PRODUCTION OF ANTISERA AGAINST CONTRACEPTIVE STEROIDS

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ABSTRACT

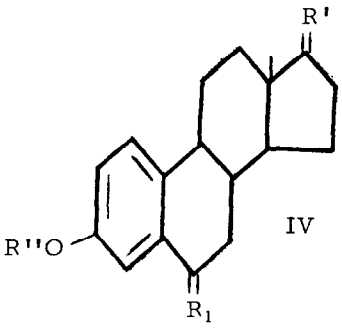
A four step synthesis of 6-(O-carboxymethyl)oximinoethynylestradiol is reported. This compound, 6-(O-carboxymethyl)oximinomestranol, the 3-(O-carboxymethyl)oximes of norethindrone and norgestrel and the 3-hemisuccinate of ethynylestradiol were synthesized and conjugated with bovine serum albumin. Rabbits were immunized at 3 dose levels of haptene (20, 66 and 200 nmoles) and eight weeks later with a booster containing 66 nmoles of haptene. The antibody titer and association constant of responding rabbits was nearly independent of dose although most antibody production occurred after the booster injection. Antibodies to mestranol crossreacted more than 100% with ethynylestradiol and to a small extent with norethindrone and norgestrel.

INTRODUCTION

Earlier (1) one of us reported a synthesis of 6-oxomestranol-6-(O-carboxymethyl) oxime, an important intermediate for the preparation of an antigen which was utilized for the production of a specific antibody against mestranol (2). In the present communication, we describe a convenient method of synthesis of the 6-(O-carboxymethyl) oxime derivative of 6-oxoethynylestradiol-17β, the 3-succinyl derivative of ethynyl estradiol-17β and the 3-carboxymethyl oxime derivatives of norethindrone and norgestrel as well as the synthesis of the corresponding bovine serum albumin conjugates required for the

production of specific antisera for mestranol ethynylestradiol, norethindrone and norgestrel in rabbits. Attempts have been made to study the relationship between the titers and doses of immunogen required for maximal response, the duration of immunization, specificities and binding affinities of the antisera produced against an antigenic hormone at different time intervals.

The sequence of the synthesis of 6-(O-carboxymethyl) oxime derivative of 6-oxoethynylestradiol-17β is similar to that described earlier (1).



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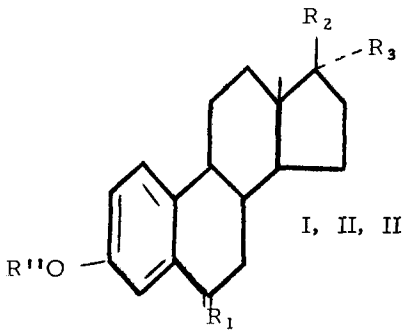

I, II, III, V

(I) R_1 = O; R'' = H; R_2 = OH; R_3 = H

(II) R_1 = O; R'' = CO-C_6H_5; R_2 = OH; R_3 = H

(III) R_1 = N-O-CH_2-COOH; R'' = CO-C_6H_5; R_2 = OH; R_3 = H

(IV) R_1 = N-O-CH_2COOH; R'' = CO-C_6H_5; R' = O

(V) R_1 = N-O-CH_2COOH; R'' = H, R_2 = OH; R_3 = C≡CH

6-Oxoestradiol (I), obtained by the method of Dean et al (3), was benzoylated by the classical method with benzoyl chloride and alkali to yield (II). The corresponding 6-(O-carboxymethyl) oxime (III), prepared by the procedure described in (1), was oxidized with Jones reagent to produce the 17-oxo-compound (IV). This compound, in turn, was converted directly to the 6-(O-carboxymethyl) oxime of 6-oxoethynylestradiol (V) with an excess of lithium acetylide-ethylene diamine complex. The overall yield of the product from I was quite satisfactory (70-75%).

After the experimental portion of this work was completed, Rao (4) reported an eight step synthesis of the same compound from 6-oxo-estradiol-3-methyl ether. By comparison, our method is much simpler than that of Rao: it requires four steps with one chromatographic separation while Rao's procedure uses eight steps and two chromatographic purifications.

