IN VIVO AND IN VITRO FORMATION OF 2,3-DIHYDROXYBENZOYLSERINE BY ESCHERICHIA COLI K_{12}

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Received October 24, 1966

During the course of studies with a methionine-vitamin B_{12} auxotroph of Escherichia coli, it was observed that the media became red in color when the organism was in the stationary phase. Preliminary purification of the red-purple compound showed that it was of low molecular weight, contained serine and iron, reacted with Folin's reagent and gave a positive test for the presence of a catechol (Arnow, 1937). When the organism was grown in a medium containing low iron, a pale yellow iron-free compound accumulated, which upon the addition of $FeSO_4$ became red in color. It appeared that the organism excreted a material into the medium which was able to complex inorganic iron to form a colored iron chelate. This iron chelate has been isolated, purified and characterized as 2,3 dihydroxybenzoylserine. An enzyme has been found which will catalyze the synthesis of this compound from 2,3 dihydroxybenzoic acid and serine. A similar compound, 2,3 dihydroxybenzoylglycine, has been isolated from Bacillus subtilus growth medium by Ito and Nielands (1958).

Isolation of 2,3-Dihydroxybenzoylserine (DHBS) from E. coli.

Escherichia coli K_{12} (strain 2276, a methionine-cyano-B_{12} auxotroph) was obtained from Dr. A. L. Taylor of the Department of Microbiology, University of Colorado Medical School, Denver, Colorado. The organism was grown at 37° in an 18-liter carboy for 36 hours under vigorous aeration in the following medium (per liter): Na_2HPO_4, 8.6 g; KH_2PO_4, 5.6 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7\ H_2O$, 0.1 g $Ca(NO_3)_2 \cdot 4\ H_2O$, 14.3 mg; thiamine hydrochloride 0.2 mg; glucose, 10 g; and 0.1 g of methionine. The cells were removed using a Sharples centrifuge and the cell free media adjusted to pH 7.4. An aliquot was removed and titrated with 0.01 M $FeSO_4$ until a maximum optical density at 500 mμ was obtained and then an appropriate amount of $FeSO_4$ was added to the rest of the medium to convert all of the material present into the iron chelate. The formation of the iron chelate stabilized the compound during the isolation procedure. DEAE-cellulose was added to the medium until at least 90% of the red color had been adsorbed. The medium containing the DEAE cellulose was then filtered through a Buchner funnel and the filtrate discarded. The red cake of DEAE was washed with H_2O, 0.1 M $(NH_4)_2CO_3$ and then eluted from the DEAE with 1 M $(NH_4)_2CO_3$. The solvent was removed by lypholization and the reddish-purple residue was dissolved in a small amount of water and centrifuged to remove any residual DEAE. The yield was 75 mg of the iron chelate per liter of medium. The material was further purified by successive passages through a Sephadex G-25 and a Sephadex G-10 column.

Characteristics and Structure

Figure 1 shows the spectrum of the isolated material. It has a broad peak between 500 mμ and 600 mμ in the visible region at neutral pH and an absorption maximum at 325-330 mμ at pH 7.4 and pH 12. A hypsochromic shift of about 20 mμ is observed in the ultraviolet spectrum when the pH is adjusted to 2. The solution is decolorized at this pH and also in the presence of 0.1 M neutral EDTA. A solution of the compound was assayed for the presence of catechol (Arnow, 1937) and then hydrolyzed overnight in a sealed evaculated tube at

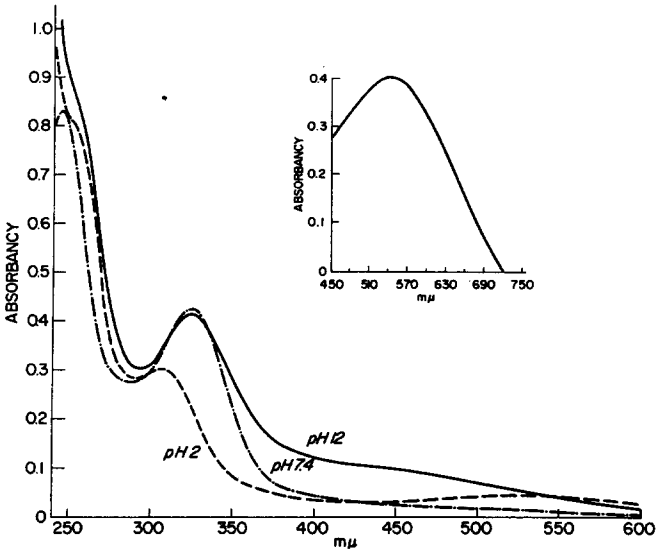
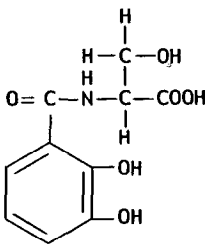


