SYNTHESIS AND PYROGENIC EFFECT OF 3α,7α-DIHYDROXY-5β-ANDROSTAN-17-ONE AND 3α-HYDROXY-5β-ANDROSTANE-7,17-DIONE

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ABSTRACT

The first chemical synthesis of 3α,7α-dihydroxy-5β-androstan-17-one and 3α-hydroxy-5β-androstane-7,17-dione is reported . In this method, the 17β-side chain of commercial chenodesoxycholic acid was degraded in 6 steps after selective protection of the hydroxyl groups : 3α-OH by a tert-butyldimethylsilyl group and 7α-OH by an acetoxy group.

The capacity of 3α,7α-dihydroxy-5β-androstan-17-one and 3α-hydroxy-5β-androstane-7,17-dione to release a pyrogen by human leukocytes was investigated by two independent methods : supernatants from leukocytes incubated with a steroid are injected to rabbits whose fever is measured, or tested by the Limulus Test (a pyrogen detection technique). The 7-keto substituted etiocholanolone still possessed pyrogenic activity, while the 7α-hydroxyl substituted one did not .

INTRODUCTION

In the course of studies on testosterone metabolism, HENRY et al. (1) have isolated and identified 3α-hydroxy-5β-androstane-7,17-dione X from urine of male guinea pigs injected with large doses of testosterone . This new metabolite is a derivative of etiocholanolone (3α-hydroxy-5β-androstan-17-one) .

It was reported earlier that the intramuscular injection of etiocholanolone produced fever only in man (2) . The production of fever is associated with an increase in the number of leukocytes in the blood, and the release of a leukocytic pyrogen . Although rabbit leukocytes are not activated by etiocholanolone, human leukocytic pyrogen produced in vitro by incubation of blood leukocytes with a steroid, when injected

into the rabbit, produces fever and then can be detected (3) .

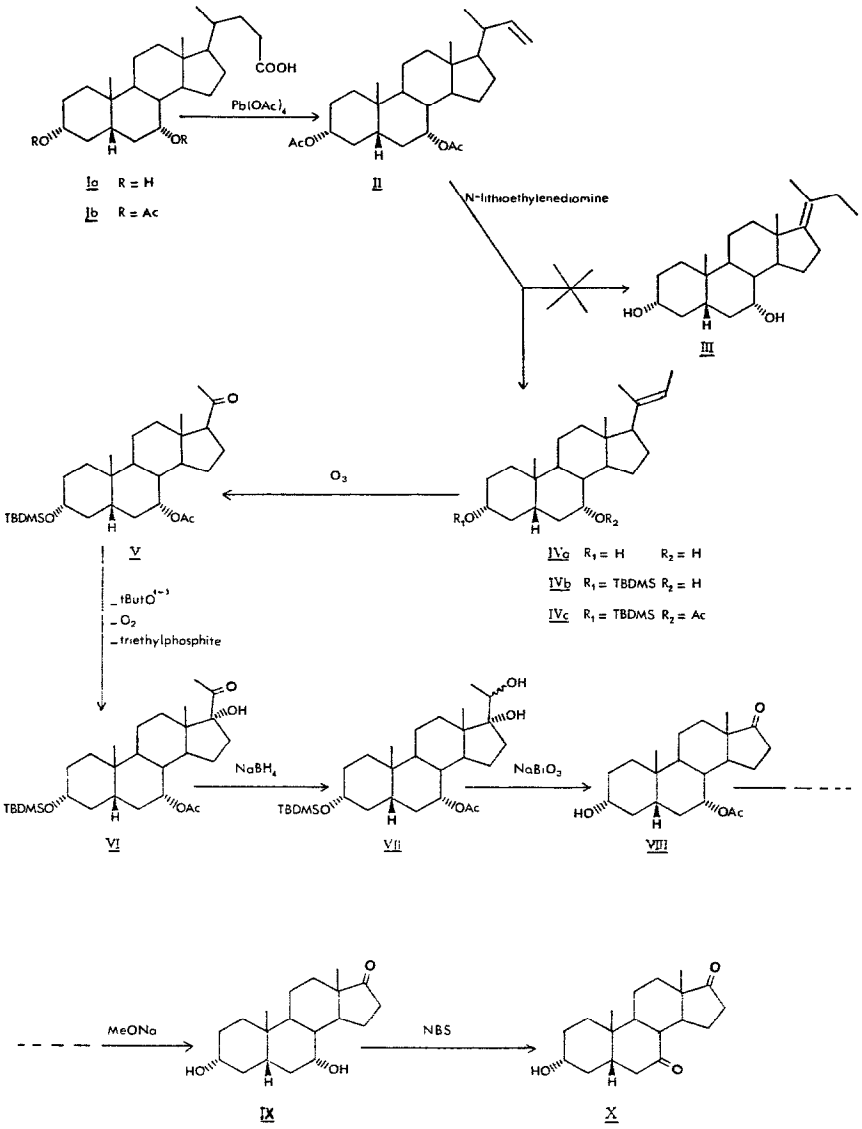
Hepatic origin of the steroid X is considered in consequence of the presence of 5β-reductase, 7α-hydroxylase and 7α-hydroxysteroid oxidoreductase in the liver . Polar metabolites having an oxygenated function at the 7-carbon are commonly found in different organs but their role in the metabolism is not known . In order to verify the structure of X and to determine its biological activity, we have investigated a chemical synthesis for this steroid and its possible hepatic precursor, 3α,7α-dihydroxy-5β-androstan-17-one IX .

