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Identification and Anti-androgenic Activity of the Metabolites of 17α-Acetoxy-6-chloropregna-4,6-diene-3,20-dione (Chlormadinone Acetate) in the Rat, Rabbit, Dog and Man[1,2]

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The metabolic fate of chlormadinone acetate (CMA), which is a new anti-androgenic agent, has been investigated with rats, rabbits, dogs and humans. Fourteen unconjugated metabolites and three conjugated metabolites were isolated from urine, feces and bile after oral administration of CMA. The structures of these metabolites were deduced from physico-chemical data and definitely characterized by direct comparison with the authentic samples (see Chart 1). There was a marked species difference in the metabolic pattern. Especially, configuration of 2-hydroxy group in the 2-hydroxylated metabolites was different among species. Further, the occurrence of 1-hydroxylation and 15-hydroxylation, new metabolic pathways in steroidal agents, was found in the rats and humans. The anti-androgenic activities of the metabolites were listed in Table III. 3β-Hydroxy CMA, one of the main metabolites in humans and rats, and its acetate were 0.7 time as active as CMA, whereas other main metabolites, 2α,3β-dihydroxy CMA and 2α-acetoxy CMA, were not effective.

Keywords——chlormadinone acetate; anti-androgen; unconjugated metabolite; conjugated metabolite; active metabolite; configuration

Chlormadinone acetate, 17α-acetoxy-6-chloropregna-4,6-diene-3,20-dione (CMA), a highly active synthetic progestational agent, was first described in 1959.[4] Brennan, *et al.* first reported in 1963 that CMA had a potent anti-androgenic action.[5] A number of anti-androgenic agents involving non-steroidal compounds came to be noticed as a new treatment for prostatic hypertrophy and prostatic carcinoma.[6] Recently CMA has been used for that

1) In this paper the following abbreviations were used: Chlormadinone acetate (CMA)=17α-acetoxy-6-chloropregna-4,6-diene-3,20-dione, 3α-hydroxy CMA=17α-acetoxy-6-chloropregna-4,6-dien-3α-ol-20-one, 3β-hydroxy CMA=17α-acetoxy-6-chloropregna-4,6-dien-3β-ol-20-one, 2α,3α-dihydroxy CMA=17α-acetoxy-6-chloropregna-4,6-diene-2α,3α-diol-20-one, 2α,3β-dihydroxy CMA=17α-acetoxy-6-chloropregna-4,6-diene-2α,3β-diol-20-one, 2β,3β-dihydroxy CMA=17α-acetoxy-6-chloropregna-4,6-diene-2β,3β-diol-20-one, 2-hydroxy Δ^1-CMA=17α-acetoxy-6-chloropregna-1,4,6-triene-3,20-dione, 1β,3α-dihydroxy CMA=17α-acetoxy-6-chloropregna-4,6-diene-1β,3α-diol-20-one, 2α,3α,15β-trihydroxy CMA=17α-acetoxy-6-chloropregna-4,6-diene-2α,3α,15β-triol-20-one, 2α,3β,15β-trihydroxy CMA=17α-acetoxy-6-chloropregna-4,6-diene-2α,3β,15β-triol-20-one, 2α,3α,17α-trihydroxy CMA=2α,3α,17α-trihydroxy-6-chloropregna-4,6-dien-20-one, 2α,3α-dihydroxy CMA 3-glucuronide=17α-acetoxy-6-chloropregna-4,6-dien-2α-ol-20-on-3α-yl-β-D-glycopyranosiduronic acid, 3α-hydroxy CMA 3-glucuronide=17α-acetoxy-6-chloropregna-4,6-dien-20-on-3α-yl-β-D-glucopyranosiduronic acid, 2-hydroxy Δ^1-CMA 2-glucuronide=17α-acetoxy-6-chloropregna-1,4,6-triene-3,20-dion-2-yl-β-D-glucopyranosiduronic acid.

2) A part of this work was presented at the 8th Symposium on Drug Metabolism and Action, Hiroshima, November, 1976.

3) Location: *a*) *1604, Shimosakunobe, Takatsu-ku, Kawasaki*; *b*) *Maebashi-shi, Gunma.*

4) H.J. Ringold, E. Gatres, A. Boweres, J. Edwards, and J. Zderic, *J. Am. Chem. Soc.*, **81**, 3485 (1959).

5) D.M. Brennan and R.J. Kraay, *Acta Endocrinol.*, **44**, 367 (1963).

6) J. Geller, A. Angist, K. Nakao, and H. Newman, *J. Am. Medical Assoc.*, **210**, 1421 (1969); B. Stoliar and D.J. Albert, *J. Urology*, **111**, 803 (1974); G.R. Prout, R.J. Irwin, B. Kliman, J. Daly, R.A. Maclaughlin, and P.P. Griffin, *J. Urology*, **113**, 834 (1975).

purpose because of its more potent anti-androgenic activity.[7] The metabolism of megesterol acetate (17α-acetoxy-6-methylpregna-4,6-diene-3,20-dione) and medroxyprogesterone acetate (17α-acetoxy-6-methylpregn-4-ene-3,20-dione), which are similar to CMA in structure, has been studied in humans and rabbits in detail by several workers.[8] On the other hand, in spite of the widespread use of CMA, little is known about its metabolism in animals including human. In this paper we will report the isolation and identification of metabolites of CMA from excreta of humans, rats, rabbits and dogs, and the anti-androgenic action of the main metabolites in the excreta. The tritium labeled CMA[9] was administered to several species of animals. Approximately 21% of the administered dose was excreted into the feces in dogs for 72 hr, while in the cases of the rats and rabbits the recovery rate in feces was 42% and 34%, respectively (Table I). The predominant fecal elimination of CMA by rats, rabbits and dogs may be attributable to the extensive biliary excretion. To elucidate the biliary excretion and metabolites of CMA, we studied on the rat, rabbit and dog. Total radioactivity of the bile sample in the CMA treated rat and rabbit was 60—80% of administered dose for 24 hr following administration, while 18% of the dose was recovered from dog bile for 24 hr (Fig. 1).

Table I. Excretion of Radioactivity in Urine, Feces and Bile after Administration of 1α-^{3}H- CMA to Several Animals

Day	Rat		Rabbit		Dog	
	Urine (%)	Feces (%)	Urine (%)	Feces (%)	Urine (%)	Feces (%)
1	8.1	29.8	6.6	3.1	1.2	0.5
2	4.6	6.2	4.5	11.5	2.6	10.3
3	1.1	6.0	7.4	8.6	1.0	10.4
4			3.5	4.0	0.3	8.3
5			4.3	3.3	0.6	1.0
6			1.8	3.9	0.3	2.5
7					0.6	1.0
Total	13.8	42.0	38.1	34.4	6.6	34.0

The pooled bile and urine were percolated directly through a column of Amberlite XAD-2 resin, respectively.[10] In the case of feces the aqueous suspensions of methanol extract were also submitted to the Amberlite XAD-2 resin column. The effluent of the column chromatography of bile was divided into two portions, one of which was used for structure elucidation of steroidal moiety of the metabolites. The steroidal fraction was hydrolyzed with β-glucuronidase and the hydrolyzate was then submitted to solvolysis in the usual manner. The liberated radioactive metabolites were then separated into six fractions by gel filtration on Sephadex LH-20 column as illustrated in Fig. 2. The amounts of the metabolites in excreta are listed in Table II.

Recrystallization of the radioactive substance in the fraction A gave metabolite I as colorless needles. The metabolite I proved to be identical with unchanged substance by direct comparison.

7) K. Shida, J. Shimazaki, E. Urano, H. Kurihara, H. Takahashi, N. Furuya, and M. Taya, *Japan. J. Urology*, **63**, 109 (1972).
8) J.M. Cooper, J.S. Elce, and A.E. Kellie, *Biochem. J.*, **104**, 57p (1967); E. Castegnaro and G. Scala, *Endocrinol.*, **24**, 445 (1962); M.L. Helmreich and R.A. Huseby, *J. Clin. Endocrinol. Metab.*, **22**, 1018 (1962).
9) T. Abe and A. Kambegawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 2824 (1974).
10) H.L. Bradlow, *Steroids*, **11**, 265 (1968).

