Chemical and Enzymatic Ketonization of 2-Hydroxymuconate, a Conjugated Enol

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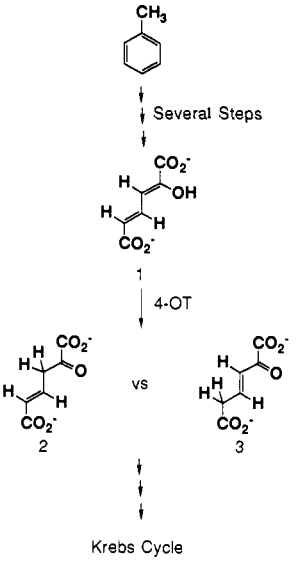
Abstract: The conjugated enol, 2-hydroxymuconate (**1**), is an unusually stable dienol that is reportedly generated in the course of bacterial catabolism of catechol by the enzymes of the meta-fission pathway. The dienol ketonizes chemically in aqueous solution and enzymatically by the action of 4-oxalocrotonate tautomerase (EC 5.3.2) to either the β,γ-unsaturated ketone **2** or its α,β-conjugated isomer **3**. Mechanistic studies for both processes remain largely unexplored. An examination of the behavior of **1** in phosphate buffer has been completed using UV, ^{1}H NMR, and ^{13}C NMR spectroscopy. The results of kinetic studies indicate that a rapid equilibrium forms between **1** and **2** before a much slower conversion to **3**. The spectroscopic results were confirmed by reduction of the isomeric ketones with sodium borohydride and subsequent identification of the products. The rapid interconversion of **1** and **2** in aqueous phosphate buffer raises the question of whether the enzyme has a preference for one isomer. The values of k_{cat}/K_M determined for **1** and a mixture of **1** and **2** indicate that 4-oxalocrotonate tautomerase is an extremely efficient catalyst apparently processing either isomer near the diffusion control limit of a small molecule and an enzyme. Further, these values suggest that **2** is a better substrate for the enzyme. A reasonable hypothesis to explain these observations is that 4-oxalocrotonate tautomerase is an isomerase that catalyzes the transformation of **2** to **3** through the intermediacy of **1**. The possible revelance of these findings to the in vivo catabolism of catechol by the enzymes of the meta-fission pathway is briefly discussed.

2-Hydroxymuconate[1] (**1**; Scheme I), a conjugated enol, or a dienol, is reportedly an intermediate generated in the course of bacterial catabolism of catechol to metabolites in the Krebs cycle by the enzymes of the meta-fission pathway.[2] It is subject to chemical ketonization in aqueous solution and enzymic ketonization by an enzyme in the pathway, 4-oxalocrotonate tautomerase[3] (EC 5.3.2.; 4-OT). As part of our efforts to understand the underlying chemical logic of this pathway, we became interested in the mechanism of ketonization in both processes.

Dienols have received close scrutiny in recent years because they are intermediates in the chemical[4] and enzymatic isomerization[5] of α,β-unsaturated ketones and their β,γ-isomers. Several factors such as alkyl substitution at the β-carbon, diene conformation and configuration, and steric hindrance can influence the rate and site of protonation of the intermediate dienol.[4h] The reactivity of a dienol is used to classify it as either slow reacting or fast reacting.[6] A 10^3-fold faster rate is observed for the uncatalyzed ketonization of simple dienols such as 1-methyl-3-*tert*-butyl-1,3-butadienol ($t_{1/2}$ = 17 ms), which is attributed to the operation of a 1,5 sigmatropic hydrogen shift.[4c,d] Moreover, these fast-reacting dienols ketonize to afford the α,β-unsaturated isomer as the major product.[4d] The slow-reacting dienols such as 1,3-cyclohexadienol ($t_{1/2}$ = 48 s) have a structural feature (e.g., a locked conformation), which precludes this intramolecular rearrangement.[4e] A further difference in mechanism is the fact that slow-reacting dienols initially ketonize to afford the β,γ-isomer followed by conversion to the α,β-unsaturated isomer.[4e,f]

Mechanisms for the chemical and enzymic ketonization of **1** remain largely unexplored. Moreover, the existing literature on fundamental aspects of these processes is conflicting. The rate of ketonization of **1** in aqueous soluton is variably reported ($t_{1/2}$ = 3–6 min).[3,7] It is further claimed that **1** ketonizes in a first-order manner to afford a single product, which Evans et al.[3] identified as the β,γ-unsaturated isomer **2**, while Dagley and others suggested that it is the α,β-isomer, **3**[8] (Scheme I). As a result of these ambiguities, three basic mechanisms can be postulated for 4-OT. In the first two mechanisms, the enzyme utilizes **1** as the substrate and catalyzes either a 1,3 proton shift to afford **2**[3] or a 1,5 proton shift to yield **3**.[8] In a third mechanism, **2** is the substrate, and the enzyme catalyzes a 1,3 allylic rearrangement of **2** to **3** through the dienolic intermediate **1**. Clearly, mechanistic studies of either the chemical or enzymatic process cannot be pursued until these issues are resolved.

Scheme I



† The University of Texas.
‡ Texas A&M University.

(1) To simplify the discussion of this compound, we use its trivial name. The systematic name for **1** is 2-hydroxy-2,4-hexadienedioate.

(2) Bayly, R. C.; Barbour, M. G. In *Microbiological Degradation of Organic Compounds*; Gibson, D. T., Ed.; Marcel Dekker: New York; 1984; pp 253–294.

(3) Sala-Trepat, J. M.; Evans, W. C. *Eur. J. Biochem.* **1971**, *20*, 400–413.

(4) (a) Whalen, D. L.; Weimaster, J. F.; Ross, A.; Radhe, R. *J. Am. Chem. Soc.* **1976**, *98*, 7319–7324. (b) Duhaime, R. M.; Weedon, A. C. *J. Am. Chem. Soc.* **1985**, *107*, 6723–6724. (c) Duhaime, R. M.; Weedon, A. C. *Can. J. Chem.* **1987**, *65*, 1867–1872. (d) Duhaime, R. M.; Weedon, A. C. *J. Am. Chem. Soc.* **1987**, *109*, 2479–2483. (e) Pollack, R. M.; Mack, J. P. G.; Blotny, G. *J. Am. Chem. Soc.* **1987**, *109*, 3138–3139. (f) Dzingeleski, G. D.; Bantia, S.; Blotny, G.; Pollack, R. M. *J. Org. Chem.* **1988**, *53*, 1540–1544. (g) Pollack, R. M.; Zeng, B.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1989**, *111*, 6419–6423. (h) Pollack, R. M.; Bounds, P. L.; Bevins, C. L. In *The Chemistry of Enones*; Patai, S., Rappoport, Z., Eds.; John Wiley & Sons Ltd.: New York, 1989, pp 559–597.

(5) (a) Bantia, S.; Pollack, R. M. *J. Am. Chem. Soc.* **1986**, *108*, 3145–3146. (b) Pollack, R. M.; Mack, J. P. G.; Eldin, S. *J. Am. Chem. Soc.* **1987**, *109*, 5048–5050. (c) Kuliopulos, A.; Mildvan, A. S.; Shortle, D.; Talalay, P. *Biochemistry* **1989**, *28*, 149–159. (d) Mildvan, A. *FASEB J.* **1989**, *3*, 1705–1714. (e) Eames, T. C. M.; Hawkinson, D. C.; Pollack, R. M. *J. Am. Chem. Soc.* **1990**, *112*, 1996–1998.

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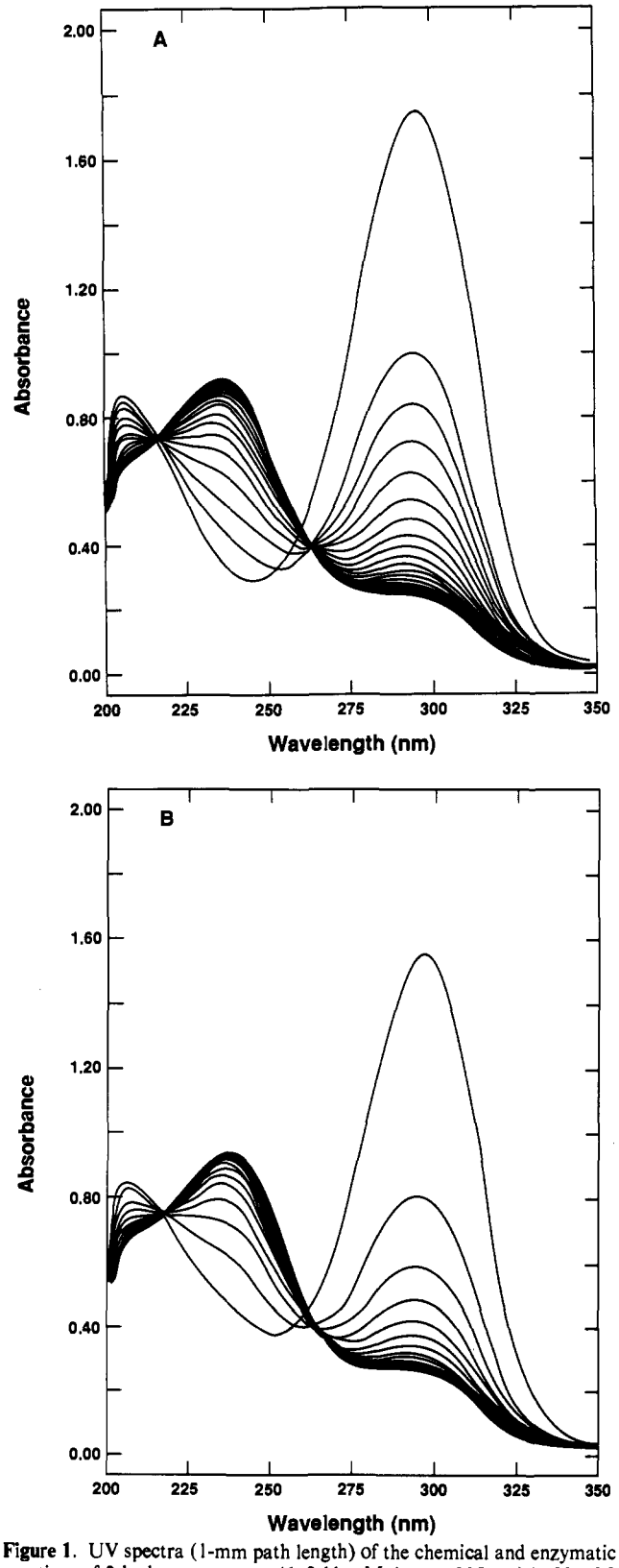


Figure 1. UV spectra (1-mm path length) of the chemical and enzymatic reactions of 2-hydroxymuconate (**1**; 0.11 mM; λ_{max} = 295 nm) in 20 mM Na_2HPO_4 (pH 7.45). (A) Buffer-catalyzed decay (6-min intervals); (B) reaction with 4-oxalocrotonate tautomerase (0.056 unit; 2-min intervals).

