 mol of the labelled ester could be introduced/mol of human growth hormone. Fig. 6 shows that when tested with antiserum HGH-R6b the immunoreactivity of human growth hormone decreases as the amount of substitution into the hormone molecule increases. A preparation with an average of 1.2 mol of residue/mol of hormone showed some loss of activity. When tested with antiserum HGH-R2, however, human growth hormone modified with an average of 2.5 mol of residue/mol of hormone reacted identically with unmodified hormone, confirming that reactions with these two antisera are affected differently by this kind of modification of the antigen.

Tracers labelled by the conjugation method are currently being used as a routine procedure in this laboratory in human growth hormone and human thyroid-stimulating hormone radioimmunoassays. In Table 1 three assay parameters for the ten most recent human growth hormone assays are compared with those of the ten previous human growth hormone assays carried out by using tracers prepared by the chloramine-T method. It can be seen that there has been an overall improvement in the assays since tracers labelled by the conjugation method have been used, resulting in assays having greater precision (reflected in the steeper slope of the assay standard curve) and higher sensitivity.

Discussion

The results presented indicate that the proposed method can be used for the preparation of ^{125}I-labelled proteins having generally similar specific radioactivities and immunoreactivities to those prepared by direct iodination by the widely used chloramine-T procedure.

The general aim in labelling proteins with ^{125}I is to provide sufficient specific radioactivity for the purpose envisaged and to achieve this with minimal alteration of the behaviour of the protein in the system in which it is to be used. For the radioimmunoassay there is no obligation to provide tracers whose reactivity is identical with unlabelled antigen, since the system depends on a comparison of the behaviour of unknowns with unlabelled standards. However, whenever a high degree of sensitivity is sought the properties of the labelled preparation are crucial and losses of affinity in the tracer diminish the sensitivity of these systems.

Several factors associated with or consequent on labelling procedures can cause alteration in the reactivity of proteins. Some proteins may be so unstable as to sustain serious damage during mere manipulation in the highly dilute and carrier-free solutions necessarily used in any labelling procedure. There is little difference between the chloramine-T procedure and our new method in this respect.

Proteins may be altered by chemical onslaught caused by contact with the oxidizing agent (chloramine-T) and subsequently by the reduction stage (by metabisulphite): the new method involves no comparable steps. There is much evidence (Hunter, 1971) for the widely held view that the main cause of iodination damage which occurs with the chloramine-T method is consequent on the direct contact between the protein being iodinated and the ^{125}I solution of high specific radioactivity. The new method is the only carrier-free system so far proposed that avoids direct exposure of the protein to the ^{125}I. A very recently developed approach in which the oxidant is Cl_2 gas that is generated within an air-tight compartment and allowed to diffuse through the vapour phase into a mixture of protein solution and ^{125}I (Butt, 1972) also avoids the contact of protein with excess of oxidizing and reducing agents, but does not obviate its direct contact with ^{125}I.

Finally, any method used to attach I to a protein must result in chemical alterations to the protein. At first sight the direct substitution of I into tyrosyl groups would appear to offer the least possible perturbation, and this may be so. However, I is a large atom, similar in size to the whole benzene ring into which it is substituted, and some configurational changes would be expected to result from its presence because of this alone. Further, the introduction of I in this way lowers the pK of the tyrosyl group. The new procedure involves a condensation with a moiety that is larger than I and results also in a greater alteration in charge; the net change being the substitution of a free amino group by an iodophenol group. We do not suggest that these changes are either smaller or preferable, a priori, to those associated with direct iodination. However, they are different and the new labelling procedure makes it possible for the first time to choose between labelling procedures that affect two distinct groups in the protein. Further, we have shown that some antisera 'see' the presence of I substituted into tyrosyl groups of the antigen and yet are indifferent to the condensation of their amino groups. Labelling by the conjugation method therefore may allow the use of some antisera which were unsuitable in radioimmunoassay systems with antigen tracer preparations labelled by the chloramine-à method. We anticipate that other workers may find similar advantages by using this labelling technique for other proteins.

Some protein hormones not so far tested with this new method are more susceptible than human growth hormone, human thyroid-stimulating hormone and human luteinizing hormone to damage by the chloramine-T procedure. Parathyroid hormone and calcitonin appear to be particularly troublesome in this respect and the radioimmunoassay of these hormones might benefit from use of the alternative approach. It should also become possible with the new method

to label peptides lacking tyrosine, for example secretin, to a high specific radioactivity for radio-immunoassay. A further advantage is the provision of an alternative to the chloramine-T method with which even the most experienced laboratories sometimes suffer difficult periods. At such times iodination yields are diminished and losses of immunoreactivity may be increased to such an extent that some preparations become unacceptable for use as tracers in highly sensitive assays.