Pyrogenic activity of various steroids, though related to the particular features of the compound, depends on a 5β-configuration . Conversion of the 3α-hydroxyl into a 3β-hydroxyl group or a ketone function, or introduction of a hydroxyl group at the 11β-position reduces pyrogenicity of etiocholanolone, but a ketone at the 11-carbon does not affect its pyrogenic activity (4) . The release of a pyrogen by human leukocytes incubated with a steroid, therefore, seemed appropriate for studying the possible effects of introduction of a hydroxyl group at the 7α-position or a ketone at C-7 of etiocholanolone . Leukocytic pyrogen was detected, first, by measure of the fever in rabbits injected with the supernatant of incubation, and then, by the Limulus Test . This qualitative test consists in a gel forming reaction between a pyrogen (protein-lipid-polysaccharide complex) and an extract of *Limulus Polyphemus* amebocytes . This test is used to detect bacterial pyrogen toxins (11, 12) .

Chemical synthesis of 3α,7α-dihydroxy-5β-androstan-17-one IX and 3α-hydroxy-5β-androstane-7,17-dione X :

Steroid IX has 3α and 7α-hydroxyl groups and a cis junction between the rings A and B . Chenodesoxycholic acid (Ia : 3α,7α-dihydroxy -5β-cholan-24-oic acid) has these characteristics, therefore it was chosen as a suitable precursor of IX which can be selectively oxidized to X . The pathway is described in scheme I .

Oxidative decarboxylation of 3α,7α-diacetoxy-5β-cholan-24-oic acid Ib with lead tetraacetate in benzene gave 3α,7α-diacetoxy-24-nor-5β-chol-22-ene II (5) . We used the method described by NARULA and DEV (6) for the migration of a Δ-22,23 double bond to Δ-20,22 or Δ-17,20 ;



Scheme I

the Δ-20,22 olefin IVa was found as the only product from the reaction of the olefin II with N-lithioethylenediamine during 15 hours refluxing in ethylenediamine . No Δ-17,20 olefin III was detected, probably due to the strain on ring D induced by the introduction of a sp^2 carbon .

During this long treatment of the olefin II, a mixture of cis and trans isomers of the olefin IVa was obtained, whereas after short treatment (15 min) only a single isomer was obtained . Under strongly basic conditions (N-lithioethylenediamine), 3α and 7α-hydroxyl groups were released from the acetoxy groups . Therefore, the 3α-hydroxyl group was protected by the action of a bulky reagent, tert-butyldimethylsilyl chloride (TBDMS-Cl) (7), and the 7α-hydroxyl group by acetylation . The use of TBDMS as protecting group prevented the formation of a weakly soluble triol during the preparation of the diol VII (See below) .

