Mono-Substituted Vit. B_{12} Amides. II. Further Inhibition Study.*† (25717)

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Methylamide, ethylamide, and anilide of the monocarboxylic acid of vit. B_{12} were inactive as B_{12}-antagonist for B_{12}-requiring organisms(1); the highest concentration tested was 1 mμg/ml. In another study these amides in concentrations above 100 mμg/ml inhibited B_{12} utilization by *Ochromonas malhamensis*(2). Here we compare microbiological activity for 4 different B_{12}-requiring organisms of mono- and dibasic acid of B_{12}, the monoacid ethylamide, and a carbanilide derivative. Preparation of mono-amides has been reported(3,4); monoacid of ethylamide and the di-(ethylamide) were prepared by reaction of 1 or 2 moles of ethylamine with dibasic acid of B_{12}. Smith also prepared the carbanil derivative by linking it with one or both of the ribose hydroxyls. We extended previous study of monosubstituted amides because a clinical trial on an adult leukemia patient indicated antagonism against B_{12} storage in the liver.

Methods. The organisms used were *Lactobacillus leichmannii,* ATCC 7830, *Escherichia coli* 113-3, ATCC 11105, *Euglena gracilis,* Z strain, ATCC 12716, and *Ochromonas malhamensis,* ATCC 11532. *L. leichmannii* was grown on prepared dry mix from Difco Labs. B_{12} assays with these organisms have been described(5,6,7). The anilide, methylamide, ethylamide, monobasic acid, dibasic acid, ethylamide monoacid, di-(ethylamide), and carbanilide derivatives of vit. B_{12} obtained as powders, were dissolved in distilled water. Solutions were stored at 4°C with volatile preservative(8).

Results. Effect of substituted B_{12} amides and acids on growth of *O. malhamensis* and *E. coli* is summarized in Tables I and II. *O. malhamensis*: The methylamide, monobasic acid, and carbanilide promoted growth comparable to cyanocobalamin. The anilide permitted some growth, the greatest at 10^4 mμg/ml. The dibasic and ethylamide monoacids, and di-(ethylamide) did not support growth; they sharply inhibited B_{12} utilization above 10^2 mμg/ml. Inhibition by anilide was not as strong; it even supported some growth at 10^4 mμg/ml. *E. coli* 113-3: Methylamide and carbanilide permitted full growth; dibasic acid permitted growth above 10^3 mμg/ml. Anilide, ethylamide, monobasic acid, dibasic acid, ethylamide monoacid, and di-(ethylamide) antagonized B_{12} utilization above 10^2 mμg/ml. For *O. malhamensis* and *E. coli* appropriate B_{12} concentrations annulled inhibitions by these compounds indicating competitive type of inhibition. With *E. coli* grown in suboptimal concentrations of DL-methionine instead of B_{12}, as concentration of amides and acids were increased, full growth was attained; in absence of methio-

TABLE I. Effect of B_{12} Amides and Acids on Growth of *Ochromonas malhamensis.**†

Compound	Conc. (mμg/ml)	B_{12} (mμg/ml) 0	.01	.1	1
Control		.1	.78	2.8	3.5
Anilide	1	.24	.86	2.5	3.3
	10	.42	.94	2.3	3.2
	10^2	.46	.84	1.1	3.8
	10^3	.54	.78	.9	2.9
	10^4	.56	.8	.86	1.0
Methylamide	1	.9	1.0	2.3	3.2
Monobasic acid	10	2.6	3.1	3.7	3.5
Carbanilide	10^2	3.2	3.9	4.0	3.8
	10^3	3.3	3.6	3.8	3.7
	10^4	3.4	3.7	3.8	3.8
Ethylamide monoacid	1	.16	.8	3.3	3.0
	10	.04	.6	3.5	2.7
Di-(ethylamide)	10^2	.02	.04	1.8	2.7
	10^3	.02	.02	.04	.8
	10^4	.02	.02	.04	.04
Ethylamide Dibasic acid	1	.14	.8	3.4	3.9
	10	.1	.7	3.0	3.6
	10^2	.12	.1	3.6	2.6
	10^3	.1	.06	.32	1.8
	10^4	.1	.1	.26	.2

* Data given were observed with first member of each group. Results with other members were similar.

