B1-3,5-diiodotyrosine Insulin: A Valid Tracer for Insulin

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Summary. Insulin, specifically substituted at the Phe^{B1} position with 3,5-diiodotyrosine, has been tested in several biological and immunological systems. Immunoreactivity was assessed using antisera specific for different parts of the insulin molecule. Biological activity in vitro was estimated on isolated rat fat cells. In vivo bioactivity (hypoglycaemia) and metabolism (metabolic and urinary clearance rates, half-life, apparent distribution space) were measured by infusion of the material into greyhounds. The results indicated that this B1-labelled insulin preparation was biologically fully active and, unlike randomly labelled preparations of iodinsulin, was metabolised with kinetics indistinguishable from those of the unlabelled hormone. We suggest that this material is a valid tracer for insulin, fulfilling the criteria of high specific activity and biological identity to the native hormone.

Key words: Iodinsulin, insulin metabolism, insulin analogues, biological activity, tracer insulin.

Much research on the mode of action and the metabolism of insulin has depended on the use of radioactive derivatives of the hormone. However the validity of iodine-labelled insulin as a tracer for such studies has been seriously questioned. Reports on the biological activity of various preparations are conflicting. Perhaps the clearest evidence against the biological identity of iodinated and native insulin is the work of Ooms et al. [1, 2] on the in vivo metabolism of the hormone. In measurements of metabolic clearance rates, unlike bioassays, the fate

of iodine-labelled molecules can be examined independently of the uniodinated species present in all heterogeneous preparations. Ooms' results indicated clearly that irrespective of the degree of iodination the metabolic clearance rate of iodinsulin was both reduced and altered in character.

The position of iodine incorporation into the tyrosine and histidine residues of insulin is not defined in most iodination methods. Variations in conditions may alter the susceptibilities of particular residues for substitution [3–5], however the A14 and/or A19 tyrosines are predominantly iodinated. A19 is an invariant residue in the insulin sequence [6] and is thought to be in an area important in maintaining the structural integrity necessary for insulin action. One way of making a valid tracer for insulin would be by labelling specifically a region of the molecule that is not involved in activity.

Insulin modified at the B-1 position appears to retain normal biological [7, 8] and metabolic characteristics (personal observation). Krail et al. [9] replaced Phe^{B1} with p-iodophenylalanine and this preparation was 62–73% active in vitro [10]. Halban et al. [11] prepared a tritiated Phe^{B1} derivative which showed full biological activity initially but appeared to deteriorate on storage. Furthermore it was of relatively low specific activity. We have examined the biological activity, immunoreactivity and the kinetics of metabolism of insulin substituted at Phe^{B1} with 3,5 diiodo¹²⁷ tyrosine.

Materials and Methods

The iodinated compound studied was prepared by replacing the phenylalanine at the N terminus of the B chain of porcine insulin with 3,5-di¹²⁷I-tyrosine (overall yield 10–15%) [12]. The reference stand-

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Fig. 1. Inhibition of ¹²⁵I insulin binding to antiserum 12 (above) and GP5 (below) by increasing concentrations of porcine insulin (●—●) and diiodoinsulin (▲—▲). Results are the mean of two determinations in one assay. The two antisera differ markedly in their ability to distinguish between insulin and the diiodinated derivative

Fig. 2. Rat fat cell lipogenesis from D3-³H-glucose: unstimulated (o) and in the presence of increasing concentrations of porcine insulin (●—●) or of diiodoinsulin (▲—▲). The results are the mean $\pm$ S.E.M. of three observations in one assay. There is no significant difference between the materials

ard in the biological assays was either porcine insulin (Novo Co., Copenhagen) or repurified bovine insulin (Dr. D. Brandenburg, Aachen). Diiodoinsulin was weighed and dissolved in hydrochloric acid (1 mmol l^{-1}) to a concentration of $0.165 \text{ mmol l}^{-1}$. Protein content was checked by micro-Kjeldahl analysis. The solution was further diluted in saline (9 g/l NaCl) containing 10 g/l human serum albumin (Blood Products Laboratory, Lister Institute, Herts) to a concentration of $3.3 \mu\text{mol l}^{-1}$, sterilised and stored frozen.

