[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA]

Products of "Low-iron Fermentation" with *Bacillus subtilis:* Isolation, Characterization and Synthesis of 2,3-Dihydroxybenzoylglycine[1,2]

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RECEIVED MARCH 31, 1958

The phenolic acid produced by *Bacillus subtilis* NRRL B-1471 in iron deficiency has been crystallized and identified by synthesis as 2,3-dihydroxybenzoylglycine.

Studies on the utilization of iron by plants, animals and microörganisms have permitted the development of a unified concept of iron metabolism which features the classification of living species as either *autosequesteric* or *anautosequesteric*.[3] In the former category iron sequestering agents can be synthesized from simple precursor molecules in the nutrient medium, while in the latter category the pre-formed organic sequestering agent must be added to the diet. Since the autosequesteric species are able to combat iron deficiency through the synthesis of abnormally large amounts of sequestering agents, such forms of life enjoy a distinct advantage in the competition for the essential, insoluble mineral elements (of which ferric ion is the prime example).

In principle it should be possible to use almost any type of living cell as an experimental subject with which to study the path of iron at the enzyme level. However, as a consequence of the plasticity of their metabolism, microörganisms exhibit certain distinct advantages for this type of investigation.

Garibaldi[4,5] has shown that *Bacillus subtilis*, when cultured under conditions of iron deprivation, excretes a ferric-ion-binding agent into the medium. Under similar conditions of growth, this clearly autosequesteric organism also produces considerable amounts of coproporphyrin III[6] and succinic acid.[7] However, from the point of view of the present investigation, the unknown ferric ion binding agent seemed to be of particular interest.

Unlike the ferrichrome compounds produced by *Ustilago sphaerogena*,[8,9] the material formed by *Bacillus subtilis* is soluble in both polar and nonpolar solvents. For this reason, as well as the fact that the compound was stable to laboratory manipulation, it appeared possible that the material might be of relatively low molecular weight and thus easily characterized. It was early recognized that the compound exhibited all of the reactions and characteristics of an *o*-dihydric phenolic acid, *i.e.*, strongly positive reactions were obtained with ferric ion and Hoepfner's reagents and the substance partitioned in favor of organic solvents at low *p*H and in favor of aqueous solvents at neutral or alkaline *p*H. Indeed it was thought for a time that the compound from *Bacillus subtilis* might be identified through the simple expedient of cochromatography of the culture supernatant with known phenolic acids.

When this procedure failed to identify the compound with any member of a large group of naturally-occurring phenolic acids, it was concluded that we were dealing with a hitherto undescribed product which would have to be isolated and characterized by more conventional methods.

For isolation purposes, *Bacillus subtilis* was cultured under aerobic conditions in one-liter Pyrex erlenmeyer flasks. At the end of the growth period the cells were separated by centrifugation and the coproporphyrin III precipitated at *p*H 3.5. The unknown phenolic acid was crystallized from the supernatant solution by a fractionation procedure based mainly on the distinctive solubility characteristics of the compound.

The purity of the recrystallized preparation was confirmed by paper chromatography and by melting point determination and the substance was then examined in the difunctional *p*H titrator.[10] Dilute aqueous solutions were acidic, *i.e.*, the *p*H was less than 2. Ionizations were detected with pK_a' values of 3.5 and 7.7, respectively. Both of these dissociations were weakened in the presence of an organic solvent and the higher of the two disappeared concomitant with the rapid browning of the solution which occurred at alkaline *p*H. From these data it was possible to conclude that both ionizations could be attributed to acidic functions, the higher one of the two being a greatly strengthened phenolate dissociation. Since the solid infrared spectrum suggested a carbonyl group in conjugation with the aromatic ring and, recalling the acidic nature of the compound, the presence of a nuclear carboxyl or substituted carboxyl group was suspected. However, the molecular weight of approximately 210 provided by the difunctional recording titrator was considerably more than that which could be derived from a simple dihydric-phenolic acid. Since a qualitative test for the elements[11] indicated the presence of nitrogen and since the molecular weight difference could be made by a residue of glycine, a small quantity of the natural product was hydrolyzed in constant boiling HCl and the split fragments subjected to paper chromatography. When the chromatograms were sprayed

(1) Abstracted from the doctoral dissertation of Takeru Ito, University of California, Berkeley, 1958, and presented in part at the 133rd meeting of the American Chemical Society, San Francisco, April, 1958.