The ozonolysis of the Δ-20,22 double bond of olefin IVc produced ketone V which upon hydroxylation by the method of GARDNER et al. (9) gave the α-ketol VI . During this reaction, the 7α-acetoxy group was unaffected by sodium tert-butoxide because of steric hindrance .

Reduction of the hydroxy-ketone VI by sodium borohydride led to the glycol VII . The hydroxy-ketone VIII was subsequently obtained by glycolytic cleavage with sodium bismuthate (9) . The 3α-hydroxyl group was simultaneously released by acetic acid .

The treatment of the hydroxy acetate VIII by sodium methanolate gave the expected 3α,7α-dihydroxy-5β-androstan-17-one IX (yield 16 % from Ia) . Oxidation of the 7α-hydroxyl group by N-bromosuccinimide (10) yielded 3α-hydroxy-5β-androstane-7,17-dione X .

To our knowledge, this is the first report on the synthesis of these two steroids .

EXPERIMENTAL

Synthesis of 3α,7α-dihydroxy-5β-androstan-17-one and 3α-hydroxy-5β-androstane-7,17-dione

Preparative t.l.c. was carried out on Merck silica gel H.F.254. Melting points were determined on a REICHERT hot stage microscope ; they were not corrected . Infrared spectra were recorded either as KBr discs or as CS_2 solutions as indicated, and are expressed in cm^{-1} . N.M.R. ^1H spectra were determined at 60 MHz for solutions in $CDCl_3$, with $(CH_3)_4Si$ as internal standard, and are expressed in ppm (δ) relative to TMS as zero .

3α-7α-Diacetoxy-24-nor-5β-chol-22-ene II

A mixture of 3α,7α-diacetoxy-5β-cholan-24-oic acid Ib (16.9 g), lead tetraacetate (33.46 g), cupric acetate (1.7 g) and anhydrous

pyridine (2 ml) in dry benzene (1 L) was stirred under reflux for 4 hr under argon . The solids were then collected on a celite filter . After removal of benzene, the residue was dissolved in ether, washed with 5 % aqueous HCl and with 10 % aqueous sodium bicarbonate . The crude extract was chromatographed on SiO_2-gel (750 g) : passage of ethyl acetate-hexane (30 : 70) eluted II (8.3 g, yield 82 % with respect to the recovered starting material), further passage of ethyl acetate-hexane first (50 : 50) and then (70 : 30) released Ib (5.6 g) . Olefin II was crystallised from methanol ; m.p. = 134°C ; $[\alpha]_D^{20}$ = - 200° (c=0.6, $CHCl_3$) ; I.R. : 1730 (νC=O, acetoxy groups), 1640 (νC=C, Δ-22,23), 1445, 905 (δ=C-H) ; N.M.R. ^1H : 0.72 (s, 18-CH_3), 0.94 (s, 19-CH_3), 1.03 (d, 21-CH_3, J = 6 Hz), 2.02 (s, 3α and 7α-CH_3CO), 4.60 (m, 3β-H), 4.86 (m, 7β-H), 4.95 to 4.75 (m, 23-CH_2), 5.45 to 5.85 (m, 22-CH) ; M.S. : $M^{+\cdot}$ = 430, m/e = 370, 255 (allylic cleavage), 256 . (Found : C : 75.15 ; H : 9.88 ; $C_{27}H_{42}O_4$ requires : C : 75.31 ; H : 9.83) .

3α,7α-Dihydroxy-24-nor-5β-chol-20(22)-ene IVa

a . Short treatment

The olefin II (10 g) was added in one lot to a stirred solution of 52 equivalents of N-lithioethylenediamine (520 ml of a 15 % solution of buthyllithium in hexane, 266 ml of ethylenediamine) at 120-125°C under argon, and the mixture refluxed for 15 min . After cooling, 52 eq. of water was added and ethylenediamine was evaporated under pressure . The solid residue was triturated with phosphate buffer (KH_2PO_4 0.025 M, Na_2HPO_4 0.025 M, pH 7) and continuously extracted with ether for 8 hr ; a solid crude extract (6.4 g, 80 %) was obtained . A final sample of IVa was purified by t.l.c. and crystallised from pentane/ether . m.p. = 178°C ; $[\alpha]_D^{20}$ = + 0.1° (c=1, $CHCl_3$) ; I.R. : 3400 (νO-H), 1660 (νC=C, Δ-20,22), 810 (δ=C-H) ; N.M.R. ^1H : 0.50 (s, 18-CH_3), 0.90 (s, 19-CH_3), 1.60 (s, 21-CH_3), 1.55 (s, 23-CH_3), 3.50 (m, 3β-H), 3.90 (m, 7β-H), 5.25 (m, 22-CH) ; N.M.R. ^{13}C : 135.1 (20-C), 119.2 (22-C) ; M.S. : $M^{+\cdot}$ = 346, m/e = 328, 310, 253 .

