To the Graduate Council:

I am submitting herewith a thesis written by Paula C. McGray entitled "Studies on Sepiapterin Synthase in Drosophila melanogaster." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

K. Bruce Jacobson, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

STUDIES ON SEPIAPTERIN SYNTHASE IN *DROSOPHILA MELANOGASTER*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Paula C. McGray

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ABSTRACT

A rapid, fluorometric assay for sepiapterin production has been developed. Excitation and emission wavelengths which minimized the fluorescent contributions of H_2NTP, NADPH, and pigments in the enzyme extract were defined. Further reductions in background fluorescence were obtained by chromatographing the enzyme extract over G25 Sephadex to reduce the amount of pteridines present and by stopping the reaction with EDTA. Fluorescence due to sepiapterin was defined as the difference in relative fluorescence intensity of sample mixture with and without EDTA added prior to incubation. Control reaction mixtures showed increased fluorescence intensity upon incubation; HPLC analysis of these mixtures showed that they did not contain sepiapterin. The fluorometric assay is limited by two photophenomena. Quenching, which was significant at relative fluorescent intensities of 250 or more, could be eliminated by appropriate dilution of the intensely fluorescent samples. Sepiapterin fluorescence was temporarily diminished by prolonged exposure to the excition wavelength or room light. A maximum fluorescent response could be ensured by holding samples in the dark for three minutes before measuring their relative fluorescence intensities.

Samples of known sepiapterin concentration were used to establish a ratio of fluorescence intensity units per μM sepiapterin. This conversion factor was used to determine relative sepiapterin concentrations in sample reaction mixtures. The validity of the fluorometric assay was tested by comparing sepiapterin concentrations measured fluorometrically and by HPLC. There was excellent correlation between

the two methods. The fluorometric assay was used to measure the relative amount of sepiapterin synthase activity in Drosophila containing the purple mutation plus zero, one, or two copies of a "suppressor of purple" mutation. Activity was not increased in flies with one copy of the suppressor mutant, however in flies with two copies, the level of activity was restored to 47% that of wild type.

GTP cyclohydrolase I, the enzyme which produces the starting material for the production of sepiapterin, has been isolated using GTP Sepharose affinity chromatography as described by Yim and Brown (1976). Experiments were performed to determine the reasons for poor GTP binding and low yields in earlier purifications. GTP binding to the activated Sepharose was markedly improved when the column material was stirred in a rotary stirring device rather than with a magnetic stirrer. Overall yield was somewhat improved following this change. Increasing the amount of $NaIO_4$ used to activate GTP for attachment to the Sepharose did not have a deleterious effect on GTP binding. Two published methods for the preparation of GTP Sepharose (Lamed et al., 1973; Jackson et al., 1973) were compared with the standard laboratory method. No differences in the amount of GTP binding or yield were observed. The rate of application of enzyme to the GTP Sepharose column was found to make no difference in the amount of enzyme bound to the column or in the amount of activity recovered.

Sepiapterin synthase has been purified by chromatography on Cibacron blue Sepharose (Krivi and Brown, 1979). Specific binding is presumed to occur between the blue dye and the NADPH-requiring activity of the enzyme. Seven millimolar NADH was found to be ineffective in

eluting activity from the column. Elution with buffer containing 1 M NaCl could remove activity from the column even after previous elution with NADPH-containing buffer. The purification described by Krivi and Brown (1979) was repeated without success.

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LIST OF ABBREVIATIONS

Biopterin	=	6-(L-*erythro*-1',2'-dihydroxypropyl)pterin
"Drosopterins"	=	neodrosopterin, drosopterin, isodrosopterin, fraction e, the aurodrosopterins. The six dipteridine-derived red eye pigments of *Drosophila melanogaster*.
EDTA	=	ethylene diamine tetraacetic acid
Fluorescein	=	spiro[isobenzofuran-1(3H),9'-[9H]-xanthen]-3-one,3' 6'-dihydroxy-[2321-07-5]
GTP	=	guanosine-5'-triphosphate
HPLC	=	high performance liquid chromatography
Isoxanthopterin	=	7-hydroxypterin
NADH	=	nicotinamide adenine dinucleotide (reduced)
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced)
Pipes	=	1,4-piperazine diethanesulfonic acid
Pterins	=	2-amino,4-hydroxy pteridines
Sepiapterin	=	2-amino-4-oxy-lactoyl-7,8-dihydropteridine
TLC	=	thin layer chromatography
"X"	=	a pterin whose exact structure is unknown, the product of the first enzymatic activity of sepiapterin synthase.

CHAPTER I

GENERAL INTRODUCTION

Sepiapterin (7,8-dihydro-6-lactoylpterin) is one of the pteridines present in the eyes of Drosophila melanogaster. The parent compound for these pterins (2-amino-4-hydroxy pteridines) is the 3'-triphosphoester of 7,8-dihydro-6-(D-erythro-1',2',3'-trihydroxypropyl) pterin (H_2NTP) which is synthesized from guanosine-5'-triphosphate (GTP) by the action of GTP cyclohydrolase I (Burg and Brown, 1968). H_2NTP is converted to sepiapterin by the two enzymatic activities comprising sepiapterin synthase (Fan et al., 1975) (Figure 1). The first of the two enzymatic activities is a Mg^{++}-requiring enzyme which converts H_2NTP to the intermediate, "X," with the concomitant loss of the three phosphate residues. This activity is the product of the purple, (pr), locus (Wilson and Jacobson, 1977b). The second activity converts "X" to sepiapterin and uses NADPH as a cofactor. "X" is also required for the biosynthesis of drosopterin and isodrosopterin, two of the six red eye pigments, or "drosopterins." The other four "drosopterins" are neodrosopterin, fraction e, and the two aurodrosopterins. Various intermediates in the conversion of H_2NTP to sepiapterin have been proposed (Krivi and Brown, 1979; Tanaka et al., 1981).

Of particular interest in the study of eye pigment biosynthesis in Drosophila is the suppression of the effect of the pr mutation by a mutation in suppressor of sable, [$su(s)^2$]. This phenomenon cannot readily be explained. The two mutations occur on different chromosomes, thus ruling out a mechanism which relies on restoration of a

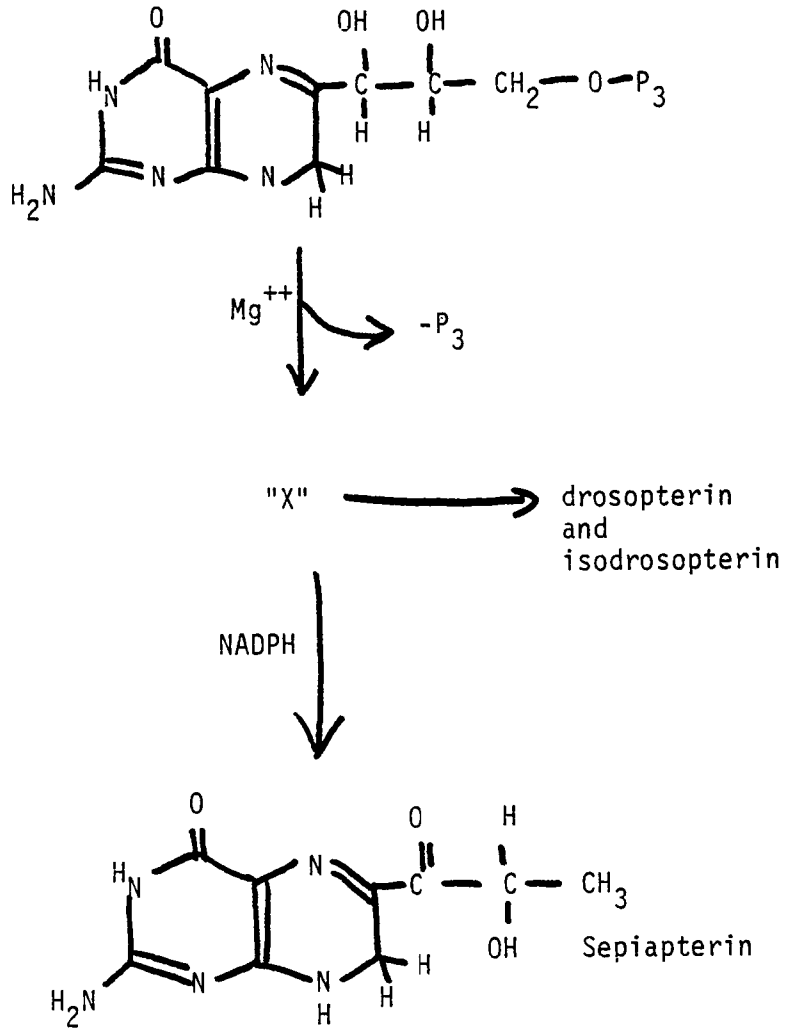

Figure 1. Conversion of H_2NTP to sepiapterin by sepiapterin synthase.

After Dorsett et al. (1979).

reading frame in DNA. Suppression due to the activation of an alternate pathway is also not a likely explanation, as $\underline{su(s)^2}$ restores the activity of four different mutations: vermilion, (v), and purple, (pr), which affect the synthesis of ommochromes and "drosopterins" respectively; sable, (s), a body color mutant; and speck, (sp), which also causes changes in body pigmentation.

Another type of suppression occurs when a tRNA is altered either in the anticodon, or so that it is recognized and "charged" (aminoacylated) by a different tRNA synthetase. In either case, substitution of a different amino acid into the polypeptide chain may occur and may rectify a mutation. Again, this is not the explanation for the effect of $\underline{su(s)^2}$ or pr. This mechanism operates at the level of translation; effects of $\underline{su(s)^+}$ on isolated, previously synthesized mutant enzyme can be observed.

