The Preparation of a Semisynthetic Tritiated Insulin with a Specific Radioactivity of up to 20 Curies per Millimole

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1. This paper describes the semisynthesis of [^3H]insulin of high specific radioactivity which is indistinguishable from native insulin in its hypoglycaemic activity towards mice.
2. The maximum specific radioactivity so far obtained is 20 Ci/mmol. At this value the recommended scale of preparation is between 1 mCi (30 μg of product) and 10 mCi (300 μg of product). Reaction volumes are then of the order of 10 μl .
3. Special problems arise when the techniques of peptide synthesis are applied to the production of such small quantities of material. We describe these problems and discuss the means of overcoming them.

Research on the mode of action of insulin has depended greatly on the use of radioiodinated derivatives of the hormone. This paper describes a tritiated derivative which may be free of some of the disadvantages of the radioiodinated product. Radioiodination is fairly easy to perform (Greenwood, 1971) and a product of high specific radioactivity (50–1000 Ci/mmol) can be obtained. However, the protein can suffer 'iodination damage'. Also, it is difficult to restrict most methods of iodination to the introduction of only one iodine atom per molecule, at a single defined site (Hamlin & Arquilla, 1974). Even if this can be done, the iodine atom is large, and may affect the behaviour of the molecule (Sodoyez *et al.*, 1973), although some authors (e.g. Freychet *et al.*, 1971) have found no loss of biological activity. [The method of Bolton & Hunter (1973), although it avoids most other disadvantages, involves the attachment of a quite bulky organic substituent in addition to the iodine atom, and the loss of one of the protein's functional groups for every iodine atom introduced.]

We have prepared a tritiated insulin which, apart from the isotopic substitution in one amino acid, is chemically indistinguishable from the native molecule. It also appears to be indistinguishable from native insulin in its hypoglycaemic activity towards mice. Apart from the advantages to be expected from the minimal nature of the chemical change, it is possible to imagine uses for the derivative in humans in which ^3H labelling would be preferable to iodination from the point of view of minimizing the radiation energy absorbed by the subject. It should also be more suitable for radioautographic work than the iodinated derivatives and may be of use in assessing the true specificity of antibodies in the immunoassay.

As with an earlier, preliminary, preparation of [^3H]insulin (Borras & Offord, 1970) and the preparation of [^{13}C]insulin (Saunders & Offord, 1972) the method is semisynthetic. Protein semisynthesis has been briefly reviewed by Offord (1973). Scheme 1 shows the reaction sequence. The disulphide bridges have been kept intact throughout, and we have therefore avoided the serious losses normally associated with their re-formation.

Our approach differs from that of Yip (1972), who tritiated proinsulin by catalytic reduction. He then activated his product by proteolysis and obtained a mixture of des[^3H]amido-insulin (70%) and [^3H]insulin (30%). Yip (1972) was not limited to the maximum specific radioactivity of commercially available [^3H]phenylalanine; his product had a specific radioactivity of 84 Ci/mmol.

Our starting material, pig insulin, is easier to obtain than Yip's (1972) and we believe that the chemical nature of our product is more certain, even leaving deamidation aside. This last point also applies to the poly[^3H]alanyl-insulin prepared by Canfield *et al.* (1972).

Experimental and Results

Materials

Pig insulin (5 $\times$ crystallized, lot no. S121042) was the gift of Dr. J. Schlichtkrull, Novo Terapeutisk Laboratorium, Copenhagen, Denmark. [^3H]Phenylalanine was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-Phenyl[2,3- ^3H]alanine and L-[4- ^3H]phenylalanine were used at specific radioactivities of between 1 Ci/mmol (obtained by isotopic dilution with non-radioactive

Scheme 1. Reaction scheme for the preparation of semisynthetic [^3H]insulin

Full details of each of the numbered steps are given in the Experimental and Results section.

