# Androst-4-ene-3,17-dione-4-$^{14}$C Metabolism by the Homogenates of the Chicken Uropygial Gland I

## Antibiotic Concentrations Necessary for Inhibition of Bacterial Growth

By EDWARD L. RONGONE and FRANK M. FERRARO

Supernatant fluid obtained from homogenates of chicken uropygial gland was incubated with androst-4-ene-3,17-dione-4-$^{14}$C in the presence of penicillin and streptomycin. Two metabolic products were isolated and identified by using preliminary thin-layer chromatography followed by gas chromatography of the free metabolites, trimethylsilyl ethers, *o*-methyloxime derivatives, and double derivatives on 1 percent SE-30 and 1 percent neopentyl glycol succinate (NGS) columns. Crystallization to constant specific activity was also used for the identification of the metabolites. The metabolites identified were 3$\alpha$-hydroxy-5$\alpha$-androstane-17-one and 5$\alpha$-androstane-3$\alpha$,17$\beta$-diol. The isolated organisms from the supernatant fluid were highly susceptible to the activity of the antibiotics added to the incubation mixture for metabolic activity. The minimal concentrations of the antibiotics required to inhibit bacterial growth under the experimental conditions of this investigation were determined.

THE PRESENCE of microorganisms in incubation preparations for metabolic activity is always of concern to investigators. Unless sterile conditions are used throughout the incubation procedure, the only alternative is to add excessive amounts of antibiotics. Since it has been previously demonstrated that the supernatant fluid obtained from homogenates of chicken uropygial gland is capable of oxidizing the C-17 hydroxyl group of testosterone (1), it is of interest to determine whether the supernatant fluid is also capable of reducing a C-17 carbonyl group. The present investigation concerns the demonstration of a 17$\beta$-hydrogenase in the supernatant fluid obtained from homogenates of chicken uropygial gland, isolation of bacteria from the incubating medium prior to the addition of antibiotics, and the determination of the minimal amounts of penicillin and streptomycin needed to prevent bacterial growth under the incubation conditions.

### EXPERIMENTAL

**Purification of Androst-4-ene-3,17-dione-4-$^{14}$C**—Androst-4-ene-3,17-dione-4-$^{14}$C was purchased from Nuclear-Chicago Corp. and purified by TLC on Silica Gel G in a chloroform–ethanol system, 49:1. The resolved androst-4-ene-3,17-dione-4-$^{14}$C was eluted from the silica gel with absolute methanol and transferred to a 3-ml. centrifuge tube. One milliliter of benzene was added to the centrifuge tube and the radioactive compound then stored at 3° until used in the incubation procedures.

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**Gas Chromatography**—A Beckman model GC4 gas chromatograph equipped with a flame-ionization detector and glass columns 1.83 m. (6 ft.) long were used. The glass columns were packed with either 1% NGS (neopentyl glycol succinate) or 1% SE-30 (silicone rubber) on 100/120 mesh siliconized Gas-Chrom P (Applied Science Laboratories, State College, Pa.). Conditions employed were: column temperature, 210°; injector temperature, 240°; detector temperature, 230°; $N_2$ input flow, 40 ml./min.; $O_2$ output flow, 200 ml./min.; $H_2$ output flow, 50 ml./min. The recorder was a 25.4-cm. (10-in.) Beckman potentiometer set at 1 mv. and a speed of 0.5 in./min. Gas-Chrom P was prepared and coated with NGS and SE-30 according to the technique of Horning *et al.* (2).

**Incubation Medium**—The uropygial gland was removed immediately after decapitation (3 lb. cockerels or hens) and freed of fat, skin, connective tissue, sebum, and the uropygial duct. The remaining gland was homogenized in cold 0.10 *M* phosphate buffer, pH 7.2 (1 g. of trimmed gland/10 ml. of buffer) and the homogenate centrifuged at 5,000 r.p.m. at 0° for 10 min. After the centrifugation, the cellular debris and the fatty layer were carefully removed and the remaining supernatant fluid was decanted into a cold flask. This supernatant consisted of mitochondria, microsomal, and phosphate-soluble material of the chicken uropygial gland. It was used immediately for the metabolic investigations. The only aseptic conditions used during this investigation were during the removal of the uropygial gland and the use of sterile phosphate buffer for homogenization of the gland.

