RADIOIMMUNOASSAY OF ARTHROPOD MOULTING HORMONE: β-ECDYSONE ANTIBODIES PRODUCTION AND ^{125}I-IODINATED TRACER PREPARATION

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1. Introduction

Ecdysones (or moulting hormones) are polyhydroxysteroids which are involved in ecdysis of arthropods. Since the identification of two hormone forms (α and β) many authors have described the chemistry and metabolism of these compounds [1]. Biological [2], physicochemical [3–6] and radioimmunological [3,7,8] tests have also been developed for their titration.

No direct data have yet been reported regarding the precise localization of intracellular sites of production and storage of ecdysones. An immunocytochemical study seemed to us particularly appropriate; its success depends on obtaining, essentially for ultrastructural studies, specific antibodies with high binding parameters. We report here the conditions of anti ecdysone antibody production and the radioimmunoassay developed with an iodinated derivative as tracer.

2. Materials and methods

2.1. *Unlabelled steroid compounds*

α and β-ecdysones were supplied by Simes (Italy) by courtesy of Dr G. Ferrari. Cholestanol, cholesterol and stigmasterol were obtained from Koch Light Laboratories and ergosterol from Sigma.

2.2. *Radiolabelled compounds*

[^{3}H]-β-ecdysone (3–4 Ci/mmol) was purchased from New England Nuclear and carrier free Na ^{125}I (in NaOH) from the Radiochemical Center, Amersham.

2.3. *Chemicals and solvents*

Carboxymethoxyamine (K and K Laboratories), isobutylchloroformiate (Sigma), tri-*n*-butylamine (Merck), bovine serum albumin (BSA)† (Behringwerke), tyramine (Eastman) were used without further purification. Thin layer chromatography (TLC) was performed on pre-coated plates of silica gel with fluorescent indicator (Merck) and developed in methanol/chloroform/water system (60 : 30 : 5, v/v/v).

2.4. *Immunogen preparation*

β-Ecdysone was linked to BSA according to Erlanger's method [9] and used as immunogen.

2.4.1. β-Ecdysone carboxymethyloxime (β-ecdysone CMO) synthesis

β-ecdysone (25 mg) dissolved in 1.5 ml of pyridine was mixed with 125 mg carboxymethoxyamine (CMA) and incubated for 8 days at room temperature. [^{3}H]-β-ecdysone was added for estimation of recovery. Pyridine was eliminated by successive washings with benzene and evaporation to dryness, ethyl acetate–30% methanol (10 : 1, v/v) solvent system was added to the residue. β-Ecdysone CMO extracted in the organic phase was purified by TLC. The chromato-

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† *Abbreviations:* TLC, thin layer chromatography; BSA, bovine serum albumin; CMO, carboxymethyloxime; CMA, carboxymethoxyamine; PBS, phosphate buffered saline.

gram showed two new u.v. absorbing and equally radioactive spots (R_f: 0.15 and R_f: 0.22). After elution with 70% ethanol (v/v) these derivatives showed u.v. maximum absorption bands at 252 and 290 nm respectively. The final recovery, estimated by the radioactivity in the two spots, was about 50%.

2.4.2. β-Ecdysone CMO-BSA coupling

The two ecdysone derivatives were pooled, dried and 5 μmol dissolved in 100 μl freshly distilled dioxane. Tri-*n*-butylamine (126 μmol) and isobutylchloroformiate (9.5 μmol) were added and the reaction mixture was left for 30 min at 0°C. Then 6 mg of BSA in 360 μl of H_2O–dioxane solution (3 : 2, v/v) were poured into the mixture. After incubation at 4°C for 4 h the conjugate was dialyzed against phosphate buffered saline (PBS) (0.1 M, pH 7.4) for 48 h at 4°C. On the basis of radioactivity of [^{3}H]-β-ecdysone the conjugate was estimated to contain 20 β-ecdysone residues per albumin molecule.

2.4.3. Immunization procedure

Adult male white rabbits were immunized with 2.6 mg of the conjugate emulsified in complete Freund's adjuvant. At two month intervals, booster injections were given intradermally and intravenously with the same amount of conjugate. Blood samples taken after booster injections were tested for antibodies binding parameters and the best ones were used for assays.

2.5. *Tracer preparation*

2.5.1. β-Ecdysone CMO tyramine synthesis

β-Ecdysone CMO was linked to tyramine by the following procedure: to β-ecdysone CMO (1.5 μmol) dissolved in freshly distilled dioxane (100 μl) were added tri-*n*-butylamine (42 μmol) and isobutylchloroformiate (64.5 μmol). The mixture was allowed to stand for 15 min at 0°C. Tyramine (10 μmol) in N/100 NaOH (20 μl) was poured in and left for 1 h at 0°C. Thin layer chromatography of the reaction mixture revealed the presence of two u.v. absorbing products (R_f: 0.72 and R_f: 0.78) which were eluted by 70% ethanol (v/v).

2.5.2. Preparation of radioactive [^{25}I]-β-ecdysone CMO tyramine

β-Ecdysone CMO tyramine was labelled with ^{125}I using the chloramine T method [10]. 10 μl of β-ecdysone CMO tyramine solution (10.6 nmol) was evaporated to dryness. Successively 4 μl of 0.5 M PBS (pH 6.5), 2 μl of Na ^{125}I (350 μCi) and 4 μl of chloramine T (3 mg/ml in 0.05 M PBS) were added and the mixture stirred for 1 min, after which the reaction was stopped by addition of 4 μl of sodium metabisulfite (15 mg/ml in 0.05 M phosphate buffer). After chromatography and autoradiography, labelled materials were isolated, extracted by 70% methanol (v/v) and stored at −20°C for further immunological tests.

