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CATABOLISM OF PTERIDINE COFACTORS

III. ON THE INTRODUCTION OF AN OXYGEN FUNCTION INTO POSITION 6 OF THE PTERIDINE RING*,**.

H. REMBOLD, H. METZGER AND W. GUTENSOHN
Max-Planck-Institut für Biochemie, München (Germany)
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SUMMARY

The formation of 6-hydroxylumazine from tetrahydrolumazine in rat liver homogenates shows the same characteristics as already described for tetrahydrobiopterin and tetrahydroneopterin.

By spectrophotometric methods, 7,8-dihydrolumazine is shown to be an intermediate in the aerobic oxidation of tetrahydrolumazine. 7,8-Dihydrolumazine is the substrate for xanthine oxidase. The kinetics of the enzyme-catalyzed formation of 7,8-dihydro-6-hydroxylumazine was measured. If the reaction is started with tetrahydrolumazine, xanthine oxidase catalysis is the rate-limiting step in the reaction sequence. The enzymic reaction shows maximal activity at pH 7.9 and a $K_m = 4.5 \cdot 10^{-4}$ M for dihydrolumazine. The conversion of 7,8-dihydrolumazine to 7,8-dihydro-6-hydroxylumazine is also effected by a nonenzymic reaction which can be distinguished clearly from the enzymic one.

In the pterin series an analogous reaction sequence is followed which results in the formation of 7,8-dihydroxanthopterin and xanthopterin from tetrahydroneopterin and tetrahydrofolic acid.

The catabolism of pteridine cofactors is discussed and summarized in a reaction scheme.

INTRODUCTION

On incubating tetrahydrobiopterin and tetrahydroneopterin in rat liver homogenates we found a degradation to the acidic products lumazine and 6-hydroxylumazine. In a previous paper the general characteristics of this reaction have been described[1]. The degradation must proceed in several steps which may be anticipated from the balance of products formed. The cleavage of the side-chain in position 6 proved to be an unspecific oxidative reaction. By the identification and enrichment

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of a specific pterin deaminase from rat liver we have characterized the enzyme that catalyzes the transition from the pterin to the lumazine series[2].

Using Test 1 as described in METHODS, we proved that the enzyme responsible for the introduction of an oxygen function into position 6 is identical with rat liver xanthine oxidase. The enzymic product is 7,8-dihydro-6-hydroxylumazine which is subsequently converted to 6-hydroxylumazine on standing. Milk xanthine oxidase also catalyses this reaction[3]. For a complete understanding of these catabolic reactions it is desirable now to study this last step in more detail.

METHODS

Hydrogenation of pteridines has already been described[1]. Folic acid was dissolved in water by adding an equimolar amount of $NaHCO_3$ and was hydrogenated in the same way[1] at neutral pH with Pt/H_2.

Formation of 6-hydroxylumazine from tetrahydrolumazine was tested as follows.

Test 1: 250 μg (1.5 μmoles) of tetrahydrolumazine are incubated together with protein in a total volume of 2 ml using 0.1 M Tris-HCl (pH 7.5) at 37° under aerobic conditions and with shaking. The reaction is stopped by adding 1 ml of 30% trichloroacetic acid. The mixture is boiled shortly and allowed to stand in air overnight. Precipitated protein is removed by centrifugation, the supernatant is brought to a volume of 4 ml and the concentration of 6-hydroxylumazine is determined spectrophotometrically at 365 mμ (ε = 5500). The absorption of lumazine (from oxidized substrate) at 365 mμ is negligible.

With purified enzyme fractions from liver or with milk xanthine oxidase Test 2 which is more rapid and accurate, can be used, as follows. Reaction mixtures with 50 μg (0.3 μmoles) of tetrahydrolumazine per ml are incubated in Tris buffer with protein as in Test 1. At intervals 2- ml samples are withdrawn and acidified with 1 ml of 1 M HCl, and the ultraviolet spectrum is taken immediately. 7,8-Dihydro-6-hydroxylumazine is determined by its absorption at 308 mμ (ε = 12300).

With pure xanthine oxidase the protein concentration was 10 μg/ml, incubation time was 20 min. For determinations with crude homogenates the protein concentration was considerably higher[1] and the incubation time had to be lengthened (up to 60 min). Initial absorption readings (zero time) serve as controls in both determinations.

