Thiamine-dependent Accumulation of Tetramethylpyrazine Accompanying a Mutation in the Isoleucine-Valine Pathway

A. L. DEMAIN, M. JACKSON, AND N. R. TRENNER

Merck Sharp & Dohme Research Laboratories, Merck & Co., Inc., Rahway, New Jersey 07065

Received for publication 24 April 1967

A mutant of Corynebacterium glutamicum was found to accumulate high concentrations of a material which crystallized upon cooling of the broth. The compound was identified as tetramethylpyrazine. The mutant was found to require isoleucine, valine, leucine, and pantothenate for growth. All four requirements probably result from the loss of a single enzyme of the isoleucine-valine pathway. Since similar mutants of Neurospora crassa accumulate acetoin, the present mutant probably forms tetramethylpyrazine from acetoin. Accumulation of tetramethylpyrazine was dependent upon addition of thiamine. This observation is consistent with the known activity of diphosphothiamine as a cofactor for the formation of acetolactate (a precursor of acetoin) from pyruvate.

Mutation of biotinless Corynebacterium glutamicum with N-methyl-N-nitroso-N'-nitroguanidine led to the production of an auxotrophic mutant with multiple nutritional requirements. [The parent organism has previously been designated as Micrococcus glutamicus. It is a biotin-requiring culture which produces glutamic acid (3).] Of unusual interest was the observation that cooling of no culture broth resulted in the appearance of massive amounts of crystals. The present paper describes experiments which show that the crystalline material is tetramethylpyrazine and the nutritional requirements are isoleucine, valine, leucine, and pantothenic acid. Accumulation and excretion of tetramethylpyrazine depended on the addition of thiamine to the medium.

Materials and Methods

Isolation of the mutant. The mutant culture, MB-1923, was obtained by treatment of the biotin-requiring C. glutamicum with N-methyl-N-nitroso-N'-nitroguanidine (K and K Laboratories, Inc., Jamaica, N.Y.) followed by replica plating. MB-1923 showed a requirement for yeast extract which was not satisfied by individual amino acids, vitamins, or derivatives of nucleic acid. The mutant was maintained on agar slants containing 1.0% (w/v) yeast extract and 1.0% (w/v) glucose, which were stored in the cold.

Media. The inoculum medium was composed of the following components per liter: glucose, 25 g; N-Z-amine (Sheffield Chemical, Norwich, N.Y.), 2.5 g; yeast autolysate, 2.0 g; (NH4)2SO4, 5.0 g; urea, 5.0 g; KH2PO4, 500 mg; K2HPO4, 500 mg; MgSO4·7H2O, 250 mg; adenine, 20 mg; FeSO4·7H2O, 10 mg; MnSO4·H2O, 10 mg; thiamine, 1 mg; and biotin, 30 µg.

The composition of the production medium was as follows (per liter): glucose (autoclaved separately), 80.0 g; (NH4)2HPO4, 9.0 g; (NH4)2SO4, 6.0 g; urea, 5.0 g; N-Z-amine, 5.0 g; CaCO3, 5.0 g; KCl, 1.5 g; K2SO4, 1.5 g; MgSO4·7H2O, 300 mg; CaCl2·2H2O, 150 mg; adenine, 200 mg; MnSO4·4H2O, 40 mg; FeSO4·7H2O, 10 mg; thiamine, 1 mg; and biotin, 15 µg. Both media were prepared with deionized, charcoal-treated water, and were dispensed at 20 ml in 250-ml Erlenmeyer flasks.

The composition of the plating medium was the same as the inoculum medium except that N-Z-amine, yeast autolysate, and adenine were omitted, and 2.0% (w/v) agar was added.

Production. A loop of growth from a slant was transferred to each flask of inoculum medium. The flasks were incubated for 23 hr at 28 C on a rotary shaker (220 rev/min, 5-cm thrust). A 0.5-ml amount of inoculum was added to each flask of production medium, which was then incubated for 5 days on the rotary shaker. Cells and insoluble calcium carbonate were removed by centrifugation at 15,000 X g for 15 min.

Cell growth. Growth was determined with a Bausch & Lomb Spectronic-20 colorimeter set at 660 mµ. Absorbance was measured in tubes with an inside diameter of 11.7 mm, and was converted to dry weight by means of a previously determined conversion.

