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Synthesis and Oncogenicity of 3-Hydroxyuric Acid*

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ABSTRACT: 3-Hydroxyuric acid has been synthesized by a method which involves unique conditions for the closure of the imidazolone ring. Administered subcutaneously it is weakly oncogenic to rats. *In vitro* xanthine oxidase reduces a small proportion of it to 3-hydroxyxanthine. The results suggest that its oncogenicity can be attributed to the reduction *in vivo* of a portion of it to 3-hydroxyxanthine.

Some chemical oncogens[1] are metabolically converted into products which are the actual initiators of the cancer process (Miller, 1970). Any metabolite of a chemical oncogen is therefore suspect of being a proximate oncogen. 3-Hydroxyxanthine and guanine 3-oxide are potent chemical oncogens (Sugiura *et al.*, 1970). Initial metabolic studies showed (Stöhrer and Brown, 1969a; Myles and Brown, 1969) that, in rats, about 95% of single doses ranging from 0.07 (Stöhrer and Brown, 1970) to 7 mg (Myles and Brown, 1969) could be accounted for in the urine within 24 hr; of this about 90% represents products which are derived *via* either oxidations or reductions catalyzed by xanthine oxidase (Stöhrer and Brown, 1969b) and by the subsequent action of uricase. From 3-hydroxyxanthine the products include: xanthine and its sequelae, uric acid and allantoin; 3-hydroxyuric acid and, from the action of uricase on it (Myles and Brown, 1969) probably *N*-hydroxyallantoins. From guanine 3-oxide the products include (Stöhrer and Brown, 1969a): a trace of 3-hydroxyxanthine; considerable guanine and its sequelae, xanthine, uric acid and allantoin, 8-hydroxyguanine 3-oxide, and 8-hydroxyguanine. Among these metabolites it was deemed that 3-hydroxyuric acid was the most important for study as a candidate for a proximate oncogen derived from 3-hydroxyxanthine.

Materials and Methods

3-Hydroxyuric Acid, Synthesis. 6-AMINO-1-BENZYLOXY-5-NITROSO- (II) AND 1-BENZYLOXY-5,6-DIAMINOURACIL (III) SULFATE. Sodium nitrite solution (3.75 g, in 50 ml of water) was slowly dropped into a suspension of finely ground 6-amino-1-benzyloxyuracil (I) (Klötzer, 1964) (12.7 g) in 0.5 N HCl (109 ml) under stirring. After stirring for 12 hr the precipitated red 6-amino-1-benzyloxy-5-nitrosouracil (14.1 g) was collected and washed thoroughly with water. The nitroso derivative was dissolved in 0.5 N sodium hydroxide (100 ml) and sodium dithionite was added in small portions (~10.2 g) until the red color faded. The gray precipitate was collected and washed with water. Stirring of the precipitate with 2 N sulfuric acid (100 ml) gave 1-benzyloxy-5,6-diaminouracil sulfate (11.1 g) as a light pink precipitate. An analytical sample was prepared by dissolving the 5,6-diaminouracil in boiling 2 N sulfuric acid and cooling immediately. It crystallized as brown needles of mp 134 (dec).

Anal. Calcd for $C_{11}H_{12}N_4O_3 \cdot 0.5H_2SO_4 \cdot H_2O$: C, 41.90; H, 4.79; N, 17.76; S, 5.08. Found: C, 42.22; H, 4.67; N, 17.65; S, 5.17.

6-AMINO-5-ETHOXYCARBONYLAMINO-1-BENZYLOXYURACIL (IV). The above diaminouracil sulfate (3.15 g), ethyl chloroformate (2.0 ml), and 1 N sodium hydroxide (30 ml) were stirred at 0° for 15 min. The ice bath was removed and the temperature was allowed to rise to 23°. The stirring was continued for about 30 min, when the acidity of the solution was pH 6. The precipitate was collected by filtration and recrystal-

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[1] The more common term "chemical carcinogen" should logically be restricted to agents that induce carcinomas. Oncogenic is an inclusive term for agents that induce any form of neoplasia (Martin, 1963; Rous, 1967).

