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HIGHLY SPECIFIC POLYCLONAL ANTISERA AGAINST ESTRIOL: CROSS-REACTIVITY RESTRICTION FOLLOWING AFFINITY CHROMATOGRAPHY

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Summary—An immunosorbent technique was developed to attenuate cross-reactivity of a polyclonal antiserum against a 4(2) (*p*-carboxyphenylazo)-1,3,5[10]-estratrien-3,16α,17β-triol–bovine serum albumin conjugate. The chromatographic separation of antiserum through stationary phases having either *p*(carboxymethyl)phenylazo-phenol or *p*(carboxymethyl)-phenylazo-2-naphthol side residues reduced the antiserum avidity, while increasing the apparent antiserum affinity and decreasing the residual cross-reactivities against heterologous ligands. The highly specific antiserum obtained allowed the development of a competitive binding assay over an extended analytical range, which opens up the possibility of direct measurement of estriol from the early pregnancy to delivery. The significance of the attenuation of antiserum cross-reactions after affinity chromatography is discussed with reference to epitope–paratope interaction in the case of small endogenous molecules like estrogens.

INTRODUCTION

The modification of a functional group in a steroid molecule has proved to be a major source of the cross-reactivity against structurally related steroids in polyclonal antisera [1]. This led to the view that cross-reactivity is an inherent property of the antiserum, arising from inappropriate hapten presentation to the immune apparatus, and hampered attempts to individuate structural factors actually responsible for the expression of cross-reactivity.

Among estrogen devoted immunogens, a high degree of specificity in recognizing D ring structures was obtained by synthesizing estrogen–azo–protein conjugates [2–6], where coupling between the steroid and the carrier molecule occurs in the A ring, mainly at C_4 [7]. These immunogens showed poor immunogenicity leading to a slow immune response and to the production of low avidity antisera. Poor discrimination for A ring substituted estrogens, cathecolestrogens or individual androgens differing from the antigen with respect only to the chemical nature of the A ring (i.e. norethisterone vs ethynylestradiol) was also evident.

On the basis of cross-reaction profiles of antibodies raised against estrogen–azo haptens, we developed an immunosorbent technique to verify if the attenuation of residual cross-reactivity onto the A ring was possible. This paper describes the effects of antiserum separation through stationary phases having either benzylazo-phenol or benzylazo-2-naph-

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Trivial names used: 1,3,5(10)-estratrien-3,16α,17β-triol, estriol; 1,3,5(10)-estratrien-3,16α,17α-triol, 17-epiestriol; 1,3,5(10)-estratrien-3,16α,17β-triol-3-sulfate, estriol-3-sulfate; 1,3,5(10)-estratrien-3,16α,17β-triol-16-glucuronide, estriol-16-glucuronide; 1,3,5(10)-estratrien-3,17β-diol, estradiol; 1,3,5(10)-estratrien-3,17β-diol-16-one, 16-ketoestradiol; 1,3,5(10)-estratrien-2,3,17β-triol-2-methyl ether, 2-methoxyestradiol; 1,3,5(10)-estratrien-3,17β-diol-3-(β-D-glucuronide), estradiol-3-glucuronide; 1,3,5(10)-estratrien-3,17β-diol-3-sulfate, estradiol-3-sulfate; 1,3,5(10)-estratrien-3,17β-diol-3-sulfate 17-glucuronide, estradiol-3-sulfate 17-glucuronide; 1,3,5(10)-estratrien-3-ol-17-one, estrone; 1,3,5(10)-estratrien-3,4-diol-17-one, 4-hydroxyestrone; 1,3,5(10)-estratrien-3,16α-diol-17-one, 16α-hydroxyestrone; 1,3,5(10)-estratrien-3-ol-17-one-3-(β-D-glucuronide), estrone-3-glucuronide; 1,3,5(10)-estratrien-2,3-diol-17-one-2-methyl ether, 2-methoxyestrone; 4-pregnene-3,20-dione, progesterone; 5β-pregnane-3α,20α-diol, pregnandiol; 5β-pregnane-3α,17α,20α-triol, pregnantriol; 4-androstene-3,17-dione, androstenedione; 4-androstene-17β-ol-3-one, testosterone; 5α-androstan-17β-ol-3-one, 5α dihydrotestosterone; 11β,17α,21-trihydroxypregn-4-ene-3,20-dione, cortisol.

thol side chains, on residual cross-reactivities of polyclonal antisera against an estriol–azobenzoyl–bovine serum albumin (E_3–azobenzoyl–BSA) conjugate raised in rabbits. The results gave rise to information on factors affecting the occurrence of cross-reactivity in polyclonal antisera against estrogens.

EXPERIMENTAL

Materials

Analytical grade reagents, estriol (E_3) and other purified steroids, as well as bovine serum albumin (BSA) fraction V, were supplied by Sigma Chemical Co. (St Louis, MO, U.S.A.). Complete and incomplete Freund's adjuvants were obtained from Difco Labs (Detroit, MI, U.S.A.); Silica Gel GF_{254} plates and solvents were from Merck (Darmstadt, Germany), Cumene from Lumac (Landgraaf, The Netherlands) and Affi Gel 102 from Bio-Rad (Richmond, CA, U.S.A.).

