The Isolation and Identification of 2,3-Dihydroxybenzoic Acid and 2-*N*,6-*N*-Di-(2,3-dihydroxybenzoyl)-L-lysine Formed by Iron-Deficient *Azotobacter vinelandii**

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ABSTRACT: Two fluorescent phenolic compounds were isolated from iron-deficient cultures of *Azotobacter vinelandii*. One was identified as 2,3-dihydroxybenzoic acid, an infrequently encountered microbial metabolite not previously reported from *Azotobacter*. The second compound was shown to be the previously unknown 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine, with final proof of structure established by synthesis. The fluorescence properties of both compounds were examined. Formation of the lysine derivative was markedly enhanced by iron deficiency in cultures with either nitrate or N_2 as the nitrogen source. The formation of 2,3-dihydroxybenzoic acid was also stimulated by iron deficiency particularly in cultures in which nitrate was used as the nitrogen source.

The iron requirement for nitrogen fixation by *Azotobacter* (Esposito and Wilson, 1956) stimulated an interest at this laboratory in the metabolic processes of this organism that are controlled or altered by iron deficiency. Although the involvement of nonheme iron in nitrogen fixation was subsequently demonstrated (Bulen *et al.*, 1965; Bulen and LeComte, 1966) at the enzyme level, other metabolic alterations induced by iron deficiency are less well defined. For example, Bulen and LeComte (1962) reported the formation of an unusual yellow-green fluorescent peptide by *Azotobacter agilis* (*Azotobacter vinelandii*) cultured on iron-deficient media. During the peptide isolation they observed the presence of several other fluorescent compounds. Two fluorescent compounds, A and B, both phenols, were subsequently isolated from the culture medium. The isolation and constitution of these two compounds and the dependence of their formation upon iron deficiency are the subjects of this report.

Experimental Section and Results

Methods. The nuclear magnetic resonance spectra were taken on a Varian A-60 instrument and the results are reported in parts per million from tetramethylsilane as the internal standard. Infrared spectra were obtained from KCl disks on a Perkin-Elmer Model 21 spectrophotometer. Amino acid analysis was performed on a Phoenix automatic amino acid analyzer. Ultraviolet spectra were taken on a Bausch & Lomb 505 spectrophotometer. Optical rotatory dispersion curves were obtained with a Cary 60 instrument. Fluorescence spectra were recorded on an Aminco-Bowman spectrophotofluorometer. Titration curves were obtained with a Radiometer TTT1c titrator and SBR2c titrigraph. All evaporations were done at or near room temperature with a rotary vacuum evaporator. Elemental microanalyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Mich.

Paper chromatography (descending) was conducted on No. 1 Whatman paper using the following solvent systems: (1) 1-butanol–pyridine–water (1:1:1, v/v), (2) 1-butanol–pyridine–water (14:3:3, v/v), (3) benzene–acetic acid (20:5, v/v) saturated with water, (4) 1-butanol–acetic acid–water (12:3:5, v/v), (5) 1-butanol–ethanol–0.025 M sodium borate (1:1:1, v/v), paper previously impregnated with the borate solution and dried, and (6) ethanol–ammonium hydroxide–water (18:1:1, v/v). Paper electrophoresis was carried out in a Spinco Model R (Durrum type) apparatus. Phenols were detected on paper with ferric nitrate solution, coupling with diazosulfanilic acid, and by the Arnow reaction (Arnow, 1937) for *o*-dihydric phenols. A Burton ultraviolet lamp (366 mμ) was used to detect fluorescent spots.

Quantitative determination of the *o*-dihydric phenols on paper electrophoretograms utilized the Arnow reagent (10 g each of sodium nitrite and sodium molybdate dihydrate in 100 ml of water). Excised paper segments containing the desired compound were swirled with 1.0 ml of ethanol–water (1:1, v/v); 1.0 ml each of 0.5 N HCl and the Arnow reagent were then added and the solution was mixed. NaOH (0.5 ml of 2.0 N) was then added and the absorbance was measured at 515 (2,3-dihydroxybenzoic acid) or 505 mμ (lysine derivative). Amounts of the compounds present were determined from standard curves prepared by subjecting known quantities of both compounds to electrophoresis and color formation.

