Naphthalene–sodium reagent prepared from 345 mg of sodium and 2.04 g of naphthalene in 30 ml of THF was added dropwise to a solution of 1 g of **5a** in 30 ml of THF as described above. The mixture was stirred for 30 min and was quenched by addition of MeOH. Work-up as described above gave 825 mg of a syrup which was charged on 15 g of silica gel and eluted with $CHCl_3$–MeOH (25: 1, v/v). The fast running fractions were collected and recrystallized from aq. MeOH to give 295 mg of **6**, mp 213—215°, powder. NMR (pyridine-$d_5$) $\delta$: 5.85 (2H, br. s, H-3′,4′). *Anal.* Calcd. for $C_{39}H_{63}N_5O_{18}$: C, 52.57; H, 7.13; N, 7.86. Found: C, 52.28; H, 7.32; N, 7.75.

Benzoylaion of **6** in pyridine gave a 2″-benzoate, mp 202—203°, powder which was identified with the sample prepared from **5a** *via* the known Tipson–Cohen method.

After the collection of fractions including **6**, the column was eluted with $CHCl_3$–MeOH (10: 1, v/v); thus, 170 mg of **5b** was obtained and identified with the authentic sample reported earlier.[8]

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# Radioimmunoassay of Pregnanediol

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Rabbit anti-pregnanediol antiserum was obtained using 3α,20α-dihydroxy-5β-pregnan-7-one O-carboxymethyloxime coupled to bovine serum albumin as the antigen. A radioimmunoassay procedure for pregnanediol utilizing the highly specific antiserum was developed and permitted the measurement of this material in body fluids.

**Keywords**——radioimmunoassay; pregnanediol; 3α,20α-dihydroxy-5β-pregnan-7-one O-carboxymethyloxime; anti-pregnanediol antiserum; progesterone metabolism

## Introduction

Progesterone is an important steroid not only as a sexual hormone but also as a key intermediate to adrenocortical and/or other hormones in the steroid biosynthesis. Although the main pathways of the metabolism of progesterone have been elucidated, many aspects of its detailed metabolism remain obscure.

Pregnanediol is a major reductive metabolite of progesterone and excretion of its glucuronide in urine has been extensively used as a parameter of circulating progesterone. The measurement of pregnanediol in urine has a definite clinical value.[2]

In the course of our research on the alternation of various steroid during the menstrual cycle, it became necessary to determine the plasma pregnanediol, especially by a simple method. We have, therefore, undertaken to develop the method of radioimmunoassay of pregnanediol and this paper describes the details of the methodology.

### Experimental

**Materials**——The haptenyl compound, 3α,20α-dihydroxy-5β-pregnan-7-one O-carboxymethyloxime, was synthesized in this laboratory by the method reported previously.[3] 5β-Pregnane-3α,20α-diol-1,2-$^3$H (45

1) Location: *Katsuraoka-cho, Otaru, Hokkaido, 047, Japan.*
2) H. Breuer, D. Hamel, and H.L. Kruskemper, "Methods of Hormone Analysis," Georg Thime Verlag, Stuttgart, 1975, Chapters, 33, 34, 35 and 36.
3) I. Yoshizawa and M. Kimura, *Yakugaku Zasshi*, **95**, 843 (1975).

Ci/mmol) was supplied from the Daiichi Pure Chemicals Co., Ltd., Tokyo. Other steroids, except 7-oxo-pregnanediol and 2-hydroxyestrone which were prepared in this laboratory, were purchased from Teikoku Hormone Mfg. Co., Tokyo. Bovine serum albumin (BSA) and Freund's complete adjuvant were purchased from the Armour Pharmaceutical Co., Kankakee, U.S.A. and the Difco Laboratories, Detroit, U.S.A., respectively. All solvents and chemicals used were of Analytical Reagent grade.