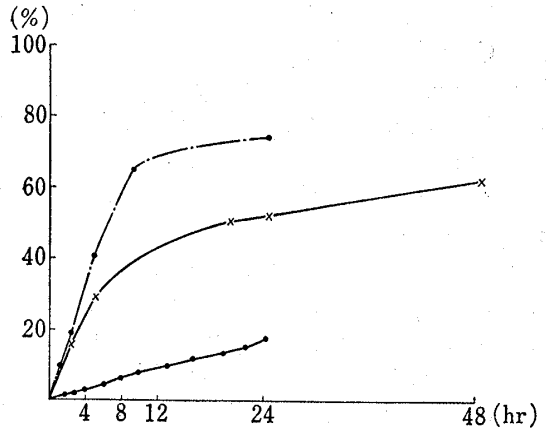


Fig. 1. Cumulative Biliary Excretion Rate after an Intravenous Injection of 1α-^{3}H-CMA in the Rats, Rabbits and Dogs

---●---, rat. —×—, rabbit. —●—, dog.

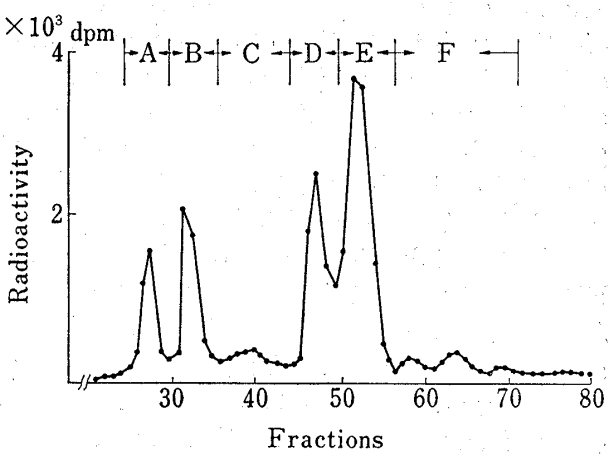


Fig. 2. Separation of Free Metabolites from Rabbit Bile after Administration of 1α-^{3}H-CMA by Column Chromatography on Sephadex LH-20

Table II. The Amount of Main Metabolites of CMA in Various Species

Metabolites isolated from excreta	Rat		Dog		Rabbit	Man	
	Feces (%)	Bile (%)	Feces (%)	Bile (%)	Bile (%)	Urine (%)	Feces (%)
I	40	10	45	7	5	5	—
II	14	n.d.	n.d.	n.d.	n.d.	10	12
III	1	14	n.d.	n.d.	n.d.	n.d.	n.d.
IV	n.d.	4	n.d.	19	20	10	n.d.
V+VI	4	n.d.	11	n.d.	15	10	10
VII	1	2	n.d.	27	2	1	
VIII	6	10	n.d.	n.d.	n.d.	n.d.	n.d.
IX	n.d.	n.d.	4	n.d.	n.d.	n.d.	n.d.
X	6	10	12	6	6	10	1
XI	17	44	n.d.	n.d.	25	30	1
XII+XIII	n.d.	2	n.d.	n.d.	n.d.	8	n·d.

n.d.: not detected.

Metabolite II from fraction B was isolated as colorless needles and showed blue staining with conc. sulfuric acid. The mass spectrum (MS) showed a parent peak at m/e 406 corresponding to increment of two mass unit. In addition, on the nuclear magnetic resonance (NMR) spectrum a signal due to 4-vinyl proton exhibited a defined AB type doublet (J=3 Hz) supporting the assignment of 3β-hydroxy group. These results led to the assumption that the metabolite II should be 3β-hydroxy CMA. Indeed, the identity of the metabolite II with the synthetic sample was justified in the usual manner.

From the mother liquor of II, metabolites III, IV, V, VI and VII were obtained by separation on preparative thin-layer chromatography (TLC). Unfortunately, metabolite II could not be obtained in the crystalline state, but it was substantially homogeneous.

This substance gave a monoacetate (III') by acetylation with pyridine-acetic anhydride. With respect to the MS II and III were indistinguishable each other in the fragmentation patterns. On the NMR spectrum a signal due to 4-vinyl proton was observed at 6.22 ppm as a doublet with coupling constant of 7 Hz. Further this substance gave only CMA by Jones oxidation. These results permitted us to assume that the metabolite III would be the 3-epimer of II, namely 3α-hydroxy CMA.

The metabolite IV exhibited a positive reaction with Blue Tetrazolium reagent. The structure responsible for reducing Blue Tetrazolium would be either 20,21-ketol or 2,3-ketol.

The MS of the acetylated derivative of IV exhibited the molecular ion peak at *m/e* 462 indicating an introduction of oxygen atom into CMA. On the NMR spectrum of the acetate a signal due to the hydrogen atom attached to carbon bearing the acetoxyl group appeared at 5.65 ppm as a quartet with coupling constants of 13 Hz and 7 Hz. Therefore, hydroxy group at C-2 was confirmed to be α configuration by NMR spectrum. The proposed structure of acetyl derivative (IV') was definitely established by direct comparison with the authentic sample.[11] The metabolite IV was assigned to be 2α-hydroxy CMA.

The metabolite V was obtained in the crystalline state upon usual acetylation. The NMR spectrum of this compound exhibited a signal at 0.84 ppm assigned to 19-methyl group. As for the MS of the metabolite V and its acetate the parent ion peak appeared at *m/e* 376 and 418, respectively. These results strongly suggested that the metabolite V would be 17α-acetoxy-5β-pregnan-3α-ol-20-one. This structure was definitely established by direct comparison with the authentic sample.

The metabolite VI was isolated as a monoacetate by acetylation in the usual manner. Based upon the physico-chemical data the metabolite VI was assumed to be a positional isomer of V, 17α-acetoxy-5β-pregnan-3β-ol-20-one. Identity with the authentic sample was confirmed by mixed melting point measurement and TLC comparison.

The metabolite VII which was isolated as colorless needles exhibited a characteristic UV absorption curve with maximum at 280 nm. The MS showed a parent peak at *m/e* 418 corresponding to an increment of 14 mass units. In addition, on the NMR spectrum a signal due to C-1 proton appeared at 6.70 ppm corresponding to the assignment of a newly introduced double bond. This finding strongly suggested that the metabolite VII would be 2-hydroxyΔ^1-CMA. The identity of the metabolite VII with the synthetic sample was definitely established by direct comparison.

Then, the purification of fraction C was carried out by preparative TLC following gel filtration on Sephadex LH-20 to yield metabolite VIII. Unfortunately the metabolite VIII could not be obtained in the crystalline state and therefore a portion was transformed into the diacetate. In the MS of VIII the molecular ion peak and a strong base peak appeared

