To the Graduate Council:

I am submitting herewith a dissertation written by Dale Louis Dorsett entitled "Biosynthesis of "Drosopterins" in Drosophila melanogaster." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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and recommend its acceptance:

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BIOSYNTHESIS OF "DROSOPTERINS" IN *DROSOPHILA MELANOGASTER*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

The biosynthesis of the six red eye pigments of Drosophila melanogaster known collectively as the "drosopterins" (neodrosopterin, fraction e, aurodrosopterin I & II, drosopterin, and isodrosopterin) was studied to clarify the role of the purple (pr) eye color mutant in the synthesis of these pigments. The pr mutant is known to accumulate much less "drosopterins" and 7,8-dihydro-6-lactoylpterin (sepiapterin) than wild-type flies. Because the sepia (se) eye color mutant accumulates much more sepiapterin than wild-type flies but no "drosopterins", it had been postulated that sepiapterin is "drosopterin" precursor (Wilson, T.G. & Jacobson, K.B. (1977) Biochem. Genet. 15, 321.). In support of this hypothesis, it had been found that the pr mutant had only 20% wild-type levels of the enzyme that catalyzes the the synthesis of sepiapterin from the 3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl)pterin (H_2-neopterin-$(P)_3$) (Yim, J.J., Grell, E.H., & Jacobson, K.B. (1977) Science 198, 1168.). Gene dosage data indicated that pr may be a lesion in the structural locus for this enzymatic activity known as sepiapterin synthase.

To test the hypothesis that sepiapterin is a "drosopterin" precursor, radioactively-labeled H_2-neopterin-$(P)_3$ was used as a substrate for sepiapterin and "drosopterin" synthesis by enzymes in Drosophila extracts to compare the requirements for biosynthesis. The reaction products were separated by two-dimensional cellulose thin-layer

chromatography. Results were obtained to indicate that sepiapterin synthase actually consists of two enzymes, the first requiring Mg^{2+} and the second, NADPH. The product of the Mg^{2+}-dependent enzyme [known hereafter as the sepiapterin synthase intermediate or (X)], and not sepiapterin, serves as a "drosopterin" precursor in the presence of either NADH or NADPH. The evidence was: (1) the pr mutant, low in accumulated sepiapterin and "drosopterins" is known to have only 20% wild-type sepiapterin synthase activity, (2) unlabeled sepiapterin did not cause isotope dilution of "drosopterin" biosynthesis from [U-^{14}C]H_2-neopterin-$(P)_3$, (3) the 600g pellet prepared from a wild-type homogenate contains "drosopterin" synthesizing activity and no sepiapterin synthetic activity, yet a heat-labile factor in this fraction stimulates sepiapterin synthesis in the 100,000g supernatants of both wild-type and pr flies, (4) sepiapterin and "drosopterin" synthesis require Mg^{2+}, and (5) sepiapterin synthesis is stimulated by NADPH while "drosopterin" synthesis responds to either NADPH or NADH. The sepiapterin synthase intermediate could not be substituted for by H_2-neopterin.

A high performance liquid chromatography (HPLC) assay for (X) was developed. The sepiapterin synthase intermediate was identified by its dependence on Mg^{2+} for enzymatic synthesis from H_2-neopterin-$(P)_3$, the decreased yield with a concomitant increase in the yield of sepiapterin in the presence of NADPH, the incorporation of ^{3}H and ^{14}C from [U-^{14}C][3'-^{3}H]H_2-neopterin-$(P)_3$ in the same ratio as sepiapterin, and the observation that the pr mutant had only 20% wild-type (X) synthetic activity.

It was found that (X) and two related compounds [(X1) and (X2)] could be synthesized nonenzymatically from H_2-neopterin-$(P)_3$ in a reaction catalyzed by Tris base, dependent on H_2-neopterin-$(P)_3$ concentration, significant at temperatures greater than 80^oC, and maximal between pH 8.5 and 9.0 (25^oC). These compounds were purified and it was found that both (X) and (X1) could serve as substrates for the enzymatic NADPH-dependent synthesis of sepiapterin. This observation, the kinetics of formation from H_2-neopterin-$(P)_3$, and the similarity of the ultraviolet absorption spectra led to the hypothesis that (X), (X1), and (X2) are isomers. None of the compounds could be reduced by $NaBH_4$ and only (X1) was sensitive to oxidation with $NaIO_4$. All three were oxidized by iodine to give rise to more fluorescent compounds which in turn could be reduced with $NaBH_4$ to give rise to the original compounds. The latter observations indicate that (X), (X1), and (X2) are dihydropterins.

Since it had been observed that the pr and se mutants both contained low amounts of quench spot (Q.S.), a pterin-like compound and no "drosopterins" while Henna-recessive-3 (Hn^{r3}) flies accumulate both Q.S. and sepiapterin but no "drosopterins" (Wilson & Jacobson, 1977), it was postulated that Q.S. is a "drosopterin" precursor. Evidence was obtained that Q.S. is an intermediate in the conversion of (X) to "drosopterins" and that there exists at least one other "drosopterin" precursor which apparently arises from H_2-neopterin-$(P)_3$ via a separate pathway. Q.S. biosynthetic activity was found in Drosophila extracts (using a two-dimensional cellulose thin-layer chromatography separation of Q.S. for the assay) and label from [U-^{14}C] but not

[3'-^{3}H]H_2-neopterin-$(P)_3$ was incorporated into Q.S. Mg^{2+} was required for Q.S. synthesis but either NADH or NADPH diminished the incorporation of label. Extracts from $\underline{pr^{bw}cn}$ heads contained only 30% of the synthetic activity found in Oregon-R heads and purified Q.S. (from Oregon-R heads), when added to $\underline{in\ vitro}$ biosynthetic reactions utilizing either Oregon-R or $\underline{pr^{bw}cn}$ extracts, stimulated the incorporation of label from [U-^{14}C]H_2-neopterin-$(P)_3$ into the "drosopterins".

The chemical properties of Q.S. were investigated. It demonstrated a molecular ion of m/z=221 with a composition of $C_9H_{11}N_5O_2$ in the mass spectrum. Q.S. has an ultraviolet absorption spectrum (maxima at 260, 360, and 384 nm at pH 6.0) and pKa values similar to pterins but it is nonfluorescent. Upon drying of Q.S. solutions, an orange compound was formed from which Q.S. could be regenerated by dissolving and diluting in H_2O. Q.S. was sensitive to oxidation by alkaline $KMnO_4$ but did not give rise to 6-carboxypterin as a product. It was oxidized by $NaIO_4$ and could be reduced with $NaBH_4$. Q.S. reacted with 2,4-dinitrophenylhydrazine to form a red precipitate. Iodine oxidized Q.S. but the products were nonfluorescent. These observations and the observation that Q.S. is an intermediate in the conversion of (X) to "drosopterins" led to two postulated structures for Q.S. in which it has retained the three carbon side chain from H_2-neopterin-$(P)_3$ but two atoms (C1' and C2') of the side chain form a four-membered ring with two atoms of the pyrazine ring (N5 and C6) by the presence of a bond between N5 and C2'. The terminal carbon of the side chain is a methyl group in both structures. One of the proposed structures is an

epoxide with an oxygen atom forming a three-membered ring with C1' and C2' and the other structure is a 2'-oxo compound. The latter is postulated to equilibrate with a hydrated form (1',2'-dihydroxy) in aqueous solution For either of these structures to be converted to "drosopterins" would require a rearrangement of the four-membered ring.

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ABBREVIATIONS

biopterin	6-(L-erythro-1',2'-dihydroxypropyl)pterin
CE	crude extract
2,4-DNPH	2,4-dinitrophenylhydrazine
"drosopterins"	class of six red dipteridine-derived eye pigments of *Drosophila melanogaster*
EDTA	ethylenediamine tetraacetic acid
GTP	guanosine-5'-triphosphate
H_2-neopterin-$(P)_3$	3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl)pterin
HPLC	high performance liquid chromatography
isoxanthopterin	7-hydroxypterin
PFE	pteridine-free extract
Pipes	1,4-piperazine diethanesulfonic acid
Q.S.	*quench spot*
sepiapterin	7,8-dihydro-6-lactoylpterin
Taps	tris(hydroxymethyl)methylaminopropanesulfonic acid
TE	Triton extract
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane
(X)	sepiapterin synthase intermediate
xanthopterin	6-hydroxypterin

CHAPTER I

INTRODUCTION

This chapter is a general introduction to the remaining chapters which are written in the form for submission to a scientific journal. Some of the material covered in this chapter will also be presented in the introductions to the individual remaining chapters. This format allows each chapter to be read as a separate entity.

The eye color mutants of Drosophila melanogaster have been used extensively in the study of inheritance in this organism as they are easily recognized and usually nonlethal. An intriguing phenomenon associated with the expression of the eye color mutants vermilion [v, 1-33.0 (map location)] (Lindsley & Grell, 1967) and purple (pr, 2-54.5) has been noticed. Suppressor of sable ($su(s)^2$, 1-0) is a mutation that suppresses certain alleles of the two eye color mutants (returning the eye color to wild-type) as well as two body color mutants, sable (s, 1-43.0 and speck (sp, 2-107.0)

The v and pr mutants affect two different types of pigments. The brown (ommochrome) and red (pteridine) pigments were first differentiated on the basis of solubility. The red pigment could be extracted from the flies with 30% ethanol, made pH 2 with concentrated HCl (Ephrussi & Herold, 1944), and the brown pigment which remained could then be extracted with methanol containing 1% HCl. The two types of pigment were also distinguished on the basis of their time of appearance in the fly during development. The brown pigment appeared at

about 53-55 hours and the the red at about 71 hours after pupation (Daneel, 1941; Hadorn & Ziegler, 1958). Only the brown pigments are affected by the v locus and only the red are affected by pr.

The pigments involved in the determination of Drosophila eye color are accessory pigments and not involved in the visual process (Goldsmith, 1964). They are located in the accessory pigment cells of the ommatidia (Figure 1) bound to protein-containing granules. Each type of pigment (ommochrome or pteridine) is bound to a different type of granule which can also be differentiated on the basis of structure and time of appearance during the development of the fly (Shoup, 1966). Accessory pigments optically isolate the individual ommatidia of the compound eye, thereby preventing the light that enters one ommatidium from straying into the neighboring ommatidium which would decrease visual acuity (Burkhardt, 1964).

The biosynthesis of ommochromes is well understood (Linzen, 1974). Xanthommatin, the major brown pigment, is derived from two molecules of 3-hydroxykynurenine (Phillips & Forrest, 1970a,b) which are synthesized from tryptophan by the sequential action of the enzymes tryptophan oxygenase (Baglioni, 1959), formamidase (Glassman, 1956), and kynurenine-3-hydroxylase (Ghosh & Forrest, 1967). Vermilion is probably a lesion in the structural locus for tryptophan oxygenase which catalyzes the conversion of tryptophan to N-formylkynurenine (Tartof, 1969). Levels of tryptophan oxygenase are restored to near wild-type levels by $su(s)^2$ and an isoacceptor of tyrosine-tRNA may be involved in the reactivation of the mutant enzyme (Jacobson, 1978).

The biosynthesis of the pteridine eye pigments is less well

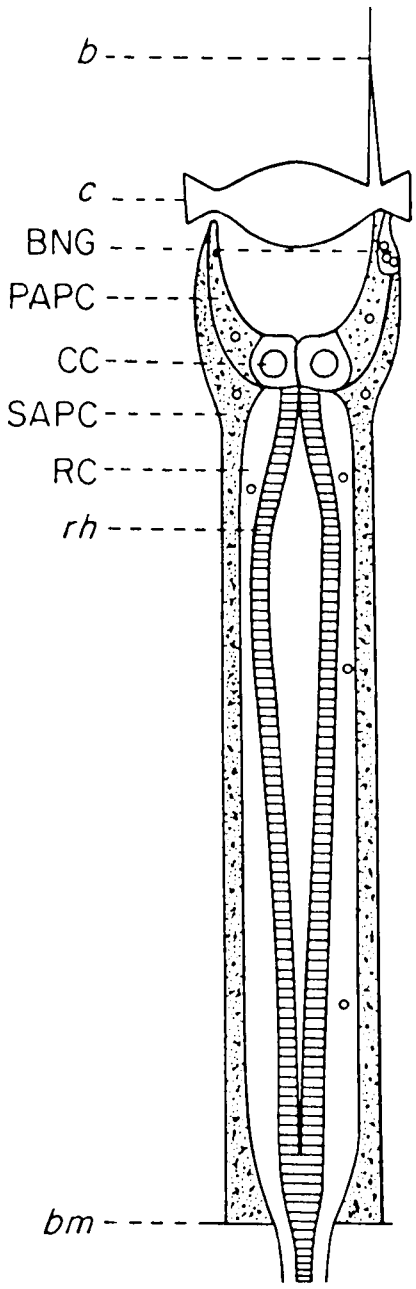
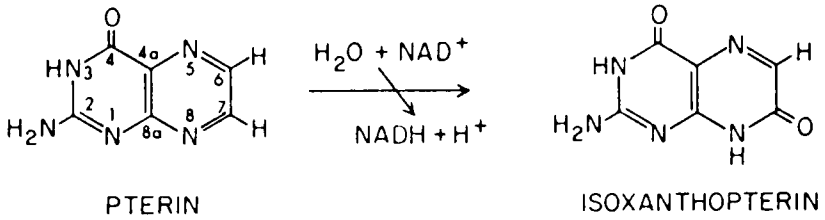


Figure 1. Structure of the Drosophila ommatidium. After Clayton, F.E.. (1954) Univ. Texas Bull. 5422, 210. b, bristle; bm, basement membrane; BNG, bristle nerve group; c, cornea; CC, cone cell; PAPC, primary accessory pigment cell; RC, retinular cell; rh, rhabdomere; SAPC, secondary accessory pigment cell.

understood. Nomenclature, biological occurence, structural determinations, and properties of the pteridines are well covered by Blakeley (1969, chapters 1, 2, and 3). The heterocyclic ring system common to pteridines can be thought of as a fusion of a pyrimidine and a pyrazine ring (pyrimido[4,5-b]pyrazine). Since most biologically occuring pteridines are derivatives of 2-amino-4-hydroxypteridine (Figure 2), this compound has been named pterin and derivatives are known collectively as pterins. The ring numbering system is also shown in Figure 2.

The pterins that are readily identified in Drosophila are pterin, isoxanthopterin (7-hydroxypterin), biopterin (6-(L-erythro-1',2'-dihydroxypropyl)pterin), sepiapterin (7,8-dihydro-6-lactoylpterin), deoxysepiapterin (7,8-dihydro-6-(1'-oxopropyl)pterin), and the six red pigments known collectively as the "drosopterins" [neodrosopterin, fraction e, aurodrosopterin I & II, drosopterin, and isodrosopterin (Schwinck & Mancini, 1973)]. It has been suggested that, except in the case of certain mutants, pterin and perhaps biopterin levels are artifactually enhanced by breakdown of the other pterins during isolation (Ziegler, 1961). As deoxysepiapterin can be formed from 7,8-dihydrobiopterin under acidic conditions, one must also consider that levels of this pterin may be artifactually enhanced (Katoh & Akino, 1966). Xanthopterin (6-hydroxypterin) and 6-carboxypterin are also found in Drosophila extracts but they are considered artifacts created by degradation of other pterins during extraction (Ziegler, 1961). Of the pterins found in Drosophila, only isoxanthopterin is not deposited in the pigment granules (Shoup, 1966). Isoxanthopterin, in contrast to other pterins, is found in significant amounts in the Malphigian tubules and



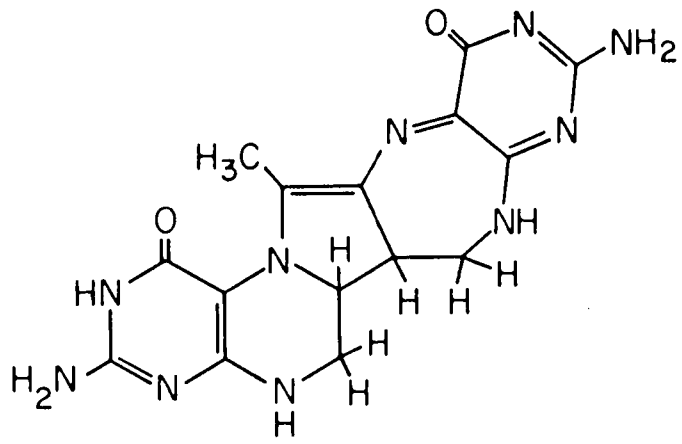
PTERIN ISOXANTHOPTERIN

Figure 2. Synthesis of isoxanthopterin from pterin by xanthine dehydrogenase.

testes (Hadorn & Mitchell, 1951).

The structures of most of the "drosopterins" are not known. A dipteridine-derived structure for drosopterin and isodrosopterin (a stereoisomeric pair) has been proposed (Theobald & Pfleiderer, 1977)(Figure 3). Although the other "drosopterins" also appear to be dipteridine-derived compounds on the basis of chemical degradation and spectral results (Rokos & Pfleiderer, 1975), their structures remain unknown. Aurodrosopterin I & II, which are more yellowish than drosopterin and isodrosopterin, are also a stereoisomeric pair. Neodrosopterin (optically inactive) is more reddish than drosopterin and isodrosopterin and gives rise to both in aqueous solutions. Fraction e has not been purified, but it is similar in color and chromatographic behavior (on cellulose thin-layer plates) to neodrosopterin (Schwinck & Mancini, 1973).

Schwinck & Mancini (1973) studied the relative amounts of "drosopterins" in various eye color mutants of Drosophila and divided them into three classes. Class I were apparently normal with regard to "drosopterins", having large amounts of all species; drosopterin, isodrosopterin, and neodrosopterin were the major pigments. The members of Class I were either wild-type flies or mutants that apparently affect only ommochrome accumulation [Oregon-R (wild-type), v, scarlet (st, 3-44.0), and cinnabar (cn, 2-57.5)]. Some members of Class I, cardinal (cd, 3-75.7), mahogany (mah, 1-64.8), and maroon (ma, 3-49.7) had enhanced levels of aurodrosopterins. Members of Class II included carnation (car, 1-62.5), ruby (rb, 1-7.5), maroon-like (mal, 1-64.8), rosy (ry, 3-52.0), and garnet (g, 1-44.4). They contained less than



DROSOPTERIN AND ISODROSOPTERIN

Figure 3. Structure of drosopterin and isodrosopterin. After Theobald, N. & Pfleiderer, W. (1977) Tetrahedron Lett. 10, 841.

half the total red pigment present in Class I flies, but fraction e was very low and the amount of the aurodrosopterins relative to the others was higher than in Class I. Class III included clot (cl, 2-16.5), raspberry (ras, 1-32.8), prune (pn, 1-0.8), and pr. They also contained less than half the total "drosopterins" present in Class I flies, but in this group both fraction e and aurodrosopterins are virtually absent. It appears that the genetic regulation of the "drosopterins" affects certain groups as well as the individual species.

Class II contains mutants that are known to have low amounts of the enzyme xanthine dehydrogenase which catalyzes the conversion of pterin to isoxanthopterin (Figure 2). Rosy is a lesion in the structural locus for xanthine dehydrogenase (Grell, 1962; Yen & Glassman, 1965) and mal also lacks xanthine dehydrogenase activity (Forrest et al., 1956). Schwinck treated several eye color mutants with allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine), an inhibitor of xanthine dehydrogenase, by osmotic uptake into pupae dissected from the puparium, feeding to larvae, and implantation into the abdomens of newly eclosed adults. Allopurinol drastically decreased the eye "drosopterin" content and also produced other characteristics of the ry mutant such as semi-lethality and excretory abnormalities (Schwinck, 1975). Since it was also found that implantation of newly eclosed ry adults with phenylalanine elevated the "drosopterin" content to nearly wild-type levels (tyrosine did not produce an effect) without affecting the level of isoxanthopterin, the conclusion was reached that isoxanthopterin is not a "drosopterin" precursor, but rather it or its precursors regulate "drosopterin" synthesis in some fashion. Since isoxanthopterin occurs

in the Malphigian tubules where it is formed by the action of xanthine dehydrogenase, easily translocates between the haemolymph and other cells (Hadorn et al., 1958), and does not bind to pigment granules, it seems that isoxanthopterin is probably an excretion product of Drosophila. It is interesting to speculate that the presence or absence of isoxanthopterin may regulate "drosopterin" synthesis because synthesis of the eye pigments themseleves could provide a means of reducing the cellular level of nitrogenous compounds.

The amounts of various pterins and an unidentified pterin-like compound, quench spot (Q.S.), have been measured in wild-type (Oregon-R, Canton-S, and Samarkand), pr and pr^{bw} (both suppressible alleles of pr), sepia (se, 3-26.0), Henna-recessive-3 (Hn^{r3}, 3-23.0), pr Hn^{r3}, and Hn^{r3} se flies with a modified two-dimensional thin-layer chromatographic procedure that utilizes cellulose layers (Wilson & Jacobson, 1977a, b). Biopterin was found to be slightly low in pr and pr Hn^{r3}, normal in pr^{bw}, high in Hn^{r3}, and even higher in se and Hn^{r3} se. Sepiapterin was found to be very low in pr, pr^{bw}, and pr Hn^{r3}, high in Hn^{r3}, and very high in se and Hn^{r3} se. The "drosopterins" were low in pr, pr^{bw}, pr Hn^{r3}, and Hn^{r3}, and virtually absent in se and Hn^{r3} se. Q.S. was low in pr, pr^{bw}, and pr Hn^{r3}, high in Hn^{r3}, and absent in se and Hn^{r3}-se. As pr, pr^{bw}, and pr Hn^{r3} had low amounts of all the compounds, the authors postulated that pr is a lesion in one of the initial steps of pterin biosynthesis. As se and Hn^{r3} se accumulated large amounts of sepiapterin but no "drosopterins", it was furthur postulated that sepiapterin is a "drosopterin" precursor. Q.S. was found to have an ultraviolet absorption spectrum similar to pterins. Because Hn^{r3}

accumulated both Q.S. and sepiapterin but little "drosopterins" while se and Hn^{r3} se contained neither Q.S. or "drosopterins", the authors suggested that Q.S. may be an intermediate in the synthesis of "drosopterins" from sepiapterin.