A special refinement in the preparation of labelled polypeptides of high specific radioactivity involves following the iodination procedure with a step in which the mono-iodinated protein is separated from the unlabelled protein. This permits the iodination reaction to be carried out with a low I/peptide ratio so as to minimize both iodination damage and the number of protein molecules that are substituted with more than 1 I atom. The mono-iodinated peptide is then isolated to provide a tracer having the requisite high specific radioactivity. The possibility of achieving this separation is related to the overall change in the charge characteristics that result from the introduction of 1 I atom into the molecule. This net change is in turn related to the molecular weight of the polypeptide. The largest molecule that appears to have been isolated in this way from the unlabelled peptide is monoiodoinsulin, which has a molecular weight of 5800 (Freychet et al., 1971). Since the change in charge caused by mono-iodination by the conjugation method is greater than that derived from direct iodination, it is to be expected that larger polypeptides could be labelled and the labelled material isolated than is possible by present methods. In preliminary experiments we have shown that human growth hormone containing 1 labelled hydroxyphenyl residue/molecule can be separated from the unlabelled hormone by polyacrylamide-gel electrophoresis.

It should be possible to develop the techniques of conjugation labelling to provide a method of introducing an I label into polypeptide chains at different amino acid residues by using attachment groups having different specificities. Thus methods for labelling proteins to high specific radioactivity at specific groups on the molecule may become available and may permit the retention of particular activities of the protein after labelling, for example immunological or biological activity of hormones, enzymic or immunological activity of enzymes.

Although labelling by the conjugation method involves more manipulations than does the chloramine-T technique, this disadvantage can be minimized by the iodination of sufficient ester at one time for the labelling of several protein preparations. Iodinated ester has been successfully used after storage in the dry state for 5 days.

Of the currently available methods for preparing iodinated hormones to high specific radioactivities,

that using iodine monochloride (McFarlane, 1958; Samols & Williams, 1961) has not been widely used, although this procedure could be arranged to avoid direct contact between the protein and ¹²⁵I solution. The unpopularity of this method may arise from the fact that some added carrier ¹²⁷I is inevitably substituted into the protein, and it also has the disadvantage of involving ¹²⁵I in a volatile form. The electrolytic procedure (Pennisi & Rosa, 1969) does not appear to be particularly suitable for use on the necessary micro-scale and has been little used. Recently, there have been several reports of successful iodination of proteins by means of the enzyme peroxidase (Marchalonis, 1969; Morrison et al., 1971) and one such report (Thorell & Johansson, 1971) has indicated that hormone proteins of high specific radioactivity can be obtained. The procedures used for testing these preparations lacked the necessary rigour, but the evidence presented did suggest that the preparations were superior to material iodinated by the chloramine-T method. The enzymic method uses only minute amounts of H_2O_2 to catalyse the reaction, no other oxidizing or reducing agents being necessary. However, as in the chloramine-T method, the protein is exposed to the potentially noxious ¹²⁵I solution.

The new procedure described here avoids the three most likely causes of iodination damage associated with the chloramine-T method: exposure to oxidizing and reducing agents and to the ¹²⁵I solution. It also offers a further and radically different alternative, which may be considered alongside the existing group of methods for the direct iodination of tyrosine residues.

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APPENDIX

Preparation of N-Succinimidyl 3-(4-Hydroxyphenyl)propionate

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To circumvent the 'iodination damage' sometimes observed when ^{125}I is introduced into proteins and peptides by direct substitution, we envisaged a procedure in which the iodination step would be carried out in the absence of protein, the label first being introduced into a moiety which would then, in a separate step, be attached to the protein; a procedure which may be called labelling by conjugation, as distinct from labelling by substitution.

The reagent used as the carrier of the ^{125}I should be readily iodinated, preferably under the standard conditions in current use for the direct iodination of proteins; the labelled reagent should be readily isolated from the iodination mixture; it should react directly and selectively with groups commonly occurring in proteins without the need for separate chemical activation and with minimal manipulation; and the bond so formed should be chemically stable.

N-Succinimidyl 3-(4-hydroxyphenyl)propionate was selected as a reagent meeting these requirements. As it is a phenol it can be iodinated under the same conditions as the tyrosine in proteins, the iodinated reagent can be simply isolated by extraction and it will react in aqueous solution with the terminal or side-chain amino groups present in most proteins and peptides to form amide bonds.

The reagent was prepared by condensation of 3-(4-hydroxyphenyl)propionic acid with N-hydroxysuccinimide by using dicyclohexylcarbodi-imide, by the general procedure of Anderson *et al.* (1964).

Experimental

Preparation of N-succinimidyl 3-(4-hydroxyphenyl)-propionate

3-(4-Hydroxyphenyl)propionic acid (1.661 g; 10 mmol) and N-hydroxysuccinimide (1.151 g; 10 mmol) [both from Fluka A. G., Buchs, Switzerland, recrystallized from ethyl acetate by addition of di-2-propyl ether] in tetrahydrofuran (7 ml) were treated at −18°C with dicyclohexylcarbodi-imide (2.475 g; 12 mmol). The mixture was stirred at −18°C for 2 h, kept at room temperature for 10 h and treated with acetic acid (0.12 ml) to destroy excess of carbodi-imide. After 1 h more the mixture was diluted with ethyl acetate (10 ml), the dicyclohexylurea was filtered