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Purification and Biosynthesis of Quench Spot, a Drosopterin Precursor in *Drosophila melanogaster*†

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ABSTRACT: Pteridine biosynthesis has been examined in extracts of the heads of *Drosophila melanogaster* by measuring the conversion of dihydroneopterin triphosphate to sepiapterin and the "drosopterins" (six eye pigments that are dipterin derivatives). These two products share a common first step in the production of an intermediate that is a branch point from which both products are formed. This first step can be catalyzed by sepiapterin synthase or by an enzyme found in particles that sediment at 600*g*. A substance named "quench spot" was found earlier to be at low levels in the purple mutants that were defective in drosopterin synthesis and to be restored to normal when a suppressor mutant, *su(s)*^{2}, restored drosopterins in purple to normal levels. The sepia mutant is also deficient in the levels of both quench spot and drosopterins. In this report we propose that quench spot is a precursor of drosopterins, but not sepiapterin, and that it is formed from the sepiapterin synthase intermediate mentioned above. An additional precursor that is formed independently of the sepiapterin synthase pathway is also proposed that would react with quench spot to form drosopterins. These proposals are based on the following: (1) quench spot biosynthesis is observed in extracts of *Drosophila* heads in which [U-^{14}C]dihydroneopterin triphosphate is the substrate; (2) Mg^{2+} is required for the synthesis of quench spot but either NADH or NADPH causes diminished incorporation of the label; (3) extracts from heads of a purple mutant (*pr*^{bw}*cn*) contain only 30% of the quench spot biosynthetic activity as compared to heads from wild type (Oregon-R); (4) quench spot has been purified from heads of wild-type *Drosophila*; (5) addition of quench spot stimulates the biosynthesis of drosopterins in an enzyme preparation from Oregon-R.

In a previous report we described the in vitro enzymatic conversion of the 3′-triphosphoester of 7,8-dihydro-6-(D-*erythro*-1,2,3-trihydroxypropyl)pterin [H_2-neopterin-$(P)_3$][1] to the six dipterin-derivative red accessory eye pigments of *Drosophila* known collectively as the "drosopterins" [neodrosopterin, fraction e, aurodrosopterins I and II, drosopterin, and isodrosopterin] (Dorsett et al., 1979). It was concluded that sepiapterin synthase, which is responsible for the conversion of H_2-neopterin-$(P)_3$ to sepiapterin, consists of two enzymes, the first of which also occurs in pellets obtained at 600*g* from head homogenates. This first enzyme utilizes Mg^{2+} as a cofactor and produces a product that can be converted to sepiapterin by the second enzyme in the presence of NADPH (Krivi & Brown, 1979; Tanaka et al., 1981). In the presence of either NADPH or NADH, the sepiapterin synthase intermediate can be converted by another set of enzymes to drosopterins. The intermediate is nonphosphorylated, but it is not H_2-neopterin (Dorsett et al., 1979; Krivi & Brown, 1979; Tanaka et al., 1981). The separation of the two activities of sepiapterin synthase from *Drosophila* (Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1981) has been reported. The purple (*pr*) eye color mutant of *Drosophila* is thought to be a lesion in the structural locus coding for the first enzyme of sepiapterin synthase (Yim et al., 1977; Dorsett et al., 1979).

Quench spot (QS) is an unidentified compound that has been found and quantitated in wild-type, *pr*, sepia (*se*), and Henna-recessive-3 (*Hn*^{r3}) flies (Wilson & Jacobson, 1977b). Purple, which contains low levels of sepiapterin and drosopterins, also has low levels of QS. Sepia contains very high amounts of sepiapterin but neither QS nor drosopterins. Henna-recessive-3 has a high level of both sepiapterin and QS but low drosopterins. These considerations lead us to hypothesize that QS is an intermediate in drosopterin biosynthesis. The data presented in this report support this hypothesis

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; H_2-neopterin-$(P)_3$, 3′-triphosphoester of 7,8-dihydro-6-(D-*erythro*-1,2,3-trihydroxypropyl)-pterin; sepiapterin, 7,8-dihydro-6-lactoylpterin; pterin, 2-amino-4-hydroxypteridine; isoxanthopterin, 7-hydroxypterin; Pipes, 1,4-piperazinediethanesulfonic acid; QS, quench spot; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; HPLC, high-performance liquid chromatography; PFE, pteridine-free extract; TE, Triton extract; TLC, thin-layer chromatography.

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and were reported recently (Jacobson & Dorsett, 1981).

Materials and Methods

Drosophila melanogaster. Oregon-R wild-type and *pr*^{bw}*cn* mutant flies were reared at 25 ± 1 °C on medium prepared as described by Lewis (1960). Adult flies were collected daily so that all were 1-day-old or less. After collection the flies were quickly frozen in liquid nitrogen; the heads were obtained as previously described (Wilson & Jacobson, 1977a). For long-term storage, the heads were kept in liquid nitrogen.