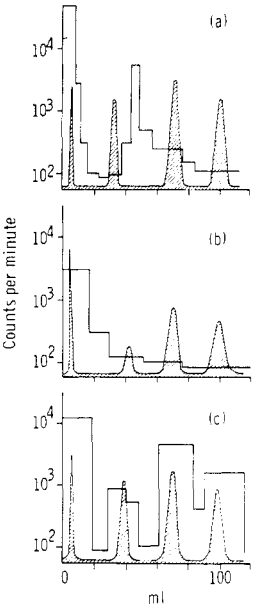


FIGURE 1: Chromatography on 5 ml of Dowex 50 (H^+), −400 mesh, with a linear gradient of 60 ml of H_2O + 60 ml of 1 N HCl as eluent. Unlabeled marker purines are added to each chromatographic run. The order of elution is: 3-hydroxyuric acid (together with uric acid and a nonpurine contaminant), 3-hydroxyxanthine, xanthine, and hypoxanthine. Shaded areas represent uv absorption. Part a shows an unknown nonpurine by-product of the enzymatic synthesis of 3-hydroxyuric acid. Part b shows the purified 3-hydroxyuric acid in a control experiment with $Na_2S_2O_4$ but without enzyme. Part c shows a typical reaction mixture of enzymatic reduction of 3-hydroxyuric acid.

lization from boiling ethanol (~100 ml) to yield the uracil (2.0 g, 62%), as light yellow crystals of mp 188 (dec).

Anal. Calcd for $C_{14}H_{16}N_4O_5$: C, 52.50; H, 5.03; N, 17.49. Found: C, 51.21; H, 5.04; N, 16.9.

6-AMINO-5-ETHOXYCARBONYLAMINO-1-HYDROXYURACIL (V). The benzyloxyuracil derivative (1.30 g) in ethanol (500 ml) was hydrogenated (1 atm) with palladium on charcoal (5%, 0.50 g) until the 1 mole of hydrogen was absorbed. The hydrogenation mixture was heated to boiling on a steam bath and filtered immediately from the catalyst. Evaporation of the filtrate *in vacuo* to 100 ml and cooling gave the *N*-hydroxyuracil (0.68 g, 73%) as a white precipitate, mp 240 dec (from ethanol).

Anal. Calcd for $C_7H_{10}N_4O_5$: C, 36.53; H, 4.38; N, 24.34. Found: C, 36.37; H, 4.57; N, 24.16.

3-HYDROXYURIC ACID (VI). 6-Amino-5-ethoxycarbonylamino-1-hydroxyuracil (1.1 g) was added to a solution of 0.42 g of sodium in 40 ml of ethanol and heated on a steam bath under reflux for 5 hr. After reaction the mixture was cooled and the sodium ethoxide was converted to sodium carbonate by slowly adding Dry Ice (~10 g). The precipitate was washed with 2% acetic acid and then with boiling ethanol to yield 3-hydroxyuric acid (0.33 g, 34%). The ultraviolet spectra and infrared spectra were identical with those of an enzymatically prepared sample (Myles and Brown, 1969).

Anal. Calcd for $C_5H_4N_4O_4 \cdot H_2O$: C, 29.71; H, 2.99; N, 27.72. Found: C, 29.64; H, 2.96; N, 27.75.

It could not be recrystallized from water without excessive loss of the oxygen from N-3, but when applied to a Dowex 50 column in water and eluted with water it crystallized as needles.

TABLE I: Tumor Induction by 3-Hydroxyuric Acid.

Compound	Dose[a]	Latent Period (Months)		No. with Tumors at Injection Site at 18 Months
		Median	Range	
3-Hydroxyuric acid	1 mg (22)	8		1[b]/5
3-Hydroxyuric acid	1 mg (22)	16	11–18	4[c]/21
Uric acid	50 mg (25)			0/50
Controls (vehicle)	0.5 ml (22)			0/20

[a] Once a week (number of weeks). [b] Sarcoma. [c] 3 fibromas, 1 pleomorphic tumor.

Oncogenesis Assays. The compound was suspended in 0.5 ml of 0.5% carboxymethylcellulose in 0.85% NaCl and injected subcutaneously in the intrascapular region in weanling Wistar rats, at the levels and frequencies indicated in Table I, and the animals were maintained and observed for 18 months as described (Teller *et al.*, 1970). Control rats were administered the vehicle alone.