For different reasons the acidic spectra were preferred for measurement in both tests.

(a) The difference between the spectrum of 7,8-dihydrolumazine and of 7,8-dihydro-6-hydroxylumazine is larger at low pH than the difference at pH 7.5 (see Table I). (b) Aerobic oxidation in acidic solution (as for example in Test 1) leads to the non-hydrogenated pteridines without any further non-enzymic hydroxylation (see below). The spectra of lumazine and 6-hydroxylumazine thus formed are also distinctly different (Table I). This type of oxidation is also useful for the identification of products by chromatographic methods. (c) Ultraviolet-absorption data for 6-hydroxylumazine and 7,8-dihydro-6-hydroxylumazine as given above are known from the literature[7,8] whereas those for 7,8-dihydrolumazine are not reported. In most experiments, however, the complete ultraviolet spectra were recorded as a control for the identity of products.

The use of dihydropteridines as substrates for xanthine oxidase proved to be difficult. Products formed by dithionite reduction are mostly obtained only in solution with an excess of reductant, and these solutions are not suitable in investigations with xanthine oxidase. An unequivocal synthesis of 7,8-dihydrolumazine has not been achieved for reasons discussed by ZONDLER AND PFLEIDERER[4]. 7,8-Dihydropterin, synthesized according to STUART *et al.*[5], is only obtained as a bisulphite addition product; we did not succeed in preparing the sodium salt of the pterin described in the paper. 7,8-Dihydropteridines useful as substrates were obtained in an indirect way as described under RESULTS.

All the other methods have been described previously[1,2]. Milk xanthine oxidase was obtained from Boehringer, Mannheim.

RESULTS

Preliminary experiments in crude homogenates

An investigation of the reaction mechanism leading to the formation of 6-hydroxylumazine is specifically possible using tetrahydrolumazine as substrate. In contrast to the previous studies[1], unlabelled tetrahydrolumazine can be used and the course of reaction can be followed spectrophotometrically. The ultraviolet-absorption readings in Table I were used in the identification of substrates, intermediates and products.

TABLE I

ULTRAVIOLET MAXIMA USED FOR IDENTIFICATION OF PTERIDINES

Pteridine	λ_{max} *1 M HCl* ($m\mu$)	λ_{max} *pH 7.5* ($m\mu$)	λ_{max} *1 M NaOH* ($m\mu$)
Tetrahydroneopterin	265	298	286
Tetrahydrolumazine	264	295	295
7,8-Dihydropterin	256	279	281
	(268)	324	324
	368		
7,8-Dihydrolumazine	264	274	277
	362	320	323
6,7,7-Trimethyl-7,8-dihydropterin[6]	252	278	280
	(270)	316	316
	350		
6-Methyl-7,8-dihydropterin[6]	252	279	282
	(271)	324	322
	361		
7,8-Dihydroxanthopterin	275	273	275
	306	310	(310)
7,8-Dihydro-6-hydroxylumazine	268	274	274
	308	311	308
Xanthopterin	268	275	255
	356	(308)	395
		380	
6-Hydroxylumazine[7]	223	226	223
	251	265	276
	365	381	394
Lumazine	325	228	236
		325	269
			346

With tetrahydrolumazine as substrate in crude homogenates the same characteristics of 6-hydroxylumazine formation (time dependence, protein dependence) are observed as already described for tetrahydrobiopterin and tetrahydroneopterin[1].

Ultraviolet spectra of 7,8-dihydropteridines

For theoretical reasons tetrahydrolumazine seems to be a less probable substrate for the xanthine oxidase reaction. 7,8-Dihydrolumazine, which is first formed from tetrahydrolumazine by a non-specific oxidation, is a more likely candidate.

Ultraviolet spectra of methyl-substituted dihydropterins with unequivocal 7,8-dihydrostructure were first described by PFLEIDERER AND ZONDLER[6]. Starting from tetrahydroneopterin and tetrahydrolumazine in Tris buffer (pH 7.5), we found spectra of this type after they had stood for a short time in air (see Table I and Fig. 1). At pH 7.5 and 14 they exactly correspond to the model compounds of PFLEIDERER[7] (7,8-dihydrolumazine shows only small shifts of its λ_{max} values compared with 7,8-dihydropterin). Differences are observed at pH 1. The marked bathochromic shift of the 324 mμ maximum is found here too (368 mμ); its extinction coefficient, however, is considerably lower than in the model compounds. This we interpret as a partial hydration of the 5,6 double bond at this pH which results in an ultraviolet spectrum more similar to that of tetrahydropteridines.

