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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Allantoin as a Free-Radical Scavenger

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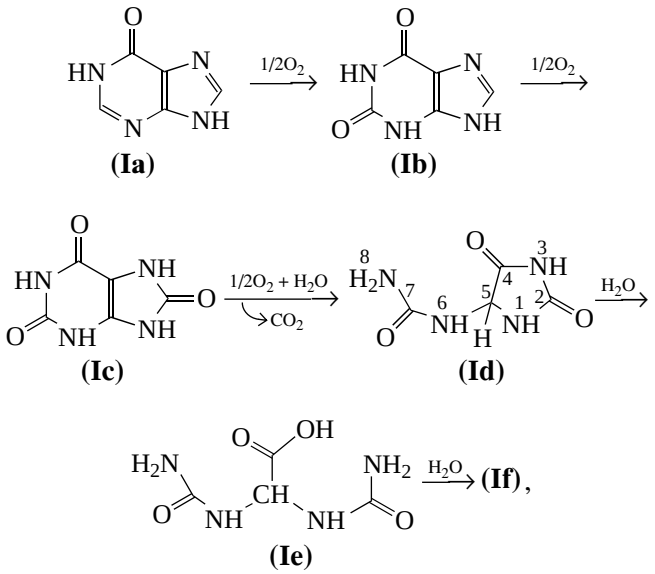
In vertebrates, free purine bases are deaminated mainly to hypoxantine (Ia) or xantine (Ib), which are further oxidized to uric acid (Ic) in the presence of oxygen. Its further conversion (Ic to If) is shown in the following scheme:



where (Ia) hypoxantine; (Ib) xantine; (Ic) uric acid; (Id) allantoin; (Ie) allantoic acid; (If) mixture of urea with glyoxilic acid.

Allantoin was shown to affect the activities of enzymes involved in regulation of the so-called reactive-oxygen-species (ROS)-dependent status of an organism [2]. The results reported in [2] support the hypothesis that intermediates of purine base catabolism are antioxidants [3]. For example, uric acid is a potent antioxidant, and *Drosophila* strains lacking the ability to synthesize uric acid are extremely sensitive to paraquat, ionizing radiation, and hyperoxia. Double mutants deficient in both Cu, Zn-superoxide dismutase and urate are synthetic lethals [4]. In model systems, uric acid protects DNA against damage from ROS [5].

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In most vertebrates, except for primates, allantoin is the immediate catabolite of uric acid. Therefore, it is of undoubted interest whether allantoin, like uric acid, can modify the genotoxic effects of ROS in humans, the more so as, owing to its higher solubility in aqueous media, allantoin may be superior to uric acid in this respect.

Peroxidation mutagenesis is commonly used to study the genetic effect of oxidative stress. For example, some researchers relate the anticancer properties of shark cartilage to its ability to suppress peroxidation mutagenesis [6]. Therefore, in this study, we assessed the effects of allantoin on the H_2O_2-induced mutagenesis and on the induction of the SOS response in *Escherichia coli*.

We used the *E. coli* strain PT-1 (a gift from L.A. Ptitsyn) obtained by transformation of the C600 strain with plasmid pPLS-1 carrying the *Lux* operon controlled by an SOS-response promoter [7]. To induce mutagenesis, H_2O_2 was used at nontoxic concentrations. Allantoin was added to the final concentration of 10^{-6} M, which proved to be the most efficient in preliminary model experiments.

To determine the frequency of rifampicin-resistant mutants [8], we prepared 1-ml aliquots of a PT-1 cell culture in an LB medium (10^4 cells/ml) and added to each aliquot the necessary volume of 10^{-3} M H_2O_2. Allantoin was added 90 min before H_2O_2. The aliquots were incubated at 37°C for 14 h, and 50 μl from each of them was then used to inoculate rifampicin-containing LB plates. The total number of cells was determined by counting the colonies grown on LB plates inoculated with 50 μl of the suspension diluted 10^6 times. Colonies were counted after incubation at 37°C for 48 h.

