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I am submitting herewith a dissertation written by Thomas Gordon Wilson entitled "Studies on the Purple Mutant of 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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STUDIES ON THE PURPLE MUTANT OF *DROSOPHILA MELANOGASTER*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

Thomas Gordon Wilson

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ABSTRACT

An improved thin-layer chromatography technique is described for the separation of fluorescent compounds found in extracts of heads of Drosophila malanogaster. Eighteen of twenty fluorescent spots are resolved, two of which are xanthurenic acid and 3-hydroxykynurenine, and the remaining spots are presumably pteridines. Of these, nine have been identified and were quantitated directly on the chromatograms with a fluorometer.

One of the spots present on the chromatogram apparently has not been described previous to this work. This spot, termed quench spot, quenches under long-wavelength ultraviolet light after chromatography in aqueous solvents; however, it fluorescences bright green after chromatography in an acidified alcoholic solvent. The characteristics of quench spot are described with respect to chromatographic properties, $KMnO_4$ oxidation, absorption spectrum, and ability to support Crithidia growth. Pteridine analyses of Drosophila mutants which have altered levels of quench spot suggest that this presumed pteridine is a component or a metabolite of a component in the drosopterin biosynthetic pathway and is located between sepiapterin and drosopterin.

A pteridine analysis of the purple (pr) mutant in Drosophila established that sepiapterin, quench spot, and

the drosopterins exist in low titers in pr. An allele of pr, purple-brown, has even lower titers of quench spot and drosopterins than does pr. Pteridine analyses of double mutants show that inclusion of pr into the genotype of another eye-color mutant has the effect of lowering the titer of a majority of the pteridines present. Such studies suggest that the affected enzymatic reaction in pr is early in the drosopterin biosynthetic pathway. Nucleotide analyses of pr and wild-type fly heads show no difference in the titers of GTP, the precursor molecule for pteridine biosynthesis. This result is interpreted to mean that sufficient GTP is present for pteridine biosynthesis. Thus, the inhibited enzymatic reaction in pr appears to lie between GTP and sepiapterin in the pteridine metabolic pathway.

Pteridine analysis or pr when suppressed by either of two suppressor loci, suppressor-of-sable [su(s)^{2}] and suppressor-of-purple [su(pr)^{e3}], demonstrates that the titers of sepiapterin, quench spot, and drosopterin are elevated to wild-type levels. This suggests that the two suppressor loci exert their effect on the deficient enzymatic reaction in pr rather than on a different enzyme in an alternate pathway.

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DROPOSILA MUTANTS

Symbol	Name
bw	brown
cl	clot
cn	cinnabar
Hn^{r3}	Henna-recessive 3
mal	maroonlike
pn	prune
pr	purple
pr^{bw}	purple-brown
ras	raspberry
ry	rosy
s	sable
se	sepia
sp	speck
st	scarlet
su(pr)	suppressor of purple
su(s)	suppressor of sable
v	vermilion
y	yellow

ABBREVIATIONS

EDTA	Ethylenediaminetetraacetic acid
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
GMP	Guanosine monophosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
TLC	Thin-layer chromatography
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

The red eye color of the vinegar fly, Drosophila melanogaster, is derived from two very distinct components, which can be separated both chemically and genetically. The color distinction between the two becomes apparent to an observer who genetically dissects the Drosophila eye color by the use of mutants. He finds that the more intensely colored component is a bright red pigment; in the wild-type eye it is dulled by the presence of the other component, a brown pigment. Much of the pioneering work in Drosophila genetics was carried out with mutants which have altered levels of one or both of these pigments and consequently display altered eye colors ranging from white to sepia.

The brown pigment, termed xanthommatin, is a condensation product of 2 molecules of 3-hydroxykynurenine (1). Since 3-hydroxykynurenine is ultimately derived from tryptophan catabolism, xanthommatin is absent in mutants, notably vermilion (v)* and cinnebar (cn), which have lesions in this pathway. It is significant to note that the brilliant transplantation experiments of Beadle and Ephussi

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*The Drosophila mutants and wild-type strains are described by Lindsley and Grell (2).

(3, 4), which provided the basis for the concept of gene action, were carried out with these mutants.

The red pigment is comprised of a group of five red to orange colored pigments called drosopterins. The chemical structures of two of these pigments, drosopterin and isodrosopterin, have been recently elucidated to be dimers of undetermined pteridine precursors (5). Pteridines are found in abundance in Drosophila eye tissue, but the metabolic relationship between the various pteridines generally remains obscure.

Electron microscopy has demonstrated both xanthommatin and pteridines to be localized within the eye tissue. These substances are bound to proteinaceous granules in the pigment cells (6). Xanthommatin-rich granules are located in both primary and secondary pigment cells, while pteridine-containing granules are found only in secondary pigment cells (7). With the exception of isoxanthopterin, all pteridines appear to be bound to carrier protein within the granules, and such binding alters the light absorption properties of at least one of the pteridines, sepiapterin. This pteridine, normally bright yellow, appears brown when bound to carrier protein (8). This color change upon protein binding is also reflected in the phenotype of the sepia (se) mutant, which accumulates sepiapterin and has, as its name implies, very dark brown eyes.

Since purple (pr), the mutant of interest in this work, appears to have a defect in the pteridine biosynthetic pathway (9), attention will be given to this pathway in the following paragraphs. An excellent review of xanthommatin and its biosynthesis appeared recently (10); therefore, no further discussion of xanthommatin will be included in this introduction.

Many Drosophila eye-color mutants exist, most of which have altered levels of pteridines relative to wild-type. The development of a procedure by Mitchell and Hadorn (9) to separate pteridines by paper chromatography and detect them as fluorescent spots under ultraviolet light has enabled investigators to visualize the altered pteridine patterns in eye-color mutants. When examined, however, few of these mutants exhibit clear-cut metabolic blocks and consequent accumulation-depletion patterns, which are so useful in unravelling metabolic pathways. Most mutants have altered pteridine levels producing complex patterns (11) which tend to confuse the investigator more than aid in reconstructing the metabolic relationship among the various pteridines.

In addition to complex pteridine patterns exhibited by eye-color mutants, other factors have hampered our understanding of the pathway. As many as 22 different fluorescent compounds—presumably all but 3 or 4 of them being

pteridines—have been detected in Drosophila extracts subjected to two-dimensional paper chromatography (12). The structures of many of these pteridines are unknown, and perhaps some of these are key intermediates in the pathway. Also, attempts to follow radiolabelled pteridines through the pathway have met with very limited success (13), due in part to the inability of exogenously introduced pteridines to penetrate the eye tissue from the haemolymph. This shortcoming merely confirms the results of imaginal disk transplantation experiments, which demonstrated that isoxanthopterin is the only pteridine which is freely diffusable from haemolymph to eye tissue (14).

Pteridines in Drosophila arise, at least in part, from guanosine triphosphate (GTP). The conversion of GTP to dihydroneopterin triphosphate has been directly demonstrated; and the enzyme, GTP cyclohydrolase, has been partially purified from Drosophila (15). Thus, Drosophila appears to be similar to procaryotes and other eucaryotes with respect to the initial biosynthetic reaction.

The conversion of dihydroneopterin triphosphate to other C-6 sidechain pteridines and ultimately to drosopterin is, unfortunately, less well-understood. It appears that, although no direct evidence exists, biopterin and/or sepiapterin are intermediates in this conversion. Two proposals have been brought forth for "initial pteridine precursor"

(dihydroneopterin triphosphate) conversion to biopterin, a process requiring several enzymatic steps; each is supported by results obtained from in vivo experiments.

The scheme proposed by Goto et al. (16) (Figure 1) envisions successive cleavages of the C-6 sidechain from dihydroneopterin phosphate to form dihydropterin. This compound can autoxidize to yield pterin, which has been identified in Drosophila (17), or it can undergo an addition reaction(s) to restore the C-6 sidechain and ultimately be converted to drosopterin. Evidence for this pathway includes the isolation of labelled 6-methylpterin from adult flies when labelled erythro-neopterin phosphate is fed to larvae and the isolation of labelled drosopterin when radioactive reduced pterin is fed to larvae (16, 18). In addition, labelled reduced pterin and hydroxymethylpterin were found to be converted into sepiapterin (19) and into biopterin (13). These experiments are open to criticism as a result of low incorporation of label into the pteridine products. Moreover, no attempt was made to grow larvae axenically; it is entirely plausable that microorganisms present in the culture medium and gut of the larvae are capable of converting the labelled test compounds to another substance(s) which can enter into pteridine biosynthesis. More decisive experiments, including identification of the substance added to the C-6 position of reduced pterin, are needed to substantiate this hypothesis.

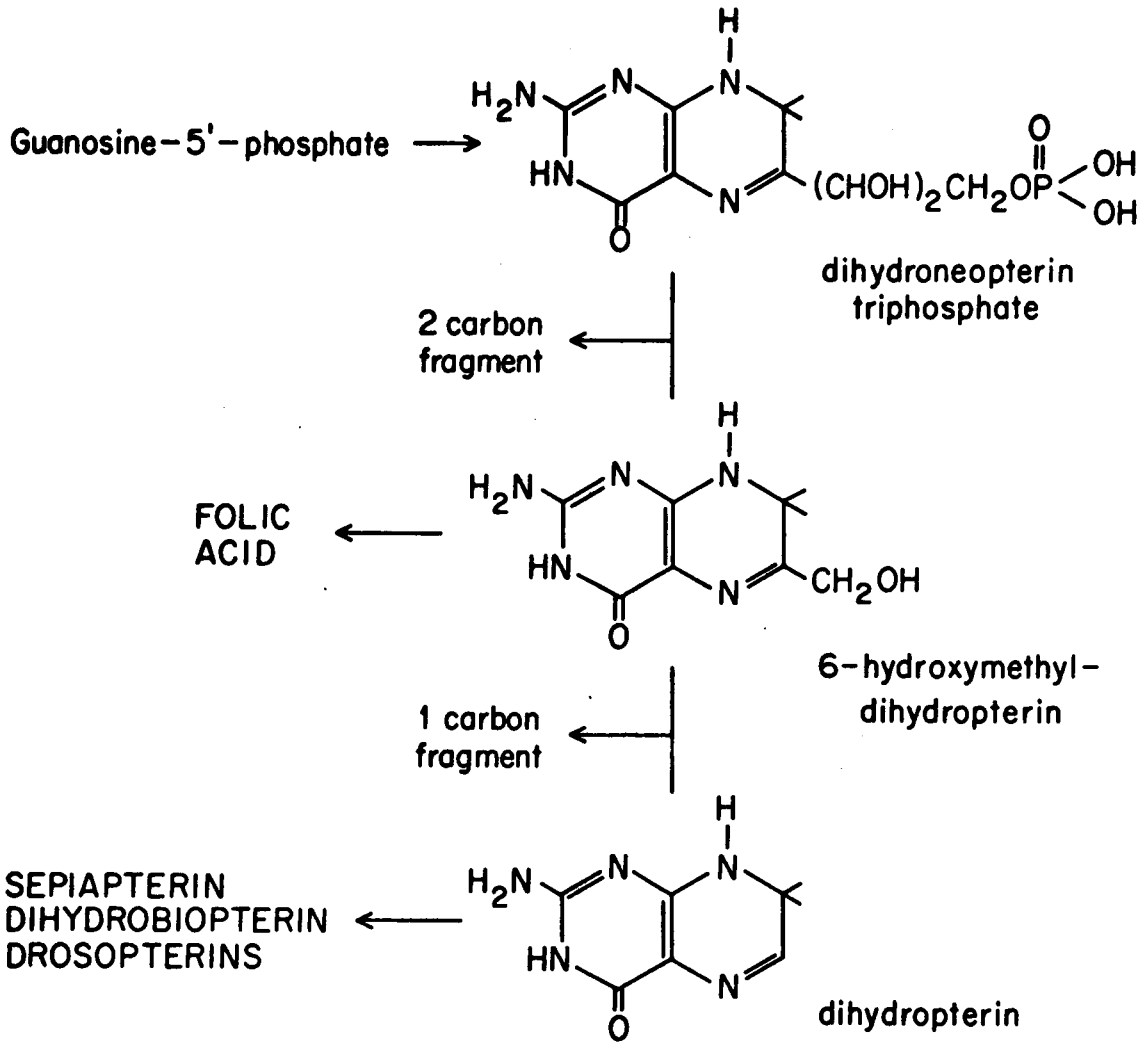


Figure 1. Pathway for the Formation of Folic Acid and Drosophila Pteridines as Proposed by Goto et al.

Another pathway postulated for biopterin biosynthesis in Drosophila is similar to those proposed for Chinese hamster cells in vitro (20) and frog skin tissue in vivo (21) (Figure 2) and appears to be more widely accepted than that of Goto et al. According to this hypothesis, biopterin is formed through alterations in the C-6 sidechain of dihydroneopterin rather than cleavage and addition. Evidence for this pathway in Drosophila stems from the findings that C-2 and C-3 of the biopterin sidechain is derived from C-5 and C-6, respectively, of glucose (22). One would expect this to occur if the carbon backbone of the sidechain of dihydroneopterin, presumably derived from a guanine nucleotide, remains intact during conversion to biopterin. Label from 2-^{14}C-glucose was not found in the sidechain of biopterin. Results from experiments with Chinese hamster cell cultures strongly suggest that sepiapterin is a precursor molecule for biopterin synthesis (20), but no evidence for this relationship exists in Drosophila.

As mentioned previously, no direct evidence exists for the hypothesis that biopterin and/or sepiapterin are precursors for drosopterin biosynthesis. The strongest evidence continues to reside in the observation that biopterin and sepiapterin accumulate to often extraordinarily high titers in Drosophila mutants (se, cl) whose drosopterin content is markedly reduced or absent (9).

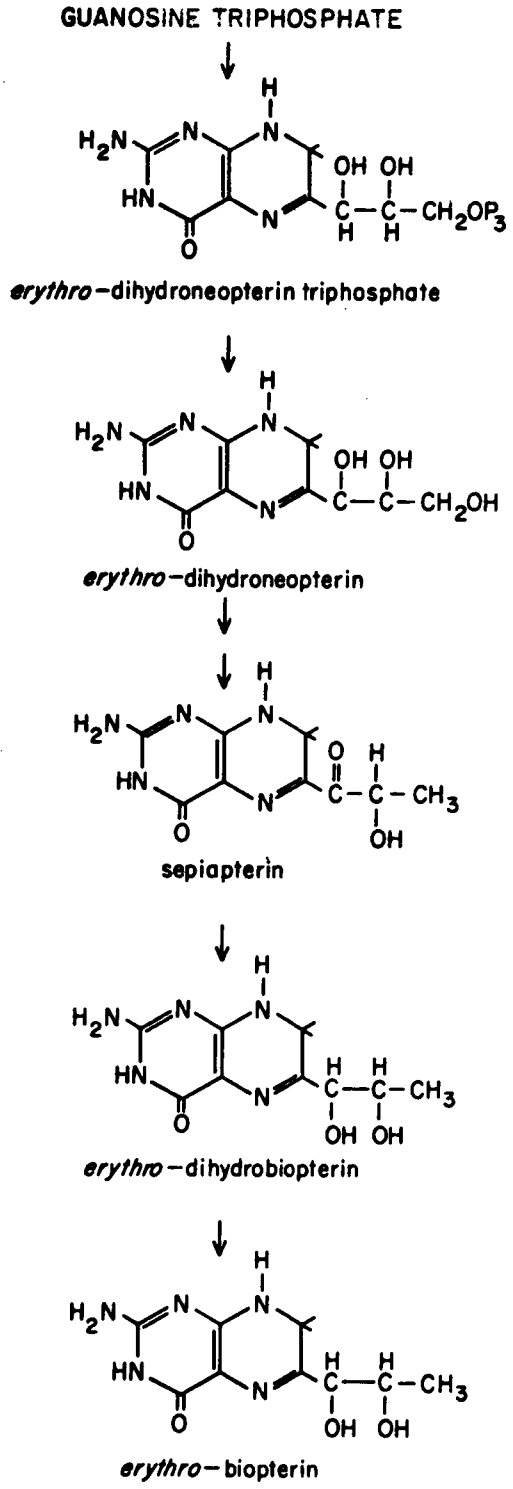


Figure 2. Hypothetical Biosynthesis Pathway of Biopterin in Frog Tissue as Proposed by Fukishima (modified).

Pteridines lacking a C-6 sidechain also exist in Drosophila; the metabolic relationship is known between two of these, pterin and isoxanthopterin. In a well-characterized reaction in Drosophila, pterin is oxidized to isoxanthopterin by xanthine oxidase (23). The rosy (ry) and maroonlike (mal) eye-color mutants lack xanthine oxidase and consequently lack isoxanthopterin (23, 24). Pterin is thought by Taira (25) to arise by nonenzymatic oxidation of tetrahydrosepiapterin.

Having reviewed the present knowledge of the pteridine biosynthetic pathway in Drosophila, I will now consider pr, the eye-color mutant of interest in this work.

Purple is a second chromosomal mutant, which was discovered by Bridges in 1912 and extensively described in 1919 (26). As its name implies, pr has a reddish-purple eye color. Purple is of particular interest because it is a suppressible locus. This phenonomenon will be considered in greater detail in a later part of the introduction.

Few studies have been done on the biochemistry of the pr mutant. Hadorn and Mitchell (9) examined the mutant with their newly developed paper chromatography technique of separating pteridines and found several fluorescent spots to be quantitatively reduced relative to wild-type. Specifically, "F5" (later identified as sepiapterin), "F4" (pterin), "F3" (isoxanthopterin, and "F1" (drosopterin) were

found in lower titers. None of the fluorescent spots was present in an amount greater than that in wild-type, indicating that pr does not accumulate any particular pteridine(s).

Pteridines in pr were further examined quantitatively after separation by paper chromatography using a different solvent system (11). These investigators found reduced levels of all pteridines and noted the xanthopterin-like spot to be completely lacking.

The fact that no pteridine was observed to accumulate in pr suggests that the metabolic block may be only a partial block in the biosynthetic pathway, since a complete block should be reflected by both an accumulation of the pteridine substrate of the affected enzyme and a total depletion of pteridines subsequent to the block. Because most or all of the pteridines are present in the pr mutant at a lower titer relative to wild-type, it would seem that the affected enzymatic step is early in the pathway leading to drosopterin biosynthesis. In fact, the first step, conversion of GTP to dihydroneopterin triphosphate, may be the affected enzymatic reaction. This postulation would not only explain the lowered titers of most or all of the pteridines, but it would also explain the observation that no pteridine accumulates, since GTP is a nonfluorescent compound and would not be visible under ultraviolet light

on the chromatograms. However, the lack of any fluorescent compound accumulation in pr may also be explained by Ziegler's hypothesis (27) that tetrahydrobiopterin, which does not fluoresce, is the precursor for both drosopterin and sepiapterin biosynthesis. If the enzymatic block in pr affects the conversion of tetrahydrobiopterin to these pteridines, tetrahydrobiopterin may indeed accumulate but go unnoticed due to its nonfluorescent property.

As mentioned previously, pr is a suppressible locus. It is suppressed by each of two recessive suppressor loci: suppressor-of-sable [su(s)], and x-chromosomal mutation (28), and suppressor-of-purple [su(pr)], which is located on the third chromosome (29). When either of these two suppressor loci are homozygous in combination with pr, the phenotype of the fly is indistinguishable from that of wild-type. Neither of the suppressor mutants per se has a phenotype different from that of wild-type.

More is known of the biochemical mechanism of suppression by su(s) than by su(pr). $su(s)^2$ suppresses sable (s), vermilion (v), and speck (sp) in addition to pr (30). v flies have bright red eyes due to a complete lack of xanthommatin; tryptophan 2,3-dioxygenase, the first enzyme in the xanthommatin biosynthetic pathway, is inactive (31). s flies are characterized by a dark body color, presumably due to abnormal melanin biosynthesis or

deposition. sp mutants display a black speck on each wing axil; one of the electrophoretic bands of tyrosinase has been found to be absent in pupae (32).

Warner et al. (32) found that su(s)^2 acts in combination with sp to restore the missing tyrosinase electrophoretic band. The mechanism by which su(s)^2 restores this band is unknown. Whether this missing band in sp is responsible for the sp phenotype is uncertain.

An in-depth investigation of the biochemical mechanism of v suppression by su(s) has been carried out. Jacobson (33) found that tryptophan 2,3-dioxygenase could be activated in v extracts by digesting with ribonuclease; this activated enzyme preparation was then inhibitable specifically by a transfer RNA isoaccepter, $tRNA_2^{Tyr}$. Twardzik et al. (34) had previously found that $tRNA_2^{Tyr}$ is absent in su(s)^{2}v flies. Recent work has shown that $tRNA_2^{Tyr}$ is indeed present in the su(s)^{2}v strain but is different from $tRNA_2^{Tyr}$ found in wild-type Drosophila and presumably is unable to completely inhibit su(s)^{2}v tryptophan 2,3-dioxygenase in vivo (35).

Recently, results have been published by Mischke et al. (36) demonstrating that high concentrations of ethylenediaminetetraacetic acid (EDTA), a metal chelating compound, was able to activate tryptophan 2,3-dioxygenase in v extracts and also enahnce the endogenous activity in wild-type extracts. The activated v enzyme preparation was not inhibitable by a

crude tRNA preparation. Whether this activated enzyme is coded for at the v locus remains to be determined; the authors concede that it could be a latent peroxidase activity which is activated by EDTA and capable of oxidizing tryptophan. If this is the case, it may not be the same enzyme as Jacobson was able to activate with ribonuclease; this would explain why Mischke et al. were unable to inhibit the activated enzyme with tRNA.

It is my objective in this work to determine the enzymatic step that is affected in the pr mutant. This will be accomplished by quantitatively examining pteridines in pr and suppressed pr to determine which pteridines have altered titers relative to wild-type and how these titers are affected by the suppressor mutations. Also the GTP titer in pr and suppressed pr will be measured to determine if it is lower than wild-type (suggesting that the enzymatic lesion occurs prior to GTP biosynthesis) or is higher than in wild-type (which would indicate that the lesion is after GTP biosynthesis). When the affected enzymatic reaction is identified in pr, it will be of interest to determine if the suppressor mutations relieve the inhibited enzymatic step by a mechanism analogous to that found in suppressed v flies.

CHAPTER II

SEPARATION, IDENTIFICATION, AND QUANTITATION OF PTERIDINES FROM *DROSOPHILA MELANOGASTER*

I. INTRODUCTION

Since the discovery of Mitchell and Hadorn (9) that *Drosophila* pteridines can be separated by paper chromatography, several other separation methods have been devised, including column chromatography (37, 38), electrophoresis (39, 40), and thin-layer chromatography (41, 42), to name a few.

Paper chromatography, however, has generally remained the method of choice when rapid, effective separation of a wide variety of pteridines (as is found in *Drosophila*) is required. The disadvantages of paper chromatography include a limited sample size and a tendency of several pteridines to diffuse to a considerable extent during the long (6-10 hours) time period required for solvent irrigation of the chromatogram.

Descimon and Barial (43) have taken advantage of cellulose thin-layer chromatography (TLC) to minimize these problems. Upon comparing cellulose TLC to paper chromatography for separating pteridines from whole body extracts of *Drosophila*, these investigators found that cellulose TLC required shorter solvent development times (30 minutes to 2

hours, depending on the solvent) and produced more compact zones of the separated pteridines. They were also able to chromatograph larger quantities of a pteridine standard, xanthopterin, on cellulose thin-layer chromatograms than on paper without the characteristic "tailing," which is indicative of a sample size too large for the resolution capacity of the cellulose support material.

I have taken advantage of Descimon and Barial's solvent systems to develop a procedure using analytical cellulose thin-layer chromatograms (160 micron thickness) for separating pteridines contained in Drosophila head extracts. This procedure results in very reproducible separation of up to 18 presumed pteridines in addition to 2 tryptophan metabolites.

Once separation of the pteridines of interest has been effected, several methods exist for their quantitation. The most common method consists of eluting the pteridine from a chromatogram and measuring the absorbance (44) or the fluorescence (45) of the resulting solution. Fluorescence values can also be obtained directly from dried chromatogram sheets (41, 46).

I have elected to quantitate various Drosophila pteridines by measuring their fluorescence directly on dried thin-layer chromatograms. In addition to the ease and rapidity with which quantitative values can be obtained, this method

is also very sensitive; detection of pteridine quantities as low as 10^{-11} moles is possible.

II. MATERIALS AND METHODS

Materials

Analytical thin-layer chromatograms (Eastman #13255) were obtained from Eastman Kodak Co. Preparative 20 X 20 cm, 250 micron-thick Avicel cellulose thin-layer chromatograms were obtained from Analtech, Inc.

Riboflavin and 3-hydroxykynurenine were obtained from CalBiochem Co. Pterin, isoxanthopterin, xanthopterin, pterin-6-carboxylic acid, folic acid, and xanthurenic acid were obtained from Sigma Chemical Co. Chromatographically purified bacterial alkaline phosphatase was obtained from Worthington Biochemicals. Uniformly labelled ^{14}(C)-guanosine with a specific activity of 423 millicuries/mmole was obtained from New England Nuclear, and 8-^{14}C-guanosine with a specific activity of 34 millicuries/mmole was purchased from Schwarz/Mann.

D-erythroneopterin was a generous gift from Dr. T. Shiota, University of Alabama at Birmingham, and L-erythrobiopterin was kindly provided by Dr. A. Maas, Smith, Kline, and French Co.

