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Urinary and Biliary Metabolites of 17α-Acetoxy-6-chloro-4,6-pregnadiene-3,20-dione in the Rabbit

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Ten metabolites of 17α-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione were isolated from urine and bile of the rabbit and their chemical structures were determined. The urinary main metabolite was 17α-acetoxy-2ξ,3ξ-dihydroxy-5ξ-pregnan-20-one. The biliary main metabolite was 17α-acetoxy-6-chloro-2ξ,3ξ-dihydroxy-4,6-pregnadien-20-one. The administered compound was metabolized through two courses, one was oxidation at C-2 and another was dechlorination at C-6, in the rabbit. A small amount of the metabolite oxidized at C-21 was identified from urine.

Recently antiandrogenic drugs came to be noticed as a new treatment for prostatomegaly or prostatic carcinoma. As an antiandrogen 17α-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione (I, chlormadinone acetate, CMA) has been investigated in this laboratory. Structures of urinary and biliary metabolites of CMA in the rabbit will be described in this publication.

Experimental[2]

Preparation of ^{3}H-CMA——A mixture of CMA (785 mg), 2,3-dicyano-5,6-dichlorobenzoquinone[3] (785 mg) and *p*-nitrophenol (157 mg) in dry benzene (20 ml) was refluxed for 6 hr. After usual treatment 1-dehydro-CMA (385 mg, mp 174°) was obtained by crystallization from MeOH. The 1-dehydro compound was submitted to catalytic hydrogenation with tritium gas by using tris(triphenylphosphin)chlororhodium[4] as a catalyst. Purification by TLC using benzene–MeOH (9: 1) as developing solvent gave 98% pure 1,2-^{3}H-CMA (3.3 Ci/mmole). After the constant specific activity of 1,2-^{3}H-CMA was obtained by mixing cold CMA, MeOH (80 ml) and 0.01 N KOH in 70% MeOH (400 ml) were added to the prepared 1,2-^{3}H-CMA (1 g). The mixture was kept at 47° for 2 days. 1-^{3}H-17α-Hydroxy-6-chloro-4,6-pregnadiene-3,20-dione (400 mg, mp 209—213°) was obtained by crystallization from MeOH. The specific activity per mmole of this compound was reduced to 51% of the starting material. It was suggested that the tritium at C-2 position was released by enolization of 3-oxo group. A mixture of the 1-^{3}H-17α-hydroxy compound (350 mg) and *p*-toluenesulfonic acid (90 mg) in acetic anhydride (1.2 ml) and acetic acid (5 ml) was kept at room temperature for 3 hr. Crystallization from MeOH gave 1-^{3}H-CMA (180 mg, 3.4 mCi/mmole). Recrystallization from MeOH was repeated to give 99% pure compound.

Synthesis of Authentic Samples——a) 17α-Acetoxy-6-chloro-2-hydroxy-1,4,6-pregnatriene-3,20-dione (IIIa): To a solution of 17α-acetoxy-6-chloro-2α-hydroxy-4,6-pregnadiene-3,20-dione[5] (IIa, 24 mg) in MeOH (2 ml) was added 1 M KOH in MeOH (0.1 ml). The solution was kept at room temperature for 5 hr and poured into water, and the material was extracted with ethyl acetate. The residue obtained after evaporation of the solvent was purified by TLC using benzene–MeOH (9: 1) as developing solvent. Crystallization

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[2] All melting points were taken on a micro hot-stage apparatus and are uncorrected. Ultraviolet (UV) spectra were measured on Hitachi Model EPS-3. Infrared (IR) spectra were run on Hitachi Model EPI-G2. Nuclear magnetic resonance (NMR) spectra were recorded on Hitachi Model R-20A spectrometer at 60 Mc using tetramethylsilan as an internal standard. Abbreviation used s=singlet, q=quartet and m=multiplet. Mass spectra were taken on Hitachi Model RMU-6E. High resolution mass spectra were measured on Hitachi Model RMU-7M in the laboratory at Hitachi Naka Works. For the preparative thin-layer chromatography (TLC) Silica gel HF (E. Merck AG) was used as an adsorbent.