Figure 1 - Absorption spectre of 2,3 dihydroxybenzoylserine at various pH values. Solutions contained 75 mμmoles/ml of the compound. The insert shows the visible spectrum of a solution containing 375 mμmoles/ml.

120° in 6N HCl. The hydrolysate was assayed for amino acids on a Beckman amino acid analyzer and serine was found to be the major amino acid present (a total of about 10-15% of aspartic and glutamic acid were also detected). No free serine was found in an unhydrolyzed control. Upon hydrolysis 0.75 μmoles of serine was recovered per micromole of catechol found in the unhydrolyzed sample. Since under the conditions of the hydrolysis approximately 15-20% of the serine would be destroyed, the serine: catechol ratio in the compound is close to unity. The catechol in the hydrolysate was extracted with ethyl acetate and shown to have a spectrum identical to that of 2,3 dihydroxybenzoic acid (K & K Lab. Plainview, New Jersey) at various pH values. In addition, paper chromatographic analysis (n-butanol, 60; acetic acid, 15; H_2O, 25) of the sample revealed a blue fluorescent spot, under ultraviolet illumination, with an R_f (0.82) identical to that of 2,3 dihydroxybenzoic acid.

An iron free permethylated sample of the metabolite was subjected to

mass spectroscopy and a parent peak having a mass of 283 was seen. This agrees with the postulated structure (Figure 2). Further verification of the structure was obtained by the use of nuclear magnetic resonance and infrared spectroscopy.



2,3, Dihydroxybenzoylserine

Enzymatic Synthesis of DHBS

E. coli, grown as described above, was suspended in 3 volumes (grams, wet weight per ml) of 0.1 M Tris pH 7.4 and disrupted in a Raytheon sonic oscillator for 10 minutes. This extract was centrifuged at 20,000 x g for 15 minutes and the supernatant solution was subjected to ammonium sulfate fractionation. The protein fraction that precipitated between 30 and 60% saturation was dissolved in 0.01 M Tris-chloride pH 7.4 and dialyzed for 16 hours against this same buffer. This fraction (40 mg protein/ml) was used for the experiments reported here.

The reaction mixture used to form DHBS from 2,3-dihydroxybenzoic acid and serine contained in a total volume of 0.2 ml; enzyme (3.2 mg protein); 10 μmoles Tris chloride pH 7.4; 100 mμmoles 2,3-dihydroxybenzoic acid, 100 mμmoles serine-3-C^{14} (CalBiochem. specific activity 1300 cpm/mμmole) and 1 μmole ATP. After incubation at 37° for 15 minutes, the reaction was stopped by the addition of 0.8 ml of 0.05 M HCl and the DHBS formed was extracted with 3 ml of ethyl acetate. Two ml of the organic phase were then removed and assayed for radioactivity in a Packard Tri-Carb spectrometer. A typical experiment is shown in Table I. The enzymatic formation of extractable radioactivity from C^{14} serine is stimulated by 2,3-dihydroxybenzoic acid and ATP, and appears to be very

TABLE I

Requirements for the Enzymatic Formation of 2,3-dihydroxybenzoylserine

	Ethyl acetate extractable radioactivity
	cpm
Complete system	10,600
- 2,3-dihydroxybenzoic acid	2,500
- ATP	2,720
- enzyme	500
Complete system 0°	450

Incubation conditions and the assay are described in the text.

specific for both of these compounds. Thus 3,4 and 2,5 dihydroxybenzoate, salicylic acid, and 3-hydroxyanthranilic acid do not serve as substrates in this reaction. In addition, ADP, dATP, GTP cannot substitute for ATP. The product has been isolated and found to possess similar chromatographic properties to purified DHBS obtained from the E. coli growth medium. As seen in Table 1 a small amount of radioactive ethyl acetate extractable material is formed in the incubation in the absence of 2,3 dihydroxybenzoate and ATP. This has not been identified but the radioactive compound (s) could readily be separated from DHBS on a DEAE-cellulose column.