At first it would seem that the results of Wilson & Jacobson (1977b) on the amount of biopterin present in the various eye color mutants is at odds with what is known about the biosynthesis of biopterin. As tetrahydrobiopterin is a cofactor in some enzymatically catalyzed hydroxylation reactions [e.g. phenylalanine hydroxylase (Kaufman, 1963)], the biosynthesis of biopterin has been examined in many organisms. An enzymatic activity (sepiapterin reductase) that reduces sepiapterin to 7,8-dihydrobiopterin was found in silkworm (Matsubara et al., 1963), Drosophila (Taira, 1961), and the livers of chicken, rat, pig, horse, and ox (Matsubara & Akino, 1964). The enzyme from Drosophila is maximally active in newly eclosed adults, but significant amounts are present throughout the life cycle (Fan & Brown, 1979). The enzyme was purified 200-fold from late larvae and early pupae. NADPH was required and the enzyme preferred oxidized sepiapterin as a substrate (Km = 10 uM as compared to 63 uM for sepiapterin). As oxidized sepiapterin was converted to biopterin (Figure 4), the enzyme was renamed biopterin synthase by these investigators. In addition to biopterin synthase, an O_2-dependent dihydropterin oxidase was also found in the extracts (Figure 4). Most dihydropterins tested would serve as substrates for the oxidase. As the pr mutant accumulates little sepiapterin and nearly normal amounts of biopterin, it would seem that there might be an alternate route for biopterin synthesis in adult

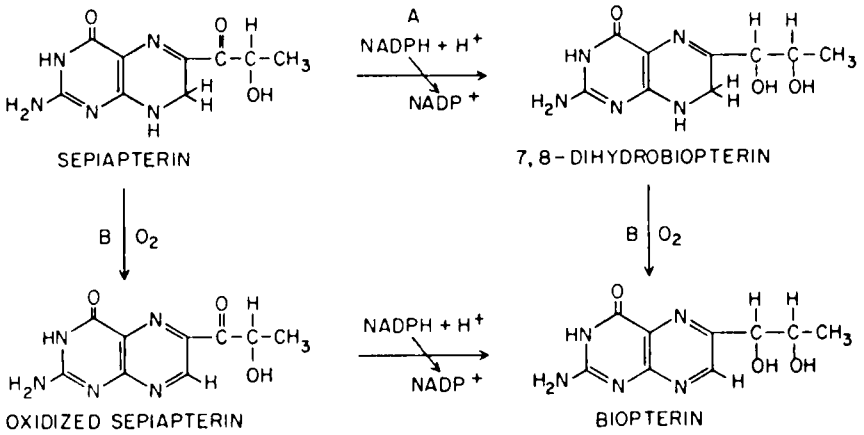


Figure 4. Reactions catalyzed by sepiapterin reductase (biopterin synthase) and dihydropterin oxidase. A, sepiapterin reductase; B; dihydropterin oxidase.

heads. It may not be necessary to postulate this alternate route, however, when one considers that pr is a leaky mutant and that biopterin levels may well be regulated since the reduced form is an enzyme cofactor. It seems possible that the pr mutant uses most of the sepiapterin it does produce to maintain the biopterin concentration. In se and Hn^{r3} flies the increased level of sepiapterin is accompanied by an increase in the level of biopterin which is consistent with the hypothesis that sepiapterin is the biopterin precursor in vivo.

Due to the obvious structural similarities, it was long postulated that guanine, guanine nucleoside, or guanine nucleotide could be the precursor of pterins. Indeed it was found that Xenopus laevis injected with [2-^{14}C]guanine incorporated the label into 6-carboxypterin isolated from their skins (Ziegler-Gunder et al., 1956). Similar results were obtained by Brenner-Holzach & Leuthardt (1959, 1961) in Drosophila. These observations were substantiated by the discovery of an enzymatic activity (GTP cyclohydrolase) in E. coli extracts during studies on the biosynthesis of folic acid (a conjugated pterin whose tetrahydro form is a cofactor in enzymatically catalyzed single carbon unit transfers).

It was known that tetrahydrofolic acid could be synthesized from 7,8-dihydro-6-hydroxymethylpterin by the sequential action of the enzymes hydroxymethyldihydropterin pyrophosphokinase (which uses ATP as the source of pyrophosphate) (Richey & Brown, 1969), dihydropteroate synthetase (which catalyzes the formation of a bond between the nitrogen atom of p-aminobenzoic acid and the side chain carbon of the pterin with the release of pyrophosphate) (Richey & Brown, 1969), an activity

that catalyzes the formation of an amide bond between the carboxyl group of the benzoic acid moiety and glutamate (Griffin & Brown, 1964), and dihydrofolate reductase (which requires NADPH and reduces the pyrazine ring) (Matthews & Sutherland, 1965). The observation had also been made that E. coli extracts were capable of synthesizing dihydrofolate if p-aminobenzoic acid, ATP, and either GMP, GDP, or GTP were supplied (Reynolds & Brown, 1964). If [U-^{14}C]guanosine was used, label was incorporated into dihydrofolate while if [8-^{14}C]guanosine was supplied, label was not incorporated into the product. GTP cyclohydrolase was detected on the basis of its ability to catalyze the release of C8 from GTP as formate (Burg & Brown, 1968). GTP is the required substrate and the product of the reaction is the 3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl)pterin (H_2-neopterin-$(P)_3$) (Figure 5). For the synthesis of dihydrofolate, the phosphates are removed by an uncharacterized phosphatase (Burg & Brown, 1968) to form 7,8-dihydroneopterin, which is cleaved to form 7,8-dihydro-6-hydroxymethylpterin by dihydroneopterin aldolase (Mathis & Brown, 1970).

GTP cyclohydrolase has been purified to homogeneity from E. coli (Yim & Brown, 1976). It has a MW of 210,000 with four identical subunits of 51,000 MW. The pH optimum for the enzyme is approximately 8.5 and the Km for GTP is 0.02 uM. With purified E. coli GTP cyclohydrolase it is possible to convert GTP to H_2-neopterin-$(P)_3$ in yields approaching 100%. [U-^{14}C]GTP is converted to [U-^{14}C]H_2-neopterin-$(P)_3$; [8,5'-^{3}H]GTP is converted to [3'-^{3}H]H_2-neopterin-$(P)_3$. GTP cyclohydrolase has been found in other bacteria (Jackson & Shiota, 1973),

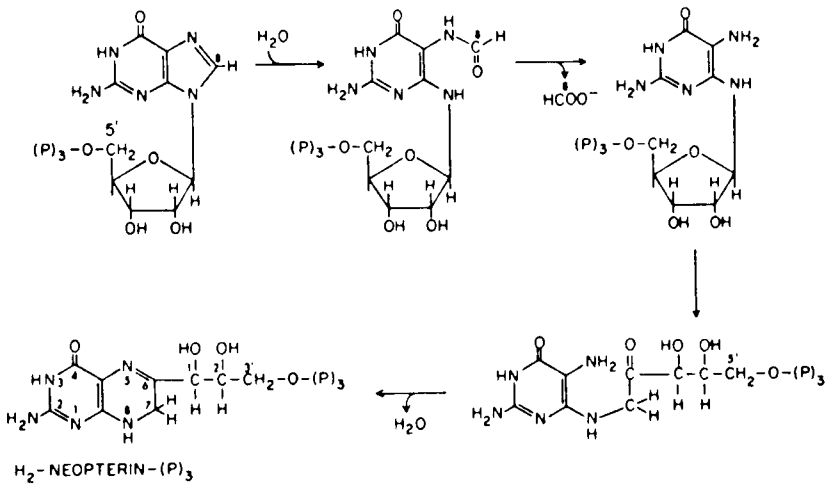


Figure 5. Postulated reaction mechanism for GTP cyclohydrolase. After Yim, J.J. & Brown, G.M. (1976) J. Biol. Chem. 251, 5087.

Saccharomyces cerevisiae (Pepperberg, 1973), and Drosophila melanogaster (Fan & Brown, 1976). Maximal levels of the enzyme occur twice during the development of the fly, with peak activities at pupation and emergence (Fan et al., 1976); the latter corresponds to the synthesis of the eye pigments and is found mainly in the head. The ras and pn mutants (both are in Schwinck's Class II) have altered developmental profiles of GTP cyclohydrolase in that the peak of activity at emergence is diminished (Evans & Howells, 1978). Gene dosage experiments indicated that neither mutant is a lesion in the structural locus for the enzyme. An autonomous temperature-sensitive allele of ras has been discovered (Grigliatti & Suzuki, 1970) that displays two temperature-sensitive periods during the development of the fly. Examination of the GTP cyclohydrolase of this mutant should be of interest.

The enzymatic synthesis of sepiapterin from H_2-neopterin-$(P)_3$ was first demonstrated in Drosophila extracts (Fan et al, 1975) (Figure 6). Sepiapterin synthase requires both Mg^{2+} and NADPH for activity. The amount of the enzyme is maximal in the fly at emergence; at this stage the enzyme is found mainly in the head. Sepiapterin synthase (with the same cofactor requirements) has also been found in chicken kidney (Tanaka et al., 1979).

Flies with the pr mutation were found to contain only 20% of the sepiapterin synthase activity found in wild-type flies (Yim et al., 1977); $su(s)^2$ pr had nearly wild-type levels of the activity. Flies with two doses of the wild-type allele of pr (pr^+) were found to have twice as much activity as flies with a single dose; flies with three doses of pr^+ had 20% more activity than flies with two doses. With

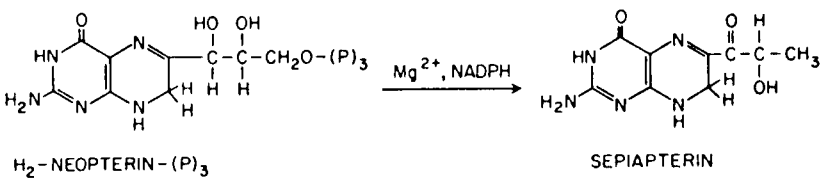


Figure 6. Synthesis of sepiapterin from H_2-neopterin-$(P)_3$ by sepiapterin synthase.

some reservations about the comparison of the three dose to two dose flies, these observations were taken as evidence that pr is a lesion in the structural locus for sepiapterin synthase.

There are interesting analogies between the v and pr mutants and their suppression by $su(s)^2$. Both are apparently lesions in structural loci coding for enzymes early in their respective pathways and the activity of both enzymes can be restored by $su(s)^2$. Both v and pr can also be suppressed by epigenetic manipulations. If pr flies are raised at 30°C they synthesize more "drosopterins" than if they are raised at 25°C. Increasing the temperature does not cause v flies to synthesize more ommochromes, but if they are raised on media containing only yeast as the nutritional source (a starvation medium) small amounts of ommochromes are synthesized. Starvation does not cause increased "drosopterin" synthesis in the pr mutant (Jacobson, unpublished observations). Furthur investigation is needed to discern if these manipulations operate through the same mechanism as the $su(s)^2$ mutation.

The intent of the investigations presented in this dissertation was to determine the requirements for biosynthesis of the "drosopterins" in order to clarify the role of the pr mutant and sepiapterin synthase in the synthesis of these pigments. The hypothesis that sepiapterin is a "drosopterin" precursor provided the framework for the initial experiments in which the requirements for in vitro synthesis of "drosopterins" from H_2-neopterin-$(P)_3$ by enzymes present in Drosophila extracts were to be compared to the requirements for in vitro synthesis of sepiapterin. As will be seen, this hypothesis required modification.

CHAPTER II

BIOSYNTHESIS OF "DROSOPTERINS" BY AN ENZYME SYSTEM FROM *DROSOPHILA MELANOGASTER*

1. INTRODUCTION

Drosophila provides certain advantages for the study of the biosynthesis of the pteridine eye pigments: first, large amounts of pteridines are found in the eyes of *Drosophila*, second, the fly is a convenient source of enzymes for the study of the synthesis, interconversion, and control of pteridine synthesis, and third, a wealth of eye color mutants are associated with the pteridine eye pigments.

The eye pigments [with the possible exception of 7-hydroxypterin (isoxanthopterin)] are deposited in protein-containing granules present in the primary and secondary pigment cells of the ommatidia (Shoup, 1966). There are specific granules for the ommochrome pigments and for the pteridine pigments. The presence of the granules may depend upon the synthesis of the pigment (Shoup, 1966).

The major pteridines present in *Drosophila* are the "drosopterins", 7,8-dihydro-6-lactoylpterin (sepiapterin), 6-(L-erythro-1',2'-dihydroxypropyl)pterin (biopterin), isoxanthopterin, and pterin. The structures of the "drosopterins" are not yet firmly established, but it appears that they are formed from two pteridine ring systems (Theobald & Pfleiderer, 1977). The structural interrelationships of "drosopterins" are of interest. Neodrosopterin is known to rearrange to a mixture of drosopterin and isodrosopterin in aqueous solutions (Rokos &

Pfleiderer, 1975). Drosopterin and isodrosopterin appear to be closely related in that they have mirror-image circular dichroism spectra (indicating that they are stereoisomers) (Rokos & Pfleiderer, 1975), but they are separable by cellulose thin-layer chromatography. Aurodrosopterin I & II are also a stereoisomeric pair (Rokos & Pfleiderer, 1975). The "drosopterin" designated as fraction e by Schwinck & Mancini (1973) has not been characterized, but its color and R_f values resemble those of neodrosopterin.

It is now becoming accepted that the 3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl)pterin (H_2-neopterin-$(P)_3$) is the precursor of all pteridines. It is synthesized from GTP by the action of GTP cyclohydrolase (Burg & Brown, 1968). This enzyme was first discovered in Escherichia coli and has been shown to be present in several organisms, including Drosophila (Fan et al., 1976). GTP cyclohydrolase occurs in two peaks of activity during the development of Drosophila (at pupariation and emergence), the latter being restricted to the head of the fly (Fan et al., 1976). Two eye color mutants, raspberry (ras) and prune (pn), have been shown to have altered developmental profiles of GTP cyclohydrolase (Evans & Howells, 1978).

Another known pteridine-synthesizing activity from Drosophila is sepiapterin synthase (Fan et al., 1975), which catalyzes the conversion of H_2-neopterin-$(P)_3$ to sepiapterin in the presence of Mg^{2+} and NADPH. The role of this enzyme in the synthesis of pteridines is implicated by the purple (pr) eye color mutant. The pr mutant accumulates less sepiapterin and "drosopterins" than wild type flies (Wilson & Jacobson, 1977b). Recently, our laboratory has reported that the pr mutant, and

the allele $\underline{pr^{bw}}$, have approximately 10-30% of the amount of sepiapterin synthase activity present in wild-type flies (Yim et al., 1977). Gene dosage data with $\underline{pr^{+}}$ support the hypothesis that $\underline{pr^{+}}$ is the structural locus for sepiapterin synthase. When pr or $\underline{pr^{bw}}$ is genetically combined with a suppressor mutation, suppressor of sable ($\underline{su(s)^{2}}$), the levels of eye pigments accumulated are restored to wild-type levels as is the sepiapterin synthase activity. Different alleles of $\underline{su(s)^{2}}$ have been found to have differing abilities to restore the sepiapterin synthase activity; when sepiapterin synthase activity is restored to approximately 30% of wild-type activity, the accumulation of "drosopterins" is the same as in wild-type flies (unpublished observations).

We have studied "drosopterin" biosynthesis to clarify the role of sepiapterin synthase in the synthesis of these pigments. In this report we describe an in vitro assay for "drosopterin" biosynthesis which utilizes [U-^{14}C]H_2-neopterin-$(P)_3$ as the substrate and present evidence that sepiapterin is not a precursor of "drosopterins"

2. MATERIALS AND METHODS

Drosophila melanogaster. Oregon-R flies were reared at 25^{o}C on medium prepared as described by Lewis (1960). Flies were collected daily, so that all were one day old or less. After collection the flies were quickly frozen in liquid nitrogen; the heads were obtained as previously described (Wilson & Jacobson, 1977a) and stored in vials under liquid nitrogen for later use. The $\underline{pr^{bw}}$ flies used in these studies were reared and collected as above, but were stored at -80^{o}C for later use.

Enzyme Extracts; Cenrifugal Fractionation. For fractionation by differential centrifugation, Oregon-R fly heads were homogenized in tube-and-pestle ground glass homogenizers (Kontes Co.) with 5 volumes of 50 mM Pipes (Calbiochem) buffer at pH 7.0. Grinding was limited to the minimum required to cause the disappearance of visible heads (approximately 10 strokes). The homogenate was centrifuged at 600g for 10 min, and the supernatant was saved for preparation of furthur fractions. The pellet consists of three layers, the uppermost having the uniform red color of the "drosopterins". This red layer was separated with a Pastuer pipet and buffer, resuspended, and centrifuged at 600g for 10 min. The pellet was washed again as above, and was finally resuspended in an amount of buffer to equal the volume of the supernatant recovered after the 100,000g centrifugation. This was done so the pellet preparations would contain roughly the same number of fly head equivalents as the 100,000g supernatant. The first 600g supernatant was centrifuged at 15,000g for 5 min; this pellet was washed twice and resuspended to the same volume as the 600g pellet. The first 15,000g supernatant was centrifuged at 100,000g for 60 min; this pellet was also washed twice and resuspended to the same volume as the 600g pellet. The 100,000g supernatant was used directly. The above preparations were made fresh for each assay. All operations were done at 0-4^{o}C. The pr^{bw} whole-fly 100,000g supernatant was obtained by homogenization of 1 g of flies in 4 mL of 50 mM Pipes at pH 7.0 with a tube-and-pestle ground glass homogenizer, centrifugation at 15,000g for 5 min, then at 100,000g for 60 min.

Preparation of Pteridine-Free Extract. As a large amount of

"drosopterin" biosynthesis occurs in the 600g and 100,000g pellets, we devised a means of recovering some of this activity in a soluble form. It was found that activity could be extracted from the pellet fractions by use of 50 mM Pipes (pH 7.0) containing 0.5-1.5 M KCl. A pteridine-free preparation was obtained by the following procedure: Oregon-R heads (2.5 g) were homogenized in 12.5 mL of 50 mM Pipes (pH 7.0) containing 0.5 M KCl. The homogenate was centrifuged at 15,000g for 10 min. The supernatant was saved, and the pellet was rehomogenized in and collected from 12.5 mL of the same buffer once, twice with buffer containing 1 M KCl, and twice with buffer containing 1.5 M KCl. The final pellet was discarded, and the proteins from the pooled supernatants were fractionated between 40 and 60% saturated $(NH_4)_2SO_4$. The pellet, collected at 15,000g for 30 min, was drained and dissolved with 1 mL of 50 mM Pipes (pH 7.0) containing 10% glycerol. The solution was applied to a Sephadex G25 (Pharmacia) column (1.7 x 13 cm) which was eluted with the buffer in which the sample was dissolved at a flow rate of 4-6 mL/h while 1 mL fractions were collected. The A_{280}-absorbing material eluted in the void volume was pooled and concentrated to 1.5 mL by vacuum dialysis (collodion bag, Schleicher and Schull, 25,000 MW cutoff). The resulting preparation had no visible pigment and was stored at -20°C until use. All operations were carried out at 0-4°C except the vacuum dialysis, which was at 23°C.

Preparation of [U-^{14}C]H_2-neopterin-$(P)_3$ and [3'-^{3}H]H_2-neopterin-$(P)_3$. [U-^{14}C]GTP (Amersham) was adjusted to a specific activity of 20 uCi/umol with unlabeled GTP (sodium salt, Sigma). It was incubated at a concentration of 670 uM with 100 mM TrisCl (pH 8.5), 100 mM NaCl, 10

mM EDTA (pH 7.5) and pure GTP cyclohydrolase I prepared from E. coli (Yim & Brown, 1976) to produce [U-^{14}C]H_2-neopterin-$(P)_3$. The incubation was carried out in the dark at 42°C for 90 min. The amount of H_2-neopterin-$(P)_3$ produced was determined from measurements of the release of [^{14}C]formate by filtration of reaction aliquots through charcoal (Burg & Brown, 1968). Conversion was consistently greater than 95%. [8,5'-^{3}H]GTP (New England Nuclear), adjusted to 45 uCi/umol, was the source of [3'-^{3}H]H_2-neopterin-$(P)_3$. An aliquot of the GTP cyclohydrolase reaction was used as the source of H_2-neopterin-$(P)_3$ for the assays discussed below. Phosphatase-treated [U-^{14}C]H_2-neopterin-$(P)_3$ was prepared by addition of 0.5 units of bacterial alkaline phosphatase (Worthington, BAPC) to 30 uL of [U-^{14}C]H_2-neopterin-$(P)_3$ prepared as above and incubation at 42°C for 30 min.

Assay of Sepiapterin and "Drosopterin" Biosynthesis. Sepiapterin and "drosopterin" biosynthesis were determined in a reaction mixture which had a total volume of 70 uL with the following components: 50 mM Pipes (pH 7.5), 143 uM [U-^{14}C]H_2-neopterin-$(P)_3$ (or 131 uM [3'-^{3}H]H_2-neopterin-$(P)_3$), Drosophila extract, and cofactors or pteridines depending upon the experiment. Mg^{2+}, when present, was at a concentration of 14.3 mM; NAD^+ (Sigma), $NADP^+$ (Sigma), NADPH (chemically reduced, Sigma), and NADH (chemically reduced, Sigma) were used at concentrations of 2.5 mM. Isotope dilution experiments were done with sepiapterin at a concentration of 4 mM, neodrosopterin at a concentration of 1 A_{500} units/mL (pH 5.5), and a mixture of drosopterin and isodrosopterin at 2 A_{500} units/mL (pH 5.5). The reactions were incubated in the dark at 42°C for 60 min, and then 0.015 umol of sepiapterin and 10

uL of a fresh fly-head extract (0.2 g Oregon-R heads homogenized in 1 mL of 30% ethanol made pH 2 with concentrated HCl, then centrifuged) as a "drosopterin" carrier were added before the reaction tubes were placed in a boiling-water bath for 5 min. After the tubes were heated, 0.5 units of bacterial alkaline phosphatase (Worthington, BAPC) was added to each, and they were then incubated at 42°C for 10 min. After the tubes were centrifuged (Brinkmann Eppendorf Centrifuge 3200), 40 uL of the resultant supernatant was subjected to two-dimensional cellulose thin-layer chromatography as previously described (Wilson & Jacobson, 1977a) except that the ammonium acetate concentration in the first solvent was increased to 2 g/100 mL. The sepiapterin and individual "drosopterin" spots were scraped into scintillation vials containing 1.5 mL H_2O, and the amount of radioactivity was determined in a scintillation counter with 15 mL of 0.28% 2,5-bis-2-[5-tert-butylbenzoxazolyl]thiophene (Packard) and 33% Triton X-100 in toluene. Kodak RP x-ray film was used for autoradiography.

Preparation of Neodrosopterin, Drosopterin, Isodrosopterin, and Sepiapterin. The "drosopterins" were prepared by the methods of Rokos & Pfleiderer (1975) except the initial extract was obtained by homogenizing 10 g of Oregon-R heads in 50 mL of 30% ethanol made pH 2 with concentrated HCl. The homogenate was centrifuged at 15,000g for 10 min. The pellet was reextracted with 50 mL of the same sovent three times. The combined extracts were concentrated by rotary evaporation to form the initial extract.

Sepiapterin was prepared by homogenization of 5 g of sepia mutant heads in 25 mL of 30% ethanol made pH 2 with concentrated HCl. The

sample was centrifuged at 15,000g for 10 min, concentrated by rotary evaporation, and applied to a Sephadex G25 column (1.7 x 40 cm) which was eluted with water. The peak sepiapterin fractions were rotary evaporated to dryness and redissolved in a minimal amount of water. The sample contained a few minor blue fluorescent contaminants as resoved by two-dimensional cellulose thin-layer chromatography.

3. RESULTS

Figure 7 shows the pattern of radioactive compounds obtained when [U-^{14}C]H_2-neopterin-$(P)_3$ was incubated with the pteridine-free extract. The radioactive compounds (1-6) correspond in position and shape of spot to sepiapterin and the "drosopterins". If the extract was heated at 100^{o}C for 5 min before incubation, no radioactivity was found in these positions. The amount of radioactivity incorporated into the "drosopterins" and sepiapterin increased with time and enzyme concentration. Radioactivity from [3'-^{3}H]H_2-neopterin-$(P)_3$ was incorporated into sepiapterin but not the "drosopterins". When [U-^{14}C]H_2-neopterin-$(P)_3$ was incubated with bacterial alkaline phosphatase to produce [U-^{14}C]H_2-neopterin, and the latter used as a substrate, no radioactivity was incorporated into the six pteridines.

When planning these investigations, we were aware of the role of sedimentable granules in Drosophila eye pigmentation (Shoup, 1966) and consequently decided to determine the activity for "drosopterin" synthesis in fractions prepared by differential centrifugation of a head homogenate. Sepiapterin and "drosopterin" synthesis appear to be distributed differently in the various centrifugal fractions (Figure 8).