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from MeOH gave an analytical sample as colorless needles (3 mg). mp 218—219°. UV $\lambda_{\max}^{\text{MeOH}}$ mμ: 286. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 1725, 1645 (C=O). NMR (2% solution in $CDCl_3$) δ: 0.75 (3H, s, 18-CH_3), 1.27 (3H, s, 19-CH_3), 2.08 (6H, s, 21-CH_3 and $OCOCH_3$), 6.26 (2H, s), 6.71 (1H, s). Mass Spectrum *m/e*: 418 (M^+), 358 (M^+-CH_3COOH), 315 ($M^+-CH_3COOH-COCH_3$). The diacetate (IIIb) was obtained on acetylation by the usual method. Crystallization from MeOH was carried out to give colorless needles (3 mg). mp 181—184°. UV $\lambda_{\max}^{\text{MeOH}}$ mμ: 265, 302. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 1765, 1733, 1712, 1665 (C=O). NMR (2% solution in $CDCl_3$) δ: 0.75 (3H, s, 18-CH_3), 1.32 (3H, s, 19-CH_3), 2.07 (3H, s, 21-CH_3), 2.09 (3H, s, $OCOCH_3$), 2.29 (3H, s, $OCOCH_3$). Mass Spectrum *m/e*: 460 (M^+), 418 (M^+-CH_2CO), 315 ($M^+-CH_2CO-CH_3CO-CH_3COOH$).

b) 2α,17α-Diacetoxy-4-pregnene-3,20-dione (VIIb): A mixture of 17α-acetoxy-4-pregnene-3,20-dione (VI, 500 mg) and lead tetraacetate (750 mg) in acetic acid (8 ml) was heated on a steam-bath for 3 hr. The mixture was treated in the same manner as described previously.[5] The 2α-acetoxy derivative was obtained by crystallization from MeOH as colorless needles (50 mg). mp 243—247°. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 1731, 1690 (C=O). NMR (2% solution in $CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.33 (3H, s, 19-CH_3), 2.05 (3H, s, 21-CH_3), 2.11 (3H, s, $OCOCH_3$), 2.19 (3H, s, $OCOCH_3$), 5.45 (1H, q, $J=5.5$ Hz, $J=13$ Hz, 2β-H), 5.77 (1H, s, 4-H).

Animals and Administration——a) Urinary Metabolites: In the first experiment, to three male rabbits (2.8—3.1 kg) was orally administered 150 mg of 1,2-^{3}H-CMA in 2% Tween-80 solution twice a day for 4 days and urine was collected every day for 7 days after the first administration. In the second experiment, to three male rabbits (3.3—3.5 kg) was orally given 200 mg of 1-^{3}H-CMA twice a day for 7 days and urine was collected for 9 days.

b) Biliary Metabolites: A single oral dose of 200 mg of 1-^{3}H-CMA was administered to two male rabbits (3.1 and 3.4 kg) which had been operated in order to set a polyethylene tubing into the common bile duct without tying the gall bladder. Bile was continuously collected during 16 hr. To other two male rabbits (4.1 and 3.5 kg) were infused 130 and 80 mg of 1,2-^{3}H-CMA into the auricular vein for 3 hr, respectively. From the beginning of infusion bile had been collected during 19 hr.

Extraction of Metabolites——After urinary metabolites were adsorbed on a column of Amberlite XAD-2[6] (6×50 cm), the column was washed with water and then eluted with MeOH. The residue obtained from evaporation of MeOH *in vacuo* was dissolved in 5 liters of acetate buffer and the solution was adjusted to pH 5.0. A thousand units of β-glucuronidase (prepared in this company) per 1 ml urine was added to the solution and it was incubated at 37° for 3 days. After the solution was adjusted to pH 1 with sulfuric acid, metabolites were extracted three times with ethyl acetate without salt. The organic layer was kept at 48° for 2 days in order to hydrolyze sulfates[7] and condensed to dryness *in vacuo*.

Bile was diluted with acetate buffer (pH 5.0) by six times. Glucuronides and sulfates were hydrolyzed in the same manner as with urine and the metabolites were extracted with ether.

