1949, v52, 202.

23. Miner, R. W., and Briggs, W., *ibid.*, 1949, v52, 63.

24. Shute, W. E., Shute, E. V., and Vogelsang, A. B., *Ann. Int. Med.*, 1949, v30, 1004.

25. Lecoq, R., Chauchard, P., and Mazoné, H., *Ann. New York Acad. Sc.*, 1949, v52, 142.

26. Lehr, D., *Bull. New York M. Coll.*, 1943, v6, 113.

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Inhibitory Effect of 5,6-Dimethylbenzimidazole and of 1,2-Dimethyl, 4,5-Diaminobenzene on Growth of *Mycobacterium tuberculosis.** (20971)

LEONARD N. HALLINGER, ROBERT SILBER, AND GERTRUDE NEUMANN.
(Introduced by Robert G. Bloch.)

From the Pulmonary and Medical Divisions, Montefiore Hospital, New York City.

The incidence of pernicious anemia has been reported to be less in patients with active pulmonary tuberculosis than in the general population(1). It occurred to us that this observation might reflect vit. B_{12} synthesis by the infecting tubercle bacilli, with consequent masking of any clinical manifestations of pernicious anemia.† Advances in the application of biologic antagonism to anti-bacterial therapy led us to investigate the influence of vit. B_{12} fragments on the growth of *Mycobacterium tuberculosis*. The primary objective was to determine whether blockade of vit. B_{12} synthesis or utilization in the tubercle bacillus might deprive the organism of an essential metabolite and thus halt its growth.

Two compounds, 5.6-dimethylbenzimidazole and 1,2-dimethyl 4,5-diaminobenzene‡ have been described by some as precursors of vit. B_{12}(3), and by others as vit. B_{12} antagonists(4). The present report is concerned with preliminary observations of their effect on the growth of a virulent strain of *Mycobacterium tuberculosis* in Dubos Medium.

Method. Twenty screw-capped tubes, containing 6.1 ml of Dubos Medium and inoculated with roughly equal numbers of tubercle bacilli (0.1 ml of inoculum), served as controls. A similar quantity of such tubes, containing in addition, one of the various concentrations of 5, 6 D or of 1, 2 D, served as the experiment. All concentrations were simultaneously tested. In detail, actively growing, 6-day-old cultures of the H37Rv strain of *Mycobacterium tuberculosis*, which had been subcultured at least 3 times in Dubos' Tween-Albumin Medium(5), were employed as inoculum. Proportionate amounts of the basal culture medium and the inoculum, as well as the glucose and the bovine albumin, were pooled, to insure uniformity of concentration of the constituents in the many culture tubes. Subsequently, 5.1 ml of the suspension were added to each of the previously sterilized tubes, which contained either 1 ml of basal medium plus the compound tested or 1.1 ml of the basal medium for the control tubes. The final concentrations of 1, 2 D and of 5, 6 D were achieved by adding appropriate 0.1 ml aliquots of their aqueous solutions to lots of 20 tubes of inoculated medium. Because the 1, 2 D solution was found to be somewhat unstable, particularly when heated, it was sterilized by passage through a bacterial filter prior to the addition of the inoculum. The

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† More recent evidence has supported our hypothesis that vit. B_{12} may be synthesized by *Mycobacterium tuberculosis*(2).

‡ Hereafter referred to as 5, 6 D and 1, 2 D respectively.

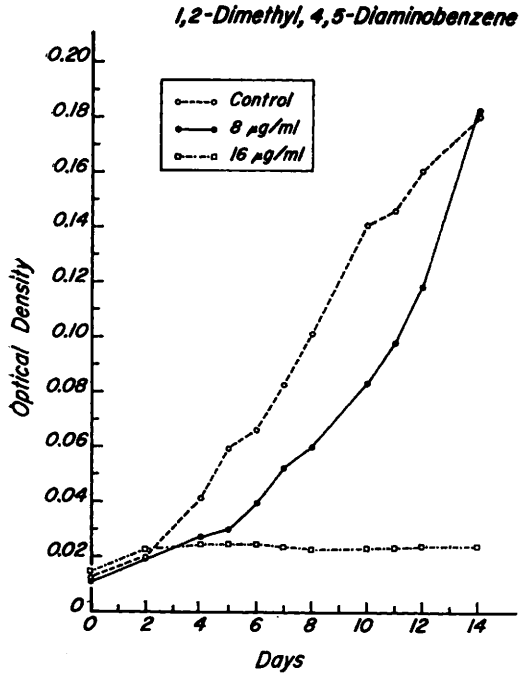

FIG. 1. Effect of 1, 2-dimethyl 4, 5-diaminobenzene, in various concentrations, on growth of *M. tuberculosis*, H37Rv, in Dubos Medium. Each plot on graph represents arithmetic average of 20 simultaneously growing cultures.