Xanthine Oxidase. Milk xanthine oxidase (Worthington Biochemical Corp.) with an activity as xanthine: O_2 oxidoreductase, determined according to Fridovich (1962), of 1.7 nmoles of uric acid formed per min per ml at 25° was used.

3-Hydroxyuric-8-^{14}C Acid. 3-Hydroxyxanthine-8-^{14}C (Stöhrer and Brown, 1969a) (500 nmoles; 12.6 × 10^6 cpm/μmole), NaH_2PO_4 (500 nmoles), EDTA (0.1 nmole) in 10 ml of H_2O (pH 7.0), and milk xanthine oxidase (0.9 unit) incubated for 1 hr at 25°. The reaction mixture was passed over a column of 10 ml of Dowex 50 (200–400 mesh, H^+ form) and the 3-hydroxyuric acid was eluted with water. To separate a nonultraviolet-absorbing impurity which elutes from the column between 3-hydroxyxanthine and xanthine (see Figure 1a) the procedure was repeated with the 3-hydroxyuric acid containing fraction. The rechromatographed 3-hydroxyuric acid fraction was concentrated *in vacuo* and dissolved in 0.6 ml of buffer. The purity of its first fraction (Figure 1a), based on paper chromatography, was 68%, the remaining 32% being another nonpurine product which is slowly formed as 3-hydroxyuric acid is air oxidized (Myles and Brown, 1969); yield 15% based on pure 3-hydroxyuric acid as separated by paper chromatography.

Paper Chromatography. NH_4Cl (3%) with Na_2EDTA (0.01%) was used, ascending, with Schleicher & Schuell No. 597 filter paper. R_F values were uric acid, 0.42; 3-hydroxyuric acid, 0.55; xanthine, 0.47; 3-hydroxyxanthine, 0.62; hypoxanthine, 0.62; and 3-hydroxyuric acid air oxidation product, 0.85.

Enzymatic Reduction of 3-Hydroxyuric Acid. The reaction was carried out in Thunberg vessels with the anaerobic techniques described (Stöhrer and Brown, 1969b). The vessels contained 0.5 unit of xanthine oxidase in the main tube and 20 mmoles of 3-hydroxyuric-8-^{14}C acid in the side arm. After nitrogen flushing, a solution of sodium phosphate (140 μmoles), EDTA (0.03 μmole), $Na_2S_2O_4$ to give an

TABLE II: Reduction of 3-Hydroxyuric Acid by Xanthine Oxidase.[a]

	3-Hydroxyuric Acid	Uric Acid	3-Hydroxyxanthine	Xanthine	Hypoxanthine
Control	68				
10 min	30	2.5	2.6	31	12
20 min	19	2.6	2.6	27	16
30 min	4	1.8	1.6	23	33

[a] The 3-hydroxyuric acid had a purity of 68%. The impurity, a nonpurine product, appearing in fraction one of the column chromatogram, remained unchanged during the reactions. The control experiment, without xanthine oxidase, was incubated for 30 min. All values are expressed as per cent of the total radioactivity.

OD_{317nm} of about 3.0, or hypoxanthine (8 μmoles) in a final volume of 2.8 ml, pH 6.5, was injected through the vent. After another nitrogen flushing, the vessels were mixed and incubated at 37°, then frozen and the enzyme inactivated by the addition of 0.2 ml of saturated trichloroacetic acid. The reaction mixtures, after appropriate time intervals, were then chromatographed on 5 ml of Dowex 50 (H^+), −400 mesh, with a linear gradient of 60 ml of H_2O and 60 ml of 1 N HCl as eluent. Unlabeled markers were added. The fraction containing uric acid was chromatographed on paper for determinations of 3-hydroxyuric acid and uric acid. The results of four experiments are given in Table II. Qualitatively similar results were obtained with hypoxanthine as the electron donor.

Radioactivity Determinations. These were carried out by scintillation counting of eluates or by measurement on paper strips as described (Stöhrer and Brown, 1969a).

