Effect of Antimetabolites on Growth of *Endamoeba histolytica*. III. 5,6-dimethylbenzimidazole and Related Compounds.* (23275)

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Hendlin and Soars(1) have studied effects of 5,6-dimethylbenzimidazole and related compounds on the growth of *Lactobacillus lactis* Dorner. They reported that 5,6-dimethylbenzimidazole, 1,2-dimethyl-4,5-diaminobenzene, and several substituted benzimidazoles inhibited vit. B_{12}-requiring microorganisms. The toxicity of the diamine was competitively antagonized by B_{12}. However, such was not the case with the 5,6-dimethylbenzimidazole and its analogues. Oginsky and Smith(2) showed that B_{12} stimulates rate of acetate oxidation by resting suspensions of *Escherichia coli* (strains which require this vitamin), whereas no stimulation occurred with benzimidazole, 2,5-dimethylbenzimidazole, and 5,6-dimethylbenzimidazole. On the other hand, Woolley(3,4) recognized 1,2 dimethyl-4,5-diaminobenzene as a probable precursor of B_{12} so an antimetabolite (1,2-dichloro-4,5-diaminobenzene) of this compound was synthesized. Dichloro-diaminobenzene was toxic to microorganisms which did not exhibit a nutritional need for B_{12}. This inhibition by the antimetabolite was competitively reversed by the dimethyl-diaminobenzene. Although a slight stimulatory activity on *Endamoeba histolytica* was observed using crude preparation of B_{12}(5), additional studies with pure crystalline B_{12} have suggested that this vitamin is not a specific requirement for the amebas; in fact, evidence has accumulated that *E. histolytica* is capable of synthesizing B_{12}.

The present studies were conducted in order to determine the effect of 5,6-dimethylbenzimidazole and related compounds on *E. histolytica* so that we may learn something about vit. B_{12} synthesis in the amebas.

Materials and methods. The NRS strain of *E. histolytica* was used. Stock cultures of this strain growing with a mixed bacterial flora were maintained on a modified Ringer-egg-serum medium. However, the effects of the compounds were studied in ameba cultures containing no viable bacteria. Detailed technics employed have been described(6,7, 8).

The following compounds were studied: 5,6-dimethylbenzimidazole, 1,2-dimethyl-4,5-diaminobenzene (obtained from Dr. David Hendlin, Research Laboratories, Merck and Co., Rahway, N.J.), 2,5-dimethylbenzimidazole, 4,5-diphenyl-2-imidazolone, 5-methylbenzimidazolone (obtained from Dr. Gustav J. Martin, Research Laboratories, National Drug Co., Philadelphia, Penna.), 1,2-dichloro - 4 - nitro - 5-hydroxybenzene, 1,2-dichloro-5-sulfanilamido-5-aminobenzene, and 1,2-dibromo - 4,5-diaminobenzene (obtained from Dr. D. W. Woolley, Rockefeller Institute for Medical Research, New York City). These compounds were tested in concentrations ranging from 0.001 mg/ml to 1 mg/ml. In cases where the compounds inhibited *E. histolytica,* reversal studies were performed with crystalline vit. B_{12} thymidine (Nutritional Biochemicals Corp., Cleveland, O.), and folic acid (from Dr. J. M. Ruegsegger, Lederle Laboratories Division, Am. Cyanamid Co., Pearl River. N.Y.)

Results. The activities of the compounds tested are listed in Table I. Only 1,2-dimethyl-4,5-diaminobenzene was without inhibitory activity against the amebas; instead this compound acted as a growth stimulant to the amebas. The inhibitory activities of 5,6-dimethylbenzimidazole, 2,5-dimethylbenzimidazole, 1,2-dichloro-4-sulfanilamido-5-aminobenzene, and 1,2-dibromo-4,5-diaminobenzene were reversed by B_{12} (0.001 mg/ml) and 1,2-dimethyl-4,5-diaminobenzene (0.1 mg/ml). Folic acid (0.1 mg/ml) and thymidine (0.1

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TABLE I. Effect of 5,6-Dimethylbenzimidazole and Related Compounds on *In Vitro* Growth of *Endamoeba histolytica* under Bacteria-Free Conditions.

