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Transformations of Steroids by Cell-free Preparations of *Penicillium lilacinum* NRRL 895. IV. Enzyme Catalyzed Acyl Transfer

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The cleavage of testosterone acetate by cell-free preparations from *P. lilacinum* was stimulated up to 2.3-fold by the addition of equimolar amounts of testosterone. Testosterone was found to be an acceptor of acyl groups derived from the acetyl esters of testosterone, epitestosterone, oestrone, epiandrosterone and desoxycorticosterone and from testosterone propionate. Epitestosterone inhibited the cleavage of testosterone acetate and did not act as an acyl acceptor to any significant degree. 4-Androstene-3,17-dione slightly stimulated the cleavage of testosterone acetate, probably acting as a modifier molecule.

Whereas the hydrolysis of steroid esters by microorganisms is well documented, reports on microbial esterification of hydroxysteroids seem to be less frequent.[1] Transformation of 4-androstene-3,17-dione into testosterone and testosterone acetate by *Saccaromyces fragilis* was demonstrated by McGuire *et al.*[2] Capek and co-workers showed that of 45 *Endomycetes* only the lactose fermenting species *S. fragilis*, *S. lactis*, *Candida pseudotropicalis*, and *Torulopsis sphaericus* were capable to acetylate testosterone. Steroids with 11α, 11β, 17α (sec), 20β, and 21-hydroxyls were not acetylated.[3] 3β-Hydroxy-C_{19}-steroids have been converted into the corresponding 3β-acetoxy compounds by *Penicillium oxalicum*.[17] 21-Acetylation of the acetonide of 9α-fluoro 16α-hydroxyhydrocortisone was demonstrated in *Trichoderma glaucum* by Holmlund *et al.*[4] Esterification of sterols with fatty acids and with succinic acid has been demonstrated in *Mycoplasma*, intestinal bacteria and *Mycobacteria*[5–8] *Aspergillus flavus* has been shown to convert oestrone into its sulphate.[9]

In the cases mentioned above, the acyl moiety was appearantly not derived from the steroid structure. The microbial formation of 17β acetates from 20-oxo-C_{21}-steroids is a result of a Baeyer-Villiger type of oxidation and must not be considered as an esterification.[1]

The steroid inducible esterase activity in cell-free preparations from *P. lilacinum* readily cleaves the ester bond of testosterone acetate.[10] When this reaction was studied, the product testosterone was found not to inhibit but instead to stimulate the reaction and to act as an acceptor of the acetyl group. The present communication describes transacylations carried out by esterase-active cell free preparations from *P. lilacinum* and the influence of some Δ^4-3-oxo-C_{19} steroids upon the esterase activity in such preparations.

MATERIALS AND METHODS

Abbreviations and trivial names. GLC: gas liquid chromatography; GC-MS: gas chromatography-mass spectrometry; TLC: thin layer chromatography; Desoxycorticosterone: 21-hydroxy-4-pregnene-3,20-dione; Epiandrosterone: 3β-hydroxy-5α-androstan-17-one; Epitestosterone: 17α-hydroxy-4-androsten-3-one; Oestrone: 3-hydroxy-1,3,5,(10)-oestratrien-17-one; Progesterone: 4-pregnene-3,20-dione; Testosterone: 17β-hydroxy-4-androsten-3-one.

Radioactive chemicals. [^{3}H]-Acetic anhydride (specific activity 0.050 Ci/mmol), [4-^{14}C]-4-

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androstene-3,17-dione (specific activity 0.0588 Ci/mmol), [7-^{3}H]-testosterone (specific activity 25 Ci/mmol) and [1,2-^{3}H]-epitestosterone (specific activity 49 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. The steroids were purified by TLC before use. Acetates of radioactive steroids or with ^{3}H-labelled acetyl groups were prepared by acetylation with acetic anhydride in pyridine, followed by purification by TLC. In the enzyme experiments the solutions of radioactive steroids were diluted with unlabelled steroids to give a final radioactivity corresponding to approx. 300 cpm per 0.3 μmol steroid in 10 μl of ethanol for desoxycorticosterone-[^{3}H] acetate and 10 000 – 50 000 cpm for the other steroids.

