Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 727-732

XANTHINE OXIDASE CATALYZED BINDING OF 1-NITROPYRENE TO DNA

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Received December 4, 1981

SUMMARY: Xanthine oxidase, a mammalian nitroreductase, catalyzed the binding of [^{3}H]1-nitropyrene to DNA. The binding was dependent on the presence of hypoxanthine and was inhibited by allopurinol, a specific xanthine oxidase inhibitor. These data support the hypothesis that nitroreduction is a necessary step in the metabolic activation of 1-nitropyrene to a bacterial mutagen.

Polycyclic aromatic hydrocarbons are environmental contaminants which pose a mutagenic and carcinogenic hazard (1-3). Certain derivatives of these compounds, the nitro polycyclic aromatic hydrocarbons, may also be ubiquitous pollutants as evidenced by studies from a number of laboratories (4-9). In addition to data suggesting their environmental occurrence, several reports have demonstrated that nitro polycyclic aromatic hydrocarbons are potent bacterial mutagens. Pitts and co-workers, for example, have shown that even in the absence of a mammalian liver homogenate metabolizing system, 3-nitroperylene and 1-, 3-, and 6-nitrobenzo[a]pyrenes were mutagenic to Salmonella typhimurium strain TA 98 (5,10). Rosenkranz et al. (7) and Lofroth et al. (6) have reported that the bacterial mutagenicity of carbon-black xerographic toners was largely due to a series of mono-, di-, tri-, and tetranitropyrenes.

It should be noted that with some nitro polycyclic aromatic hydrocarbons, most notably 1-nitropyrene, the bacterial mutagenicity decreased when mammalian liver homogenates were included in the incubation (9). Furthermore, Wang and co-workers (8) have shown that 1-nitropyrene is less mutagenic in nitroreductase deficient strains (S. typhimurium TA 98 NR) than in normal tester strains (TA 98). These data suggest that the bacteria actively participate in the formation of the ultimate mutagen and that this reactive metabolite is

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formed through reduction of the nitro moiety. Nitroreduction would presumably generate arylhydroxylamines, species which have been shown to react with DNA, especially under slightly acidic conditions (11-14). In support of this concept, rat liver cytosol and microsomes catalyzed the reduction of nitrofurans to protein binding metabolites (15). Furthermore, bacterial and mammalian nitroreductases have been implicated in the metabolic activation of 4-nitroquinoline-1-oxide (16) and niridazole (17) to mutagens.

In order to assess the importance of nitroreduction in the mutagenic activation of 1-nitropyrene, we have examined the ability of a mammalian nitroreductase, xanthine oxidase, to catalyze the binding of this nitro polycyclic aromatic hydrocarbon to DNA.

MATERIALS AND METHODS

[4,5,9,10-^{3}H]1-nitropyrene (sp. act. 117 mCi/mmol; radiochemical purity >99%) was obtained from Robert W. Roth, Midwest Research Institute, Kansas City, MO. Bovine milk xanthine oxidase (EC 1.2.3.2), hypoxanthine, calf thymus DNA (Type I), Bis-Tris, and allopurinol were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were at least reagent grade.

The xanthine oxidase catalyzed binding of 1-nitropyrene to DNA was performed as follows: to a 50 mM potassium phosphate buffer, pH 5.8, was added 0.5 mg/ml hypoxanthine and then 2 mg/ml calf thymus DNA. The solution was purged with argon for 15 min and then [^{3}H]1-nitropyrene (stock solution of 1 mg/ml in dimethylsulfoxide) was added to give a 20 μM final concentration. Following addition of xanthine oxidase at 0.0, 0.05, 0.1, or 0.5 U/ml, the mixtures were incubated under argon at 37°C with shaking for time intervals up to 4 hrs. The incubations were terminated by addition of an equal volume of water-saturated chloroform:isoamyl alcohol:phenol (24:1:25), followed by vigorous mixing and centrifugation. This extraction sequence was repeated two additional times and then the DNA was precipitated from the aqueous phase by addition of three volumes ice-cold ethanol. The DNA was dissolved in a 5 mM Bis-Tris-HCl (pH 7.1) buffer containing 0.1 mM EDTA and again precipitated by adding ice-cold ethanol. After the DNA was redissolved in the Bis-Tris buffer, the DNA content was determined spectrophotometrically with a Gilford model 2400-2 spectrometer and the extent of [^{3}H]1-nitropyrene binding was established with a Searle Mark III scintillation counter using Scintisol (Isolabs, Akron, OH). Inhibition experiments were conducted in the same manner, but with 0.74, 2.2, and 4.4 μM allopurinol included in the incubation. The results are expressed as the mean $\pm$ standard deviation for at least three separate incubations.

