this work. They also wish to thank Dr. Kazuo Iwai, Assistant Porfessor of Research Institute for Food Science, University of Kyoto, for the gift of leucovorin.

Summary

Sixty-eight substituted azaindolizines were tested for their activity on *Lactobacillus casei* and *Streptococcus faecalis*. These azaindolizines could be divided into three classes according to the pattern of growth inhibition, substituent present, and the position of the substituent in azaindolizine molecule. Compounds in Group 1 and 2 which have sulfur-containing substituents and halogen groups, respectively, were not affected appreciably by folic acid, leucovorin, thymine, or purines, while Group 2 compounds which have amino or closely related groups were antagonized by folic acid, leucovorin, and thymine but not by purines.

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32. Tadashi Okabayashi : Action of Substituted Azaindolizines on Microörganisms. II.[1] Action of Halogenated Azaindolizines on *Escherichia coli*.

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It has already been shown[1] that substituted azaindolizines may be divided into three groups according to their structure and their action on lactic acid bacteria. It was also demonstrated that there is a considerable discrepancy between their inhibitory action on lactic acid bacteria and their antineoplastic activities. The discrepancy between these two activities was the most prominent in Group 3 compounds which have halogens substituted in the azaindolizine ring. Since some halogenated azaindolizines were reported to cause a considerable damage to the growth of experimental animal tumors,[2] although they exhibited only a little growth inhibition of lactic acid bacteria, it seemed desirable to study their mode of action using some other appropriate microbial system. The present paper deals with the fact that halogenated azaindolizines inhibit the growth of *Escherichia coli* and that the inhibition is reversed by tyrosine and other amino acids.

Table I. Growth Inhibition of *E. coli* Strains by 7-Methyl-5,6-dichloro-1,3,8-triazaindolizine (0307)

0307(γ/cc.)	100	20	4	0.8	0.16	0
ATCC 9637	−	−	−	+	+	+
1011	−	−	−	+	+	+
O-20	−	−	−	+	+	+
287	−	−	−	+	+	+
Comm. W	−	−	−	+	+	+
Comm. MT	−	−	−	+	+	+
K-12	−	−	−	+	+	+

\+ Full growth of test organism observed. − No growth observed.

* 192 Imafuku, Amagasaki, Hyōgo-ken (岡林 直).

1) Part I. T. Okabayashi, H. Kanô, Y. Makisumi : This Bulletin, 8, 157(1960).

2) S. Mineshita, *et al.* : Ann. Repts. Shionogi Research Lab., **9**, 1(1959).

In Table I, the growth inhibition of seven strains of *Escherichia coli* by 7-methyl-5,6-dichloro-1,3,8-triazaindolizine (designated as compound 0307) is presented. This compound was reported by Mineshita, *et al.*[2] to be one of the most powerful carcinostatic agents from more than 100 kinds of substituted azaindolizines. This table clearly shows that the agent is a powerful inhibitor on all *E. coli* strains tested. Preliminary experiments with this agent showed that *E. coli* K-12 is the most profitable strain with regard to the ease of handling and to superiority in examining the reversible effect of metabolites. This strain was therefore used throughout this work.

Further, preliminary experiments showed that the growth-inhibitory activity of 0307 on *E. coli* K-12 was much weaker in nutrient media than in salt-glucose medium. It was considered, therefore, that some of the nutrients might suppress the activity of this agent. Thus, the effect of addition of various amino acids, vitamins, purines, and pyrimidines was examined.

Table II gives a list of substances that were found to be ineffective in relieving the inhibition of 0307.

Table II. Compounds found to be Ineffective in Reversing the Inhibition of 0307

Purines and pyrimidines : Adenine, guanine, xanthine, hypoxanthine, uracil, thymine, thymidine.
Vitamins : Folic acid, leucovorin (synthetic citrovorum factor), thiamine, biotin, riboflavin, Ca pantothenate, PABA, nicotinic acid, vitamin B_{12}.
Amino acids : Alanine, glycine, methionine, proline, glutamic acid, aspartic acid, arginine, lysine, histidine, phenylalanine.