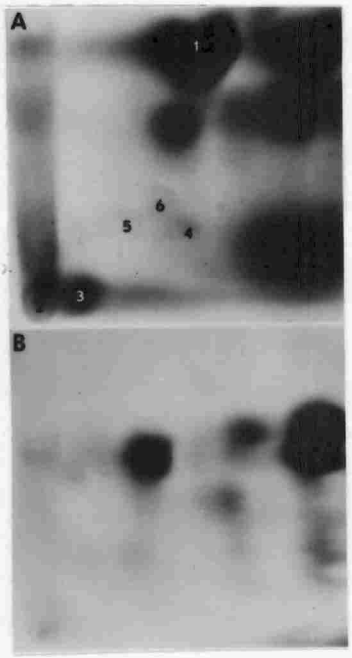


Figure 7. Autoradiogram of a thin-layer separation of 40 uL of a reaction which used pteridine-free extract, Mg^{2+}, and NADPH. 1, sepiapterin; 2, neodrosopterin; 3, fraction e; 4, aurodrosopterins; 5, drosopterin; 6, isodrosopterin.

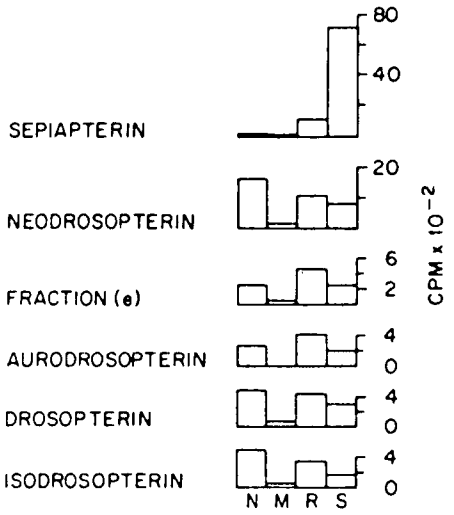


Figure 8. Ability of various subcellular fractions to support sepiapterin and "drosopterin" biosynthesis. Aliquots (15 uL) of each fraction were used in the standard assay. Mg^{2+}, NADPH, $NADP^{+}$, and NAD^{+} were the cofactors used. N, 600g pellet; M, 15,000g pellet; R, 100,000g pellet; S, 100,000g supernatant.

The 600g pellet contains a red layer that is enriched in the "drosopterins" and shows considerable "drosopterin" synthesizing activity yet essentially no sepiapterin synthesis. Since Yim et al. (1977) had shown that the pr mutant is low in sepiapterin synthase and the "drosopterins", it was assumed that sepiapterin is a precursor of the "drosopterins". To test if sepiapterin synthase was limiting in the 600g pellet, a mixture of the pellet and the 100,000g supernatant was assayed. The "drosopterin" synthesis was additive but, surprisingly, sepiapterin synthase activity was nearly twice the sum of the individual activities (Figure 9). Since the stimulatory effect could be eliminated by heating the 600g pellet in a boiling-water bath for 5 min, we believe the stimulatory effect is due to an enzyme. Krivi and Brown have fractionated sepiapterin synthase into two proteins, one that is Mg^{2+}-dependent and one that is NADPH-dependent (Brown, et al., 1979). If these two proteins represent two steps of sepiapterin synthesis, we propose that only the first is necessary for "drosopterin" synthesis. The 600g pellet would contain the enzyme that catalyzes the first step, but not that for the second, while the 100,000g supernatant would contain both. If the enzyme for the first step is limiting for sepiapterin synthesis in the 100,000g supernatant, the mixing the 600g pellet with the 100,000g supernatant would result in synergistic synthesis of sepiapterin. As the pr^{bw} mutant accumulates less "drosopterins" than the wild-type and shows lowered sepiapterin synthase levels, the above hypothesis would predict that pr^{bw} is lacking the enzyme for the first step of sepiapterin synthase. When the Oregon-R 600g pellet was mixed with 100,000g supernatant from a whole-fly pr^{bw} extract, a synergistic

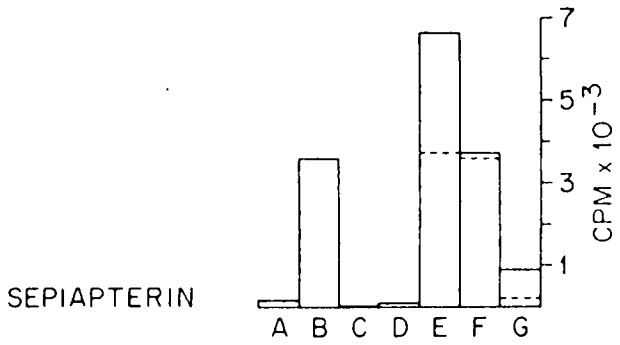


Figure 9. Stimulation of sepiapterin synthase in Oregon-R head and $\underline{pr^{bw}}$ whole-fly 100,000g supernatants by an Oregon-R head 600g pellet. The assay employed 15 uL of each extract; Mg^{2+}, NADPH, $NADP^{+}$, and NAD^{+} were the cofactors used. A, Oregon-R 600g pellet; B, Oregon-R head 100,000g supernatant; C, heat-inactivated A; D, $\underline{pr^{bw}}$ whole-fly 100,000g supernatant; E, A + B; F, C + B; G, A + D; (——), obtained; (---), additive.

incorporation into sepiapterin was seen, supporting our hypothesis. Furthur evidence for the common precursor hypothesis came from studies with the pteridine-free extract as a source of enzyme.

When these studies were initiated, the 600g pellet and 100,000g supernatant of crude head extracts were employed. Although "drosopterin" synthesis could be detected, the crude extracts presented several technical problems: storage at -20°C resulted in loss of activity, large amounts of pteridines present precluded the possibility of performing meaningful isotope dilution experiments, and the presence of various cofactors interfered with determination of cofactor requirements. These difficulties led us to develop the procedure for the preparation of the pteridine-free extract described in the Methods section. The pteridine-free extract was stored for at least three weeks at -20°C without significant loss of activity.

By use of this soluble system, the various cofactor requirements were determined (Figure 10). Sepiapterin synthesis requires both Mg^{2+} and NADPH. When Mg^{2+} and NADH are present in a reaction, only slight conversion of H_2-neopterin-$(P)_3$ to sepiapterin is seen, yet synthesis of the "drosopterins" occurs at the same level as if NADPH were present. This is evidence that sepiapterin is not a "drosopterin" precursor. When Mg^{2+} is left out of the reaction, however, both sepiapterin and "drosopterin" synthesis are decreased, suggesting that a common enzymatic step exists for the production of both classes of pteridines. When NADPH and NADH are both left out of a reaction, sepiapterin synthesis is negligible and "drosopterin" synthesis is reduced. Since neodrosopterin synthesis, in contrast to the other

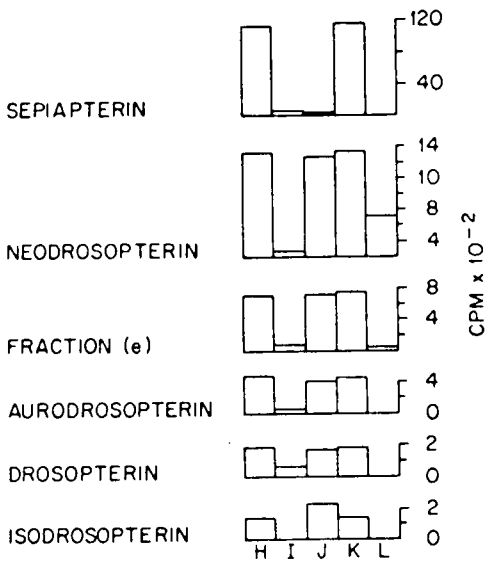


Figure 10. Effects of cofactors on sepiapterin and "drosopterin" biosynthesis. Aliquots (30 uL) of the pteridine-free extract were used in the standard assay. H, Mg^{2+} + NADPH + NADH; I, NADPH + NADH; J, Mg^{2+} + NADH; K, Mg^{2+} + NADPH; L, Mg^{2+}.

"drosopterins", is only reduced by little more than 50% in the absence of NADPH and NADH, it is possible that there is an enzyme-dependent background at the neodrosopterin position. A possible reaction scheme in agreement with the above results is shown in Figure 11.

Isotope dilution experiments provide additional support for the common precursor hypothesis. When unlabeled sepiapterin is present during "drosopterin" biosynthesis, no dilution of the isotope entering the "drosopterins" is seen. While it is possible that there is no equilibration between the unlabeled sepiapterin and enzymatically produced sepiapterin or that sepiapterin is converted by a first-order reaction under the conditions used, the most likely explanation is that sepiapterin is not a precursor of the "drosopterins".

Isotope dilution experiments were also done in an attempt to answer questions about the interconversion of the various "drosopterin" species. No decreases in the amount of radioisotope-labeled "drosopterins" synthesized were seen when either unlabeled neodrosopterin or unlabeled drosopterin and isodrosopterin were added to the reactions. As drosopterin and isodrosopterin can arise nonenzymatically from neodrosopterin in aqueous solutions (Rokos & Pfleiderer, 1975), kinetic considerations could explain the failure of neodrosopterin to dilute the isotope entering drosopterin and isodrosopterin. Because the structural relationship between the two forms of aurodrosopterins is analogous to that between drosopterin and isodrosopterin (Rokos & Pfleiderer, 1975), and because fraction e is similar to neodrosopterin in color and in chromatographic behavior, we are also considering the hypothesis that fraction e nonenzymatically rearranges to the

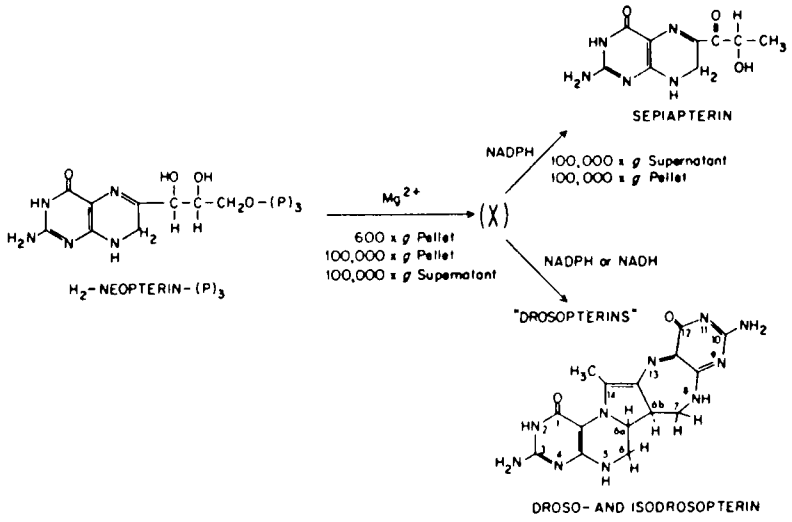


Figure 11. Postulated reaction sequence for sepiapterin and "drosopterin" biosynthesis and the differential centrifugation fractions in which the steps are recovered.

aurodrosopterins. Schwinck & Mancini (1973) have reported that fraction e and aurodrosopterins act genetically as a group. The pteridine-free extract synthesizes relatively more fraction e and aurodrosopterins than the crude extracts, with neodrosopterin and fraction e being the major products. While this does not reflect the relative amounts of "drosopterins" found *in vivo*, the possibilities may be raised for the nonenzymatic conversion of neodrosopterin and fraction e to other "drosopterins" in the fly. If such nonenzymatic conversion occurs during extraction of the "drosopterins", the distribution measured after chromatography may not represent the distribution *in vivo*.

4. DISCUSSION

In the past, hypotheses about "drosopterin" biosynthesis have relied upon genetic results and quantitation of the "drosopterins" accumulated in various mutants. Schwinck & Mancini (1973) divided several eye color mutants into three classes based upon the relative amounts of different "drosopterins" resolved by thin-layer chromatography on cellulose layers. They proposed that the biosynthesis and accumulation of different "drosopterins" are under genetic control.

The *in vitro* assay of "drosopterin" biosynthesis has become possible because of three major developments: (1) H_2-neopterin-$(P)_3$ is recognized as the precursor of pteridines and this precursor is readily produced by use of purified GTP cyclohydrolase from *E. coli* (Yim & Brown, 1976); (2) high resolution, fairly rapid, reproducible separations of pteridines is possible using cellulose thin-layer chromatography (Wilson & Jacobson, 1977a); (3) sepiapterin synthase was

discovered (Fan et al., 1975) and its role in "drosopterin" biosynthesis was recognized (Wilson & Jacobson, 1977b; Yim et al., 1977).

Several lines of evidence support the idea that "drosopterin" biosynthesis is enzyme mediated: (1) the substrate required is H_2-neopterin-$(P)_3$ and not H_2-neopterin; (2) Mg^{2+} is required; (3) NADPH or NADH greatly stimulates synthesis of "drosopterins"; (4) the activity is heat labile, precipitable in ammonium sulfate, nondialyzable, and elutes in the void volume of a Sephadex G25 column.

Both genetic and biochemical evidence support the idea that sepiapterin synthesis and "drosopterin" synthesis are related. In the pr mutant, the levels of sepiapterin and "drosopterins" are greatly reduced (Wilson & Jacobson, 1977b). This mutant also has low amounts of sepiapterin synthase (Yim et al., 1977). In this study when the 100,000g supernatant of the pr mutant was supplemented with the 600g pellet enzymes of wild-type, a superadditive rate of sepiapterin synthesis occured. Since the 600g pellet enzymes of wild type make little or no sepiapterin but do make "drosopterins", it seems likely that a common enzyme in the 600g pellet is involved in the synthesis of sepiapterin and the "drosopterins". This, along with the finding that adding nonradioactive sepiapterin to the reaction mixture caused no reduction in the rate or amount of "drosopterin" synthesis, indicates that sepiapterin is not a precursor to "drosopterins". Brown et al. (1979) reported that sepiapterin synthase can be separated into two protein fractions, both of which are required for sepiapterin production. It appears that their Mg^{2+}-requiring fraction is the same we found in the 600g pellet.

Since it had been previously proposed that $\underline{pr^+}$ is the structural locus for sepiapterin synthase (Yim et al., 1977), it is logical, in light of the evidence presented in this report, to now propose that $\underline{pr^+}$ is the structural locus for the enzyme that catalyzes the Mg^{2+}-requiring step. The effect of gene dosage for $\underline{pr^+}$ was examined by comparing sepiapterin synthase activities. A ratio of 2.0 was found when flies with two doses of $\underline{pr^+}$ were compared with flies with one dose, but the ratio was only 1.2, instead of 1.5, when three doses were compared with two doses. This might be expected if $\underline{pr^+}$ is the structural locus for the Mg^{2+}-requiring step that synthesizes the precursor, since the possibility exists that with three doses of this enzyme, the second step (NADPH-requiring) becomes limiting.

In a possible scheme for the biosynthesis of "drosopterins" it is necessary to recognize that (1) the 3'-hydrogens of H_2-neopterin-$(P)_3$ do not appear in the final products and (2) there are three carbons less in drosopterin than there are in two molecules of H_2-neopterin-$(P)_3$. The removal of the 3'-hydrogens could be a consequence of (1) the loss of the 3'-carbon or larger portions of the side chain of H_2-neopterin-$(P)_3$, or (2) the oxidation of the 3'-carbon. As the 3'-hydrogens do appear in sepiapterin, it is necessary to consider such alterations as occuring at some stage subsequent to the production of the common precursor. The mechanism by which the number of carbon atoms is reduced by three in the formation of "drosopterins" must also be sought. Identification and structure determination of the "drosopterin"-sepiapterin precursor should shed light on these questions.

CHAPTER III

BIOSYNTHESIS, NONENZYMATIC SYNTHESIS, AND PURIFICATION OF THE SEPIAPTERIN SYNTHASE INTERMEDIATE

1. INTRODUCTION

The enzymatic conversion of the 3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl)pterin (H_2-neopterin-$(P)_3$) to 7,8-dihydro-6-lactoylpterin (sepiapterin) occurs in the presence of Mg^{2+} and NADPH. Recently it has been proposed that sepiapterin synthase from Drosophila melanogaster (Dorsett et al., 1979; Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1979) consists of two enzymes, the first requiring Mg^{2+} and the second requiring NADPH. In the case of Drosophila (Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1979), the separation of these two activities has been reported. The product of the Mg^{2+}-dependent enzyme appears to be a precursor of the Drosophila red eye pigments, the "drosopterins", as well as sepiapterin (Dorsett et al., 1979).

In this report we describe an assay for the sepiapterin synthase intermediate (X) (product of the Mg^{2+}-dependent enzyme) using HPLC. A nonenzymatic method for the conversion of H_2-neopterin-$(P)_3$ to the intermediate (X) and related compounds [(X1), (X2)] is described as well as the purification and partial characterization of these compounds. The implications of these studies with regard to the structure of the intermediate and mechanism of sepiapterin synthase is discussed.

2. MATERIALS AND METHODS

Drosophila melanogaster. Oregon-R and $pr^{bw}cn$ flies were reared at 25°C on medium prepared as described by Lewis (1960). 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) and stored in vials under liquid nitrogen for later use.

Crude Enzyme Extract (CE). Heads (1 g) were homogenized in 5 mL of 50 mM Pipes (pH 7.0) (Calbiochem) with a ground glass homogenizer (Kontes). The resulting homogenate was centrifuged at 15,000*g* for 10 min, and the supernatant was centrifuged at 100,000*g* for 60 min. The protein precipitating between 40 and 60% saturated $(NH_4)_2SO_4$ (4°C) was collected at 15,000*g* for 30 min, redissolved in 0.2 mL of 50 mM Pipes (pH 7.0), and filtered through 20 mL of Sephadex G25 (medium) (Pharmacia) by use of the centrifuge procedure of Neal & Florini (1973). The preparation was stored at -20°C.

Pteridine-free Extract (PFE). PFE was prepared as previously described (Dorsett *et al.*, 1979) except the pellet resulting from 60% saturated $(NH_4)_2SO_4$ was redissoved in 1 mL of 50 mM Pipes (pH 7.0) containing 10% glycerol and desalted by filtering through 20 mL of Sephadex G25 (medium) by the centrifuge procedure of Neal & Florini (1973). The extract was stored at -20°C.

H_2-neopterin-$(P)_3$. H_2-neopterin-$(P)_3$ was prepared using purified *E. coli* GTP cyclohydrolase as described by Yim & Brown (1976). The reaction contained 1.67 mM GTP, 0.11 M TrisCl (pH 8.5), 0.11 M NaCl,

0.011 M EDTA (pH 7.5), and enough enzyme to achieve at least a 90% conversion in 90 min at 42°C. The completed GTP cyclohydrolase reaction was used directly as the source of H_2-neopterin-$(P)_3$. All H_2-neopterin-$(P)_3$ concentrations were calculated assuming 100% conversion. [U-^{14}C]GTP was obtained from Amersham and [8,5'-^{3}H]GTP from New England Nuclear. H_2-neopterin-$(P)_3$ was treated with bacterial alkaline phosphatase as previously described (Dorsett et al., 1979).

High Performance Liquid Chromatography (HPLC). A Waters Liquid Chromatograph with a 3.6 mm x 30 cm uBondapak C18 reverse phase column was used to separate sepiapterin and (X) from the reactions. The column was eluted at 2 mL/min with 10% methanol (Burdick and Jackson, Inc.) at a temperature of 35°C. The pterins were quantitated using a Schoeffel SF 770 UV monitor at 260 nm set to 0.1 aufs with a 1 mV recorder and a Schoeffel FS 970 fluorescence monitor (excitation at 360 nm, Corning 7-54 excitation filter, 418 nm cutoff emission filter) set to 0.1 uA (4 sec time-constant) with a 1 mV recorder. Calibration of the peaks was accomplished using [U-^{14}C]H_2-neopterin-$(P)_3$ as a substrate (specific activity, 20.3 uCi/umol) and comparing the amount of radioactivity in the peak to the area of the peak as determined by absorption or fluorescence.

Preparation of Purified (X), (X1), and (X2). Aliquots (500-1000 uL) of GTP cyclohydrolase reaction that had gone to completion were heated at 100°C for 40 min and then centrifuged. The (X), (X1), and (X2) in the supernatant were separated by HPLC (as above except the column was eluted with 5% methanol) and fractions containing the individual species were lyophilized to dryness. The pterins were

redissolved in approximately 100 uL H_2O and the purity checked by HPLC. Occasionally the (X1) fraction had to be rechromatographed due to significant impurities. While this resulted in some loss of (X1), the second purification was always sufficient to obtain a homogeneous compound. All compounds were stored at -20°C.

Enzymatic Synthesis of (X) and Sepiapterin. The exact conditions (enzyme, substrate, cofactors present, and reaction time) are shown for the individual experiments in Table I. All reactions were carried out in a total volume of 70 uL with 50 mM Pipes (pH 7.5) at 42°C. The reactions were stopped by heating at 100°C for 5 min, the resultant precipitate was removed by centrifugation (Brinkmann Eppendorf Centrifuge 3200) and aliquots were analyzed for (X) and sepiapterin by HPLC. When radioactive H_2-neopterin-$(P)_3$ was used as a substrate, fractions were collected in scintillation vials and the radioactivity was determined in a scintillation counter after adding 0.28% 2,5-bis-2-[5-tert-butyl-benzoxalyl]thiophene (Packard) and 33% Triton X-100 in toluene. When present, Mg^{2+} was used at a concentration of 14.3 mM and NADPH at a concentration of 2.5 mM.

Nonenzymatic Synthesis of (X), (X1), and (X2). The exact conditions for the experiments are given in the Figure legends. Aliquots of the reactions were analyzed by HPLC after centrifugation.

Phosphate Analysis. Assays were carried out in a total volume of 100 uL containing 1 mM H_2-neopterin-$(P)_3$, 0.067 M TrisCl (pH 8.5), 0.067 M NaCl, 0.0067 M EDTA (pH 7.5), and up to 20 uL of either 0.25 M NaOH or 0.25 M HCl. The reactions were run at 100°C for 10 min, cooled in ice and analysed for phosphate by the method of Sumner (1944).

Aliquots of H_2O (700 uL), 100 uL of 6.6% ammonium molybdate in 7.5 N H_2SO_4, and 100 uL of $FeSO_4$ solution (2.5 g of $FeSO_4 \cdot 7\ H_2O$ dissolved in 25 mL H_2O plus 0.5 mL of 7.5 N H_2SO_4) were added to each reaction. The solutions were mixed and incubated at room temperature for 15 min; the absorbance at 660 nm was determined. Phosphate concentrations were calculated from a standard curve.

Side Chain Loss Analysis. Reactions were run with a total volume of 50 uL containing the following components: 1 mM [3'-^{3}H]H_2-neopterin-$(P)_3$ (prepared from [8,5'-^{3}H]GTP, specific activity, 2 mCi/mmol); 0.067 M TrisCl (pH 8.5), 0.067 M NaCl, 0.0067 M EDTA (pH 7.5), and up to 9 uL of 0.25 M NaOH. The reactions were run at 100°C for 10 min, cooled in ice, and centrifuged. H_2O (0.5 mL) was added to each reaction and the amount of side chain released was determined by filtering through Pasteur pipette columns containing 0.5 x 1 cm acid-washed charcoal at the bottom and 0.5 x 1 cm DEAE-Sephadex A120 (equilibrated with 50 mM Pipes, pH 7.0) following the procedure of Yim et al. (1980). The columns were washed two times with 0.5 mL H_2O, and the radioactivity present in the effluent was determined by scintillation counting.

Degradation of (X) at Various pH Values. The intermediate (X) was generated by incubating 1.67 mM H_2-neopterin-$(P)_3$ [0.11 M TrisCl (pH 8.5), 0.11 M NaCl, 0.011 M EDTA (pH 7.5)] at 100°C for 10 min before cooling in ice. Aliquots (15 uL) of the heated H_2-neopterin-$(P)_3$ were diluted to 25 uL with up to 5.5 uL of 0.25 M NaOH or 0.25 M HCl and H_2O. The reactions were incubated at 60°C for 120 min and analyzed by HPLC.

