Carboxy-migration facilitated by Bacterial Hydroxylation of 4-Hydroxybenzoate

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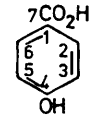
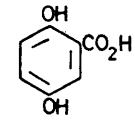
Summary Gentisic acid, accumulated from 4-hydroxybenzoic acid by bipyridyl-inhibited cells of *Bacillus stearothermophilus*, is formed by hydroxylation effecting intramolecular *ortho* migration of a carboxy-group with no observed exchange with hydrogen carbonate.

GENTISIC ACID, (2,5-dihydroxybenzoic acid, II) is a widely distributed product of plant[1] and fungal[2] biosynthesis as well as an intermediate formed by bacterial degradation of such compounds as salicylic acid,[3] 3-hydroxybenzoic acid,[4] β-naphthol,[5] and *m*-cresol.[6] Its recent identification as a product formed by strains of *Bacillus* utilizing 4-hydroxybenzoate (I)[7] and 3-(4-hydroxyphenyl)propionate[8] has led to the suggestion that a carboxy-migration occurs as a consequence of the reaction introducing the second hydroxy-group. Evidence for a carboxy-rearrangement has now been obtained using a strain of *Bacillus stearothermophilus*



(I)



(II)

isolated from decomposing grass cuttings. This organism grew aerobically with 4-hydroxybenzoate as principal carbon source at 65 °C; washed cells rapidly oxidized both 4-hydroxybenzoate and gentisate. Other possible intermediates such as catechol and protocatechuic acid[9] were not attacked. In the presence of αα′-bipyridyl (10^{-3} M) cells oxidized 4-hydroxybenzoate to give *ca.* 35% conversion into gentisic acid recovered by ether extraction, preparative t.l.c., and crystallization. Complete identity

with authentic gentisic acid was established by m.p. (205 °C decomp.), i.r., and mass spectrometry of the acid and its trimethylsilyl derivative.† Compounds such as 3-hydroxybenzoate were not detected.

Repeated attempts to obtain active cell extracts capable of effecting gentisate formation have been uniformly unsuccessful although the presence of high induced levels of enzymes of gentisate catabolism were readily demonstrated.[10] Accordingly the formation of gentisate was investigated by determining the isotope content of gentisate formed by intact cells from 4-hydroxybenzoate specifically labelled with ^{2}H or ^{13}C. After [2-^{2}H]-4-hydroxybenzoate‡ (96% enrichment) and [3-^{2}H]-4-hydroxybenzoate§ (96% enrichment) were separately oxidized by bipyridyl-inhibited cells, accumulated gentisic acid was recovered by extraction and t.l.c. for analysis of its deuterium content by g.l.c.–mass spectrometry as its trimethylsilyl derivative.† There was a 50% loss of the label from C-2 but negligible loss from C-3. These results are consistent with migration of the carboxy not the hydroxy group. Similar experiments with [7-^{13}C]-4-hydroxybenzoate¶ (90% enrichment) showed that carboxy-migration proceeds without loss or dilution of isotope even when incubations were carried out in the presence of a five-fold molar excess of unlabelled sodium hydrogen carbonate. By simultaneous incubation of equimolar [7-^{13}C]-4-hydroxybenzoate and [3-^{2}H]-4-hydroxybenzoate no significant intermolecular transfer occurred as revealed by the complete absence of double label in gentisic acid. These findings are in accord with a reaction mechanism involving an intramolecular *ortho* migration of a carboxy-group as a consequence of C-1 hydroxylation. NIH shift migrations of hydrogen, chlorine, bromine, and methyl substituents are generally attributed to rearrangement of arene oxide intermediates. When an ionizable group is present *ortho* or *para* to the site of hydroxylation, however, substituent loss rather than migration is the rule.[11] Such is also the case when hydroxylations of salicylate, *p*-anisate, and vanillate occur at the carbon carrying the carboxy-group.[12] The hydroxylation effecting conversion of 4-hydroxyphenylacetic acid into homogentisate[13] and the example reported here both provide exceptions to this generalization questioning the participation of arene oxide intermediates in these cases. Investigation of the mechanism by which oxygen introduction facilitates carboxy-migration must await isolation of the enzyme system involved. Reactions such as this, and the related formation of 4-methoxygentisate from vanillic and ferulic acids,[14] present alternative degradative routes for the bacterial catabolism of 4-hydroxy-substituted acids previously recognized as undergoing degradation *via* catechol and protocatechuate.[9]

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† G.l.c. of trimethylsilyl derivatives was carried out using an LKB 9000A gas chromatograph–mass spectrometer; mass spectra were obtained at 70 eV and isotopic enrichment determined after correcting for the natural abundance of ^{13}C, ^{29}Si, and ^{30}Si.

‡ Synthesized by deuteriation of 2-bromo-4-nitrotoluene, acetylation to give [2-^{2}H]-*N*-acetyl-[^{2}H]aminotoluene, permanganate oxidation, and hydrolysis followed by diazotization and boiling.

§ Synthesized by deuteriation of 3-bromo-4-hydroxybenzoate in D_2O–tetrahydrofuran–triethylamine with palladium on carbon.

¶ Synthesized by oxidation of 90% [7-^{13}C]benzoate (BioRad Laboratories, California) with ferrous sulphate, ethylenediaminetetraacetic acid, and ascorbic acid (B. B. Brodie, J. Axelrod, P. A. Shore, and S. Udenfriend, *J. Biol. Chem.*, 1954, **208**, 741) and separated from the accompanying salicylic 3-hydroxybenzoic, and gentisic acids by preparative t.l.c. and paper chromatography.

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