Table III. Effect of Tyrosine on Inhibition of 0307

tyrosine(γ/cc.) \ 0307(γ/cc.)	100	20	4	0.8	0
1000	+	+	+	+	+
200	−	+	+	+	+
40	−	−	+	+	+
8	−	−	−	+	+
1.6	−	−	−	−	+
0	−	−	−	−	+

Table III, which illustrates the effect of addition of *l*-tyrosine, demonstrates that the addition of this amino acid caused a marked decrease in the growth inhibitory activity of 0307, and that the degree of reversal depends upon the concentration of tyrosine. Thus the addition of 1000 γ/cc. of *l*-tyrosine caused more than a hundred-fold decrease in the activity of 0307.

In Table IV, effect of 11 kinds of halogenated azaindolizines in the presence of various concentrations of tyrosine is shown. This table illustrates rather clearly the relationship among structure, growth inhibition, and reversal by tyrosine with the exception of 5-methylthio-6-chloro-7-methyl-1,3,8-triazaindolizine (0390). Compounds which were most inhibitive and most prominently reversed by tyrosine were those that have two halogen atoms substituted in their 5- and 6-positions (03138, 03120, 0307, and 03115). Introduction of a methyl group in 7-position reduced the activity to some extent (0307, 03115). The activity of compounds that have only one halogen atom in 5-position (0325, 03118, 0391) was much smaller than that of dihalogenated compounds. Compounds that have halogen in 6-position but not in 5-position had only a little or no effect, except for 0390 (03114, 03110, 0308). For the moment it is obscure why 0390, which has a halogen atom in 6-position and methylthio group in 5-position, inhibits the growth of *E. coli* and is reversed by tyrosine.

Table V demonstrates the effect of cystine and tryptophan. These two amino acids

TABLE IV. Growth Inhibition of *E. coli* K-12 by Halogenated Azaindolizines in the Presence of Various Concentrations of Tyrosine

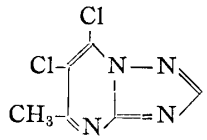
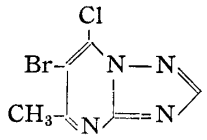
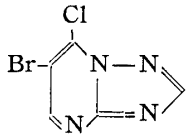
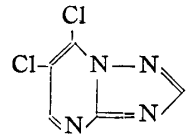
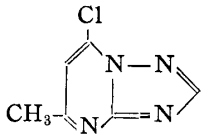
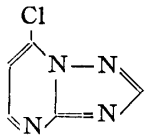
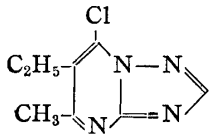
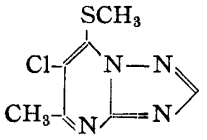
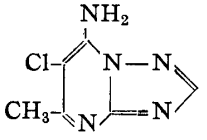
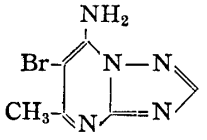
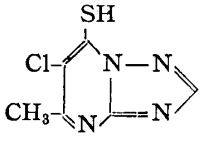
	Tyrosine(γ/cc.)	Min. growth-inhibitory concn.(γ/cc.)					
		1000	200	40	8	0.16	0
	0307	100<	100	40	4	0.8	0.8
	03115	100<	100	20	4	—	4
	03120	100<	100<	20	4	—	0.8
	03138	100<	100<	20	4	—	0.8
	0325	100	20	—	—	—	20
	03118	100	100	20	20	—	20
	0391						100<
	0390	20	20	4	0.8	—	0.8
	03114						100<
	03110						100<
	0308						100<

Table V. Effect of Cystine and Tryptophan on Inhibition of 0307

Amino acid(γ/cc.) \ 0307(γ/cc.)		100	20	4	0.8	0
l-Cystine	500	−	+	+	+	+
〃	150	−	−	+	+	+
〃	25	−	−	−	+	+
dl-Tryptophan	500	−	−	+	+	+
Control		−	−	−	+	+

had not so marked an effect as tyrosine but still had a significant effect when a large amount of one or the other of amino acids was added to the medium.

