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^{125}I-Labelling of Erythropoietin without Loss of Biological Activity

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Erythropoietin, the hormone that regulates erythropoiesis in mammals, was ^{125}I-labelled by using the catalytic properties of lactoperoxidase and the H_2O_2-generating properties of glucose oxidase. This methodology, both rapid and simple, not only produced hormone preparations with high specific radioactivity but also did not substantially alter the biological integrity of erythropoietin when it was assayed *in vivo*.

A glycoprotein hormone, erythropoietin, is acknowledged to be the prime mover of erythropoiesis in mammals (Fisher, 1972; Gordon, 1974). Erythropoietin has been ^{125}I-labelled in investigations of its biochemical characteristics (Lowy *et al.*, 1960; Keighley & Lowy, 1971; Goldwasser & Kung, 1972), in attempts to develop an erythropoietin radioimmunoassay (Fisher *et al.*, 1972; Garcia, 1972), and in a study on the rate of plasma clearance of erythropoietin (Roh *et al.*, 1972). However, the use of chloramine-T in these ^{125}I-labelling protocols (Greenwood *et al.*, 1963; McConahey & Dixon, 1966; Hunter & Greenwood, 1962) either seriously impaired the erythropoiesis-stimulating properties of erythropoietin (Fisher *et al.*, 1972), or abolished it altogether (Lowy *et al.*, 1960; Keighley & Lowy, 1971). The present paper describes the ^{125}I-labelling of erythropoietin while maintaining the integrity of its biological activity. The method uses an enzyme, lactoperoxidase, which in the presence of H_2O_2, catalyses the substitution of iodine for hydrogen on the phenolic side chains of exposed tyrosine residues of proteins (Phillips & Morrison, 1970). The H_2O_2-generating system of glucose and glucose oxidase was used because it generated controlled, continuous and small quantities of this reactive chemical (Hubbard & Cohn, 1972).

Materials and Methods

Erythropoietin of two different sources and potencies was used: (1) sheep plasma erythropoietin (Step III) from animals with induced haemolytic anaemia (Connaught Laboratories Ltd., Willowdale, Ont., Canada) with a specific biological activity of 2.77 International Reference Preparation (IRP) units/ml of protein, and (2) human urinary erythropoietin (H-14 TaLSL) [erythropoietin was collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina, further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, CA, U.S.A., under Research Grant HE-10880 (National Heart and Lung Institute)] purified from patients with severe anaemia secondary to hookworm (*Necator americanus*) infestation and possessing a specific activity of 318 IRP units/mg. A portion (8mg) of a preparation of sheep plasma erythropoietin (22 IRP units) or 155μg of a preparation of erythropoietin from human urine (36 IRP units) were dissolved in 0.05M-potassium phosphate buffer (pH7.2) with 5μM-glucose, 3.6munits of glucose oxidase (Sigma Chemical Co., St. Louis, MO, U.S.A.; type V), 10–100μCi of carrier-free Na^{125}I (New England Nuclear Corp., Boston, MA, U.S.A.), and 3.6munits of lactoperoxidase (Sigma). ^{125}I labelling was conducted at 37°C for 10–40min. The reaction was terminated by the addition of 10vol. of cold 50μM-sodium thiosulphate. The labelled protein was separated from free ^{125}I by gel-filtration chromatography on a column (1cm×13cm) of Sephadex G-25, equilibrated with 0.05M-potassium phosphate buffer, pH7.2, and eluted in 0.4ml portions with the same buffer. The radioactivity of each fraction was assessed in a gamma counter (Packard Instrument Co., La Grange, IL, U.S.A.). Pooled fractions were freeze-dried and stored frozen at −5°C until assayed for erythropoietic activity in hypertransfused plethoric (Jacobson *et al.*, 1957), or exhypoxic polycythaemic (Cotes & Bangham, 1961) CF_1 virgin female mice. The samples were reconstituted with an appropriate volume of sterile water to re-establish osmoticity and then injected intraperitoneally into groups of no less than five mice. After 2 days, each mouse received 0.5μCi of ^{59}Fe intravenously, and the percentage of ^{59}Fe radioactivity incorporated into the erythrocyte pool was determined 48h later. In certain instances, these values were converted into equivalent units of erythropoietin by reference to a standard curve (determined empirically for each assay) for the Inter-

national Reference Preparation (IRP) of erythropoietin (Camiscoli & Gordon, 1970).