Unlabelled steroids. 4-Androstene-3,17-dione, desoxycorticosterone acetate, epiandrosterone acetate, progesterone, testosterone and its acetate and propionate were obtained from Sigma Chemical Co., St Louis, Mo. Epitestosterone and its acetate were obtained from Steraloids Inc., Pawling, N.Y. and desoxycorticosterone from Ikapharm, Ramat-Gan, Israel. They were checked for purity by TLC and/or GLC.

Other chemicals. All other chemicals were of reagent grade and treated as described previously.[10]

Chromatographic systems. TLC was carried out on Silica gel GF_{254} (system 1)[11] and on Al_2O_3 GF_{254} (0.5 % ethanol in benzene as solvent). GLC on OV-17 and GC-MS were performed as previously described.[11]

Growth of organism and preparation of cell free extracts. P. lilacinum NRRL 895 was grown on Czapek-Dox medium, induced, washed and frozen as described previously.[11] Progesterone was used as induced (70 mg in 2 ml of dimethylformamide per 300 ml of culture). 0.06 M Tris-HCl pH 7.2 was used as buffer in the washing of the cells and in the preparation of cell free extracts.

Cell free extracts were prepared using high speed grinding with glass beads according to Hedenskog *et al.*[12] All procedures were carried out at +4°C. 7 – 9 g of frozen cells were thawed and suspended in buffer to a final volume of 21 ml. The suspension was ground for 30 min with an equal volume of glass beads (diameter 0.45 – 0.50 mm) in a 28 mm i.d. glass tube with a loop stirrer of 20 mm diameter, rotating at approx. 1500 – 2000 rpm. The supernatant was decanted and the beads were washed with two small portions of buffer which were combined with the first supernatant. Cell debris was removed by centrifugation at 6000 rpm for 15 min, yielding about 30 ml of crude supernatant. After centrifugation at 100 000 *g* for 60 min, the resulting supernatant was frozen in suitable aliquots at −22°C. After thawing at room temperature the extract was centrifuged at 6000 rpm for 15 min in order to remove inactive protein precipitated during the freezing and thawing procedure. The clear extract was diluted with 0.06 M Tris-HCl pH 7.2 (usually to a fourfold dilution) and used in the enzyme experiments.

Incubation of steroids with cell-free extracts. The procedures for incubation and for extraction of the reaction mixture were the same as those previously described.[13] Due to short reaction times (2 min) the incubations were carried out on a vortex mixer. Each steroid was added in 10 μl of ethanol and in each series the ethanol concentration was equalized. The enzyme solution was preincubated with the steroid additive, usually for 45 s (for technical reasons), and the steroid ester substrate was then added. All incubations were carried out in duplicates.

The esterase activity in these preparations is unstable at room temperature, loosing approx. 10 – 20 % of its original activity in 40 – 60 min.[10] Therefore reference incubations with enzyme and steroid ester were run at the beginning and at the end of the series. The decrease in esterase activity was assumed to be linear and was corrected for in the calculations.

Steroid analysis. The relative amount of steroid alcohol and steroid ester was determined by TLC and liquid scintillation counting as described previously.[13] It was checked that side reactions (*e.g.* alcohol dehydrogenation) did not take place to any detectable degree. The variation between individual samples in duplicate incubations was calculated as $S.D. = (\sum d^2/2N)^{\frac{1}{2}}$. In two series, one with a mean testosterone concentration of 31.6 mol % (4.2 – 49.3 %, N = 61) and another with a mean concentration of 80.3 mol % (51.9 – 95.8 %), N = 20) the S.D. was ± 1.5 mol %.

