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Kinetics and Mechanism of Allantoin Racemization

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The kinetics and mechanism of racemization of allantoin have been examined; racemization proceeds via two independent pathways that can be separately monitored. One pathway involves proton exchange at C5 with solvent. The other pathway occurs via intramolecular attack of N8 on C4 to form a symmetrical bicyclic intermediate, which can decompose to form either enantiomer of allantoin. The intramolecular pathway proceeds more rapidly from the allantoin anion than from neutral allantoin. This result is explained by conformational analyses based on experimental NMR data and computational results, which suggest that the ureido arm of anionic allantoin adopts a *cis*-conformation, allowing intramolecular attack. Neutral allantoin adopts a *trans*-conformation. The proton exchange pathway is buffer-catalyzed and also proceeds more rapidly at basic pH, although it is suggested that the reaction occurs from neutral allantoin. The relatively slow rate of racemization, particlarly at physiological pH, suggests that nonenzymatic racemization of allantoin is not a viable mechanism for the *in vivo* generation of (*S*)-allantoin.

INTRODUCTION

Allantoin (5-ureidohydantoin) serves as an important carrier of metabolic nitrogen in tropical leguminous plants such as soybeans. In these plants nitrogen is fixed from the atmosphere through the action of nitrogenase, which reduces gaseous dinitrogen to ammonia. The ammonia is used to synthesize purines which are converted to urate **(1)** and further oxidized through the ureide pathway to produce allantoin **(3)** and allantoate **(4),** the so-called ureides (Scheme 1).

The chemistry of the ureide pathway is surprisingly rich. Although it is widely believed that allantoin is the product of the urate oxidase reaction (*1,2*) it has recently been demonstrated that it is not the immediate product of the enzymatic reaction, and the details of the biogenesis of allantoin remain unknown. The true product of the urate oxidase reaction is 5-hydroxyisourate **(2),** which quantitatively decomposes to allantoin under neutral aqueous conditions *in vitro* (*3,4*). The conversion of 5-hydroxyisourate to allantoin involves hydrolysis of the purine ring and an unusual 1,2 carboxylate shift (*3*). It is unclear whether those transformations which are observed *in vitro* are a part of the ureide pathway. Nonenzymatic decomposition of optically active 5-hydroxyisourate which is produced by urate oxidase generates

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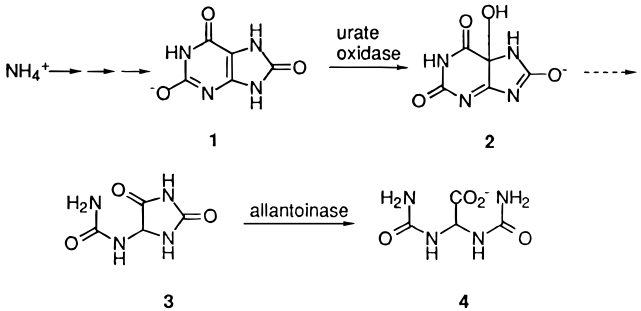


