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Mono-Substituted Vit. B_{12} Amides. I. A Microbiological Study.*† (24791)

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Smith(1) suggested that the labile amide groups of the B_{12} molecule function in enzyme reactions involving B_{12}. There are 3 of these, probably those on the propionamide chains. The amide linkages can be hydrolyzed with dilute acid or alkali. The acids can then be converted to variously substituted amides by reaction with methylamine, ethylamine, or aniline(1,2). Many substituted amides have been reported to have anti-Vit. B_{12} activity (3). Since Vit. B_{12} is essential for growth of many organisms and man, it could be supposed that a specific antagonist of Vit. B_{12} might stop cell growth. This report compares the microbiological activity of these 3 mono-substituted amides for 4 different B_{12}-requiring organisms.

Methods. The B_{12}-requiring organisms used are: *Lactobacillus leichmannii* ATCC No. 4797, *Escherichia coli* 113-3, *Euglena gracilis, Z* strain, and *Ochromonas malhamensis*. Technics for using these organisms for B_{12} assay have been reported(4,5,6). The methylamide, ethylamide, and anilide derivatives of the monocarboxylic acid of Vit. B_{12}, obtained in dry form, were dissolved in distilled water and diluted appropriately.

Results. The effect of mono-substituted amides on growth of the 4 organisms is given in Table I. As B_{12}-substitutes, the amides are practically inactive for *E. coli* and *O. malhamensis*. Their slight activity may be related to some B_{12} contamination of the amides. The amides support half-maximal growth for *L. leichmannii* in low concentrations, and almost full growth in higher concentrations. The ethylamide and methylamide do not support full growth of *E. gracilis*, but they are more than 3 times as effective as the anilide as B_{12}-substitutes. Additions of amides to various concentrations of B_{12} showed that they neither inhibited nor enhanced B_{12} utilization, since growth was unchanged from that without amide addition.

When *E. coli* is grown in the presence of the amide and suboptimal levels of methionine, the 2 are utilized synergistically; methionine and B_{12} combinations are additive (Table II). When combined with methionine, methylamide, ethylamide and anilide are respectively 30, 100, and 300 times less effective than B_{12} for growth of *E. coli*. In concentrations greater than 30 μg/ml of methionine, growth stimulation by added amide was not noticeable because adequate concentra-

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TABLE I. Effect of B_{12}-amides on Growth of B_{12}-requiring Microorganisms.*

Organism	Compound	Concentration (mμg/ml) 0	.001	.01	.1	.3	1	10
E. coli 113-3	B_{12}	0	0	0	.4	.78	1.0	1.3
	Methylamide	0	0	0	.08	.12	.12	.28
	Ethylamide	0	0	0	0	0	0	0
	Anilide	0	0	0	0	0	0	0
L. leichmannii	B_{12}	.1	.1	.32	.46	.54	.54	
	Methylamide	"	"	.14	.3	.36	.4	
	Ethylamide	"	"	.2	.4	.54	.54	
	Anilide	"	.16	.16	.24	.36	.44	
E. gracilis	B_{12}	.1	.22	.78	3.8	5.2	7.2	
	Methylamide	.12	.2	.48	1.8	2.0	3.0	
	Ethylamide	.1	.1	.34	"	2.9	3.8	
	Anilide	"	"	.12	.44	.78	2.7	
O. malhamensis	B_{12}	.16	.24	.5	1.6	3.0	5.4	
	Methylamide	.1	.2	.2	.3	.3	.3	
	Ethylamide	.2	"	"	.24	"	.26	
	Anilide	.18	"	.22	"	.32	.28	

* Growth given in optical density units, as measured with a Welch Densichron equipped with a red-sensitive probe.

tions of methionine completely replaces B_{12} for growth(6).

Discussion. It has been reported that both methylamide and anilide of B_{12} antagonize growth-promoting effects of B_{12} for *E. coli* 113-3(7). In the present study, neither ethylamide, methylamide, nor anilide inhibited utilization of B_{12} by any of the 4 organisms; these compounds cannot be regarded as B_{12}-antagonists on the basis of these results.

