untreated mice, and that the LD_{50} for mice treated with impure penicillin was 9 times as great. This finding has been confirmed by repeated experiments of the same kind using a single batch of endotoxin. Results are shown in Table 2.

TABLE 1

PROTECTIVE ACTION OF PENICILLIN AGAINST BACTERIAL ENDOTOXIN

Dose of endotoxin (*S. aertrycke*) (ml)	Numbers of mice killed by each dose of endotoxin		
	Saline control	Crystalline penicillin (mostly G)	Impure penicillin
.8	—	—	10/10
.4	—	—	6/10
.2	—	9/10	5/10
.1	9/10	5/10	0/10
.05	7/10	5/10	—
.025	5/10	2/10	—
.0125	3/10	—	—
LD_{50}*	.025 ml	.082 ml	.235 ml

* Computed by the Reed-Muench formula.

From these figures it is apparent that in a large series of experiments the LD_{50} of endotoxin for mice treated with impure penicillin is 5 times as great as that for control mice and more than twice as great as the LD_{50} for mice treated with crystalline penicillin.

TABLE 2

AVERAGE LD_{50} OF ENDOTOXIN (*S. aertrycke*) IN TREATED AND UNTREATED MICE

Control mice* (untreated)	Mice treated with:	
	crystalline penicillin	impure penicillin
.05 ml	.11 ml	.26 ml
Averages computed from:		
33 experiments 1,113 mice	9 experiments 300 mice	43 experiments 1,436 mice

* This control group includes mice receiving no injections before endotoxin, as well as mice receiving, instead of penicillin, injections of various control materials, e.g. physiological saline solution, corn-steep liquor, aleuronat suspension, etc.

That this protection is not due to the control of intercurrent infections has been demonstrated by routine autopsy cultures on a representative number of mice dying after injection of endotoxin.

The total dose of penicillin used in these experiments was 15,000 units/mouse. Greater amounts provided no additional protection. The penicillin was given in 3 doses at 20, 18, and 2 hrs before injection of endotoxin. Treatment after endotoxin or with a single large dose of penicillin seems to afford less protection.

The impure penicillin preparation[2] which we employ loses its protective activity when treated with sufficient heat or penicillinase to inactivate its penicillin, but its protective activity can be completely restored by the addition of crystalline penicillin in the amount originally present. Other investigators (*3, 4, 5, 7, 8, 9*) have recently reported that certain impurities associated with penicillin enhance its activity in the control of infections. We have not yet determined whether those are the same as the heat-stable impurity factor which we have herein described.

Addendum: Since the completion of the experiments recorded in Table 2, 28 more experiments have been performed using the same impure penicillin with certain improvements designed to reduce its toxicity and to increase its protective activity. The average LD_{50} of endotoxin for the entire group of animals treated with impure penicillin has thereby been raised to about 8 times the LD_{50} for the control animals.

Statistical analysis, using the method of the standard error, shows that a difference of this magnitude between the means of these two sets of data is likely to occur by chance less than one time in a million.

[2] An intermediate fraction in the purification of penicillin, kindly supplied by the Abbott Laboratories.

References

1. BOOR, A. K., and MILLER, C. P. *Science,* 1945, **102,** 427–428.
2. BOOR, A. K., MILLER, C. P., and HAWK, W. D. *Fed. Proc.,* 1947, **6,** 240.
3. GROUPÉ, V., and RAKE, G. *J. Immunol.,* 1947, **57,** 17–29.
4. HOBBY, G. L., BURKHART, B., and HYMAN, B. *Proc. Soc. exp. Biol. Med.,* 1946, **63,** 296–301.
5. HOBBY, G. L., LENERT, T. F., and HYMAN, B. *J. Bact.,* 1947, **54,** 305–323.
6. MILLER, C. P., and BOOR, A. K. *Proc. Soc. exp. Biol. Med.,* 1946, **61,** 18–20.
7. RAKE, G., DUNHAM, W. B., and DONOVICK, R. *J. inf. Dis.,* 1947, **81,** 122–129.
8. RANDALL, W. A., PRICE, C. W., and WELCH, H. *Amer. J. publ. Hlth,* 1947, **37,** 421–425.
9. WELCH, H., RANDALL, W. A., and PRICE, C. W. Unpublished data presented at the Conference on Antibiotic Research held at Washington, D. C., January 31 and February 1, 1947, under the auspices of the Antibiotics Study Section of the National Institute of Health.

The Folic Acid Activity and Antagonism of Two Structurally Related Derivatives of Benzimidazole[1]

PRADISTH C. EDWARDS, DOROTHY STARLING, ALBERT M. MATTOCKS,[2] and HOWARD E. SKIPPER

Biochemistry Division, Southern Research Institute, Birmingham, Alabama

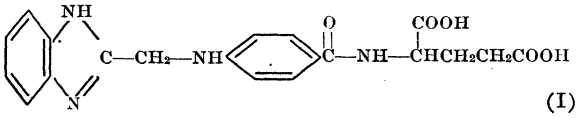
Folic acid is an essential growth factor for *Streptococcus faecalis* R. and other lactic acid bacteria. However, these microorganisms are known to develop without folic acid provided certain pyrimidine and purine bases are present. Thymine can be considered to replace folic acid in the nutrition of *Str. faecalis,* though much higher concentrations are required (*8*). Spies has shown that large doses of thymine elicit a hematopoietic response

[1] Presented at Chattanooga Section, American Chemical Society National Meeting in Miniature, October 11, 1947.

[2] Present address: School of Pharmacy, Western Reserve University, Cleveland, Ohio.