Pteridine-Free Extract (PFE). PFE was prepared as previously described (Dorsett et al., 1979), except that the redissolved protein was desalted by filtering through 20 mL of Sephadex G-25 (medium) (Pharmacia) by using the centrifuge procedure of Neal & Florini (1973) instead of chromatography on a Sephadex G-25 column. The centrifuge procedure obviated the vacuum dialysis concentration that would have been required if the extracts had been desalted by chromatography.

Triton Extract (TE). With a ground-glass homogenizer (Kontes), 1.0 g of heads (Oregon-R or *pr*^{bw}*cn*) was homogenized in 5.0 mL of 50 mM 1,4-piperazinediethanesulfonic acid (Pipes; pH 7.0) containing 1% Triton X-100. The homogenate was centrifuged at 15000*g* for 10 min, and that supernatant was subsequently centrifuged at 100000*g* for 60 min. The supernatant was carefully removed with a Pasteur pipet so as not to disturb the lipid layer at the top of the tube, filtered through Sephadex G-25 (medium) by using the centrifuge procedure of Neal & Florini (1973), and then incubated with 2.5 mL of washed Bio-Beads (SM2) (Bio-Rad) with gentle shaking at 0 °C for 60 min to remove the Triton X-100. The protein that precipitated between 40 and 60% saturated $(NH_4)_2SO_4$ (4 °C) was collected at 15000*g* for 30 min, redissolved in 0.5 mL of 50 mM Pipes (pH 7.0) containing 10 mM 2-mercaptoethanol and 10% glycerol, filtered through Sephadex G-25 (medium) by using the centrifuge procedure, and stored in liquid nitrogen. The QS synthesizing activity was stable for approximately 1 week under these conditions.

H_2-neopterin-(P)$_3$. H_2-neopterin-(P)$_3$ was prepared from guanosine 5′-triphosphate (GTP) (sodium salt; Sigma Chemical Co.) as previously described (Dorsett et al., 1979) by using pure GTP cyclohydrolase I prepared from *Escherichia coli* (Yim & Brown, 1976). Depending on the experiment, [U-^{14}C]GTP (Amersham) or [8,5′-$^3H_2$]GTP (New England Nuclear) was used at the specific activities described below. H_2-neopterin-(P)$_3$ was treated with bacterial alkaline phosphatase as previously described (Dorsett et al., 1979).

[U-^{14}C]Sepiapterin. For production of sepiapterin, 117 μL of 0.67 mM [U-^{14}C]H_2-neopterin-(P)$_3$ (20.3 μCi/mol) was incubated with 157 μL of H_2O, 157 μL of Oregon-R PFE, 39 μL of 35 mM NADPH (Sigma; chemically reduced), 39 μL of 0.2 M $MgCl_2$, and 39 μL of 0.7 M Pipes (pH 7.5) at 42 °C for 60 min in the dark. The reaction was stopped by incubation at 100 °C for 5 min, and the resulting precipitate was removed by centrifugation. The [U-^{14}C]sepiapterin was isolated by high-performance liquid chromatography (HPLC) as described below. Fractions containing sepiapterin were pooled and evaporated to dryness at 40 °C by a stream of dry nitrogen. The sepiapterin was redissolved in 100 μL of H_2O and stored at −20 °C.

Pterin, 7-Hydroxypterin (Isoxanthopterin), and 7,8-Dihydropterin. Pterin and isoxanthopterin were purchased from Sigma Biochemical Co. For preparation of 7,8-dihydropterin, 1.0 mg of pterin was suspended in 400 mL of H_2O, and 200 μL of 2 N KOH was added to bring it into solution. Twelve milligrams of zinc dust was added, and the mixture was incubated in the dark (with occasional stirring) for 15 min before incubation at 55 °C for 5 min. The pH was adjusted to 6 with 4 N HCl, and the precipitate was removed by centrifugation. One molar K_2HPO_4 was added slowly until a precipitate formed. After incubation in an ice bath for 60 min, the precipitate was removed by centrifugation before the supernatant was tested for the presence of Zn^{2+} (Feigel & Oesper, 1954). When all of the Zn^{2+} had been removed, the 7,8-dihydropterin was separated by HPLC. Fractions containing 7,8-dihydropterin were pooled, and the methanol was removed with a stream of dry nitrogen. The ultraviolet absorption spectrum at pH 13 (Blakeley, 1969) was determined to confirm identity and calculate concentration.

