Anal. Calcd. for $C_{17}H_{14}BrNO_3$: C, 56.7; H, 3.9. Found: C, 57.0; H, 4.2.

N-[β-(3-Coumarinyl)-β-oxoethyl]-quinolinium bromide formed orange-brown prisms from water or from acetic acid–ethyl acetate, dec. *ca.* 210°.

Anal. Calcd. for $C_{20}H_{14}BrNO_3$: C, 60.6; H, 3.6. Found: C, 61.0; H, 3.9.

3-Carbethoxy-N-[β-3-(coumarinyl)-β-oxoethyl]-pyridinium bromide formed nearly colorless plates from water containing a little hydrobromic acid, dec. *ca.* 190°.

Anal. Calcd. for $C_{19}H_{16}BrNO_5$: C, 54.5; H, 3.8. Found: C, 55.1; H, 4.3.

4-Carbethoxy-4-[β-3-(coumarinyl)-β-oxoethyl]-pyridinium bromide formed fine nearly colorless needles from alcohol, dec. *ca.* 170°.

Anal. Calcd. for $C_{19}H_{16}BrNO_5$: C, 54.5; H, 3.8. Found: C, 54.3; H, 4.2.

Summary

The substance obtained by the action of bromine on 3-acetylcoumarin and described previously as 3-acetyl-4-bromocoumarin is actually 3-bromoacetylcoumarin, for it yields a substituted thiazole when it is treated with thiourea. It reacts with aniline to form an anilino derivative whose red color is anomalous, and with pyridine to form an addition compound which is not cleaved by aqueous alkali.

MINNEAPOLIS, MINNESOTA RECEIVED DECEMBER 12, 1949

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

Studies in Enzyme Inhibition. I. Action of Some Simple Pterines on Xanthine Oxidase

BY H. G. PETERING AND J. A. SCHMITT

Wieland and Liebig[1] showed that xanthine oxidase catalyzes the oxidation of xanthopterine to leucopterine about half as rapidly as it does hypoxanthine or xanthine. Recently Kalckar and Klenow[2] and Kalckar, Kjeldgaard and Klenow[3] found that 2-amino-4-hydroxy-6-formylpterine (I) inhibits the enzymatic oxidation of hypoxanthine, xanthine and xanthopterine. The activity reported by Kalckar, *et al.*,[2,3] for I is of such very high order as to suggest great specificity.

We have investigated the relationship of structure to the inhibitory action of a number of simple pterines on the xanthine oxidase system of milk and rat liver, and wish to report the results of one phase of our work which indicates the highly specific chemical nature of this inhibition.

Experimental

Materials.—The xanthine oxidase preparations were either a crude milk enzyme made according to the method of Dixon and Kodoma[4] or a rat liver preparation obtained by homogenization in a Waring Blendor.

Hypoxanthine (Schwarz Laboratories) and xanthine (Eastman Kodak Co.) were used without further purification. The pterines and 2,6-diaminopurine were synthesized by methods which will be published elsewhere.

Procedure.—All studies were made with the Warburg respiration apparatus at 38°. With the milk enzyme the method of Ball[5] was closely followed. With rat liver homogenates a procedure similar to that of Potter and Elvehjem[6] was used. The inhibitor was dissolved in 0.15 ml. of 0.01 *N* sodium hydroxide and either added directly to the enzyme at the beginning of the experiment or mixed with the xanthine solution in the side arm.

Results

Preliminary experiments with the milk enzyme confirmed the reports of Wieland and Liebig[1] and Kalckar, *et al.*,[2,3] in that the enzyme preparation which was active with hypoxanthine and xanthine activated the oxidation of xanthopterine and its activity was markedly inhibited by I. In addition it was found that the enzyme would activate the oxidation of 2-amino-4-hydroxypterine at a rate about twice the rate of the oxidation of xanthopterine.

A more detailed study with a number of different pterines revealed important differences in the effect of these compounds on the enzymatic oxidation of hypoxanthine. These data are given in Table I. The strikingly effective inhibition of hypoxanthine oxidation by 2-amino-4-hydroxy-6-hydroxymethylpterine (II), which is similar in its activity to I is readily seen. The data with milk xanthine oxidase shown in Table I are insufficient to make a direct comparison of the activities of I and II with the less active compounds; such a comparison would require information on the minimum inhibitor concentration for optimum activity against this type of enzyme preparation. However, the data of Table I are qualitatively consistent with other data on liver homogenates presented below, wherein such a comparison was possible.

