SELECTIVE REVERSIBLE INHIBITION OF MICROBIAL GROWTH WITH PYRITHIAMINE

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Since it has been shown recently (1) that pyrithiamine,[1] the pyridine analog of thiamine, will produce symptoms of thiamine deficiency in mice, it was of interest to determine whether this substance would be effective as an inhibitor of the growth of other living things. Robbins (2) has shown that two fungi grew very poorly when this compound was added to the basal medium. In the present study, many species of bacteria, yeasts, and fungi have been tested quantitatively for ability to grow in the presence of pyrithiamine. It was found that without exception those organisms which needed thiamine in the medium for maximal growth were prevented from growing by small amounts of pyrithiamine, and that those which did not need thiamine were unaffected even by considerable concentrations of the pyrithiamine in the medium.

Those organisms which were not affected by pyrithiamine did not synthesize large amounts of thiamine. Even when grown in a medium which contained considerable pyrithiamine, such organisms failed to produce increased amounts of thiamine. The failure of pyrithiamine to inhibit such organisms, therefore, could not be attributed to increased production of thiamine. And thus the explanation of sulfonamide fastness of certain bacteria by the increased production of the structurally-related *p*-amino benzoic acid (3) did not find analogy here. Furthermore, it was demonstrated that such insusceptible organisms grown either in the presence or absence of pyrithiamine did not produce detectable amounts of an antagonist other than thiamine which would be capable of reversing the inhibition of a susceptible organism.

EXPERIMENTAL

Materials.—2-Methyl-3-hydroxyethyl-pyridine and pyrithiamine[2] were synthesized according to the directions of Tracy and Elderfield (4). The organisms employed had the following sources. *Saccharomyces cerevisiae* (5), *Endomyces vernalis* (6), *Clostridium butylicum* (7), hemolytic steptococcus H69D (8), and *Lactobacillus casei* were

[1] Pyrithiamine is 1-[(4-amino-2-methyl)-5-pyrimidylmethyl]-2-methyl-3-(β-hydroxyethyl) pyridinium bromide hydrobromide (1).

[2] We wish to thank Dr. John C. Keresztesy, of Merck & Co., Inc., for samples of 2-methyl-4-amino-5-bromomethyl-pyrimidine used in the synthesis of pyrithiamine, and of crystalline biotin and cocarboxylase used in some of the media.

the strains previously used in this laboratory. The other lactic acid organisms and the *Propionibacterium pentosaceum* were supplied by Dr. W. H. Peterson of the University of Wisconsin, and the fungi by Dr. W. J. Robbins (9) of the New York Botanical Gardens. *Salmonella gallinarum* was obtained from Dr. L. F. Rettger of Yale University. We wish to thank these gentlemen for their cooperation.

Media.—All of the yeasts and fungi were grown in a medium composed of glucose 20 gm., $(NH_4)_2SO_4$ 3 gm., KH_2PO_4 2 gm., $MgSO_4 \cdot 7H_2O$ 0.25 gm., $CaCl_2 \cdot 2H_2O$ 0.25 gm., $ZnSO_4$ 1 mg., $MnCl_2$ 1 mg., $FeCl_3$ 0.5 mg., $CuSO_4 \cdot 5H_2O$ 0.1 mg., KI 1 mg., *l*-asparagine 100 mg., inositol 5 mg., calcium pantothenate 0.5 mg., pyridoxine 0.02 mg., biotin 0.01 mg., thiamine 0.01 mg., H_2O to make 1 liter.

Staphylococcus aureus was grown in a solution of glucose 2.5 gm., K_2HPO_4 5 gm., salts B (10) 2.5 cc., NaCl 2.5 gm., acid-hydrolyzed casein (5) 2.5 gm., tryptophane 25 mg., uracil 5 mg., nicotinic acid 1 mg., thiamine 0.01 mg., biotin 0.001 mg., in 1 liter of water adjusted to pH 7.2.

The lactic and propionic acid bacteria were grown in the medium of Landy and Dicken (11) with thiamine omitted. The hemolytic streptococci were cultured in a medium similar to that described previously (8, 12) but with thioglycollate instead of reduced iron. The media for *Clostridium butylicum* (7) and for *Salmonella gallinarum* (13) have been described. In the case of the latter organism, 0.01 γ of thiamine per cc. was used.

Escherichia coli was grown in a solution of glucose 10 gm., K_2HPO_4 4 gm., KH_2PO_4 2 gm., NaCl 3 gm., salts B (10) 4 cc., acid-hydrolyzed casein (5) 2 gm., in 1 liter of water adjusted to pH 7.

Tests showed that, in the experiments outlined below, it made no difference whether the thiamine was sterilized by filtration and added to the sterile media, or mixed with the media before sterilization in an autoclave, except with those fungi which required intact thiamine for growth, *i.e.*, those not able to grow on thiazole and pyrimidine halves[3] of the thiamine molecule. All media for the cultivation of organisms not requiring thiamine as a growth stimulant did not have thiamine added.

