THE CONVERSION OF DIHYDRONEOPTERIN TRIPHOSPHATE TO SEPIAPTERIN BY AN ENZYME SYSTEM FROM *Drosophila melanogaster*

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Summary: The enzyme system for the synthesis of the pteridine pigment, sepiapterin, from 2-amino-4-hydroxy-6-(D-erythro-1',2',3'-trihydroxypropyl)7,8-dihydropteridine triphosphate (dihydroneopterin triphosphate) has been found in extracts of *Drosophila melanogaster*. $NADP^+$ or NADPH and Mg^{2+} are required for this enzymatic transformation. No sepiapterin is produced when dihydroneopterin is supplied as substrate in place of dihydroneopterin triphosphate.

Introduction: Dihydroneopterin[1] triphosphate (H_2-neopterin-PPP) is known to be produced from GTP in the presence of the enzyme GTP cyclohydrolase (or GTP cyclohydrolase I to distinguish it from a similar enzyme, GTP cyclohydrolase II (1)), known to occur in bacteria (2-4), plants (5) and *Drosophila melanogaster* (6). H_2-neopterin-PPP is known to be a precursor of the pterin portion of folic acid (7,8) and other pterins (9) in bacteria, and the view has been expressed (10) that this compound (or perhaps its dephosphorylated form, H_2-neopterin) is a key intermediate in the biosynthesis of all naturally-occurring pterins. This view has received some support from recent observations by Fukushima, *et al.* (11) that suggest it is an intermediate in the biosynthesis of biopterin in Syrian golden hamsters and by the report of Fan and Brown (6) on the presence of GTP cyclohydrolase in *Drosophila melanogaster*. The latter animal contains large quantities of several kinds of pterins present mainly as eye pigments. These include xanthopterin, isoxanthopterin, pterin, sepiapterin and drosopterin (the major pterin eye pigment). In the present paper we provide further support for the role of H_2-neopterin-PPP as a key intermediate with the finding that one of

[1] Dihydroneopterin or H_2-neopterin is the trivial name for 2-amino-4-hydroxy-6-(1',2',3'-trihydroxypropyl)7,8-dihydropteridine. Pterin is the trivial name for 2-amino-4-hydroxypteridine.

these pigments, sepiapterin, can be synthesized from H_2-neopterin-PPP in the presence of an enzyme system from Drosophila melanogaster. The nature of this enzymatic transformation becomes apparent from the formulas of the compounds shown later in Fig. 3 of this paper.

Materials and Methods: [U-^{14}C]GTP was purchased from Amersham-Searle Corp., phosphocellulose and ECTEOLA-cellulose from Gallard Schlesinger, and piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES) buffer from Calbiochem. Purified GTP cyclohydrolase I from E. coli was supplied by John Yim of this laboratory. Sepiapterin was prepared (12) from sepia mutants of Drosophila melanogaster.

Crude extracts of Drosophila melanogaster, strain Oregon-R (obtained from Dr. Linda Hall), were prepared as described earlier (6). Radioactive H_2-neopterin PPP was prepared enzymatically by incubation (overnight at 42° in a total volume of 1.0 ml) of 100 μM [U-^{14}C]GTP (10^7 cpm), 100 mM PIPES buffer (pH 7.5), 100 mM KCl, 5 mM EDTA (pH 7.5), and 10 mM 2-mercaptoethanol with enough purified GTP cyclohydrolase I to achieve at least 90% conversion of GTP to H_2-neopterin-PPP (measured as described earlier (6)). The standard reaction mixture for the assay of sepiapterin synthesis contained per 0.2 ml total volume: 75 mM PIPES buffer (pH 7.5), 20 mM $MgCl_2$, 3.3 mM NADPH, 0.1 ml of the incubated reaction mixture described above as a source of H_2-neopterin-PPP, and Drosophila extract. After incubation for 1 hour at 42°, 4 units of alkaline phosphatase (Worthington) and 0.1 μmole of sepiapterin were added and the incubation was continued for an additional 30 minutes. Each mixture was heated at 100° for 5 minutes and subjected to centrifugation. The resulting supernatant material was applied to a phosphocellulose column (1.1 x 50 cm). The column was developed at 4° with water. Fractions of 5.0 ml were collected at a rate of 15 ml per hour. A portion of each fraction was analyzed for radioactivity in a scintillation counter (Packard 3320). The fractions containing standard sepiapterin were yellow. Sepiapterin synthesis was estimated from the radioactivity in the region of elution from the column of standard sepiapterin.

