Preparation and Properties of [Monoiodotyrosine]-Insulin

By D. A. BIHLER* and J. W. S. MORRIS†

Department of Pathology, California College of Medicine, University of California Irvine,
Irvine, Calif. 92664, U.S.A.

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Iodinated insulins have found widespread use in radioimmunoassay and as biological tracers (Haugaard *et al.*, 1954; Berson *et al.*, 1956; Welsh *et al.*, 1956; Edelman & Schwartz, 1966; Tomasi *et al.*, 1967). Consequently, biological and immunological properties of iodinated insulins have been the subject of considerable investigation (De Zoeten & Van Strik, 1961; Izzo *et al.*, 1964; Rosa *et al.*, 1967; Glover *et al.*, 1967; Arquilla *et al.*, 1968; Brunfeldt *et al.*, 1968; Massaglia *et al.*, 1969; Freychet *et al.*, 1971b; Garratt *et al.*, 1972). Previous studies have been hampered by the lack of adequate separation techniques for the preparation of iodinated insulins. We present a method of preparation and purification of a biologically active mono-iodinated insulin. This work was presented in part at the 7th. Congress of the International Diabetes Federation, Buenos Aires, Argentina, on 23–28 August 1970.

Iodination of insulin was performed by a method based on work of Mayberry *et al.* (1964) on the iodination of tyrosine derivatives. In a typical preparation 130 μ mol (1.5 equiv.) of I_2 in 2 ml of ethanol was allowed to exchange with 500 μ Ci of carrier-free $Na^{125}I$ (New England Nuclear Corp., Boston, Mass., U.S.A.). This solution was then added to 87 μ mol (500 mg) of bovine insulin (Elanco no. 1CB98W; Eli Lilly and Co., Indianapolis, Ind., U.S.A.). The reaction was carried out in 0.11 M-sodium phosphate buffer, pH 7.4, at an insulin concentration of 2.5 mg/ml at 23°C. The iodinating mixture was added to the magnetically stirred insulin solution in 100 μ l portions over a 2 min interval. The stirring was continued for 30 min. In all preparations 47–50% of the radioactivity was precipitable in 1 M-trichloroacetic acid, indicating that the reaction was 94–100% complete. The pH was adjusted to 5.2, and the resulting precipitate was collected by centrifugation and washed three times with water.

To separate the iodinated insulin derivatives advantage was taken of the fall of the phenolic pK from 10.1 to 8.6 that accompanies introduction of an iodine atom in the *ortho* position of tyrosine

(Sokolovsky *et al.*, 1967). At pH 8.6 [monoiodotyrosine]-insulin should have a charge difference of $-\frac{1}{2}$ relative to insulin. A further fall in phenolic pK from 8.6 to 6.8 accompanies the formation of 3,5-diiodotyrosine. Introduction of a second iodine atom into either an iodotyrosine residue or a tyrosine residue should result in an additional charge difference at pH 8.6 of $-\frac{1}{2}$ relative to insulin. Therefore separation of iodinated insulins based on net charge at pH 8.6 should result in fractions of the same degree of iodination. Accordingly, the reaction mixture was separated on a column (4 cm $\times$ 100 cm) of DEAE-Sephadex A-25 at 4°C. The column was equilibrated with a buffer containing 0.14 M-NaCl, 0.01 M-tris-HCl, pH 8.60 $\pm$ 0.02, and 7 M-urea. Elution at 60 ml/h was performed with a salt gradient (0.14–0.4 M-NaCl) in the tris-urea buffer. The E_{276} and radioactivity (Packard model 3002 scintillation spectrometer) of each fraction (20 ml) was determined (Fig. 1a). Corresponding peaks were promptly pooled and dialysed at 4°C against 0.01 M-ammonium formate buffer, pH 5.2. The resulting precipitates were collected by centrifugation, washed with water and freeze-dried.

The specific radioactivities [c.p.m./ E_{276} unit (in 0.01 M-HCl)] of the peaks I–IV were in the proportions 1.24:2.36:3.00:3.95. Neutron-activation analysis revealed iodine contents of 3.1 ± 0.3 , 4.4 ± 0.2 , 6.6 ± 0.4 and $9.1 \pm 0.5\%$ for fractions I–IV respectively. The theoretical values for mono-, di-, tri- and tetra-iodoinsulin are 2.15, 4.17, 6.13 and 8.02%.

Peak I from a separation similar to that shown in Fig. 1(a) was rechromatographed to obtain purified [monoiodotyrosine]-insulin (Fig. 1b). Two distinct fractions were obtained. The first fraction (F_I), evidently unchanged insulin, crystallized in high yield from 0.01 M-sodium acetate–1 mM-ZnSO₄–5% (w/v) NaCl, pH 5.9. The second fraction (F_{II}), [monoiodotyrosine]-insulin, did not crystallize under these conditions. The absorption spectra of fraction F_{II} were fully consistent with the presence of 1 equivalent of L-3-iodotyrosine. A batch of [monoiodotyrosine]-insulin, prepared as described above, had an iodine content of $2.05 \pm 0.15\%$ when dried to constant weight.

