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Preparation of Specific Antisera to 3β-Hydroxy-5-cholenoic Acid by Immunization with Conjugates Attached to Protein through the C-3 Position[1)]

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3β-Hydroxy-5-cholenoic acid–bovine serum albumin (BSA) conjugates were prepared by coupling of the haptens with the carrier protein through a 3-hemisuccinoyl or 3-hemiglutaroyl bridge. The antisera elicited by these new immunogens showed high affinity constants (K_a = 4.88×10^8 and 8.75×10^8 M^{-1}) and excellent specificity to 3β-hydroxy-5-cholenoic acid, exhibiting little or no cross-reactivities with cholesterol (0.001%) and related compounds. Though the antiserum obtained with the hemisuccinate-BSA conjugate was somewhat more specific than the other, they showed substantially the same reactivity, and should be useful for direct radioimmunoassay of 3β-hydroxy-5-cholenoic acid in biological fluids without any chromatographic separation step prior to assay.

Keywords——radioimmunoassay; specificity; cross-reaction; antiserum; hapten; 3β-hydroxy-5-cholenoic acid; 3β-hemisuccinoyloxy-5-cholenoic acid *N*-succinimidyl ester; 3β-hemiglutaroyloxy-5-cholenoic acid *N*-succinimidyl ester; bovine serum albumin conjugate; cholesterol

Oxidation of the cholesterol side chain is one of the more significant transformations in bile acid biosynthesis. Since the oxidative cleavage of the side chain in the minor pathway precedes the transformation of the ring structure initiated by 7α-hydroxylation of cholesterol, the key intermediate of this metabolic pathway is 3β-hydroxy-5-cholenoic acid (3β-hydroxychol-5-en-24-oic acid) with a ring structure identical to that of cholesterol, this compound is then further converted to bile acids.[2)] Recently, much work has been done to determine the content of this intermediate in body fluids,[3)] especially in infants with biliary atresia[4)] and in patients with cholestasis.[5)] Radioimmunological measurements have been developed to investigate the possibility that this new bile acid could play an important role in the etiology and pathophysiology of hepatobiliary diseases.[6,7)] However, the antisera used in these studies were not satisfactory as regards specificity and a chromatographic separation step was required prior to assay. In the antigens used to elicit the antisera, the carboxylic side chain in 3β-hydroxy-5-cholenoic acid was used for coupling with the protein. Therefore the resulting antisera showed a significant cross-reactivity to cholesterol. In order to elicit an antibody with enhanced specificity to the side chain structure we decided to prepare a hapten which could be linked to the immunogenic carrier at the C-3 position. The present paper describes a convenient sysnthesis of a new type of haptens which are capable of coupling with the protein selectively at that position, the preparation of the bovine serum albumin (BSA) conjugate, and the properties of antisera raised by these immunogens.

Our initial effort was directed to the synthesis of new haptens, 3β-hemisuccinoyloxy-5-cholenoic acid *N*-succinimidyl ester and the analogous hemiglutaroyloxy derivative. The regioselective introduction of an *N*-succinimidyl linkage at the C-3 position in the synthetic route was performed and regeneration of the carboxylic side chain was achieved without

removal of the C-3 ester group. 3β-Hydroxy-5-cholenoic acid (**1**) obtained from hyodeoxycholic acid[8] was treated with 2,2,2-trichloroethanol in methylene chloride and pyridine in the presence of *N*, *N'*-dicyclohexylcarbodiimide (DCC) to give the trichloroethyl ester (**2**) in quantitative yield. Refluxing of **2** with succinic anhydride in pyridine afforded the 3-hemisuccinate (**3**), which underwent esterification with *N*-hydroxysuccinimide in 95% dioxane to give the *N*-hydroxysuccinimidyl ester (**5**). The desired *N*-succinimidyl derivative (**7**) was readily afforded by removal of the protecting group at C-24 of the ester (**5**) by brief exposure to zinc dust in acetic acid. The preparation of the hemiglutarolyoxy analogue (**8**) was also achieved in a similar fashion. Chart 1 shows the synthetic route to these haptens.