(2) This research was sustained by grants from the Office of Naval Research and from the U. S. Public Health Service.

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(7) J. B. Neilands, unpublished experiments.

(8) J. B. Neilands, THIS JOURNAL, **74**, 4846 (1952).

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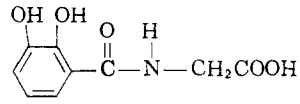
with phenol- and amino acid-detecting reagents, glycine and 2,3-dihydroxybenzoic acid were found. The isolated product was thus tentatively identified as 2,3-dihydroxybenzoylglycine (I).



I

Inspection of the literature revealed the interesting fact that, although well known in the chemical literature, 2,3-dihydroxybenzoic acid has only seldom been encountered as a natural product. Bray, *et al.*,[12] have found chromatographic evidence for the occurrence of 2,3-dihydroxybenzoic acid in a bound form in rabbit urine. The substance has been established definitely as a constituent of the tree *Populus balsamifera*.[13] The *o*-dihydric phenolic structure is rare even among microbial products; here the principal representatives apparently are citromycetin, flavipin, dihydrofuscin and viridicatin.[14] Since the glycine conjugate of 2,3-dihydroxybenzoic acid appeared to be unknown in the chemical as well as the biochemical literature, it was concluded that positive identification of I could only be achieved by synthesis.

The 2,3-dihydroxybenzoic acid, which is not available commercially, was prepared by two independent methods. The hydroxyl groups were protected by reaction with ethyl chlorocarbonate and the carboxyl function was converted to the acid chloride. When the resultant 2,3-dicarboethoxybenzoyl chloride reacted with ethyl glycinate in the manner used for the successful synthesis of the glycine conjugate of protocatechuic acid,[15] extensive decomposition of the phenol partner ensued and a product could not be obtained. Finally, compound I was synthesized in satisfactory yield by the dicyclohexylcarbodiimide method of Sheehan and Hess.[16]

The identity of natural and synthetic I was established by infrared analysis, by paper chromatography with various solvent systems and by mixed-melting point determination.

The biosynthesis and the further metabolism of I in *B. subtilis* will be the subject of future investigation in this Laboratory.

Experimental[16a]

Growth of *Bacillus subtilis*.—*Bacillus subtilis* NRRL B-1471 was cultured in the manner previously described[9] for the growth of *U. sphaerogena*. In order to obtain good growth and subsequent good yield of the iron-chelating compound, iron-free yeast extract may be added to the normal medium at a level of 0.1%.

Isolation of 2,3-Dihydroxybenzoylglycine.—After 3 or 4 days' growth or when the pink coloration due to the accumulation of coproporphyrin III in the medium became maximum, the cells were centrifuged out. The supernatant was adjusted to *p*H 3.5 and the porphyrin separated by centrifugation. The solution was then concentrated under reduced pressure to one-tenth of the original volume. The concentrate was acidified with dilute HCl and extracted several times with ethyl acetate. The solvent layer was evaporated as much as possible and the residue taken into a large volume of water. Any insoluble material was removed by decantation or filtration. The filtrate was then fractionated with lead acetate according to the method of Rudkin and Nelson.[17] That is, the filtrate was adjusted to *p*H 8 to 9, and 20% lead acetate solution was added until no further precipitate appeared. The precipitate was collected and treated with several large volumes of 5% acetic acid until the extracts yielded a negative ferric chloride reaction. The extracts were combined and adjusted to *p*H 8 to 9 in order to re-precipitate the lead salt. The latter was decomposed with a relatively large volume of very dilute sulfuric acid and the white precipitate of lead sulfate removed by filtration. The filtrate was made 2 *M* in KH_2PO_4 and extracted with ethyl acetate two or three times. The extract was concentrated and two volumes of petroleum ether were added in order to precipitate impurities. On the addition of several further volumes of petroleum ether an amorphous, highly purified preparation of I was obtained. This was dissolved in the least amount of dilute ammonium hydroxide and dilute HCl then was added slowly until a definitely acidic *p*H was obtained. The colorless needles were centrifuged off and dried at 100° under reduced pressure. The yield was of the order of 50 mg. per l. of medium.

Chemical and Physical Properties of I.—The crystalline compound obtained as above melted at 210–211° with some reddish coloration. Qualitative tests[11] were positive for nitrogen but negative for sulfur, chlorine and phosphorus.

