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Preparation of an Ecdysone Immunogen for Radioimmunoassay Work*

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A highly improved procedure for the preparation of ecdysone-protein conjugates for immunological work is reported. Bovine thyroglobulin is succinylated and the succinylated protein is coupled to β-ecdysone with 1-ethyl-3-(-dimethylaminopropyl)-carbodiimide in the presence of the acylation catalyst 4-dimethylaminopyridine. The antiserum obtained using this immunogen provides a radioimmunoassay sensitive to 25 pmol of β-ecdysone. The anti-ecdysone antibody cross-reacts with α-ecdysone but not with cholesterol or progesterone. This procedure reverses the standard strategy for synthesizing ester linkages in hapten-protein conjugates and should have widespread applicability for the preparation of other such conjugates for immunological work.

The primary obstacle in the development of radioimmunoassays for ecdysone has been the preparation of an ecdysone-protein conjugate. In our hands, published procedures for the conjugation of ecdysones to proteins (1–4) have been deficient for a number of reasons, including lengthy protocols, low yields, difficult separations, annoying side products, and chemical destruction of the hapten. These problems led us to re-examine existing methodology for the coupling of haptens to proteins in search of improvement. We report here a new strategy for the preparation of an ecdysone-protein conjugate suitable for immunological work that reverses the standard approach for the formation of ester linkages between haptens and proteins using the most recent chemical technology. The advantages of this strategy are its facility, efficacy, economy, and potentially wide applicability to other haptens and proteins as well.

EXPERIMENTAL PROCEDURES

Materials—Bovine thyroglobulin (type I), bovine serum albumin, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were purchased from Sigma. Succinic anhydride and 4-dimethylaminopyridine were obtained from Mallinckrodt. β-Ecdysone was purchased from Simes S.P.A. Milano, Italy. Freund's complete and incomplete adjuvants were purchased from the Colorado Serum Co. Laboratories, Norton, Mass. Three New Zealand white rabbits were purchased from Pine Acre, Norton, Mass.

All water used was distilled and then passed through a Millipore filter. Dimethyl sulfoxide was distilled at reduced pressure from calcium hydride under nitrogen and stored over molecular sieves. All other reagents were used as obtained from the manufacturer.

Protein Succinylation—Succinic anhydride (210 mg, 2.1 mmol) was added in small increments to a solution of thyroglobulin (70 mg, 14.6 μmol of lysine residues) (5) while the pH was maintained at 7 with 1 N NaOH. The solution was incubated at room temperature for 30 min., the pH was adjusted to 2.5 with 1 N HCl, and the solution was dialyzed against Millipore water (3 × 3 liters) overnight. The contents of the dialysis bag were lyophilized to yield succinylated thyroglobulin (61 mg).

Preparation of Ecdysone-Protein Conjugate—The succinylated thyroglobulin (61 mg) was dissolved in dry dimethyl sulfoxide (15 ml); to the solution was then added β-ecdysone (30 mg), 4-dimethylaminopyridine (1 mg), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (422 mg). The reaction mixture was incubated at room temperature for 4½ h and then dialyzed against Millipore water (3 × 3 liters) overnight.

Solubilization of the Ecdysone-Protein Conjugate—During dialysis, a portion of the ecdysone-protein conjugate precipitated. Nevertheless, upon completion of the dialysis, the contents of the dialysis bag were lyophilized, yielding 69 mg of conjugate, which was then completely dissolved in Millipore water (5 ml) by slowly adjusting the pH of the solution to 7 by the addition of pH 10 buffer (potassium carbonate/borate-hydroxide, 50 mM). The buffered solution was then dialyzed as before, and the dialysate was lyophilized and stored at −20°C. This buffered ecdysone-protein conjugate dissolved readily in water at pH 7.

Rabbit Inoculation and Serum Collection and Assay—Rabbits were inoculated and boosted essentially by the procedure of Horn *et al.* (4). Serum was collected at regular intervals and a standard radioimmunoassay curve for β-ecdysone was constructed from results obtained using the assay method of Borst and O'Connor (1). Using the same method, the antiserum was tested for cross-reactivity with cholesterol and progesterone.

