The preparation of tritiated insulin specifically labelled by semisynthesis at glycine-A1

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We have prepared and characterized semisynthetic [Gly^{A1-3}H]insulin. The preparation was carried out at specific radioactivities ranging from 1Ci/mmol to 44Ci/mmol. The largest quantity prepared in any one synthesis was 3.5 mCi. Chemical degradation showed that the label was in its expected position in the molecule. The semisynthetic product behaved authentically on reversed-phase h.p.l.c. and radioimmunoassay. It gave the expected profiles of biological activity as regards depression of blood sugar concentration in rats and stimulation of conversion of glucose into lipid in isolated rat fat-cells. We discuss some applications for which this tracer would be particularly suited. An expanded version of this paper, containing full experimental details of the semisynthesis and characterization of [Gly^{A1-3}H]insulin, has been deposited as Supplementary Publication SUP 50129 (30 pages) at the British Library (Lending Division), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1985) **225**, 5.

INTRODUCTION

Much of the current knowledge about the mechanism of insulin action and the pharmacokinetics of injected insulin has been obtained with the use of radioactive insulin derivatives. One such derivative can be produced by the replacement of phenylalanine-B1 with its tritiated analogue by using semisynthesis (Halban & Offord, 1975). This isotopic substitution represents a minimal alteration to the hormone and gives a chemically defined product. At physiological concentrations the biological activity and the metabolism of this tritiated insulin have been shown to be indistinguishable from that of the native hormone in all systems so far studied (Halban *et al.*, 1976; Morishima *et al.*, 1985). An alternative derivative is insulin labelled with radioactive iodine, usually by covalent linkage to tyrosine side chains. Insulin has four such residues, and high degrees of iodination are known to alter its biological activity (Izzo *et al.*, 1964). When iodination is restricted to the incorporation of a single iodine atom into tyrosine-A14 a derivative with full biological activity can be obtained (Glieman *et al.*, 1979). However, the iodine atom is comparable in size with the phenyl ring of an aromatic amino acid and is very hydrophobic. Therefore it cannot be certain that such a tracer will behave authentically in every situation. In spite of this difficulty, a great deal of research has had to be carried out with the use of this type of labelling.

One of the advantages of [Phe^{B1-3}H]insulin is that its label is in a known position, and this fact can be exploited during investigation of the tritiated species found in plasma after its injection (Davies *et al.*, 1980). However, with this tracer, a few chemical alterations to the hormone would be difficult to detect by chromatographic and electrophoretic techniques. One such alteration would be the loss of one or a few amino acid residues from the amino terminus of the A-chain of insulin, since such

a change would not alter greatly the mass or charge of the molecule. In order to overcome this problem we have tritiated insulin at glycine-A1. We have used the techniques developed for the semisynthesis of [Phe^{B1-3}H]insulin together with other semisynthetic processes.

METHODS AND RESULTS

Semisynthesis and characterization of [Gly^{A1-3}H]insulin

Full experimental details of the semisynthesis and characterization of [Gly^{A1-3}H]insulin have been deposited as Supplementary Publication SUP 50129 at the British Library (Lending Division). The following is an abbreviated description.

The reaction scheme for the synthesis of [Gly^{A1-3}H]insulin is shown in Scheme 1. Protection of the amino group of glycine-A1 with the t-butyloxycarbonyl (Boc) group and purification of this product by ion-exchange chromatography (Saunders & Offord, 1977) is followed by protection of the amino groups of phenylalanine-B1 and lysine-B29 with the methanesulphonylethylloxycarbonyl (Msc) group (Tesser & Balvert-Geers, 1975; Rees & Whittle, 1980). The Msc group is stable in strong acid, allowing the removal of the Boc group from the insulin derivative without deprotection of the other amino groups. Glycine-A1 can then be cleaved from the molecule by the Edman reaction (Saunders & Offord, 1977). The product of these reactions can be used to couple to Boc-[³H]glycine hydroxysuccinimide ester, prepared, with minor modifications, as described by Halban & Offord (1975). The coupling occurs specifically at the amino group of isoleucine-A2. Removal of the protecting groups gives an insulin derivative in which the glycine-A1 has been replaced by a tritiated glycine residue. The product was purified by reversed-phase h.p.l.c. Chemical characterization (performate oxidation,

Abbreviations used: Boc-, t-butyloxycarbonyl-; Msc-, methanesulphonylethylloxycarbonyl-.