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

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TABLE II. Effect of B_{12} Amides and Acids on Growth of *E. coli* 113-3.* Growth measured as in Table I.

Compound	Conc. (mμg/ml)	B_{12} (mμg/ml) 0	.1	1	10
Control		0	.42	.84	.96
Methylamide	1	.12	.66	.88	.94
Carbanilide	10	.66	.76	.88	.92
	10^2	.72	.74	.76	.88
	10^3	.74	.74	.76	.8
	10^4	.72	.76	.76	.78
Anilide	1	.0	.48	.88	.92
Ethylamide	10	.0	.54	.9	.92
Ethylamide mono-acid	10^2	.0	.14	.88	.92
	10^3	.0	.06	.38	.88
	10^4	.0	.06	.08	.44
Di-(ethylamide)	1	.06	.38	.94	1.0
	10	.06	.36	.96	.98
	10^2	.06	.4	.96	1.0
	10^3	.06	.2	.94	.96
	10^4	.04	.02	.76	.96
Dibasic acid	1	.1	.4	.94	1.0
	10	.1	.54	.98	1.0
	10^2	.2	.64	.6	.94
	10^3	.74	.68	.8	.96
	10^4	.84	.82	.86	.92
Monobasic acid	1	.0	.66	.92	.92
	10	.0	.12	.78	.96
	10^2	.0	.06	.18	.86
	10^3	.0	.06	.08	.16
	10^4	.0	.06	.06	.06

* Data given were observed with first member of each group. Results with other members were similar.

nine the amides permitted no growth (Table III).

All preparations were about as active for *E. gracilis* and *L. leichmannii* as was true B_{12}.

Discussion. Methylamide and anilide of B_{12} antagonize growth of *E. coli* 113-3 and *O. malhamensis*(2,9). In this study the anilide, but not the methylamide, inhibited. Inhibition of *O. malhamensis* by ethylamide confirms Ford(2). Previously(1) we had seen no inhibition because concentrations of the antagonists above 1 mμg/ml were not used. Growth promotion by methylamide does not agree with previous results(1). This compound stimulates both growth of *E. coli* and *O. malhamensis*. The fresh batch, reported here, may have been contaminated with small amounts of true B_{12}; this was not so with the previous preparation(1). To test this assumption, the preparation of methylamide sent by Smith, upon which previous observations(1) were based, was restudied. As before, no stimulation was noted.

The stimulation produced by monobasic acid for *O. malhamensis* without comparable stimulation for *E. coli* rules out contamination by true B_{12} (cyanocobalamin) or other cobalamins active for higher animals(10); perhaps *O. malhamensis* converts this derivative to true B_{12} or else uses B_{12} with such an altered cyanocorphinamide.

Methionine results with *E. coli* agree with our earlier ones(1). Suboptimal levels of methionine act synergistically with B_{12} amides and acids enhancing *E. coli* growth. Methionine supplies methyl groups which may permit B_{12}-antagonists to act as true B_{12} in syntheses requiring methyl group transfer (11). Hence the B_{12}-antagonists, at least in *E. coli* 113-3, act by inhibiting methyl group synthesis; if preformed methyl groups are supplied, the bacterium retains its ability to transfer these groups.

Assuming no B_{12} contamination, carbanilide activity for *O. malhamensis* and *E. coli* shows that both organisms can convert or utilize this compound as well as true B_{12}. The reason dibasic acid antagonizes B_{12} utilization for *O. malhamensis* and not for *E. coli* is obscure.

The activity noted here for various antagonists against B_{12}-requiring organisms appears

TABLE III. Effect of B_{12} Amides and Acids on *E. coli* 113-3, Grown on DL-methionine.*†

Compound	DL-methionine (μg/ml) 1	3	10
Control	.08	.14	.52
Anilide	.14	.26	.6
Ethylamide	.22	.42	.66
Monobasic acid	.26	.52	.7
	.44	.62	.78
	.42	.62	.9
Methylamide	.4	.52	.68
Dibasic acid	.7	.74	.84
Carbanilide	.8	.74	.84
	.78	.8	.86
	.8	.8	.88
Ethylamide monoacid	.12	.26	.54
Di-(ethylamide)	.14	.36	.64
	.18	.4	.66
	.16	.44	.66
	.18	.46	.68

* Data given were observed with first member of each group. Results with other members were similar.