Drosophila Culture

Drosophila melanogaster were reared in half-pint milk bottles on medium prepared as described by Lewis (47). Culture conditions included a temperature regimen of 26°±1°C and a 12-hr light-12-hr dark cycle, the light being provided by a 50 watt incandescent bulb located approximately one meter from the culture bottles.

In order to study incorporation of radiolabelled guanosine into pteridines, Hn^{r3} flies were reared axenically on a defined medium. Hn^{r3} females were allowed to oviposit for a four hour period on 2% agar gel which contained 15-20% grape juice. Eggs were then collected, dechorionated, and surface sterilized according to the method of Keith and Goldin (48). Approximately 40 sterilized eggs were placed on the surface of 20 ml of sterile, defined medium (49) with the following modifications: the agar concentration was decreased to 1.75%, crude azolecithin was substituted for purified lecithin, and penicillin and streptomycin were omitted from the medium. Other than radiolabelled guanosine, no RNA or ribonucleosides were added. Two 20-ml defined medium preparations were prepared, one containing 7.5 µCi of uniformly labelled ^{14}C-guanosine and the other containing 7.5 µCi of 8-^{14}-C-guanosine. Hn^{r3} larvae were allowed to grow on these medium preparations under the growth conditions previously described. No evidence of bacterial or fungal contaminations was observed.

Preparation of Drosophila Head Extract for TLC

Drosophila heads were obtained in quantity by a modification of the procedure of Moorefield (50). Lightly-etherized flies were rapidly frozen in 25-ml vials containing liquid nitrogen. The vials were placed in a -85°C freezer until all liquid nitrogen had boiled off. After tightly capping the vial, it was forcefully shaken for 20-30 seconds, a procedure which resulted in complete decapitation of the frozen flies. Heads were separated from bodies by sieving the vial contents through a large mesh tea strainer (EKCO #53461), which restrained bodies and wings, superimposed on a small mesh strainer (ECKO #534351), which restrained heads and passed leg fragments. The strainers were precooled to -85°C to prevent adherence to fly fragments to them.

Each fly head preparation was rapidly weighed on an analytical balance and homogenized in 2:1 propanol : 3.5% NH_4OH v/v in a glass tube and pestle homogenizer at a ratio of 0.4 ml solvent per 10 mg heads. In certain instances 2-mercaptoethanol was included in the homogenizing solvent as a 0.5% v/v addition. The homogenate was then transferred to a 10 X 75 mm glass tube and centrifuged at 3600 X g for 10 minutes.

Preparation of Axenically Cultured Drosophila Head Extract

Three day old Hn^{r3} adults cultured axenically as previously described were decapitated with a razor blade. Heads were homogenized in 2:1 propanol : 3.5% NH_4OH at a ratio of 5 heads per 30 µl solvent in small glass tube and pestle homogenizers. After centrifugation at 3600 X g for 10 minutes, 30 µl aliquots of supernatant was subjected to 2-dimensional TLC in the standard manner described below.

TLC of Drosophila Head Extract

Approximately 18 µl of head extract supernatant was applied onto a circular area approximately 0.5 cm in diameter in the lower left-hand corner at a distance of 3 cm from each edge of an analytical thin-layer chromatogram. The chromatogram was dried with a stream of warm air during sample application. The chromatogram was then subjected to ascending chromatography in 1:1 isopropanol : 2% ammonium acetate until 80% of the sheet was irrigated, a process requiring 6 hours at 19-23°C. After drying, the chromatograph was turned 90° counterclockwise and subjected to ascending chromatography in 3% ammonium chloride until the entire sheet was irrigated (1 hour). If 2-mercaptoethanol was included in the homogenizing solvent, it was also included in both chromatography solvents at a concentration of 0.1% v/v. After the chromatogram was dried, the pteridines of interest were immediately quantitated.

In order to separate the "quench spot" for quantitation, 1-dimensional TLC in another solvent system was carried out. A volume of 9-10 µl of supernatant was applied to a 0.5 cm diameter area 3 cm from the edge of a 4 X 20 cm strip from an analytical thin-layer chromatogram. The chromatogram was developed in an ascending direction in 4:1:1 butanol : glacial acetic acid : H_2O. Four to five hours were required for complete irrigation of the sheet.

Quantitation of Pteridines

Following either 2-dimensional or 1-dimensional TLC, pteridines to be quantitated were circled with a pencil while illuminated under long wavelength ultraviolet light. The sheet was then cut into 4-cm strips, and each strip was mounted with tape on the chromatogram door of a Turner model 110 fluorometer. The strip was illuminated with light from a general purpose lamp filtered through a #760 (filter numbers are according to the Turner catalog) narrow band-pass filter with a transmittance maximum at approximately 360 nm. Secondary filters utilized included #2A, a sharp-cutoff filter passing light above 410 nm; #NDI, a 10% neutral density filter; and one of three filters specific for the particular pteridine being quantitated. To measure sepiapterin and the quench spot, #58, a narrow band-pass filter with a transmittance maximum at approximately 530 nm was used with an aperature

setting of 1X (quench spot) or 3X (sepiapterin). To quantitate drosopterins, #23A, a narrow band-pass filter passing light above 560 nm, was utilized in conjunction with the 1X aperature. Finally, biopterin, pterin, and isoxanthopterin were measured with a #47B filter, which is a narrow band-pass filter with a transmittance maximum of around 440 nm, and an aperature setting of 3X.

After blanking the fluorometer against a nonfluorescing section of the chromatogram strip, the fluorescence value was recorded.

Preparation of Quench Spot Substance

A quantity of heads of Hn^{r3}, in which the quench spot substance accumulates, was homogenized in 2:1 propanol : 3.5% NH_4OH in a glass tube and pestle homogenizer at a ratio of 0.3 ml solvent per 10 mg heads. After centrifugation at 3600 X g for 10 minutes, the supernatant was streaked across a line 3 cm from the edge of a preparative 250 micron-thick cellulose thin-layer chromatogram. Up to 1 ml of supernatant could be streaked on each chromatogram without loss of resolution. The chromatogram was developed in an ascending direction in 4:1:1 butanol : glacial acetic acid : H_2O and dried after complete irrigation. The quench substance, which was identified by its Rf value of approximately 0.6 and its green fluorescent color, was scraped from the plate

and eluted twice with 3 ml of distilled H_2O over a period of 1 hour. After low-speed centrifugation to pellet the cellulose particles, the supernatant was evaporated to near-dryness in a stream of nitrogen gas. The residual volume (approximately 200 µl) was streaked across a line 3 cm from the edge of an analytical thin-layer chromatogram; the chromatogram was subjected to ascending chromatography in distilled H_2O. The quench substance, which had no fluorescent color after TLC in this solvent, was scraped from the sheet and eluted twice from 1 ml of distilled H_2O. Quench substance prepared in this manner was free from fluorescent impurities when chromatographed in either 4:1:1 butanol : glacial acetic acid : H_2O isopropanol : 5% boric acid.

Preparation of Sepiapterin

In order to prepare a quantity of sepiapterin, advantage was taken of Dewey and Kidder's observation (38) that dihydropteridines separate from fully oxidized pteridines during chromatography on Sephadex. Forty mg of se fly heads were homogenized in 2 ml of 0.5 N perchloric acid in a glass tube and pestle homogenizer. After centrifugation at 10,000 X g for 20 minutes, the supernatant was extracted twice with 20% w/v alamine dissolved in Freon-TF[2] (51) to extract the perchloric acid. The aqueous phase was then applied to a 2.3 X 50 cm Sephadex G-25 column equilibrated with distilled

H_2O, and the column was eluted with distilled H_2O at a flow rate of approximately 1 ml/min. The yellow sepiapterin band, which separated from two blue fluorescent bands, was collected. Two-dimensional TLC of an aliquot from this sepiapterin fraction revealed only a trace contamination of a blue fluorescent compound.

Alkaline Phosphatase Digestion

Oregon-R fly head extract for alkaline phosphatase digestion was prepared by homogenizing heads in 0.1 N NaOH in a glass tube and pestle homogenizer at a ratio of 0.4 ml NaOH per 10 mg heads. After centrifugation at 3600 X g for 10 minutes, the supernatant was titrated to pH 8.0 with 1M tris-HCl. The supernatant was divided into two fractions: bacterial alkaline phosphatase was added to one fraction, and the other fraction served as an untreated control. After incubation at room temperature for 1 hour, aliquots from both fractions were applied to analytical TLC sheets, which were then subjected to 2-dimensional TLC as previously described.

Miscellaneous Methods

Alkaline potassium permanganate oxidation of pteridines was carried out according to the procedure of Kidder and Dewey (52). Both L-erythrobiopterin and pterin-6-carboxylic acid were used as standards and were carried through the

$KMnO_4$ oxidation procedure in the same manner as the pteridine of interest. After the oxidation reaction was completed, the MnO_2 product was removed by low-speed centrifugation, and aliquots of each supernatant were applied to analytical TLC sheets and developed sequentially in two dimensions in the following solvent systems: 1:1 isopropanol : 2% ammonium acetate and 3% ammonium chloride. The Rf value of oxidized pteridine in these solvent systems was compared with those of the standards.

Catalytic hydrogenation of the quench substance was carried out in subdued light for two hours in a 12-ml conical centrifuge tube. Hydrogen gas was continuously bubbled through a solution of quench substance in 0.1 N HCl to which 20 mg/ml of platinum oxide had been added. Afterwards, PtO_2 was removed by low-speed centrifugation.

Absorbance spectra were recorded with either a Beckman Acta III or Acta V recording spectrophotometer. Absorbance measurements at a fixed wavelength were determined on a Gilford spectrophotometer.

Molar Extinction coefficients used to quantitate various pteridines are presented in Table 1.

The solvent systems utilized for separation of pteridines by TLC are shown in Table II.

All procedures subsequent to weighing of the fly heads were carried out either under red light or in darkness.

TABLE I

PTERIDINE MOLAR EXTINCTION COEFFICIENTS

Pteridine	Molar Extinction Coefficient	Wave-length (nm)	Solvent	Reference
Pterin	7,100	360	1% NH_4OH	53
Isoxanthopterin	14,200	339	0.1N NaOH	54
Biopterin	8,300	360	0.1N NaOH	55
Sepiapterin	10,400	420	pH 6.8	56

TABLE II

TLC SOLVENT SYSTEMS

Designation	Solvent Composition
A	1:1 isopropanol : 2% ammonium acetate
B	3% ammonium chloride
C	4% sodium citrate
D	2:1 propanol : 1% NH_4OH
E	4:1:1 butanol : glacial acetic acid : H_2O
F	4:1 isopropanol : 5% boric acid
G	2:1:1 ethanol : 5% boric acid (pH 8 with NH_4OH) : 3% ammonium chloride

III. RESULTS

Identification of Fluorescent Spots

The appearance of a typical thin-layer chromatogram under long wavelength UV light after 2-dimensional TLC of an Oregon-R head extract is shown in Figure 3. From eighteen to twenty fluorescent spots are resolved, two of which are tryptophan metabolites, and the remaining are presumed pteridines or pteridine derivatives.

The identity of most of these fluorescent spots has been determined by one or more of the following criteria: characteristic fluorescent color, Rf values in various solvent systems, behavior in various Drosophila mutants, chemical modification, and comparison with an authentic standard.

Two spots have been identified as tryptophan metabolites. One of these, spot K, appears to be xanthurenic acid, based on the similarity of its chromatographic behavior with that of authentic xanthurenic acid (Table III). In addition, the light blue fluorescent color of spot K is indistinguishable from that of xanthurenic acid.

The other tryptophan metabolite, spot P, is presumed to be 3-hydroxykynurenine, based on the similarity of their fluorescent colors and Rf values (Table IV). Also, spot P, but not spot K, is absent in cn flies, a mutant whose conversion of kynurenine to 3-hydroxykynurenine is known to be defective (57).

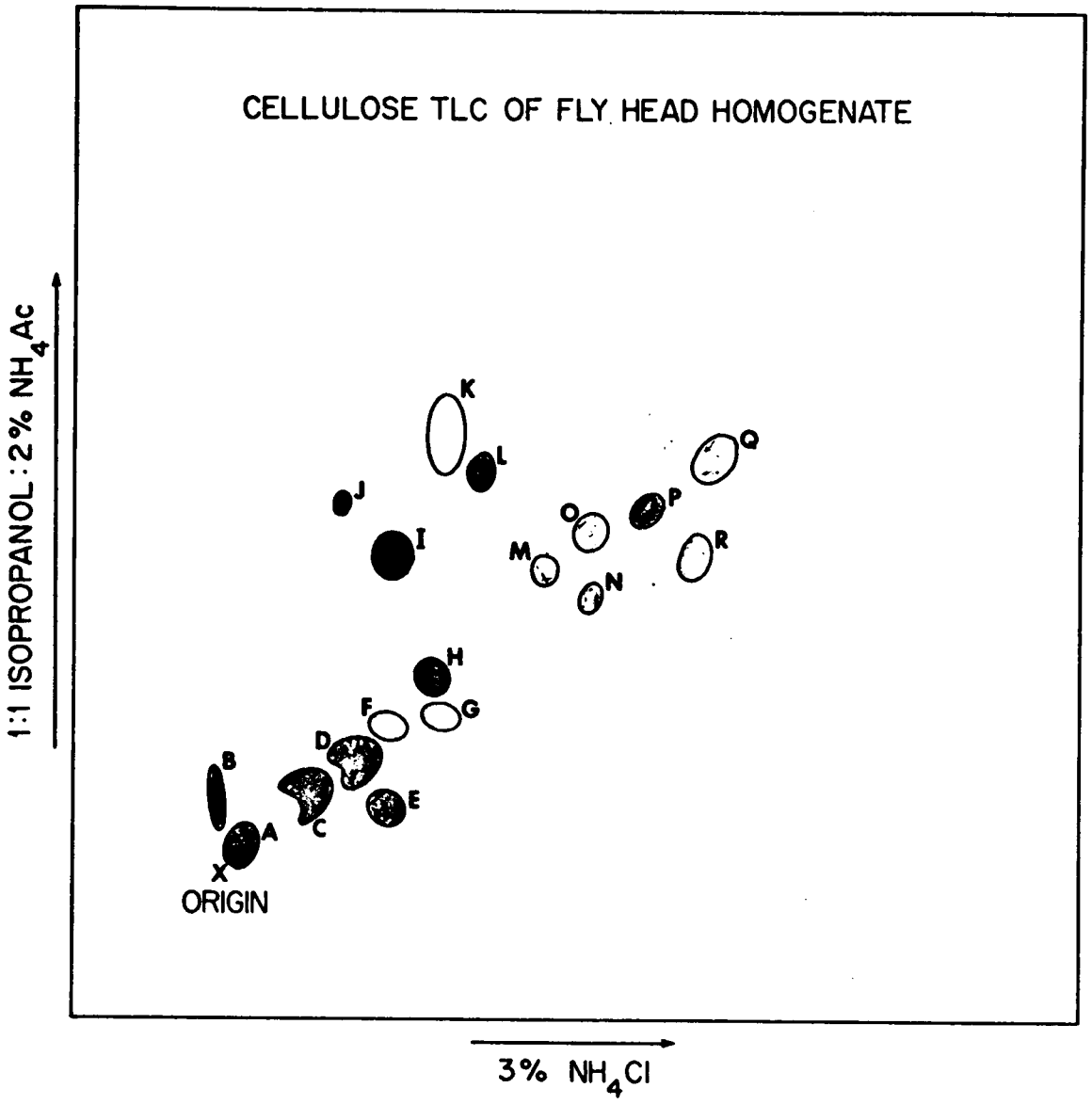


Figure 3. Cellulose Thin-Layer Chromatogram of the Fluorescent Spots from Drosophila Head Extract.

TABLE III

COMPARISON OF RF VALUES OF SPOT K WITH XANTHURENIC ACID AFTER CELLULOSE TLC

Solvent	Rf Value	
	Spot K	Xanthurenic Acid
A	.70	.73
B	.28	.27
A + 0.1% 2-Mercaptoethanol	.84	.84
B + 0.1% 2-Mercaptoethanol	.28	.26

TABLE IV

COMPARISON OF RF VALUES OF SPOT P WITH 3-HYDROXYKYNURENINE AFTER CELLULOSE TLC

Solvent	Spot P	3-Hydroxykynurenine
A	.55	.55
B	.55	.53

In addition, both of these spots are present in the bw mutant, which lacks most or all pteridines. Spots K and P are absent in v, a mutant incapable of biosynthesizing xanthommatin. Moreover, both spots are present in small amounts in $su(s)^2v$ flies, in which xanthommatin biosynthesis is partially restored.

Spots A-D constitute the drosopterin fraction. These pteridines are readily recognized by their red or orange fluorescent color and their red color in visible light. Drosopterins are absent in the bw mutant (58) and in the se mutant (11), as are compounds A-D. Although bw lacks almost all pteridines, se lacks primarily the drosopterin fraction and actually accumulates many other pteridines.

Spot A appears to be identical to neodrosopterin. This identification is based on its red fluorescent color and very low Rf value in both aqueous and alcoholic solvents, properties which are characteristic of neodrosopterin (39, 59).

Spot B corresponds to drosopterin fraction (3) described by Schwinck and Mancini (59). It is present in a relatively small quantity and appears more as a streak than as a distinct spot on the TLC sheet. It is absent in pn^2 and ras^2, as is fraction (e) (59).

Spot E corresponds to drosopterin fraction (3) (59) judging from its yellowish-orange fluorescent color and its absence in pn^2 and ras^2. This drosopterin fraction has

recently been designated aurodrosopterin (60), a term which will be adopted in this work.

Spots C and D correspond to drosopterin and isodrosopterin, respectively. The two can be distinguished based on a larger drosopterin (59), and spot C, fraction in wild-type flies, in agreement with Schwinck and Mancini's observation. In addition, both spot C and drosopterin possess smaller Rf values in 6% acetic acid than do compound D and isodrosopterin.

Spot H has been identified as isoxanthopterin. Spot H has a fluorescent color and Rf value (Table V) identical to that of authentic isoxanthopterin. Spot H is absent in $\underline{ry^2}$, a mutant previously shown to lack isoxanthopterin (61).

TABLE V

COMPARISON OF RF VALUES OF SPOTS H AND O WITH PTERIDINE STANDARDS AFTER CELLULOSE TLC

Spot/Standard	Solvent System A	Solvent System B
H	.23	.26
Isoxanthopterin	.23	.28
O	.46	.46
Pterin	.46	.45

Fluorescent spots I and J appear to be sepiapterin and isosepiapterin, respectively. The fluorescent color of both spots is bright yellow, as are those of sepiapterin and isosepiapterin. Moreover, both compounds accumulate in large amounts in the se mutant, as do sepiapterin and isosepiapterin (9). The two pteridines are distinguished on the basis of their Rf values, sepiapterin migrating slower in 1:1 propanol : 2% ammonium acetate, but faster in 3% ammonium chloride than does isosepiapterin (62). In addition, spot I gives a product chromatographically identical to pterin-6-carboxylic acid after $KMnO_4$ oxidation, a result which rules out the possibility that spot I is riboflavin. Viscontini and Mohlmann (62) found riboflavin, a yellow fluorescent compound, to possess Rf values similar to sepiapterin in three different solvent systems.

Spot O appears to be pterin, based on its similarity to authentic pterin. The fluorescent colors of the two compounds after TLC are identical. Likewise, the Rf values of spot O in two solvent systems are similar to those of pterin (Table V).

Spot Q has been identified as biopterin. Its light blue fluorescent color and Rf values (Table VI) are similar to those of authentic L-erythrobiopterin. In addition, material from spot Q gives a $KMnO_4$ oxidation product which possesses an Rf values similar to that of pterin-6-carboxylic acid.

TABLE VI

COMPARISON OF RF VALUES OF SPOT Q WITH L-ERYTHROBIOPTERIN AFTER CELLULOSE TLC

Solvent	Rf Value	
	Spot Q	L-erythrobiopterin
A	.57	.58
B	.60	.60
F	.35. .20 (minor)	.38
G	.51. .34 (minor)	.53

When spot Q is chromatographed in solvents containing borate, which other investigators have found effective in separating the erythro and threo isomers of biopterin (52, 63), the resulting Rf values obtained indicates that spot Q is the erythro isomer of biopterin (Table VI). A minor component—perhaps 10% of the major component—of spot Q separates in solvents F and G and may be threobiopterin, but this possibility has not been further investigated.

Spot R may be neopterin, but the identification is less certain than for other pteridines. The Rf values of spot R and D-erythroneopterin after cellulose TLC are similar (Table VII) yet some doubt remains as to whether the two compounds are identical with respect to chromatographic behavior. Spot R is present in small amounts in Oregon-R

flies relative to other pteridines, and it does not seem to accumulate with age after eclosion. Compound R gives a $KMnO_4$ oxidation product that is chromatographically indistinguishable from pterin-6-carboxylic acid.

TABLE VII

COMPARISON OF RF VALUES OF SPOT R WITH D-ERYTHRONEOPTERIN AFTER CELLULOSE TLC

Solvent	Rf Value	
	Spot R	D-erythroneopterin
A	.43	.46
B	.58	.55

Spots M and N have not been identified. Both are light blue in fluorescent color. In some Drosophila mutants, particularly se, these spots accumulate and are smeared together, an observation which may be indicative of another fluorescent spot chromatographing in the same region. Sharper resolution of these presumed pteridines can be achieved by developing the chromatograms in 4% sodium citrate instead of 3% ammonium chloride in the second dimension.

Spots F and G have not been identified, but they appear to be alkaline degradation products, arising during homogenization of fly heads, of some unknown compound. These spots

are not detectable on thin-layer chromatograms after chromatography of a wild-type head extract if the heads are homogenized in 2:1 propanol : 3% ammonium acetate. If the centrifugation pellet from this homogenization is itself homogenized in 2:1 propanol : 3.5% NH_4OH, spots F and G do not appear after TLC of the supernatant. This observation indicates that the precursor for F and G is in fact extracted in 2:1 propanol : 3% ammonium acetate. Moreover, if NH_4OH is added to the 2:1 propanol : 3% ammonium acetate homogenization supernatant to produce a final NH_4OH concentration of 3.5%, spots F and G appear after TLC of this ammonical supernatant. These experiments suggest that F and G are indeed alkaline degradation products.

Degradation of the unknown precursor to compounds F and G can be prevented by the inclusion of 0.5% 2-mercaptoethanol or ascorbic acid in the homogenizing solvent. 2-mercaptoethanol appears to prevent the formation of F and G rather than chemically reduce F and G to nonfluorescing compounds, as shown in Table VIII. Once F and G are formed, they cannot be reduced by 2-mercaptoethanol to nonfluorescing products.

The possibility that 2-mercaptoethanol is simply modifying the NH_4OH present in the homogenizing solvent, thereby preventing F and G formation, is discounted by two observations. First, the pH of 3.5% NH_4OH which contains

0.2% 2-mercaptoethanol is 11.5. Second, inclusion of 0.5% 2-mercaptoethanol in 2:1 propanol : 3.5% NH_4OH and subsequent homogenization of wild-type fly heads results in complete extraction of all pteridines relative to that obtained by homogenization in 2:1 propanol : 3.5% NH_4OH; as will be shown in a later part of this section, pteridine extraction is dependent upon NH_4OH concentration in the homogenizing solvent. Incomplete extraction occurs when heads are homogenized in lower NH_4OH concentrations or in 2:1 propanol : 3% NH_4AC.

TABLE VIII

EFFECT OF 2-MERCAPTOETHANOL ON FORMATION OF SPOTS F AND G

Presence of 2-Mercaptoethanol		Presence of
Homogenization Solvent	TLC Solvent	Spots F and G
+	+	-
-	-	+
+	-	-
-	+	+

Interestingly, the precursor for compounds F and G is apparently absent in extracts of several Drosophila mutants examined. The presence of F and G could not be detected after

TLC of bw, v, cn, st, or ry^2. However, F and G are present in small amounts in $su(s)^2v$. The se mutant exhibits enhanced titers of spots F and G. In this case, inclusion of 2-mercaptoethanol in the homogenization and chromatography solvents decreases, but does not eliminate, the breakdown of precursor to F and G. It was also noted that the precursor substance of F and G disappears with age after eclosion of wild-type flies. Spots F and G are most pronounced in newly eclosed flies and barely perceptible in flies greater than 6 days old.

Spot L, which will be referred to as quench spot, appears as a weak quenching spot under long wavelength UV light. However, when chromatographed in certain other solvent systems, it fluoresces dull to bright green (Table IX).

TABLE IX

FLUORESCENT COLOR OF QUENCH SPOT IN VARIOUS CHROMATOGRAPHIC SOLVENTS

Solvent	Fluorescent Color
3% NH_4CL	none
2:1 propanol : 2% NH_4OH	dull green
4:1:1 butanol : acetic acid : H_2O	bright green
4:1:1 isobutanol : acetic acid : H_2O	bright green
4:1:1 butanol : propionic acid : H_2O	bright green
4:2 butanol : H_2O	none
5:1 H_2O : acetic acid	none

The ability of quench spot to fluoresce bright green can be reversed by rechromatography in 3% ammonium chloride to yield apparently the original quench spot with its characteristic Rf value in 3% ammonium chloride. Thus, it does not appear that a permanent alteration of the compound occurs when it is chromatographed in 4:1:1 butanol : acetic acid : H_2O. As can be seen in Table IX, the bright green fluorescent color after chromatography in 4:1:1 butanol : acetic acid : H_2O is not peculiar to butanol or acetic acid. The same fluorescent color and very similar Rf values are obtained when quench substance is chromatographed in 4:1:1 butanol : propionic acid : H_2O or 4:1:1 isobutanol : acetic acid : H_2O. As shown also in Table IX, both alcohol and acid are necessary components of the solvent system in order for the quench spot to fluoresce.