Identification of steroids. The fractions containing [7-^{3}H] testosterone acetate formed by acetyl transfer from unlabelled steroid acetates (with exception of epitestosterone acetate) were isolated individually by TLC on Silica gel GF_{254}, rechromatographed on Al_2O_3 GF_{254} and analyzed by GC-MS and GLC using OV-17 as stationary phase. Crystallization to constant specific activity was also carried out after addition of 30 mg of carrier testosterone acetate. The [7-^{3}H]testosterone acetate fractions obtained from the acetates of desoxycorticosterone, epiandrosterone and oestrone were pooled and crystallized. The [7-^{3}H]testosterone acetate obtained from [7-^{3}H]testosterone and unlabelled testosterone acetate was crystallized separately.

Due to difficulties in the separations of the 17-epimeric acetyl esters, the [7-^{3}H]testosterone acetate obtained by transacetylation from unlabelled epitestosterone acetate was only tentatively identified by its TLC R_F-value.

[7-^{3}H] testosterone propionate obtained by transacylation from non radioactive testosterone propionate was isolated by TLC on Silica gel GF_{254} and by rechromatography on Al_2O_3 GF_{254}. In the latter system the acetates and propionates of testosterone separated fairly

Table 1. Transacylation of [7-^{3}H]testosterone by cell-free preparations from *P. lilacinum*.

Experiment	Acyl donor	% of [7-^{3}H]testosterone transacylated
1[a]	Testosterone acetate	19.8
1[a]	Testosterone propionate	4.9
1[a]	Desoxycorticosterone acetate	6.3
1[a]	Epiandrosterone acetate	10.4
1[a]	Oestrone acetate	21.0
2[a]	Testosterone acetate	20.5
2[b]	Epitestosterone acetate	4.2

[a] Initial concentration of [7^{3}H]testosterone 3.47×10^{-4} M, of steroid ester 3.04×10^{-4} M, preincubation time 45 s, incubation time 2.0 min. Temp. 30°C. Hydrolysis of [7-^{3}H] testosterone acetate without additive 31.2 %. [b] Conditions as in Note *a*, hydrolysis of [7-^{3}H] testosterone acetate and [1,2-^{3}H]-epitestosterone acetate without additive 36.2 and 30.8 %, respectively.

well (R_F-value for testosterone acetate 0.45, for testosterone propionate 0.53). The propionate was identified by crystallization to constant specific activity.

RESULTS

Acylation of testosterone. When [7-^{3}H]testosterone was incubated together with steroid acetates and with testosterone propionate, transfer of the acyl group from the ester substrate to testosterone could be demonstrated (Table 1). All the esters used are hydrolyzed by the esterase preparation.[10] TLC, GLC, and GC-MS properties for the testosterone acetate and propionate formed by transacylation were identical to those of authentic reference compounds. The results from the crystallizations to constant specific radioactivity are shown in Table 2.

When desoxycorticosterone-[^{3}H]acetate was incubated together with unlabelled testosterone, significant amounts of radioactivity could be recovered only in the testosterone acetate and desoxycorticosterone acetate fractions.

Ethyl acetate and sodium acetate did not serve as acetyl donors, even after prolonged incubation (20 min). Boiled extracts did not catalyze transacylations.

When [1,2-^{3}H]epitestosterone was incubated with enzyme preparation and testosterone acetate or epitestosterone acetate, significant amounts of [1,2-^{3}H]epitestosterone acetate could not be detected.

Effect of Δ^4-3-oxo-C_{19} steroids on the cleavage of [7-^{3}H]testosterone acetate and [1,2-^{3}H]epitestosterone acetate. The cleavage of testosterone acetate was clearly stimulated by testosterone and to a less degree also by 4-androstene-3, 17-dione (Tables 3–5). It was inhibited by epitestosterone (Tables 3 and 5). The hydrolysis of epitestosterone acetate was inhibited by epitestosterone and to a smaller degree also by testosterone. It was slightly stimulated by 4-androstene-3,17-dione (Table 3).