Kjeldahl Analysis

The nitrogen contents of diiodoinsulin and of bovine insulin ($\times 6$ recrystallised, Boots Co. Ltd., Nottingham) were estimated by micro-Kjeldahl analysis [13].

Immunoreactivity

Immunoreactivity was assessed using a double-antibody radioimmunoassay [14] and two different antisera known to react with different sites on the insulin molecule [15]. Antiserum 12 was insensitive to modifications in the B-1 region but reacted with lower affinity to analogues modified in the A1, B30 area. Conversely, GP5 antiserum distinguished insulins modified in the B-1 region from native insulin but was relatively insensitive to alterations elsewhere in the molecule.

Bioactivity

a) In vitro: Isolated fat cells were prepared by a modification [16] of the method of Rodbell [17] from the epididymal fat tissue of 100–120 g male Wistar rats. Aliquots of the cell pool were incubated in Krebs-Ringer bicarbonate buffer containing human serum albumin (10 g l^{-1}), glucose (0.55 mmol l^{-1}) and $0.1 \mu\text{Ci } 3\text{-}^3\text{H-glucose}$ (Radiochemical Centre, Amersham) in 1.0 ml incubates. Dilutions of insulin and diiodoinsulin were included in the assay and incorporation of tritium into toluene-extractable lipids determined [18].

Data from the linear portion of the log dose-response curves were analysed using parallel-line bioassay techniques as described by Finney [19].

b) In vivo: Experiments were performed in intact, anaesthetised greyhounds by a priming-dose, constant infusion technique [20] based on that used in humans by Sönksen et al. [14]. Standard bovine insulin or diiodoinsulin were infused at equimolar

Fig. 3. Canine plasma glucose response to equimolar infusions of bovine insulin ($\blacksquare$ — $\blacksquare$, mean $\pm$ S.E.M., $n = 6$) and of diiodoinsulin (o—o, individual data, 3 experiments). The infusion was started at $t = 30$ min. Its rate was doubled every thirty minutes until $t = 150$ min when the pump was stopped. Diiodoinsulin is shown to have the same activity as bovine insulin

rates (total dose $0.37 \text{ nmol/kg} \pm 0.043 \text{ SEM}$) to achieve four different steady state concentrations spanning the normal physiological range. Blood and urine samples were collected at regular intervals. Plasma glucose was measured with a Beckman glucose analyser.

Metabolism

The greyhound experiments described above allowed calculations of the parameters of the *in vivo* metabolism of insulin and diiodoinsulin [14]. Serum and urine concentrations of the two materials were measured by radioimmunoassay using antiserum 12 and standards of bovine or diiodoinsulin. Urine samples were dialysed against 0.05 mol l^{-1} phosphate buffer, pH 7.5 for twenty four hours before measurement. The metabolic and urinary clearance rates were calculated at the four steady state concentrations of hormone achieved, and the half-decay time ($T_{1/2}$) and apparent distribution space derived.

Results

Micro-Kjeldahl

The nitrogen content of the diiodoinsulin preparation was found to be 14.6% (mean of 3 assays). This compares with a nitrogen content of 14.7% for the bovine insulin.

Immunoreactivity

Diiodoinsulin was indistinguishable from standard porcine insulin in its ability to compete with ^{125}I -insulin for binding to antiserum 12 (Fig. 1, top). The lower graph of Figure 1 illustrates that the diiodinated derivative was considerably less effective than insulin in competing with labelled insulin for binding to GP5 antiserum. This is consistent with an insulin modified in the B1 position.

