[CONTRIBUTION FROM THE DEPARTMENTS OF PHYSIOLOGY AND PEDIATRICS OF THE SCHOOL OF MEDICINE AND THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF PENNSYLVANIA]

Benzimidazoles as Specific Inhibitors of Vitamin B_{12} or Thymine in Bacterial Mutants[1,2]

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Substituted benzimidazoles have been tested on six microbial systems to determine the specificity of their inhibition of growth. SH in the 4-position produced a compound with specific inhibition of the vitamin B_{12} requiring mutant and this inhibition was reversed by excess vitamin B_{12}. Compounds with $-NO_2$ in the 4-position and to a less extent in the 6-position were also inhibitory to the vitamin B_{12} mutant and the inhibition was reversed by methionine. Alkyl or chloride substitution on the 5- and/or 6-positions produced competitive inhibitors of thymine. Compounds with two or three alkyl groups on positions 2, 5 and 6 especially were inhibitory to *L. casei*, the growth of which was limited by folic acid.

Introduction

Vitamin B_{12} has been shown to be essential for growth of many organisms, including man. Therefore it seems possible that a specific inhibitor of vitamin B_{12} might have a carcinostatic effect. 5,6-Dimethylbenzimidazole is one component of the vitamin B_{12} molecule. Benzimidazoles with other substituents might be expected to act as analogs of the vitamin B_{12} benzimidazole. Hoover and Day[3] have synthesized a series of derivatives of benzimidazole with substituent groups, amino, nitro, chloro, mercapto and methyl mercapto, mainly on the 4- and 6-positions. These compounds and some of a series of benzimidazoles found by Tamm, *et al.*,[4] to be inhibitory to the development of certain viruses, have been investigated to determine the inhibition of growth of six microbial systems.

Methods and Materials.—The 49 substituted benzimidazole compounds tested in this study are listed in Table I, which also includes the source from which they were procured and the molecular weights.

The organisms used were the vitamin B_{12} or methionine requiring *E. coli* 113-3 or B68, purine-less *E. coli* B96, thymine-less *E. coli* $15T^-$ and *L. casei* ATCC7469 with either limiting folic acid or limiting riboflavin. The media, procedures for culture and for assay were adaptations of methods described previously.[5] Results are expressed in terms of per cent. inhibition of growth compared with the control, as determined by optical density readings on the Klett–Summerson photoelectric colorimeter or by change in *p*H of the cultures.

Screening of compounds was accomplished by incubation of non-aerated cultures containing three levels of the drug (0.01, 0.1 and 1.0 mg./ml.) and a concentration of the metabolite which permitted the control culture to attain half of the maximum growth. Where inhibition was noted, further tests were set up with increased concentration of metabolite and appropriate concentrations of the drug to determine whether the inhibition could be overcome and thus whether the drug was acting as a specific competitor of the metabolite.

Studies of the effect of the compounds on the growth, cell division and synthesis of nucleic acids in cultures of the wild type *E. coli* B will be reported in a subsequent communication.

Results

The importance of both the substituent and its position on the benzimidazole molecule is clearly evident from consideration of the results obtained with the compounds in the different mutant systems.

The compounds which were most inhibitory to, and reversed by, vitamin B_{12} are listed in Table II, which expresses the inhibition in terms of the concentration of drug producing 50% inhibition of growth as compared with the controls, at the levels of metabolite indicated.

Ten of these benzimidazoles acted as specific inhibitors of the vitamin B_{12} requiring mutant, in that the inhibition was reversed by increased amounts of vitamin B_{12} or methionine.

The nitro and the mercapto groups in position 4 or 6 were in competition with the vitamin B_{12}. Methionine was most effective in counteracting the inhibition caused by the nitro group in the 4-position (Fig. 1), was as effective as increased vitamin B_{12} against inhibition caused by the nitro group in