Bacterial Growth. A. vinelandii O was maintained in liquid culture as previously described (Bulen, 1961). Cells were cultured at 30° in 3 or 6 l. of medium under high aeration using 150 ml of a 16-hr culture as inoculum. For iron-deficient cultures the usual 3 ppm of

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iron was omitted and purified sucrose containing less than 5×10^{-5} % iron was used. When present, nitrate was added as the potassium salt to give 300 ppm of nitrogen and the inoculating cultures were previously adapted to nitrate for 24 hr.

Isolation and Purification of Compound A. The initial lot of material was isolated from a Dowex 50 column fraction obtained during purification of a yellow-green fluorescent peptide (Bulen and LeComte, 1962). The same material was later obtained by continuous ether extraction of the acidified culture medium. The crude solid, resulting from evaporation of the solvent, was purified by preparative chromatography on Whatman No. 3MM paper using solvent system 3. The blue fluorescent band (R_F *ca.* 0.5) was cut out, and the product eluted with acetone–water (1:1, v/v). Evaporation of the solvent and sublimation of the residue under vacuum (100°) gave colorless crystals of compound A. The yield of A by this procedure was 10–20 mg/l. when the bacteria were grown on iron-deficient nitrate medium for 24 hr. The second phenol, B(R_F *ca.* 0.1), was also observed on these chromatograms and subsequently isolated and identified.

Properties and Structure of Compound A. After vacuum drying overnight, A melted at 204–205°. Rapid heating was necessary because of sublimation. The phenolic nature of the compound was indicated by formation of a blue-violet adduct with iron(III) and an orange-brown color with diazosulfanilic acid. An *o*-dihydric phenol structure was demonstrated by a positive Arnow reaction (yellow in acid, red in base). The ultraviolet spectra of A had maxima at 315 and 246 mμ at pH 1, 306 and 238 mμ at pH 7, and 329 and 259 mμ at pH 13. The large bathochromic shift in alkaline solution is typical of phenols. A titration curve in 15% ethanol–water revealed a carboxyl group (pK_a = 3.2, equiv wt 154) and two phenol groups (end point barely distinguishable). A high-wavelength carbonyl stretch (6.0 μ) in the infrared region suggested that the carboxyl was *ortho* to a phenolic group (Bellamy, 1958), a concept supported by the low pK_a value. The simplest structure fitting the above data was 2,3-dihydroxybenzoic acid. The elemental microanalysis was consistent with this formulation. *Anal.* Calcd for $C_7H_6O_4$: C, 54.55; H, 3.92. Found: C, 54.58; H, 4.04. The infrared and ultraviolet spectra were superimposable on those of authentic 2,3-dihydroxybenzoic acid.[1] A was converted into 2,3-dimethoxybenzoic acid by methylation using the methyl iodide–silver oxide method (Walker *et al.*, 1962) followed by saponification. The product, after recrystallization from hexane–benzene, melted at 121–123° and gave the same infrared spectrum as 2,3-dimethoxybenzoic acid.