RESULTS AND DISCUSSION

Mammalian nitroreductases exist in both the cytosol and endoplasmic reticulum. The cytosolic enzymes have been identified as xanthine oxidase, aldehyde oxidase and DT-diaphorase (18-20), while the microsomal nitrore-

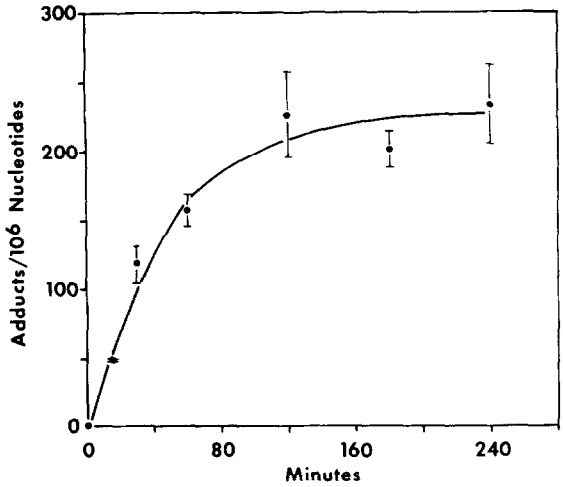

Figure 1. Xanthine Oxidase Catalyzed Binding of 1-Nitropyrene to DNA. The incubation consisted of: 50 mM potassium phosphate buffer (pH 5.8), 2 mg/ml calf thymus DNA, 3.7 mM hypoxanthine, 0.5 U/ml xanthine oxidase, and 20 μM [^{3}H]1-nitropyrene. At the times indicated, the incubations were terminated and the extent of binding determined as outlined in Materials and Methods. Each point represents the mean ± standard deviation of at least three separate incubations.

ductases are possibly NADPH- and NADH-dependent cytochrome P-450 reductase (15,18,19) and/or cytochrome P-450 (21-22). Although bacterial nitroreductases have not been as thoroughly investigated, E. coli contains both O_2-sensitive and O_2-insensitive enzymes (23).

Incubation of [^{3}H]1-nitropyrene with xanthine oxidase and hypoxanthine resulted in nitropyrene becoming covalently bound to added calf thymus DNA. The extent of binding was linear for approximately 30 min and reached a plateau at 120 min (Figure 1). Assuming that the nitropyrene bound to the DNA had the same specific activity as the initial substrate, then under these incubation conditions the final binding level reached 200-250 adducts/10^6 nucleotides.

Adduct formation was dependent upon the presence of xanthine oxidase and increased as the enzyme concentration was increased (Figure 2). In the absence of hypoxanthine, no binding was detected. Xanthine could replace hypoxanthine in the incubation; however, the extent of adduct formation was much lower. This may be due to the lower solubility of xanthine compared to hypoxanthine. Inclusion of allopurinol, a specific xanthine oxidase

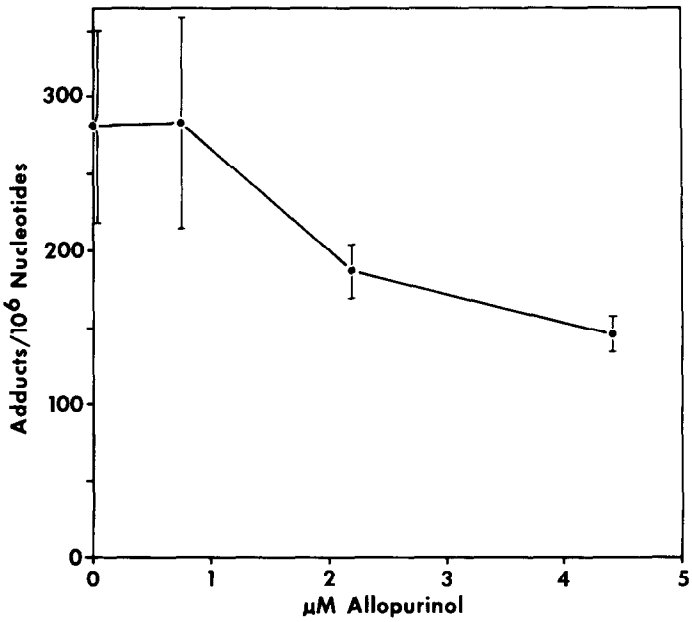

Figure 2. The Effect of Xanthine Oxidase Concentration of 1-Nitropyrene Binding to DNA. The incubation conditions are as described for Figure 1, with incubation times of 0.5 hr (▲) or 1.0 hr (●), at the enzyme concentrations indicated.

inhibitor in the incubation, decreased the binding of 1-nitropyrene to DNA in a concentration-dependent manner (Figure 3).