It was observed early in these studies that the amount of catechol (and iron) reacting material found in the medium was inversely related to the concentration of iron in the medium in which the organism was grown. This same observation has been reported in a variety of organisms which produce phenols

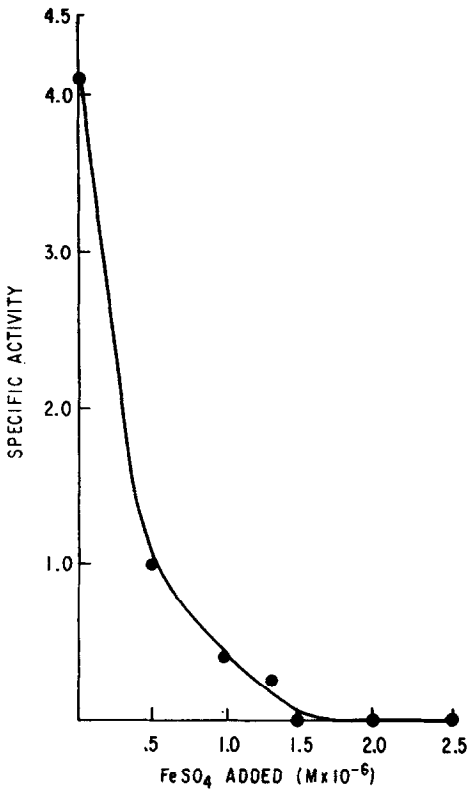


Figure 3 - Effect of iron concentration on the specific activity of the enzyme which synthesizes 2,3 dihydroxybenzoylserine. The organism was grown for 16 hours at 37° in a New Brunswick gyrating shaker in 2 liter flasks containing 500 ml of the medium described in the text (which contained about 1.5×10^{-6}M endogenous iron). Additional iron was added as indicated. The cells were harvested by centrifugation, washed with 5 ml of 0.1 M Tris pH 7.4, recentrifuged and then suspended in 3 volumes (grams wet weight/ml) of 0.1 M Tris pH 7.4. The cells were then disrupted by sonication for 10 seconds using a Branson sonifier and centrifuged at 15,000 x g for 5 minutes. The supernatant solution was dialyzed for 16 hours against 0.01 M Tris pH 7.4 and used for these experiments. The incubation conditions and assay are described in the text except that 1.5 to 1.7 mg of protein were used in these experiments.

and catechols, such as 2,3-dihydroxybenzoylglycine (Ito and Nielands, 1958), 2,3-dihydroxybenzoic acid (Dyer et al, 1964) and salicylic acid (Ratledge and Winder, 1962). No data, however, is available which indicates that any relationship exists between the iron concentration in the medium and the enzyme system that may be involved in synthesizing the hydroxylated product. Figure 3 shows that when increasing amounts of iron (as $FeSO_4$), are added to the growth medium there is a sharp decline in the specific activity of the enzyme which catalyze the synthesis of DHBS from 2,3-dihydroxybenzoic acid and serine. When an active enzyme preparation was incubated, in vitro, with these low levels of iron, no significant inhibition of enzyme activity was observed.

Although 2,3-dihydroxybenzoic acid has been found in the culture medium from a number of organisms (Arcamone, et al., 1961; Terui, et al., 1961; Pittard, et al., 1961; and Dyer, et al., 1964), its occurrence as an aminoacyl conjugate has only been reported by Ito and Neilands (1958), who isolated 2,3-dihydroxybenzoyl glycine from the culture medium of Bacillus subtilus. The finding of a glycine derivative in these earlier studies in addition to the present studies in which the serine derivative has been isolated raises the possibility that other amino acid conjugates of 2,3 dihydroxybenzoic acid may be found elsewhere. Along these lines Pittard, et al. (1961) have described the isolation of an unidentified compound from Aerobacter aerogenes with properties suggesting the presence of o-dihydroxy, amino and carboxylic acid groupings and whose spectral characteristics are similar to those reported here.

Acknowledgements

We wish to thank Drs. Robert Colburn and Walter Lovenberg of the National Institutes of Health for performing the iron and amino acid analyses.

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