**Incubation**—Eleven milliliters of the supernatant fluid was used per flask. Thirty micrograms of androst-4-ene-3,17-dione-4-$^{14}$C, (200,000 c.p.m.) and 2 mg. each of triphosphopyridine nucleotide (TPN), citrate, and isocitrate were added to the supernatant fluid. The pH was readjusted to 7.2 and 1 ml. was removed and examined for bacteria. Penicillin (100,000 units) and streptomycin (166 mg.) were then added to the remainder and this mixture was incubated in a Warner-Chilcott

model 2156 water bath at 37° for 24 hr. Control mixtures lacking supernatant fluid or androst-4-ene-3,17-dione-4-$^{14}$C or antibiotics were prepared and incubated simultaneously. Experimental tubes were prepared either in duplicate or triplicate. After incubation, all tubes were checked for the presence of bacteria. Identification of the steroid metabolites was made only on tubes which were free of bacteria. The purity of the substrate, androst-4-ene-3,17-dione-4-$^{14}$C, was rechecked immediately before its addition to the incubation medium by TLC on silica gel as previously described. The radiochromatogram scan of the radioactive substrate demonstrated only one radioactive peak, thereby indicating that the substrate was chemically pure.

**Extraction and Identification of Steroid Metabolites**—The materials and methods for the extraction and identification of the steroid metabolites are standard procedures which have been previously reported (1, 3). Procedures used for the identification of the steroid metabolites were TLC, GLC, and crystallization to constant specific activity.

**Isolation of Bacteria**—An aliquot of the supernatant fluid, used as the enzyme source, was examined for the presence of bacteria by flooding 1 ml. on the surface of a blood agar plate. The plate was incubated at 37° for 24 hr. The large number of colonies present could be divided on the basis of colonial morphology into four distinct types. Samples of each different colony type were isolated and transferred to brain heart infusion for identification.

**Enumeration of Bacterial Populations**—A series of dilutions of the supernatant fluid, ranging from $10^1$ to $10^7$, were prepared through a 10-fold sequence. One-milliliter portions of the dilutions were flooded on the surface of agar plates prepared as described by Hentges (4) and incubated at 37° for 24 hr. Bacterial colonies that developed on the agar were counted with an electronic colony counter.

The population sizes of a 24-hr. broth suspension of each isolated organism were determined by the same technique. Since the suspensions presented counts considerably higher than those obtained from the supernatant fluid, each was diluted with sterile saline so that the number obtained by plate counts was comparable to that previously determined for the supernatant fluid. These concentrations of organisms were used for antibiotic sensitivity studies.

**Determination of Survival Time of Isolated Bacteria in Antibiotic Broth**—Ten milliliters of each suspension of organisms received 100,000 units of penicillin and 166 mg. of streptomycin. These concentrations correspond with the experimental conditions used in the initial work of Rongone *et al.* (1). The suspensions were incubated at 37° in a water bath shaker, and at 0.5-hr. intervals 1-ml. samples were removed and flooded on the surface of dried agar plates which were incubated at 37° for 24 hr.

**Antibiotic Susceptibility of Isolated Bacteria**—*Test Tube Serial Dilution Technique*—The technique as described in Bailey and Scott (5) was used with final antibiotic concentrations of 250, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.2, and <0.2 units or mcg./ml. of broth culture medium. Each tube was inoculated with the isolated bacteria and the series incubated at 37° for 18–24 hr. Tubes were examined macroscopically for evidence of turbidity and/or sediment. The lowest concentration of the antimicrobial agent which prevented growth was considered as the minimal inhibitory concentration (MIC) and expressed as units or mcg./ml.