**Conjugation of Hapten to BSA**——To a stirred solution of hapten (407 mg) in dry dioxane (30 ml) were added ($n$-$C_4H_9)_3N$ (180 mg) and isobutyl chlorocarbonate (120 mg) under ice-cooling. After thirty minutes was added dropwise a solution of BSA (1.2 g) in $H_2O$ (30 ml)-dioxane (30 ml) containing 1 N NaOH (1.3 ml) under ice-cooling. The mixture was stirred for 4 hr with maintaining a pH of about 7. The resulting solution was dialyzed against cold running water overnight and the turbid protein solution was brought to pH 4.6 with 1 N HCl. The suspension was allowed to stand at 0° and centrifuged at 3000 rpm for 10 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed against cold running water for 3 days. Lyophilization of the solution afforded BSA-conjugate (1.3 g) as a fluffy powder.

**Determination of Steroid Numbers of Antigen**——The number of steroid molecules linked to a BSA molecule was calculated by the following procedure, which will be published in detail elsewhere.[4] About fifty milligrams of antigen was treated with 1-fluoro-2,4-dinitrobenzene to give the 2,4-dinitrophenylate of the antigen, which was refluxed in 6 N HCl (10 ml) for 24 hr. After cooling, the reaction mixture was extracted with ether (10 ml × 5). The amount of steroid in the ether layer was determined by gas liquid chromatography and 2,4-dinitrophenyllysine in the aqueous layer was calculated by spectrophotometric analysis as described by Erlanger, *et al.*[5] The results of the triplicated experiments showed that the antigen synthesized had 25—29 steroid molecules per molecule of BSA.

**Radioactivity Measurement**——Samples were counted in Aloka-LSC-501 liquid scintillation spectrometer employing Bray's scintillant, composed of 2,5-diphenyloxazole (4 g), 1,4-bis(5-phenyl-2-oxazolyl)benzene (200 mg), naphthalene (60 g), MeOH (100 ml), ethylene glycol (20 ml), and sufficient dioxane to make the total volume 1 liter. For quenching corrections the channel ratio and external standard methods were employed.

**Preparation of Antiserum**——The purified antigen was shaken vigorously with Freund's complete adjuvant to provide an emulsion containing 0.5 mg of antigen/ml. This material was administered subcutaneously to adult New Zealand rabbits at 4 sites near the axial and pelvic lymph nodes. The schedule of injections was 0.5 ml to each site once a week for 6 weeks, followed by a similar booster shot 3 weeks later. Intravenous injections of 0.5 mg of the antigen in 1 ml of saline were then continued at monthly intervals.

Table I. Cross-Reaction of Similar Steroids with Anti-pregnanediol Antiserum

| | |
|---|---|
| Pregnanediol (5β-Pregnane-3α,20α-diol) | 100.0% |
| 7-Oxo-pregnanediol | 143.5 |
| 5β-Pregnane-3α,20β-diol | 0.62 |
| 5β-Pregnane-3β,20β-diol | 0.11 |
| 5α-Pregnane-3α,20α-diol | 0.02 |
| 5α-Pregnane-3α,20β-diol | 0.01 |
| 5α-Pregnane-3β,20α-diol | 0.01 |
| 5α-Pregnane-3β,20β-diol | 0.01 |
| Pregnanetriol (5β-pregnane-3α,17α,20α-triol) | 0.02 |

Steroids which showed cross-reaction less than 0.001%:

Bile Acids: 5β-Cholanic acid-3α,7α,12α-triol
5β-Cholanic acid-3α,7α-diol
5β-Cholanic acid-3α,7β-diol
5β-Cholanic acid-3β,7α-diol
5β-Cholanic acid-3β,7β-diol
5β-Cholane-3α,7α,24-triol
5β-Cholane-3β,7α,24-triol
5β-Cholane-3β,7β,24-triol
5β-Cholane-3β,24-diol

Estrogens: Estrone, Estradiol, Estriol, 2-Hydroxyestrone

Androgens: Androsterone, Epiandrosterone, Testosterone

Corticoids: Cortisone, Cortisol, Corticosterone, Prednisone Prednisolone, Aldosterone, α-Cortol

4) I. Yoshizawa, R. Ohuchi, and A. Nakagawa, "in preparation."
5) B.F. Erlanger, F. Borek, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **234**, 1090 (1959).