It would seem that some product of the $\underline{su(s)^+}$ gene has the ability to interact with the enzyme which makes "X" from H_2NTP. The combination of an extract from $\underline{su(s)^+}$ $\underline{pr^{bw}}$ cn flies with an enzyme from $\underline{su(s)^2}$ $\underline{pr^{bw}}$ cn flies resulted in inhibition of sepiapterin synthase activity. Increasing the amount of $\underline{su(s)^+}$ $\underline{pr^{bw}}$ cn in the system, either by adding increasing amounts of extract or by obtaining extract from flies with 1, 2, 3, or 4 gene copies of the $\underline{su(s)^+}$ gene resulted in increasing inhibition (Grell, Jacobson, and Wobbe, unpublished). If the $\underline{su(s)^+}$ product is absent, the activity of sepiapterin synthase is increased; thus the activity of enzyme from $\underline{su(s)^2}$ pr cn was restored to approximately 90% of wild type from a value of 20-30% of wild type observed in $\underline{su(s)^+}$ pr cn flies.

Sepiapterin titers in $\underline{su(s)^2}$ $\underline{pr^+}$ flies were approximately two times those of wild type (Oregon R) flies (Wilson and Jacobson, 1977b), suggesting the possibility that the $\underline{su(s)^+}$ product exerts a net inhibitory effect on both mutant and wild type enzyme. Alternatively, an altered product made by $\underline{su(s)^2}$ flies could increase the level of activity in $\underline{pr}$ mutants to approximately that of wild type. This product would have to also enhance the activity of normal sepiapterin synthase resulting in increased sepiapterin titers in $\underline{su(s)^2}$ $\underline{pr^+}$ flies (Wilson and Jacobson, 1977b).

Attempts have been made to isolate an $\underline{su(s)^+}$ product. The assay for $\underline{su(s)^+}$ is based on an enhancement of wild type sepiapterin synthase activity and/or an inhibition of $\underline{su(s)^2}$ $\underline{pr^{bw}}$ $\underline{cn}$ enzyme activity. Results of earlier work (Wobbe, unpublished) indicated that the $\underline{su(s)^+}$ substance could be fractionated with ammonium sulfate and was excluded from G50 but not G100 Sephadex, indicating a molecular weight of 20-2000 K. The presence of NaCl in the reaction mixture reduced the effects of the $\underline{su(s)^+}$ extract. These effects could be restored by desalting the extract over G25 or G50 Sephadex (Wobbe, unpublished). This salt treatment enhanced the stimulation of Oregon R enzyme, suggesting that $\underline{su(s)^+}$ does exert an inhibitory effect even on wild type enzyme.

The material presented in this thesis has been organized into three sections. The first describes the development of a fluorometric assay for sepiapterin synthase activity, the second examines the isolation and purification of GTP cyclohydrolase I, and the third

reports attempts to purify sepiapterin synthase by affinity chromatography using Cibacron blue Sepharose.

CHAPTER II

DEVELOPMENT OF A RAPID, FLUOROMETRIC ASSAY FOR SEPIAPTERIN PRODUCTION

INTRODUCTION

Analysis of sepiapterin titers and of sepiapterin synthase activity in Drosophila melanogaster has provided information on developmental changes in the production of this pterin (Tobler et al., 1979; Fan et al., 1976; Wilson and Jacobson, 1977b; and Krivi and Brown, 1979), position effect variegation of the purple (pr) gene (Tobler et al., 1979), location and effects of mutations in pteridine biosynthetic pathways (Wilson and Jacobson, 1977b; Yim et al, 1977; Dorsett et al., 1979) and effects of suppressor mutations on the pr mutation (Wilson and Jacobson, 1977b; Yim et al., 1977). Sepiapterin has been measured with thin layer chromatography (TLC) (Wilson and Jacobson, 1977a) and high performance liquid chromatography (HPLC) (Dorsett, 1980). These analyses, while providing quantitative measures of sepiapterin concentration, are time consuming. As an aid to further studies involving the production of sepiapterin, a rapid flurometric assay for sepiapterin has been developed.

MATERIALS AND METHODS

Materials

GTP (sodium salt) was purchased from Sigma Chemical Company; [8-^{14}C] GTP was obtained from New England Nuclear. Pipes (Ultrol grade) was purchased from Calbiochem. G25 Sephadex was from Pharmacia.

All other chemicals were reagent grade. GTP cyclohydrolase I was purified as described by Yim and Brown (1976) and used in the preparation of H_2NTP according to Dorsett et al. (1979). Crude extracts containing sepiapterin synthase activity were prepared following the procedure of Krivi and Brown (1979) or a procedure adapted from Yim et al. (1977). Sepiapterin, prepared according to Dorsett et al. (1979), was kindly provided by Dr. K. B. Jacobson.

Drosophila melanogaster

All stocks were grown at 25°C with a 12:12 photoperiod on medium described by Lewis (1960). Flies were harvested daily and kept at -80°C until use. Heads were separated and collected according to Wilson and Jacobson (1977a). The assay was developed using enzyme obtained from Oregon R. Stocks homozygous for the purple mutation, pr, cinnabar, cn, and suppressor of purple, $su(pr)^{e3}$, were crossed with pr cn that were wild type at the suppressor locus to generate flies which were heterozygous for the suppressor. Sepiapterin production in these three types, pr cn; +, pr cn; $su(pr)^{e3}$, and pr cn; $su(pr)^{e3}/+$ was evaluated using the fluorometric assay. All mutations are described in Lindsley and Grell (1968).

Sepiapterin Synthase Assay

The standard reaction mixture for the biosynthesis of sepiapterin contained in 75 µl total volume 5 µl each of 0.7 M Pipes pH 7.5, 0.2 M $MgCl_2$, and 35 mM NADPH, 15 µl of H_2NTP freshly prepared from GTP and having a concentration of $\geq$ 0.6 mM, and between 7.5 and 45 µl of sepiapterin synthase according to experimental design. When necessary,

mixtures were adjusted to volume with 50 mM Pipes pH 7.5 made 10% in glycerol. Reaction mixtures were incubated at 42°C in the dark and were stopped by the addition of 50 µl 0.1 M EDTA pH 7.5. Extraneous fluorescence was estimated with controls in which EDTA was added before the reactions were started.

Measurement of Sepiapterin

a) Fluorometric

Sepiapterin was quantitated in an Aminco Bowman spectrofluorophotometer using an exciting wavelength of 460 nm and monitoring emission at 550 nm with slit settings of 4, 3, 4, and 5 mm for the lamp, entrance, exit, and phototube, respectively. Relative fluorescence intensity was calibrated using a fluorescent, uranium-doped glass standard with the entrance and exit slits reduced to a setting of 1 mm. The excitation and emission maxima for this glass were 365 and 530 nm, respectively. More precise calibration was obtained by measuring the relative fluorescence intensity of a known concentration of a freshly made solution of purified sepiapterin. The extinction coefficient of sepiapterin at 420 nm is 10.4 l/mmol cm (Matsubara et al., 1966). Fluorescence due to sepiapterin was defined as the difference in intensity of the sample and control reaction mixtures.

b) HPLC

HPLC determination of sepiapterin was performed by Dr. K. B. Jacobson using a Zorbax C-8 column and 8% methanol for isocratic elution according to the method of Dorsett (1980). The amount of sepiapterin

present in any given sample was determined by comparing the area under the peak to that of a known amount of sepiapterin.

Determination of Excitation and Emission Spectra

Excitation, (ex), and emission, (em), spectra for compounds contained in the standard sepiapterin reaction mix were obtained using an Aminco Bowman spectrofluorophotometer attached to a linear X,Y recorder which plotted relative fluorescent intensity vs. wavelength. Spectra were determined at 18°C with the following slit settings: lamp = 4 mm, entrance = 2 mm, exit = 3 mm, and phototube = 5 mm, except for measurement of sepiapterin, where the entrance slit was increased to 4 mm and the exit and phototube slits reduced to 2 mm.

RESULTS

The development of a flurometric assay for sepiapterin was initiated by Dr. K. B. Jacobson by defining excitation and emission wavelengths which could be used to monitor sepiapterin production with minimal interference from other fluorescent components in the reaction mixture, i.e., NADPH, H_2NTP, and the enzyme extract from Drosophila. Figure 2 gives excitation and emission spectra for sepiapterin. Excitation and emission maxima occurred at 420 and 550 nm, respectively. NADPH fluorescence could be largely avoided by using an exciting wavelength of greater than about 420 nm and/or by monitoring emission at wavelengths approaching 600 nm (Figure 3). Fluorescence due to H_2NTP could be partially avoided by exciting at 460 nm and monitoring emission near 600 nm (Figure 4).

Figure 2. Fluorescence of 12.6 μM sepiapterin in 70 mM Pipes pH 7.5.

Relative fluorescence intensity is given at 0.3% of full scale.