2.6. *Standard radioimmunoassay procedure*

Phosphate buffer (0.1 M PBS, pH 7.4, 0.1% gelatin, 0.1% sodium azide) was used routinely to dilute the reagents in the assay. In polypropylene test tubes, standard steroid or buffer (0.1 ml), ^{125}I-labelled β-ecdysone (0.1 ml) (18 000 dpm) and diluted antiserum (0.1 ml) were successively added. The mixture was stirred gently and incubated for 2 h at room temperature and then for 15 min at 0°C. One ml of dextran charcoal mixture (Norit A 2.5 g, Dextran T 70 0.25 mg in 100 ml buffer) was added to each tube. Twenty minutes later, the tubes were centrifuged at 2200 *g* for 15 min at 4°C. The precipitate was counted in a gamma spectrometer.

3. Results and discussion

3.1. *Immunoreactivity of the two ^{125}I-labelled ecdysone derivatives*

The two forms of ecdysone CMO (R_f: 0.15 and R_f: 0.22) described above gave two β-ecdysone CMO tyramine derivatives (R_f: 0.72 and R_f: 0.78) and iodinated materials (R_f: 0.80 and R_f: 0.84). The immunoreactivities of these labelled materials were tested with different antisera dilution. The first one (R_f: 0.80) presented a binding capacity three times higher than the other (R_f: 0.84) (table 1). All the investigations were carried out with the most immunoreactive compound.

3.2. *Sensitivity and specificity of radioimmunoassay*

Fig.1 shows the standard curves obtained using tritiated and iodinated tracers. The latter shows that 70 pg of unlabelled β-ecdysone reduces the amount

Table 1
Binding capacity of the two [^{125}I]-β-ecdysone CMO tyramine to ecdysone antiserum obtained one week after the first booster injection

Bo	Initial dilution of antiserum (%)			
	1/10	1/100	1/10 000	1/50 000
1	89	86	62	25
2	31	29	18	9

Bo: percentage of bound radioactivity.
1 : tracer R_f: 0.80.
2 : tracer R_f: 0.84.

of label bound by 50%. The minimum detectable level calculated following Rodbard's method [11] is 5 pg.

The parallelism of the two curves suggested that the affinity for the antibody was neither impaired by introduction of a tyramyl group on the β-ecdysone molecule nor by its iodination. Consequently the sensitivity of the assay should depend only on the specific radioactivity of the tracer. In our conditions this sensitivity was improved a hundred times when the specific radioactivity rose from 3 Ci/mmol for the tritiated tracer to approximately 400 Ci/mmol for the iodinated product [12].

Cross reactions were performed with α-ecdysone, cholesterol, cholestanol, stigmasterol and ergosterol. As was expected, the lack of a hydroxyl group on the C_{20} atom of the steroid molecule does not prevent α-ecdysone from competing with β-ecdysone from 20% to 100% depending on individual variations of animals in immunological responses. On the other hand there was no significant inhibition of label binding with the other steroids added in concentration 1 up to 10^5 pg.

This sensitive and specific tool could be used either for a quantitative assay or for immunocytochemical and physiological studies.

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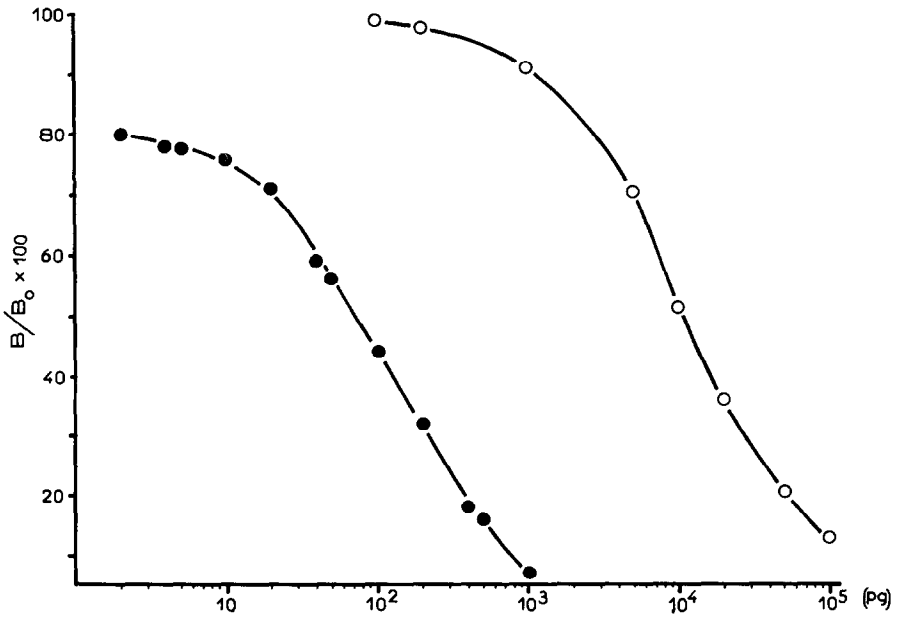

Fig.1. Inhibition of labelled β-ecdysone binding to anti ecdysone serum in the presence of increasing amounts of unlabelled β-ecdysone. (o——o) [^{3}H]-β-ecdysone, 1/100 antiserum dilution. (•——•) [^{125}I]-labelled-β-ecdysone, 1/20 000 antiserum dilution. B: bound radioactivity in presence of competitor. Bo: bound radioactivity in absence of competitor.

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