Blood (—10 ml) was obtained from the ear vein prior to and 3, 6, 9 and 12 weeks following the first injection, and the plasma was separated and frozen. Antibody titers determined by pregnanediol binding assays showed maximal titers in the 9- and 12-week collections and these were used in the subsequent work. The above plasma was treated first with BSA[6] and then with Rivanol,[7] to provide purified antiserum of approximately 5-fold dilution. The antiserum preparation was stored frozen (−30°) and its binding activity remained unchanged for at least 5 months. The antiserum was thawed and diluted as needed with pH 7 phosphate buffer. Dilution of 1: 2000, 1: 3000, 1: 4000 and 1: 5000 were prepared and their dose response curves were determined. The standard curve obtained with 1: 4000 dilution was best suited for assay purposes and is presented in Fig. 1.

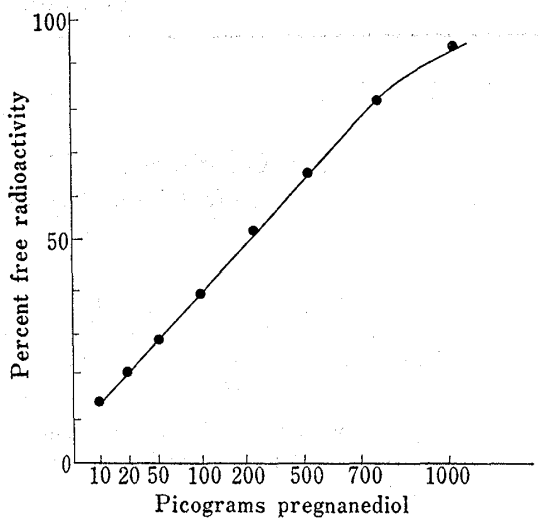


Fig. 1. Standard Curve Using Anti-pregnanediol Antiserum I: 4000 Dilution

**Assay Procedure**——The assay procedure involved the addition of 0.2 ml of the pregnanediol-1,2-$^3$H (*ca.* 4000 cpm, in pH 6 phosphate buffer solution) to 1 ml of the 1: 4000 antiserum solution and the subsequent addition to 0.1 ml of a pH 7 phosphate buffer solution containing standard or unknown pregnanediol. After incubation for 3 hr at room temperature, with occasional shaking, 0.2 ml of an aqueous suspension containing 0.1 mg of charcoal coated with 1% dextran was added. After another 2 hr at room temperature the tubes were centrifuged and the two 0.5 ml aliquots of supernatant were removed for counting. The percentage of the free pregnanediol in the sample was determined with the following equation:

$$\% \text{ free steroid} = \frac{B}{A} \times \frac{1.5}{0.5} \times 100$$

where $A$ = count/min added to each samples, $B$ = count/min recovered. Water blanks were obtained with each series of determinations and never exceeded 5 pg.

**Cross-Reaction Studies**——Cross-reaction of anti-pregnanediol antiserum with selected potential competitors was determined by using the above assay procedures with different concentrations of the materials tested. The relative amounts required to reduce the initial binding of pregnanediol-1,2-$^3$H by half, where the mass of non-labeled pregnanediol was arbitrarily chosen as 100%, were calculated by the standard curve. The results are shown in Table I.

## Results and Discussion

The steroidal hapten, 3α,20α-dihydroxy-5β-pregnan-7-one O-carboxymethyloxime, was covalently linked to BSA by the mixed anhyride technique and satisfactory numbers of steroid molecules were coupled to BSA. The serum sample obtained from the immunized rabbits showed an increased binding activity to pregnanediol, though there was considerable individual variation. After three or four months following the injection several samples showed a significant increase in the binding activity.

The standard curve obtained with 1: 4000 dilution of the antiserum is shown in Fig. 1. The plot of percent radioactivity against the logarithm of inert pregnanediol added shows a linear relationship of less than 5% for each point.

The results of the cross-reaction studies of various compounds with anti-pregnanediol rabbit antiserum are given in Table I. It is particularly gratifying that the only compound tested with which significant cross-reaction occurred was 7-oxo-pregnanediol which is not present in the body fluids or in urine.

The utilization of this highly specific antiserum for the measurement of pregnanediol in the biological fluids will be the subject of our future communication.

6) D.H. Campbell, J.S. Garvey, N.Y. Cramer, and D.H. Sussdorf, "Methods in Immunology," W.A. Benjamin Inc., New York, 1963, p. 136.
7) G.E. Abraham, *J. Clin. Endocr. Metab.*, **29**, 870 (1969).