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Specificity of *Lactobacillus fermenti-36* for the Assay of Thiamine

2. COMPETITIVE INHIBITION ON THE GROWTH OF Lactobacillus fermenti-36 BY PYRITHIAMINE

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In the preceding paper we have shown that for the successful assay of thiamine with *Lactobacillus fermenti*-36, the organism should be maintained in a dependent state by stab-culturing it in a thiamine-rich agar medium. We have also shown that the thiamine requirement of this organism was independent of the amino-acid composition of the medium. The essentiality of this vitamin for this organism has been questioned only by Shankman, Camien, Block, Merrifield, and Dunn (1947) but has been verified in several laboratories (Sarett and Chaldelin, 1944; Barton-Wright, 1945; Cheldelin, Bennett and Kornberg, 1946; Fitzgerald and Hughes, 1949; Hoover and Jayasuriya, 1950).

For further confirmation of the essentiality of thiamine for *Lactobacillus fermenti*-36, we investigated the effect of the well known antimetabolite, pyrithiamine, on the growth of this organism. Pyrithiamine was shown to evoke symptoms of thiamine deficiency in mice by Woolley and White (1943a) and to inhibit the growth of several species of fungi, and bacteria (Robbins, 1941; Woolley and White, 1943b). The latter studied the inhibitory power of pyrithiamine for a number of microorganisms grown in the presence of 0·01 μg thiamine per ml. of the growth medium. They expressed their results in terms of Inhibition Index values, the index being defined as the ratio of the amount of antimetabolite to metablite which would produce half maximal inhibition. They made use of these inhibition indices to classify the organisms studied according to their sensitivity towards thiamine. Thus those which required intact thiamine for growth had indices ranging from 2 to 5, those which could in addition utilise the primidine and thiazole moieties gave values ranging from 200 and 500, while those which did not need thiamine for growth had indices well over 40,000. Sarett and Cheldelin (1944) claimed that the pyrimidine and thiazole moieties could not replace thiamine for growth of *Lactobacillus fermenti*-36 during an incubation period of 18 hours. This organism has not been

studied by Woolley and White (1943b), who have, however, given an index value of 200 for *Saccharomyces cerevisiae*, which has been used for thiamine assay since the work of Williams, McMahan and Eakin (1941). They have, incidentally, confirmed that pyrimidine and thiazole moieties could replace thiamine for the growth of *Saccharomyces cerevisiae*, which cannot be regarded as an ideal organism for the assay of thiamine. We, therefore, studied the pyrithiamine-thiamine relationship in the case of *Lactobacillus fermenti*-36 in order to ascertain, on the classificatory basis of Woolley and White (1943b), the degree of specificity of thiamine for this organism.

Experiment

Organism:—*Lactobacillus fermenti*-36 (NCTC).

Media:—All media employed in these experiments were similar to those described in the preceding paper.

Pyrithiamine:—1-[(4-amino-2-methyl) -5- pyrimidyl-methyl] -2-methyl-3-(β-hydroxy-ethyl) -pyrimidinium bromide hydrobromide. A sample of 100 mg. was made available to us through the courtesy of Dr. Karl Folkers, Associate Director of Research and Development Division, Merck & Company Inc., New Jersey.

Stock solution of pyrithiamine :—Five milligrammes of pyrithiamine were dissolved in 500 ml. distilled water. This solution contained 10 μg pyrithiamine per ml.

Standard solution of pyrithiamine :—Fifty ml. of the stock solution of pyrithiamine were made up to 500 ml. with distilled water. The solution contained 1·0 μg pyrithiamine per ml. and was sterilised by Seitz filtration prior to its addition to the sterilised assay tubes.

Stock solution of thiamine :—Fifty milligrammes of thiamine hydrochloride, dried under vacuo for 24 hours, were dissolved in 500 ml. of 25 per cent. (v/v) ethanol to which one drop of concentrated hydrochloric acid had been added. This solution contained 100 μg thiamine per ml.

Standard solution of thiamine :—One ml. of the stock solution of thiamine was diluted to a litre with distilled water. This solution contained 0·10 μg thiamine per ml.