Having confirmed the effect of I on xanthine oxidase of milk and noted differences between several types of pterines, this inhibition was studied more extensively with the rat liver enzyme. In this work a larger series of compounds was used and these compounds were studied over a wider range of inhibitor concentration. Data for these experiments are given in Table II and Figs. 1–3.

The data in Table II may be more readily evaluated and a number, which is reasonably indicative of the relative inhibition index of the compound, may be obtained if the inhibition curve for I shown in Fig. 1 is used as a reference and the

(1) Wieland and Liebig, *Ann. Chem.*, **555**, 146 (1944).
(2) Kalckar and Klenow, *J. Biol. Chem.*, **172**, 349 (1948).
(3) Kalckar, Kjeldgaard and Klenow, *ibid.*, **174**, 771 (1948).
(4) Dixon and Kodoma, *Biochem. J.*, **20**, 1104 (1926).
(5) Ball, *J. Biol. Chem.*, **128**, 5 (1939).
(6) Potter and Elvehjem, *ibid.*, **114**, 495 (1936).

TABLE I

INHIBITION OF CRUDE MILK XANTHINE OXIDASE BY PTERINES

Expt.	Inhibitor	Molar ratio, inhibitor: substrate[a]	μl. O_2/hr./g. enzyme preparation[b]	% inhibition
1	None	....	864	...
2	2-Amino-4-hydroxy-6-formylpterine (I)	1:1000	0	100
3	2-Amino-4-hydroxy-6-hydroxymethylpterine (II)	1:10	0	100
4	2-Amino-4-hydroxy-6-carboxypterine	1:10	300	65
5	2-Amino-4-hydroxy-7-carboxypterine	1:10	624	28
6	2-Amino-4-hydroxy-6-methylpterine	1:10	708	18
7	2-Amino-4-hydroxy-7-methylpterine	1:10	720	18

[a] Hypoxanthine (3.33×10^{-3} *M*) was the substrate; temp. 38°; *p*H of phosphate buffered enzyme preparation was 7.4–7.5. [b] Values calculated on the basis of the first ten minutes of the experiment; 50 mg. of enzyme preparation was used in every case. Inhibitors were added at zero time.

ratio of concentration of I to that of the given compound at the same degree of inhibition is calculated. These ratios are relative to the latter compound and their values show the impact of substitution at the C_6 and C_7 position in the pterine ring on the relative effectiveness of the compounds as xanthine oxidase inhibitors.

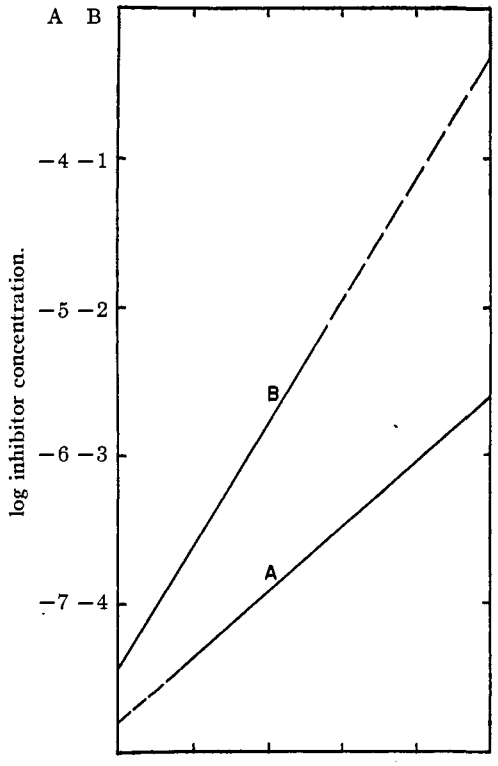


Fig. 1.—Relationship of inhibitor concentration to degree of activity against liver enzyme: (A) 2-amino-4-hydroxy-6-formylpteridine (I); (B) 2-amino-4-hydroxy-6-methylpteridine; xanthine (3.75×10^{-3} *M*) as substrate.

Figure 2 shows the type of curve obtained in studying the liver enzyme. It is readily seen that the highly active inhibitors greatly retard endogenous respiration. Figure 2 shows II is active whether it was added before or after the endogenous respiration period.