RESULTS

The ultraviolet spectrum of the ecdysone-thyroglobulin conjugate (0.1 mg/ml in H_2O, blanked against thyroglobulin) displayed a sharp absorbance maximum at 249 nm (absorbance = 0.22). This corresponds to a hapten density of 118 molecules of ecdysone/molecule of thyroglobulin, calculated using 24,000 as the extinction coefficient for ecdysone (6) and 670,000 as the molecular weight of bovine thyroglobulin (5). Comparable densities were obtained when radioactive ecdysone was coupled to thyroglobulin and values were calculated based on the radioactivity of the conjugate. The maximum

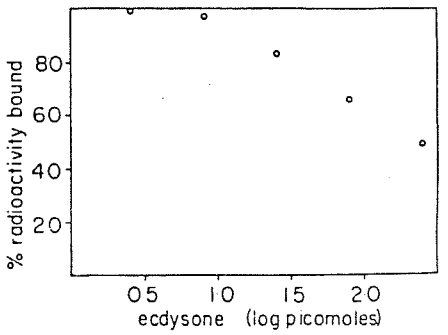

FIG. 1. **Standard curve run according to the method of Borst and O'Connor (1).** Each assay contained 25,000 cpm of β-[^{3}H]ecdysone and varying amounts (3 to 30 pmol) of unlabeled ecdysone.

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number of sites available for conjugation is 140/molecule of thyroglobulin, based on the lysine content of the protein. Control experiments demonstrated that ecdysone itself was unaffected by the reaction conditions used for coupling.

Antiserum obtained from rabbits inoculated with the conjugate was of satisfactory sensitivity, although with lower than expected titre. A standard curve (Fig. 1) showed that half of the bound radioactive ecdysone was displaced with 25 pmol of unlabeled material. Some cross-reactivity was observed with α-ecdysone, but not with either cholesterol or progesterone.

DISCUSSION

Since 1959, when Erlanger (8) first achieved a successful preparation of steroid antigens, the strategy for synthesizing ester linkages between haptens and proteins has remained essentially unchanged. The traditional approach, followed by previous investigators, has been to covalently attach a linker to the hapten through a functional group on the hapten, and then to couple the hapten-linker derivative to an immunologic carrier such as thyroglobulin (Figure 2). We have reversed this approach, attaching the linker first to the thyroglobulin, and only then coupling the thyroglobulin-linker derivative to the hapten.

Although conjugates have occasionally been made in which a hapten is coupled to a derivatized carrier (9), such coupling has required the reaction of a strong nucleophile (*e.g.* an amine or a thiol) with activated derivative. Of course, such a reaction is impossible in the case of ecdysone and many other haptens, where the only significant nucleophile is an alcohol

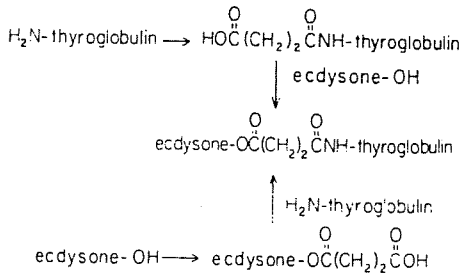

FIG. 2. **Strategy for the synthesis of ecdysone-protein conjugates.** The standard strategy for the synthesis of ecdysone-protein conjugates (*lower path*) first involves the formation of a succinylated hapten. The approach reported here (*upper path*) first involves the formation of a succinylated protein.

function. It is important to note that, because of the poor nucleophilicity of alcohols, our synthetic approach would not be feasible without dimethylaminopyridine. Dimethylaminopyridine has only recently been recognized to be a potent acylation catalyst (10), and its use in esterification procedures in nonaqueous media permits the synthesis of conjugates in high yield after short reaction time.

Reversing the standard strategy for the synthesis of hapten-protein conjugates offers significant advantages in the case of ecdysone. The succinylation of proteins in aqueous solution is extremely simple and highly efficient, producing yields approaching 100%. The succinylated protein can easily be separated from reactants and by-products simply by dialyzing overnight; no chromatographic step is required, a distinct advantage over the traditional approach where hapten-linker derivatives often must be purified with difficulty. Furthermore, addition of the ecdysone last is economical, since it is the most expensive component of the conjugate.

While the exact procedure for synthesizing hapten-protein conjugates will depend on the precise nature of the hapten, all of the advantages described above are potentially realizable should this strategy be applied to a wide range of haptens.

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