Procedure for the determination of Inhibition Index :—A series of tubes containing the basal medium, 0·10 μg thiamine, and appropriate amounts of distilled water was autoclaved at 10 lb. pressure for 10 minutes and to these tubes were added graded doses of the sterilised (by Seitz filtration) standard pyrithiamine solution. The tubes were inoculated with *Lactobacillus fermenti* and incubated at 37°C for 18 hours. The turbidity measurements were made photoelectrically (Klett-Summerson colorimeter). In another series of experiments, the above procedure was repeated with double the quantity of thiamine. For full details and results, reference is made to Tables I and II. All levels were carried in triplicates.

TABLE I

Effect of graded doses of pyrithiamine on the growth of *Lactobacillus fermenti*-36 in a medium containing 0·01 μg thiamine per ml.

ml. per tube				*Turbidity value***
double-strength basal medium, (Hoover and Jayasuriya, 1950)	*standard thiamine solution*	*distilled water*	*standard pyrithiamine solution**	
5	0	5	0	0
5	1	4	0	257
5	1	3·4	0·6	190
5	1	3·3	0·7	162
5	1	3·2	0·8	110
5	1	3·1	0·9	75

*Pyrithiamine content at the five levels employed were 0, 0·06, 0·07, 0·08, and 0·09 μg per ml. of the growth medium.

**Turbidity was determined in a Klett-Summerson colorimeter, using No. 54 filter against a blank containing 5 ml. basal medium and 5 ml. distilled water.

TABLE II

Effect of graded doses of pyrithiamine on the growth of *Lactobacillus fermenti*-36 in a medium containing 0·02 μg thiamine per ml.

ml. per tube				*Turbidity*
double-strength basal medium, (Hoover and Jayasuriya, 1950)	*standard thiamine solution*	*distilled water*	*standard pyrithiamine solution**	
5	0	5	0	0
5	2	3	0	274
5	2	2·2	0·8	256
5	2	2·0	1·0	239
5	2	1·8	1·2	218
5	2	1·6	1·4	175
5	2	1·4	1·6	102

*Pyrithiamine content at the six levels tested were 0, 0·08, 0·10, 0·12, 0·14, and 0·16 μg per ml. growth medium.

Results and Discussion

The turbidity readings were plotted against pyrithiamine concentrations. *c.f.* Fig. 1