Isolation and Purification of Compound B. Cultures (6 l.; iron-deficient nitrate medium) were freed from cells by centrifugation and the medium was chilled and divided into 3-l. portions. NaCl (300 g) and concentrated HCl (12 ml) were added to each portion in a large separatory funnel from which the liquid could be withdrawn from the floating denatured protein. The liquid was extracted twice with ethyl acetate (1000 and 500 ml), the second volume being used in the first extraction of the next portion of medium. The denatured protein was then rinsed and the organic solvent fractions were combined. The extract was evaporated to dryness and the residue was dissolved in 75 ml of fresh ethyl acetate and washed twice with water. Acidic material was extracted into 0.1 M potassium phosphate (pH 7.0; 40, 40, and 20 ml), and the buffer solution was washed twice with ethyl acetate. After acidification with HCl, the aqueous solution was extracted twice with ethyl acetate (35-ml portions) and the organic layers were evaporated to give a brown gum. This was fractionated by the lead acetate technique for *o*-dihydric phenols (Rudkin and Nelson, 1947). The brown gum was dissolved in sufficient 0.1 M NH_4OH (*ca.* 60 ml) to give a solution of pH 8.5. Lead acetate (20 ml of 15%) was added and the precipitated lead salts were separated by centrifugation. The precipitate was stirred with portions of 5% acetic acid until only a small amount of very dark residue remained which was removed by filtration. The light yellow filtrate was brought to pH 8.5 with concentrated NH_4OH, and the precipitate was separated by centrifugation, washed with 60 ml of water, and suspended in 50 ml of water. After acidification (concentrated HCl) the crude product was again extracted into ethyl acetate (three 30-ml portions), and the combined organic layers were washed with water, and then evaporated. The residue (mainly 2,3-dihydroxybenzoic acid and compound B) was dried under vacuum, dissolved in 85 ml of benzene–ethyl acetate (4:1, v/v) equilibrated with 0.5 M aqueous formic acid, and chromatographed on a 4.8 × 2.5 cm column of silica gel. This column was prepared from 10 g of dried silica gel using 2.0 ml of 0.5 M formic acid to provide the immobile phase and was eluted with the benzene–ethyl acetate mixture. The 2,3-dihydroxybenzoic acid eluted in the first 150 ml and compound B eluted in the 300–480-ml fraction (detection by the Arnow test). The eluate containing B was evaporated and the residue was dried under vacuum (Drierite–KOH) for 24 hr to give a porous solid. This was dissolved in 5 ml of ethyl acetate and added slowly to 100 ml of cold benzene which precipitated B as an amorphous white solid (550 mg). The solid was dried under vacuum at 65° for 72 hr.

Properties of Compound B. Compound B melted at 82–87°, appeared amorphous upon microscopic examination, and could not be induced to crystallize from a variety of solvents. It was nearly insoluble in water, benzene, chloroform, and ether, but was quite soluble in alcohol, ethyl acetate, acetone, and trifluoroacetic acid. The product, even after the prolonged drying described, contained 3.0% benzene as shown by nuclear magnetic resonance spectroscopy (see below). Despite the broad melting range, B appeared to be homogeneous. The compound migrated as a single spot on paper chromatograms. The R_F values were 0.79, 0.60, 0.06, 0.86, and 0.51 in solvent systems 1–5, respectively. A single component was indicated by paper electrophoresis in 0.1 M sodium acetate buffers (pH 4.0–5.5).

[1] Aldrich Chemical Co. This material was recrystallized from water, sublimed, and dried under vacuum overnight.

Compound B exhibited a blue fluorescence under ultraviolet light and gave positive Arnow and iron(III) reactions, the colors being essentially the same as with 2,3-dihydroxybenzoic acid. An acidic reaction was given with bromocresol green. No reaction was given with ninhydrin or Ehrlich's aldehyde reagent.

The ultraviolet spectra of B (similar to those of 2,3-dihydroxybenzoic acid) had maxima at 310 and 246 mμ at pH 1, 311 and 246 mμ at pH 7, and 336 and 260 mμ (sh) at pH 13.

A titration curve for B (3.90 mg in 1.50 ml of 50% ethanol) showed a group with a pK_a of 4.8, probably carboxyl. After correction for the benzene impurity (see below) an equivalent weight of 423 was obtained. Only two phenolic (pK_a = *ca.* 9) groups appeared to be titrated in this solvent.

The nuclear magnetic resonance spectrum of B in trifluoroacetic acid showed the following peaks: 1.67–2.47 (6 H, broad multiplet), 3.80 (2 H, broad triplet), 5.03 (1 H, broad multiplet), 6.75–7.79 (7 H, complex multiplet), and 8.35 (1 H, broad doublet). A spike at 7.33 was also seen and appeared to be from residual benzene. The benzene content (3.0 wt %) was obtained by examining the spectrum in acetone which separated this peak from the complex aromatic grouping. The peak position corresponded exactly to that of benzene.