material) and 20Ci/mmol. Most of the work described here was carried out with the 2,3- ^3H -labelled amino acid, code TRK 266, batch 2, at 20Ci/nmol. Several batches of [^3H]phenylalanine gave relatively large quantities of a discoloured substance on being dried down, which interfered with the synthesis. If the sample was resuspended in 10 μl of water, the contaminant could be partially removed by centrifugation in the bench centrifuge. Dimethylformamide (laboratory-reagent grade, supplied by Fisons Scientific Apparatus, Loughborough, Leics., U.K.) was purified by being stood over K_2CO_3 for 24 h, then filtering and refluxing under reduced pressure over phthalic acid (analytical-reagent grade, supplied by BDH Chemicals, Poole, Dorset, U.K.) for 1 h before fractional redistillation under reduced pressure at 45–55°C. *N*-Ethylmorpholine (laboratory reagent, BDH) was purified by fractional redistillation, and phenyl isothiocyanate (puriss. grade, supplied by Koch–Light Laboratories, Colnbrook, Bucks., U.K.) by fractional redistillation under reduced pressure at 92°C. 1,2-Dimethoxyethane (BDH, laboratory reagent) and diethyl ether (BDH, analytical-reagent grade) were both dried over sodium metal and 1,2-dimethoxyethane was further purified by fractional redistillation. The absence of contamination by peroxides was checked with KI before use. Pyridine (BDH, analytical-reagent grade) was purified by fractional redistillation over BaO with exclusion of moist air. Trifluoroacetic acid was supplied by Koch–Light (puriss. grade) and fractionally redistilled before use. *t*-Butyl-azidoformate was also supplied by Koch–Light and used without further

purification. *NN'*-Dicyclohexylcarbodi-imide (crystalline, puriss. grade, Koch–Light) was dissolved in diethyl ether and filtered. The solution was evaporated to dryness and subjected to exhaustive desiccation under reduced pressure (oil pump) before use. *N*-Hydroxysuccinimide [crystalline, supplied by Sigma (London) Chemical Co., London S.W.6, U.K.] was recrystallized from ethyl acetate after filtration, by the method of Anderson *et al.* (1964). The melting point was 99–100°C. Ampholines for polyacrylamide-gel isoelectric focusing were supplied by LKB, Croydon, Surrey, U.K., and the gels were polymerized with *NNN'*-tetramethylethylenediamine (laboratory-reagent grade, BDH) and potassium persulphate. Silica-gel plates (SIL N-HR pre-coated plastic sheets) for t.l.c. were supplied by Macherey-Nagel and Co., Duren, Germany. Urea (BDH, analytical-reagent grade) was prepared as an 8M solution, which was filtered through macerated Whatman no. 3 paper and then passed down an 'Amberlite' MB3 monobed resin (BDH) column. Bovine serum albumin (fraction V) was a Pentex-grade product supplied by Miles Laboratories, Kankakee, Ill., U.S.A. DEAE-cellulose (DE52, pre-swollen microgranular anion-exchanger) for column chromatography was supplied by Whatman Biochemicals, Maidstone, Kent, U.K., and Sephadex G-25 by Pharmacia (G.B.) Ltd., London W.5, U.K. Chromatographic materials were prepared according to the supplier's instructions. Radioactive samples were counted in a Beckman liquid-scintillation counter (model LS-230) by using 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (Koch–Light;

5g/l) as the scintillant in toluene–Triton (2:1, v/v). Triton X-100 and toluene (scintillation-grade) were both supplied by BDH. Cultured human lymphocytes (strain Bristol 8, from G. D. Searle and Co., High Wycombe, Bucks., U.K.) were grown at 37°C under air + CO₂ (95:5) in RPMI 1640 culture medium (Gibco Bio-Cult, Paisley, U.K.) supplemented with 10% foetal calf serum (Gibco Bio-Cult) and penicillin (100 i.u./ml)–streptomycin (100 µg/ml) also supplied by Gibco Bio-Cult. Cell numbers were determined by using a haemocytometer. Cell viability was determined by exclusion of Trypan Blue (supplied by Flow Laboratories, Irvine, Ayrshire, U.K.). All other reagents were analytical-reagent grade.