Treatment of (X), (X1), and (X2) with I_2, $NaBH_4$, and $NaIO_4$.

Oxidations of (X), (X1), or (X2) in a solution containing 0.1% I_2 and 0.2% KI were carried out in the dark at room temperature for up to 30 min; the excess I_2 was titrated out with a 1% ascorbic acid solution before analysis of the reaction by HPLC. To test the sensitivity of the iodine oxidation products to $NaBH_4$, aliquots of the iodine reactions were made 0.05 M $NaBH_4$ with a 0.5 M $NaBH_4$ in 0.1 NaOH solution, and incubated in the dark at rooom temperature for various time up to 30 min before HPLC analysis. The unoxidized forms of (X), (X1), or (X2) were treated with the same concentrations of $NaBH_4$ and NaOH. Periodate oxidations of (X), (X1), or (X2) were carried out in 0.02 M $NaIO_4$ (pH 5). The reactions were incubated for various times up to 60 min at room temperature in the dark before analyzing aliquots of the incubation mixtures by HPLC.

3. RESULTS

The discovery of the nonenzymatic conversion of H_2-neopterin-$(P)_3$ to the sepiapterin synthase intermediate (X) occured when the GTP cyclohydrolase reaction products were heated at 100°C. The detection of (X) by HPLC was based on its chromatographic identity to the intermediate produced by the partially purified enzyme. Indeed on furthur study two other chromatographic peaks were found to be associated with (X) when it was produced nonenzymatically; these forms, (X1) and (X2), are not present in significant amounts when (X) is produced enzymatically. An HPLC chromatogram of nonenzymatically synthesized (X), (X1), and (X2) along with added sepiapterin is shown in Figure 12. Enzymatically synthesized (X) eluted in the same position as the nonenzymatically

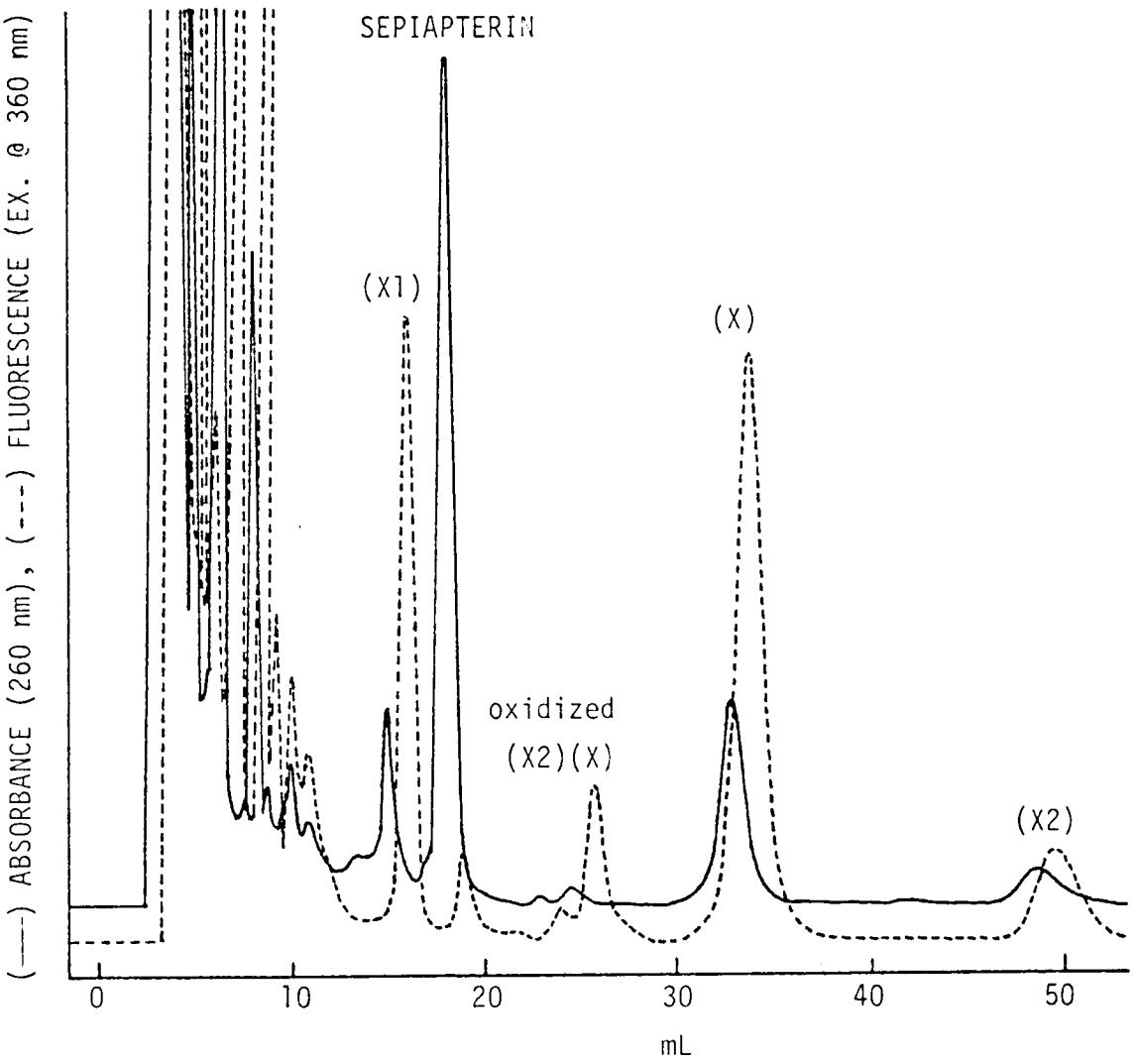


Figure 12. Separation of (X), (X1), (X2), and sepiapterin by HPLC. The sample contained 1 nmol of sepiapterin plus 10 uL of a GTP cyclohydrolase reaction that had been allowed to go to completion before heating at 100°C for 40 min. The HPLC was performed as described in the text except the fluorometer was set to the 1.0 uA range instead of 0.1 uA.

produced compound and demonstrated the same absorbance to fluorescence ratio.

Biosynthesis of (X) and Sepiapterin. Several lines of evidence support the hypothesis that the enzymatically synthesized (X) is the sepiapterin synthase intermediate. Treatment of H_2-neopterin-$(P)_3$ with bacterial alkaline phosphatase or heating of the enzyme extract at 100^oC for 5 min prior to use prevents the synthesis of (X) (Table I). Synthesis of (X) requires Mg^{2+} whereas the recovery of (X) decreases when NADPH is added; this decrease is concomitant with an increase in the synthesis of sepiapterin (Table I). The pr^{bw}cn mutant contains only 20% of the sepiapterin synthase present in Oregon-R (wild-type) and is thought to be a lesion in the structural locus for the Mg^{2+}-dependent first enzyme of sepiapterin synthase (Yim et al., 1977; Dorsett et al., 1979; Krivi & Brown, 1979). With respect to (X) biosynthesis the pr^{bw}cn mutant exhibited only 20% of the activity found in Oregon-R (Table I). Enzymatically produced (X) incorporates ^{14}C from [U-^{14}C]H_2-neopterin-$(P)_3$ and ^3H from [3'-^3H]H_2-neopterin-$(P)_3$ in the same ratio as sepiapterin (Table I).

More direct evidence that (X) is the intermediate, and additional evidence that (X) produced nonenzymatically is the same compound as (X) produced enzymatically, was obtained using nonenzymatically synthesized (X) as a substrate for the enzymatic synthesis of sepiapterin. The conditions for the synthesis of (X) from H_2-neopterin-$(P)_3$ nonenzymatically is examined in detail below. As mentioned above, when a GTP cyclohydrolase reaction that has gone to completion is heated at 100^oC, three related compounds [(X), (X1), (X2)] are formed. It was found

TABLE I: Biosynthesis of (X) and Sepiapterin[a].

Enzyme (uL)		Substrate (mM)		Mg^{2+}	NADPH	RT[b] (min)	Amount of Product (X)	Amount of Product SEP
Substrate, Enzyme, and Cofactor Requirements							nanomoles / 30 uL	
Oregon-R PFE	10	Pase-H_2-neopterin-$(P)_3$	0.143	+	0	15	0	0
Oregon-R PFE	10	Pase-H_2-neopterin-$(P)_3$	0.143	+	+	15	0	0
ΔOregon-R PFE	10	H_2-neopterin-$(P)_3$	0.143	+	0	15	0	0
Oregon-R PFE	10	H_2-neopterin-$(P)_3$	0.143	0	0	15	0.011	0
Oregon-R PFE	10	H_2-neopterin-$(P)_3$	0.143	+	0	15	0.069	0
Oregon-R PFE	10	H_2-neopterin-$(P)_3$	0.143	+	+	15	0.039	0.163
Comparison of Oregon-R and $pr^{bw}cn$ Activities							nanomoles / 30 uL	% Oregon-R activity (X) / SEP
Oregon-R PFE	5	H_2-neopterin-$(P)_3$	0.286	+	0	10	0.033	0	100.0	--
$pr^{bw}cn$ PFE	5	H_2-neopterin-$(P)_3$	0.286	+	0	10	0.007	0	21.2	--
Oregon-R PFE	5	H_2-neopterin-$(P)_3$	0.286	+	+	10	0.021	0.066	--	100.0
$pr^{bw}cn$ PFE	5	H_2-neopterin-$(P)_3$	0.286	+	+	10	0.004	0.013	--	19.7

TABLE I (continued)

Comparison of Radioactivity incorporated from [U-^{14}C]H_2-neopterin-(P)$_3$[b] into (X) and Sepiapterin							dpm / 30 uL				^{3}H dpm / ^{14}C dpm	
							^{3}H	^{14}C	^{3}H	^{14}C	(X)	SEP
Oregon-R PFE	5	[U-^{14}C][3'-^{3}H][c] H_2-neopterin-(P)$_3$	1.56	+	0	30	2783	2194	--	--	1.27	--
Oregon-R PFE	15	[U-^{14}C][3'-^{3}H] H_2-neopterin-(P)$_3$	0.78	+	+	30	--	--	12890	9539	--	1.35

Synthesis of Sepiapterin from ΔH_2-neopterin-(P)$_3$							nanomoles / 30 uL	
Oregon-R CE	15	H_2-neopterin-(P)$_3$	0.954	0	+	60	--	0.059
Oregon-R CE	15	ΔH_2-neopterin-(P)$_3$	0.954	0	+	60	--	0.252
Oregon-R CE	15	ΔH_2-neopterin-(P)$_3$	0.954	0	0	60	--	0.039
ΔOregon-R CE	15	ΔH_2-neopterin-(P)$_3$	0.954	0	+	60	--	0.030

TABLE I (continued)

Synthesis of Sepiapterin from Purified (X) and (X1)					nanomoles / 30 uL	
Oregon-R PFE 30 (X)	0.013	0	+	90	--	0.298
Oregon-R PFE 30 (X)	0.013	0	0	90	--	0
ΔOregon-R PFE 30 (X)	0.013	0	+	90	--	0
Oregon-R PFE 30 (X1)	0.004	0	+	90	--	0.081
Oregon-R PFE 30 (X1)	0.004	0	0	90	--	0
ΔOregon-R PFE 30 (X1)	0.004	0	+	90	--	0

[a]Reaction conditions are described in the text.

[b]Abbreviations: RT, reaction time; SEP, sepiapterin; Pase-H_2-neopterin-$(P)_3$, phosphatase-treated H_2-neopterin-$(P)_3$; ΔOregon-R PFE, Oregon-R PFE heated at 100°C for 5 min; ΔH_2-neopterin-$(P)_3$, GTP cyclohydrolase reaction heated at 100°C for 40 min; ΔOregon-R CE, Oregon-R CE heated at 100°C for 5 min, +, present in the reaction; 0, not present in the reaction; --, not measured.

[c]Specific activity, 2.03 uCi/umol ^{14}C and 37.69 uCi/umol ^{3}H.

that such heated H_2-neopterin-$(P)_3$ solutions could support enzyme-dependent synthesis of sepiapterin in the presence of NADPH and absence of Mg^{2+} (Table I). The lack of a Mg^{2+} requirement indicated that heated H_2-neopterin-$(P)_3$ solutions could substitute for the Mg^{2+}-dependent enzyme of sepiapterin synthase. Furthurmore, it was found that either (X) or (X1) purified from the heated H_2-neopterin-$(P)_3$ solutions could serve as substrates for the NADPH-dependent enzymatic synthesis of sepiapterin (Table I). As the nonenzymatically produced (X) appears to be identical to the enzymatically produced compound by HPLC elution and by the relationship between absorbance and fluorescence, we propose that (X) is the actual sepiapterin synthase intermediate and that (X1) is probably an isomer produced under the nonenzymatic reaction conditions. The third substance, (X2), was produced in smaller amounts than (X) or (X1) and upon storage at $-20^{o}C$ it became converted to (X1); (X2) was not tested as a substrate for sepiapterin synthesis.

Nonenzymatic Synthesis of (X), (X1), and (X2). The time course for the nonenzymatic synthesis of (X), (X1), and (X2) from H_2-neopterin-$(P)_3$ is shown in Figure 13. The concentration of (X) reached a maximum by 40 min and then fell off; (X2) reached a maximum at 60 min and then declined; (X1) reached a maximum at 100 min and then declined as the amount of oxidized (X1) increased. Small amounts of oxidized (X) and oxidized (X2) were also produced during the reaction (identity of the oxidized forms was confirmed by iodine oxidations of purified (X), (X1), and (X2) as described below). These results suggest the following reaction sequence: H_2-neopterin-$(P)_3$ is converted to (X) which gives rise to (X2) which is converted to (X1) which in turn is

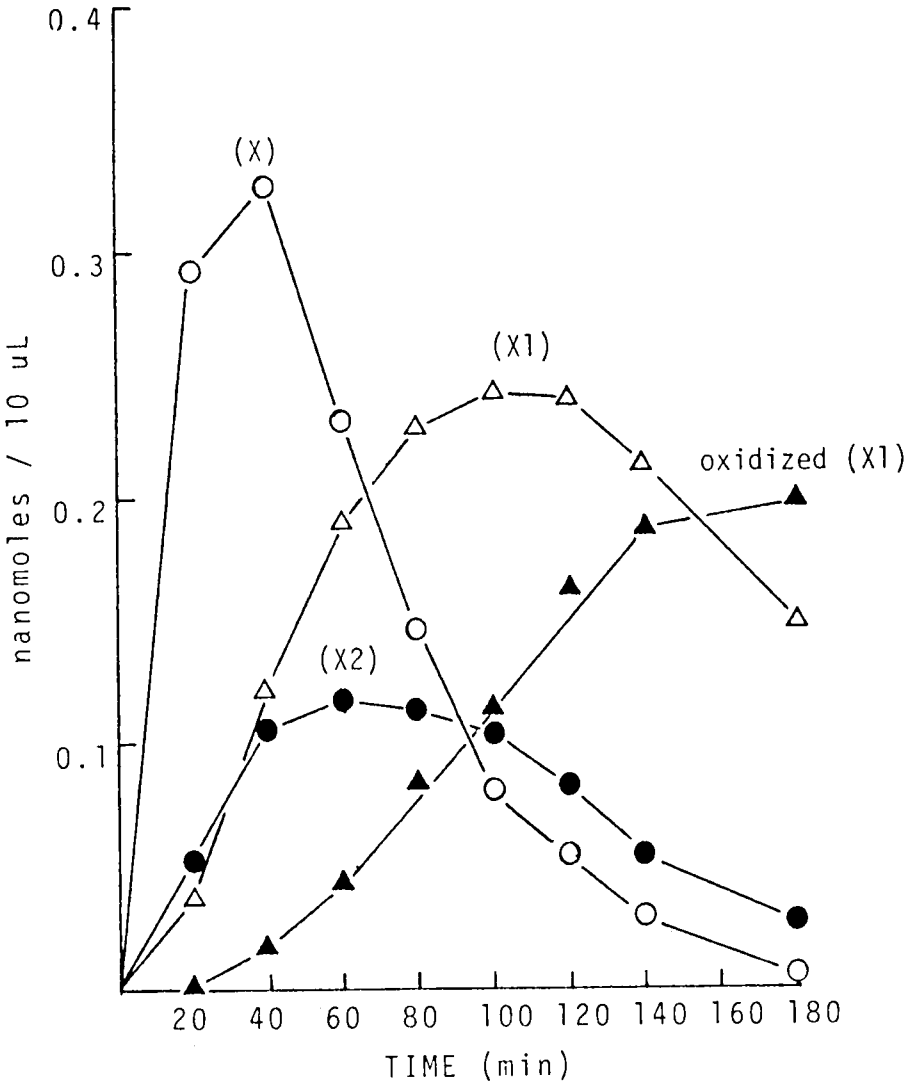


Figure 13. Time course of nonenzymatic synthesis of (X), (X1), (X2), and oxidized (X1). O, (X); ●, (X2); △, (X1); ▲, oxidized (X1). Reactions contained 1.67 mM H_2-neopterin-$(P)_3$, 0.111 M TrisCl (pH 8.5), 0.011 M EDTA (pH 7.5), and 0.111 M NaCl. The reactions were carried out at 100°C in the dark and aliquots were analyzed by HPLC at the times indicated. Amounts of oxidized (X1) were calculated assuming its absorption coefficient at 260 nm is the same as for (X1).

oxidized. Support for this hypothesis comes from the observations that: (1) purified (X) gives rise to small amounts of (X2) and (X1) even during storage at -20°C; and (2) as much as 50% of purified (X2) can be converted to (X1) by storage at -20°C for a week. Because of these interrelationships, and the observation that (X1) can serve as a substrate for the enzymatic synthesis of sepiapterin, we believe that (X), (X1), and (X2) are isomers and (X1) is apparently the most stable form.

The requirements for the nonenzymatic synthesis of (X) were studied for two main reasons: (1) to optimize the conditions so enough purified (X), (X1), and (X2) could be produced for characterization; and (2) to determine the mechanism of the nonenzymatic synthesis as an aid in understanding the enzymatic mechanism for the synthesis of (X).

The rate of nonenzymatic synthesis increased rapidly with temperature above 80°C (Figure 14). The reaction was first order with respect to H_2-neopterin-$(P)_3$; ionic strength has no effect on the reaction rate in the range tested (0.067 to 0.167 M NaCl).

The nonenzymatic synthesis of (X) demonstrates a remarkable dependence upon pH (Figure 15). At pH values below the maximum (pH 8.5 to 9.0), the pH dependence can be explained by comparison with the effect of TrisCl (pH 8.5) at various concentrations (Figure 16). At higher concentrations of TrisCl (pH 8.5) the reaction rate levels off. By extrapolation (lower concentrations of TrisCl (pH 8.5) are not possible without either modifying the GTP cyclohydrolase reaction or diluting the H_2-neopterin-$(P)_3$ to such an extent that the nonenzymatic synthesis of (X) is very slow), the concentration of Tris base to produce half

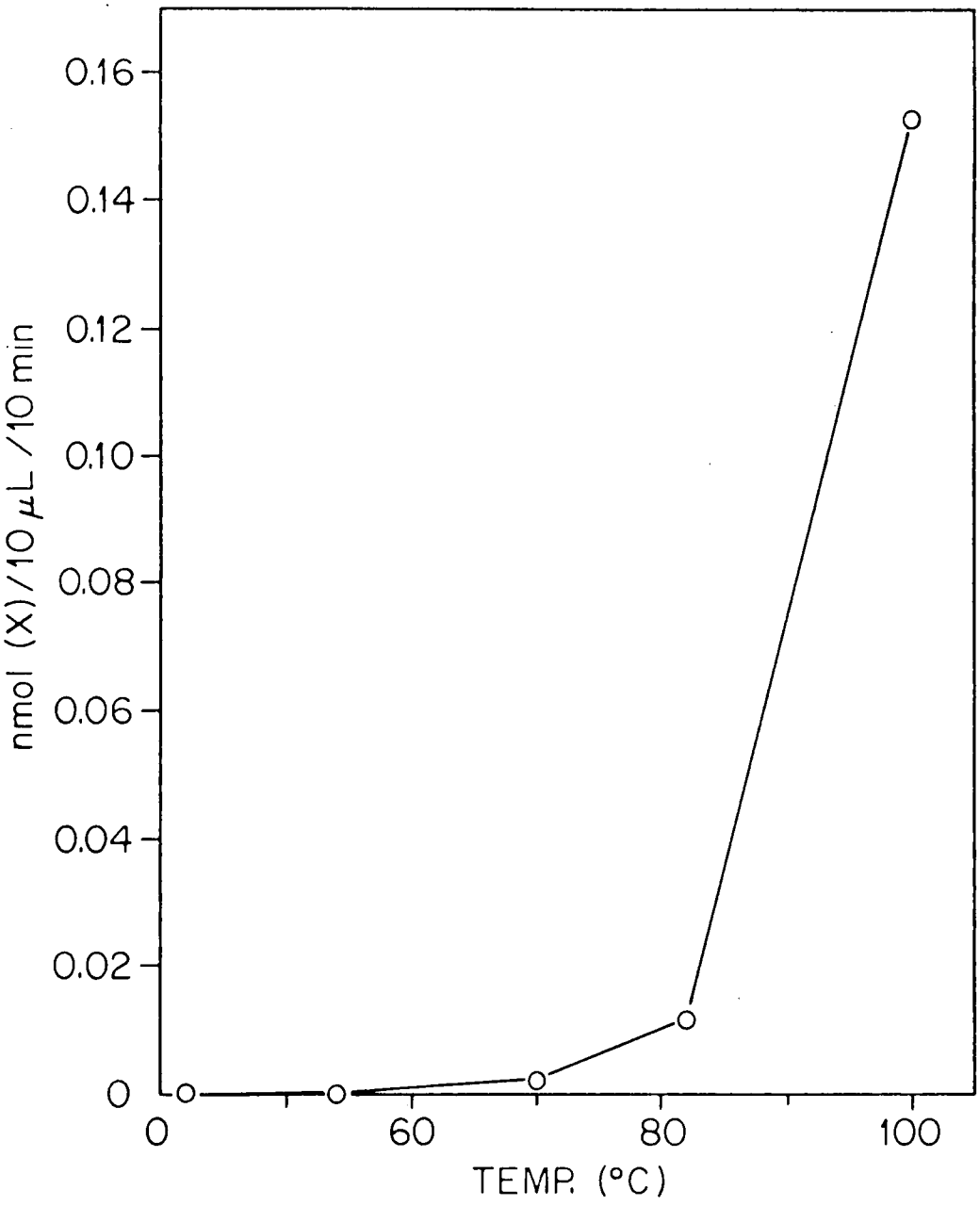


Figure 14. Rate of nonenzymatic synthesis of (X) as a function of temperature. Reactions contained 1.67 mM H_2-neopterin-$(P)_3$, 0.111 M TrisCl (pH 8.5), 0.011 M EDTA (pH 7.5), and 0.111 M NaCl. The reactions were incubated for 10 min at the temperatures indicated.