Methylation of Compound B. A methanol solution of B (64 mg) was treated with etheral diazomethane for 4 hr at 0° and the solvent evaporated. A colorless gum resulted which would not crystallize. The nuclear magnetic resonance spectrum ($CDCl_3$) showed five methyl groups (3.77, 3.87, 3.88, 3.90, and 3.97, all singlets), in addition to the protons described for the parent compound. Two amide protons were clearly evident at 8.08 (broad triplet) and 8.65 (broad doublet), along with six aromatic hydrogens (6.92–7.78, complex multiplet). Obviously in the spectrum of the parent compound one of the amide peaks was obscured by the aromatic protons in the 6.75–7.59 region. The triplet and doublet character of the amide proton peaks in the methylated derivative suggested that the nitrogens were attached to CH_2 and CH groups, respectively.

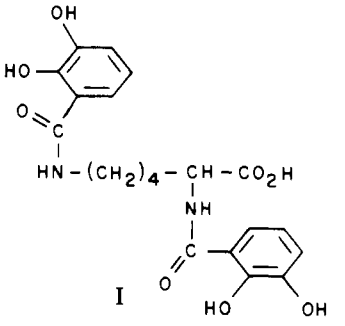
Hydrolysis of the methylated material with 0.1 N NaOH in methanol–water at 25° for 1 hr gave the free acid as a colorless gum which would not crystallize. The nuclear magnetic resonance spectrum ($CDCl_3$) of this showed only four methyl groups (3.77 methyl ester peak gone) and a new peak at 10.02 (1 H singlet, carboxylic acid). From this it was concluded that B contained one carboxyl and four phenol groups. In addition, the combined nuclear magnetic resonance data indicated two amide, six aromatic, and nine aliphatic protons for B.

Acid Hydrolysis of Compound B. The presence of amide groups in compound B was indicated by nuclear magnetic resonance. Compound B (9.01 mg) was hydrolyzed with 6 N HCl (3 ml) under N_2 at 105° for 23 hr. Examination of the reaction mixture by paper chromatography showed a blue fluorescent component, which gave the same color reactions as 2,3-dihydroxybenzoic acid, and a ninhydrin-positive component as the main products. The presence of some partially hydrolyzed material (*ca.* 20–25%) was also evident.[2] Both the phenolic and amino acid components showed identical chromatographic properties as 2,3-dihydroxybenzoic acid and lysine in solvents 1–4 and 6. The phenolic component was sublimed out of the crude mixture and gave an infrared spectrum identical with that of 2,3-dihydroxybenzoic acid. 2-*N*,6-*N*-Di-(2,3-dihydroxybenzoyl)-L-lysine (I) (mol wt 418) was consistent with the titration and nuclear magnetic resonance data for compound B. To test this, a quantitative acid hydrolysis of B was carried out. Compound B (10.675 mg) was hydrolyzed as above but for 57 hr to ensure complete reaction, and the lysine content was determined on the amino acid analyzer. The amount of 2,3-dihydroxybenzoic acid was determined by the Arnow procedure after paper electrophoresis in 0.1 N sodium acetate (pH 4.5) for 2 hr at 400 V. The results, shown in Table I, were consistent with the proposed structure.

TABLE I: Yields of 2,3-Dihydroxybenzoic Acid and Lysine from Hydrolysis of Compound B.

	Found[a]	Calcd[a]	% Theory
2,3-Dihydroxybenzoic acid	47.6	49.6	96.0
Lysine	24.1	24.8	97.2

[a] In micromoles.



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Synthesis of 2-N,6-N-Di-(2,3-dihydroxybenzoyl)-L-lysine. Final proof that B was indeed 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine came from synthesis of this previously unknown compound. A solution of dicyclohexylcarbodiimide (3 mmoles) in 4 ml of acetonitrile was added to a solution of 2,3-diacetoxybenzoic acid[3] (6 mmoles) and pyridine (6 mmoles) in 26 ml of the same solvent. Anhydride formation was rapid and in 10 min the solid dicyclohexylurea (yield 94%) was filtered and rinsed with a little solvent. Finely ground L-lysine methyl ester dihydrochloride[4]

[2] A minor spot which gave positive ninhydrin and *o*-diphenol tests was seen. This material, probably 6-*N*-(2,3-dihydroxybenzoyl)-L-lysine, could be eluted from the chromatogram and further hydrolyzed to give more 2,3-dihydroxybenzoic acid and lysine.