Purification of QS. Oregon-R heads (15 g) were homogenized in 50% 2-propanol–1% 2-mercaptoethanol (75 mL) for 5 min by using a Virtis homogenizer at half speed in the dark at room temperature. The supernatant obtained after centrifugation at 15000*g* for 5 min was saved, and the pellet was rehomogenized in 75 mL of solvent and centrifuged. The pooled supernatants were concentrated by rotary evaporation (45 °C) to remove the 2-propanol. The sample was loaded onto a carboxymethylcellulose column (Whatman CM-23; 8.5 × 13 cm; precycled as described by the manufacturer and washed with 3 L of 0.5 M ammonium formate and then 4 L of distilled water prior to use). Elution was carried out at 4 °C in the dark with water at a flow rate of 4 mL/min while 20-mL fractions were collected. The A_{260} of the fractions revealed three major, poorly resolved peaks, with the third containing QS [as identified by the ultraviolet spectrum and thin-layer chromatography (TLC) (Wilson & Jacobson, 1977a)]. This peak (fractions 36–50) was pooled and concentrated (rotary evaporation at 60 °C) to a volume of 3 mL. The sample was applied to a second carboxymethylcellulose column (Whatman CM-32; 2.5 × 75 cm; precycled as described by the manufacturer and washed with 1 L of 0.5 M ammonium formate and 2 L of distilled water prior to use). QS was eluted in the dark at 4 °C with a 660-mL linear gradient (0–50 mM ammonium formate) while 10-mL fractions were collected. QS was identified by the ultraviolet spectrum, and the fractions containing QS (74–80) were pooled and lyophilized to dryness. The dry powder was usually a yellowish green color but at times was orange. When either colored material was dissolved in water, the resulting solution was the typical yellowish green color; the orange-colored material apparently represents some type of chemical rearrangement. The yield was 0.3 mg of QS. QS was estimated by HPLC to contribute greater than 95% of the sample's absorption at 260 nm. The spectrum of the purified sample in water was identical with that obtained prevously (Wilson & Jacobson, 1977a).

Sepiapterin, Drosopterin, and QS Carrier. Sepiapterin was purified from sepia mutant heads as previously described (Dorsett et al., 1979). Drosopterin and QS carrier was prepared by homogenizing (ground glass) 0.2 g of Oregon-R heads in 1.0 mL of 30% ethanol adjusted to pH 2 with concentrated HCl. The homogenate was clarified by centrifugation and could be stored at −20 °C for several months without loss of drosopterins or QS.

Assay of QS, Sepiapterin, and Drosopterin Biosynthesis. The exact conditions of the assays whose results are presented in the tables and figures are given in the legends. The protocol described below was used for all assays. QS, sepiapterin, and drosopterin biosyntheses were determined in a total reaction volume of 70 μL with the following components: *Drosophila* extract (PFE and/or TE), 50 mM Pipes (pH 7.5) (except in the determination of the optimum pH for QS synthesis),

[U-^{14}C]- and/or [3′-^{3}H]H_2-neopterin-(P)$_3$ (concentration and specific activities are described for each experiment in the table and figure legends), and various cofactors or pteridines, depending upon the experiment. When present, $MgCl_2$ was used at a concentration of 14.3 mM; NADPH or NADH (Sigma; chemically reduced) was used at a concentration of 2.5 mM. The reactions were carried out in the dark at 42 °C for various times up to 60 min before being stopped by heating at 100 °C for 5 min. Any precipitate was removed by centrifugation (Brinkmann Eppendorf Centrifuge 3200), and 15 μL of carrier (preparation described above; supplemented with sepiapterin when necessary) was added before subjecting 40 μL of the solution to two-dimensional cellulose TLC as previously described (Wilson & Jacobson, 1977a), except that the ammonium acetate concentration in the first solvent was increased to 2 g/100 mL of solvent. QS exhibits no fluorescence under 365-nm illumination at 25 °C but a greenish yellow fluorescence at −196 °C which was accomplished by immersing the thin-layer plates in liquid nitrogen. QS and sepiapterin each occur at unique positions on the chromatogram whether they are derived from the carrier preparation or from highly purified fractions. Similarly, the drosopterins occupy the same unvarying positions whether fresh or stored carrier was the source. The appropriate pterin derivatives were scraped into scintillation vials containing 1.5 mL of water, and the amount of radioactivity was determined in a scintillation counter after 15 mL of 0.28% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene (Packard) and 33% Triton X-100 in toluene were added. Kodak RP X-ray film was used for autoradiography of the thin-layer plates.