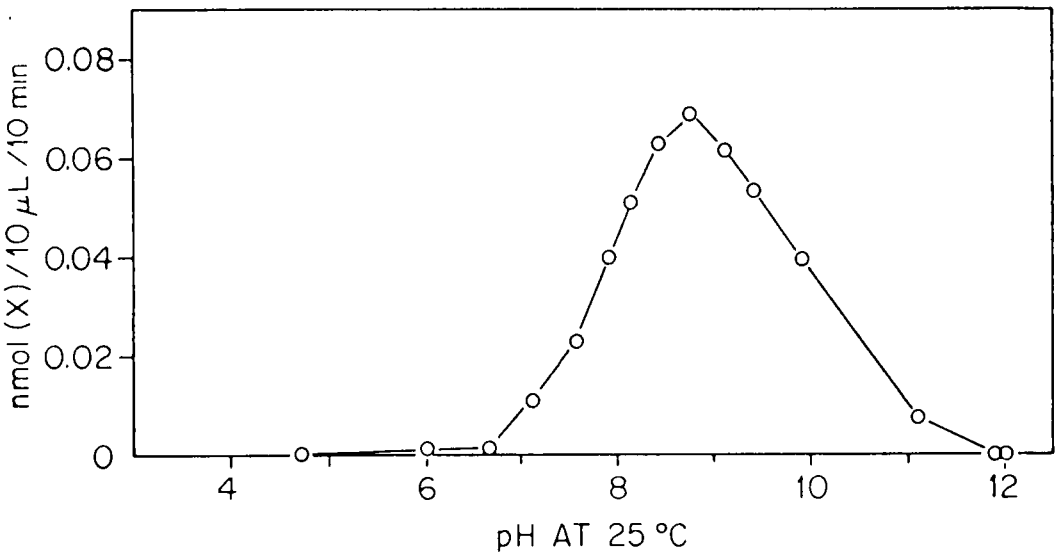


Figure 15. Rate of nonenzymatic synthesis of (X) as a function of pH. Reactions were run with a total volume of 50 uL containing 1 mM H_2-neopterin-$(P)_3$, 0.067 M TrisCl (pH 8.5), 0.067 M NaCl, 0.0067 M EDTA (pH 7.5), and varied amounts (up to 20 uL) of either 0.25 M NaOH or 0.25 M HCl. The pH was measured at 25°C with a glass electrode before the reaction which occured at 100°C for 10 min.

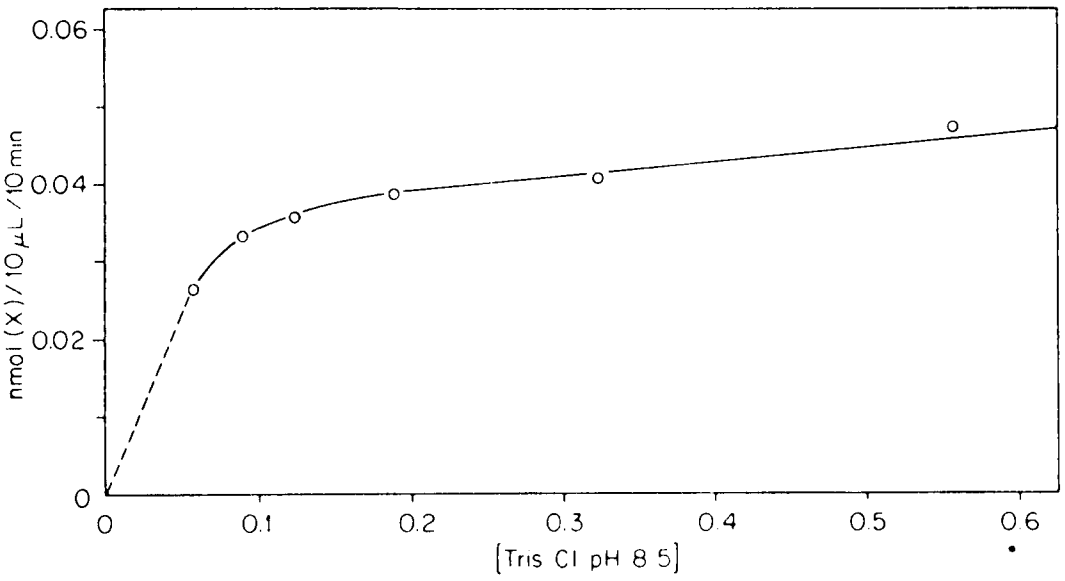


Figure 16. Rate of nonenzymatic synthesis of (X) as a function of TrisCl (pH 8.5) concentration. Reactions were run at 100°C for 10 min in a total volume of 60 uL containing 0.835 mM H_2-neopterin-$(P)_3$, 0.056 M NaCl, 0.0056 M EDTA (pH 7.5), and TrisCl (pH 8.5) at various concentrations.

the maximal velocity is estimated to be 0.02 M. This corresponds very well to the pH curve where the calculated concentration of Tris base to produce one-half the maximal rate is 0.016 M. This leads us to hypothesize that Tris base is a catalyst for the nonenzymatic synthesis of (X). It should be noted that while the pH values are reported at 25°C and the reactions are run at 100°C, the pK of Tris will shift the same extent in both the pH-dependence and Tris-concentration experiments. Therefore, while the actual concentration of Tris base at 100°C necessary to give half the maximal velocity is not determined, the experiments can be compared on the basis of the Tris base concentrations at 25°C.

To understand why the reaction rate falls off at higher pH values, we considered five distinct possibilities. (1) The phosphate groups of H_2-neopterin-$(P)_3$ may be hydrolyzed at high pH. (2) The side chain of H_2-neopterin-$(P)_3$ may be labile at high pH. (3) Degradation of (X) could increase with pH. (4) Tris acid may play a role in the reaction. (5) The ionization of the pterin moiety of H_2-neopterin-$(P)_3$ could decrease the reaction rate.

The first four possibilities were examined as follows. (1) Phosphate analysis revealed no significant loss of phosphate above pH 9.4 and below pH 7; a small amount of phosphate is released between pH 7 and 9.4. While some of may be accounted for by the synthesis of (X), the amount is stoichiometrically ten-fold greater than could be accounted for by the synthesis of (X) alone. The amount still represents no more than 5% of the H_2-neopterin-$(P)_3$ present; since phosphate is not detected in reactions carried out at pH values greater than 9.4, it

is concluded that phosphate loss does not account for the decrease in the rate of (X) synthesis at higher pH. (2) Analysis of side-chain release using [3'-^{3}H]H_2-neopterin-$(P)_3$ revealed no significant loss of side chain at any pH tested. (3) Significant degradation of (X) (60^oC for 120 min) did not accur between pH 4 and 11. (4) The addition of ethanolamine (pKa, 9.5) to the reactions (which would contribute more protonated amine than Tris at higher pH) did shift the pH for half maximal velocity somewhat [Figure 17(A)], but one would predict the pH value for half maximal velocity would be shifted about 1.2 pH units if the protonated amine participated in the reaction. The maximum of the pH curve was also not broadened as would be expected if this were the case. Replotting the pH curves as rate of (X) synthesis vs. total unprotonated amine ([Tris base] or [Tris base] + [ethanolamine base]) reveals a correlation between the two curves at unprotonated amine concentrations below the maximum velocity and no correlation at unprotonated amine concentrations above the maximum velocity [Figure 17(B)]. When the data is replotted as reaction velocity vs. total protonated amine ([Tris acid] or [Tris acid] + [ethanolamine acid]), there is no correlation between the two curves [Figure 17(C)]. These observations reinforce the hypothesis that the reaction is catalyzed by unprotonated amine and the protonated amine is not involved.

When one considers the pKa values of various 7,8-dihydropterins, it seems likely that H_2-neopterin-$(P)_3$ would have a pKa of approximately 10. Since the inflection point in the pH curve occurs around pH 10, we hypothesize that when the pterin moiety of H_2-neopterin-$(P)_3$ is ionized, the reaction to form (X) does not occur. Since the pKa in

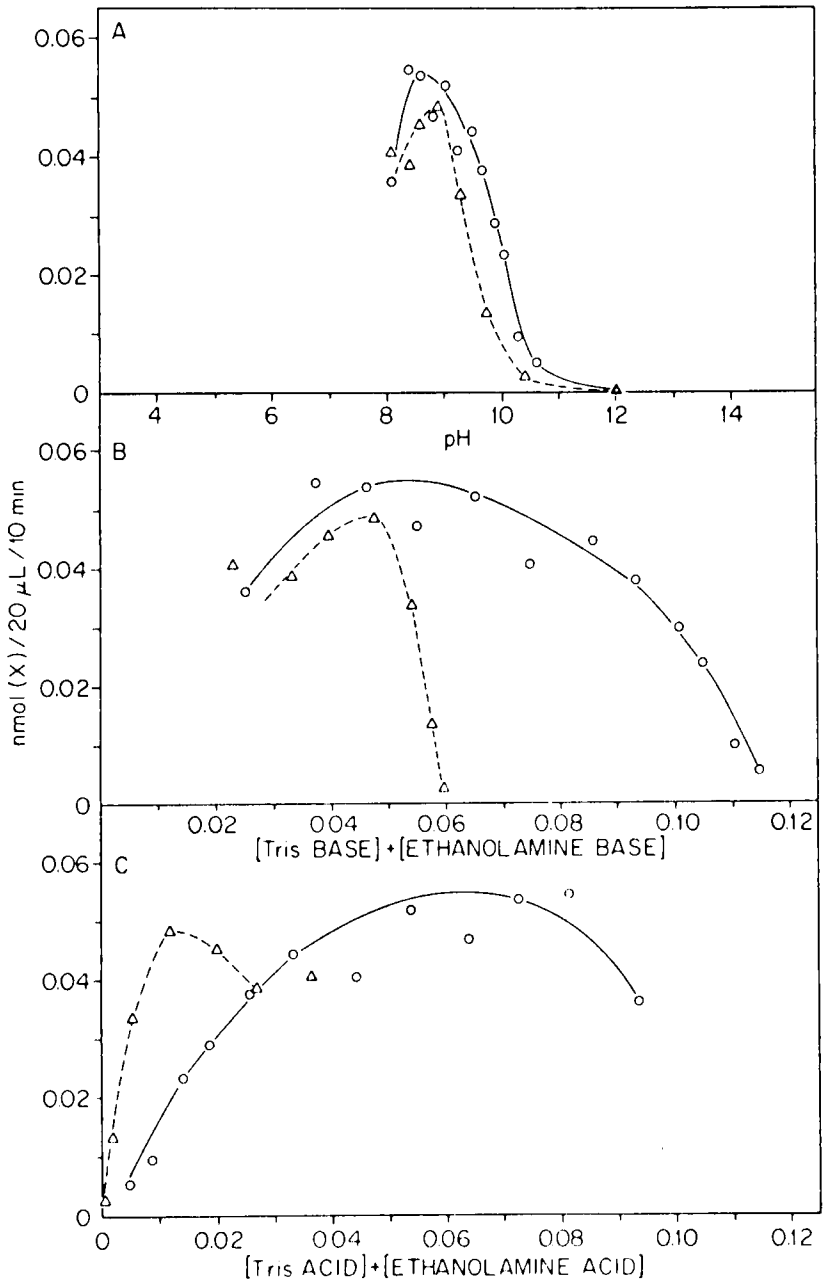


Figure 17. Comparison of rate of nonenzymatic synthesis of (X) as a function of pH with either TrisCl or TrisCl and ethanolamine buffers. O, TrisCl + ethanolamine; Δ, TrisCl; A, rate plotted as a function of pH; B, rate plotted as a function of total nonprotonated amine concentration; C, rate plotted as function of total protonated amine concentration. Reactions occured in a total volume of 56 uL containing the following components: 0.89 mM H_2-neopterin-$(P)_3$, 0.06 M NaCl, 0.006 M EDTA (pH 7.5), 0.06 M TrisCl (pH 8.5) or 0.06 M TrisCl (pH 8.5) + 0.06 M ethanolamine. The pH was varied by the addition of up to 16 uL of either 0.25 M HCl or 0.25 M NaOH. The reactions were run at 100°C for 10 min.

question is probably due to the 3-amido (4-hydroxy) moiety of the pterin (Blakeley, 1969, pp. 61-65), one predict that if the 3-methyl derivative of H_2-neopterin-$(P)_3$ was used as the substrate for the reaction, an inflection point at pH 10 would not be seen.

Properties of (X), (X1), and (X2). The ultraviolet absorption spectra of (X), (X1), and (X2) at pH 7.0 and 11.7 are shown in Figure 18. The long wavelength absorption maxima of all three compounds undergo a bathochromic shift with increasing pH, indicating that each of the three has a pKa value between 7.9 and 11.7 as do most 6-alkylpterins. The spectra are unusual for two reasons. First, the long wavelength absorption maxima of most 6-alkyl-7,8-dihydropterins (e.g., 6-methyl-7,8-dihydropterin and 7,8-dihydrobiopterin) usually do not show a bathochromic shift with increasing pH (Blakeley, 1969, pp. 65-72). Secondly, few 6-alkyl-7,8-dihydropterins exhibit an absorption maxima at wavelengths greater than 365 nm while (X), (X1), and (X2) all show maxima at 380 nm or greater. Sepiapterin is also an exception to these general rules (it demonstrates a bathochromic shift with increasing pH, and an absorption maximum at 420 nm at pH 6.8). These observations clearly indicate that the three unknowns are closely related to each other structurally, and also that they are similar in their properties to sepiapterin.

As enzymatically synthesized (X) incorporates label from [3'-^{3}H]-H_2-neopterin-$(P)_3$ and as (X) and (X1) both can serve as precursors for the synthesis of sepiapterin, we assume that they retain the three carbon side chain from H_2-neopterin-$(P)_3$. We also assumed that at least two of the oxygen substituents of the side chain are retained and we

Figure 18. Ultraviolet absorption spectra of (X), (X1), and (X2) at pH 7.0 and 11.7. (——), pH 7.0; (---), pH 11.7. Molar absorption coefficients were obtained by comparing $\underline{A}_{260}$ peaks of the compounds generated by heating a solution of [U-^{14}C]H_2-neopterin-$(P)_3$ at 100°C for 40 min with the $\underline{A}_{260}$ peaks obtained with aliquots of the purified compounds. The nanomoles per unit peak area were calculated assuming (X), (X1), and (X2) were the same specific activity as the [U-^{14}C]H_2-neopterin-$(P)_3$ they were generated from. The errors in this method may be significant but it provides a method of comparison.

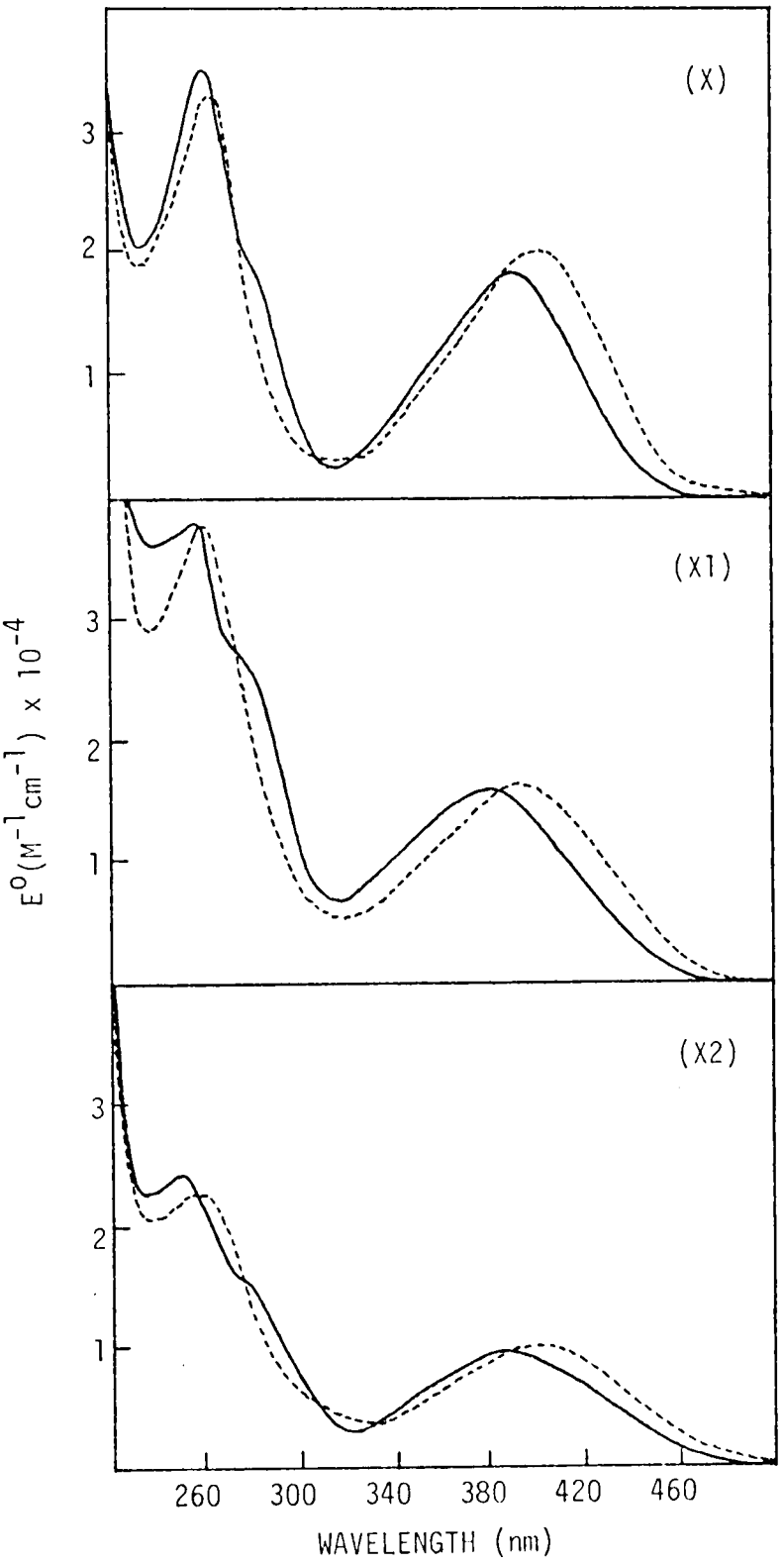

(X)
(X1)
(X2)
3
2
1
$E^{o}(M^{-1}cm^{-1}) \times 10^{-4}$
260
300
340
380
420
460
WAVELENGTH (nm)

Figure 18.

expected that (X), (X1), and (X2) should be sensitive to oxidation with $NaIO_4$. Only (X1), however, is sensitive to periodate oxidation. As (X) and (X2) are apparently precursors of (X1), we believe that all three contain potentially periodate-sensitve groups but perhaps structural constraints prevent the oxidation of (X) and (X2).

None of the compounds were reduced by treatment with $NaBH_4$ indicating that no carbonyls are present in the side chain. This also indicates that the periodate-sensitive group in (X1) is probably due to vicinal hydroxyl groups.

Recently, iodine oxidations of pterins has been developed as means of oxidizing the pyrazine ring (Fukushima & Nixon, 1979). All three compounds were oxidized by iodine to give rise to compounds that are also found in the nonenzymatic reactions during the synthesis of (X), (X1), and (X2). These compounds are more fluorescent than the parent compounds as would be expected from the oxidation of the pyrazine ring. As the parent compounds are fluorescent they are probably dihydropterins. The iodine oxidation products can be reduced with $NaBH_4$ to regenerate the parent compounds which is again evidence that the parent compounds [(X), (X1), (X2)] are dihydropterins.

4. DISCUSSION

Two different mechanisms have been proposed for the conversion of H_2-neopterin-$(P)_3$ to sepiapterin, and the validity of both will be tested by identification of the intermediate (X) (Krivi & Brown, 1979; Tanaka et al., 1979). Attempts to isolate and characterize the enzymatically formed intermediate have been unsuccessful. In this study we

have developed a sensitive assay for (X) (which should aid in purification of the Mg^{2+}-dependent enzyme of sepiapterin synthase) and have demonstrated that H_2-neopterin-$(P)_3$ can be converted nonenzymatically to a substance that is the same as (X) by the following criteria: (1) chromatographic behavior with C18 reverse phase chromatography; (2) absorption and fluorescent characteristics; and (3) utilization by sepiapterin synthase to produce sepiapterin. We have provided evidence that (X) can isomerize to at least two other forms [(X1), (X2)], at least one of which [(X1)] can serve as a substrate for sepiapterin synthesis. The nonenzymatic method also allowed synthesis of large amounts for purification and characterization.

As evidence obtained by Krivi & Brown (1979) suggested that NADPH is used and regenerated in the conversion of (X) to sepiapterin (i.e., H_2-neopterin-$(P)_3$, (X), and sepiapterin are all at the same oxidation state), they proposed a mechanism in which H_2-neopterin-$(P)_3$ is converted to (X) by a base-catalyzed, nonhydrolytic loss of the phosphate groups (Figure 19). Our evidence indicates that Tris base is a catalyst in the nonenzymatic synthesis of (X) from H_2-neopterin-$(P)_3$ and agrees with their hypothesis in this respect. Their proposed structure for the intermediate, however, does not fit the chemical data we have obtained on (X) to date. The structure they have proposed (7,8-dihydro-6-(1'-hydroxy-2'-oxopropyl)pterin) (Figure 19) should react readily with both $NaIO_4$ and $NaBH_4$ while (X) does not. The intermediate structure proposed by Tanaka et al. (1979) (7,8-dihydro-6-(1',2'-dioxopropyl)pterin) would also react readily with $NaIO_4$ and $NaBH_4$. While we do not have sufficient evidence at this time to establish a structure for

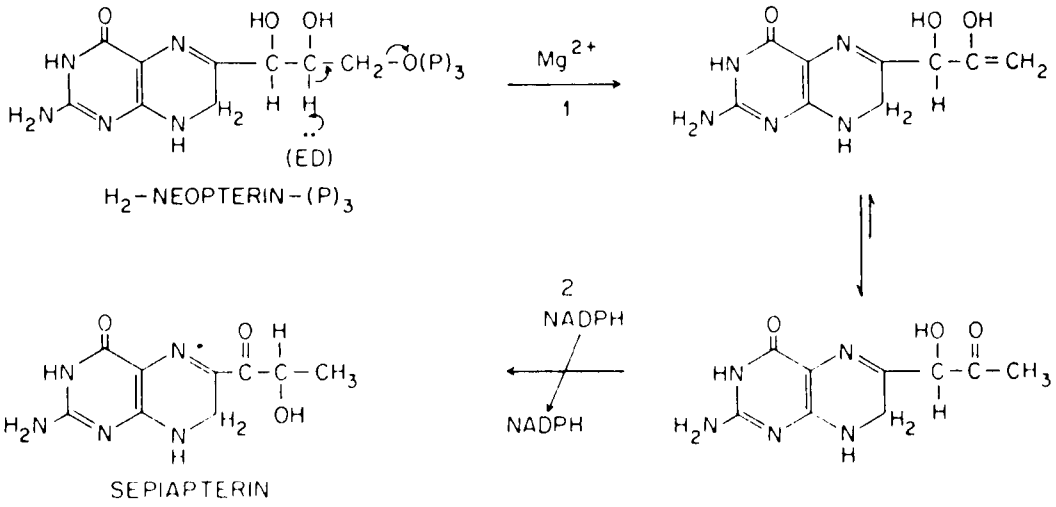


Figure 19. Postulated reaction mechanism for sepiapterin synthase. After Krivi, G.G. & Brown, G.M. (1979) Biochem. Genet. 17, 371.

(X), it seems that both of the recently proposed structure are unlikely. Furthur analysis of purified (X), (X1), and (X2) should resolve this problem. Given sufficient GTP cyclohydrolase, the nonenzymatic synthesis of (X) could be scaled up to produce large amounts of (X) for future structural studies.