Purification and Identification of Urinary Metabolites——In the first experiment, an oily mixture of free metabolites was submitted to gradient elution chromatography on celite[8] (4.4×67 cm, celite No. 545) using iso-octane (1 liter) and ethyl acetate (1 liter) as eluting solvents. After the gradient elution, the celite column was washed with 1 liter of MeOH as shown in Fig. 1. Peaks a, b, c, d and e contained 3, 11, 9, 17 and 32 per cent of the radioactivity charged on the column, respectively.

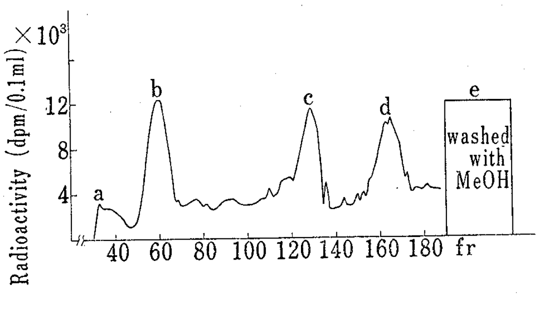

Fig. 1. Celite Gradient Elution Chromatography of Urinary Metabolites of 1,2-^{3}H-CMA

Each fraction was 10 ml and 0.1 ml of the fraction was counted.

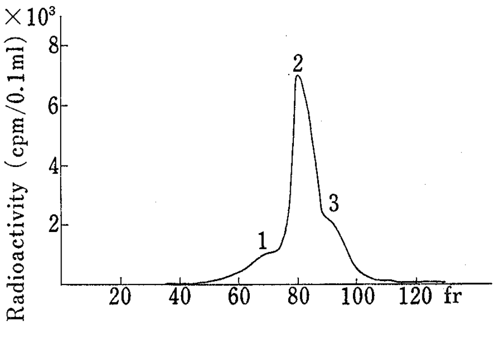

Fig. 2. Sephadex LH-20 Chromatography of Urinary Metabolites of 1-^{3}H-CMA

Each fraction was 10 ml and 0.1 ml of the fraction was counted.

In the second experiment, the mixture of metabolites was chromatographed on a Sephadex LH-20 column (3.5×120 cm) instead of celite using 0.6% acetic acid in MeOH as eluting solvent. Figure 2 exhibits the elution pattern on Sephadex LH-20. Radioactivities of peaks 1, 2 and 3 were 8, 32 and 9 per cent of that

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6) H.L. Bradlow, *Steroids*, **11**, 265 (1968).
7) S. Burstein and S. Lieberman, *J. Am. Chem. Soc.*, **80**, 5235 (1958).
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in total urine. Peak 2 was charged on a silica gel column (2.2×32 cm, Wakogel C-200) using $CHCl_3$ as solvent. After elution with $CHCl_3$ Fr. 2-1 (11% of radioactivity in urine) was obtained. Fr. 2-2 (8%), Fr. 2-3 (3%) and Fr. 2-4 (6%) were eluted with 1% MeOH–$CHCl_3$, 2% MeOH–$CHCl_3$ and 5% MeOH–$CHCl_3$, respectively.

Unchanged CMA (I)——Peak a was submitted to preparative TLC using benzene–MeOH (9: 1) as developing solvent and a main radioactive band was collected. Further purification on preparative TLC using $CHCl_3$–MeOH (19: 1) yielded an oily material which was crystallized from MeOH to give unchanged CMA (I). The identity of the material with an authentic sample was proved by TLC, UV and mass spectra (M^+ *m/e* 404).

17α-Acetoxy-6-chloro-2α-hydroxy-4,6-pregnadiene-3,20-dione (IIa)——Two radioactive substances were isolated from Peak b by means of preparative TLC using benzene–MeOH (9: 1) after developing repeatedly. The more polar metabolite was acetylated with pyridine–acetic anhydride at room temperature and crystallized from EtOH to give colorless prisms. Recrystallization from MeOH yielded 2α,17α-diacetoxy-6-chloro-4,6-pregnadiene-3,20-dione (IIb) as colorless prisms (2.4 mg, mp 227—230°), which was identical with the synthetic substance5) in mixed melting point and NMR, mass, UV and IR spectra.

