INFLUENCE OF THE SITE OF CONJUGATION ON THE SPECIFICITY OF ANTIBODIES TO PROGESTERONE

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Abstract

In order to determine how the site on the molecule used for conjugation influences the specificty of the resulting antiserum, progesterone was conjugated to bovine serum albumin (BSA) through substituents on the A(C3), B(C6), C(C11), and D(C20) rings for use as a hapten to elicit antibody formation in rabbits. Specificty of the antisera was determined by testing the ability of 24 representative steroids to displace radioactive progesterone in a radioimmunoassay procedure. Progesterone-tyrosine methyl ester (TME) conjugates were radioiodinated and used as the radioactive form of the hormone and radioactivity bound to antibody was separated from free radioactivity by a double antibody procedure. Immunization with progesterone conjugated at C20 resulted in the formation of antibodies which could not distinguish between progesterone and other Δ^4-3-ketosteroids with structures similar in the A, B, and C ring (namely 17-hydroxyprogesterone, 20α and 20β-hydroxy-4-pregnen-20-one, deoxycorticosterne and testosterone). Immunization with progesterone-3-BSA resulted in the formation of antisera which were fairly specific for progesterone while immunization with progesterone conjugated at the 11 or 6 positions resulted in antisera which were very specific for progesterone. It was concluded that steroid hormones should be conjugated to protein at sites on the B or C ring of the molecule for the production of specific antisera.

INTRODUCTION

Although numerous investigators[1,2,3,4] have reported the successful development of antisera to progesterone, the specificity of the antisera has been extremely variable. In a previous communication[2] we have suggested that the site through which the steroid molecule is conjugated to protein influences the specificity of the antisera produced against that steroid-protein conjugate.

The purpose of this paper is to summarize the results regarding specificity of the antisera obtained when the progesterone molecule was conjugated to protein through substituents on the A(C3), B(C6), C(C11) or D(C20) ring. The specificity of these antisera was studied using radioimmunoassay procedures.

MATERIALS AND METHODS

Conjugation to Bovine Serum Albumin (BSA) or Tyrosine Methyl Ester (TME)

Progesterone was obtained from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. All solvents and chemicals were Reagent grade. Pyridine was dried over a molecular sieve, succinic anhydride was sublimed in vacuo, and dioxane distilled from $LiAlH_4$. Crystalline bovine serum albumin was obtained from Pentex Inc. (Kankakee, Ill.). Melting points were taken in a Hoover-Thomas apparatus and are uncorrected. Infrared spectra were obtained with Nujol emulsions in a Perkin-Elmer Model 137 double beam spectrophotometer. Ultra-violet (U.V.) spectra were obtained using a Beckman DB spectrophotometer in 0.05 M Tris buffer at pH 8.5.

Progesterone-20. Progesterone-20-(O-carboxymethyl) oxime was prepared from 3β-hydroxy-5-pregnen-20-one as described by Erlanger et al.[1]. The progesterone-20-oxime derivative had a m.p. of 164-166°, U.V. adsorption maximum at 249 mu (extinction coefficient (E) = 15,890) and I.R. bands at 1725 cm^{-1} (C = O of carboxyl) and 1635 $^{-1}$ (conjugated C = O). The progesterone-20-oxime derivative was conjugated to BSA and TME with the mixed anhydride procedure[1]. The BSA conjugate showed a U.V. adsorption maximum at 248 mu and it was calculated[5] that 14 steroid molecules were linked to each BSA molecule.

Progesterone-11α. Progesterone-11α-chloroformate was prepared from 11α-hydroxyprogesterone by the general method used by Miescher, et al.[6] to prepare testosterone-17-chloroformate. The compound had a U.V. maximum at 246 mu (E = 15,600), infrared adsorption at 1635 cm^{-1} (conjugated C = O) and 1685 cm^{-1} (C = O at C20) and no adsorption at 3200 - 3650 cm^{-1} (no 11α-OH). The 11α-chloroformate derivative of progesterone was conjugated to BSA using the Schotten-Baumann reaction[1]. The conjugate showed a U.V. maximum at 242 mu and by U.V. analysis contained 36 steroid molecules per protein molecule.