CHAPTER IV

PURIFICATION AND BIOSYNTHESIS OF QUENCH SPOT, A "DROSOPTERIN" PRECURSOR IN DROSOPHILA MELANOGASTER

1. INTRODUCTION

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-$(P)_3$) to the six dipteridine-derived red accessory eye pigments of Drosophila known collectively as the "drosopterins" (neodrosopterin, fraction e, aurodrosopterin I & II, drosopterin, and isodrosopterin) (Dorsett et al., 1979). It was concluded that sepiapterin synthase, the enzymatic activity responsible for the conversion of H_2-neopterin-$(P)_3$ to sepiapterin (7,8-dihydro-6-lactoylpterin), consists of two enzymes. The 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. In the presence of either NADPH or NADH, the sepiapterin synthase intermediate can be converted to "drosopterins". The intermediate is nonphosphorylated but it is not H_2-neopterin (Dorsett et al., 1979; Krivi & Brown, 1979; Tanaka et al., 1979). The separation of the proteins that carry out the two activities of sepiapterin synthase from Drosophila (Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1979) 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 component of sepiapterin synthase (Yim et al., 1977; Dorsett et al., 1979).

Quench spot (Q.S.) is an unidentitfied, pterin-like compound that has been found and quantitated in wild-type, pr, and Henna-recessive-3 (Hn^{r3}) flies (Wilson & Jacobson, 1977b). Purple, which contains low levels of sepiapterin and "drosopterins", also has low levels of Q.S. Sepia contains very high amounts of sepiapterin but no Q.S. or "drosopterins". Henna-recessive-3 has a high level of both sepiapterin and Q.S. but no "drosopterins". These considerations led us to hypothesize that Q.S. is an intermediate in "drosopterin" biosynthesis.

The results presented in this report support this hypothesis. Q.S. is apparently an intermediate in the conversion of the sepiapterin synthase intermediate to "drosopterins" and is the source of only one pterin unit of the dipteridine-derived "drosopterins"; the other pterin unit apparently arises from H_2-neopterin-$(P)_3$ by a pathway which is distinct from the sepiapterin synthase pathway.

2. MATERIALS AND METHODS

Drosophila melanogaster. Oregon-R wild-type and $pr^{bw}cn$ mutant flies were reared at 25°C on medium prepared as described by Lewis (1960). Adult flies were collected daily so that all were one 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 vials under 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 G25 (medium)

(Pharmacia) using the centrifuge procedure of Neal & Florini (1973) instead of chromatography on a Sephadex G25 column. The centrifuge procedure obviated the vacuum dialysis required if the extracts has been desalted by chromatography.

Triton X-100 Extract (TE). Using a ground glass homogenizer (Kontes) 1.0 g of heads (Oregon-R or $pr^{bw}cn$) were homogenized in 5.0 mL of 50 mM Pipes (pH 7.0) containing 1% Triton X-100. The homogenate was subjected to centrifugation at 15,000g for 10 min and the supernatant was subjected to furthur centrifugation at 100,000g for 60 min. The supernatant was removed carefully with a Pasteur pipet so as not to disturb the lipid layer at the top of the tube, filtered through Sephadex G25 (medium) using the centrifuge procedure of Neal & Florini (1973), and then incubated with 2.5 mL of washed BioBeads (SM2) (BioRad) with gentle shaking at $0^{o}C$ for 60 min to remove the Triton X-100. The protein that precipitated between 40 and 60 % saturated $(NH_4)_2SO_4$ ($4^{o}C$) was collected by centrifugation at 15,000g for 30 min, redissolved in 0.5 mL of 50 mM Pipes (pH 7.0) that also contained 10 mM 2-mercaptoethanol and 10% glycerol, and filtered through Sephadex G25 (medium) using the centrifuge procedure. The extract was stored in vials under liquid nitrogen. The Q.S. synthesizing activity was stable for approximately a week under these conditions.

H_2-neopterin-$(P)_3$. H_2-neopterin-$(P)_3$ was prepared from GTP (sodium salt, Sigma) as previously described (Dorsett et al., 1979) using pure GTP cyclohydrolase I prepared form E. coli (Yim & Brown, 1976). [U-^{14}C]GTP (Amersham) or [8,5'-^{3}H]GTP (New England Nuclear) were added to the GTP depending upon the experiment to produce

[U-^{14}C]H_2-neopterin-$(P)_3$ and [3'-^{3}H]H_2-neopterin-$(P)_3$ respectively. The specific activities used are described below. H_2-neopterin-$(P)_3$ was treated with bacterial alkaline phosphatase as previously described (Dorsett et al., 1979).

[U-^{14}C]sepiapterin. An aliquot (117 uL) of 0.67 mM [U-^{14}C]H_2-neopterin-$(P)_3$ (20.3 uCi/umol) was incubated with 157 uL H_2O, 157 uL Oregon-R PFE, 39 uL 35 mM NADPH (Sigma, chemically reduced), 39 uL 0.2 M $MgCl_2$, and 39 uL 0.7 M Pipes (pH 7.5) at 42^{o}C for 60 min in the dark to produce sepiapterin. The reaction was stopped by incubation at 100^{o}C for 5 min and the resulting precipitate was removed by centrifugation. [U-^{14}C]sepiapterin was separated by HPLC as described below. Fractions containing sepiapterin were pooled and evaporated to dryness at 40^{o}C by a stream of dry nitrogen. The sepiapterin was redissolved in 100 uL of H_2O and stored at -20^{o}C.

Pterin, Isoxanthopterin, and 7,8-dihydropterin. Pterin and isoxanthopterin were purchased from Sigma Biochemical Co. The method used by Kaufman (1967) to reduce biopterin to 7,8-dihydrobiopterin was modified to prepare 7,8-dihydropterin from pterin. Pterin (1.0 mg) was suspended in 400 uL H_2O and 200 uL of 2 N KOH was added to bring it into solution. Zn dust (12 mg) was added and the mixture was incubated in the dark (with occasional stirring) for 15 min before incubating at 55^{o}C for 5 min. The pH was adjusted to 6 with 4 N HCl and the precipitate was removed by centrifugation. K_2HPO_4 (1 M) was added slowly until a precipitate formed. After incubating in an ice bath for 60 min, the precipitate was removed by centrifugation before testing the supernatant for the presence of Zn^{2+} (Feigl & 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, p.66) was determined to confirm identity and calculate concentration.

Purification of Quench Spot. Using a Virtis homogenizer, 15 g of Oregon-R heads were homogenized in 75 mL of 50% isopropanol and 1% 2-mercaptoethanol in water for 5 min at half speed in the dark at room temperature. The supernatant obtained after centrifugation at 15,000g for 5 min was saved and the pellet rehomogenized in 75 mL of solvent before centrifugation. The final pellet was discarded and the pooled supernatants were concentrated by rotary evaporation (45°C) to remove the isopropanol. The sample was loaded onto a carboxymethyl-cellulose column (Whatman CM 23, 8.5 x 13 cm, precycled as described by the manufacturer, washed with 3 L of 0.5 M ammonium formate and then 4 L of distilled water prior to use). Elution was carried out in the dark with water at a flow rate of 4 mL/min while collecting 20 mL fractions. The profile obtained by measuring the A_{260} of the fractions revealed three major, poorly resolved peaks with the third containing Q.S. [as identified by the ultraviolet spectrum and thin-layer chromatography (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 carboxymethyl-cellulose column (Whatman CM 32, 2.5 x 75 cm, precycled as described by the manufacturer, washed with 1 L of 0.5 M ammonium formate and 2 L of distilled H_2O prior to use). Q.S. was eluted in the dark at 4°C with a 660 mL linear

gradient (0-50 mM ammonium formate) while collecting 10 mL fractions (Figure 20). Q.S. was identified by the ultraviolet absorption spectrum and the fractions containing Q.S. (74-80) were pooled and lyophilized to dryness. Upon lyophilization an orange substance was formed. The lyophilization flask was washed 20 times with 50 uL aliquots of water which were pooled. The orange substance was dissolved in the water while much of the Q.S. remained insoluble. Q.S. was recovered by centrifugation, washed with 50 uL 95% ethanol and 50 uL diethyl ether prior to drying in a vacuum dessicator. The yield was 0.3 mg of Q.S. Q.S. 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 in shown in Fiqure 21.

Sepiapterin, "Drosopterin", and Q.S. Carrier. Sepiapterin was purified from sepia mutant heads as previously described (Dorsett et al., 1979). "Drosopterin" and Q.S. carrier was prepared by homogenizing (ground glass) 0.2 g of Oregon-R heads in 1.0 mL of 30% ethanol made pH 2 with concentrated HCl. The homogenate was clarified by centrifugation and stored at -20^{o}C.

Assay of Q.S., Sepiapterin, and "Drosopterin" Biosynthesis. The exact conditions of the assays whose results are presented in Tables and Figures are given in the legends. The protocol described below was used for all assays. Q.S., sepiapterin, and "drosopterin" biosynthesis were determined in a total reaction volume of 70 uL 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 Q.S. synthesis), [U-^{14}C] and/or [3'-^{3}H]H_2-neopterin-$(P)_3$, and various cofactors or

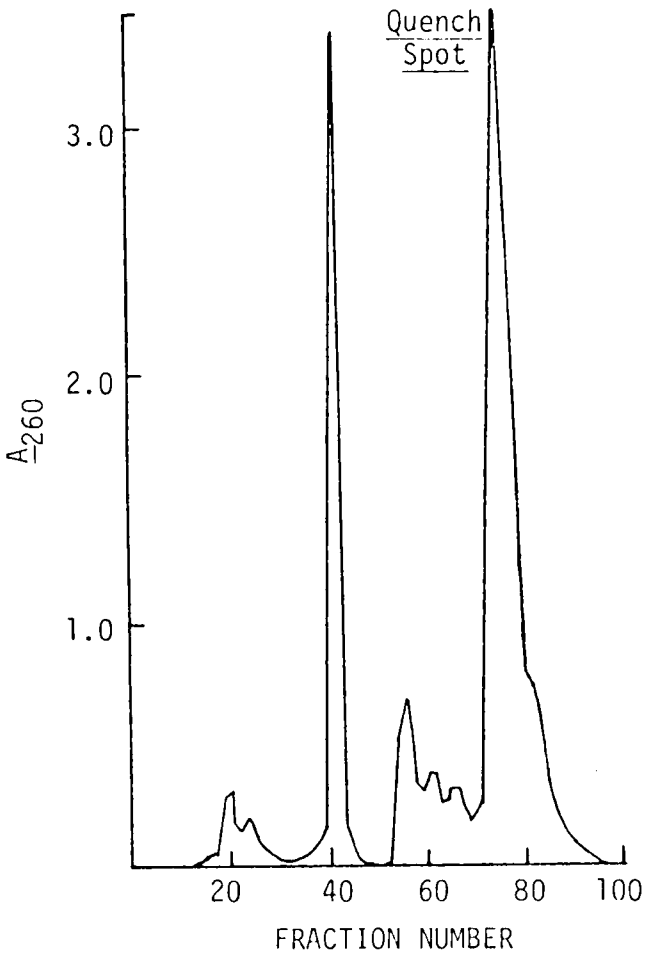


Figure 20. Isolation of quench spot by CM-cellulose chromatography.

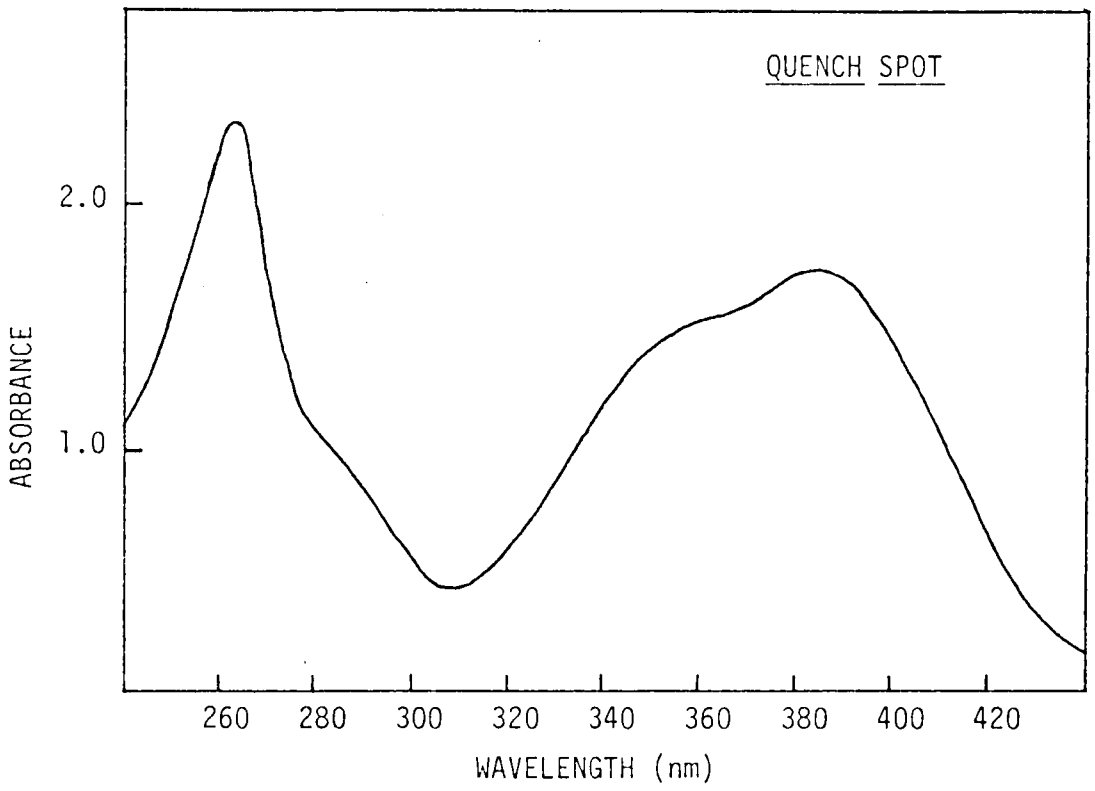


Figure 21. Ultraviolet absorption spectrum of purified quench spot in water.

pteridines depending upon the experiment. $MgCl_2$, when present, was used at a concentration of 14.3 mM; NADPH or NADH (Sigma, chemically reduced) 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 stopping by heating at 100°C for 5 min. Any precipitate was removed by centrifugation (Brinkmann Eppendorf Centrifuge 3200) and 15 uL of carrier (preparation described above, supplemented with sepiapterin when necessary) was added before subjecting 40 uL of the solution to two-dimensional cellulose thin-layer chromatography as previously described (Wilson & Jacobson, 1977a), except the ammonium acetate concentration in the first sovent was increased to 2 g per 100 mL. Q.S. was visualized by immersing the thin-layer plates in liquid nitrogen and viewing with a long wavelength ultraviolet lamp. The Q.S., sepiapterin, and individual "drosopterin" spots were scraped into scintillation vials containing 1.5 mL of water, and the amount of radioactivity was determined in a scintillation counter after adding 15 mL of 0.28% 2,5-bis-2-[5-tert-butylbenzoxalzolyl]thiophene (Packard) and 33% Triton X-100 in toluene. Kodak RP x-ray film was used for autoradiography of the thin-layer chromatograms.

High Performance Liquid Chromatography (HPLC). A Waters Liquid Chromatograph with a 3.6 x 30 cm uBondapak C18 column was used to purify [U-^{14}C]sepiapterin and 7,8-dihydropterin and also to check the purity of Q.S. The column was operated at a temperature of 35°C and a flow rate of 2 mL/min. Elution of 7,8-dihydropterin was with 5% methanol in water; sepiapterin was eluted with 20% methanol; Q.S. was eluted with 20% methanol containing 10 mM ammonium phosphate (pH 8.0). The

ultraviolet absorption and fluorescence of the pteridines were detected with a Schoeffel SF 770 UV monitor at 260 nm and a Schoeffel FS 970 fluorescence monitor (excitation at 360 nm, Corning 7-54 excitation filter, 418 nm cutoff emission filter) set to appropiate sensitivities.

3. RESULTS

Quench Spot Biosynthesis. If Q.S. is an intermediate in "drosopterin" biosynthesis, then we would expect that H_2-neopterin-$(P)_3$ could be used as a substrate for Q.S. biosynthesis mediated by Drosophila enzymes. While such enzymatic activity was found in the pteridine-free extracts (PFE) used earlier for in vitro "drosopterin" biosynthesis, the amount of labeled Q.S. found was small and insufficient to test the conditions that affect the synthesis of Q.S. Greater activity could be acheived in the Triton X-100 extract (TE). Using this exract it was found that label from [U-^{14}C] but not [3'-^{3}H]H_2-neopterin-$(P)_3$ was incorporated into Q.S. [Table II(b)]. Treatment of H_2-neopterin-$(P)_3$ with phosphatase prior to use as a substrate prevented the synthesis of Q.S. The correspondence of the radioactive spot to the visible Q.S. on the thin-layer plates was demonstrated by autoradiography. Under the reaction conditions used in Table II, the synthesis of Q.S. 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 22).

Certain cofactors affect the rate of Q.S. accumulation. Earlier we had shown that "drosopterin" biosynthesis was dependent on Mg^{2+} and NADPH. We found that Mg^{2+} also stimulates the synthesis of Q.S. from

TABLE II: Biosynthesis of Quench Spot: 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	
			Radioactivity of Q.S. TLC spot (dpm)	
Enzyme Extract	Rate of Q.S. synthesis (^{14}C cpm/30 min)	% Oregon-R activity[c]	^{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 uL of enzyme extract, 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 uCi/umol). Reactions were for 30 min at 42°C.

[b]Reactions contained 15 uL of enzyme extract, 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 uCi/umol ^{14}C, 38 uCi/umol ^{3}H). Reactions were for 30 min at 42°C.

[c]Values in both experiments are averages of duplicate reactions and no background corrections have been made. The per cent Oregon-R activity was calculated, however, after subtracting the heat-inactivated Oregon-R TE values as background.

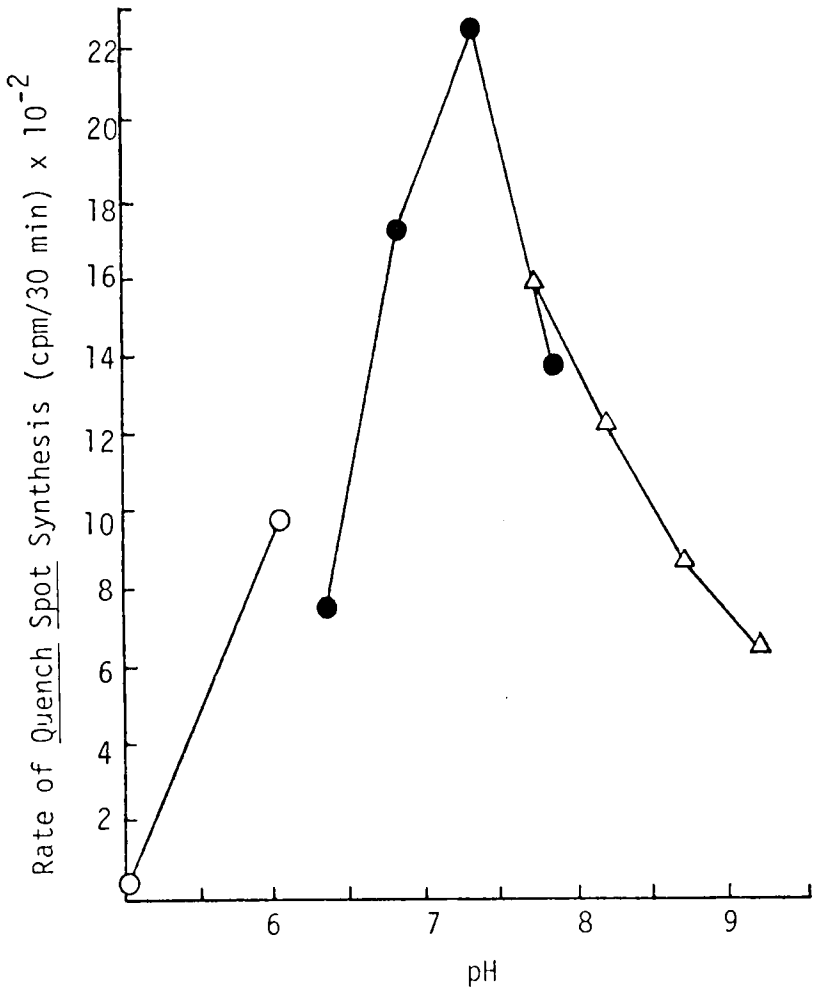


Figure 22. Effect of pH on quench spot biosynthesis. O, potassium acetate buffer; O, Pipes buffer; Δ, Taps buffer. Each reaction contained 40 uL Oregon-R TE, 14.3 mM $MgCl_2$, 0.142 mM [U-^{14}C]H_2-neopterin-$(P)_3$, and the various buffers at 50 mM. The reactions were carried out for 30 min at 42°C.

H_2-neopterin-$(P)_3$ whereas NADPH or NADH decreased the amount of Q.S. recovered from a reaction (Table III). The decreased accumulation of Q.S. in the presence of NADPH or NADH is consistent with the hypothesis that Q.S. is a "drosopterin" precursor as "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 enzyme of sepiapterin synthase (Dorsett et al., 1979). The Mg^{2+} requirement for Q.S. biosynthesis then is consistent with the hypothesis that (X) is either a precursor or product of Q.S. As the pr^{bw}cn mutant (1) has decreased Q.S., sepiapterin, and "drosopterin" levels in vivo , (2) contains only 20% of the sepiapterin synthase activity compared to wild-type 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 Q.S. biosynthetic activity present in pr^{bw}cn and Oregon-R TE. The pr^{bw}cn extracts contained only 28% of the Q.S. biosynthetic activity found in Oregon-R extracts [Table II(a)].

Whether Q.S. preceeds 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 no ^{3}H was incorporated into Q.S. [Table II(b)], it seems unlikely that Q.S. could function as a sepiapterin precursor. This observation and the result that pr^{bw}cn extracts are low in Q.S. synthetic activity are consistent with the hypothesis that (X) is a

TABLE III: Effect of Mg^{2+}, NADPH, and NADH on the Biosynthesis of Quench Spot and other Pteridines from [U-^{14}C]H_2-neopterin-$(P)_3$[a].

Product	Radioactivity of Pteridine TLC Spots (cpm)			
	No cofactors	Mg^{2+}	Mg^{2+} and NADPH	Mg^{2+} and NADH
quench spot	403	1850	540	789
sepiapterin	449	1718	15454	1719
"drosopterins"	540	537	925	1493

[a]Each reaction contained 20 uL Oregon-R PFE, 20 uL Oregon-R TE, 50 mM Pipes (pH 7.5), 0.142 mM [U-^{14}C]H_2-neopterin-$(P)_3$ (20.3 uCi/umol) 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 analyzed for the incorporation of label into the various pteridines. The values for "drosopterins" are the sum of the values obtained for the individual species. All values are averages of duplicated reactions; no background corrections were made.

precursor of Q.S. Recently we have developed an HPLC assay for (X) (Dorsett et al., 1980) and have shown directly that $pr^{bw}cn$ TE and PFE contain only 20% of the (X) synthetic activity found in the respective Oregon-R extracts (unpublished results).