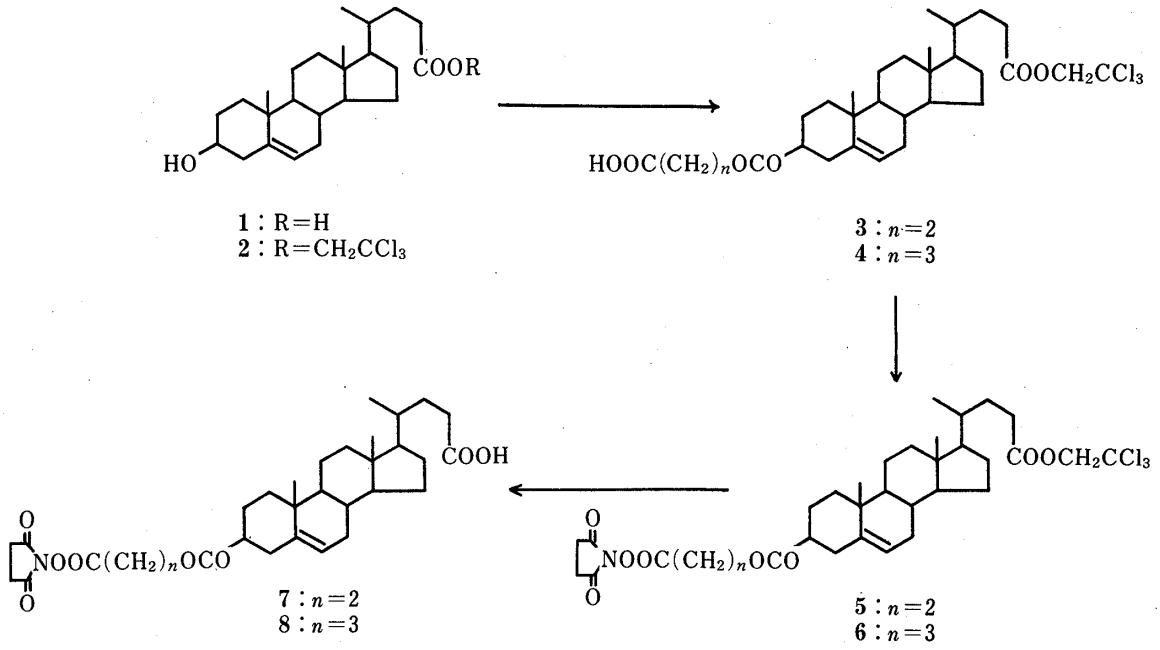

Chart 1

The haptenic derivatives (**7** and **8**) were covalently coupled to BSA in dioxane and 0.01 M phosphate buffer (pH 7.4) according to Hosoda *et al.*[9] The number of bile acid residues incorporated into each BSA molecule was spectrophotometrically determined on the basis of coloration with concentrated sulfuric acid to be 14 for **7** and 15 for **8**.

Rabbits were immunized with these conjugates emulsified with complete Freund's adjuvant. The titer was determined from the ability of the antibody to bind a certain amount of labeled [^{3}H]3β-hydroxy-5-cholenoic acid. These antibodies were tested to determine the 50% binding level at several dilutions after each bleeding. The serum obtained from the rabbit immunized with each conjugate for six months showed satisfactorily increased activity towards 3β-hydroxy-5-cholenoic acid. The dose-response curves were constructed with 1 : 1000 dilution of the antisera. When logit transformation was used to construct the curve, plots of logit per cent bound radioactivity *vs.* logarithm of the amount of unlabeled 3β-hydroxy-5-cholenoic acid showed a linear relationship in the range of 0.5 to 50 ng. The binding affinity was determined by incubating a constant amount of antiserum with increasing amounts of the labeled antigen. The ratio of the bound to the free antigen (B/F) observed with each antiserum was plotted against the concentration of antigen according to the method of Scatchard.[10] These antisera exhibited high affinity for 3β-hydroxy-5-cholenoic acid with association constants of 4.88×10^8 and 8.75×10^8 M^{-1}, respectively (Fig. 1).