To obtain progesterone-11α-hemisuccinate, a solution of 1 gm of 11α-hydroxyprogesterone and 1 gm succinic anhydride in 10 ml of pyridine was refluxed for 4.5 hrs. The reaction was taken to dryness under reduced pressure. The semi-solid residue was dissolved in ethyl acetate and washed three times with water. The ethyl acetate solution was extracted with 2 x 25 ml of 5% NaOH, the combined basic extract was washed twice with 50 ml of ethyl acetate and made acidic (pH 2.0) with concentrated hydrochloric acid. The oil which separated was taken up in ethyl acetate, dried over $MgSO_4$ and the solvent removed under reduced

pressure. The residue solidified and was recrystallized from benzene-hexane (1:1). The resulting compound had a m.p. of 165-167° and produced a single spot (Rf = 0.4) following thin layer chromatography on silica gel (methylene chloride:acetone, 8:2). 11α-hydroxyprogesterone exhibited an Rf = 0.6. The compound showed maximum U.V. adsorption at 245 mu (E = 16,000), infrared adsorption at 1635 cm^{-1}, 1685 cm^{-1}, and 2960 cm^{-1} (succinyl ester group). The progesterone-11α-hemisuccinate was conjugated to TME using the mixed anhydride procedure[1].

Progesterone-3. 20β-hydroxy-4-pregnen-3-one 3-(O-carboxymethyl oxime) was obtained by preparing a solution of 1 gm 20β-hydroxy-4-pregnen-3-one and 1 gm (O-carboxymethyl) hydroxylamine hydrochloride in 52 ml of ethanol and 4.65 ml 2N KOH which was refluxed for 3 hrs. The solvents were evaporated at reduced pressure, the solid residue was dissolved in 75 ml water, pH was adjusted to 8.5 with dilute KOH and this solution was washed twice with 100 ml ethyl acetate. The pH adjusted to 2.0 with dilute hydrochloric acid, the resulting solid residue was taken up in ether and dried over $MgSO_4$ which was subsequently evaporated under reduced pressure. The residue was recrystallized from ethyl acetate. The derivative had a m.p. of 184-186°, no infrared adsorption at 1626-1640 cm^{-1} (no conjugated C = O) and no adsorption band was noted at 3650 cm^{-1} (20β-OH).

20β-hydroxy-4-pregnen-3-one 3-(O-carboxymethyl) oxime methyl ester was obtained by treating a solution of 680 mg (1.8 m moles) of 20β-hydroxy-4-pregnen-3-one 3-(O-carboxymethyl) oxime in 50 ml of ether with a solution of diazomethane in 15 ml ether obtained from 1.2 gm (10.0 m moles) of N-nitroso-N-methyl urea. The mixture was allowed to stand 14 hrs. at room temperature. Excess diazomethane was decomposed by adding four drops of 1N acetic acid. The mixture was evaporated to dryness under reduced pressure and the residue was recrystallized from methanol. The m.p. of the compound was 163-166° and infrared adsorption bands were seen at 1750 cm^{-1} (C = O ester) and 3650 cm^{-1} (20β-OH).

To obtain progesterone-3-(O-carboxymethyl) oxime, 3 gm of 20β-hydroxy-4-pregnen-3-one (O-carboxymethyl) oxime methyl ester was dissolved in 450 ml acetone and the solution was cooled to 10°. Jones reagent[7] was added dropwise until the appearance of a green color while keeping the solution at 15° under a stream of nitrogen. A solution of 26 ml methanol in 2600 ml water was added and the solid was collected by filtration, washed with water, dried and recrystallized from acetone-hexane (1:1). The compound had a m.p. of 113-114°, an I.R. adsorption band at 1685 cm^{-1} (20C = O) and no band at 3200-2650 cm^{-1} (no OH). A mixture of 2.2 gm progesterone-3-(O-carboxyemthyl) oxime methyl ester, 78.5 ml methanol and 8.7 ml N NaOH was allowed to stand at room temperature for 3 hours. The volume was reduced by evaporation under reduced pressure, water was added and the solution was washed with ether. The aqueous solution was adjusted to pH 2 with 1N hydrochloric acid, cooled for 16 hours and the solid collected by filtration. The compound was washed with water, dried and recrystallized from acetone-hexane (1:1). The resulting crystals had a m.p. of 169-170 with decomposition, I.R. adsorption bands at 1730 cm^{-1} (C = O of carboxy group) and 1685 cm^{-1} (20 C = O), and a U.V. adsorption maximum at 251 mu (E = 15,500).

