¹²⁵I-Labeling of Erythropoietin without Loss of Biological Activity

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(Received 3 May 1976)

Erythropoietin, the hormone that regulates erythropoiesis in mammals, was ¹²⁵I-labelled by using the catalytic properties of lactoperoxidase and the H₂O₂-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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-labelling of erythropoietin while maintaining the integrity of its biological activity. The method uses an enzyme, lactoperoxidase, which in the presence of H₂O₂, catalyses the substitution of iodine for hydrogen on the phenolic side chains of exposed tyrosine residues of proteins (Phillips & Morrison, 1970). The H₂O₂-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 (8 mg) of a preparation of sheep plasma erythropoietin (22 IRP units) or 155 µg of a preparation of erythropoietin from human urine (36 IRP units) were dissolved in 0.05 M-potassium phosphate buffer (pH 7.2) with 5 µM-glucose, 3.6 munits of glucose oxidase (Sigma Chemical Co., St. Louis, MO, U.S.A.; type V), 10–100 µCi of carrier-free Na¹²⁵I (New England Nuclear Corp., Boston, MA, U.S.A.), and 3.6 munits of lactoperoxidase (Sigma). ¹²⁵I labelling was conducted at 37°C for 10–40 min. The reaction was terminated by the addition of 10 vol. of cold 50 µM-sodium thiosulphate. The labelled protein was separated from free ¹²⁵I by gel-filtration chromatography on a column (1 cm × 13 cm) of Sephadex G-25, equilibrated with 0.05 M-potassium phosphate buffer, pH 7.2, and eluted in 0.4 ml 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) CF₁ 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 0.5 µCi of ⁵⁹Fe intravenously, and the percentage of ⁵⁹Fe radioactivity incorporated into the erythrocyte pool was determined 48 h 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 μ l) of 125 I-labelled H-14 TaLSL preparations of human erythropoietin from urine, with known biological activity, were subjected to electrophoresis for 14–20 h in 7.5% (w/v) polyacrylamide slab gels containing the anionic detergent, sodium dodecyl sulphate in 0.1M-potassium phosphate buffer, pH 7.2, 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 16 h, stained with 0.25% Coomassie Blue for 5 h, 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 integrating 1.0 mm² 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 3 mol 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 2 mol of iodine was examined with Connaught Step-III sheep plasma erythropoietin. The radioactivities of samples iodinated for 10, 20 or 40 min are listed in Table 2. 125 I-labelling for 10 min was as effective as 40 min 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 40 min.

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 (8 mg) 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)	$10^{-4} \times$ Radioactivity (c.p.m./10 μ l)
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 ¹²⁵I-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 ¹²⁵I-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 Na¹²⁵I. These samples, dissolved in either water or 0.5% bovine serum albumin/0.9% NaCl, but not ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-labelling of Connaught Step-III sheep plasma erythropoietin

A preparation (8 mg) of sheep plasma erythropoietin was ¹²⁵I-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./10 μl)	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 ¹²⁵I-labelled sheep plasma erythropoietin which had been incubated for various lengths of time (i.e. 10, 20 and 40 min) 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	
0.05 IRP unit of H-14 TaLSL erythropoietin		5.47 ± 0.18	
0.20 IRP unit of H-14 TaLSL erythropoietin		14.82 ± 1.45	
0.80 IRP unit of H-14 TaLSL erythropoietin		27.05 ± 1.15	

Table 4. *Effect of ^{125}I -labelling of H-14 TaL SL 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 $\pm$ 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	$10^{-6} \times$ 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
Erythrocytes incorporating ^{59}Fe (%)						
Assay standards						
0.9% NaCl						
0.05 IRP unit of H-14 TaL SL erythropoietin		0.73 ± 0.06				
		3.38 ± 0.42				
0.20 IRP unit of H-14 TaL SL erythropoietin		13.92 ± 2.92				
0.80 IRP unit of H-14 TaL SL erythropoietin		25.71 ± 2.37

EXPLANATION OF PLATE I

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.

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 (O) 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 erythropoietin, 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 1b). Peaks 1 and 2 represent glucose oxidase (186000 mol.wt.) and lactoperoxidase (85000 mol.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 (2.77 IRP units/mg of protein) from sheep plasma and purified erythropoietin (318 IRP units/mg protein) extracted from human urine. The specific radioactivity was high (e.g. 129×10^4 – 252×10^4 c.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.

The technical assistance of Mrs. K. Larrabee and Mr. H. Galbraith is gratefully acknowledged. This work was supported by the following grants: no. CA-08480, during a tenure at St. Jude Children's Research Hospital, Memphis, TN; no. CA-17085, from the National Institutes of Health; no. CH-3 from the American Cancer Society and the Gar Reichman Foundation.

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