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IDENTIFICATION OF 5,6,7,8-TETRAHYDROPTERIN AND 5,6,7,8-TETRAHYDROBIOPTERIN IN *Drosophila melanogaster*

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SUMMARY: Using reversed-phase high-performance liquid chromatography with electrochemical detection we have demonstrated the occurrence of 5,6,7,8-tetrahydropterin and 5,6,7,8-tetrahydrobiopterin in *Drosophila melanogaster*. The former is the first time that has been detected *in vivo*. The identification has been based on the retention times, hydrodinamic voltagrams and the differential concentration in three strains of *Drosophila melanogaster*. Compared to the wild type, the *Punch*^{2} mutant has diminished levels of both pteridines, whereas *Henna-recessive*^{3} lacks completely tetrahydropterin and has increased levels of tetrahydrobiopterin, as expected according to their biochemical lesions.

The role of H_4biopterin as a cofactor for the aromatic amino acid hidroxylases in mammals is well documented and measurements of cofactor levels in different tissues have been reported by several groups (1, 2). However, very little is known about the hydroxylation system in insects. In *Drosophila melanogaster* there are indirect evidences that H_4biopterin could be the cofactor for the aromatic amino acid hydroxylases (3, 4). Recently, it has been hypothesized that another tetrahydropteridine, *viz.* H_4pterin, is involved in the hydroxylation of phenylalanine in Drosophila; this was based on the *in vivo* stimulation of the synthesis of pterin by phenylalanine, the lethal effects of the latter on the Hn^{r3} mutant (which has impaired the biosynthesis of pterin), and the high levels of phenylalanine in this mutant (5).

Abbreviations: H_4, tetrahydro; H_2, dihydro; DTT, dithiothreitol; DTE, dithioerythreitol; HPLC, high-performance liquid chromatography.

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In spite of the above considerations, neither H_4biopterin nor H_4pterin had ever been detected in Drosophila. In the present paper we have been able to demonstrate the occurrence of both tetrahydropteridines using HPLC with electrochemical detection. Furthermore, the analysis of two pteridine biosynthesis mutants (Hn^{r3} and Pu^{2}) has served to corroborate the identity of both tetrahydropteridines according to the expected concentrations of these compounds *in vivo*.

MATERIALS AND METHODS

Chemicals: Bovine liver dihydrofolate reductase, octyl sodium sulfate, N-acetyl-serotonin, DTT and DTE were purchased from Sigma Chem. Co. H_4Biopterin and H_4pterin were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). H_2Neopterin triphosphate was synthesized with GTP cyclohydrolase I that had been purified from *Escherichia coli* using affinity chromatography on UTP-Sepharose (6).

Preparation of synthetic standards and Drosophila extracts: Because of the high instability of tetrahydropteridines at neutral pH, all synthetic standard solutions were prepared in 1 M HCl containing 1 g/l DTE, and stored frozen. Prior to injection into the HPLC system, the standard solutions were diluted 10-fold in water.

For the preparation of Drosophila extracts, 40 heads (from 20 males and 20 females, 5 days old) were homogenized in 0,125 ml of 0.1 M HCl containing 1 gr/l of DTE. After centrifugation for 10 min at 15,000 g, the clear supernatant was collected and mixed with one-tenth volume of 30% TCA. The precipitated proteins were separated centrifuging for 3 min at 15,000 g. The supernatant was filtered through a 0,4 µm pore filter, and immediately stored on ice.

Synthesis of 6-lactoyl-5,6,7,8-tetrahydropterin: 6-Lactoyl-H_4pterin was prepared as described by Smith and Nichol (7) using commercial dihydrofolate reductase and sepiapterin. The reaction product was characterized by the UV spectrum and its reconversion to the dihydropteridine precursor upon oxidation with iodine.

Preparation of rat kidney enzyme extract for the synthesis of 6-(1'-hydroxy-2'-oxopropyl)-H_4pterin: Rat kidney (8.44 g) was homogenized in 4 volumes of 10 mM phosphate buffer (pH 7.0) and centrifuged at 27,000 g for 60 min. The supernatant was fractionated with ammonium sulfate; the fraction precipitating between 35 and 55% saturation was redissolved in 2 ml of homogenizing buffer and dialyzed overnight against 1 l of 10 mM phosphate buffer (pH 6.0) containing 1 mM EDTA, according to Takikawa *et al.* (8).