If the quench material is chromatographed in 3% ammonium chloride, it will fluoresce bright yellow if the TLC sheet is cooled with liquid nitrogen and viewed under long wavelength ultraviolet light. This property is useful for detecting small amounts of quench spot after TLC in an aqueous solvent.

Quench spot is oxidized, but not to pterin-6-carboxylic acid, upon treatment with alkaline $KMnO_4$. Even if the oxidation is allowed to proceed gently at room temperature, quench spot is apparently destroyed. No fluorescent spot

can be detected after TLC of the spent $KMnO_4$ reaction, even in 4:1:1 butanol : acetic acid : H_2O or when the chromatogram is viewed in liquid nitrogen.

The absorbance spectrum of a quench spot preparation is shown in Figure 4. As can be seen, the substance exhibits multiple absorbance peaks, one in the far UV region and two in the near UV. When a quench spot preparation is catalytically hydrogenated, all absorbance peaks are lost (Figure 4); such a reduced preparation is not autoxidizable, even after standing at room temperature for several hours or stored at -20°C for 2 weeks. If an aliquot of this reduced preparation is chromatographed on cellulose TLC sheets in 3% ammonium chloride, no quench spot or fluorescent products can be detected.

The quench spot does have activity for growth support of Crithidia, but it has very low activity compared with biopterin. A sample of fluorescently pure quench spot having an absorbance of 260 nm of 1.3 was analyzed by Dr. T. Shiota in a Crithidia assay (64). He found the preparation at that concentration to have Crithidia activity equivalent to 7 ng/ml of erythrobiopterin. This value is an average of 2 determinations, one of 8 ng/ml and the other of 6 ng/ml. Making a rough assumption that the quench substance has a molar extinction coefficient near that for xanthopterin (6500 at 392 nm) (65) and using that extinction coefficient

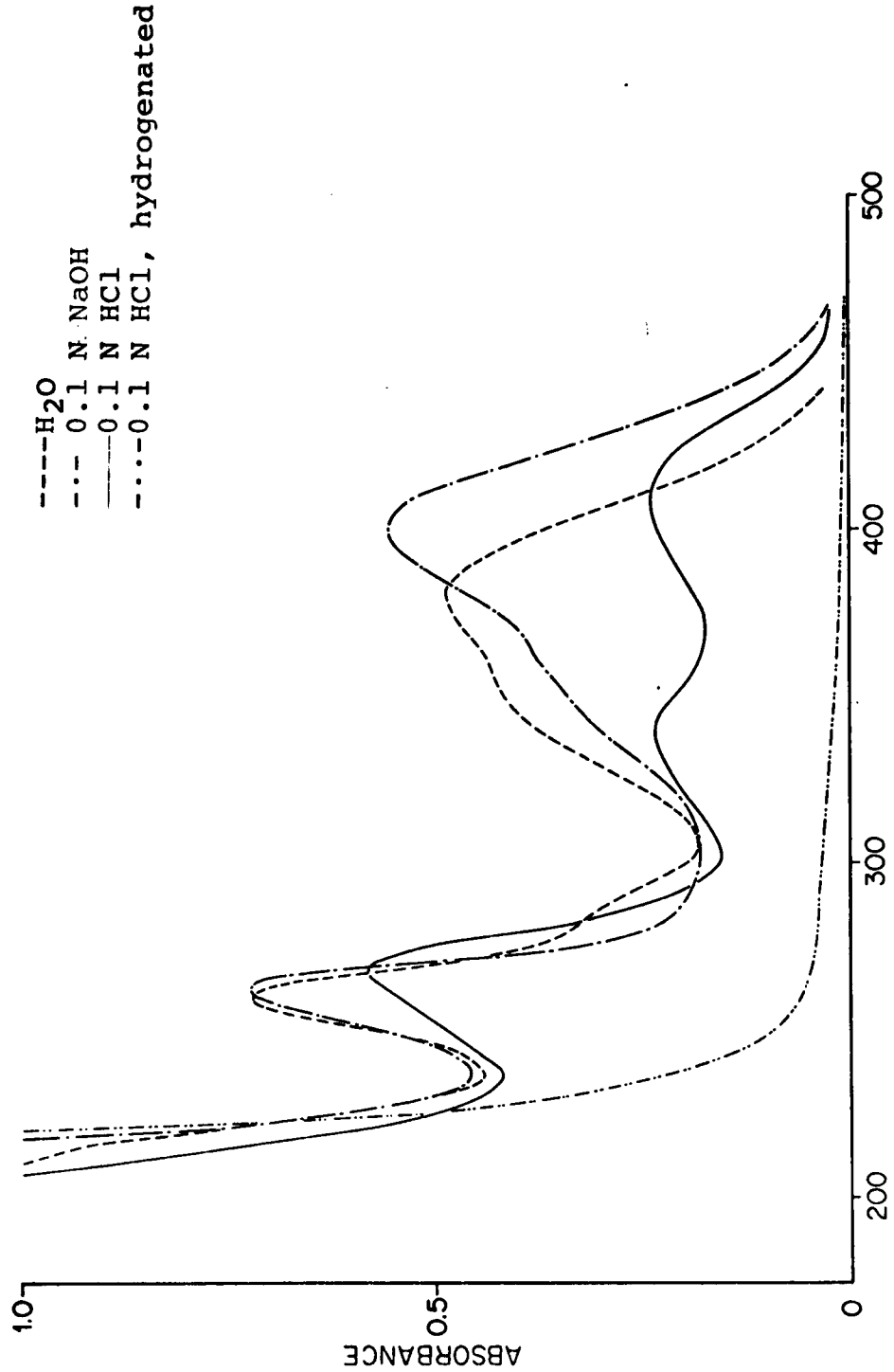


Figure 4. Absorption Spectrum of Quench Spot

at 260 nm plus a molecular weight estimate of 200 g/mole, a concentration of 4 X 10^4 ng/ml can be calculated for the quench spot sample. Therefore, biopterin is 6 X 10^3 more active than quench spot in supporting Crithidia half-maximal growth. This result assumes that the quench spot preparation was free from contaminants active in supporting Crithidia growth. Although no fluorescent contaminants were present in the preparation, the possibility that a nonfluorescing contaminant was responsible for the Crithidia activity cannot be excluded. Nevertheless, quench spot has very low, if any, Crithidia activity relative to that of biopterin.

When Hn^{r3} flies were axenically reared on medium containing uniformly labelled guanosine, radioactivity was recovered in the eluted quench spot after 2-dimensional TLC of a head extract from 3-day-old adults (Table X).

TABLE X

RADIOACTIVITY RECOVERED FROM ELUATES OF VARIOUS PTERIDINES AFTER TLC ANALYSIS OF Hn^{r3} CULTURED ON MEDIUM CONTAINING RADIOACTIVE GUANOSINE

Radiolabelled Compound	Pteridine	CPM Minus Background In Eluate
U-^{14}C-guanosine	sepiapterin	15
U-^{14}C-guanosine	biopterin	19
U-^{14}C-guanosine	quench spot	62
8-^{14}C-guanosine	sepiapterin	20
8-^{14}C-guanosine	biopterin	18
8-^{14}C-guanosine	quench spot	23

Hn^{r3} flies reared on medium containing 8-^{14}C-guanosine incorporated one-third of the radiolabel in the quench spot as did those reared on U-^{14}C-guanosine. Since the 8-carbon is eliminated from guanosine during pteridine biosynthesis, the experiment suggests that guanosine may be a precursor for quench spot biosynthesis and that quench spot may be a pteridine or pteridine derivative. Due to the fact that the amount of labelled guanosine incorporated was very low and also the fact that no label from U-^{14}C-guanosine was incorporated into biopterin or sepiapterin in amounts greater than those from 8-^{14}C-guanosine, this interpretation is subject to question. More definitive experiments are needed in order to conclude that quench spot is definitely a pteridine or pteridine derivative.

The following compounds were individually chromatographed on TLC sheets: kynurenine, folic acid, riboflavin, pterin-6-carboxylic acid, and xanthopterin. No fluorescent spots corresponding to any of these compounds were observed after TLC of head extracts of Oregon-R or any of the various eye-color mutants examined.

Phosphorylated Pteridines

Since the first pteridine in the biosynthetic pathway is dihydroneopterin triphosphate (15), it was of interest to determine if any of the unidentified fluorescent spots are

phosphorylated pteridines. Suzuki and Brown (66) found that a nonspecific bacterial alkaline phosphatase preparation was capable of completely hydrolyzing dihydroneopterin triphosphate to dihydroneopterin. An amount (0.2 mg) of this enzyme which completely hydrolyzed 0.4 mg (in 0.4 ml of 0.1 M tris-HCl, pH 8.0) of both GTP and GMP to guanosine in 1 hour was added to an Oregon-R head extract prepared as described in Materials and Methods. After a 2-hour incubation period, the extract was subjected to 2-dimensional TLC. No significant difference could be detected between the pteridine pattern of the phosphatase-treated extract and that of an untreated extract.

Quantitation of Pteridines

Using a Turner fluorometer with the filter combinations described previously, the following pteridines can be rapidly and easily quantitated: biopterin, sepiapterin, pterin, isoxanthopterin, quench spot, drosopterin, isodrosopterin, neodrosopterin, and aurodrosopterin.

As shown in Figure 5, the standard curves for fluorescence measured *versus* aliquot size of Oregon-R head extract for pterin, isoxanthopterin, biopterin, and sepiapterin exhibit linearity in the concentration range normally measured. The curves extrapolate to the origin or reasonably close to it.

Figure 6 shows a standard curve for the quench spot after chromatography in 4:1:1 butanol : acetic acid : H_2O.

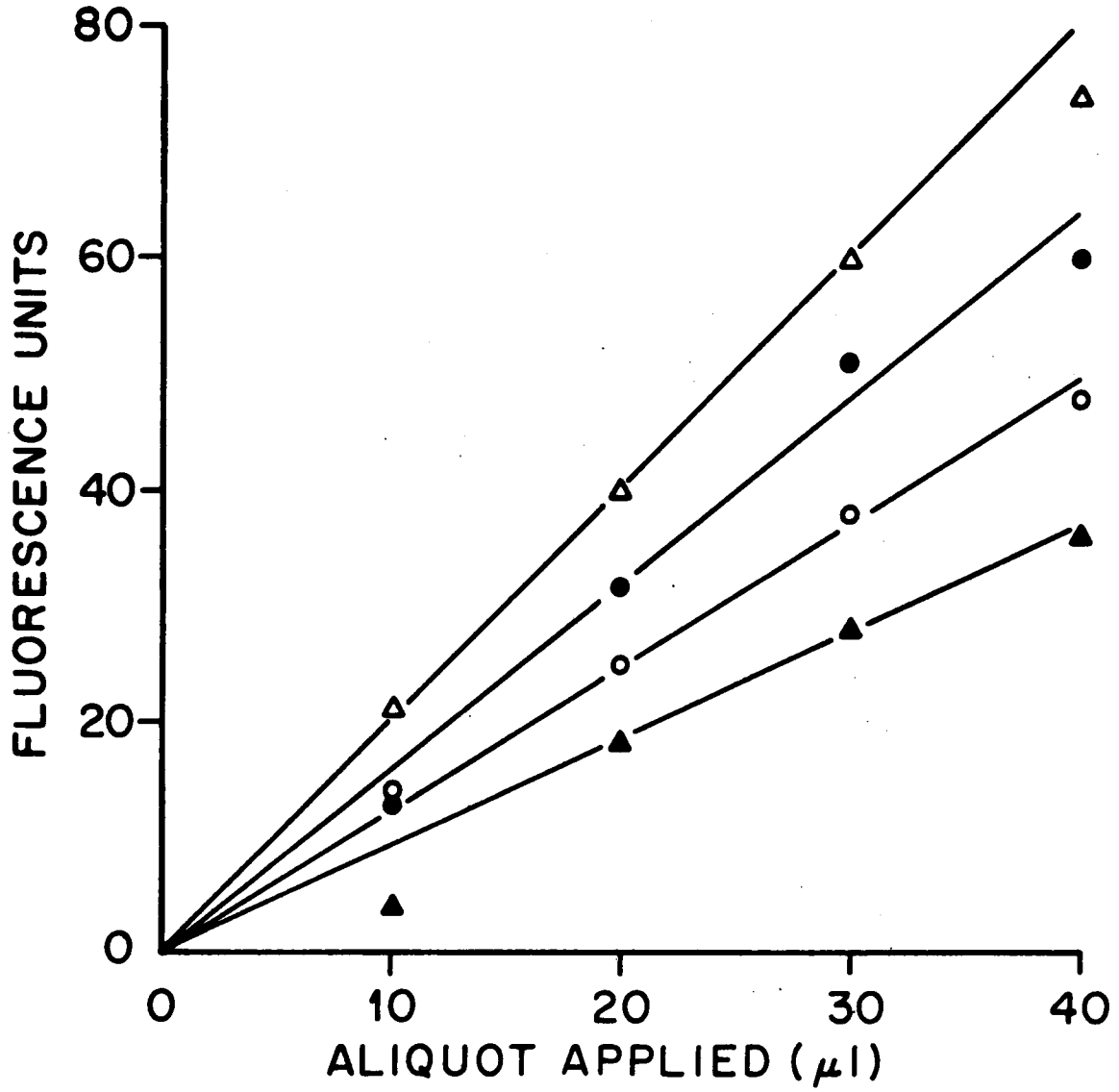


Figure 5. Fluorescence Units versus Aliquot Applied for Pterin, Isoxanthopterin, Biopterin, and Sepiapterin.

▲ Isoxanthopterin
● Pterin
o Sepiapterin
▲ Biopterin

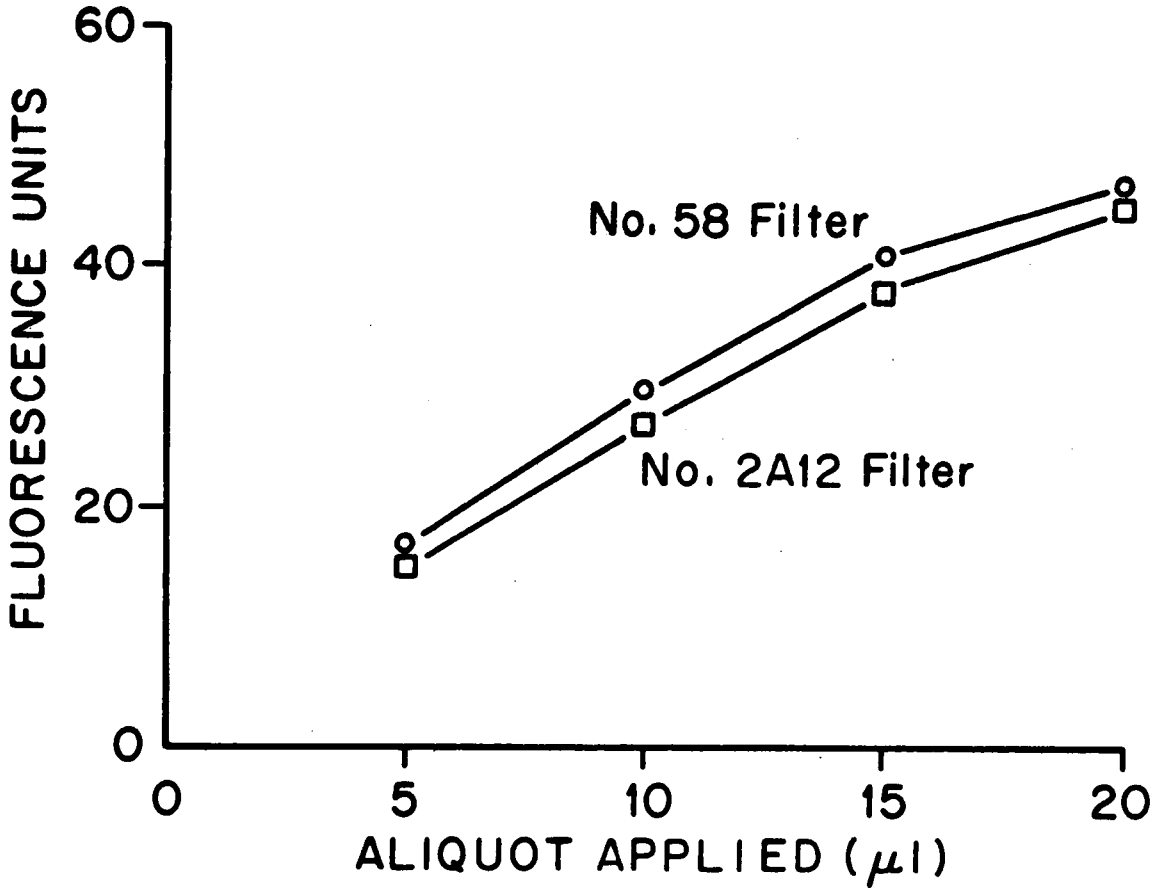


Figure 6. Fluorescence Units versus Aliquot Applied for Quench Spot.

o #58 Filter
□ #2A12 Filter

Filter number denotes the secondary filter used. Number 58 filter was afterwards selected for all remaining quench spot determinations.

The curve deviates from linearity to a slight degree at fluorescence values less than 40 and to a marked degree at values greater than 40. The curve does not intersect zero. When quantitating quench spot, particularly in $\underline{Hn^{r3}}$ flies, it is necessary to dilute the sample in order that the fluorescence value will be less than 40.

Figure 7 shows standard curves for the drosopterins. Linearity is excellent in the desired concentration range. For these substances also the curves do not intersect zero.

Using isoxanthopterin, pterin, and biopterin standards in addition to isolated sepiapterin prepared as described in Materials and Methods, it is possible to obtain an estimate of the molar concentrations of these pteridines using known extinction coefficients (Table 1, page 25). The fluorometric values can then be quantitated by reading the fluorometric value for a known amount of one of these pteridines that has been applied to a TLC sheet and developed in 3% ammonium chloride. The correlation of molar quantity and fluorometric value for these pteridines is shown in Table XI. Also, the average amount of each of these pteridines in a newly eclosed Oregon-R fly head has been calculated. This calculation is based on an average fly head weight of 1.5×10^{-4}g.

Factors Affecting Pteridine Extraction and Stability

Complete extraction of Drosophila head pteridines requires the presence of ammonium hydroxide in the

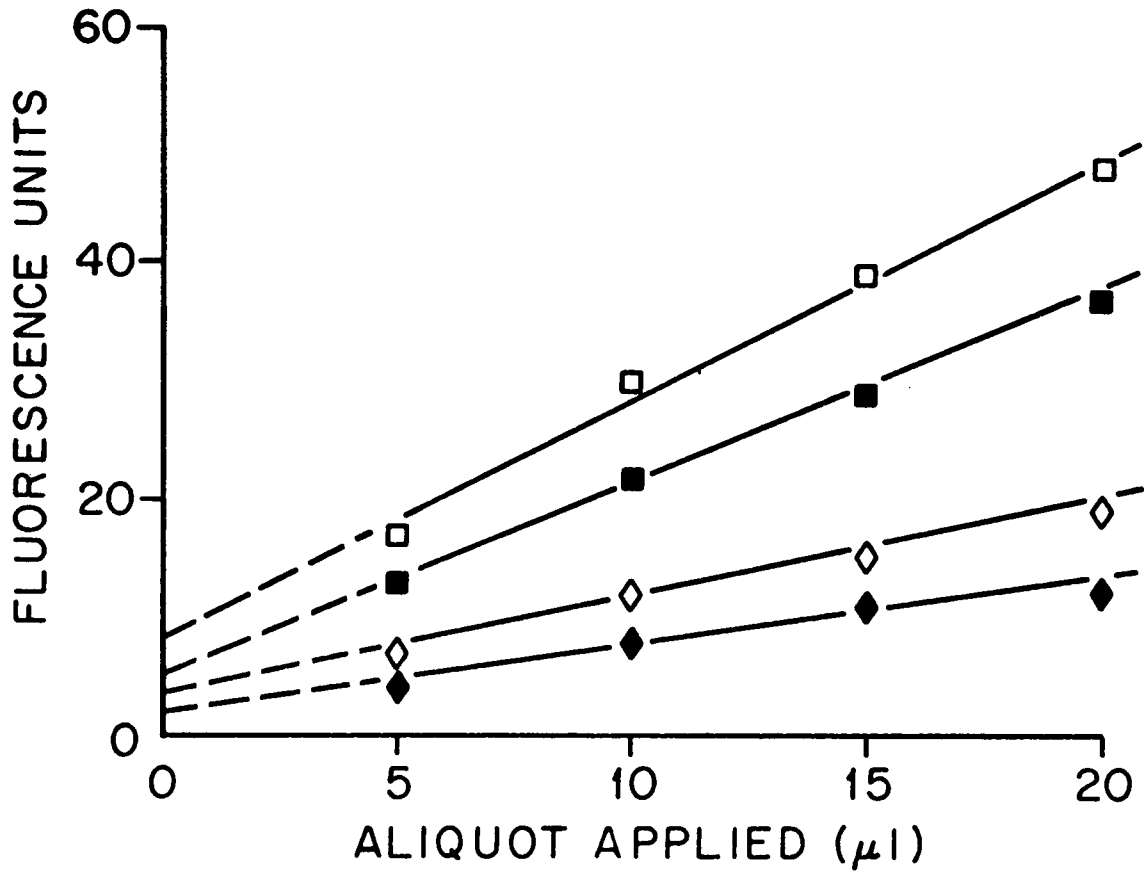


Figure 7. Fluorescence Units versus Aliquot Applied for Drosopterins.

□ Drosopterin
■ Isodrosopterin
◊ Neodrosopterin
◆ Aurodrosopterin

Each curve was fitted to the experimental values by least squares analysis.

TABLE XI

CORRELATION OF MOLAR QUANTITY AND FLUOROMETRIC READING FOR FOUR PTERIDINES

Pteridine	Quantity Applied to TLC Sheet (pico-moles)	Fluorometric Value	Average Fluorometric Value Per Oregon-R Fly Head	Average Quantity Per Oregon-R Fly Head (pico-moles)
isoxanthopterin	57	18	11	40
pterin	220	32	7	50
biopterin	160	32	6	30
sepiapterin	450	45	10	100

homogenizing solvent. Figures 8 and 9 show that pteridine extractability is a function of NH_4OH concentration at low NH_4OH titers; concentrations higher than 3% do not increase extraction. If 2-mercaptoethanol is included in the homogenization and TLC solvents, higher fluorometric values are obtained for sepiapterin and drosopterins. The values obtained in Table XII demonstrating this effect were obtained on a split sample of Oregon-R fly head preparation; half of the sample was homogenized and chromatographed in the solvents described in Materials and Methods; the other half was homogenized and chromatographed in the same solvents which contained 2-mercaptoethanol in concentrations specified in Materials and Methods.

It is necessary to quantitate pteridines soon after TLC is complete. Figure 10 shows the fluorometric decay of the drosopterin fraction after separation by TLC, even when the chromatograms are stored in the dark. Figure 11 shows that pterin, isoxanthopterin, and biopterin are reasonably stable, while sepiapterin exhibits a more rapid degradation.

It is likewise not possible to spot TLC sheets and store them for any length of time prior to solvent development. Figures 12 and 13 show that degradation, particularly of the drosopterin fraction, is significant over a 5-day period. Interestingly, biopterin increased in fluorescent value over this time period.

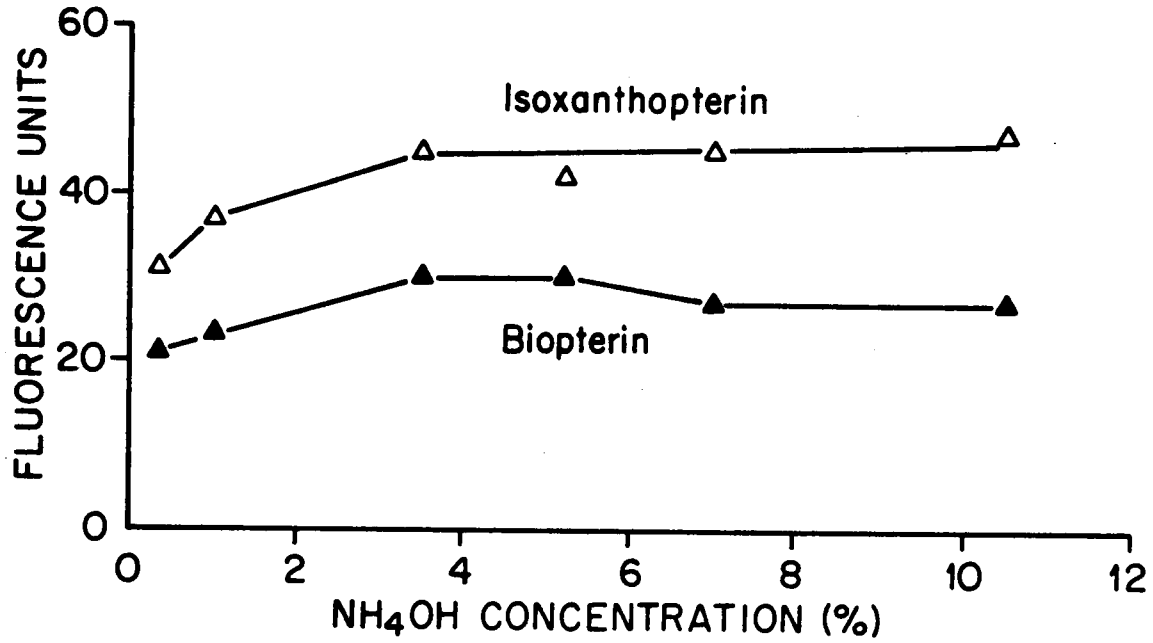


Figure 8. Extractability of Isoxanthopterin and Biopterin as a Function of NH_4OH Concentration in the Homogenizing Solvent.