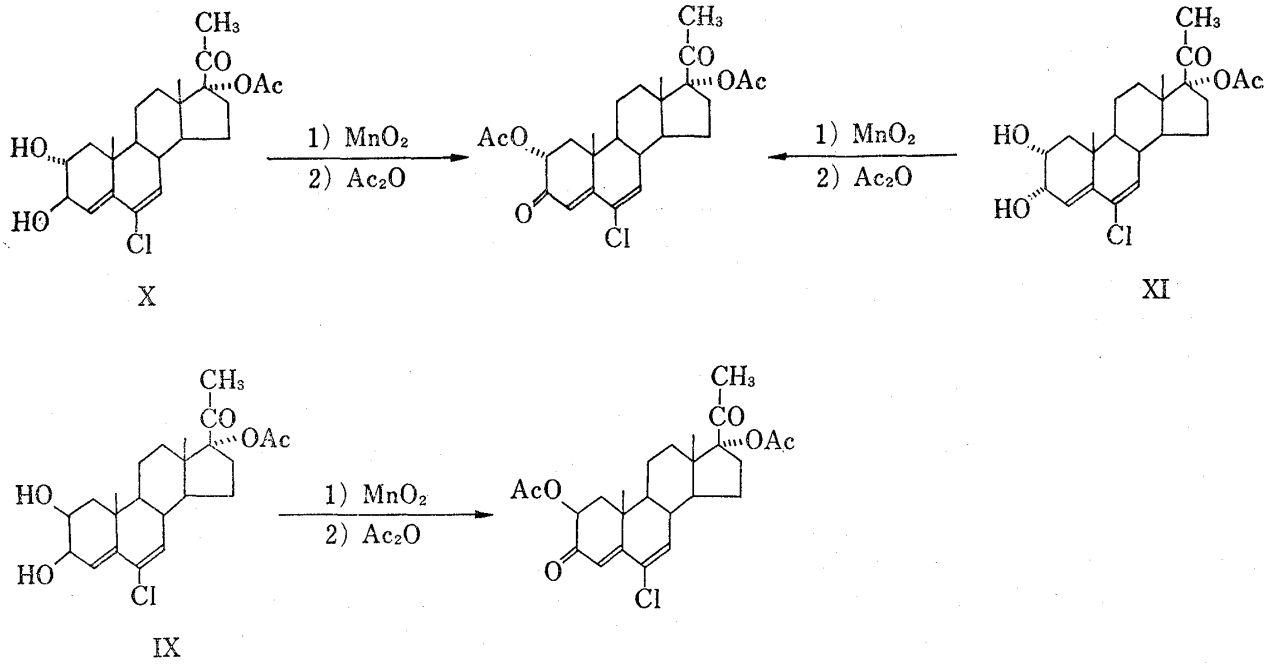
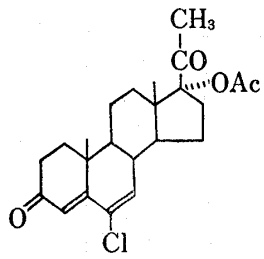
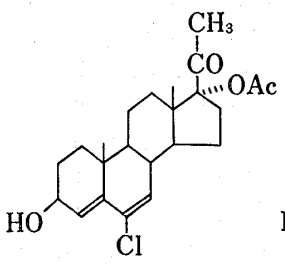


Chart 1

11) T. Abe and A. Kambegawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 1295 (1973).



I (CMA)



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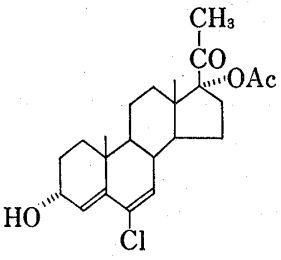

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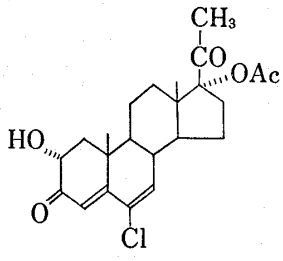

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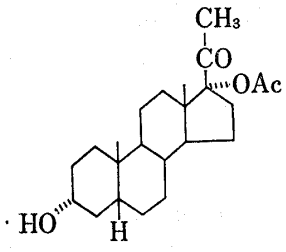

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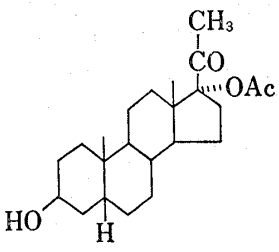

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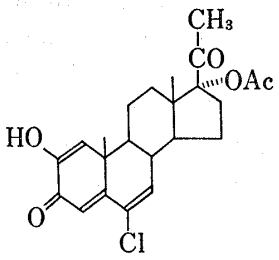

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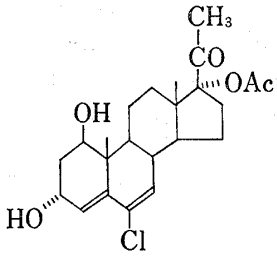

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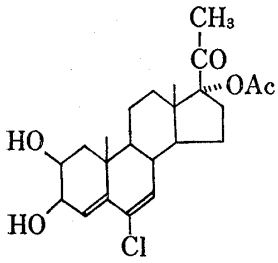

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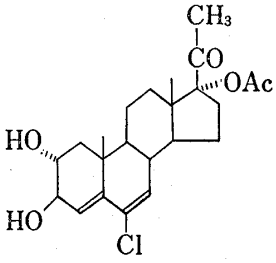

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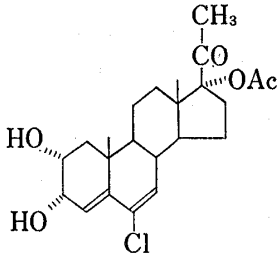

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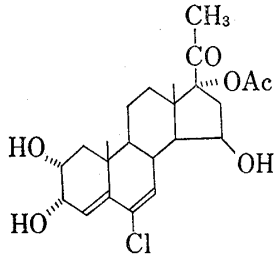

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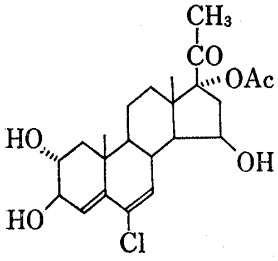

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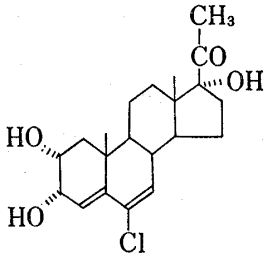
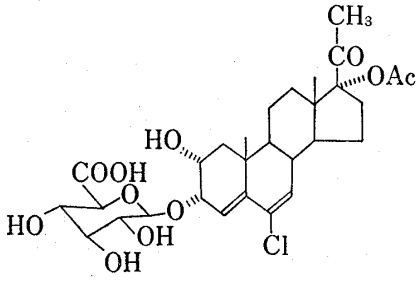
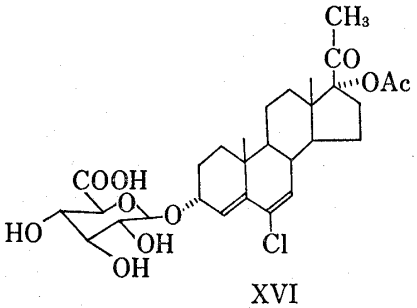


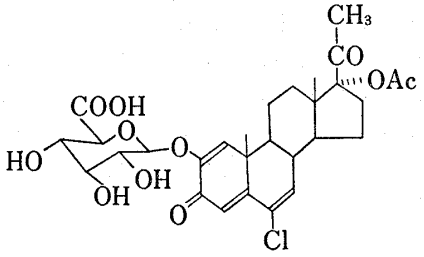
XIV



XV



XVI



XVII

Chart 2

at *m/e* 436 and 301, respectively. This evidence strongly favored the structure VIII having two hydroxy groups in the A or B ring. In the NMR spectrum of VIII, C-4 vinyl proton appeared at 6.24 ppm with coupling constant of 6 Hz indicating α configuration of 3-hydroxy group. Further, the position and configuration of another hydroxy group was elucidated by NMR spectrum. The 19-methyl group of this compound occurred at lower frequency (62 Hz) than that of the 3α-hydroxy CMA and only 18-methyl group shifted to the higher field in the acetate. These results permitted us to assign the structure 1β,3α-dihydroxy CMA to metabolite VIII. This compound was formed only in the rat, but not in the rabbit, dog and human.