Samples (25 μl) of ^{125}I-labelled H-14 TaLSL preparations of human erythropoietin from urine, with known biological activity, were subjected to electrophoresis for 14–20h in 7.5% (w/v) polyacrylamide slab gels containing the anionic detergent, sodium dodecyl sulphate in 0.1M-potassium phosphate buffer, pH7.2, with 0.1% sodium dodecyl sulphate (Shapiro *et al.*, 1967). By using the migration of Bromophenol Blue as the reference point within each gel, the separated proteins (their molecular weights estimated by reference to standards of bovine serum albumin, ovalbumin, glucose oxidase, lactoperoxidase and cytochrome *c*), were fixed in 20% (v/v) sulphosalicylic acid for 16h, stained with 0.25% Coomasie Blue for 5h, de-stained with 7% (v/v) acetic acid, and dried on absorbent paper *in vacuo*. Kodak X-ray film was placed over the dessicated slab and radioautograms were exposed for 20–65 days. The photographically developed grains were scanned with an Artronix scanning densitometer coupled with a PC-12/7 computer (Artronix Co., St. Louis, MO, U.S.A.) scanning and intergrating 1.0mm^2 areas and plotting results as a percentage of maximum absorbance.

Results

Three different concentrations of ^{125}I were used to label Connaught Step-III sheep plasma erythropoietin. A typical elution profile of a Sephadex G-25 separation is represented in Fig. 1. The first peak contained virtually all of the labelled protein, whereas the second and larger peak contained the free ^{125}I. The radioactivities of each of the three samples (i.e., equivalent to 1, 2 and 3mol of iodine) is presented in Table 1. Although there was an increase in radioactivity as a function of iodine concentration, the erythropoietic-stimulating property of erythropoietin was not impaired (Fig. 2). For each assay the mouse received material which, before ^{125}I-labelling, contained 0.1 IRP unit of erythropoietin. After ^{125}I labelling, the erythropoietin activity was retained. Further, the biological activity of erythropoietin was retained over the threefold range of iodine concentration.

The effect of the duration of ^{125}I-labelling with 2mol of iodine was examined with Connaught Step-III sheep plasma erythropoietin. The radioactivities of samples iodinated for 10, 20 or 40min are listed in Table 2. ^{125}I-labelling for 10min was as effective as 40min incubation. Two different dilutions of the ^{125}I-labelled samples were made and bioassayed for erythropoietin activity in exhypoxic polycythaemic mice (Table 3). No diminution of erythropoietin biological potency was observed during ^{125}I-labelling incubations of up to 40min.

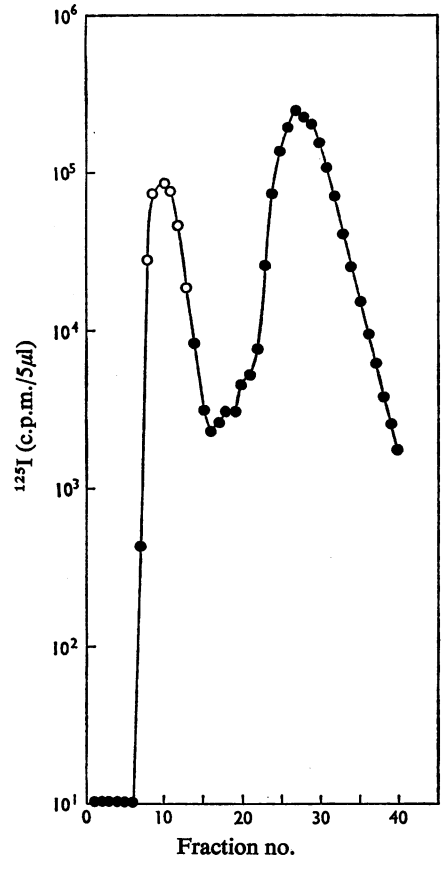


Fig. 1. *Sephadex G-25 separation of ^{125}I-labelled Connaught Step-III sheep plasma erythropoietin*

The pooled fractions (○) were assayed for erythropoietic activity.

Table 1. *Effect of iodine concentration on the ^{125}I-labelling of Connaught Step-III sheep plasma erythropoietin*

A preparation (8mg) of sheep plasma erythropoietin was ^{125}I-labelled as described in the Materials and Methods section, with three different concentrations of iodine. The erythropoietic activity after ^{125}I-labelling is depicted in Fig. 2.