The results of our investigation indicate that the chemical and enzymic ketonization of **1** is more complicated than previously thought. In aqueous solution, we observe the formation of a rapid equilibrium between **1** and **2** before a much slower conversion to **3**. This result is in accord with the observed behavior reported for other slow-reacting dienols.[6] More interestingly, though, examination of the values of k_{cat}/K_M determined for **1** and a mixture of **1** and **2** reveals that 4-OT is an extremely efficient catalyst apparently processing either isomer near the diffusion control limit for the association of a small molecule and an enzyme.[9] Furthermore, a comparison of these values indicates that **2** is a better substrate for the enzyme. This finding suggests that 4-OT may be an isomerase that catalyzes the transformation of **2** to **3** most reasonably through the intermediacy of **1**. In light of these findings, a revision of portions of the currently accepted version of the meta-fission pathway may be in order.

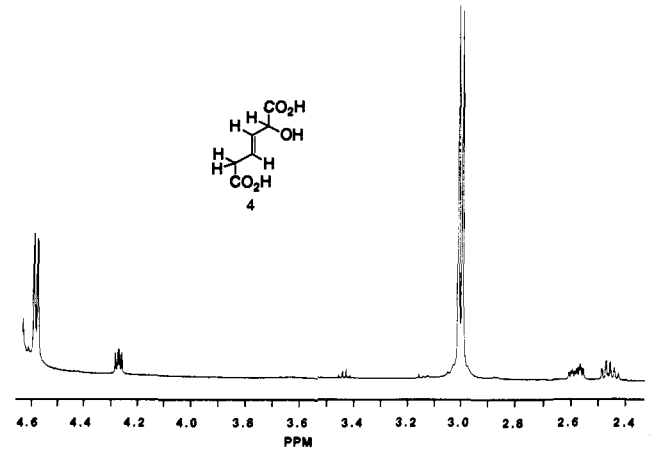
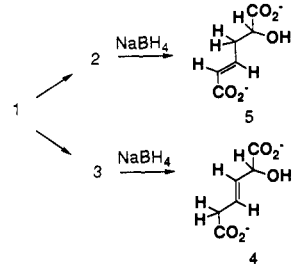


Figure 2. ^1H NMR (500-MHz) spectrum of 2-hydroxy-3-*trans*-hexenedioate (**4**) generated by the $NaBH_4$ reduction of 2-oxo-3-*trans*-hexenedioate (**3**), which is the product of 4-oxalocrotonate tautomerase catalyzed ketonization of 2-hydroxymuconate (**1**).

Scheme II



Results

Identification of the Product of the 4-OT-Catalyzed and Chemical Decay of 1. The enzyme-catalyzed and chemical ketonizations of 2-hydroxymuconate (**1**) result in the formation of the same product. At pH 7.45, (20 mM Na_2HPO_4/NaH_2PO_4) UV analysis reveals (Figure 1A) facile chemical decay of **1** (λ_{max} = 295 nm) accompanied by the formation of a stable product (λ_{max} = 236 nm) with two isosbestic points at 263 and 217 nm.[10] Addition of 4-OT (0.056 unit) from *Pseudomonas putida mt-2* to an identical mixture affords the same spectral changes (Figure 1B) although at a faster rate. These spectral changes are identical with those reported previously.[3,7]

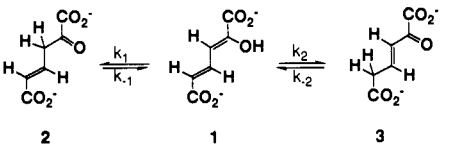
(6) Dzingeleski, G. D.; Blotny, G.; Pollack, R. M. *J. Org. Chem.* **1990**, *55*, 1019–1023.
(7) Harayama, S.; Rekik, M.; Ngai, K.-L.; Ornston, L. N. *J. Bacteriol.* **1989**, *171*, 6251–6258.
(8) (a) Dagley, S. In *The Bacteria*; Gunsalus, I. C., Ed.; Academic Press: New York, 1978; Vol. 6, pp 305–388. (b) Sparnins, V. L.; Chapman, P. J.; Dagley, S. *J. Bacteriol.* **1974**, *120*, 159–167.
(9) (a) Walsh, C. *Enzymatic Reaction Mechanisms*: W. H. Freeman and Co.: San Francisco, CA, 1977; pp 33–35. (b) Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: San Francisco, CA, 1985; pp 111–112, 150.
(10) The λ_{max} observed at 206 nm is either a second absorption band of **1** or is indicative of the presence of **2**. Typically, an α,β-unsaturated acid (trans) absorbs at 205 nm: Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrophotometric Identification of Organic Compounds*, 4th ed.; John Wiley & Sons: New York, 1981; p 320.

Table I. Rate Constants for the Phosphate-Catalyzed Ketonization of 2-Hydroxymuconate[a]

K_{obs}	k_0,[b] $\times 10^{-4}$ s^{-1}	k_{OH^-}, $\times 10^2$ M^{-1} s^{-1}	$k_{H_2PO_4^-}$, $\times 10^{-2}$ M^{-1} s^{-1}	$k_{HPO_4^{2-}}$, $\times 10^{-2}$ M^{-1} s^{-1}
k_1	16.0 ± 2.9	14.7 ± 5.3	15.9 ± 2.39	23.7 ± 1.3
k_{-1}	16.5 ± 2.4	15.6 ± 4.4	23.3 ± 1.97	36.0 ± 1.1
k_2	1.29 ± 0.25	2.01 ± 0.45	3.36 ± 0.21	3.58 ± 0.11
k_{-2}	0.0857 ± 0.0285	0.27 ± 0.05	0.567 ± 0.024	0.495 ± 0.013

[a] The rate constants are derived from eq 1 and are defined in Scheme III. [b] 30.0 °C, ionic strength 0.2 (NaCl), 0.8% methanol. Errors are standard deviations.

Scheme III



2 1 3

The product with a λ_{max} at 236 nm is identified as 2-oxo-3-hexenedioate (**3**) by its immediate conversion to 2-hydroxy-3-*trans*-hexenedioate (**4**) with $NaBH_4$ (Scheme II). The reduced product **4** is the major product isolated by anion-exchange chromatography as determined by ^1H NMR and ^{13}C NMR spectroscopy.[11] Compound **4** is readily distinguished from the other possible product, **5**, that can result from $NaBH_4$ reduction of **2** (Scheme II), by characteristic coupling patterns for the methylene protons (C-5 of **4** and C-3 of **5**) and the C-2 protons in a ^1H NMR spectrum. Analysis of the upfield region (2.3–4.7 ppm) of a ^1H NMR spectrum of the isolated product is consistent with the predicted coupling pattern of **4** (Figure 2). The methylene protons on C-5 of **4** are coupled to the single proton on C-4 resulting in a doublet (3.03 ppm). Likewise, the signal assigned to the proton on C-2 is also observed as a doublet (4.58 ppm) because it is coupled to a single proton on C-3. In contrast, the methylene protons of compound **5** are on C-3 adjacent to both C-4 and the chiral center at C-2, giving rise to separate signals (2.40–2.62 ppm) because they are diastereotopic. Accordingly, the signal corresponding to the proton on C-2 of **5** is a doublet of doublets (4.28 ppm). Finally, the downfield portion of the spectrum (not shown) is characterized by the trans coupled (J = 15.6 Hz) olefinic protons at 5.62 and 5.80 ppm located on C-3 and C-4, respectively, indicative of a 1,2-reduction.[12] Complete ^1H–^1H coupling constants and ^{13}C NMR chemical shifts can be found in the Experimental Section and are fully consistent with the structure of **4**. Therefore, the final product of enzymic ketonization of **1** is **3**, the conjugated isomer. Because nonenzymic and enzymic ketonization of **1** results in an identical final UV spectrum, it can be inferred that the product of chemical ketonization of **1** is also **3**. Hence, the final product in both processes is **3**.

Two additional features of the UV spectra warrant further comment. First, the initial spectra recording the nonenzymatic decay of **1** to **3** does not coincide at the isosbestic point with the remaining spectra. In addition, ketonization of **1** does not go to completion as there is some absorbance remaining at 295 nm at equilibrium. These observations clearly indicate that the decay of **1** is not a simple first-order process and it is not irreversible.