For the synthesis of 6-(1'-hydroxy-2'-oxopropyl)-H_4pterin the reaction mixture contained the following components: 15 µM H_2neopterin triphosphate, 8 µM $MgCl_2$, 0,1 M Tris/HCl buffer (pH 7.4), 10 mM DTT, 1 mM NADPH, 100 µM or 10 µM N-acetyl-serotonin, and enzyme extract, in a final volume of 100 µl. After incubation at 37°C for 60 min, the reaction was terminated by the addition of

10 µl of 30% TCA. As a positive control for the reaction, N-acetyl-serotonin was omitted and H_4biopterin synthesis measured (9).

HPLC analysis of tetrahydropteridines: The chromatographic system consisted of a Waters Model 510 pump, Waters Universal Model U6K manual injector, Guard-Pak µBondapak C_{18} precolumn (Waters) and an analytical 5 µm column Nova-Pak C_{18} (150x3.9 mm ID, Waters). Amperometric detection was performed with a Methrom 656/641 electrochemical detector using a glassy carbon working electrode and setting the potentiometer at 250 mV.

The mobile phase consisted of 20 mM phosphate buffer (pH 3.5), 60 µM EDTA, 0.5 mM octyl sodium sulfate and 3% methanol. After filtering and degassing in vacuo, DTE was added to give a concentration of 0.16 mM (10, 11). The flow rate was mantained at 1.0 ml/min with a pressure of 1.3 psi.

RESULTS

A typical HPLC chromatogram of Drosophila heads using electrochemical detection with an applied potential of 250 mV is show in Fig. 1. Since this low potential is only suitable for oxidation of readily oxidable compounds, such as tetra-hydropteridines, the identification of the various peaks was started with the comparison of the retention times with those of synthetic tetrahydropteridines.

Synthetic H_4pterin and H_4biopterin eluted at 9.5 and 14.1 min respectively. The enzymatic synthesis of non-commercially available intermediates of the H_4biopterin pathway (Fig. 2), was carried out from H_2neopterin triphosphate according to published procedures. The results are shown in Fig. 3. Since N-acetyl-

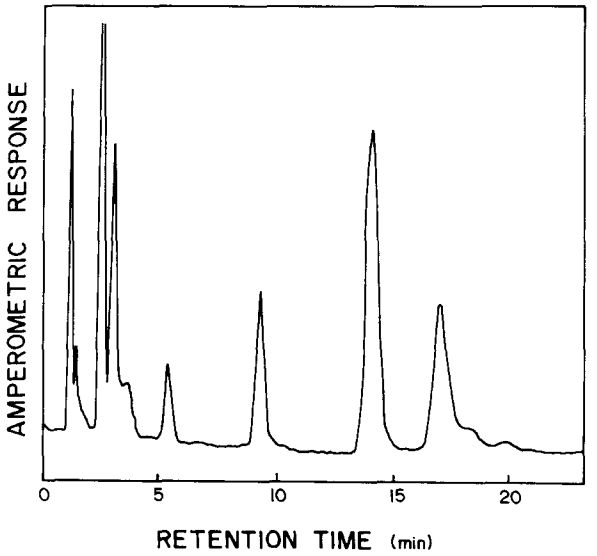

Fig. 1. Chromatogram of 20 µl of an extract of Drosophila heads.

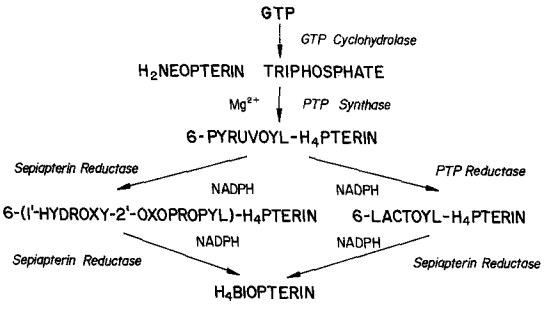

Fig. 2. Proposed pathway for the biosynthesis of H4biopterin in mammals. PTP: 6-pyruvoyl-H4pterin

serotonin is a strong inhibitor of sepiapterin reductase, its inclusion in the reaction mixture gives rise to the accumulation of intermediates in the synthesis of H4biopterin (7); in our case, the peak at 18.2 min is presumably 6-(1'-hydroxy-2'-oxopropyl)-H4pterin, since 6-lactoyl-H4pterin appears at 15.9 min. When we excluded NADPH in the reaction mixture, we were unable to detect 6-pyruvoyl-H4pterin. This suggests that in our chromatographic conditions this intermediate is unstable and therefore we could not detect it in Drosophila extracts.

