The Effects of 8-Azaguanine and 6-Mercaptopurine on Purine Catabolism in the Rat*

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Various purine analogs active as carcinostatic agents have previously been shown to act as potent inhibitors of xanthine oxidase in vitro in both homogenate and isolated enzyme systems (4, 5). 8-Azaguanine (8-AG) has been demonstrated to be an in vitro inhibitor of both xanthine oxidase and uricase (4, 9). Elion et al. have reported that 6-mercaptopurine (6-MP) is a substrate for xanthine oxidase and is oxidized to thiouric acid (3). It seemed possible, therefore, that 6-MP could competitively interfere with the interaction of this enzyme with its physiologic substrate.

It is the purpose of this study to determine whether these in vitro relationships are of exclusive interest to the enzymologist or whether they occur in vivo and therefore deserve the attention of those interested in ascertaining the mechanism of action of carcinostatic agents. With isotopically labeled compounds used to follow biochemical events in vivo, it is possible to elucidate the effects of drugs on these metabolic reactions.

In rats, xanthine is oxidized by xanthine oxidase to uric acid, which is then further oxidized and decarboxylated by uricase to allantoin (Chart 1). In this reaction sequence, the number 6 carbon of xanthine is converted to CO₂. With xanthine-6-C¹⁴, the rate at which the radioactive carbon atom appears in the expired CO₂ provides a direct measure of xanthine catabolism and thus permits study of the in vivo effects of drugs on the sum of the xanthine oxidase- and uricase-catalyzed reactions. The use of uric acid-6-C¹⁴ permits elucidation of the in vivo effects of drugs on the uricase-catalyzed reaction alone.

**MATERIALS AND METHODS**

A crude preparation of xanthine-6-C¹⁴—which had been prepared from guanine-6-C¹⁴ (Isotope Specialties Co.) by deamination with nitrous acid and therefore contained a mixture of xanthine, guanine, and nitroso intermediary compounds—was purified in this laboratory with a Dowex-50 ion exchange column in the acid form. The xanthine was separated from all contaminants by elution with 0.6 N HCl. The purity of the isolated radioactive xanthine was verified with paper chromatographic techniques, with isopropanol:2 N HCl (4:6 v/v) as the developing solvent. The isolated xanthine-6-C¹⁴ was dissolved in 0.9 per cent saline and the pH adjusted to 9.0 with NaOH. One ml. of this xanthine solution contained 918,000 counts/min.

Radioactive uric acid was prepared enzymatically from the xanthine-6-C¹⁴. Five ml. of the purified xanthine-6-C¹⁴ was added to 5.0 ml. of 0.1 M glycyl-glycine buffer, pH 8.0; 0.5 ml of 10 per cent catalase; 10 ml. of water; and 0.1 ml. of purified cream xanthine oxidase. The reaction mixture was incubated overnight at 31°C. in a Dubnoff apparatus under an atmosphere of air. The mixture was then deproteinnzzed by being shaken in two successive treatments with 2 ml. of chloroform: octanol (4:1 v/v). After removal of the protein gel by centrifugation, the supernatant was adjusted to
pH 8.5 with concentrated ammonium hydroxide. The uric acid-6-C\textsuperscript{14} was then purified by means of a Dowex-1 column in the formate form. With the use of a gradient system in which 1 m sodium formate flows into a 200-ml reservoir of water, it was possible to obtain uric acid free of contaminants. The purity of the isolated radioactive uric acid was verified by ultraviolet spectrophotometry and by paper chromatographic techniques. The final solution of uric acid-6-C\textsuperscript{14} prepared in 0.9 per cent NaCl contained 990,000 counts/min/ml.

Male Sprague-Dawley rats, weighing between 350 and 420 gm., were maintained on stock Purina laboratory diet. For the xanthine-6-C\textsuperscript{14} metabolism studies, two animals received 8-AG, 5 mg/100 gm body weight intraperitoneally (I.P.), daily for 6 days. Two animals received 8-AG, 5 mg/100 gm I.P., only once. One animal received a single dose of 8-AG, 1 mg/100 gm I.P. Two animals received 5 mg/100 gm body weight I.P. of 6-MP daily for 6 days. One control animal was given injections I.P. of 0.05 N NaOH (1.0 ml/100 gm body weight) daily for 6 days. Two other control animals were not treated. The 8-azaguanine solution was freshly prepared for each experiment as 5 mg/ml in 0.05 N NaOH. The 6-MP was made up as 3 mg/ml in 0.05 N NaOH. For the uric acid-6-C\textsuperscript{14} metabolism studies, three animals were given injections I.P. of a solution of 5 mg/ml 8-azaguanine (5 mg/100 gm body weight); four control animals were given intraperitoneal injections of 0.05 N NaOH (1 ml/100 gm body weight).

