Enzymatic Determination of Itoic Acid, a *Bacillus subtilis* Siderophore, and 2,3-Dihydroxybenzoic Acid

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A specific enzymatic method to determine the amounts of itoic acid, a *Bacillus subtilis* siderophore, and 2,3-dihydroxybenzoic acid (2,3-DHBA) was devised. A sample was incubated first with hippurate hydrolase and then with 2,3-DHBA-3,4-dioxygenase. Itoic acid was estimated from the increase in A_{374}. The incubation with the first enzyme was omitted for the determination of 2,3-DHBA.

Amino acid conjugates of 2,3-dihydroxybenzoic acid (2,3-DHBA) make up a major class of siderophores (10–12). The first siderophore to be chemically identified is 2,3-dihydroxybenzoylglycine (7), commonly known as itoic acid (4, 9, 10, 13). To study the biosynthetic mechanism of itoic acid, a specific method for determination of the amount of itoic acid, especially in the presence of 2,3-DHBA, is needed. Although the amounts of *o*-dihydroxybenzenes can be determined by a colorimetric method (1) and the amounts of siderophores in general can be determined by another colorimetric method (15), these methods are definitely not specific enough for the purpose. In the following report, a specific enzymatic method for determining the amounts of itoic acid and 2,3-DHBA is described.

Pseudomonas putida (ATCC 12633) containing hippurate hydrolase (*N*-benzoylglycine amidohydrolase [EC 3.5.1.32]) was grown overnight in a medium containing 0.1% sodium hippurate, 0.05% NH_4NO_3, 0.06% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, and a 1:1 mixture of distilled water and tap water, pH 7.0. The cells were harvested by centrifugation, washed with 17 mM potassium phosphate buffer (pH 7.0), and suspended in the buffer to make the concentration of the cells 20 to 40 mg (dry weight) per ml. The cell extract containing 2,3-DHBA-3,4-dioxygenase (2,3-dihydroxybenzoate:oxygen 3,4-oxidoreductase [decyclizing; EC 1.13.11.14]) was prepared by sonication from *Pseudomonas fluorescens*, which was isolated from soil in the presence of 2,3-DHBA as the sole source of carbon, as described previously (5). The organism is similar or perhaps identical to a strain designated 23D-1 by Ribbons (14). *Bacillus subtilis* (ATCC 13933, NRRL B-1471) was grown in an iron-deficient growth medium as described previously (7).

Itoic acid was chemically synthesized by a method previously described (7). 2,3-DHBA was purchased from Aldrich Chemical Co. Yeast extract was a product of BBL, Division of BioQuest. Other chemicals were obtained from Fisher Scientific Co.

A 5.0-ml sample mixture (such as appropriately diluted culture fluid of *B. subtilis*) containing 0 to 40 μM itoic acid, 0.2 ml (4 to 8 mg [dry weight]) of freshly harvested cells of *P. putida*, and 0.8 ml of 0.1 M potassium phosphate buffer (pH 7.0) was incubated at room temperature for 1 h to hydrolyze itoic acid completely to 2,3-DHBA and glycine (Fig. 1). The cells were removed by centrifugation. To 2.9 ml of the supernatant, 0.1 ml (0.3 to 0.5 mg of proteins) of *P. fluorescens* cell extract was added, and the increase in A_{374} due to the formation of α-hydroxy-*cis*,*cis*-muconic semialdehyde

(Fig. 1) was monitored until no more increase was observed. The reaction was complete within 1 to 2 min. Afterward, absorbance decreased (although very slowly) with some extracts because of the presence of other enzymes that degrade the product of the dioxygenase reaction. From the increase in absorbance, the total amounts of itoic acid and 2,3-DHBA were estimated by comparison with the increase in absorbance that occurred when 2.9 ml of 2,3-DHBA (0 to 25 μM) in 17 mM potassium phosphate buffer (pH 7.0) was incubated with 0.1 ml of *P. fluorescens* extract. *P. putida* was replaced by the same volume of the phosphate buffer to determine the amount of 2,3-DHBA alone. The amount of 2,3-DHBA found was subtracted from the total amounts of itoic acid and 2,3-DHBA.