When simultaneous injections of Drosophila extracts with synthetic standards were performed, the peaks that co-eluted with the standards were those at 9.5 min and 14.1 min, corresponding to H4pterin and H4biopterin. A further characterization of these two peaks included their hydrodinamic voltagrams (Fig. 4). The voltagrams showed half-maximal oxidation potentials of around 200 mV and were characteristic of tetrahydropterins (9, 12). Drosophila extracts also had another very small peak with a retention time that coincided with that of the 2'-oxo intermediate. Confirmation of its identity is under progress.

Additional confirmation of the identity of the chromatographic peaks from Drosophila with H4pterin and H4biopterin was obtained with mutants affecting the biosynthesis of pteridines. Two mutant strains were used: Pu^2 and Hn^{r3}. *Punch* is the structural gene for GTP cyclohydrolase and the Pu^2 mutant has only 19-33% the activity of the wild type enzyme (13). The *Henna-recessive* gene has been proposed to be involved in the synthesis of H2pterin from 6-pyruvoyl-H4pterin, and Hn^{r3} is one of the most drastic alleles (14). The results are summarized in Table 1 and agree well with those expected for these mutants if

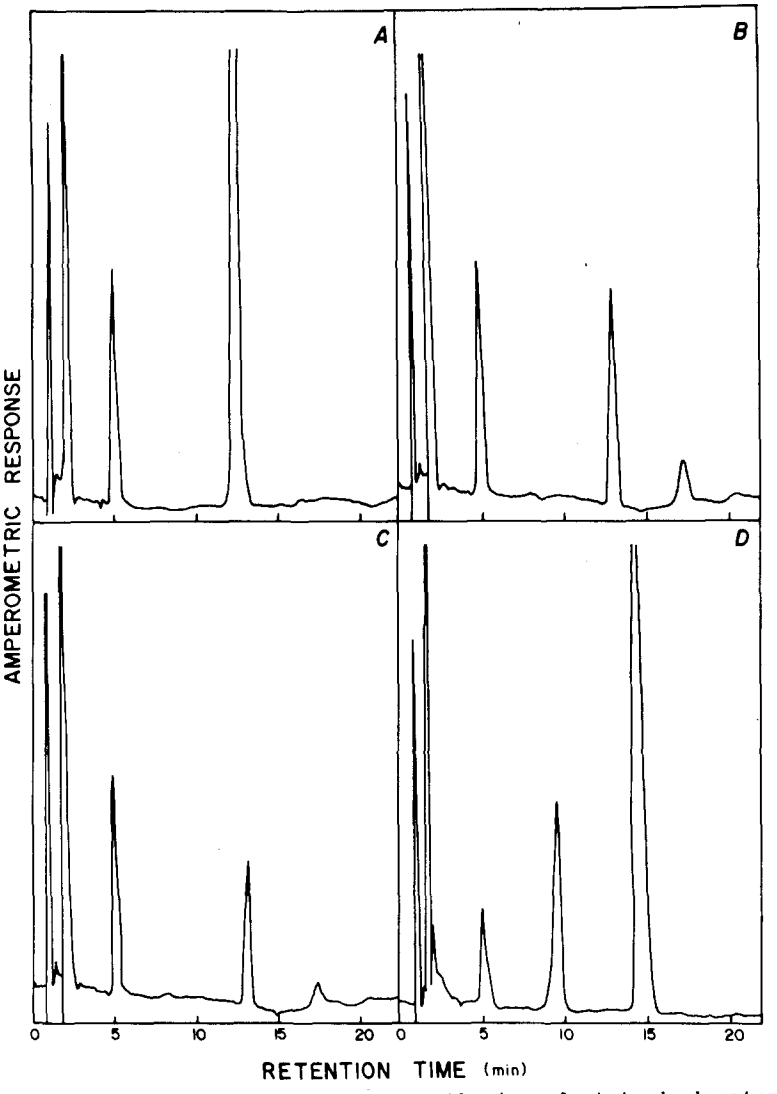

Fig. 3. Results of the enzymatic synthesis of tetrahydropteridine intermediates. Assays were carried out as described in Materials and Methods. (A) No N-acetyl-serotonin added. (B) 10 μM of N-acetyl-serotonin. (C) 100 μM of N-acetyl-serotonin. (D) Synthesis of 6-lactoyl-H_4pterin from sepiapterin using bovine liver dihydrofolate reductase.

the peaks at 9.5 min and 14.1 min were H_4pterin and H_4biopterin respectively.