The progesterone-BSA and TME conjugates were prepared by the mixed anhydride procedure[1]. The U.V. spectrum in 0.05 M Tris buffer (pH 8.5) showed a maximum adsorption at 252 mu and it was calculated that 28 steroids were bound per mole BSA.

Progesterone-6β. Progesterone-6β-hemisuccinate was prepared from 6β-hydroxyprogesterone[7] using succinic anhydride and pyridine as described previously. The resulting compound was recrystallized from ethyl acetate:iso-octane (1:1), had a m.p. of 160-161°, showed I.R. adsorption bands at 1635 cm^{-1} (carboxylate anion), 1725 cm^{-1} (20 C = O), and 2950 cm^{-1} (OH stretching), and a U.V. adsorption maximum at 236 mu (E = 13,000). The BSA and TME conjugates were prepared by the mixed anhydride procedure[5]. U.V. analysis indicated 28 steroid molecules were bound per mole BSA.

Progesterone-6α. Progesterone-6α-hemisuccinate was prepared from 6α-hydroxyprogesterone[8]. Attempts to recrystallize the produce failed and it was analyzed as an oil which showed I.R. adsorption bands at 1635 cm^{-1} (carboxylate anion), 1725 cm^{-1} (20 C = O), and 2950 cm^{-1} (OH stretching), and a U.V. maximum at 241 mu (E = 16,000). The BSA conjugate prepared as above showed a U.V. maximum at 242 mu and it was calculated that 22 steroid molecules were bound to each BSA molecule.

Immunization of Rabbits. Groups of six rabbits were immunized (two at 0.26 mg, two at 1.26 mg and two at 6.25 mg) with each of the progesterone-BSA conjugates. The antigen was emulsified in complete Freund's adjuvant and injected into the footpads and multiple subcutaneous sites. Each rabbit was injected with the same dose three times with three week intervals between the injections. Weekly bleedings (50 ml) were begun two weeks after the final injection.

Preparation of the anti-rabbit gamma globulin (RGG) used in these studies has been described previously[9].

Radioimmunoassay Procedures

Radioiodination. The progesterone-TME conjugates were put into solution in glass redistilled methanol at a concentration of 1 mg/ml. At the time of radioiodination 2.5 ul of this solution were added to a 1 ml serum bottle (Fisher Scientific) and 50 ul of 0.5 M phosphate buffer (pH 7.5) were added. One mCi of high specific activity $Na^{125}I$ (New England Nuclear Corporation, Boston, Mass.) was added, the vial was stoppered and its contents mixed gently. Sixty ug of chloramine-T in 30 ul of 0.05 M phosphate buffer were added and the reaction mixture was gently mixed by finger tapping for exactly 2 minutes. The reaction was stopped by adding 120 ug of sodium metabisulfite in 50 ul of a 16% sucrose solution. The progesterone-TME-^{125}I was separated from free radioactivity by electrophoresis in polyacrylamide gel. Twenty-five ul aliquots of the radioiodination reaction mixture were layered beneath the buffer on the surface of columns of 7.5% polyacrylamide gel (5 x 63 cm) and subjected to electrophoresis for 75 minutes with a 220 volt gradient (3 mAmp/gel) in a continuous buffer system of 0.011 M boric acid, 0.0032 M disodium EDTA and 0.007 M tris at pH 8.9. Following electrophoresis autoradiograms were obtained using Kodak n-screen X-ray film and the segment which contained the progesterone-TME-^{125}I