HPLC. A Waters liquid chromatograph with a 3.6 mm × 30 cm μBondapak C_{18} column was used to purify [U-^{14}C]-sepiapterin and 7,8-dihydropterin and also to check the purity of QS. The column was operated at a temperature of 35 °C and a flow rate of 2 mL/min. Elution of 7,8-dihydropterin was accomplished with 5% methanol in water, sepapterin was eluted with 20% methanol, and QS was eluted with 20% methanol containing 10 mM ammonium phosphate (pH 8.0). Ultraviolet absorption and fluorescence of the pteridines were detected with a Schoeffel SF770 UV monitor at 260 nm and a Schoeffel FS970 fluorescence monitor (excitaiton at 360 nm; Corning 7-54 excitation filter; 418-nm cutoff emission filter) set to appropriate sensitivities.

Results

QS Biosynthesis. If QS is an intermediate in drosopterin synthesis, then we would expect that H_2-neopterin-(P)$_3$ could be used as a substrate for QS biosynthesis mediated by *Drosophila* enzymes. Although such enzymatic activity was found in the PFE used earlier for in vitro drosopterin synthesis, the amount of labeled QS found was small and insufficient to test the conditions that affect the synthesis of QS. Greater activity could be achieved in the TE. Using this extract, it was found that label from [U-^{14}C]H_2-neopterin-(P)$_3$ was incorporated into QS whereas label from [3′-^{3}H]H_2-neopterin-(P)$_3$ was not (Table I). Treatment of H_2-neopterin-(P)$_3$ with phosphatase prior to use as a substrate virtually eliminated the synthesis of QS (data not shown). The correspondence of the radioactive spot to the visible QS on the thin-layer plates was demonstrated by autoradiography. Under the reaction conditions used in Table I, the synthesis of QS ceased by 60 min, so reactions were conducted for 30 min or less when it was necessary to quantitate the enzyme. The synthetic activity has a pH optimum of approximately 7.3 (Figure 1).

Certain cofactors affect the rate of QS accumulation. Earlier we had shown that drosopterin biosynthesis was dependent on Mg^{2+} and either NADPH or NADH; sepiapterin formation requires Mg^{2+} and NADPH. We found that Mg^{2+} also stimulates the synthesis of QS from H_2-neopterin-(P)$_3$, whereas NADPH or NADH decreased the amount of QS recovered from a reaction (Table II). The decreased accumulation of QS in the presence of NADPH or NADH is consistent with the hypothesis that QS is a drosopterin precursor, since drosopterin accumulation increases in the presence of these cofactors.

The biosynthesis of sepiapterin and drosopterins requires the conversion of H_2-neopterin-(P)$_3$ to an intermediate (X) by the Mg^{2+}-dependent first enzyme of sepiapterin synthase

Table I: Biosynthesis of QS: Comparison of Oregon-R and *pr*^{bw}*cn* Mutant Activities[a] and Comparison of ^{3}H and ^{14}C Incorporation from [U-^{14}C,3′-^{3}H]H_2-neopterin-(P)$_3$[b]

	Oregon-R and *pr*^{bw}*cn* comparison		^{3}H and ^{14}C comparison	
	rate of QS synthesis (^{14}C cpm/30 min)	% Oregon-R act.[c]	radioact of QS TLC spot (dpm)	
enzyme extract			^{3}H	^{14}C
Oregon-R TE	369	100	248	567
pr^{bw}*cn* TE	153	28		
heat-inactivated Oregon-R TE (background)	67		244	31

[a] Each reaction contained 20 μL of enzyme extract (240 μg of protein), 14.3 mM $MgCl_2$, 50 mM Pipes (pH 7.5), and 0.142 mM [U-^{14}C]H_2-neopterin-(P)$_3$ (20.3 μCi/μmol). Reactions were for 30 min at 42 °C. [b] Reactions contained 15 μL of enzyme extract (180 μg of protein), 14.3 mM $MgCl_2$, 50 mM Pipes (pH 7.5), and 0.29 mM [U-^{14}C,3′-^{3}H]H_2-neopterin-(P)$_3$ (5.0 μCi/μmol ^{14}C; 38 μCi/μmol ^{3}H). Reactions were for 30 min at 42 °C. [c] Calculated after subtracting heat-inactivated Oregon-R TE values as background. Values in both experiments are averages of duplicate reactions: no background corrections have been made.