The effect of allantoin on the SOS response of *E. coli* was assessed using the SOS-*lux* test. The final concentration of H_2O_2 was 10^{-6} M. Allantoin (150 μl) was added to 3-ml aliquots of the bacterial suspension to the final concentration of 10^{-5} M either 1.5 h before the H_2O_2 treatment, simultaneously with H_2O_2, or 1.5 h after its addition (i.e., after a 1.5-h incubation in the presence of H_2O_2). In control experiments, distilled water instead of allantoin was added to the culture samples to record spontaneous bioluminescence from *E. coli*. The optical signal was recorded using a chemiluminometer designed on the basis of a PFT spectrometer (model 220298, Germany). The degree of

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Table 1. Rif^r mutant frequency in *E. coli* treated with H_2O_2 and allantoin

H_2O_2, concentration, M	Number of Rif^r mutants per 10^7 cells	
	no allantoin	allantoin
0	2.4 ± 0.38	1.2 ± 0.26
10^{-7}	3.2 ± 0.32*	1.08 ± 0.29
10^{-6}	17.2 ± 0.28*	1.0 ± 0.17

* $p < 0.05$ vs. control.

luciferase induction (the induction factor for Ic) was determined as the ratio of the luminescence intensity in the presence of allantoin to that in the control.

As can be seen from Table 1, H_2O_2 at both concentrations used (10^{-7} and 10^{-6} M) induced mutations in the *E. coli* tester strain. Allantoin added 1.5 h before the H_2O_2 treatment decreased the mutant frequency at any H_2O_2 concentration.

Interestingly, the background mutant frequency decreased by half in the presence of allantoin. A similar effect was reported for mammals [2]. Table 2 shows how allantoin affected the SOS induction in H_2O_2-treated *E. coli* cells. Bacterial cells treated with H_2O_2 developed a marked SOS response, whereas the activity of the SOS system in control cells was low. Incubation of bacterial cells in the presence of allantoin for 1.5 h prior to the H_2O_2 treatment eliminated this effect: the induction factor remained as low as it was in control cells.

If allantoin was added 1.5 h after the treatment of *E. coli* cells with H_2O_2, the level of the SOS response slightly decreased. If allantoin and H_2O_2 were added simultaneously, no changes in the induction factor could be detected.

The nonspecific SOS response plays a key role in protecting *E. coli* cells against genotoxic damage by various agents and proceeds through simultaneously expressing 20 genes in a highly coordinated manner [9].

The fact that allantoin inhibited both mutagenesis and the SOS response to H_2O_2 treatment indicates its ability to directly protect DNA from damage induced by the hydroxyl radical, as uric acid usually does. To assess the correspondence between the biological effects of allantoin and the mechanisms of protection it can afford, we performed quantum chemical calculations of the key stage of free radical attacks on allantoin by some ROSs.

As is obvious from the scheme given above, free radicals can interact with allantoin (Id) and the known free radical scavengers hypoxantine, xantine, and uric acid [1] (Ia, Ib, and Ic in the scheme, respectively) through different mechanisms. In uric acid, NH bonds are the only possible target of an attack by free radicals. However, in hypoxantine, xantine, and especially allantoin, CH bonds may also be their target and compete with NH bonds in this respect.

For the model reaction,

$$(\mathrm{Id}) + \mathrm{R}^{\bullet} \rightarrow (\mathrm{Id})^{\bullet} + \mathrm{HR}, \quad \text{where} \quad \mathrm{R}^{\bullet} = \mathrm{HO}^{\bullet}, \mathrm{HO}_2^{\bullet},$$

we performed nonempirical energy calculations using the MP2(fc) (second-order Möller–Plesset frozen-core) method at the level of a 6-31G** basis set, as described in more detail previously [10].

Table 2. Effect of allantoin on the induction of the SOS response in H_2O_2-treated *E. coli* cells (as assessed by the induction factor in the SOS-lux test)

Experimental conditions	No H_2O_2	Allantoin 1.5 h before H_2O_2	Allantoin and H_2O_2 added simultaneously	Allantoin 1.5 h after H_2O_2
Control (H_2O)	1	1	1	1
H_2O_2		8.91 ± 0.10	5.9 ± 0.31	5.14 ± 0.14
Allantoin	1.07 ± 0.11	0.95 ± 0.12	5.40 ± 0.06	4.12 ± 0.06
p_1			<0.01	<0.001
p_2		<0.001		<0.01

Note: p_1, significance of difference from the control; and p_2, significance of difference from the values observed in H_2O_2-treated cells in the absence of allantoin.