was located, pressed between two glass slides and the fragments of gel were eluted for 2-24 hours in 0.01 M phosphate buffered saline (PBS) pH 7.0 with 0.1% gelatin. The elute was then further diluted in PBS-0.1% gelatin for use in the assay so that 100 ul contained approximately 30,000 CPM (mass = approximately 150 pg; specific activity = approximately 100 Ci/mmol.). It was also possible to separate free radioiodine from the progesterone-TME-^{125}I by placing the reaction mixture on a 15 x 1 cm column of Sephadex G25 prepared and eluted with PBS-0.1% gelatin. With this system the free iodine was eluted at 9-12 ml and the progesterone-TME-^{125}I was retained by the Sephadex gel and eluted in a broad peak at 15-20 ml.

Radioimmunoassay procedure. A double antibody radioimmunoassay procedure modified from the procedure used for protein hormones[9] was used in these studies. All antisera were diluted 1:400 with .05 M EDTA PBS and were further diluted to a concentration which bound approximately 50% of the appropriate progesterone-TME-^{125}I with 1:400 normal rabbit serum which had been diluted with .05 M EDTA PBS. All steroid preparations were obtained from Sigma Chemical Co. (St. Louis, Mo.) and used as supplied. All standard steroids were put into solution (10 mg/ml) in dimethyl sulfoxide and then appropriate dilutions (at least 1000 fold) were made in assay buffer (PBS-0.1% gelatin). Inhibition curves were run in duplicate at 4 to 11 dose levels, with each of 24 steroids to assess their relative activity in each of the five radioimmunoassay systems. In the radioimmunoassay 500 ul of combined standard solution plus assay buffer were allowed to incubate with 200 ul of the diluted antibody and 100 ul of the appropriate progesterone-TME-^{125}I for 4-6 hours at 4°C. In all cases the progesterone-TME-^{125}I used had the TME conjugated through the same position as the BSA conjugate used to produce the antiserum. Anti-RGG (200 ul of the appropriate dilution) was then added and the reaction mixture was allowed to incubate for 12-25 hours at 4°C. Three ml of cold PBS were added and the assay tubes were centrifuged for 30 min, the supernatant decanted and the precipitates were counted in a Nuclear Chicago gamma spectrometer for a sufficient period of time to minimize counting error (± 1%).

To determine the usefulness of the radioimmunoassay procedure for quantification of progesterone in serum several experiments were performed. Progesterone was quantitated by radioimmunoassay in a series of samples, kindly provided by Dr. R. Erb, Purdue University, in which the progesterone content had been determined by double isotope derivate and competitive protein binding techniques. In an additional 82 samples obtained from sheep at different times during the estrous cycle the progesterone concentration was determined with or without purification of the sample by thin-layer chromatography as described previously[10]. This chromatographic system has been shown to clearly separate progesterone from 20α-hydroxy-4-pregnene-3-one, 20β-hydroxy-4-pregnen-3-one, 17-hydroxyprogesterone, and 3β-hydroxy-5-pregnen-20-one. In all cases approximately 50 pg of tritiated progesterone (50.3 Ci/mmol) were added to 1 ml of serum and allowed to equilibrate with endogenous progesterone. The sample was then extracted twice with 10 ml of petroleum

ether (Distilled-in-glass solvents, Burdick-Jackson Laboratories, Mushegan, Mich.). The petroleum ether extract was washed with 2 ml distilled water, taken to dryness, 2 ml of assay buffer (PBS - .1% gel) were added and 300 and 30 ul aliquots were assayed in duplicate and 200 ul were counted to determine recovery of the tritiated progesterone. All values are corrected for procedural losses. Samples collected from humans, horses, cattle and dogs were also quantified with and without thinlayer chromatographic purification of the samples. In all cases samples were obtained from three females during the follicular phase of the cycle, from three females during the luteal phase of the cycle, three pregnant females and three males.