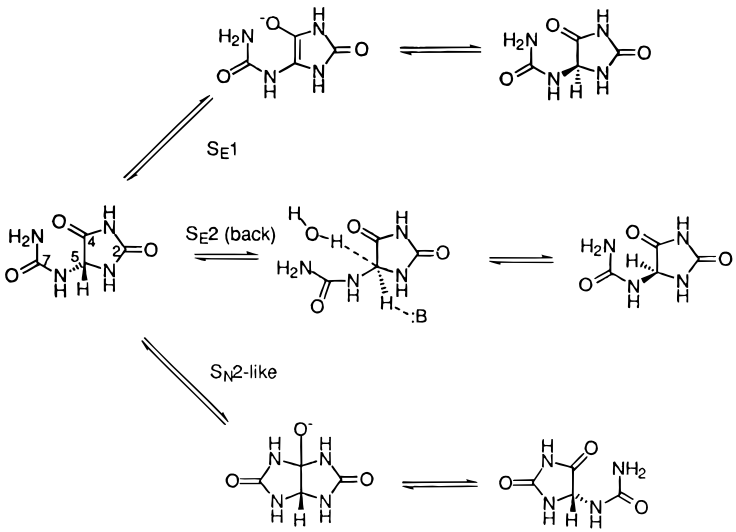
SCHEME 1

racemic allantoin (*5*), and it has been reported that allantoinase, which catalyzes the conversion of allantoin the allantoate, is specific for the *S*-enantiomer of allantoin (*6*). Given the metabolic importance of the ureide pathway, and the presumption that flux through it should be rapid and efficient, two scenarios for the metabolic conversion of 5-hydroxyisourate to allantoate can be envisioned: that there exist hitherto undescribed enzymes that mediate the stereospecific conversion of 5-hydroxyisourate to *S*-allantoin or that allantoinase draws off the *S*-enantiomer from the pool of racemic allantoin, which reequilibrates through nonenzymatic or enzymatic chemistry. There have been no reports of an allantoin racemase occurring in plants, to our knowledge. In order to evaluate whether nonenzymatic racemization of allantoin could constitute a viable biochemical pathway, we have examined the kinetics and mechanism of that reaction.

Like other 5-substituted hydantoins, allantoin could racemize via an S_E2-type reaction or an S_E1 mechanism involving a carbanion intermediate that is stabilized by tautomerization (Scheme 2) (*7*). Allantoin also has the unique possibility of racemizing via formation of a symmetrical bicyclic intermeidate formed by attack of the primary amide nitrogen at C4 (*8*). The former mechanisms involve cleavage of the C5-H bond and can be monitored by measuring the rate at which the C5 proton exchanges with solvent D_2O. In the latter mechanism the C5-H bond is not cleaved, but if this mechanism is operative the rate of racemization can be determined by measuring the rate at which C2 and C7 of allantoin interconvert. We have determined the pH-dependence of proton exchange and of C2–C7 interconversion, as well as the pH-dependence of allantoin racemization. In order to rationalize the kinetics data, we have examined the conformation of neutral allantoin and its anion by NMR and computational methods.

EXPERIMENTAL SECTION

Allantoin was purchased from Sigma Chemical Co. and used without further purification. [2-^{13}C]Allantoin and [7-^{13}C]allantoin were generated by the urate oxidase-catalyzed oxidation of [8-^{13}C]urate and [2-^{13}C]urate, respectively. The syntheses of regiospecifically-labeled [^{13}C]urates have been described (*3*). Recombinant thioredoxin-urate oxidase fusion protein was purified as described (*18*). Catalase was purchased from Sigma Chemical Co. Enzyme-catalyzed urate oxidations were typically performed by dissolving 0.03 g (0.17 mol) [^{13}C]urate in 8 ml 100 mM phosphate buffer in D_2O



SCHEME 2

at pD 7.2; approximately 5 units of urate oxidase and 1200 units of catalase were added, and the solution was gently bubbled with O_2 at room temperature for 5 h. The enzymes were removed by ultrafiltration, and the filtrate containing [^{13}C]allantoin was evaporated to dryness under reduced pressure. (-)Allantoin was purified from a culture of *Pseudomonas aeruginosa*, strain PAO1, grown on racemic allantoin as the sole carbon source as described (*19*).

NMR spectroscopy. 1H NMR spectra of allantoin were acquired in a 50:50 (v/v) mixture of d_6-DMSO and $CDCl_3$ with 0.12 Hz spectral resolution using a Bruker AVANCE DRX500 spectrometer. A selective 1D saturation experiment was performed by irradiating either the N1-H resonance or the N8-H resonance and observing the coupling pattern of the C5 proton. The NOESY spectrum of allantoin was recorded in d_6-DMSO using a mixing time of 0.8 s and a repetition time of 1.24 s. The three-bond proton–proton coupling constants were obtained from the 1D spectrum of allantoin. The values of $^3J_{CH}$ (C4-C5-N1-H1) and $^3J_{CH}$ (C4-C5-N6-H6) were obtained from the analysis of the 1H NMR spectrum of [4-^{13}C]allantoin in d_6-DMSO. Values of $^3J_{C(O)NCH}$ were obtained from the analysis of ^{1}H and ^{13}C NMR spectra of [2-^{13}C]allantoin and [7-^{13}C]allantoin in D_2O at pD 7.0 and 10.9. The ^{13}C NMR spectra that provided the heteronuclear coupling information were recorded with a spectral resolution of 0.075 Hz using a gated decoupling pulse program. NOEs detected between pairs of protons in allantoin were classified as strong, medium, or weak; the NOEs were compared to a reference NOE observed between C5-H and N1-H. In all conformers of allantoin the distance between these two protons is 2.6 ± 0.1 Å, and the observed NOE was classified as strong.