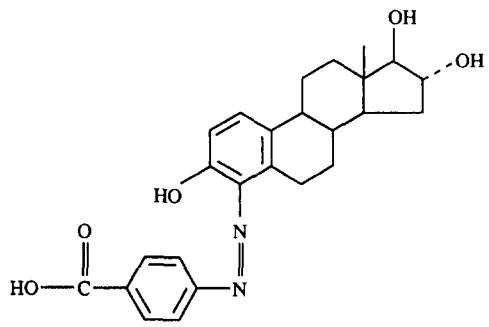
Standards and radionuclides

Stock solutions of E_3 and other conjugated steroids tested for cross-reactivity were prepared by dissolving 1.0 mg hapten in 10 ml ethanol. Aliquots of the stock solutions were dried under a nitrogen stream and the residues were dissolved by overnight incubation in appropriate volumes of 100 mM phosphate buffer, pH 7.4, to prepare 1×10^{-2} M solutions. The conjugated steroids were dissolved directly in the same buffer solution. The concentrations were checked by u.v. spectra (Uvidec-510, Jasco Ltd, Japan) and solutions stored as indicated by the manufacturer for individual steroids. Before use, the standard solutions were serially diluted to concentrations ranging from 1×10^{-6} to 1×10^{-12} M.

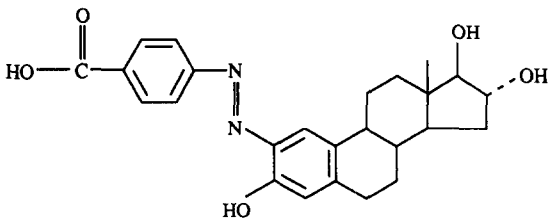
[2,4,6,7-^{3}H(N)]E_3 (sp. act. 3885.0 GBq/mmol) was obtained from Dupont de Nemours GmbH (New England Nuclear), Germany. The radiochemical purity of the isotope was judged to be >95%, as checked by TLC on Silica Gel GF_{254} (solvent system, toluene–ethanol, 9:1) and chromatography on Whatman No. 1 paper, treated with 30% formamide in acetone (solvent system, butyl acetate–ethyl acetate–water, 85:15:5). The isotope was stored at −20°C in ethanol. Before use, aliquots of radioligand solution were evaporated under a nitrogen stream and dissolved in the assay buffer to a final concentration of 1×10^{-9} M.

Synthesis of E_3–azobenzoyl–BSA conjugate

4(2)(*p* - Carboxyphenylazo) - 1,3,5[10] - estratrien-3,16α,17β-triol (E_3–azobenzoic acid) was synthesized as described previously [2], with minor modifications. Under appropriate conditions, a diazonium ion enters the A ring of an estratriene mainly at C_4 and to a minor extent at C_2 (Fig. 1) [7,8]. In this study, isomers were not separated. E_3–azobenzoic acid was prepared by dropwise addition (within 10 min and under stirring in an ice-water bath) of a refrigerated diazonium salt solution (prepared by adding 69 mg of sodium nitrite in 2 ml water to 137 mg of *p*-aminobenzoic acid in 4 ml distilled water and 2.5 ml hydrochloric acid 1 N) to 288 mg of E_3, dissolved in 10 ml distilled water, 3 ml NaOH 1 N, 2 ml dioxane and 2 ml pyridine. After 30 min of stirring in the absence of light, the mixture was adjusted to pH 5.5 and the product was extracted with 1-butanol. The unreacted E_3 was removed with CH_2Cl_2, the solution was acidified with HCl (pH 4.0) and further extracted with CH_2Cl_2–1-butanol (1:1 v/v). The organic layer was then washed with NaCl saturated water until neutrality of the aqueous layer was obtained. TLC on Silica Gel



4-*p* carboxyphenylazo-1,3,5 [10]-estratrien-3,16α,17β, triol



2-*p* carboxyphenylazo-1,3,5 [10]-estratrien-3,16α,17β, triol

Fig. 1. Structural formulas of estriol-4(2)-azobenzoyl isomers. Isomers were not separated before conjugation to BSA by the mixed anhydride method, in order to increase exposure modes of the phenolic hydroxyl. Coupling with the carrier proteins occurs mainly at the ε-amino group of *Lys* residues via a carbamide linkage.

GF_{254} plates, with a water saturated 1-butanol eluent, gave $R_f = 0.68$. The reaction products were dried under reduced pressure.

