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Specificity of Antiserum raised against Estetrol using 6-O-Carboxymethyloxime-Bovine Serum Albumin Conjugate[1]

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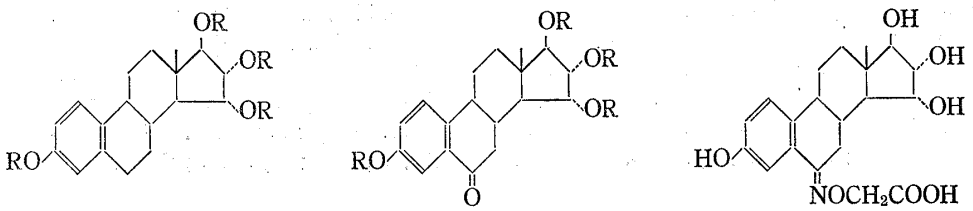
In order to obtain the specific antiserum used for radioimmunoassay of estetrol a new hapten-carrier conjugate was prepared from 6-ketoestetrol O-carboxymethyloxime by coupling with bovine serum albumin employing the mixed anhydride technique. The specificity of anti-estetrol antiserum elicited in the rabbit by immunization with this antigen was tested by cross-reaction studies with the closely related steroids. The results indicated that highly specific antiserum to estetrol would be produced by antigen whose steroidal moiety is coupled to a protein through the position remote from the inherent functional groups.

Estetrol (estra-1,3,5(10)-triene-3,15α,16α,17β-tetraol) (Ia) was isolated first from urine of human newborn.[3] In recent years considerable attentions have been directed to the physiological role of this novel estrogen in the feto-placental unit.[4] The plasma level of estetrol may serve as a possibly superior index of fetal viability to that of estriol which has been widely accepted as a measure of feto-placental function. Although radioimmunoassay systems employing estetrol 3-carboxymethyl ether and 16,17-dihemisuccinate as haptens were proposed for measurement of estetrol,[5,6] the satisfactory results have not yet been attained in respect of the specificity. For the purpose of obtaining much more specific antiserum for the assay of estetrol we have attempted to prepare the 6-O-carboxymethyloxime as a new hapten.



Ia: R=H
Ib: R=Ac

IIa: R=H
IIb: R=Ac

III

Chart 1

Oxidation of estetrol tetraacetate (Ib)[7] with chromium trioxide in acetic acid[8] provided the 6-keto derivative (IIb) in 27% yield. Upon treatment with alkali under the mild conditions hydrolysis of the acetoxyl groups was effected to furnish 6-ketoestetrol (IIa). Condensation with O-carboxymethylhydroxylamine in the usual manner afforded the desired 6-O-carboxy-

1) Part CX of "Studies on Steroids" by T. Nambara; Part CIX: T. Nambara, T. Iwata, and K. Kigasawa, *J. Chromatog.*, **118**, 127 (1976).
2) Location: *Aobayama, Sendai.*
3) A.A. Hagen, M. Barr, and E. Diczfalusy, *Acta Endocrinol.*, **49**, 207 (1965).
4) D. Tulchinsky, F.D. Frigoletto, Jr., K.J. Ryan, and J. Fishman, *J. Clin. Endocrinol. Metab.*, **40**, 560 (1975) and references quoted therein.
5) J. Fishman and H. Guzik, *J. Clin. Endocrinol. Metab.*, **35**, 892 (1972).
6) M.E. Giebenhain, G.E. Tagatz, and E. Gurpide, *J. Steroid Biochem.*, **3**, 707 (1972).
7) J. Fishman and H. Guzik, *J. Org. Chem.*, **33**, 3133 (1968).
8) B. Longwell and O. Wintersteiner, *J. Biol. Chem.*, **133**, 219 (1940).

Table I. Replicate Analyses of Authentic Estetrol added to Deionized Distilled Water[a)]

	Estetrol		Standard deviation	Standard error	Coefficient of variation (%)	Regression equation
	Added (pg)	Found (pg)				
Anti-estetrol antiserum (1: 7000 dilution)	0	15	3.21	1.44	20.8	$Y=0.90X+6$
	20	19	4.24	1.90	22.3	
	50	43	5.59	2.50	13.2	
	100	93	7.53	3.37	8.2	
	300	275	20.74	9.27	7.9	
	1000	998	76.61	34.26	7.7	

a) number of determination (n)=5

methyloxime (III) in a reasonable yield. The oxime derivative was covalently linked to bovine serum albumin (BSA) by the mixed anhydride technique.[9)] As judged from the ultraviolet absorption it proved that satisfactory number of steroid molecules were joined to each BSA.