△ Isoxanthopterin
▲ Biopterin

Oregon-R head preparations were homogenized in 2:1 propanol: NH_4OH at the indicated percentages of NH_4OH. Twenty microliter aliquots were then chromatographed, and the pteridines quantitated.

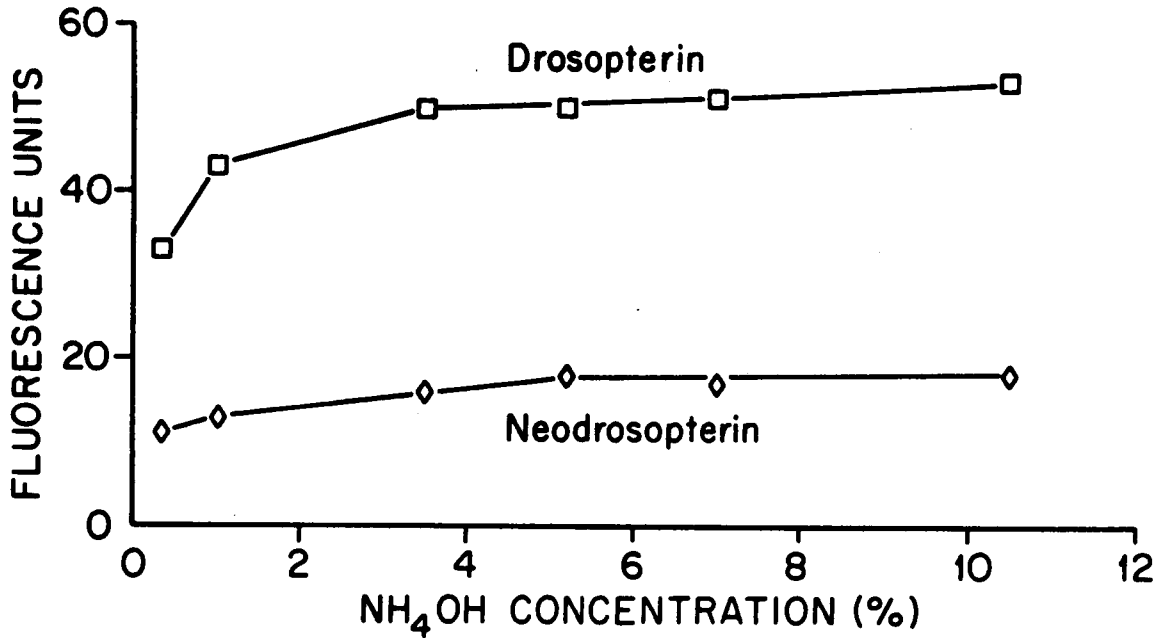


Figure 9. Extractability of Drosopterin and Neodrosopterin as a Function of NH_4OH Concentration in the Homogenizing Solvent.

□ Drosopterin
◇ Neodrosopterin

Oregon-R head preparations were homogenized in 2:1 propanol: NH_4OH at the indicated percentages of NH_4OH. Twenty microliter aliquots were then chromatographed, and the pteridines quantitated.

TABLE XII

EFFECT OF 2-MERCAPTOETHANOL ON THE FLUOROMETRIC VALUES OBTAINED FOR OREGON-R HEAD EXTRACT PTERIDINES

Pteridine	2-Mercaptoethanol	
	Present	Absent
biopterin	32	32
sepiapterin	42	33
pterin	42	39
isoxanthopterin	56	57
drosopterin	56	51
isodrosopterin	38	36
aurodrosopterin	17	12
neodrosopterin	25	21

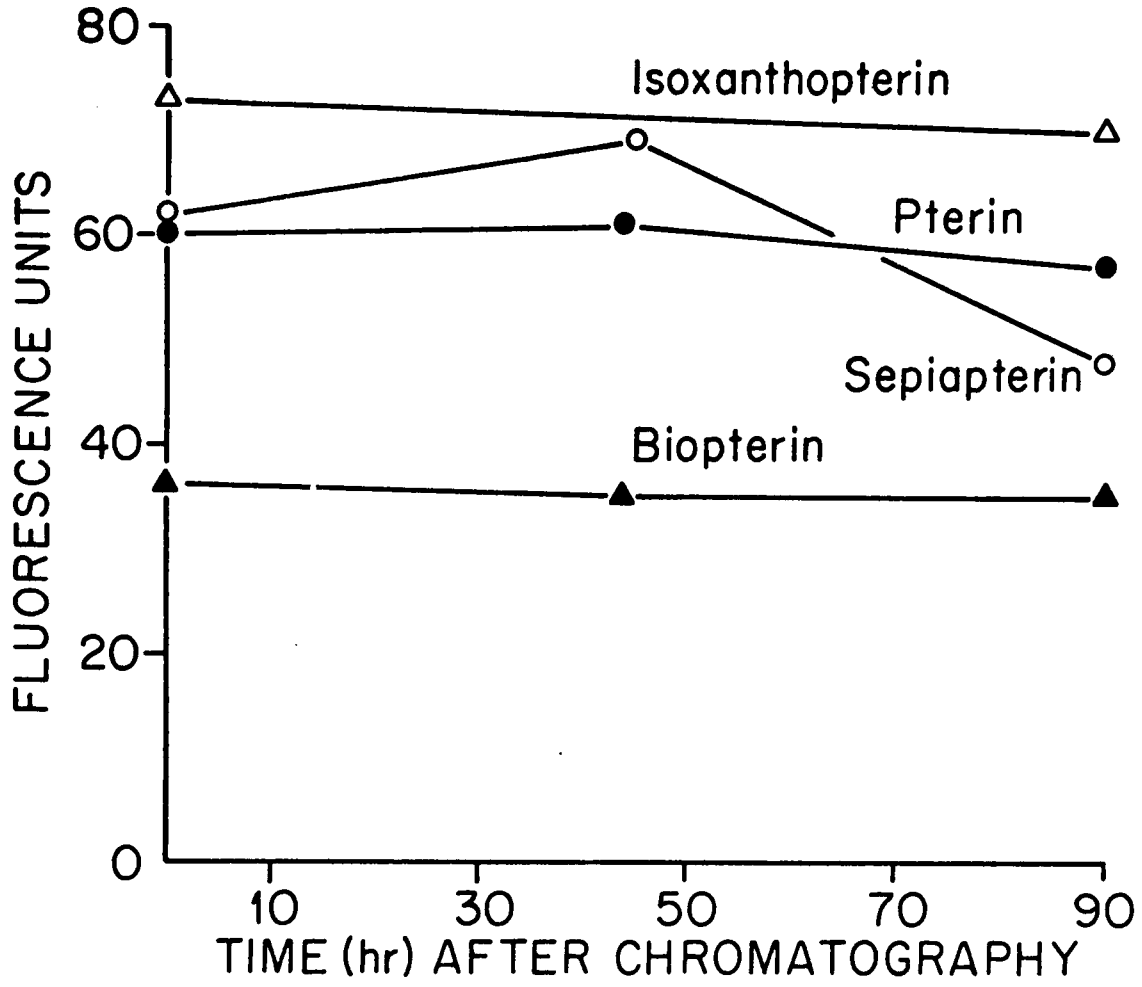


Figure 10. Stability of Isoxanthopterin, Pterin, Sepiapterin, and Biopterin After Thin-Layer Chromatography.

△ Isoxanthopterin
● Pterin
○ Sepiapterin
▲ Biopterin

Thin-layer chromatograms were stored at room temperature in the dark after chromatography. Pteridines on the chromatogram were quantitated at the indicated time intervals after chromatography.

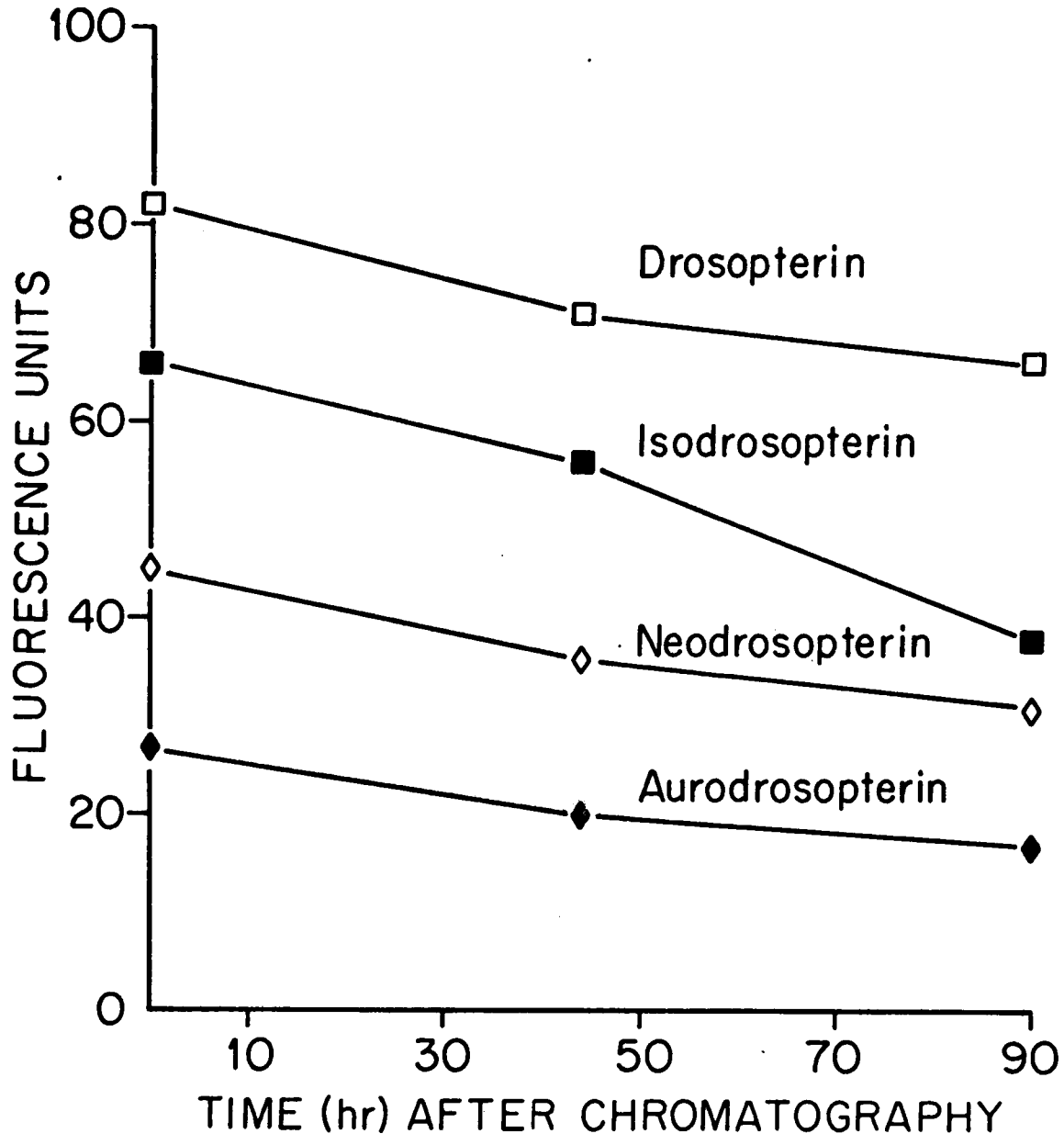


Figure 11. Stability of Drosopterins After Thin-Layer Chromatography.

- □ Drosopterin
- ■ Isodrosopterin
- ◊ Neodrosopterin
- ◆ Aurodrosopterin

Thin-layer chromatograms were stored at room temperature in the dark after chromatography. Pteridines on the chromatogram were quantitated at the indicated time intervals after chromatography.

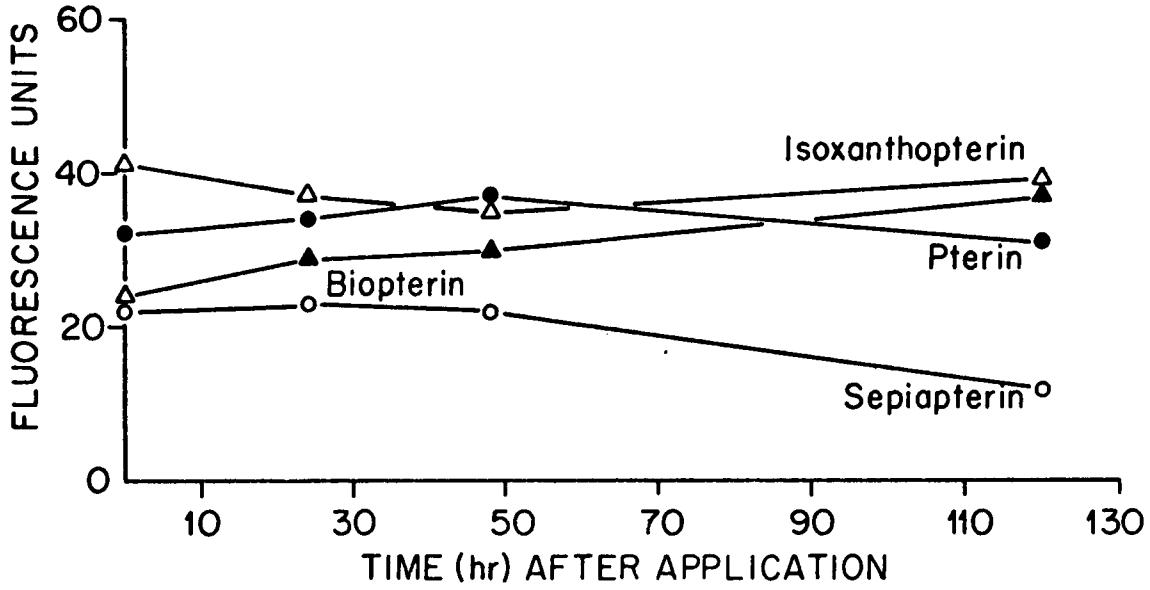


Figure 12. Stability of Isoxanthopterin, Biopterin, Pterin, and Sepiapterin as a Function of Time Before Chromatography.

△ Isoxanthopterin
▲ Biopterin
● Pterin
○ Sepiapterin

Twenty microliters of an Oregon-R head extract was spotted on each of four thin-layer chromatograms. The chromatograms were stored in the dark at room temperature. At each of the indicated time intervals, a chromatogram was developed in solvent in two dimensions and the pteridines quantitated.

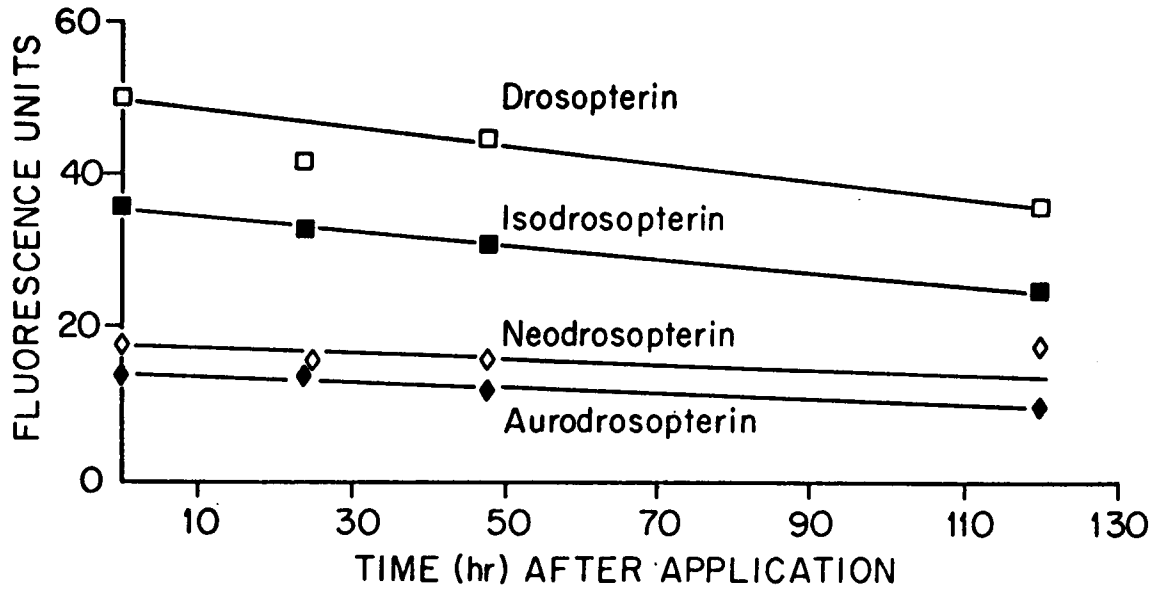


Figure 13. Stability of Drosopterins as a Function of Time Before Chromatography.

- □ Drosopterin
- ■ Isodrosopterin
- ◇ Neodrosopterin
- ◆ Aurodrosopterin

Twenty microliters of an Oregon-R head extract was spotted on each of four thin-layer chromatograms. The chromatograms were stored in the dark at room temperature. At each of the indicated time intervals, a chromatogram was developed in solvent in two dimensions and the pteridines quantitated.

IV. DISCUSSION

The utilization of analytical TLC sheets coupled with the solvent systems employed by Descimon and Barial (43) permits the investigator to separate Drosophila head pteridines in a relatively rapid and highly reproducible manner. Complete separation is apparently achieved, with the possible exception of the stereoisomers of biopterin. Inclusion of borate in the second-dimension solvent may separate the threo and erythro isomers of this pteridine. I have not investigated this possibility.

The advantage of chromatographing fly heads instead of whole flies is that detection of minor fluorescent spots, presumably pteridines, is possible. I am able to detect 18-20 spots, whereas Descimon and Barial (43) noted 11 on their chromatograms, Taira (25) found nine, and Narayanan and Weir (45) noted ten. Kiriazis and Friedman (12) were able to obtain 23 spots by their technique, but these workers found only four drosopterin spots. TLC of head extracts yields 5 drosopterins in Schwinck and Mancini's solvent systems (59) and in mine. When the structures of these minor components of head extracts are determined, they may yield invaluable information concerning the drosopterin biosynthetic pathway in Drosophila.

The positions of the various pteridines on the chromatogram after TLC generally agree with the results which other workers have obtained after 2-dimensional chromatography (25, 43). However, several discrepancies in identification of the fluorescent spots were noted when Descimon and Barial's results (43) were compared with the present results. Specifically, kynurenine appears in the same position on their chromatogram as xanthurenic acid does on the chromatogram obtained in my work. Also, I find isosepiapterin to have a smaller Rf value than sepiapterin in 3% ammonium chloride, whereas they note a larger Rf value for isosepiapterin relative to sepiapterin. These authors identify as isodrosopterin the spot which I have labelled aurodrosopterin. Also, they find riboflavin on the chromatogram, a substance that I was unable to detect. Differences in experimental procedures may account for these discrepancies.

Quantitation of the pteridines by direct measurement of their fluorescence on the TLC sheets is less time consuming and easier than elution of each pteridine from the chromatogram and quantitation in solution. Unusually, ten pteridines were quantitated on each chromatogram; six chromatograms (a total of 60 spots) could be quantitated in about two hours. No difficulty was found with pteridine instability provided the pteridines were quantitated within two hours after TLC.

The lack of effect of alkaline phosphatase digestion on the pteridine pattern seen after TLC does not indicate that that no phosphorylated pteridines exist in adult Drosophila. It was subsequently found during an analysis of Drosophila head nucleotide pools (see Chapter III) that endogenous phosphatases act very rapidly after decapitation to dephosphorylate nucleotides. Consequently, it is entirely possible that during the few seconds of thawing that the heads undergo during the weighing procedure, any phosphorylated pteridines would be dephosphorylated. The result from the phosphatase digestion experiment does indicate, however, that none of the unknown fluorescent compounds seen after TLC are phosphorylated pteridines. Dephosphorylation of a phosphorylated pteridine would substantially alter its Rf value, yet the pteridine pattern proved to be virtually unchanged after phosphatase digestion of the head homogenate. Although the alkaline phosphatase preparation used in this work dephosphorylated GTP and GMP, it was not tested for its ability to digest a phosphorylated pteridine; however, a similar enzyme preparation was used by Suzuki and Brown (66) to dephosphorylate dihydroneopterin triphosphate.

It is clear that the green fluorescent spots F and G are alkaline degradation products arising during homogenization. The fact that 2-mercaptoethanol and ascorbic acid prevent their formation suggests that oxygen or some oxidizing

agent may be involved in the degradative process. The identity of the compound which is degraded is unknown. The observation that spots F and G are absent or present in only trace amounts in $\underline{ry^2}$ and mal would seem to indicate that xanthine oxidase is involved in the formation of the parent compound. The absence or near-absence of these spots in v, cn, and st cannot be readily explained at present.

Spots F and G appear to be analogous to the "xanthopterin-like" and possibly xanthopterin spots reported by other workers (11, 67). Ziegler-Gunder and Hadorn (68) recognized that these compounds were alkaline degradation products and concluded that sepiapterin was probably the parent compound. Although spots F and G are elevated in mutants (se, $\underline{Hn^{r3}}$) which also have elevated sepiapterin levels, these two spots are absent or near-absent in other mutants which have wild-type sepiapterin levels (v, cn, st, $\underline{ry^2}$, mal). In addition, a preparation of sepiapterin which was purified as described in Materials and Methods, homogenized in 2:1 propanol : 3.5% NH_4OH, and subjected to TLC remained intact with no evidence of breakdown to spots F and G. Therefore, I have no evidence that spots F and G are degradation products of sepiapterin.

The superior resolution achieved on cellulose TLC sheets in these solvent systems has made it possible for a new compound—spot L, the quench spot—to be described.

Evidence indicating it is a pteridine include its absence in bw flies, its UV absorbance spectrum, and possibly its ability to support Crithidia growth, albeit poor. The fact that it was labelled with U-^{14}C-guanosine, but not with 8-^{14}C-guanosine, also indicates that it is a pteridine; however, this interpretation must be treated with caution, as will be discussed presently.

Albert (69) and his associates have described the phenomenon of covalent hydration of pteridines. Since addition of water across a C=N bond destroys ring aromaticity, it would be expected that any fluorescence due to ring aromaticity would also be abolished upon hydration. From Table IX, page 36, it can be seen that the quench spot exhibits no fluorescence after chromatography in aqueous solvents but fluoresces after chromatography in alcohol-containing solvents at low pH values. Therefore, it is possible that this compound is readily hydrated in aqueous solvents, but it loses covalently bound water in alcoholic solvents at low pH values, thus reestablishing aromaticity and consequent fluorescence. It does not seem likely that quench spot is a reduced, nonfluorescent pteridine which simply becomes oxidized in 4:1:1 butanol : acetic acid : H_2O, because the change from quenching to fluorescent property is fully reversible upon rechromatography of the fluorescing substance in an aqueous solvent.

Although only a small amount of radiolabel from U-^{14}C-guanosine was incorporated into the quench spot, the amount was substantially greater than that incorporated from 8-^{14}C-guanosine. This important control experiment rules out the possibility that the radioactivity found in the quench spot area of the chromatogram was due to co-chromatography of quench spot with some unrelated labelled guanosine metabolite. It also discounts the possibility that the quench spot itself is a guanosine metabolite unrelated to a pteridine, because the carbon-8 of guanosine is extricated during pteridine biosynthesis. The observation that biopterin and sepiapterin incorporated such low levels of radioactivity from U-^{14}C-guanosine so as to be indistinguishable from those levels incorporated from 8-^{14}C-guanosine is disappointing. These results reflect the independence which Drosophila maintains with regard to exogenous sources of guanosine and presumably other nucleosides. The label found in quench spot, but not in biopterin or sepiapterin, may indicate that quench spot is present in Drosophila in much greater quantities than are biopterin and sepiapterin. This relative abundance would not be evident upon chromatography in an acidified alcoholic solvent if the fluorescent yield of quench spot is lower than those for other pteridines.

The evidence in this work suggests that quench spot is a pteridine or pteridine derivative, but the results are

by no means conclusive. A qualitative analysis of quench spot is needed to firmly establish its identity.

CHAPTER III

LOCALIZATION OF THE PURPLE MUTATION OF *DROSOPHILA MELANOGASTER* IN THE DROSOPTERIN BIOSYNTHETIC PATHWAY

I. INTRODUCTION

Few studies have been done to determine the mechanism of suppression in *Drosophila*. The most detailed study concerned the involvement of tRNA in the partial restoration of activity of tryptophan 2,3-dioxygenase in *su(s)*^{2}*v* flies (33). *su(s)*^{2} also suppresses *sp*, *pr*, and *s*, but only *sp* has been examined biochemically to determine the mechanism of suppression. The missing isoenzyme band of tyrosinase in *sp* flies was found to be restored in *su(s)*^{2}*;sp* (32); however, the possible involvement of tRNA in inhibition of the tyrosinase band was not assessed. The enzymatic lesions in *pr* and *s* have not been determined, and no biochemical studies have been done on *su(s);pr* or *su(s) s*.