E_3–azobenzoic acid was conjugated to BSA by the mixed anhydride method. E_3–azobenzoic acid (200 μmol) was dissolved in 3 ml *N,N*-dimethylformamide and equimolar amounts of triethylamine and isobutyl chloroformate were added while stirring in an ice-water bath. 30 Min later, the mixture was added dropwise to a refrigerated alkaline solution of BSA in distilled water and *N,N*-dimethylformamide (5:2 v/v) under stirring. When turbidity became evident, the coupling reaction was stopped by lowering the pH to below 4.5 with HCl. The E_3-azobenzoyl–BSA conjugate was purified by gel filtration through Sephadex G25 (Pharmacia, Sweden), using water saturated 1-butanol as the mobile phase. The first colored peak was collected and pooled. This eluate was dialyzed against distilled water, and finally lyophilized. The number of E_3–azobenzoic acid residues bound per molecule of carrier protein was estimated by u.v. light absorption to be 22.

Immunization

Immunization was carried out in 5 adult (3–5 kg) male New Zealand rabbits by multiple site dorsal s.c. injections (0.2 ml) of the immunogen. Animals were immunized with 1 mg antigen in 1 ml of 100 mM phosphate buffer, pH 7.4, emulsified with an equal amount of Freund's complete adjuvant. Booster injections (1 mg) were given every 30 days, in the same buffer solution, emulsified with an equal amount of incomplete adjuvant. At the third month of immunization, the use of the oil component was omitted in 2 animals to evaluate the role of the non-specific enhancement of the immune apparatus in eliciting the antibody response to the immunogen. Blood was obtained from the ear marginal vein (20–30 ml). The rabbits were bled every 30 days, 15 days after administration of the immunogen. Clotting was assisted by maintaining the tube at 37°C for 1 h before transfer to a cold room for clot contraction. After 2 h, the tubes were centrifuged at 4000 rpm for 10 min and the serum removed. Serum samples were then heated at 56°C for 1 h, allowed to reach room temperature and stored at −20°C.

Synthesis of Affi Gel 102 conjugates

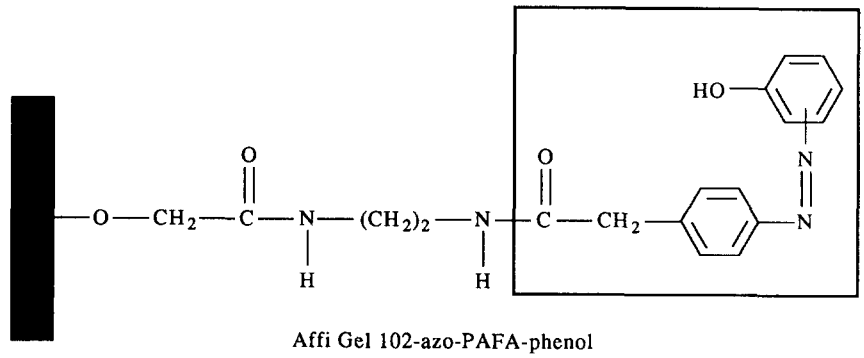
p(Carboxymethyl)phenylazo-phenol (azo-PAFA-phenol) was synthesized according to the procedure described above. Refrigerated solution (10 ml) containing an equimolar amount of sodium nitrite was added dropwise to 210 mg of *p*-aminophenyl acetic acid (PAFA), dissolved in 14 ml of distilled water and 3.5 ml HCl 1 N and cooled in ice-water bath. The diazonium salt solution, after 30 min standing in an ice-water bath under stirring, was added to 65 mg of phenol dissolved in 1.75 ml NaOH 1 N and 2.25 ml distilled water. After 30 min incubation, the yellow-brownish azo-PAFA-phenol solution was acidified with acetate buffer 1 M, pH 4.0. A red-brown precipitate was collected and dried. The purity of the crystallized product was checked by TLC on Silica Gel GF_{254} plates with water saturated 1-butanol as the eluent ($R_f = 0.82$).

The same procedure was applied to synthesize *p*(carboxymethyl)phenylazo-2-naphthol (azo-PAFA-2-naphthol), starting with 99 mg of 2-naphthol. Upon acidification, the dark-red solution of azo-PAFA-2-naphthol gave a magenta-red precipitate which was collected and dried. Its purity was checked as described above ($R_f = 0.75$).