Fraction D was separated into two radioactive metabolites by preparative TLC. Recrystallization of the less polar (*Rf* 0.60) one from methanol gave metabolite IX as colorless needles and the more polar fraction gave metabolite X as colorless needles. Both metabolites exhibited the parent ion peak at *m/e* 422 on the MS and were converted into the diacetate by usual acetylation. In the NMR spectra IX and X exhibited a signal assignable to the 18-methyl group at 0.70 ppm. The 19-methyl proton of the metabolite IX resonated at the higher frequency than that of the compound X. These findings were in accord with anticipated deshielding of the 19-methyl group due to its 1,3-diaxial interaction with 2β-hydroxy function. Further, these compounds were converted into 2α- and 2β-hydroxy-3-ketone by oxidation with manganese dioxide,[12] respectively as illustrated in Chart 1. The results indicated that these metabolites would be isomeric 2α,3β-dihydroxy and 2β,3β-dihydroxy CMA. Actually the metabolite IX could be evidently assigned to 2β,3β-dihydroxy CMA by comparison with the synthetic sample. The identity of metabolite X with the corresponding authentic sample was also rationalized by usual criteria.

The metabolite XI of the radioactive substance in the fraction E was isolated as colorless needles. This compound was transformed into the diacetate by acetylation. With regard to the MS compound XI and its acetate showed a parent ion at *m/e* 422 and 506, respectively and their fragment patterns were similar to those of metabolite X and its acetate. The NMR spectrum of XI exhibited a doublet signal at 5.95 ppm with coupling constant of 6 Hz indicating the α configuration of 3-hydroxy group. In order to elucidate configuration of 2-hydroxy group, XI was oxidized with manganese dioxide to the 2α-hydroxy CMA which in turn was converted to the crystalline acetate by usual acetylation. This derivative was identified as 2α-acetoxy CMA by comparison with the authentic sample. Based on these data this metabolite was concluded to be 2α,3α-dihydroxy CMA.

Metabolites XII, XIII and XIV were separated from fraction F by the preparative TLC. Both metabolites XII and XIII showed a molecular ion peak at *m/e* 438 on the MS and were transformed into the triacetates (XII′ and XIII′) by usual acetylation. To assign the position of one of the three hydroxy groups NMR spectra of metabolites and their acetates were determined. The difference in the chemical shift between the protons attached to the same carbon atom bearing hydroxyl and acetoxyl groups was found to be 0.85 ppm. This characteristic value indicated the location of the oxygen function in the five-membered ring.[13] Further, the chemical shift of 18-methyl group was observed at the lower field (0.30 ppm) than that of CMA by the effect of the axial 15β-hydroxy group and on acetylation the signal of 18-methyl group was shifted slightly to the higher field. With respect of the remaining hydroxy groups in XII, a signal of 19-methyl group at 1.12 ppm and a doublet signal of 4-vinyl proton at 6.12 ppm with coupling constant of 5 Hz strongly supported the 2α,3α-dihydroxy structure. These results permitted us to assign the structure 2α,3α,15β-trihydroxy CMA to the metabolite XII. As for the structure of metabolite XIII, the signals

12) F. Sondheimer, O. Mancera, M. Urquiza, and Rosenkranz, *J. Am. Chem. Soc.*, 77, 4145 (1955).
13) N.S. Bhacca and D.H. Williams, "Application of NMR Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, 1964.

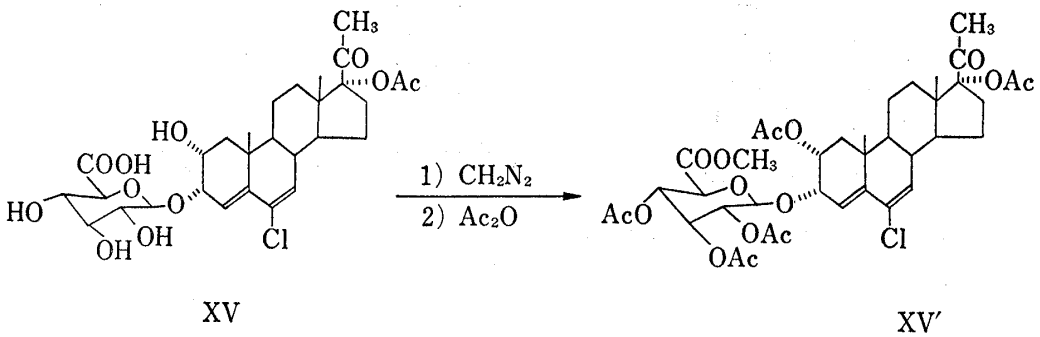
assignable to 19-methyl and 4-vinyl proton appeared at 0.95 and 5.90 ppm, respectively in the acetate as in the case of 2α,3β-diacetoxy CMA. On the basis of these data the metabolite XIII was suggested to be 2α,3β,15β-trihydroxy CMA.

The last metabolite XIV was found to possess a characteristic UV absorption with maximum at 242 nm and the molecular ion peak at *m/e* 380 on the MS. This compound was transformed into the triacetate (XIV′) by acetylation in the presence of *p*-toluenesulfonic acid as a catalyst. From these results, the structure of the metabolite XIV was concluded to be 2α,3α,17α-trihydroxy CMA.

Conjugated Metabolites

The metabolites excreted in the bile and urine following administration of CMA were mostly present as conjugated forms. It has been generally assumed that conjugation of steroids is prerequisite for their biological inactivation and subsequent excretion into the bile and urine. However, the recent studies reveal that the conjugates of physiologically active substance are not always the end product and in certain case the conjugate possesses more potency than the free.[14] In addition, some of steroid sulfates act as the intermediary metabolites.[15] A particular interest in these respects prompted us to explore the conjugated metabolite formed from CMA. The next effort was directed to the isolation and characterization of biliary conjugated metabolite in the rat and dog.

The conjugated substance obtained by Amberlite XAD-2 resin chromatography was submitted to the gel filtration on Sephadex LH-20 to give steroid conjugates. The metabolites XV and XVI were isolated by preparative TLC from the radioactive eluate. The metabolite XV exhibited a positive reaction with naphthoresorcinol and underwent facile hydrolysis by beef liver β-glucuronidase to yield XI and glucuronic acid. In order to elucidate the attached position of a glucuronyl moiety to the steroid nucleus, the metabolite XV was treated with diazomethane and then with pyridine–acetic anhydride to give the acetate–methyl ester (XV′). The attached position of glucuronic acid was deduced by comparison with NMR spectra of compounds XV and XV′. The proton signal of C-2 in XV′ shifted downfield with 0.73 ppm due to the introduced acetyl group in the steroidal moiety, whereas the chemical shift of C-3 proton in XV′ was not affected. From these results metabolite XV would be assigned to the 2α,3α-dihydroxy CMA 3-glucuronide.



XV XV′

Chart 3

The less polar fraction described above was similary purified by Sephadex LH-20 column chromatography to provide the metabolite XVI. This compound exhibited a positive reaction with naphthoresorcinol and was hydrolyzed with β-glucuronidase to give 3α-

14) H. Yoshimura, S. Ida, K. Oguri, and T. Tsukamoto, Proc, 1st Symp. on Drug Metabolism and Action, Chiba, 1970, p. 107.

15) S. Honma and T. Nambara, Proc. 5th Symp. on Drug Metabolism and Action, Shizuoka, 1973, p. 241; J-Å Gustafasson and M. Ingerman-Sandberg, *J. Biol. Chem.*, **249**, 1940 (1974); J. Fishman, I. Yoshizawa, and L. Hellman, *Steroids*, **22**, 401 (1973).

hydroxy CMA (III). Derivatization in the usual manner yielded the acetate-methyl ester (XVI'), which exhibited fragment ions due to the aglycone and glucuronic acid at *m/e* 406 and 317, respectively. On the NMR spectrum the anomeric proton appeared at 4.70 ppm with a coupling constant of 7 Hz indicating the β-glucuronide linkage. These evidences together led us to conclude that the metabolite XVI should be 3α-hydroxy CMA 3-glucuronide.

On the other hand, the isolation of conjugated metabolite from dog bile was difficult. Therefore, the crude metabolite obtained by Sephadex LH-20 column chromatography was converted to the acetate-methyl ester (XVII') by treatment with diazomethane and then usual acetylation. A portion of the crude metabolite was hydrolyzed with β-glucuronidase to give 2-hydroxy Δ^1-CMA. The derivative XVII' showed the UV absorption curve with maximum at 284 nm and on the NMR spectrum an olefinic proton signal assignable to the C-1 due to 1-ene. With regard to the MS fragment ions appeared at *m/e* 418 and 317 indicating steroid and sugar moiety. The results indicated that the metabolite XVII would be 2-hydroxy Δ^1-CMA 2-glucuronide.