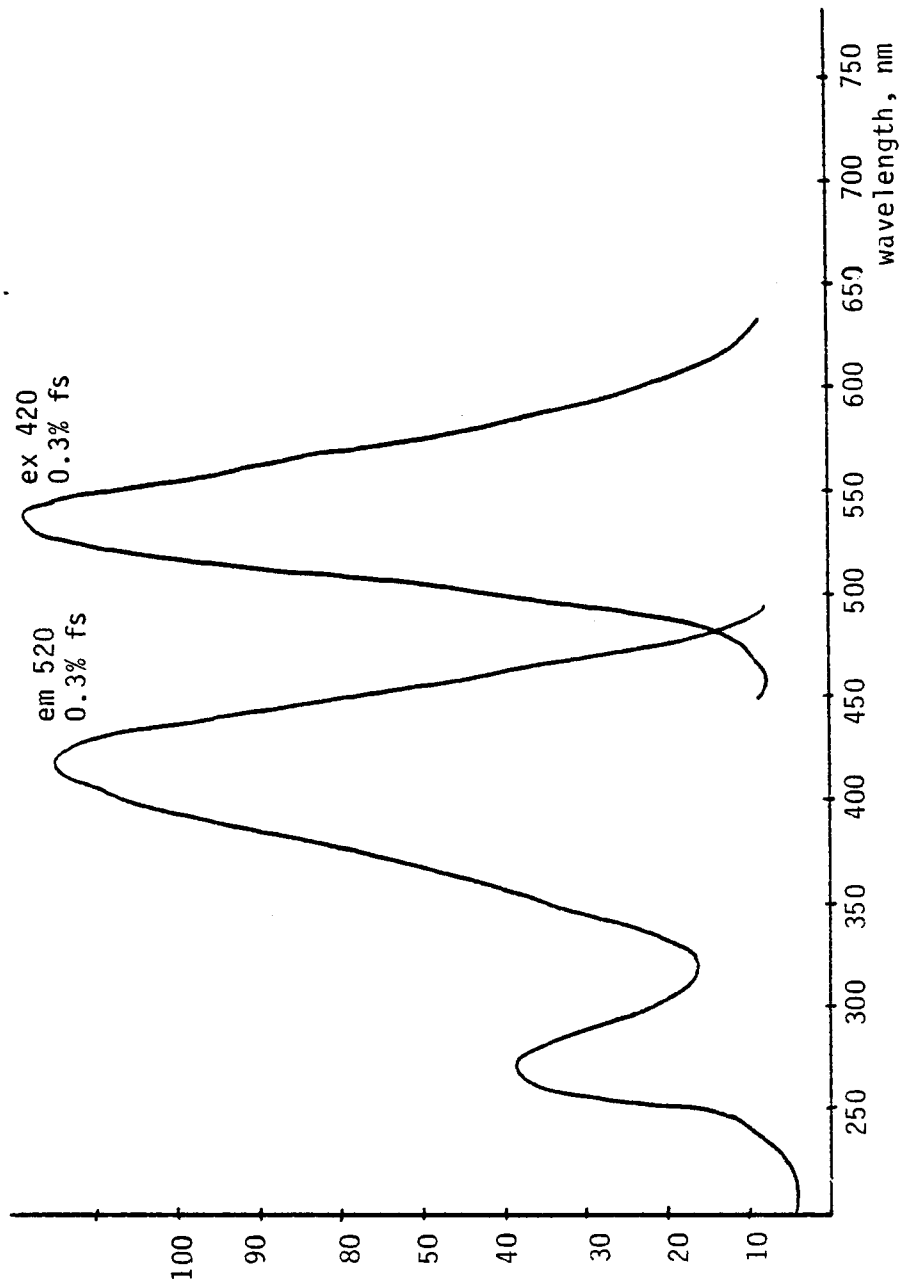
ex 420
0.3% fs
em 520
0.3% fs
100
90
80
50
40
30
20
10
250
300
350
400
450
500
550
600
650
700
750
wavelength, nm

Figure 2.

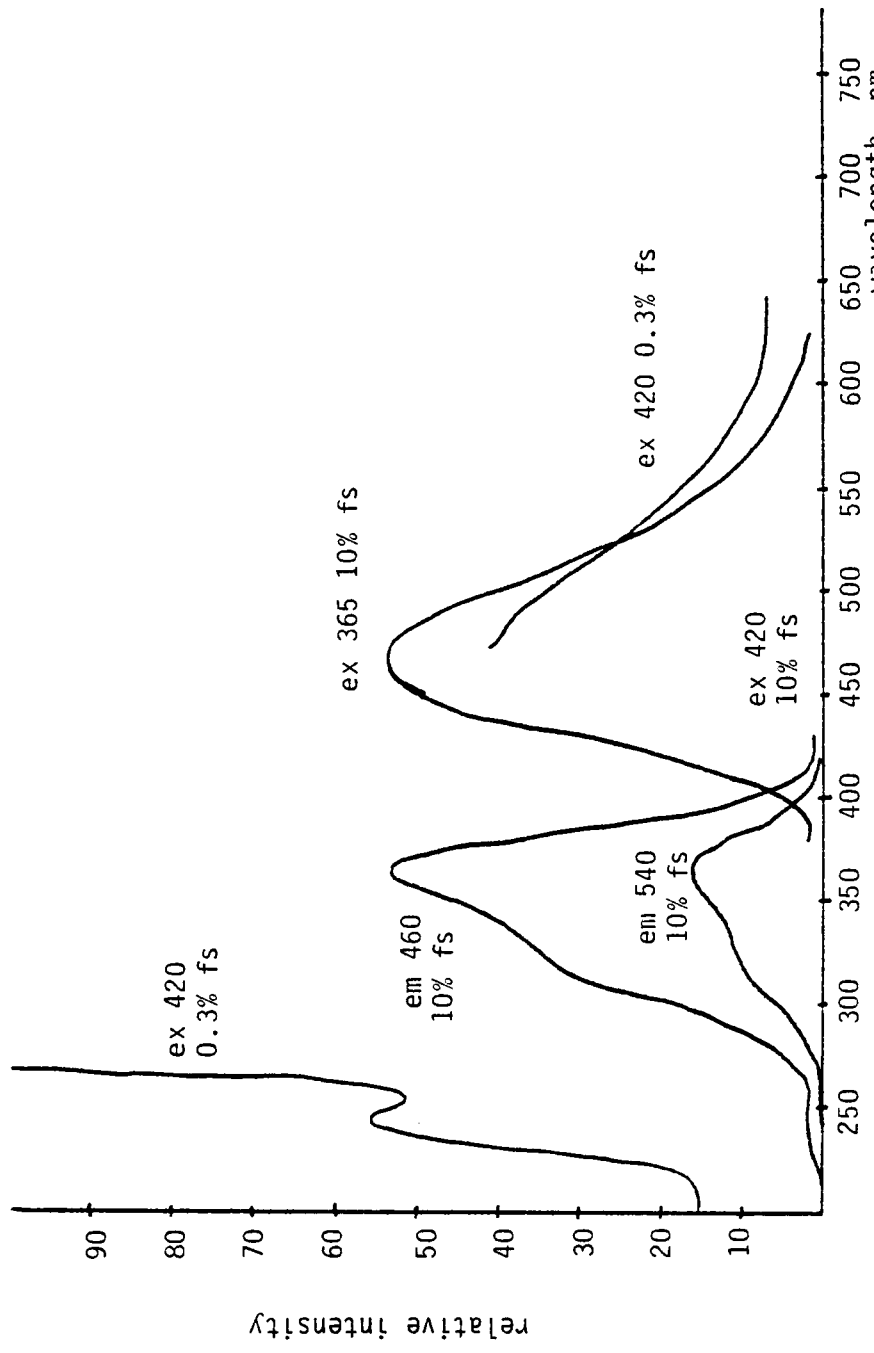

Figure 3. Fluorescence of 0.7 mM NADPH in 70 mM Pipes pH 7.5.

Figure 4. Fluorescence of H_2NTP at pH 7.5.

H_2NTP was generated from 5 mM GTP according to Yim and Brown (1976) then made up in the standard sepiapterin synthase reaction mixture without NADPH. The final concentration of H_2NTP was $\leq$ 0.357 mM.

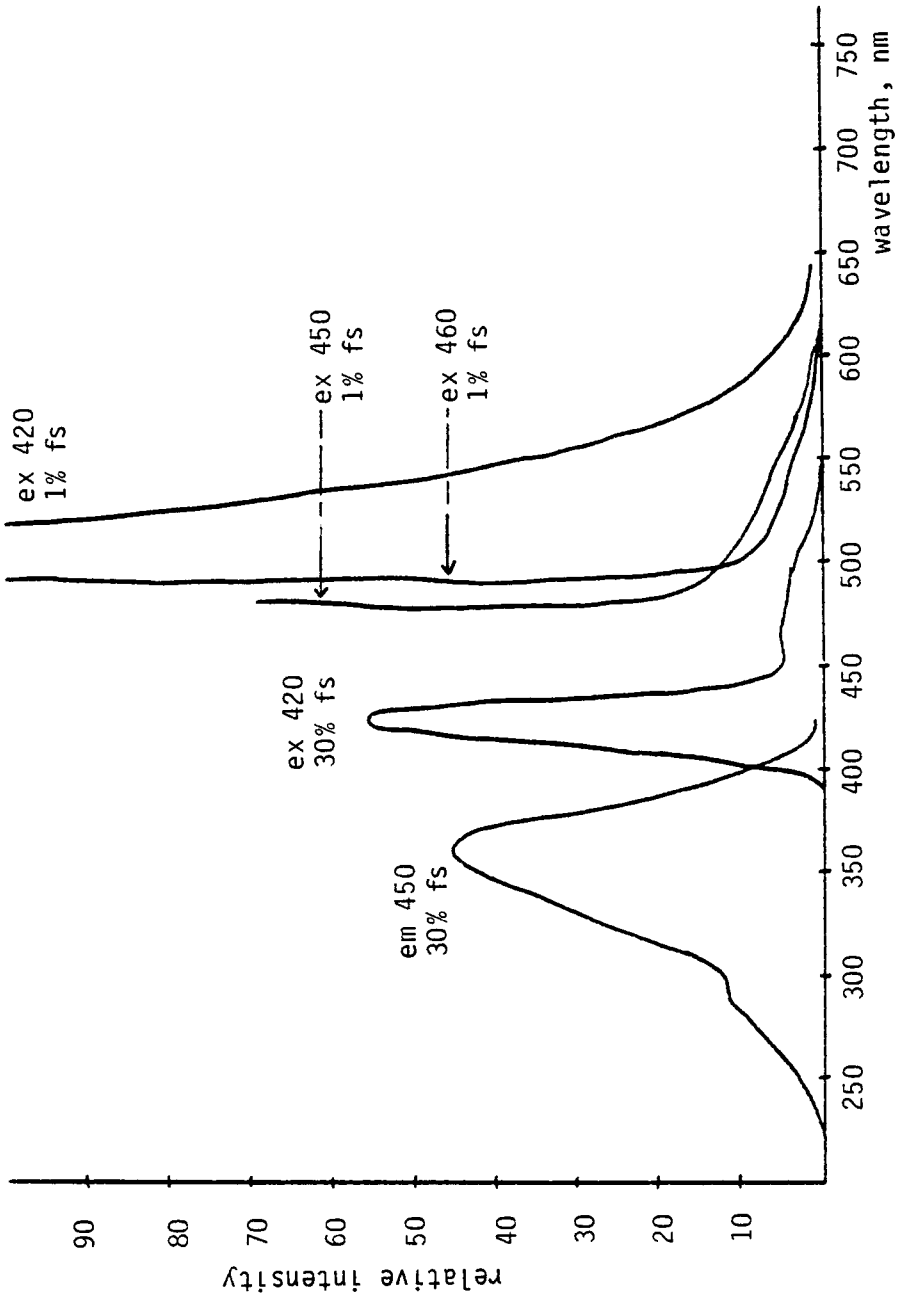
ex 420
1% fs
ex 450
1% fs
ex 460
1% fs
ex 420
30% fs
em 450
30% fs
relative intensity
wavelength, nm
10
20
30
40
50
60
70
80
90
250
300
350
400
450
500
550
600
650
700
750

Figure 4.