17-αAcetoxy-6-chloro-2-hydroxy-1,4,6-pregnatriene-3,20-dione (IIIa)——The less polar metabolite isolated from Peak b was supposed to be the diosphenol of CMA on the basis of the NMR spectra. When the metabolite was acetylated by the usual method, colored powders were obtained by crystallization from acetone–hexane. Further purification gave 2,17α-diacetoxy-6-chloro-1,4,6-pregnatriene-3,20-dione (IIIb, mp 182—184°), which was identical with the authentic sample in melting point and NMR, mass and UV spectra.

17α-Acetoxy-6-chloro-2α,3β-dihydroxy-4,6-pregnadien-20-one (IVa)——Peak c was submitted to preparative TLC using $CHCl_3$–MeOH (9: 1) as developing solvent and a main radioactive band was collected. Crystallization from MeOH gave light yellow needles (mp 248—249°). This metabolite was identical with the synthetic compound5) in mixed melting point and NMR, mass, UV and IR spectra. The triacetoxy compound (IVb) was also identical with the authentic triacetate in physical properties and in reverse isotope dilution method.

6-Chloro-17α,20ξ,21-trihydroxy-4,6-pregnadien-3-one (Va)——When preparative TLC using $CHCl_3$–MeOH (10: 1) as developing solvent was carried out twice, a major radioactive substance was obtained from Peak d. As it was difficult to crystallize the metabolite was acetylated by pyridine–acetic anhydride at room temperature. Repeated crystallization from MeOH or EtOH gave 4 mg of 6-chloro-20ξ,21-diacetoxy-17α-hydroxy-4,6-pregnadien-3-one (Vb) as colorless prisms. mp 179—180°. UV $\lambda_{\max}^{\text{MeOH}}$ mμ: 286. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 3510 (OH), 1745, 1675 (C=O). NMR (1% solution in $CDCl_3$): 0.83 (3H, s, 18-CH_3), 1.14 (3H, s, 19-CH_3), 2.03 (3H, s, $OCOCH_3$), 2.10 (3H, s, $OCOCH_3$), 4.1—4.4 (2H, m, 21-CH_2), 5.30 (1H, q, $J=3$ Hz, $J=8$ Hz, 20-H), 6.32 (2H, s, 4 and 7-H). Mass Spectrum *m/e*: 464 (M^+), 404 ($M^+ - CH_3COOH$), 344 ($M^+ - 2CH_3COOH$), 326 ($M^+ - 2CH_3COOH–H_2O$), 301 ($M^+ - 2CH_3COOH - H_2O - 25$).

To a solution of Va (about 2 mg) in MeOH (0.5 ml) was added HIO_4 solution (5 mg in 0.2 ml of H_2O) and the mixture was kept at room temperature for 18 hr. After the usual treatment 6-chloro-4,6-androstadiene-3,17-dione was obtained as amorphous solid after crystallization from hexane–ether. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1}: 1735, 1660 (C=O). Mass Spectrum *m/e*: 318 (M^+), 300 ($M^+ - H_2O$), 283 ($M^+ - 35$).

17α-Acetoxy-4-pregnene-3,20-dione (VI)——Fr. 2-1 was submitted to Sephadex LH-20 chromatography (1.6×95 cm) using benzene–MeOH (85: 15) as eluting solvent. The main peak was submitted to preparative TLC using benzene–MeOH (19: 1) as developing solvent to yield two major radioactive bands. One of them was purified three times by preparative TLC using benzene–MeOH (9: 1), benzene–MeOH (19: 1) and benzene–ether (5: 1) as developing solvents to afford oily 17α-acetoxy-4-pregnene-3,20-dione (VI), which was the same as the authentic sample in NMR and mass spectrum and in reverse isotope dilution method.

17α-Acetoxy-2α-hydroxy-4-pregnene-3,20-dione (VIIa)——Another radioactive band obtained from Fr. 2-1 by means of Sephadex LH-20 and preparative TLC was submitted to preparative TLC using benzene–MeOH (9: 1) once more. The main metabolite was acetylated by the usual method to yield 2α,17α-diacetoxy-4-pregnene-3,20-dione (VIIb), which was identical with the synthetic compound in NMR, mass and UV spectra.