Iodine (mol equivalent)	10^{-4} × Radioactivity (c.p.m./10μl)
1	3.8
2	11.0
3	26.7

Since (*a*) erythropoietin in a relatively crude preparation (i.e. Connaught Step III) was not inactivated by the lactoperoxidase enzyme-catalysed procedure, and (*b*) the material in this preparation was ^{125}I-

labelled, more highly purified hormone was ^{125}I-labelled. Erythropoietin from human urine (H-14 TaLSL, hereafter called 'H-14 erythropoietin'), was dissolved in either sterile double-distilled water or in 0.5% bovine serum albumin in sterile 0.9% NaCl. These were either ^{125}I-labelled as described in the Materials and Methods section or incubated for the same 20min interval in the presence of all reagents (i.e. buffer, lactoperoxidase, glucose and glucose oxidase) except Na^{125}I. These samples, dissolved in either water or 0.5% bovine serum albumin/0.9% NaCl, but not ^{125}I-labelled, served as controls for the incubation interval. The 0.5% bovine serum albumin/0.9% NaCl medium was used as one of the diluents, since it was suggested that it provided protection for the active moiety of erythropoietin (P. P. Dukes, personal communication).

The Sephadex G-25 elution profiles for the two ^{125}I-labelled samples are shown in Fig. 3. The increased radioactivity seen with H-14 erythropoietin dissolved in 0.5% bovine serum albumin is probably

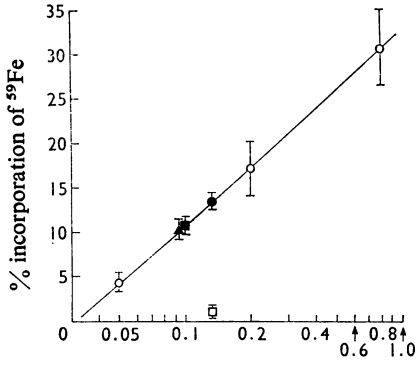


Fig. 2. *Bioassay of ^{125}I-labelled Connaught Step-III sheep plasma erythropoietin*

The symbols ■, ● and ▲ signify 1, 2 and 3 molar equivalents of iodine. □, Saline control; ○, erythropoietin standards with bars indicating ±S.E.

Table 2. *Effect of the period of incubation at 37°C on the ^{125}I-labelling of Connaught Step-III sheep plasma erythropoietin*

A preparation (8mg) of sheep plasma erythropoietin was ^{125}I-labelled with an equivalent of 2mol of iodine for various lengths of time. There was no difference in the specific radioactivity (c.p.m./IRP unit of erythropoietin). The biological activities are recorded in Table 3.

Period of ^{125}I-labelling (min)	10^{-4}×Radioactivity (c.p.m./10μl)	10^{-6}×Specific ^{125}I radioactivity (c.p.m./IRP unit of erythropoietin
10	9.0	1.29
20	8.3	1.47
40	10.8	1.65

Table 3. *Effect of the period of incubation at 37°C on the biological activity of Connaught Step-III sheep plasma erythropoietin*

The biological activity of ^{125}I-labelled sheep plasma erythropoietin which had been incubated for various lengths of time (i.e. 10, 20 and 40min) as described in the Materials and Methods section was determined. There was no loss of erythropoietic activity. Values are means±S.E. of five samples.

^{125}I-labelling period (min)	Erythropoietin activity before ^{125}I-labelling (IRP unit)	Erythrocytes incorporating ^{59}Fe after ^{125}I-labelling (%)	Erythropoietin activity after ^{125}I-labelling (IRP unit)
10	0.2	14.82±1.32	0.18
20	0.2	15.60±1.04	0.20
40	0.2	16.40±1.60	0.20
10	1.0	28.02±1.43	0.96
20	1.0	27.30±1.37	1.00
40	1.0	29.09±2.05	1.00