As shown by a typical experiment (Fig. 2), the A_{374} was linear with 2,3-DHBA when it was treated with a *P. fluorescens* extract. It was approximately 95% of the expected increase in absorbance calculated from the molar extinction coefficient (29,700 at pH 7.0) (2). A typical experiment with known amounts of itoic acid showed that approximately 97% of the itoic acid added was detected by this method (data not shown). Hence, it may not be necessary to run the standard assay with a known amount of 2,3-DHBA every time. Without the standard assay, underestimation may be only about 8%. Such a high rate of recovery of itoic acid observed indicates that *P. putida* does not degrade 2,3-DHBA. Moreover, itoic acid (and catechol and protocatechuic acid as well) does not serve as a substrate for 2,3-DHBA-3,4-dioxygenase (4a). These properties are essential for this method to work.

The minimum amount of *P. putida* required to accomplish complete hydrolysis of itoic acid within 30 to 60 min was
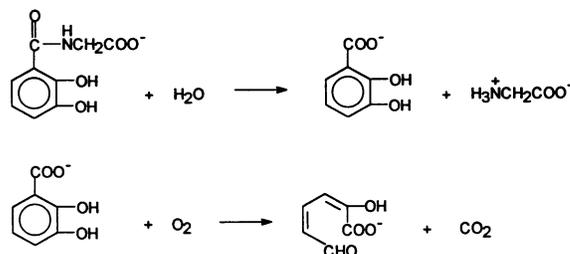

FIG. 1. The reactions involved in the assay for itoic acid and 2,3-DHBA catalyzed by hippurate hydrolase (first equation) and 2,3-DHBA-3,4-dioxygenase (second equation).
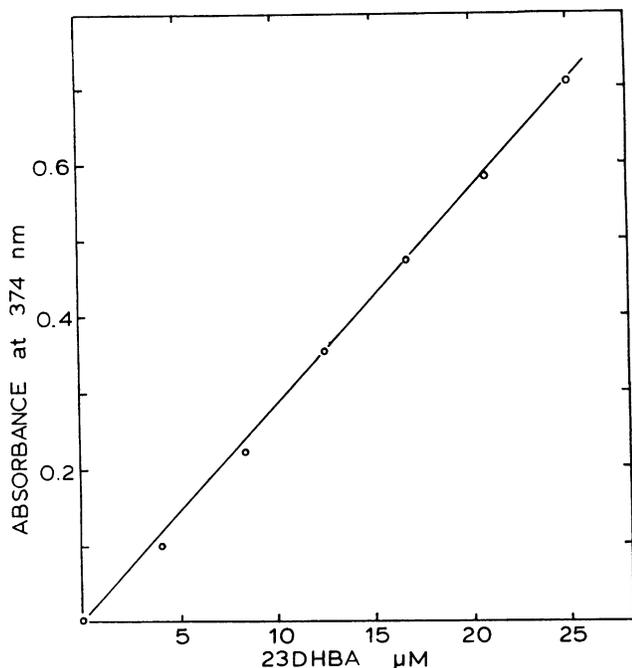
FIG. 2. Increase in absorbance that occurred when 2,3-DHBA was treated with 2,3-DHBA-3,4-dioxygenase. Various amounts of 2,3-DHBA were treated with a *P. fluorescens* extract (0.16 mg of protein per ml), and the increase in A_{374} was measured.

approximately 0.7 mg (dry weight) per ml of the reaction mixture (Fig. 3).

The method was applied to estimate the amounts itoic acid and 2,3-DHBA in samples of *B. subtilis* culture fluid containing no added iron (but containing usually about 0.3 µM

FIG. 4. Accumulation of itoic acid and 2,3-DHBA in iron-deficient culture fluid of *B. subtilis* during a 5-day growth period. Empty circles, itoic acid; filled circles, 2,3-DHBA; triangles, itoic acid in experiments in which iron (18 µM) was added at 72 h. 23DOB, 2,3-DHBA.