Although it is known that several pteridines are present in *pr* flies in lowered titers relative to wild-type, it is uncertain whether the enzymatic lesion is located in the drosopterin biosynthetic pathway or in another pathway controlling the biosynthesis of some metabolite or cofactor required for drosopterin biosynthesis. Once the enzymatic lesion is determined, any involvement of tRNA in the inhibition of the enzyme can be determined.

The most feasible approach for determining the enzymatic lesion in pr is a careful examination of the pteridine pattern of pr flies. If any pteridines are found in reduced quantities, this would be indicative of an enzymatic block prior to the biosynthesis of these compounds. Any pteridine(s) found in an elevated quantity relative to wild-type would suggest that the lesion is located after its biosynthesis. Upon localization of the affected enzymatic step in pr, an *in vitro* assay can then be developed to determine if the activity of the enzyme is reduced relative to wild-type. A lowered amount of activity would suggest that the primary lesion in pr is located at that site; a wild-type level of activity would indicate that the lesion is located at another site and that the effect on this enzymatic reaction is indirect.

It is also of interest to determine if two characteristics of v suppression by su(s) are also characteristic of pr suppression. One characteristic is that some alleles (v, v^2) are suppressible by su(s), but other alleles (v^{36f}, v^{51a}) are insuppressible (70). The allele pr^{bw} can be likewise tested for its suppressibility by su(s).

The other characteristic is that v flies reared on starvation medium exhibit a partial restoration of xanthommatin biosynthesis (71), thereby mimicking su(s) v in this

respect. If pr shows a similar phenomenon of increased drosopterin biosynthesis when reared on starvation medium, this might indicate that the same enzyme inhibition mechanism operates in both pr and v flies.

II. MATERIALS AND METHODS

Chemicals

Aminex A-28 anion exchanger and Bio-Gel P-2 were obtained from Bio-Rad Laboratories. Alamine 336 (tricaprylyl tertiary amine) was obtained from McKerson Corp., Minneapolis, Minnesota. Freon-TF[2] (CCl_2FCCIF_2) was purchased from DuPont. All nucleoside and nucleotide standards were obtained from Sigma Biochemicals.

Culture of Drosophila

Drosophila was routinely cultured as described in Chapter II.

In order to determine the effect of starvation on brown and red eye pigment synthesis, mutants of interest were reared on starvation medium as described by Tatum and Beadle (71). A yeast concentration of 0.5% was found to be sufficient to produce a partial resumption of xanthommatin biosynthesis in v;bw flies; therefore, that concentration was utilized in all starvation medium preparations.

Quantitation of Pteridines

Pteridines were quantitated fluorometrically after TLC of Drosophila head extracts. These procedures are described in Chapter II. In Figure 1, page 6; Figure 2, page 8; Figure 3, page 27, Figure 4, page 39, Figure 5, page 43; Figure 6, page 44; and Figure 7, page 46, the error bars indicate twice the average standard error for all points on each curve (Figures 5-7) or all points on all curves on the graph (Figures 1-4).

Preparation of Drosophila Heads for Nucleotide Analysis

Drosophila cultures were cleared of adults shortly before the onset of the light phase of the light-dark cycle. Adults were collected four hours later and immediately frozen in liquid nitrogen. Heads were collected from 200-400 mg of flies by the procedures described in Chapter II and immediately homogenized in 0.6 ml of ice-cold 0.5N perchloric acid in a glass tube and pestle homogenizer. The homogenate was centrifuged at 10,000 X g for 20 minutes. The supernatant was then extracted twice with an equal volume of 20% w/v Alamine in Freon -TF[2] (72), a procedure which extracts the perchloric acid from the supernatant. A 0.2 ml aliquot of the extracted supernatant was placed on a 0.6 X 90 cm column of Bio-Gel P-2 which had been prepared as described in Khym (72) and equilibrated with 0.02M potassium tetraborate titrated to pH 8.8 with formic acid. The sample was eluted

with equilibrating buffer at a flow rate of approximately 0.5 ml/min. The eluate was monitored at 254 nm with a Savant detector. The first peak eluting from the column, which contained the nucleotide fraction of the homogenate supernatant, was collected and immediately fractionated over an Aminex A-28 column.

A 2-ml aliquot of the nucleotide fraction eluting from the P-2 column was pumped onto a 0.6 X 12 cm column of Aminex A-28 which had been washed as described by Khym (51) and equilibrated with 0.01 M sodium citrate titrated to pH 8.3 with HCl. The column temperature was maintained at 70 $\pm$ 1°C with a Haake thermoregulated circulator. The column was eluted with a gradient from 0.01 M to 0.5 M sodium citrate, pH 8.3 (72) at a pressure (approximately 50 psi) required to maintain a flow rate of 0.4 ml/min. The eluate was monitored at 254 nm with a Savant detector connected to a Leeds-Northrup recorder.

The Aminex column could be utilized again without carrying it through the wash procedure provided that at least 300 ml of 0.5 N sodium citrate, pH 8.3 was passed through the column at 70°C.

Nucleotide peaks were quantitated by determining the area under the curve by weighing each peak tracing and comparing the weight with that of a known quantity of AMP passed through the column.

Drosophila Crosses

su(s)^2 flies were obtained from a stock of su(s)^{2}v by crossing y^2 females with su(s)^{2}v/Y males. F_1 females were crossed with FM7/Y males, and wild-type females with the genotype su(s)^2/FM7 were selected from the F_2 generation. These females were mated to FM7/Y males; from this cross su(s)^2/FM7 females and su(s)^2/Y males were selected and crossed to each other to yield su(s)^2 female and su(s)^2/Y male progeny.

su(s)^2;pr was obtained by first introducing an FM7 balancer chromosome into a stock of pr flies. This was done by crossing pr males with FM7;Pin^{YT}/CyO females. From this cross FM7/Y;CyO/pr males and Fm7/+;CyO/pr females were selected and crossed. Finally, homozygous or hemizygous FM7;pr progeny were selected. The eye color of FM7;pr flies is orange due to the v locus in the FM7 inversion chromosome.

FM7;pr females were crossed to su(s)^2/Y;CyO/+ males, which, had been selected from the F_1 progeny of the cross su(s)^2 with FM7/Y;Pin^{YT}/CyO. su(s)^2/Y;pr males were selected and crossed. From this final cross su(s)^2;pr females and su(s)^2/Y;pr males were obtained, both of which had a wild-type phenotype. Females were test-crossed with pr males to ensure that the pr chromosome was indeed present in the suppressed stock.

Stocks of su(s)^2;pr^{bw} and su(s)^2;pd were constructed in the same manner as was su(s)^2;pr. Female progeny from

the final cross of each were test-crossed with either $\underline{pr^{bw}}$ or pd males.

$\underline{su(s)^2v}$ was obtained from a stock of $\underline{su(s)^2v;bw}$ by mating $\underline{su(s)^2v;bw}$ females with $\underline{FM7/Y;CyO/Pin^{YT}}$ males. F_1 females of the genotype $\underline{su(s)^2v/FM7;CyO/bw}$ were mated to Oregon-R males. From this cross $\underline{su(s)^2v/Y;Cyo/+}$ males were selected and mated to $FM7;Pin^{YT}/CyO$ females. Females having the genotype $\underline{su(s)^2v/FM7;CyO/+}$ and males of the genotype $\underline{FM7/Y;CyO/+}$ were selected and crossed. $\underline{su(s)^2v/Y}$ male progeny were mated to $\underline{su(s)^2v/FM7}$ female progeny to obtain $\underline{su(s)^2v}$ and $\underline{su(s)^2v/Y}$ flies. $\underline{su(s)^2v/Y}$ males were test-crossed with $\underline{v}$ females to ensure that $\underline{su(s)^2v/v}$ progeny had a $\underline{v}$ phenotype.

$\underline{Pu^2}$ was genetically combined with $\underline{su(s)^2}$ by mating $\underline{su(s)^2}$ females with $\underline{Pu^2/+}$ males. Of the F_1 males, which were of the genotype $\underline{su(s)^2/Y;Pu^2/+}$ or $\underline{su(s)^2/Y;+/+}$, half exhibited the $\underline{Pu^2}$ phenotype. This indicates that $\underline{su(s)^2}$ does not suppress $\underline{Pu^2}$.

$\underline{su(pr)^{e3};pr}$ was obtained from a stock of $\underline{su(pr)^{e3};pr\ cn}$ by first introducing a TM3 balancer chromosome into a stock of pr flies. pr males were crossed to TM3/+ females. F_1 TM3/+;pr/+ were crossed to their TM3/+;pr/+ siblings. F_2 TM3/+;pr flies were selected. Concurrently with these crosses, $\underline{su(pr)^{e3};pr\ cn}$ males were crossed to TM3/+;CyO/+ females. F_1 $\underline{TM3/su(pr)^{e3};CyO/pr\ cn}$ males were then mated to

Tm3/su(pr)^{e3};CyO/pr cn siblings. F_2 su(pr)^{e3};CyO/pr cn males were selected and crossed with TM3/+;pr females. From this cross TM3/su(pr)^{e3};CyO/pr males were selected and crossed with TM3/su(pr)^{e3};CyO/pr females. Finally, su(pr)^{e3};pr progeny were selected. The pr phenotype was suppressed; a test-cross of su(pr)^{e3};pr with pr yielded progeny which displayed a pr phenotype.

pr;se was obtained by mating se females with CyO/+ males; F_1 CyO/+;se/+ males were selected and mated to sibling CyO/+;se/+ females. F_2 CyO/+ progeny possessing the se phenotype, and CyO/+;se genotype, were selected. Female CyO/+;se were crossed with pr;TM3/+ males. F_1 CyO/pr;TM3/se males were mated to siblings of the same genotype. F_2 pr;se progeny were selected. Females of this genotype were test-crossed with either pr or se males.

pr;Hn^{r3} was constructed in the same manner as was pr;se.

pn^2;pr was constructed in a similar manner to su(s)^2;pr. pn^2 males were mated to FM7;CyO/Pin^{YT} females, and pn^2/FM7; CyO/+ progeny were selected. These females were crossed with FM7/Y;pr males. From this cross pn^2/FM7;CyO/pr females and pn^2/Y;CyO/pr males were selected and crossed. Homozygous and hemizygous pn^2;pr progeny were selected from this final cross. Females were test-crossed with either pn^2 or pr males.

se Hn^{r3} was constructed by initially mating se male flies with Hn^{r3} females. F_1 females were crossed with TM3/+ males; F_2 males having the TM3 balancer chromosome were selected. Of these males 137 were individually mated to 2 or 3 se females, then to 2 or 3 Hn^{r3} females. One male produced both se and Hn^{r3} progeny, indicating that its genotype was TM3/se Hn^{r3}. Unfortunately, this male died before it could be mated to a TM3/+ balanced female. Therefore the se Hn^{r3} chromosome was rescued from the stock of se/se Hn^{r3} flies issuing from the test-cross. Several se/se Hn^{r3} males were crossed with TM3/+ females. F_1 TM3 males, having a genotype of either TM3/se or TM3/se Hn^{r3}, were selected and individually mated to 2 or 3 Hn^{r3} females. Several males which produced Hn^{r3} progeny were then mated to TM3/+ females. From this cross male and female TM3 flies, having a genotype of TM3/+ or TM3/se Hn^{r3}, were selected and crossed. Progeny having a se phenotype, and se Hn^{r3} genotype, were selected; females were test-crossed to either se or Hn^{r3} males.

III. RESULTS

Comparison of Wild-Type Drosophila Strains

The eye-color mutants utilized in this work have been isolated from various wild-type Drosophila strains. Before one can validly compare pteridine titers among eye-color mutants of interest, it is necessary to either ensure that

wild-type strains have identical titers of pteridines or utilize only mutants which have a common complement of chromosomes (isogenic). The latter choice requires that one induce the desired mutants from a single wild-type strain, which is a time-consuming process. I have compared pteridine quantities from three wild-type Drosophila strains to determine if any significant differences exist. When Oregon-R, Canton-S, and Samarkand were examined (Table XIII), no significant differences in pteridine quantities were noted, with the possible exception of isoxanthopterin.

Therefore, I am assuming that there are no significant differences in head pteridine titers among wild-type Drosophila strains and that any quantitative differences noted between two or more eye-color mutants are due solely to the loci in question and not to differences in background chromosomes.

Comparison of pr and pr^{bw} with Oregon-R

When pteridines from pr and pr^{bw} flies were quantitated and compared with those from Oregon-R flies of the same age, several pteridines were found in reduced quantities (Table XIV).

As can be seen from Table XIV, sepiapterin, quench spot, and the drosopterins are markedly depressed in pr and pr^{bw} relative to wild-type. Sepiapterin is almost completely

TABLE XIII

COMPARISON OF HEAD PTERIDINE QUANTITIES
FROM THREE WILD-TYPE STRAINS
OF DROSOPHILA

Pteridine	Quantity Measured (fluorescence units) Oregon-R	Canton-S	Samarkand
Biopterin	25	26	27
Sepiapterin	18	19	20
Pterin	23	22	27
Isoxanthopterin	30	35	24
Drosopterin	43	45	43
Isodrosopterin	29	32	28
Aurodrosopterin	13	15	15
Neodrosopterin	18	19	15

Each pteridine quantity is derived from a single determination in this experiment; however, the quantities for Oregon-R agree well with subsequent determinations on this strain of flies.

TABLE XIV

COMPARISON OF HEAD PTERIDINES FROM
pr, pr^{bw}, AND OREGON-R FLIES

Pteridine	Quantity Measured (fluorescence units) pr	pr^{bw}	Oregon-R
Biopterin	23 (2.0)	32 (4.5)	26 (1.5)
Sepiapterin	trace	trace	19 (1.0)
Pterin	8 (1.0)	37 (0.0)	22 (1.5)
Isoxanthopterin	30 (3.5)	27 (1.0)	41 (1.0)
Drosopterin	15 (0.0)	10 (0.5)	43 (2.5)
Isodrosopterin	12 (0.5)	8 (0.5)	35 (2.5)
Aurodrosopterin	2 (0.5)	1 (0.5)	12 (0.5)
Neodrosopterin	11 (0.0)	7 (1.0)	18 (0.5)
Quench Spot	21 (1.0)	13 (1.0)	58 (0.0)

Flies were 0-1 days old. Each value is an average of separate analyses made on two samples drawn from the same culture of flies. Standard errors are included in parentheses.

absent, being present in trace amounts barely discernable to the eye on the chromatograms. Although pterin is depressed in pr and elevated in pr^{bw} relative to Oregon-R, there seems to be little difference between the two mutants in the titers of isoxanthopterin, a pteridine which is derived from pterin metabolically.

It can also be noted from Table XIV that pr and pr^{bw}, although allelic to each other, possess different amounts of pteridines. The metabolic block in pr^{bw} appears to be stronger than in pr.

A further distinction was noted between pr and Oregon-R. Although the spots were not quantitated, spots F and G (see Chapter II) were present in much lower titers in pr and pr^{bw} relative to wild-type when these flies were homogenized and chromatographed in the absence of 2-mercaptoethanol. These green fluorescent spots are postulated to be alkaline degradation products of some unknown compound.

A new fluorescent spot was found in pr, but not pr^{bw}, in trace amounts. It possesses an Rf value of 0.60 in 1:1 isopropanol : 2% ammonium acetate and an Rf value of 0.45 in 3% ammonium chloride on cellulose thin-layer chromatograms. This light blue fluorescent spot persisted in stocks of $su(s)^{2};pr$, $pn^{2};pr$, and $pr;Hn^{r3}$. It was not detected in any other mutant analyzed in this work. No attempt was made to identify this spot.

Since this new fluorescent spot is not present in pr^{bw} flies, it does not seem likely that the spot is a consequence of the pr locus. Although biopterin and pterin are found in 0-1 day-old flies in higher quantities than in Oregon-R, it does not seem that these pteridines are found in large enough quantities to indicate that they accumulate as a result of a metabolic block subsequent to their biosynthesis. None of the unidentified fluorescent spots on chromatograms of pr appeared to accumulate in quantities significantly larger than those of wild-type.

Pteridine Analyses of Double Mutants

The effect of the pr locus when in genetic combination with several other eye-color mutations was determined by comparing the pteridine patterns of the single mutants with that of the double mutant. A detailed developmental analysis was carried out on pn^2, se, pn^2;pr, and pr;se in addition to pr. pn^2 has lower titers of several pteridines but no larger accumulations of any pteridine, resembling pr in this respect. However, se accumulates several pteridines in considerable quantities and is devoid of all drosopterins.

A comparison of pn^2, pr, and pn^2;pr with regard to their titers of quench spot, drosopterin, and pterin are shown in Figures 14-16. These pteridines in pn^2;pr are depressed to levels below those found in either of the single mutants.

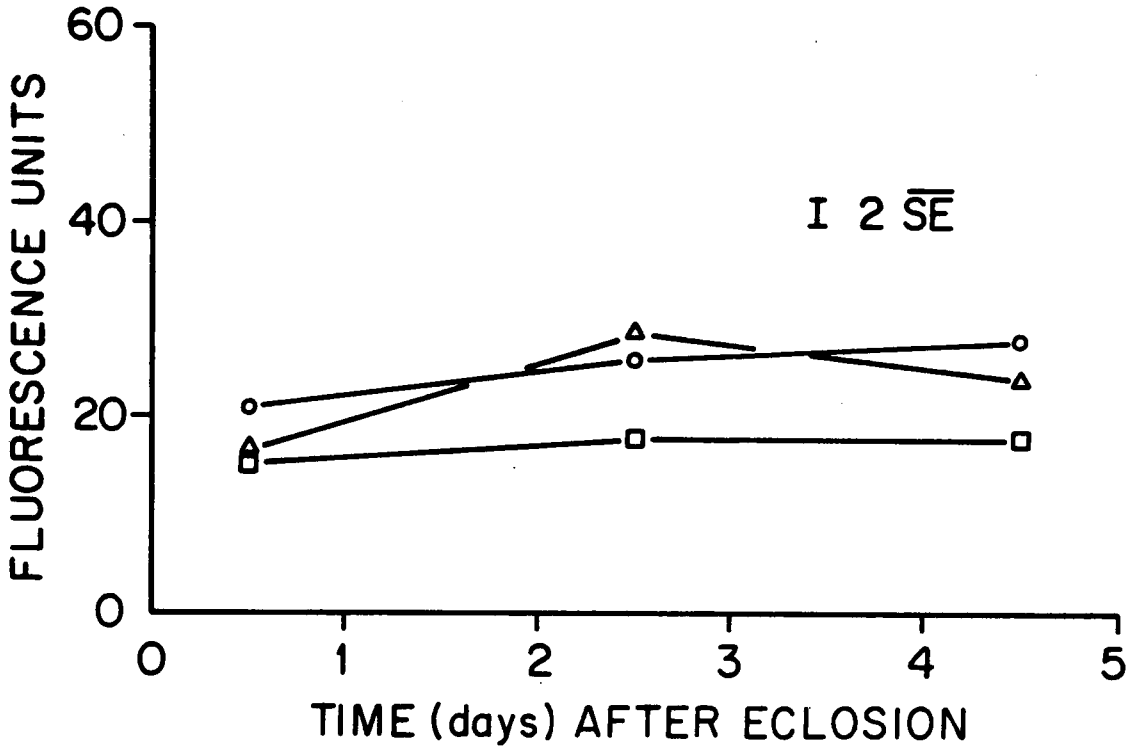


Figure 14. Titers of Quench Spot in pr, pn^2, and pn^2;pr Adults as a Function of Age.

- ○ pr
- △ pn^2
- □ pn^2;pr

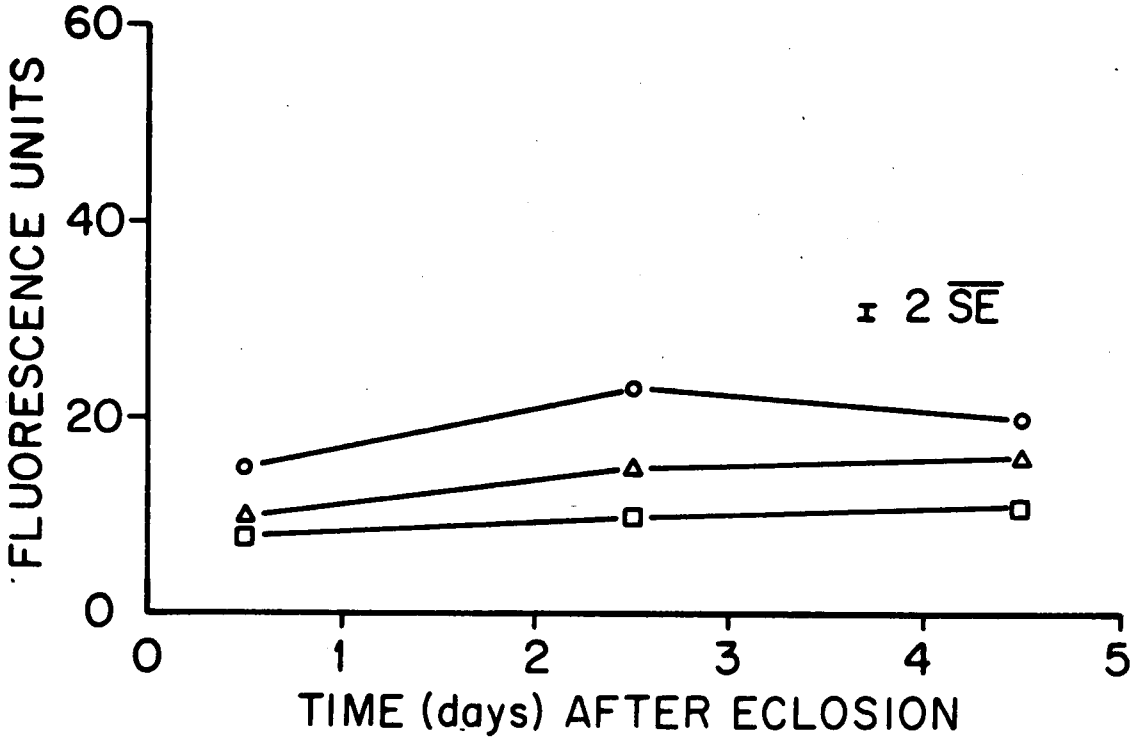


Figure 15. Titers of Drosopterin in pr, pn^2, and pn^2;pr Adults as a Function of Age.

○ pr
△ pn^2
□ pn^2;pr

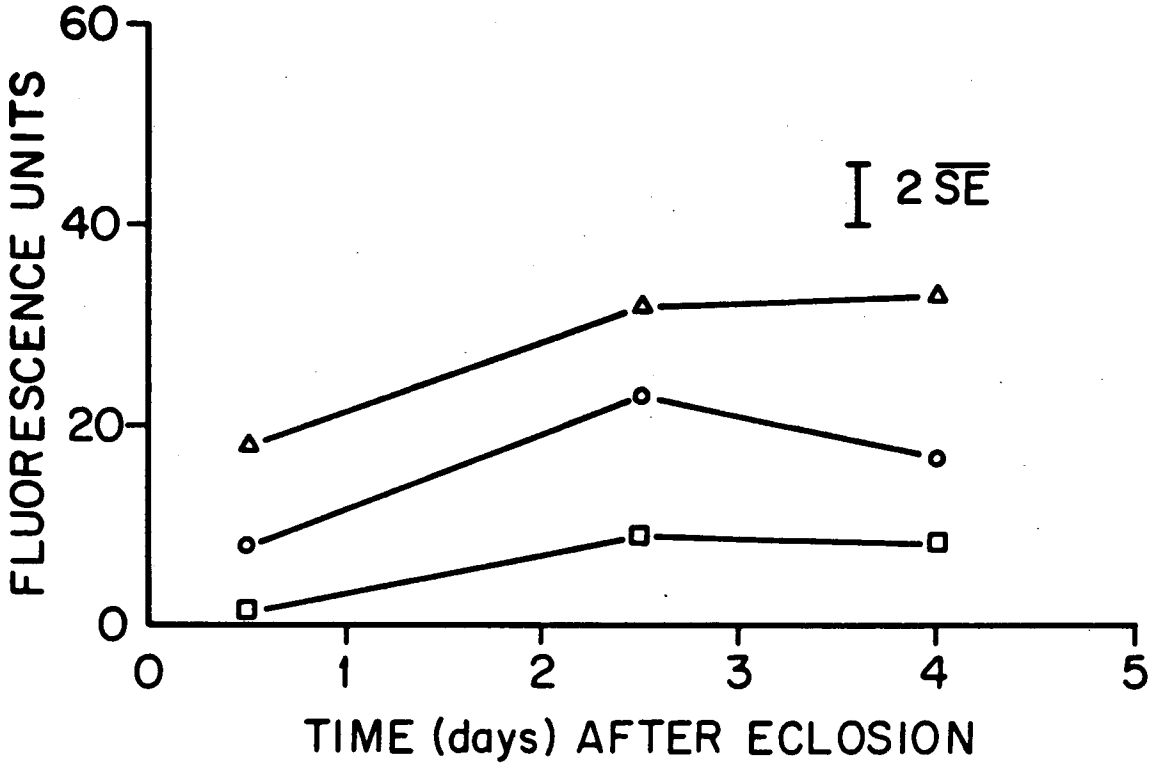


Figure 16. Titers of Pterin in pr, pn^2, and pn^2;pr Adults as a Function of Age.

o pr
Δ pn^2
□ pn^2;pr

Biopterin, on the other hand, which is present in $\underline{pn^2}$ in an amount greater than wild-type, is depressed in $\underline{pn^2;pr}$ to a level somewhat intermediate between those found in $\underline{pn^2}$ and $\underline{pr}$ (Figure 17). Thus, although $\underline{pr}$ has a wild-type titer of biopterin, it acts to depress the elevated titer of this pteridine in $\underline{pn^2}$ when genetically combined with $\underline{pn^2}$. Sepiapterin is present in $\underline{pn^2}$ in an amount nearly equivalent to that of wild-type. In $\underline{pn^2;pr}$ the level is reduced to a trace amount, similar to the level in $\underline{pr}$.

