Journal of Bacteriology
Vol. 88, No. 4, p. 1024-1029 October, 1964
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INHIBITION OF THE BIOSYNTHESIS OF THE PYRIMIDINE PORTION OF THIAMINE BY ADENOSINE

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Received for publication 8 June 1964

Abstract

Moyed, H. S. (Harvard Medical School, Boston, Mass.). Inhibition of the biosynthesis of the pyrimidine portion of thiamine by adenosine. J. Bacteriol. **88**:1024–1029. 1964.—The bacteriostatic effects of adenosine and several other purines on *Aerobacter aerogenes* can be overcome by either thiamine or the pyrimidine portion of thiamine. Adenosine causes almost complete cessation of the synthesis of the pyrimidine and consequently also of thiamine. However, synthesis of deoxyribonucleic acid, ribonucleic acid, and protein persists in the absence of thiamine synthesis until a three- or fourfold increase has occurred, indicating that *A. aerogenes* has a surplus supply of either thiamine or the pyrimidine. The failure of cells to continue production of the thiazole portion of thiamine when the synthesis of the pyrimidine is blocked indicates that control over the thiazole is exerted by the thiazole itself rather than by the intact thiamine molecule. Bacteria blocked in the synthesis of the pyrimidine either as the result of mutation or because of inhibition by adenosine excrete an intensely fluorescent, but as yet unidentified, compound. The fluorescent compound bears the nutritional relationship to the pyrimidine characteristic of that between an intermediate and an end product: an excess of the pyrimidine prevents its formation, whereas a deficiency of the pyrimidine greatly stimulates its formation. Adenosine inhibition of the synthesis of the pyrimidine is partially relieved by histidine or succinate. It is suggested that these compounds either bypass the blocked reaction or participate in the detoxification of adenosine.

Bacteriostasis by adenine as well as by several other purines, and its antagonism by thiamine, were first observed by Brooke and Magasanik (1954). Since then, the phenomenon has been encountered by other investigators studying the nutrition of enteric organisms (Yara, 1955). The site of the antithiamine effect of adenine is unknown. However, it might reasonably be attributed to an inhibition of either the synthesis or the utilization of thiamine. M. S. Brooke (*personal communication*) found that both histidine and succinate could replace thiamine in overcoming adenine toxicity. More recently, similar results were obtained by Neidhardt (1963). It was suggested that histidine and succinate overcome the requirement for thiamine by providing products of reactions in which thiamine has a catalytic role (Neidhardt, 1963). This does not seem to be a completely satisfactory explanation, particularly in the case of histidine. First of all, thiamine is not directly involved in the synthesis of histidine, nor is histidine converted by a known alternative pathway to products of thiamine-dependent reactions. Succinate, on the other hand, conceivably could have a slight sparing effect. The cell would not have to provide whatever portion of its carboxylic acid supply it normally obtains in the thiamine-dependent decarboxylation of α-ketoglutarate. However, a supply of succinate could not relieve the cell of carrying out the major thiamine-dependent reactions: transketolase catalyzed reactions; acetyl coenzyme A formation from pyruvate; and several steps in the synthesis of isoleucine, valine, and leucine (Umbarger and Davis, 1962). Thus, the basis of adenine's antithiamine effect is unknown. Unfortunately, its reversal by histidine or by succinate does not presently contribute to an understanding of the problem, because the basis for neither the histidine-adenine nor the succinate-adenine antagonism has a satisfactory explanation. The possibility of defining these problems more precisely made it seem worthwhile to explore an antithiamine effect of adenosine on *Aerobacter aerogenes* observed during the course of work on an unrelated problem.

MATERIALS AND METHODS

Chemicals. B_1 pyrimidine (4-amino-5-hydroxymethyl-2-methyl pyrimidine), the pyrimidine portion of thiamine, and B_1 thiazole (2-hydroxyethyl-3-methylthiazole hydrochloride), the thiazole portion of thiamine, were gifts of Bernard D. Davis. All other chemicals were obtained from commercial sources.

Bacteria. The prototrophic strain of *A. aerogenes* strain 1033 and the purine-thiamine auxotroph, strain PD 1, were from the collection of Boris Magasanik. The thiamine auxotroph, strain M 26-43, of *Escherichia coli* W was from the collection of Bernard D. Davis. The methionine auxotroph of *E. coli* K-12 was obtained from Frederick C. Neidhardt. These strains were grown at 37 C with vigorous aeration in a mineral salts-glucose medium with the appropriate supplement. Absorbancy of the cultures at 420 mμ was used as an indicator of growth. Measurements of growth rates were carried out under the following conditions. The inoculum was an exponential culture; the starting optical density at 420 mμ was between 0.05 and 0.06; growth rates were determined from measurements of optical density during a 2-hr interval starting with the third hour of incubation.