The increase in testosterone acetate cleavage depended on the amount of initially added free testosterone (Table 4). The figures clearly indicate that the effect was due to alcoholysis (transacylation) and thus the net amount of tritiated + unlabelled testosterone acetate in the mixture was not significantly affected. In

Table 2. Results from crystallizations to constant specific activity of [7-^{3}H]testosterone acetate and [7-^{3}H]testosterone propionate obtained by transacylation from unlabelled steroid esters.

Crystallization	Specific activity, cpm/mg		
	[7^{3}H]Testosterone acetate from unlabelled testosterone acetate	[7^{3}H]Testosterone acetate from other unlabelled steroid acetates (pool)	[7^{3}H]Testosterone propionate
First	220	194	33.5
Second	226	191	30.5
Third	214	191	31.2

Table 3. Effect of Δ^4-3-oxo-C_{19} steroids on the cleavage of [7-^{3}H]testosterone acetate and [1,2-^{3}H]epitestosterone acetate by cell-free preparations from *P. lilacinum*.

Experiment [a]	Additive [b]	% relative ester cleavage
3 T	none	100 (14.6 % hydrolysis)
3 T	testosterone	226
3 T	epitestosterone	56
3 T	4-androstene-3,17--dione	170
3 E	none	100 (13.6 % hydrolysis)
3 E	testosterone	86
3 E	epitestosterone	66
3 E	4-androstene-3,17--dione	115

[a] Initial concentration of [7-^{3}H]testosterone acetate (3T) or [1,2-^{3}H]epitestosterone acetate (3E) 3.04×10^{-4} M, incubation time 2.0 min, temp. 26°C. [b] Initial concentration 3.47×10^{-4} M. Preincubation time 45 s.

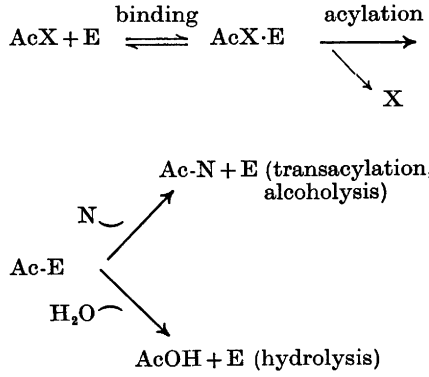
separate experiments the effect was found to be independent upon preincubation time (0 – 600 s).

Addition of equimolar amounts of 4-androstene-3,17-dione together with the 17-hydroxysteroids did not significantly influence the effects of testosterone (transacylation or stimulation of [7-^{3}H]testosterone acetate cleavage). The inhibitory effect of epitestosterone was slightly diminished by 4-androstene-3,17-dione (Table 5). When [4-^{14}C]4-androstene-3,17-dione was incubated with unlabelled testosterone acetate, no radioactivity could be detected in the testosterone acetate fraction. It was also found that the 17-oxo-group of [4-^{14}C]4-androstene-3,17-dione was not reduced in this system.

DISCUSSION

Acyl group transfer catalyzed by hydrolytic enzymes is well known.[14] However, transacylation between steroid esters and alcohols does not seem to have been reported to occur in microbial steroid metabolism.

The increase in testosterone acetate cleavage caused by testosterone is appearently due to "alcoholysis" since it corresponds to the amount of transacetylated steroid alcohol (Table 4). It is generally assumed that the enzyme catalyzed ester cleavage starts with a rapid binding step and proceeds *via* an acyl enzyme intermediate according to the following equation:[14]



where AcX is the ester, E the enzyme, AcX·E the enzyme substrate complex, X the alcohol, Ac – E the acyl-enzyme intermediate and N a nucleophile such as testosterone. If the acylations step is fast, the acyl-enzyme can be supplied as fast as it reacts further. Thus the rate of ester cleavage will be determined by the deacylation steps, *i.e.* hydrolysis or alcoholysis. The total rate of ester cleavage will increase linearly with increasing concentration of nucleophile.[15] The results presented in Table 4 clearly indicate that this is the case for the testosterone acetate cleavage.