17α-Acetoxy-2ξ,3ξ-dihydroxy-5ξ-pregnan-20-one (VIIIa) and Its Isomers——Fr. 2-2 was charged on a Sephadex LH-20 column (1.6×95 cm) using benzene–MeOH (85: 15) as eluting solvent and two peaks were obtained. The latter main peak was repeatedly purified by means of preparative TLC using $CHCl_3$–MeOH (19: 1) or benzene–MeOH (9: 1) as developing solvents to give three major metabolites, all of which had the same molecular ion peaks *m/e* 392 in the mass spectrum. The most purified metabolite of them had no absorption in the UV spectrum and was supposed to be 17α-acetoxy-2ξ,3ξ-dihydroxy-5ξ-pregnan-20-one (VIIIa). NMR (1% solution in $CDCl_3$) δ: 0.61 (3H, s, 18-CH_3), 0.86 (3H, s, 19-CH_3), 2.04 (3H, s, 21-CH_3), 2.27 (3H, s, $OCOCH_3$), *ca.* 3.3 (2H, m, 2 and 3-H). Mass Spectrum *m/e*: 392 (M^+), 350 ($M^+ - CH_2CO$), 332 ($M^+ - CH_3COOH$), 289 ($M^+ - CH_3COOH–CH_3CO$), 271 ($M^+ - CH_3COOH–CH_3CO–H_2O$), 253 ($M^+ - CH_3COOH–CH_3CO–2H_2O$). Triacetoxy compound (VIIIb) was yielded on acetylation by the usual method. Mass Spectrum *m/e*: 476 (M^+).

The Enol Compound of 17α-Acetoxypregnane-2,3,20-trione (IXa)——When Peak 3 was submitted to preparative TLC using $CHCl_3$–EtOH (19: 1) as developing solvent, a less polar radioactive band was separated. Crystallization from MeOH gave the enol compound of 17α-acetoxypregnane-2,3,20-trione (IXa) as colorless needles, mp 203—204°. High Resolution Mass Spectrum *m/e*: 388.2228 (M^+) indicating $C_{23}H_{35}O_5$ as molecular formula. Mass Spectrum *m/e*: 388 (M^+), 346 (M^+-CH_2CO), 328 (M^+-CH_3COOH), 285 (M^+-CH_3COOH-CH_3CO), 267 (M^+-CH_3COOH-CH_3CO-H_2O). UV $\lambda_{max}^{95\% EtOH}$ mμ: 273 (pH 7 and 3), 320 (pH 9). IR ν_{max}^{KBr} cm^{-1}: 1728, 1670 (C=O). NMR (1% solution in $CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.18 (3H, s, 19-CH_3), 2.05 (3H, s, 21-CH_3), 2.10 (3H, s, $OCOCH_3$), 6.07 (1H, s, 1 or 4-H). Diacetate (IXb) was obtained by the usual method. NMR (0.5% solution in $CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.25 (3H, s, 19-CH_3), 2.04 (3H, s, 21-CH_3), 2.11 (3H, s, $OCOCH_3$), 2.24 (3H, s, $OCOCH_3$). Mass Spectrum *m/e*: 430 (M^+), 388 (M^+-CH_2CO).

Purification and Identification of Biliary Metabolites——The oily mixture extracted with ether from the bile after hydrolysis was purified on a Sephadex LH-20 column (2×87 cm) using benzene–MeOH (85: 15) as eluting solvent. Metabolites IIa, IIIa and IVa were isolated from the bile obtained after oral administration by using the same techniques as described above. Metabolites I, IIa and IVa were also isolated from the bile obtained after intravenous administration. When the bile obtained after intravenous administration was treated carefully, another metabolite was found in the fraction containing IVa. It was crystallized from acetone to give 17α-acetoxy-6-chloro-2ξ,3ξ-dihydroxy-4,6-pregnadien-20-one (Xa). UV λ_{max}^{MeOH} mμ: 236.5, 244.5, 253. NMR (2% solution in CD_3OD) δ: 0.70 (3H, s, 18-CH_3), 1.14 (3H, s, 19-CH_3), 2.01 (3H, s, 21-CH_3), 2.08 (3H, s, $OCOCH_3$), 5.87 (1H, 4 or 7-H), 6.09 (1H, d, *J*=3.5 Hz, 4 or 7-H). Mass Spectrum *m/e*: 422 (M^+). Triacetoxy compound (Xb) was clearly different from 2α,3β- or 2β,3β-triacetoxy derivatives[5] on TLC using benzene–ether (5: 1) as developing solvent.