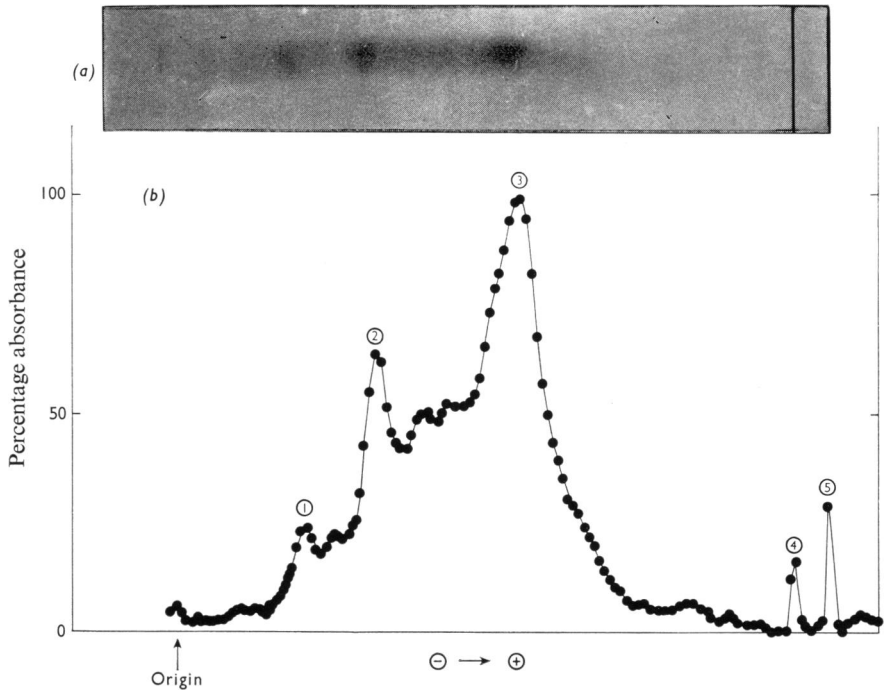
Assay standards	Erythrocytes incorporating ^{59}Fe (%)
0.9% NaCl	1.09±0.18
0.05IRP unit of H-14 TaLSL erythropoietin	5.47±0.18
0.20IRP unit of H-14 TaLSL erythropoietin	14.82±1.45
0.80IRP unit of H-14 TaLSL erythropoietin	27.05±1.15

Table 4. *Effect of ^{125}I-labelling of H-14 TaLSL erythropoietin from human urine on its biological activity*

A sample (155 μg) of human urinary erythropoietin was ^{125}I-labelled as described in the Materials and Methods section and then assayed for erythropoietic activity in the exhypoxic polycythaemic mouse assay. The biological activity was retained in the range of 85–95%. Values are means ± S.E. of five samples.

Sample	Labelled with $Na^{125}I$ (100 μCi)	Erythropoietin activity before 20 min incubation (IRP unit)	Erythrocytes incorporating ^{59}Fe after 20 min incubation (%)	Erythropoietin activity after 20 min incubation (unit)	Retention of biological activity	10^{-6} × Specific ^{125}I radioactivity (c.p.m./IRP unit of erythropoietin)
H-14 erythropoietin in twice-distilled water	No	1.0	26.30 ± 2.07	0.90	90%	—
H-14 erythropoietin in 0.5% bovine serum albumin	No	1.0	27.45 ± 2.42	0.96	96%	—
H-14 erythropoietin in twice-distilled water	Yes	1.0	26.61 ± 3.04	0.85	85%	1.63
H-14 erythropoietin in 0.5% bovine serum albumin	Yes	1.0	28.02 ± 2.98	0.95	95%	2.52

Assay standards	Erythrocytes incorporating ^{59}Fe (%)
0.9% NaCl	0.73 ± 0.06
0.05 IRP unit of H-14 TaLSL erythropoietin	3.38 ± 0.42
0.20 IRP unit of H-14 TaLSL erythropoietin	13.92 ± 2.92
0.80 IRP unit of H-14 TaLSL erythropoietin	25.71 ± 2.37



EXPLANATION OF PLATE 1

Radioautogram (a) of slab-gel electrophoresis in the presence of sodium dodecyl sulphate of ^{125}*I-labelled H-14 TaLSL erythropoietin from human urine, and (b) the plot of its densitometric scan*

The three peaks represent (1) glucose oxidase, (2) lactoperoxidase, and (3) erythropoietin. Peaks (4) and (5) represent two lines inked on the radioautogram for scanning reference.