Sample reaction mixtures were set up to test the effects of NADPH, H_2NTP, and enzyme extract fluorescence on the fluorometric measurement of sepiapterin (Table 1). The results indicated that the presence or absence of NADPH or the enzyme extract had relatively little effect on the fluorescence observed but that H_2NTP posed a significant background problem. Dr. Jacobson chose excitation and emission wavelengths of 460 and 550 nm respectively as the standard assay values.

Interference by H_2NTP fluorescence was not severe except when a reaction mixture containing H_2NTP was boiled, in which case a marked increase in fluorescence occurred (Figure 5). As an alternative to boiling, the reaction could be stopped by chelating the Mg^{++} in the reaction mixture with EDTA. Addition of EDTA to the reaction mixture had virtually no effect on the background fluorescence.

Despite this reduction in total background fluorescence, there was still a severe background problem. The effect of incubation on background fluorescence is shown in Table 2. The addition of twice as much EDTA, i.e., six times the amount of Mg^{++} in the reaction mix, did not decrease the background, suggesting that all the Mg^{++} was being chelated and that something other than sepiapterin production was responsible for this increase. Analysis of control reaction mixtures with HPLC confirmed that there was no sepiapterin being produced.

The contribution of each reaction mix component to background fluorescence was tested (Figure 6). There was an appreciable increase in background fluorescence in the absence of one or both cofactors required for sepiapterin synthesis. Somewhat lower levels were obtained

Table 1. Relative Fluorescence Intensity of Sepiapterin Synthase Reaction Components[a]

Reaction Mix	#1 µl	2 µl	3 µl	4 µl	5 µl	6 µl
Buffer[b] + NADPH	-	30	-	30	-	30
Buffer - NADPH	30	-	30	-	30	-
H_2NTP[c]	30	-	-	30	30	-
50 mM Pipes pH 7.5	70	100	100	90	90	120
Enzyme[d]	20	20	20	-	-	-
Relative Fluorescence Units (0.3% fs) Emission at 550 nm						
Exciting at 450 nm	63	9	13	35	38	-2
at 470 nm	62	17	27	40	38	-2

Effect of the Presence or Absence of:	Reaction Mixtures Used for Evaluation	Difference in Relative Intensity At 450 nm	At 470 nm
H_2NTP	1-3	50	35
	4-6	37	42
NADPH	2-3	- 4	-10
	4-5	- 3	- 2
Enzyme	1-5	25	24
	2-6	11	19

[a]Sample reaction mixes were prepared, incubated for 20 min. at 42°C in the dark, then boiled to stop the reaction. The experimental designed allowed each fluorescent component to be evaluated in the presence or absence of the others.

[b]Buffer consisted of equal parts 0.7 M Pipes pH 7.5, 0.2 M $MgCl_2$, and either 35 mM NADPH or H_2O.

[c]H_2NTP was prepared from 5 mM GTP according to Yim and Brown (1976).

[d]Enzyme used in these tests was a pteridine-free extract prepared as in Dorsett et al. (1979).

Figure 5. Effect of H_2NTP on sepiapterin response curve.

Solutions containing a known concentration of purified sepiapterin were prepared in the standard reaction mix buffer or in a reaction mix buffer that did not contain H_2NTP. One set of solutions was placed in a boiling water bath for five minutes. Fluorescence was then measured under standard assay conditions.

Legend:
- ■ reaction mix with H_2NTP
- ● reaction mix with H_2NTP, boiled
- □ reaction mix without H_2NTP
- ○ reaction mix without H_2NTP, boiled

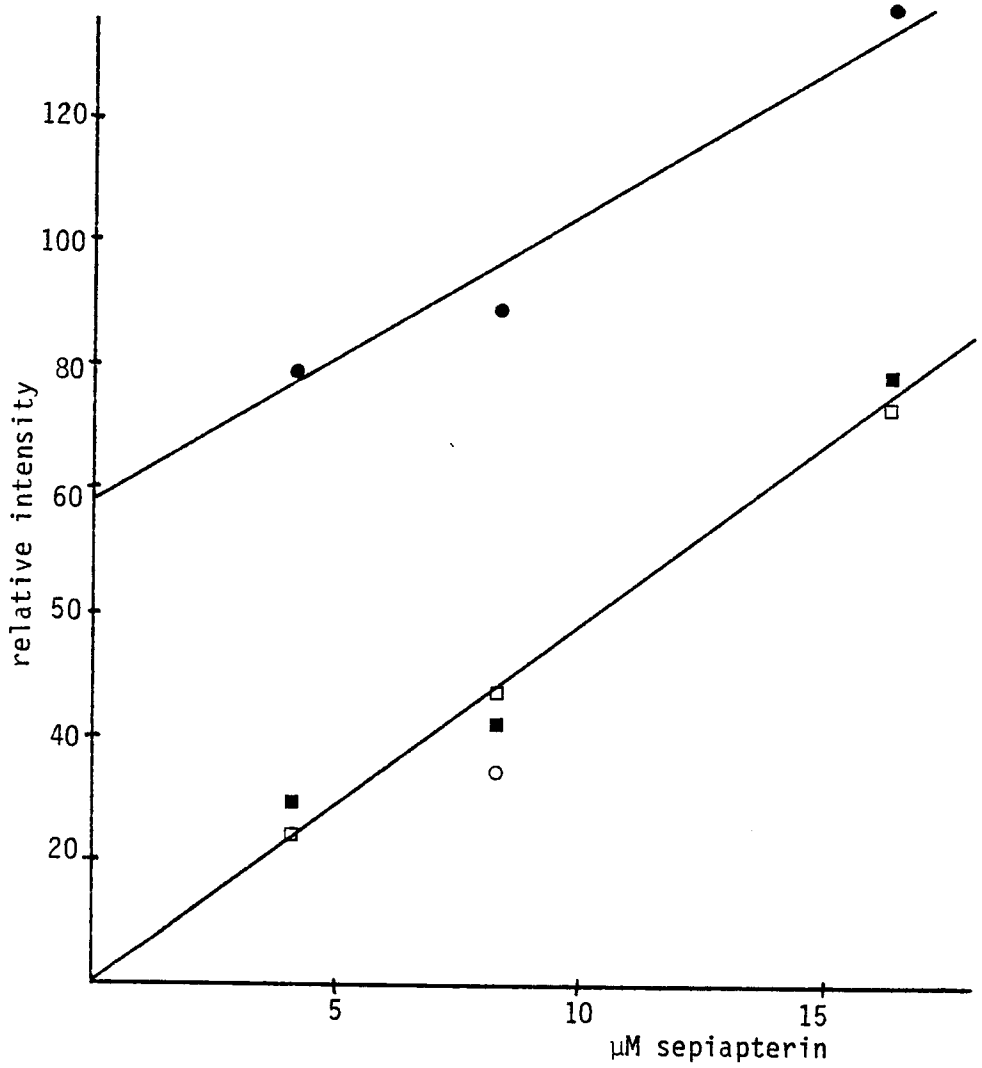
120
100
80
60
50
40
20
relative intensity
5
10
15
µM sepiapterin

Figure 5.

Table 2. Increase in Background Fluorescence upon Incubation

Reaction Conditions	Relative Fluorescence Intensity[a]	(Background/Positive Control) X 100%
1. Positive Control: Standard reaction mixture incubated 20 minutes at 42°C and stopped with EDTA	69	-
2. Background (incubated): Standard reaction mixture stopped with EDTA at time zero, then incubated at 42°C for 20 minutes.	53	77%
3. Background (on ice): Standard reaction mixture stopped with EDTA at time zero and kept on ice for 20 minutes.	27	39%

[a]Measured at ex 560 and em 550 nm using a standard of 5.77 μM Sepiapterin set to 45.

Figure 6. Contribution of reaction components to background fluorescence.

All reaction mixtures were made up as per the standard reaction mix; components omitted were replaced by H_2O. The mixtures were incubated at 42°C for the time indicated and were held on ice following incubation. Component omitted: X = Mg^{++}; O = NADPH; △ = Mg^{++} and NADPH; ● = H_2NTP; + = enzyme; □ = boiled enzyme. A positive control (not shown) had values of 10.5 at t = 0, 97 at t = 30 min. and 132 at t = 60 min.