Anti-androgenic Activity of Metabolites

The metabolites of drug have often been observed to have more potent activity than the parent compound or other pharmacological properties.[16] The metabolites and their derivatives of CMA, each administered orally at dose of 20 mg/kg, exhibited significant activities in inhibiting the weight increase of seminal vesicle and ventral prostate induced by testosterone propionate. The activities of metabolites were listed in Table III. The data revealed that on the basis of the weight of ventral prostate, 3β-hydroxy CMA and its acetate were 0.7 times as active as CMA, whereas 2α,3β-dihydroxy and its acetate were not effective.

Table III. Anti-androgenic Activities of CMA and Its Metabolites Administered to Castrated Rat

Compound	Organ	
	Prostate	Seminal vesicle
CMA (I)	46.4±1.81 mg	30.4±1.29 mg
3β-Hydroxy CMA (II)	54.4±0.98	36.4±1.21
3β-Acetoxy CMA (II')	55.6±2.50	39.6±1.47
2α,3β-Dihydroxy CMA (X)	63.0±3.05	41.0±1.79
2α,3β-Diacetoxy CMA (X')	69.0±2.93	47.4±2.93
2α-Acetoxy CMA (IV')	64.2±3.18	
Control	23.4±1.89	12.4±1.12
Testosterone propionate	68.2±1.66	45.6±2.56

a) Figures express mean ±S.D. ($n=5$).
Testosterone propionate (50 μg) and samples (2 mg/head) were injected sc simultaneously to castrated rats for 3 days.

Discussion

There can be a marked species difference in the execrtion of radioactivity of CMA. The rabbits excreted 34—38% of the dose into urine and feces in 7 days, whereas the dogs and rats excreted most of the dose into the feces in 7 days. The slower disappearance and the lower metabolic clearance rate of CMA than natural steroids which are corticoid, testosterone[17]

16) S. Honma, A. Kambegawa, A. Izumi, and S. Nishida, Abstract of 7th Sym. on Drug Metab. and Action, Sapporo, 1975, p. 67; B. Katchen and S. Buxbaum *J. Clin. Endocrinol. Metab.*, **41**, 373 (1975); M. Himori, A. Izumi, and Y. Hiramatsu, *Arch. Internat. Pharmaco. Therapie*, **220**, 4 (1976).
17) C.M. Stowe, *Ann. Rev. Pharmacol.*, **8**, 337 (1968).

and progesterone,[18] may be due to a considerable extent of enterohepatic circulation and to a characteristic metabolites of CMA on enterohepatic circulation. Indeed, the autoradiography showed that large amount of radioactivity appeared in the liver and intestine at 24 hr after a single oral dose of CMA-^{14}C. Further, the metabolites isolated from bile were a considerable amount of conjugated form and the radioactive substances were reabsorbed to the extent of 60—80% of the dose from intestine.[19] From these findings, it was likely that the slower metabolic clearance rate was attributable to the enterohepatic circulation. The species difference in the excretion patterns involving biliary and renal clearance may be dependent upon the nature of metabolites.

Most of the metabolites of CMA were excreted in the bile as glucuronide conjugates and free metabolites were a small amount. Indeed, main biliary metabolites in the rat and dog were glucuronide conjugates (XV, XVI, XVII) and other conjugated forms, glucoside and sulfate, were not detected. The 3α-hydroxy CMA, one of the main metabolites, was exclusively conjugated with glucuronic acid but there was no detectable amount of its isomer, 3β-hydroxy CMA, in rat bile in spite of detailed examination. On the other hand, 3β-hydroxy CMA was one of the main metabolites isolated from rat feces, and human urine and feces. It is to be noted that 3α-hydroxy group would be converted to the corresponding 3β-hydroxy epimer by an enzyme system in the rat intestine. Epimerization of steroidal alcohol has infrequently been cited in the literature, and studies showing the interconversion of the epimeric 3β,7α-dihydroxy-5-androsten-17-one, 16α-hydroxyestrone and 3α,17β-dihydroxy-17α-ethynylestr-5(10)-ene have recently appeared.[20]

Generally speaking, Δ^4-3-ketosteroids are first reduced on the double bonds of Δ^4-3-ketone in the presence of NADPH followed by reduction of carbonyl group to give 3α- and 3β-hydroxy saturated steroids. Ringold, *et al.*[21] reported that introduction of halogen substituents into a steroidal Δ^4-3-ketone at the C-2, C-4 and C-6 positions interfered with hydrogenation of double bond yielding the Δ^4-3-hydroxy metabolite. The findings are manifested by the fact that the metabolism of 4-chlorotestosterone in human and rat liver also predominantly produced the Δ^4-3-hydroxy derivatives.[22] This metabolic scheme readily accounts for the formation of the 3α- and 3β-hydroxy CMA from the substrate.

There are at least three forms on oxidative metabolism of CMA, namely 1-, 2- and 15-hydroxylations. The occurrence of hydroxylation at C-2, C-1 and C-15 decreased in the sequential order. Configuration of 2-hydroxy group in the 2-hydroxylated metabolites which were predominantly formed from CMA in all the animals examined was different among species. The main metabolites in the rat, rabbit and man were 2α,3β-dihydroxy, 2α,3α-dihydroxy compounds and their intermediate, 2α-hydroxy CMA. The positional isomer, 2β,3β-dihydroxy CMA, was isolated only in the dog, but not in other animals. Biliary 2α,3α-dihydroxy CMA primarily appeared in the conjugated form, almost certainly as a glucuronide. The conjugated position on the steroid nucleus has definitely been determined to be C-3 by NMR. It is noteworthy that 15β-hydroxy metabolites isolated from the human and rat, and 1β,3α-dihydroxy CMA formed only in the rat have been unequivocally identified on the basis of spectral data. To our knowlegde these are the first recorded instances of the biotransformation in synthetic C_{21} progestins which are similar in structure to CMA. However, the occurrence of 15-hydroxylation of estrogen and androgen has already been reported

18) A.A. Sandberg, W.R. Slaunwhite, and R.Y. Kirdani, "Metabolic Conjugation and Metabolic Hydrolysis," Vol. II, ed. by W.H. Fishman, Academic Press, New York, 1970, p. 123.
19) G.K. Hanson and L.J. Fisher, *Drug Metab. Disposition*, **2**, 159 (1974).
20) R.I. Freudenthal, C.E. Cook, M. Twine, R. Rosenfeild, and M.E. Wall, *Biochem. Pharmacol.*, **20**, 1507 (1971).
21) H.J. Ringold, S. Ramachandran, and E. Forchielli, *Biochem. Biophys. Acta*, **82**, 143 (1964).
22) E. Castegnaro and G. Sala, *Steroid Lipids Res.*, **4**, 184 (1973); L. Staka, L. Siekmann, H.O. Hoppen, and Breuer, *Arzneim. Forsch.*, **19**, 2022 (1969).

in the rat and human.[23] With respect to the finding of 1-hydroxylation, testosterone and cortisol were easily transformed into corresponding steroid derivatives.[24]

The dechlorinated metabolites were obtained to the extent of 10—30% of excreted metabolites in the examined animals. Recently Maynad, *et al.*[25] have reported an interesting finding that 4-dechlorination of the 4,6-dichloroprogestational steroid was enhanced under anaerobic conditions in the presence of glutathione. A similar reaction, the reductive dechlorination of DDT to DDD, has been observed in several animals.[26]

Finally, it should be emphasized that 3β-hydroxy CMA is about 0.70 times potent as CMA in the activity of anti-androgenic action in the rat, while the other main metabolite, 2α,3β-dihydroxy CMA, exhibits no potency. The blood level of active metabolite II is similar to that of unchanged CMA in the case of oral administration. In prostate, one of the target organs of CMA, this substrate is biotransformed exclusively into an active metabolite, 3β-hydroxy CMA, but not to other metabolites. Honma, *et al.*[27] have shown that an average of 20—30% of total radioactivity in the prostate is recovered in the cell nuclei *in vivo*. And the active metabolite accounts for at least 10—15% of the nuclear radioactivity recovered from prostate after administration of CMA. It is evident from these data that the pharmacological activity of CMA is attributable to a considerable extent to the active metabolite in the prostate.