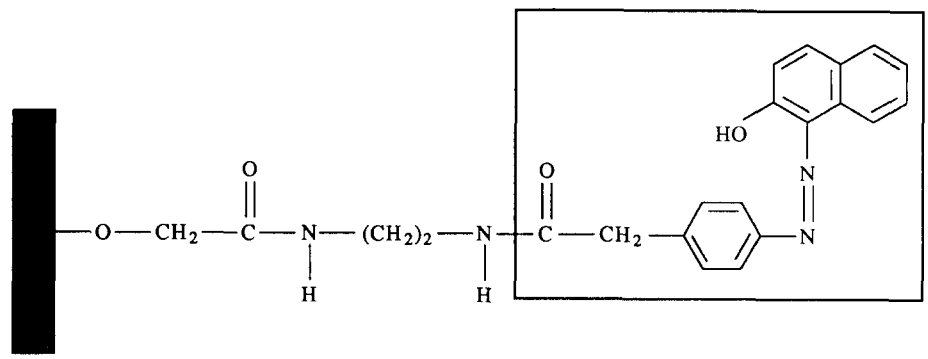
Azo-PAFA-phenol (200 mequiv) and azo-PAFA-2-naphthol (200 mequiv) were conjugated to Affi Gel 102 (2 ml, 15–65 mequiv/ml) by the carbodiimide method (Fig. 2). Affi Gel 102 was washed with 500 mM NaCl and diluted with 3 vol of the same solution. Azo-PAFA-phenol was dissolved in 3 ml dimethylformamide and 4 ml distilled water (pH 4.4). 10 mg/ml gel *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride and the Affi Gel 102 were added and incubated overnight with gentle stirring. After coupling, the gel was washed extensively with the same mixture used for the solubilization of the azo-PAFA-derivative, poured into a glass column (5 ml vol) and perfused with NaCl 500 mM in 50 mM phosphate buffer, pH 8.5, until the eluted liquid was free from azo-compounds, and finally equilibrated with 100 mM phosphate buffer, pH 7.4. The same synthesizing procedure was used for azo-PAFA-2-naphthol. In this case, extensive washing of the gel with toluene and alkaline NaCl solution is needed to remove unreacted azo-PAFA-2-naphthol.

Affinity chromatography of antisera

Aliquots of antisera (1 ml) were applied to either Affi Gel 102-azo-PAFA-phenol or Affi Gel 102-azo-PAFA-2-naphthol columns, allowed to diffuse into the stationary phase (2 ml)



Affi Gel 102-azo-PAFA-phenol



Affi Gel 102-azo-PAFA-2-napthol

Fig. 2. Structural formulas of Affi Gel 102-azo-PAFA-phenol and Affi Gel 102-azo-PAFA-2-naphthol conjugates. The dashed area indicates the agarose matrix. The rectangle encloses the azo-PAFA-phenol and azo-PAFA-2-naphthol residues, which mimick parts of the hapten molecule. A similarity is apparent between the agarose hydrophilic spacer and the lateral chain of *Lys*.

and recycled 15 times throughout the gel in 1.5 h with the use of a peristaltic pump. The mobile phase was eluted from the column with 100 mM phosphate buffer, pH 7.4, and monitored by u.v. (280 nm) (LKB 2238 Uvicord S II). Complete recovery of antisera was obtained within 3 vol of the effluent. The volume of the collected mobile phase was measured and accounted for in the calculation of dilutions required for the binding assays. Desorption of bound antibodies from the Affi Gel 102-azo-PAFA-phenol was accomplished by stepwise application of 100 mM sodium citrate buffer, pH 4.0, to the column. 500 μl fractions were serially collected into glass vials containing 100 μl of 1 M Tris–HCl, pH 8.5, and tested for their binding capacity against [2,4,6,7-^{3}H(N)]E_3 (23,300 dpm). Attempts at desorbing retained antibodies from Affi Gel 102-azo-PAFA-2-naphthol were made either as above or with NaCl 2 M in 50 mM phosphate buffer, pH 7.4.

Characterization of antisera

The characterization included the determination of optimum dilutions (titers), affinity constants and specificities of the antisera raised against the E_3–azobenzoyl–BSA conjugate, as well as of the same antisera following affinity chromatography through the Affi Gel 102-azo-PAFA-phenol or Affi Gel 102-azo-PAFA-2-naphthol columns. To test antibody titer, a conventional labeled antigen immunoassay procedure was followed. 23,300 dpm of [2,4,6,7-^{3}H(N)]E_3 solution in 100 mM phosphate buffer, pH 7.4, were added to each reaction tube. The antiserum dilution that could bind 40–50% of the radioligand in 300 μl of the reaction mixture was regarded as optimal for RIA. Standard curves were constructed by setting up duplicate assay tubes containing 100 μl antiserum, 100 μl radioligand and 100 μl unlabeled E_3 to the appropriate dilutions. The specificities of antisera were tested by cross-reaction studies with purified steroids. In accordance with Abraham's criteria [8], the cross-reactivity was expressed as the percentage ratio between the mass of the unlabeled antigen and that of the heterologous ligand, required to displace 50% of antibody-bound [^{3}H]antigen. The assay tubes were agitated on a Vortex mixer and incubated overnight at 4°C. Bound/free separation was accomplished by adding 0.2 ml