After injection of the hapten-carrier conjugate for three months the serum sample obtained from the immunized rabbit showed significantly increased binding activity to estetrol. The standard curve was constructed with 1: 7000 dilution of the rabbit serum raised against the 6-O-carboxymethyloxime-BSA conjugate and was used for the replicate analyses and cross-reaction studies. As illustrated in Fig. 1 the plot of per cent bound radioactivity *versus* the logarithm of inert estetrol showed a linear relationship. The precision of the method was examined with various known amounts of authentic estetrol ranging from 20 pg to 1 ng. The replicate determinations of each sample gave the satisfactory results with a regression line as listed in Table I.

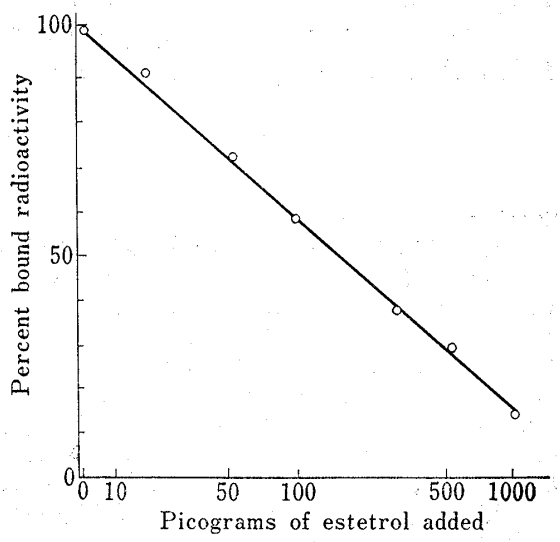


Fig. 1. Standard Curve for Estetrol in Deionized Distilled Water Using Anti-Estetrol Antiserum (1: 7000 dilution)

Table II. Per Cent Cross-Reaction of Anti-Estetrol Antiserum with Selected Steroids

Steroid	Cross-reaction (%)	Steroid	Cross-reaction (%)
Estetrol	100	Estriol 16-glucuronide	<0.001
6-Ketoestetrol	72	Estradiol	<0.001
15β-Hydroxy-16-epiestriol	0.014	16-Ketoestradiol	<0.001
15α-Hydroxyestradiol	0.08	2-Hydroxyestradiol	0.025
15α-Hydroxyestrone	0.06	2-Methoxyestradiol	0.015
Estriol	1.2	Estrone	<0.001
16-Epiestriol	0.2	2-Hydroxyestrone	<0.001
17-Epiestriol	0.01	Testosterone	<0.001
6-Ketoestriol	0.11	Progesterone	0.01

9) S.A. Berson and R.S. Yalow, *J. Clin. Invest.*, **36**, 873 (1957); G. Mikhail, C.H. Wu, M. Ferin, and R.L. Vande Wiele, *Acta Endocrinol.*, **64**, Suppl., 147, 347 (1970).

The specificity of antiserum was assessed by testing the ability of some steroids closely related to estetrol to compete for binding sites on the antibody. The results of cross-reaction studies with anti-estetrol antiserum are collected in Table II. It is evident from the data that this antiserum is much more specific for estetrol than the others so far reported. None of the steroids tested in this system showed more than 1.2% of the relative activity of estetrol. In particular, much less cross-reactivity with the 15- and 16-oxygenated steroids appears to be advantageous for the assay of estetrol in the presence of related estrogens involving the feto-placental unit. Significant cross-reaction with 6-ketoestetrol (72%) seems to be inevitable, since the hapten is coupled to a protein through a derivative at C-6. The similar pattern of specificity has been observed with antiserum raised against estradiol when the 6-O-carboxymethyloxime-protein conjugate was used as an antigen.[10] From the present results together with the previous findings[11] it is concluded that steroid hormones should be conjugated to protein in such a way that both A and D rings are left available as antigenic determinant for the production of specific antiserum.