(Found : C : 79.37 ; H : 10.82 ; $C_{23}H_{38}O_2$ requires : C : 79.71 ; H : 11.05) .

b . Long treatment

Same as in "a" but the mixture was refluxed for 15 hr . A mixture of cis and trans isomers of IVa was obtained . N.M.R. ^1H : 0.53 (d, 18-CH_3), 0.87 (s, 19-CH_3), 1.60 (m, 21-CH_3), 1.57 (d, 23-CH_3), 3.50 (m, 3β-H), 3.90 (m, 7β-H), 5.27 (q, 22-CH) ; N.M.R. ^{13}C : 135.1 and 119.2 (respectively 20-C and 22-C of the isomer

isolated after a short treatment), 130.9 and 128.8 (20-C and 22-C of the other isomer) .

3α-Tert-butyldimethylsilyloxy-7α-acetoxy-24-nor-5β-chol-20(22)-ene IVc

3α,7α-Dihydroxy-24-nor-5β-chol-20(22)-ene IVa (5 g) was treated with tert-butyldimethylsilyl chloride (3.27 g) and imidazole (3.75 g) in dimethylformamide (25 ml) (7) . The crude extract was acetylated with pyridine and acetic anhydride . The product was chromatographed on SiO_2-gel (500 g) ; elution with hexane/ether (90 : 10) gave olefin IVc (4.9 g, 68 %), m.p. = 113-114°C (ether/methanol) .

$[\alpha]_D^{20}$ = + 5.3° (c=1, $CHCl_3$) ; I.R. : 1740 (νC=O), 1670 (νC=C) ; N.M.R. ^1H : 0.03 (s, $Si(CH_3)_2$), 0.50 (s, 18-CH_3), 0.87 (s, 19-CH_3 and C-$(CH_3)_3$), 1.62 (s, 21-CH_3), 1.57 (s, 23-CH_3), 2.03 (s, 7α-$COOCH_3$), 3.45 (m, 3β-H), 4.85 (m, 7β-H), 5.25 (m, 22-CH) ; M.S. : $M^{+\cdot}$ = 502, m/e = 445, 442, 385, 311 .

(Found : C : 73.80 ; H : 10.61 ; $C_{31}H_{54}O_3Si$ requires : C : 74.10 ; H : 10.76) .

3α-Tert-butyldimethylsilyloxy-7α-acetoxy-5β-pregnan-20-one V

Ozone was passed through a solution of the olefin IVc (6.5 g) in dichloromethane/methanol (5 : 2) at - 70°C, until the mixture was blue . After treatment with a saturated acetic acid solution of potassium iodide, the mixture was washed with aq. 10 % sodium thiosulfate, aq. 10 % sodium bicarbonate and extracted with ether . Crystallisation from methanol yielded the ketone V (6.1 g, 96 %), m.p. = 120°C,

$[\alpha]_D^{20}$ = + 50.4° (c=0.8, $CHCl_3$) ; I.R. (CS_2) : 1740 (νC=O, acetoxy group), 1710 (νC=O, 20-CO) ; N.M.R. ^1H : 0.03 (s, $Si(CH_3)_2$), 0.63 (s, 18-CH_3), 0.92 (s, 19-CH_3 and C-$(CH_3)_3$), 2.03 (s, 7α-$COCH_3$), 2.13 (s, 21-CH_3), 3.55 (m, 3β-H), 4.90 (m, 7β-H) ; M.S. : m/e = 433, 373, 299 .

(Found : C : 70.90 ; H : 10.18 ; $C_{29}H_{50}O_4Si$ requires : C : 71.02 ; H : 10.20) .

