A FLUORESCEIN-LABELED DERIVATIVE OF ESTRADIOL WITH BINDING AFFINITY TOWARDS CELLULAR RECEPTORS

by

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SUMMARY

1,3,5,[10]-Estratriene-3,17β-diol-6-iminooxyacetic acid (I) and fluorescein amine condense in the presence of dicyclohexylcarbodiimide to give a fluorescent derivative of estradiol: 9{ p-(1,3,5[10]-Estratriene-3,17β-diol-6-iminooxyacetylamino) o-carboxyphenyl}-6-hydroxy-3-isoxanthenone (II). (II) can be prepared and isolated on a micro scale by thin layer chromatography. The results show that (II) is useful as a probe for estrogen interaction with cellular receptors and with antibody to estradiol.

INTRODUCTION

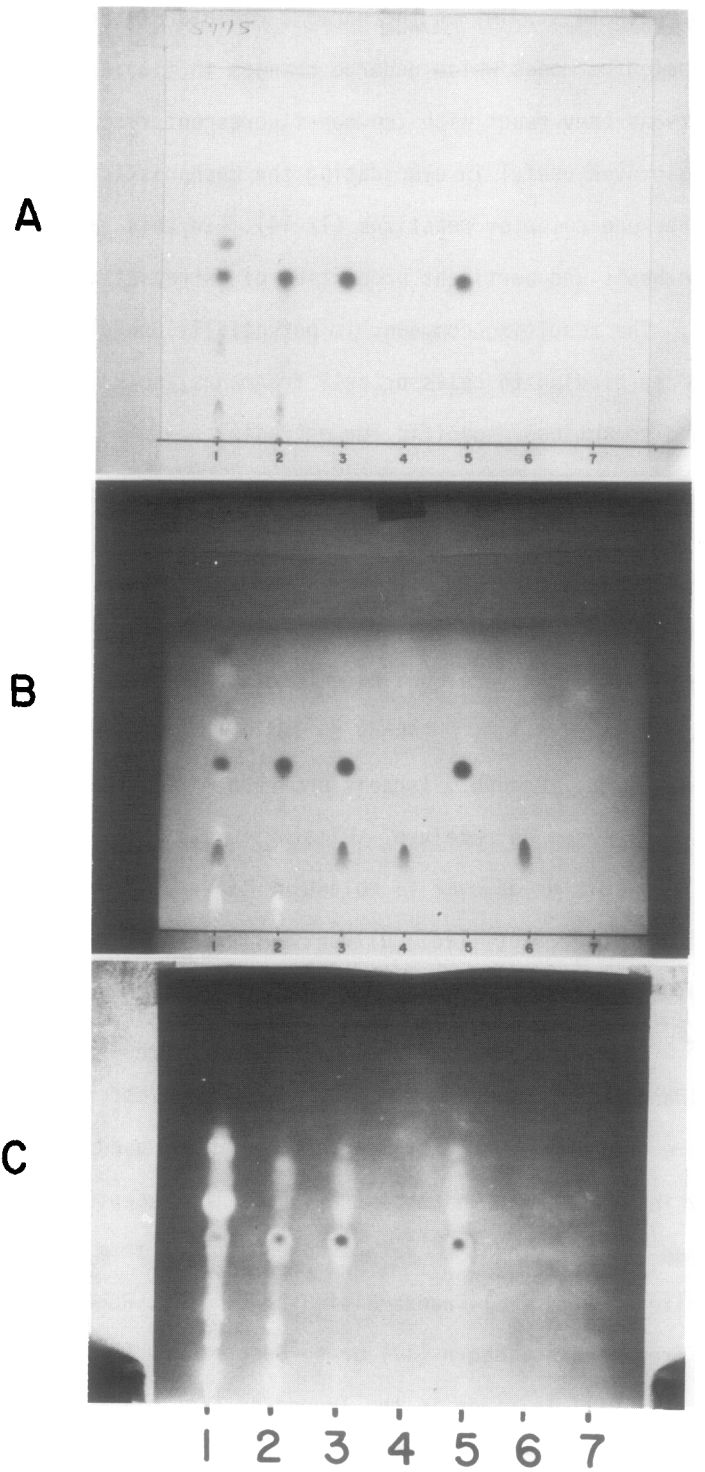
In recent years the binding of estrogens to specific cellular receptors (1-6) and the binding of peptide hormones to their specific membrane receptors (7-11) has been studied by means of radiolabeled hormones. Workers have been particularly interested in determining both the number of specific receptor sites per cell and the magnitude of the binding affinity. However, certain aspects of the radiolabeling approach are cumbersome and hamper the investigator's efforts to properly characterize the binding process. In particular, the necessity of physically separating the bound and free forms of the labeled hormone in order to make binding measurements may perturb equilibrium conditions, makes the experiments slow and laborious and limits kinetic studies to very slow processes.