"Drosopterin" Biosynthesis in the Presence of Quench Spot. While compound (X), the sepiapterin synthase intermediate, appears to be a precursor to Q.S. and to "drosopterins" (Dorsett et al., 1979), it remains to be demonstrated that Q.S. is a "drosopterin" precursor. Since Q.S. is made in small amounts and is somewhat labile during thin-layer chromatography and extraction, it was not possible to isolate large enough amounts of [^{14}C]Q.S. to test if the label could be incorporated into the "drosopterins". As Q.S. can be purified from the heads of flies, the problem could be approached by asking whether unlabeled Q.S. added to a reaction synthesizing "drosopterins" could cause a decrease in the label incorporated into the "drosopterins" from [U-^{14}C]H_2-neopterin-$(P)_3$. Much to our surprise, the addition of Q.S. did not diminish but stimulated the incorporation of label into all the "drosopterins" (Figure 23). This can be interpreted to mean that Q.S. is a "drosopterin" precursor. To arrive at this interpretation we assume that Q.S. is the limiting component in the in vitro reaction and that another precursor (or precursors), arising via another pathway from H_2-neopterin-$(P)_3$ is also required for "drosopterin" synthesis. The addition of Q.S., therefore, would allow greater incorporation of the putative precursor(s) into the "drosopterins". The possibility that a contaminant in the Q.S. preparation caused the stimulation in "drosopterin" biosynthesis was tested by comparing the ability of Q.S. preparations

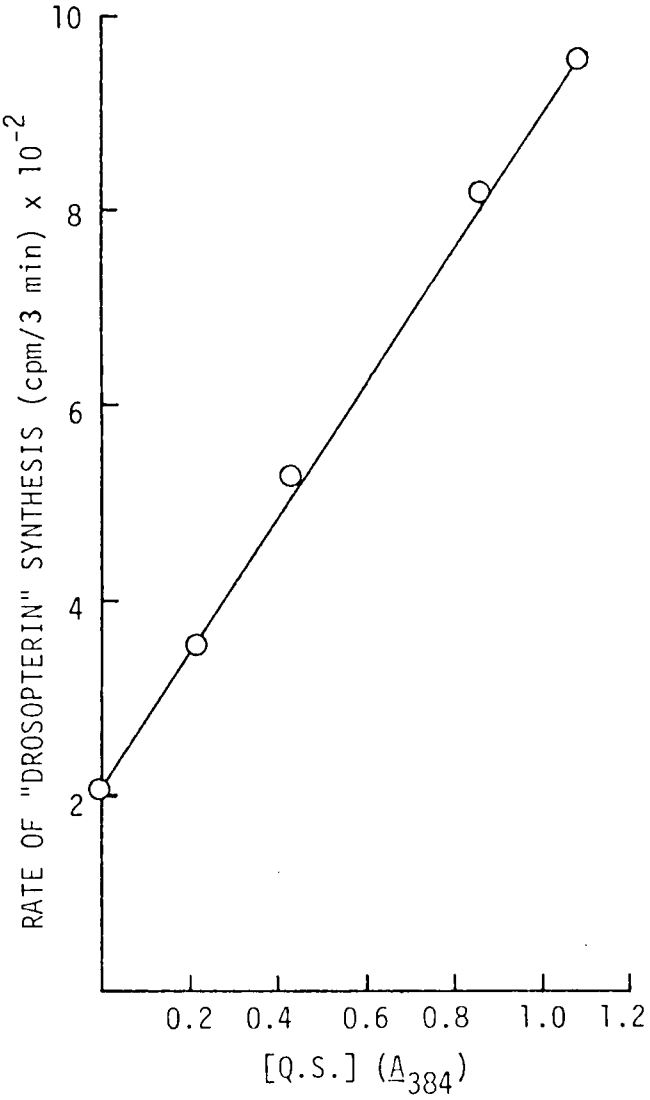


Figure 23. Effect of quench spot on the rate of "drosopterin" biosynthesis. Each reaction contained 15 uL Oregon-R PFE, 14.3 mM $MgCl_2$, 2.5 mM NADPH, 50 mM Pipes (pH 7.5) and 0.142 mM [U-^{14}C]H_2-neopterin-(P)$_3$ (20.3 uCi/umol). Reactions were carried out for 3 min at 42^{o}C. The values are the sum of the incorporation into the individual "drosopterins". Reactions containing heat-inactivated Oregon-R PFE were used to determine the values for background correction.

at various stages of purification to cause the stimulation of "drosopterin" synthesis. The amount of stimulation remained proportional to the amount of Q.S. present (as estimated by the absorbance at 384 nm).

The possibility that Q.S. may be acting as a cofactor in "drosopterin" synthesis is an alternative hypothesis that may be considered. This seems unlikely as (1) Q.S. 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 Q.S.

We have demonstrated that $\underline{pr^{bw}cn}$ flies have a lesion in the structural locus for the Mg^{2+}-dependent, first enzyme of sepiapterin synthase which produces (X), the sepiapterin synthase intermediate (Yim et al., 1977; Dorsett et al., 1979). The evidence shows that Q.S. is an intermediate in the conversion of (X) to "drosopterins". If this hypothesis is correct, then addition of Q.S. to $\underline{pr^{bw}cn}$ extracts should overcome the metabolic block in these extracts for the synthesis of "drosopterins" and therefore allow greater incorporation of label from [U-^{14}C]H_2-neopterin-$(P)_3$ (via the other "drosopterin" precursor) into the "drosopterins". Indeed, Q.S. did stimulate (to nearly the same degree as reactions utilizing Oregon-R PFE) the incorporation of label from [U-^{14}C]H_2-neopterin-$(P)_3$ into "drosopterins" mediated by $\underline{pr^{bw}cn}$ PFE. This may also indicate that the additional "drosopterin" precursor does not arise from the sepiapterin synthase intermediate as (X) is synthesized in low amounts by the $\underline{pr^{bw}cn}$ extracts.

Since the hypothesis predicts that Q.S. should not cause a

decrease of incorporation of label from [U-^{14}C]H_2-neopterin-$(P)_3$ into sepiapterin, we were surprised to see as much as a 50% decrease in the amount of labeled sepiapterin recovered upon the addition of Q.S. to a reaction. This decrease in labeled sepiapterin, however, was shown not to be due to isotope dilution by the following experiment. [U-^{14}C]sepiapterin was added to in vitro sepiapterin and "drosopterin" synthesizing reactions using unlabeled H_2-neopterin-$(P)_3$ as substrate. Even in the absence of enzyme the addition of Q.S. resulted in a substantial loss of radiolabeled sepiapterin; this indicates that a reaction was occuring between sepiapterin and Q.S. (Table IV). The "missing" label was not found in the "drosopterins" but in an unidentified spot on the thin-layer chromatogram (detected by autoradiography) which had previously been described as a possible degradation product of sepiapterin (spot G, Wilson & Jacobson, 1977a). The reaction between Q.S. and sepiapterin was not furthur characterized, but these results point to a possible pitfall in the interpretation of even positive isotope dilution results when actual specific activities cannot be measured.

To test the possibility that pterin, 7,8-dihydropterin, or isoxanthopterin might be "drosopterin" precursors, isotope dilution experiments with these compounds were performed in Q.S. stimulated reactions using [U-^{14}C]H_2-neopterin-$(P)_3$ as substrate. No change in the amount of ^{14}C recovered in the "drosopterins" occured in the presence of any of these compounds (pterin and 7,8-dihydropterin were used at a concentration of 0.4 mM; isoxanthopterin was used at a concentration of 0.26 mM).

Some aspects of in vitro "drosopterin" synthesis in the presence

TABLE IV: Effect of Quench Spot on [U-^{14}C]sepiapterin Recovery[a].

Quench Spot	Enzyme	Radioactivity of Pteridines (cpm)		
		Sepiapterin	"Drosopterins"	Quench Spot
0	+	8535	310	61
0	0	7716	400	63
+	+	4234	283	49
+	0	4931	468	34

[a]Each reaction contained 20 uL Oregon-R PFE or 20 uL 50 mM Pipes (pH 7.0) containg 10% glycerol, 20 uL quench spot solution (A_{384}, 3.1) or 20 uL H_2O, 50 mM Pipes (pH 7.5), 14.3 mM $MgCl_2$, 2.5 mM NADPH, 0.142 mM H_2-neopterin-$(P)_3$, and [U-^{14}C]sepiapterin (20.3 uCi/umol). Reactions were carried out at 42^{0}C for 60 min. No background corrections have been made. "Drosopterin" values are the sum of the values for the individual species. (+), present in the reaction; (0), not present in the reaction.

of Q.S. should be mentioned. Mg^{2+} and NADPH are both still required for "drosopterin" synthesis in the presence of Q.S. (data not shown). Under the assay conditions used, total "drosopterin" synthesis appeared to level off at 9 to 12 min but this is not true for individual "drosopterins". Incorporation into drosopterin and isodrosopterin reaches a maximum and then decreases after 12 min while incorporation into neodrosopterin, fraction e (assayed together) and aurodrosopterins continue to increase (Figure 24). 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 linearly dependent upon H_2-neopterin-$(P)_3$ concentration when Q.S. is present. Saturation was not acheived with a H_2-neopterin-$(P)_3$ concentration of 0.4 mM. The reaction rate is also dependent upon the concentration of enzyme and Q.S. While the molar concentration of Q.S. could not be calculated, addition of 25 uL of a Q.S. solution with an absorbance of 3.1 at 384 nm did not acheive saturation. Furthur investigation with this system could possibly answer questions regarding the interconversion of "drosopterins" as well as identify other "drosopterin" precursors.

4. DISCUSSION

The proposed pathway for "drosopterin" biosynthesis is illustrated in Figure 25. Q.S. 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 Q.S. present in the eye color mutants

Figure 24. Time course of "drosopterin" biosynthesis in the presence of quench spot. O, sum of incorporation into all "drosopterins; ●, sum of incorporation into neodrosopterin, fraction e, and aurodrosopterins; Δ, sum of incorporation into drosopterin and isodrosopterin. Each reaction contained 15 uL Oregon-R PFE, 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 uCi/umol), and 25 uL of quench spot solution (A_{384}, 3.1). Reactions using heat-inactivated Oregon-R PFE were used to determine values for background correction.

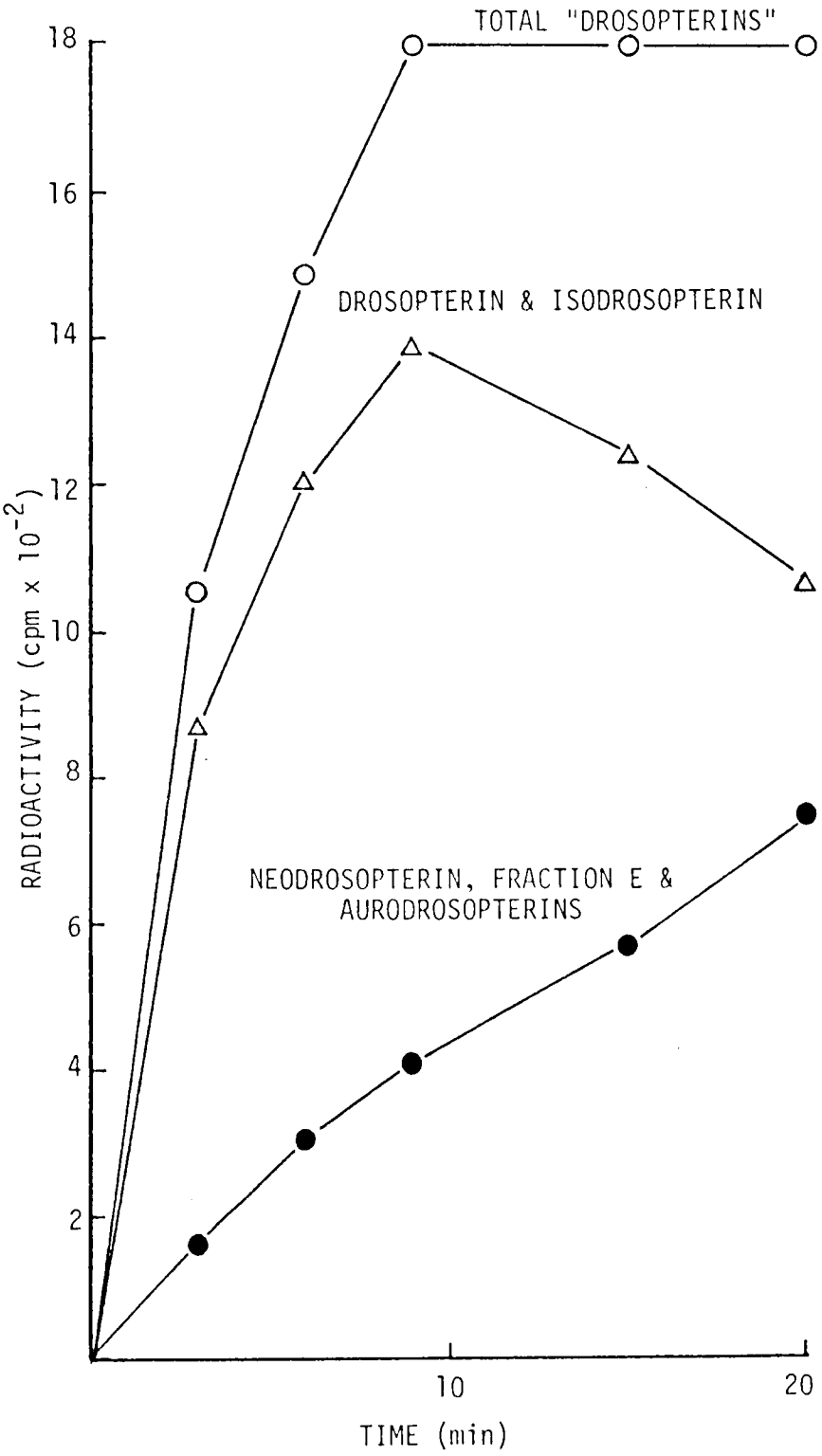

TOTAL "DROSOPTERINS"
DROSOPTERIN & ISODROSOPTERIN
NEODROSOPTERIN, FRACTION E &
AURODROSOPTERINS
RADIOACTIVITY (cpm x 10^{-2})
18
16
14
12
10
8
6
4
2
10
20
TIME (min)

Figure 24.

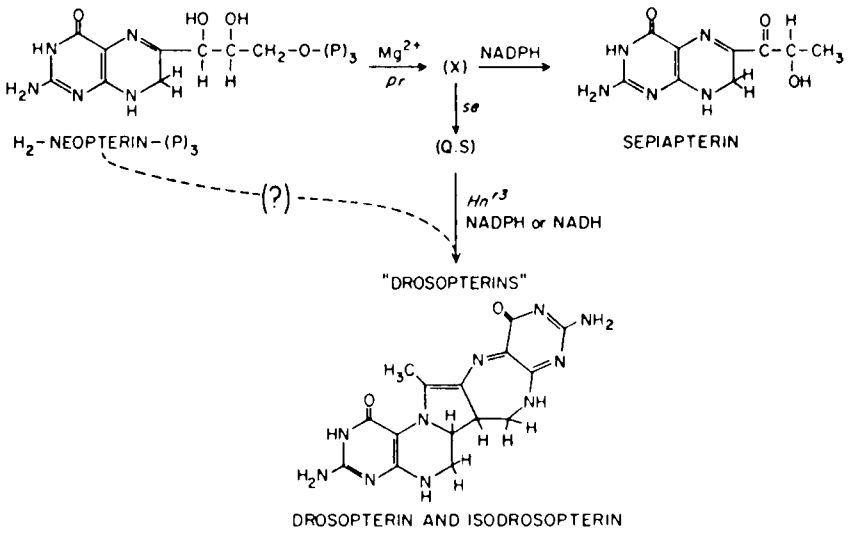


Figure 25. Postulated reaction sequence for sepiapterin, quench spot, and "drosopterin" biosynthesis.

pr, se, and Hn^{r3} are consistent with the proposed scheme; possible steps at which the latter two mutants are defective are indicated in Figure 25. The level of Q.S. 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 in all three mutants. The evidence that pr is a lesion in the structural locus 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 are uknown, but the effect is to block interconversion of the pteridines in vivo in a manner consistent with the proposed biosynthetic scheme. It should be possible to devise assays for the enzymatic steps which are proposed to be defective in these mutants.

It is known that under the proper conditions, 7,8-dihydropterin will react with α-keto-β-hydroxybutyric acid to form drosopterin and isodrosopterin. Indeed it has been proposed that this is how "drosopterins" are synthesized in Drosophila (Masada, et al., 1979). The failure that we observed of 7,8-dihydropterin to cause isotope dilution in the enzymatically catalyzed "drosopterin" synthesis makes this mechanism seem unlikely. It is also difficult to explain the role of Q.S. in "drosopterin" synthesis with this mechanism. Additionally, the extracts used in the experiments designed to measure "drosopterin" synthesis have been through ammonium sulfate precipitation and Sephadex G25 chromatography, and would not be expected to 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 be a lesion in the structural locus for xanthine dehydrogenase, which catalyzes the the conversion of pterin to isoxanthopterin (Grell, 1962). Although ry flies contain low amounts of "drosopterins", our evidence indicates that isoxanthopterin is not a "drosopterin" precursor as the presence of either pterin or isoxanthopterin has 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 the production of "drosopterins" to near normal levels without affecting isoxanthopterin levels. We have found that the addition of phenylalanine to the reaction mixture had 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 Q.S. by chemical and mass spectral analysis. While this is still in progress, it seems clear that Q.S. has nine carbon atoms as detected by high resolution mass spectral analysis (Dorsett, Jacobson, Sethi, & McCloskey, unpublished observations). This indicates that Q.S. retains the three carbon side chain from H_2-neopterin-$(P)_3$. As drosopterin contains fifteen carbon atoms, the other "drosopterin" precursor need only contain six carbon atoms. This suggests that the other "drosopterin" precursor does not need to retain the side chain from H_2-neopterin-$(P)_3$. This was the reason for considering pterin, 7,8-dihydropterin, and isoxanthopterin as possible "drosopterin" precursors. On the basis of the

isotope dilution experiments it seems unlikely that they are precursors, so it may be that either the precursor retains at least a portion of the side chain or that it is not a pterin. An enzymatic activity has been found to occur in Drosophila heads which removes the three carbon side chain from H_2-neopterin-$(P)_3$ (Yim et al., 1978). The product of this reaction was isolated by chromatography; by chromatographic and spectral characteristics it was different from pterin, 7,8-dihydropterin, isoxanthopterin, xanthopterin, 6-carboxypterin, 6-carboxaldehydepterin, 6-hydroxymethylpterin and 6-methylpterin (Yim et al., 1980). 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 assy "drosopterin" biosynthesis. It remains to be seen whether the product of side chain release activity is a "drosopterin" precursor.

CHAPTER V

CHEMICAL AND MASS SPECTRAL ANALYSIS OF QUENCH SPOT

1. INTRODUCTION

In previous investigations we have provided evidence that the enzymatic conversion of the 3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl)pterin (H_2-neopterin-$(P)_3$) to 7,8-dihydro-6-lactoylpterin (sepiapterin) occurs via a nonphosphorylated intermediate (X) which also serves as a precursor of the six red eye pigments of Drosophila melanogaster known collectively as the "drosopterins" (neodrosopterin, fraction e, aurodrosopterin I & II, drosopterin, and isodrosopterin) (Dorsett et al., 1979). Furthur investigations have revealed that quench spot (Q.S.), a pterin-like compound found in the heads of Drosophila (Wilson & Jacobson, 1977a,b), is an intermediate in the conversion of (X) to "drosopterins" (Chapter IV). The other pterin unit arises from H_2-neopterin-$(P)_3$ via an unidentified pathway. The postulated biosynthetic scheme is shown in Figure 25 (p. 86).

In this report we present the results of chemical and mass spectral analysis designed to determine the structure of Q.S. Two alternative structures are also presented and discussed in light of the observations presented here.

2. MATERIALS AND METHODS

Q.S. was purified from the heads of Oregon-R (wild-type strain of Drosophila melanogaster) as previously described (Chapter IV, p. 68).

Determination of Q.S. pKa Values. Aliquots (20 uL) of aqueous Q.S. solution ($\underline{A}_{384}$ = 3.4) were added to 300 uL aliquots of HCl or NaOH solutions ranging in pH from 1 to 13 and the absorption at 405 nm (the greatest change in absorption with pH occurs at this wavelength) was determined. It was assumed that the addition of the Q.S. solution did not significantly alter the pH.

High Performance Liquid Chromatography (HPLC). After treatment with various reagents (described below) Q.S. was quantitatively measured by an HPLC separation using a Waters Liquid Chromatograph with a DuPont Zorbax C8 column (4 mm x 25 cm). The column was operated at 35^{o}C and elution of Q.S. was accomplished with 15% methanol (Burdick and Jackson, Inc.) containing 10 mM ammonium phosphate (pH 7.4) at a flow rate of 2 mL/min. Q.S. and various products were detected with a Schoeffel SF 770 ultraviolet monitor (260 nm, 0.2 aufs with a 1 mV recorder). A Schoeffel FS 970 fluorescence monitor (excitation at 360 nm with a Corning 7-54 excitation filter, 418 nm cutoff emission filter, sensitivity = 6.8, 0.2 uA scale with a 1 mV recorder) was also used to detect any fluorescent products.

Treatment of Q.S. with Alkaline $KMnO_4$. Oxidation of Q.S. with $KMnO_4$ was performed by incubation of a 10 uL aliquot of aqueous Q.S. solution ($\underline{A}_{384}$ = 3.1) with 2 uL of 0.1 M $KMnO_4$ and 2 uL of 0.1 N NaOH in capped polypropylene tubes for 60 min at 42^{o}C in the dark. The

excess $KMnO_4$ was reduced to MnO_2 by titration with a 1% ascorbate solution until the color of $KMnO_4$ disappeared. The MnO_2 was removed by centrifugation (Brinkmann Eppendorf Centrifuge 3200) and the supernatant was analyzed by HPLC. In the reagent control, 2 uL of H_2O replaced the $KMnO_4$ solution. The oxidation of sepiapterin to 6-carboxypterin was performed as a positive control. Treatment of sepiapterin was also used as a positive control for the reactions described below.

Oxidation of Q.S. with $NaIO_4$. Aliquots (10 uL) of Q.S. solution ($\underline{A}_{384}$ = 3.1) were incubated with 10 uL H_2O, 1 uL 0.01 N HCl, and 4 uL 0.1 M $NaIO_4$ for various times up to 30 min at room temperature in the dark before analysis by HPLC. Water replaced the $NaIO_4$ solution in the reagent control.

Reaction of Q.S. with 2,4-dinitrophenylhydrazine (2,4-DNPH). A saturated solution of 2,4-DNPH was made in concentrated H_2SO_4 and then 15 uL were diluted with 20 uL H_2O and 70 uL 95% ethanol. The excess 2,4-DNPH was removed by centrifugation to give rise to the working solution. An aliquot (20 uL) of Q.S. solution ($\underline{A}_{384}$ = 3.1) was incubated with 2 uL 2 N KOH (to keep the pH above 2 when the 2,4-DNPH solution is added) and 2 uL of the 2,4-DNPH solution at 42°C for 20 min in the dark. The precipitate was removed by centrifugation and the supernatant was analyzed by HPLC. The 2,4-DNPH solvent replaced the solution as a reagent control.

Reduction of Q.S. with $NaBH_4$. An aliquot (10 uL) of Q.S. solution ($\underline{A}_{384}$ = 3.1) was incubated with 1 uL of $NaBH_4$ solution (0.1 M $NaBH_4$ in 0.1 N NaOH) for various times up to 15 min at room temperature in the dark prior to analysis by HPLC. NaOH solution (0.1 N) replaced the

$NaBH_4$ solution as a reagent control.

Oxidation of Q.S. with Iodine. Oxidation of Q.S. with iodine was performed by incubation of 10 uL aliquots of Q.S. solution ($\underline{A}_{384}$ = 3.1) with 2 uL of either H_2O, 0.01 N HCl, or 10 mM potassium acetate (pH 5.0), and 2 uL of iodine solution (1% I_2 in 2% KI) for up to 30 min at room temperature in the dark. The reactions were stopped by titration with a 1% ascorbate solution until the color of iodine was no longer present before analysis by HPLC. A reagent control was obtained by replacing the iodine solution with H_2O. Treatment of the iodine oxidation products with 2,4-DNPH, $NaBH_4$, or $NaIO_4$ was performed as described for Q.S. after titration of the excess iodine with ascorbate.