Preparation

The numbering of the following sections corresponds to that used in Scheme 1. Although the description is lengthy, because of the importance of a number of minor points of technique, the overall process is easy to carry out. If stocks of the product of step (i) are held, [^3H]insulin can be prepared in 4 working days.

(i) The di-protected des-Phe^{B1} derivative was prepared in batches of about 50 mg by the method of Geiger *et al.* (1971), except that the solvent (R. Geiger, personal communication) used for the introduction of the Boc (t-butoxycarbonyl) groups was methanol–5% (w/v) NaHCO₃ (4:1, v/v). Also, the intermediate [N^{α} -Boc-Gly^{A1}, N^{ϵ} -Boc-Lys^{B29}]-insulin was purified from other side products by chromatography on DEAE-cellulose by using the system reported by Borrás & Offord (1970), as was the final derivative of this step. The purity of the products was checked by dansylation and isoelectric focusing in polyacrylamide gels. The dialysed freeze-dried material deteriorated on storage unless it was kept sealed under N₂ at –20°C in the dark. It was found convenient to freeze-dry the material in a number of ampoules for this purpose. To ensure accuracy in dispensing the protein for the coupling step (see below) each ampoule contained exactly 0.6 mg. Only 30–300 µg of this was used for the synthesis (see below) and the rest was discarded.

(ii) The standard methods for the introduction of the Boc groups [e.g. Schnabel (1967) and Schwyzler *et al.* (1959)] were found to be unsuitable at the small scale (of the order of 25 nmol) dictated by the cost of the ^3H -labelled amino acid. We did not find the use of large excesses of solvents and azidoformate satisfactory; all the quantities had to be scaled down, with the result that the reaction volume was as low as 5 µl.

The normal methods of stirring and pH control were not possible at this level, but the following procedure proved satisfactory. The amino acid was dried down in a glass tube of 2 cm internal diameter

which had been extended by sealing a drawn-out Durham tube (6 mm internal diameter tapering to zero in 22 mm) to an opening at the base. In this way 5 ml of stock solution of the radioactive amino acid could be freeze-dried, the amino acid washed down to the drawn-out end, and redissolved for reaction in as little as 5 µl with only minor losses. Where the amino acid was contaminated with insoluble material this vessel could be used in a bench centrifuge (see above).

The dried material was redissolved in water (25 nmol/µl) and 1.5 vol. each of dimethylformamide and ethanol were then added. The final concentration of amino acid was 6.25 nmol/µl. *N*-Ethylmorpholine was added until a 0.1 µl sample of the mixture gave colours on moist narrow-range pH papers corresponding to pH 8.5 (royal blue on BDH 'pH 6–8', barely perceptible violet on BDH '8–10'). The quantity of base required is about 1 µl/100 nmol of amino acid, and little harm seems to be caused by a moderate excess. This is in contrast with the problems posed by the use of triethylamine (R. E. Offord, unpublished work). Then 50 nmol of *t*-butylazidoformate was added per nmol of amino acid and the vessel was stoppered. The mixture was dried down after 20 h at 37°C and 0.1 µl of 0.05 M-HCl added per nmol of amino acid. The *N*^α-Boc-phenylalanine was extracted with 3×4 vol. of ethyl acetate. The acid treatment is thought to be necessary to ensure that the carboxyl group is in the un-ionized form for step (iii), as well as to facilitate the extraction.

The extracted material was compared with authentic non-radioactive standards by t.l.c. on silica gel in chloroform–methanol (9:1, v/v). The radioactive material was located by scanning with a Berthold model LB 2723 windowless gas-flow counter. Phenylalanine has *R_F* 0, whereas the Boc derivative has *R_F* 0.1–0.2 in the carboxylate form and 0.5–0.6 in the carboxyl form. The radioactive product moved as a single spot to an *R_F* corresponding to the carboxyl form, and yields of 90% could be obtained. Small departures from the above procedure led to a drastic fall in the yield. Step (ii) was, in fact, the most troublesome of all, and the only one likely to give any serious difficulty.

