PTERIDINE-REQUIRING DIHYDROOROTATE HYDROXYLASE FROM CRITHIDIA FASCICULATA*

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Summary. Dihydroorotic acid is converted to orotic acid in Crithidia by hydroxylation and subsequent dehydration. The hydroxylase is soluble, stable to acid (pH 4.0), destroyed by alkali (pH 11) and by heat (55° for 3 min.). Activity is rapidly lost upon standing at 4° and upon freezing. Its activity is optimum at pH 7.4. Its isoelectric point is 6.2. It has an absolute dependence on O_2 and a reduced pteridine. Pteridine reductases are present in cell extracts which, in the presence of NADH, permits the efficient use of biopterin (the oxidized form of the naturally occurring pteridine in this organism) as a cofactor for the hydroxylase.

A decade has passed since the suggestion was made (1) that, in the trypanosomid (kinetoplastid) flagellate, Crithidia fasciculata, the reaction in the biosynthetic pathway leading to pyrimidines, in which an unconjugated pteridine was implicated, probably was the dehydrogenation of orotic acid. This suggestion was made on purely theoretical grounds to account for the observed sparing effect of pyrimidines on biopterin, a nutritional requirement of the flagellate. The reaction was visualized as a hydroxylation of dihydroorotic acid, analogous to the hydroxylation of phenylalanine (2), producing an intermediate which, upon dehydration would give rise to orotic acid. The status of this problem up to 1967 has been reviewed (3).

We have now obtained evidence clearly demonstrating that the formation of orotic acid from dihydroorotic acid in Crithidia is dependent upon aerobic conditions, a reduced pteridine, reduced

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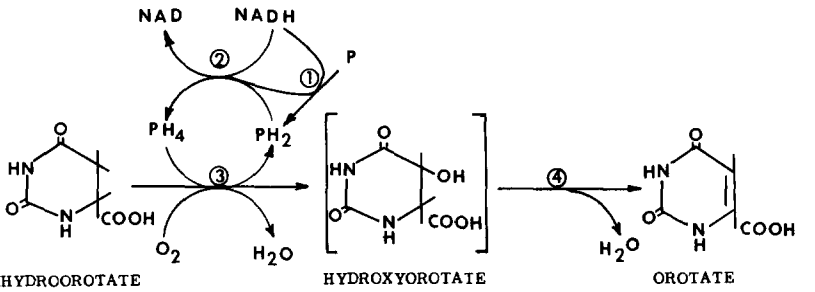

Fig. 1. Scheme for the biosynthesis of orotic acid from dihydroorotic acid in Crithidia fasciculata. Circled numbers represent the following enzymes: 1 = pteridine reductase; 2 = dihydropteridine reductase; 3 = dihydroorotate hydroxylase; 4 = a dehydrase (this reaction may be spontaneous). P = pteridine (oxidized as in biopterin); PH_2 and PH_4 = dihydro- and tetrahydropteridine.

pyridine nucleotide and a hydroxylase. The reactions are represented in Figure 1. We have not isolated the intermediate.

MATERIALS AND METHODS

All isotopically labeled compounds used were products of New England Nuclear Corp. Our sample of (4-^{14}C)dihydroorotic acid (DHO)**, a racemic mixture, had a specific activity of 3.03 mCi/mmole. Before use it was purified by paper chromatography and diluted to 5 x 10^6 cpm/ml. $DMPH_4$ was obtained from Calbiochem. Sources of all other materials, culture methods for obtaining Crithidia in the exponential phase, harvesting of the cells and preparing the cell extracts have been described elsewhere (4). The basal medium contained no pyrimidine.

Assays for the OA synthesizing enzymes were made by incubating the enzyme preparations with radioactive substrate (DHO), phosphate buffer and the various additions as noted, for 1 hr at 37° in subdued light. The reaction was stopped with a drop of glacial acetic acid and an aliquot of each, together with appro-

**Abbreviations used: DHO, dihydroorotic acid; OA, orotic acid; CAA, carbamylapartic acid (ureidosuccinic acid); $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine; NADH, reduced nicotinamide adenine dinucleotide.

