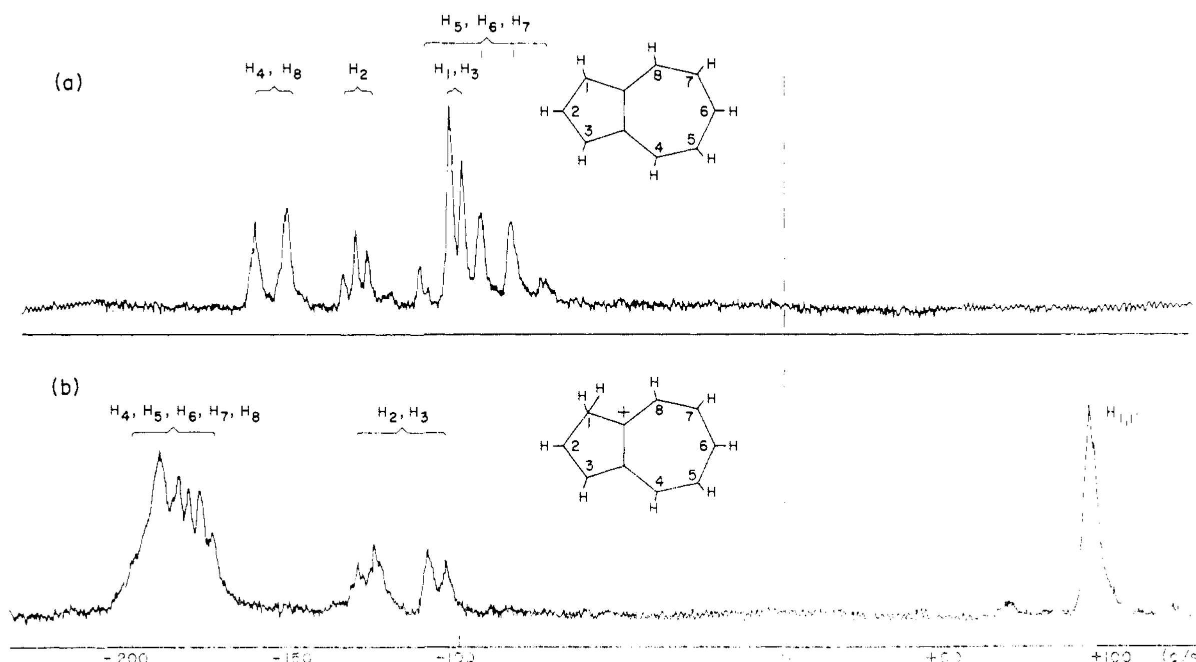

Fig. 1.—Proton magnetic resonance spectrum at 60 Mc./s. of (a) 7 mole % solution of azulene in CCl_4, and (b) 7 mole % solution of azulene in CF_3COOH. External reference signal (dotted line) is CH_2Cl_2.

solution of azulene in trifluoroacetic acid. It is evident that under these conditions the azulene is converted completely to the protonated ion. The signal at high field, +93 c./s., is characteristic of methylene protons which are assigned to the 1 (or 3) position. The quadruplet centered at −117 c./s., which is equal in intensity to that of the methylene proton signal, is a typical AB spectrum for two non-equivalent protons and arises from protons 2 and 3 on the 5-membered ring. A partially resolved spin-coupling ($J \sim 1$ c./s.) between these protons and the two methylene protons is evident. The chemical shifts of protons 2 and 3 are only slightly altered from their values in pure azulene. The corresponding changes in the signals of the protons in the 7-membered ring are more pronounced; in the ion these protons tend to be more nearly equivalent and show a marked decrease in screening. These changes are consistent with the conclusion that the electron deficiency in the ion resides almost exclusively on the 7-membered ring, where it tends to be shared with all the carbon atoms in the ring. In the formula in Fig. 1b, as suggested by Heilbronner and Simonetta,[5] a positive charge is indicated as localized on the "branch" carbon atom, but may in fact be more delocalized in the 7-membered ring and tend to approach the configuration of the tropylium ion.

From the width of the proton resonance lines it can be concluded that the mean "life-time" of the protonated azulene ion is at least of the order of one second and probably greater.

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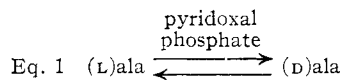
RECEIVED DECEMBER 22, 1959

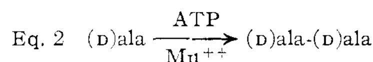
(5) E. Heilbronner and M. Simonetta, *Helv. Chim. Acta*, **35**, 1049 (1952).