[3] Prepared from 2,3-dihydroxybenzoic acid by the pyridine–acetic anhydride method (Simokoriyama, 1941).

[4] Pierce Chemical Co., Rockford, Ill.

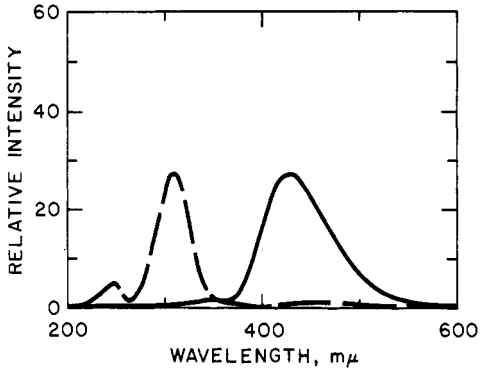

FIGURE 1: Activation (---) and fluorescence (—) spectra of 2,3-dihydroxybenzoic acid (concentration 7.72×10^{-5} M) in pH 7.0 phosphate buffer.

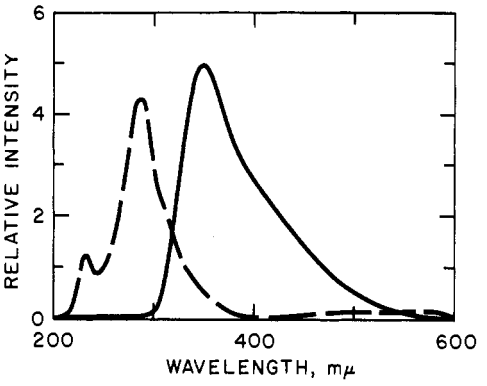

FIGURE 2: Activation (---) and fluorescence (—) spectra of di-(2,3-dihydroxybenzoyl)-L-lysine (concentration 3.86×10^{-5} M) in pH 7.0 phosphate buffer.

(1.5 mmoles) and triethylamine (6 mmoles) were added to the filtrate with stirring. Little solid remained after several minutes. After 2 hr the reaction mixture was evaporated to dryness and the residue was partitioned between ethyl acetate (35 ml) and 1 N HCl (7 ml). The organic layer was washed with water and the solvent was evaporated. The resulting colorless gum was shaken with 1 N NaOH (35 ml) under N_2 for 3.5 hr to remove the ester and acetyl groups. The hydrolysis mixture was filtered into concentrated HCl (4 ml) and extracted twice with ethyl acetate (30-ml portions), and the combined extracts were washed with water. The solvent was evaporated and the residue was chromatographed on silica gel in the same manner described for purification of B, except that the solvent system was chloroform–ethyl acetate (3:1, v/v) saturated with 0.5 M aqueous formic acid. The eluate between 150 and 300 ml contained the desired product and was evaporated to dryness. The residue was dried under vacuum overnight (KOH–Drierite) yielding a porous solid which was crushed and dried for an additional 72 hr at 65°. This material (mp 81–86°), like compound B, would not crystallize. The synthetic compound was indistinguishable from B by ultraviolet and nuclear magnetic resonance spectroscopy, paper chromatography, paper electrophoresis, and titration. Both compounds gave rotatory dispersion curves with a negative Cotton effect (trough, 272 mμ; peak, 250 mμ) when examined in 0.1 N HCl. The curves were superimposable, thus establishing the L configuration in the natural compound. Nuclear magnetic resonance showed the presence of residual ethyl acetate (2.0 wt %). *Anal.* Calcd for $C_{20}H_{22}N_2O_8$: C, 57.41; H, 5.30; N, 6.70. Found: C, 57.45; H, 5.25; N, 6.62.