as a contaminant) at different hours of growth. The result indicated that very little 2,3-DHBA was excreted into the culture fluid, and there was a long delay before itoic acid excretion began rapidly (Fig. 4). When iron was added (18 µM) in the growth medium, virtually no itoic acid or 2,3-DHBA was detected in the culture fluid (data not shown). When iron was added at the time at which itoic acid began to appear in the culture fluid, the itoic acid content continued to increase for a number of hours before it declined (Fig. 4). The greatest amount of itoic acid was approximately 38 µM (8 mg/liter) in the culture fluid in this particular experiment (Fig. 4). This amount is not one that is typically accumulated under an optimum iron-deficient condition. As much as 250 mg of itoic acid per liter of *B. subtilis* culture fluid accumulates, as estimated by a nonspecific color reaction (9). It was noticed that the pink that develops in iron-deficient culture as the organism grows was not as intense as is usually observed. The A_{403}, at which the colored accumulated compound coproporphyrin III absorbs maximally, was much lower than usual and was 0.258 when itoic acid accumulation was highest. Coproporphyrin III, which accumulates along with itoic acid (3, 7), gives an intense pink under an optimum iron-deficient condition. The color is a simple visual qualitative measure of accumulation not only of coproporphyrin III but also of itoic acid, and it indicates optimum iron deficiency (6). Under this condition, *B. subtilis* produces as high as 1.5 in A_{403} (4), which is much higher than the value found in the present study. The low accumulations of itoic acid and coproporphyrin III in this particular experiment
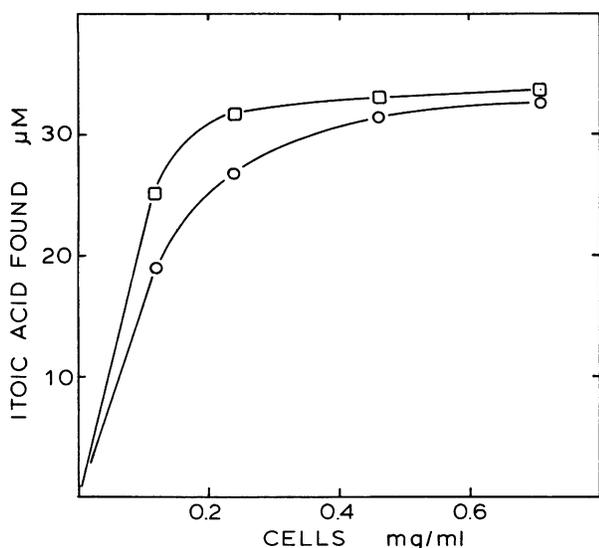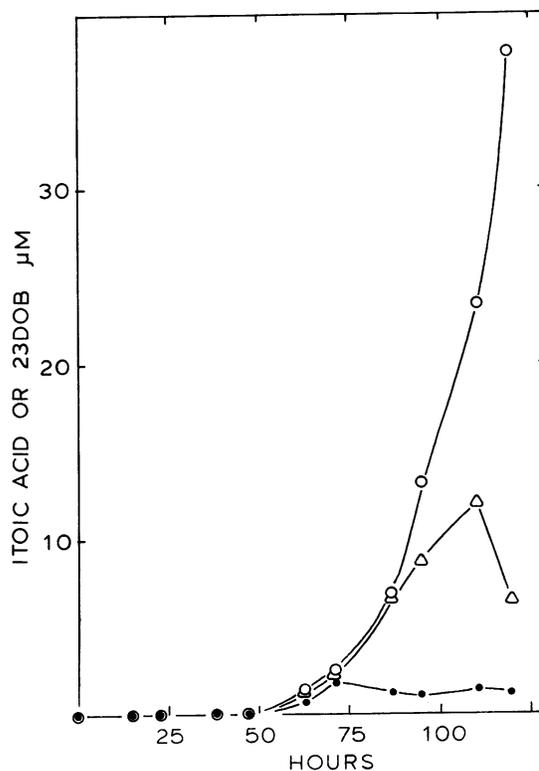
FIG. 3. Effects of the cell content and the time of incubation with *P. putida* on the extent of itoic acid hydrolysis. Itoic acid (35 µM) was assayed as described in the text. The abscissa shows the concentrations of *P. putida* cells in the incubation mixtures. The *P. fluorescens* extract used subsequently was 0.16 mg/ml of the incubation mixtures. Squares, 60 min; circles, 30 min.

indicate that the content of contaminant iron, which could vary from one experiment to another, was probably higher than expected. That itoic acid was detected under such a condition, which was less favorable for its accumulation, shows that the method devised in the present study is a sensitive one.