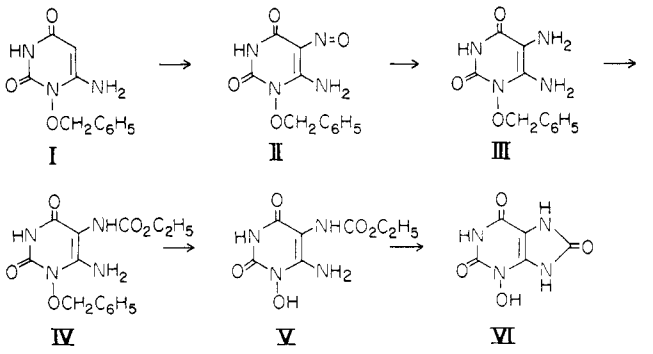
Characterizations of Products. All radioactive products accompanied the respective unlabeled markers both on column chromatography and on subsequent paper chromatography of fractions from the column. 3-Hydroxyxanthine was also characterized by complete ultraviolet spectra of a sample obtained from a preparative experiment. The radioactive 3-hydroxyxanthine, as obtained by enzymatic reduction of 3-hydroxyuric acid, was reduced with hot 57% HI to yield xanthine with unchanged specific activity after column chromatography.

Results

An unambiguous total synthesis of 3-hydroxyuric acid confirms the assigned structure (VI) and makes the compound more readily available. It was accomplished as shown in Scheme I.

All conventional methods failed to cyclize an imidazolone ring on 5,6-diamino-1-benzyloxyuracil (III) or the corresponding 5,6-diamino-1-hydroxyuracil, with deoxygenation a frequent complication. By first forming the 5-ethoxycarbamyl derivative IV, then debenzylating to V, an intermediate was obtained which could be cyclized. In hot hexamethyldisilazane or in hot xylene the cyclization of V was accompanied by deoxygenation and uric acid was produced. With

SCHEME I

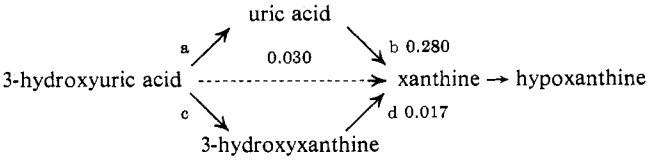


4 moles of hot sodium ethylate, cyclization to 3-hydroxyuric acid proceeded in 34% yield; with less than 4 moles of sodium ethylate much lower yields resulted. Cyclization of pyrimidines under basic conditions is common (Brown, 1962) but this appears to be the first instance of a cyclization in base of the imidazolone ring of uric acid.

An initial oncogenesis assay, with only 5 rats because of scarcity of the compound, led to one tumor in 5 rats, an equivocal result (Table I). A repeat test with 21 rats resulted in a 19% incidence at a dose level of 1.0 mg, which is to be compared with a 100% incidence at 0.5-mg doses and 30% at 0.1-mg doses, on the same dosage schedule, with 3-hydroxyxanthine (Sugiura *et al.*, 1970). Controls with the vehicle and with uric acid (Table I), as well as with xanthine (Sugiura *et al.*, 1970), were all negative.

The reduction of 3-hydroxyuric acid by xanthine oxidase has already been shown to occur at an initial rate about twice that of 3-hydroxyxanthine and about one-ninth that of uric acid (Stöhrer and Brown, 1969a,b). Two routes are possible (Scheme II). Route a–b would form uric acid which would then be further reduced to xanthine and hypoxanthine. Route c–d would first reduce the 8-carbon to form 3-hydroxyxanthine, which on further reduction would also give xanthine and hypoxanthine. From the results in Table II, which show the composition of the reaction mixture after various time intervals, it is obvious that both pathways are involved. Hypoxanthine continues to increase while both uric acid and 3-hydroxyxanthine reach steady concentrations during the first 20 min. From the rates of reduction of 3-hydroxyxanthine and uric acid, it appears that route a–b, with uric acid as first product of reduction predominates, since uric acid is further reduced at a rate of 16 times that of 3-hydroxyxanthine and yet reaches about the same steady concentration.

