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PTERIDINES AS SUBSTRATES OF MAMMALIAN XANTHINE OXIDASE

I. THE END-PRODUCT OF THE ENZYMIC OXIDATION OF PTERIDINES*

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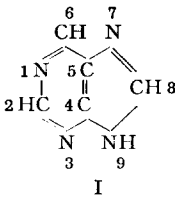
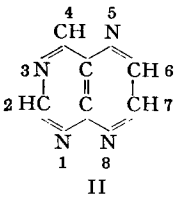
The chemical and biochemical interrelationship of purines (I) and pteridines (II) has been recognised in the past.



I



II

For example, ALBERT[1] has shown that on incubation of 2-hydroxypurine with glyoxal in aqueous solution, 2-hydroxypteridine is formed, and that similar transformations can be carried out with other purines. ZIEGLER-GÜNDER, SIMON AND WACKER[2] injected guanine, labelled at carbon atom 2, intradermally into *Xenopus* larvae and isolated afterwards labelled pteridines from their skin. These observations make it probable that in the cell certain metabolites are formed, such as *e.g.* 4,5-diaminopyrimidines, which may undergo cyclisation to a variety of heterocyclic systems (pteridines, alloxazines[3]).

All naturally occurring pteridines carry substituents in the 2- and 4-position of

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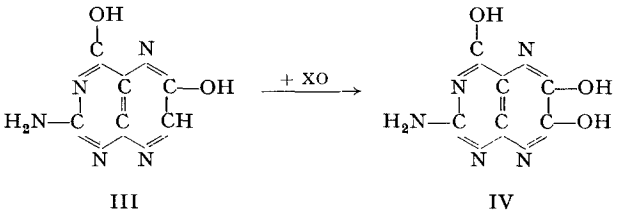
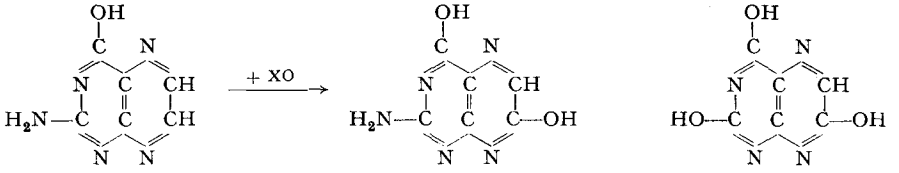
the pyrimidine ring, equivalent to position 2 and 6 in the purine skeleton. The coincidence of substitution suggests that purines and pteridines may be attacked by the same enzymes. Indeed, through the work of WIELAND AND LIEBIG[4], of KALCKAR AND KLENOW[5] and of LOWRY, BESSEY AND CRAWFORD[6] it became clear that xanthine oxidase (XO) can attack both heterocyclic systems. Thus, xanthopterine (= 2-amino-4,6-dihydroxypteridine, III) is converted by this enzyme to leucopterine (IV), by introduction of a hydroxyl group in position 7. However, 2-amino-4-hydroxy-pteridine (V) is oxidized to isoxanthopterine (VI), and after insertion of the 7-hydroxyl, the enzymic reaction stops. Comparison of structure I and II does not indicate, whether a 2,4,6- or a 2,4,7-trisubstituted pteridine would correspond to the end-product of purine oxidation, *viz.* uric acid.



III IV



V VI VII

The fact that leucopterine can be obtained by enzymic oxidation *in vitro*[5], but is also found as a terminal metabolite in the wings of butterflies[7], may suggest that IV is the end-product of oxidation and thus plays in pteridine metabolism a role similar to uric acid in purine oxidation. In this connection it should be recalled that uric acid has also been isolated from butterflies[8].

Since oxidation of pteridine and its mono-and dihydroxy-derivatives by XO has not yet been investigated, we have undertaken an enzymic study to answer the following questions: a) What is the end-product of interaction of pteridines with XO? b) What is the pathway of oxidation of individual derivatives? c) Is the substrate specificity of XO in the pteridine series comparable to that of purine derivatives? d) Does there exist a common reaction mechanism for the attack of XO on purines and pteridines?

The present paper is mainly concerned with the first problem. An extension of our experiments to the more general problems b–d will be presented in a future publication.