The effect of $\underline{pr}$ in genetic combination with $\underline{se}$ is to depress the levels of biopterin, pterin, and sepiapterin, all of which accumulate markedly in $\underline{se}$. As shown in Figures 18-20, the titers of these pteridines occur at intermediate levels in $\underline{pr;se}$ relative to those of $\underline{pr}$ and $\underline{se}$. The titer of sepiapterin in $\underline{pr;se}$ approximates that of $\underline{pr}$ more than that of $\underline{se}$, but it is greater than the titer found in wild-type. No detectable quantities of quench spot or any of the drosopterins can be found in $\underline{se}$ flies; the same is true in $\underline{pr;se}$.

The effect of $\underline{pr}$ in genetic combination with $\underline{Hn^{r3}}$ on biopterin, sepiapterin, pterin, and quench spot titers is similar to that noted for $\underline{pr}$ in combination with $\underline{pn^2}$ (Table XV).

The titers of these pteridines in the double mutant are reduced to those found in $\underline{pr}$, or lower. However, the titers

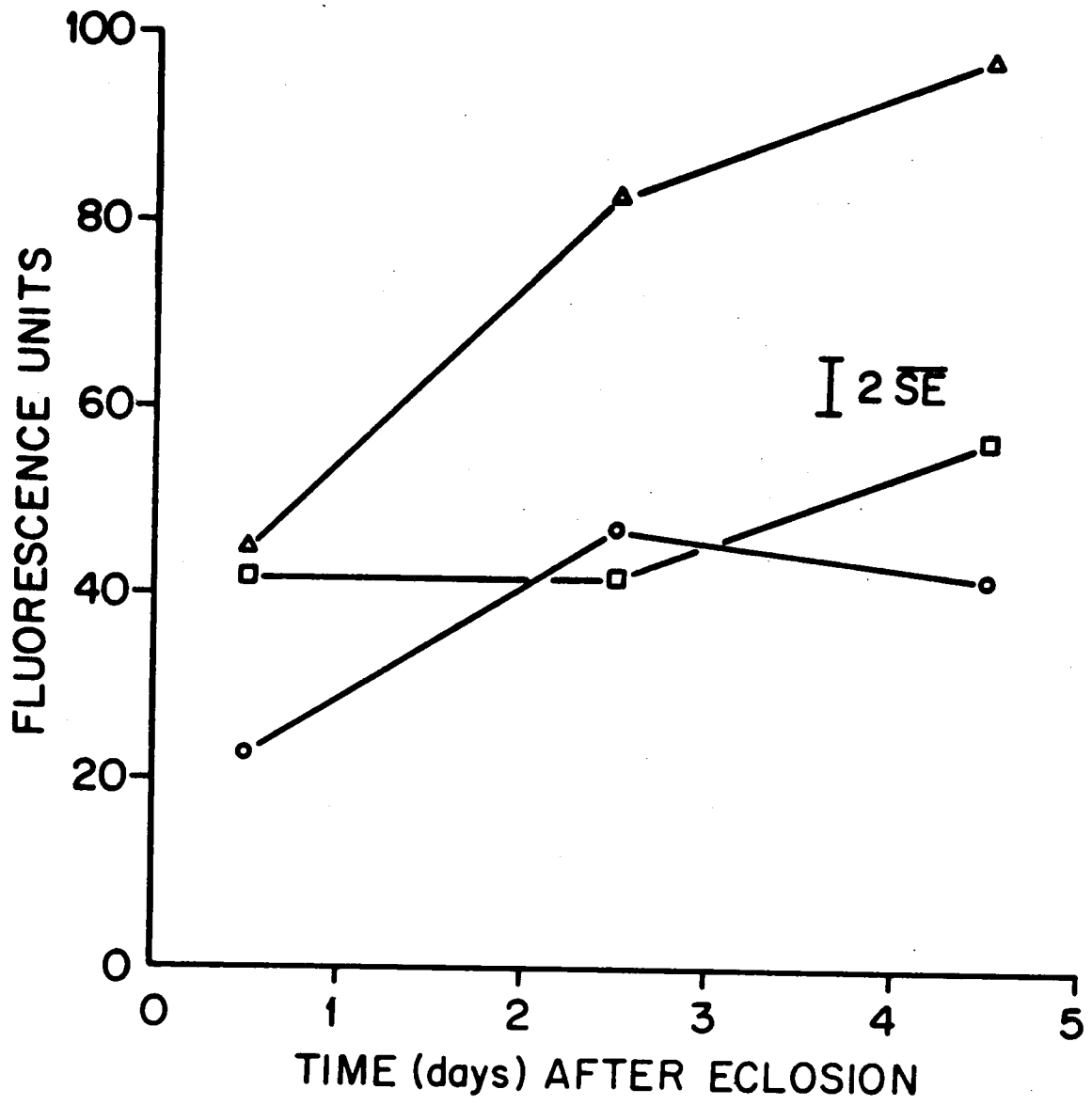


Figure 17. Titers of Biopterin in pr, pn^2, and pn^2;pr Adults as a Function of Age.

- ○ pr
- △ pn^2
- □ pn^2;pr

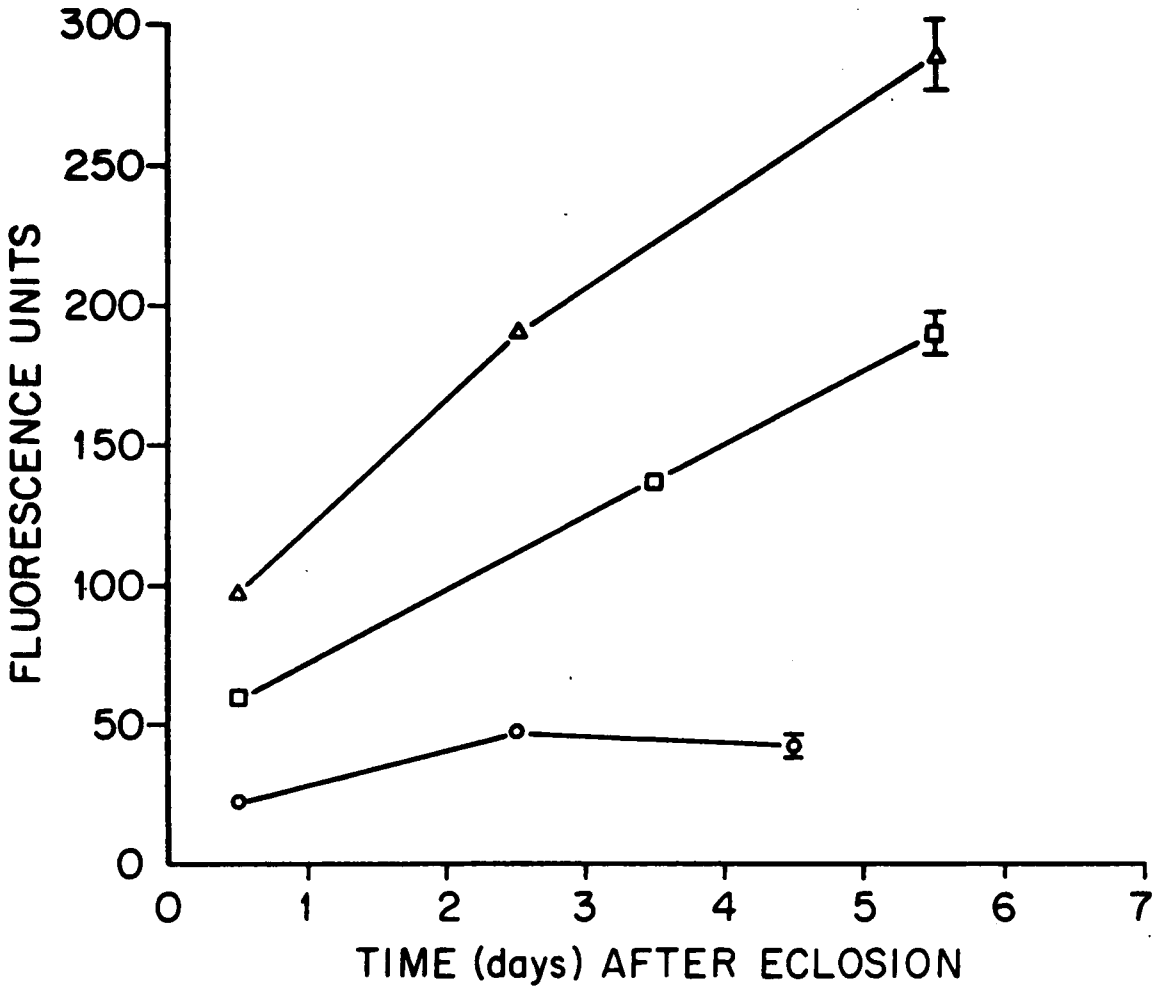


Figure 18. Titers of Biopterin in pr, se, and pr;se Adults as a Function of Age.

○ pr
△ se
□ pr;se

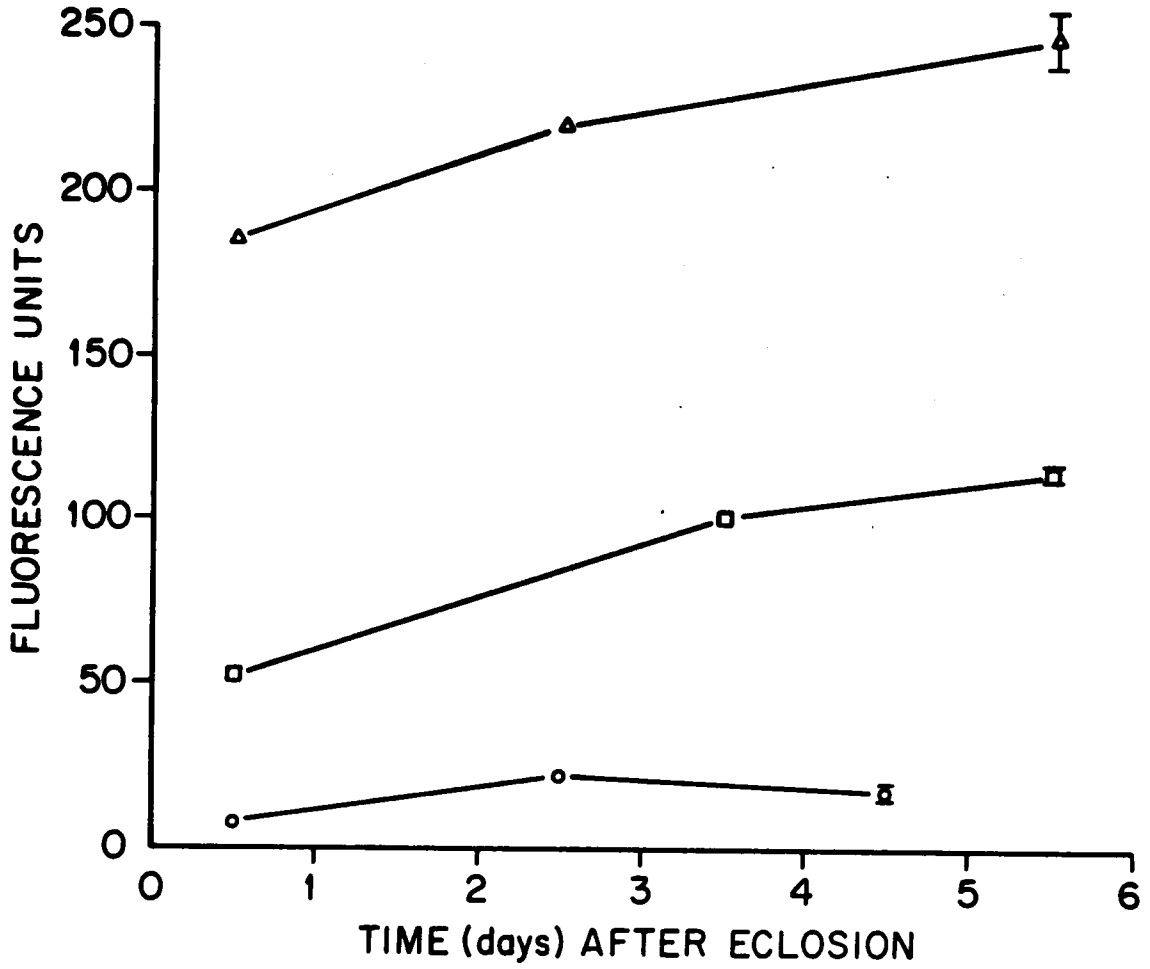


Figure 19. Titers of Pterin in pr, se, and pr;se Adults as a Function of Age.

o pr
Δ se
□ pr;se

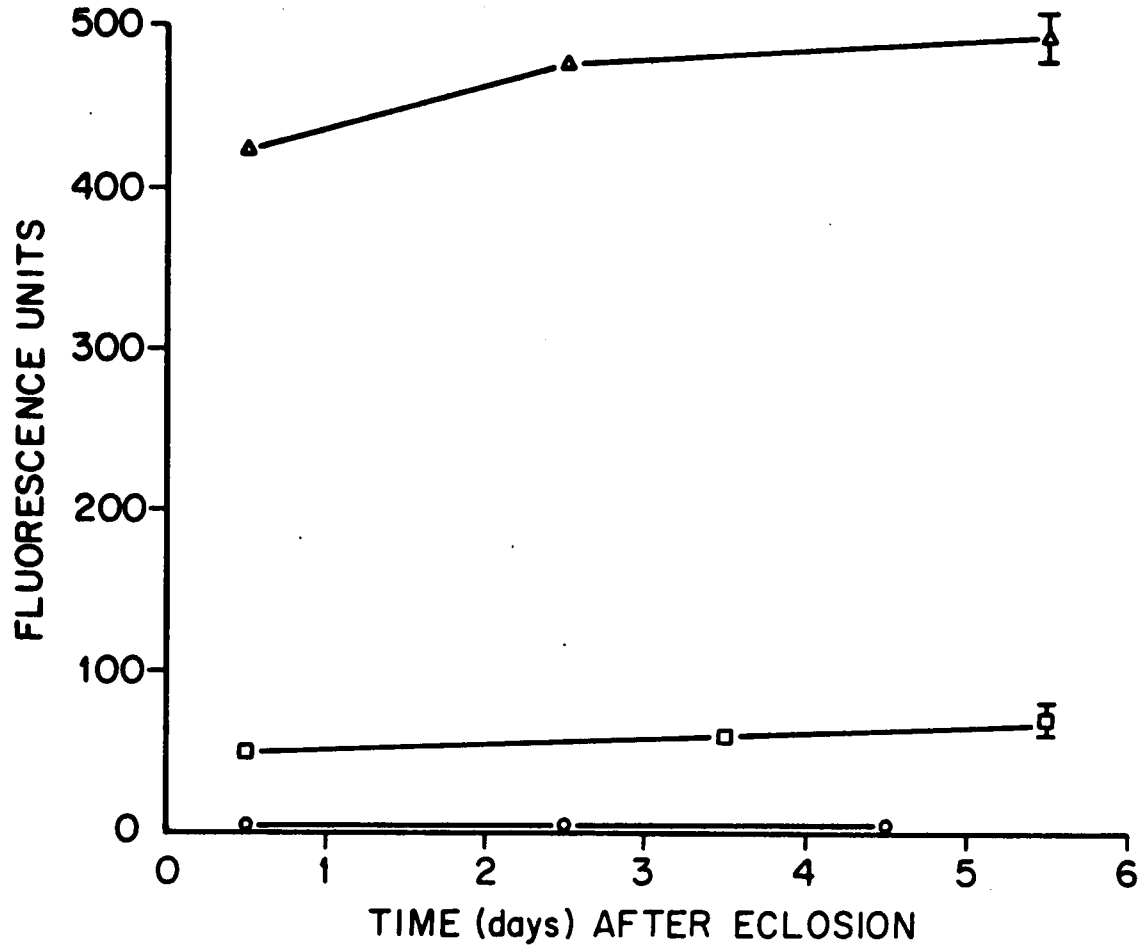


Figure 20. Titers of Sepiapterin in pr, se, and pr;se Adults as a Function of Age.

o pr
Δ se
□ pr;se

TABLE XV

COMPARISON OF HEAD PTERIDINE QUANTITIES FROM *pr*, *Hn^{r3}*, AND *pr;Hn^{r3}* FLIES

Pteridine	Quantity Measured (fluorescence units) pr	Hn^{r3}	pr;Hn^{r3}
Biopterin	23 (2.0)	64 (8.0)	20 (0.5)
Sepiapterin	trace	70 (2.0)	trace
Pterin	8 (1.0)	ND	ND
Isoxanthopterin	30 (3.5)	ND	17 (1.0)
Drosopterin	15 (0.0)	6 (0.5)	10 (0.5)
Isodrosopterin	12 (0.5)	5 (1.0)	8 (0.5)
Aurodrosopterin	2 (0.5)	ND	1 (0.0)
Neodrosopterin	11 (0.0)	2 (0.0)	7 (1.0)
Quench Spot	21 (1.0)	82 (22.0)	11 (0.0)

ND=not detectable on chromatogram.

All flies were 0-1 day old.

Standard errors (N=2) included in parentheses.

of the drosopterins in pr;Hn^{r3} are found at intermediate levels between pr and Hn^{r3}. This is clearly different from the situation found in pn^{2};pr for the drosopterins. Quench spot is elevated in 0-1 day-old Hn^{r3} relative to wild-type, and it accumulates in Hn^{r3} older than two days to a quantity twice that found in wild-type. Of the various eye-color mutants analyzed in this work, Hn^{r3} is the only one which accumulates quench spot.

Since se is devoid of detectable amounts of quench spot, and Hn^{r3} accumulates this presumed pteridine with aging, it was of interest to determine its fate in se Hn^{r3}. As shown in Table XVI, quench spot is not detectable in extracts from se Hn^{r3}. Quantities of other pteridines in se Hn^{r3} very closely resemble those in se.

Nucleotide Analysis of pr

The fact that many of the pteridines in pr and double mutants containing pr are depressed suggests that the affected enzymatic reaction in pr is early in the drosopterin biosynthetic pathway. Since no pteridine accumulates in pr, the affected enzymatic reaction may be the initial reaction; i.e., conversion of GTP to dihydroneopterin triphosphate. If this is the case, one would expect GTP to accumulate, but this nucleotide would not be evident on the thin-layer chromatogram under UV light, since it does not fluoresce.

TABLE XVI

COMPARISON OF HEAD PTERIDINE QUANTITIES IN *se*, *Hn^{r3}*, AND *se Hn^{r3}* FLIES

Pteridine	Quantity Measured (fluorescence units)		
	se	*Hn^{r3}*	*se Hn^{r3}*
Biopterin	96 (12.0)	64 (8.0)	95 (5.0)
Sepiapterin	426 (10.0)	70 (2.0)	445 (35.0)
Pterin	120 (0.0)	ND	79 (7.0)
Isoxanthopterin	14 (6.0)	ND	trace
Drosopterin	ND	6 (0.5)	ND
Isodrosopterin	ND	5 (1.0)	ND
Aurodrosopterin	ND	ND	ND
Neodrosopterin	ND	2 (0.0)	ND
Quench Spot	ND	82 (22.0)	ND

ND=not detectable on chromatograms.

All flies were 0-1 days old.

Standard errors (N=2) included in parentheses.

Therefore, I analyzed the GTP levels in pr and wild-type fly heads to determine if pr accumulates GTP relative to wild-type.

Initially, fly head preparations were weighed and then homogenized in perchloric acid at a ratio of 0.1 g/ml so that GTP titers could be quantitatively compared in pr and wild-type head preparations.

The nucleotide fraction of the homogenate supernatant was separated by chromatography over a Bio-Gel P-2 column. A typical elution pattern is presented in Figure 21. The initial peak, which contains the nucleotide fraction, was collected from point A to point B. The collected volume, usually about 2 ml, was immediately subjected to Aminex A-28 chromatography.

The chromatogram obtained upon Aminex A-28 chromatography of the initial peak from a P-2 column is shown in Figure 22. The peaks on the chromatogram are predominantly adenosine and guanosine nucleotides. Each peak was identified by comparison with authentic standards. Table XVII compares the elution positions of standards applied to the column with peak positions from Aminex chromatograms of Oregon-R head extracts.

It should be noted at this point that Aminex A-28 columns of various lengths, ranging from 9 to 12 cm, were used in the following nucleotide determinations. Differences in length affect the nucleotide elution positions; therefore,

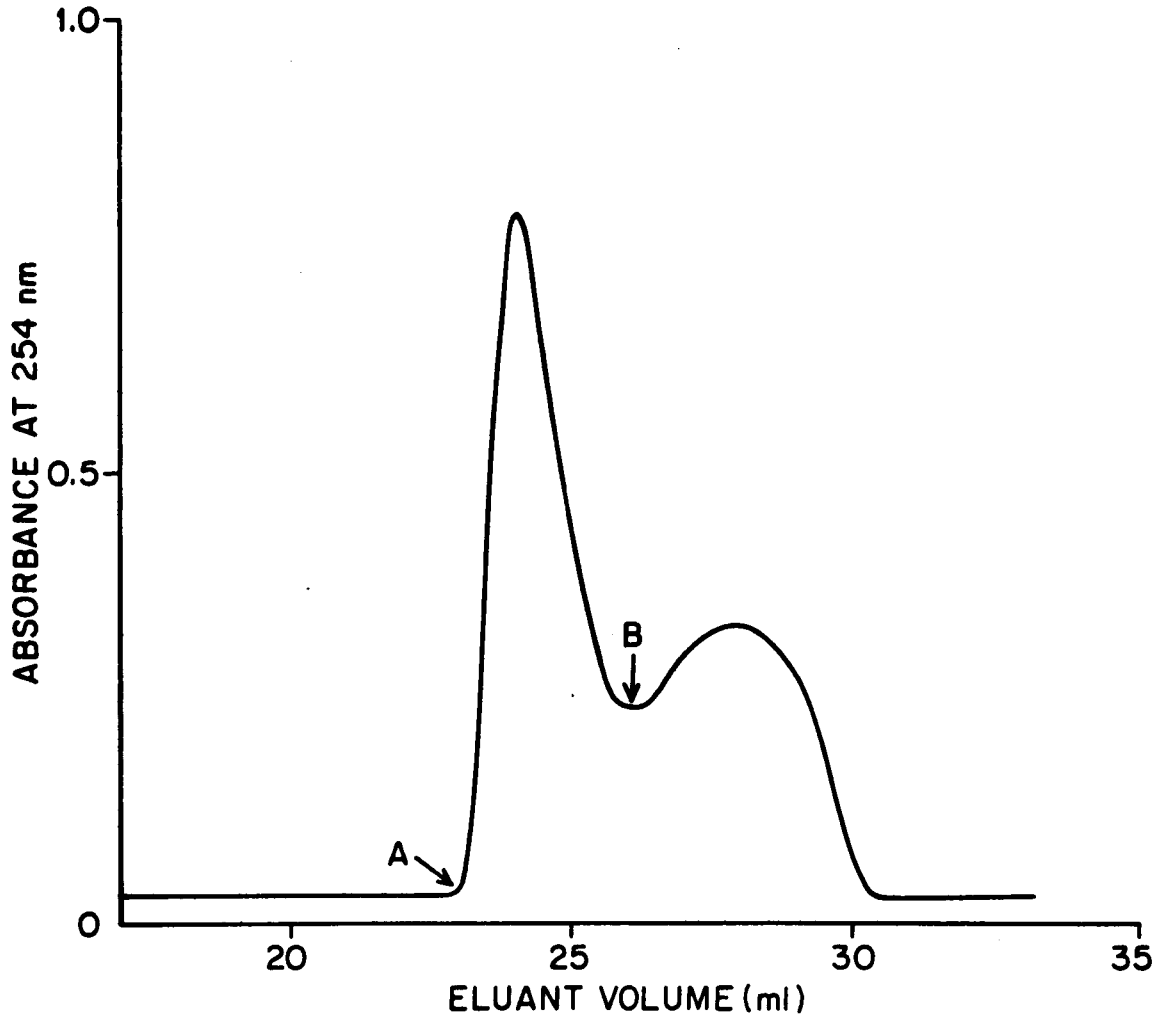


Figure 21. Elution of Drosophila Head Extract from a Bio-Gel P-2 Column.