The specificity of the resulting antisera was assessed by testing the ability of the related compound to compete for binding sites on the antibody. The per cent cross-reactions of

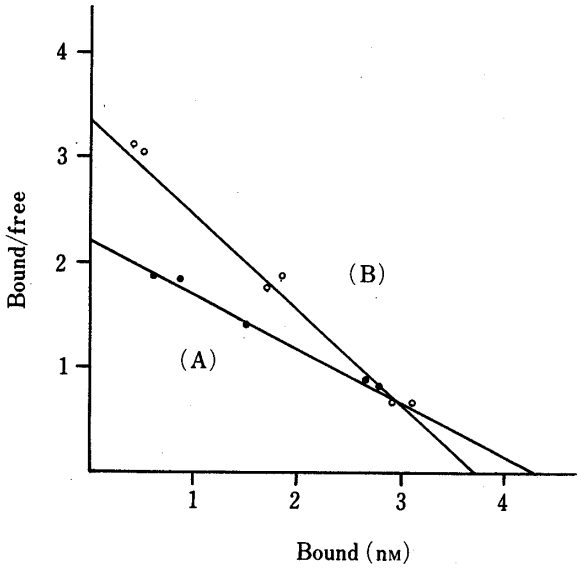

Fig. 1. Scatchard Plots for Antisera against 3β-Hemisuccinoyloxy-5-cholenoic Acid–BSA Conjugate (A) and 3β-Hemiglutaroyloxy-5-cholenoic Acid–BSA Conjugate (B)

(A): $K_a = 4.88 \times 10^8\ M^{-1}$.
(B): $K_a = 8.75 \times 10^8\ M^{-1}$.

TABLE I. Per Cent Cross-Reactivity of Anti-3β-hydroxy-5-cholenoic Acid Antisera with Related Steroids

Compounds	% cross-reactivity 3-HS[a)]	3-HG[a)]
3β-Hydroxy-5-cholenoic acid	100	100
3β, 12α-Dihydroxy-5-cholenoic acid	1.11	1.33
Lithocholic acid	<0.001	<0.001
Chenodeoxycholic acid	0.010	0.036
Deoxycholic acid	<0.001	0.013
Ursodeoxycholic acid	0.003	0.015
Cholic acid	<0.001	<0.001
Glyco-3β-hydroxy-5-cholenoic acid	0.145	0.429
Glycolithocholic acid	<0.001	<0.001
Glycochenodeoxycholic acid	0.002	0.002
Glycodeoxycholic acid	<0.001	<0.001
Glycocholic acid	<0.001	<0.001
Tauro-3β-hydroxy-5-cholenoic acid	0.706	1.08
Taurolithocholic acid	<0.001	<0.001
Taurochenodeoxycholic acid	<0.001	<0.001
Taurodeoxycholic acid	<0.001	<0.001
Taurocholic acid	<0.001	<0.001
Testosterone	<0.001	<0.001
5α-Dihydrotestosterone	0.004	0.003
Dehydroepiandrosterone	0.004	0.003
Estrone	<0.001	<0.001
Estradiol	<0.001	<0.001
Estriol	<0.001	<0.001
Pregnenolone	<0.001	<0.001
Progesterone	<0.001	<0.001
Hydrocortisone	<0.001	<0.001
Corticosterone	<0.001	<0.001
Cholesterol	<0.001	<0.001

a) 3-HS = 3β-Hemisuccinoyloxy-5-cholenoic acid–BSA, 3-HG = 3β-hemiglutaroyloxy-5-cholenoic acid–BSA.

antisera were determined by the method of Abraham.[11] The cross-reactions of anti-3β-hydroxy-5-cholenoic acid antisera with 27 closely related compounds are listed in Table I.

