BIOSYNTHESIS OF 5-HYDROXYTETRACYCLINE

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In a recent report (Miller et al., 1964) evidence was presented for a common pathway in the biosynthesis of the tetracycline antibiotics. It was shown that Streptomyces rimosus T1686B can under certain circumstances accumulate late intermediates involved in the biosynthesis of TC*, OTC and DMTC. In that report the terminal reactions of tetracycline biosynthesis were carried out with cell-free extracts of Streptomyces aureofaciens. We have now extended those studies to include reactions specifically required for the biosynthesis of OTC and have determined the branching point for the TC and OTC biosynthetic pathways. In these studies we have used cell-free sonic extracts of Streptomyces rimosus ATCC 13224 which carry out the sequence of reactions involved in the biosynthesis of OTC from ATC. The requirements for these reactions are given in Table I.

The data show that the requirements for the conversion of ATC to OTC by cell-free extracts of S. rimosus are O_2 and NADPH. Addition of an external source of glucose-6-phosphate dehydrogenase was unnecessary.

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*Abbr. used: TC, tetracycline; OTC, 5-hydroxytetracycline; DMTC, 6-demethyltetracycline; DMCTC, 6-demethyl-7-chlorotetracycline; ATC, anhydrotetracycline; DHTC, 5a(11a)-dehydrotetracycline; AOTC, 5-hydroxy-anhydrotetracycline; DOTC, 6-deoxytetracycline; DHOTC, 5a(11a)-dehydro-5-hydroxytetracycline; DOOTC, 6-deoxy-5-hydroxytetracycline; DHCTC, 5a(11a)-dehydro-7-chlortetracycline; 5a-epi-TC, 5a-epi-tetracycline; CTC, 7-chlorotetracycline.

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Table I. Requirements for the Synthesis of OTC from ATC.

	OTC formed (μg/ml)
Complete system*	42
minus cell-free extract	0
" ATC	0
" $NADP^+$ + glucose-6-P	0
" O_2	0

*The complete system contained per ml: cell-free extract, 10 mg (dry wt.); tris buffer, pH 7.0, 100 μmoles; ATC·HCl, 100 μg; $NADP^+$, 0.5 μmoles; and glucose-6-P, 9.0 μmoles. Samples without oxygen were prepared in Thunberg tubes by evacuating and then flushing the tubes with argon. Samples were incubated on a shaker at 28° for 30 minutes and reactions were terminated with $HClO_4$ (0.7% final conc.). Bioassays were performed on portions of the supernatant; other portions were extracted with o-chlorophenol (0.25 ml/ml). These o-chlorophenol extracts were chromatographed in the following systems: (A) n-butanol:NH_4OH:water; 4:1:5 (Kelly and Buyske, 1960), strips were wetted with 0.1% ethylenediaminetetraacetic acid and dried before use; and (B) water-saturated n-butanol, strips were wetted with 0.1 M phosphate buffer, pH 7.0, containing 0.1% ethylenediaminetetraacetic acid and dried before use. Identication was by ultraviolet fluorescence and bioautography with Bacillus subtilis.

With this cell-free system it was feasible to investigate the biochemical pathway from ATC to OTC. The possibilities for a sequence of 2 oxidations and 1 reduction are shown in Figure 1. With the exception of DHTC and DHOTC, the detection and identification of which have not been reported, all the compounds in Figure 1 are known. We now report the isolation of microgram quantities of DHTC. DHTC was prepared from ATC using cell-free extracts of Streptomyces aureofaciens E-504*, a mutant derived from a DMCTC-producing strain. This mutant lacks a cofactor necessary for the reduction of the 5a,11a-double bond. When cell-free extracts of this organism were incubated with ATC (complete system in Table I) a new compound was produced (R_f 0.13 in system B). Microgram quantities of this compound were isolated by paper chromatography in

*Culture obtained from Chemical Production Section, Lederle Laboratories.

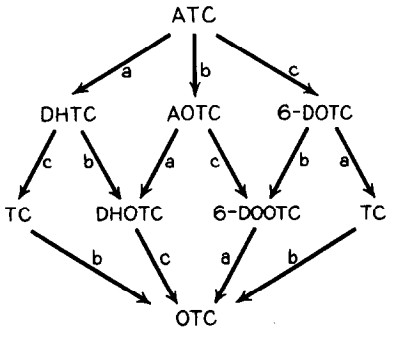

Figure 1. Possible pathways for the conversion of ATC to OTC. a, 6-hydroxylation; b, 5-hydroxylation; c, 5a,11a-reduction.

system B. The absorption spectrum resembles that of DHCTC (McCormick et al., 1958a) with a peak at approximately 400 mμ. The compound was quite unstable and attempts to crystallize it resulted in degradation. Catalytic hydrogenation (Pd/C) converted the compound to TC and 5a-epi-TC (McCormick et al., 1958a). With the cell-free system previously described by Miller et al. (1964) the compound was converted to TC with NADPH as the only requirement. These data are sufficient to establish that the R_f 0.13 component is DHTC.

With the exception of DHOTC, the compounds listed in Figure 1 were tested as possible intermediates in the biosynthesis of OTC. Results in Table II show that only ATC and DHTC were converted to OTC. Therefore, these data establish 6-hydroxylation as the first reaction in the sequence. The inability of TC to serve as substrate established 5-hydroxylation as the second reaction and, as a consequence, 5a,11a-reduction is the terminal reaction. The other pathways are excluded. DHTC is thus a common intermediate in the biosynthesis of both TC and OTC and this finding is consistent with the fact that DHCTC has been shown to be an intermediate in CTC biosynthesis (McCormick et al., 1958b).