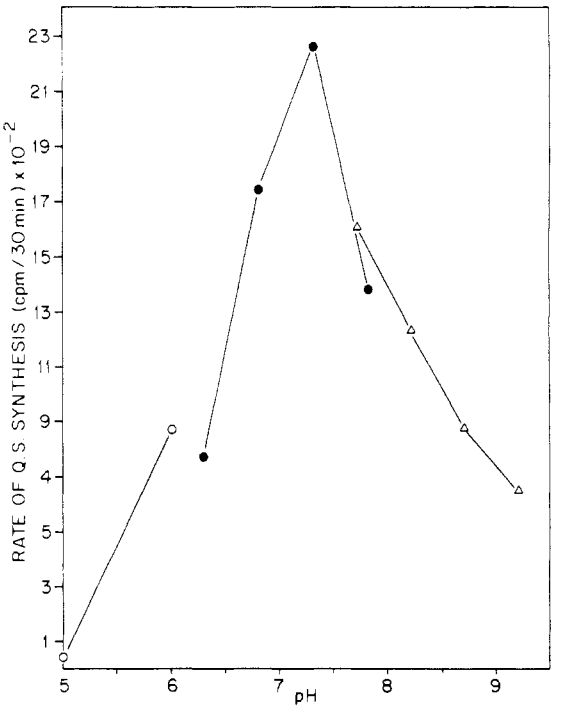

FIGURE 1: Effect of pH on the rate of QS biosynthesis. Each reaction contained 40 μL of Oregon-R TE (480 μg of protein), 14.3 mM $MgCl_2$, 0.142 mM [U-^{14}C]H_2-neopterin-(P)$_3$, and various buffers at concentrations of 50 mM [(○) potassium acetate; (●) Pipes; (△) Taps]. Reactions were carried out for 30 min at 42 °C.

Table II: Effect of Mg^{2+}, NADPH, and NADH on Biosynthesis of QS and Other Pteridines from [U-^{14}C]H_2-neopterin-$(P)_3$ [a]

product	radioact of pteridine TLC spots (cpm)			
	no cofactors	Mg^{2+}	Mg^{2+} and NADPH	Mg^{2+} and NADH
QS	403	1850	540	789
sepiapterin	449	1718	15454	1719
drosopterins	540	537	925	1493

[a] Each reaction contained 20 μL of Oregon-R PFE (340 μg of protein), 20 μL of Oregon-R TE (490 μg of protein), 50 mM Pipes (pH 7.5), 0.142 mM [U-^{14}C]H_2-neopterin-$(P)_3$ (20.3 μCi/μmol), and cofactors. Mg^{2+} was used at a concentration of 14.3 mM; NADPH or NADH was used at a concentration of 2.5 mM. Reactions were for 60 min at 42 °C and were analyzed for the incorporation of label into the various pteridines. The values for drosopterins are the sums of the values obtained for the individual species. All values are averages of duplicate reactions, and no background corrections were made.

(Dorsett et al., 1979). The Mg^{2+} requirement for QS biosynthesis is, then, consistent with the hypothesis that X is either a precursor or a product of QS. Since the *pr*^{bw}*cn* mutant (1) has decreased QS, sepiapterin, and drosopterin levels in vivo, (2) contains only 20% wild-type sepiapterin synthase activity, and (3) probably contains a lesion in the structural locus for the first enzyme of sepiapterin synthase (Wilson & Jacobson, 1977b; Yim et al., 1977; Dorsett et al., 1979), we compared the QS biosynthetic activity present in TE from either *pr*^{bw}*cn* or Oregon-R. The *pr*^{bw}*cn* extracts contained only 28% of the QS biosynthetic activity found in Oregon-R extracts (Table I).

Whether QS precedes or is derived from X can be considered by examining the disposition of the hydrogens on the 3′ carbon of H_2-neopterin-$(P)_3$. In previous studies we found that sepiapterin incorporates label from [3′-^{3}H]H_2-neopterin-$(P)_3$ (Yim et al., 1977; Dorsett et al., 1979). Since QS does not (Table I), it is unlikely that QS could function as a sepiapterin precursor. This observation and the result that *pr*^{bw}*cn* extracts are low in QS-synthesizing activities taken together are consistent with the hypothesis that X is a precursor of QS. Recently we have developed an HPLC assay for X and have shown directly that *pr*^{bw}*cn* TE and PFE contain only 20% of the X biosynthetic activities found in the respective Oregon-R extracts (unpublished results).

Drosopterin Biosynthesis in the Presence of QS. Although compound X, the sepiapterin synthase intermediate, appears to be a precursor to both QS and drosopterins (Dorsett et al., 1979), it remains to be demonstrated that QS is a drosopterin precursor. Since QS is made in small amounts and is somewhat labile during TLC and extraction, it was not possible to isolate large enough amounts of [^{14}C]QS to test whether the label could be incorporated into the drosopterins. Since QS can be purified from the heads of flies, the problem could be approached by asking whether unlabeled QS added to a reaction in which drosopterins were synthesized could cause a decrease in the label incorporated into the drosopterins from [U-^{14}C]H_2-neopterin-$(P)_3$. Contrary to expectation, the addition of QS stimulated the incorporation of label into all the drosopterins from [U-^{14}C]H_2-neopterin-$(P)_3$ (Figure 2). This can be interpreted to mean that QS is a drosopterin precursor if QS is considered to be the limiting component in the in vitro reaction and that still another precursor (or precursors), arising via another pathway from H_2-neopterin-$(P)_3$, is also required for drosopterin synthesis. The addition of QS, therefore, would allow greater incorporation of the putative precursor(s) into the drosopterins. The possibility that a contaminant in the