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Scheme 1. Semisynthesis of [Gly^{A1-3}H]insulin

For experimental details see the text and Supplementary Publication SUP 50129. Abbreviations: Boc₂O, di-*t*-butyl dicarbonate; ONSu, hydroxysuccinimide ester; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate. Key to pathway components: (I) insulin; (II) [N^α-Boc-Gly^{A1}]insulin; (III) [N^α-Boc-Gly^{A1}, N^ε-Msc-Phe^{B1}, N^ε-Msc-Lys^{B29}]insulin; (IV) [N^α-Msc-Phe^{B1}, N^ε-Msc-Lys^{B29}]insulin; (V) [N^α-Msc-Phe^{B1}, N^ε-Msc-Lys^{B29}, des-Gly^{A1}]insulin; (VI) [Gly^{A1-3}H]insulin.

Edman degradation; see Supplementary Publication SUP 50129) of the product shows that the label is indeed present at the *N*-terminus of the A-chain. Fig. 1 shows the elution profile of the tritiated insulin on reversed-phase h.p.l.c. after storage for 2 months in a solution that had been sterilized by membrane filtration (Sartorius 11107,

Fig. 1. H.p.l.c. of [Gly^{A1-3}H]insulin

[Gly^{A1-3}H]insulin was mixed with 10 µg of unlabelled pig insulin and applied to a reversed-phase h.p.l.c. column (0.4 cm × 25 cm; C18, particle size 5 µm). It was eluted with a gradient of acetonitrile. (a) Absorbance at 214 nm; (b) radioactivity profile determined by collecting fractions of the column effluent and determining their radioactivities in a liquid-scintillation counter. Peaks: I, solvents; II, pig insulin; III, methyl *p*-hydroxybenzoate.

0.2 µm pore size) and saturated with the bacteriostat methyl *p*-hydroxybenzoate.

The overall yield in the preparation was 3% to 7% with respect to radioactive amino acid. From 50 mCi of [³H]glycine starting material, 3.5 mCi of tritiated insulin was prepared at 17 Ci/mmol. This is equivalent to 1.2 mg of product.

Biological characterization of [Gly^{A1-3}H]insulin

Radioimmunoassay and measurement of blood sugar depression concentration in rats. These assays were carried out by Dr. P. Halban and Mr. A. Gjinovci of the Institut de Biochimie Clinique, Geneva, Switzerland. Comparisons were made with authentic unlabelled insulin. For these assays the concentration of tritiated insulin was calculated from the specific radioactivity of the tritiated glycine used for coupling (with, where necessary, a correction made for the protein-chemical purity of the material being tested).

On the linear part of the curve, the ratio between the radioimmunological activity of the tritiated insulin and

Fig. 2. Depression by [$\text{Gly}^{\text{A1-3}}\text{H}$]insulin of blood sugar concentration in rats

Rats were injected intravenously with [$\text{Gly}^{\text{A1-3}}\text{H}$]insulin ($8 \mu\text{g/kg}$ body wt.) ($\square$) or the same dose of Actrapid insulin ($\circ$). Blood samples were taken at the time intervals indicated and the plasma glucose concentrations were determined by an enzymic method. The graph shows the mean and range of single determinations in three animals.

Fig. 3. Stimulation by [$\text{Gly}^{\text{A1-3}}\text{H}$]insulin of the incorporation of ^{14}C from [^{14}C]glucose into lipids in isolated rat fat-cells

Isolated rat fat-cells were incubated in the presence of [^{14}C]glucose and either [$\text{Gly}^{\text{A1-3}}\text{H}$]insulin ($\square$) or unlabelled pig insulin ($\circ$). Lipids were extracted into a toluene-based scintillant and the radioactivities determined in a liquid-scintillation counter. The graph shows the mean and range of three or four determinations at each concentration.

that of the authentic insulin was 1.04:1 ($n = 3$, individual values 1.0, 1.0 and 1.13). The corresponding ratio for the assay of depression of blood sugar concentration was 1.04:1. The individual values obtained in this comparison were: authentic insulin 208, 272 and 283; tritiated insulin

234, 260 and 298 (units as defined in Halban *et al.*, 1981). Further details of this comparison are given in Fig. 2.