Anal. Calcd. for $C_9H_9O_5N$: C, 51.16; H, 4.30; N, 6.64. Found: C, 51.68; H, 4.49; N, 6.20.

The compound is readily soluble in methanol, ethanol, ethyl acetate and hot water. It is sparingly soluble in ether and in cold water and is insoluble in benzene, chloroform and carbon tetrachloride.

In the presence of ferric ion, I is purple at neutral *p*H, green at acidic *p*H, colorless in strongly acidic solution, reddish-purple at slightly basic *p*H and red in alkaline solution. I gives a positive Hoepfner's nitrous acid reaction,[18] indicative of the *o*-dihydric phenolic structure.

The infrared spectrum, taken in 2% KBr pellet, is indistinguishable from that of the synthetic compound. The ultraviolet spectrum in methanol shows two peaks, as does 2,3-dihydroxybenzoic acid. However, in the case of I, the larger band is intensified and shifted some 5 mμ to longer wave lengths

	2,3-Dihydroxybenzoylglycine (I)		2,3-Dihydroxybenzoic acid	
Max. (λ, mμ)	314	250	314	245
ϵ_{mM}	3.0	7.5	3.0	6.1
Min. (λ, mμ)	277	240	271	234

Electrometric titration of the aqueous solution at 25° gave apparent pK_a values of 3.5 and 7.7 and a molecular weight (based on pK_{a_1}) of 210, ±2%.

For the hydrolysis of I, approximately one milligram of the compound was dissolved in 0.1 ml. of 6 *N* HCl in a Pyrex glass tube and sealed under reduced pressure. The tube was autoclaved overnight at 15 lb. pressure. For chromatographic analyses, the following solvent systems were employed: (1) *n*-butanol, 4; acetic acid, 1; water, 5; (2) benzene, 2; acetic acid, 2; water, 1; (3) methanol, 20; water, 5; pyridine, 1; (4) *t*-butyl alcohol, 10; methyl ethyl ketone, 10; water, 5; diethylamine, 1. The paper chromatograms were analyzed by (1) ultraviolet illumination, (2) spraying with 1% aqueous ferric chloride, (3) spraying with acidic sodium nitrite followed (after drying) with 1 *N* NaOH, (4) spraying with ninhydrin reagent. The R_f values are given.

For I only a single discrete spot was detected in each of the four solvent systems. Compound I gave a strong yellow fluorescence under ultraviolet light, a blue color with ferric chloride spray and a red color on a green background with the nitrous acid reagents.

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(15) T. Kametaka, *Ber.*, **42**, 1482 (1909).

(16) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

(16a) Melting points uncorrected. Microanalyses by Chemistry Department, University of California, Berkeley. Ultraviolet and infrared spectra were measured with the Process and Instruments and the Baird Associates spectrophotometers, respectively.

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Solvent system	2,3-Dihydroxybenzoylglycine (I)	Hydrolyzed I: Glycine	Hydrolyzed I: 2,3-Dihydroxybenzoic acid
(1)	0.80	0.18	0.90
(2)	.29	.32	.39
(3)	.67	.42	.70
(4)	.39	.18	.67

The hydrolyzed sample of I gave two discrete spots in each of the four solvent systems. The two spots were identified as glycine and 2,3-dihydroxybenzoic acid by cochromatography with authentic specimens. Glycine was identified with ninhydrin spray and the 2,3-dihydroxybenzoic acid gave under ultraviolet light a brilliant blue fluorescence which could be seen even in the lighted room. The latter compound also gave the ferric chloride and nitrous acid reactions.

Synthesis of 2,3-Dihydroxybenzoic Acid.—A good synthetic method[19] is available for this compound. However, because of their technical simplicity, the methods of Horri, *et al.*,[20] and Kawai,[21] were adopted.

The ultraviolet absorption characteristics of 2,3-dihydroxybenzoic acid were reported above. Electrometric titration in water at 25° provided apparent pK_a values of 2.8 and 10.1 and a molecular weight of 152, ±2%.