The results suggest the following sequence (Figure 4). During the oxidation of hypoxanthine to xanthine, xanthine oxidase (a molybdoflavoprotein) becomes reduced. The 1-nitropyrene is a substrate for the reduced enzyme, resulting in the re-oxidation of the enzyme and the formation of N-hydroxy-1-aminopyrene. As has been noted with other arylhydroxylamines (11-14), this species then reacts with nucleophilic sites in the DNA to form a covalent adduct. Although not indicated on the scheme, xanthine may also participate in this sequence by becoming oxidized to uric acid. In addition, it appears that N-hydroxy-1-aminopyrene may be further reduced or undergo auto-oxidation because examination of the incubation medium by reversed-phase high pressure liquid chromatography indicated the presence of 1-aminopyrene, which increased in concentration in a manner that paralleled the DNA binding. N-Hydroxy-1-aminopyrene was not detected by this chromatographic procedure, but its inherent reactivity may have precluded its determination.

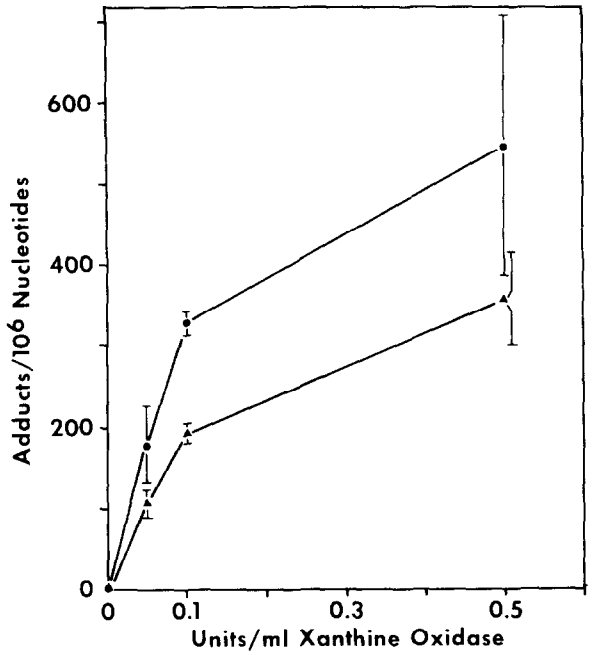

Figure 3. Allopurinol Inhibition of Xanthine Oxidase Catalyzed Binding of 1-Nitropyrene to DNA. The incubation conditions are as described for Figure 1, with a 1.0 hr incubation, at the allopurinol concentrations indicated.

In summary, these experiments lend support to the suggestions that nitroreduction of 1-nitropyrene yields a species that readily reacts with DNA and, therefore, may be responsible for the bacterial mutagenic activity observed with this compound. Furthermore, our data suggest that xanthine oxidase may be used for the efficient *in situ* generation of arylhydroxylamines, reactive species which may resist preparation by standard synthetic techniques.

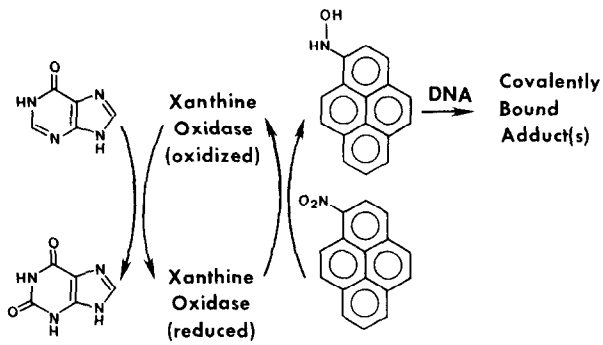

Figure 4. Proposed Pathway for Xanthine Oxidase Catalyzed Binding of 1-Nitropyrene to DNA.

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