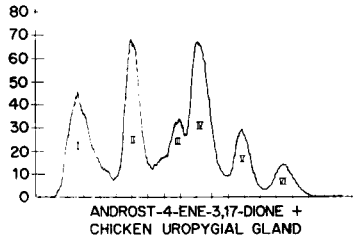


*Fig. 1—A radiochromatogram scan of a residue obtained from an incubation mixture from cockerel glands including penicillin and streptomycin, phosphate buffer, and androst-4-ene-3,17-dione.*

## RESULTS AND DISCUSSION

**Steroid Metabolism**—Analysis of control mixtures, which were devoid of the enzyme preparation, by spectrophotometric procedures at 240 mμ, resulted in recovery of 96–98% of added androst-4-ene-3,17-dione-4-$^{14}$C. UV analysis at 240 mμ of the residue obtained from the incubation medium containing substrate, cofactors, and enzyme preparation demonstrated that 65% of the conjugated system in Ring A was destroyed during the incubation procedure. This compared favorably with the metabolism of testosterone (3) by the homogenates of the chicken uropygial gland suggesting that the compounds are metabolized at a similar rate. Although maximum metabolism of steroids by the supernatant fluid obtained from the homogenates of the chicken uropygial gland under the present conditions appeared to require from 6–8 hr., as indicated by spectrophotometric analysis, 24 hr. was chosen as a matter of convenience and to insure that metabolic reactions would proceed to their last product in the isolated pathways. Only the C-19 steroids identified which possessed radioactivity were considered to be metabolites. Figure 1 demonstrates a radiochromatogram scan of a residue obtained from an incubation mixture containing the supernatant fluid, penicillin, streptomycin, phosphate buffer, and androst-4-ene-3,17-dione-4-$^{14}$C on a thin-layer plate. The numbered peaks represent the different radioactive products present in the residue. The numbered peaks in this figure correspond to the same Roman numerals in Figs. 2–4. Table I shows the $R_f$ values of the radioactive peaks

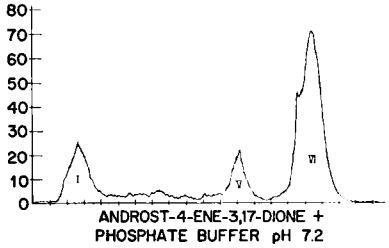


*Fig. 2—A radiochromatogram scan of a residue obtained from an incubation mixture which contained phosphate buffer and androst-4-ene-3,17-dione only.*

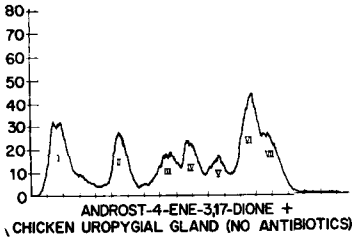


*Fig. 3—A radiochromatogram scan of a residue obtained from an incubation mixture which contained cockerel glands, phosphate buffer, androst-4-ene-3,17-dione, but no antibiotics.*

in Figs. 1–4. It can be seen that the $R_f$ values of the corresponding numbered peaks are not identical in all instances. This may be readily explained in that thin-layer plate chromatography is not as reproducible as other methods of chromatography. Therefore, in order to determine whether the given numbered peaks were identical or similar to each other, the radioactive peaks were eluted from the thin-layer plates. Aliquots of corresponding numbers from each plate were mixed and rechromatographed as before. In all instances only one radioactive peak was detected demonstrating that the peaks with identical numbers were similar to each other. Figure 2 is a radiochromatogram of a control sample of androst-4-ene-3,17-dione-4-$^{14}$C without enzyme or antibiotics. It is obvious that there is a small amount of natural steroid breakdown which occurs during the incubation or extraction procedure since only Peak VI was present in the radiochromatogram scan of the substrate immediately prior to its addition to the incubation medium. Therefore, Peaks I and V were not considered to be enzymatic metabolites of pregnenolone. It is readily observed from Fig. 1 that three radioactive metabolites were partially resolved, two of which have been identified by gas chromatography procedures. Figure 3 demonstrates a radiochromatogram of a residue obtained from an incubation mixture from cockerels which contained no antibiotics. It is readily seen that the relative heights of the peaks in Fig. 1 differ from those of Fig. 3 indicating that bacterial enzymes or bacterial toxins may be the cause of this difference. The possibility also exists that the antibiotics used in this experiment may have been responsible for the slight difference between Figs. 1 and 3. Therefore, an incubation was performed in which the steroid substrate was incubated with phosphate buffer and