Synthesis of 3-succinylethynylestradiol was performed in the usual way : the product was characterized by elemental analysis and physical measurements. Norethindrone and norgestrel were derivatized to their corresponding 3-oximes which were characterized by elemental analysis and physical data.

All steroid derivatives thus synthesized were conjugated with bovine serum albumin by the carbodiimide method. The extent of conjugation was determined by differential UV-absorption at two wave lengths.

Immunization followed essentially the method described by Vaitukaitis et al (5). It was very frustrating that an adequate titer could not be obtained from those rabbits immunized with either ethynylestradiol-3-conjugate with or without castration. Therefore, no further study was made with these antisera of low titers. Due to the non-availability of radioactive norgestrel having a high specific activity, titration of the antisera produced by norgestrel antigen was done using the structurally related radioactive norethindrone. Further studies with these antisera were not done since the results of studies using a "wrong" radioactive ligand may not ultimately be significant. However, a complete study of the antisera involving all three animals immunized with mestranol antigen at each of three dose levels and of the antisera from one of the animals immunized with norethindrone antigen at each of the three dose levels was made. The results are given in Table 1 and 2.

There has been relatively little study of the immune response that produced the key reagent for RIA. In regard to the quantity of antigen injected, Odell and Hescox (6) found that increasing doses of hFSH up to 127 I. U. produced increasing titers of antibody. Beyond 127 I. U. there was no further increase. Vaitukaitis, et al. (5) reported that injection of 20 and 50 μg of hCGα and β, respectively, and of 75 μg of testosterone-3-(O-carboxymethoxy) oxime conjugate produced high antibody titers in 2-3 months. Abraham (7), who used sheep rather than rabbits, reported that 1-2 mg of conjugate gave optimum response. Either lesser or greater amounts gave poorer results.

In regard to response time and the effect or prolonged immunization, there is also little agreement. Abraham (7) feels that titer plateaus at 6-8 months, but Forest, et al (8) using rabbits noted a great deal of variability with peaks or plateaus occurring from 3 months to over 1 year. This observation agrees with that of Honing, et al. (9) who used sheep. Odell et al (10) found that titers plateaued at 8-9 months and Vaitukaitis et al (5) at 2-3 months. While Abraham (7) reports that affinity (association constant) increases throughout a one year period, Forest et al (8) found that in 4/5 cases there was little or no increase after the third month. Specificity improved with time (7-9), especially during the first 3-6 months.

Abraham (7) finds that the haptene protein ratio is critical, i.e., if the ratio is less than twenty, he obtains a poor or no response. Many investigators have produced antibodies with antigens having ratios of 5-15.

We measured response in terms of titer, association constant and cross-reactivity as a function of time after immunization with three dose levels of each antigen. While dose is commonly based on the conjugate, it made more sense to us to base the dose on the haptene. Therefore, 20, 66 and 200 nmoles (ca. 8, 25 and 80 μg) of steroid derivate contained in a variable amount of BSA conjugate (0.1 - 3 mg), which reflected the incorporation of haptene, was injected initially. All booster injections, however, contained 66 nanomoles of haptene.

Observation of figures 1-3 shows that titer response was largely independent of the dose used for original injection. There was a slightly poorer response to 20 nmoles of norethindrone (Figure 2). In most instances, the response prior to the booster at eight weeks was minimal. Thus, these observations confirm and extend the observations of Vaitukaitis et al (5) that only small doses of steroid haptene are required to produce antibodies. The failure of 18 rabbits to respond to two different preparations of ethynylestradiol is apparently an immunological vagary.

Examination of Tables 1 and 2 also shows that the association constants of the antibodies formed to mestranol and norethindrone did not change appreciably with dose or time. Apparently large doses of haptenic determinant do not induce response from a different type of clonal cell. Idiotypic specificity may change during prolonged immunization (see Nisonoff, et al (11)), but there is no evidence that such small changes will significantly alter the association constant.