The utilization of this highly specific antiserum for determination of estetrol in biological fluids will be the subject of a future communication.

Experimental

Synthesis of Hapten[12]

3,15α,16α,17β-Tetrahydroxyestra-1,3,5(10)-trien-6-one Tetraacetate (IIb)——To a solution of estetrol tetraacetate (Ib)[7] (1.3 g) in AcOH (10 ml) was added CrO_3 (2 g) dissolved in H_2O (1 ml)–AcOH (14 ml) dropwise and stirred at room temperature for 1.5 hr. The resulting solution was poured onto ice-water, neutralized with 5% $NaHCO_3$, and extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. An oily residue obtained was chromatographed on silica gel (100 g). Elution with hexane–AcOEt (2: 1) and recrystallization of the eluate from MeOH gave IIb (300 mg) as colorless needles. mp 224—225° (Lit. mp 224—228°).[13] IR ν_{max}^{KBr} cm^{-1}: 1760 (C=O), 1690 (conjugated C=O). NMR (5% $CDCl_3$ solution) δ: 0.95 (3H, s, 18-CH_3), 2.03, 2.09, 2.38 (12H, s, 3-, 15α-, 16α-, 17β-$OCOCH_3$), 4.90—5.02, 5.18—5.34 (3H, m, 15β-, 16β-, 17α-H), 7.20—7.40 (2H, m, 1-, 2-H), 7.72 (1H, d, J=4 Hz, 4-H).

3,15α,16α,17β-Tetrahydroxyestra-1,3,5(10)-trien-6-one (IIa)——To a solution of IIb (400 mg) in MeOH (20 ml) was added dropwise 15% methanolic KOH (1 ml) under a stream of N_2 gas and allowed to stand at room temperature for 2 hr. After neutralization with 5% HCl the resulting solution was evaporated, diluted with H_2O, passed through a column of Amberlite XAD-2 resin, and washed with distilled water. Elution with MeOH and recrystallization of the eluate from MeOH gave IIa (150 mg) as colorless needles. mp 165—170°. $[\alpha]_D^{10}$ +35.4° (c=0.11, MeOH). *Anal.* Calcd. for $C_{18}H_{22}O_5 \cdot H_2O$: C, 64.27; H, 7.19. Found: C, 64.34; H, 7.26. IR ν_{max}^{KBr} cm^{-1}: 1690 (conjugated C=O). NMR (4% CD_3OD solution) δ: 0.79 (3H, s, 18-CH_3), 7.38 (1H, d, J=4 Hz, 4-H).

3,15α,16α,17β-Tetrahydroxyestra-1,3,5(10)-trien-6-one O-Carboxymethyloxime (III)——To a solution of IIa (70 mg) in MeOH (4 ml) were added O-carboxymethylhydroxylamine·HCl (100 mg) and 1 M AcONa (40 ml) and allowed to stand at 35° for 12 hr. After evaporation of MeOH the residue was diluted with H_2O, passed through a column of Amberlite XAD-2 resin, and washed with distilled water. Elution with MeOH and recrystallization of the eluate from MeOH–AcOEt gave III (49 mg) as amorphous substance. mp 223° (decomp). *Anal.* Calcd. for $C_{20}H_{25}O_7N \cdot 3^1/_2H_2O$: C, 52.85; H, 7.10; N, 3.08. Found: C, 53.15; H, 5.59; N, 3.02. $[\alpha]_D^{10}$ +37.5° (c=0.28, MeOH). IR ν_{max}^{KBr} cm^{-1}: 1600 (>C=N-, -COOH). NMR (4% CD_3OD solution) δ: 0.75 (3H, s, 18-CH_3), 4.55 (3H, m, 16β-H, =$NOCH_2$-).

Animals——Domestic strain male albino rabbits weighing 2.5—3.0 kg were used.

Materials——Estetrol-2,4-^{3}H (52 Ci/mmole) was supplied from New England Nuclear, Boston, and was purified by thin-layer chromatography (TLC) prior to use. Nonradioactive estetrol, 15α-hydroxyestrone,

10) S.L. Jeffcoate and J.E. Searle, *Steroids*, **19**, 181 (1972); K. Wright, D.C. Collins, and J.R.K. Preedy, *ibid.*, **21**, 755 (1973).