Procedure for Determination of Antimicrobial Potency.—For each organism studied, the following method was used to determine quantitatively the degree of inhibition of growth produced by pyrithiamine. A series of tubes (or flasks) of appropriate basal medium was prepared with graded doses of pyrithiamine ranging from 5 mg. per cc. to 0.01 γ per cc. Each tube was then inoculated with 1 drop of a suspension of cells which had been washed 3 times, and diluted until turbidity was just discernible. When good growth had occurred in the blank tubes which contained no pyrithiamine, the extent of growth in each tube was measured quantitatively by titration of the acid produced by those species which produced acid, or by determination of the turbidity in an Evelyn photoelectric colorimeter (5), or, in the case of the fungi, by weighing the dried mycelium from each flask. Some data so obtained for *Endomyces vernalis* are shown in Table I. The extent of growth was plotted against the amount of pyrithiamine and, by interpolation on the curve so obtained, the amount of pyrithiamine re-

[3] Throughout this paper, pyrimidine and thiazole halves of thiamine refer to 2-methyl-4-amino-5-ethoxymethyl-pyrimidine, and 4-methyl-5-hydroxyethyl-thiazole, respectively.

quired to reduce growth to half maximum was found. Since the various media used for organisms which required thiamine or its component parts for growth always contained 0.01 γ of thiamine per cc., the amount of pyrithiamine obtained from the curve represented the amount required to antagonize the effect of 0.005 γ of thiamine. Therefore, an inhibition index of 10 denoted that the organism was inhibited half maximally, under the conditions studied, by 0.05 γ of pyrithiamine per cc. These values for the various species are tabulated in Table II. The media used for the organisms not requiring thiamine did not contain added thiamine. However, most of them contained inadequately defined material (either casein hydrolysate or a concentrate of folic acid). Therefore, thiamine was determined in these media by the biological method described below. No thiamine was detected in any of the media. Since the method was only sensitive to 0.001 γ of thiamine per cc., this value was assumed for the thiamine content of these media. It is probable that the actual content of thiamine or its components was less. The values in Table II for the organisms under discussion were calculated for this amount of thiamine in the media.

TABLE I

Effect of Pyrithiamine and of Pyrithiamine Plus Thiamine on the Growth of Endomyces vernalis

Pyrithiamine	Thiamine	Turbidity reading*
γ *per cc.*	γ *per cc.*	
0	0.01	66
400	0.01	97
40	0.01	98
10	0.01	95
5	0.01	95
1	0.01	92
0.5	0.01	75
0.1	0.01	66
0.05	0.01	65
1	1	64

* The turbidity readings are expressed as per cent of incident light transmitted by the culture.

From the data in Table II, it can be seen that the thiamine requirement of an organism was correlated with its susceptibility to pyrithiamine. Those organisms whose growth was not stimulated by thiamine or its component parts were not prevented from growing by large amounts of pyrithiamine. Those which were stimulated by the pyrimidine and thiazole parts of thiamine or by the thiazole portion alone were inhibited and showed a ratio of pyrithiamine to thiamine required for inhibition of about 1000 to 1. Those which needed only the pyrimidine portion of thiamine were inhibited by smaller concentrations of pyrithiamine and exhibited a pyrithiamine to thiamine ratio of about 100 to 1. Finally, those which required intact thiamine were inhibited by minute amounts of pyrithiamine, and showed a ratio of about 10 to 1. In this

connection, it was of interest that mice, which needed intact thiamine, showed a ratio of pyrithiamine to thiamine of between 40 and 3 to 1 (1).

Reversal of Pyrithiamine Effect with Thiamine.—For each organism which was inhibited by pyrithiamine, it was found that growth was again made possible when sufficient thiamine was added. Some of the data for *Endomyces vernalis* which bear on this point are shown in Table I. With this yeast which required only the pyrimidine portion of thiamine, the pyrimidine alone was effective in counteracting the action of pyrithiamine.