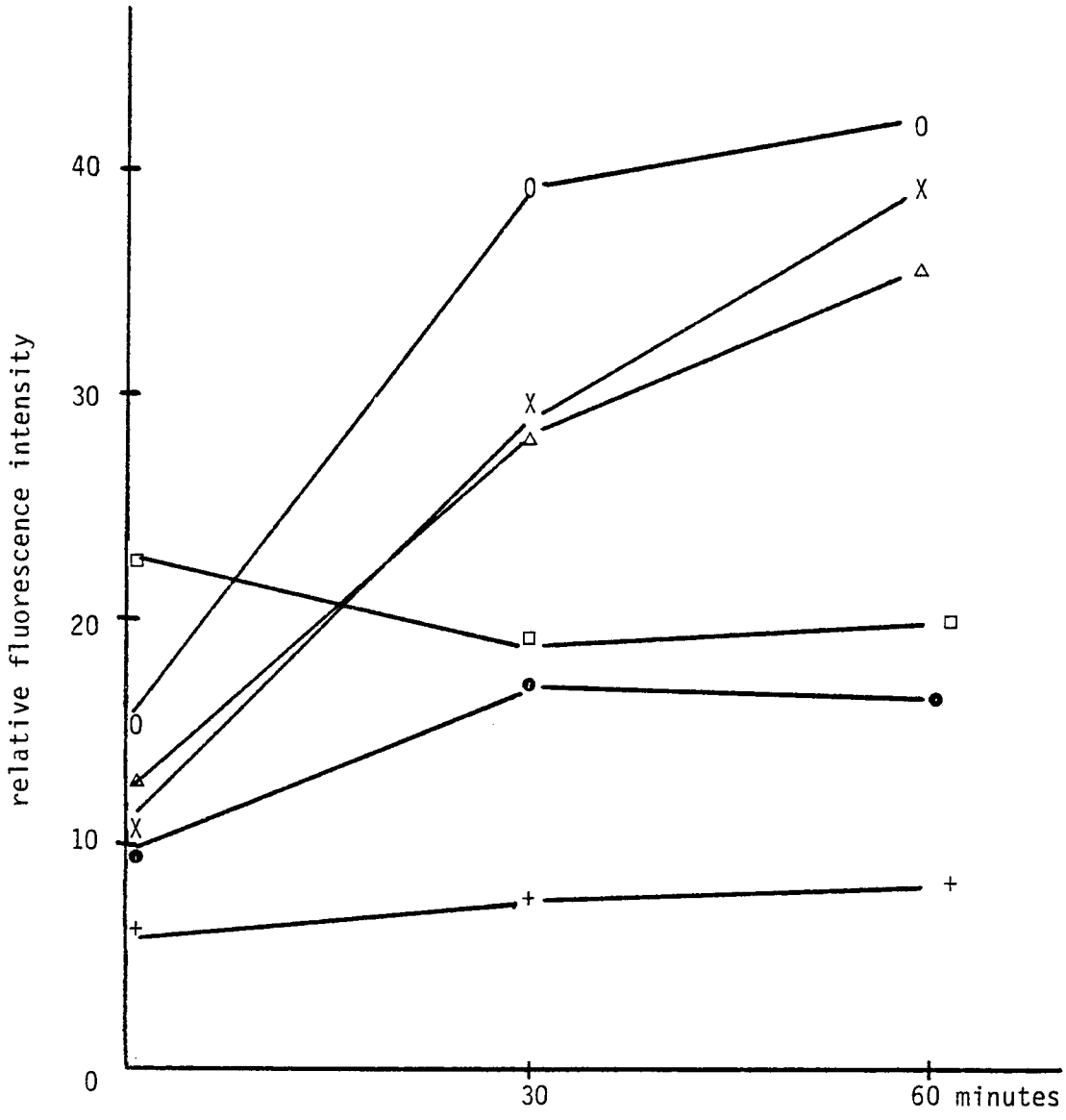

Figure 6.

if the substrate was missing or if the enzyme was inactivated by boiling before it was added to the reaction mixture. The least background resulted when enzyme was omitted. As none of the reaction mixtures showed zero fluorescence at time zero, the contribution of individual components at the concentration in which they occur was tested. There were small increases in background due to H_2NTP and NADPH; the most significant contribution was from the crude enzyme. A noticeable decrease in this background could be obtained by passing the enzyme through a G25 Sephadex column. This column retained pteridines which contributed to the background fluorescence of the enzyme.

An accurate determination of the amount of sepiapterin present cannot be made without consideration of three phenomena associated with the exposure of sepiapterin to light. The first, a photodegradation of sepiapterin to pterin-6-carboxylate has been described by Pfleiderer (1975). A second light-induced reaction of sepiapterin that resulted in decreasing fluorescence intensity was observed upon prolonged exposure to 460 nm light. This decrease was not apparent in the first half minute of exposure. Recovery time following a 10 minute exposure was observed to be 3 minutes. Samples were therefore routinely kept in the dark 3 minutes before being placed in the spectrofluorophotometer and their relative fluorescence intensities were read without delay. A third photoeffect, quenching, occurred at sepiapterin concentrations above approximately 14.5 μM (Figure 7) and could easily be avoided by making known dilutions of those samples exhibiting relative fluorescence intensities of more than 250 units.

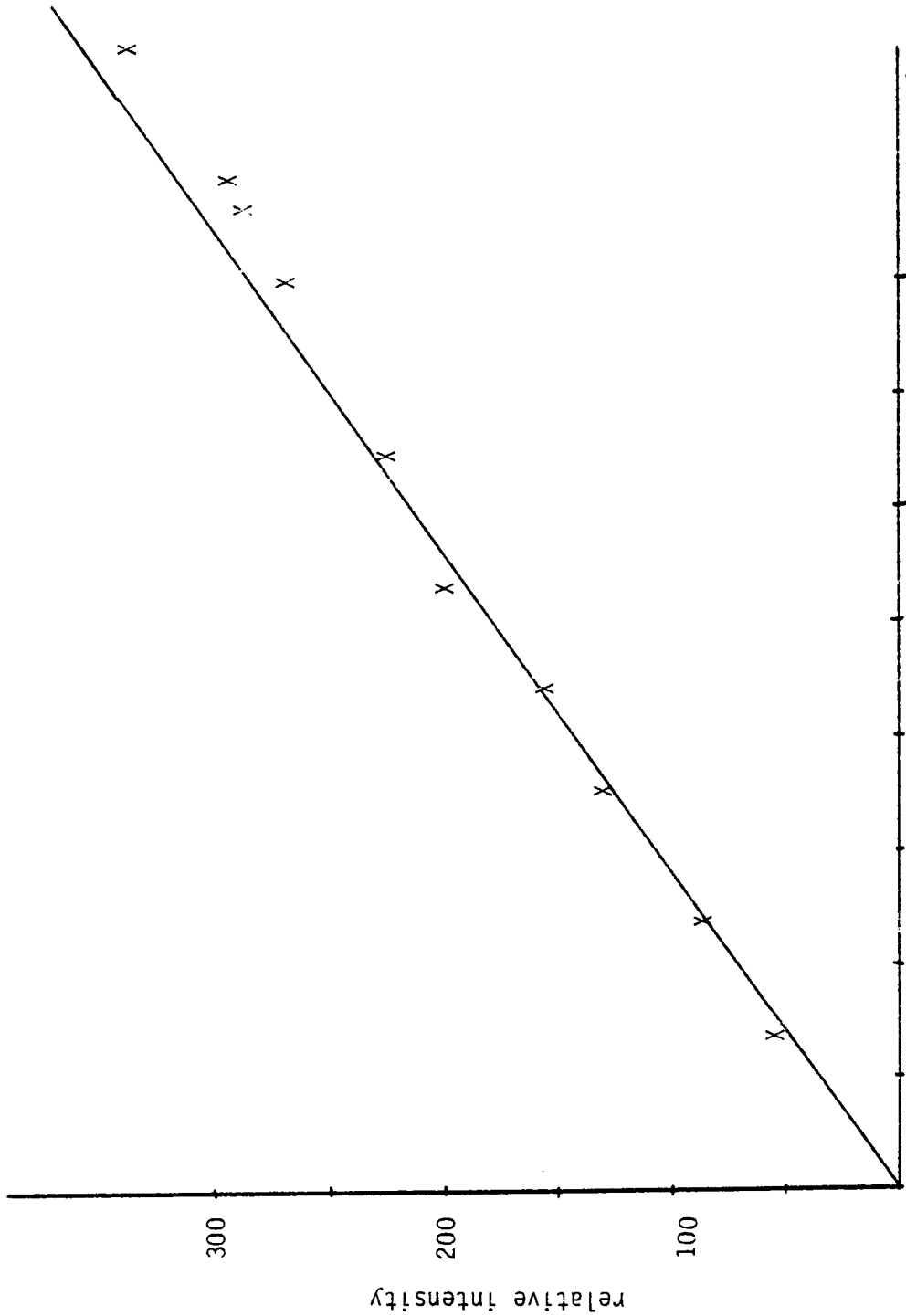

Figure 7. Sepiapterin response curve showing quenching above ~ 250 units.

Estimation of sepiapterin concentration from relative fluorescence intensity is dependent on careful calibration of machine sensitivity. While the fluorescent glass standard could be used for a coarse adjustment of machine sensitivity, it was too highly fluorescent to allow precise calibration of sensitivity in the normal reading range of 250 units or less. Sepiapterin solutions of known concentration could be used for fine adjustment of machine sensitivity, however these solutions could not be frozen and thawed repeatedly or stored frozen without significant reduction in fluorescence intensity. The time course of degradation and the degradation products were not determined. Fluorescein, (spiro [isobenzofuran-1(3H), 9'-[9H]-xanthen]-3-one, 3', 6'-dihydroxy'[2321-07-5]) was investigated as a possible fluorescent standard; the compound is stable and is fluorescent at the wavelengths chosen for monitoring sepiapterin.

Several factors made it difficult to obtain accurate determinations of fluorescein concentration in the normal reading range. The molar absorptivity of fluorescein is 251 l/mol cm at 492 nm in a basic ethanol solution; this solvent absorbed CO_2 from the air which then precipitated as a salt of the base used. Within 15 minutes enough precipitate had formed that an accurate reading could not be obtained. Fluorescein in basic ethanol exhibited a large, persistent photoreaction upon exposure to room light. Fluorescein solutions were intensely fluorescent at the wavelengths used; dilution of the basic ethanol solution in H_2O to normal reading range cleared up any precipitate and eliminated the photoeffect. The ratio of final fluorescein concentration to fluorescent intensity was not consistent;

therefore fluorescein was abandoned as a potential machine standard and a freshly made sepiapterin solution was used for fine calibration of the spectrofluorophotometer.

A graph of relative fluorescence intensity vs. sepiapterin concentration was constructed using 42 samples of known sepiapterin concentration from the non-quenched portion of the sepiapterin response curve. Least squares analysis gave a slope of 17.05 $\pm$ 0.39 fluorescence intensity units per µM sepiapterin (Figure 8). This slope was used to determine the sepiapterin concentration of any sample from its relative fluorescent intensity.