tubes were incubated at 37°C and shaken daily. Growth was determined turbidimetrically at 650 λ, with a Coleman Jr. Spectrophotometer, according to the method of Schaefer *et al.*(6). This was usually recorded daily. Each experiment was repeated at least 3 times.

Results. Fig. 1 illustrates a single typical graph of the effect of 1, 2 D on the growth of *Mycobacterium tuberculosis H37Rv*, expressed in units of optical density. It will be noted that with the lesser concentration, 8.0 µg/ml, there is partial inhibition of growth for the first 4 to 5 days. Doubling the quantity of 1, 2 D, 16.0 µg/ml, produces complete cessation of growth by the second to third day. The fluctuations thereafter are within the range of experimental error. Subcultures taken from these tubes on the twelfth or fourteenth days yielded viable organisms when cultured on Dubos Medium. Slightly higher concentrations were needed to inhibit growth when it was sterilized by Selas filtration rather than autoclaving.

A greater concentration of 5, 6 D was needed to achieve comparable inhibition (Fig. 2). With this compound viable organisms could not be subcultured at the end of the experiment.

Discussion. Antimetabolites are substances whose activity appears to be due to structural similarity between their molecules and those of biologically important substances such as vitamins, hormones, or amino acids. Because of this structural similarity, they may become incorporated into the metabolism of the organism at the reaction site(s) usually occupied by the metabolite they resemble. Their lack of biologic specificity prevents them from performing the exacting role usually required of the metabolite, and the reactions are blocked. These reactions are important in such functions as energy metabolism, growth, and reproduction, and the deficiency state thus produced may be manifested by cessation of growth or death of the organism.

The study of the metabolism of vit. B_{12} is comparatively recent. Degradation of this vitamin by acid hydrolysis has yielded 5, 6 D

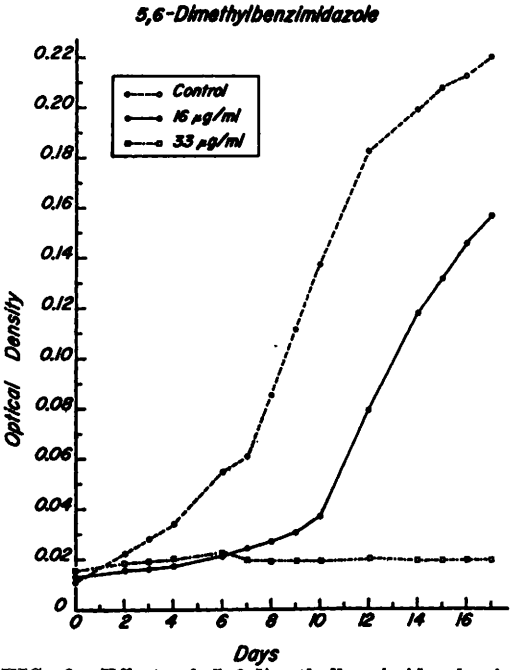

FIG. 2. Effect of 5, 6-dimethylbenzimidazole, in various concentrations, on growth of *M. tuberculosis*, H37Rv, in Dubos Medium. Each plot on graph represents arithmetic average of 20 simultaneously growing cultures.

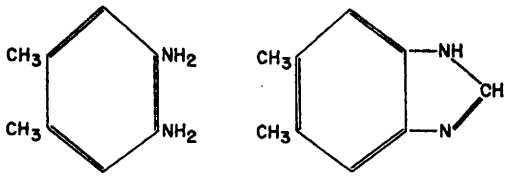

FIG. 3. An illustration of structural similarity between 1, 2-dimethyl 4, 5-diaminobenzene and 5, 6-dimethylbenzimidazole.