TABLE II

Inhibitory Power of Pyrithiamine for Various Microbial Species

Organism	Inhibition index $\frac{\text{Pyrithiamine}}{\text{Thiamine}}$	Thiamine requirement
Ceratostomella fimbriata	7	Intact thiamine
Ceratostomella from London plane tree	19	" "
Ceratostomella pennicillata	10	" "
Phytophthora cinnamomi	12	" "
Chaloropsis thielavoides	11	" "
Endomyces vernalis	130	Pyrimidine
Mucor ramannianus	800	Thiazole
Saccharomyces cerevisiae	800	Pyrimidine and thiazole
Staphylococcus aureus	2000	" "
Salmonella gallinarum	1000	" "
Neurospora crassa	Greater than 400,000	None
Escherichia coli	" " 2,000,000	"
Clostridium butylicum	" " 2,000,000	"
Lactobacillus arabinosus	" " 40,000	"
Lactobacillus casei	" " 5,000,000	"
Lactobacillus delbruckii	" " 5,000,000	"
Lactobacillus mesenteroides	" " 5,000,000	"
Lactobacillus pentoaceticus	" " 5,000,000	"
Streptococcus lactis R	" " 5,000,000	"
Propionibacterium pentosaceum	" " 5,000,000	"
Hemolytic streptococcus H69D	" " 4,000,000	"

Effect of 2-Methyl-3-Hydroxyethyl-Pyridine.—In order to determine if the pyridine portion of pyrithiamine would inhibit growth, the effect of 2-methyl-3-hydroxyethyl-pyridine was tested on *Endomyces vernalis*, an organism requiring only the pyrimidine part of thiamine, and against *Mucor ramannianus* (14), one which needs only the thiazole portion. Since such fungi synthesized the other half of thiamine and coupled it with the required half to make thiamine (15), it was thought that perhaps they would join the 2-methyl-3-hydroxyethyl-pyridine to the pyrimidine portion of thiamine and thus produce

pyrithiamine. However, neither organism was inhibited by any amount of the pyridine up to 100 γ per cc.

Stimulation of Growth by Pyrithiamine.—Several organisms grew better in media containing subinhibitory amounts of pyrithiamine than they did in the same media without pyrithiamine. This effect was noted with *Mucor ramannianus*, *Lactobacillus delbruckii*, *Propionibacterium pentosaceum*, *Streptococcus lactis* R, and *Lactobacillus pentoaceticus*. The amount of stimulation was slight but definite. Representative data for *Mucor ramannianus* are shown in Table III.

Attempts to Learn Why Some Organisms Were Not Inhibited by Pyrithiamine. (*a*) *Synthesis of Thiamine by Certain Bacteria.*—In order to learn if the resistance of organisms which did not require thiamine was due to their ability to

TABLE III

Stimulation and Inhibition of Growth of Mucor ramannianus by Pyrithiamine

Pyrithiamine	Dry weight of mycelium
γ per cc.	*mg. per 10 cc.*
0	7.3
0.1	8.8
0.2	9.3
0.4	9.8
0.8	9.4
1	9.8
5	4.3
10	2.7
40	2.7

synthesize large amounts of this vitamin, thiamine production by several of these organisms was measured. The method depended on the fact that *Endomyces vernalis* needed thiamine or its pyrimidine component in order to grow. The method has been outlined briefly (6) and will now be described more fully.

The basal medium was the one listed earlier in this paper for growth of yeasts and fungi, from which thiamine was omitted. The tests were conducted in 50 cc. Erlenmeyer flasks containing 10 cc. of medium. The standard curve was constructed from the responses observed in a series of flasks containing from 0.001 γ to 0.01 γ of thiamine per cc. The flasks were inoculated with 1 drop of inoculum prepared as previously described (5), and incubated at 37° for 24 hours. Turbidity of the contents of the various flasks was determined photometrically. A standard curve was then constructed and used to evaluate the thiamine content of the unknowns in the usual manner. The data from which a typical response curve was drawn are shown in Table IV. In this table is also shown the response to cocarboxylase. The response curve rose too steeply to allow of great accuracy of results, but the method had distinct

advantages over other microbiological procedures for estimation of thiamine. It was rapid, sensitive to small amounts of thiamine, and, since it was a growth test, required no elaborate apparatus. No treatment with phosphatases was required, since cocarboxylase was as active (on a molecular basis) as thiamine. The organism grew as well in the basal medium plus thiamine as it did when plant extracts were added.

Organisms to be tested for synthesis of thiamine were grown in 1 liter quantities of the appropriate media described earlier in this paper. The cells were separated and washed by centrifugation, dried at 60° under reduced pressure, and extracted with hot water in a manner similar to that used in the usual methods of analysis for thiamine (16). These extracts, as well as the culture centrifugates, were assayed for thiamine as described above. The results are

TABLE IV

Growth Response of Endomyces vernalis to Graded Amounts of Thiamine and Cocarboxylase

Thiamine	Cocarboxylase	Turbidity reading
γ per cc.	*γ per cc.*	
0	0	82
0.001	0	79
0.002	0	76
0.004	0	73
0.006	0	68
0.008	0	66
0.01	0	66
0	0.0015	80
0	0.0045	76
0	0.0075	70
0	0.015	64

shown in Table V. It will be noted that all of the organisms tested synthesized thiamine, and that most of the vitamin was found in the cells. However, the amount formed was not sufficient to make it probable that the synthesis of thiamin was great enough to account for the failure of pyrithiamine to inhibit growth, unless the latter substance, when present, caused marked stimulation of the synthesis of thiamine.