EXPERIMENTAL

Melting point determinations were made on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra were determined in nujol using a Perkin-Elmer Infracord spectraphotometer, Model 137 B. Ultraviolet spectra were recorded with a Cary Model 14 spectrophotometer. The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

Synthesis of 6-Carboxymethyloxime of 6-oxoethynylestradiol (EE-6-oxo)

Benzoylation of 6-oxoestradiol: To an ice-cold solution (yellow in color) of 6-oxoestradiol (2.6 gm) in 10% potassium hydroxide solution (75 ml), benzoyl chloride (7 ml) was added in 0.5 ml aliquots with vigorous shaking. Colorless crystalline material began to separate.

It was worked up in the usual way and the solid was crystallized from a mixture of acetone and methanol, yield 2.75 gm, mp. 215-16°; ν_{max}, 3600 (OH), 1760 (carbonyl), 1680 (C-6-carbonyl),1620, 1570 and 1470 (phenyl) cm-1; $\lambda_{max}^{ethanol}$, 255 nm (ξ=8800) and 300 nm (ξ=2100). Found: C, 76.5; H, 6.6. Calcd for $C_{25}H_{26}O_4$: C, 76.9, H, 6.7%.

Preparation of 6-oxoestradiol-3-benzoate-6-(O-carboxymethyl) oxime (III): A mixture of 6-oxoestradiol-3-benzoate (II) (500 mg) in methanol (170 ml), carbomethoxylamine hemihydrochloride (1 gm) and 2 M sodium acetate solution (25 ml) was stirred at room temperature for 48 hours. It was worked up as previously described (1). It was recrystallized from methanol as colorless shining crystals; yield 540 mg, mp. 165-67°d; ν_{max}, 3400 (broad, OH), 1740 (broad, carboxyl), 1620, 1590 and 1570 (phenyl) cm^{-1}. $\lambda_{max}^{ethanol}$, 258 nm (ξ=12,900) and 305 nm (ξ=3,100). Found: C, 68.7; H, 6.5; N, 2.7. Calcd. for $C_{27}H_{29}O_6N$, $\frac{1}{2}$ H_2O: C, 68.6; H, 6.4; N, 2.9%. The identity of the product was further confirmed by hydrolysing the material with potassium hydroxide solution at room temperature to 6-(O-carboxymethyl) oxime of 6-oxoestradiol, mp. 208-10°; mixed mp with authentic sample same, Lt. (4) mp. 199-200°.

Preparation of 6-oxoestrone-3-benzoate-6-(O-carboxymethyl) oxime (IV): III (463 mg) dissolved in acetone (50 ml, distilled from potassium permanganate) was oxidized with Jones reagent (0.25 ml) at 0°. The crude oily product was dissolved in dry ether and crystallized from a mixture of ether and hexane; yield 400 mg; mp. 194-95°d. ν_{max}, 3400 (broad, OH), 1750 (C-17 CO), 1735 (broad, carboxyl), 1615, 1580 and 1490 (phenyl) cm^{-1}. $\lambda_{max}^{ethanol}$, 258 nm (ξ=11,700) and 305 nm (ξ=3,900). Found, C, 70.2; H, 5.8; N, 2.8. Calcd. for $C_{27}H_{27}O_6N$: C, 70.3; H. 5.9; N, 3.0%.

It was observed that Jones reagent must be slightly in excess of the theoretical amount as judged from the color of the reaction mixture; then the excess is consumed by the addition of a few drops of methanol. If this procedure is not followed, the oily product cannot by crystallized.