Bioactivity

The relative abilities of porcine insulin and diiodoinsulin to stimulate rat fat cell lipogenesis are shown in Figure 2. The two preparations appeared identical. Calculation of the results from three separate experiments indicated that the diiodo derivative had a mean potency 108.8% that of insulin (95% fiducial limits 129.9–96.4%).

Similarly the material had full biological activity *in vivo* when assessed in three dogs. The fall in plasma glucose concentration on infusion of diiodoinsulin was indistinguishable from that caused by insulin (Fig. 3).

Metabolism

Calculation of the metabolic clearance rate at four different steady-state concentrations (Fig. 4) indicated that the metabolism of diiodoinsulin and the

Fig. 4. Relationship between metabolic clearance rates (MCR) of insulin ($\square$ — $\square$, mean $\pm$ 2 S.D. $n = 9$) and of diiodoinsulin ($\circ$ — $\circ$, individual data, 3 experiments) and plasma steady state concentration. The two materials were indistinguishable and there was a fall in MRC with increasing hormone concentration in both instances, indicating that both are metabolised by a similar saturable pathway

Table 1. Half-decay time ($T^{1/2}$) apparent distribution space and urinary clearance rates of bovine insulin and diiodoinsulin in dogs. The two materials are not significantly different by unpaired 't' test in any of these respects

	Bovine Insulin	Diiodoinsulin	P
$T^{1/2}$ (min) mean $\pm$ S.E.M.	4.8 ± 0.5 $n = 7$	5.9 ± 0.3 $n = 3$	> 0.1
apparent distribution space — ml/kg mean $\pm$ S.E.M.	114 ± 13 $n = 7$	115 ± 4 $n = 3$	> 0.98
Urinary clearance rate ml/min/kg mean $\pm$ S.E.M.	0.115 ± 0.025 $n = 31$	0.06 ± 0.006 $n = 12$	> 0.1

native material were indistinguishable. As with unlabelled insulin, the metabolic clearance rates fell with increasing plasma concentrations of the derivative and the values from all three experiments lay within the normal range for unlabelled insulin. In addition, $T^{1/2}$ and apparent distribution space were not significantly different for the two materials. Urinary clearance showed no tendency to change with increasing plasma concentration and again, insulin and diiodoinsulin were indistinguishable in this respect (Table 1).

Discussion

Evidence that iodinated insulins are biologically active and metabolised normally has been contradictory. Since the demonstration of a direct relation-

ship between the degree of iodination and either loss of bioactivity [21, 22], or reduction in metabolism [1, 2, 23] it has been common practice to use "lightly" iodinated insulin in tracer studies. However Arquilla et al. [24] demonstrated that the bioactivity of insulin labelled with one iodine atom per molecule or less was still impaired. Similarly, Ooms [1, 2] reported a reduced metabolic clearance rate and some loss of saturability of clearance of insulin labelled with less than 0.2 atoms per mole.

Randomly iodinated insulin preparations consist of a mixture of molecules with varying degrees of substitution and, in all but heavily iodinated preparations, there is a significant proportion of molecules that are unsubstituted. Great care is required lest the biological activity of the preparation is ascribed to the iodinated insulin when it is in fact due to the unlabelled molecules present. In an attempt to overcome this problem Freychet et al. [25] purified monoiodoinsulin from a crude iodination mixture and demonstrated full activity of the product in vitro. However others have found purified monoiodoinsulins to be only partially active [26–28] or completely inactive [29]. The biological activities of some of these monoiodoinsulin preparations [26, 27, 29] correlated directly with the percentage of their radioactivity in the A14 position, consistent with the observation by Garratt et al. [30] that iodination of A19 resulted in a reduced activity of the hormone. However the preparation of Linde and Hansen [28] showed the reverse trend. The monoiodoinsulin of Sodoyez et al. [31] was fully active biologically and in receptor binding and may prove to be a valid insulin tracer.