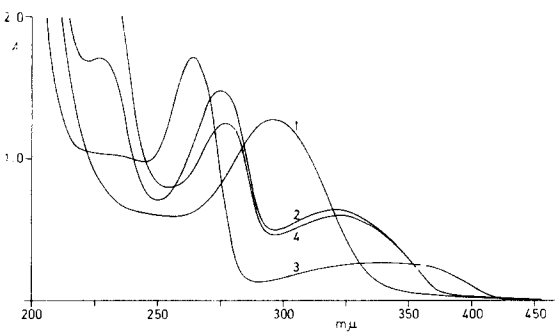


Fig. 1. Ultraviolet spectra, 1, tetrahydrolumazine (25 μg/ml) in 0.1 M Tris–HCl (pH 7.5); 2, same sample after 20 min standing at room temperature; 3, same sample as 2 at pH 1; 4, same sample as 2 at pH 14.

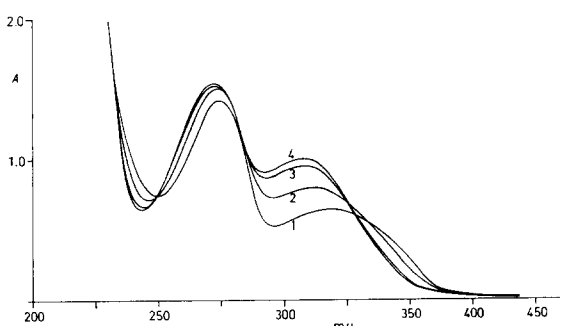


Fig. 2. Ultraviolet spectra. 1, dihydrolumazine (25 μg/ml) in Tris–HCl (pH 7.5), obtained from tetrahydrolumazine as shown in Fig. 1. Spectra 2, 3, and 4 were recorded 5, 10, and 15 min after addition of 66 μg xanthine oxidase/ml to 1.

7,8-Dihydropteridines as substrates of xanthine oxidase

Tetrahydrolumazine is oxidized to 7,8-dihydrolumazine by standing in air as described above. When xanthine oxidase is then added to the solution the ultraviolet spectrum changes to that of 7,8-dihydro-6-hydroxylumazine (Fig. 2). This change is also observed in the absence of enzyme, but then proceeds more slowly (see below).

The course of the enzymic reaction followed by acidic spectra, as described in METHODS, is shown in Fig. 3A, and spectra of the same samples after aerobic oxidation in Fig. 3B.

Non-enzymic formation of 7,8-dihydro-6-hydroxylumazine and 6-hydroxylumazine

In former experiments with crude homogenates, attempts were made to obtain blank values by incubating tetrahydrolumazine with heat-denatured homogenate or in pure buffer. Under such conditions 6-hydroxylumazine is also formed *via* 7,8-dihydro-6-hydroxylumazine in considerable quantities. A comparison with the reaction catalyzed by pure xanthine oxidase is given in Figs. 4A and 4B. The non-enzymic oxidation of tetrahydrolumazine results in the formation of material absorbing at 308 mμ after 10 min. But this cannot be 7,8-dihydro-6-hydroxylumazine, as can be seen from the spectrum and as is clearly shown by the oxidized samples. Fig. 4 unequivocally demonstrates that xanthine oxidase participates in the reaction and that, in the absence of enzyme, hydroxylation starts only after about 20 min and proceeds linearly. Thus after short times of incubation (up to 20 min, as described in Test 2) only the initial rate of the enzymic reaction is observed.