11) T. Nambara, M. Takahashi, Y. Tsuchida, and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 2176 (1974).

12) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra were run on a JASCO Model IR-S spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi Model R-20A spectrometer at 60 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, and m=multiplet.

13) J. Fishman and H. Guzik, *J. Labelled Comp.*, **6**, 341 (1970).

and 15α-hydroxyestradiol were prepared in this laboratory by the known methods[7,14] and their homogeneity was checked by TLC. Other steroids were kindly donated from Teikoku Hormone Mfg. Co., Tokyo. BSA, bovine gamma-globulin (Miles Laboratories, Detroit) were purchased, respectively. All solvents and chemicals used were of analytical grade.

Conjugation of 6-Ketoestetrol O-Carboxymethyloxime to BSA——To a stirred solution of 6-ketoestetrol O-carboxymethyloxime (III) (15 mg) in dimethylformamide (DMF) (0.6 ml) were added $(n\text{-}C_4H_9)_3N$ (0.018 ml) and then isobutyl chloroformate (4.5 μl) under ice-cooling. Twenty minutes later this was added to a solution of BSA (48 mg) in H_2O (1.2 ml)-DMF (0.9 ml) containing 1 N NaOH (0.05 ml) under ice-cooling and stirred maintaining a pH of *ca.* 7 for 3 hr. The resulting solution was dialyzed against cold running water overnight and the turbid protein solution was brought to pH 4.5 with 1 N HCl. After allowing to stand at 4° overnight the suspension was centrifuged at 3000 rpm for 10 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the manner as the above. Lyophilization of the solution afforded the conjugate (50 mg) as a fluffy powder. Measurement of absorbance at 264 nm with aq. solution of conjugate, hapten, and BSA revealed that 12 molecules of hapten were bound to each mole of protein.

Radioactivity Measurement——The samples were counted on a Aloka Model 651 liquid scintillation spectrometer employing Bray's scintillant, composed of 2,5-diphenyloxazole (4 g), 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (200 mg), naphthalene (60 g), MeOH (100 ml), ethylene glycol (20 ml), and sufficient dioxane to make the total volume 1 liter.[15] For quenching corrections the external standard method was employed.

Immunization of Rabbits——Three rabbits were immunized with the 6-O-carboxymethyloxime-BSA conjugate. The antigen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). This emulsion was injected into rabbits subcutaneously at the multiple sites over the scapulae and in the thighs. This procedure was repeated at intervals of two weeks for further 2 months and then once a month. The rabbits were bled from the ear vein, 5—10 ml of blood being collected a week after the 5 th injection. The antiserum was separated by centrifugation at 3000 rpm for 10 min and stored at −80°. The antiserum was thawed and diluted with 0.05 M borate buffer (pH 8.0) containing 0.06% BSA and 0.05% bovine gamma-globulin. Dilutions of 1: 7000, 1: 10000, and 1: 13000 were prepared and their dose response curves were constructed. The standard curve obtained with 1: 7000 dilution proved to be most suitable for the assay.

Assay Procedure——To a test sample containing estetrol were added estetrol-^{3}H (*ca.* 1×10^4 dpm) and diluted antiserum (0.25 ml) and incubated at room temperature for 30 min. After addition of 50% $(NH_4)_2SO_4$ (0.25 ml) the resulting mixture was allowed to stand at room temperature for 10 min and centrifuged at 3000 rpm for 10 min. A 0.2 ml aliquot of the supernatant was used for radioactivity counting. The percentage of bound estetrol in the sample was determined with the following equation:

$$\%\ \text{bound estetrol} = \frac{A - P\times\dfrac{0.5}{0.2}}{A}\times 100$$

where A=disintegration/min added to each sample, P=disintegration/min recovered.

Cross-Reaction Study——The specificity of antiserum elicited by immunization with the 6-O-carboxymethyloxime-BSA conjugate was tested by cross-reaction with 17 kinds of steroids related to estetrol. The relative amounts required to reduce the initial binding of estetrol-^{3}H by half, where the mass of nonlabeled estetrol was arbitrarily chosen as 100%, were calculated by the standard curve.

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14) H. Hosoda, K. Yamashita, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **23**, 3141 (1975).
15) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).