Thus the biological activities of purified monoiodoinsulins prepared by random iodination methods appear to differ, possibly in relation to the amount of iodine incorporated into A19 tyrosine, and it is impossible to escape from the conclusion that these materials fail to meet all the requirements for a valid tracer for insulin. The selective approach described here overcomes the inherent problems of random iodination. The immunoreactivity results indicated a pure "insulin" preparation modified at the B-1 position. The substituted material was fully active in an in vivo and an in vitro bioassay and exhibited normal metabolic and urinary clearance, half-life and apparent distribution space in dogs. When ^{125}I iodine is used in the synthesis, this derivative will have the high specific activity required for physiological studies, (maximum theoretical specific activity = $733 \mu\text{Ci}/\mu\text{g}$). (3,5-diiodotyrosine) B1 insulin therefore fulfills all the necessary criteria for a valid tracer for insulin.

It may be argued that it is valid to use a tracer

whose properties are not all identical to those of the native hormone in radioimmunoassays or receptor binding assays where the relative abilities of two unlabelled species in displacing a radioactive derivative are being compared. However in studies on the mode of action or the metabolism of a hormone, the behaviour of the labelled species is being examined directly and it is essential that it behaves identically to the native hormone. It will be interesting to note whether the parameters of many mathematical models for insulin metabolism, or the calculations of insulin receptor numbers and binding characteristics will be altered when a valid tracer is used in their measurement.