(iii) The ethyl acetate layer was dried down exhaustively on the oil pump to free it from all traces of the reagents used in step (ii). The Boc derivative was dissolved in 1,2-dimethoxyethane (25 nmol/µl) carrying one equivalent of *N*-hydroxysuccinimide. After cooling to 2–4°C an equal volume of 1,2-dimethoxyethane carrying 1.1 equivalents of *NN'*-dicyclohexylcarbodi-imide was added. After 20 h at 2–4°C the esterification reaction had gone to completion. The *R_F* of the product (a single spot) in the system mentioned above was 0.7–0.8, which is that of authentic non-radioactive material. Crystals of dicyclohexylurea were visible in the mixture. It is

Fig. 1. Isoelectric focusing of ^3H -labelled derivatives of insulin

Isoelectric focusing in polyacrylamide gels (7.3% acylamide, 0.19% NN' -methylenebisacrylamide) between pH 5 and 7. The gels were scanned for radioactivity as described in the Experimental and Results section. (a) Coupled product ($[N^{\alpha}\text{-Boc-Gly}^{\text{A1}}, N^{\alpha}\text{-Boc-}^3\text{H]Phe}^{\text{B1}}, N^{\alpha}\text{-Boc-Lys}^{\text{B29}}\text{]insulin}$). (b) Deprotected product ($[\text{Phe}^{\text{B1}}, ^3\text{H]insulin}$) run after having been dried down. There is evidence of deamidation (see the Experimental and Results section). (c) A deprotected product that had never been dried down after purification by gel filtration. The positions are shown of the bands corresponding to authentic samples of native insulin (○) and of (1) mono-, (2) di- and (3) tri-acyl insulin.

particularly important that all reagents used in step (iii) should be dry.

(iv) We found that the active ester from step (iii) decomposed if it was dried down. (We have not investigated whether or not this is a radiochemical phenomenon, although it did not occur with non-radioactive material made by a scaled-up version of the procedure.) The product is therefore used directly by addition of a solution of the insulin derivative (6 ng/ μl in dimethylformamide) from step (i). We used a tenfold molar excess of ester over protein. The 1,2-dimethoxyethane was removed at once by gentle blowing with N_2 . The pH of this mixture was adjusted to greater than 8.5 by the addition of N -ethylmorpholine (approx. 0.1 $\mu\text{l}/\mu\text{l}$ of reaction solution).

The coupled product was precipitated after 24 h at room temperature by the addition of 5 vol. of diethyl ether followed by centrifugation of the stoppered vessel on a bench centrifuge. A Durham tube drawn to a point was used as the reaction vessel for coupling and this permitted the centrifugation of the small volumes of coupling solution used.

In spite of the small quantities involved, the precipitation proved to be an efficient means of separating the protein from excess of active ester (see Fig. 2 below). It ought to be possible to reuse radioactive material obtained from the ether supernatant

Fig. 2. Gel filtration of $[\text{}^3\text{H}]$ insulin

Gel filtration (Sephadex G-25) of the product of step (v) ($[\text{Phe}^{\text{B1}}, ^3\text{H]insulin}$) (○) and of the supernatant from step (iv) (●) after ether precipitation of the protein. (The radioactive peak in the region of fraction 17 presumably corresponds to $[\text{}^3\text{H}]$ phenylalanine derivatives.) Fraction size was 1.7 ml.

(probably after total hydrolysis to the free amino acid), thereby considerably decreasing the cost of the semisynthesis.

The product migrates on isoelectric focusing in polyacrylamide gels as a single symmetrical peak to the expected position, that occupied by triacyl insulins. In spite of the very high self-shielding of such gels, it was possible to locate 50–100 μCi of material in the gels by scanning with the Berthold counter (Fig. 1a).