Mass Spectra. High resolution (70 eV, thermal vaporization), isobutane chemical ionization, and field desorption mass spectra of Q.S. were obtained by Dr. S.K. Sethi and Dr. J.A. McCloskey of the University of Utah with samples of Q.S. provided by the author.

3. RESULTS

The ultraviolet absorption spectrum of Q.S. in H_2O is shown in Figure 21 (p. 71). Q.S. is nonfluorescent in solution. To determine the pKa values of Q.S., the change in absorption at 405 nm with pH was examined (Figure 26); the pKa values were approximated as 2.7 and 10.7. Below pH 2 Q.S. is rapidly degraded (irreversibly) and therefore the absorption measurements made below this pH are inaccurate. The absorption decreases as Q.S. is degraded, so the estimated pKa may actually be greater than 2.7.

There is evidence that Q.S. can exist in more than one form.

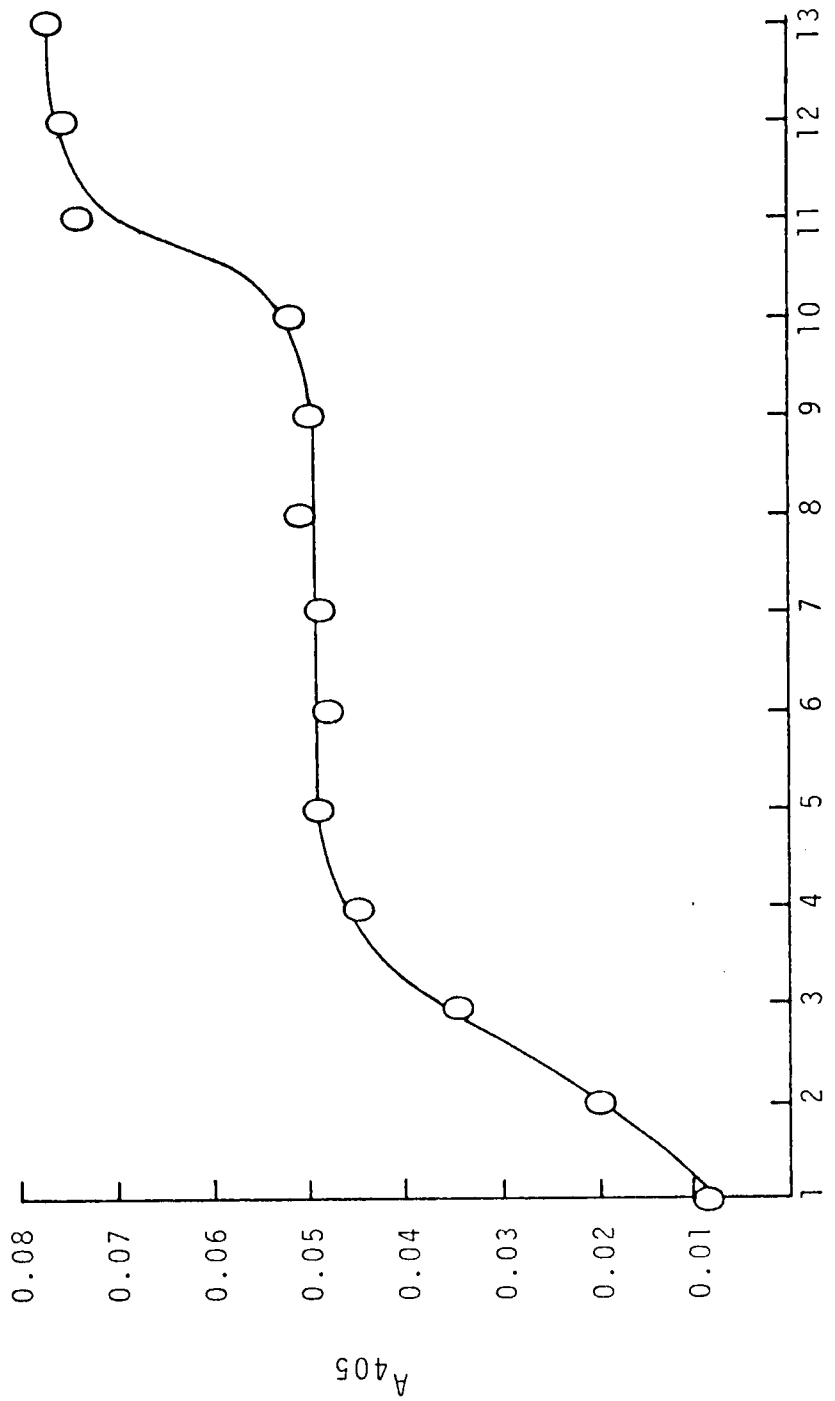


Figure 26. Effect of pH on the absorbance of quench spot at 405 nm.

During the purification of Q.S. it was found that if solutions of Q.S. were dried by lyophilization, rotary evaporation, or with a stream of dry nitrogen, an orange colored compound was formed. This compound was found to be more soluble in water than Q.S., yielding an orange solution. When the solution was diluted to obtain the ultraviolet absorption spectrum, the orange color disappeared and the ultraviolet spectrum indicated that only Q.S. was present. As Q.S. can be regenerated from the orange compound, it is apparently not an oxidation product.

As H_2-neopterin-$(P)_3$ and (X) are precursors of Q.S., we suspected that the side chain of these compounds might be retained in Q.S. and therefore examined the sensitivity of Q.S. to oxidation with alkaline $KMnO_4$. Q.S. was lost after oxidation, but no 6-carboxypterin (which was the product of the oxidation of sepiapterin and the expected product from all 6-alkylpterins) was produced. No fluorescent products or $\underline{A}_{260}$-absorbing products were seen in the HPLC analysis. The reagents used for the oxidation appear as a large $\underline{A}_{260}$-absorbing peak early in the chromatogram; it is possible that an early-eluting nonfluorescent product may have been produced but was obscured by the reagent peak.

While the oxidation of Q.S. with alkaline $KMnO_4$ indicated that it may have retained the side chain from H_2-neopterin-$(P)_3$, the lack of the expected product made other tests necessary. As the side chain of H_2-neopterin-$(P)_3$ contains periodate-sensitive groups, the oxidation of Q.S. with $NaIO_4$ was attempted. Q.S. was readily oxidized by $NaIO_4$ (as was sepiapterin in which case the product was 6-carboxypterin) but no fluorescent or $\underline{A}_{260}$-absorbing products were detected by HPLC (again, an

$\underline{A}_{260}$-absorbing product might be obscured by the reagent peak).

To determine if the periodate-sensitive group contained a carbonyl group, the ability of Q.S. to react with 2,4-DNPH and $NaBH_4$ was tested. Q.S. formed a red precipitate upon reaction with 2,4-DNPH (as did sepiapterin) and like sepiapterin was quickly reduced by $NaBH_4$ (3 min). A nonfluorescent, $\underline{A}_{260}$-absorbing product was detected by HPLC after $NaBH_4$ reduction. This product, which has a retention time half that of Q.S., was sensitive to oxidation with both iodine and $NaIO_4$.

Recently, oxidation of reduced pterins with iodine have been described (Fukushima & Nixon, 1979), so the ability of iodine to oxidize Q.S. was tested. Under the conditions used, Q.S. was oxidized in 30 min to form two nonfluorescent products which were detected by HPLC. These products had retention times of about 30% the Q.S. retention time on the column. While the earlier-eluting product was predominant by $\underline{A}_{260}$ absorption, the amount of the other product could be increased (at the expense of the earlier-eluting product) by increasing the pH at which the oxidation was performed. The presence of acetate buffer inhibited the production of both products. A time course of the oxidation reaction revealed that the two products are independent (i.e., one is not a precursor of the other. As oxidation of pterins with iodine should produce fluorescent products (as did the oxidation of sepiapterin) by increasing the aromaticity of the pyrazine ring, these products are anomalous. Also, neither reacted with either 2,4-DNPH or $NaBH_4$ and only the earlier-eluting product could be oxidized with $NaIO_4$.

A 70 eV mass spectrum of Q.S. is shown in Figure 27. The composition of each fragment was determined by a high resolution mass spectrum

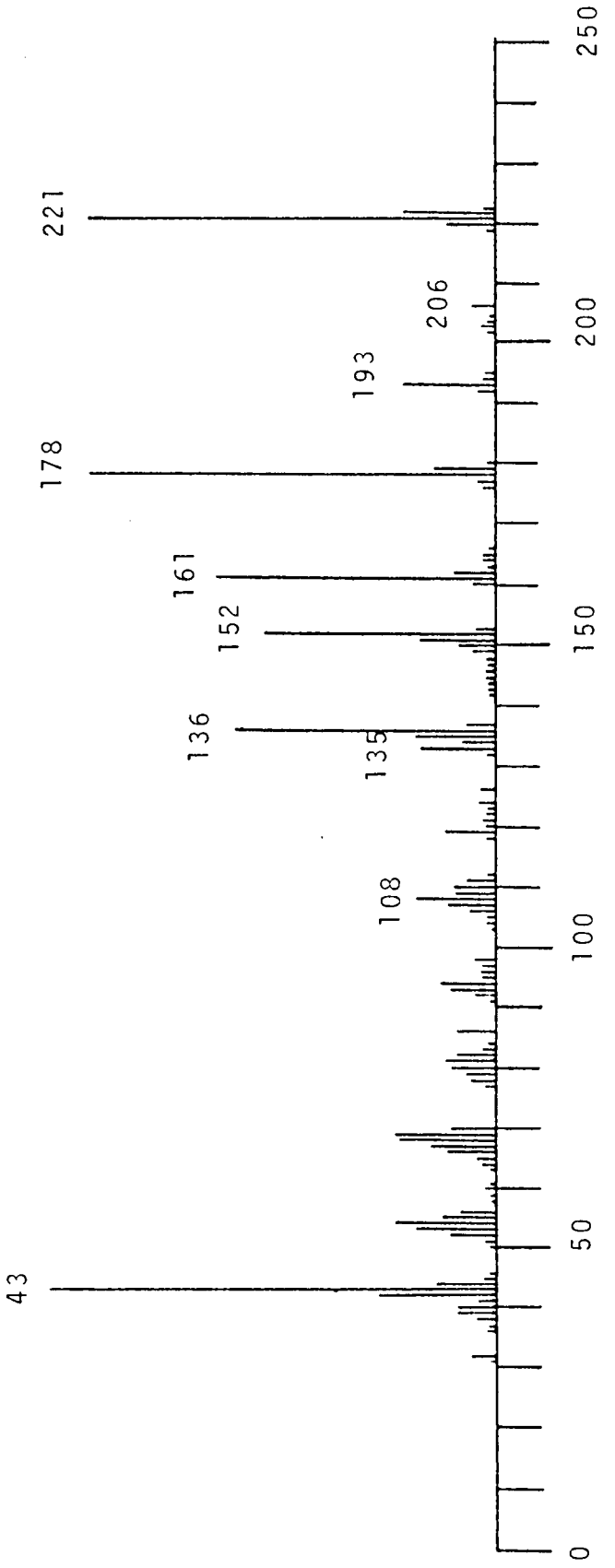


Figure 27. Mass spectrum (70 eV) of quench spot.

(Table V). The m/z=221 fragment is the molecular ion, as in an isobutane chemical ionization spectrum the expected protonated molecule (m/z=222) and adduct ions (M+39, M+41, and M+43) were observed. The field desorption mass spectrum demonstrated fragments at m/z=221 and m/z=222, which provides evidence that thermal vaporization did not cause decomposition of a larger molecule to produce m/z=221.

4. DISCUSSION

Several lines of evidence indicate that Q.S. is a pterin: (1) H_2-neopterin-$(P)_3$ is its precursor, (2) it has an ultraviolet absorption spectrum and pKa values similar to pterins (Blakeley, 1969, pp. 62, 66-71), and (3) the molecular ion in the mass spectrum has a composition ($C_9H_{11}N_5O_2$) indicating that it has retained all the carbon (including the side chain carbons) and nitrogen atoms from H_2-neopterin-$(P)_3$.

The absence of fluorescence from Q.S. indicates the lack of aromatic character in the pyrazine ring. While tetrahydropterins would be nonfluorescent, they are usually unstable and very easily oxidized. When pure, Q.S. has been stored for up to six months in aqueous solutions (at -20^{o}C) without significant degradation. A very small amount of a product (detected by HPLC and identified by elution position and chemical properties), that is also produced by iodine oxidation, was found in the stored solutions. The iodine oxidation of Q.S. is not rapid and the two products formed are anomalous in that they are not fluorescent (which would be expected form an oxidized pyrazine ring). Iodine oxidations of pterins have been examined only recently and anomalous products have been seen (Fukushima & Nixon, 1979). The

TABLE V: Composition of Quench Spot Mass Spectrum Fragments from High Resolution Mass Spectral Analysis.

Found mass	Calculated mass	Ion composition
221.0897	221.0913	$C_9H_{11}N_5O_2$
206.0687	206.0678	$C_8H_8N_5O_2$
193.0966	193.0964	$C_8H_{11}N_5O$
178.0717	178.0729	$C_7H_8N_5O$
161.0452	161.0463	$C_7H_5N_4O$
152.0578	152.0572	$C_5H_6N_5O$
151.0542	151.0494	$C_5H_5N_5O$
136.0508	136.0511	$C_6H_6N_3O$
135.0671	135.0671	$C_6H_7N_4$
133.0509	133.0514	$C_6H_5N_4$
108.0537	108.0562	$C_5H_6N_3$
43.0160	43.0180	C_2H_3O

reaction mechanism is unknown, but an opening of the pyrazine ring is being considered as a possibility (J. Nixon, personal communication).

The absence of fluorescence, the stability of Q.S., and the observation that alkaline $KMnO_4$ oxidation of Q.S. does not yield 6-carboxypterin as a product lead to the hypothesis that the pyrazine ring of Q.S. is substituted at another position in addition to C6. Knowing that Q.S. retains the side chain from H_2-neopterin-$(P)_3$, and that Q.S. is a precursor of "drosopterins", it was postulated that the side chain forms a four-membered ring that includes N5 and C6 of the pyrazine ring and two side chain carbons (C1' and C2') (Figure 28). The ring structure is similar to the five membered ring present in drosopterin and isodrosopterin (Figure 25, page 86) in that both N5 and C6 of the pterin unit are substituted and there is a methyl group to N5.

While the mass spectrum revealed that a three carbon side chain is present in Q.S., it indicated that only a single oxygen atom is present in this side chain. Assuming this to be true there are two possibilities that are consistent with both the periodate sensitivity of Q.S. and its reactivity with the carbonyl reagents (2,4-DNPH and $NaBH_4$). If Q.S. has structure I shown in Figure 28, the mass spectrum could be explained by ring contraction with subsequent loss of CH_3CO (Figure 29) [this mechanism is common in alicyclic epoxides (Porter & Baldas, 1971)]. Epoxides are sensitive to periodate under acidic conditions and could be expected to react with $NaBH_4$ and 2,4-DNPH.

The second possibility is to postulate an equilibrium between a hydrated form (structure II) and a ketone (structure III) in solution. This may provide an explanation for the reversible formation of an

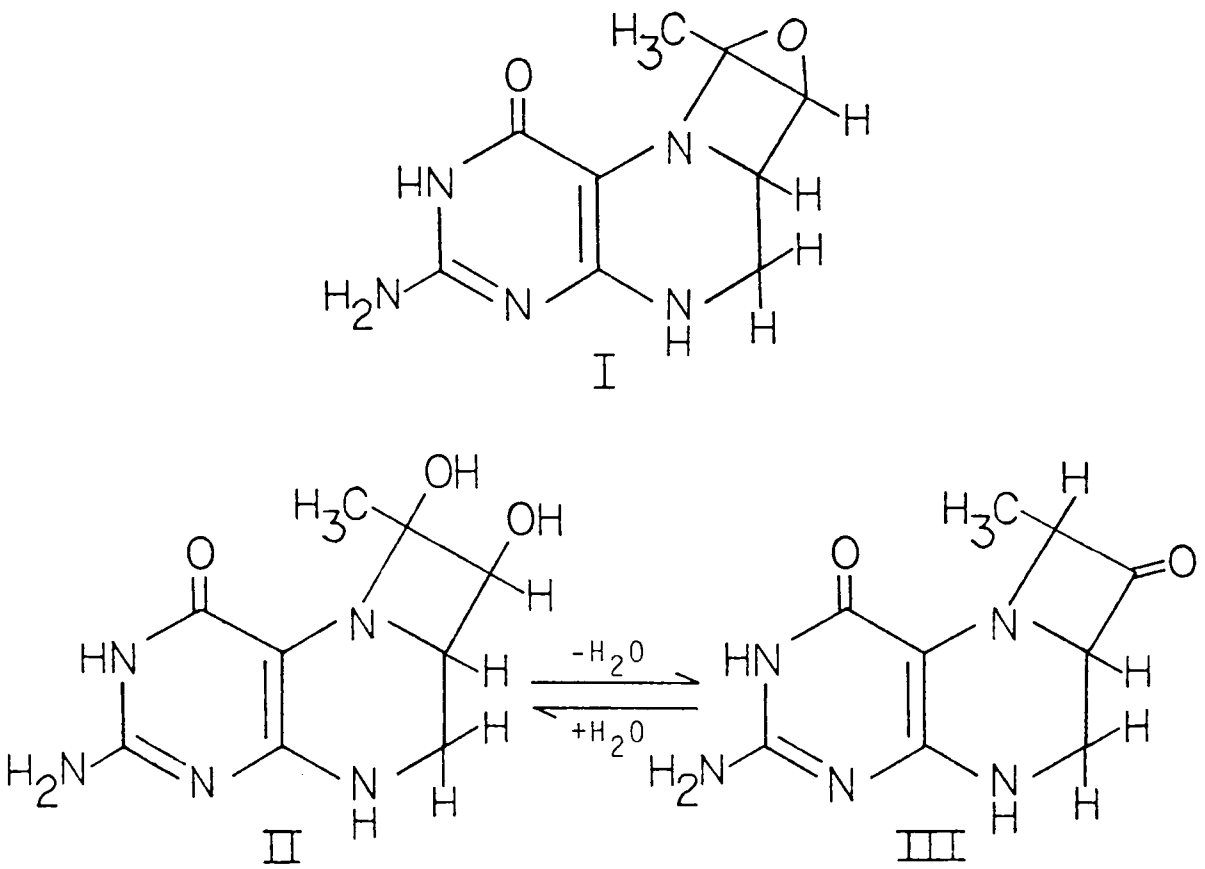


Figure 28. Possible structures of quench spot.

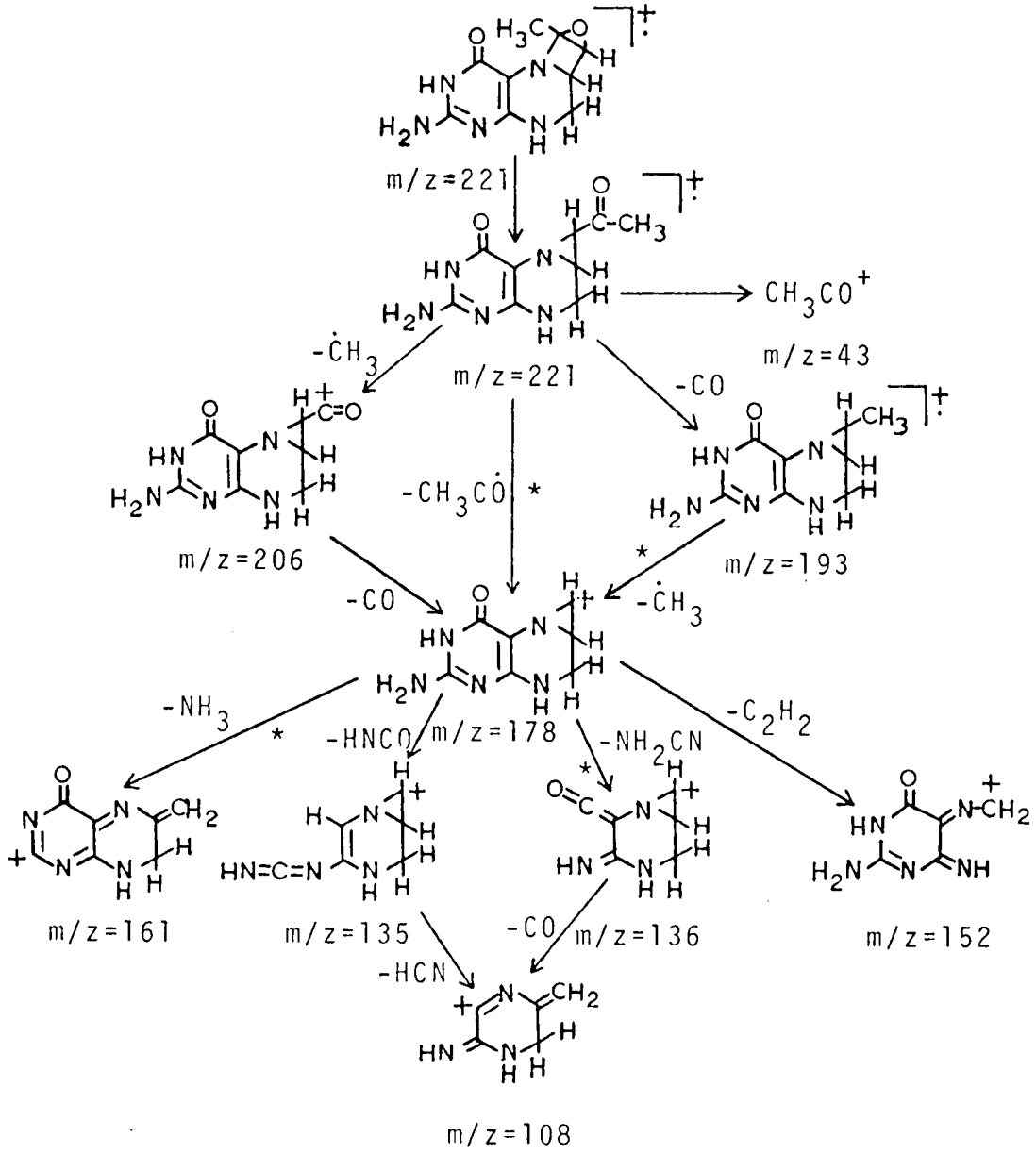


Figure 29. Postulated fragmentation pattern for the proposed epoxide structure for quench spot.

orange compound when Q.S. solutions are dried. The mass spectrum, although more consistent with the epoxide structure (I), could be explained by assuming that the dehydrated form (III) is generated during thermal vaporization and this form then generates the spectrum. The hydrated form (II) has vicinal hydroxyl groups and would therefore be sensitive to oxidation with $NaIO_4$. The ketone form (III) would react with both $NaBH_4$ and 2,4-DNPH, and these reactions could go to completion by continuous removal of the carbonyl form by its reaction with these reagents.

The proposed structures for Q.S. raise a question about the mechanism of synthesis that was not considered when the biosynthesis of Q.S. was under investigation. The precursor of Q.S. is the sepiapterin synthase intermediate (X) which is either at the same oxidation level as H_2-neopterin-$(P)_3$ and sepiapterin ($C_9H_{11}N_5O_2$) (Krivi & Brown, 1979) or more oxidized than both (Tanaka et al., 1979). In either case, a reduction is necessary for the conversion of (X) to Q.S. We found the greatest Q.S. synthetic activity in an extract made with buffer containing Triton X-100, so it is possible that a membrane component, possibly a cytochrome, is required for Q.S. biosynthesis. Final determination of the structure of Q.S. and investigation of its biosynthesis should resolve these questions.

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