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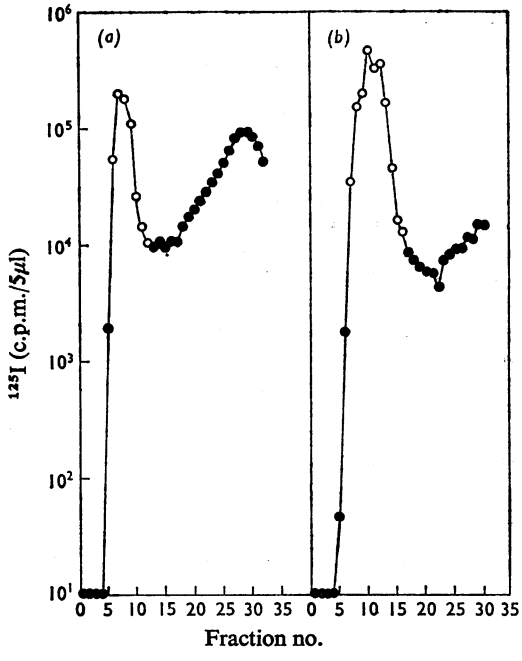

Fig. 3. *Sephadex G-25 separation of ^{125}I-labelled H-14 TaLSL erythropoietin from human urine*

Erythropoietin was dissolved in either sterile double-distilled water (*a*) or 0.5% bovine serum albumin in sterile 0.9% NaCl (*b*). The pooled fractions (○) were assayed for erythropoietic activity.

due to the ^{125}I-labelling of bovine serum albumin. The results of the bioassay for erythropoietin activity as shown in Table 4 are consistent with the previous data, which demonstrated that this procedure does not inactivate erythropoietin, and extend those observations to include purified erythropoietin.

^{125}I-labelled H-14 erythroprotein, dissolved in water and concentrated by Sephadex G-25 chromatography, was further subjected to slab-gel electrophoresis in the presence of sodium dodecyl sulphate to differentiate molecular species as a function of molecular weight. A radioautogram of such a gel is shown in Plate 1(*a*), together with the profile of its densitometric scan normalized to percentage absorbance (Plate 1*b*). Peaks 1 and 2 represent glucose oxidase (186000mol.wt.) and lactoperoxidase (85000mol.wt.) respectively. These two reagents used in ^{125}I-labelling were also labelled, but to a lesser extent than erythropoietin as resolved by peak 3. Its electrophoretic mobility was consistent with a mol.wt. of 40000–45000.

Discussion

Proteins labelled with ^{125}I have been useful in a broad range of biochemical investigations. The original methodology of Hunter & Greenwood (1962), using chloramine-T oxidation, or a modification thereof, have enabled high specific labelling of hormones (Greenwood *et al.*, 1963; Hunter & Greenwood, 1962), bacterial flagellins (Ada *et al.*, 1964) and immunoglobulin (McConahey & Dixon, 1966; Marchalonis & Nossal, 1969) with carrier-free ^{125}I. These methods suffer certain disadvantages, since the ^{125}I-labelling is conducted in the presence of relatively high concentrations of chloramine-T, a powerful oxidizing reagent that may denature proteins. The limitations imposed by chloramine-T, in which Fisher *et al.* (1972) lost 46–65% of the erythropoietin activity, in conjunction with a report by Keighley & Lowy (1971) of its total inactivation of erythropoietin, gave impetus to the search for a more gentle means of ^{125}I-labelling of erythropoietin.

Studies of enzyme-catalysed ^{125}I-labelling by the peroxidases (Phillips & Morrison, 1970; Hubbard & Cohn, 1972; Marchalonis, 1969), especially lactoperoxidase, have demonstrated their use in protein labelling. Molecular H_2O_2, an obligate reactant of the ^{125}I-labelling reaction, is usually added in portions, but temporary excesses can have untoward effects on proteins (Schenkein *et al.*, 1972). In seeking to avoid these problems, glucose oxidase, glucose and O_2 were used to generate H_2O_2 at a controllable rate and obviated the possible adverse effects caused by direct addition of H_2O_2 (Hubbard & Cohn, 1972; Schenkein *et al.*, 1972).

The present paper describing studies with lactoperoxidase-catalysed ^{125}I-labelling and glucose oxidase production of H_2O_2, documents the ^{125}I-labelling of a relatively crude preparation of erythropoietin (2.77IRP units/mg of protein) from sheep plasma and purified erythropoietin (318IRP units/mg protein) extracted from human urine. The specific radioactivity was high (e.g. 129×10^4–252×10^4c.p.m./IRP unit of erythropoietin) and the biological activity of the hormone was retained (e.g., 85–100%). It is hoped that the deployment of this easy and rapid method of erythropoietin labelling will provide a tool with which the mechanism(s) of erythropoietin-induced erythropoiesis may be investigated and with which a sensitive radioimmunoassay may be developed.

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