Preparation of 6-oxoethynylestradiol-6-(O-carboxymethyl) oxime (V): To a solution of lithium acetylide-ethylenediamine complex (1.8 gm) in dry dimethylsulfoxide (15 ml) was added a solution of IV (800 mg) in dry dimethylsulfoxide (10 ml) with stirring in a nitrogen atmosphere at 15-20°. After stirring overnight, the reaction mixture was decomposed carefully with water; the resulting solution was stirred at room temperature for 8-10 hours. A semisolid mass, obtained after acidification with dilute hydrochloric acid, was extracted with ethyl acetate, washed with warm water and dried over anhydrous sodium sulfate. The sticky residue obtained upon evaporation was chromatographed on silica gel. The fraction eluted with 8% ether in benzene was exaporated, yielding a crystalline material which was recrystallized from a mixture of benzene and hexane; yield 580 mg, mp. 155-58°d; lit (5) m.p. 155-57°d; ν_{max}, 3400 (broad, OH), 3290 (ethynyl), 1740 (broad, carboxyl), 1620, 1590, 1490 (phenyl) cm^{-1}. $\lambda_{max}^{ethanol}$, 258 nm (ξ=11.900) and 305 nm (ξ=3,600). Found: C, 68.6; H, 6.6; N, 3.3. Calcd for $C_{22}H_{25}O_5N$: C, 68.9; H, 6.8; N, 3.6%.

Synthesis of 3-succinylethynylestradiol (EE-3-SUC). A mixture of ethynylestradiol (2.9 g), succinic anhydride (1.2 g) and dry pyridine (20 ml) was heated at 100°C for two hours. The oily matter obtained after evaporating the solvent under suction was dissolved in ethyl-acetate. The solution was washed twice with water and then extracted with sodium bicarbonate solution (5%). The alkaline extract was immediately acidified (pH 3.0) with dilute hydrochloric acid yielding a colorless focculent precipitate which was filtered, washed with water and crystallized from dilute methanol (charcoal). Crystalline precipitate, 2.34 g, m.p. 153-54°. ν max, 3400 (broad, OH), 1730 (broad, carboxyl), 1615, 1585, 1775 (phenyl) cm^{-1}. $\lambda_{max}^{ethanol}$, 268 nm (ξ=900). Found: C, 73.1; H, 7.3. Calcd. for $C_{24}H_{28}O_5$; C, 72.7; H, 7.1%.

Synthesis of norethindrone-3(O-carboxymethyl) oxime (NE-3-OXO). A mixture of norethindrone (2.5 g), carboxymethoxylamine hemihydrochloride (2.5 g), ethyl alcohol (125 ml) and 2 N sodium hydroxide solution (10.5 ml) was refluxed for 3 hours. It was worked up as previously described (1). The oil, produced upon removal of the solvent, crystallized when it was triturated with dry ether. It was finally crystallized as colorless crystals from benzene containing a few drops of methanol, 1.9g, m.p. 179-80°. ν max, 3380 (broad, OH), 3270 (ethynyl), 1705 (carboxyl), 1625, 1455, 1465 (phenyl) cm^{-1}. $\lambda_{max}^{ethanol}$, 250 nm (ξ=11,600). Found: C, 71.3; H, 8.0; N, 3.7. Calcd. for $C_{22}H_{29}O_4N$: C, 71.2; H, 7.8; N. 3.8%.

Synthesis of norgestrel-3(O-carboxymethyl) oxime (NG-3-OXO): This was prepared from norgestrel (2.5 g), carboxymethoxylamine hemihydrochloride (2.5 g), ethyl alcohol (125 ml) and 2 N sodium hydroxide solution following the procedure described for the preparation of the norethindrone derivative. Colorless crystals, 1.78 g, m.p. 130-32 C. ν max, 3375 (broad, OH), 3275 (ethynyl), 1700 (carboxyl), 1630, 1450, 1470 (phenyl) cm^{-1}. $\lambda_{max}^{ethanol}$, 250 nm (ξ=22,000). Found: C, 71.5; H, 8.4; N, 3.5. Calcd. for $C_{23}H_{31}O_4N$: C, 71.7; H, 8.1; N, 3.6%.

Synthesis of 6-carboxymethyloximinomestranol (M-6-OXO): The synthesis of this compound has already been described (1).