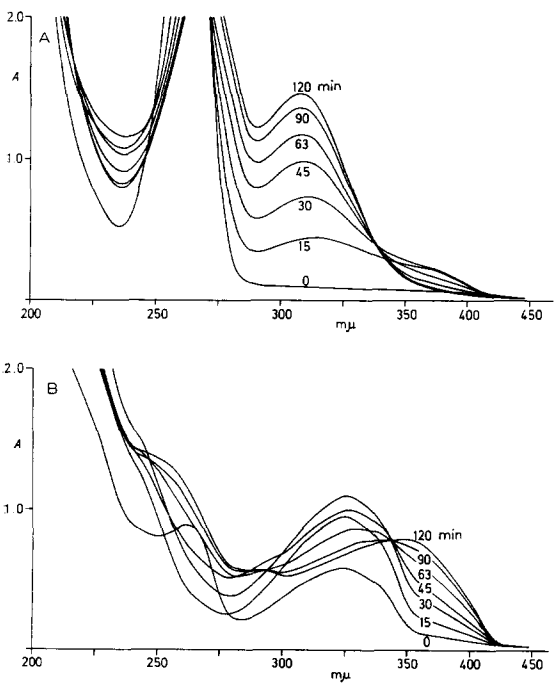


Fig. 3. A. Conversion of tetrahydrolumazine by xanthine oxidase, followed by ultraviolet spectra in 0.33 M HCl and obtained as described in Test 2. Time intervals are given in the figure. B. The same sample as in A, after oxidation in air. Samples at 0 and 15 min are not completely oxidized.

Is xanthine oxidase catalysis the rate limiting step?

Starting with tetrahydrolumazine in our test system, oxidation to the quinonoid dihydro derivative, which has been discussed by many authors[9-12], or tautomerization of the latter to 7,8-dihydrolumazine, could be the rate-limiting step, and the initial rates measured would not be those of the enzymic reaction. Conversion to 7,8-dihydrolumazine is completed after about 20 min, as shown in Fig. 1. Consequently,

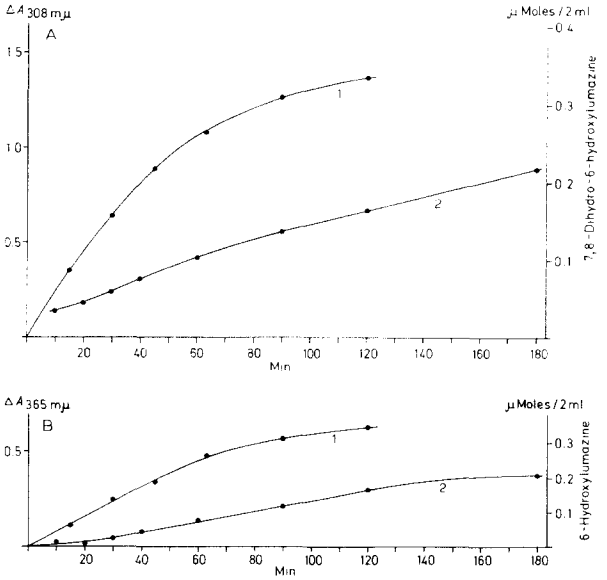


Fig. 4. A. Kinetics of the enzyme-catalyzed and of the non-specific formation of 7,8-dihydro-6-hydroxylumazine from tetrahydrolumazine. Conditions as in test 2. Curves derived from spectra as in Fig. 3A. B. Same kinetics as in A and derived from the oxidized samples, from spectra as in Fig. 3B. Curve 1, with xanthine oxidase; Curve 2, without xanthine oxidase.

if this step were rate limiting, the production of 7,8-dihydro-6-hydroxylumazine should also stop after 20 min, which it does not, as is shown in Figs. 3 and 4. This is further strengthened by the experiment shown in Fig. 5. Delayed addition of the enzyme to the reaction mixture does not bring about marked differences in initial velocities. If conversion to 7,8-dihydrolumazine were rate limiting, one should expect increased initial rates after about 20 min. In contrast, the initial velocities decrease slowly with time which is probably due to substrate depletion by the non-enzymic hydroxylation. So xanthine oxidase catalysis is the rate-limiting step in the reaction sequence under the conditions of our test system.

The enzymic reaction shows a sharp maximal activity at pH 7.9. A Lineweaver–Burk plot results in a $K_m = 4.5 \cdot 10^{-4}$ M for 7,8-dihydrolumazine.

Analogous reactions in the pterin series

From the presence of 7,8-dihydroxanthopterin in the degradation products of tetrahydrobiopterin and tetrahydroneopterin[1] it is evident that an analogous reaction sequence as described above for the lumazine is also followed in the pterin series. The intermediate in this system would be 7,8-dihydropterin, which is formed by a non-specific cleavage of the side chain[1]. The following experiments answer the ques-

tions whether xanthine oxidase is also involved in this reaction sequence and whether the final oxidation of 7,8-dihydroxanthopterin to xanthopterin can be observed under the conditions given for incubation and analysis.

