

Inhibition of Synthesis of Vitamin B₁₂ and of Riboflavin by 1,2-Dichloro-4,5-diaminobenzene in Bacterial Cultures. (18328)

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It has been shown recently(1) that 1,2-dichloro-4,5-diaminobenzene is a rather selective poison, in that it inhibits the growth of many kinds of living things which can synthesize riboflavin or vit. B₁₂, or both, and is not harmful to those species which cannot form either of these substances. The demonstration arose from the realization that both of these vitamins probably had a common precursor, namely 1,2-dimethyl-4,5-diaminobenzene, and that a properly constituted analogue of this metabolite, such as 1,2-dichloro-4,5-diaminobenzene should exert such a selective action because it would inhibit the synthesis of these two vitamins in those cells which were endowed with the metabolic machinery for conducting such syntheses. By choosing a common metabolic precursor of two vitamins concerned intimately in cell division, rather than a precursor of one such substance, it was postulated that an agent might be realized which would act even in the presence of the products of the inhibited reactions. This actually proved to be the case with 1,2-dichloro-4,5-diaminobenzene because both riboflavin and vit. B₁₂ did not prevent its action.

The working hypothesis just described involves some assumptions because 1,2-dimethyl-4,5-diaminobenzene, the postulated common precursor of both vitamins, has not been demonstrated to be the actual starting point for such biosyntheses. The only evidence in favor of such a view has been that this substance can be recognized in the formulations of these two vitamins, and that it has a trace of riboflavin activity for *Lactobacillus casei*, and of vit. B₁₂ potency for 2 other microorganisms(1). The fact that it will antagonize the growth-inhibiting effects of 1,2-dichloro-4,5-diaminobenzene in a competitive

fashion has suggested that the latter substance is exerting its toxicity by interference with the functioning of the dimethyldiamine for the synthesis of riboflavin and vit. B₁₂. The present investigation was undertaken in order to determine whether any evidence of a more direct nature for this postulate could be found.

The 1,2-dichloro-4,5-diaminobenzene inhibited the formation of vit. B₁₂ and of riboflavin by growing cultures of *Bacillus megatherium* strain WRRL B-938.[†] When this organism was cultured aerobically in flasks at 30° for 48 hours, in the riboflavin-free Landy-Dicken medium(2) previously described for testing toxicity of this compound (1) half-maximal inhibition of growth was produced by 7-8 µg of the dihydrochloride per cc. For measurement of any possible effect on the formation of riboflavin and of vit. B₁₂ in such cultures, an amount of the analog was chosen which was just insufficient to cause detectable inhibition of growth. This, and graded dilutions lower than this, were added to the medium before inoculation. After seeding and growth had occurred, microbiological assays for the two vitamins in question were performed on the heated, and suitably diluted cultures. Riboflavin was determined with the aid of *Lactobacillus casei*, and vit. B₁₂ with *Euglena gracilis*(3). Results of a typical experiment are shown in Table I. There it can be seen that quantities of the analog which were too small to cause inhibition of growth, reduced the concentration of these 2 vitamins which this organism formed. The reduction in vit. B₁₂ was greater than in riboflavin. A situation similar to this inter-

[†] This organism was very kindly supplied by Dr. D. Perlman of E. R. Squibb and Sons, Inc.

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* With the technical assistance of N. Smith and E. A. Singer.

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TABLE I. Effect of 1,2-Dichloro-4,5-diaminobenzene Dihydrochloride on the Synthesis of Vitamin B₁₂ and of Riboflavin by *B. megatherium*.

Analog in the growth medium, μg per cc	Light transmission (growth) %	Vit. B ₁₂ synthesized, $\text{m}\mu\text{g}$ per cc	Riboflavin synthesized, μg per cc
0	50	.083	.14
10	87	—	—
5	53	.045	—
3	52	—	.10
2	43	.038	—
1	42	.060	—

ference with vit. B₁₂- and riboflavin-production was found by Miller(4) who showed that sulfanilamide and its congeners reduced the amount of folic acid which cultures of *E. coli* formed. In this case, however, the degree of inhibition was greater.

When 1,2-dimethyl-4,5-diaminobenzene was added to growing cultures of *B. megatherium*, (in the absence of the dichloro-analog), a stimulation in synthesis of vit. B₁₂ was observed. Thus, when 10 μg of this postulated metabolic precursor were present per cc during growth under the conditions just defined, approximately twice as much of the vitamin was formed as in the unsupplemented basal medium (0.14 millimicrogram compared to 0.083 millimicrogram). Although the dimethyldiamine has a slight activity as vit. B₁₂ for *E. gracilis*(1), control experiments showed that the amount employed in the test just described was insufficient to detect. The increase observed was therefore probably due to newly synthesized vit. B₁₂.

When these experiments were completed,

the publication of Hendlin and Ruger(5) appeared which showed that additions of cobalt ions to bacterial culture media increased the amount of vit. B₁₂ synthesized by various microorganisms grown in them. Because cobalt salts had not been added to the basal medium used in the present investigation the experiments were repeated with cobalt nitrate (10 μg per cc) in the culture fluid. Under these conditions the organism formed 0.45 millimicrogram of vit. B₁₂ per cc. The presence of 5 μg of the dichlorodiaminobenzene dihydrochloride reduced this production to 0.07 millimicrogram of vit. B₁₂, and 2 μg of this same compound resulted in the appearance of 0.23 millimicrogram of the vitamin. In the presence of cobalt therefore, the inhibiting effect of this analog on synthesis of this vitamin was magnified. Cobalt ions did not detectably alter the growth-retarding potency of the dichlorodiamine.

Summary. The amounts of vit. B₁₂ and of riboflavin which were formed during growth of *Bacillus megatherium* were decreased by 1,2-dichloro-4,5-diaminobenzene when the latter was present in quantities insufficient to inhibit growth. 1,2-Dimethyl-4,5-diaminobenzene caused stimulation of the production of vit. B₁₂. These facts have been viewed as being compatible with the previously expressed postulates that the dimethyldiamine is a precursor of riboflavin and of vit. B₁₂, and that the dichloro-analog exerts its selective toxicity, at least in part, by inhibition of biosynthetic processes in the formation of these vitamins.

5. Hendlin, D., and Ruger, M. L., *Science*, 1950, v111, 541.

4. Miller, A. K., *Proc. Soc. Exp. Biol. Med.*, 1944, v57, 151.

Received November 8, 1950. P.S.E.B.M., 1950, v75.