Conjugation of Steroid Derivatives with Bovine Serum Albumin (BSA)

The conjugation of the steroid derivatives with BSA was done by the carbodiimide method. The steroid (100-200 mg) dissolved in dilute dioxane (1:1) was added to a cold aqueous solution of BSA (45-90 mg in 15 ml water). The mixture was stirred slowly in the cold; the pH of the solution was kept at 7.0 - 7.2 by occasionally adding dilute sodium hydroxide. After stirring for 24 hr. in the cold; a cloudy solution was obtained which was centrifuged. The clear supernatant was dialyzed against cold water for 48 hours and then lyophilyzed. The conjugates were analysed by differential UV-absorption at two wave-lengths to determine the extent of conjugation. The values found are as follows: EE-3-SUC, 24; EE-6-OXO, 12; M-6-OXO, 5; NE-3-OXO, 13; NG-3-OXO, 5 moles/mole BSA.

Immunization of Rabbits: New Zealand, white, male rabbits were immunized following the method described by Vaitukaitis, et al. (5). The sterile saline solution of each of the five conjugates, emulsified with an equal volume of Freund's complete adjuvant, was used for multiple site injections in the back prior to Bordetella pertussis vaccine injection at a separate site. Each antigen was used at three dose levels to three groups of rabbits containing three animals in each group except EE-3-SUC antigen where two dose levels were used. On the eighth week after immunization, a booster dose was given. Even after waiting for 18 weeks, the animals had not responded well (titers were all in the hundreds except a few animals with M-6-OXO antigen). It was decided to castrate them on the 20th week followed by a booster dose the next week.

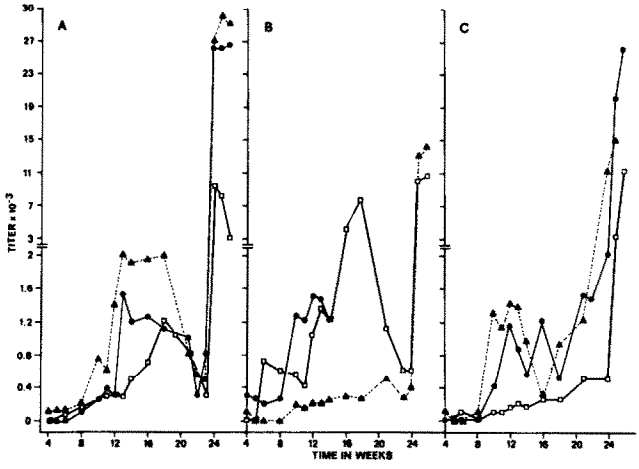

Figure 1. The antibody response to 6-oxomestranol conjugate (5 moles/mole BSA) injected in Freund's complete adjuvant at three dose levels; 1A, 20 nmoles (7.94 µg) of steroid contained in 284 µg of BSA conjugate; 1B, 66 nmoles (26.2 µg) of steroid contained in 937 µg of BSA conjugate; and 1C, 200 nmoles (79.4 µg) of steroid contained in 2.84 mg of BSA conjugate. Each rabbit received a booster injection of 66 nmoles 8 and 21 weeks after injection and were castrated at 20 weeks after injection. Each curve represents one animal.

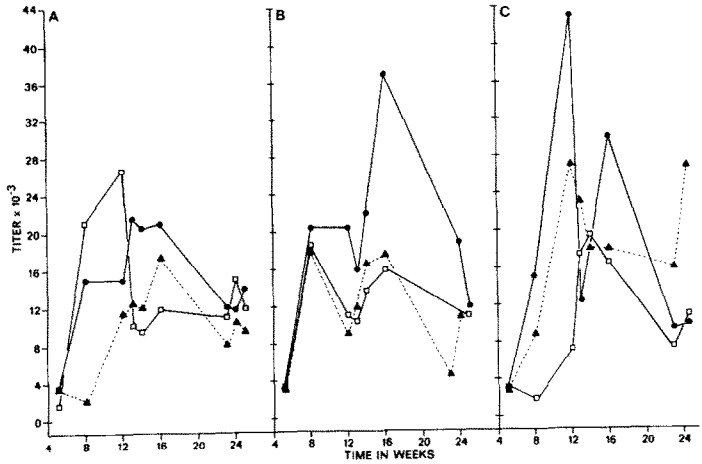

Figure 2. The antibody response to norethindrone-3(O-carboxymethyl) oxime-conjugate (13 moles-mole BSA) injected in Freund's complete adjuvant at three dose levels: 2A, 20 nmoles (7.4 µg) of steroid contained in 114 µg of BSA conjugate; 2B, 66 nmoles (24.5 µg) of steroid contained in 375 µg of BSA conjugate and 2C, 200 nmoles (74 µg) of steroid contained in 1.14 mg of BSA conjugate. Other details are the same as those given in Figure 1.