Fluorescence Spectra of 2,3-Dihydroxybenzoic Acid and 2-N,6-N-Di-(2,3-dihydroxybenzoyl)-L-lysine. The fluorescence spectra of both 2,3-dihydroxybenzoic acid and 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine were recorded (Figures 1 and 2) because such data might be useful in the future detection of these compounds by this technique. 2,3-Dihydroxybenzoic acid exhibited a maximum at 430 mμ (310-mμ activation) and 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine a maximum at 350 mμ (290-mμ activation). The intensity of fluorescence for 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine was much less than that of 2,3-dihydroxybenzoic acid.

Effect of Iron on the Formation of 2,3-Dihydroxybenzoic Acid and Its Lysine Derivative by A. vinelandii. The formation of 2,3-dihydroxybenzoic acid and its lysine derivative was followed as a function of time in 3-l. cultures with normal and deficient levels of iron using either N_2 or nitrate as the nitrogen source. Portions of the cultures (50 ml) were withdrawn periodically and the cells removed by centrifugation. The medium was acidified with concentrated HCl (0.2 ml) and extracted with three 20-ml portions of ethyl acetate. The organic extract was dried with sodium sulfate and the solvent was evaporated. The residue was dissolved in acetone (2.0 ml) and stored at −20° until analyzed. An aliquot of the acetone solution was subjected to paper electrophoresis in 0.5 N acetic acid for 4 hr at 450 V. The strip was air dried and the fluorescent segments containing the 2,3-dihydroxybenzoic acid and the lysine derivative were cut out and analyzed by the Arnow procedure. The results are given in Table II. The formation of 2,3-dihydroxybenzoic acid was relatively low in all media, but a significant increase in its formation on nitrate medium was induced by iron deficiency. Iron deficiency was necessary for the formation of 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine in either medium. Accumulated levels are no doubt influenced by air oxidation accompanying the vigorous aeration of the cultures.

Discussion

The formation of 2,3-dihydroxybenzoic acid by *Azotobacter* has not been reported previously. Its formation from normal carbohydrate sources by *Streptomyces griseus* (Dyer *et al.*, 1964), by washed cell suspensions of tryptophan and tyrosine auxotrophs of *Aerobacter aerogenes* (Pittard *et al.*, 1961, 1962), and by *Escherichia coli* (Ratledge, 1964) has been observed. *A. aerogenes*, both the wild type and a mutant blocked after the synthesis of anthranilic acid, can also form

TABLE II: Effect of Iron on the Formation of 2,3-Dihydroxybenzoic Acid and 2-*N*,6-*N*-Di-(2,3-dihydroxybenzoyl)-L-lysine.[a]

Nitrogen Source		Yield, 2,3-Dihydroxybenzoic Acid (μmoles/50 ml)					
	Time (hr):	8	12	16	20	24	28
N_2	+Fe	0.0	0.0	0.6	0.7	0.7	0.6
	−Fe	0.0	0.1	1.0	1.3	0.9	0.9
Nitrate	+Fe	0.1	0.3	0.4	0.1	0.1	0.1
	−Fe	0.1	0.8	1.6	2.2	2.5	2.9
		Yield, 2-*N*,6-*N*-Di-(2,3-dihydroxybenzoyl)-L-lysine (μmoles/50 ml)					
N_2	+Fe	0.0	0.1	0.1	0.0	0.0	0.0
	−Fe	1.9	4.7	5.8	6.6	7.0	6.6
Nitrate	+Fe	0.0	0.1	0.1	0.1	0.1	0.1
	−Fe	0.4	2.6	5.1	6.6	7.7	5.8

[a] Logarithmic growth ceases between 8 and 12 hr. Growth (log optical density) of Fe-deficient and nitrate cultures after 28 hr expressed as per cent of N_2-grown cells supplied with 3 ppm of Fe was: N_2 (−Fe), 82%; NO_3^- (+Fe), 96%; NO_3^- (−Fe), 86%. N_2 and NO_3^- cultures were not grown concurrently.

2,3-dihydroxybenzoic acid from quinic acid (Ratledge, 1964, 1967).