Experimental

General Procedure——All melting points were taken on a micro hotstage apparatus and are uncorrected. Ultraviolet (UV) and infrared (IR) spectra were run on Hitachi Model EPS-3 and EPI-G2 spectrometers, respectively. Nuclear magnetic resonance (NMR) spectra were recorded on Hitachi Model R-20A spectrometer at 60 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, t=triplet, q=quartet and m=multiplet. MS measurement was run by direct insertion technique on Hitachi Model RMU-6E spectrometer.

Thin-Layer Chromatography (TLC)——TLC was carried out on Silica gel HF_{254} plate employing several solvent systems.

Gel Filtration——Sephadex LH-20 (Pharmacia Fine Chem., Inc.) was previously equilibrated with eluent for 18 hr and then used.

Radioactive Counting——Counting was carried out on Aloka Model LSC 651 liquid scintillation spectrometer. For toluene-soluble samples toluene containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (100 mg/l) was used as a scintillator. Aqueous samples were counted in a scintillator, composed of dioxane (1 l), naphthalene (100 g), 2,5-diphenyloxazole (8 g) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (250 mg). For quench correction the channel ratio and external standards were employed.

Material——1α-^{3}H-Chlormadinone acetate was synthesized in our laboratory.[9] The labeled steroid was purified by TLC on silica gel HF_{254}. The final purity of labeled steroid was over 99% and its specific activity was 3.4 Ci/mmol. Unlabeled chlormadinone acetate (mp 212—214°) was synthesized in Teikoku Hormone Mfg. Co.

Assay of Anti-androgenic Activity——The male rats weighing 55—65 g were orchiectomized and drug treatment was begun after 14 days. The rats were supplemented with daily sc injections of 50 μg/head of testosterone propionate in sesame oil and of 2 mg/head of samples in sesame oil for 3 days. Twenty-four hr after the last treatment seminal vesicle and prostates were removed and weighed.

Animal——The adult male rabbits weighing about 3 kg, the adult Wistar rats weighing about 300 g and the adult male dogs weighing about 10—15 kg were housed in the metabolic cages respectively that designed to separate feces and urine. Seven patients with prostatic carcinoma employed in these studies were hospitalized during periods of the treatment.

Administration of CMA and Collection of Urine and Feces——A suspension of 1α-^{3}H-CMA (2 mg, 5 μCi) with Tween 80 in saline (1 ml) was orally administered to each of ten rats and nonlabeled steroid (20 mg/head)

23) H.C. Ford, R. Wheeler, and L.L. Engel, *Eur. J. Biochem.*, **57**, 9 (1975): A.A. Hagen, M. Barr, and E. Diczfalusy, *Acta Endocrinol.*, **49**, 207 (1965).
24) B.P. Lisboa and J.-A. Gustafsson, *Eur. J. Biochem.*, **6**, 419 (1968).
25) D.E. Maynard, O. Gurny, M. Carson, R.A. Lemahie, M.A. Schwatz, M.K. Taylor, and R.W. Kierstead, *Biochemistry*, **10**, 355 (1971).
26) P.J. Bunyan, J.M.J. Page, and A. Taylor, *Nature* (London), **210**, 1048 (1966).
27) S. Honma, S. Yugi, and A. Kambegawa, "in preparation."

was similarly administered to twenty rats. The urine and feces were collected during 4 days. In dogs a solution of 1α-^{3}H-CMA (50 μCi, 1.45 mg) dissolved in 20% DMSO-saline was injected intravenously to two dogs and the mixture of nonlabeled steroid and food was given to each of three dogs. The urine and feces were collected daily to 7 days. The nonlabeled CMA (80 mg) enclosed in gelatine capsule was orally given to seven patients for 2 days. The urine and feces were collected daily until 4 days after final dose.

Biliary Excretion of Animals——The male rats, rabbits and dogs were anesthesized with sodium pentobarbital and cannulated to the bile duct with polyethylene tube (PE-50, PE-200 Clay Adams, Parsippany, N.J.) by surgical operation. A solution of 1α-^{3}H-CMA (50—78 μCi, 0.18—1.5 mg) dissolved in 20% DMSO-saline was injected intravenously. A suspension of nonlabeled steroid (100 mg/kg) with Tween 80 in saline was orally given to rats and rabbits. In dogs the nonlabeled CMA (150 mg/kg) enclosed in gelatine capsule was orally given to three dogs. These animals were housed in the metabolic cage for collection of the bile at various intervals of time during 48 hr.

Extraction of Fecal Metabolites——The fecal samples (human 2876 g, dog 2050 g, rat 1340 g) were extracted in Soxhlet apparatus employing successive extraction 3 times with methanol (1.5 vol.) and 50% aqueous methanol (2 vol.). The methanolic extracts were combined, evaporated and applied as aqueous solution to a column on Amberlite XAD-2 resin as described below.

Separation of Free Metabolites——The pooled bile (2—3 l) and urine (4 l) were percolated through a column packed with Amberlite XAD-2 (6×55 cm), washed with 1.5 volumes of water at a flow rate of 400 ml/hr, and then eluted with methanol (3 l) at a flow rate of 1 l/hr, respectively. A half portion of radioactivity effluent in the bile was used for structure elucidation of the aglycones. After evaporation of the solvent a gummy substance obtained was redissolved in distilled water (200 ml), adjusted to pH 4.5 with 0.1 M acetate buffer (20 ml) and then incubated with beef-liver β-glucuronidase 500000 units at 37° for 48 hr. The incubated fluid was extracted twice with two-fold volume of ethyl acetate and then the organic layer was washed with water and dried over anhyd. Na_2SO_4. The remaining aqueous layer was brought to pH 1.0 with 20 N H_2SO_4, saturated with NaCl (1/5 wt/vol.) and then extracted with two-fold volume of ethyl acetate. The organic phase was combined and allowed to stand for 48 hr at 37°. The extract was washed with water, dried over anhyd. Na_2SO_4 and evaporated. The deconjugated metabolites derived from glucuronide and sulfate fractions were combined and separated by column chromatography on Sephadex LH-20 into six fractions as shown in Fig. 1. Each fraction, if necessary, was submitted to preparative TLC.

Identification of Metabolites

Chlormadinone Acetate (CMA, Metabolite I)——The fraction A was evaporated and then crude product obtained was recrystallized from MeOH to give metabolite I as colorless needles, mp 212—214°. Comparison with the authentic sample showed the identity of two samples in every respect.

17α-Acetoxy-6-chloropregna-4,6-dien-3β-ol-20-one (3β-Hydroxy CMA, Metabolite II)——The fraction B was evaporated and then crude product obtained was recrystallized from MeOH to give metabolite II as colorless needles, mp 233—235°. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 6.08 (1H, d (J=3 Hz), 4-H). MS m/e: 406 (M^+). Mixed melting point on admixture with the authentic sample showed no depression, and IR, NMR and MS of two samples were entirely identical.

17α-Acetoxy-6-chloropregna-4,6-dien-3α-ol-20-one (3α-Hydroxy CMA, Metabolite III)——The mother liquor of metabolite II from fraction B was further purified by preparative TLC employing $CHCl_3$–MeOH (20:1) as solvent. Eluate of the adsorbent corresponding to the spot (Rf 0.40) was further purified by gel filtration on Sephadex LH-20 employing 20% methanol–benzene as eluent to give metabolite III as colorless oil. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 4.32 (1H, m, 3β-H), 6.22 (1H, d (J=6 Hz), 4-H). MS m/e: 406 (M^+), 301. Treatment of metabolite III with Ac_2O and pyridine in the usual manner gave the monoacetate. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 6.22 (1H, d (J=6 Hz), 4-H). MS m/e: 448 (M^+). Oxidation of metabolite III (10 mg) with Jones reagent in acetone (1 ml) solution gave CMA as colorless needles, mp 210—214°. From these results metabolite III was established to be 3α-hydroxy CMA.