TABLE II. Growth of *E. coli* in Amide-Methionine Combinations.*

Compound (mμg/ml)		DL-methionine (μg/ml) 0	1	3	10	30
Control	0	0	.1	.22	.58	.96
B_{12}	.1	.4	.48	.6	.98	1.4
	.3	.78	.78	.92	1.16	1.52
	1	1.0	1.0	1.0	1.4	1.6
	3	1.3	1.3	1.3	1.46	1.68
	10	1.4	1.4	1.52	1.5	1.66
Methylamide	.1	.0	.16	.24	.62	1.0
	.3	.0	.24	.36	.7	1.0
	1	.08	.5	.5	.74	1.12
	3	.12	.68	.64	.9	1.2
	10	.28	.78	.9	.96	1.2
Ethylamide	.1	0	.14	.24	.54	.96
	.3	0	.18	.24	.54	.96
	1	0	.24	.4	.64	1.0
	3	0	.26	.5	.72	1.16
	10	0	.38	.66	.82	1.16
Anilide	.1	0	.1	.22	.6	.98
	.3	0	.1	"	.62	.96
	1	0	.1	"	"	1.0
	3	0	.24	.28	.66	1.16

* Growth given in optical density units, as measured with a Welch Densichron equipped with a red sensitive probe.

The alteration of a single amide group on one of the 3 propionamide chains did, however, make the B_{12} molecule practically inert for *E. coli* and *O. malhamensis*: The results with *O. malhamensis* are consistent with its specificity for true B_{12} (cyanocobalamin) or other cobalamins active for higher animals(8). As previously noted, this specificity suggests that *O. malhamensis* is an especially useful organism for testing B_{12}-antagonism(9).

The reason sub-optimal levels of methionine act synergistically with methylamide and ethylamide to enhance *E. coli* growth (Table II) is obscure. Perhaps the amides cannot perform some of the metabolic functions of B_{12} in the absence of a source of labile methyl groups.

Summary. The methylamide, ethylamide, and anilide of the monocarboxylic acid of Vit. B_{12} were inactive as B_{12}-antagonists despite their previous designation as "anti-Vit. B_{12}" substances. They satisfied the B_{12} requirement of *E. gracilis* and *L. leichmannii,* but not that of *E. coli* and *O. malhamensis*. Combinations of sub-optimal levels of methionine plus methylamide, or ethylamide, gave greater growth of *E. coli* than this level of methionine alone. Addition of amide to Vit. B_{12} neither inhibited nor enhanced growth over that with B_{12} alone.

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Agglutination of Erythrocytes *in vivo* in Mice after Injection of *Ascaris* Extracts, Related to Immunization with Human Erythrocytes. (24792)

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In previous studies(1,2), it was found that extracts from the isolated tissues of *Ascaris lumbricoides*, such as cuticle, muscle, intestine, eggs, sperm, and coelomic fluid, possess certain immunologic properties briefly summarized as follows: 1. When such extracts are added in specific amounts to human serums of Groups O and B, a_2 isoagglutinin titer is reduced to negative. 2. When injected intravenously into dogs and guinea pigs that contain a_2 isoagglutinins in their plasma, when tested against human erythrocytes of subgroup A_2, *in vivo* agglutination of erythrocytes occurs, leading to formation of large clumps of erythrocytes, seen chiefly in the liver. When dogs are utilized, there occur severe reactions suggestive of anaphylaxis and followed by death. 3. When injected intravenously into normal mice with no a_2 titers in their plasma, agglutination of erythrocytes was not observed in liver and kidney, but agglutination takes place when animals develop a_2 titers by a previous artificial immunization with human erythrocytes of subgroup A_2. It has been postulated that upon injection of ascaris tissue extract, the substance with A_2 isoagglutinogen-like property, adheres to the erythrocytes. Agglutination is then accomplished by union of a_2 antibody in animal's plasma with erythrocytes which had the A_2-like antigen induced by the substance present in the extract. Further studies were therefore necessary to determine the specificity of the reaction; that is, whether by using the erythrocyte-tissue extract combination agglutination of erythrocytes would be also observed in mice immunized against human erythrocytes of subgroup A_1, and Groups B and O, as seen in those immunized against erythrocytes of subgroup A_2. In addition, the pathological reactions in the liver and other organs of all animals needed more detailed consideration. Mice of A strain, of both sexes, and weighing 20 to 30 g, were injected intraperitoneally with 1% suspension of human erythrocytes of Groups B and O, and subgroups A_1 and A_2, in physiological saline. Each animal received injections of 1 cc of the particular cell suspension for 3 consecutive days, followed by another series of 3 injections administered after interval of 4 days. Ten to 15 days after last injection, the mice were injected into tail vein, with 1 mg of ascaris tissue extract suspended in 0.5 cc of saline. The extract prepared from the cuticle was the only one utilized in this study, since previous experiments showed that extracts from other ascaris tissues elicited the same reactions(2). Mice were killed 24 hours following injection. Normal mice, as well as mice injected with A_2 cells alone, and with cuticular extract alone, were utilized as controls. Organs of all animals were fixed in Zenker-formol and stained with hematoxylin and eosin after paraffin embedding.