The reliability of the fluorometric assay was tested by measuring the amount of sepiapterin by this method and by HPLC. HPLC has been the method of choice for accurate determination of sepiapterin concentrations. The standard error for the measurement of sepiapterin solutions used as machine standards was less than 10%. The fluorometric assay was compared with the HPLC assay after first measuring the sepiapterin concentration of reaction mixtures fluorometrically, then taking an aliquot of the reaction mix and analyzing for sepiapterin using HPLC. Sepiapterin concentrations were calculated for both methods and were compared using least squares linear regression. Figure 9 is a plot of sepiapterin concentration estimated fluorometrically vs. concentration determined using HPLC for 20 reaction mixtures. The regression coefficient was 0.96, indicating very good correlation between the two methods. The slope was not 1 but 0.59; thus while sepiapterin concentrations determined by these two methods followed the same trends, their absolute values were different.

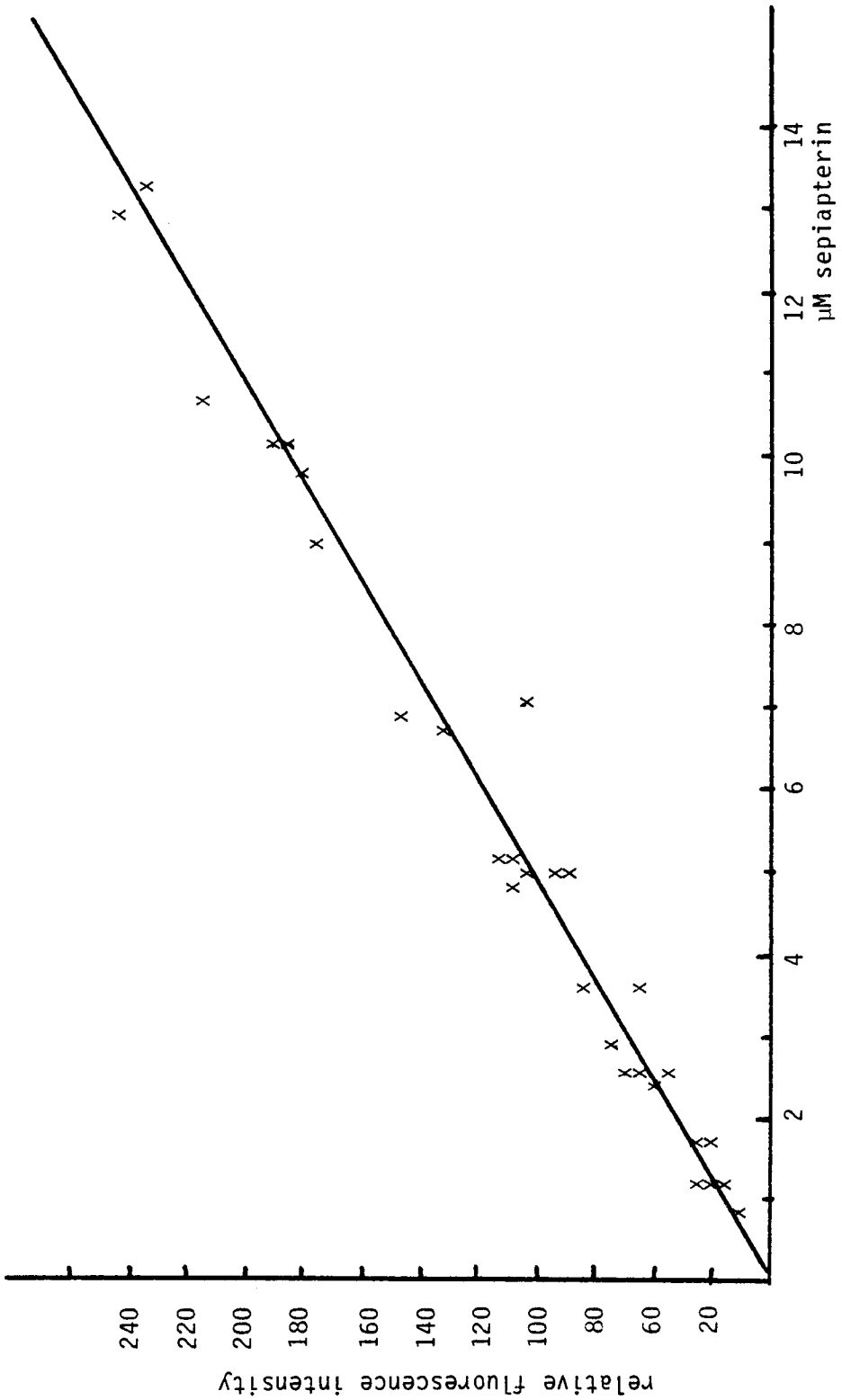

Figure 8. Least squares analysis of fluorescent units per μM sepiapterin.

Figure 9. Comparison of sepiapterin concentrations determined using HPLC to values obtained fluorometrically.

This line was calculated as a linear regression of the data shown.

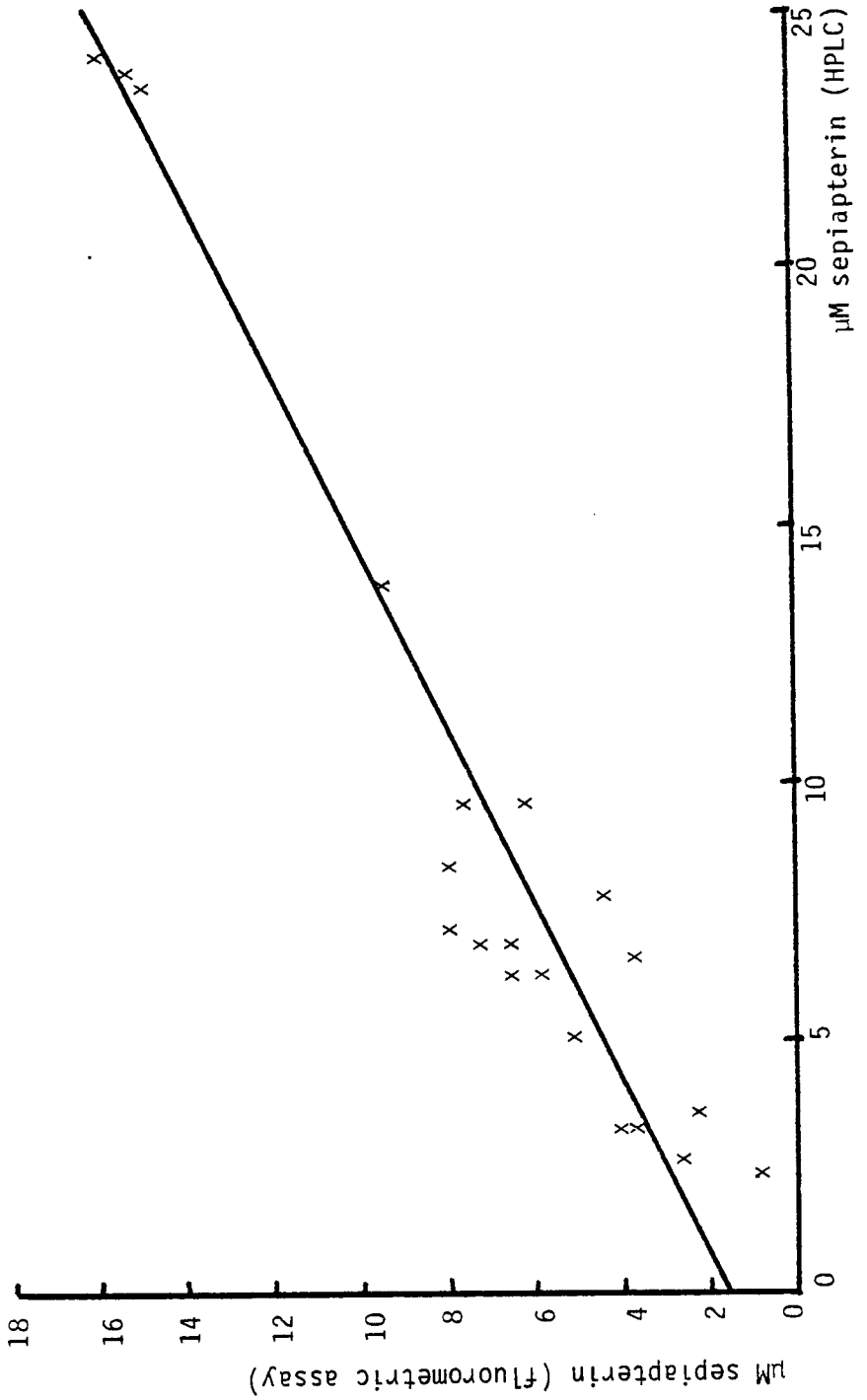
μM sepiapterin (fluorometric assay)
μM sepiapterin (HPLC)
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25

Figure 9.

As a practical application of this fluorometric assay, the effect of a suppressor of purple mutant, [$\underline{su(pr)^{e3}}$], on $\underline{pr}$ was investigated. Previous work (Griffith, unpublished) had shown that sepiapterin, extracted from $\underline{Drosophila}$ heads and quantitated by TLC (Wilson and Jacobson, 1977a), was present in trace amounts in $\underline{pr\ cn}$ flies with zero and one copy of $\underline{su(pr)^{e3}}$ but that two copies of $\underline{su(pr)^{e3}}$ restored the sepiapterin titers to 56% of wild type (Table 3). The amount of sepiapterin synthase activity in $\underline{pr\ cn}$ flies with zero, one, or two copies of $\underline{su(pr)^{e3}}$ as well as from Oregon R was determined by the assay procedure developed here and by HPLC (Table 4). Sepiapterin synthase activity did not increase with a single copy of $\underline{su(pr)^{e3}}$; with two copies of this allele the level was elevated to approximately 47% of wild type. Both the levels of sepiapterin synthase activity measured with the fluorometric assay and the sepiapterin titers measured by Griffith (unpublished) showed that two copies of $\underline{su(pr)^{e3}}$ were necessary to partially restore sepiapterin synthase activity in $\underline{pr\ cn}$ flies. This clearly demonstrated the utility of the fluorometric assay.

DISCUSSION

A rapid fluorometric assay for sepiapterin production has been developed. This assay is considerably faster than either HPLC or TLC and gives a general indication of the amount of sepiapterin in a reaction mixture. Potential applications for this assay include rapid screening for sepiapterin synthase activity during purification of the enzyme or of the $\underline{su(s)^{+}}$ product and analysis of relative sepiapterin titers.