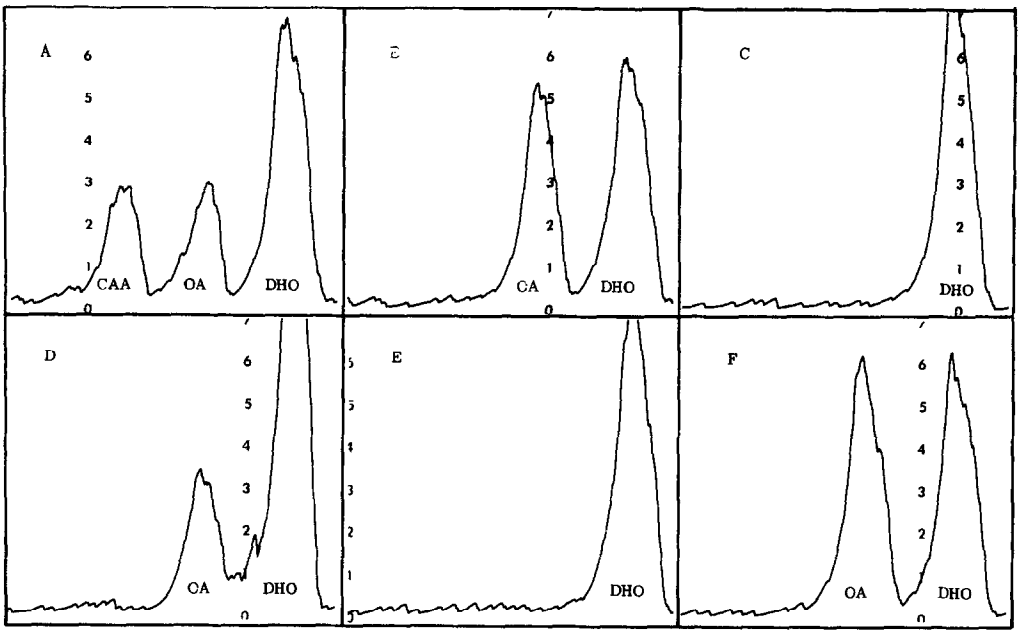

Fig. 2. Traces showing radioactivity of scans (origin, not included in figures, to left) of chromatograms, developed in isopropanol:ammonia:H_2O (7:1:2 v/v), of reaction mixtures all of which contained, in a total volume of 0.25 ml, 0.4 µmoles of (4-^{14}C)DHO (4 X 10^5 cpm), 2.5 µmoles phosphate buffer (pH 7.4). A contained crude cell extract (supernatant fraction of 100,000 X g sonicate, 56 µg protein); B contained acid-treated extract (6.1 µg protein); C-F contained acid-treated and dialyzed extract (3.2 µg protein). A-C contained no further additions; D contained $DMPH_4$ (5 µg); E contained biopterin (0.25 µg); F contained both biopterin (0.25 µg) and NADH (0.1 mg).

priate authentic samples, were placed in lanes on a large (46 X 57 cm) sheet of Whatman #1 paper. Either radioactive or unlabeled authentic samples were used, the latter being visualized with UV light (OA) or by treating with p-dimethylaminobenzadehyde, before (CAA) or after hydrolysis (DHO), following the method of Fink, et al. (5). After development in either n-butanol:acetic acid:H_2O (20:3:7 v/v) or in isopropanol:ammonia:H_2O (7:1:2 v/v) in a descending direction, the lanes were cut out and the position of the radioactive peaks determined by use of a Tracerlab 4π scanner. The peaks were quantitated by determining their areas by planimetry.

Anaerobic conditions, sufficient for our experiments, were obtained by adding the substrate (DHO) to the side arm of a small (10 ml) Thunberg tube and all other components of the reaction to the main tube. The tubes were then evacuated and sealed, connected to a nitrogen source, re-opened and immediately sealed again. After tipping in the substrate the tubes were incubated with their various controls.

Extracts of cell sonicates were prepared (4) from the high speed (100,000 X g for 3 hrs) supernatant fraction. These extracts contained the DHO hydroxylase as well as the pteridine reductases. The hydroxylase contained sufficient reduced pteridine for OA production. This cofactor was removed by membrane dialysis (Amicon, PM10) against 50 or more volumes of buffer containing dithioerythritol (50 µg/ml). This extract contained a dihydropyrimidinase which competed for the substrate in the isotopic assay. Its activity was destroyed by acid treatment. The pH of the extract was adjusted to 4.0 with HCl and the copious precipitate which formed was removed by centrifugation. The pH was rapidly brought to 7.0 with NaOH and the second precipitate which formed was centrifuged out. Protein determinations were made by the method described by Lowry, et al. (6) or spectrophotometrically (7). In some cases this extract, after concentration by membrane filtration (Amicon, PM10) was subjected to electrofocusing (LKB Instruments, Inc.). The fractions were assayed for DHO hydroxylase activity, as above, but with $DMPH_4$ added. This technique was of limited value at this stage of the investigation as the hydroxylase proved to be very unstable when subjected to this much purification.

RESULTS AND DISCUSSION

When undialyzed cell extracts are incubated with DHO both OA

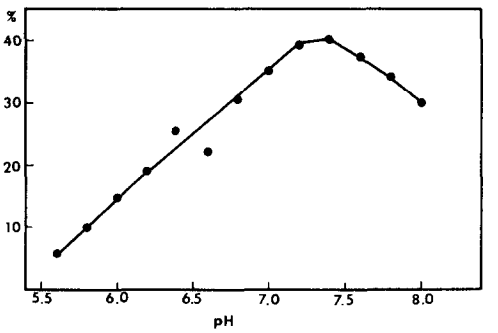

Fig. 3. Percent conversion of DHO to OA in relation to pH. Reaction mixtures contained cell extract as in A of Fig. 2, 0.8 μmoles of (4-^{14}C)DHO (8 X 10^5 cpm), 10 μmoles of phosphate buffer at varying pH values. Total volumes 0.5 ml.