The dihedral angles in allantoin and the corresponding three-bond coupling constants were evaluated using Karplus-type relationships (*20*). The expected coupling constants between C5-H and N1-H and between C5-H and N6-H in each conformer were calculated using Eq. [1] (*21*). The expected value of the heteronuclear coupling between C4 and N1-H and between C4 and N6-H was calculated using Eq. [2] (*22*).

The expected values of the coupling constants between C5-H and C2 and between C5-H and C7 were calculated with Eq. [3] (*23*).

$$^3J_{HCNH} = 6.4 \cos^2\alpha - 1.4 \cos \alpha + 1.9 \quad [1]$$

$$^3J_{C(O)CNH} = 9.5 \cos^2\beta - 4.4\cos \beta - 0.8 \quad [2]$$

$$^3J_{C(O)NCH} = 3.96 \cos^2\theta - 1.83 \cos \theta + 0.81 \quad [3]$$

Proton exchange assay. The exchange of the proton at C5 of allantoin with solvent deuterium was studied at 22°C over the pH range 5.7 to 10.0 by integrating the allantoin proton resonance at 5.40 ppm relative to the signal from the internal standard 3-(trimethylsilyl) propionate-2,2,3,3,–d_4 in 99.9% D_2O. Under these experimental conditions proton loss from C5 is essentially irreversible. The pH of the solution was maintained at the desired value using sodium phosphate. It was determined that phosphate catalyzed the allantoin proton exchange with solvent; therefore, the reaction was conducted at several different phosphate concentrations between 10 and 125 mM at each pH value. The decrease in intensity of the C5 proton signal followed first order kinetics; the observed first order rate constant was linearly dependent on phosphate concentration, so the rate constant for the uncatalyzed exchange at each pH value was obtained by linear extrapolation to zero phosphate concentration. The pH dependence of the rate constants for the uncatalyzed reaction was fitted to Eq. [4], where k_{max} is the plateau value of the rate constant K_a is the acid dissociation constant governing the observed process.

$$\log k = \log [k_{max}/(1 + [H^+]/K_a)] \quad [4]$$

^{13}C Exchange assay. The exchange of ^{13}C between C2 and C7 of allantoin was monitored by ^{13}C NMR at 22°C. Solutions containing 25 mM [7-^{13}C]allantoin were prepared in phosphate buffer in 90% D_2O; control experiments established that phosphate does not catalyze the interconversion of C2 and C7. The rate of exchange was determined in the pH range 6.9 to 9.6. The exchange process followed first order kinetics. The observed rate constant for exchange from C2 to C7 was the same as for exchange from C7 to C2 (data not shown). The microscopic rate constant for conversion of [7-^{13}C]allantoin to [2-^{13}C]allantoin is identical to the microscopic rate constant for the reverse process, and the microscopic rate constants are equal to half of the observed rate constant. The pH dependence of the observed rate constants was fitted to Eq. [4].

Circular dichroism assay. The loss of optical activity from solutions of (–)-allantoin was monitored by circular dichroism spectropolarimetry. Solutions of 6 mM (–)-allantoin were prepared in sodium phosphate buffers in the pH range 6.5 to 10.0. The phosphate buffer concentrations were 10, 100, and 200 mM; however, it was determined that the rate of loss of optical activity was independent of the phosphate concentration. CD spectra were obtained at 25°C in 1-mm pathlength quartz cuvettes. The maximum Cotton effect for allantoin was observed to vary from 210 nm at pH 6.5 to 232 nm at pH 10, and the rate of loss of optical activity at each pH value was measured by monitoring the CD signal at the signal maximum. The observed CD intensities decreased exponentially over time, and the first order rate constants determined at each pH value were fitted to Eq. [4].