The antisera were found to be sufficiently specific for 3β-hydroxy-5-cholenoic acid. The cross-reactivities of these two antisera were substantially the same, but the antiserum elicited by the hemisuccinate-BSA conjugate was somewhat more specific than the other one. All of the major human bile acids and other steroids exhibited negligible competition for the antibodies ranging from 0.001% to 0.016% relative to 3β-hydroxy-5-cholenoic acid. 3β,12α-Dihydroxy-5-cholenoic acid, which was found as a minor bile acid in the serum of patients with cholestasis,[12] exhibited the greatest activity among the bile acid tested in each serum, but these values were only 1.11% and 1.33%. Surprisingly, the cross-reactivity with cholesterol, which has the same ring structure as 3β-hydroxy-5-cholenoic acid, was less than 0.001%. Minder and his co-workers[7] reported that the antiserum raised by immunizing with 3β-hydroxy-5-cholenoic acid 24-thyroglobulin adduct exhibited a high degree of cross-reaction with cholesterol (5.6%). During the preparation of this paper, a specific antiserum with less than 0.01% cross-reactivity to cholesterol has been prepared by immunization with C-19-oxime-BSA conjugate.[13] In this work, we have shown that antiserum capable of recognizing the side chain in 3β-hydroxy-5-cholenoic acid could be obtained by immunizing animals with an antigen in which the hapten is coupled to the carrier protein through the C-3 position, remote from the carboxylic side chain. These antisera should be useful for direct assay without any chromatographic separation step prior to the actual radioimmunoassay, and their utilization for the measurement of 3β-hydroxy-5-cholenoic acid in biological fluids will be reported in the near future.

Experimental

All melting points were taken on a Mitamura micro hot-stage apparatus and are uncorrected. Optical rotations were determined with a Union Giken PM-201 polarimeter in $CHCl_3$. Ultraviolet (UV) spectra were measured on a Shimadzu model UV-200 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi model R-40 spectrometer at 90 MHz using tetramethylsilane as an internal standard. Abbreviations: s = singlet, d = doublet, and m = multiplet. For column chromatography, silica gel (Merck Kiesel gel 60, 70—230 mesh) was used.

Chemicals and Reagents——[3α-^{3}H]3β-Hydroxychol-5-en-24-oic acid (4.5 mCi/mg) was kindly provided by Professor Shoichi Nakagawa, Hokkaido University. BSA and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, Mo) and Iatron laboratories (Tokyo), respectively. The reference bile acids and steroid specimens were either synthesized in our laboratory or were obtained commercially. All solvents and chemicals were of analytical reagent grade.

2,2,2-Trichloroethyl 3β-Hydroxychol-5-en-24-oate (2)——DCC (1.6 g) and then 2,2,2-trichloroethanol (6 ml) were added to a solution of 3β-hydroxychol-5-en-24-oic acid[7] (**1**, 600 mg) in pyridine (6 ml) and CH_2Cl_2 (15 ml). The mixture was stirred at room temperature for 5 h and poured into a large amount of H_2O. After extraction with AcOEt, the organic solution was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo*. The residue was chromatographed with benzene–ether (5 : 1) as the eluent and the product was recrystallized from MeOH to give **2** (541 mg) as colorless needles. mp 122—123 °C. $[\alpha]_D^{15}$ −7.4° (c = 0.54). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.93 (3H, d, J = 6 Hz, 21-CH_3), 0.97 (3H, s, 19-CH_3), 3.46 (1H, m, 3α-H), 4.67 (2H, s, –$COOCH_2CCl_3$), 5.26 (1H, m, C_6-H). *Anal.* Calcd for $C_{26}H_{39}Cl_3O_3$: C, 61.78; H, 7.70. Found: C, 61.68; H, 7.74.

2,2,2-Trichloroethyl 3β-Hemisuccinoyloxychol-5-en-24-oate (3)——A solution of **2** (500 mg) and succinic anhydride (1 g) in pyridine (5 ml) was refluxed for 1.5 h. After evaporation of pyridine *in vacuo*, the residue was diluted with ether, and the insoluble material was removed by filtration. The filtrate was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo*. The crude product was chromatographed with hexane–AcOEt (1 : 1) as the eluent and recrystallized from MeOH to give **3** (450 mg) as colorless needles. mp 166—168 °C. $[\alpha]_D^{15}$ −15.7° (c = 0.45). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.95 (3H, d, J = 6 Hz, 21-CH_3), 1.00 (3H, s, 19-CH_3), 2.58 (4H, s, –$COCH_2CH_2CO$–), 4.56 (1H, m, 3α-H), 4.67 (2H, s, –$COOCH_2CCl_3$), 5.30 (1H, m, C_6-H). *Anal.* Calcd for $C_{30}H_{43}Cl_3O_6$: C, 59.45; H, 7.15. Found: C, 59.65; H, 7.22.