Table VI. Effect of Mixture of Amino Acids on Inhibition of 0307

Amino acids[a] \ 0307(γ/cc.)	500	100	20	4	0.8	0
a + b + c + d + e	−	+	+	+	+	+
b + c + d + e	−	+	+	+	+	+
a + c + d + e	−	+	+	+	+	+
a + b + d + e	−	+	+	+	+	+
a + b + c + e	−	−	+	+	+	+
a + b + c + d	−	−	+	+	+	+
c	−	−	+	+	+	+
control	−	−	−	−	+	+

a) a *l*-Glutamic acid (125 γ/cc.), *l*-aspartic acid (50 γ/cc.).
b *l*-Arginine, *l*-lysine, *l*-histidine (50 γ/cc.).
c *l*-Tyrosine(50 γ/cc.).
d *dl*-Serine(100 γ/cc.), *l*-alanine (50 γ/cc.), *dl*-isoleucine (100 γ/cc.), *l*-leucine (50 γ/cc.), *dl*-valine (100 γ/cc.), *l*-tryptophan (50 γ/cc.).
e *dl*-Methionine (100 γ/cc.), *l*-proline (50 γ/cc.), *l*-threonine (50 γ/cc.), *l*-phenylalanine (50 γ/cc.).

Table VI illustrates the effect of addition of a mixture of various amino acids. In this experiment, 16 kinds of amino acid were divided into 5 groups and the effect of each group was examined. As shown in this Table, addition of a mixture of 16 amino acids caused a marked decrease in the inhibitory action of 0307. It is noteworthy that the exclusion of tyrosine from the mixture did not cause any decrease with regard to the ability of reversal (the 4th line in the Table). From the consideration of these fact that the exclusion of group (d) and (e) caused at least some decrease (5th and 6th lines in the Table) suggests that, besides amino acids such as tyrosine, cystine, and tryptophan, a mixture of certain amino acids also causes reversal.

Table VII. Effect of Mixture of Amino Acids on Inhibition of 03120

Amino acids[a] \ 03120(γ/cc.)	100	20	4	0.8	0.16	0
a + b + c + d	−	+	+	+	+	+
b + c + d	−	+	+	+	+	+
a + c + d	−	+	+	+	+	+
a + b + d	−	+	+	+	+	+
a + b + c	−	+	+	+	+	+
a	−	−	+	+	+	+
a + c	−	+	+	+	+	+
control	−	−	−	−	+	+

a) a *dl*-Serine (200 γ/cc.), *dl*-threonine (200 γ/cc.), *l*-leucine (100 γ/cc.), *dl*-iseleucine (200 γ/cc.).
b *l*-Glutamic acid (250 γ/cc.), *l*-aspartic acid (100 γ/cc.).
c *l*-Tyrosine (50 γ/cc.).
d *l*-Arginine (100 γ/cc.), *l*-lysine (100 γ/cc.), *l*-histidine (100 γ/cc.).

The relationship between halogenated azaindolizines and a mixture of amino acids is very complicated, and many points remain to be investigated. In this paper only one of the most typical data is given. Table Ⅶ, which shows a relation between 5-chloro-6-bromo-7-methyl-1,3,8-triazaindolizine (03120) and a mixture of amino acids, demonstrates that the mixture of four amino acids, i.e. *dl*-serine, *dl*-threonine, *l*-leucine, and *dl*-isoleucine, also suppresses the activity of the agent (the 6th line in the Table). Exclusion of any one of these amino acids causes a loss of the activity to overcome the inhibition.

According to the data reported by Mineshita, *et al.*,[2] most of the compounds that were shown here to inhibit the growth of *E. coli* and to have their inhibition reversed by tyrosine exerted at least some inhibition on experimental animal tumors, but there is no strict correlation between carcinostatic activity and inhibition on *E. coli*. They indicated that 0307 has the most powerful carcinolytic activity among more than 100 derivatives of azaindolizines, and that compounds such as 03120 and 03138, which were shown here to have the most powerful activities on *E. coli*, were not so powerful inhibitors as 0307. It is worthwhile to note, however, that there seems to be rather a close relationship between the inhibition on *E. coli* and on Hella cells.[3]

The fact that the growth inhibition of *E. coli* by halogenated azaindolizines is reversed by tyrosine and cystine or tryptophan is interesting from the standpoint of their mode of action. Up to date, however, no evidence has been presented that suggests halogenated azaindolizines act as antimetabolites of tyrosine or of some other amino acids.