Table 3. Sepiapterin Titers in the Heads of *pr cn* Flies with Zero, One, or Two Copies of *su(pr)*^{e3} [a]

Genotype	Relative Fluorescence[b]	Average Value
pr cn	trace	
pr cn; *su(pr)*^{e3}/+	trace	
pr cn; *su(pr)*^{e3}	3,2	2.5
Oregon R	5,4	4.5

[a] After Griffith, unpublished.

[b] Values are from two separate experiments.

Table 4. Specific Activity of Sepiapterin Synthase from *pr cn*, *su(pr)^{e3}/+*; *pr cn*, *su(pr)^{e3}*, *pr cn*, and Oregon R Stocks

Genotype	Specific Activity[a] Based on Relative Activity Determined with	
	Fluorometric Assay	HPLC
pr cn	47	37
su(pr)^{e3}/+; *pr cn*	40	34
su(pr)^{e3}; *pr cn*	70	72
Oregon R	136	173

[a]Specific activity = μmol sepiapterin produced per minute per mg protein at 42°C in the standard reaction mixture. Protein concentration was determined by the method of Lowry et al. (1951) with a bovine serum albumin standard.

Excitation and emission wavelengths of 460 and 550 nm respectively were chosen to monitor the fluorescence of sepiapterin. Absorbance at 460 nm was only half that at 420 nm; maximum absorbance was sacrificed in order to minimize the fluorescent contribution of H_2NTP and NADPH.

A certain percentage of the total fluorescence observed was due to compounds other than sepiapterin. The two major sources of this background fluorescence were pigments in the crude enzyme and the products of reactions which could proceed in the absence of Mg^{++} and/or NADPH. The contribution of enzyme pigments could be reduced by chromatographing the crude enzyme over G25 Sephadex. H_2NTP and, to a slight extent, NADPH also contributed to background. Fluorescence due to sepiapterin production was measured as the difference between the relative fluorescence intensity of a reaction which has been blocked at the first step in the enzymatic synthesis of sepiapterin by removal of the Mg^{++} cofactor with EDTA before incubation and one in which sepiapterin synthesis had been allowed to proceed. When corrected in this manner, the concentration of sepiapterin in a reaction mixture could be reliably estimated from its relative fluorescence intensity. This measurement was dependent on careful calibration of machine sensitivity; a stable, low-intensity fluorescence standard would be beneficial. The amount of sepiapterin measured fluorometrically correlated well with, but was not identical to, the amount measured with HPLC.

This assay has been used to examine the levels of sepiapterin synthase activity in pr cn flies with zero, one, and two doses of $su(pr)^{e3}$. Results showed that two doses of this suppressor allele were

necessary to produce a measurable increase in enzyme activity. Griffith (unpublished), who measured sepiapterin titers in the heads of these same genotypes using TLC, reported only trace amounts of sepiapterin in flies with zero or one copy of suppressor. Two copies of suppressor restored both enzyme activity and sepiapterin titers to about half those of wild type. In similar studies done on other suppressors of pr (Yim, unpublished), a single copy of suppressor significantly increased sepiapterin synthase activity. The reason for the observed behavior of $su(pr)^{e3}$ is not clear.

Sepiapterin synthesis occurs in two steps: H_2NTP is converted to the intermediate, "X," by the pr gene product, then "X" is converted to sepiapterin (see Figure 1, page 2). If the first step in sepiapterin synthesis is not rate limiting, the production of sepiapterin will not be an accurate measure of activity at the pr locus. Data from previous studies (cited below) can be used to obtain a rough estimate of the relative rates of the first and second steps in sepiapterin synthesis.

Krivi and Brown (1971), using a partially purified enzyme from Oregon R flies, measured the amount of α-^{32}P released in the conversion of H_2NTP to "X" and found it to be identical to the amount of sepiapterin produced. If the first step were not rate limiting, the amount of α-^{32}P released might be expected to be greater than the amount of sepiapterin produced.

Dosage studies on the pr locus (Yim, 1977) indicated that while Drosophila with two copies of pr^+ had twice the sepiapterin synthase activity of those with one copy, flies with three copies of pr^+ had only 1.21 instead of 1.5 times the activity of those with two copies. This

data can be interpreted to mean that while the first enzymatic step was rate limiting at one or two doses of $\underline{pr}^{+}$, the second step became rate limiting when the amount of the first enzyme is increased from two to three doses.

Wobbe (unpublished) found that addition of an extract from $\underline{su(s)}^{+}$; $\underline{pr}$ $\underline{cn}$ flies to sepiapterin synthase made from Oregon R resulted in a superadditive increase in sepiapterin production. As the $\underline{pr}$ mutation affects the first enzyme, these results demonstrated an increase in activity due at least partially to the addition of the second enzyme and argued that the second step in sepiapterin synthesis was rate limiting in this case.

Neither the first nor the second step of sepiapterin biosynthesis has been shown to be clearly rate limiting in any of these studies. This enzyme system could, then, be manipulated so that either step was rate limiting. Experiments in which sepiapterin production is used to monitor activity at the $\underline{pr}$ locus would best be done in an excess of the second enzyme.

Another potential drawback to any assay system using sepiapterin production to measure activity at the $\underline{pr}$ locus is that "X," the product of the first reaction, may be preferentially used for "drosopterin" biosynthesis. If this were the case, sepiapterin levels would not be as responsive as "drosopterin" or "drosopterin" precursors to changes in activity at this locus. Griffith (unpublished) reported increases in "drosopterin" titers in flies which were $\underline{su(pr)}^{e3}/+$; $\underline{pr}$ $\underline{cn}$, while two doses of suppressor were required to increase $\underline{pr}$ activity to a point where an increase in sepiapterin or biopterin levels could be

seen (Figure 10). Dorsett (1980) has developed conditions for the separation of "X" on HPLC. Activity at pr might be more accurately analyzed by an assay which measures the production of "X."

Figure 10. Titer of drosopterin and pteridines in Oregon R, $\underline{su(pr)^{e3}}$; $\underline{pr\ cn}$, $\underline{su(pr)^{e3}/+}$; $\underline{pr\ cn}$, and $\underline{pr\ cn}$.

Each value represents the average of two separate analyses made on two samples. A = Oregon R, B = $\underline{su(pr)^{e3}}$; $\underline{pr\ cn}$, C = $\underline{su(pr)^{e3}/+}$; $\underline{pr\ cn}$, D = $\underline{pr\ cn}$. No explanation has been offered for the decreased titers of neodrosopterin and pterin in wild type flies. After Griffith (unpublished).

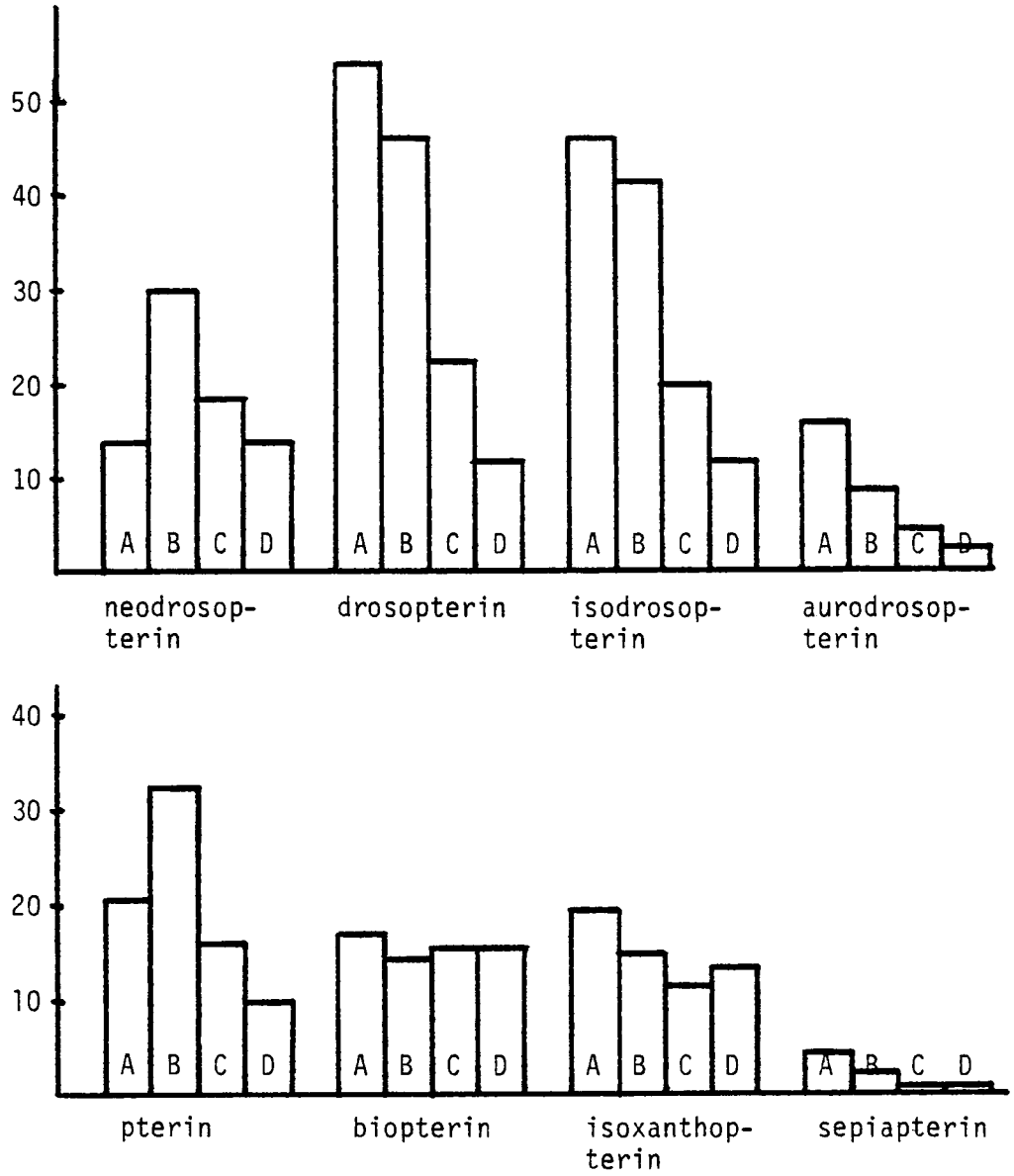
50
40
30
20
10
A B C D
neodrosop-
terin
drosopterin
isodrosop-
terin
aurodrosop-
terin
40
30
20
10
pterin
biopterin
isoxanthop-
terin
sepiapterin

Figure 10.