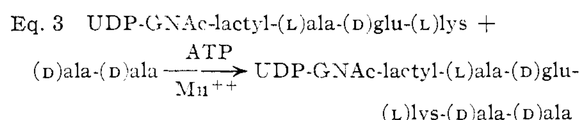
COMPETITIVE INHIBITION OF ENZYMATIC REACTIONS BY OXAMYCIN

Sir:

Inhibition of *S. aureus* by oxamycin ((D)cycloserine, (D)4-amino-3-isoxazolidone), which results in accumulation of the uridine nucleotide, UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys,[1] is competitively reversed by (D)alanine.[2] The antibiotic is a structural analog of this natural substrate. (D)-alanine is formed and incorporated into a uridine nucleotide through the reactions[3]


Eq. 1 (L)ala $\underset{}{\overset{\text{pyridoxal phosphate}}{\rightleftharpoons}}$ (D)ala


Eq. 2 (D)ala $\xrightarrow[\text{Mn}^{++}]{\text{ATP}}$ (D)ala-(D)ala


Eq. 3 UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys + (D)ala-(D)ala $\xrightarrow[\text{Mn}^{++}]{\text{ATP}}$ UDP-GNAc-lactyl-(L)ala-(D)glu-(L)lys-(D)ala-(D)ala

The purpose of this communication is to report that oxamycin is a competitive inhibitor of the enzymes catalyzing reactions 1 and 2.

Reaction 1 is catalyzed by alanine racemase.[4] Oxamycin is a competitive inhibitor of the reaction in both directions (Fig. 1). The Michaelis constants (K_m) for (D)alanine ($6.1 \times 10^{-3}M$) and for (L)alanine ($6.5 \times 10^{-3}M$) are nearly identical. Similarly, K_i for oxamycin measured in the direction of (D)alanine formation ($0.6 \times 10^{-4}M$) is

(1) Abbreviations: UDP, uridine diphosphate; GNAc-lactyl, an ether of acetylglucosamine and lactic acid (acetylmuramic acid).

(2) J. L. Strominger, R. H. Threnn and S. S. Scott, THIS JOURNAL, **81**, 3803 (1959).

(3) E. Ito and J. L. Strominger, *J. Biol. Chem.*, **235**, Feb., (1960).

(4) W. A. Wood and I. C. Gunsalus, *ibid.*, **190**, 403 (1951).

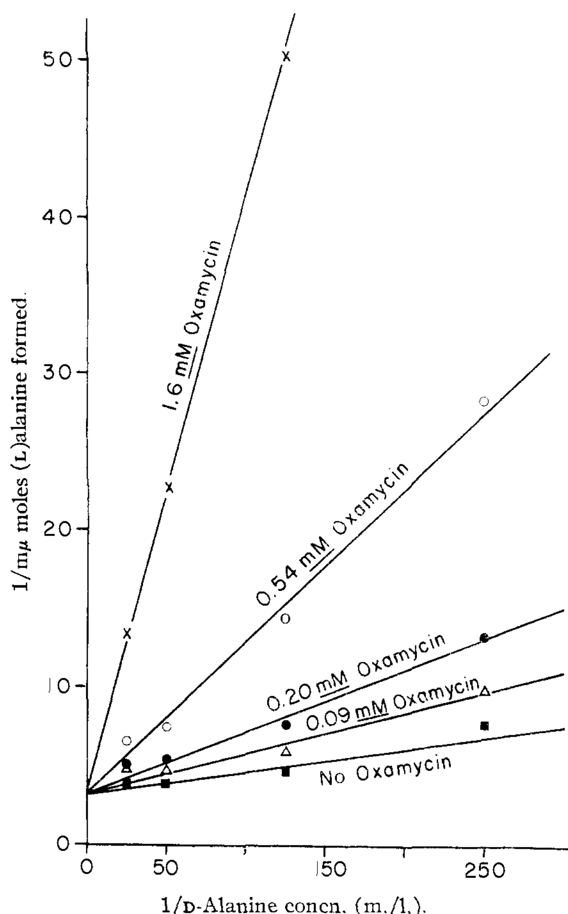


Fig. 1.—Competitive inhibition of alanine racemase by oxamycin. Data are plotted according to Lineweaver and Burk[5] ($1/v$ *vs.* $1/s$). v is expressed as the amount of product formed in the 30 min. assay.

about the same as K_i measured in the direction of (L)alanine formation ($1.0 \times 10^{-4}M$). Pyridoxal phosphate, over a wide range of concentrations, had no effect on the inhibition of the reaction by oxamycin.