Six minutes after the administration of the last drug or saline dose, each animal received 0.25 ml/100 gm body weight of either xanthine-6-C\textsuperscript{14} (913,000 counts/min/ml) or uric acid-6-C\textsuperscript{14} (990,000 counts/min/ml) and was placed immediately into a modification of the metabolic apparatus described by Mackenzie et al. (8). During the CO\textsubscript{2} collection period, the animals had access to neither food nor water. The expired C\textsuperscript{14}O\textsubscript{2} was collected at a flow rate of 700 ml/min in a series of gas washing bottles containing 2.5 x N NaOH at 10, 30, 60, 180, 300 minutes and 24 hours after isotope administration. For the determination of specific activity, a Warburg apparatus was employed for quantitating the expired CO\textsubscript{2}. Sulfuric acid was added in excess from the side-arm to liberate quantitatively the trapped CO\textsubscript{2}. For the estimation of the radioactivity present as C\textsuperscript{14}O\textsubscript{2}, duplicate 10-ml aliquots of samples from each period were placed into modified 25-ml Erlenmeyer flasks, the side-arms of which contained 4 ml of 10 N H\textsubscript{2}SO\textsubscript{4}. The flasks were connected with a U-shaped tube to a 5-dram liquid scintillation counting bottle (Kimble #7600) containing 0.3 ml. Hyamine hydroxide (10) and 5.0 ml of a toluene solution containing 0.3 per cent 2,5-diphenyl-oxazole and 0.03 per cent 1,4-di[2-(5-phenyl-oxazolyl)]benzene (1). This system was evacuated, and the acid was then tipped into the alkali containing the expired C\textsuperscript{14}O\textsubscript{2}. Overnight diffusion of the liberated C\textsuperscript{14}O\textsubscript{2} assured the quantitative transfer of the C\textsuperscript{14}O\textsubscript{2} into Hyamine counting mixture. The samples thus obtained were counted in a Tri-Carb liquid scintillation spectrometer with an efficiency of 47 per cent and with a background of 15 counts/min. Sufficient counts were made to reduce the probable counting error to less than ± 5 per cent. The results are expressed as specific activity (counts/min/μl CO\textsubscript{2}) and as the total cumulative expired counts, expressed in terms of percentage of the total counts administered to each animal.

RESULTS

In the control animals following the injection of labeled xanthine, there was rapid production of C\textsuperscript{14}O\textsubscript{2} (Chart 2): by 60 minutes about 80 per cent of the injected dose was expired; at the end of 5 hours more than 96 per cent of the injected dose was recovered as expired C\textsuperscript{14}O\textsubscript{2}. The specific activity of the expired C\textsuperscript{14}O\textsubscript{2} was highest between 10 and 30 minutes, when an average of 2.4 counts/min/μl CO\textsubscript{2} was obtained (Chart 3). After 1 hour, the specific activity fell rapidly. It can thus be seen that administered xanthine was metabolized rapidly to allantoin within an hour after administration. In contrast to the controls, the 8-AG-treated rats given labeled xanthine manifested a marked inhibition in C\textsuperscript{14}O\textsubscript{2} expiration, particularly during the first 30 minutes (Chart 2). By 60 minutes, only approximately 10 per cent of the injected counts were recovered, and after 24 hours only a total of 30 per cent of administered counts had been expired. The average specific activity of expired C\textsuperscript{14}O\textsubscript{2} of 8-AG-treated animals reached maximum values between the 30- and 60-minute period (Chart 3), indicating the existence of a transient lag in xanthine catabolism induced by...
8-AG. The 8-AG administration had no effect on the total quantity of nonradioactive CO₂ expired by these animals. The animals treated with a single injection of 8-AG metabolized xanthine-6-C¹⁴ to C¹⁴O₂ identically as did animals that had been pretreated with the drug for 6 days.

The injection of a smaller dose of 8-AG (1 mg/100 gm) demonstrated that the degree of inhibition of xanthine breakdown was proportional to the magnitude of the dose of 8-AG. With the smaller dose, there was diminished inhibition of C¹⁴O₂ expiration, 50 per cent of the administered C¹⁴ being recovered (Chart 2); the maximal specific activity was reduced to one third of normal or twice that of animals treated with 5 mg 8-AG/100 gm (Chart 3).

In animals treated with 3 mg 6-MP/100 gm body weight for each of 6 consecutive days, the 5–10 per cent weight loss was comparable to that of the 8-AG-treated rats. In duplicate animals, there was no lag period in xanthine catabolism as evidenced by maximal specific activities which were reached during 10 and 30 minutes as in the control animals, but only two thirds of control values were attained (Chart 5). There was a diminished linear rate of C¹⁴O₂ expiration: 60 per cent of the injected counts were recovered after 1 hour, with no more than 75 per cent of the administered counts finally recovered (Chart 2).

To determine whether the xanthine-6-C¹⁴ which was not catabolized to CO₂ in the drug-treated animals was incorporated into the liver nucleic acids via anabolic pathways, estimations of C¹⁴ in the isolated DNA and RNA were made. The liver nucleic acids of neither drug-treated nor control animals revealed any significant C¹⁴ incorporation.