Computational methods. Conformations of allantoin were evaluated computationally as follows. Allantoin consists of a relatively rigid imidazoline ring and a more flexible ureido

sidechain attached to C5. A grid search with 20 degree spacing over the conformational space of the ureido sidechanin was performed with a Tripos force field (*24*), using the program SYBYL (Tripos, Inc.). The energies of 5832 conformations were evaluated and six local minima were identified. Full molecular mechanics optimizations starting from these minima converged to six conformations that were further studied with the AM1 semi-empirical quantum mechanical model (*25*), using the program MOPAC (Version 6.0, U.S. Air Force Academy). The AM1 geometries were used as starting points for optimization at the HF/6-311+G(d,p) level with Gaussian 94 (*26*). Initial geometries for the optimization of the allantoin anions were obtained by removing a proton from N3 of the neutral conformer.

RESULTS AND DISCUSSION

It seems clear that racemization of allantoin can occur via two distinct pathways which can be monitored independently. Figure 1 illustrates that C2 and C7 of allantoin interconvert over time under physiologically relevant conditions. The most likely mechanism for this interconversion is via the intermediacy of the bicyclic species shown in

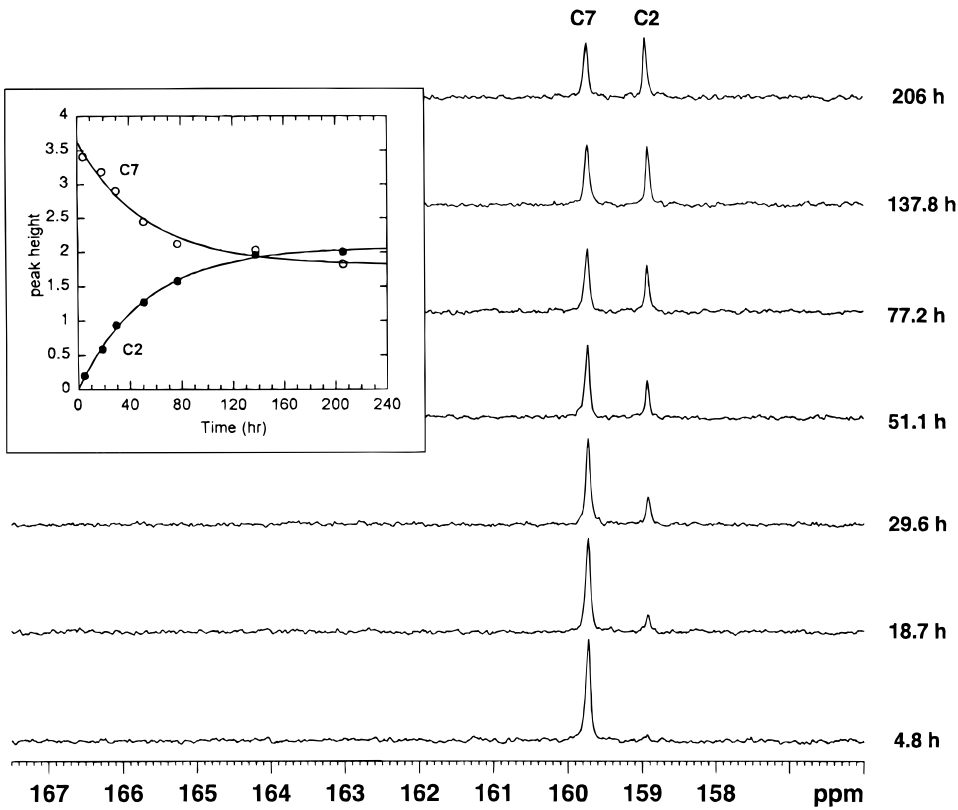


FIG. 1. Isotopic exchange between C2 and C7 of allantoin at pD 7.4. Starting material was [7-^{13}C]allantoin. The lines through the experimental points were obtained by fitting the data to the equation describing a first order process.