However, this information was anomalous in that nucleotide accumulation in whole cells was not reversed by (L)alanine and was *competitively* reversed by (D)alanine.[2] A second point of inhibition was, therefore, sought and it has been found that reaction 2, the dipeptide synthesizing system,[3] is similarly inhibited by the antibiotic (Fig. 2). K_m for (D)alanine is $3–5 \times 10^{-3} M$ while K_i for oxamycin is $2–4 \times 10^{-5} M$. The ratio K_m/K_i = about 100 emphasizes the efficiency of the antibiotic as a competitor for the substrate.

Reaction 3, the addition of the dipeptide to the uridine nucleotide,[3] was not at all inhibited, even by high concentrations of the antibiotic ($4 \times 10^{-3}M$) at $1 \times 10^{-3}M$ (D)ala-(D)ala. The active penetration of C^{14}-(D)alanine into whole cells of *S. aureus* was similarly not affected when the D-alanine concentration in the medium was 10^{-4}, 10^{-5} or $10^{-6}M$ at an oxamycin concentration of $10^{-3}M$.

Thus, the inhibition of these enzymatic reactions by oxamycin provides, for the first time, a definition

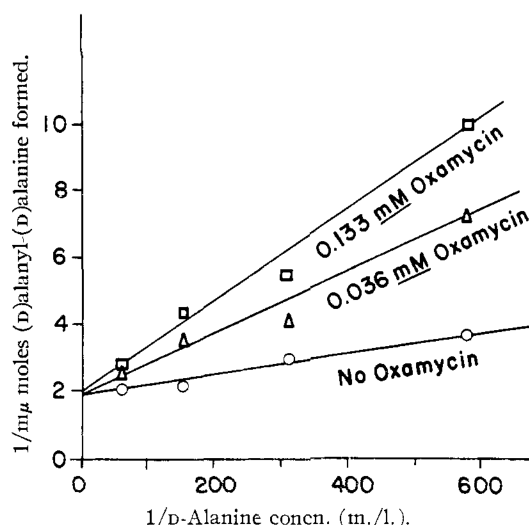


Fig. 2.—Competitive inhibition of the dipeptide synthesizing enzyme by oxamycin. v is expressed as the amount of product formed in the 15 min. assay.

of the mechanism of action of an antibiotic at an enzymatic level. Although competitive inhibition of enzymatic utilization of natural substrates has previously been demonstrated for substances which are not selectively toxic for bacterial cells (*e.g.*, ref.[5,6]), special interest in inhibition of alanine racemase and of the dipeptide synthesizing enzyme lies in the fact that the inhibited reactions are found uniquely in bacterial cells. Hence, the substance is a useful chemotherapeutic agent.[7]

(5) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).
(6) B. Levenberg, I. Melnick and J. M. Buchanan, *J. Biol. Chem.*, **225**, 163 (1957).
(7) Blood levels in human beings undergoing therapy with oxamycin are between 10^{-4} and 10^{-3} *M*. Thus, the levels obtained are sufficiently high to inhibit these enzymatic reactions *in vivo*.
(8) Supported by NSF Grant G-7619 and NIAID Grant E-1902.

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RECEIVED DECEMBER 7, 1959

NUCLEAR MAGNETIC RESONANCE STUDIES USING PYRIDINE SOLUTIONS

Sir:

Nuclear Magnetic Resonance spectroscopy has become very useful as a method for identification of unknown steroids.[1] The problem of interpretation of spectra and assignment of structures of complex molecules often is acute because of superposition of resonance lines arising from similar constituents. An observation is recorded herein which helps resolve such absorptions and facilitates correct assignments.

(1) J. N. Shoolery and M. T. Rogers, THIS JOURNAL, **80**, 5121 (1958); W. E. Rosen, J. B. Ziegler, A. C. Shabica and J. N. Shoolery, *ibid.*, **81**, 1687 (1959); G. Slomp and B. R. McGarvey, *ibid.*, **81**, 2200 (1959); R. L. Hirschmann, G. A. Bailey, R. Walker and J. M. Chemerda, *ibid.*, **81**, 2822 (1959); H. L. Slates and N. L. Wendler, *ibid.*, **81**, 5474 (1959); A. L. Nussbaum, F. E. Carlon, D. Gould, E. P. Oliveto, E. B. Hershberg, M. L. Gilmore and W. Charney, *ibid.*, **81**, 5230 (1959).