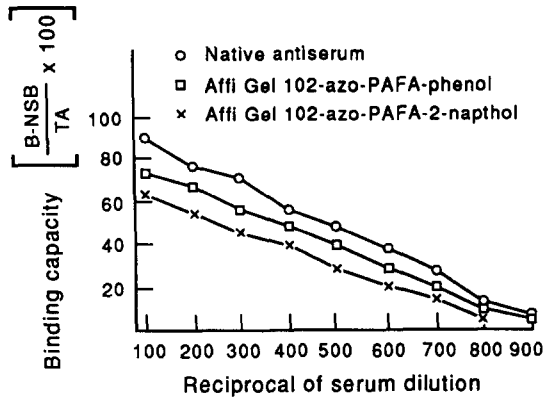


Fig. 3. Effects of affinity chromatography on antiserum avidity. The binding capacity was reduced after antiserum separation through Affi Gel 102-azo-PAFA-phenol or Affi Gel 102-azo-PAFA-2-naphthol columns. Elution of antiserum from columns was performed with 100 mM phosphate buffer pH 7.4, monitored by continuous flow u.v. detection at 280 nm and stopped when optical absorbance returned to baseline. Sample dilution of collected antiserum fractions was taken into account for calculation of further dilutions with the same buffer. The binding capacity was estimated by adding 100 μl of 1×10^{-9} M [2,4,6,7-^{3}H(N)]E_3 solution in 100 mM phosphate buffer pH 7.4 to 100 μl of serially diluted antiserum. After overnight incubation at 4°C, the bound/free separation was accomplished by adding 0.2 ml of a suspension of dextran-coated charcoal (0.5% w/v activated charcoal, 0.05% w/v dextran) in 100 mM phosphate buffer pH 7.4, stirring on ice and centrifuging at 2200 *g* for 20 min at 4°C.

of a suspension of dextran-coated charcoal in phosphate buffer (0.5% w/v activated charcoal, 250–350 mesh, 0.05% w/v dextran), stirring on ice and centrifuging at 2200 *g* for 20 min at 4°C. The supernatant was decanted into a counting vial, 3 ml scintillation cocktail (Cumene) added and the radioactivity counted (1500 Packard Tri-Carb Analyzer, Packard Inst.).

RESULTS

Antibody production

The E_3–azobenzoyl–BSA was administered monthly to rabbits and antibody production was followed 15 days after each immunization by measuring the binding capacities for [^{3}H]E_3 in each serum. A weak response was observed, with titers of antisera no greater than 1:400 vs a radioligand concentration of 1×10^{-9} M, together with a long latency period, lasting $\approx$ 200 days. No further enhancement of the antibody response was observed after the 6th booster injection. When the use of the Freund's incomplete adjuvant was interrupted, the antibody response disappeared within 2 months.

Immunosorption of antisera

The binding capacities of individual antisera were reduced by recycling affinity chromatography through either Affi Gel 102-azo-PAFA-phenol or Affi Gel 102-azo-PAFA-2-naphthol (Fig. 3).

The Affi Gel 102-azo-PAFA-phenol stationary phase was sensitive to pH changes and allowed recovery of entrapped antibodies which retained the ability to selectively bind [2,4,6,7-^{3}H(N)]E_3 (Fig. 4). Only minimal desorption of antibodies from Affi Gel 102-azo-PAFA-2-naphthol resin was obtained by stepwise application of an acidic buffer to the column. An increase in the ionic strength of the mobile phase was equally ineffective, although two fractions left the column, as detected by continuous flow u.v. monitoring at 280 nm. They were devoid of binding capacity against the radioligand and were left unidentified.

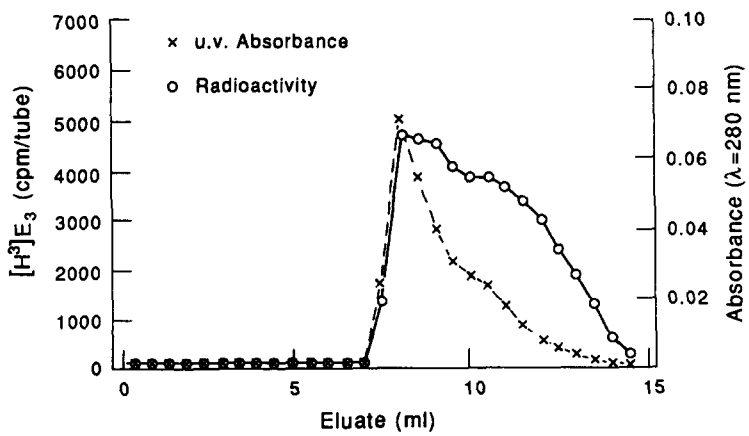


Fig. 4. Elution profile of retained antibodies from the Affi Gel 102-azo-PAFA-phenol column. The collection of the eluate was started at the time of the stepwise application to the column of 100 mM sodium citrate buffer, pH 4.0. 500 μl fractions, serially collected into glass vials containing 100 μl of 1 M Tris–HCl, pH 8.5, were tested for their binding capacity against [2,4,6,7-^{3}H(N)]E_3 (23,300 dpm).

RIA procedure

Unusually extended standard curves were obtained when native antisera were applied to RIA. The addition of 1×10^{-6}–1×10^{-12} M unlabeled E_3 to aliquots of antisera against E_3–azobenzoyl–BSA at optimum dilution titer, in the presence of 1×10^{-9} M radioligand and under the assay conditions described, produced inhibition of binding of the labeled steroid; the degree of inhibition increased with the amount of cold steroid over the range 5×10^{-7}–5×10^{-11} M, with a low detection limit of 5×10^{-11} M [Fig. 5(A)].