TABLE II

INHIBITION OF RAT LIVER XANTHINE OXIDASE BY PTERINES

Inhibitor	Molar ratio, inhibitor: substrate[a]	% inhib.[b]	Inhib. effect[c]
2-Amino-4-hydroxy-6-formylpterine (I)	1:100	100	
	1:1000	100	100
	1:10,000	61	
	1:100,000	16	
2-Amino-4-hydroxy-6-hydroxymethylpterine (II)	1:10	100	
	1:100	100	100
	1:1000	100	
2-Amino-4-hydroxy-6-formylpterine oxime	1:1000	80	27
2-Amino-4-hydroxy-6-carboxypterine	1:10	40	0.03
	1:1000	0	
2-Amino-4-hydroxy-6-methylpterine	1:1	48	
	1:10	24	.005
	1:100	0	
2-Amino-4-hydroxy-7-carboxypterine	1:10	36	
	1:1000	0	.03
2-Amino-4-hydroxy-7-methylpterine	1:1	46	.005
Xanthopterine	1:1	88	.04
2-Amino-4-hydroxypterine	1:1	75	.02
	1:100	10	
2,4-Diaminopterine	1:100	0	.000
Leucopterine	1:10	4	.000
2,6-Diaminopurine	1:1	26	.002

[a] Xanthine (3.75×10^{-3} *M*) was the substrate; temp. 38°; *p*H of buffered homogenate mixture 7.4–7.5. [b] The % inhibition was calculated from a comparison of the rate of oxygen uptake after addition of the substrate, as shown by the enzyme with and without inhibitor present. The inhibitors were added at the beginning of the endogenous respiration period. This period was 80–100 minutes prior to addition of xanthine and was necessary for accurate rate determinations. The rates of enzyme activity were calculated from the oxygen uptake during the first 60 minutes after addition of substrate. 250 mg. of fresh liver was used in each flask. [c] Relative to activity of 2-amino-4-hydroxy-6-formylpterine.

It has been found that neither 2-amino-4-hydroxy-6-methylpterine nor 2,6-diaminopurine can act as substrates for xanthine oxidase *in vitro* although both have a low order of inhibitory activity and the latter compound has been shown by Bendich and Brown[7] to be converted by rat liver

(7) Bendich and Brown, *J. Biol. Chem.*, **176**, 1471 (1948).

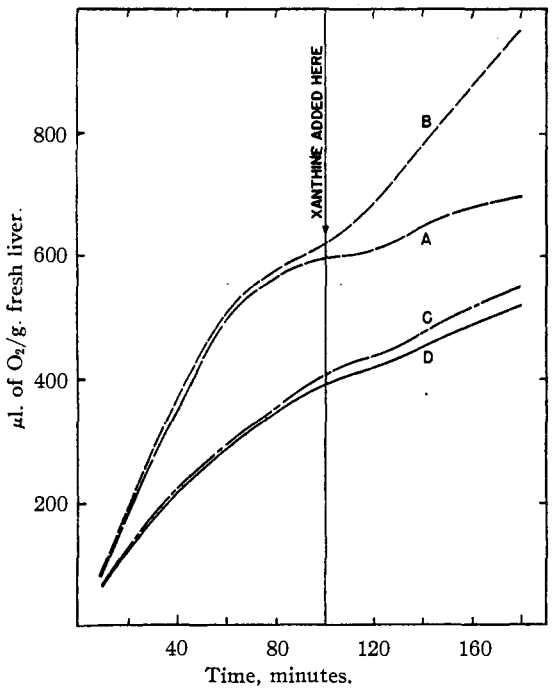


Fig. 2.—Typical experiment with liver xanthine oxidase, inhibitor added at zero time: (A) endogenous respiration; (B) A + xanthine; (C) 2-amino-4-hydroxy-6-formylpteridine (I) (3.75×10^{-6} *M*); (D) C + xanthine; xanthine (3.75×10^{-3} *M*) as substrate.