† No growth without DL-methionine.

to reflect subtle differences in B_{12} metabolism. One might postulate that the greatest use of these compounds would be in clinical entities embodying highly deranged B_{12} metabolism, as in leukemia and liver disease(12,13,14).

Summary. The anilide, ethylamide, ethylamide monoacid, and di-(ethylamide) derivative of B_{12} inhibit cobalamin utilization by *O. malhamensis* and *E. coli.* The monobasic acid inhibits B_{12} utilization by *E. coli,* but not by *O. malhamensis:* the reverse is true for the dibasic acid. Methylamide and carbanilide derivatives are utilized as well as true B_{12}; all compounds are as active as true B_{12} for *E. gracilis* and *L. leichmannii.* In *E. coli,* suboptimal levels of methionine in presence of B_{12} amides and acids give increasing growth with increasing concentrations of B_{12} derivatives; neither methionine nor B_{12} acid and amide, when added alone, permit growth.

1. Baker, H., Frank, O., Pasher, I., Hutner, S. H., Herbert, V., Sobotka, H., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 825.

2. Ford, J. E., *J. Gen. Microbiol.,* 1959, v21, 693.

3. Smith, E. L., *Nature,* 1958, v181, 305.

4. ———, in *The Chemistry and Biology of Purines,* ed. G. E. W. Wolstenholme, C. M. O'Connor, Little, Brown and Co., 1957, p160.

5. Baker, H., Sobotka, H., Pasher, I., Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 636.

6. Hutner, S. H., Bach, M. K., Ross, G. I. M., *J. Protozool.,* 1956, v3, 101.

7. Burkholder, P. R., Burkholder, L. H., *Science,* 1956, v123, 1071.

8. Hutner, S. H., Bjerknes, C. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 393.

9. Cuthbertson, W., Gregory, J., O'Sullivan, P. Pegler, H. I., *Biochem. J.,* 1956, v2, 15P.

10. Hutner, S. H., Cury, A., Baker, H., *Anal. Chem.,* 1958, v30, 849.

11. Johnson, B. C., Holdsworth, E. S., Porter, J. W. G., Kon, S. K., *Brit. J. Nutrition,* 1957, v11, 313.

12. Rachmilewitz, M., Izak, G., Hochman, A., Aronovitch, J., Grossowitz, N., *Blood,* 1957, v12, 804.

13. Rachmilewitz, M., Aronovitch, J., Grossowitz, N., *J. Lab. Clin. Med.,* 1956, v48, 339.

14. Baker, H., Brill, G., Pasher, I., Sobotka, H., *Clin. Chem.,* 1958, v4, 27.

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Influence of ACTH upon Avian Species and Osteoporosis.* (25718)

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During previous investigations(1,2,3), osteoporosis was found in the culls of a flock of White Leghorn hens bred for heavy egg production. Endocrine interactions and role of pituitary, adrenals, and gonads in pathogenesis of this disorder are obscure. In the human, *species in which osteoporosis is common,* it is debatable whether exogenous ACTH is a causative agent or whether it only aggravates a pre-existing condition. Adrenal cortex increases in size and weight during reproduction in birds, due to hyperplasia and hypertrophy and occurs presumably through increased secretion of ACTH. In White Leghorn hens and roosters, the cortex represents 43% of weight of both adrenal glands(4). Bovine ACTH increases size of adrenal cortex, and prolongs survival of hypophysectomized birds; cortisone sustains the animal for indefinite period. Both ACTH and cortisone are powerful suppressors of bone growth in the chick(5). Pituitary - adrenocortical - gonadal hormone interactions have been demonstrated in birds(4,6,7) by the following experiments: (a) castration causes enlargement of adrenal cortex; (b) unilateral ovariectomy is followed by hypertrophy of opposite vestigial ovary if adrenals are intact but not if adrenal glands are also excised; (c) adrenal cortex may react to injections of small or large doses of estrogen or androgen in various ways, directly and indirectly through liberation or suppres-

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