JOURNAL OF BACTERIOLOGY, Aug. 1967, p. 323-326
Copyright © 1967 American Society for Microbiology
Vol. 94, No. 2
Printed in U.S.A.
factor. For measurement of absorbance, the whole broth was diluted in 0.02 N HCl to dissolve the insoluble calcium carbonate.

Ultrasound absorption. Measurements were done in a Beckman spectrophotometer, model DB, with the use of a cuvette of 1-cm light path.

Nuclear magnetic resonance analysis. Spectra were determined by use of a standard Varian Associates A-60 spectrometer.

Infrared analysis. Infrared spectra were determined with the Perkin-Elmer model 421 spectrometer.

RESULTS

Crystal formation. After growth of the mutant culture in the complex seed medium, it was able to grow in the production medium, which was chemically defined with the exception of the enzymatic digest of casein. After removal of cells and insoluble calcium carbonate, it was noted that cooling of the broth in a freezer resulted in the formation of crystals. No crystals were formed by the parent culture.

Crystals were obtained by filtration of cold centrifuged broth from six production flasks through Whatman no. 1 paper. The crystals were washed with cold water and were dried in air. The weight of the dried crystals was 115 mg.

Identification as tetramethylpyrazine. When crystals which had been dried over CaCl₂ were dissolved in water at 10 μg/ml, the ultraviolet spectrum showed two peaks at 280 and 293 μm, each of which had an absorbance of 0.70. In 0.1 N NaOH, the results were similar. When dissolved in 0.1 N perchloric acid, a single sharp peak at 300 μm with an absorbance of 0.88 was observed. When 60 μg of crystals were spotted on paper, they failed to show color when sprayed with ninhydrin.

The primary clue to the identity of the crystalline product was its nuclear magnetic resonance spectrum in conjunction with its elemental composition, which suggested an empirical formula of C₄H₈N₄. The spectrum in deuterochloroform was characterized by a single sharp resonance band at 7.65 τ. This clearly indicated that all twelve protons were equivalent and were probably located as methyl groups attached to unsaturated carbon atoms in a symmetrical arrangement. All eight carbon atoms were thus accounted for. Further consideration of the ultraviolet and infrared spectra of both the base and the hydrochloride led to the concept of a tetramethylated pyrazine. These spectra and the melting points of the base and hydrochloride agreed well with literature values (4, 5).

Identification of nutritional requirements for growth. When discs saturated with individual amino acids, vitamins, or nucleic acid derivatives were placed on the surface of basal agar seeded with MB-1923, no growth resulted. The parent culture grew well on the basal agar medium. It was soon found that the mutant grew with a mixture of amino acids and vitamins. Elimination experiments demonstrated that good growth on agar occurred only when isoleucine, valine, leucine, and Ca pantothenate were present. Omission of any of the four components severely limited growth. Although Ca pantothenate was a requirement for growth on agar, it was not routinely added to the fermentation medium. Apparently, the inoculum and the N-Z-Amine supplied ample quantities of the vitamin. Omission of thiamine and adenine from the basal agar medium did not affect the results.

Dependence of tetramethylpyrazine formation on thiamine. Since the above nutritional tests on agar had shown that neither adenine nor thiamine was required for growth, we attempted to eliminate both from the liquid fermentation medium. Surprisingly, despite good growth, no crystals were formed. We then eliminated adenine and thiamine separately (Table 1) and found the vitamin to be required specifically for production of tetramethylpyrazine. Removal of adenine alone improved growth markedly but had only a slight effect on formation of the pyrazine. Omission of thiamine alone resulted in a decrease in growth and in the complete elimination of tetramethylpyrazine production. These growth effects are presumably due to the well-known toxicity of adenine for microbial growth and its reversal by thiamine (7).

DISCUSSION

Although pyrazines have been only rarely found as products of microbial metabolism, a small amount of tetramethylpyrazine was isolated by Kosuge and Kamiya (6) from broths of a strain of Bacillus subtilis. The compound is apparently responsible for the characteristic odor of fermented soybean or "natto." These workers (1) postulated that tetramethylpyrazine is derived from 2 moles of acetoin (acetyl methylcarbinol) and 2 moles of ammonia. Our finding that a block in the isoleucine-valine pathway leads to the accumulation and excretion of a large amount of tetramethylpyrazine supports the role of acetoin as a precursor, as discussed below.