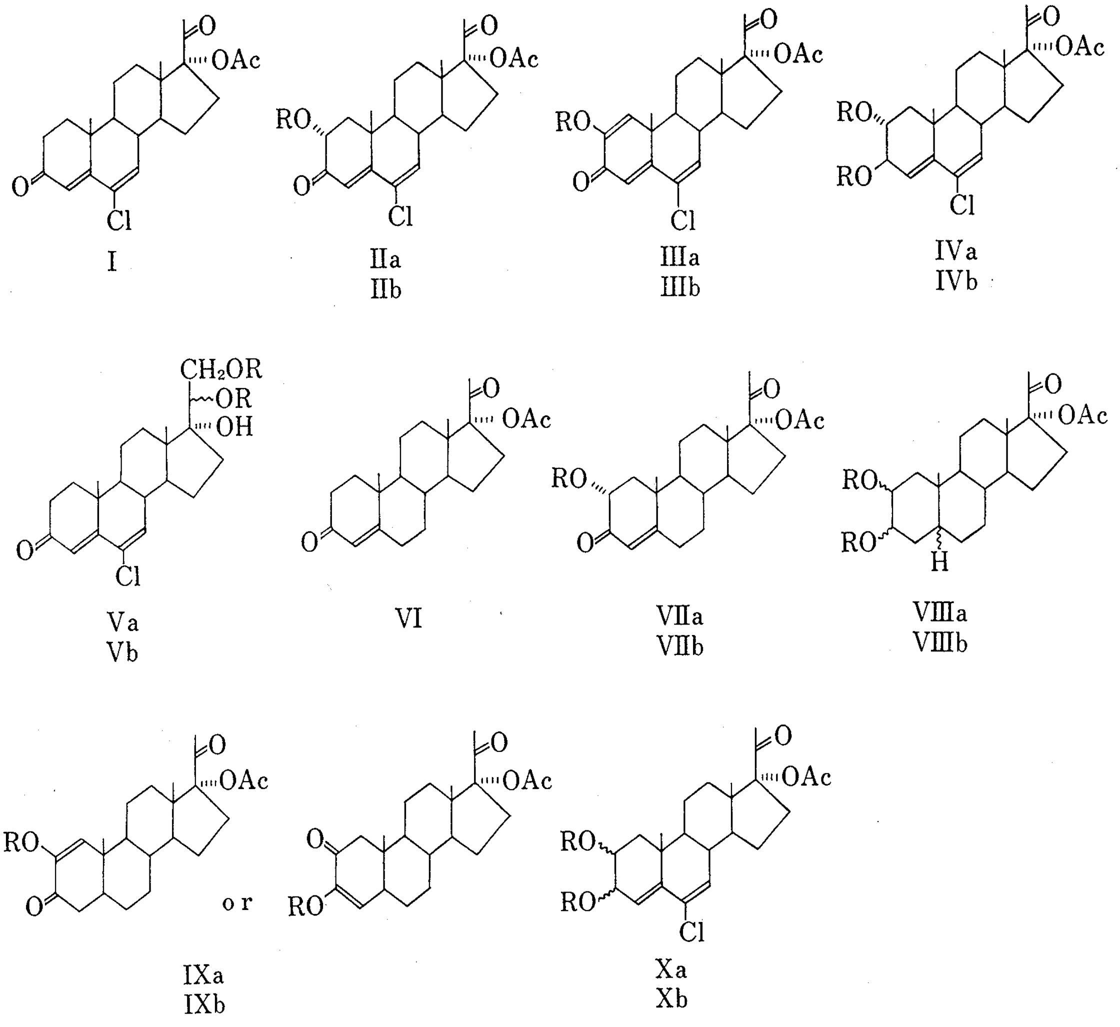

Fig. 3. Structures of Urinary and Biliary Metabolites of CMA in the Rabbit

a: R=H, b: R=Ac

Result and Discussion

When the urine of the rabbit treated with 1,2-^{3}H-CMA was hydrolyzed and ethyl acetate was used as an extraction solvent, 43 per cent of radioactivity in the urine was transferred into the organic layer and 15 per cent was retained in the aqueous layer. Most of the radioactivity (42%) in the urine was excluded by washing in the step of extraction with XAD-2 resin. The loss of radioactivity must be that of the tritium at C-2 position released by enolization of 3-oxo group in CMA or by metabolic change at C-2 position *in vivo*. On the other hand, 70 per cent of the radioactivity in urine was extracted into the ethyl acetate layer and 20 per cent remained in the aqueous layer in case of the rabbit treated with 1-^{3}H-CMA. In both cases 15—20 per cent of the radioactivity retained in the aqueous layer was not identified.

Table I. Reverse Isotope Dilution Method of Metabolite IVa as Its Acetate (IVb)

Recrystallization		Specific activity	
No.	from	Precipitate dpm/mg	Filtrate dpm/mg
1	MeOH	12000	38800
2	MeOH	10900	20600
3	MeOH	10600	12100

To IVb was added 15 mg of synthetic 2α,3β,17α-triacetoxy-6-chloro-4,6-pregnadien-20-one.

Table I indicates the results of the reverse isotope dilution method of metabolite IVa after converting to its acetate (IVb). The reason why much radioactivity escaped into the first filtrate suggests the presence of the isomer (Xa) in urine. Xa was contained about three times of IVa in bile. Because Xa was more difficult to crystallize than IVa, Xa could not be isolated from urine. Xa is neither 2α,3β- nor 2β,3β-dihydroxy compound and the hydroxylation at C-2 in CMA in the rabbit seems to produce mainly 2α-configuration. Therefore Xa is expected to be 2α,3α-dihydroxy derivative. The possibility of 2β,3α-configuration, however, is not excluded.

Table II exhibits the identity of the metabolite VI with 17α-acetoxyprogesterone by reverse isotope dilution method.