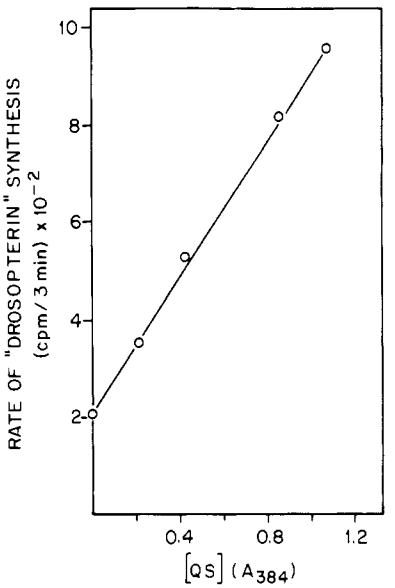

FIGURE 2: Effect of QS on the rate of drosopterin biosynthesis. Each reaction contained 15 μL of Oregon-R PFE (250 μg of protein), 14.3 mM $MgCl_2$, 2.5 mM NADPH, 50 mM Pipes (pH 7.5), and 0.142 mM [U-^{14}C]H_2-neopterin-$(P)_3$ (20.3 μCi/μmol). Reactions were carried out for 3 min at 42 °C. The values are the sums of the incorporation into the individual drosopterins. Reactions containing heat-inactivated Oregon-R PFE were used to determine the values for background correction.

QS preparation caused the stimulation in drosopterin biosynthesis was tested by comparing the ability of QS preparations at various stages of purification to cause such stimulation. The amount of stimulation remained proportional to the amount of QS present (as estimated by the absorbance at 384 nm).

The possbility that QS may be acting as a cofactor in drosopterin synthesis is an alternative hypothesis that may be considered. This seems unlikely since (1) QS occurs in relatively large amounts in various eye color mutants (Wilson & Jacobson, 1977b) and (2) the sepiapterin synthase intermediate (X), which has previously been demonstrated to be a drosopterin precursor (Dorsett et al., 1979), is also a precursor of QS.

Since our hypothesis predicts that QS would not cause a decrease of incorporation of label from [U-^{14}C]H_2-neopterin-$(P)_3$ into sepiapterin, we were surprised to see decreases as great as 50% in the amount of labeled sepiapterin recovered upon the addition of QS to a reaction. This decrease in labeled sepiapterin, however, was shown by the following experiment not to be due to isotope dilution. When [U-^{14}C]sepiapterin was added to in vitro sepiapterin- and drosopterin-synthesizing reactions using unlabeled H_2-neopterin-$(P)_3$ as the substrate, it was found that even in the absence of enzyme, the addition of QS resulted in a substantial loss of radiolabeled sepiaterin. This indicates that a reaction was occurring between sepiapterin and QS. The "missing" label was found not in the drosopterins but in an unidentified spot on the thin-layer chromatogram (detected by autoradiography), which has previously been described as a possible degradation product of sepiapterin [spot G (Wilson & Jacobson, 1977a)]. The reaction between QS and sepiapterin was not further characterized, but these results must be considered in the interpretation of isotope dilution results when actual specific activities cannot be measured.

So that the possibility that pterin, 7,8-dihydropterin, or isoxanthopterin might be drosopterin precursors could be tested, isotope dilution experiments with these compounds were

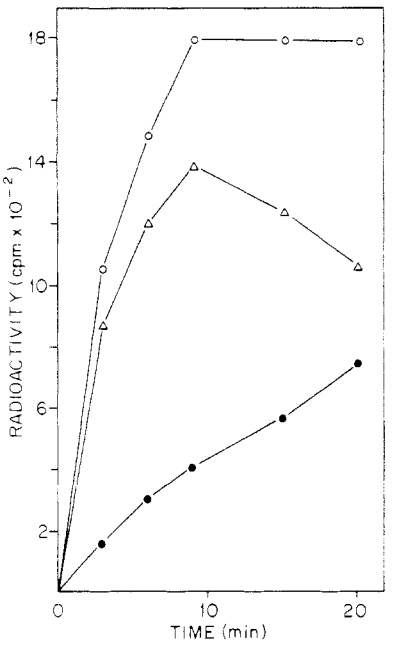

FIGURE 3: Time course of drosopterin biosynthesis [(○) sum of incorporation into all drosopterins; (●) sum of incorporation into neodrosopterin, fraction e, and aurodrosopterins; (△) sum of incorporation into drosopterin and isodrosopterin]. Each reaction contained 15 μL of Oregon-R PFE (260 μg of protein), 14.3 mM $MgCl_2$, 2.5 mM NADPH, 50 mM Pipes (pH 7.5), 0.142 mM [U-^{14}C]H_2-neopterin-$(P)_3$ (20.3 μCi/μmol), and 25 μL of QS solution (A_{384} = 3.1 in water). Reactions using heat-inactivated Oregon-R PFE were used to determine values for background correction.