Biological assays. Strain PD 1 requires a purine and thiamine for growth (Brooke and Magasanik, 1954). Presumably, the defect in thiamine synthesis is at a step leading to B_1 pyrimidine, because this portion of the vitamin satisfies the thiamine requirement. PD 1 was therefore used as an assay organism for the detection of the thiamine or B_1 pyrimidine content, or both, of culture media and of cells. Strain M 26-43, which requires either thiamine or B_1 thiazole for growth, was used in combination with strain PD 1 for the assay of B_1 thiazole. Cells were extracted by heating in a boiling-water bath for 5 min. Extracts and spent culture media were sterilized by filtration, and portions were added aseptically to assay tubes (200 by 22 mm) containing 6 ml of sterile medium. The tubes were inoculated with about 5×10^4 cells of the assay organism, and then were incubated in a nearly horizontal position on a reciprocating shaker for 16 hr at 37 C. Tubes with known amounts of thiamine were included in each assay.

Chemical determinations. Standard procedures were used for the determination of the ribonucleic acid (RNA; Schneider, 1945), deoxyribonucleic acid (DNA; Ceriotti, 1952), and protein (Lowry et al., 1951) content of cells which had been extracted at 0 C for 16 hr with a volume of 5% trichloroacetic acid twice that of the culture from which the cells had been obtained.

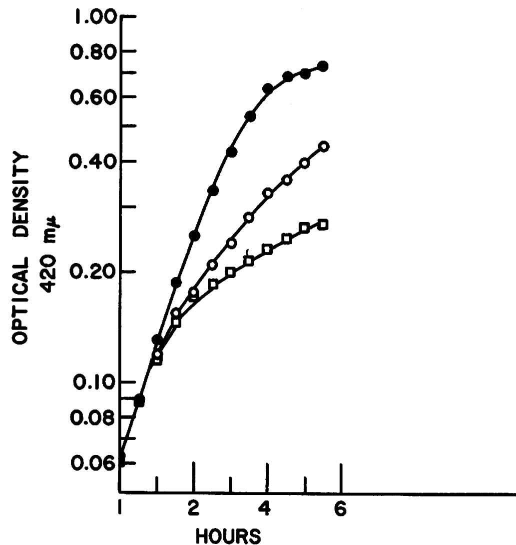

FIG. 1. *Bacteriostatic effect of adenosine on Aerobacter aerogenes 1003. Symbols: ●, no additions; ○, 100 μg/ml of adenosine; □, 1,000 μg/ml of adenosine.*

RESULTS

Antithiamine effect of adenosine and other purines. A small amount (6 μg/ml) of adenosine, nontoxic by itself, was found to greatly enhance the bacteriostatic effect of psicofuranine (Moyed, 1961), an inhibitor of guanylic acid biosynthesis (Slechta, 1960). When tested in larger amounts, adenosine was found to have a bacteriostatic effect of its own which was expressed in most experiments after one division cycle (Fig. 1). However, this effect of adenosine, in contrast to its potentiation of psicofuranine's action, can be reversed by thiamine. The amount of thiamine required for reversal is of the same order as that required by thiamine auxotrophs (Table 1). Both adenine and inosine were also bacteriostatic, although somewhat less potent than was adenosine (Table 2). As in the case of adenosine, the inhibitory effects of inosine and adenine were overcome by small amounts of thiamine. Hypoxanthine, guanosine, and guanine (observed in a

TABLE 1. *Reversal of bacteriostatic action of adenosine by thiamine (B_1)**

Addition (amt, µg/ml)	Growth (generations/hr)
None	1.34
Adenosine (300)	0.46
Adenosine (300) + B_1 (0.004)	0.60
Adenosine (300) + B_1 (0.010)	0.68
Adenosine (300) + B_1 (0.020)	0.78
Adenosine (300) + B_1 (0.060)	1.32

* *Aerobacter aerogenes* 1033 was used in this experiment.