17α-Acetoxy-6-chloropregna-4,6-dien-2α-ol-3,20-dione (2α-Hydroxy CMA, Metabolite IV)——The mother liquor of metabolite II was submitted to the preparative TLC employing $CHCl_3$–MeOH (20:1) as solvent. Eluate of the adsorbent corresponding to the spot (Rf 0.36) gave the metabolite IV as an amorphous substance. Treatment of metabolite IV with Ac_2O-pyridine in the usual manner, followed by recrystallization from MeOH gave 2,17-diacetate as colorless needles, mp 230—231°. NMR ($CDCl_3$ solution) δ: 0.72 (3H, s, 18-CH_3), 1.30 (3H, s, 19-CH_3), 5.65 (1H, q (J=13, 7 Hz), 2β-H). MS m/e: 462 (M^+). Direct comparison of metabolite IV and the 2α,17-diacetate with the authentic specimens showed the identity of two samples, respectively.

17α-Acetoxy-5β-pregnan-3α-ol-20-one (Metabolite V)——The mother liquor of metabolite II was purified by preparative TLC employing $CHCl_3$–MeOH (20:1) as solvent. Elution of the adsorbent corresponding to the spot (Rf 0.30) and acetylation of the eluate with Ac_2O–pyridine in the usual manner gave the diacetate as colorless needles, mp 228—232°. MS m/e: 418 (M^+). Mixed melting point on admixture with the authentic sample showed no depression, and IR spectra were entirely identical.

17α-Acetoxy-5β-pregnan-3β-ol-20-one (Metabolite VI)——The mother liquor of metabolite II was submitted to preparative TLC employing $CHCl_3$–MeOH (20:1) as solvent. Elution of the adsorbent corre-

sponding to the spot (*Rf* 0.30) and then acetylation of metabolite VI with Ac_2O–pyridine in the usual manner gave the diacetate, mp 86—88.5°. Direct comparison of metabolite VI and the diacetate with the authentic samples showed entire identity of two samples, respectively.

17α-Acetoxy-6-chloropregna-1,4,6-trien-2-ol-3,20-dione (2-Hydroxy Δ^1-CMA, Metabolite VII)——The mother liquor of metabolite II was submitted to the preparative TLC using $CHCl_3$–MeOH (20: 1) as solvent. Eluate of the adsorbent corresponding to the spot (*Rf* 0.40) gave an amorphous substance. NMR ($CDCl_3$ solution) δ: 0.74 (3H, s, 18-CH_3), 1.27 (3H, s, 19-CH_3), 6.70 (1H, s, 1-H). MS *m/e*: 418 (M^+). Treatment of the metabolite VII with Ac_2O–pyridine in the usual manner gave the diacetate as colorless needles, mp 182—184°. MS *m/e*: 460 (M^+). Direct comparison with metabolite VII and its acetate with the authentic specimens showed identity of two samples, respectively.

17α-Acetoxy-6-chloropregna-4,6-diene-1β,3α-diol-20-one (1β,3α-Dihydroxy CMA, Metabolite VIII)——Fraction C was further purified by preparative TLC employing $CHCl_3$–MeOH (20: 1) as developing solvent. Eluate of the adsorbent corresponding to the spot (*Rf* 0.20) was submitted to the gel filtration on Sephadex LH-20 employing 20% methanol–benzene as eluent to give metabolite VIII. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 5.95 (1H, s, 7H), 6.24 (1H, d (J=6 Hz), 4-H). MS *m/e*: 422 (M^+). Treatment of the metabolite VIII with Ac_2O–pyridine in the usual manner gave the diacetate as oil. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 1.12 (3H, s, 19-CH_3), 5.00—5.45 (1H, m, 1α-H), 5.60—5.80 (1H, m, 3β-H). MS *m/e*: 446 (M^+-60). From these results metabolite VIII should be 1β,3α-dihydroxy CMA.

17α-Acetoxy-6-chloropregna-4,6-diene-2β,3β-diol-20-one (2β,3β-Dihydroxy CMA, Metabolite IX)——Fraction D was further purified by preparative TLC employing $CHCl_3$–MeOH (10: 1) as developing solvent. Eluate of the adsorbent corresponding to the spot (*Rf* 0.40) was recrystallized from MeOH to give metabolite IX as colorless needles, mp 229—231°. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 1.24 (3H, s, 19-CH_3), 5.98 (1H, d (J=3 Hz), 4-H). MS *m/e*: 422 (M^+). To a solution of metabolite IX (10 mg) in $CHCl_3$ (1.5 ml) MnO_2 (30 mg) was added and the mixture was stirred for 30 min at room temperature. The filtrate was evaporated to dryness and acetylated with Ac_2O–pyridine in usual manner to give oil residue. Purification on TLC using benzene–ether (5: 1) as developing solvent gave the corresponding acetate (1 mg), mp 230—234°. MS *m/e*: 462 (M^+). Direct comparison of metabolite IX and its derivative with the authentic samples showed identity of the two samples, respectively.

17α-Acetoxy-6-chloropregna-4,6-diene-2α,3β-diol-20-one (2α,3β-Dihydroxy CMA, Metabolite X)——Fraction D was further purified by preparative TLC employing $CHCl_3$–MeOH (10: 1) as developing solvent. Eluate of the adsorbent corresponding to the spot (*Rf* 0.30) was recrystallized from MeOH to give metabolite X as colorless needles, mp 262—264°. NMR (DMSO solution) δ: 0.60 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 5.70—5.90 (2H, m, 4-H, 7-H). MS *m/e*: 422 (M^+). To a solution of metabolite X (10 mg) in $CHCl_3$ (2 ml) MnO_2 (30 mg) was added and the mixture was stirred for 30 min at room temperature. The filtrate was evaporated to dryness and acetylated with Ac_2O–pyridine to give oily residue. Purification on TLC using benzene–ether (5: 1) as developing solvent gave the corresponding acetate. Recrystallization of the crude product from MeOH gave 2α-acetoxy CMA (2 mg) as colorless needles, mp 229—232°. MS *m/e*: 462 (M^+). Mixed melting point on admixture with the authentic sample showed no depression and IR, NMR, MS of two samples were entirely identical.

17α-Acetoxy-6-chloropregna-4,6-diene-2α,3α-diol-20-one (2α,3α-Dihydroxy CMA, Metabolite XI)——Recrystallization of fraction E from MeOH gave metabolite XI as colorless needles, mp 233—235°. NMR (DMSO solution) δ: 0.60 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 5.95 (1H, d (J=6 Hz), 4-H). MS *m/e*: 422 (M^+). To a solution of metabolite XI (20 mg) in $CHCl_3$ (3.5 ml) MnO_2 (40 mg) was added and the mixture was stirred at room temperature for 1 hr. The filtrate was evaporated to dryness and the oily product obtained was then acetylated with Ac_2O–pyridine. The acetylated compound was purified by TLC as described above and then recrystallized to give 2α-acetoxy CMA as colorless needles, mp 231—233°. From identity of the acetate with the authentic sample metabolite XI was definitely characterized as 2α,3α-dihydroxy CMA.