It seems pertinent to assume that the lability of the halogen in 5-position of substituted azaindolizine molecule is accountable for their physiological activities, but, for the moment, there is no direct evidence to connect their physiological activitiy with the lability of the halogen.

Experimental

Test Organisms—Strains of *E. coli* used in this study were *E. coli* ATCC 9637 wild type, *E. coli* 1011 (tryptophan-less derived from ATCC 9637), *E. coli* O-20 wild type, *E. coli communior* wild type, *E. coli communior* MT (tryptophan-less), and *E. coli* K-12 wild type. The former 4 strains were obtained from Prof. Ryoji Hayashi of Yamaguchi Medical College through Prof. Ryohei Takata of University of Kyoto, and the latter 3 strains were obtained through the courtesy of Mr. Ken'ichi Kotera, Research Institute for Microbial Diseases, University of Osaka. These strains were incubated on bouillion broth at 37° for 16~24 hr., and diluted washed suspensions were used as inoculum.

Basal Medium—Composition of 1 L. of basal medium was as follows : Glucose 0.2 g. (autoclaved separately), K_2HPO_4 7.0 g., KH_2PO_4 3.0 g., Na citrate 0.5 g., $MgSO_4$ 0.1 g., and $(NH_4)_2SO_4$ 1.0 g. (pH 7.0). This medium has the same composition as that described by Davis, *et al.*[4]

Samples—Samples of substituted azaindolizines were obtained from Dr. Hideo Kanô of this Research Laboratory. Almost all of the vitamins, purines, pyrimidines, and amino acids were obtained from commercial source. Leucovorin was obtained through the courtesy of Dr. Kazuo Iwai, Assistant Professor of Research Institute for Food Science, University of Kyoto. Samples of tyrosine, cystine and tryptophan used were mainly levorotatory isomers, but at the same time, synthesized *dl*-form was also used to eliminate the effect of contaminated impurities.

Experimental Procedure—Two cc. of the minimal medium (double strength) and calibrated amount of water were placed in test tubes, the tubes were covered with loose fitting aluminium cap, and sterilized. After cool, aliquots of azaindolizine solutions, sterilized by filtration, and glucose solution were mixed aseptically and the final volume was brought to 5 cc. Each tube was seeded with one drop of the foregoing inoculum. The content of the tube was mixed well by mannual shaking and incubated at 37°. After incubation for 48 hr., the growth of the test organism was observed.

3) N. Ishida, *et al.* : Unpublished data.
4) B. D. Davis, E. S. Mingioli : J. Bacteriol., **60**, 17(1950).

Preliminary experiments revealed that the action of halogenated azaindolizines was bactericidal. Full growth of the microörganisms was observed at concentrations lower than that determined as being bactericidal. The data show that *E. coli* can overcome the inhibitory action of these compounds at concentrations lower than the bactericidal level. The data given in this experiment was the results observed with the naked eye.

In the case where inhibition was noted, further tests were set up with increased concentrations of the metabolite and appropriate concentration of the test compound to determine whether the compounds were overcome by metabolites.

The author is greatly indebted to Dr. Ken'ichi Takeda, Director of this Laboratory, and to Mr. Eitaro Masuo of this Laboratory for their helpful advice and encouragement throughout this work.

Summary

Halogenated azaindolizines were found to inhibit the growth of *E. coli* strains in salt-glucose medium and to have the inhibition reversed by tyrosine or some other amino acids. Compounds which were most inhibitory and most prominently reversed by tyrosine were those that have two halogen groups in 5- and 6-positions of the azaindolizine molecule. The activity of compounds that have only one halogen substituted in 5-position was much smaller than that of dihalogenated compounds. Most of the compounds that have only one halogen in 6-position did not exert any inhibition on the growth of *E. coli*.

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