Compounds assayed	Stimulatory to amebas	Amebacidal activity	Minimum conc. amebacidal, mg/ml	Compounds able to reverse inhibition	Compounds unable to reverse inhibition
5,6-dimethylbenzimidazole		+	.1	DMDAB (partial), B_{12} (partial)	Folic acid, thymidine
1,2-dimethyl-4,5-diaminobenzene (DMDAB)	+				
2,5-dimethylbenzimidazole		+	.1	*Idem*	*Idem*
4,5-diphenyl-2-imidazolone		+	1	Folic acid (partial), thymidine (partial)	DMDAB, B_{12}
5-methylbenzimidazolone		+	1		Folic acid, thymidine, DMDAB, B_{12}
1,2-dichloro-4-nitro-5-hydroxybenzene		+	1		*Idem*
1,2-dichloro-4-sulfanilamido-5-aminobenzene		+	.5	DMDAB (complete), B_{12} (complete)	Folic acid, thymidine
1,2-dibromo-4,5-diaminobenzene		+	.5	*Idem*	*Idem*

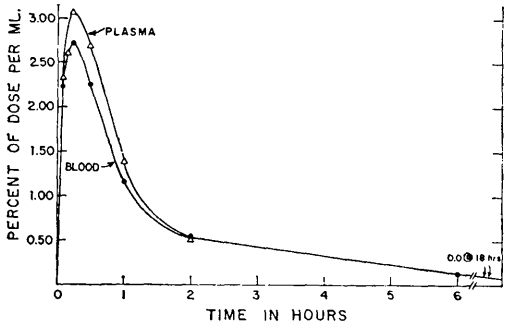
mg/ml) were ineffective in reversing this inhibition. Reversal of 5-methylbenzimidazolone and 1,2-dichloro-4-nitro-5-hydroxybenzene was not possible when folic acid, thymidine, B_{12}, or dimethyldiamine were added. Interestingly, the inhibtory activity of 4,5-diphenyl-2-imidazolone was reversed with folic acid and thymidine but not by B_{12} or the dimethyldiamine.

Discussion. It appears from these data that 1,2-dimethyl-4-5 diaminobenzene is a precursor of vit. B_{12} in *E. histolytica.* The amebas, apparently, are able to convert this intermediate to B_{12}, yet are unable to accomplish the total synthesis of this dimethyldiaminobenzene since this substance added to bacteria-free amebas produced a great increase in amebic growth and multiplication. 5,6-dimethylbenzimidazole is thought by some workers to be a precursor of B_{12} especially in view of the fact that it has been identified as an acid degradation product of B_{12}(9). On the other hand, Emerson *et al.*(10) found this compound to be without B_{12} activity for *L. lactis* Dorner. Furthermore, Oginsky and Smith(2) reported that 5,6-dimethylbenzimidazole inhibited the ability of B_{12} to stimulate the rate of oxidation of acetate in *E. coli.* This compound also inhibited *L. lactis*(1); the dimethyldiamine and its ribityl analogue inhibited *Euglena gracilis*(11). Although the mechanism of this inhibition is not clearly understood at this time, Hendlin(12) suggests that the inhibitory effect of 5,6-dimethylbenzimidazole might result from its interference with adsorption or absorption of the vitamin.

The fact that the inhibition is reversed by B_{12} and also by the dimethyldiamine indicates that this compound, the dimethylbenzimidazole, prevents the utilization of B_{12} in the amebas or prevents the conversion of B_{12} precursors into the vitamin.

The inhibition observed by 2,5-dimethylbenzimidazole, 1,2-dichloro-4-sulfanilamido-5-aminobenzene and 1,2-dibromo-4,5-diaminobenzene may well have been due to the blockade of conversion of the dimethyldiamine to B_{12} since reversal was possible by supplying the precursor or the vitamin. Other workers have also reported on the inhibitory activity of 2,5-dimethylbenzimidazole(10). These substituted benzene compounds are possibly antimetabolites of the dimethyldiamine as postulated by Woolley(3,4,13).