performed in QS-stimulated reactions by using [U-^{14}C]H_2-neopterin-$(P)_3$ as the substrate. No change in the amount of ^{14}C label recovered in the drosopterins occurred in the presence of any of these compounds (data not shown) (pterin and 7,8-dihydropterin were used at a concentration of 0.4 mM; isoxanthopterin was used at a concentration of 0.26 mM).

Both Mg^{2+} and NADPH are required for drosopterin synthesis in the presence of QS, but not all members of this group are produced at the same rate. Under the assay conditions used, total drosopterin synthesis appeared to level off at 9–12 min, but this is not true for individual drosopterins. Incorporation into drosopterin and isodropterin reached a maximum and then decreased after 12 min, while incorporation into neodrosopterin, faction e (assayed together), and aurodrosopterins continued to increase (Figure 3). This raises the possibility that drosopterin and isodrosopterin may be the precursors of the other four drosopterins, in contrast to our previous suggestion that neodrosopterin is a precursor of drosopterin and isodrosopterin (Dorsett et al., 1979). The synthesis of drosopterins is also dependent upon H_2-neopterin-$(P)_3$ concentration when QS is present. Saturation was not achieved with a H_2-neopterin-$(P)_3$ concentration of 0.4 mM. The reaction rate is also dependent upon enzyme and QS concentration. Although the QS concentration could not be calculated, addition of 25 μL of a QS solution with an absorbance of 3.1 at 384 nm did not achieve saturation (Figure 2). Further investigation with this system could possibly answer questions regarding the interconversion of drosopterins as well as identify other drosopterin precursors.

Discussion

The proposed pathway for drosopterin biosynthesis is illustrated in Figure 4. QS stimulates the incorporation of label from [U-^{14}C]H_2-neopterin-$(P)_3$ into all six drosopterins, indicating that it is a common precursor. The amounts of QS present in the eye color mutants *pr*, *se*, and *Hn*^{r3} are consistent with the proposed scheme. The level of QS is elevated in *Hn*^{r3} but low in *pr* and *se*. The sepiapterin level is low in *pr* but high in *se* and *Hn*^{r3}, and the drosopterins are low or absent in all three mutants. The evidence that *pr* is a lesion in the structural locus coding for the first step of sepiapterin synthase has been discussed elsewhere (Yim et al., 1977; Dorsett et al., 1979). The nature of the defects in *Hn*^{r3} and *se* is unknown, but the net effect is to block interconversion of the pteridines in vivo at the reaction steps shown in the proposed scheme.

It is known that under the proper conditions, 7,8-dihydropterin will react with α-keto-β-hydroxybutryic acid to form drosopterin and isodrosopterin, and it has been proposed that this is how drosopterins are synthesized in *Drosophila* (Masada et al., 1979). The failure of 7,8-dihydropterin to cause isotope dilution in drosopterin synthesis makes this mechanism unlikely. It is also difficult to explain the role of QS in drosopterin synthesis with this mechanism. Additionally, the extracts used in this report for drosopterin synthesis have been obtained through ammonium sulfate precipitation and Sephadex G-25 chromatography and would not be expected to