(v) The precipitate from step (iv) was dissolved in trifluoroacetic acid (6 ng of protein/ μl). Deprotection is complete after 45 min at room temperature. We have found that, in contrast with large-scale syntheses with non-radioactive material, the drying down of the acid solution leads to loss of side-chain amide groups from the $[\text{}^3\text{H}]$ insulin. Bands corresponding to $[\text{}^3\text{H}]$ insulin lacking one, two and three amide groups are seen on isoelectric focusing (Fig. 1b). The deprotection was therefore carried out in trifluoroacetic acid containing 1% bovine serum albumin and after 45 min the acid solution was added to 100 vol. of 50 mM- $(\text{NH}_4)_2\text{HPO}_4$ (adjusted to pH 7.3 with H_3PO_4). The buffer solution was prepared with 5% (w/v) bovine serum albumin. The pH fell to 2 and was restored to pH 7.3 with 1 M- NH_3 . Any active ester that survived the preceding operations should have been destroyed before reacting further with the $[\text{}^3\text{H}]$ insulin.

The buffered solution was then subjected to gel filtration in 5 mM-sodium phosphate buffer (pH 7.3) on Sephadex G-25 (column dimensions 0.8 cm $\times$

66cm) to separate the protein from the small molecules (Fig. 2). The recovery of radioactivity was approximately quantitative. The final concentration of the [^3H]insulin was 1 $\mu\text{g}/\text{ml}$ (i.e. about 3×10^6 c.p.m./ml at high specific radioactivity). The serum albumin concentration was checked spectrophotometrically and adjusted, if necessary, to 0.5% (w/v).

The deprotected material gave a single, symmetrical band of radioactivity on isoelectric focusing, which ran at the expected position of native insulin (Fig. 1c). The u.v. spectrum of a sample prepared as above, except that bovine serum albumin had not been used at any stage, was taken in a micro-cell in a Unicam SP.800 spectrophotometer. It closely resembled that of native insulin and showed an overall protein yield of 90%.

When serum albumin was present, the yield had to be assessed by radioactivity. The preparative methods outlined above make it possible to conclude that the only appreciable losses of protein are likely to be in the gel-filtration step (all the previous operations are carried out in the same tube, without transfers, and Fig. 2 shows that the supernatant from the ether precipitation is free of protein). The losses on gel filtration can be estimated by comparing the total radioactivity of the column effluent with that of the sample before it was applied. As in the u.v. determination, the calculated overall yield is 90%. The specific radioactivity of the product can then be derived and is, within experimental error, that of the [^3H]phenylalanine.

Chemical characterization of the product

The chemical characterization of 30 μg of protein is difficult. All the above steps were therefore carried out with non-radioactive or isotopically diluted material at a slightly larger scale. Sufficient material was obtained for end-group determination of the products of steps (iv) and (v) and the amino acid analyses have been undertaken of the products of analogous but still larger-scale preparations. All results were satisfactory. The scaled-up products of steps (iii) and (v) were crystallizable. The melting point of the active ester was 150–152°C [crystallized from ether–light petroleum (b.p. 60–80°C)] compared with 152–153°C quoted in the literature (Anderson *et al.*, 1964).

Biological characterization of the product

Fig. 3 compares the hypoglycaemic activity of our product with that of native insulin at two dilutions. The concentrations of [^3H]insulin were standardized for binding experiments by means of radioactivity, as above. Separate experiments (P. J. R. Phizackerley & M. R. Richards, unpublished work) were carried out with native insulin to ensure that these doses

Fig. 3. Bioassay of [^3H]insulin by blood-glucose depression in mice

[^3H]Insulin (●) and native pig insulin (○) were assayed by intravenous injection into mice. Blood samples were taken immediately before injection and after 10 and 20 min by orbital bleeding and the glucose concentrations determined. A solution of 0.9% NaCl (Δ) was used as a control. Each point is the mean of ten independent observations, and the S.E.M. is shown by bars. The assay was carried out at the two insulin concentrations indicated. (These assays were carried out by P. J. R. Phizackerley and M. R. Richards.)