RESULTS AND DISCUSSION

Of the 30 rabbits immunized with progesterone-BSA conjugates 26 produced antisera which bound usable quantities of progesterone-TME-^{125}I at an initial dilution (200 ul) of 1:400 or greater (final dilution = 1:2000 or greater). Eighteen of the antisera bound 50% or more of the progesterone-TME-^{125}I at initial dilutions of 1:6000 or higher and two of the antisera bound 50% or more of the radioactive progesterone at a 1:30,000 initial dilution. There was no evidence that the dose of antigen used for immunization influenced the resultant antibody titer. Titers in all rabbits immunized with progesterone-20-BSA were low, in fact, three of the four animals which had unusable titers were in this group. It seems likely that the failure of these rabbits to produce usable antisera was due to the low steroid to protein molecular ratio (14) in this conjugate. We have previously reported[2] that conjugates with steroid to protein molecular ratios of less than 10 were not efficacious for antibody production while conjugates with ratios of 20 or greater were very effective. The quantity of conjugate injected was constant in this study, therefore the amount of progesterone injected was less with progesterone-20-BSA than for the other conjugates

Table 1 Relative Activity of Selected Steroids in Five Different Progesterone Radioimmunoassays

Steroids	Progesterone-20	Progesterone-3	Progesterone-11α	Progesterone-6α	Progesterone-6β
Progesterone	1.00	1.00	1.00	1.00	1.00
3β-hydroxy-5 pregnen-20-one	.011	.133	.005	.0016	.034
11α-hydroxyprogesterone	.071	.134	.345	.0075	.0092
11β-hydroxyprogesterone	.02	.08	.08	.03	.015
17-hydroxyprogesterone	.981	.046	.0122	.0026	.0032
5α-pregnane-3,20-dione	.46	.61	.13	.27	.30
20β-hydroxy-4-pregnen-3-one	.965	.003	.0011	.0021	.0012
20α-hydroxy-4-pregnen-3-one	.336	.001	.0014	.0017	.0016
5β-pregnane-3α,20α-diol	.003	<.0001	<.0001	<.00001	.0004
5α-pregnane-3β, 20β, diol			<.0001	<.00001	.0001
Deoxycorticosterone	.965	.016	.007	.0015	.0017
Corticosterone	.004	.004	.002	.00014	.00004
Aldosterone	.071	.0004	.0001		
Cortisone	.056	.0002	.0003	<.00001	<.00001
Hydrocortisone	.004	.0002	<.0001	<.00001	<.00001
Testosterone	.952	.0005	.0004	.00003	.00003
4-androstene-3,17-dione			<.0001	.00005	.00002
5-androstene-3β-17β-diol			<.0001	.00002	.00002
3β-hydroxy-5-androsten-17-one			<.0001	.00002	.00002
17β-hydroxy-5α-androstan-3-one			<.0001	.00001	.00001
Estradiol-17α	.0004	<.0001	<.0001	<.00001	<.00001
Estradiol-17β	<.0001	<.0001	<.0001	<.00001	<.00001
Estrone	<.0001	<.0001	<.0001	<.00001	<.00001
Estriol	<.0001	<.0001	<.0001	<.00001	<.00001

which had 22-36 steroid molecules per protein molecule. Others have obtained high titered antibodies using progesterone-20-BSA as antigen[3,5]. There were no apparent differences in the antigenicity of the four conjugates with steroid to protein molecular ratios of 22 to 36.

The specificity of the resulting antisera was assessed by ascertaining the ability of 24 different steroids to compete with the appropriate progesterone-TME-^{125}I for binding to antibody (Table 1). Anti-progesterone-20-BSA serum was not able to discriminate between steroids with structures similar to that of progesterone in the A, B, and C rings (i.e., 17-hydroxyprogesterone, 20β-hydroxy-4-pregnene-3-one, deoxycorticosterone, testosterone). The somewhat decreased relative activity of 20α-hydroxy-4-pregnen-3-one is probably due to the spacial configuration of this molecule at carbon 20.