Thin layer chromatography was carried out (ascending technique) at room temperature with 13255 cellulose Eastman Chromagram sheets (5 x 10 cm). Protein was determined by the method of Lowry, et al. (13) with bovine serum albumin as the standard.

Results: Preliminary experiments indicated that a radioactive substance with the chromatographic properties of sepiapterin could be produced enzymatically by incubation of radioactive H_2-neopterin-PPP with a crude extract of Drosophila melanogaster. Similar experiments revealed that the addition of $NADP^+$ or NADPH stimulated the production of the putative sepiapterin by 2- to 3-fold and that $MgCl_2$ was also stimulatory. The results of an experiment devised to measure the enzymatic conversion of H_2-neopterin-PPP to this radioactive substance in the presence of a crude extract are given in Fig. 1. In this experiment, both NADPH and $MgCl_2$ were included in the incubation mixture to promote maximal synthesis of the product. The data presented in the figure represent the elution pattern

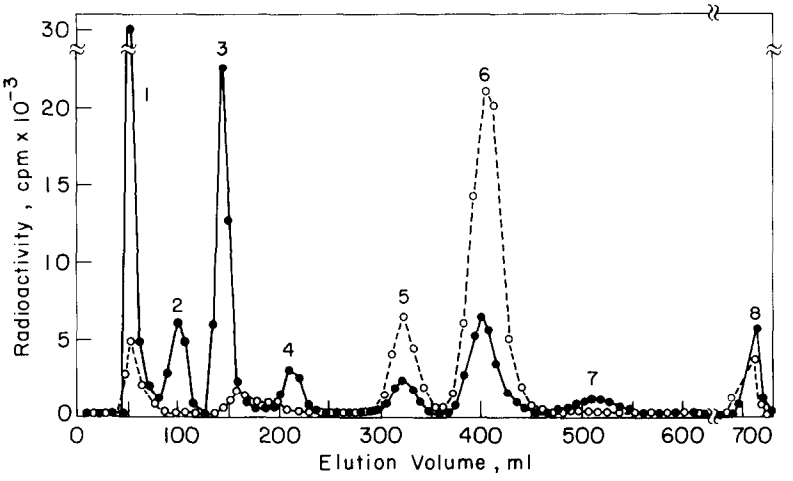

Fig. 1. Elution pattern from phosphocellulose of an incubated reaction mixture. The standard reaction mixture was prepared to contain either a crude extract of Drosophila (2.8 mg of protein) or heated (at 100° for 5 minutes) extract. After incubation (1 hour at 42°) and treatment with phosphatase, sepiapterin (0.1 μmole) was added to each mixture as a marker. The mixtures were applied to individual columns and each was developed with water (650 ml) and then with 1% NH_3. Portions (1.5 ml) of fractions were analyzed for radioactivity. The standard sepiapterin appeared as a yellow substance in the fractions collected in the 135-150 ml region. The elution pattern of the reaction mixture containing unheated extract is represented with closed circles (•) and that of the control mixture, containing heated extract, with open circles (o).

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from a phosphocellulose column of radioactive compounds present after incubation of ^{14}C-labeled H_2-neopterin-PPP with heated and unheated extract. Peak 3 of the elution pattern coincides with the position of elution of standard sepiapterin. Peaks 5 and 6 coincide with the positions of elution of guanosine and neopterin, respectively. The guanosine must have been derived (by treatment with phosphatase) from unreacted GTP that remained as a contaminant in the H_2-neopterin-PPP preparation, and neopterin (Peak 6) was produced from unreacted H_2-neopterin-PPP by the phosphatase treatment and oxidation during the development of the column. Peak 2 contained xanthine, presumably produced enzymatically from GTP. All of these radioactive substances were identified by thin layer chromatography. Peak 1 coincided with the void volume of the column and contained several radioactive components of unknown identities. Peaks 4, 7 and 8 also contained small quantities of unknown radioactive compounds. Approximately

94% of the radioactivity applied to the column was recovered in Peaks 1-8. The radioactivity associated with the enzymatically-produced materials present in Peaks 1, 3, 4 and 7 (418,000 cpm) approximately equalled the amount of H_2-neopterin PPP consumed during the incubation. Of the radioactivity present in Peaks 1, 3, 4 and 7, 54% was accounted for in Peak 3, the putative sepiapterin peak.