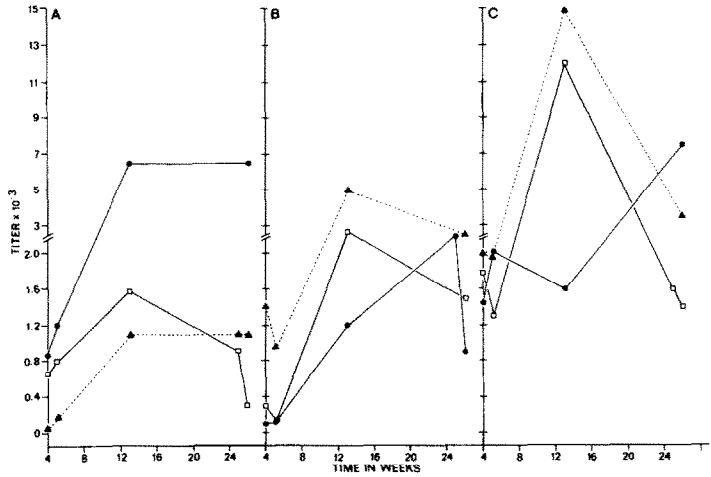

Figure 3. The antibody response to norgestrel-3(O-carboxylmethyl) oxime-conjugate (5 moles/mole BSA) injected in Freund's complete adjuvant at three dose levels: 3A, 20 nmoles (7.7 µg) of steroid contained in 284 µg of BSA conjugate; 3B, 66 nmoles (25.4 µg) of steroid contained in 936 µg of BSA conjugate and 3C, 200 nmoles (77 µg) of steroid contained in 2.84 mg of BSA conjugate. Other details are the same as those given in Figure 1.

Characterization of Antisera: From time to time after immunization, small amounts (10 ml) of blood were drawn for checking the response to immunization. When a sufficient titer in a particular blood was found, a larger amount of blood was drawn in order to characterize the antisera by determining its specificity and its association constant (Kass). The results are given in Tables 1 and 2 and figures 1-3.

Antibody against two ethynylestradiol antigens was not characterized due to their low titers. Titration of the antibody against norgestrel used tritiated norethindrone. Since we could not obtain radioactive norgestrel of high specific activity, the characterization of its antibody was also not performed.

ACKNOWLEDGEMENT

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2. The abbreviations, trivial names and systematic nomenclature of the compounds used are as follows:

ethynylestradiol: 17α-ethynyl-1, 3, 5 (10)-estratriene-3, 17β-diol
6-oxoestradiol: 6-oxo-1, 3, 5 (10)-estratriene-3, 17β-diol
6-oxoestradiol-3-benzoate: 6-oxo-17β-hydroxy-1, 3, 5 (10)-estratrien-3-yl-benzoate
6-oxoestradiol-3-benzoate-6-(O-carboxymethyl) oxime: 6-(O-carboxymethyl)oximino-17β-hydroxy-1, 3, 5 (10)-estratrien-3-yl-benzoate
6-oxoestrone-3-benzoate-6-(O-carboxymethyl) oxime: 6-(O-carboxymethyl)oximino-17-oxo-1, 3, 5 (10)-estratrien-3-yl benzoate
6-oxoethynylestradiol-6-(O-carboxymethyl) oxime: 6-(O-carboxymethyl)oximino-17α-ethynyl-1, 3, 5 (10)-estratriene-3, diol
3-succinylethynylestradiol: 17β-hydroxy-17α-ethynyl-1, 3, 5 (10)-estratrien-3-yl hemisuccinate
norethindrone: 17β-hydroxy-17α-ethynyl-4-estren-3-one
norethindrone-3-(O-carboxymethyl)oxime: 3-(O-carboxymethyl) oximino-17α-ethynyl-4-estren-17β-ol
norgestrel: 17β-hydroxy-17α-ethynyl-18-methyl-4-estren-3-one
norgestrel-3-(O-carboxymethyl) oxime: 3-(O-carboxymethyl)oximino-17α-ethynyl-18-methyl-4-estren-17β-ol
mestranol: 17β-hydroxy-17α-ethynyl-1, 3, 5 (10)-estratrien-3-yl methyl ether
6-carboxymethyloximinomestranol: 17β-hydroxy-6-(O-carboxymethyl)oximino-17α-ethynyl-1, 3, 5 (10)-estratrien-3-yl methyl ether
BSA: bovine serum albumin