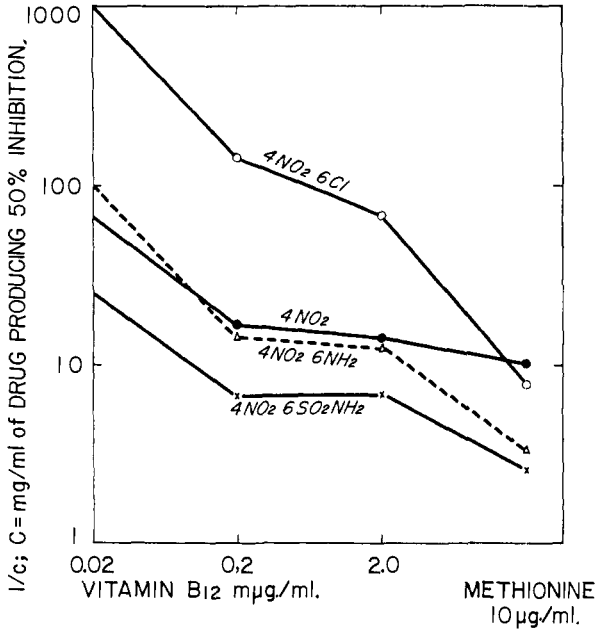

Fig. 1.—Reversal of 4-nitrobenzimidazole inhibition of growth of *E. coli* 113-3 by vitamin B_{12} or methionine. Cultures of *E. coli* 113-3 were incubated in 5 ml. of medium for 24 hr. without aeration. They contained concentrations of compounds and vitamin B_{12} or methionine as indicated. Growth was determined as turbidity with 660 mμ filter.

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(2) This investigation was supported by a grant (PHS C-2189) from the National Institutes of Health, Public Health Service.

(3) J. Hoover and A. Day, THIS JOURNAL, **76**, 4144 (1954); **77**, 4324, 5652 (1955).

(4) I. Tamm, K. Folkers, C. H. Shunk, D. Heyl and F. L. Horsfall, *Exp. Med.*, **98**, 245 (1953); I. Tamm, K. Folkers and C. H. Shunk, *J. Bact.*, **72**, 54, 59 (1956); I. Tamm, *ibid.*, **72**, 42 (1956).

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Table I

Benzimidazoles

Code no.	Position of substituent 1	2	4	5	6	Salt	Mol. wt.	Source[a]
1	.....	....	...	...	....	...	118	C
2	.....	Iso-C_3H_7	...	...	....	...	160	P
3	.....	*n*-C_3H_7	...	...	....	...	160	P
4	.....	$-CH_2CH{=}CHCH_3$	...	...	....	$\cdot H_2O$	190	P
5	.....	C_2H_5	...	...	....	...	146	P
6	.....	CH_3	...	...	....	...	132	P
7	.....	....	...	CH_3	....	...	132	E
16	.....	CH_3	...	CH_3	....	...	146	M
17	.....	....	...	CH_3	CH_3	...	146	M
18	.....	C_4H_9	...	CH_3	....	...	188	M
19	β-D-Ribofuranosyl	....	...	Cl	Cl	...	318	M
20	β-D-Glucosyl	....	...	...	....	...	296	P
25	.....	....	...	OCH_3	....	...	148	M
26	.....	....	...	NH_2	....	$\cdot 2HCl$	206	P
28	.....	....	NH_2	...	NH_2	$\cdot 2HCl$	221	P
30	.....	....	NH_2	...	....	$\cdot 2HCl$	206	P
33	.....	....	NH_2	...	NO_2	...	178	P
38	.....	....	...	NO_2	....	...	163	P
42	.....	C_2H_5	...	CH_3	....	...	160	M
43	.....	CH_3	...	CH_3	CH_3	...	160	M
44	.....	....	...	Cl	Cl	...	187	M
48	.....	....	NO_2	...	Cl	...	198	P
54	.....	....	Cl	...	NO_2	...	198	P
55	.....	....	NH_2	...	Cl	$\cdot HCl$	204	P
56	.....	....	SH	...	NO_2	$\cdot HCl$	232	P
57 or 57B	.....	....	Cl	...	NH_2	$\cdot HCl \cdot H_2O$	222	P
58	.....	$-CH_2CH(NH_2)COOH$	...	...	....	...	205	P
63	.....	....	SH	...	NO_2	...	195	P
66	.....	....	SH	...	NH_2	$\cdot 2HCl$	238	P
67	.....	....	NH_2	...	SO_2OH	...	213	P
68	.....	....	...	Cl	....	...	153	P
73	.....	....	NO_2	...	SO_2NH_2	...	242	P
88	.....	....	NH_2	...	SH	$\cdot 2HCl$	238	P
90	.....	....	Cl	...	Cl	...	187	P
91	.....	....	NO_2	...	NH_2	...	178	P
99	.....	....	NH_2	...	SO_2NH_2	$\cdot HCl$	249	P
136	.....	....	SCH_3	...	NO_2	...	209	P
147	.....	....	SCH_3	...	NH_2	$\cdot 2HCl \cdot {}^1/_2C_6H_6$	291	P
148	.....	....	NO_2	...	....	...	163	P
190	β-D-Ribofuranosyl	....	Cl	Cl	Cl	...	354	M
163	.....	$-CH_2CH_2COOH$	...	...	....	...	190	P
164	Diethyl-N-4-(2-benzimidazolyl)-ethylaminobenzoyl glutamate HCl						466	P
172	N-(1-Benzyl-2-benzimidazolylmethyl)-pyridinium chloride						336	P
174	N-(1-Benzyl-2-benzimidazolylmethyl)-3-carboxamidopyridinium-chloride						379	P
213	1,4-Bis-(5-methyl-2-benzimidazolyl)-butane						318	P
214	Diethyl-N-4-(5-chloro-2-benzimidazolyl)-isopropylaminobenzoyl glutamate						515	P
215	N-(α-2-Benzimidazolylethyl)-pyridinium chloride						260	P
216	N-(2-Benzimidazolylmethyl)-3-carboxamidopyridinium chloride						289	P
217	Diethyl, β-hydroxyethyl,-2-benzimidazolylmethylammonium chloride						284	P