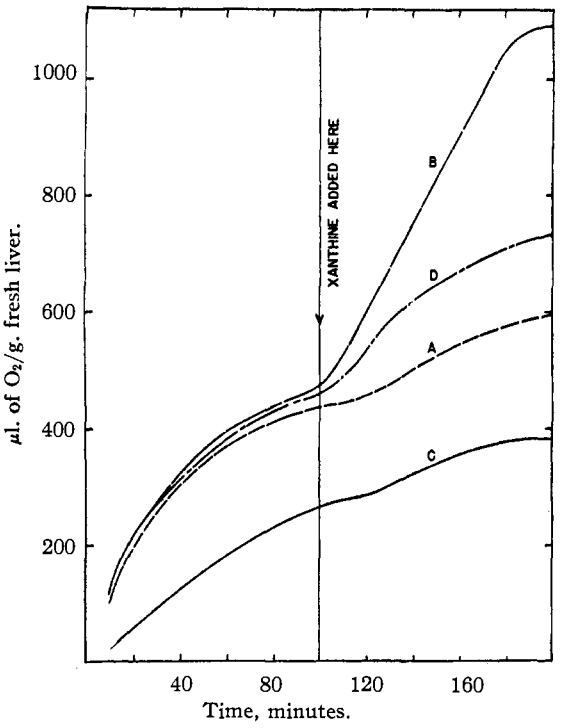


Fig. 3.—Effect of adding inhibitor and xanthine simultaneously at 100 minutes: (A) endogenous respiration; (B) A + xanthine (3.75×10^{-3} *M*); (C) 2-amino-4-hydroxy-6-hydroxymethylpteridine (II) (3.75×10^{-5} *M*) added at zero time and xanthine (3.75×10^{-3} *M*) at 100 minutes; (D) 2-amino-4-hydroxy-6-hydroxymethylpteridine (II) (3.75×10^{-5} *M*) and xanthine (3.75×10^{-3} *M*) added at 100 minutes.

to allantoin. 2,6-Diaminopurine was included since it has been mentioned as a purine antagonist which is related to the enzymic activity of folic acid.[8] On the other hand, xanthopterine and 2-amino-4-hydroxypterine, which have a low order of xanthine oxidase inhibitory activity, are oxidized in the presence of both the milk and liver enzymes at much lower rates than is xanthine, while leucopterine which has little or no inhibitory activity against xanthine oxidase is not a substrate.

These facts are of importance in studying the relationship of structure to adsorption on the enzyme surface and in formulating a theory of the mechanism involved in the inhibition of xanthine oxidase by pterines.

Discussion

The specificity of the structure of the pterines which inhibit xanthine oxidase of rat liver is of considerable interest, since this enzyme system may be of significance in the metabolism of purines and pterines. It is a striking fact that II appears to be as active as I whereas the more reduced 2-amino-4-hydroxy-6-methylpterine and the more oxidized 2-amino-4-hydroxy-6-carboxypterine have a much lower order of activity toward the milk enzyme and very little inhibitory action toward the rat liver enzyme.

(8) Hitchings, Elion, Vanderwerff and Falco, *J. Biol. Chem.*, **174**, 765 (1948).

Since Kalckar, *et al.*,[3] reported that the activity of I is destroyed by 2,4-dinitrophenylhydrazine and diphenylhydrazine, the high inhibition shown by 2-amino-4-hydroxy-6-pterinealdoxime is of considerable interest. It indicates that carbonyl reagents in general probably do not destroy the inhibitory activity of the 6-aldehyde, but that the effect by Kalckar, *et al.*,[3] with the hydrazines mentioned above may be related to the insolubility of the hydrazones formed. In addition the 6-aldoxime and II are generally more stable in solution than is the 6-aldehyde, and therefore they have been found to be useful in studying the significance of xanthine oxidase in several biological systems.

Those compounds which were markedly inhibitory also showed a general depressive activity toward the endogeneous respiration. It is not certain whether all of this endogenous respiratory inhibition is due only to action against endogenous oxidation of xanthine or hypoxanthine. From unpublished work in this Laboratory it is known that these inhibitors do not affect cytochrome C oxidase or tyrosinase and from the report of Kalckar, *et al.*,[3] it is known that I does not inhibit uricase or triosephosphate oxidase. It should also be noted that the active inhibitors

are not affected by incubation at 38° during the endogeneous respiration period.

The inhibitory activity of I and II is of an order rarely encountered with organic enzyme inhibitors. This fact would indicate that the inhibitors are acting against a highly active catalytic portion of the enzyme. In considering the nature of the inhibition shown by the compounds discussed, it should be noted that I and II completely inhibit xanthine oxidase at molar ratios of inhibitor/substrate of 1/1000 to 1/3000 while other pterines, such as xanthopterine, are highly active only in molar concentrations equal to the xanthine substrate.