It is not known whether the mechanism by which the amount of itoic acid decreases when iron is added to iron-deficient growing culture of *B. subtilis* is autooxidation or metabolic degradation or both.

The effect of a high concentration of coproporphyrin III in *B. subtilis* culture fluid on the measurement of A_{374} would be minimal. Under the optimum iron-deficient condition at which high accumulation of coproporphyrin III occurs, the absorption spectrum of the culture fluid shows that the minimum occurs at near this wavelength and is approximately only 0.7 (4). Moreover, under the same condition, itoic acid accumulation is also high, and the culture fluid must be diluted several-fold before it is subjected to the assay.

The complete loss of its activity by sonication (4a) indicates that hippurate hydrolase in *P. putida* ATCC 12633 is probably particle bound and that it is different from the water-soluble enzyme purified from another strain of *P. putida* (8). The specificity of the enzyme of the present study appears relatively low, as manifested by the fact that it catalyzes hydrolysis of itoic acid. Low specificity is also the case for the soluble enzyme. It is possible that in addition to itoic acid, other amino acid conjugates of 2,3-DHBA, of which there are many (10, 12), are detected by this method. Nevertheless, the method reported here is the most specific one for itoic acid and 2,3-DHBA among presently available methods.

REFERENCES

1. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
2. **Dagley, S., W. C. Evans, and D. W. Ribbons.** 1960. New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. Nature (London) **188**:560–566.
3. **Garibaldi, J. A., and J. B. Neilands.** 1956. Formation of iron-binding compounds by micro-organisms. Nature (London) **177**:526–528.
4. **Ito, T.** 1987. Lunar soil and Zagami meteorite inhibit biosynthesis of itoic acid, a siderophore. Experientia **43**:1012–1013.
4a.**Ito, T.** Unpublished observations.
5. **Ito, T., A. R. Ayers, and R. T. Powell.** 1991. 2,3-Dihydroxybenzoate dioxygenase from *Pseudomonas fluorescens* catalyzes simultaneous oxygenation and decarboxylation of 2,3-dihydroxybenzoate. J. Elisha Mitchell Sci. Soc. **107**:49–55.
6. **Ito, T., and K. Behfar.** 1992. Formation of a membrane protein in *Bacillus subtilis* grown in an iron-limited medium. J. Elisha Mitchell Sci. Soc. **108**:19–24.
7. **Ito, T., and J. B. Neilands.** 1958. Products of "low iron fermentation" with *Bacillus subtilis*: isolation, characterization and synthesis of 2,3-dihydroxybenzoylglycine. J. Am. Chem. Soc. **80**:4645–4647.
8. **Miyazawa, E., J. Yano, T. Hamakado, Y. Kido, K. Nishimoto, and Y. Motoki.** 1985. Crystallization and properties of N-benzoylglycine amidohydrolase from *Pseudomonas putida*. Agric. Biol. Chem. (Tokyo) **49**:2881–2886.
9. **Neilands, J. B.** 1961. Early stages in the metabolism of iron, p. 194–206. *In* J. E. Falk, R. Lemberg, and R. K. Morton (ed.), Haematin enzymes. Pergamon Press, New York.
10. **Neilands, J. B.** 1973. Microbial iron transport compounds (siderochromes), p. 167–202. *In* G. L. Eichhorn (ed.), Inorganic biochemistry, vol. 1. Elsevier Scientific Publishing Co., Amsterdam.
11. **Neilands, J. B.** 1974. Iron and its role in microbial physiology, p. 3–34. *In* J. B. Neilands (ed.), Microbial iron metabolism. Academic Press, Inc., New York.
12. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
13. **Peters, W. J., and R. A. J. Warren.** 1968. Itoic acid synthesis in *Bacillus subtilis*. J. Bacteriol. **95**:360–366.
14. **Ribbons, D. W.** 1966. Bacterial oxidation of 2,3-dihydroxybenzoic acid—a new oxygenase. Biochem. J. **99**:30P–31P.
15. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47–56.