ENTEROBACTIN, AN IRON TRANSPORT COMPOUND FROM SALMONELLA TYPHIMURIUM

J. R. Pollack and J. B. Neilands

Department of Biochemistry

University of California

Berkeley, California 94720

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Enterobactin, a physiologically active iron sequestering agent, is excreted into the medium by Salmonella typhimurium under low iron conditions. It is a cyclic polyester consisting of three residues of 2,3-dihydroxy-N-benzoylserine.

Low molecular weight compounds known at this time to be involved in microbial transport and metabolism of iron contain hydroxamate or phenolate groups which are capable of coordination with a central ferric ion. Ferrichrome (1) and 2,3-dihydroxybenzoylglycine (2) are representative of these two types of substance, respectively. The mycobactins (1), from mycobacteria, are unusual in that both hydroxamate and phenolate groups are found on the same molecule.

In this communication we describe a new member of the phenolate series, obtained from S. typhimurium LT2, for which we propose the name enterobactin and the structure shown in Fig. 1.

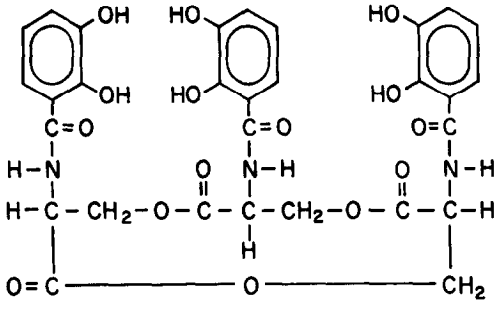

Fig. 1. Enterobactin.

S. typhimurium was grown aerobically at 37° for 15 hours from a 1% inoculum in the following medium: K_2HPO_4, 7g; KH_2PO_4, 4g; $(NH_4)_2SO_4$, 2g; $MgSO_4 \cdot 7H_2O$, 200 mg; $MnSO_4 \cdot H_2O$, 17 mg; glucose, 5g; and water to one liter. The cell-free culture supernatant was concentrated 20-fold *in vacuo* at 40° and then extracted with an equal volume of ether. The ether extract was discarded and the culture supernatant adjusted to pH 2.0 with 12 N HCl. It was then extracted with an equal volume of ether; this ether extract was washed with 0.1 M sodium citrate, pH 5.5, and dried over anhydrous $MgSO_4$. The ether extract was taken to dryness *in vacuo* and the residue treated with hot benzene-dioxane. The insoluble fraction was collected by centrifugation and resuspended in water. Filtration and drying over P_2O_5, followed by precipitation from ethanol-water, gave crystalline enterobactin, m.p. 202-203°. The yield was about 6 mg per 10 liters of culture.

Enterobactin was examined for growth factor activity in several mutants of S. typhimurium which are unable to synthesize the substance (3). These mutants grow normally upon the addition to the medium of large amounts of iron salts or certain chelating agents. Enterobactin proved to be 100 times as active, on a molar basis, as 2,3-dihydroxy-N-benzoyl-L-serine, which has been isolated from Escherichia coli (4,5) and Aerobacter aerogenes (5). The latter compound has recently been shown to promote the uptake of iron from the medium by E. coli (6); 2,3-dihydroxybenzoylglycine has a similar effect with Bacillus subtilis (7).

Enterobactin is soluble in acetone, dioxane, dimethylsulfoxide, and methanol. It is very sparingly soluble in water. It behaves as a neutral substance on paper electrophoresis at pH 5 and displays the bright blue fluorescence under ultraviolet light characteristic of the 2,3-dihydroxybenzoyl nucleus. Hydrolysis in 6 N HCl at 105° for 20 hours followed by analysis with paper chromatography revealed the

presence of serine and 2,3-dihydroxybenzoic acid. The infrared spectrum of enterobactin showed a strong absorption at 1760 cm^{-1} which is characteristic of a strained ester group C=O stretch.

The following nuclear magnetic resonance spectrum at 220 MHz was obtained for enterobactin in deuterodimethylsulfoxide with tetramethylsilane as the internal standard: δ = 8.90, doublet (amide H); δ = 7.30, doublet (aromatic H); δ = 6.96, doublet (aromatic H); δ = 6.71, triplet (aromatic H); δ = 4.91, complex (alpha H); δ = 4.64, complex (methylene H); and δ = 4.41, complex (methylene H). All peak areas were equivalent. This nmr spectrum was very similar to that reported for 2,3-dihydroxy-N-benzoyl-L-serine in deuteroacetone (5).

At pH 6.5 the ferric complex of enterobactin migrated to the anode on paper electrophoresis at a rate approximately equivalent to that of ferrichrome A, an anion with a triple negative charge (1). This suggests that free phenolic hydroxyls at both the 2 and 3 positions act as ligands since if the carbonyl and the phenolic hydroxyl at the 2 position of the 2,3-dihydroxybenzoyl moiety were the ligands, one would expect a neutral ferric complex.

Microanalysis of enterobactin gave C, 54.06%; H, 4.19%; N, 6.11%. Theoretical for $C_{30}H_{27}N_3O_{15}$: C, 53.84%; H, 4.07%; N, 6.28%.

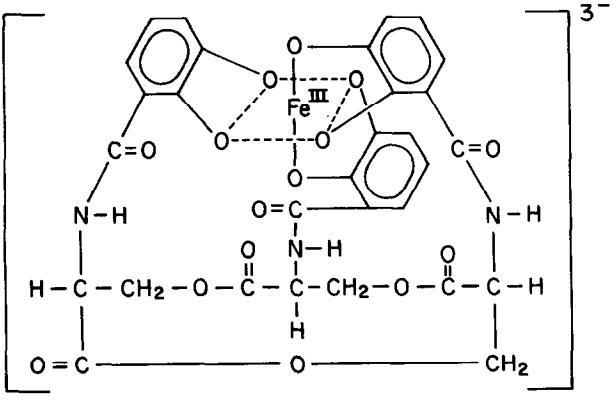

Fig. 2. Ferric enterobactin.

The largest major ion found by mass spectrometry of enterobactin had a m/e of 223, which corresponds to $C_{10}H_9NO_5$. However, sedimentation equilibrium in water and D_2O (8) gave in duplicate runs molecular weights of 685 and 605, which agree with the molecular weight of 669 for $C_{30}H_{27}N_3O_{15}$.

From these data we conclude that the structure of enterobactin is as shown in Fig. 1. Inspection of molecular models suggests the structure given in Fig. 2 for ferric enterobactin.

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References

1. Neilands, J. B., Science 156, 1443 (1967).
2. Ito, T. and J. B. Neilands, J. Am. Chem. Soc. 80, 4645 (1958).
3. Pollack, J. R., B. N. Ames and J. B. Neilands, unpublished results.
4. Brot, N., J. Goodwin and H. Fales, Biochem. Biophys. Res. Commun. 25, 454 (1966).
5. O'Brien, I. G., G. B. Cox and F. Gibson, Biochim. Biophys. Acta 177, 321 (1969).
6. Wang, C. C. and A. Newton, J. Bacteriol. 98, 1142 (1969).
7. Peters, W. J. and R. A. J. Warren, Biochim. Biophys. Acta 165, 225 (1968).
8. Edelstein, S. J. and H. K. Schachman, J. Biol. Chem. 242, 306 (1967).