Table 3. Energetic characteristics of the (Id), (Id)$^{\bullet}$, R$^{\bullet}$, and RH systems as calculated by the MP2(fc) method at the level of a 6-31G** basis set for the gas phase and by the scheme described Minkin et al. [12] for an aqueous environment

Characteristic	System						
	(Id)	(Id)$^{\bullet}$	(Id)$^{\#}$	$HO^{\bullet}$	$HO_2^{\bullet}$	H_2O	H_2O_2
Total energy (gas phase), atomic units	−599, 14165	−598, 50570	−674, 67862	−75, 53180	−150, 50748	−76, 21906	−151, 14832
Solvatation energy, kcal/mol	−29.3	−28.3	−31.0	−5.0	−5.7	−19.8	−8.9

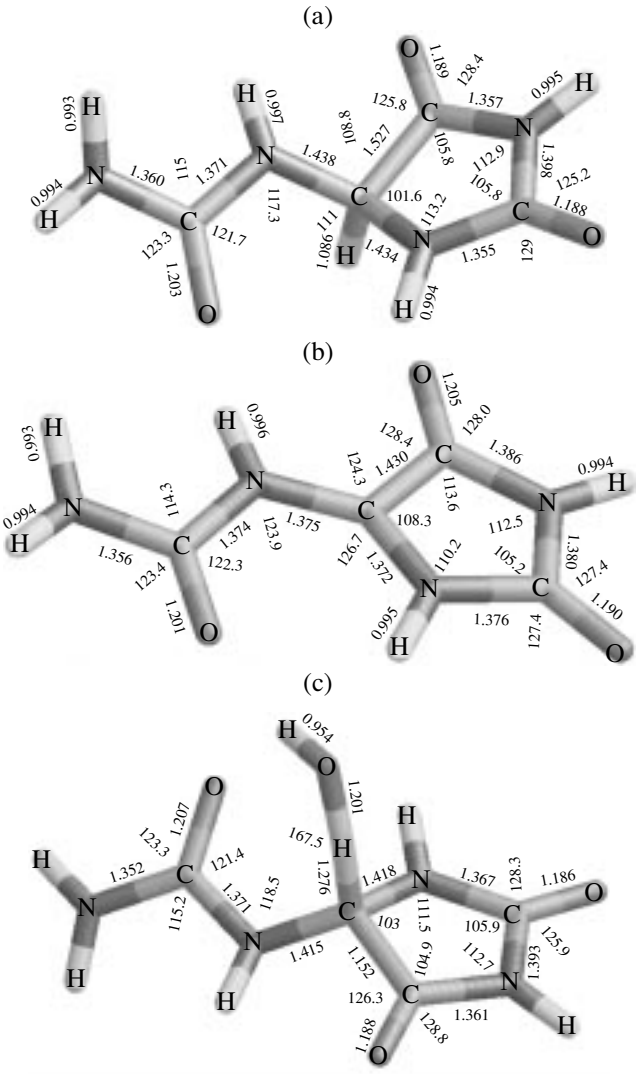

Fig. 1. Geometrical characteristics of (a) allantoin (Id), its (b) radical (Id)˙, and (c) intermediate (Id)#, as calculated by the MP2(fc) method at the level of a 6-31G** basis set. Bond lengths are in Angstroems (Å), and valence angles in degrees.

Figure 1a shows the geometry calculated for allantoin. These results are consistent with the data of X-ray analysis of its structure [11]. The radical configuration of allantoin that is most stable energetically is formed when a hydroxyl radical attacks the CH bond of the C atom at position 5 (C^5-radical), whereas all the N-radicals of allantoin are energetically much less favorable in the gas phase and in nonpolar solvents (Fig. 1b, Table 3).

The processes of NH bond breakage in the cyclic part of allantoin are energetically unfavorable. Compared with the C^5 radical, the N^1 and N^2 radicals are less stable (by 21.0 and 27.1 kcal/mol, respectively).The N^6- and N^8-radicals are also energetically less favorable (by 16.7 and 18.3 kcal/mol, respectively). Moreover, all the NH bonds of allantoin are known to be protected in condensed media by almost linear hydrogen bridges N–H··O [10], providing one more argument in favor of the suggestion that it is the CH bond of the C atom at position 5 that is the target of radical attacks.

As is seen from Table 3, the greatest energy advantage found in the gas phase and in nonpolar solvents equals 14.9 kcal/mol; in aqueous environments, this value was estimated at 28.7 kcal/mol. Obviously, the π-bond conjugation resulting in the formation of the united electronic system allows a planar radical of allantoin to be thermodynamically stable.

In addition, we identified the intermediate state of the (Id) → (Id)˙ process with the geometrical characteristics shown in Fig. 1c. It is an expected result that the free radical attack on allantoin proceeds through the formation in the intermediate state, (Id)#, of an almost linear hydrogen bond C^5–H··O, for which the attack angle is about ~168° [13]. The Hessian matrix calculated for this system has one negative eigenvalue equal to 3200 cm^{-1}. The energy barrier for breaking hydrogen from allantoin is very low: 3.2 and 6.5 kcal/mol in the gas and aqueous phases, respectively (Table 3).

Thus, both the experimental and theoretical results strongly suggest that allantoin behave as a potent antioxidant in its reactions with ROSs.

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