Samples of tetrahydroneopterin and tetrahydrofolic acid were incubated in 10 ml of 0.1 M Tris–HCl (pH 7.5) in the presence and absence of xanthine oxidase (3 mg protein), respectively. After 2 h the reaction was stopped by boiling the mixtures and the products were analyzed by chromatography on Dowex 1X8 and phosphocellulose as described earlier[1]. The results are compiled in Table II.

When tetrahydroneopterin is used as a substrate and the reaction is followed by ultraviolet spectra at pH 7.5, the same sequence of events can be observed as for tetrahydrolumazine (Figs. 1 and 2): first a rapid conversion to the spectrum of

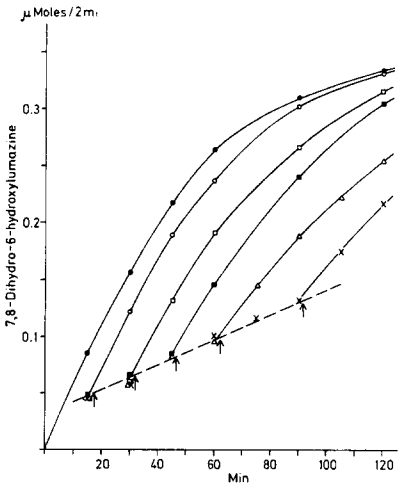


Fig. 5. Delayed addition of xanthine oxidase. Conditions as in Test 2. In different experiments the enzyme was added at different times (arrows) after the start. Final concentrations of xanthine oxidase were the same in each run.

TABLE II

INCUBATION OF TETRAHYDRONEOPTERIN AND TETRAHYDROFOLIC ACID IN TRIS–HCl (pH 7.5) WITH AND WITHOUT XANTHINE OXIDASE

Substrate	*Products (µmoles)*			
	With xanthine oxidase		*Without xanthine oxidase*	
Tetrahydroneopterin (4.2 µmoles)	Isoxanthopterin	0.43	Unknown product	0.28
	Xanthopterin + Dihydroxanthopterin*	1.77	Xanthopterin + Dihydroxanthopterin*	1.11
			Pterin	0.70
Tetrahydrofolic acid (9.7 µmoles)	Isoxanthopterin	0.30		
	Xanthopterin + Dihydroxanthopterin*	4.27	Xanthopterin + Dihydroxanthopterin*	1.94
			Pterin	1.07

* Mixtures of xanthopterin and dihydroxanthopterin could not be separated by our chromatographic procedure. These compounds are identified by comparison of their ultraviolet spectra and R_F values with those of authentic compounds.

7,8-dihydropterin and after addition of xanthine oxidase a further change to the spectrum of 7,8-dihydroxanthopterin.

Inhibition experiments

According to models proposed by FRIDOVICH AND HANDLER[13] and MASSEY *et al.*[14], and KOMAI *et al.*[15] the active site of xanthine oxidase responsible for the oxidation of purines and aldehydes is specifically blocked by *p*-chloromercuribenzoate and by CN^-. The following experiments show that this also applies to our system.

Xanthine oxidase was preincubated in 0.01 M CN^- at pH 7.8 for 15 and 30 min, respectively. It was then diluted, and the remaining activity was found to be of the same order of magnitude both with tetrahydrolumazine (Test 2) and xanthine as substrates. Inhibition of the xanthine oxidase reaction by 1.6 mM *p*-chloromercuribenzoate is complete, whereas the non-enzymic hydroxylation remains unaffected.