Kinetics of Nonenzymic Ketonization of 1. The ketonization of **1** in aqueous phosphate buffer is monitored at 30.0 °C. (μ = 0.2, NaCl, 0.8% methanol) at pH values 7.00, 7.45, and 8.00 by observing the decay in UV absorbance at 295 nm in different concentrations of phosphate buffer (0.002–0.05 M). In all cases, a rapid drop in absorbance followed by a much slower decrease to equilibrium is observed. A representative experiment (20 mM Na_2HPO_4/NaH_2PO_4, pH 7.45) is shown in Figure 3. The initial drop in absorbance corresponds to the rapid ketonization of **1** to form the unconjugated ketone **2**. The subsequent slower decrease

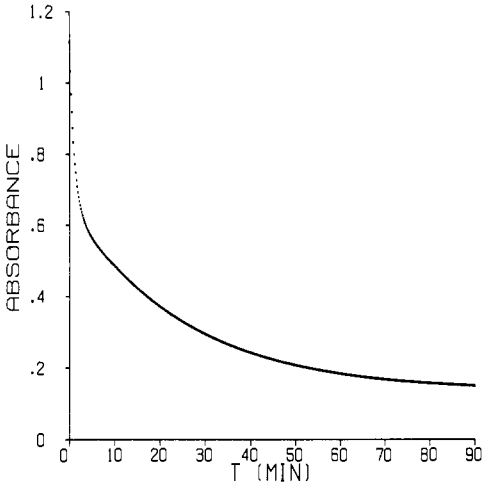


Figure 3. Plot of absorbance vs time for the reaction of 2-hydroxymuconate (**1**) in 20 mM Na_2HPO_4 buffer (pH 7.45, 0.8% methanol, μ = 0.2 M, NaCl, 30.0 °C).

in absorbance results from the slower formation of **3** from **1**. The kinetic parameters for the mechanism shown in Scheme III are estimated by nonlinear least-squares regression analysis of the absorbance data generated for each individual experiment.[13] From this analysis, it is estimated that an equilibrium mixture contains 11.3% **1**, 8.2% **2**, and 80.5% **3**. In all cases, an excellent fit to the data is obtained. The kinetic constants obtained for all experiments are fit to eq 1 by multiple linear regression to estimate the kinetic constants listed in Table I.

$$k_{obs} = k_0 + k_{OH^-}[OH^-] + k_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^{2-}}[HPO_4^{2-}] \quad (1)$$

The results clearly indicate that $H_2PO_4^-$, HPO_4^{2-}, and hydroxide ion have a catalytic effect on each step of the mechanism although the effect is greater on k_1 and k_{-1} (the interconversion of **1** and **2**). The values of k_0 determined for k_1 and k_2 in Table I provide an upper limit on the uncatalyzed rate constant for ketonization of **1** to **2** and **1** to **3**, respectively. These k_0 values can only be considered an upper limit because the rate due to the concentration of hydroxide ion, hydronium ion, and other buffer species cannot be determined within the narrow pH range studied.[14] Nonetheless, the measured k_0 values are substantially lower than the uncatalyzed rate constants reported for all slow-reacting dienols investigated except for (*E*)-1-hydroxy-1,3-butadiene.[6]

^1H NMR and ^{13}C NMR Detection of 1 and 3. A ^1H NMR spectrum of **1** in phosphate buffer made up in ^2H_2O shows initially three sets of signals corresponding to the three protons of **1** (Figure 4A). The doublet furthest upfield at 5.84 ppm (J = 15 Hz) is assigned to the proton on C-5 while the doublet at 5.95 ppm (J = 12 Hz) is assigned to the proton on C-3. A heteronuclear multiple-bond correlation (HMBC) NMR experiment confirms these assignments. Most notably, a strong correlation exists between C-2 and the downfield signal (H-3).[15] The proton on

(11) The minor component present in this mixture is **5**, which results from $NaBH_4$ reduction of **2**. There is a small quantity of unconjugated ketone in the equilibrium mixture of **1**–**3**.

(12) A 1,2-reduction has been reported in a similar system: Marcotte, P.; Walsh, C. *Biochemistry* **1978**, *17*, 5620–5626.

(13) Veng-Pederson, P. *J. Pharmacokinet. Biopharm.* **1977**, *5*, 513–531.

(14) The multivalent buffer and narrow pH range were chosen for study in order to correlate these results with those reported earlier and to enable comparison to physiological conditions. A statistically better fit of the data is obtained by including a contribution from hydroxide ion. A full analysis of the effect of different buffers and pH is under investigation.

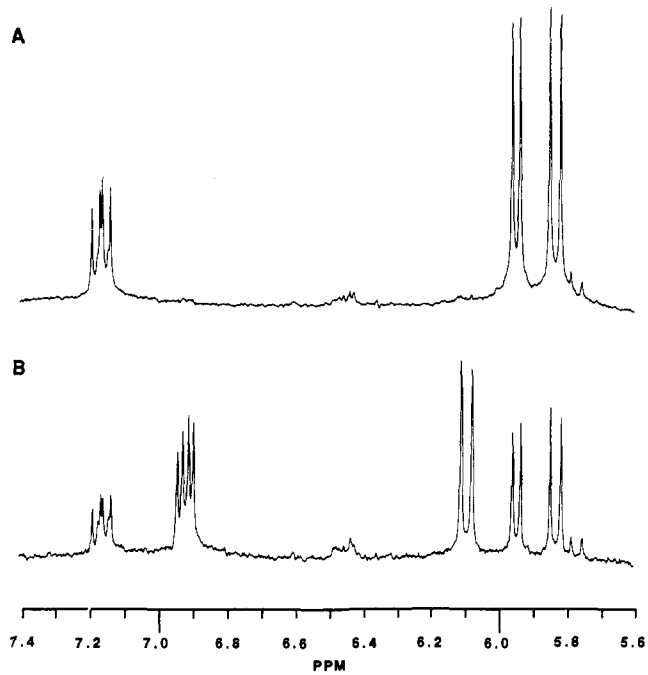


Figure 4. ^1H NMR (500-MHz, ^2H_2O) spectra indicating the chemical and enzymatic reactions of 2-hydroxymuconate (**1**) in 100 mM K[$^2H_2$]PO_4 (pD = 7.49, 15% v/v ethanol). (A) Buffer-catalyzed decay after 5 min; (B) reaction with 4-oxalocrotonate tautomerase (2 units) after 5.5 min. Identical results were obtained with a methanolic solution (6% v/v) of 2-hydroxymuconate (**1**).

C-4 gives rise to the downfield doublet of doublet at 7.16 ppm (J = 12 and 15 Hz). The coupling constants (15 Hz) for the protons on C-4 and C-5 indicate a trans configuration. The stable conformation of **1** is presumably s-trans around the C-3–C-4 bond.[16]

Compound **3** is generated rapidly by the addition of **1** to a NMR tube containing 4-OT in phosphate buffer made up in ^2H_2O. After 5.5 min, the ^1H NMR spectrum (Figure 4B) reveals the formation of a new set of signals, which can be reasonably assigned to the protons of **3** in accord with the results of the UV spectra (Figure 1) and with the results of the $NaBH_4$ trapping experiment. Thus, the proton on C-4 shifts upfield to 6.91 ppm while the proton on C-3 shifts downfield to 6.11 ppm. The observed coupling constant (J = 16 Hz) between the protons on C-3 and C-4 indicates that the configuration of the newly formed bond of **3** is trans—inconsistent with a cyclic transition state required for a 1,5 sigmatropic rearrangement. Finally, the proton on C-5, now a sp^3 center in **3** is observed upfield at 3.00 ppm as a broadened doublet (not shown). Incorporation of a solvent deuteron at C-5 of **3** accounts for the observed broadening of the C-5 proton via geminal ^1H–^2H coupling while simplifying the coupling pattern of the proton on C-4 to a doublet of doublets.

The stability of **3** in aqueous solution permits the aquisition of a ^{13}C NMR spectrum. The most stable feature is the upfield shift observed for the carbonyl carbon of **3**. Model chemistry predicts that conjugation of a carbonyl carbon results in an upfield shift of the resonance corresponding to that carbon.[17] Overall, the chemical shifts observed in this spectrum are consistent with the structure of **3** and can be found in the Experimental Section.