[a] Source: C = Commercial. P = Chemistry Department, University of Pennsylvania. E = Eastman. M = Merck.

the 6-position, and was less effective than vitamin B_{12} when the growth was inhibited by the 4-mercapto substitution (Fig. 2). Methylation of the mercapto group decreased it inhibitory capacity.

While inhibition by 6-nitrobenzimidazole was reversed by increased vitamin B_{12}, the slope of the reversal lines was less than with the 4-nitro compounds. Substitution of chlorine on the 4-position on the 6-nitrobenzimidazole changed the pattern of inhibition, decreasing the ability of vitamin B_{12} to reverse the inhibition as compared with the 6-nitro compound (Fig. 3). The substituent on position 4 would seem to have more influence on the pattern of activity than the substituent on position 6.

Inhibitions by 4,6-dichlorobenzimidazole and 5,6-dimethylbenzimidazole were partially relieved by vitamin B_{12} or methionine. The inhibition of growth of *E. coli* 113-3 due to the other alkyl substituted benzimidazoles was not reversible by vitamin B_{12} or methionine. However, methionine was partially effective in reversing these same compounds in the purine-less B96 system.

Table III includes compounds which inhibited

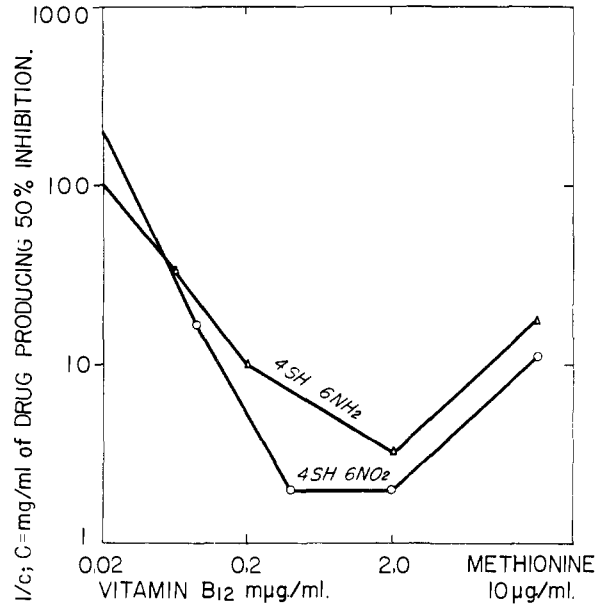

Fig. 2.—Inhibition of growth of *E. coli* 113-3 with 4-mercaptobenzimidazole compounds and reversal by vitamin B_{12}. Cultures of *E. coli* 113-3 were grown as in Fig. 1. They contained concentrations of 4-SH-benzimidazoles and vitamin B_{12} or methionine as indicated.

the growth of *E. coli* 113-3, but whose action was not at all or only slightly reversed by increased vitamin B_{12} or methionine.