3α-Tert-butyldimethylsilyloxy-7α-acetoxy-17-hydroxy-5β-pregnan-20-one VI

The ketone V (4.7 g) was treated with sodium hydride (1.46 g) in dimethylformamide (20 ml), t-butanol (9.7 ml) and triethyl phosphite (2.4 ml) . Oxygen was passed through the mixture for 1 hr at - 25°C. Extraction of the solution with ether furnished an oil (7.9 g) ; a small part of it was purified by crystallisation from pentane/ether . An analytical sample of VI was obtained . m.p. : 152-153°C,

$[\alpha]_D^{20}$ = - 6° (c=1.7, $CHCl_3$) ; I.R. : 1710 (νC=O, 20-C), 1735 (νC=O, acetoxy group), 3500 (νO-H) ; N.M.R. ^1H : 0.05 (s, $Si(CH_3)_2$), 0.68 (s, 18-CH_3), 0.93 (s, 19-CH_3 and $C(CH_3)_3$), 2.04 (s, 7α-$COCH_3$), 2.25 (s, 21-CH_3),

2.93 (s, 17α-OH), 3.50 (m, 3β-H), 4.9 (m, 7β-H) ; M.S. : $M^{+\cdot}$ = 506, m/e = 446, 315, 297 .

(Found : C : 68.78 ; H : 9.75 ; $C_{29}H_{50}O_5Si$ requires : C : 68.77 ; H : 9.88) .

3α-Tert-butyldimethylsilyloxy-7α-acetoxy-5β-pregnane-17,20ζ-diol VII

Crude ketol VI (7.9 g) in methanol/water (100 : 20) was reduced with sodium borohydride (7 g) for 40 hr at room temperature . Workingup in the usual way afforded an oil which was separated by t.l.c. (hexane/ethyl acetate 65 : 35), diol VII (2.5 g, 51 % yield from V) crystallised from pentane/ether . m.p. = 268°C ; I.R. : 3540, 3380 to 3280 (νO-H), 1740 (νC=O, acetoxy group) ; N.M.R. ^1H : 0.05 (s, $Si(CH_3)_2$), 0.80 (s, 18-CH_3), 0.90 (s, $C(CH_3)_3$), 0.95 (s, 19-CH_3), 2.03 (s, 7α-$COCH_3$), 3.96 to 4.45 (20-CH, 17α-OH and 20-OH), 4.93 (m, 7β-H) ; M.S. : m/e = 463, 403, 385 .

(Found : C : 68.75 ; H : 10.16 ; $C_{29}H_{52}O_5Si$ requires : C : 68.46 ; H : 10.30) .

3α-Hydroxy-7α-acetoxy-5β-androstan-17-one VIII

Glycol VII (6 g) in acetic acid (120 ml) was treated with sodium bismuthate (60 g) in water (120 ml) for 72 hr at room temperature. The mixture was filtered, washed with aq. 10 % sodium thiosulfate and worked-up in the usual way . The acetate VIII could not be crystallised (3.3 g, 80 %) . I.R. (CS_2) : 3400 (νO-H), 1745 (νC=O, 17-CO), 1735 (νC=O, acetoxy group) ; N.M.R. ^1H : 0.86 (s, 18-CH_3), 0.97 (s, 19-CH_3), 2.05 (s, 7α-$COCH_3$), 3.50 (m, 3β-H), 5.00 (m, 7β-H) ; M.S. : $M^{+\cdot}$ = 348, m/e = 288, 270, 252 .

3α,7α-Dihydroxy-5β-androstan-17-one IX

Acetate VIII (0.405 g) was saponified with sodium methanolate (0.9 g) in anhydrous methanol (20 ml) . Pure diol IX (purified by t.l.c.) could not be crystallised (0.340 g, 95 %) .

$[\alpha]_D^{20}$ = + 43° (c=1.06, $CHCl_3$) ; I.R. : 3400 (νO-H), 1745 (νC=O, 17-CO) ; N.M.R. ^1H : 0.86 (s, 18-CH_3), 0.92 (s, 19-CH_3), 2.88 (m, 3α-OH and 7α-OH), 3.40 (m, 3β-H), 3.95 (m, 7β-H) ; M.S. : $M^{+\cdot}$ = 306, m/e = 288, 270 .