The presence of large amounts of pyrithiamine did not stimulate the synthesis of thiamine by *E. coli*. This organism was grown in 100 cc. portions of the synthetic medium described for it above, to which had been added 500 γ of pyrithiamine per cc. The entire culture was assayed for thiamine, both before and after treatment with taka-diastase, according to a modification[4] of the method of Emmett *et al.* (17). Pyrithiamine alone did not give a color in this test. Only 2 γ of thiamine were found per 100 cc. of culture. It

[4] Private communication from Dr. Emmett.

was clear that pyrithiamine had not stimulated the bacteria to form more thiamine than they did normally.

(*b*) *Absence of Other Antagonists to Pyrithiamine.*—When grown in the presence of pyrithiamine, *E. coli* did not form a substance which prevented the action of pyrithiamine on *Endomyces vernalis*. The basal medium for *Endomyces vernalis* described at the beginning of this paper was supplemented with 1 γ of pyrithiamine per cc. This was just sufficient to cause 75 per cent inhibition of growth. To such a medium was added either a Berkefeld filtrate or the heated and filtered culture of *E. coli* grown in pyrithiamine as above. The inhibition of *Endomyces* was not relieved by these materials. Indeed, the extra pyrithiamine present in the preparations completed the inhibition of growth which had been begun by the pyrithiamine in the test medium.

TABLE V

Synthesis of Thiamine by Various Bacteria As Determined by Endomyces Method

Organism	Dry weight of cells	Thiamine synthesized	
		In culture filtrate	In cells
	mg. per cc. of culture	*γ per cc.*	*γ per cc. of culture*
Escherichia coli	0.24	0.008	0.03
Lactobacillus arabinosus	0.42		0.0037
Clostridium butylicum	0.23		0.0064
Lactobacillus casei	0.30	less than 0.001	0.01
Hemolytic streptococcus H69D	0.16		0.0032

DISCUSSION

It is clear from the foregoing data that pyrithiamine is an inhibitor with marked selective properties. Some organisms were able to grow at a normal rate in concentrations of the substance more than 500,000 times the amount sufficient to prevent the growth of other organisms. The growth of yeasts, fungi, bacteria, and mice was inhibited, while that of other fungi and bacteria was not affected. The basis of this selective action appeared to be related to the ability of the particular organisms to thrive in the absence of an external source of thiamine. The more exacting a given species was in its requirements for thiamine, the more susceptible it was to the action of pyrithiamine. Thus, those species which needed intact thiamine were about 10 times as sensitive as those which needed only the pyrimidine portion of thiamine, and about 100 times as sensitive as those stimulated by the pyrimidine and thiazole halves of the vitamin.

The inhibition of growth by pyrithiamine may be regarded as the production of thiamine deficiency by the structurally related pyrithiamine because the

inhibition was overcome by the addition of thiamine. Since thiamine was a vitamin active in such low concentration, and since the ratio between thiamine and pyrithiamine was so low, pyrithiamine was an exceedingly active inhibitor.

Some of the characteristics of pyrithiamine action have been observed with inhibitors related structurally to other vitamins. Thus Snell (18) and McIlwain (19) observed that the sulfonic acid analog of pantothenic acid was effective only against bacteria which required pantothenic acid, and McIlwain (20) noted that pyridine-3-sulfonic acid caused reversible inhibition only of those species which needed nicotinic acid. In these instances, such a wide range of selectivity among species as can be shown for pyrithiamine could not be shown, partly because of the reasons given in the preceding paragraph. The resistance of certain bacteria to sulfanilamide has been credited to the ability of the resistant strains to synthesize more of the structurally related *p*-aminobenzoic acid (3). Because of the experiments described above, the ability to synthesize thiamine did not of itself seem to offer an adequate explanation of resistance to pyrithiamine. The reason for the selective action of this compound, therefore, is not clear.

SUMMARY

Growth of many microbial species was inhibited by pyrithiamine, the pyridine analog of thiamine. Growth of many other species was not influenced. In a series of bacteria, yeasts, and molds, it was found that inhibition of growth occurred only in those in which growth was stimulated by thiamine or its component pyrimidine and thiazole portions. The amount of pyrithiamine required for inhibition was correlated with the type of thiamine requirements of various species. The least amount was needed to inhibit organisms which required intact thiamine. Those which could use the pyrimidine and thiazole portions were not so readily inhibited. In the case of the former organisms, half maximal inhibition was produced by as little as 0.03 γ per cc. In all instances, the inhibition was overcome by sufficient amounts of thiamine. The synthesis of thiamine by insusceptible species was studied, and it was concluded that formation of thiamine or other antagonistic substance did not provide an adequate explanation of the resistance of these species to the action of pyrithiamine.

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