To confirm that the radioactive material present in Peak 3 was sepiapterin, the material was subjected to chromatographic analyses. For this purpose, the fractions constituting the peak were combined, concentrated to dryness, and the residue was redissolved in 0.075 ml water. Portions (0.004 ml, 11,000 cpm) of the concentrated material were subjected to thin layer chromatography (Table I). The coincidence of R_F values of standard sepiapterin and the enzymatic product indicate that the product was sepiapterin. In another experiment to be presented later in this paper, we obtained spectrophotometric evidence that supports the conclusion that this radioactive product was sepiapterin. For convenience, in the remainder of this paper the radioactive product present in Peak 3 of Fig. 1 will be referred to as sepiapterin.

The presence in crude extracts of sepiapterin and pyridine nucleotides

Table I. Identification by Thin Layer Chromatography of the Enzymatic Product as Sepiapterin

	R_F values in solvent system					
Compound	1	2	3	4	5	6
Sepiapterin	0.40	0.39	0.24	0.48	0.49	0.40
Enzymatic product	0.40	0.39	0.24	0.49	0.50	0.40

Solvent systems: 1, 1-propanol-1% NH_3 (2:1); 2, 1-propanol-ethylacetate-H_2O-(7:1:2); 3, 3% NH_4Cl; 4, 1-butanol-acetic acid-H_2O (4:1:2); 5, 1-propanol-1% ammonium acetate (1:1); 6, ethanol-ammonium borate (pH 8.0, 5% as boric acid)-3% NH_4Cl (2:1:1). Standard sepiapterin was observed as yellow fluorescent zones under ultraviolet light. To locate the radioactive enzymatic product, appropriate areas were scraped from the plate and transferred to vials, each containing 1.7 ml of H_2O and 5.0 ml of ScintiVerse (Fisher Scientific Co.), and radioactivity was measured in a scintillation counter.

interferred with (a) the spectrophotometric analysis for the production of sepia pterin and (b) an assessment of the degree and kind of pyridine nucleotide requirement. To circumvent these difficulties, we fractionated the crude extract with ammonium sulfate and found that most of the enzymatic activity for the synthesis of sepiapterin was recovered in the protein fraction that precipitated between 40 and 60% saturation and that no sepiapterin was present in this fraction. This fraction was used in the remainder of the experiments to be reported below.

The synthesis of sepiapterin from H_2-neopterin-PPP was linear with protein concentration up to 14 mg/ml. Other observations are: (a) H_2-neopterin cannot replace H_2-neopterin-PPP as substrate, (b) no sepiapterin is produced if the ammonium sulfate fraction is omitted from the reaction mixture, (c) the sepiapterin produced enzymatically is not phosphorylated, (d) Mg^{2+} is an absolute requirement, and (e) no sepiapterin is produced unless either $NADP^+$ or NADPH is added to reaction mixtures. Neither NAD^+ nor NADH can satisfy this pyridine nucleotide requirement.

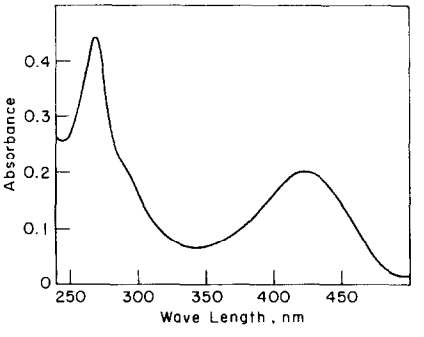

Fig. 2. Absorption spectrum of the enzymatic product. Six standard reaction mixtures were prepared (see text). After incubation, the mixtures were heated and subjected to centrifugation to remove insoluble material. The combined supernatant fractions were applied to a standard phosphocellulose column and the column was developed with H_2O. The fractions constituting the region of elution of sepiapterin were combined (40 ml) and concentrated to 3.0 ml. The resulting yellow solution was applied to an ECTEOLA-cellulose column (1.1 x 55 cm). The column was developed with H_2O. The yellow fractions were combined (20 ml), concentrated (1 ml) and subjected to chromatography on a second phosphocellulose column. The resulting yellow fractions were combined (40 ml) and concentrated to 1 ml. The spectrum of this material in H_2O was determined with a Perkin-Elmer spectrophotometer, Model 202.