(Found : C : 74.87 ; H : 9.63 ; $C_{19}H_{30}O_3$ requires : C : 74.47 ; H : 9.87) .

3α-Hydroxy-5β-androstane-7,17,dione X

Diol IX (0.215 g) was oxidized by N-bromosuccinimide (0.250 g, 1.25 eq.) in aqueous acetone for 10 min at room temperature . A mixture of IX (0.046 g) and X (0.108 g) was separated by t.l.c. (Yield 64 % with respect to the recovered starting material) . Dione X crystallised from pentane/ether . m.p. = 189-190°C .

$[\alpha]_D^{20}$ = + 8° (c=0.4, $CHCl_3$) . I.R. : 3410 (νO-H), 1745 (νC=O, 17-CO), 1710 (νC=O, 7-CO), 1070 (νC-O) ; N.M.R. ^1H : 0.96 (s, 18-$\underline{CH_3}$), 1.23 (s, 19-$\underline{CH_3}$), 3.60 (m, 3β-$\underline{H}$) ; M.S. : $M^{+\cdot}$ = 304, m/e = 192 . (Found : C : 74.89 ; H : 9.54 ; $C_{19}H_{28}O_3$ requires : C : 74.96 ; H : 9.27) .

Pyrogenic activity of 3α,7α-dihydroxy-5β-androstan-17-one and 3α-hydroxy-5β-androstane-7,17-dione

Methods for preparation of steroid solutions, leukocytes and incubation have been reported previously (3) . Buffer and other solutions were filtered through an ultra-fine membrane (Pellicon, exclusion of substances of MW > 10 000, Millipore) and transfered with non-pyrogenic plastic syringes . The final concentration of the steroid solutions was : etiocholanolone (47 µg/ml), 3α,7α-dihydroxy-5β-androstan-17-one (45 µg/ml), 3α-hydroxy-5β-androstane-7,17-dione (46 µg/ml) . Leukocytes were counted by the Trypan blue exclusion test and the concentration was adjusted to 2.8 10^7 ml .

Incubation

All incubations were done in 25 ml stoppered sterile flasks . The "Incubation-Steroid" flasks contained leukocyte preparation, 1 ml, autologous serum, 6 ml, penicillin, 6,000 U, heparin, 90 U and glucose 13.5 mg . Leukocyte preparation and autologous serum were omitted from the "Control-Steroid" flasks . The "Control-Buffer" flasks were identical to "Control-Steroid", except that the steroid was also omitted . "Control-Leukocytes" flasks did not contain steroids . Final volume of the incubation mixture was adjusted to 7.35 ml . After incubation, cell-free supernatants were prepared from each mixture . The different supernatants obtained are : "Control-Buffer" a ; "Control-Leukocytes" b ; "Control-etiocholanolone" c : "Incubation-etiocholanolone" d ; "Control-3α-hydroxy-5β-androstane-7,17 dione" e ; "Incubation-3α-hydroxy-5β-androstane-7,17,dione" f ; "Control 3α,7α-dihydroxy-5β-androstan-17-one" g ; "Incubation 3α,7α-dihydroxy-5β-androstan-17-one" h . (Table 1).

Injection to rabbits and temperature recording

26 New-Zealand male rabbits (Evis-Ciba Breeders) each weighing 2.1-2.5 kg were maintened under the same regimen . On the day of treatment, animals were starved and kept in restraining cages ; the room temperature was 20° C. Rectal temperature was recorded with a milliampere-remeter every 15 min . Recordings began 3 hr before injection and ended 4 hr after . Each rabbit received 5 ml of an appropriate supernatant and 2 ml of pyrogen-free physiological serum . Animals divided into 4 groups (I to IV) of 6 each were first injected with supernatant as follows : I with a ; II with b ; III with c ; IV with d . They were then rested for 72 hr before the next injection : I with f ; II with g ; III with h ; IV with e (Table 1) .

Fever Index

For each rabbit, Δt was calculated as the difference between the rectal temperature before and after injection . The area under fever

curves (expressed in mm^2) was measured 30 min, 1 hr, 1 hr 30 min, 2 hr and 3 hr after injection . For each group, the average fever index was calculated at each time point .