Two hundred microliters of an Oregon-R head extract was placed on the column. The nucleotide-containing fraction was collected from point A to point B.

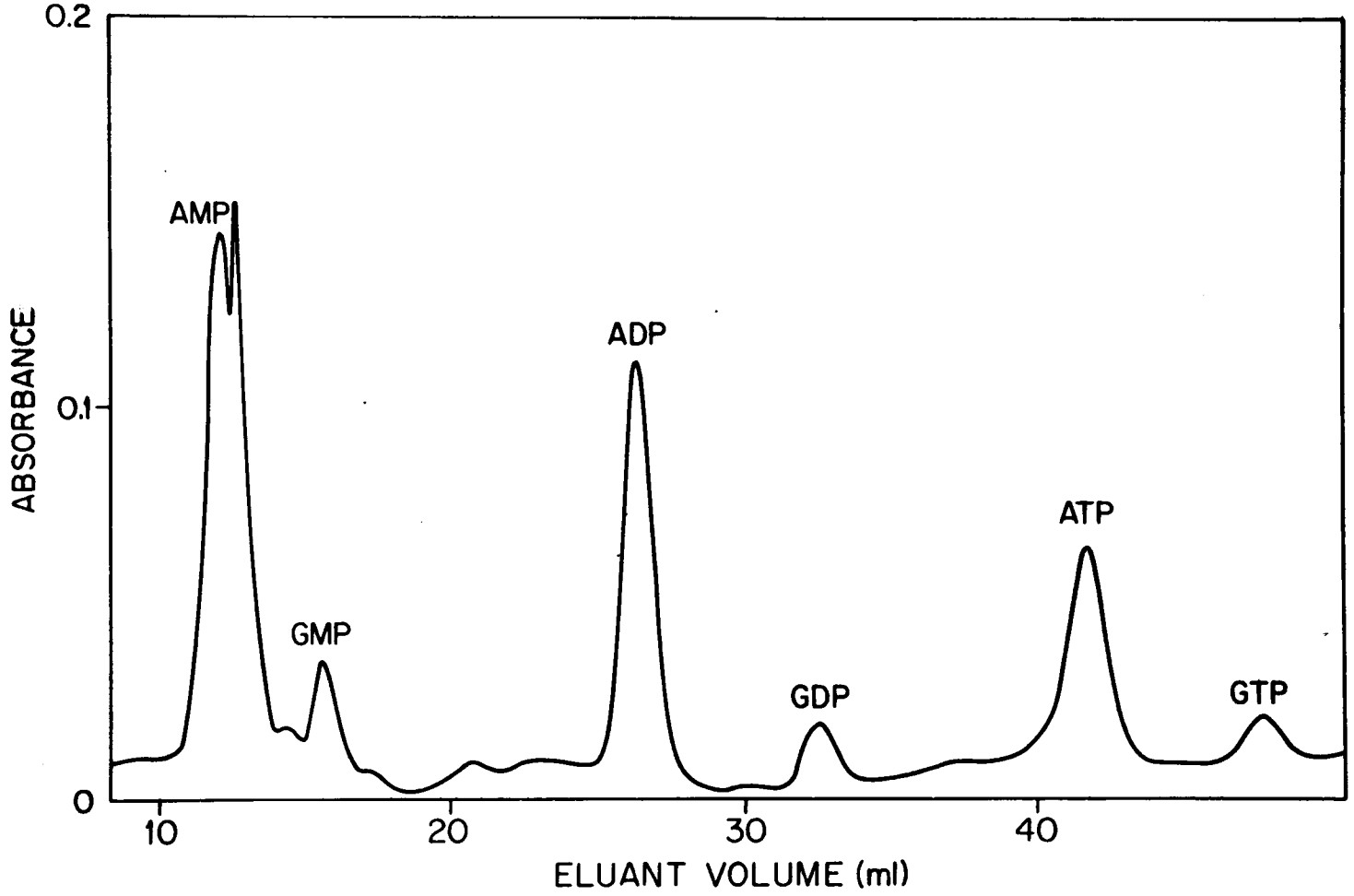


Figure 22. Nucleotide Elution Profile from an Aminex A-28 Column of a Thawed Drosophila Head Preparation.

Two milliliters of a nucleotide peak which was collected from a Bio-Gel P-2 column was placed over the Aminex column. Oregon-R flies were used for the head preparation.

TABLE XVII

COMPARISON OF THE ELUTION POSITIONS OF NUCLEOTIDE STANDARDS WITH PEAK POSITIONS OBTAINED AFTER AMINEX CHROMATOGRAPHY OF AN OREGON-R HEAD EXTRACT

Column Length (cm)	Standard	Presumed Peak	Elution Position (ml)
12	-	AMP	16.2
12	AMP	-	16.0
12	-	ADP	31.0
12	ADP	-	31.8
10	-	GMP	16.8
10	GMP	-	16.9
10	-	GDP	31.5
10	GDP	-	31.7
10	-	ATP	40.0
10	ATP	-	40.1
10	-	GTP	45.1
10	GTP	-	45.4

The peaks from head extract chromatograms are labelled as in Figure 22. An aliquot of 2-4 µg of each standard was applied to the column. A longer column (approximately 12 cm) was used when AMP and ADP were compared with an Oregon-R head homogenate, hence the discrepancy in elution positions of these nucleotides with those eluting from the 10 cm column.

one can compare the relative elution positions of the various nucleotides within an experiment, but not between experiments. For example, the elution position of ATP in Table XVII should not be compared with that shown in Figure 22.

In addition, when 2 µg each of ADP and GDP were added to an Oregon-R extract and chromatographed over Aminex, the standards co-chromatographed with presumed peaks ADP and GDP, respectively.

Further evidence for the identity of these peaks was provided by phosphatase digestion of a pr head extract prior to Aminex chromatography. Fifty micrograms of Worthington chromatographically purified alkaline phosphatase was added to a 1-ml aliquot of the nucleotide peak collected from the Bio-Gel P-2 column. After a 3-hour digestion at 22°C, the aliquot was boiled and centrifuged to remove the phosphatase. Aminex chromatography of the supernantant (Figure 23) revealed two peaks (peaks A and C) with elution positions similar to adenosine and guanosine which had been chromatographed either as authentic standards or as phosphatase digests of ATP and GTP (Table XVIII).

The AMP shoulder peak seen in Figure 22 is likewise not digested and appears as peak D, Figure 23. This result suggests co-chromatography of some UV-absorbing compound with ADP. However, all other peaks are totally eliminated by phosphatase digestion.

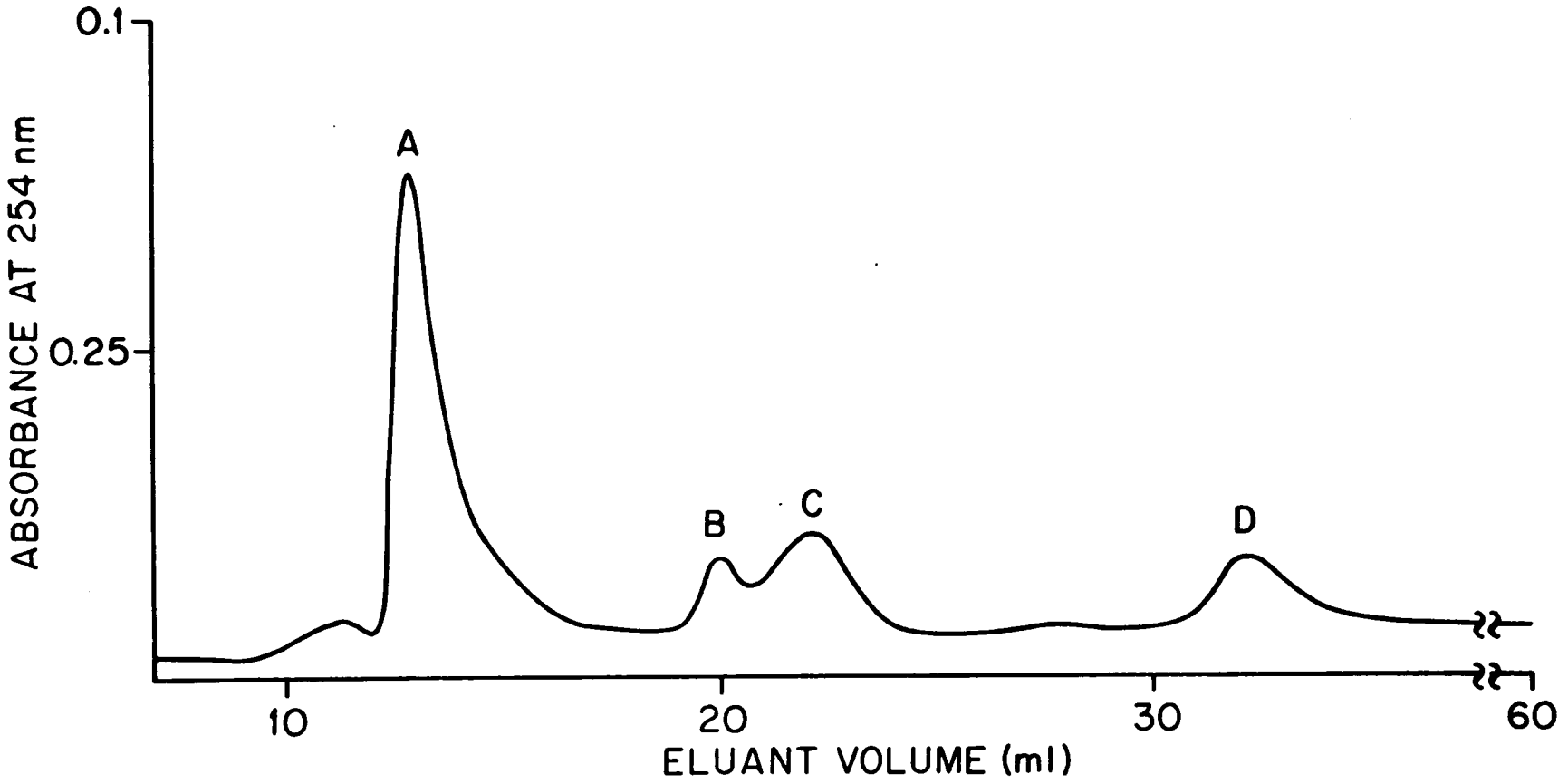


Figure 23. Nucleotide Elution Profile from an Aminex A-28 Column of an Alkaline Phosphatase Digested Drosophila Head Preparation.

Two milliliters of a nucleotide peak which was collected from a Bio-Gel P-2 column was mixed with 50 µg of alkaline phosphatase subsequent to Aminex chromatography. Peak identification is discussed in the text.

TABLE XVIII

COMPARISON OF ELUTION POSITIONS OF ADENOSINE AND GUANOSINE WITH THOSE OF THE MAJOR PEAKS OBTAINED AFTER ALKALINE PHOSPHATASE DIGESTION OF A pr HEAD EXTRACT

Standard/Peak	Elution Position (ml)
peak A	12.7
peak C	21.9
phosphatase digested ATP	12.5
phosphatase digested GTP	22.0
adenosine	11.4
guanosine	21.9

When nucleotides from fly head extracts were quantitated in this manner, reproducible results were difficult to obtain. It was subsequently found that during the few seconds required to weigh the fly head preparations, the heads became sufficiently thawed so that an endogenous phosphatase(s) acted to generate mono- and dinucleotides from GTP and ATP. If a frozen head preparation is immediately homogenized in ice-cold perchloric acid, a nucleotide pattern such as the one shown in Figure 24 is obtained. The predominant nucleotides are ATP and GTP (peaks F and G); the mono- and dinucleotides are present in essentially negligible quantities. The AMP-shoulder peak in Figure 22, page 90, is present as a single peak on the Aminex chromatogram (peak B, Figure 24). It should be noted that the small peak between D and E in Figure 24 is not GDP; this nucleotide chromatographs to position E on the chromatogram.

Therefore, nucleotide analysis of pr, su(s)^2;pr, and two wild-type strains were carried out according to the modified procedure described in Materials and Methods. Since the head preparations are not weighed, nucleotide titers in each mutant can not be determined. Therefore, the results are given by ATP/GTP ratios. These are shown in Table XIX. There appears to be no significant difference between the ATP/GTP ratios of pr and either of the two wild-type strains.

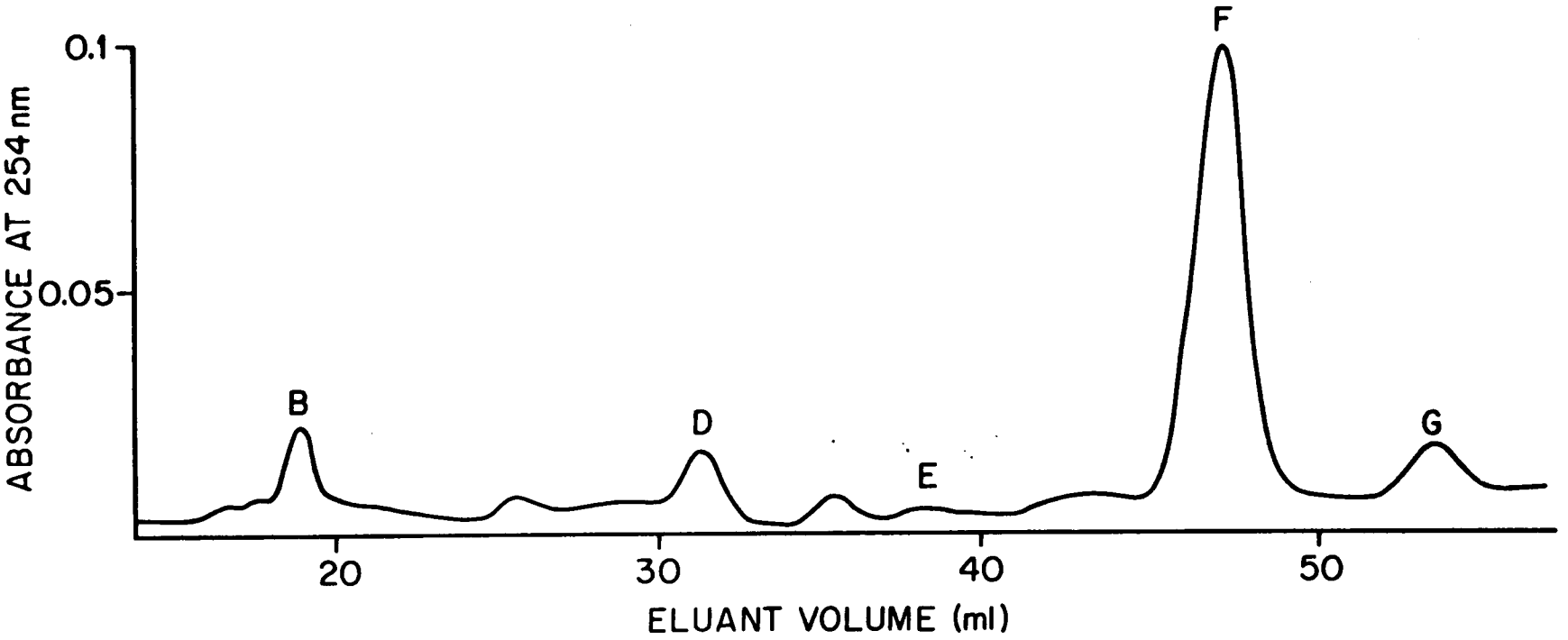


Figure 24. Nucleotide Elution Profile from an Aminex A-28 Column of a Frozen Drosophila Head Preparation.

Two milliliters of a nucleotide peak which was collected from a Bio-Gel P-2 column was placed over the Aminex column. Oregon-R flies were used for the head preparation.

TABLE XIX

ATP/GTP RATIOS IN HEAD EXTRACTS OF *pr*, *su(s)*^{2};*pr*, OREGON-R, AND SAMARKAND FLIES

Mutant/Strain	ATP/GTP + SE
Samarkand	8.0 ± 0.4
Oregon-R	7.9 ± 0.4
pr	7.4 ± 0.7
su(s)^2;*pr*	9.2 ± 0.1

All flies were 0-4 hours old. Each ratio is an average of two determinations, except *pr*, which is an average of three. The means for *su(s)*^{2};*pr* and Samarkand differ significantly at P=0.05 but not P=0.02 (Student's T-test).

Characteristics of the Suppression of pr

When su(s)^2 was crossed with pr^{bw} to obtain su(s)^2;pr^{bw}, the pr^{bw} eye color was completely suppressed. Although su(s)^2 suppresses both pr and pr^{bw}, it is not a general suppressor of eye-color mutants. su(s)^2 failed to suppress bw, Pu^2, and pd when in genetic combination with these mutants.

Stocks of v reared on starvation medium exhibit a phenotype resembling that of su(s) v. Presumably, limited xanthommatin biosynthesis is resumed in these flies. However, when cn pr flies are reared on starvation medium, the phenotype of the adult is indistinguishable from that of cn pr flies reared on normal medium. v;bw flies reared on the same starvation medium show a phenotype similar to su(s)^2 v;bw. Therefore, pr flies reared on starvation medium do not show a discernable phenotype response to the low yeast concentration as does v;bw.

A pteridine analysis was carried out on su(s)^2, su(s)^2;pr, and su(pr)^{e3};pr. In Figures 25-29 a comparison of these mutants with pr and Oregon-R is shown for five pteridines. Each point on the curves obtained for su(s)^2, su(s)^2;pr, and su(pr)^{e3};pr represents a single analysis. Each point for pr and Oregon-R represents an average of two separate determinations.

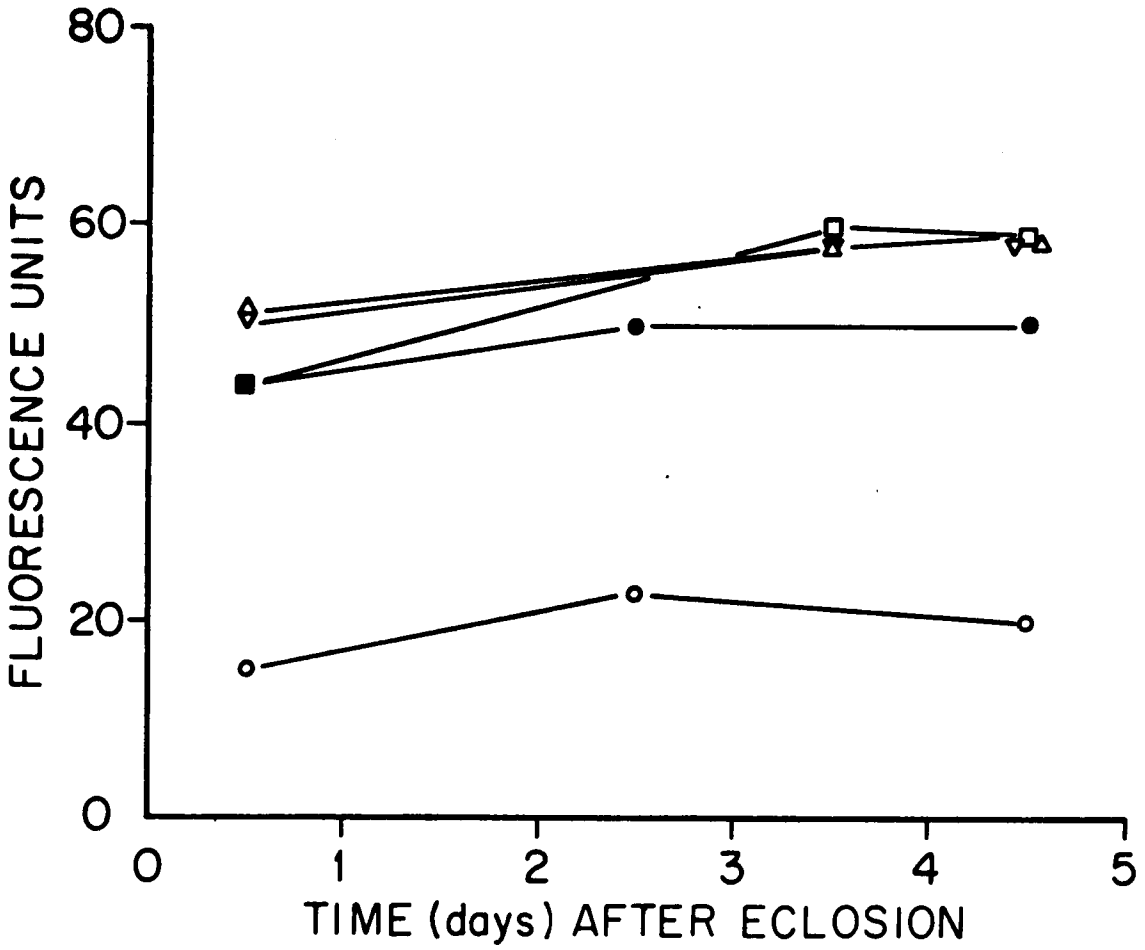


Figure 25. Titers of Drosopterin in pr, Suppressed pr. and Oregon-R Adults as a Function of Age.

- ○ pr
- △ su(pr)^{e3};pr
- □ su(s)^{2},pr
- ▽ su(s)^{2}
- ● Oregon-R

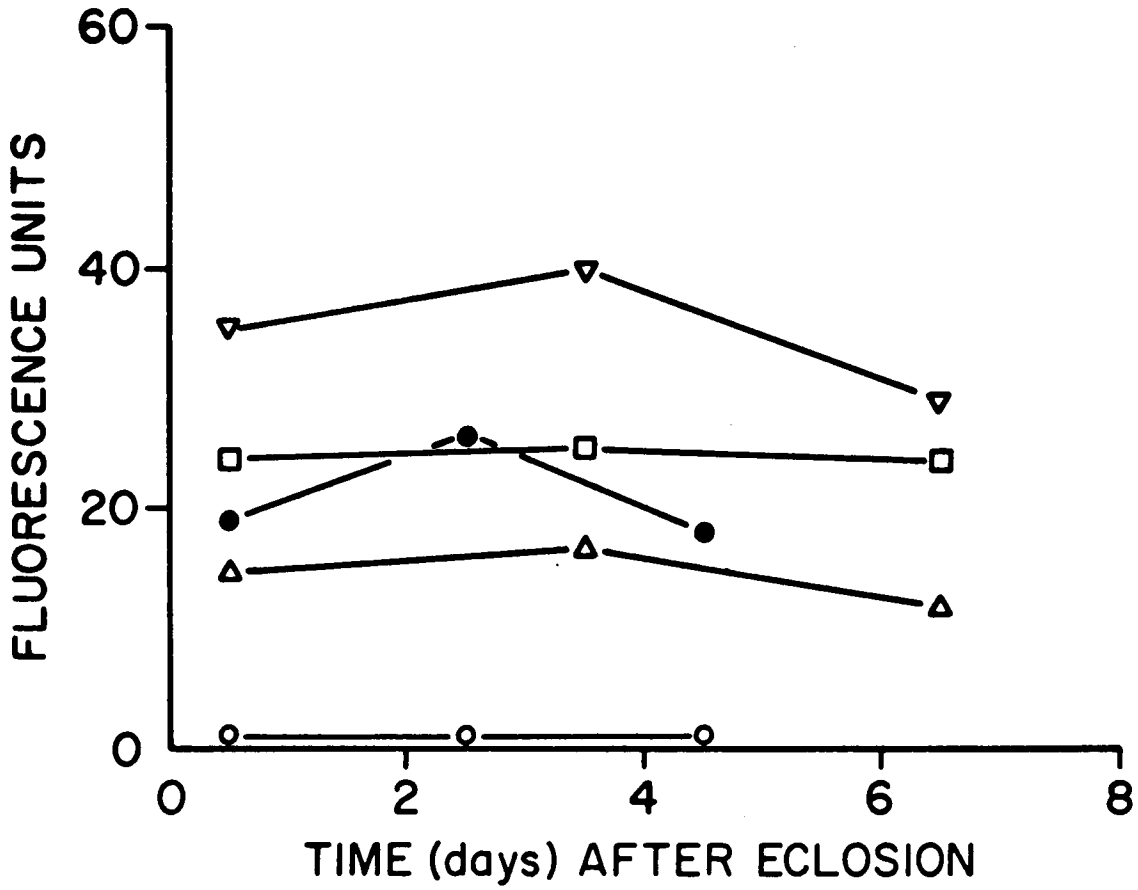


Figure 26. Titers of Sepiapterin in pr, Suppressed pr, and Oregon-R Adults as a Function of Age.

