The Isolation and Identification of an Intermediate Involved in the Biosynthesis of Drosopterin in Drosophila melanogaster*

Gregory J. Wiederrecht, Duncan R. Paton, and Gene M. Brown
From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

A compound that is involved in the biosynthesis of the drosopterin eye pigments has been isolated from the heads of Drosophila melanogaster. Analyses of this compound by chemical, mass spectral, and proton nuclear magnetic resonance techniques in conjunction with biochemical considerations provide evidence for the structure 2-amino-4-oxo-6-acetyl-7,8-dihydro-3H,9H-pyrimido[4,5-b][1,4]diazepine (PDA). At least three eye pigments (drosopterin, isodrosopterin, and aurodrosopterin) are synthesized when PDA and 2-amino-4-oxo-(D-erythro-1’,2’,3’-trihydroxypropyl)-7,8-dihydropteridine triphosphate (dihydroleopterin triphosphate) are incubated with Mg2+ and protein fractions prepared from Drosophila heads. The synthesis of aurodrosopterin, in addition, requires reduced pyridine nucleotide. Other evidence suggests that dihdroleopterin triphosphate is a biosynthetic precursor of PDA.

The eye color phenotype of Drosophila melanogaster has been the subject of many investigations since the discovery of the first eye color mutant about 70 years ago. Two classes of pigments, the brown ommochromes and the red “drosopterins,” together with the pteridine, sepiapterin, have been recognized as the compounds responsible for the typical eye color phenotype in Drosophila. Lederer (1) first reported the isolation of a red pigment from Drosophila in 1940. Subsequent studies have shown the presence of a number of red pigments (“drosopterins”) that share similar properties (2, 3), and the five “drosopterins” that have been separated by thin layer chromatography (4) are usually referred to as drosopterin, isodrosopterin, neodrosopterin, aurodrosopterin, and “fraction e.”

In contrast with the ommochrome biosynthetic pathway, which has been well characterized both biochemically and genetically (5, 6), little is known concerning the biosynthesis of the “drosopterins.” Work on the degradation of drosopterin and isodrosopterin to pterin-6-carboxylic acid (7), the chemical synthesis of “drosopterin” from reduced pterin and 2-keto-β-hydroxybutyric acid (8, 9), and one report of the in vitro synthesis of radioactive drosopterin from 3H-labeled reduced pterin fed to wild type larvae (10) have suggested that these pigments are similar to or are derived from pteridine compounds. Structural analysis of the “drosopterins,” however, has proven to be difficult because of low yields and instability of the compounds. Early proposals considered the structure to be similar to that of the 6-substituted pteridines (11) or, in order to account for differences in physical and chemical properties, to that of 6-substituted dipteridonyl derivatives (12, 13). The most recent proposal (14) suggests that drosopterin and isodrosopterin contain both pteridine and pyrimidodiazepine ring systems within their structures. To date, no further information has been given on the other members of the “drosopterin” group.

Evidence has been presented (15) that dihydroleopterin triphosphate (H2-NTP) is a product of the action of GTP cyclohydrolase I on GTP and the probable precursor of all pteridines in Drosophila (16), is also a precursor of the “drosopterins.” We report in this paper that a heat-stable protein fraction from Drosophila heads when incubated with H2-NTP, Mg2+, and NADH supports a low level of synthesis of drosopterin, isodrosopterin, and neodrosopterin and that the addition of a boiled extract prepared from heads causes a large stimulation of the synthesis of these pigments. The present report also describes the isolation and identification of a compound from the boiled extract that is required as a substrate, along with H2-NTP, in the biosynthesis of the “drosopterins.”

EXPERIMENTAL PROCEDURES

Materials—Deuterium oxide (99.7%), [1H3]diamethyloxide was obtained from Merck Isotopes; [1-14C]GTP from Amersham-Searle; Pipes and biotineropt from Calvin; and other pteridines, pyridine nucleotides, Sephadex G-10-80 and G-25-20, and ECTEOLA-cellulose from Sigma.