TABLE II

CONCENTRATIONS OF SUBSTITUTED BENZIMIDAZOLES PRODUCING 50% INHIBITION OF GROWTH OF *E. coli* 113-3 REQUIRING VITAMIN B_{12} OR METHIONINE

Code no.	Position 2	4	5	6	Concn. of drug, μg./ml. Vitamin B_{12}, mμg./ml. 0.02	0.2	2.0	Methionine 10 μg/ml.
48		NO_2		Cl	1, 3	7, 12, 23	10, 13	130
66		SH		NH_2	8, 10, 60	100	300	..
63		SH		NO_2	5, 10	200	500	90
148		NO_2		...	10, 20	60	70	100
91		NO_2		NH_2	10, 20	65	65	270
73		NO_2		SO_2NH_2	17, 35	120	125	320
54		Cl		NO_2	40	100	120	200
33		NH_2		NO_2	50	220	230	200
38		..		NO_2	50	200	260	210

TABLE III

CONCENTRATION OF SUBSTITUTED BENZIMIDAZOLES PRODUCING 50% INHIBITION OF GROWTH OF *E. coli* 113-3

Code no.	Position of substituent 2	4	5	6	Concn. of drug, μg./ml. Vitamin B_{12}, mμg./ml. 0.02	0.2	2.0	Methionine 10 μg./ml.
90	..	Cl	..	Cl	40	50	70	65
18	C_4H_9	..	CH_3	..	75	65	50	..
44	..	..	Cl	Cl	80	..	..	..
17	..	..	CH_3	CH_3	20	140	150	170
43	CH_3	..	CH_3	CH_3	50	..	..	..
68	..	..	Cl	..	100, 150	150	150	150
42	C_2H_5	..	CH_3	..	300	300	300	300
55	..	NH_2	..	Cl	250, 150	300	200	250
28	..	NH_2	..	NH_2	100	..	..	200
57 B	..	Cl	..	NH_2	500, 900	400	400	400
88	..	NH_2	..	SH	200	300	200	500
1	Unsubstituted				..	500	350	450
16	CH_3	..	CH_3	..	>500	>500	>500	..

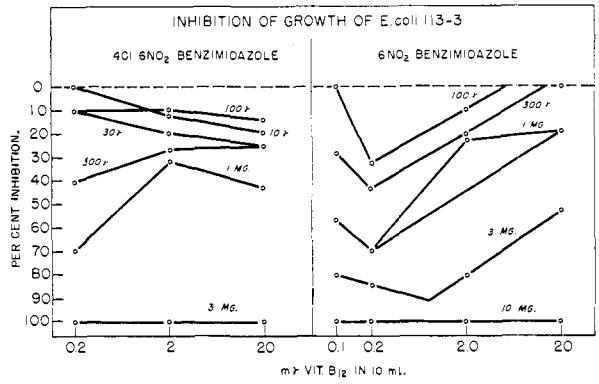

Fig. 3.—Patterns of growth of *E. coli* 113-3 inhibited by 4-chloro- 6-nitrobenzimidazole and 6-nitrobenzimidazole. Concentrations indicated are amounts in 10 ml. of medium.

Growth of the pyrimidine requiring mutant *E. coli* 15T⁻ was inhibited by the compounds listed in Table IV. The action of some of these was reversed by increased concentrations of thymine. The pattern of inhibition and reversal was different from that with the vitamin B_{12} requiring organism inhibited by the nitro or mercapto compounds. The most effective compounds were those substituted with methyl or chloro on 5- and/or 6-positions. The 5,6-dichlorobenzimidazole showed a pattern of inhibition and reversal which is graphed in Fig. 4. Inhibition of *E. coli* 15T⁻ by 4-nitro-, 6-chloro-