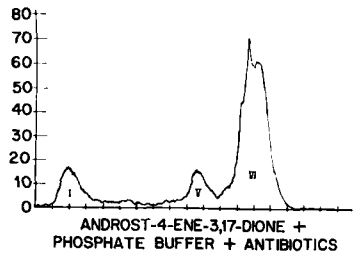


*Fig. 4—A radiochromatogram scan of a residue obtained from an incubation mixture which contained androst-4-ene-3,17-dione, phosphate buffer, penicillin, and streptomycin.*

TABLE I—THE $R_f$ VALUES OF THE RADIOACTIVE PEAKS

| Peak Number | Fig. 1, $R_f$ | Fig. 2, $R_f$ | Fig. 3, $R_f$ | Fig. 4, $R_f$ |
|---|---|---|---|---|
| I | 0.15 | 0.10 | 0.11 | 0.11 |
| II | 0.47 | — | 0.35 | — |
| III | 0.66 | — | 0.47 | — |
| IV | 0.75 | — | 0.58 | — |
| V | 0.89 | 0.67 | 0.70 | 0.78 |
| VI | 0.97 | 0.91 | 0.90 | 0.95 |

penicillin and streptomycin (Fig. 4). Figure 4 demonstrates that under the experimental conditions, the antibiotics used *per se* had no effect on the metabolism of androst-4-ene-3,17-dione-4-$^{14}$C since Figs. 2 and 4 are almost identical. Fabian (6) has shown that strains of *Staphylococcus aureus* are capable of reducing the C-17 carbonyl of androst-4-ene-3,17-dione.

Table II shows the relative retention times to cholestane of authentic steroids and the radioactive metabolites which were isolated by gas chromatographic techniques on 1% NGS columns. The table demonstrates that the relative retention times of the isolated metabolites are identical or almost identical to those of the authentic steroids. Table III shows the steroid numbers of the authentic steroids and the isolated steroid metabolites calculated from data obtained by gas chromatographic techniques on 1% SE-30 columns. The table demonstrates that the steroid numbers of the isolated metabolites are identical or almost identical to those of the authentic steroids.

This table also demonstrates, without doubt, that the supernatant fluid obtained from homogenates of chicken uropygial gland is capable of converting androst-4-ene-3,17-dione into 3$\alpha$-hydroxy-5$\alpha$-androstane-17-one and 5$\alpha$-androstane-3$\alpha$, 17$\beta$-diol. Further confirmation of the identification of the two metabolites was obtained by crystallization to constant specific activity techniques.

It was previously demonstrated that the homogenates of the chicken uropygial gland are capable of oxidizing a C-17 hydroxyl to a C-17 carbonyl. This investigation demonstrated the presence of a 17$\beta$-hydrogenase. It also demonstrated that the homogenates of the uropygial gland from cockerels and hens destroyed equally well the conjugated system in Ring A of androst-4-ene-3,17-dione-4-$^{14}$C.

TABLE II—IDENTIFICATION OF PRODUCTS FROM REDUCTION STUDIES. RELATIVE RETENTION TIMES OF C-19 STEROIDS TO CHOLESTANE ON 1% NGS

| Compd. | Androst-4-ene-3,17-dione-4-$^{14}$C | 3$\alpha$-Hydroxy-5$\alpha$-androstane-17-one | 5$\alpha$-Androstane-3$\alpha$,17$\beta$-diol |
|---|---|---|---|
| TMSi[a] | | | |
| Authentic | — | 0.915 | 0.312 |
| Isolated | — | 0.915 | 0.312 |
| O.M.O.[b] | | | |
| Authentic | 2.88<br>3.26 | 2.75 | — |
| Isolated | 2.86<br>3.23 | 2.74 | — |
| D.D.[c] | | | |
| Authentic | — | 0.625 | — |
| Isolated | — | 0.625 | — |

[a] TMSi = Trimethylsilyl ether derivatives. [b] O.M.O. = o-methyloxime derivatives. [c] D.D. = Double derivatives of trimethylsilyl ether and o-methyloxime.