Animal Procedures—Wild type D. melanogaster, strain Oregon-R, was used for the preparation of enzyme fractions and boiled extract. The maintenance of a breeding population of flies and the collection of eggs were as described previously (17). Eggs were transferred to ventilated plastic containers containing standard Drosophila food (18) that had been seeded with a thick suspension of baker’s yeast. At 8 days after the beginning of oviposition, pupae were washed from the containers with distilled water, dried at 25 °C, and allowed to eclose in small wooden cages provided with trays of food. The adults (1–2 days old) were killed by freezing them at −20 °C and then were stored at −80 °C until used.

Preparation of Enzyme and Boiled Extract—The heads of frozen flies were removed from the bodies by mechanical shock, separated from body parts by a sieving procedure (19), and homogenized in a buffer solution (1/4, w/v) containing 100 mm Pipes, 10% glycerol, and 10 mm β-mercaptoethanol, pH 7.5. Protease activity was inhibited by 0.01 M PMSF.

Three procedures were used in the isolation of drosopterin. The first was a simple precipitation at pH 3.0. The second was a treatment of the boiled extract with 6 M HCl at 100 °C for 1 h. The third was a treatment of the boiled extract with 0.1 M HCl at 100 °C for 30 min, followed by vacuum dialysis against 5% acetic acid, pH 3.0. This treatment was repeated twice.

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the addition of 5 ml of a phenylmethylsulfonyl fluoride solution (40 mM in absolute ethanol) to every 95 ml of buffer. After filtration through Teflon mesh (Spectranex, 74-μm mesh, Spectrum Medical Industries, Inc.) to remove debris, the homogenate was divided into 10- to 15-ml portions and heated for 2 min with gentle shaking at 75 °C. The heat-treated homogenate was cooled in an ice water bath for 1 h at 0.01°C. Aliquots (1 g) were used for isolation and any residual pigments. The dialyzed protein solution was stored at -80 °C.

Boiled extracts of fly heads were prepared by homogenizing heads (1-4, w/v) with 100 mM Pipes buffer, pH 7.5. The homogenate, after filtration, was heated for 2 min at 75 °C, cooled in an ice water bath, and centrifuged for 1 h at 40,000 X g. The resultant supernatant solution was placed in a boiling water bath for 15 min and, after again cooling in an ice water bath, centrifuged to remove any precipitated protein. Boiled extract was stored at -80 °C until used.

Assay for Enzyme Activity—"Drosopterin"-synthesizing activity was assayed by the following procedure. [U-14C]H2-NTP was synthesized in GTP by and an assay mixture containing H2-NTP and the enzyme preparation. (15) for the preparation of phosphocellulose was used to prepare the phosphocellulose from Sephadex G-25-80. Sephadex G-25-80 (Pharmacia, 7.8-dihydropteridine) was isolated according to Krivi and Brown (16). 7.8-Dihydropteridine and 7.8-dihydrobiop- terin were prepared by the procedure of Kaufman (23).

RESULTS AND DISCUSSION

Evidence that H2-NTP is the precursor of the "drosopterin" eye pigments was obtained with our finding that incubation of a heat-treated extract of Drosophila heads with [3H]-labeled H2-NTP results in the incorporation into "drosopterin" of 10-13% of the total radioactivity added to the incubation mixture. On further fractionation of the protein by ammonium sulfate, "drosopterin"-synthesizing activity was detected in the fraction precipitating between 40% and 70% saturation only when MgCl2 (15 mM) and NADH (2.5 mM) were included in the reaction mixture. However, with the level of synthesis observed (a maximum of 1-2%), it became clear either that enzymes competing for H2-NTP had been concentrated in the protein fraction or that something else was required. Evidence in favor of the latter alternative was provided with the observation that the addition of a boiled extract of Drosophila heads stimulated "drosopterin" synthesis to a maximum of approximately 10% conversion of H2-NTP. Treatment of the boiled extract with activated charcoal eliminated the stimulatory property even when NADH was also included in the reaction mixture. We concluded, therefore, that an aromatic compound, probably used as a substrate for "drosopterin" synthesis, was present in the boiled extract.