Table II. Reverse Isotope Dilution Method of Metabolite VI

Recrystallization		Specific activity dpm/mg
No.	from	
1	MeOH	3068
2	acetone	2296
3	EtOH	2156

To metabolite VI was added 22.34 mg of authentic 17α-acetoxy-4-pregnene-3,20-dione.

Three isomers including VIIIa were isolated from Fr. 2-2 and another isomer from Fr. 2—3. These four isomers were apparently different in *Rf* values of TLC using $CHCl_3$–MeOH (19:1) as developing solvent. Although mass spectra of these isomers were essentially the same, their chemical structures except VIIIa were not fully established. The positions of the two hydroxyl groups in VIIIa were tentatively assigned to C-2 and C-3 on the ground of the comparison with the identified metabolites.

The fact that the absorption of diosphenol (IXa) in UV spectrum was shifted depending on pH had been reported in case of cholestane series.[9] Standing IIa in $CHCl_3$ for a long time gave the diosphenol IIIa. Therefore, the posibility that the diosphenols IIIa or IXa are artifacts is not excluded.

Further 15 metabolites were isolated from urine, but they were not identified. The presence of highly polar metabolites hydroxylated at side chain of D ring is expected.[10]

On the basis of the results in this investigation, it is concluded that CMA is metabolized through two courses, one is oxidation at C-2 and another is dechlorination at C-6 of 4,6-diene-3-one, in the rabbit. Although the oxidation at C-2 is common,[11] the dechlorination at C-6 of 4,6-diene-3-one is little known.[12] No dechlorinated metabolites have been reported in 17α-acetoxy-6-chloro-1α,2α-methylene-4,6-pregnadiene-3,20-dione[13] nor in 17α-acetoxy-6-chloro-16-methylene-4,6-pregnadiene-3,20-dione.[14] A dechlorination enzyme, however, is supposed to be present in rat liver microsomes.[15] It would be interesting to study the mechanism and the meaning of the dechlorination in CMA.

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