Limulus Test

The pyrogen detection kit (Industrie Biologique Française, 16 Bd du Gal Leclerc - 92115 CLICHY) contained an extract of *Limulus Polyphemus* amebocytes, a gram negative bacterial endotoxin for use as a positive control, pyrogen-free distilled water for a negative control, an inhibition control for testing the inhibitory potential of the supernatant towards positive control, and special microtubes . The quality of the reagents was checked using the controls . Tests were made on all the 8 supernatants prepared as described above . The formation of a gel in the microtube was an indication of a positive test .

RESULTS

Production of fever in rabbits

To determine whether 3α,7α-dihydroxy-5β-androstan-17-one and 3α-hydroxy-5β-androstane-7,17-dione could induce release of pyrogen from human leukocytes *in vitro*, we incubated these steroids with white blood cells preparation . The cell-free supernatants were then injected to the rabbits . Results are given in Table 1 . Supernatants from Controls (a, c, e, g) were inactive : the fever indexes were negligible over the test period . Maximal febrile response was noted 1 hr after injection of the supernatant from "Incubation-Steroid" mixture (Figure 1, Curves d, f, h) . Fever indexes, for each steroid supernatant and "Control-Leukocytes" were expressed in arbitrary area unit at this time, statistically analysed (mean ± SE, N=6) and compared with one another (Cochran *t* test) : these values are represented in Table 1 .

The supernatant derived from "Control-Leukocytes" (Fig. 1, Curve b) had a weak activity ($\Delta t = + 0.4°$) ; it could be an artifact due to the presence of some leukocytes in the supernatant as a result of incomplete sedimentation during centrifugation of the incubation-suspension . Hence, the fever index of "Control-Leukocytes" was considered as the basal level instead of that of each "Control-Steroid" (c, e, g) . The fever indexes of etiocholanolone (d) and of 3α-hydroxy-5β-androstane-7,17-dione (f) were greater ($p < 0.05$) than that of "Control-Leukocytes" (b) ; the fever index of 3α,7α-dihydroxy-5β-androstan-17-one (h) did not differ significantly from that of "Control-Leukocytes" .

These results thus show that only etiocholanolone and 3α-hydroxy

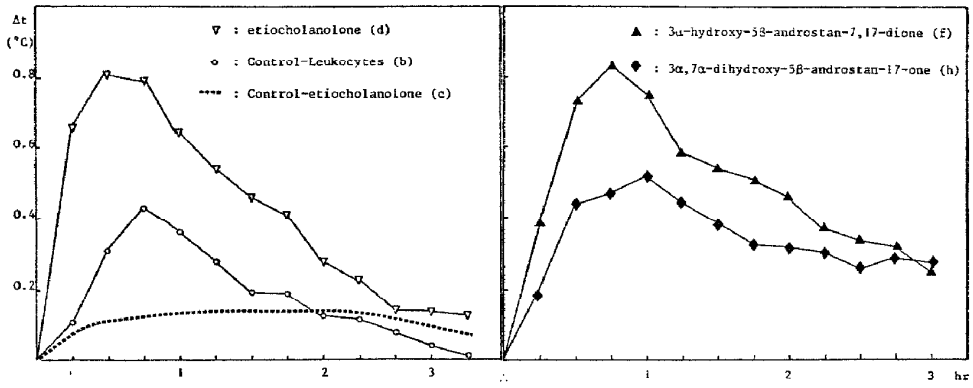


Figure 1 : Average fevers in rabbits (N=6) after injection of supernatants from etiocholanolone alone (c), from 2.8 10^7 leukocytes incubated alone (b), with etiocholanolone (d), 3α,7α-dihydroxy-5β-androstan-17-one (h) and 3α-hydroxy-5β-androstane-7,17-dione (f) .

-5β-androstane-7,17-dione, but not 3α,7α-dihydroxy-5β-androstan-17-one, produce a leukocytic pyrogen when incubated with white blood cells .