The following well known pteridines were tested for the ability to replace the boiled extract requirement: sepiapterin, pterin, xanthopterin, isoxanthopterin, biop- terin, dihydrobiop- terin, and dihydropterin. None of these caused a stimulation of synthesis either alone or in the presence of a limiting volume of boiled extract. The likely possibility that the compound responsible for the stimulation had not yet been identified stimulated us to purify the active component in the boiled extract. A summary of the isolation procedure that was developed is as follows. Boiled extract (40 ml) was applied to a column of Sephadex G-10-80 (3.5 X 80 cm) and eluted with deionized distilled water at a flow rate of 90 ml/h. Fractions 160-200 (13 ml each) contained activity and these were concentrated to 20 ml. The concentrated material was applied to a column of diethylaminoethyl-cellulose (2.8 X 100 cm), and the column was developed with deionized distilled water. The yellow fractions contained the activity; these were concentrated, and concentrated to 10 ml and the material was chromatographed further on a phosphate-Sephadex (2.2 X 110 cm) column with a solvent containing 62 mM pyridine and 870 mM acetic acid. The yellow fractions (120-150, 11 ml each) were combined and concentrated to 20 ml and this material was passed through a Sephadex G-10-80 column, as before, to remove contaminating pyridine and acetic acid. The pale yellow fractions were combined, concentrated, and stored at -80 °C. All column procedures were performed at 2°C in the dark. The purity of the compound, as determined by high pressure liquid chromatography, was greater than 95%.

Although the compound is yellow like sepiapterin, unlike sepiapterin, it quenches UV light at 360 nm. The UV spectrum of the compound, presented in Fig. 1, does not resemble the spectrum of any known pteridine, but does resemble the spectrum of an unidentified compound prepared from head extracts by Wilson and Jacobson (24). The isolated compound, in the presence of 15 mM MgCl2, can substitute completely for the boiled extract requirement for the conversion of H2-NTP to "drosopterin," and synthesis of "drosopterin" is linear with increasing amounts (measured by absorbance at 384 nm) of the compound.
Several attempts failed to show that the compound can be converted to pterin-6-carboxylic acid by oxidation with permanganate or by subjection to intense light. These results suggest that it is not a 6-substituted pterin (25). The presence of a conjugated carbonyl group was indicated when an orange-colored dinitrophenylhydrazone resulted from treatment (26) of the compound with a solution of dinitrophenylhydrazine. Neither biopterin nor pterin was converted to a phenylhydrazone under the same conditions.

Further information was provided by proton NMR spectroscopy. Samples were prepared as described under "Experimental Procedures." The proton NMR spectrum shown in Fig. 2 integrates to 11 protons. On the addition of a small amount of deuterium oxide to the samples to reveal the presence of exchangeable protons, the resonances at 6.64, 5.73, and 6.10.64 (integrating to 2:1:1, respectively) disappeared. The chemical shifts of these peaks, their broadness, and their disappearance in the presence of deuterium oxide indicate that the unknown contains two imino groups and one amino group. A comparison of this portion of the spectrum with the one published for sepiapterin (27) reveals that the three resonances are at positions characteristic for these groups in sepiapterin. This suggests that the unknown compound contains the 2-amino-4-oxopyrimidine ring of pteridines as well as the imino group present in the pyrazine ring. The intense singlet at 6.31 and the two multiplets at 62.75 and 63.17 integrate to 3:2:2, respectively. The appearance of a CH3 signal at 62.31 is consistent with the presence of a methyl group adjacent to a carbonyl group in the structure (28). In order to resolve the two methylene multiplets, the resonances at 62.75 and 63.17 were subjected to homonuclear decoupling. Irradiation at 62.75 caused the multiplet at 63.17 to collapse to a doublet. When the multiplet at 63.17 was irradiated, the resonance at 62.75 and the unresolved triplet at 67.53 both collapsed to singlets. These results support a —NH—CH2—CH2— structure in the compound. Two minor singlets at 61.13 and 61.72 (not shown in Fig. 2) and the singlets at 62.07, 62.2, and 62.7 are thought to be the result of minor contaminants because they did not integrate consistently from sample to sample and were completely missing from some of the spectra.