^1H NMR Proof for the Formation of 2 during Chemical Ketonization of 1. The phosphate-catalyzed ketonization of **1** can be monitored by ^1H NMR spectroscopy in order to provide

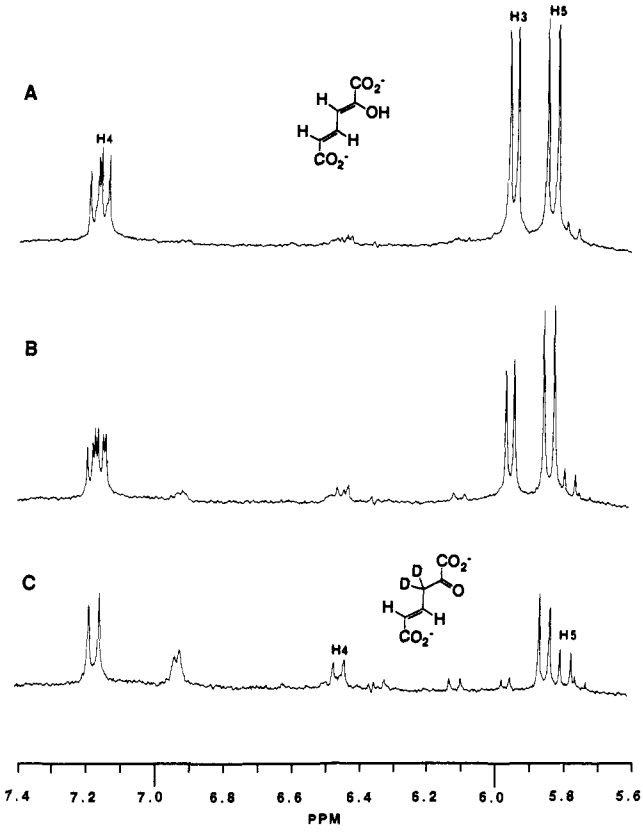


Figure 5. ^1H NMR (500-MHz, ^2H_2O) spectra indicating the time course of the buffer-catalyzed decay of 2-hydroxymuconate (**1**) in 100 mM K[$^2H_2$]PO_4 (pD = 7.49, 15% v/v ethanol). (A) After 5 min with the proton assignments shown for **1**; (B) after 15 min; and (C) after 60 min with the proton assignments shown for **2**. Identical results were obtained by using a methanolic solution (6% v/v) of 2-hydroxymuconate (**1**).

confirmatory evidence for the mechanism shown in Scheme III. The ^1H NMR spectra (Figure 5A–C) recording the fate of **1** during its nonenzymic decay in ^2H_2O are consistent with the mechanism. Initially, the proton at C-4 of **1** is observed as a doublet of doublets (7.16 ppm) due to coupling to adjacent protons at C-3 and C-5 (Figure 5A). After 15 min, phosphate-catalyzed decay of **1** results in the appearance of a new doublet, superimposed upon the doublet of doublet (Figure 5B). After 1 h,[18] only the new doublet remains (Figure 5C). Moreover, concomitant with the appearance of the new doublet is the disappearance of the upfield doublet assigned to the proton on C-3 of **1** (5.95 ppm).

The key to interpretation of the spectra is the identification of the species giving rise to the new doublet. Such a signal can only result by deuteriation at the C-3 or C-5 of **1**. The latter possibility is ruled out as the major contributor to the formation of [^2H]-**1** by the loss of the doublet at 5.95 ppm (Figure 5C) corresponding to the proton on C-3 and not the doublet (5.84 ppm) corresponding to the proton on C-5 of **1**. Thus, the new doublet indicates that [3-^2H]-**1** is the major species present.[19] Rapid exchange of the proton on C-3 of **1** with a solvent deuteron through the intermediate [3-^2H]-**2** affords [3-^2H]-**1**. The formation of [3-^2H]-**1** from [3-^2H]-**2** is favored as a result of an isotope effect and the greater stability of the dienol **1**. Observation of a selective exchange of a single proton on **1** with a solvent deuteron is consistent with the nearly 12-fold difference in rates observed for k_1 and k_2 in Scheme III.

Rapid exchange of the proton on C-3 of **1** is accompanied by the appearance of a set of ^1H NMR signals that can reasonably

(15) These assignments are also consistent with those reported for the 1-hydroxy-1,3-butadiene system: Capon, B.; Guo, B. *J. Am. Chem. Soc.* **1988**, *110*, 5144–5147.

(16) The s-trans conformation is the stable conformation reported for butadiene, 1-hydroxy-1,3-butadiene, and several alkoxybutadienes.[15]

(17) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy: High Resolution Methods and Applications in Organic Chemistry and Biochemistry*; VCH: New York, 1987; pp 114, 215–226.

(18) The slower rate observed for the phosphate-catalyzed ketonization of **1** in the NMR experiments (in ^2H_2O) is presumably due to a solvent isotope effect on the reaction.

(19) There is some reversion of **3** to **1** as indicated by the decrease in intensity of the signal corresponding to the proton on C-5 of **1**. However, this reversion is clearly much less than that observed for **2** to **1**.

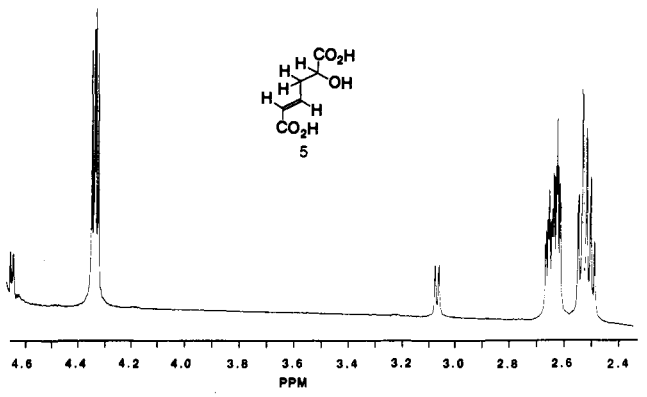


Figure 6. ^{1}H NMR (500-MHz) spectrum of 2-hydroxy-4-*trans*-hexenedioate (**5**) generated by the $NaBH_4$ reduction of 2-oxo-4-*trans*-hexenedioate (**4**), which is the product of phosphate-catalyzed decay of 2-hydroxymuconate (**1**).

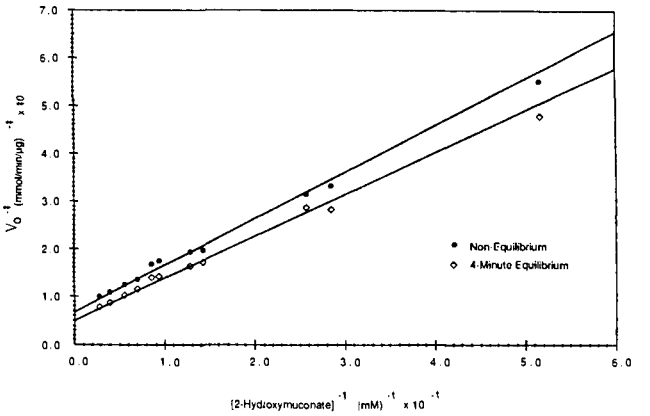


Figure 7. Comparison of the initial velocities determined in the nonequilibrium experiment (●) and the equilibrium experiment (◇) at pH 7.3 and 30 °C.

be assigned to **2** (Figure 5C). The proton on C-5 of **2** produces a small doublet (5.80 ppm) just upfield of the doublet corresponding to the proton on C-5 of **1**. The proton on C-4 of **2**, which initially gives rise to a poorly defined multiplet (6.46 ppm; Figure 5A), is more clearly observed after 60 min as a doublet centered at 6.46 ppm (Figure 5C). Finally, the C-3 proton of **2** is observed as a broadened doublet at 3.39 ppm (not shown) and is present only in early spectra following the decay of **1**. The broadening and eventual loss of the signal corresponding to the proton on C-3 along with the simplification of the signal assigned to the proton on C-4 of **2** is consistent with the exchange of these methylene protons with solvent deuterons by the interconversion of **1** and **2**.[20]

Finally, a comparison of spectra recorded at similar time points in the enzymatic and nonenzymatic decay of **1** (Figure 4) clearly shows that **3** is produced more rapidly in the presence of enzyme. This result is in accord with the results of the UV and $NaBH_4$ trapping experiments. Clearly, the presence of 4-OT in the meta-fission pathway accelerates a kinetically unfavorable reaction (i.e., production of **3**), which prevents an unwanted, but a kinetically favorable reaction (interconversion of **1** and **2**).

Chemical Trapping of 2 in the Chemical Ketonization of 1. The presence of **2** during the chemical ketonization of **1** can be confirmed chemically by trapping the unstable β,γ-unsaturated isomer through its immediate conversion to 2-hydroxy-4-*trans*-hexenedioate (**5**) with $NaBH_4$ (Scheme II). Our strategy for the trapping of **2** is based on its generation from phosphate-catalyzed decay of **1** so that the contaminating presence of **3** is unavoidable. However, the nearly exclusive formation of **5** can be achieved by the addition of $NaBH_4$ at short intervals (3 min) because of the nearly 12-fold difference in the rate of formation of **2** and **3** from **1**. Moreover, conversion of **2** and **3** to their respective products with $NaBH_4$ at intervals effectively removes them from the equilibrium mixture to allow complete ketonization of **1**.

After repeated additions of $NaBH_4$ to a solution containing buffer and **1**, the reduced alcohol, 2-hydroxy-4-*trans*-hexenedioate (**5**), is the major product (94%) recovered by ion-exchange chromatography as determined by ^{1}H and ^{13}C NMR analysis. The upfield region (2.30–4.70 ppm) of its ^{1}H NMR spectrum (Figure 6) shows the characteristic coupling pattern for **5**. The diastereotopic methylene protons on C-3 of **5** appear as separate and distinctly different signals at 2.52 and 2.66 ppm. The upfield signal presents a quintet of triplets while the downfield signal appears to be two overlapping octets. The difference in the appearance of these signals presumably reflects a different degree of allylic coupling.[21] The proton on C-2 results in a doublet of doublets at 4.34 ppm.[22] The signals corresponding to the olefinic protons (not shown) along with the complete ^{1}H–^{1}H coupling constants and the ^{13}C NMR chemical shifts can be found in the Experimental Section and are fully consistent with the structure of **5**. Finally, intergration of the signals produced by the methylene protons on **4** and **5** reveals the presence of **5** in a nearly 15-fold excess, which is in good agreement with the rate constants determined from the study of the solution kinetics.

Table II. Kinetic Parameters Derived from Nonequilibrium and Equilibrium Experiments

substr	K_M,[a] μM	k_{cat}, s^{-1}	k_{cat}/K_M, M^{-1} s^{-1}
1	144.9 ± 11.8	1.39×10^6	9.57×10^9
1 and **2**	175.7 ± 12.2	1.87×10^6	1.06×10^{10}
2	189	2.88×10^6	1.52×10^{10}

[a] Errors are standard deviations.