- ○ pr
- △ su(pr)^{e3};pr
- □ su(s)^{2};pr
- ▽ su(s)^{2}
- ● Oregon-R

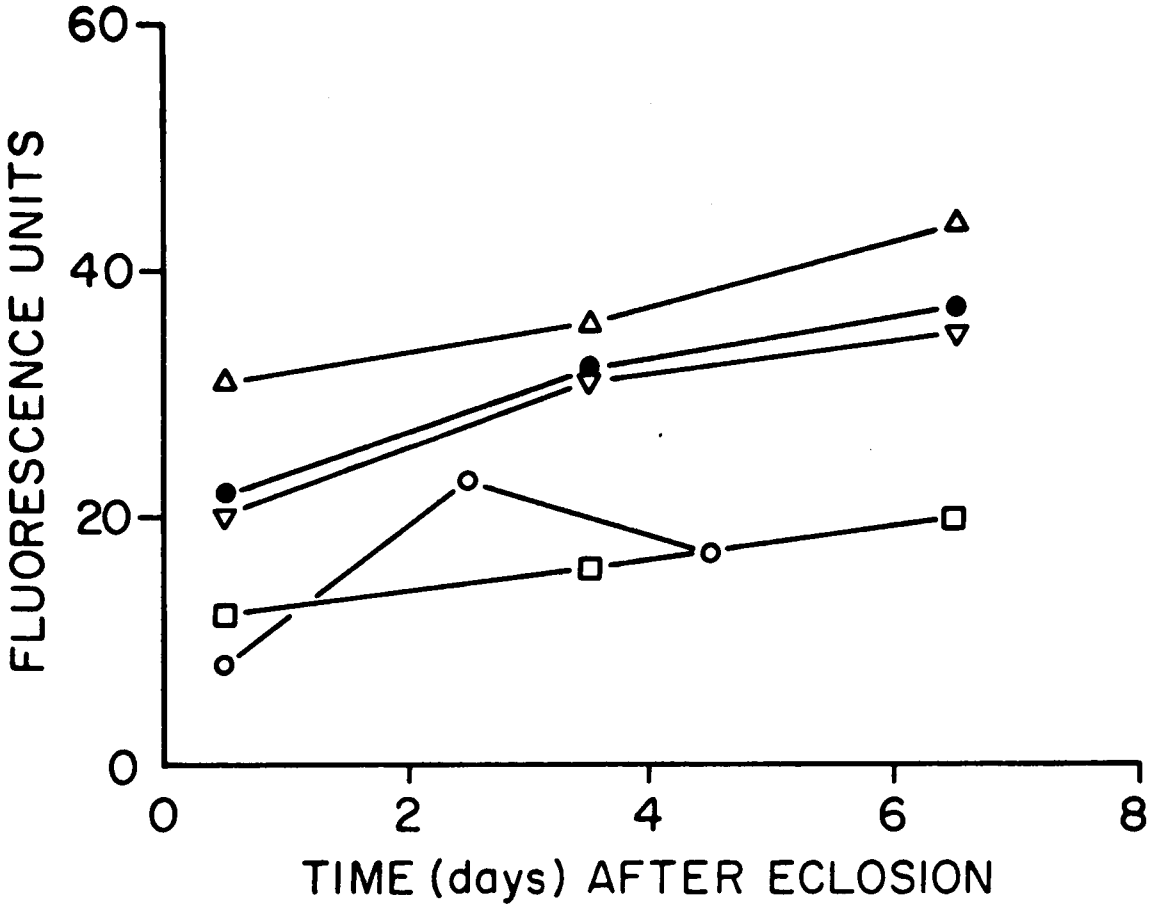


Figure 27. Titers of Pterin in pr, Suppressed pr, and Oregon-R Adults as a Function of Age.

- ○ pr
- △ su(pr)^{e3};pr
- □ su(s)^{2};pr
- ▽ su(s)^{2}
- ● Oregon-R

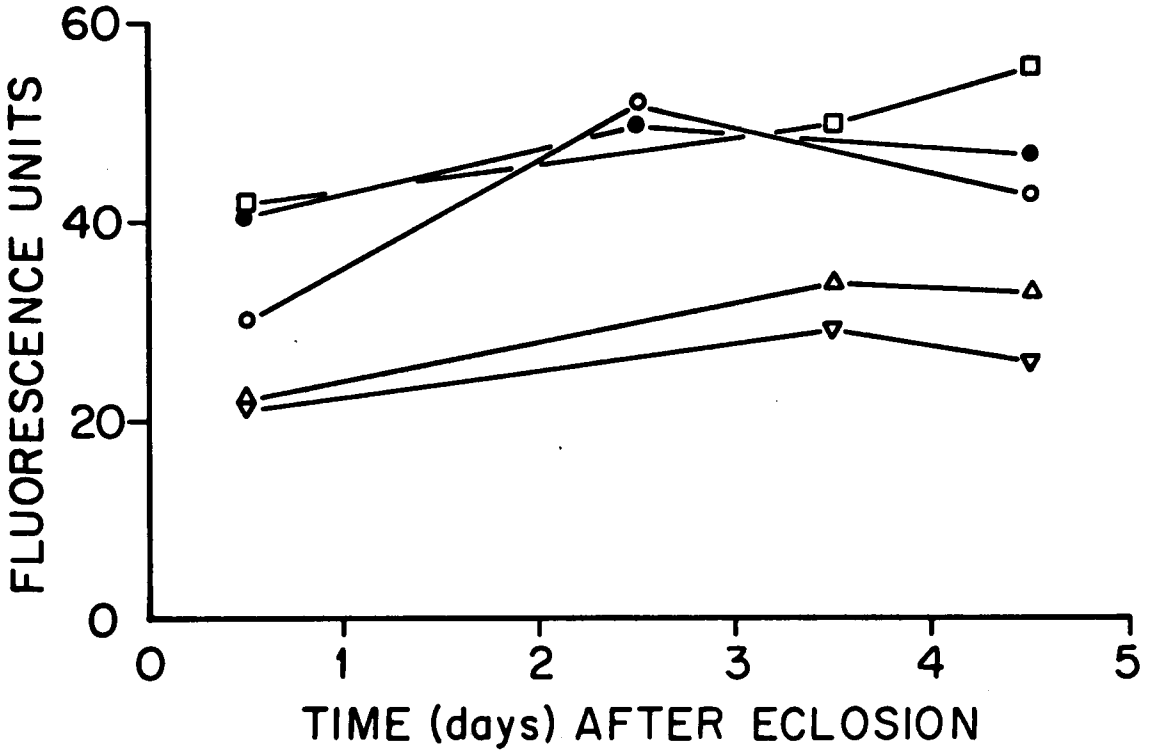


Figure 28. Titers of Isoxanthopterin in pr, Suppressed pr, and Oregon-R Adults as a Function of Age.

- o pr
- Δ su(pr)^{e3};pr
- □ su(s)2;pr
- ▽ su(s)^{2}
- ● Oregon-R

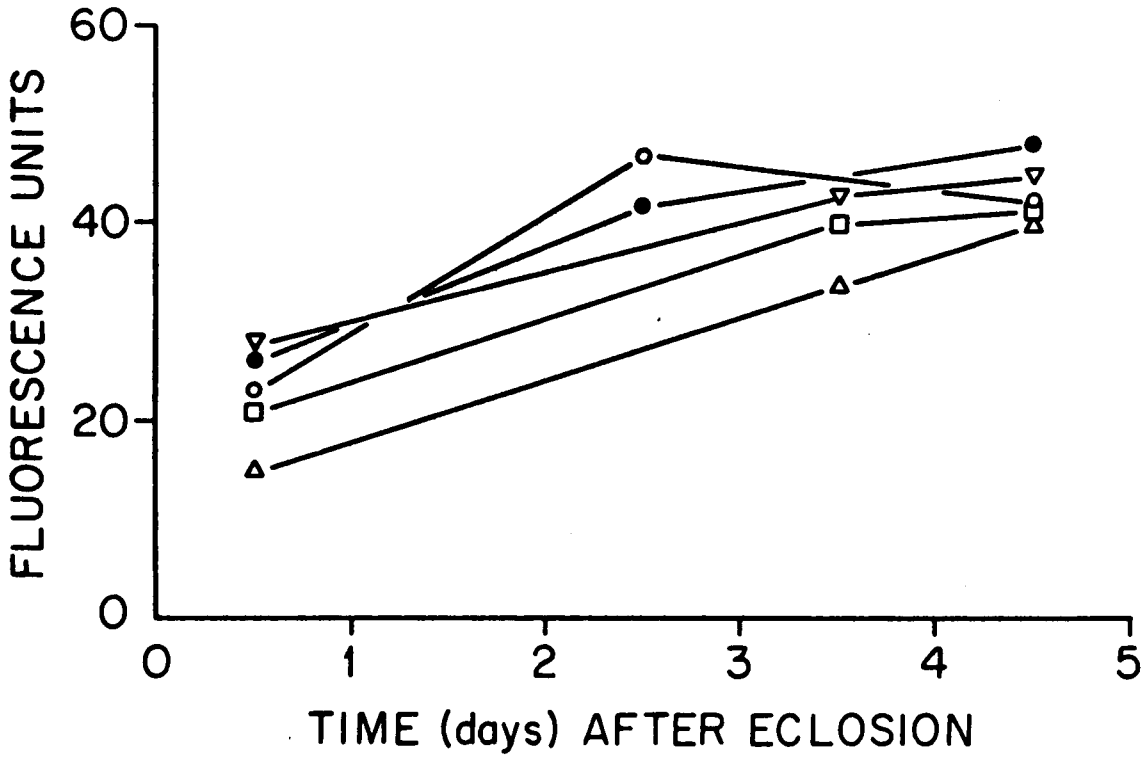


Figure 29. Titers of Biopterin in pr, Suppressed pr, and Oregon-R Adults as a Function of Age.

- ○ pr
- △ su(pr)^{e3};pr
- □ su(s)^{2};pr
- ▽ su(s)^{2}
- ● Oregon-R

Figure 25, page 99, compares drosopterin titers in these mutants. As can be seen, both $\underline{su(s)^2;pr}$ and $\underline{su(pr)^{e3};pr}$ have essentially wild-type levels of drosopterin. The same is true for isodrosopterin, aurodrosopterin, and neodrosopterin.

Figure 26, page 100, shows that sepiapterin is elevated from trace amounts in pr to near wild-type amounts in $\underline{su(s)^2;pr}$ and $\underline{su(pr)^{e3};pr}$. $\underline{su(s)^2;pr}$ appears to have a somewhat higher titer of sepiapterin than does $\underline{su(pr)^{e3};pr}$.

Titers of pterin and isoxanthopterin are shown in Figures 27 and 28, pages 101 and 102 respectively. The level of pterin in $\underline{su(s)^2;pr}$ is lower than $\underline{su(pr)^{e3};pr}$ and approximates the level in pr. Isoxanthopterin titers show erratic behavior in the mutants, and the results are difficult to rationalize.

Figure 29, page 103, shows that quantitative differences in biopterin content exist among the mutants, particularly $\underline{su(pr)^{e3};pr}$ and Oregon-R. However, the differences are not large enough to suggest that biopterin plays a key role in suppression of pr by either of the two suppressor mutations.

A comparison of pteridine quantities in 0-1 day old pr, Oregon-R, and suppressed pr is presented in Table XX. These values have been lifted from Figures 25-29, pages 99-103 and are presented in tabular form to emphasize the

TABLE XX

COMPARISON OF HEAD PTERIDINE QUANTITIES IN pr, OREGON-R, AND SUPPRESSED pr

	Quantity Measured (fluorescence units)			
Pteridine	pr	Oregon-R	su(s)^{2};pr	su(pr)^{e3};pr
Biopterin	22	26	21	15
Sepiapterin	trace	19	24	15
Pterin	8	22	12	31
Isoxanthopterin	30	41	42	22
Drosopterin	15	44	44	51
Quench Spot	+	+++	+++	+++

All flies were 0-1 days old. Quench spot was visually estimated on the chromatograms viewed in liquid nitrogen under UV light.

differences in pteridine quantities existing among pr, wild-type, su(s)^{2};pr, and su(pr)^{e3};pr.

Although quench spot was not quantitated in these mutants, visual examination of the chromatograms in liquid nitrogen under UV light showed this presumed pteridine to occur in su(s)^{2};pr and su(pr)^{e3};pr at levels comparable to that of wild-type (Table XX, page 105).

Spots F and G are restored to wild-type levels in su(s)^{2};pr, but these spots in su(pr)^{e3};pr are found in amounts comparable to pr. Another difference between the two suppressed pr mutants is that spots M and O are at wild-type levels in su(s)^{2};pr but are found in elevated quantities in su(pr)^{e3};pr.

IV. DISCUSSION

In order to study the mechanism of suppression of pr by su(s), it is necessary to locate the position of the metabolic block in pr flies. Once the affected enzymatic reaction in pr is identified, studies can then be initiated to determine if the enzyme is inhibited in a manner analogous to tryptophan 2,3-dioxygenase in v flies (33). Experiments can then be designed to study the mechanism of suppression of pr by the su(s) locus.

The results obtained from this work do not indicate which enzymatic reaction in the drosopterin biosynthetic

pathway is affected in pr. However, the results suggested two properties of pr: it appears to be a leaky mutant, and the metabolic block may occur early in the drosopterin biosynthetic pathway.

pr appears to be a leaky mutant; in other words, the metabolic block is partial instead of complete. Thus, a definite amount of the drosopterin fraction is biosynthesized, but this amount is lower than that of wild-type flies. The low amount of drosopterins in pr is predominantly responsible for the altered eye color relative to wild-type.

Two other pteridines also were found to be noticeably affected in pr. Sepiapterin is present in only a trace amount, and quench spot is considerably less than the amount found in wild-type. The observation that sepiapterin is low suggests that the position of the metabolic block in pr is early in the pathway.

The very low level of sepiapterin in pr indicates that the metabolic block occurs prior to sepiapterin biosynthesis. Unfortunately, little research has been done in Drosophila to determine the enzymatic steps leading to sepiapterin biosynthesis. However, work done on Rana catesbeiana (21) indicates that radiolabelled dihydroneopterin triphosphate, the first pteridine in the pathway, is metabolized in vivo to sepiapterin. The number of enzymatic steps required for this conversion is unknown. If the same pathway for

sepiapterin biosynthesis is functional in Drosophila, then dihydroneopterin triphosphate biosynthesis or its conversion to sepiapterin are likely suspects for the location of the enzymatic lesion in pr.

No large accumulation of any fluorescent spot occurs in pr. This observation suggests several possibilities: the accumulated product in pr is not fluorescent, or the accumulated fluorescent product is rapidly degraded in vivo or in vitro to a nonfluorescent product, or the fact that pr is a leaky mutant precludes the likelihood of any large product accumulation. Unfortunately, the results from this work do not indicate which of these possibilities is occurring in pr flies.

Pteridine analyses carried out on the double mutants containing pr also suggest that the affected enzymatic reaction in pr is early in the biosynthetic pathway. In mutants which accumulate pteridines, such as se and Hn^{r3}, inclusion of pr in the genotype resulted in a lowering of these accumulated pteridines. The fact that pterin, quench spot, and drosopterin are reduced in pn^{2};pr to titers lower than either pr or pn^{2} is not readily explained but again underscores the effect of the pr locus on the titers of these pteridines.

If the lesion in pr is located prior to or at the site of the first enzyme, which converts GTP to dihydroneopterin

triphosphate, then any accumulated product would not be evident under UV light on the chromatogram.

Since GTP is the probably metabolic precursor to the pteridines, the virtual absence of sepiapterin in pr could be due to a deficiency of GTP biosynthesis. Accordingly, the amount of GTP in pr was measured and compared to that in Oregon-R. No significant difference was noted. This result indicates that the metabolic block in pr is probably after GTP biosynthesis.

The result from the nucleotide analysis does not necessarily rule out the possibility that the block in pr is immediately after GTP biosynthesis, however. Since GTP is a substrate in a wide array of metabolic reactions, including nucleic acid and protein synthesis, any accumulation of it owing to a block in the reaction which converts GTP to dihydroneopterin triphosphate might be expected to be rapidly depleted for purposes other than pteridine biosynthesis. Thus, GTP might not accumulate, even though a portion of it is not utilized for pteridine biosynthesis.

In Rana catesbeiana (21) and also Chinese hamster cells (20), sepiapterin appears to be a precursor for biopterin biosynthesis. This is evidenced in both systems by the observation that addition of unlabelled sepiapterin prevents biopterin from acquiring label from radioactive GMP or dihydroneopterin triphosphate. This type of experiment has

not been done in Drosophila; however, an enzyme has been isolated in Drosophila which reduces sepiapterin to tetrahydrosepiapterin (73). It is postulated that tetrahydrosepiapterin spontaneously tautomerizes to dihydrobiopterin, which can then oxidize to biopterin. Indeed, the biopterin found on thin-layer chromatograms after chromatography of fly extracts may reflect in vivo dihydrobiopterin which oxidizes to biopterin during homogenization or TLC.

Since it seems likely that sepiapterin is also a precursor for biopterin synthesis in Drosophila, a perplexing question arises regarding the pteridine titers measured in pr. It was found that although sepiapterin occurs in trace amounts in pr, biopterin is found in wild-type amounts. If biopterin is indeed synthesized from sepiapterin, it would be expected that biopterin would also be found in trace amounts in pr. Although this observation is not readily explained, several possibilities can be presented. It may be that biopterin is synthesized from dihydroneopterin triphosphate by more than one pathway. If this is the case, then a lower rate of biopterin synthesis in the sepiapterin pathway may be compensated for by a higher rate of biosynthesis in the other pathway. The titer of biopterin could be regulated by feedback inhibition, a well-documented phenomenon in biological systems (74).

Another possibility presupposes that conversion of sepiapterin to biopterin is a side reaction in the drosopterin biosynthetic pathway. In this case, sepiapterin would be metabolizable to either biopterin or to another pteridine which then is converted ultimately to drosopterin. If sepiapterin is preferentially utilized by the enzyme which converts sepiapterin to biopterin, then the trace amount of sepiapterin biosynthesized in pr would be initially utilized for biopterin synthesis. After a certain titer of biopterin is reached (wild-type titer), then sepiapterin would be shunted to drosopterin biosynthesis. Again, these branch-point enzymes could be controlled by biopterin feedback regulation.

Biosynthesis of pterin is thought to be a side reaction in drosopterin biosynthesis (75), in which the C-6 sidechain of one of the pteridine components is removed. Pterin is then oxidized to isoxanthopterin in a well-characterized reaction. Although the level of pterin is very low in pr, it occurs in pr^{bw} in an amount greater than wild-type. Since the titers of pterin in pr and pr^{bw} are very different it seems that pterin levels are unrelated to those of drosopterin in pr and pr^{bw}.

As mentioned previously, quench spot is also found in a low titer in pr. This observation suggests that quench spot may be a component or a metabolite of a component in the

drosopterin biosynthetic pathway. The studies done with se, Hn^{r3}, and se Hn^{r3} support this contention.

Since se is totally devoid of quench spot and drosopterins but accumulates other pteridines, especially sepiapterin, it could be hypothesized that the metabolic block in se is after sepiapterin and prior to biosynthesis of quench spot and drosopterins. Furthermore, Hn^{r3} has lowered drosopterins relative to wild-type and accumulates quench spot and sepiapterin. It appears, therefore, that the metabolic block in Hn^{r3} is between quench spot and the drosopterins. This block would be a partial block since some drosopterin biosynthesis does occur and since quench spot accumulation is not large as is sepiapterin in se flies.

Given these hypotheses, it would be expected that the se Hn^{r3} mutant would be very similar to se, since the first mutation in the pathway would predominate in the double mutant. This appears to be the case; se Hn^{r3} is devoid of quench spot and drosopterins and has biopterin and sepiapterin titers nearby identical to se.

The results from analyses of se, Hn^{r3}, and se Hn^{r3} indicate that quench spot is either a precursor or a metabolite of a precursor of drosopterin. Quench spot appears to be biosynthesized from sepiapterin in an unknown number of reactions.

Some of the data is also consistent with the hypothesis that quench spot is a metabolite of drosopterin. However, to account for the observation that quench spot is elevated in $\underline{Hn^{r3}}$ while drosopterin is depressed, one would have to postulate an enhanced degradation rate of drosopterin caused by the $\underline{Hn^{r3}}$ mutation. It would then be difficult to explain the increases in sepiapterin and biopterin in $\underline{Hn^{r3}}$.

Taira (76) found that sepiapterin reductase is depressed in $\underline{Hn^{r3}}$ flies. He attributed the inhibition to a dializable inhibitor present $\underline{in\ vivo}$ which he characterized as having the same chromatographic properties as biopterin but was more photostable than biopterin (77). This inhibitor is clearly distinct from quench spot, which readily separates from biopterin during TLC in aqueous solvents such as 3% ammonium chloride or 4% sodium citrate. If quench spot is biosynthesized from sepiapterin, as I contend, then it is difficult to explain the accumulation of quench spot if one accepts Taira's conclusion that sepiapterin reductase is the affected reaction in $\underline{Hn^{r3}}$. Additional studies, including a determination of the structure of quench spot, are needed to reconcile these differences.

Unfortunately, the results of the starvation medium experiments did not lend themselves to a conclusive interpretation. Although the phenotype of $\underline{cn\ pr}$ was not noticeably altered when these flies were reared on

starvation medium, it cannot be concluded that the inhibition mechanism of pr is different from that of v. It is possible that changes in cn pr too subtle to be recognizable by the eye-color occurred. Such changes, perhaps an increase in drosopterin biosynthesis, might be detected upon pteridine analysis of starved cn pr flies, this result would argue for an enzyme inhibition mechanism in pr similar to the one occurring in v flies since brown eye pigment synthesis is restored in the v by the effect of starvation (71).

Genetic suppression can occur by an effect of the suppressor locus on the inhibited enzymatic reaction or by an effect on another enzymatic reaction. An example of the former method is the partial relief from inhibition which su(s) affords tryptophan 2,3-dioxygenase in v flies (23). In this case, a tRNA isoacceptor species is presumably altered by the su(s) locus and is no longer able to completely inhibit tryptophan 2,3-dioxygenase.

An example of a suppressor locus exerting its effect on an enzyme different from the inhibited enzyme was recently published. Bahn (78) found that suppressor of rudimentary acts to relieve rudimentary of its pyrimidine requirement in the diet by inhibiting dihydrouracil dehydrogenase, an enzyme which degrades uracil and thymine. Thus, in suppressed rudimentary flies, the small amounts of uracil and thymine synthesized are accumulated instead of degraded, and addition

of uracil and thymine to the medium is unnecessary for viability of the flies.

It would seem that if $\underline{su(s)^2}$ is exerting its effect on the inhibited enzyme in pr, then the pteridine titers in $\underline{su(s)^2;pr}$ would closely match those in wild-type. If $\underline{su(s)^2}$ is affecting a different enzyme, such as inhibiting an enzyme which degrades drosopterin, for example, then it might be expected that the drosopterin titer would be equal to that of wild-type, but sepiapterin and quench spot might remain at pr levels in the $\underline{su(s)^2;pr}$ fly.

With this reasoning in mind, the fact that drosopterin, sepiapterin, and quench spot are elevated to wild-type levels, or nearly so, suggests that $\underline{su(s)^2}$ exerts its effect on the inhibited enzymatic reactions in pr. Although the titer of pterin remains low in $\underline{su(s)^2;pr}$, the fact that it is at wild-type titer in $\underline{pr^{bw}}$ indicates that this pteridine may not be involved directly in drosopterin biosynthesis.

Likewise, the titers of sepiapterin, quench spot, and drosopterin are elevated to wild-type levels in $\underline{su(pr)^{e3};pr}$. Although the titer of sepiapterin in $\underline{su(pr)^{e3};pr}$ does not appear to be significantly different from that in Oregon-R, it is present at a level approximately 60% that of $\underline{su(s)^2;pr}$. Whether this difference is reflective of different mechanisms of suppression of pr by the two suppressor loci is unknown.

CHAPTER IV

CONCLUSION

The purpose of this research was to identify the enzyme in the drosopterin biosynthetic pathway which is affected in the pr mutant. Although I was not able to localize the lesion in pr to a specific enzyme, I did determine that the lesion occurs early in the pathway. Pteridine analyses of pr and double mutants containing pr revealed that the block must occur prior to sepiapterin. Nucleotide analysis suggested that the pathway leading to GTP biosynthesis is unaffected in pr, indicating that sufficient GTP is available for optimal pteridine biosynthesis. Therefore, it would seem that the affected enzymatic reaction in pr occurs between GTP and sepiapterin.

In addition to indicating the general location of the block in pr, pteridine analyses of the various mutants also contributed to an understanding of drosopterin biosynthesis. A new compound, quench spot, was resolved upon thin-layer chromatography of Drosophila head extracts. Based on both biochemical and genetic evidence, it is hypothesized that quench spot is a pteridine or a metabolite of a pteridine which occurs in the drosopterin biosynthetic pathway at a position between sepiapterin and drosopterin.

Also, pteridine analyses indicated that biopterin either may be biosynthesized by several routes or may be a side reaction in the drosopterin biosynthetic pathway. Regulation of the titer of this pteridine appears to be complex.

Finally, analyses of the pteridine patterns of suppressed pr flies indicated that the pteridines which are depressed in pr are elevated to wild-type levels in suppressed flies. Differences found in the titers of several pteridines in su(s);pr and su(pr)^{e3};pr may be reflective of different suppression mechanisms by the two suppressor mutations.

This research provides a foundation for subsequent studies to determine the affected enzymatic reaction in the pr mutation. When this enzyme is identified, it should be possible to determine how the enzyme is inhibited and also how inhibition relief is obtained in suppressed pr. Results from such studies will add to our understanding of the mechanism of suppression in Drosophila.

Subsequent studies are indicated for a better understanding of drosopterin biosynthesis. Determination of the chemical structures of the unidentified pteridines, particularly quench spot, will aid in the identification of the specific enzymatic reactions in the drosopterin biosynthetic pathway. In addition, a study of the metabolism of radiolabelled pteridines will indicate the origin and fate of various pteridines, particularly pterin and biopterin.

It is evident that pteridine biosynthesis and metabolism is under strict regulation in Drosophila. Further investigations should lead not only to an understanding of pteridine regulation, but also to a better understanding of enzyme regulation in general.

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