were on the linear part of the dose–response curve. The particular assays shown were carried out on the day after the completion of step (v) above. Other assays, using fewer mice, were satisfactory on this and other batches. Lower activity was found for chemically damaged material. After an interval of 12 weeks, during which the material had been stored frozen but with occasional thawing and refreezing the assays were repeated in as near an identical fashion as possible. The results showed a marked decrease of biological activity, which corresponds to the fact that after 9 weeks our product only stimulated respiration in isolated adipocytes by about 50% of the expected amount (J. Ellis & R. H. Jones, personal communication). The mode of storage could be improved; it is often unwise to freeze molecules labelled at high specific radioactivity.

Binding studies were carried out by the method of Gavin *et al.* (1973). Cells were harvested by centrifugation in a bench centrifuge from the culture medium and resuspended in buffer containing

25mM-Tris, 120mM-NaCl, 1.2mM-MgSO₄, 2.5mM-KCl, 10mM-glucose, 1mM-EDTA and 1% bovine serum albumin, adjusted with HCl to pH7.6. The cells were incubated in the presence of the [³H]-insulin and the amount of insulin bound was determined by layering samples (1 ml) after incubation on 0.8ml of the assay buffer containing 2% bovine serum albumin, which was contained in Pasteur pipettes sealed at the narrow end. The cells were spun down and the portion carrying the pellet (a length of tube of about 20mm per 10⁷ cells) was snapped off and the cells were expelled into a counting vial. Non-specific binding (defined as the radioactivity associated with the cell pellet after incubation with labelled insulin in the presence of a large excess of unlabelled insulin) was assessed by incubation in the presence of 20 μM native unlabelled insulin. The non-specific component of the radioactivity found in the pellet varied from 5 to 20%. Binding of the [³H]insulin (500pM) was linear in the range 5 × 10⁵–2 × 10⁷ cells/ml.

Discussion

We believe that our material is worthy of further evaluation. Assays *in vivo* appear promising even in the crude unfractionated product, in contrast with the unfractionated products of most other routes to modified insulins. If it were necessary to purify the material further, immunological methods would probably be best. Since the half-life of ³H is so long, it would also be worthwhile to seek the optimum conditions for long-term storage.

Although the specific radioactivity of our product is high, it is only one-tenth or less of that of a typical iodinated insulin. Nonetheless, our material appears to be sufficiently radioactive for the investigation of the behaviour of insulin at physiological concentration both *in vivo* and *in vitro*. At a counting efficiency of 15%, a dose of 2 μg/kg (50 μ-i.u./kg) body wt. would give a usefully measurable radioactivity in body fluids. Iodinated insulin has been used *in vitro* to determine the number of insulin receptors of various cells and membrane fractions and the strengths and velocities of the binding processes involved [see, e.g. Desbuquois & Cuatrecasas (1973), Cuatrecasas (1974) and Roth (1973)]. Some of these authors used insulin at a concentration of about 1 pM, but the significant parts of the titrations of the binding sites have usually come at considerably higher concentrations. We have found it possible to investigate the interaction between our product and cultured lymphocytes at concentrations of 80pM and above (P. A. Halban & R. E. Offord, unpublished work). For example the time-course of binding at 22°C was as shown in Fig. 4. Maximal binding occurs at about 20min, which corresponds well to previous observations (e.g. those of Gavin *et al.*, 1973). The

Fig. 4. Time-course of [³H]insulin binding to cultured human lymphocytes

Cultured human lymphocytes (between 5 × 10⁶ and 6 × 10⁶ cells/ml) were incubated at 22°C in the presence of 160pM-insulin (a) and 1.6nM-insulin (b). Each point indicates the mean ± S.E.M. of three independent observations, corrected for non-specific binding. Separate determinations of the non-specific binding were carried out for each point on the two lines by using 20 μM-native insulin.

cells were found to be viable by exclusion of Trypan Blue after 180min in the assay buffer in the presence of the [³H]insulin, both with or without excess of unlabelled insulin.

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