Substrate Specificity of 4-OT for 1 and 2. The rapid interconversion of **1** and **2** in buffered solution raises the question of which isomer is the better substrate for the enzyme. The question is most readily answered by determining the specificity constant (k_{cat}/K_M) of the enzyme for each isomer.[23] While this value is easily obtained for **1** by using a "nonequilibrium" experiment, a straightforward determination of a k_{cat}/K_M value for **2** is complicated by the fact that **2** has never been isolated or synthesized. It is therefore necessary to use an "equilibrium" experiment in order to ascertain the value for **2**.

The basis of these experiments is as follows. In a nonequilibrium experiment, isomer **1** is added to a cuvette containing buffer (20 mM Na_2HPO_4, pH 7.3; 30 °C) and a quantity of enzyme. The rate of increase in product (**3**) is monitored at 236 nm for 11 concentrations of substrate ranging from 19 to 350 μM. The observed rate is due to enzymic ketonization of **1**. In an equilibrium experiment, a quantity of enzyme is added to a cuvette containing isomer **1**, which has been previously allowed to decay in the buffer for 4 min. This time interval allows the system to reach *kinetic* equilibrium where the greatest quantity of **2** is observed while not allowing the system to reach complete or *thermodynamic* equilibrium where the greatest quantity of **3** is observed. The 4-min equilibration results in a mixture of isomers **1** (55.8%) and **2** (34.6%) and a small amount of **3** (9.6%). The rate of increase of product (**3**) is again monitored at 236 nm for 11 different concentrations of total substrate ranging from 19 to 350 μM. However, the observed rate in this experiment results from enzymic processing of **1** and **2**.

The kinetic parameters, K_M and k_{cat}, for **1** and a mixture of **1** and **2** are determined from a double-reciprocal plot of the data from the nonequilibrium experiment and the data from the equilibrium experiment (Figure 7). These values and the cor-

(20) The other signals present in the spectrum (Figure 5C, 6.11 and 6.95 ppm) correspond to the protons on **3**. The incorporation of a solvent deuteron at C-3 of **2** accounts for the observed broadening of the corresponding signal via geminal ^{1}H–^{2}H coupling as described above for the broadened signal observed for C-5 of **3**.

(21) Becker, E. D. *High Resolution NMR*, 2nd ed.; Academic Press: Orlando, FL, 1980; pp 102–105.

(22) The other signals present in this spectrum correspond to the protons on C-2 and C-5 of **4**.

(23) Fersht, A. R. ref 9b, pp 105–106.

responding specificity constants (k_{cat}/K_M) are reported in Table II.[24] The kinetic parameters for **2** (Table II) are obtained from these experiments by using a velocity equation derived for a model in which two substrates (**1** and **2**) compete for the same active site.[9b]

A comparison of the results of the nonequilibrium experiment to those in the equilibrium experiment provides insight into the nature of the 4-OT reaction. The initial rate observed in the equilibrium experiment (**1** and **2**) is always faster than the initial rate observed in the corresponding nonequilibrium experiment (**1**) even though the concentration of **1** and **2** is less than the concentration of **1** (~9%). Moreover, there is no observable lag time in the production of **3** when the enzyme processes either **1** or a mixture of mixture of **1** and **2**. The turnover numbers for **1** and **2** are on the order of 10^6 s^{-1} with **2** being processed ~2 times faster than **1**. The specificity constants determined for **1** and **2** indicate that the reaction is diffusion controlled using either substrate although the value of k_{cat}/K_M for **2** is ~60% faster than that determined for **1**. These combined observations are most reasonably explained by a hypothesis in which **1** is an intermediate in the enzyme-catalyzed transformation of **2** to **3**.

Finally, these data allow an estimation of the rate acceleration produced by 4-OT. The rate enhancement can be determined by comparing the rate of spontaneous conversion of **1** to **3** (k_2 in Scheme III) to the reaction of 4-OT with **1**. It is necessary to use k_2 in this analysis because the nonenzymatic rate of isomerization of **2** to **3** is not known. However, comparison of the rate constants for the conservation of **2** to **1** and **1** to **3** suggests that k_2 is the slow step in the overall process. Hence, the rate of chemical ketonization of **1** to **3** in 20 mM sodium phosphate buffer at pH 7.3 is 8.65×10^{-4} s^{-1}. The ratio of K_{cat} for **1** to **3** to k_2 is $\sim 1.6 \times 10^9$.

Discussion

The results clearly indicate that the behavior of 2-hydroxymuconate (**1**) in aqueous solution and in the presence of 4-OT is considerably more complex than previously thought. Its nonenzymic behavior is fully consistent with the properties observed in other slow-reacting dienols.[6] Further, our investigation into the nature of the 4-OT reaction suggests that 4-OT may be an isomerase catalyzing an allylic rearrangement of **2** to **3** through the dienol intermediate **1**. The role we propose for **1** and 4-OT in this mechanism is also consistent with literature precedent.[5] Finally, our results raise questions about whether **1** and other dienol compounds widely believed to be intermediates generated during the course of catechol degradation by the enzymes of the meta-fission pathway are, in fact, the intermediates generated or if they result from the harsh conditions of isolation.

Studies of the chemical decay of 2-hydroxymuconate (**1**) in aqueous solution indicate that a rapid kinetic equilibrium is formed between **1** and **2** before a much slower conversion to **3**. At thermodynamic equilibrium, a mixture of isomers **1**–**3** results with **3** being the predominant species in aqueous solution. The behavior we describe is in marked contrast to earlier reports indicating a first-order decay to either **2** or **3**.[3,8] Presumably, the high concentration of phosphate buffer used (83 mM) results in the rapid formation of a mixture of **1** and **2**. The reported rate constants probably result from the apparent first-order decay of a mixture of **1** and **2** to **3**.

The behavior of **1** in aqueous solution is fully consistent with the observed behavior of other slow-reacting dienols.[6] Three observations are generally used to determine if a dienol is a slow-reacting dienol.[4e,f,6] First, protonation is favored on the α-carbon rather than the β-carbon. Second, the compound is reasonably stable in aqueous solution and ketonizes unusually slowly compared to simple enols and other dienols. Finally, there is a structural feature in the dienol that prevents the operation of a 1,5 sigmatropic rearrangement. The unavailability of this pathway to the molecule is the major cause for its slower uncatalyzed rate of ketonization.

(24) These calculations assume that 4-OT is a tetramer.

Our kinetic, NMR, and chemical trapping experiments show that protonation of the dienolate ion of **1** occurs more rapidly at the α-carbon rather than the γ-carbon (~12-fold).[25] The partitioning factor (k_α/k_γ) for **1** is somewhat greater than the corresponding factor determined for (*Z*)- and (*E*)-1-hydroxy-1,3-butadiene (<4-fold)[6,15] and substantially less than the factor determined for 1,3-cyclohexadienol (~50-fold).[4e,6] The difference in the rate constants is attributed to the relative charge density at the α- and γ-carbon. In a number of studies, Whalen,[26] Pollack,[4g,6] and Capon[15] suggested that the major influence on the charge density at these carbons is the planarity of the π-system. If the two double bonds are nearly or completely planar to each other, as in the case of the hydroxybutadienes,[15] then the negative charge is very effectively delocalized throughout the π-system, and protonation at the α-carbon is only slightly favored. If, on the other hand, there is a significant twist about the single bond between the two double bonds, as in the case of 1,3-cyclohexadienol, then the negative charge is localized at the α-carbon so that protonation at the α-carbon is greatly favored.[6] A priori, significant twisting of the C-3, C-4 bond in **1** is not expected. However, slight twisting in combination with other factors such as the steric hindrance to protonation at $C_{(\gamma)}$ and the electronic effect of the carboxylate group at $C_{(\gamma)}$ can easily explain the observed rate differences.

Our kinetic studies also confirm that **1** is an unusually stable dienol with a slow rate of ketonization. The stability of **1** is presumably due to its extensive conjugation similar to the conjugated steroidal trienol 3-hydroxy-3,5,7-estratrien-17-one.[4f] The slow rate of uncatalyzed ketonization of **1** can be explained by its inability to undergo a 1,5 sigmatropic rearrangement. Presumably, **1** exists in solution in the s-trans conformation in order to place the charged carboxylate groups at a favorable distance. The cyclic transition state necessary for a 1,5 sigmatropic shift requires the s-cis conformation. The close proximity of the carboxylate groups in this conformation makes it sufficiently high in energy so that it is an unlikely conformation. Likewise, the conformation of (*E*)- and (*Z*)-1-hydroxy-1,3-butadiene[15] and the structure of the steroidal trienol[4f] and 1,3-cyclohexadienol[4e] preclude the cyclic transition state necessary for such an intramolecular rearrangement.