TABLE 2. *Bacteriostasis by other purines and reversal by thiamine (B_1)**

Addition (amt, µg/ml)	Growth (generations/hr)
None	1.22
Adenosine (300)	0.44
Adenosine (300) + B_1 (0.1)	1.33
Adenine (300)	0.92
Adenine (300) + B_1 (0.1)	1.32
Inosine (300)	0.78
Inosine (300) + B_1 (0.1)	1.24
Hypoxanthine (300)	1.26
Guanosine (300)	1.22

* *Aerobacter aerogenes* 1033 was used in this experiment.

TABLE 3. *Effects of components of thiamine (B_1) on bacteriostasis by adenosine**

Addition (amt, µg/ml)	Growth (generations/hr)
None	1.44
Adenosine (500)	0.88
Adenosine (500) + B_1 (0.01)	1.42
Adenosine (500) + B_1 thiazole (0.01)	0.88
Adenosine (500) + B_1 pyrimidine (0.01)	1.43

* *Aerobacter aerogenes* XXXV was used in this experiment. B_1 thiazole = 2-hydroxyethyl-3-methylthiazole hydrochloride; B_1 pyrimidine = 4-amino-5-hydroxymethyl-2-methylpyrimidine.

separate experiment) were not bacteriostatic. Adenosine appears to be an antimetabolite of B_1 pyrimidine rather than of the entire thiamine molecule, since its toxic effect could be overcome by either thiamine or B_1 pyrimidine (Table 3). B_1 thiazole, on the other hand, had no effect. A comparison of the response of the adenosine-inhibited cultures to graded amounts of thiamine and of B_1 pyrimidine indicated that the latter, as might be expected, is the more effective on a weight basis in overcoming the inhibition (Fig. 2).

Inhibition of thiamine biosynthesis by adenosine. To distinguish between the equally likely possibilities that adenosine inhibits the utilization or the synthesis of B_1 pyrimidine, measurements were made of the thiamine content of normal and adenosine-inhibited cultures (Table 4). Both the cells and the culture filtrates were assayed. There was no significant level of thiamine or the components in any of the filtrates. Measurements of the major cellular constituents did not reveal a differential effect of adenosine on any one of them. However, the thiamine-protein ratio was reduced threefold. This threefold reduction occurred while the cells underwent slightly less than a threefold increase, thus indicating that thiamine synthesis was almost completely inhibited. The similar thiamine content found by assays with the thiamine-B

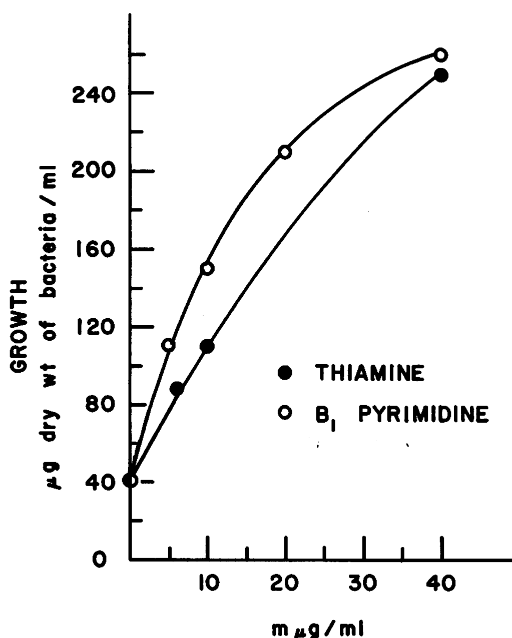

FIG. 2. *Response of adenosine-inhibited culture of Aerobacter aerogenes 1033 to thiamine and the pyrimidine portion of thiamine. B_1 pyrimidine = 4-amino-5-hydroxymethyl-2-methylpyrimidine. Adenosine = 300 µg/ml. Growth is expressed as micrograms (dry weight) of bacteria, based on a previous calibration relating the turbidity of a culture at 420 mµ to its dry weight.*

pyrimidine auxotroph and the thiamine-B_1 thiazole auxotroph showed that B_1 thiazole did not continue to be synthesized when the synthesis of the other component, B_1 pyrimidine, was inhibited.