Synthesis of I.—The procedure of Sheehan and Hess[16] was followed: 1.0 gram of 2,3-dihydroxybenzoic acid and 0.7 to 0.8 g. of glycine ethyl ester were dissolved in 5 to 6 ml. of tetrahydrofuran. One and a half gram of dicyclohexylcarbodiimide in the minimum amount (3 to 5 ml.) of tetrahydrofuran was added. The reaction mixture was let stand overnight at room temperature in an atmosphere of N_2. A small amount of acetic acid was added to decompose the excess condensing reagent. The insoluble urea was removed and the solvent evaporated. The residue was dissolved in ethyl acetate and washed with a small amount of dilute HCl. Ethyl acetate was then evaporated to dryness.

The residue, mainly 2,3-dihydroxybenzoylglycine ethyl ester, was dissolved in 20 to 30 ml. of 1 *N* NaOH. The solution was stirred at room temperature for 4 hr. in an atmosphere of N_2 to prevent the decomposition of the product in the basic solution. The insoluble material was filtered out and the filtrate was acidified with 4 to 6 ml. of dilute H_2SO_4 to obtain a strongly acidic *p*H. The solution was extracted with ethyl acetate 2 or 3 times and the solvent evaporated to dryness. The residue was dissolved with the aid of dilute NH_4OH, and then (in the ice-bath) dilute HCl was added slowly to precipitate the product. The precipitate, weighing 500 mg., was recrystallized several times from the minimum amount of hot water and dried at 100° under vacuum (m.p. 210–211° with reddish coloration). A mixed melting point with the isolated product gave no depression.

Anal. Calcd. for $C_9H_9O_5N$: C, 51.16; H, 4.30; N, 6.64. Found: C, 51.09; H, 4.38; N, 6.81.

Synthetic I proved indistinguishable from natural I when examined in the following ways: paper chromatography in the various solvent systems described above, color reactions with aqueous ferric chloride, the reaction with nitrous acid, solubility characteristics, ultraviolet spectrum, infrared spectrum, apparent pK_a values and molecular weight (by titration).

Acknowledgments.—The authors are indebted to Albert N. Booth, F. H. Carpenter and James Cason for gifts of chemicals and to Keith Freeman for interpretation of infrared data.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF MICHIGAN]

The Thermal Breakdown of Diaryltetrazoles

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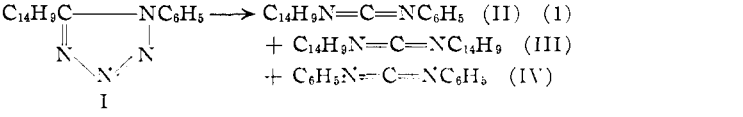
RECEIVED APRIL 5, 1958

The thermal decomposition of diaryltetrazoles containing phenyl, α- and β-naphthyl and 1-phenanthryl groups has been studied. The products are diarylcarbodiimides, 2-aryl-arimidazoles or both. Unsymmetrical diarylcarbodiimides have been found to undergo a redistribution reaction when strongly heated, giving rise to the two related symmetrical carbodiimides. The von Braun–Rudolf tetrazole synthesis has been found beset with hitherto unsuspected side-reactions that lead to ureas (*via* carbodiimides) and anilinotetrazoles. These can apparently be avoided by using aqueous sodium azide in place of anhydrous hydrogen azide solutions.

The tetrazole ring system is a relatively stable one in the absence of heat-sensitive substituents. Although there have been casual references to decomposition of tetrazoles when strongly heated, surprisingly little has been reported regarding the products. 5-Diazotetrazole was reported in 1893[2] to give cyanogen and nitrogen, and in 1897, 5-phenyl- and 5-*p*-anisyltetrazole were reported to decompose on heating to give the corresponding 3,5-diaryl-1,2,4-triazoles and diaryl-*sym*-tetrazines.[3] No further work was reported until 1953, when pyridotetrazole and its 6- and 8-nitro derivatives were studied; only the last gave an identifiable product, a pyridofuroxan.[4]

A chance observation by one of us[5] that 5-(1-phenanthryl)-1-phenyltetrazole (I) decomposed when heated above its melting point to a gas and crystalline products, one of which is N-phenyl-N-1-phenanthrylcarbodiimide (II), led us to investigate this neglected field. We have since found that the unsymmetrical carbodiimide is the primary product of decomposition, resulting from a carbon-to-nitrogen rearrangement accompanying loss of nitrogen, and that the other products are the two corresponding symmetrical carbodiimides III and IV, which arise from a subsequent redistribution reaction. These previously unreported reactions



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