Claviceps paspali form 2,3-dihydroxybenzoic acid from tryptophan (Arcamone *et al.*, 1961; Tyler *et al.*, 1964) and *Aspergillus niger* form it from tryptophan (Subbarao *et al.*, 1967) or anthranilate (Terui *et al.*, 1961) with 3-hydroxyanthranilate as an intermediate.

Earlier work of Pittard *et al.* (1962) suggested that 2,3-dihydroxybenzoic acid and other *o*-dihydric phenols arose from the breakdown of intermediates in aromatic biosynthesis and recent work has demonstrated its formation from chorismate by cell-free extracts of *E. coli* and *A. aerogenes* (Young *et al.*, 1967a,b) with 2,3-dihydro-2,3-dihydroxybenzoic acid being formed as an intermediate.

Conjugate forms of 2,3-dihydroxybenzoic acid with lysine (2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine, for example) have not been observed previously. The glycine and serine conjugates are formed by *Bacillus subtilis* (Ito and Neilands, 1958) and by a methionine-B_{12} auxotroph (K_{12}) of *E. coli* (Brot *et al.*, 1966), respectively.

Conditions of reduced iron availability enhance the formation of 2,3-dihydroxybenzoic acid and markedly stimulate the formation of the lysine derivative by *A. vinelandii*. Apparently iron deficiency does not reduce N_2 fixation sufficiently to limit the amount of lysine available for 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine under these conditions since N_2-grown cells produce about the same levels of the conjugate as cells supplied with nitrate. The glycine and serine conjugates of 2,3-dihydroxybenzoic acid previously mentioned were formed by *B. subtilis* and *E. coli* K_{12} in iron-deficient media. Also *S. griseus* (Dyer *et al.*, 1964) and *A. aerogenes* (Ratledge, 1967) formed 2,3-dihydroxybenzoic acid in iron-deficient media. A single relationship between iron deficiency and the formation of 2,3-dihydroxybenzoic acid or its conjugates is not evident from the available information. Iron in the growth medium of *E. coli* K_{12} reduced the specific activity of cell extracts catalyzing the synthesis of 2,3-dihydroxybenzoylserine from 2,3-dihydroxybenzoic acid and serine (Brot *et al.*, 1966). Iron in the growth medium of *A. aerogenes* 62-I markedly reduced the ability of cell extracts to convert chorismate into 2,3-dihydroxybenzoic acid, but so did cobalt, citrate, and salicylic acid (Young *et al.*, 1967a).

The fluorescent properties of 2,3-dihydroxybenzoic acid and 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine should be useful in detecting the presence of these compounds. The wavelengths of maximum intensity, 430 and 350 mμ, respectively, are sufficiently separated to permit detection of the lysine derivative even in the presence of 2,3-dihydroxybenzoic acid when the latter is not present in sufficient concentration to mask the lysine derivative. Fluorescent detection could be useful in examining body fluids or tissue extracts for 2,3-dihydroxybenzoic acid or 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine in suspected cases of metabolic abnormalities, perhaps including those related to the function of iron-containing enzymes.

The method of synthesis of 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine by *A. vinelandii* remains to be demonstrated but should prove informative. The compound most nearly related to its structure is ornithuric acid (*N*,*N*-dibenzoylornithine) which is synthesized by chicken kidney enzymes *via* the formation of an acyl coenzyme A intermediate (Marshall and Koeppe, 1964).

A partly purified and dialyzed enzyme preparation from the *E. coli* K_{12} mutant will catalyze the formation of 2,3-dihydroxybenzoylserine in a reaction specific for 2,3-dihydroxybenzoic acid and ATP but apparently not requiring coenzyme A (Brot *et al.*, 1966). This suggests different methods of synthesis by the fowl and bacterial mutant. Amide-bond formation in 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine synthesis would be expected to involve an attack by lysine on a carboxyl-activated 2,3-dihydroxybenzoic acid molecule. An examination of this activation whether *via* acyl coenzyme A formation, an enzyme bound adenylate (with release of pyrophosphate), or a glutamine or glutathione synthetase type activation (with release of phosphate) will contribute to our knowledge of the biochemistry of amide- or small-peptide-bond formations.

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