From its chemical properties and its NMR spectrum, we conclude that the compound is a 2-amino-4-oxo-dihydropyrimidodiazepine with an acetyl substituent on the diazepine ring system, as shown in Fig. 2. For comparison, the proposed structure for drosopterin (14) is also shown in an inset in Fig.

2. Corroboration for the proposed structure was obtained by the mass spectral data of Fig. 3. The parent ion peak at 221 m/e gives the expected molecular weight for the compound. Furthermore, the daughter ion peak at 178 m/e can be interpreted as an ion corresponding to the molecular ion minus the —COCH3 side chain. The proposed structure is also supported by high resolution mass spectral data: C11H11N3O2 calculated 221.09127; measured value 221.09115. For convenience, the compound will be referred to by the abbreviation "PDA" in the rest of this paper.

Our data for the structure of the compound do not allow one to distinguish between the 6-substituted or 8-substituted acetyl derivatives. Support for the proposed 6-substituted structure (as shown in Fig. 2) comes from experiments conducted to determine its biosynthetic precursor. Because both H2-NTP and PDA contain a pyrimidine ring and a total of 9 carbon atoms, it seemed possible that PDA is derived from H2-NTP. No synthesis of PDA from 14C-labeled H2-NTP could be detected when the standard enzyme preparation was used in incubation mixtures. However, the use of protein fractions prepared by ammonium sulfate precipitation from an unheated crude extract resulted in the conversion of [U-14C]H2-NTP to radioactive PDA in a 2-h incubation period. The formation of PDA was detected by autoradiography. For this purpose, 0.01-ml portions of an incubated reaction mixture were spotted on cellulose thin layer chromatography plates and each plate was developed in two dimensions with equal volumes of 2-propanol and 2% ammonium acetate (first dimension) and then with 3% ammonium chloride (24). PDA, which has an Rf of 0.57 in the first solvent and an Rf of 0.60 in the second, was detected in all samples, indicating that it was synthesized from H2-NTP.

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![Fig. 1. Ultraviolet-visible spectrum of the unknown compound in distilled deionized water determined with the use of a Perkin-Elmer 552 spectrophotometer.](image)

![Fig. 2. Proton NMR spectrum of the unknown compound. The spectrum was obtained in [1H3]MeSO at 290 MHz. Proton resonances derived from various groups are labeled. From these and other data, the proposed structure for the unknown compound is 2-amino-4-oxo-6-acetyl-7,8-dihydro-3H,9H-pyrimido[4,5-b][1,4]diazepine, as shown and abbreviated as PDA. For comparison, the structure proposed for drosopterin (14) is shown in the inset. DMSO, dimethyl sulfoxide.](image)

![Fig. 3. Low resolution mass spectrum of the compound.](image)
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in the second solvent, can be identified easily by its ability to quench UV light. Radioactive zones were identified by exposure of the plates to film for 10 days. The observation that radioactivity was found to coincide with the PDA spot on the plates indicates that PDA is derived enzymatically from H₂-NTP. Theoretical considerations of the pathway from H₂-NTP to PDA suggest that the 8-substituted structure for PDA is the most likely.

It was stated in a previous section of this paper that only drosopoterin, isodrosopoterin, and neodrosopoterin are synthesized in the presence of the heat-treated protein fraction. However, the experiment described above with the use of an unheated extract showed that radioactive aurodrosopoterin, as well as PDA, was synthesized from H₂-NTP. Thus, the enzymes responsible for the synthesis of this pigment would appear to be relatively heat-labile. The unheated and heated enzyme preparations were tested again to determine which of the "drosopterins" are synthesized in the presence of PDA in place of boiled extract. Incubations that contained [U-¹⁴C]Hz-NTP, PDA, and various cofactors were carried out as described previously, and samples were subjected to two-dimensional thin layer chromatography for autoradiography. Radioactivity was found to coincide with the PDA spot on the plates.

The isolation and identification of PDA and its participation in the synthesis of PDA, as well as PDA, was synthesized from H₂-NTP. Thus, the enzymes responsible for the formation of the other pigments. The role of PDA in the synthesis of neodrosopoterin and "fraction e" is still uncertain. The most recent proposal for the structure of neodrosopoterin and aurodrosopoterin suggests that they are dipteridinyl compounds.

REFERENCES


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