Effects of succinate and histidine. The reversal of adenosine toxicity for *A. aerogenes* 1033 by histidine and by succinate was not a readily reproducible phenomenom. The effects of both compounds ranged from negligible to partial (Table 5). Histidine proved to be more effective than succinate. Neither the histidine nor the succinate used in these and subsequent experiments contained detectable levels of thiamine or B_1 pyrimidine. In more recent experiments than those shown in Table 5, it was not possible to demonstrate an effect by succinate. On the other hand, histidine, as before, partially overcame inhibition by adenosine. This protective effect of histidine could be attributed to the fact that cultures exposed to both histidine and adenosine were able to synthesize two or three times as much thiamine as were cultures exposed to adenosine alone. Antagonism of adenosine inhibition by both succinate and histidine could be demonstrated in a methionine auxotroph of *E. coli* as previously reported by Neidhardt (1963). It was found that histidine and succinate could partially restore the ability of this organism to synthesize thiamine in the presence of adenosine. The relative effectiveness of histidine and succinate in relieving bacteriostasis was roughly proportional to the extent of the restoration of thiamine synthesis (Fig. 3).

TABLE 4. *Effect of adenosine on synthesis of cell components*[a]

Component	With adenosine	Without adenosine
DNA	7[b]	15
RNA	41	126
Protein	114	248
Thiamine[c]	0.004	0.021
Thiamine[d]	0.003	0.021

[a] *Aerobacter aerogenes* XXXV was incubated in a mineral salts-glucose medium with and without 500 mg/ml of adenosine for 2 hr. B_1 pyrimidine = 4-amino-5-hydroxymethyl-2-methyl-pyrimidine; B_1 thiazole = 2-hydroxyethyl-3-methylthiazole hydrochloride.

[b] Results expressed as micrograms per milliliter of culture.

[c] Assay organism responds to thiamine or to B_1 pyrimidine.

[d] Assay organism responds to thiamine or to B_1 thiazole.

TABLE 5. *Effects of succinate and histidine on bacteriostasis by adenosine**

Expt	Addition (amt, μg/ml)	Growth (generations/hr)
1	None	1.22
	Adenosine	0.49
	Adenosine (300) + succinate (20)	0.78
	Adenosine (300) + succinate (200)	0.78
	Adenosine (300) + histidine (20)	0.94
	Adenosine (300) + histidine (200)	0.88
2	None	1.29
	Adenosine (300)	0.56
	Adenosine (300) + succinate (20)	0.65
	Adenosine (300) + succinate (200)	0.65
	Adenosine (300) + histidine (20)	0.64
	Adenosine (300) + histidine (200)	0.65

* *Aerobacter aerogenes* 1033 was used in these experiments.

Excretion of a fluorescent compound during adenosine inhibition. A substance with blue fluorescence (activation λ_{max} = 400 mμ; emission λ_{max} = 460 mμ) was detected in the medium of adenosine-inhibited cultures of *A. aerogenes* 1033. A similarly fluorescent substance was found in the medium of strain PD 1, the purine-thiamine auxotroph of *A. aerogenes*, which is defective in the synthesis of B_1 pyrimidine. This material was not detected in the medium of the thiamine auxotroph whose defect is in the synthesis of B_1 thiazole. In addition to identical fluorescence spectra, the fluorescent substances found in the medium of the adenosine-inhibited cultures of the auxotroph had the following properties in common: thiamine or B_1 pyrimidine in excess of that required for maximal growth prevented their excretion; when mixed, they migrated as a single fluorescent spot in paper chromatography with HCl-isopropanol-water, formic acid-isopropanol-water, or NH_4OH-isopropanol-water as solvents; they appeared to

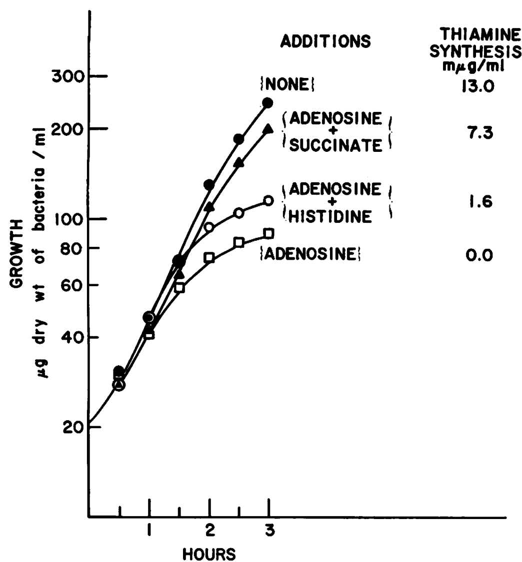

FIG. 3. *Effect of succinate and histidine on thiamine synthesis by adenosine-inhibited cultures of Escherichia coli. Adenosine = 300 μg/ml; succinate = 20 μg/ml; histidine = 20 μg/ml. Growth is expressed as micrograms (dry weight) of bacteria, based on a previous calibration relating the turbidity of a culture at 420 mμ to its dry weight.*