Abbreviations: (I): 1,3,5,[10]-estratriene-3,17β-diol-6-iminooxyacetic acid or estradiol-6-iminooxyacetic acid. (II): 9{ p-(1,3,5 [10]-estratriene-3,17β-diol-6-iminooxyacetylamino) o-carboxyphenyl} 6-hydroxy-3-isoxanthenone which may also be called estradiol-6-iminooxyacetyl fluorescein amine.

A new approach to studies on the hormone-receptor interaction makes use of fluorescent-labeled hormones which undergo changes in fluorescence polarization and/or intensity as they react with the non-fluorescent receptor. Similar methodology has proven useful in elucidating the mechanistic details of antigen-antibody, and hormone-receptor reactions (12-14). In this paper we report a micro scale synthesis and pertinent properties of estradiol chemically coupled to fluorescein. The resulting compound is potentially useful for in vitro studies of hormone binding to cells or cell fragments, solubilized receptor preparations and to antibody specific for estradiol.

MATERIALS AND METHODS

Fluorescein amine, isomer I (Sigma, Chemical Co., St. Louis, Mo.) was purified by thin layer chromatography on 5766 plates in chloroform-ethanol (7:3 by volume). Dicyclohexylcarbodiimide (Aldrich Chemical Co., Milwaukee, Wis.), (I) Steraloids, Pawling, N.Y.), antibody to 17β-estradiol-6-BSA and to 17β-estradiol 17-BSA (Miles-Yeda Ltd., Rehovot, Israel) and rabbit serum albumin (Pentex, Inc., Kankakee, Ill.) were used as received. Dimethylformamide (Baker Chemical Co., Phillipsburg, N.J.) was dried over #4 molecular sieves. Other solvents were of reagent grade and were redistilled. Diluent buffer, pH 7.0:0.15 M NaCl, 0.01 M $Na_2H\ PO_4$ and 0.005 M NaH_2PO_4; Tris buffer, pH 7.7:0.0067 M Tris HCl, 0.0033 M Tris base, 0.0015 M EDTA·Na_4 and 0.001 M NaN_3. Precoated thin layer chromatography plates (#5766) and sheets (#5775) (E.M. Laboratories, Elmsford, N.Y.) were ethanol extracted before use. A modified (15) Kober method was used to test for the steroid moiety in (II). Protein concentration of the receptor preparations was by the Lowry method (16). Cytosol receptors were prepared from frozen uteri of normal New Zealand White rabbits (Pel-Freeze Biologicals, Inc., Rogers, Arkansas) according to Korenman and Sanborn (17) or to Puca et al. (18). The procedure for the preparation of (II) is given in the legend of Figure 1. An analogous compound, N-acetyl fluorescein amine, was made in similar fashion by using acetic acid in place of (I).


A
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C
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Fluorescence polarization measurements were made in a fluorescence polarometer with a time constant of about 1 sec and with a cell thermostatted to ± 1°. Scatchard plots were obtained from polarization values (12). Rate constants for the dissociation of complexes were obtained by the dilution jump technique (19,20) in which a solution of a dissociable complex is quickly diluted by a large factor and the polarization is then monitored as a function of time.

