that the transient state must be brief for the steady-state method to be applicable. With enzymic reactions the relative duration of the transient state may be suppressed simply by increasing the substrate/enzyme ratio, and deviations from the steady state arising from the explosive accumulation of enzymic intermediates are also precluded.

For the mechanism of Michaelis and Menten for irreversible one-substrate reactions, the error equations derived in this study help define the errors of the steady-state method in terms of experimental observables and assess what may constitute a sufficiently high substrate/-enzyme ratio. For other enzymic mechanisms, it also would not be unexpected that the relative errors δ_c and δ_p will both increase with enzyme concentration. Since for all enzymic mechanisms the initial steady-state velocity is predicted to be strictly proportional to enzyme concentration,[2,3] the following operational criterion appears applicable. If velocity *vs.* enzyme concentration is observed to be linear, the errors of the steady-state theory may be *tentatively* accepted as within the errors of experimental observations. On the other hand, if experimental precision permits the detection of a nonlinear relationship of velocity *vs.* enzyme concentration unexplained by experimental factors, the adequacy of the steady-state theory over the nonlinear range of enzyme concentrations *must* be questioned.

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Communications to the Editor

Biosynthesis of the Tetracyclines. VII.[1] 4-Hydroxy-6-methylpretetramid, an Intermediate Accumulated by a Blocked Mutant of *Streptomyces aureofaciens*[2]

Sir:

In our continuing study of the biosynthetic pathways to the tetracycline antibiotics, we have found the phenomenon of cosynthesis[3] to be a useful tool both in characterizing new blocked mutants of the tetracyclines-producing *Streptomyces* and in demonstrating the presence of transferable intermediates in the biosynthetic process. This phenomenon has been used in the study of a new *Streptomyces aureofaciens*, strain V655, a dark-green-pigmented, spontaneous variant isolated directly from a 7-chlorotetracycline-producing parental strain. Significant cosynthetic activities of this new strain with two other blocked *S. aureofaciens* mutants are presented in Table I. The cosynthetic production of 7-chlorotetracycline in the mixed fermentation of strain V655 with the inherently nonchlorinating, blocked mutant T219 confirms that V655 has the chlorination potential indicated by its derivation from a chlorinating parental strain. This result, in conjunction with the further observation that V655 accumulated no chlorinated product of itself (as shown by fermentation in the presence of radioactive chloride ion, $^{36}Cl^-$), shows that the primary metabolic block in V655 is earlier than the chlorination step. The elaboration of 7-chlorotetracycline in mixed fermentation of V655 with the nonmethylating, blocked mutant ED1369 suggests the transfer of a 6-methyl-containing, unchlorinated intermediate from V655 to ED1369 and completion of the 7-chlorotetracycline molecule by the latter mutant. Addition of heat-killed, mature V655 mash to growing ED1369 again resulted in appearance of 7-chlorotetracycline, although in smaller amount, showing that a stable intermediate had accumulated in the V655 fermentation. The appearance of antibacterial activity in ED1369 fermentations upon adding V655-derived fractions was used as a biological assay method, by means of which we were able to isolate the active component of V655 fermentation mashes in pure form. In the accompanying paper[4] this substance is characterized as 1,3,-4,10,11,12-hexahydroxy-6-methylnaphthacene-2-carbox-

Table I. Cosynthesis of Tetracycline Antibiotics

	Strain		
	T219	ED1369	V655
Principal antibiotic product of parental strain	TC[a]	7-Chloro-6-demethyl-TC	7-Chloro-TC
Assay[b] when grown alone (μg./ml.)	<1.0	<1.0	2–5
Assay in mixed fermentation with strain V655 (μg./ml.)	460	370	—
Antibiotic produced in mixed fermentation with strain V655	7-Chloro-TC	7-Chloro-TC	—

[a] TC = tetracycline. [b] Antibacterial activity as determined by *Staphylococcus aureus* turbidimetric assay.