TABLE III—STEROID NUMBERS FOR COMPOUNDS IDENTIFIED FROM INCUBATION MIXTURES[a]

| Compd. | Androst-4-ene-3,17-dione-4-$^{14}$C | 3α-Hydroxy-5α-androstane-17-one | 5α-Androstane-3α,17β-diol |
|---|---|---|---|
| Free | | | |
| Authentic | 24.57 | 23.50 | 23.65 |
| Isolated | 24.54 | 23.47 | 23.64 |
| TMSi | | | |
| Authentic | — | 23.60 | 24.51 |
| Isolated | — | 23.62 | 24.50 |
| O.M.O. | | | |
| Authentic | 25.62 | 24.20 | — |
| Isolated | 25.59 | 24.20 | — |
| D.D. | | | |
| Authentic | — | 24.30 | — |
| Isolated | — | 24.31 | — |

[a] Steroid numbers are obtained from data resulting from chromatography on 1% SE-30 columns. Steroid numbers were determined by the method of Vanden Heuvel and Horning (7).

Again it is of interest to note that the only metabolites identified belong to the 5α-androstane family. This tends to suggest that the formation of the 5α-androstane series is the major route of metabolism for steroids, if not the only route, by the homogenates of the chicken uropygial gland.

**Identification of Isolated Bacteria**—By means of colonial morphology and microscopic examination the bacteria present in the supernatant fluid were identified as: (*a*) *Staphylococcus aureus*, (*b*) *Staphylococcus epidermidis*, (*c*) aerobic spore-forming bacilli, and (*d*) Gram-negative pleomorphic rods (morphology highly variable).

*S. aureus* and the spore-forming bacilli were β-hemolytic, whereas *S. epidermidis* and the Gram-negative rods were nonhemolytic. The coagulase test revealed *S. aureus* to be coagulase-positive and *S. epidermidis* coagulase-negative.

It should be noted, that aseptic conditions were used to remove the chicken uropygial gland to prepare it for metabolic investigations. The bacteria isolated could possibly have been laboratory or environmental contaminants rather than part of the normal flora of the supernatant fluid.

**Survival Time**—This portion of the project was concerned with the effect of antibiotics on the survival of isolated bacteria. Approximately 2,200/ml. of each of the species of isolated bacteria was used to inoculate brain heart infusion containing the antibiotics.

The 2,200 bacteria/ml. was chosen as the bacterial concentration for the survival time study because at the end of the incubation period the total bacterial count was 2,200/ml. It was observed that this bacterial contamination was found in approximately 80% of the incubating tubes. One-half hour after inoculation, no viable bacteria could be isolated from any of the samples. This indicates that the concentration of antibiotics used with these suspensions rapidly destroys any contaminating bacteria.

**Antibiotic Susceptibility**—*Test Tube Serial Dilution Technique*—This quantitative method was used to determine as accurately as possible susceptibilities of the cultures to measured amounts of penicillin and streptomycin. The data presented in Table IV are based on the minimum concentrations of the above-mentioned antibiotics inhibiting growth of the organisms under the experimental conditions used. It can be seen that a minimum concentration of 100 units/ml. of penicillin and 250 mcg./ml. of streptomycin were sufficient to destroy any organisms isolated.

TABLE IV—MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTICS AGAINST ISOLATED ORGANISMS

| Organisms | Penicillin, units/ml. | Streptomycin, mcg./ml. |
|---|---|---|
| *Staphylococcus aureus* | 0.20 | 3.12 |
| *Staphylococcus epidermidis* | 1.56 | 50.00 |
| Aerobic spore-forming bacilli | 6.25 | 6.25 |
| Gram-negative rods | 100.00 | 250.00 |

The addition of excessive amounts of antibiotic mixture to the supernatant fluid insured favorable conditions for metabolic investigations. Although the concentrations used were far in excess of the concentrations necessary for inhibition of all isolated bacteria, the large amounts of added antibiotics had no apparent metabolic effect under the experimental conditions of this investigation.

In prolonged incubations of biological materials, bacterial growth may significantly affect metabolic results. These bacterial effects may be avoided by determining and using the lowest amounts of antibiotics required to suppress the growth of likely contaminants.

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### Keyphrases

Androst-4-ene-3,17-dione-4-$^{14}$C—*in vitro* metabolism
Uropygial gland homogenates, chicken—17β-hydrogenase
Bacterial contamination—uropygial gland homogenates
Antibiotic addition—uropygial gland homogenates
TLC—separation
GLC—separation, identity
Radiochromatography—identity
UV spectrophotometry—analysis