be phosphate esters because they formed barium salts which are soluble in water but insoluble in 80% ethanol, and strongly acidic [0.002 N HCl is necessary for their removal from a Dowex-1 chloride column and 1.0 M ammonium formate (pH 3.0) is required to remove them from a Dowex-1 formate column]. The purification of the compound from culture filtrates was followed by making measurements of fluorescence. Unfortunately, determinations of the phosphate content of the most highly purified sample revealed that, from 40 liters of culture filtrate, less than 0.5 μmole of the compound had been isolated. Thus, the compound must be intensely fluorescent, which of course made it possible to detect; however, the extremely low yields of this compound have so far thwarted further chemical characterization.

Discussion

Many normal metabolites have been observed to cause bacteriostasis in certain circumstances. Most frequently, these effects have been attributed to competition with an essential metabolite for entry into the cell (Kepes and Cohen, 1962), to erroneous incorporation into macromolecules (Matthews, 1958), and to inhibition of the biosynthesis of another metabolite, either by spurious feedback effects (Davis and Feingold, 1962) or by competition with an essential intermediate of similar structure for an enzyme surface (Davis, 1955). The bacteriostatic effects of adenosine and several related purines can now be attributed to the latter cause, inhibition of the biosynthesis of thiamine. The claim that adenosine acts by inhibiting the synthesis of pyrimidine portion of thiamine rather than by competitively inhibiting its incorporation into thiamine is based on the following considerations. (i) B_1 pyrimidine is about twice as effective as thiamine on a weight basis in overcoming the inhibition, whereas, if there were a competitive relationship between B_1 pyrimidine and adenosine, thiamine might be expected to be more effective. (ii) The bacteriostatic effect can be overcome by thiamine or by B_1 pyrimidine in the same amounts required by auxotrophs incapable of synthesizing either thiamine or B_1 pyrimidine. (iii) Neither the thiamine nor the B_1 pyrimidine content of adenosine-inhibited cultures increased over that of the inoculum.

Normally, both *A. aerogenes* and *E. coli* appear to have a surplus supply of thiamine or B_1 pyrimidine, or both, since there is a three- to fivefold increase in the major constituents of the cell, protein, RNA, and DNA after synthesis of B_1 pyrimidine has been inhibited. On the other hand, the synthesis of the thiazole portion of thiamine does not continue under these circumstances. This suggests that the pathway leading to B_1 thiazole is controlled independently by B_1 thiazole itself rather than by the intact thiamine molecule.

In the absence of enzymological studies, it is not possible to distinguish with certainty between the possibilities that adenosine has a pseudofeedback effect at the first step on the pathway to B_1 pyrimidine, or that it is a competitor with an intermediate further along the pathway. However, the latter possibility might better account for the accumulation of an unidentified fluorescent compound during inhibition by adenosine. In applying this argument, it is first necessary to consider the evidence as to whether the fluorescent compound is an intermediate, or at least derived from an intermediate in the synthesis of B_1 pyrimidine. (i) The same compound is accumulated by mutants blocked in the synthesis of B_1 pyrimidine. (ii) Both with the mutants and

with adenosine-inhibited cultures, this compound bears the usual nutritional relationship of an intermediate to the end product; an excess of B_1 pyrimidine prevents its formation, whereas a deficiency greatly stimulates its formation. (iii) It does not appear to be a substrate of a thiamine-dependent reaction which accumulates simply as a result of thiamine deficiency; it is not produced by mutants blocked either in the synthesis of B_1 thiazole or in the last steps of thiamine synthesis, the condensation of B_1 thiazole and B_1 pyrimidine.

Histidine and succinate antagonize the bacteriostatic effect of adenosine in proportion to the amount of thiamine they allow to be produced, despite the presence of adenosine. These observations tend to rule out the possibility that the antiadenosine effects of histidine and succinate stem from an ability of these compounds to reduce the cell's requirement for thiamine by providing products of thiamine-catalyzed reactions. The possibility that succinate or histidine yields intermediates beyond the reaction blocked by adenosine has not been eliminated. Similarly, and perhaps more profitably, the possibility has to be considered that histidine and succinate might act by preventing the conversion of adenosine to the actual inhibitor of the synthesis of the pyrimidine.

ACKNOWLEDGMENTS

This work was supported in part by the Hastings Foundation and by Public Health Service grant GM 11269.

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