Antisera obtained after chromatography through Affi Gel 102-azo-PAFA-phenol allowed further enlargement of RIA standard curves with elevation of the upper detection limit to 5×10^{-6} M [Fig. 5(B)]. This finding is consistent with the disappearance from antisera of antibodies with poor affinities against the homologous ligand. Indeed, following chromatography, the apparent affinity constant of antiserum changed from $K_{app} = 8.25 \times 10^7$ to $K_{app} = 4.44 \times 10^8\ M^{-1}$. On the other hand, chromatography through Affi Gel 102-azo-PAFA-2-naphthol led to an increase of the low detection limit to 5×10^{-9} M [Fig. 5(C)], suggesting that antibodies with higher affinities against the ligand were also removed from native antiserum by the stationary phase, as indicated by the apparent affinity constant ($K_{app} = 2.09 \times 10^8\ M^{-1}$).

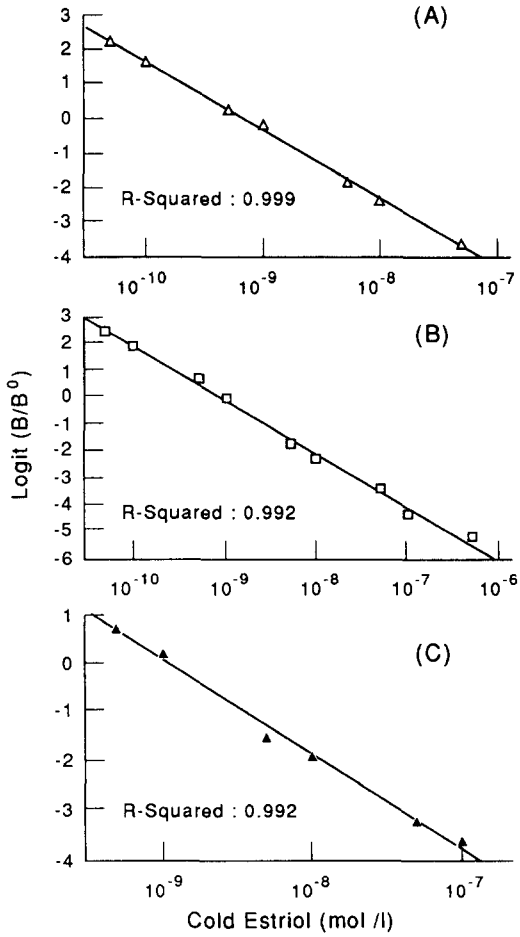


Fig. 5. Logit–log plots of standard curves of RIA performed with the use of native antiserum (A) and antisera obtained after chromatographic separation through Affi Gel 102-azo-PAFA-phenol (B) or Affi Gel 102-azo-PAFA-2-naphthol stationary phases (C). Standard curves were constructed by setting up duplicate assay tubes containing 100 μl antiserum at optimum titre, 100 μl of a 1×10^{-9} M [2,4,6,7-^{3}H(N)]E_3 solution in 100 mM phosphate buffer pH 7.4 (23,300 dpm) and 100 μl unlabeled E_3 to the appropriate dilutions within the range 1×10^{-6}–1×10^{-12} M. After overnight incubation at 4°C, the bound/free separation was accomplished by adding 0.2 ml of a suspension of dextran-coated charcoal (0.5% w/v activated charcoal, 0.05% w/v dextran) in 100 mM phosphate buffer pH 7.4, stirring on ice and centrifuging at 2200 *g* for 20 min at 4°C. The plots include the lower and upper detection limits of the assay, defined as concentrations of unlabeled ligand which can be distinguished from the zero standard and the non-specific binding at a 99.73% confidence limit.

Cross-reactions

The specificity of the antisera, examined with [2,4,6,7-^{3}H(N)]E_3 in competition with any one of 25 other steroids, is shown in Table 1. Antisera raised against the E_3–azobenzoyl–BSA showed a marked ability to distinguish strictly related estrogens, differing from each other in their substituents and structure of the cyclopentanoic ring of the molecule (estradiol, estrone, 16α-hydroxyestrone, 17-epiestriol and 16-ketoestradiol). Absence of cross-reactivity was also observed for either A and/or D ring substituted estrogens, differing from E_3 in the D ring (4-hydroxyestrone, estrone-3-glucuronide, 2-methoxyestrone, 2-methoxyestradiol, estradiol-3-sulfate, estradiol-3-glucuronide, estradiol-3-sulfate-17-glucuronide). With the exception of 16-ketoestradiol, the cross-reactions of these steroids fell outside the low detection limit of the assay, since 50% displacement of radioligand was not attained even at the highest steroid concentration tested. Similarly, the cross-reactions against the pregnane and androstane antigens tested were not detectable, with the exception of 5-androstene-3β,16α,17β-triol. Among estriol metabolites, a minimal cross-reactivity was observed for estriol-16-glucuronide, whereas relevant cross-reaction was observed for estriol-3-sulfate.