DISCUSSION

There is evidence that Drosophila phenylalanine hydroxylase requires tetrahydropterin as a cofactor (3), and that this insect has the enzymes required for the synthesis of H_4biopterin *in vitro* (4). The occurrence of H_4biopterin in Drosophila had already been reported, based on a study on the pteridines in the Malpighian tubules using thin-layer chromatography (15). However,

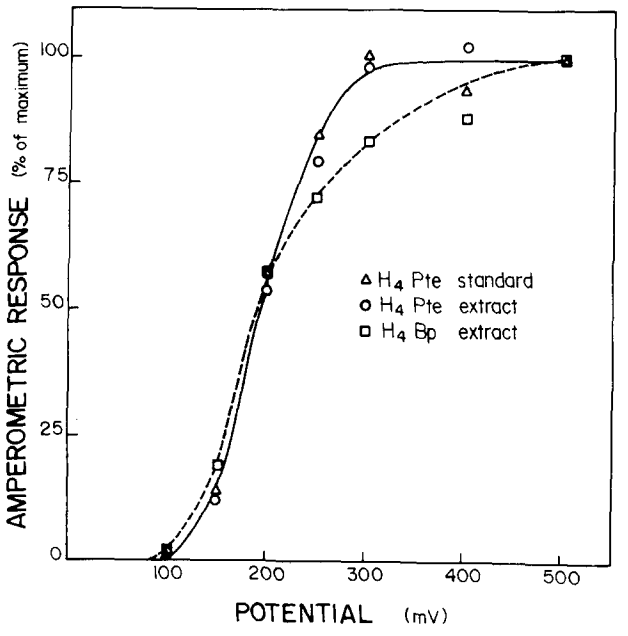

Fig. 4. Hydrodinamic voltagrams of synthetic H_4pterin and Drosophila H_4pterin and H_4biopterin.

considering the instability of such a reduced pterin and the conditions used by the authors, it is highly improbable that H_4biopterin could had been detected using thin-layer chromatography, even as an oxidized compound different from H_2biopterin or biopterin. In fact, our attempts to reproduce their results have failed using synthetic H_4biopterin, although we have found a spot with chromatographic and fluorescent properties similar to the one they claimed to be H_4biopterin, which we have identified as 6-lactoylpterin; this compound has the very characteristic property of becoming fluorescent, on cellulose or paper chromatography, after 5 sec of irradation with 365 UV ligth (14).

In the present work we have been able to demonstrate conclusively the occurrence of H_4biopterin in Drosophila, as well

Table 1. Levels of H_4pterin and H_4biopterin in the wild type (Oregon R) and two mutant strains of *Drosophila melanogaster*

	H_4pterin[a]	H_4biopterin[a]
Or-R	5.8	33.4
Pu^2	2.2	13.4
Hn^{r3}	N.D.[b]	75.0

[a] Concentration expresed in pmol/fly head.
[b] Not detected.

as that of another tetrahydropteridine not found before in any other organism, identified as H_4pterin. The identification has been based on the retention times, hydrodinamic voltagrams and its differential concentration in three strains of *Drosophila melanogaster*. The relative values found for H_4pterin and H_4biopterin in the Pu^2 mutant are around 40%, and correlated well with the biochemical lesion and the values previously reported for pterin and biopterin in this mutant (14). Hn^{r3}, proposed to block the conversion of 6-pyruvoyl-H_4pterin into H_2pterin and reported not to have any detectable pterin and increased amounts of biopterin (14), shows indeed higher concentration of H_4biopterin than the wild type and no detectable H_4pterin. These results make very unlikely the possibility of H_4pterin being a degradation product of another unstable tetrahydropteridine, and thus being produced under the isolation conditions.

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