The nonenzymic behavior of **1** impinges directly on studies of the nature of the 4-OT reaction. The finding that a significant concentration of **2** rapidly accumulates in buffered solution from the ketonization of **1** compelled us to examine the question of whether one isomer is a better substrate for the enzyme. The results of kinetic studies into this question demonstate that the rate of product formation (**3**) is always faster when 4-OT acts on a mixture of **1** and **2** than when only **1** is present. This effect is noticeable for the wide variety of substrate concentrations used in the study. The faster rate is observed in spite of the fact that the total concentration of **1** and **2** in an equilibrium experiment is less than the concentration of **1** in the corresponding nonequilibrium experiment. These observations are reflected in the values of k_{cat}/K_M determined for **1** and **2**, which indicate that the enzyme will utilize either substrate although the β,γ-unsaturated ketone is the better substrate. The magnitude of the two values suggests that the reaction is diffusion controlled using either substrate.[9]

These results are best explained by hypothesizing that 4-OT is an isomerase that catalyzes the conversion of **2** to **3** through the dienol intermediate **1**. The higher rates of enzymic activity measured in the presence of **2** reflected in a higher value of k_{cat}/K_M are the basis of our contention that **2** is a better substrate. The intermediacy of **1** in the mechanism is based on two observations: it is an intermediate in the chemical interconversion of **2** and **3** and it is processed by the enzyme without a detectable lag time

(25) This analysis assumes a mechanism in which the dienolate ion of **1** is generated by either a hydroxide ion or a water molecule, followed by protonation at either $C_{(\alpha)}$ or $C_{(\gamma)}$. This is a reasonable assumption under these experimental conditions.[4f]

(26) Whalen, D. L.; Weimaster, J. F.; Ross, A. M.; Radhe, R. *J. Am. Chem. Soc.* **1976**, *98*, 7319–7324.

at the diffusion-controlled limit.[5a]

Although there is little additional experimental evidence to support this hypothesis, it is chemically reasonable and substantiated by literature precedent. As an isomerase, 4-OT belongs to the class of isomerases that catalyzes a 1,3 allylic rearrangement.[27] The most extensively studied enzyme in this group is 3-oxo-Δ^5-steroid isomerase (KSI).[5] There is a considerable body of evidence to support the existence of an enolic intermediate in the enzymic mechanism.[5] Kinetic studies of the solution chemistry of substrates for KSI implicate dienol intermediates in the chemical isomerization of the β,γ-unsaturated ketones to the α,β-unsaturated ketones.[4g,5b] Further, it has been demonstrated that KSI will convert the steroidal trienol, 3-hydroxy-3,5,7-estradien-17-one, and the dienol of 5-androstene-3,17-dione to their respective α,β-unsaturated ketone products at rates comparable to those observed in the normal mechanism (the diffusion limit).[5a,d] Finally, an intermediate in the KSI reaction is consistent with the fact that a concerted, suprafacial, 1,3 proton transfer is symmetry forbidden.[28] The stereochemical course of the 4-OT reaction is under investigation.

The turnover number for **1** is ~50% as great as that determined for the formation of **3** from **2** while the second-order rate constant for the reaction of 4-OT and **1** is ~63% of the same rate constant determined for the reaction of **2** with 4-OT. These rates do not satisfy the criterion for the steady-state definition of kinetic competence of a freely dissociable intermediate.[29] There are two possible explanations for the less than expected rate. The dienol present in solution may either have a different double-bond configuration or a different state of protonation (dienol vs dienolate) than the dienol generated on the enzyme's surface. The second possibility is that the catalytic groups on the enzyme are not in the appropriate protonation state for turnover of **1**.[27,30]

The second-order rate constant for the reaction of 4-OT with either isomer is near the diffusion-controlled limit. While estimates for this limit vary, a range of 10^9–10^{11} is generally accepted.[9] Our rather large magnitude for k_{cat}/K_M may stem from the fact that 4-OT is a small protein, so that an encounter of enzyme and substrate is more likely to result in a productive turnover. The rate enhancement afforded by 4-OT ($\sim 10^9$) can be considered "average" for an enzyme.[9a] Interestingly, a comparable rate enhancement has been reported for KSI.[5c] The modest rate enhancement observed for these enzymes may reflect the fact that the nonenzymatic reaction is reasonably facile in aqueous solution.

If 4-OT does indeed prove to be an isomerase and utilizes **2** as its physiological substrate, then it is likely that **2** is the intermediate generated in vivo during the course of bacterial degradation of catechol by the enzymes of the meta-fission pathway. The metabolic precursor to the substrate for 4-OT is purportedly 2-hydroxymuconate semialdehyde (2-hydroxy-6-formyl-2,4-hexadienoate), a dienol that results from ring cleavage of catechol.[2] A NAD^+-dependent dehydrogenase oxidizes the aldehyde to the acid to generate the substrate for 4-OT. Although ring cleavage of catechol is accompanied by the observation of a characteristic yellow color (λ_{max} = 375 nm), there are no data regarding the amount of the β,γ-unsaturated ketone present or whether the dienol or the ketone is the substrate for the dehydrogenase.[3] Experiments are in progress to determine the substate for the dehydrogenase.

Identification of **1** and other dienols in the meta-fission pathway was established by their isolaton from a mixture of catechol and cell extract after treatment with concentrated acid. In our hands, the addition of ^2HCl to a solution at equilibrium containing **1**–**3** results only in the isolation of **1**.[31] Hence, the harsh conditions of isolation may have resulted in the mistaken identification of intermediates. Our results clearly demonstrate that future studies of the meta-fission pathway must pay close attention to the nonenzymatic chemistry of the intermediates in the meta-fission pathway in order to interpret correctly the enzymatic chemistry.

Experimental Section

Materials. All chemicals and solvents were obtained from Aldrich Chemical Co. with the exception of 2-hydroxymuconate, which was prepared according to the method of Lapworth.[32] Biochemicals and buffers were obtained from Sigma Chemical Co. DEAE cellulose (DE-52) was purchased from Whatman. Hydroxyapatite (Bio-Gel HTP), Dowex 1-X8, and electrophoresis chemicals were obtained from Bio Rad Laboratories. Centricon (10000 MW cutoff) centrifugal microconcentrators and the PM-10 and YM-5 ultrafiltration membranes were obtained from Amicon.

Methods. Protein concentrations were determined colorimetrically by the Bradford method using bovine serum albumin as a standard.[33] HPLC was performed on a Waters system using a Waters Protein Pak DEAE 5PW anion-exchange column (10-μm particle size), a Bio Rad Bio-Gel phenyl 5-PW hydrophobic column, and a Pharmacia Superose 12 (HR 10/30) gel filtration column. NMR spectra were obtained on a Bruker AM 500-MHz spectrometer. Chemical shifts were standardized to the $^1H^2HO$ resonance at 4.70 ppm. The nonenzymic kinetic results were obtained on a Beckman DU-7 spectrophotometer connected to a Zenith portable computer for data collection. The enzyme kinetic results were obtained on a Perkin-Elmer fast scan UV/vis spectrophotometer Model 553.

Purification of 4-Oxalocrotonate Tautomerase from *Pseudomonas putida mt-2*. Cultures of *P. putida mt-2* (ATCC 33015) are grown on minimal salts medium supplemented with 10 mM *m*-toluate at 30 °C as described elsewhere.[34] The purification scheme is adapted from the procedure used by Harayama et al. to purify a recombinant 4-OT cloned into *Escherichia coli*.[7] Because the procedure is extensively modified, it is included in detail here. In a typical procedure, frozen cells (30 g) are suspended in 60 mL of 10 mM ethylenediamine dihydrochloride (pH 7.3), which is made 1 mM in 6-aminocaproic acid, 0.5 mM in phenylmethanesulfonyl fluoride, 50 μM in leupeptin, and 0.067 μM in aprotonin to limit proteolysis. The cells are disrupted at 4 °C with 10 pulses (1 min each) by a Heat Systems W-385 sonicator equipped with a 0.5-in. tapped horn delivering approximately 330 W/pulse. The solution is centrifuged at 27000*g* for 30 min to remove cell debris. The supernatants are combined and centrifuged at 100000*g* for 60 min.

The extract is loaded onto a DE-52 cellulose anion-exchange column (22.5 × 5 cm) equilibrated in ethylenediamine buffer. The column is washed with 200 mL of buffer and elution is carried out with a NaCl gradient (0.0–0.5 M NaCl, 2 L total volume). The activity elutes in a broad peak centered at approximately 0.125 M NaCl. Active fractions are pooled and concentrated in a 200-ml Amicon filtration cell 10–15-fold (60 psi N_2) using a PM-10 membrane (10000 MW cutoff).

After brief centrifugation to remove insoluble material, the concentrate is injected (250-mg portions) into a Waters Protein Pak (DEAE-5PW) anion-exchange column (15 × 2.15 cm) equilibrated with ethylenediamine buffer at a flow rate of 5 mL/min. The column is washed with buffer for 10 min and elution is carried out with a NaCl gradient (0.0–0.4 M NaCl) in 60 min. The eluant is monitored by UV detection (280 nm) and collected in 10-mL fractions and assayed for tautomerase activity. Active fractions are pooled and concentrated ~40-fold in a 50-mL Amicon filtration cell using a PM-10 membrane.

The resulting concentrate is loaded onto a hydroxyapatite column (55 × 2.5 cm) equilibrated in 10 mM K_2HPO_4 buffer (pH 6.8). The column is washed with buffer (50 mL) and elution is achieved with a K_2HPO_4 buffer gradient (0.0–0.25 M, 1 L total volume). The eluant is collected in 5-mL fractions. The enzyme typically elutes at 0.13 M K_2HPO_4. Active fractions are pooled and concentrated 100-fold by ultrafiltration in a 50-mL Amicon filtration cell.