RESULTS AND DISCUSSION

The validity of the structure for the fluorescent estradiol derivative (II) is based upon the method of synthesis of the compound and upon several other lines of evidence presented below. The chromatographic detection of (II) as a new fluorescent spot arising in the complete reaction mixture but not in the controls is shown in Figure 1. The yield of (II) is 14% based upon either (I) or FNH_2. This separation also provides the basis for the isolation and purification of (II) from the reaction mixture. The R_f's of (II) on 5775 plastic TLC strips in various solvents are as follows: about zero in both chloroform and

Figure 1. Photographs of a thin layer chromatogram showing the isolation of (II) from the reaction mixture. Illumination of chromatogram-A: visible light, B: short wavelength ultra-violet and C: long wavelength ultra-violet. Stock solutions of the reactants at the following concentrations were made up in dimethylformamide: fluorescein amine, 50 mg/ml; (I) 40 mg/ml and dicyclohexylcarbodiimide, 100 mg/ml. Acetone-HCl (0.0074M) was made by mixing 5 ul of concentrated HCl with 8.1 ml acetone. Mixtures were made as shown below and after 30 min reaction at room temperature, 5 ul per spot was applied to a 20 x 20 cm 5775 sheet. Development was in a chloroform-95% ethanol mixture, 7:3 by volume. (II) can be seen as a spot ($R_f \sim 0.5$) present in 1 but not in the others. This spot was eluted with 95% ethanol and the solution was stored at -20°. The procedure may be scaled up as necessary and thick plates (5766) may be used for the isolation.

Composition of Reaction Mixtures (ul)

Track	1	2	3	4	5	6	7
(I)	5	0	5	5	0	5	0
Dimethylformamide	0	5	2	4	7	6	9
Fluorescein amine	4	4	4	0	4	0	0
Acetone-HCl	50	50	50	50	50	50	50
Dicyclohexylcarbodiimide	2	2	0	2	0	0	2

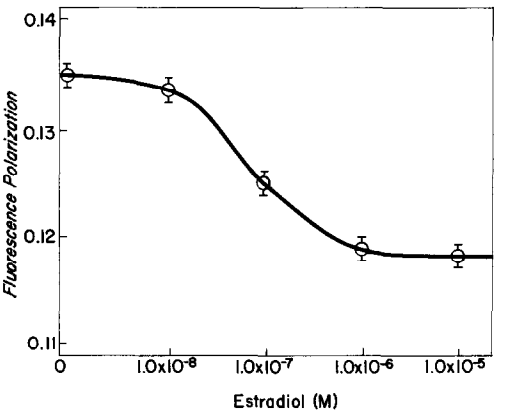

Figure 2. Specificity of the reaction between (II) and anti-estradiol as shown by inhibition of the reaction by estradiol. For each point the cuvette contained initially 200 ul of anti-serum and 3 ml of estradiol in diluent buffer to give the various estradiol concentrations indicated on the abscissa. The mixture was incubated at room temperature for 30 min and then a constant amount of (II) (20 µl of 3.0 x 10^{-8} M) was added. After 20 min the polarization of fluorescence was read. Inhibition is indicated by the decrease in polarization with increasing estradiol levels. In this test a change of 0.001 polarization units is produced by a 3 ng/ml change in estradiol concentration.

benzene, 0.52 in ethyl acetate, 0.53 in a mixture of 7 chloroform and 3 ethanol (95%) 0.86 in dioxane, 0.88 in 95% ethanol, 0.96 in tetrahydrofuran and 0.97 in acetone and in 1,2-dimethoxyethane.

The absorption spectrum of (II) is consistent with a 1:1 mole ratio of the fluorescein and estradiol moieties (21). THe positive Kober test also indicates the presence of estradiol in (II). The emission spectrum of (II) in the visible region is very similar to that of free fluorescein or of N-acetyl fluorescein amine.