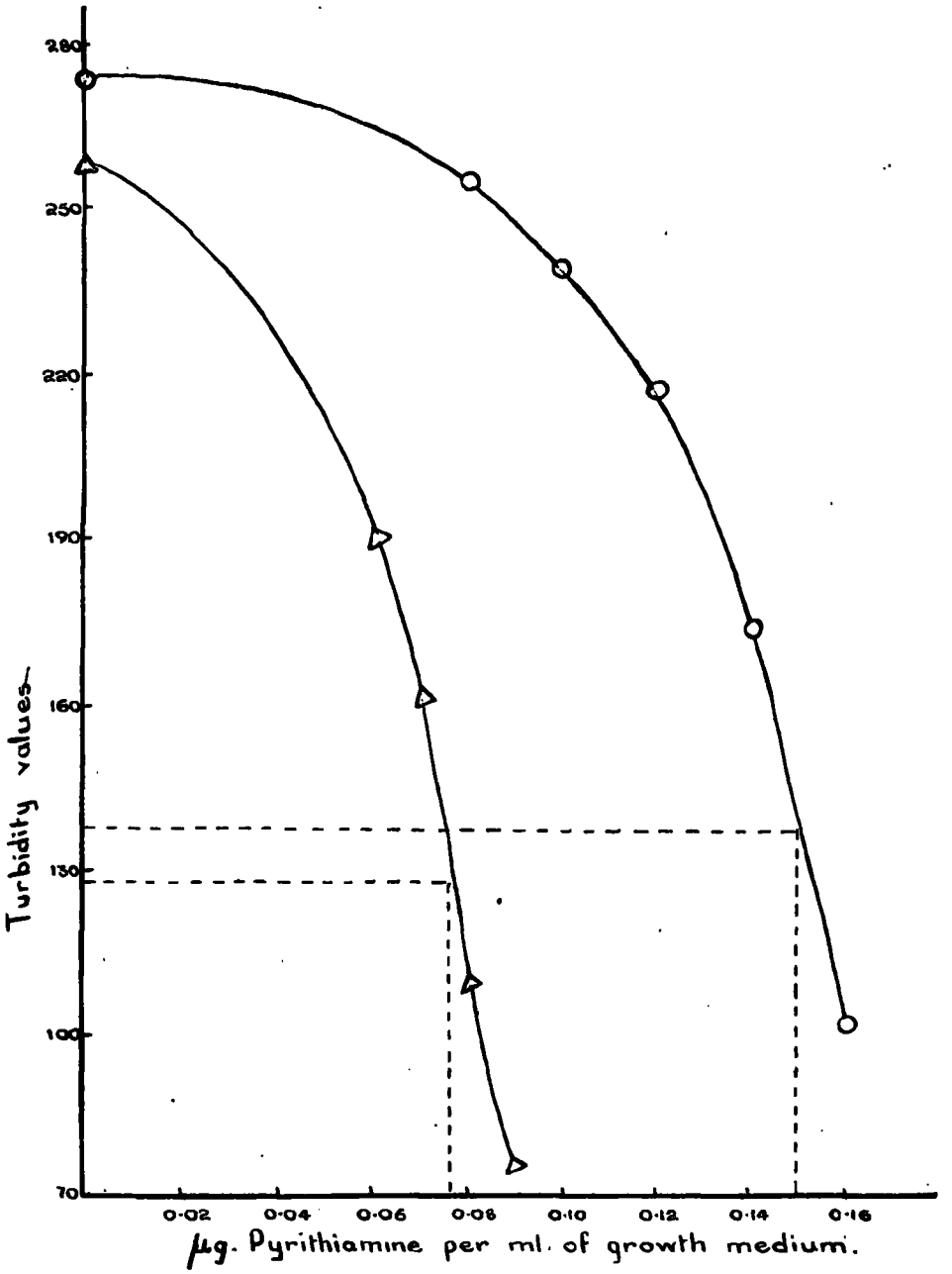

FIGURE 1: Response of *Lactobacillus fermenti*-36 towards graded doses of pyrithiamine when grown in a medium containing 0·01 µg and 0·02 µg thiamine per ml.

△—Response in the presence of 0·01 µg thiamine per ml.

0—Response in the presence of 0·02 µg thiamine per ml.

By interpolation on the curves so obtained, the amount of pyrithiamine required to reduce growth to half maximum was found at each level of thiamine. The values so obtained were 0·0755 and 0·15 μg per ml. at thiamine levels of 0·01 and 0·02 μg respectively. The Inhibition Indices were therefore 7·55 and 7·50.

According to Woolley (1952) antagonism is truly competitive when the inhibition index is constant despite changes of concentrations of the metabolite. If the index is not constant, the antagonism is not strictly competitive. Since our inhibition index was constant at the two levels of thiamine, it can be inferred that for *Lactobacillus fermenti*-36 the antagonism of pyrithiamine to thiamine is strictly competitive in nature. Further for organisms which required intact thiamine for growth, Woolley and White (1943b) obtained inhibition indices below 5, while for those which did not need the vitamin, the values were well above 40,000. An intermediate class of microorganisms, which could in place of thiamine utilise the pyrimidine and thiazole moieties, gave values above 200. Our index of 7·5 for *Lactobacillus fermenti*-36 may be therefore regarded as a pointer to the extreme specificity of this organism for thiamine. We conclude that this organism is ideally suited to assay thiamine in biological materials.

Summary

1. The antagonism of pyrithiamine to thiamine was studied with *Lactobacillus fermenti*-36.

2. The antagonism is strictly competitive in nature.

3. The low inhibition index value is an indication of the extreme specificity of this organism for thiamine.

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