The concentrate is made 2 M in $(NH_4)_2SO_4$, centrifuged, and injected into a phenyl 5-PW column (75 × 7.5 mm) equilibrated with 10 mM ethylenediamine buffer (pH 7.3) made 2 M in $(NH_4)_2SO_4$ at a flow rate of 0.5 mL/min. The column is washed with buffer for 10 min and elution

(27) Schwab, J. M.; Henderson, B. S. *Chem. Rev.* **1990**, *90*, 1203–1245.
(28) Woodward, R. B.; Hoffmann, R. *The Conservation of Orbital Symmetry*; Verlag Chemie: Weinheim, Germany, 1979, pp 114–132.
(29) (a) Cleland, W. W. *Biochemistry* **1990**, *29*, 3194–3197. (b) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131–1149.
(30) There are other examples in the literature in which a chemically reasonable intermediate does not satisfy the criterion of kinetic competence. Such examples are found in the formyltetrahydrofolate synthetase reaction,[29b] the ribulose 1,5-bisphosphate carboxylate reaction,[29b] the malate dehydrogenase reaction,[29a] and the pyruvate kinase reaction: Kuo, D. J.; O'Connell, E. L.; Rose, L. A. *J. Am. Chem. Soc.* **1979**, *101*, 5025–5030.
(31) Whitman, C. P.; Watson, R. J.; Stolowich, N. J., unpublished results, 1990.
(32) Lapworth, A. J. *J. Chem. Soc.* **1901**, *79*, 1265–1284.
(33) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248–254.
(34) (a) Murray, K.; Duggleby, C. J.; Sala-Trepat, J. M.; Williams, P. *Eur. J. Biochem.* **1972**, *28*, 301–310. (b) Stanier, R. Y.; Palleroni, N. J.; Doudoroff, M. *J. Gen. Microbiol.* **1966**, *43*, 159–271.

is carried out with a $(NH_4)_2SO_4$ gradient (2.0–0.0 M, 30 mL total volume) in 60 min collecting 2-mL fractions. Fractions with tautomerase activity are pooled and concentrated 100-fold by ultrafiltration using a YM-5 membrane (5000 MW cutoff).

After dialysis of the concentrate into 50 mM Na_2HPO_4 buffer (pH 7.0) made 0.15 M in NaCl, the solution is injected (100–200-μL portions) into a Superose 12 (Pharmacia) gel filtration column equilibrated in the same buffer at a flow rate of 0.5 mL/min. The eluant is collected in 0.5-mL fractions. Active fractions are pooled and concentrated in a 10-mL Amicon filtration cell using a YM-5 membrane. The final specific activity of 4-OT ranges from 1900 to 4700 units/mg. Typically, 450 μg of protein is obtained from 30 g of frozen cells. A unit of enzyme produces 1 mmol of **3**/min at pH 7.3 at 30 °C.

The native molecular weight for 4-OT is determined by chromatography on the Superose 12 column of the purified protein using bovine serum albumin (dimer 134000 Da, monomer 67000 Da), ovalbumin (43000 Da), β-lactoglobulin (35000 Da, carbonic anhydrase (29000 Da), horse heart cytochrome *c* (12500 Da), and aprotinin (6500 Da) as standards. A plot of $(V_e - V_0)/(V_t - V_0)$ vs log molecular mass for the standards, where V_e is the elution volume, V_0 is the void volume, and V_t is the total volume, gives a linear curve, which is used to predict the molecular mass. These calculations indicate that 4-OT has a molecular mass of 22500 Da.

Polyacrylamide gel electrophoresis under denaturing conditions is performed on 15% gels as described.[35] 4-OT migrates as a single band with an apparent subunit size of 3600 Da. This estimation is not accurate because the smallest molecular weight standard in this system is aprotonin (6500 Da). Consequently, tricine sodium dodecyl sulfate–polyacrylamide gels (22.5% acrylamide) are performed under strongly denaturing conditions (8 M urea) using a peptide molecular weight kit consisting of intact sperm whale myoglobin (17000 Da) and four cyanogen bromide fragments of 14600, 8200, 6400, and 2600 Da.[36] Under these conditions, the enzyme migrates as a single band with an apparent molecular mass of 2800 Da. The 35 residues of the N-terminal amino acid sequence of 4-OT give a molecular weight of 3865, making the possibility of an octameric structure unlikely.[37] A reasonable possibility to explain these data is the suggestion that 4-OT is a tetramer. The structure of 4-OT is under active investigation.

Identification of the Product of the 4-OT-Catalyzed and Chemical Decay of 1. Wavelength scans are performed from 350 to 200 nm with a Perkin-Elmer 553 spectrophotometer at 30 °C. The buffer concentration used in both the chemical and enzyme-catalyzed ketonization of **1** is 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7.45; μ = 0.2 M NaCl). Chemical ketonization of **1** is performed by adding a quantity of **1** (14 μL) from a stock solution (8.0 mM) in methanol to 1.0 mL of buffer (final CH_3OH concentration is 1.4%). It is mixed by inversion, and spectra are recorded every 6 min until equilibrium is achieved. Enzyme-catalyzed ketonization of **1** is done by adding a quantity of 4-OT from *P. putida* (4 μL, 0.012 μg; specific activity 4700 units/μg) to 1.0 mL of buffer followed by the addition of a quantity of **1** (14 μL) from the same stock solution. After mixing by inversion, spectra are recorded every 2 min until equilibrium is obtained.

Conversion of 3 to 2-Hydroxy-3-*trans*-hexenedioate (4). 2-Hydroxymuconate (**1**; 15.2 mg, 0.096 mmol) in methanol (1.5 mL) is added in portions (0.15 mL) to a solution containing 4-OT from *P. putida* (44 units) in 20 mM KH_2PO_4 buffer (25 mL, pH 7.28). The ketonization process is monitored spectrophotometrically (λ_{max} = 236 nm) and is complete in approximately 5 min. The mixture is then treated with $NaBH_4$ (2 equiv). The final pH of the solution is 8.3. After the pH is adjusted to 8.0 with HCl, the solution is subjected to chromatography on a Dowex-1 (formate) column (0.8 × 7.5 cm), eluting with a formic acid gradient (0 to 2 M formic acid, 60 mL total volume). **4** elutes at approximately 1.4 M formic acid. Appropriate fractions are pooled and evaporated to dryness under mechanical vacuum to yield 12 mg (78%) of **4**. **4**: ^{1}H NMR (^2H_2O) δ 3.03 (2 H, d, J = 7.8 Hz, H-5), 4.58 (1 H, d, $J_{2,3}$ = 6.25 Hz, H-2), 5.62 (1 H, dd, $J_{2,3}$ = 6.25 Hz, $J_{3,4}$ = 15.6 Hz, H-3), 5.80 (1 H, ddd, $J_{3,4}$ = 15.6 Hz, $J_{4,5}$ = 7.8 Hz, H-4); ^{13}C NMR (^2H_2O) δ 39.7 (C-5), 73.5 (C-2), 129.6 (C-4), 132.7 (C-3), 178.5, 178.9 (C-1, C-6). The spectra also reveal the presence of **5**. The ratio of the intergrals corresponding to the methylene protons in **4** and **5** indicates that **5** makes up ~15% of the recovered product.

Kinetics of Nonenzymic Ketonization of 1. Buffers are made by the addition of calculated aliquots of 100 mM stock solutions of NaH_2PO_4 and Na_2HPO_4, plus the calculated amount of a 0.5 M NaCl solution necessary to maintain constant ionic strength, followed by dilution with H_2O in volumetric flasks. The ionic strength of all buffers is calculated to be 0.2 M. If necessary, small amounts of HCl or NaOH are added to adjust the pH of the buffer solution before use. For the higher buffer concentrations (10, 20, and 50 mM), the pH values of the solutions did not change significantly when measured before and after a run (<0.02 pH unit). In the case of low buffer concentrations (2 and 5 mM), there is a significant drop in pH (~0.3 unit). The pH reported in all cases is the final pH.

Decay of **1** is followed at 295 nm in a Beckman DU-7 spectrophotometer, which is connected to a Zenith portable computer. Kinetic runs are initiated by the addition of 6.8–8.8 μL of a stock solution of **1** in CH_3OH to 1.0 mL of the desired buffer, which is equilibrated at 30 °C prior to use. An identical solution without **1** is used in a reference cell. The final concentration of CH_3OH ranges from 0.6 to 0.9% and the final concentration of **1** is generally 56–59 μM. Buffer and **1** are mixed by inversion. It generally takes 10 s to mix buffer with **1** and to start the run. The rate constants obtained are reproducible in multiple runs. Stock solutions of **1** are freshly made after 24 h. Statistically identical results are obtained by using a solution of **1** made up in ethanol.

All reactions are monitored to a minimum of 5 half-lifes. The observed final absorbance readings, reflecting the equilibrium concentration of **1**, vary from 0.100 to 0.134 with the lower values being observed for the longer time runs and at pH 8.0. The variance is presumably due to the nonenzymic decomposition of **3** by decarboxylation. The model does not take this into consideration.

Absorbance readings for the decomposition of **1** are collected every 10 s and fit to the kinetic model (Scheme III) by nonlinear regression analysis.[13] The program assumes a 10-s delay before the first reading. In each case the model fits are excellent (correlation coefficient >0.9999). The individual sets of rate constants (k_1, k_{-1}, k_2, and k_{-2}) determined from these analyses are then fitted to eq 1 over the measured pH range and buffer concentration. The narrow pH range precludes a reliable estimate of k_{H^+} and k_{OH^-}. Buffer species other than H_2PO_4 and HPO_4 are assumed to be present at insignificant concentrations.

^{1}H NMR and ^{13}C NMR Detection of 3. 2-Hydroxymuconate is synthesized according to the procedure of Lapworth.[32] The proton and carbon NMR spectra of **1** in methanol-d_4 show only the presence of isomer **1**. Signals corresponding to **2** or **3** are not observed. Likewise, an initial proton NMR spectra of **1** in D_2O indicates only the presence of **1**. Dienol **1** is stored as a powder in a desiccator at room temperature and shows no evidence of decomposition after several months.