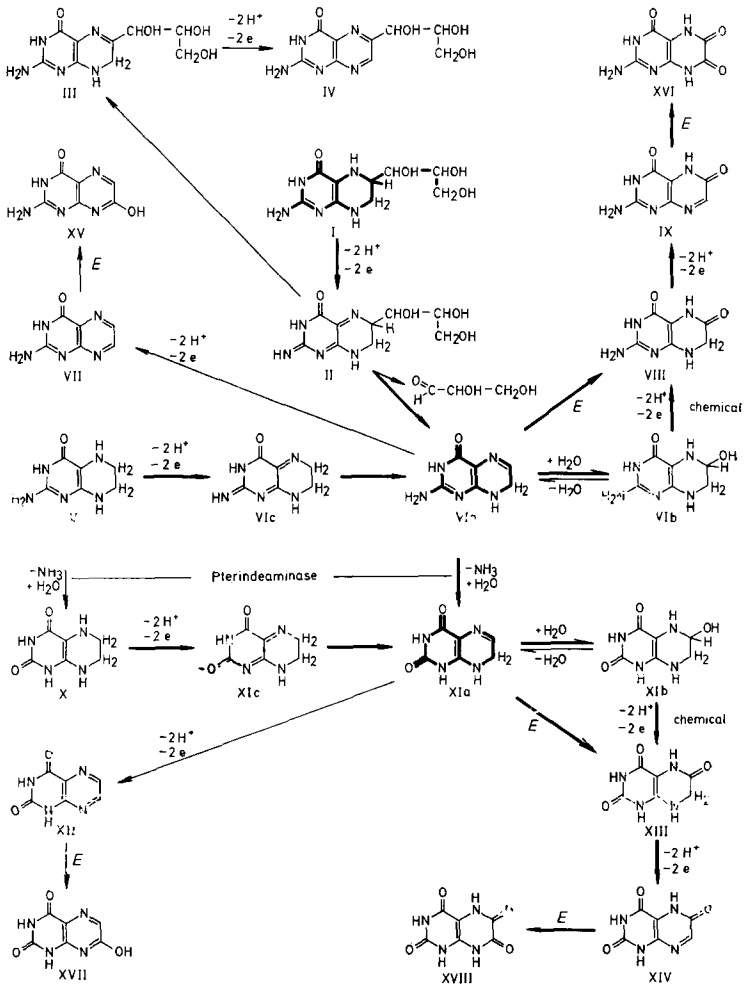
DISCUSSION

The results of this and of the previous papers[1–3] can now be summarized in a reaction scheme for the oxidative degradation of hydrogenated pteridine cofactors (for example for tetrahydroneopterin, I).

Quinonoid dihydropteridines (II, VIc, XIc) have been discussed by many authors as primary oxidation products of tetrahydro derivatives[9–12], whereas others postulate different mechanisms[16]. We could not observe these quinonoid intermediates in our system. However, the assumption of a quinonoid dihydroneopterin (II) would most easily explain our results. This compound has two possibilities of tautomerization; first to 7,8-dihydroneopterin (III) which would give rise to neopterin (IV) after further oxidation, and second to 7,8-dihydropterin (VIa) by the irreversible cleavage of the side chain[1].

Both 7,8-dihydro compounds (VIa and XIa) are converted to their 6-hydroxy derivatives (VIII and XIII) by xanthine oxidase. This reaction can also take place in the absence of enzyme. In contrast to our previous suggestions[3] and based on our recent results, we propose two different pathways for these reactions in the scheme. INOUE *et al.*[17] have shown for quite a number of pteridines that xanthine oxidase preferentially attacks non-hydrated C=N double bonds, whereas hydroxylation by chemical agents starts from hydrated compounds. This could explain the lag phase and the lower yield of the non-enzymic hydroxylation in our system. A hydration of the 5,6 double bond of VIa and XIa appears to be possible at neutral pH at least in a low equilibrium concentration; in acidic medium the ultraviolet spectrum (Fig. 1) indicates a hydration. Oxidation of the hydrated species, VIb and XIb to VIII and XIII, can easily be understood as proceeding *via* a quinonoid intermediate which subsequently tautomerizes.

Thus the presence of xanthopterin (IX) in biological materials can be explained in two ways. Tetrahydrofolic acid or tetrahydrobiopterin may be present originally, and then transformed to xanthopterin by non-specific reactions during the isolation procedure, following the scheme indicated. Alternatively, degradation is effected in the organism by the action of xanthine oxidase. Dihydroxanthopterin (VIII) formed is then excreted as such or as xanthopterin. In this way the deposition of xanthopterin in many insects can be explained.



In the absence of a deaminating enzyme—*i.e.* during oxidations in pure buffer or by the use of pure xanthine oxidase—only the upper part of the reaction scheme is followed. In a crude homogenate or on the addition of a deaminase fraction[2] one passes from the pterin to the lumazine series. The only difference is that here oxidation of XIII to 6-hydroxylumazine (XIV) proceeds considerably faster than that of VIII to IX and is always quantitative. Thus only two enzymes are involved in the reaction scheme. Indeed a pterin deaminase fraction[2] and pure xanthine oxidase are sufficient to obtain 6-hydroxylumazine from tetrahydroneopterin in high yields.