In order to obtain enough of the enzymatic product for a spectrophotometric analysis, six of the standard reaction mixtures were prepared, and to each was added an amount of ammonium sulfate fraction equal to 24 mg of protein. No standard sepiapterin was included. The processing of the reaction mixtures to purify the putative sepiapterin product was as described in the legend to Fig. 2. The absorption spectrum of the purified yellow product, given in Fig. 2, is identical with that reported by Tsusue and Akino (14) for sepiapterin. This, along with the chromatographic evidence presented in Table I, strongly indicates that the enzymatic product is sepiapterin.

Discussion: The observation that Drosophila melanogaster contains GTP cyclohydrolase (6), the enzyme that catalyzes the conversion of GTP to H_2-neopterin-PPP, and the results reported in this paper on the enzymatic formation of sepiapterin from H_2-neopterin-PPP lend further support to the contention that the latter compound is a key intermediate in the biosynthesis of all naturally-occurring pterins. Several pterins other than sepiapterin are known to occur in substantial quantities in Drosophila. Whether or not these pterins are made enzymatically from H_2-neopterin-PPP is presently under investigation in our laboratory.

Our observation that H_2-neopterin is not converted to sepiapterin by the

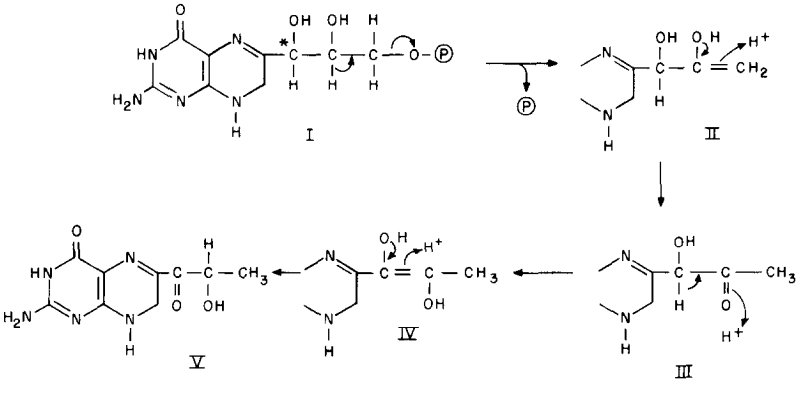

Fig. 3. Possible reaction mechanism for the enzymatic conversion of a phosphorylated form of H_2-neopterin (I) to sepiapterin (V). Ⓟ represents either one, two, or three phosphate residues. The carbon in compound I marked with an asterisk (*) is carbon 1'.

enzyme system of Drosophila shows that a phosphorylated form of H_2-neopterin is necessary and suggests that the removal of one or more of the phosphate groups occurs coincidently with the formation of sepiapterin, but we do not yet know how many of the three phosphate groups need to be retained to allow the H_2-neopterin moiety to be converted directly to sepiapterin since neither H_2-neopterin-P nor H_2-neopterin-PP has been available, and we have not yet fractionated the enzyme system enough to decide how many enzymes might be involved.

A plausible reaction pathway for the formation of sepiapterin would include, as a first step, the removal of a phosphate (or pyrophosphate or triphosphate) group to produce compound II, shown in Fig. 3. By rearrangement, this putative enzyme-bound intermediate could then be converted through intermediates III and IV to give sepiapterin (V). Although this reaction mechanism does not include an oxidation or a reduction and thus provides no role for a pyridine nucleotide, the removal of phosphate in the initial step might be expected to be facilitated by the prior oxidation of the OH group on carbon 1' to a keto group. Such an oxidation might require $NADP^+$, but if this occurs one might expect that a reduction should occur later to regenerate the $NADP^+$ since no oxidation or reduction occurs in the overall proposed reaction. More precise knowledge about the pyridine nucleotide requirement and the mechanism of the reaction must await the purification of the enzyme or enzymes involved in the conversion of H_2-neopterin-PPP to sepiapterin.

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