The residual cross-reactivities against the estrogen molecules were reduced by chromato-

Table 1. Comparison of specificity of native antiserum and the antisera obtained after chromatographic separation through Affi Gel 102-azo-PAFA-phenol or Affi Gel 102-azo-PAFA-2-naphthol stationary phases

	Cross-reaction (%)[a]				
	Native	azo-PAFA-phenol	Δ(%)[b]	azo-PAFA-2-naphthol	Δ(%)[b]
E_3	100	100	0	100	0
17-Epiestriol	ND[c]	ND	—	ND	—
Estriol-3-sulfate	5.2	1.0	−80.7	3.1	−40.3
Estriol-16-glucuronide	0.49	7.1×10^{-2}	−105	8.8×10^{-2}	−82.0
Estradiol	ND	ND	—	ND	—
16-Ketoestradiol	3.8×10^{-2}	1.0×10^{-2}	−73.6	2.5×10^{-2}	−34.2
2-Methoxyestradiol	ND	ND	—	ND	—
Estradiol-3-glucuronide	ND	ND	—	ND	—
Estradiol-3-sulfate	ND	ND	—	ND	—
Estradiol-3-sulfate-17-glucuronide	ND	ND	—	ND	—
Estrone	ND	ND	—	ND	—
4-Hydroxyestrone	ND	ND	—	ND	—
16α-Hydroxyestrone	ND	ND	—	ND	—
Estrone-3-glucuronide	ND	ND	—	ND	—
2-Methoxyestrone	ND	ND	—	ND	—
Progesterone	ND	ND	—	ND	—
Pregnandiol	ND	ND	—	ND	—
Pregnantriol	ND	ND	—	ND	—
Androstenedione	ND	ND	—	ND	—
5-Androstene-3β,16α,17β-triol	0.28	0.30	+7.1	0.28	0
5-Androstene-3β,16α,17α-triol	ND	ND	—	ND	—
5-Androstene-3β,16β,17β-triol	ND	ND	—	ND	—
Testosterone	ND	ND	—	ND	—
5α-Dihydrotestosterone	ND	ND	—	ND	—
Cortisol	ND	ND	—	ND	—

[a]Defined as $100 \times A/B$, where A is the mass of unlabeled estriol and B is the mass of the heterologous ligand required to produce 50% inhibition of the binding of tritiated estriol by antibody.
[b]Defined as $-100 \times (A - B)/A$, where A is the cross-reaction (%) with the native antiserum and B is the cross-reaction (%) after chromatographic separation through Affi Gel-azo-PAFA-phenol or Affi Gel 102-azo-PAFA-2-naphthol stationary phases.
[c]Not detectable, <0.01%.

graphic separation of the antisera through stationary phases having either azo-PAFA-phenol or azo-PAFA-2-naphthol side residues (Table 1). The Affi Gel 102-azo-PAFA-phenol column gave the greatest changes in cross-reactivity, whereas Affi Gel 102-azo-PAFA-2-naphthol was less effective, probably on account of its ability to remove some antibodies with high affinity against the primary antigen. Even in the case of heterologous ligands whose cross-reactivities fell outside the range of detectability of the assay, a right-shift was evident in the displacement curves.

DISCUSSION

The E_3–azobenzoyl–BSA acted as a weak immunogen, eliciting low avidity antisera after an unusually extended latency period. In addition, we found the presence of nonspecific adjuvant necessary, in contrast to reports of negative effects on the titers of antisera elicited against several arylazohaptens [9].

Immunogenicity, which is determined by the availability of antigenic determinants to which T cells are reactive, appears to be an inherent property of the carrier molecule. In most cases, T cell recognition resulting in the activation of antigen specific T helper cells (T-dependent antigens) seems essential for effective antibody as well as T effector cell mediated responses [10]. The number and location of T cell determinants appears to be almost exclusively defined by oligopeptides which are characterized by common conformational features. They enable processed forms of the immunogen to interact with self-glycoproteins, encoded for by the major histocompatibility complex (MHC) and expressed on the surface of antigen presenting cells. The occurrence of low avidity antisera following immunization with E_3–azobenzoyl–BSA could be related to the destruction of the azo-bond, due to the azobenzene reductase, which is known to be specific for hydroxylated azocompounds, and would have the effect of liberating the estrogen from the carrier as an amine derivative [11], so decreasing the number of the acquired epitopes.