2,2,2-Trichloroethyl 3β-Hemiglutaroyloxychol-5-en-24-oate (4)——Treatment of **2** (460 mg) with glutaric anhydride (1 g) in pyridine (5 ml) was carried out in the same manner as described for **3**. The crude product was chromatographed with hexane–AcOEt (1 : 1) and recrystallized from MeOH to give **4** (386 mg) as colorless needles.

mp 153.5—156 °C. $[\alpha]_D^{15}$ −26.8° (c=0.56). ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.95 (3H, d, J=6 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 2.20—2.56 (6H, m, –$COCH_2CH_2CH_2CO$–), 4.56 (1H, m, 3α-H), 4.68 (2H, s, –$COOCH_2CCl_3$), 5.32 (1H, m, C_6-H). *Anal.* Calcd for $C_{31}H_{45}Cl_3O_6$: C, 60.04; H, 7.31. Found: C, 60.05; H, 7.11.

2,2,2-Trichloroethyl 3β-Hemisuccinoyloxychol-5-en-24-oate *N*-Succinimidyl Ester (5)——1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (200 mg) and *N*-hydroxysuccinimide (120 mg) were added to a solution of **3** (420 mg) in 95% dioxane (2.5 ml). The reaction mixture was stirred at room temperature for 5 h. The resulting solution was diluted with AcOEt, washed with H_2O, and dried over anhydrous Na_2SO_4. The solution was passed quickly through an Al_2O_3 (10 g) layer on a sintered-glass funnel, and the filtrate was evaporated to dryness *in vacuo*. Recrystallization of the crystalline product from MeOH gave **5** (370 mg) as colorless needles. mp 185—187 °C. $[\alpha]_D^{15}$ −22.8° (c=0.66). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.95 (3H, d, J=6 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 2.78 (4H, s, *N*-succinimidyl), 2.55—3.00 (4H, m, –$COCH_2CH_2CO$–), 4.60 (1H, m, 3α-H), 4.68 (2H, s, –$COOCH_2CCl_3$), 5.32 (1H, m, C_6-H). *Anal.* Calcd for $C_{34}H_{46}Cl_3NO_8$: C, 58.07; H, 6.59. Found: C, 57.45; H, 6.65.

2,2,2-Trichloroethyl 3β-Hemiglutaroyloxychol-5-en-24-oate *N*-Succinimidyl Ester (6)——Treatment of **4** (400 mg) with *N*-hydroxysuccinimide (120 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (200 mg) in 95% dioxane (3 ml) was carried out in the same manner as described for **5**. The crude product was chromatographed with benzene–ether (3 : 1) as the eluent and recrystallized from acetone–MeOH to give **6** (386 mg) as colorless needles. mp 168—170.5 °C. $[\alpha]_D^{15}$ −21.5° (c=0.42). ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.95 (3H, d, J=6 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 2.16—2.73 (6H, m, –$COCH_2CH_2CH_2CO$–), 2.78 (4H, s, *N*-succinimidyl), 4.56 (1H, m, 3α-H), 4.68 (2H, s, –$COOCH_2CCl_3$), 5.32 (1H, m, C_6-H). *Anal.* Calcd for $C_{35}H_{48}Cl_3NO_8$: C, 58.61; H, 6.74. Found: C, 58.80; H, 6.81.

3β-Hemisuccinoyloxychol-5-en-24-oic Acid *N*-Succinimidyl Ester (7)——Zinc dust (450 mg) was added to a solution of **5** (400 mg) in AcOH (5 ml), and the reaction mixture was stirred at room temperature for 1 h. After removal of the excess zinc dust by filtration, the filtrate was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo*. The crude product was chromatographed with benzene–ether (2 : 1) as the eluent and recrystallized from MeOH to give **7** (214 mg) as colorless needles. mp 166—168 °C. $[\alpha]_D^{15}$ −17.9° (c=0.50). ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.93 (3H, d, J=6 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 2.78 (4H, s, *N*-succinimidyl), 2.55—3.00 (4H, m, –$COCH_2CH_2CO$–), 4.60 (1H, m, 3α-H), 5.32 (1H, m, C_6-H). *Anal.* Calcd for $C_{32}H_{45}NO_8 \cdot 1/2H_2O$: C, 66.19; H, 7.81. Found: C, 66.12; H, 7.89.