SCHEME II: Alternative Pathways for the Reduction of 3-Hydroxyuric Acid by Xanthine Oxidase.[a]



[a] The relative rates are given in terms of ΔA/min of the electron donor, sodium dithionite (Stöhrer and Brown, 1969b). Hypoxanthine has not previously been identified among the products of such a reduction.

Discussion

On a preparative scale the enzymatic synthesis of 3-hydroxyuric acid suffers from the fact that dilute solutions of it, like those of uric acid (Griffiths, 1952; Stahl, 1969) are susceptible to air oxidation. A total synthesis of it required unusual conditions for cyclization of the imidazolone ring to avoid simultaneous deoxygenation at N-3.

In a biological assay the low incidence of tumors at a relatively high dose (Table I) indicates that 3-hydroxyuric acid and its major metabolites, presumably *N*-hydroxyallantoins produced by the action of uricase, are quite weak oncogens.

A small amount of the highly oncogenic 3-hydroxyxanthine is produced by the action of xanthine oxidase *in vitro*. The knowledge that similar reduction products of purine *N*-oxide derivatives are produced *in vivo* (Stöhrer and Brown, 1969b), and that xanthine oxidase activity is present in subcutaneous tissues (Myles and Brown, 1969), suggests that the tumors observed are most probably due to a small amount of 3-hydroxyxanthine arising *in vivo* from the 3-hydroxyuric acid.

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Modification of Chromatin with Acetic Anhydride*

Robert T. Simpson

ABSTRACT: Acetylation of rabbit liver chromatin with increasing excesses of acetic anhydride leads to increasing modification of tyrosyl and lysyl residues of chromatin proteins, reaching a limiting value at the acetylation of 54 amino acid residues/100 base pairs. Only slight alterations in the thermal stability of chromatin, no detectable alterations in the conformations of protein or DNA, and no dissociation of chromatin proteins from the nucleoprotein complex occur consequent to the modification reaction. Only a portion (*ca.* 25%) of the lysyl residues of the proteins of chromatin is acetylated, while all the tyrosyl residues appear to react. Polyacrylamide gel electrophoresis demonstrates that some lysyl residues of all the three main histone fractions react with the acylating agent in the native chromatin complex. All histone molecules are acetylated, and the modification reaction appears to possess a high degree of specificity in terms of the numbers of lysyl residues available for modification for each individual histone class. This modification should facilitate the localization of those lysyl residues of histones which bind to DNA.

The compositions of the proteins of chromatin are such that an approximate equivalence exists between the positively charged basic groups of the histones and nonhistone proteins, on the one hand, and the negatively charged phosphates of DNA, on the other, suggesting that the nucleoprotein might exist as a stoichiometric complex, with all the basic amino acids electrostatically linked to DNA phosphate (Bonner *et al.*, 1968). Other evidence has, however, made such a suggestion less likely. Thus, a significant proportion of the basic groups of chromatin can be titrated in native nucleoprotein (Walker, 1965). Furthermore, a large portion of the phosphate groups are accessible to interaction with calcium (Simpson and Sober, 1970), cationic dyes (Simpson, 1970), or polylysine (Itzahki, 1970; Clark and Felsenfeld, 1971).

Recently, primary structure investigations of the several classes of histones have generated a surprising result, that the basic groups of these proteins are neither randomly arranged nor repetitively spaced throughout the length of the peptide chain, but rather are preferentially localized in large segments of high concentration of basic residues. Thus, the amino-terminal regions of the arginine-rich (FIV) (DeLange *et al.*, 1969; Ogawa *et al.*, 1969) and the slightly lysine-rich (FII) (Iwai *et al.*, 1970; Hnilica *et al.*, 1970) histones are much more basic than the carboxyl-terminal regions of these molecules. In the case of the lysine-rich histones, partial sequence studies also indicate asymmetry in basic residue distribution, although, in this case, the carboxyl-terminal portion of the molecule is the more highly basic (Bustin and Cole, 1969, 1970). Taken together, these observations have suggested that the basic regions of the histones might serve as a DNA binding region, lying in close proximity to the DNA phosphate backbone, while the relatively less basic regions of the histones are less firmly attached to the nucleic acid, perhaps indeed, free in solution to interact with other proteins or

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