In addition, the metabolic clearance of [$\text{Gly}^{\text{A1-3}}\text{H}$]insulin in dogs has been determined at various plasma concentrations of insulin (J. G. Davies, T. Watarai, S. Pye & J. Radziuk, unpublished work). The metabolic clearance rate in each case was very similar to that determined with [$\text{Phe}^{\text{B1-3}}\text{H}$]insulin, which had already been shown to be a valid tracer in this system (Morishima *et al.*, 1985).

Isolated rat fat-cells. The stimulation of the incorporation of [^{14}C]glucose into lipid in isolated rat fat-cells was determined by the method of Moody *et al.* (1974). The activity of the tritiated insulin seems not to differ noticeably from that of a standard insulin preparation (Fig. 3).

DISCUSSION

A new tritiated tracer for insulin has been prepared. The label was specifically incorporated into the A1 position of the molecule by the semisynthetic replacement of glycine with tritiated glycine. The specific radioactivity of the tracer depends on that of the commercially available tritiated glycine. Thus during this study the highest specific radioactivity obtained was 44Ci/mmol. Specific radioactivities of around 20 Ci/mmol are adequate for most tracer studies *in vivo* (Berger *et al.*, 1979). On the other hand, analysis of receptor interactions is difficult at such specific radioactivities, but even here some useful results can be obtained (Misbin *et al.*, 1980).

The long half-life of tritium means that optimum storage conditions are important. Two months' storage under the best conditions found so far left 95% of the radioactivity still in the form of intact insulin as assayed by h.p.l.c. The effect of longer storage has not yet been investigated.

We believe that the tritiated derivative merits evaluation as a tracer for insulin. It represents the first tracer to be labelled only at the N-terminus of the A-chain of insulin. The label is thus present in part of the proposed receptor-binding region (Pullen *et al.*, 1976); it must therefore not disturb the structure of this region of the molecule, or the behaviour of the tracer could be severely altered with respect to insulin. Given that this condition appears to be satisfied in the present study, the fate of a part of the molecule particularly concerned with biological activity can now be followed. Thus the tracer may prove particularly useful in studies of insulin degradation.

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REFERENCES

- Berger, M., Halban, P. A., Assal, J.-P., Offord, R. E., Vranic, M. & Renold, A. E. (1979) *Diabetes* **28**, Suppl. 1, 53-57

- Davies, J. G., Offord, R. E., Halban, P. A. & Berger, M. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Peptides* (Brandenburg, D. & Wollmer, A., eds.), pp. 517–523, Walter de Gruyter, Berlin and New York
- Glieman, J., Sonne, O., Linde, S. & Hansen, B. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1183–1190
- Halban, P. A. & Offord, R. E. (1975) *Biochem. J.* **151**, 219–225
- Halban, P. A., Karakash, C., Davies, J. G. & Offord, R. E. (1976) *Biochem. J.* **160**, 409–412
- Halban, P. A., Berger, M., Gjcinovci, A. & Renold, A. E. (1981) *Diabetes Care* **4**, 238–243
- Izzo, J. L., Roncone, A., Izzo, M. J. & Bale, W. F. (1964) *J. Biol. Chem.* **239**, 3749–3754
- Misbin, R. I., Davies, J. G., Offord, R. E., Halban, P. A. & Mehl, T. D. (1980) *Diabetes* **29**, 730–735
- Moody, A. J., Stan, M. A., Stan, M. & Glieman, J. (1974) *Horm. Metab. Res.* **6**, 12–16
- Morishima, T., Bradshaw, C. G. & Radziuk, J. (1985) *Am. J. Physiol.* **248**, E203–E208
- Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Glieman, J. & Gammeltoft, S. (1976) *Nature (London)* **259**, 369–373
- Rees, A. R. & Whittle, M. R. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Peptides* (Brandenburg, D. & Wollmer, A., eds.), pp. 327–335, Walter de Gruyter, Berlin and New York
- Saunders, D. J. & Offord, R. E. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 1469–1474
- Tesser, G. I. & Balvert-Geers, I. C. (1975) *Int. J. Pept. Protein Res.* **7**, 295–305

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