The high reactivity of (II) with antibody to estradiol gives a direct indication for the presence of the estradiol moiety. This reactivity was found to depend upon the point of attachment of the estradiol molecule to the carrier protein in producing the immunogen. When the carrier was attached to the 6 position of estradiol a high reactivity was observed with (II) together with a strong inhibition by estradiol (Figure 2). However, when the estradiol moiety was coupled via the 17-OH position in the immunogen, the resulting anti-

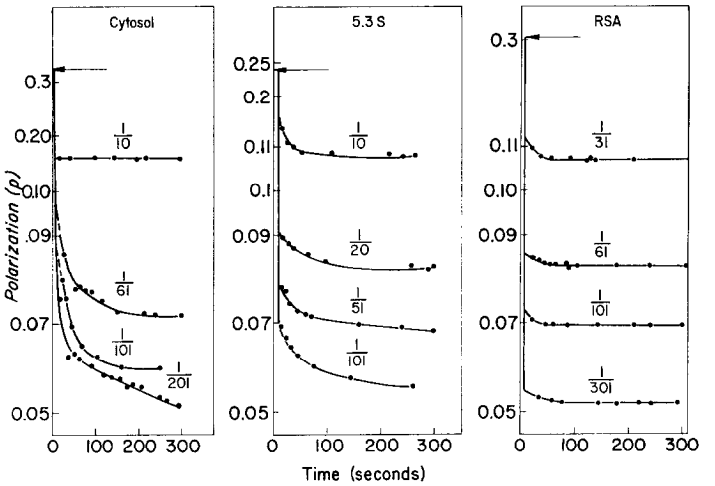

Figure 3. "Dilution jump" experiments showing the kinetics of the dissociation of complexes of (II) with cytosol receptors or with rabbit serum albumin. Initial concentrations were as follows: cytosol, 8.3 mg/ml protein with 2.4 x 10^{-8} M (II); 5.3S fraction, 1.9 mg/ml with 3.8 x 10^{-8} M (II); rabbit serum albumin, 2 mg/ml with 3.6 x 10^{-7} M (II). The buffer contained 0.1 M Tris, 0.015 M EDTA and 0.4 M KCl and the preliminary incubation to form the complex was for 1 hr at 4°C. The dilutions in the dilution jumps are indicated on the figure and the polarization was followed at 4°C after the jump. The values of the intial polarizations prior to the dilution jump are indicated by the arrows. The polarization values of these solutions are close P_b, the polarization of completely bound (II), indicating that only a small fraction of the (II) was not bound in the original incubation mixture prior to the dilution jump. The cytosol and 5.3S cytosol receptors were prepared according to Puca et al. (18).

body reacted only weakly with (II) and the inhibition by unlabeled estradiol was also weak. Similarly, the binding of (II) by uterine cytosol receptors as shown by fluorescence polarization measurements (21) and the inhibition of this reaction by estradiol and by diethylstilbestrol indicates the presence of an estradiol moiety in (II). As controls, it should be noted that neither fluorescein nor N-acetyl fluorescein amine bind appreciably to either anti-estradiol or to cytosol receptors.

Equilibrium measurements of the binding of (II) to cytosol receptors (21) gave highly curved Scatchard plots indicative of either site heterogeneity or cooperativity. In such instances, dilution jump measurements are especially useful to gain information on the high affinity sites. By this method, slowly dissociating complexes are isolated in time and can be observed after other complexes

have dissappeared (19,20). Dilution jump results for (II)-receptor complexes are shown in Figure 3. As a comparison control for a low affinity type of binding, the dissociation of complexes between (II) and rabbit serum albumin is also shown. The high values of the intial polarizations (indicated by arrows in Figure 3) show that most of the (II) was bound before the dilution jump. Simple inspection of these curves show that the dissociation of complexes involving receptors has a very slow component not seen in the serum albumin complexes. Estimates of k_{-1} for the sites tightly binding (II) in cytosol and the 5.3S fraction of cytosol indicate values of the order of 10^{-2} to 10^{-3} sec^{-1}. In contrast, the complexes formed between (II) and serum albumin dissociate very rapidly and reflect much lower binding affinity.

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