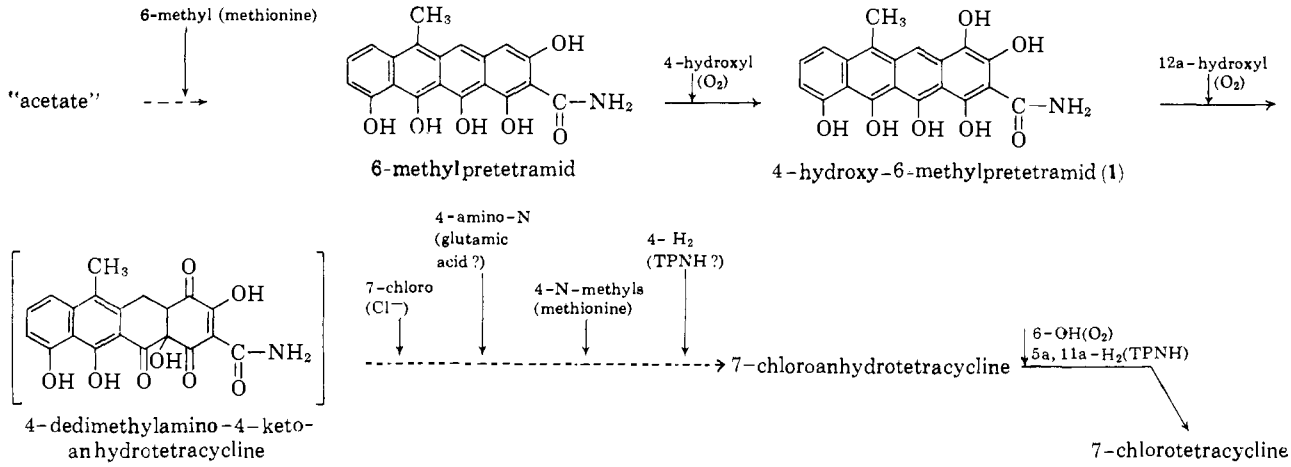
(1) Paper VI: J. R. D. McCormick, J. Reichenthal, S. Johnson, and N. O. Sjolander, *J. Am. Chem. Soc.*, **85**, 1694 (1963).

(2) The results of this work were included in summary in a paper presented at the Congress for Antibiotics, Prague, Czechoslovakia, June 1964.

(3) J. R. D. McCormick, U. Hirsch, N. O. Sjolander, and A. P. Doerschuk, *J. Am. Chem. Soc.*, **82**, 5006 (1960).

(4) J. R. D. McCormick and E. R. Jensen, *ibid.*, **87**, 1794 (1965).

Chart I



amide, or, in accordance with the nomenclature which we have suggested for this family of compounds,[5] 4-hydroxy-6-methylpretetramid (**1**). This compound was isolated from V655 freeze-dried, whole mash by extraction into tetrahydrofuran and was crystallized from that solvent by evaporation. It was obtained as orange needles of no definite melting point. Analyses indicated the composition $C_{20}H_{15}NO_7$. When the compound was dissolved in dimethyl sulfoxide and added to a 24-hr. ED1369 fermentation, up to 75% of the theoretical amount of 7-chlorotetracycline was formed on further incubation.

The fermentation conditions for the accumulation of **1** were generally those found best for other *S. aureofaciens* fermentations,[6] and the amount accumulated was about 500 μg./ml.

The discovery of this mutant-produced, naphthacenic intermediate is full confirmation of the postulated role of completely aromatic naphthacene derivatives as intermediates in the biosynthesis of the tetracyclines.[5] The details of structure of **1**, *i.e.*, the presence of the 4-hydroxyl and the 6-methyl groups and the absence of the 7-chloro substituent, confirm our earlier conclusions about the relative locations of the introduction of the 6-methyl and 7-chloro substituents[5] and strongly suggest the manner of introduction of the dimethylamino group. Our current assessment of this portion of the pathway from "acetate" to 7-chlorotetracycline is shown in Chart I.

(5) J. R. D. McCormick, S. Johnson, and N. O. Sjolander, *J. Am. Chem. Soc.*, **85**, 1692 (1963).