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TABLE 1

Antibody Production Against the 6-Carboxymethyl oxime of 6-oxomestranol

Animal No.	Dose of Steroid (nmoles)	Time after Immunization (weeks)	% Crossreactivity[1]							Kass nM^{-1}	Titer
			E_1	E_2	E_3	EE	NE	NG	P		
14	20	18	0.3	2.6	0.3	120	5.1	0.6	---	1.5	2,000
		21	0.4	0.5	0.4	112	0.8	---	---	1.0	800
		25	0.1	5.8	0.1	223	---	---	4.1	0.6	30,000
15		21	0.3	0.4	0.3	104	8.2	5.2	---	1.1	1,000
		25	0.3	0.3	0.3	108	6.5	0.3	0.3	0.9	26,000
16		18	2.9	0.9	0.4	178	4.9	4.6	0.4	1.0	1,200
		21	4.3	0.7	0.4	169	0.9	4.4	0.4	1.0	800
		25	3.1	1.5	2.5	105	---	---	1	0.4	8,000
18	66	18	0.1	0.1	0.1	114	6.0	9.2	---	0.9	7,500
		21	0.3	0.3	0.3	132	6.5	6.5	---	0.4	1,100
		25	0.7	2.6	0.7	118	4.4	1.3	---	0.8	10,000
19		21	0.3	1.2	0.3	140	24.2	53.1	---	1.9	500
		25	0.4	0.7	0.3	100	8.0	6.2	0.4	1.1	13,000
20	200	21	0.2	1.4	0.2	116	5.4	0.2	---	0.9	1,200
		25	0.1	1.6	0.1	124	27	---	0.6	0.7	15,000
21		21	0.2	0.2	0.2	143	10.7	26.0	---	1.5	500
		25	0.4	0.9	0.4	100	11.0	14.0	---	0.8	3,000
22		18	0.4	0.4	0.4	133	6.2	0.2	---	0.9	500
		21	0.6	0.6	0.6	153	5.1	0.4	0.5	0.5	1,500
		25	0.4	0.6	0.4	150	16.5	5.4	---	0.5	20,000

[1]Crossreaction is the ratio of weight of compound to weight of mestranol that will produce a value of 50% for B/Bo (7).

TABLE 2

Antibody Production Against the Norethindrone-3 (O-Carboxymethyl) Oxime

Animal No.	Dose of Steroid (nmoles)	Time after Immunication (weeks)	% Crossreactivity[1]					Kass nM^{-1}	Titer
			EE	E_1	E_2	E_3	P		
24	20	5	11.9	1.0	1.0	1.0	1.0	2.4	1,500
		13	4.8	0.3	0.3	0.3	0.3	3.0	10,000
		24	2.7	0.3	0.3	0.3	0.3	4.3	15,000
26	66	5	3.1	0.6	0.6	0.6	0.6	1.4	3,000
		13	1.7	0.2	0.2	0.2	0.2	2.1	16,000
		24	2.6	0.3	0.3	0.3	0.3	2.8	19,000
31	200	5	3.4	0.5	0.5	0.5	0.5	2.3	3,000
		13	4.3	0.1	0.1	0.1	0.1	0.6	23,000
		24	6.5	0.4	0.4	0.4	0.4	2.4	27,000

[1]Crossreactivity is defined in Table 1.