Fever indexes of supernatants from "Incubation-Steroid" (d, f, h) are compared with one another in order to determine the effect of the introduction of an oxygenated function at the 7-carbon of etiocholanolone in the production of a leukocytic pyrogen . The fever index of 3α,7α-dihydroxy-5β-androstan-17-one (h) was diminished and significantly different ($p = 0.05$) from that of etiocholanolone (d), whereas the fever index of 3α-hydroxy-5β-androstane-7,17-dione (f) was not ($p > 0.05$) (Table 1) . Thus, etiocholanolone and 3α-hydroxy-5β-androstane-7,17-dione have nearly the same pyrogenic property, whereas 3α,7α-dihydroxy-5β-androstan-17-one has lost it . Fever indexes of the supernatants b, f, h expressed as a percentage of d were : etiocholanolone (d) 100 ; 3α-hydroxy-5β-androstane-7,17-dione (f) 86 ; 3α,7α-dihydroxy-5β-androstan-17-one (h) 58 : control-leukocytes (b) 39 .

Limulus Test

The Limulus Test is a pyrogen detection method which uses an amebocyte lysate of *Limulus Polyphemus* . In vitro, this extract reacts with bacterial endotoxin (11) and with pyrogen (12) to form a gel .

Table 1 : Pyrogenic activity of supernatants (a to h) from controls and from etiocholanolone (47 µg/ml), 3α-hydroxy-5β-androstane-7,17-dione (46 µg/ml) and 3α,7α-dihydroxy-5β-androstan-17-one (45 µg/ml) incubated with leukocytes ($2.8\ 10^7$/ml) : Limulus Test and fever indexes (in arbitrary area unit) in rabbit .

Flasks	Limulus Test	Fever Index at 1 hr
Control Buffer (a)	-	< 100
Control Leukocytes (b)	-	1953 ± 1767 °
Control Etiocholanolone (c)	-	100
Incubation Etiocholanolone (d)	+	4991 ± 2132 $p < 0.05$ °°
Control 3α-hydroxy-5β-androstane-7,17-dione (e)	-	< 100
Incubation 3α-hydroxy-5β-androstane-7,17-dione (f)	+	4298 ± 1339 $p < 0.05$ °°
Control 3α,7α-dihydroxy-5β-androstan-17-one (g)	-	< 100
Incubation 3α,7α-dihydroxy-5β-androstan-17-one (h)	+	2911 ± 759 NS °°

° : mean ± SE (N=6)
°° : p (t test) vs Control Leukocytes supernatant (b) NS (no significant)

Results are summarized in Table 1 . As can be seen, the tests were negative for the supernatants a, b, c, e, g and positive for the supernatants d, f, h .

DISCUSSION

Previous studies (3) have shown that etiocholanolone can activate human blood leukocytes, in vitro, to release an endogenous pyrogen ; injection of this pyrogen contained in the supernatant from leukocytes incubated with etiocholanolone causes fever in rabbits . The current investigation confirms this observation . We also investigated the pyrogenic activity of this supernatant by the Limulus Test . Positive result obtained in this method provides evidence that the Limulus Test is a valid technique for detecting a leukocytic pyrogen induced by a steroid . However, this method is qualitative but the results must be corroborated and assayed by quantitative determination of the fever index in rabbit .

3α-Hydroxy-5β-androstane-7,17-dione has been isolated from urine

of male guinea pigs which have received injections of large doses of testosterone (1) ; it represented 25 % of the urinary metabolites . This steroid has no androgenic activity . Our studies show that it has pyrogenic activity though less than that of etiocholanolone ; the introduction of a ketone function at C-7 of etiocholanolone slightly diminishes this property (86 % with respect to the fever index of etiocholanolone, and positive Limulus Test) . Introduction of a 7α-hydroxyl group in etiocholanolone diminishes even more the pyrogenic property with regard to the fever index (58 %), even though a positive reaction is observed with the Limulus Test . Thus, the Limulus Test is not as sensitive as the fever index in the rabbit .

Therefore, it appears that a keto function at C-7 or at C-11 (4) of etiocholanolone has the same action on the pyrogenic effect . The property to release a leukocytic pyrogen is maintained in 3α-hydroxy -5β-androstane-7,17-dione, whereas it is abolished in 3α,7α-dihydroxy-5β-androstan-17-one .

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