(6) (a) J. R. D. McCormick, J. Reichenthal, U. Hirsch, and N. O. Sjolander, *ibid.*, **84**, 3711 (1962); (b) J. J. Goodman, U. S. Patent 3,050,446 (1962).

(7) The partial biosynthetic scheme presented here encompasses conclusions drawn from the following works as well as from the present paper: R. Robinson, "Structural Relations of Natural Products," Oxford Press, 1955; S. Gatenbeck, *Biochem. Biophys. Res. Commun.*, **6**, 422 (1961); J. F. Snell, A. J. Birch, and P. L. Thomson, *J. Am. Chem. Soc.*, **82**, 2402 (1960); ref. 5, this paper; A. P. Doerschuk, J. R. D. McCormick, J. J. Goodman, S. A. Szumski, J. A. Growich, P. A. Miller, B. A. Bitler, E. R. Jensen, M. Matrishin, M. A. Petty, and A. S. Phelps, *J. Am. Chem. Soc.*, **81**, 3069 (1959); P. A. Miller, A. Saturnelli, J. H. Martin, L. A. Mitscher, and N. Bohonos, *Biochem. Biophys. Res. Commun.*, **16**, 285 (1964); J. R. D. McCormick, P. A. Miller, S. Johnson, N. Arnold, and N. O. Sjolander, *J. Am. Chem. Soc.*, **84**, 3023 (1962).

J. R. D. McCormick, Ursula Hirsch Joachim, Elmer R. Jensen, Sylvia Johnson, Newell O. Sjolander

Lederle Laboratories, American Cyanamid Company Pearl River, New York

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Biosynthesis of the Tetracyclines. VIII.[1] Characterization of 4-Hydroxy-6-methylpretetramid[2]

Sir:

As presented in the previous communication[1] 4-hydroxy-6-methylpretetramid (**1**), a substance biologically convertible to 7-chlorotetracycline, was isolated from the mash of a *Streptomyces aureofaciens* mutant, V655. The compound was separated by extraction with tetrahydrofuran and was crystallized from this solvent by evaporation. Product of high purity was obtained as orange needles by recrystallization from hot phenol in the presence of hydriodic and hypophosphorous acids to prevent oxidation. The pure substance melted with decomposition over the range 260–310°. Analyses indicated the composition to be $C_{20}H_{15}NO_7$ (found: C, 63.1; H, 4.13; N, 3.78). The infrared absorption spectrum was strongly reminiscent of that of 6-methylpretetramid (desdimethylaminoterrarubein[3]) but was distinguished by the presence of a strong sharp maximum at 970 cm.$^{-1}$. The absorption spectrum in sulfuric–boric acid[4] was very characteristic [λ_{max} (ϵ): 280 (22,100), 316 (42,100), 467 (15,400), and 520 mμ (16,500)], and again was similar to, but easily distinguished from, that of 6-methylpretetramid under the same conditions [λ_{max} (ϵ): 263 (22,900), 278 (21,600), 341 (14,200), 400 (14,700), and 512 mμ (13,700)].

Like the earlier known pretetramid derivatives, crystalline **1** was essentially insoluble in most organic solvents but dissolved readily in dimethyl sulfoxide containing 1% magnesium acetate tetrahydrate, forming an amber solution. The resulting solution was stable in the absence of oxygen, but in the presence of air was oxidized. The uptake of oxygen was initially rapid, but slowed and finally came to halt after about 6 hr. at 25°. The final solution was purple, and the absorp-

(1) Paper VII: J. R. D. McCormick, U. H. Joachim, E. R. Jensen, S. Johnson, and N. O. Sjolander, *J. Am. Chem. Soc.*, **87**, 1793, (1965).

(2) A preliminary report of the material of this communication formed part of a paper presented at the Congress for Antibiotics, Prague, Czechoslovakia, June 1964.

(3) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings, and R. B. Woodward, *J. Am. Chem. Soc.*, **75**, 5455 (1953).

(4) The solvent was 99 parts concentrated (97%) sulfuric acid and 1 part saturated aqueous sodium tetraborate. Spectrophotometric solutions were allowed to stand 0.5 hr. at room temperature before spectra were run.