On the other hand, the extended latency observed in the immune response requires further comment. Antigenicity depends on the appropriate appearance of epitopes recognizable to a B cell. In E_3–azobenzoyl–BSA, the azene double bond at the C_4 or C_2 positions induces coplanarity of the ring A and parts of ring B of the estratriene with the phenyl ring of benzoic acid which in turn is linked to the protein by a carbamide linkage. This structural feature al-

lows the hapten to protrude from the carrier with minor influences on the steric configuration of the D ring that identify the individual estrogen. Being so, the uncommon latency for immune response to E_3–azobenzoyl–BSA may be interpretated as a consequence of the *self* characteristic of the hapten which may cause the encounter with a B cell's recognizable paratopes to be an event of low probability. Experience with protein immunogens has shown the relevance of the phylogenetical relationship of the immunogen to the host. A closer relationship leads to a weaker response, which however is more specific [12]. Since the C_{16-17} region of the immunogenic determinant may strongly influence the antibody hapten affinity [13], and is probably of paramount importance in determining the specificities of antibodies against steroids [14, 15], it may be assumed that the D ring is the most important molecular site for the expression of the phylogenetical relationship between an individual estrogen and its host.

In terms of specificity, the elicited antisera showed interesting properties. Steroid–protein conjugates containing azo-estrogens have previously been shown to produce antisera with rather high cross-reactivities for norandrostanes differing from the homologous antigen in the A ring chemical nature, for example estradiol-17β and 19-nortestosterone or ethynylestradiol and norethisterone [3, 5, 16–18]. This puzzling cross-reactivity was speculatively attributed to the existence of an internal hydrogen bond in *o*-hydroxyazo-conjugated steroids and to the conversion of these haptens to quinoid structures [5]. It has been suggested that the use of adjuvants containing mineral oils for emulsification of the antigens may produce tautomeric structures [9]. In this present study, only a weak cross-reactivity was observed against 5-androstene-3β,16α,17β-triol, a C_{19} androgen sharing with estriol the same D ring configuration. On the contrary, no evidence of cross-reactions with androstane molecules having D ring substituents and configurations dissimilar from E_3 was observed. The cross-reaction profile we observed is contrary to the above mentioned hypothesis of tautomerism of azobenzoylsteroids as a cause of cross-reactivity with androstanes, but it supports the view that the D ring region is a primary locus of antigen recognition by antibodies elicited against steroid–protein conjugates containing azo-estrogens.

Indeed, a marked discriminating ability for structural differences on the D ring was observed, with cross-reactivities $<0.01\%$ against strictly related estrogens such as estradiol, estrone, 16α-hydroxyestrone and 17-epiestriol. These differ only in individual ketonic or hydroxyl groups, the latter by their relative configuration. 16-ketoestradiol was an exception, with a cross-reactivity of the order of 0.1%. Similarly, no evidence of cross-reaction was found for the sulfate-, glucuronide-, methoxy- and catechol-estrogen metabolites tested, which differ from E_3 in the structure of the D ring. The most relevant cross-reactivity was observed for the conjugated estriol metabolite, estriol-3-sulfate. The presence of a glucuronide substituent onto the cyclopentanoic D ring, as in estriol-16-glucuronide, led to a marked loss of cross-reactivity of the heterologous antigen, probably on account of steric hindrance.

Separation of the antiserum with stationary phases having side residues which mimic the A side of the primary antigen reduced antiserum avidity by entrapping some antibodies, increased the apparent antiserum affinity and decreased the residual cross-reactivities against the heterologous C_{18} ligands. Considering that in both stationary phases, parts of the hapten molecule protrude into the environment, antibodies with good affinity against the estrogen molecule should be expected to interact, if the A and D rings were equally weighted in the recognition process. The increase of the apparent affinity constants and the changes in antiserum specity we observed are consistent with the view that antibodies with low affinity were actually removed by immunosorption. This finding seems to suggest that antibody clones differing in the directionality of epitope recognition are produced, also in the case of low molecular weight molecules like estrogens. This interpretation is apparently in contrast with the evidence that the size of paratope onto the hypervariable domains of immunoglobulins is of the same order of magnitude as the size of the steroid hapten [14, 15, 19]. Nevertheless, in E_3–azobenzoyl–BSA, coplanarity of the ring A and parts of ring B of the estratriene with the phenolic ring from *p*-aminobenzoic acid allows the hapten to protrude from the carrier with minor influences on the steric structure of the ring D and on the central hydrophobic domain of the estrogen, favouring the recognition of the hapten from the D side of the molecule.

Lastly, the interesting binding properties of antisera permitted the development of a competitive binding assay over a very extended analytical range (a 10^4-fold concentration range, with a low detection limit of 5×10^{-11} M). This enables the measurement of E_3 from the early pregnancy to delivery, without the need to perform extraction or dilution procedures of unknown serum samples. Assuming a saliva–serum ratio of 8–12% [20], direct assessment of unconjugated E_3 concentration in saliva starting from the early weeks of gestation should in theory be possible. By the application of enrichment extraction to plasma or saliva samples, the assessment of serum and salivary E_3 levels both in the phollicular and luteal phases of the menstrual cycles, as well as in adult eugonadal men is in progress in our laboratories.

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