References

- Ooms, H. A., Arnould Y., Rosa U., Pennis, G. F., Franckson, J. R. M.: Clearances metaboliques globales de l'insulin cristalline et d'insulines substituées au radioiode. *Pathol. Biol. (Paris)* **16**, 241–245 (1968)
- Ooms, H. A.: *Emploi des insulines radiodées comme traceurs en biologie*, Vol. 1. Bruxelles: Editions Arscia 1973
- Springell, P. H.: An unreactive tyrosine residue in insulin and the exclusive iodination of the A chain. *Nature* **191**, 1372–1373 (1961)
- Massaglia, A., Rosa, U., Rialdi, G. A.: Iodination of insulin in aqueous and organic solvents. *Biochem. J.* **115**, 11–18 (1969)
- Lambert, B., Jacquemin, C.: About insulin iodination. II. Comparative study of enzymic and chemical methods. *Biochimie* **56**, 1191–1196 (1974)
- Blundell, T., Dodson, G., Hodgkin, D., Mercola, D.: Insulin: The structure in the crystal and its reflection in chemical and biological properties. *Adv. Protein Chem.* **26**, 279–402 (1972)
- Brandenburg, D.: Dis-Phe^{B1}-Insulin, ein kristallines Analogon des Rinderinsulins. *Hoppe-Seylers Z. Physiol. Chem.* **350**, 741–750 (1969)
- Lindsay, D. G., Shall, S.: The acetylation of insulin. *Biochem. J.* **121**, 737–745 (1971)
- Krail, G., Brandenburg, D., Zahn, H., Geiger, R.: [B1-p-Jod-phenylalanin] Insulin, ein einheitliches Monojodinsulin. *Hoppe-Seylers Z. Physiol. Chem.* **352**, 1595–1598 (1971)
- Gliemann, J., Gammeltoft, S.: The biological activity and the binding affinity of modified insulins determined on isolated rat fat cells. *Diabetologia* **10**, 105–113 (1974)
- Halban, P. A., Offord, R. E.: The preparation of a semi-synthetic tritiated insulin with a specific radioactivity of up to 20 curies per millimole. *Biochem. J.* **151**, 219–225 (1975)
- Tectz, V., Echert, H. J., Geiger, R.: (B1-3,5-Diiodotyrosine)-Insulin. *Proc. 14th Eur. Peptide Sympos.*, Belgium, 263–267. Bruxelles: Editions de l'Université de Bruxelles 1976
- Ballentine, R.: In: Colowick, Kaplan (Eds.): *Methods in Enzymology*, Vol. III, 985–995. New York: Academic Press 1957
- Sönksen, P. H., Tompkins, C. V. T., Srivastava, M. C., Nabarro, J. D. N.: A comparative study on the metabolism of human insulin and porcine proinsulin in man. *Clin. Sci. Mol. Med.* **45**, 633–654 (1973)
- Thomas, J. H., Dron, D. I., Jones, R. H.: Immunospecificity studies with chemically modified insulins. *Diabetologia* **11**, 379 (1975) (Abstract)
- Gliemann, J.: Assay of insulin-like activity by the isolated fat cell method: I. Factors influencing the response to crystalline insulin. *Diabetologia* **3**, 382 (1967)
- Rodbell, M.: Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**, 375–380 (1964)
- Moody, A. J., Stan, M. A., Stan, M., Gliemann, J.: A simple free fat cell bioassay for insulin-like activity. *Horm. Metab. Res.* **6**, 12–16 (1974)
- Finney, D. J.: *Statistical methods in biological assay*. Second edition, pp 99–138. London: Charles Griffin & Co. Ltd. 1964
- Jones, R. H., Dron, D. I., Ellis, M. J., Sönksen, P. H., Brandenburg, D.: Biological properties of chemically modified insulins. 1. Biological activity of proinsulin and insulin modified at A1 and B29. *Diabetologia* **12**, 601–608 (1976)
- De Zoeten, L. W., Vonsrik, R.: A study of the biological activity of iodinated insulin. *Rec. Trav. Chim. Pays-Bas* **80**, 927–930 (1961)
- Izzo, J. L., Roncone, A., Izzo, M. J., Bale, W. F.: Relationship between degree of iodination of insulin and its biological electrophoretic and immunochemical properties. *J. Biol. Chem.* **239**, 3749–3754 (1964)
- Izzo, J. L., Roncone, A., Izzo, M. J., Foley, R., Bartlett, J. W.: Degradation of ¹³¹I-insulins by rat liver studies in vitro. *J. Biol. Chem.* **247**, 1219–1226 (1972)
- Arquilla, E. R., Ooms, H., Mercola, K.: Immunological and Biological properties of iodoinsulin labelled with one or less atoms of iodine per molecule. *J. Clin. Invest.* **47**, 474–487 (1968)
- Freychct, P., Roth, J., Neville, D. M.: Monoiodoinsulin: Demonstration of its biological activity and binding to fat cells and liver membranes. *Biochem. Biophys. Res. Commun.* **43**, 400–408 (1971)
- Bihler, D. A., Morris, J. W. S.: Preparation and properties of (monoiodotyrosine) insulin. *Biochem. J.* **130**, 321–324 (1972)
- Hamlin, J. L., Arquilla, E. R.: Monoiodoinsulin. Preparation, purification and characterisation of a biologically active derivative substituted predominately on tyrosine A14. *J. Biol. Chem.* **249**, 21–32 (1974)
- Linde, S., Hansen, B. A.: Preparation and characterisation of monoiodoinsulin. *Int. J. Pept. Protein Res.* **6**, 157–163 (1974)
- Lambert, B., Sutter, B. C. H., Jacquemin, C. I.: Effect of iodination on the biological activity of insulin. *Horm. Metab. Res.* **4**, 149–151 (1972)
- Garratt, C. J., Harrison, D. M., Wicks, M.: The effect of iodination of particular tyrosine residues on the hormonal activity of insulin. *Biochem. J.* **126**, 123–131 (1972)
- Sodoyez, J. C., Sodoyez-Goffaux, F., Goff, M. M., Zimmerman, A. E., Arquilla, E. R.: (¹²⁵I) or carrier-free (¹²⁵I) monoiodoinsulin. Preparation, physical, immunological and biological properties, and susceptibility to "insulinase" degradation. *J. Biol. Chem.* **250**, 4268–4277 (1975)

Received: October 15, 1976, and in revised form: February 2, 1977

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