The reaction scheme is completed by those metabolites produced by the action of xanthine oxidase upon non-hydrogenated pteridines. Thus xanthine oxidase converts pterin (VII), xanthopterin (IX), lumazine (XII), and 6-hydroxylumazine (XIV) to isoxanthopterin (XV), leucopterin (XVI), 7-hydroxylumazine (XVII) and 6,7-dihydroxylumazine (XVIII), respectively[18–20].

In this way the origin of all essential metabolites isolated from incubations with tetrahydrobiopterin and tetrahydroneopterin *in vitro* can be explained. The high

O_2 consumption of the overall reaction is equally well understood. The balances of products described earlier[1] not only demonstrate the activity of the two enzymes involved (pterin deaminase and xanthine oxidase), but they also reflect the O_2 supply of the mixture during the incubation and its termination by boiling. This especially applies to those products – biopterin (neopterin), pterin, dihydroxanthopterin and xanthopterin – that are produced in high yield by non-enzymic reactions.

That this catabolism is not confined to systems *in vitro* has recently been shown in insects. The results of HARMSEN[21] and DESCIMON[22] on pteridine metabolism in Pierids are in excellent agreement with this scheme. Moreover, the experiment of HARMSEN[21] further stresses the importance of O_2 pressure as discussed above.

In combination with reactions leading to a loss of the C-6 side-chain xanthine oxidase seems to play a role in the catabolism of pteridine cofactors similar to its role in purine metabolism.

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REFERENCES

1 H. REMBOLD, H. METZGER, P. SUDERSHAN AND W. GUTENSOHN, *Biochim. Biophys. Acta*, 184 (1969) 386.
2 H. REMBOLD AND F. SIMMERSBACH, *Biochim. Biophys. Acta*, 184 (1969) 589.
3 H. REMBOLD AND W. GUTENSOHN, *Biochem. Biophys. Res. Commun.*, 31 (1968) 837.
4 H. ZONDLER AND W. PFLEIDERER, *Chem. Ber.*, 99 (1966) 2984.
5 A. STUART, H. C. S. WOOD AND D. DUNCAN, *J. Chem. Soc.*, (1966) C 285.
6 W. PFLEIDERER AND H. ZONDLER, *Chem. Ber.*, 99 (1966) 3008.
7 W. PFLEIDERER, *Chem. Ber.*, 90 (1957) 2604.
8 A. ALBERT AND S. MATSUURA, *J. Chem. Soc.*, (1962) 2162.
9 S. KAUFMAN, *J. Biol. Chem.*, 239 (1964) 332.
10 M. VISCONTINI AND A. BOBST, *Helv. Chim. Acta*, 48 (1965) 816.
11 D. J. VONDERSCHMITT AND K. G. SCRIMGEOUR, *Biochem. Biophys. Res. Commun.*, 28 (1967) 302.
12 K. H. NIELSEN, *European J. Bioch.*, 7 (1969) 360.
13 I. FRIDOVICH AND P. HANDLER, *J. Biol. Chem.*, 231 (1958) 899.
14 V. MASSEY, P. E. BRUMBY AND H. KOMAI, *J. Biol. Chem.*, 244 (1969) 1682.
15 H. KOMAI, V. MASSEY AND G. PALMER, *J. Biol. Chem.*, 244 (1969) 1692.
16 A. W. WAHLEFELD AND L. JAENICKE, *Ann. Chem.*, 715 (1968) 52.
17 Y. INOUE AND D. D. PERRIN, *J. Chem. Soc.*, (1962) 2600.
18 O. H. LOWRY, O. A. BESSEY AND E. J. CRAWFORD, *J. Biol. Chem.*, 180 (1949) 389.
19 O. H. LOWRY, O. A. BESSEY AND E. J. CRAWFORD, *J. Biol. Chem.*, 180 (1949) 399.
20 F. BERGMANN AND H. KWIETNY, *Biochim. Biophys. Acta*, 33 (1959) 29.
21 R. HARMSEN, *J. Insect Physiol.*, 15 (1969) 2239.
22 H. DESCIMON, *Compt. Rend., Ser. D*, 268 (1969) 416.