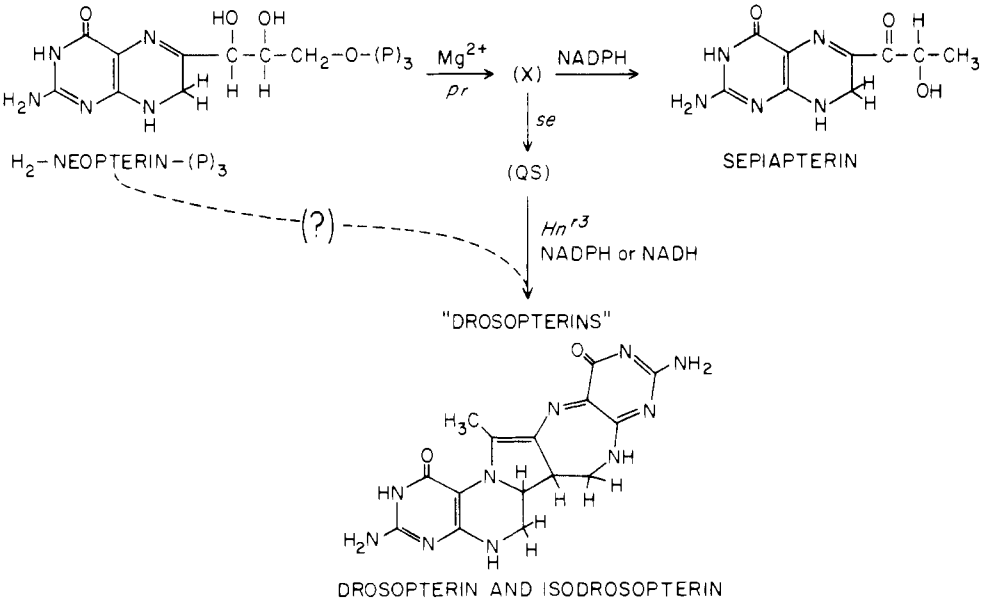

FIGURE 4: Postulated reaction sequence for sepiapterin, QS, and drosopterin biosynthesis.

contain very much if any α-keto-β-hydroxybutyric acid. Since the nonenzymatic reaction is known to produce only drosopterin and isodrosopterin, the origin of the other four species would require additional explanation.

The rosy (*ry*) mutant has low levels of drosopterins and has been shown to have a lesion in the structural locus for xanthine dehydrogenase, which catalyzes the conversion of pterin to isoxanthopterin (Grell, 1962). Although *ry* flies contain low amounts of drosopterins, the evidence to date indicates that isoxanthopterin is not a drosopterin precursor, since the presence of either isoxanthopterin or pterin had no measurable effect on the in vitro synthesis of drosopterins. This agrees with the conclusions of Schwinck (1975), who had found that implantation of *ry* pupae or adults with phenylalanine crystals stimulated production of drosopterins to near-normal levels without affecting isoxanthopterin titers. We have found that phenylalanine has no effect on drosopterin synthesis in vitro (unpublished observations). Perhaps xanthine dehydrogenase and phenylalanine regulate drosopterin synthesis in an indirect fashion in vivo.

We are currently investigating the structure of QS by chemical and mass spectral analysis. Although this is still in progress, it seems clear that QS has nine carbon atoms as detected by high-resolution mass spectral analysis (K. B. Jacobson, D. Dorsett, S. Sethi, and J. A. McCloskey, unpublished observations). This indicates that QS retains the three carbons from the side chain from H_2-neopterin-$(P)_3$. Since drosopterin contains 15 carbon atoms (Theobald & Pfleiderer, 1978), the other drosopterin precursor need only contain 6 carbon atoms. Thus the other drosopterin precursor need not retain the side chain from H_2-neopterin-$(P)_3$. We tested pterin, 7,8-dihydropterin, and isoxanthopterin as possible drosopterin precursors but, on the basis of the isotope dilution experiments, they seem not to be. An enzymatic activity has been found to occur in *Drosophila* heads that removes the three-carbon side chain from H_2-neopterin-$(P)_3$ (Yim et al., 1981). The product of this reaction was isolated by chromatography; by chromatographic and spectral characteristics it was different from pterin, 7,8-dihydropterin, isoxanthopterin, xanthopterin, 4-carboxypterin, 6-formylpterin, 6-(hydroxymethyl)pterin, and 6-methylpterin (Yim et al., 1981). The possibility remains, then, that this compound is not a pterin but is modified in some fashion. We demonstrated that the side-chain release activity is present in significant amounts in the fly extracts used to assay drosopterin biosynthesis. It remains to be seen whether the product of side-chain release activity is a drosopterin precursor.

While this report was being prepared, two papers appeared that are important to recognize. Tanaka et al. (1981) have proposed a structure for the sepiapterin synthase intermediate that has, on the 6 position of the pterin, a side chain with the structure $-C(=O)C(=O)CH_3$. Wiederrecht et al. (1981) have isolated a substance from *Drosophila* heads that stimulates the in vitro synthesis of drosopterins from H_2-neopterin-$(P)_3$. The ultraviolet and mass spectral and biochemical properties of this substance are identical with those that have been determined for QS (K. B. Jacobson, D. Dorsett, J. D. McCloskey, and S. Sethi, unpublished observations). From the data presented herein, it would appear that the sepiapterin synthase intermediate is the immediate precursor to QS which contains a seven-membered diazepine ring. Presumably the first carbonyl of the side chain intrudes into the pyrazine ring, thus producing the diazepine structure. We demonstrated that the ^3H on the terminal carbon of H_2-neopterin-$(P)_3$ is not incorporated into quench spot or drosopterins but is incorporated into sepiapterin. This observation is compatible with a structure of the sepiaterin synthesis intermediate that involves a double bond between the two terminal carbons of the side chain that isomerized with some alternate structure.

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