MATERIALS AND METHODS

Substrates

Pteridine and its various oxy derivatives, used in this investigation, were all synthesised by ALBERT and coworkers[9–11]. They were analytically pure, as evidenced by their ultraviolet absorption spectra.

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Enzymes

Highly purified milk xanthine oxidase was supplied by Prof. F. BERGEL and Dr. R. C. BRAY of the Chester Beatty Institute of Cancer Research, London, England*. This preparation, when diluted 1:800, produced 1 γ/ml of uric acid per min when the reaction was carried out at pH 8.0 with $6.5 \cdot 10^{-5}$ M xanthine as substrate.

Since oxidation of pteridines proceeds rather slowly, it was necessary to add catalase to all runs, in order to prevent inactivation of XO by hydrogen peroxide. The enzyme used was a commercial product of Worthington Biochemical Corporation, containing 5000 units/ml.

Methods of analysis

The progress of enzymic oxidation was followed spectrophotometrically. Since the absorption maxima of many of the pteridines involved are very similar, it was not yet possible to identify in all cases the intermediates and thus to establish the pathway of the enzymic reaction.

Isolation of intermediates or reaction products proved to be a most difficult task. Because the pteridines are extremely weak bases, their quantitative adsorption on cation exchangers, similar to the method used for purification of basic purines (DIKSTEIN, BERGMANN AND CHAIMOVITZ[12]), was not feasible. Paper-chromatographic separation of pteridine mixtures by known procedures has not proved satisfactory in our hands. In all solvents reported in the literature, which we have tested, the R_F values of various oxidation stages were very similar. In addition, many pteridine derivatives are sensitive to atmospheric oxidation or light, so that their recovery after development of the chromatogram often fails. Furthermore, no staining method, applicable to *all* pteridine derivatives, is known. The best method for the detection of individual spots is ultraviolet fluorescence, using light of about 254 mμ.

In the present study we have confined ourselves to the isolation and identification of the final products of the enzymic reactions.

Experimental procedure

All substrates tested were incubated at 27° with an appropriate dilution of XO in 10^{-3} M phosphate buffer, pH 8.0, and the spectral changes recorded periodically. After the reaction had stopped, the solution was concentrated *in vacuo* and the residue dissolved in a few drops of water for spotting on Whatman No. 1 paper. The descending chromatogram was developed with the following mixture: Dimethylformamide, 50 ml; glacial acetic acid, 40 ml; water, 10 ml. This solvent gave an excellent separation of *e.g.* 2,4-di- and 2,4,7-trihydroxypteridine (see Fig. 3), but was not yet satisfactory for the separation of all pteridine mixtures. It had the advantage of protecting the substances tested—at least partially—against atmospheric oxidation during the drying period. In addition, with this solvent, all pteridines were found to give well-defined spots, without appreciable trailing. R_F values were determined with the aid of ultraviolet fluorescence. For measuring the shifts of λ_{max} with pH, the method of BERGMANN AND DIKSTEIN[13] was applied, using the following buffers: pH —3 to +3, sulfuric acid; pH 4.0 to 6.5, 0.1 M acetate; pH 6.5 to 8.0, 0.1 M phosphate; pH 8.0 to 11.0, 0.1 M borate; pH > 11.0, sodium hydroxide.

In the presence of catalase alone and in the absence of XO, none of the substrates used underwent a change.

RESULTS

It was found that with all susceptible substrates, the same end-product was obtained. As a representative example, we reproduce in Fig. 1 the spectral changes taking place during the oxidation of 2,4-dihydroxypteridine. The end-product has practically the same absorption spectrum as the original substrate, but a higher extinction. Since only 3 oxidation products are possible (*viz.* oxidation at position 6, 7 or both), it becomes probable at once that the enzymic reaction leads to 2,4,7-trihydroxypteridine (VII), which is the only derivative, possessing an absorption spectrum almost identical with that of the starting material, but exhibiting higher extinction[11]. The influence of pH on the long-wave absorption maximum of VII is shown in Fig. 2. It is apparent that 2,4,7-trihydroxypteridine possesses 3 dissociation constants: it forms a cation below pH — 1, a mono-anion with a pK of 3.0 and a bis-anion with a

* We wish to express our sincere thanks to Prof. BERGEL and Dr. BRAY for the generous gift of this enzyme preparation.