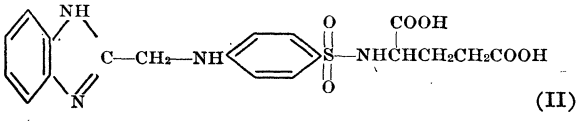
in patients with pernicious anemia (*7*). Orotic acid has been reported to replace folic acid in part for *Lactobacillus casei* (*1*). Martin, Moss, and Avakian (*5*) showed N-[{4-(4-quinazoline)-amino}-benzoyl]-glutamic acid to be a growth factor, the potency of which approximated 0.1–0.01 that of folic acid in the case of bacteria.

A number of compounds which are antagonistic to folic acid have been reported. N-[4-({(2-amino-4-hydroxy-7-methyl-6-pteridyl)-methyl}-amino)-benzoyl]-d(−)-glutamic acid, the most extensively studied of the folic acid antagonists, has been shown to have a remarkable *in vivo* action, producing a typical deficiency syndrome in rats (*3*). Several synthetic pterins have recently been shown to possess marked antibacterial activity which is antagonized competitively by folic acid (*2*). Quinoxaline has also exhibited certain inhibiting effects on the growth of *Str. faecalis* R. (*4*).



(I)

N-[4-({(2-benzimidazolyl)-methyl}
-amino)-benzoyl]-glutamic acid



(II)

N-[4-({(2-benzimidazolyl)-methyl}
-amino)-benzenesulfonyl]-glutamic acid

In our searches for other molecules having folic acid activity or antagonism, two compounds which show interesting relationships of structure to biological action have been synthesized (see Formulas I and II).

The above compounds have been assayed for effects on growth of *Str. faecalis* by the method of Mitchell and Snell (*6*).

The close structural similarity of benzimidazole to purine and its competitive action with amino purines (*10*) led us to the use of this nucleus. Compound I, in which the pyrimido-(4,5)-pyrazine (or pterin) nucleus of pteroylglutamic acid has been substituted by the benzimidazole nucleus, still retains a certain degree of growth-promoting activity. Compound II, differing from Compound I only in the substitution of a sulfonyl for a carbonyl group in the *p*-aminobenzoic acid moiety of the molecule, reverses its biological activity and becomes a metabolite antagonist. Supporting data are presented in Table 1. This particular reversal of activity with change in structure is reminiscent of the classic *p*-aminobenzoic acid-sulfanilamide antagonism of Woods (*9*) and throws more doubt on the specificity of the pteridin nucleus for the folic acid system.

TABLE 1

Folic acid concentration (μ/15 ml)		Effect of Compound I: Amount added (μ/15 ml)		Effect of Compound I: Acid production (ml)* 24 hrs	Effect of Compound I: Acid production (ml)* 48 hrs	Effect of Compound II: Amount added (μ/15 ml)		Effect of Compound II: Acid production (ml) 24 hrs	Effect of Compound II: Acid production (ml) 48 hrs
0		0		0.45	1.06	0		0.45	1.06
0		4×10^{1}	(1×10^{-7})	0.75	2.24	4×10^{1}	(1×10^{-7})	0.63	1.37
0		1.9×10^{3}	(5×10^{-6})	3.10	5.80	2.2×10^{3}	(5×10^{-6})	0.21	0.16
1×10^{-3}	(2.3×10^{-12})†	0		1.75	3.33	0		1.75	3.33
1×10^{-3}	(2.3×10^{-12})	2×10^{2}	(5×10^{-7})	2.70	3.19	2.2×10^{3}	(5×10^{-6})	2.21	4.66
1×10^{-3}	(2.3×10^{-12})	4×10^{2}	(1×10^{-6})	3.24	5.01	4.3×10^{2}	(1×10^{-6})	0.48	0.91
1×10^{-3}	(2.3×10^{-12})	8×10^{2}	(2×10^{-6})	4.70	6.45	8.6×10^{2}	(2×10^{-6})	0.35	0.81
2×10^{-3}	(4.5×10^{-12})	0		3.19	3.87	0		3.19	3.87
5×10^{-3}	(1.1×10^{-11})	0		4.22	5.13	0		4.22	5.13
5×10^{-3}	(1.1×10^{-11})	8×10^{2}	(2×10^{-6})	6.05	8.03	8.6×10^{2}	(2×10^{-6})	0.63	...
8×10^{-3}	(1.8×10^{-11})	0		4.37	5.66	0		4.37	5.66
8×10^{-3}	(1.8×10^{-11})	8×10^{2}	(2×10^{-6})	6.20	8.17	8.6×10^{2}	(2×10^{-6})	0.78	...
1.5×10^{-2}	(3.4×10^{-11})	0		4.45	6.12	0		4.45	6.12

* Acid production measured in ml of 0.05N NaOH/15 ml of culture required to bring to pH 6.8. Turbidimetric measurements were also made and were in good agreement with acid production values.

† The figures in parentheses are the corresponding molar quantities.

References

1. Chattaway, F. W. *Nature, Lond.*, 1944, **153**, 250.
2. Daniel, L. J., Norris, L. C., Scott, M. L., and Henser, G. F. *J. biol. Chem.*, 1947, **169**, 689.
3. Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H. *J. biol. Chem.*, 1947, **169**, 427.
4. Hall, D. A. *Biochem. J.*, 1947, **41**, 294.
5. Martin, G. J., Moss, J., and Avakian, S. *J. biol. Chem.*, 1947, **167**, 737.
6. Mitchell, H. K. and Snell, E. E. *Univ. Texas Publ. 4137*, 1941. P. 36.
7. Spies, T. D. *Experiences with folic acid.* Chicago: Year Book Publishers, 1947. P. 60.
8. Stokes, J. L. *J. Bact.*, 1944, **48**, 201.
9. Woods, D. D. *Brit. J. exp. Path.*, 1940, **21**, 74.
10. Woolley, D. W. *J. biol. Chem.*, 1944, **152**, 225.