and CAA are produced (Fig. 2, A), while acid-treated, undialyzed extract yields only OA (Fig. 2, B). Dialysis renders the acid-treated extract inactive (Fig. 2, C; Table 1) but OA production can be partially restored by the addition of high concentrations of fully reduced pteridine ($DMPH_4$) (Fig. 2, D; Table 1). The addition of oxidized pteridine (6-biopterin) to the dialyzed extract has no effect (Fig. 2, E; Table 1) unless a reduced pyridine nucleotide (NADH) is also present (Fig. 2, F; Table 1). Catalytic amounts of biopterin, together with NADH, results in 100% of the natural isomer of the substrate being converted to OA. This reaction is irreversible as evidenced by the fact that none of the preparations act on added OA. We have never been able to achieve much more than a 50% yield of OA with $DMPH_4$, an unnatural pteridine, and the addition of NADH does not increase the conversion (Table 1). These results can only mean that dialysis removes a natural pteridine cofactor together with the electron donor (NADH) necessary for the initial production of a dihydropteridine and cycling of the tetrahydropteridine (Fig. 1). The results also indicate that there are pteridine reductases present in this type of preparation.

TABLE 1

Effect of reduced pyridine nucleotide and pteridines (oxidized and reduced) on the production of OA from DHO. All reaction mixtures contained 0.4 μmoles of (4-^{14}C)DHO (4 X 10^5 cpm), 2.5 μmoles of phosphate buffer (pH 7.4), acid treated, dialyzed, 100,000 X g supernatant fraction of sonicated cells (2.3 μg protein) in a total volume of 0.25 ml.

ADDITIONS	PERCENT CONVERSION TO OA
None	0
NADH (0.1 mg)	0
6-Biopterin (0.25 μg)	0
$DMPH_4$ (5 μg)	52.2
6-Biopterin + NADH	101.2
$DMPH_4$ + NADH	51.4

TABLE 2

Effect of oxygen on the production of OA from DHO. Reaction mixtures all contained 3.4 μmoles of (4-^{14}C)DHO (2.4 X 10^6 cpm), 15 μmoles of phosphate buffer (pH 7.0), 15 μg $DMPH_4$, 0.2 ml of a high speed (100,000 X g) supernatant fraction of cell sonicate in a total volume of 1.7 ml. All reaction mixtures were incubated 1 hr at 37° under the following conditions: A in small (10 X 40 mm) tubes; B in 25 ml Erlenmeyer flasks (for increased aeration); C in Thunberg tubes under nitrogen.

	Percent OA formed	
	Exp. 1	Exp. 2
A	24.8	23.2
B	52.8	43.2
C	3.0	8.0

That the production of OA from DHO in Crithidia is oxygen-dependent is illustrated in Table 2. This was expected, as it had been earlier shown (8), by the use of a coupled system which

evolved $^{14}CO_2$ from (carboxyl-^{14}C)DHO during OMP decarboxylation, that the series of reactions (DHO $\longrightarrow$ OA $\xrightarrow{PRPP}$ OMP $\xrightarrow{CO_2}$ UMP) proceeded under oxygen but not under nitrogen or argon.

The intermediate compound which must be formed from DHO by hydroxylation (hydroxyorotate, Fig. 1) apparently does not accumulate as we have seen no evidence of a third compound on the appropriate chromatograms. This would mean that our preparations either contain a powerful hydroxyorotate dehydrase or the dehydration is spontaneous and rapid.

Regarding the properties of the DHO hydroxylase of Crithidia our observations lead us to conclude the following: it (along with the pteridine reductases) is freely soluble, it is relatively unstable at 4° (all activity is lost within two weeks upon refrigerator storage), it is very unstable to freezing when extracted into buffer although good recovery has been achieved from cells following lyophilization. Protection against freeze-destruction appears to be offered by cell materials although no protection was achieved by adding bovine serum albumin to extracts. Alkali treatment (pH 11) completely destroys the enzyme, as does heat (55° for 3 min.). Under the conditions of our experiments we are unable to rule out a role for metal ions. The optimum pH for activity is 7.4 and the isoelectric point, as indicated by electrofocusing (10% ampholine, pH 3-10 range), is about 6.2.

The production of OA from DHO in Crithidia fasciculata is carried out in an entirely different manner from what has been found in the anaerobic microorganism, Zymobacterium oroticum (9-13), where the DHO dehydrogenase is an iron-containing flavoprotein requiring NAD as a cofactor. This dehydrogenase, as are those of Corynebacterium (14), Escherichia coli (15-16), Pseudo-

monas (17) and rat liver mitochondria (18) is particle bound. So far no organism other than Crithidia has been found to have a pteridine-requiring DHO hydroxylase, just as no other organism, except other trypanosomid (kinetoplastid) flagellates, has been found to have a nutritional requirement for an unconjugated pteridine (3), or alternatively, synthesized biopterin from the pteridine moiety of folic acid (19).

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