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pK of 9.7. The oxidation product of 2,4-dihydroxypteridine gave the following values for λ_{max}: pH 2.3–326 mμ; pH 8.0–328 mμ; and pH 10.5–336 mμ. In addition, the paper chromatogram, reproduced in Fig. 3, reveals the similarity of the oxidation product with authentic 2,4,7-trihydroxypteridine. All pertinent data are summarised in Table I.

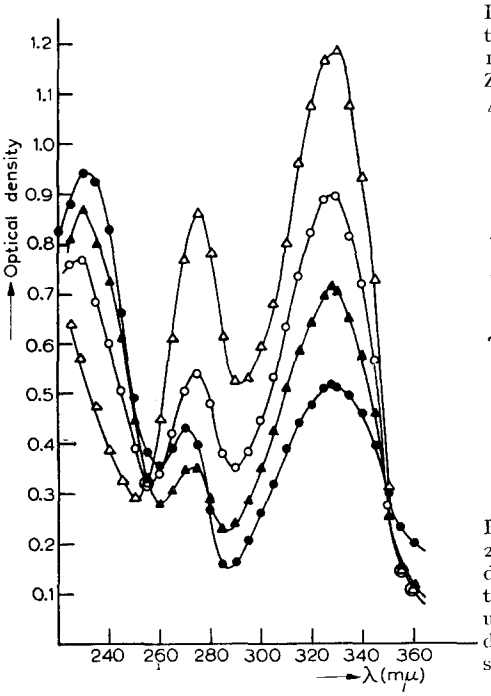

Fig. 1. Spectral changes during the enzymic oxidation of 2,4-dihydroxypteridine. Xanthine oxidase: 1:400; substrate: $6.1 \cdot 10^{-5}$ M; pH 8.0; t 27°. ●—● Zero time. ▲—▲ After 15 min. ○—○ After 30 min. △—△ After 90 min (representing the final stage of the reaction).

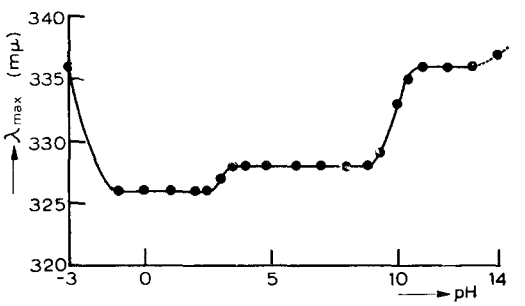

Fig. 2. The long-wave absorption maximum of 2,4,7-trihydroxypteridine as a function of pH. The dashed portion of the curve above pH 13 indicates, that the small rise in λ_{max}, observed at pH 14, is unreliable, since the substance undergoes a slow decomposition. The curve indicates 3 dissociation steps: below pH —1; between pH 2.5 and 3.5; and between pH 9–11.

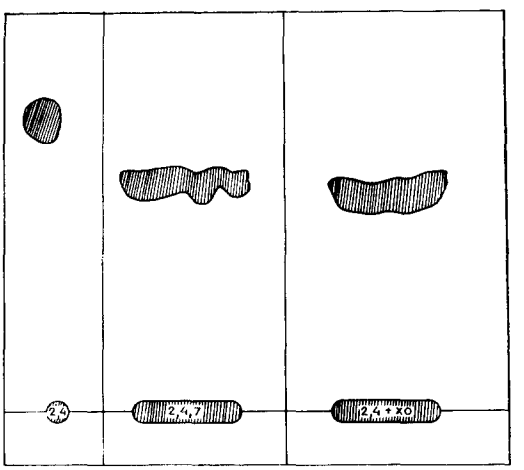

Fig. 3. Paperchromatographic identification of the oxidation product of 2,4-dihydroxypteridine. Solvent front 37.5 cm (after 12 h). The spots were located by fluorescence in ultraviolet light.