Following the injection of labeled uric acid to control animals, there was a rapid production of C¹⁴O₂ so that, by 60 minutes, 69 per cent of the injected dose was expired (Chart 4). At the end of 5 hours, 79 per cent of the injected dose was recovered, and by 24 hours 81 per cent of the administered radioactivity was recovered. The specific activity of the expired C¹⁴O₂ (Chart 5) was highest during the first 10 minutes, when it averaged 2.8 counts/min/μl CO₂. After the first 10 minutes, the specific activity fell rapidly and by 1 hour was down to 1.2 counts/min/μl CO₂. Animals treated with 5 mg of 8-azaguanine/100 gm body weight, 6 minutes prior to the injection of labeled uric acid, showed rapid production of C¹⁴O₂ essentially identical with that of the control animals. Thus, by 60 minutes, approximately 65 per cent of the injected dose was expired; at the end of 5 hours, 74 per cent was expired; and at the end of 24 hours 76 per cent of the injected dose was recovered as expired C¹⁴O₂ (Chart 4). In contrast to that in the control animals, the specific activity (Chart 5) of expired C¹⁴O₂ of 8-azaguanine-treated animals reached maximum values of 2.3 counts/min/μl CO₂ between 10 and 30 minutes, reflecting the existence.
of a brief transient lag in at least one of the animals (Chart 4). Determination of urinary radioactivity excreted by the animals indicated that, in the 24-hour urine collection, the total counts excreted were in all cases less than 1 per cent of the injected dose.

**DISCUSSION**

It has been shown by Feigelson and Davidson (4) and Norris and Roush (9) that 8-azaguanine inhibits in *vivo* xanthine oxidase and uricase, respectively.¹ The present findings indicate a strong inhibition in *vivo* of xanthine catabolism by 8-azaguanine. In view of the fact that inhibitory effects of 8-azaguanine on uric acid catabolism in *vivo* are negligible, it follows that the inhibitory action of 8-azaguanine in *vivo* is on the xanthine oxidase-catalyzed portion of the reaction sequence.

While this investigation has shown the pharmacologic effect of 8-azaguanine on xanthine catabolism in animals, the significance of this mechanism in carcinostasis remains uncertain. It is evident that, since a drug may influence many biochemical reactions, the demonstration of a specific biochemical lesion induced by a pharmacologic agent is not conclusive evidence of a causal relationship between this lesion and the gross pharmacologic effects of this drug. Since tumors, however, are known to contain considerably lower concentrations of xanthine oxidase than liver (9), one may anticipate a more complete drug-induced inhibition of this enzyme system in tumors than in liver.

6-Mercaptopurine has been shown to be a relatively weak inhibitor of xanthine catabolism in *vivo*. Since 6-MP is also a substrate for xanthine oxidase (9), support is provided for the hypothesis that a drug which is a substrate for an enzyme system serves also as an inhibitor of the metabolism of the physiologic substrate of that enzyme.

The brevity of the lag in the conversion of parenterally administered xanthine-C¹⁴ to expired C¹⁴O₂ in nontreated animals indicates great rapidity of absorption and metabolism of this labeled xanthine. This rapid absorption and metabolism provide the rationale for the previous findings (7) and those herein reported, which indicate that isotopically labeled xanthine is essentially not utilized as a precursor for nucleic acids.

Both xanthine-6-C¹⁴ and uric acid-6-C¹⁴ are rapidly metabolized to C¹⁴O₂ and allantoin. However, there is a detectable lag period of 10 minutes in the rate of expiration of C¹⁴O₂ when xanthine-C¹⁴ is ad-

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¹ **ADDENDUM:** Personal communication (9/12/58): Dr. A. H. Roush indicates that an 8-azaxanthine contaminant in the 8-azaguanine was responsible for the observed uricase inhibition in *vivo*. He found that purified 8-azaguanine did not inhibit uricase in *vivo*.

This further confirms the hypothesis that in *vivo* enzyme inhibitions are indicators of in *vivo* events.
Pharmacologic doses of 8-azaguanine and 6-mercaptopurine were found to inhibit xanthine catabolism in vivo in the rat. The rate of expiration of C14O2 after parenteral administration of xanthine-6-C14 or uric acid-6-C14 provides a direct measure of xanthine and uric acid catabolism including the oxidation of xanthine to uric acid by xanthine oxidase and the further oxidative deamination of uric acid to allantoin and CO2 by uricase. Pharmacologic doses of 8-azaguanine did not inhibit the in vivo catabolism of uric acid-C14 while they exerted strong inhibition on the catabolism of xanthine-C14. The in vivo effect of 8-azaguanine was, therefore, localized to the xanthine oxidase system. In control animals, the rate of uric acid catabolism was more rapid than that of xanthine catabolism, suggesting the latter to be a rate-limiting reaction.

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