The activation caused by 4-androstene-3,17-dione is probably analogous to the modifier action of acetone and other non hydroxylic compounds on esterases from pig and beef liver.[14,15] Greenzaid and Jencks found that nucleophiles such as methanol as well as modifier molecules (acetone, dioxane) increased the activity of pig liver esterase.[15] However, the modifiers were found to specificially suppress the degree of methanolysis. From this the authors postulated an alcohol binding site which might be occupied by the alcohol as well as by the modifier. The lack of effect of 4-androstene-3,17-dione on the transacylation

Table 4. Relation between increased cleavage of [7-^{3}H]testosterone acetate and transacylation of [7-^{3}H]testosterone by cell-free preparations from *P. lilacinum*.

Experiment	Substrate	Added testosterone or [7-^{3}H]testosterone, μmol	Increased in liberated [7-^{3}H]testosterone, μmol	Transacylated[7-^{3}H] testosterone, μmol
4[a]	[7-^{3}H]testosterone acetate	0.087	0.025	
»	»	0.174	0.033	
»	»	0.347	0.068	
»	testosterone acetate	0.087		0.020
»	»	0.174		0.034
»	»	0.347		0.073

[a] Initial steroid ester concentration 3.04×10^{-4} M, preincubation time 45 s, incubation time 2.0 min. Temp. 26°C. Hydrolysis of [7-^{3}H]testosterone acetate without additive 21.0 %.

of testosterone does not necessarily speak against the existence of such an alcohol binding site in the preparation from *P. lilacinum*. It might be due to differences in affinity towards the 17 β-alcohol and the 17-ketone.

Epitestosterone was found to suppress the hydrolysis of testosterone acetate as well as of epitestosterone acetate. It was also not possible to demonstrate transacylation of epitestosterone. This latter finding can be easily explained by the steric differences between the epimeric 17-alcohols. Wynne and Shalitin have studied the influence of the structure of alcohols upon their action of beef liver esterase activity.[16] They assumed the alcohol binding site to be a narrow cleft "padded" with hydrophobic groups. This will fit well with the different behaviour of the two epimeric 17-hydroxysteroids: see Fig. 1. The less favourable configuration of the 17α-hydroxyl might also

Table 5. Influence of the addition of 4-androstene-3,17-dione together with testosterone or epitestosterone.

Experiment[a]	Substrate	Additive	% relative ester cleavage	% of [7-^{3}H]testosterone transacylated
5	[7-^{3}H]testosterone acetate	testosterone	183	
»		4-androstene-3,17-dione	126	
»		testosterone + 4-androstene-3,17-dione	195	
»		epitestosterone	45	
»		epitestosterone + 4--androstene-3,17-dione	70	
»	testosterone acetate	[7-^{3}H]testosterone		11,2
»		[7-^{3}H]testosterone + 4-androstene-3,17-dione		9,2

[a] Initial concentration of additive 3.47×10^{-4} M, of steroid ester 3.04×10^{-4} M. Preincubation time 45 s, incubation time 2.0 min. Temp. 24°C. Hydrolysis of [7-^{3}H]testosterone acetate without additive 9.4 % (= 100 % relative ester cleavage).

explain its resistence towards enzymatic acetylation by yeasts as shown by Capek *et al.*[3]