17α-Acetoxy-6-chloropregna-4,6-diene-2α,3α,15β-triol-20-one (2α,3α,15β-Trihydroxy CMA, Metabolite XII)——Fraction F was submitted to preparative TLC using $CHCl_3$–MeOH (10: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.18) gave an amorphous substance. Further purification by Sephadex LH-20 column chromatography with 30% methanol–benzene as eluent gave metabolite XII as colorless oil. NMR ($CDCl_3$ solution) δ: 1.00 (3H, s, 18-CH_3), 1.20 (3H, s, 19-CH_3), 4.40—4.60 (1H, m, 15α-H). MS *m/e*: 438 (M^+). Treatment of metabolite XII with Ac_2O–pyridine in the usual manner gave the triacetate as colorless oil. MS *m/e*: 504 (M^+-60). NMR ($CDCl_3$ solution) δ: 0.92 (3H, s, 18-CH_3), 1.12 (3H, s, 19- CH_3), 5.00—5.45 (3H, m, 15α-, 2β-, 3β-H), 6.12 (1H, d (J=5 Hz), 4-H). From these evidences metabolite XII was assigned the structure 2α,3α,15β-trihydroxy CMA.

17α-Acetoxy-6-chloropregna-4,6-diene-2α,3β,15β-triol-20-one (2α,3β,15β-Trihydroxy CMA, Metabolite XIII)——Fraction F was submitted to preparative TLC using $CHCl_3$–MeOH (10: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.18) gave a yellow oily material. Further purification by Sephadex LH-20 column chromatography with 30% methanol–benzene gave metabolite XIII as colorless oil. Treatment of metabolite XIII with Ac_2O–pyridine in the usual manner, followed by purification on Sephadex LH-20 column chromatography gave the 2α,3β,15β-triacetate as colorless oil. MS *m/e*: 504 (M^+—

60). NMR ($CDCl_3$ solution) δ: 0.90 (3H, s, 18-CH_3), 1.18 (3H, s, 19-CH_3), 5.80—5.95 (2H, m, 4,7-H). From these results metabolite XIII was assigned the structure 2α,3β,15β-trihydroxy CMA.

6-Chloro-2α,3α,17α-trihydroxypregna-4,6-dien-20-one (2α,3α,17α-Trihydroxy CMA, Metabolite XIV)——Fraction F was submitted to the preparative TLC using $CHCl_3$–MeOH (1: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.15) gave an oily substance. Further purification by Sephadex LH-20 column chromatography with 30% methanol–benzene gave metabolite XIV as oil. MS *m/e*: 380 (M^+). UV λ_{max}^{MeOH}: 242 nm. To a solution of metabolite XIV (*ca.* 4 mg) in glacical acetic acid (1 ml)–acetic anhydride (0.5 ml) *p*-TsOH (2 mg) was added and the mixture was then allowed to stand for 2 hr. The resulting solution was diluted with ether, washed with 5% $NaHCO_3$ and water, dried over anhyd. Na_2SO_4 and evaporated. A portion of the residue thus obtained was submitted to TLC using benzene–ether (5: 1) as developing solvent. The sample proved to be identical with 2α,3α-diacetoxy CMA in *Rf* value.

Separation of Conjugated Metabolite——One portion of the effluent obtained by chromatography on Amberlite XAD-2 was submitted to column chromatography (3×90 cm) on Sephadex LH-20 using 60% methanol–0.2 M AcOH as eluent. The radioactive fraction obtained (1.2 g) was further purified by Sephadex LH-20 column chromatography using $CHCl_3$-MeOH-H_2O (6:4:1) as eluent.

17α-Acetoxy-6-chloropregna-4,6-dien-2α-ol-20-one-3α-yl-β-D-glucopyranosiduronic Acid (2α, 3α-Dihydroxy CMA 3-Glucuronide, Metabolite XV)——The gummy product (460 mg) was submitted to the preparative TLC using $CHCl_3$–MeOH–HCOOH (15: 7: 3) as developing solvent and the eluate was further purified to give metabolite XV by column chromatography (2×92 cm) on Sephadex LH-20 using 60% methanol–0.2 M AcOH as eluent. The eluate showed a positive reaction with naphthoresorcinol reagent. NMR (CD_3-OD solution) δ: 0.68 (3H, s, 18-CH_3), 1.12 (3H, s, 19-CH_3), 4.20—4.38 (1H, m, 3β-H), 4.42—4.62 (1H, m, 2β-H), 5.90 (1H, s, 7-H), 6.08 (1H, d (J=6 Hz), 4-H). The conjugate XV (1 mg) was dissolved in acetate buffer (0.1 M, pH 4.5) and incubated with beef-liver β-glucuronidase (10000 units) at 37° for 48 hr. The incubation mixture was extracted with EtOAc and then the extract was submitted to TLC. The hydrolyzate proved to be identical with 2α,3α-dihydroxy CMA by TLC comparison with metabolite XI. To a solution of the conjugate (10 mg) in MeOH (10 ml) ether solution of CH_2N_2 was added and then allowed to stand for 2 hr at room temperature. After decomposition of the excess reagent with AcOH the resulting solution was evaporated to give oily product, which in turn was acetylated with Ac_2O–pyridine in usual manner. After evaporation of the solvent an oily residue obtained was recrystallized from MeOH to give the acetate–methyl ester (XV′) as colorless needles, mp 118—121°. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 3.75 (3H, s, OCH_3), 3.95—4.20 (2H, m, 3β-H, pyranose-5-H), 5.12—5.40 (2H, m, 2β-H, pyranose-H), 5.97 (1H, s, 7-H), 6.02 (1H, d (J=6 Hz), 4-H). MS *m/e*: 617 (M^+−2×AcOH, $COCH_3$), 464 (aglycone), 446 (base), 317. These results permitted to assign 2α,3α-dihydroxy CMA 3-glucuronide to metabolite XV.

17α-Acetoxy-6-chloropregna-4,6-dien-20-one-3α-yl-β-D-glucopyranosiduronic Acid (3α-Hydroxy CMA 3-Glucuronide, Metabolite XVI)——The gummy product was submitted to the preparative TLC using $CHCl_3$–MeOH–HCOOH (15: 7: 3) as developing solvent and the eluate was further purified to give metabolite XVI by column chromatography on Sephadex LH-20 (2×97 cm) using 60% methanol–0.2 M AcOH as eluent. Metabolite XVI showed a positive reaction with naphthoresorcinol. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 5.95 (1H, s, 7-H), 6.20 (1H, d (J=6 Hz), 4-H). Metabolite XVI was treated with beef-liver β-glucuronidase in the manner as described in XV. The incubation mixture was extracted with EtOAc and then the extract was submitted to TLC. The hydrolyzate proved to be identical with 3α-hydroxy CMA by TLC comparison. This substance was treated with CH_2N_2 and then with Ac_2O–pyridine in the usual manner as described above to give the acetate–methyl ester as oily substance. MS *m/e*: 662 (M^+−AcOH), 406 (aglycone), 317. NMR ($CDCl_3$ solution) δ: 0.70 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 3.78 (3H, s, OCH_3), 4.70 (1H, d (J=8 Hz), pyranose-1-H), 5.95 (1H, d (J=6 Hz), 4-H). These data were evidence that metabolite XVI should be 3α-hydroxy CMA 3-glucuronide.

17α-Acetoxy-6-chloropregna-1,4,6-trien-3,20-dione-2-yl-β-D-glucopyranosiduronic Acid (2-Hydroxy Δ^1-CMA 2-Glucuronide, Metabolite XVII)——The eluate obtained by gel filtration on Sephadex LH-20 was further purified by silica gel column chromatography using $CHCl_3$–MeOH–H_2O (15: 3: 1) as eluent. The crude substance obtained was treated with CH_2N_2 and then Ac_2O–pyridine in the usual manner as described above. After evaporation of the solvent an oily residue obtained was submitted to preparative TLC. Elution of the main radioactive fraction with acetone gave the acetate–methyl ester as oily product. MS *m/e*: 691 (M^+−$COCH_3$), 418 (aglycone), 317. NMR ($CDCl_3$ solution) δ: 0.77 (3H, s, 18-CH_3), 1.26 (3H, s, 19-CH_3), 3.82 (3H, s, OCH_3), 6.63 (1H, s, 1-H). From these findings this metabolite would be 2-hydroxy Δ^1-CMA 2-glucuronide.

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