The antiserum against progesterone-3-BSA was reasonably specific for progesterone. However, this antiserum did not clearly discriminate structural differences in the A and B rings of steroid molecules similar to progesterone. For example, 3β-hydroxy-5-pregnen-20-one had 13% of the relative activity of progesterone, while 5α-pregnane-3,20-dione was 61% as active as progesterone. 11α-hydroxyprogesterone was 13% as reactive as progesterone while 11β-hydroxyprogesterone was 8% as reactive. The antiserum against progesterone-11α-BSA was more specific than either the anti-progesterone-20-BSA or anti-progesterone-3-BSA serum. None of the steroids tested in this system had more than 1% of the relative activity of progesterone except 11α-hydroxyprogesterone (35%), 11β-hydroxyprogesterone (8%) and 5α-pregnane-3,20-dione (13%).

There were no significant differences in specificity between the anti-progesterone-6α-BSA and progesterone-6β-BSA serum. The only steroid tested which had significant activity (> 3%) in either system was 5α-pregnane-3,20-dione. The high level of crossreactivity of 5α-pregnane-3,20-dione was anticipated since the only difference in the structure of this molecule compared to progesterone occurs very near the site used to conjugate the progesterone molecule to BSA.

Since our preliminary report[2] several investigators[11-17] have developed specific antisera to different steroids by conjugation to protein through a position in the B or C rings. In all cases these antisera are more specific than those obtained when conjugation was done through the A or D rings.

When the samples which had their progesterone concentrations estimated using the double isotope derivative method and the competitive protein binding method were subjected to radioimmunoassay using the anti-progesterone-11-BSA system, without chromatographic purification of the sample, the agreement between the three different methods was excellent (Table 2). However, the lowest progesterone concentration in these samples was 3 ng/ml which is relatively high. The results obtained with the ovine, bovine, equine, canine and human samples with and without chromatographic purification of the samples are shown in Table 3. There was excellent agreement between the two estimates, even for sample with low levels of progesterone (0.2 - 2.0 ng/ml). Since this chromatographic system separates progesterone in a relatively pure form it was concluded that chromatographic purification of samples collected from normal females during different reproductive states or from males was

not necessary using the progesterone-11α radioimmunoassay system. This appeared to be true for all five species tested. It was also possible to quantitatively recover exogenous progesterone (.5 - 10 ng) from 1 ml of serum from pregnant humans or sheep. This exhaustive validation of the assay procedure was not done for any of the other progesterone antisera.

Table 2 Levels of Progesterone in Porcine Serum as Determined by Three Methods (ng/ml)

Sample[1]	DID[2]	CPB[3]	RIA
147	3.6	3.1	3.7
165	5.0	5.3	4.9
146	7.7	3.1	7.4
164	7.0	4.2	4.2
166	8.1	11.6	9.1
75	9.6	8.2	8.6
144	14.7	10.5	11.2
151	20.7	24.2	19.4
167	30.7	20.7	31.8

[1]Samples provided by Dr. R. E. Erb, Purdue University where the progesterone concentrations were estimated with the double isotope derivative[2] and competitive protein binding[3] techniques.

Table 3 Comparison of Serum Progesterone Concentrations by Radioimmunoassay With and Without Purification of the Sample by Thin-layer Chromatography

Species	Number of Samples	Range ng/ml	Correlation Coefficient	Slope
Ovine	37	0.20 - 2.00	.97	1.03
	45	2.00 - 16.00	.94	.97
Bovine	12	0.10 - 11.0	.98	.93
Equine	12	0.10 - 20.4	.98	.86
Canine	12	0.10 - 15.6	.86	1.19
Human	12	0.10 - 15.1	.98	.87

It was concluded from this study that to obtain specific antisera against progesterone the steroid molecule should be conjugated to the protein through positions on the B or C ring. This method of conjugation leaves the A and D rings and their substituents available to incur specificity to the antibody.

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