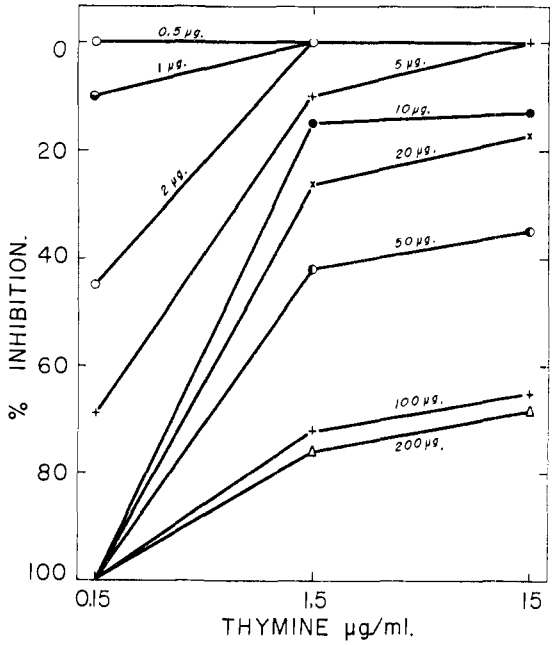

Fig. 4.—Reversal of 5,6-dichlorobenzimidazole inhibition of growth of *E. coli* 15T⁻ by increased thymine. Concentrations of 5,6-dichlorobenzimidazole and thymine as indicated are amounts per ml. of medium.

benzimidazole was not reversible by increased thymine but partially reversed by methionine. Compounds which are substituted on the 2-position, that is the carbon of the imidazole ring, were inactive against the thymine requiring organism.

TABLE IV

CONCENTRATION OF SUBSTITUTED BENZIMIDAZOLES PRODUCING 50% INHIBITION OF *E. coli* 15T⁻ REQUIRING THYMINE

Code no.	Position of substituent 1	2	4	5	6	Concn. of drug, μg./ml. Thymine μg./ml. 0.15	1.5	Reversal
17	....	.....	..	CH_3	CH_3	0.01–30	80–120	+
44	....	.....	..	Cl	Cl	2.5	60	+
7	....	.....	..	CH_3	..	1, 12	>100	+
25	....	.....	..	OCH_3	..	1, 15, 100	5–500, 600	+
68	....	.....	..	Cl	..	20	190	+
190	Ribose	.....	Cl	Cl	Cl	30	30	−
63	....	.....	SH	..	NO_2	60	...	
1	....	.....	..	..	..	140	650	+
48	....	.....	NO_2	..	Cl	80, 140	80, 140	−, M[a]
30	....	.....	NH_2	..	...	70, 170	500	+
55	....	.....	NH_2	..	Cl	200[b]	...	
26	....	.....	..	NH_2	..	200[b]	...	
66	....	.....	SH	..	NH_2	200[b]	...	
38	....	.....	..	NO_2	..	300[b]	...	
172	Benzyl	Methylpyridinium	..	CH_3	..	300[b]	...	

[a] Partially reversed by methionine. [b] Estimated from preliminary screening.

TABLE V

CONCENTRATIONS OF SUBSTITUTED BENZIMIDAZOLES PRODUCING 50% INHIBITION OF *E. coli* B96 REQUIRING PURINE

Code no.	Position of substituent 2	4	5	6	Concn. of drug (μg./ml.) Adenine-SO_4 5 μg./ml.	Adenine-SO_4 20 μg./ml.	Guanine-HCl 5 μg./ml.	Guanine-HCl 20 μg./ml.	Reversed by[a]
213	Dimer on butane	..	..	..	1		8		
					10	5	20	5	
					20[b]	10	5	2	
18	C_4H_9	..	CH_3	..	<20	<20	<20	<20	M,T,R
					55	40			
48	....	NO_2	..	Cl	30				
					15	12			M
					20	12	30	20	(R inhibits)
148	....	NO_2	..	..	30		30–100		
					15	30			M
							(100 stim.)	>100	(R inhibits)
44	....	..	Cl	Cl	30		15	15	M,R
					20	15			(FA inhibits)
38	....	..	NO_2	..	30		..	..	
					180	130			
33	....	NH_2	..	NO_2	30		..	..	
					(100 stim.)	140			
90	....	Cl	..	Cl	30		..	..	
					60	60			
73	....	NO_2	..	SO_2NH_2	60		..	..	M
					70	70			
						150			
91	....	NO_2	..	NH_2	70	70			
					150	150	300	200	M
68	....	..	Cl	..	140	120			
					120	70	<50	60	M,R
42	C_2H_5	..	CH_3	..	150	..	..	..	
1	....	..	..	..	350				
					700	500	..	..	
26	....	NH_2	..	NH_2	300			..	
					>1000	>1000	>1000	..	
66	....	SH	..	NH_2	300	..	300	..	
54	....	Cl	..	NO_2	350				
					200	150	..	..	
16	CH_3	..	CH_3	..	300	..	..	..	

[a] M = methionine. T = thymine. R = riboflavin. FA = folic acid. [b] Stimulated at 5 μg./ml.—results on this compound were variable.