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TABLE 1

PHYSICAL PROPERTIES OF 2,4-DI- AND 2,4,7-TRIHYDROXYPTERIDINE

Compound	λ_{max} *at pH 8.0*	$\frac{\varepsilon_{max_1}}{\varepsilon_{max_2}}$	R_F	*Fluorescence*
2,4-Dihydroxypteridine	328 270	1.2	0.71	blue-white
2,4,7-Trihydroxypteridine	328 275	1.4	0.54	blue-violet
Enzymic oxidation product of 2,4-dihydroxypteridine	328 275	1.37	0.53	blue-violet

Since only a single step is involved in the enzymic oxidation of 2,4-dihydroxypteridine, we can plot the increase of optical density at $\lambda_{max} = 329$ mμ as function of time. As shown in Fig. 4, the rate is linear for the first 15 min; from the slope of the straight line, the initial reaction velocity can be derived, which is about 14 times smaller than that of xanthine under identical conditions.

We have also found that pteridine itself and its 2-, 4- or 7-monohydroxy derivatives are ultimately converted into VII, whereas the 6-hydroxy compound is completely refractory to enzymic attack.

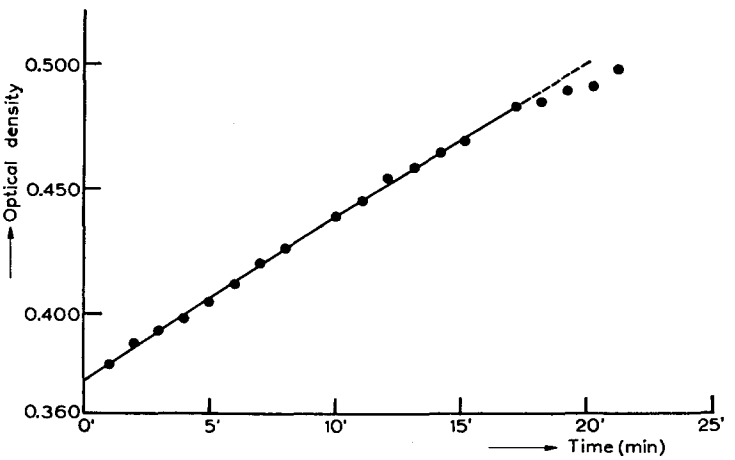

Fig. 4. Rate of enzymic oxidation of 2,4-dihydroxypteridine. Since λ_{max} of 2,4-di- and 2,4,7-trihydroxypteridine at pH 8.0 are practically identical, the ordinate represents the increase in optical density at 329 mμ. Xanthine oxidase: 1:400; substrate: $6.1 \cdot 10^{-5}$ M; pH 8.0; t 27°.

DISCUSSION

The present experiments show that the pteridine nucleus is attacked by mammalian XO in a manner, similar to purine oxidation. The end-product of this interaction is 2,4,7-trihydroxypteridine (VII), which is related to isoxanthopterin. On the other hand, the 6-hydroxyl group in 6-hydroxypteridine prevents enzymic attack, but this

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effect is overcome by further substitution in the pyrimidine ring, as evidenced by the enzymic conversion of xanthopterin (III) into leucopterin (IV). These observations make it almost certain that the 6-hydroxyl in III does not originate through interaction of its precursor with XO.

In an earlier paper[14], we proposed a hypothesis about the mechanism of action of mammalian XO. It was assumed that the dehydrogenation step involved essentially the nitrogens in position 3 and 7 of the purine skeleton (I). In analogy, one would expect the reacting entity in II to comprise N^1 and N^5, so that a new hydroxyl group in the pyrazine ring would become attached to carbon atom 6. The fact that the enzyme attacks position 7 instead, raises doubts as to the validity of our previous hypothesis, unless oxidation of purines and pteridines proceeds by different mechanisms. In order to clarify the situation, it now becomes very important to compare the pathways of oxidation of individual pteridines with those of the corresponding purines. The results of such studies will be reported in a future paper.

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The authors wish to express their sincere thanks to Prof. ADRIAN ALBERT, Department of Medical Chemistry, The Australian National University, Canberra, Australia, for the generous gift of all pteridines, used in the present investigation and for his continuous help and advice during all stages of this work.

SUMMARY

1. The oxidation by mammalian xanthine oxidase of pteridine, its monohydroxy derivatives and of 2,4-dihydroxypteridine has been investigated. All these compounds, with the exception of 6-hydroxypteridine, are attacked by the enzyme.

2. In all cases the same end-product is obtained, which has been identified as 2,4,7-trihydroxypteridine.

3. These observations indicate that the 6-hydroxyl group in xanthopterin can not originate through interaction of its precursor with xanthine oxidase.

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