Although no conclusive data are available, one possible mode of action is the antagonism of a prosthetic group of unidentified structure. The existence of a prosthetic group other than isoalloxazine-adenine dinucleotide has been postulated by Corran, *et al.*[9] Another possibility is the competition of all inhibitors regardless of activity with the substrate for the active catalytic surface of the enzyme.

(9) Corran, Dewan, Gordon and Green, *Biochem. J.*, **33**, 1694 (1939).

Summary

The inhibitory action of a number of simple pterines on xanthine oxidase from milk and rat liver has been studied. It has been shown that 2-amino-4-hydroxy-6-hydroxymethylpterine is as active an inhibitor as 2-amino-4-hydroxy-6-formylpterine, and the oxime of the latter compound has high inhibitory activity.

It has been shown that 2-amino-4-hydroxypterine is a better substrate than xanthopterine for the milk enzyme and that its action in this respect is similar to xanthine or hypoxanthine.

The data are self-consistent for both enzyme preparations and therefore may be of general importance.

A discussion of the data and their implication is given.

KALAMAZOO, MICH. RECEIVED MAY 2, 1949

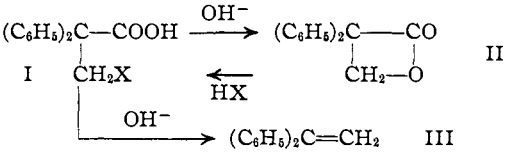
[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, THE ABBOTT RESEARCH LABORATORIES]

The Preparation and Behavior of α,α-Diphenyl-β-propiolactone

BY HAROLD E. ZAUGG

Many of the classic papers which have been published on theoretical organic chemistry have been concerned with reactions of β-halogen acids with various anions under a number of conditions.[1] These reactions are characterized by kinetic complexity and by the relatively large number of products formed. β-Alkoxy- and hydroxy-acids, α,β-unsaturated acids, β-lactones and ethylenes are among the products which have been reported from these reactions in aqueous or alcoholic solution.

2,2-Diphenyl-3-bromopropanoic acid (I, X = Br)[2] appeared to provide a structure which, on reaction with bases under suitable conditions, could be expected to give only two products, the β-lactone and the ethylene. Preparation of this acid according to the published procedure[2] called for purification through the sodium salt, m. p. 79–80°. Repetition of this method yielded a product, m. p. 91–92°, containing no bromine, which proved to be the β-lactone II. Indeed, treatment of the bromo acid with an equivalent amount of aqueous sodium hydroxide at room temperature for only thirty minutes gave the β-lactone in 60 to 70% yields. The expected[3] by-product, 1,1-diphenylethylene (III), was always obtained in appreciable amounts, regardless of wide variation in reaction conditions.



Treatment of the β-lactone II with hydrochloric, hydrobromic and hydriodic acids in acetic acid solution resulted in formation of the three β-halogen acids I (X = Cl, Br, I, respectively). The reactivities of the mineral acids increased in the order, HCl < HBr < HI, but in all cases yields were good.

Each of these β-halogen acids was treated with an equivalent amount of aqueous sodium hydroxide (1.5 equivalents for the iodo acid) for thirty minutes at room temperature. The chloro acid gave 12% of II, no III and an 85% recovery of the acid; the bromo acid gave 65% of II, 24% (calculated by difference) of III, and the only recovered acid (11%) was that which had not been converted to the sodium salt; the iodo acid gave 25% of II, 52% of III and no recovered acid. The much slower rate of reaction of the chloro acid did not favor ethylene formation, for after three days, when the reaction was essentially complete at room temperature, a 95%

(1) (a) Walden, *Ber.*, **29**, 133 (1896); (b) Senter, *et al.*, *J. Chem. Soc.*, 1070 (1915); 1847 (1925); (c) Holmberg, *J. prakt. Chem.*, **88**, 553 (1913); (d) Olson, *et al.*, THIS JOURNAL, **56**, 1294 (1934); **60**, 2687 (1938); *J. Phys. Chem.*, **41**, 267 (1937); (e) Kohler, *et al.*, THIS JOURNAL, **56**, 729, 200 (1934); **60**, 2142 (1938); **63**, 1531 (1941).

(2) Wegmann and Dahn, *Helv. Chim. Acta*, **29**, 415 (1946).

(3) Johansson and Hagman, *Ber.*, **55**, 647 (1922).