The compounds which had methyl groups on 5 or 5 and 6 and the 5-methoxy inhibited to about the same extent over a wide range of concentrations.

Table V includes the compounds which inhibited growth of the purine requiring *E. coli* B96. With none of these was the inhibition reversed by in-

creased amounts of adenine or guanine, though inhibition was not always to the same extent when guanine rather than adenine was the limiting purine. Reversal of inhibition by some compounds could be obtained by addition of thymine, methionine or riboflavin, that is, by the metabolite to which the compound showed specific antagonism in the other mutant systems. Thus the inhibition of growth of the purine requiring mutant by the 4-nitrobenzimidazoles was reversed by addition of methionine to this system but not by addition of B_{12}, thymine, folic acid, purines or riboflavin. The inhibition by 5,6-dichlorobenzimidazole or 2-butyl-5-methylbenzimidazole was partially reversed by methionine, thymine or riboflavin but was increased by the addition of folic acid. Riboflavin was most effective in reversing inhibition of B96 by 5-chlorobenzimidazole.

Cultures of *L. casei* in which growth was limited to 50% of the maximum by limitation of the concentration of folic acid in the medium responded with decreased growth to the presence of certain of the 2-alkyl substituted benzimidazoles (Table VI) at concentrations which were not inhibiting to the *E. coli* mutants.

TABLE VI

CONCENTRATIONS OF SUBSTITUTED BENZIMIDAZOLES PRODUCING 50% INHIBITION OF GROWTH OF *Lactobacillus casei* WITH LIMITED FOLIC ACID

Code no.	Position of substituent 2	4	5	6	Drug concn. μg./ml. with 0.3 mμg./ml. of folic acid
17	..	..	CH_3	CH_3	<10
16	CH_3	..	CH_3	..	<10
18	C_4H_9	..	CH_3	..	<10
43	CH_3	..	CH_3	CH_3	10–100
42	C_2H_5	..	CH_3	..	20, 600nr[a]
163	C_2H_4COOH	..	..	..	30

[a] nr = no reversal.

Compounds of Table I which do not appear in any of the other tables did not produce 50% inhibition of growth at levels below 300 μg./ml. in any system.

Discussion

Benzimidazoles have been found to have pharmacological activity of several kinds and the effect of substituted derivatives has been studied in some instances.

In animals benzimidazole causes a reversible flaccid muscle paralysis and prevents the convulsions caused by strychnine, metrazol or electric shock. In lower doses benzimidazole causes polydipsia and polyuria in rats by specific inhibition of renal tubular resorption of water.[6]

Domino, Unna and others[7] studied the effect of benzimidazole derivatives on this depressant action, which they described as depressing interneuronal activity without affecting monosynaptic reflex arcs. Substitution in the 2-position increased the toxicity and paralyzing action. The toxicity increased with increasing number of carbon atoms in an alkyl chain up to five, while the greatest degree of paralysis was caused by 2-amino- or 2-methylaminobenzimidazole.

Benzimidazole with a mercapto in the 2-position was more goiterogenic than thiouracil; that is, it decreased the iodine content and increased the weight of the thyroid of rats.[8] Additional substitution of methyl or halogen on the 5-position decreased the activity. The unsubstituted benzimidazole was inactive.

The compounds found by Tamm and Folkers[4] to be most active in inhibiting the growth of viruses in fertile eggs or in mice were the di- or trialkyl or halogen substituted benzimidazoles, including those substituted in the 2-position and their 1γD-ribofuranosyl derivatives.

Hendlin and Soars[9] found the inhibition of growth of *Lactobacillus lactis* Dorner by 5,6-dimethylbenzimidazole was increased by alkyl substitution in the 2-position and the toxicity was increased with increasing number of carbons in the alkyl group up to the butyl.

If any correlation of our results with these pharmacological properties is permissible, the toxicity and neurological and goiterogenic effects of benzimidazole are increased by substitution in the 2-position, which in our experience show inhibition of the *L. casei*, the growth of which was limited by folic acid. Whether this characteristic may be comparable to the effect of the folic acid analogs, aminopterin and amethopterin, in affecting tumor growth or whether the benzimidazole derivatives showing specific inhibition of vitamin B_{12}, methionine or thymine may be less toxic and more effective as tumor inhibitors, remains to be determined.

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(9) D. Hendlin and M. H. Soars, *J. Bact.*, **62**, 633 (1951).