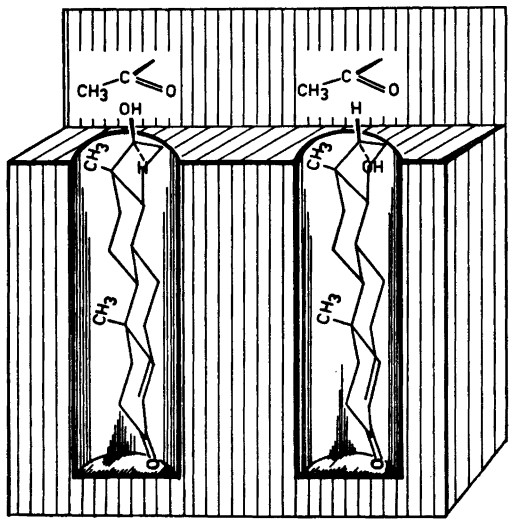


Fig. 1. Differences between testosterone and epitestosterone in reactivity: The 17β-hydroxyl group has a far more favourable position towards the acetyl group in the acetyl-enzyme complex.

While the nucleophile testosterone does not affect the hydrolysis of testosterone acetone to any considerable degree but stimulates the total disappearance rate by alcoholysis, its action upon the cleavage epitestosterone acetate is quite different. Transacylation of testosterone from epitestosterone acetate occurs to only about one fifth of that observed in the testosterone-testosterone acetate system. The hydrolysis is partly inhibited and this results in a slight suppression of the total cleavage rate. Similar observations have been made previously by Greenzaid and Jencks, using highly purified preparations of pig liver esterase.[15] While the addition of methanol causes a slight suppression of the hydrolysis of phenyl acetate but an increase on total cleavage due to alcoholysis, the hydrolysis of smaller acetates such as methyl acetate was greatly suppressed which in some cases resulted in a decrease of the total cleavage rate. These findings together with differences in response to organophosphorus inhibitors led to the conclusion that at least two different types of active sites with differences in substrate specificity must exist. This might also be true for the esterase active preparations from *P. lilacinum*, especially when its adaptive nature is considered. It must also be kept in mind that this preparation is rather crude and might contain several esterase active proteins with different substrate specificity.

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REFERENCES

1. Charney, W. and Herzog, H. L. *Microbial Transformations of Steroids, A Handbook*, Academic, New York and London 1967.
2. McGuire, J. S., Maxwell, E. S. and Tomkins, G. M. *Biochim. Biophys. Acta 45* (1960) 392.
3. Capek, A., Tadra, M. and Tuma, J. *Folia Microbiol. (Prague) 9* (1964) 380.
4. Holmlund, C. E., Feldman, L. I., Rigler, N. E., Nielsen, B. E. and Evans, R. H. *J. Amer. Chem. Soc. 83* (1961) 2568.
5. Smith, P. F. *J. Bacteriol. 77* (1959) 682.
6. Lynn, R. J. and Smith, P. F. *Ann. N. Y. Acad. Sci. 79* (1960) 493.
7. Rosenfeld, R. S. *Arch. Biochem. Biophys. 112* (1965) 621.
8. Schubert, K. and Kaufmann, G. *Biochim. Biophys. Acta 106* (1965) 592.
9. Schubert, K., Kaufmann, G. and Hörhold, C. *Biochim. Biophys. Acta 176* (1969) 163.
10. Carlström, K. and Krook, K. *Acta Chem. Scand. 27* (1973) 1240.
11. Carlström, K. *Acta Chem. Scand. 26* (1972) 1718.
12. Hedenskog, G., Enebo, L., Vendlova, I. and Prokes, B. *Biotech. Bioeng. 11* (1969) 37.
13. Carlström, K. *Acta Chem. Scand. 27* (1973) 1622.
14. Krisch, K. *Carboxylic Ester Hydrolases (Review)*. In Boyer, P. D., Ed., *The Enzymes*, 3rd Ed., Academic, New York and London 1971, Vol. V, p. 43.
15. Greenzaid, P. and Jencks, W. P. *Biochemistry 10* (1971) 1210.
16. Wynne, D. and Shalitin, Y. *Eur. J. Biochem. 31* (1972) 554.
17. Krachy, S. and Mizuba, S. *U.S. Pat. 3597, 320, 03* Aug. 1971.

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