The essentials of isoleucine-valine biosynthesis in microorganisms are shown in Fig. 1 (9). Since the same enzymes are used for the isoleucine and valine paths and since the immediate precursor of valine, α-ketoisovalerate, is necessary for synthesis of leucine and pantothenate, it is evident that formation of all four products depends on
Table 1. Dependence of tetramethylpyrazine formation on thiamine

<table>
<thead>
<tr>
<th>Omission from medium</th>
<th>Growth</th>
<th>Tetramethylpyrazine production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance</td>
<td>Dry wt</td>
</tr>
<tr>
<td>None</td>
<td>mg/ml</td>
<td>g/ml</td>
</tr>
<tr>
<td>Adenine</td>
<td>17</td>
<td>5.9</td>
</tr>
<tr>
<td>Thiamine</td>
<td>40</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* Whole broth measured in 0.02 N HCl at 660 mµ in a Spectronic-20 colorimeter.
* Calculated from absorbance.
* Visual observation upon cooling centrifuged broth.
* Centrifuged broth measured in 0.1 N perchloric acid at 300 µ in a Beckman DB spectrophotometer.
* Calculated from absorbance.

These enzymes. Thus, the requirements for isoleucine, valine, leucine, and pantothenate are probably the result of a single mutational block. Most microorganisms with such mutations require only isoleucine and valine; presumably, they can deaminate the added valine back to α-ketosovalerate and satisfy their needs for leucine and pantothenate. Mutant MB-1923 apparently cannot carry out such a reaction.

As shown in Fig. 1, acetolactate is also a source of the acetoin formed by some microorganisms. In Aerobacter aerogenes, the condensation reaction is actually carried out by two enzymes with similar activities but with different controls (2). The “biosynthetic” enzyme is inhibited and repressed by its end product, valine. The “degradative” enzyme is not controlled by amino acids. Its function is to participate in the formation of a neutral end product (acetoin) of glucose disimilation.

The mutant described in the present study probably is deficient in the reductoisomerase. Such mutants of Neurospora crassa (10, 11) accumulate acetoin in their growth medium. Similarly, an Escherichia coli mutant blocked at the reductoisomerase step accumulates acetolactate and acetoin (8). Presumably, MB-1923 produces high levels of acetoin in the presence of amino acids with the aid of the “degradative” condensing enzyme and α-acetolactate decarboxylase, and then converts the acetoin to tetramethylpyrazine (Fig. 2). It is possible that the “biosynthetic” enzyme rather than the “degradative” condensing enzyme is responsible for the production of acetoin in MB-1923. In this case, either not enough valine is present for inhibition or repression, or both, or the “biosynthetic” enzyme in this organism may not be sensitive to feedback effects. It should be emphasized that the present studies do not prove that the genetic block is at the reductoisomerase step or that the block is the reason for accumulation of tetramethylpyrazine. Further work is needed to demonstrate that the tetramethylpyrazine carbons are derived from pyruvate and to determine the effect of limiting valine, isoleucine, leucine, or pantothenate.

The requirement of thiamine for accumulation of tetramethylpyrazine fits in well with the above concept. Extracts of E. coli require diphosphothiamine for maximal formation of acetolactate from pyruvate (8). The reaction apparently involves formation of “active acetaldehyde” from one of the pyruvate molecules, followed by transfer of the acetal group to another molecule of pyruvate (9). “Active acetaldehyde” appears to be an acetaldiphosphothiamine complex. Thus, although MB-1923 grows well in the presence of amino acids and in the absence of added thiamine, the rate of formation of acetolactate (and of tetramethylpyrazine) could be expected to depend on the level of thiamine added to the medium.

The concentration of tetramethylpyrazine...
excreted by MB-1923 is very impressive. Whereas the \textit{B. subtilis} strain of Adachi et al. (1) produced about 5 mg/liter in 15 days, we have observed after 5 days the production of as much as 3 g/liter with \textit{C. glutamicum}.

\textbf{ACKNOWLEDGMENT}

The technical assistance of Richard J. Prevoznak is gratefully acknowledged.

\textbf{LITERATURE CITED}