Table II. Effects of Cell-Free Extracts of S. rimosus on Various Tetracycline Derivatives.

Substrate	OTC Produced
ATC	+
DHTC	+
AOTC	-
DOTC	-
DOOTC	-
TC	-

Reaction mixtures were the same as in Table I, except that the above substrates were used. o-Chlorophenol extracts were prepared and chromatographed in system A.

The results in Table II imply that DHOTC is an intermediate in the conversion of ATC to OTC. While it has not been possible to isolate DHOTC, evidence for its existence has been obtained. A reaction mixture (Table I, 2 mg ATC·HCl/10 ml) was incubated for 20 minutes after which proteins were removed with 4 volumes of acetone. Chromatography of the reaction mixture on sheets of Whatman 3 MM paper in system B revealed the presence of residual ATC (R_f 0.61), some OTC (R_f 0.44) and an intense band corresponding to DHTC (R_f 0.13). The R_f 0.13 material was eluted and catalytically hydrogenated (Pd/C). Chromatography of the reduction mixture (system A) revealed the presence of both OTC and TC. This synthesis of OTC and TC must mean that the R_f 0.13 material is a mixture of DHOTC and DHTC. The nonseparation of DHOTC and DHTC is expected inasmuch as OTC and TC do not separate in this system. These results provide direct evidence for the participation of both DHTC and DHOTC as intermediates in the biosynthesis of OTC.

From the data presented in Tables III and IV, it can be shown that NADPH and O_2 are needed for 5-hydroxylation, a requirement previously demonstrated for 6-hydroxylation by Miller et al. (1964). These requirements strongly suggest that the oxygens at C-6 and C-5 of the tetracycline antibiotics are both derived from molecular oxygen.

Table III. Stoichiometry of NADPH Requirements for the Terminal Reactions in the Biosynthesis of Tetracyclines.

Reaction	NADPH
	moles/mole product
(1) DHCTC ⟶ CTC	1.0 (1.0)
(2) ATC ⟶ TC	2.1 (2.0)
(3) ATC ⟶ OTC	2.7 (3.0)

Reaction (1) and (2) were carried out with cell-free extracts of S. aureofaciens; reaction (3), with S. rimosus. Conditions were similar to those described in Table I. Figures given for NADPH in parentheses are theoretical values.

Table IV. Requirements for the Synthesis of OTC from DHTC.

	Biologically active product
Complete system*	OTC
minus cell-free extract	none
" DHTC	none
" $NADP^+$ + glucose-6-P	none
" O_2	TC
" O_2 and $NADP^+$ + glucose-6-P	none

*The complete system was the same as that given in Table I except that the substrate was DHTC (approximately 50 μg/ml). Incubation was at 28° for 2 hours. The products were identified by methods given in Table I.

The small amount of TC synthesized from DHTC in the absence of O_2 (Table IV) is consistent with the fact that some TC synthesis by S. rimosus has been observed (Perlman et al., 1960).

Together with the N-methylation reactions previously reported (Miller et al., 1964), the reactions shown in Figure 2 represent that portion of the pathway for the biosynthesis of the tetracycline antibiotics which has been firmly established. With the exception of OTC, all of the compounds shown can be obtained from their preceding intermediates by reactions in cell-free extracts. The biosynthesis of OTC from DHOTC cannot be unequivocally demonstrated until a method is found for obtaining DHTC-free DHOTC.

Von Wittenau et al. (1963) speculated that DHTC might serve as a common intermediate for the biosynthesis of both TC and OTC, and further

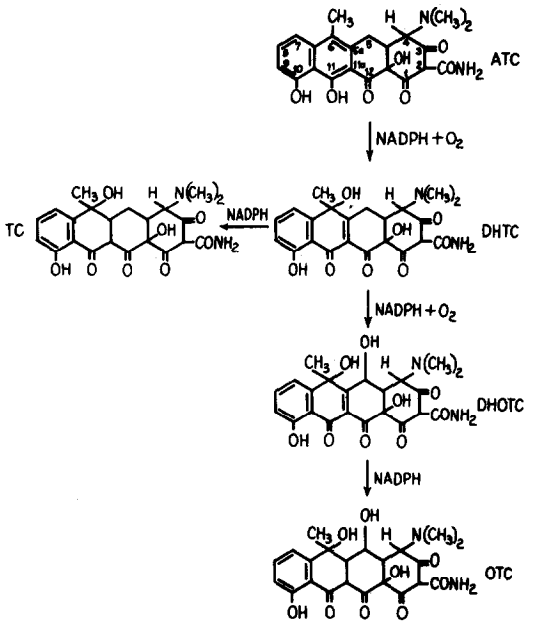

Fig. 2 Terminal Reactions in the Biosynthesis of OTC.

suggested a 5,5a-position for the double bond in the dehydro intermediate rather than the 5a,11a-position previously indicated for DHCTC by McCormick et al. (1958a). The 5,5a-position is more attractive from the point of view of providing structurally similar sites for the similar oxidations occurring at C-6 and C-5. However, there is no evidence for a 5,5a-double bond in the tautomer of DHTC which exists at pH 7, the pH of the cell-free extracts. The fact that DHTC serves as a common intermediate for the biosynthesis of both TC and OTC permits a greater biosynthetic economy (i.e., fewer intermediates) than if ATC served as the substrate for C-5-oxidation (McCormick et al., 1962).

One possibility for the synthesis of OTC from DHTC not considered in Figure 1 involves the direct addition of water to DHTC. The requirement for oxygen in that synthesis (Table IV) is not consistent with the addition of water. Thus, not only has a pathway for OTC biosynthesis been clearly demonstrated, but also all possible alternative pathways have been ruled out by experiment.

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