(7) and also a dimethylbenzimidazole riboside which on further breakdown was found to contain the 1, 2 D moiety(8) (Fig. 3). Certain observations with reference to these fragments have been made.

Woolley(3) believes that they are precursors of vit. B_{12}, and has demonstrated that 1, 2 D has the ability to counteract, in a competitive fashion, the toxicity of 1,2-dichloro 4,5-diaminobenzene which he had developed as an antimetabolite to both riboflavin and vit. B_{12}. Emerson *et al.*(9) have shown 1, 2 D and 5, 6 D to possess vit. B_{12} activity for the rat. Conversely, they have been thought to be vit. B_{12} antagonists by Hendlin and Soars who demonstrated growth inhibition of *Lactobacillus lactis Dorner* and other vit. B_{12} requiring organisms, with addition of 1, 2 D and 5, 6 D to the culture medium(4). The growth inhibitory effects of the benzimidazole nucleus (which is parent to both these compounds) has been adduced to operate in the area of vit. B_{12}-nucleic acid metabolism, since purines and vit. B_{12} can reverse this inhibition in some species of microorganisms(10).

While the evidence implicating these compounds in the metabolism of vit. B_{12} is indirect and circumstantial, the present report would seem to demonstrate yet another species in which vit. B_{12} is a metabolite, and in which these compounds produce a biologic effect. The results which we have obtained lead us to suspect that 1, 2 D and 5, 6 D may act as antimetabolites rather than precursors of vit. B_{12}, and either block its synthesis or compete for this vitamin's role(s) in the intermediary metabolism of the tubercle bacillus. The possibility also exists that these compounds are not active in the form wherein they were added to the culture medium, but rather underwent chemical change in the Dubos Medium to become a new substance, the active inhibitor.§ We recognize too, that another mechanism, yet unknown, may be the effective one. Further studies are planned in the hope of developing a new class of compounds of possible therapeutic value.

Conclusions. Two compounds, 5,6-dimethylbenzimidazole and 1,2-dimethyl 4,5-diaminobenzene, which have been previously implicated in vit. B_{12} metabolism, were found to inhibit the growth of *Mycobacterium tuberculosis, strain H37Rv,* in Dubos Medium.

§ 1, 2 D is known to be somewhat unstable in aqueous solution(11). The color change noted on autoclaving, and even at 37°C after 10 to 15 days in solution, perhaps results from a chemical reaction with one or more of the constituents of the culture medium. Some of the resultant products may possibly be similar to those synthesized by Woolley(12), with substituted or added alkyl groups on the parent nucleus. It may be that the superior inhibitory activity of 1, 2 D is due, in part, to one or more of these substituted compounds, rather than the parent compound. Since 1, 2 D and 5, 6 D are closely related (Fig. 3), it is probable that the mode of action of these and their questionably substituted forms are similar.

1. Baron, M., *J.A.M.A.,* 1933, v100, 1590.
2. Peterson, A., and Pope, H., *J. Bact.,* 1952, v64, 25.
3. Woolley, D. W., *J. Exp. Med.,* 1951, v93, 13.
4. Hendlin, D., and Soars, M., *J. Bact.,* 1951, v62, 633.
5. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.,* 1947, v56, 334.
6. Schaefer, W. B., Marshak, A., and Burkhart, B., *J. Bact.,* 1949, v58, 549.
7. Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.,* 1949, v71, 2951.
8. Brink, N. G., Holly, F. W., Shunk, C. H., Peel, E. W., Cahill, J. J., and Folkers, K., *ibid.,* 1950, v72, 1866.
9. Emerson, G., Brink, N. G., Holly, F. W., Koniuszy, F., Heyl, D., and Folkers, K., *ibid.,* 1950, v72, 3084.
10. Tamm, I., Folkers, K., Shunk, C. H., Heyl, D., and Horsfall, F. L., Jr., *J. Exp. Med.,* 1953, v98, 245.
11. Folkers, K., personal communication.
12. Woolley, D. W., and Pringle, A., *J. Biol. Chem.,* 1952, v194, 729.

Received March 11, 1954. P.S.E.B.M., 1954, v85.