Oxidative sulphytolysis (Bromer *et al.*, 1967) followed by separation on Dowex 50 (Morris *et al.*, 1970) revealed that 95% of the radioactivity was present on the A chain. Chymotryptic hydrolysis (Aoyama *et al.*, 1965) demonstrated that the distribution of

* Present address: Department of Environmental Interactions, California College of Medicine, University of California Irvine, Irvine, Calif. 92664, U.S.A.

† Present address: California College of Medicine, University of California Irvine, Irvine, Calif. 92664, U.S.A.

Fig. 1. Chromatography of insulin and derivatives

(a) Ion-exchange chromatography of the iodinated reaction mixture. The iodinated reaction mixture was separated on a column (4cm $\times$ 100cm) of DEAE-Sephadex A-25 at 4°C. The column was equilibrated with a buffer containing 0.14M-NaCl, 0.01M-tris-HCl, pH8.60 $\pm$ 0.02, and 7M-urea. Elution at 60ml/h was performed with a salt gradient (---, 0.14–0.4M-NaCl) in the tris-urea buffer and 20ml fractions were collected. (b) Rechromatography of iodinated insulin. [Monoiodotyrosine]-insulin was further purified on a column (1.5cm $\times$ 60cm) of DEAE-Sephadex A-25 at 4°C. The column was equilibrated with a buffer containing 0.10M-NaCl, 0.01M-tris-HCl, pH8.60 $\pm$ 0.02, and 7M-urea. Elution at 40ml/h was performed with the same buffer at constant ionic strength and 10ml fractions were collected. —, E_{276} ; ----, radioactivity.

radioactivity between tyrosine residues A19 and A14 was 1:1. Gel filtration (on Sephadex G-50) of the monosubstituted derivative was performed in 7M-urea–0.01M-NaCl–0.05M-tris-HCl, pH7.5 (Boesel

& Carpenter, 1970). Results gave no indication of intermolecular cross-linking, as has been noted in unseparated reaction mixtures of iodinated insulins (Csorba & Gattner, 1970).

Table 1. *Biological and immunological activities of insulin and derivatives*

Biological activity was measured by the mouse convulsion assay (Young & Louis, 1947), and is expressed as the mean $\pm$ the 95% confidence interval for the numbers of mice in parentheses. Immunological activity was measured by passive immune haemolysis inhibition (Arquilla, 1972).

Insulin or derivative	Biological activity (i.u./mg)	Immunological activity (%)
Unchanged insulin (F_I)	24.1 ± 4.0 (150)	100
[Monoiodotyrosine]-insulin (F_{II})	16.8 ± 2.8 (150)	6
[Mono-3-nitrotyrosine]-insulin	12.8 ± 2.7 (270)	4*
[Di-3-nitrotyrosine]-insulin	5.3 ± 0.8 (210)	9*

* Morris *et al.* (1970).

The biological activities of unchanged insulin (F_I), [monoiodotyrosine]-insulin (F_{II}) and [mono- and di-3-nitrotyrosine]-insulins (Morris *et al.*, 1970) were measured by the mouse convulsion assay (Table 1). The results presented are based on weight, and have not been corrected for water content. The biological activity of unchanged insulin (F_I) compared favourably with the biological activity of the crystalline starting material (26.0 i.u./mg). Previous determinations (Morris *et al.*, 1970) of the biological activity of [mono- and di-3-nitrotyrosine]-insulin, based on a blood-sugar depression assay, differ significantly from results of the mouse convulsion assay presented here.

The ability of [monoiodotyrosine]-insulin, [mono-3-nitrotyrosine]-insulin, [di-3-nitrotyrosine]-insulin and unchanged insulin to combine with purified guinea-pig anti-insulin antibodies was measured by passive immune haemolysis inhibition (Arquilla, 1972) (Table 1).

We have prepared and characterized [monoiodotyrosine]-insulin, which was found to be a mixture of the isomers with tyrosine residues A14 and A19 substituted. Results on the localization of the sites of substitution are in accord with results based on unseparated iodinated insulins (Massaglia *et al.*, 1969; De Zoeten & Havinga, 1961). This insulin derivative is biologically active, in agreement with reports on heterogeneous iodinated insulin preparations characterized by an average degree of substitution (Rosa *et al.*, 1967). Our results on biological activity substantiate recent findings on the biological activity of purified [monoiodotyrosine]-insulin (Freychet *et al.*, 1971a). Immunological characteristics as measured by passive immune haemolysis inhibition correspond well with results obtained in this laboratory on [3-nitrotyrosine]-insulins, which also possess predominant A-chain tyrosine residue substitution. In addition, the [monoiodotyrosine]-insulin was found to have a maximum insulin contamination of 6%, as indicated by its immunological activity.

Our results indicate that substitution of A-chain tyrosine residues has a marked effect on immunological activity and a relatively minor effect on biological activity. In this regard, it can be expected that mono[^{125}I]iodo-insulin should be a reliable competitor in a 'receptor site' assay. This view is supported by the results of Freychet *et al.* (1971b), which demonstrate that only biologically active insulin derivatives could effectively displace ^{125}I -labelled insulin bound to liver or fat-cells. In contradistinction, poor correlation was found between the activity in the radioimmunoassay and the biological activity of the insulin derivatives tested.

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