3β-Hemiglutaroyloxychol-5-en-24-oic Acid *N*-Succinimidyl Ester (8)——Treatment of **6** (290 mg) with zinc dust (440 mg) in AcOH (4 ml) was carried out in the same manner as described for **5**. The crude product was chromatographed with benzene–ether (3 : 1) as the eluent and recrystallized from MeOH to give **8** (140 mg) as colorless needles. mp 166—170.5 °C. $[\alpha]_D^{15}$ −21.5° (c=0.42). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.92 (3H, d, J=6 Hz, 21-CH_3), 1.00 (3H, s, 19-CH_3), 2.16—2.76 (6H, m, –$COCH_2CH_2CH_2CO$–), 2.81 (4H, s, *N*-succinimidyl), 4.56 (1H, m, 3α-H), 5.32 (1H, m, C_6-H). *Anal.* Calcd for $C_{33}H_{47}NO_8$: C, 67.66; H, 8.08. Found: C, 67.60; H, 8.26.

Conjugation of Haptens with BSA——BSA (90 mg) in 0.01 M phosphate buffer (pH 7.4) (2.1 ml) was added to a solution of a hapten (**7** or **8**, 45 mg) in dioxane (3.7 ml) and the mixture was stirred at 4 °C for 2 d. The resulting solution was dialyzed against cold running water overnight. After addition of acetone and 5% $NaHCO_3$, the suspension was centrifuged at 3000 rpm for 10 min. This procedure was repeated until the free bile acid was removed completely. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the same manner as described above. Lyophilization of the solution afforded the bile acid-BSA conjugate (*ca.* 70 mg) as a fluffy powder. The molar ratio of the hapten to the protein in the conjugate was spectrophotometrically (at 420 nm) determined by the use of the color reaction with conc. H_2SO_4 to be 14 for **7** and 15 for **8**.

Immunization of Rabbits——Two female domestic albino rabbits were used for immunization with each conjugate. The antigen (2 mg) was dissolved in sterile isotonic saline (1 ml) and emulsified with complete Freund's adjuvant (1 ml). The emulsion was injected into rabbits subcutaneously at multiple sites over the scapulae. This procedure was repeated at intervals of one week for a further one month and then once a month. The rabbits were bled one week after the booster injection. The sera were separated by centrifugation at 3000 rpm for 15 min and stored at −20 °C. The antisera were thawed and diluted with 0.01 M phosphate buffer (pH 7.4) containing 0.9% NaCl, 0.1% gelatin, and 0.01% NaN_3.

Measurement of Radioactivity——Radioactivity counting was carried out on a Aloka model 903 liquid scintillation spectrometer employing EX-H (Dozin Chemical Institute) as a scintillator.

Assay Procedure——A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing 0, 0.5, 1, 5, 10 and 50 ng of unlabeled 3β-hydroxychol-5-en-24-oic acid in buffer (0.1 ml) and [^{3}H]3β-hydroxychol-5-en-24-oic acid (10000 dpm) in buffer (0.1 ml). The diluted antiserum (0.1 ml) was added to this solution, and the mixture was incubated at 4 °C overnight. After addition of dextran (0.03%, w/v)–charcoal (1%, w/v) solution (0.5 ml), the resulting solution was vortex-mixed and was allowed to stand at 0 °C for 15 min, then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was transferred by decantation into a vial containing a scintillation cocktail (10 ml), and the radioactivity was counted for 20 min in each sample.

Cross-Reaction Study——The specificity of antisera raised against the 3β-hydroxychol-5-en-24-oic acid–BSA conjugates were tested by determining the reactivities with 27 kinds of purified compounds related to 3β-

hydroxychol-5-en-24-oic acid (Table I). The relative amounts required to reduce the intial binding of [^{3}H]3β-hydroxychol-5-en-24-oic acid by half, where the mass of unlabeled 3β-hydroxychol-5-en-24-oic acid was arbitrarily taken as 100%, were calculated from the standard curves.

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