Since reversal of inhibition was not possible with folic acid and thymidine, it may be surmised that, in *E. histolytica* at least, B_{12} does not function in thymidine synthesis. The logic here is that if a vitamin acts in a cell at some stage in the synthesis of essential metabolites, then it is to be expected that addition



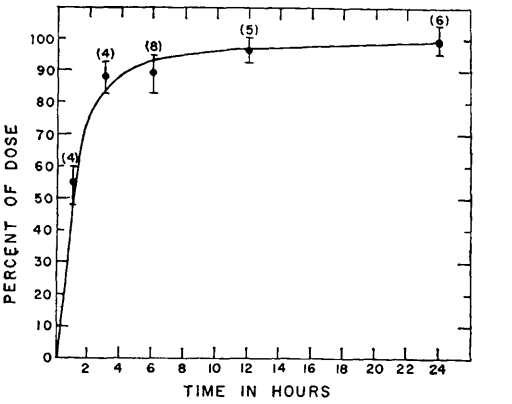


FIG. 1 (top). Conc. of radiophosphorus in blood and plasma following intraper. inj. of $KP^{32}F_6$.

FIG. 2 (bottom). Excretion of radiophosphorus in urine following intraper. inj. of $KP^{32}F_6$.

radiophosphorus. Two rats were injected intravenously with 1 mg of $KP^{32}F_6$ dissolved in 0.2 ml of physiological saline, and sacrificed by decapitation at the end of 10 minutes. The heart, liver, lungs, kidneys, spleen, and a portion of thigh muscle were excised, blotted, and weighed. The samples were ignited for 3 hours at 550°C, dissolved in 1N HCl, and analyzed for radiophosphorus. Recovery of labelled phosphorus added as PF_6^- to 6 control tissue samples was 95.9 ± 1.6% (mean ± S.E.).

Results. Concentration of radioactivity in whole blood and plasma reached a maximum at 15 minutes, then fell precipitously (Fig. 1). No detectable activity was found in the blood 18 hours after injection. Concentration values were consistently higher in plasma than in blood, and may reflect differences in water content. The findings are consistent with the belief that blood cells are freely permeable to the PF_6^- ion.

Ten minutes after intravenous injection the mean concentration of radiophosphorus in the tissues, expressed as percent of dose per gram of fresh weight, was: liver, 0.57; spleen, 0.55; heart, 0.51; lungs, 0.97; kidney, 1.67; and muscle, 0.29.

The high radiophosphorus content of kidney seems in accord with the role of this organ in the excretion of the label. Thus 55% of the dose had been cleared at the end of one hour, and 88% by the end of 3 hours (Fig. 2). The 24-hour urine contained all of the injected radiophosphorus. The rapidity of clearance suggests that tubular reabsorption was minimal, if not absent.

In 8 urine samples selected at random, the ratio of calculated-to-found fluoride was 0.98 ± 0.03 (mean ± S.E.). This finding substantiates the physiological stability of PF_6^-. Similarly, the quantitative recovery of injected radiophosphorus in urine can only be explained by the inability of the rat to hydrolyze PF_6^- to $PO_4^{\equiv}$ and F^-.

The physiological stability of this ion, coupled with its quantitative excretion in the urine, suggests consideration of radiophosphorus-tagged hexafluophosphate as a possible tool in renal function studies.

Summary. 1. Distribution and excretion of radiophosphorus labelled hexafluophosphate ion has been investigated in the rat. 2. The concentration of PF_6^- in whole blood and plasma reached a maximum at 15 minutes, fell precipitously, and none was found 18 hours after its injection. 3. Evidence is presented that PF_6^- is not hydrolyzed by the rat. 4. Injected PF_6^- is rapidly and quantitatively excreted in the urine.

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