To a NMR tube containing 100 mM K[$^2H_2$]PO_4 in ^2H_2O (0.395 mL, pD = 7.49) is added a quantity of 4-OT (30 μL, 0.9 μg, 2 units). The enzyme is exchanged into ^2H_2O by repeated centrifugation of the enzyme in K[$^2H_2$]PO_4 buffer in a Centricon-10 microconcentrator. The reaction is initiated by the addition of **1** (0.86 mg/0.075 mL, 15% v/v ethanol) from a stock solution made in ethanol-d_5. The concentration of **1** in the NMR tube is 10.9 mM. The first NMR spectrum is recorded 3.5 min after addition of substrate and every 2 min thereafter until completion. Identical results are obtained by using **1** from a stock solution made in methanol-d_4. The concentration of methanol in these experiments cannot exceed 6% without significant loss of enzyme activity. **1**: ^{1}H NMR (^2H_2O) δ 5.84 (1 H, d, J = 15 Hz, H-5), 5.95 (1 H, d, J = 12 Hz, H-3), 7.16 (1 H, dd, J = 12, 15 Hz, H-4); ^{13}C NMR (CD_3OD) δ 109.0 (C-3), 122.2 (C-5), 139.6 (C-4), 148.2 (C-2), 166.6 (C-1), 170.5 (C-6). **3**: ^{1}H NMR (^2H_2O) δ 3.00 (1 H, br m), 6.11 (1 H, d, J = 16 Hz), 6.91 (1 H, dd, J = 9, 16 Hz); ^{13}C NMR (^2H_2O) δ 42.7 (C-5), 128.8 (C-3), 151.2 (C-4), 172.8 (C-1), 178.3 (C-6), 198.1 (C-2).

^{1}H NMR Proof for the Formation of 2 during Chemical Ketonization of 1. To a NMR tube containing 100 mM K[$^2H_2$]PO_4 in ^2H_2O (0.425 mL, pD 7.49) is added a quantity of **1** (1.05 mg/0.075 mL, 15% v/v ethanol) from a stock solution made in ethanol-d_5. The concentration of **1** in the NMR tube is 13.3 mM. After addition of **1**, spectra are recorded every 5 min for 2 h. Identical results are obtained by using **1** from a stock solution made in methanol-d_4. **2**: ^{1}H NMR (^2H_2O) δ 3.39 (1 H, br m, H-3), 5.80 (1 H, d, H-5), 6.46 (1 H, m to d, H-4).

Conversion of 2 to 2-Hydroxy-4-*trans*-hexenedioate (5). A solution of **1** (15.0 mg; 0.095 mmol) dissolved in CH_3OH (1.5 mL) is added in portions (0.15 mL) to a solution of 20 mM KH_2PO_4 aqueous buffer (25 mL, pH 7.28). The solution is treated with small quantities of $NaBH_4$ (2 equiv) at 3-min intervals for 27 min. An aliquot is removed from the solution and analyzed by UV spectroscopy. There is no appreciable UV absorbance in the 200–300-nm region. The final pH of the solution is 9.80. After the pH is adjusted to 8.1 with HCl, the solution is subjected to anion-exchange chromatography as described above to afford a mixture of **4** and **5** (14.0 mg, 94%). From the ratio of the integrals corresponding to the methylene protons of **4** and **5**, it is estimated that 94% of the mixture is **5**. **5**: ^{1}H NMR (^2H_2O) δ 2.52 (1 H, m, H-3), 2.66 (1 H, m, H-3), 4.34 (1 H, dd, H-2), 5.85 (1 H, d, $J_{4,5}$ = 15 Hz, H-5), 6.87 (1 H, dt, H-4); ^{13}C NMR (^2H_2O) δ 40.1 (C-3), 72.9 (C-2), 127.6 (C-5), 149.4 (C-4), 173.8 (C-6), 179.6 (C-1).

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Substrate Specificity of 4-OT for 1 and 2. 4-OT activity is assayed spectrophotometrically at 30 °C by following either the rate of disappearance of substrate (**1**) at 295 nm ($\epsilon = 24.1 \times 10^3$ M^{-1} cm^{-1}) or the rate of appearance of product (**3**) at 236 nm ($\epsilon = 6.58 \times 10^3$ M^{-1} cm^{-1}). All initial velocities reported in the nonequilibrium and equilibrium experiments are determined by the latter assay. In a nonequilibrium experiment, the assay mixture contains 20 mM Na_2HPO_4 buffer (1.0 mL, pH 7.3) and an aliquot (1 μL) of sufficiently dilute enzyme to obtain a linear rate. The assay is initiated by the addition of a quantity (1–10 μL) of **1** from either a 20 or a 50 mM stock solution made up in ethanol. In an equilibrium experiment, the assay mixture contains 20 mM Na_2HPO_4 buffer (1.0 mL, pH 7.3) and a quantity of substrate (1–10 μL) from the same stock solution. After 4 min, an aliquot (1 μL) of the same dilute enzyme is added to the mixture. The cuvettes are mixed by inversion. A cuvette containing only buffer is used as a blank in both experiments. At each substrate concentration, a nonequilibrium velocity is obtained immediately following the determination of an equilibrium velocity. No lag time in the production of **3** is observed in either experiment. The initial velocities are measured from linear portion of the first 5–10 s of the tracing except when very low concentrations of substrate are used. In this case, the initial velocity is measured from the first 15–20 s of the tracing. The stock solutions of **1** are made up just prior to the start of the experiment. Detectable decomposition of the solution is not observed during the lifetime of an experiment. Dilutions of enzyme are made 2 h prior to the start of an experiment. Inconsistent results are obtained otherwise. No significant inhibition of the enzyme by ethanol is observed at ethanol concentrations below 2.5% (v/v). All results are reproducible in multiple runs.

Analysis of Enzyme Kinetic Data. The data from the nonequilibrium and equilibrium experiments are fitted by using a statistical program known as MacEnzkin run on a Macintosh II computer. The program fits the initial velocity data as a function of substrate concentration to the best rectangular hyperbola by an iterative nonlinear least-squares method. It is a kind gift from Professor J. Westley and J. D. Ozeran (The University of Chicago).

For a mechanism in which two substrates A and B compete for the active site of the enzyme, the rate of product formation is described by eq 2,[9b] where A is the concentration of **1** at 4 min, B is the concentration

$$v = (V/K)_A(A) + (V/K)_B(B)/1 + (A)/K_A + (B)/K_B \quad (2)$$

of **2** at 4 min, K_A and K_B are the Michaelis constants for **1** and **2**, and V is the maximal velocity measured when the enzyme is fully saturated with substrate. The kinetic parameters K_B and $(V/K)_B$ in eq 2 are obtained from eqs 3 and 4, where K_{A+B} and $(V/K)_{A+B}$ are the kinetic

$$K_B = \%\ B/1/(K_{A+B}) - \%\ A/K_A \quad (3)$$

$$(V/K)_B = (V/K)_{A+B} - \%\ A(V/K)_A/\%\ B \quad (4)$$

constants observed in the equilibrium experiment, % A is the percentage of A present at 4 min, and % B is the percentage of B present at 4 min. Because the 4-OT reaction is not inhibited by the product, **3**, it is not necessary to include a product inhibition term in this analysis.

Acknowledgment. We gratefully acknowledge the Donors of the Petroleum Research Fund, administered by the American Chemical Society, the Biomedical Research Support Grant, administered by the University Research Institute at the University of Texas at Austin, and the National Institutes of Health (Grant GM 41239) for support of this research. In addition, we thank Professor I. A. Scott (Texas A&M University) for use of the Bruker AM 500-MHz NMR spectrometer. Finally, we thank Drs. Ralph Pollack, Albert Mildvan, W. W. Cleland, and Norman Oppenheimer for helpful discussions and suggestions.

Biosynthesis of Pyrroloquinoline Quinone. 2. Biosynthetic Assembly from Glutamate and Tyrosine

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Abstract: The biosynthesis of pyrroloquinoline quinone (PQQ), the prosthetic group of quinoproteins, was studied in the methylotrophic bacterium, *Methylobacterium extorquens* sp. AM1. Using ^{13}C-labeled precursors and NMR spectroscopy, we have elucidated the biosynthetic origin of PQQ. In an initial series of feeding experiments, *M. extorquens* AM1 was grown on [1-^{13}C]- or [2-^{13}C]ethanol, and the resulting ^{13}C enrichments in PQQ were compared to the labeling patterns in amino acids. These data revealed that PQQ is biosynthesized from two amino acids: one molecule of glutamate and one molecule of either tyrosine or phenylalanine. Direct incorporation of tyrosine was observed by using [^{13}C]tyrosine labeled in the phenol side chain and at the methylene position. Moreover, a double-labeling experiment with [^{15}N,^{13}C]tyrosine demonstrated that the pyrrole nitrogen is derived from the α-amino group of tyrosine. Therefore, carbons 2′, 2, 3, 3a, 4, 5, 5a, 9a, 1a and nitrogen 1 of PQQ are derived from tyrosine; the pyrrole ring forms via the intramolecular cyclization of the tyrosine. Carbons 7′, 7, 8, 9, 9′, and, in all probability, nitrogen 6 are derived from glutamate. Using this information, we have proposed biosynthetic pathways for the assembly of PQQ from tyrosine and glutamate.

Pyrroloquinoline quinone (PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione) is one of several *o*-quinones that serve as prosthetic groups in some redox enzymes. *o*-Quinone prosthetic groups are known in alcohol dehydrogenases,[1] amine oxidases,[2] and amine dehydrogenases.[3] These prosthetic groups can be divided into two classes based on the presence or absence of a covalent linkage between cofactor and enzyme. The covalently linked *o*-quinone cofactors are apparently the product of post-translational modification of amino acyl residues present in the parent protein. The best characterized of this class of compounds is 6-hydroxy-dopa, or topa, which has recently been identified at the active site of bovine serum amine oxidase,[4] suggesting that

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