CHAPTER III

STUDIES ON THE ISOLATION AND PURIFICATION OF GTP CYCLOHYDROLASE I

INTRODUCTION

GTP cyclohydrolase I is used to generate H_2NTP, the parent compound of the pterins. Studies on the isolation and purification of this enzyme were initiated because the standard laboratory method for obtaining this enzyme did not work reproducibly in my hands. A number of parameters were investigated; subsequent improvements in GTP binding efficiency and overall yield were observed.

MATERIALS AND METHODS

Materials

GTP was obtained from Sigma Chemical Company and [8-^{14}C]GTP or [8-^{3}H]GTP from either Amersham or New England Nuclear. Cyanogen bromide was purchased from Aldrich and $NaIO_4$ from Sigma Chemical Company. Diethyl adipate was from Matheson, Coleman and Bell, and hydrazine from Eastman Kodak Company. Biogel A-0.5m, 100-200 mesh was purchased from BioRad, Sepharose 4B and 6B from Pharmacia. Darco G60 activated charcoal was obtained from the Atlas Powder Company. Ammonium sulfate (ultra pure) was from Schwarz/Mann. All other chemicals were reagent grade. E. coli, Whitkin strain, was grown in minimal medium to mid-log phase, harvested, and stored in 500 g aliquots at -80°C.

Assay for GTP Cyclohydrolase I

The assay used to measure cyclohydrolase I activity was developed by Burg and Brown (1968). GTP is converted to H_2NTP with the loss of the number eight carbon as formate (Figure 11). The extent of the reaction could be determined by measuring the amount of radioactive formate produced from 8-labeled GTP. The standard reaction mixture contained equal volumes of buffer (made of equal volumes 1 M NaCl, 1 M Tris HCl pH 8.5, and 0.1 M EDTA pH 7.5), labeled GTP, at a concentration of 1-4 mM, and enzyme. Typically, the final volume was 45 µl. After incubation at 42°C, the reaction was stopped by the addition of 250 µl of 0.4 M formate and the mixture transferred to cotton-plugged Pasteur pipettes in which 1 cm charcoal columns had been poured. The cyclic compounds (GTP and H_2NTP) were retained on the charcoal under acidic conditions and the labeled formate was washed into scintillation vials with a total of 2 ml of H_2O. This was sufficient to remove most of the formate from 1 cm charcoal columns. More extensive washing was required with longer columns; it was, therefore, important that the column heights be the same (Lee, unpublished). One mole of labeled formate is produced for each mole of H_2NTP; if the specific activity of the GTP were known, the amount of H_2NTP produced in the reaction could be calculated. Enzyme units for cyclohydrolase were expressed as nmol H_2NTP produced per minute at 42°C in the standard reaction mixture.

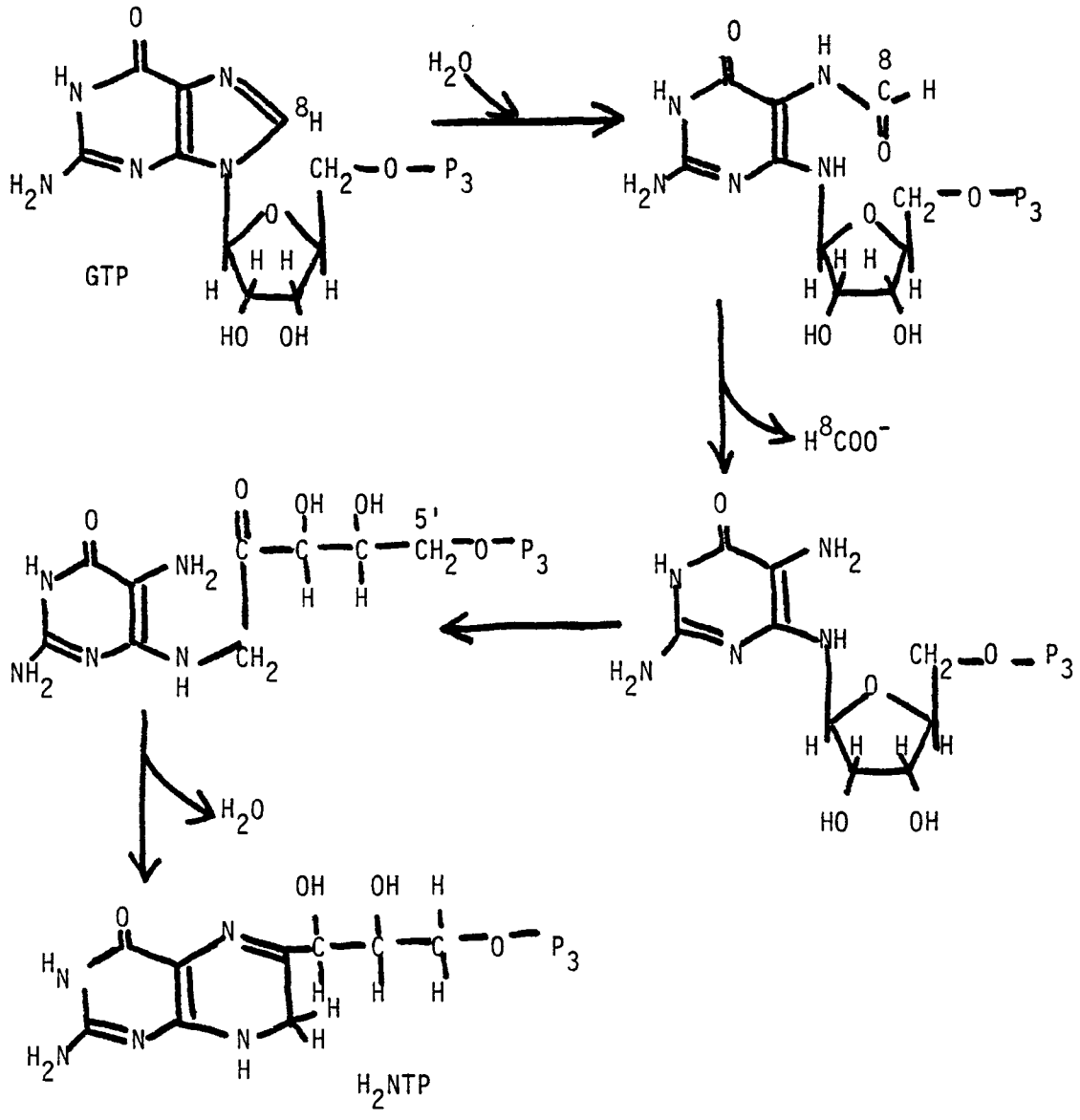

Figure 11. Biosynthesis of H_2NTP from GTP by GTP cyclohydrolase I.

Postulated reaction mechanism from Yim and Brown (1976).

Preparation of GTP Sepharose Affinity Column

a) Standard Laboratory Method

This procedure has been derived from Yim and Brown (1976). Sepharose 4B, suspended in 4 volumes H_2O and stirred with a glass rod, was activated with cyanogen bromide (250 mg per ml Sepharose) at 4°C. The pH was maintained at 11.0 ± 0.3 by addition of 5 N NaOH. When the reaction had subsided somewhat, the activated Sepharose was washed with 20 volumes cold 0.1 M $NaCO_3$ buffer, pH 9.5, and resuspended in 5 volumes of this buffer. Adipate dihydrazide, prepared according to Lamed et al. (1973), was added at 300 mg per ml Sepharose. The coupling reaction was allowed to proceed overnight at 4°C in the dark on a rotary stirring device. [$8^{14}C$]GTP (2 µmol per ml Sepharose at 5.59 µCi per mmol GTP) was activated for coupling to the Sepharose hydrazide by peroxidation in 0.1 M NaAc, pH 5.0 (0.2 ml per µmol GTP) using a 1:1 molar ratio of GTP to $NaIO_4$ for 30 minutes at room temperature in the dark. Excess $NaIO_4$ was reacted with a fivefold excess of ethylene glycol; formaldehyde generated in this reaction was bubbled off with a He/butane mixture. The oxidized GTP was coupled to the Sepharose hydrazide, which had been washed with 6 volumes each of 0.2 M NaCl and H_2O and equilibrated with 3 volumes 0.1 M NaAc, pH 5.0, by mixing on a rotary stirrer for 2.5 hours at room temperature in the dark. The GTP Sepharose was washed with 6 volumes of 2 M NaCl to remove adventitiously bound GTP, then rinsed with 20 volumes H_2O and equilibrated with column buffer. The amount of GTP bound per ml Sepharose was determined by measuring the cpm for a weighed amount of GTP Sepharose.

This was converted to dpm by measuring the counting efficiency. The specific activity of the GTP added was used to express the result in terms of μmol GTP bound per ml Sepharose.

b) Method of Lamed, Levin, and Wilchek (1973)

This method was followed exactly as described in Lamed et al. (1973).

c) Method of Jackson, Wolcott, and Shiota (1973)

This method was modified to use adipate dihydrazide as the spacer. The specific activity of the GTP used was changed to 1.18 μCi per mmol GTP and [8-^{14}C]GTP rather than [γ-^{32}P]GTP was used as the label. All other details of the preparation remained unchanged.

Preparation of GTP Cyclohydrolase I

GTP cyclohydrolase I was prepared by a method adapted from Yim and Brown (1976). An aliquot of *E. coli B* was thawed overnight at 4°C or else broken into chunks and thawed in 1 l room temperature buffer (50 mM Potassium phosphate, 5 mM EDTA, pH 7.0; buffer A). This suspension was passed through a Sigma peristalsic pump (finger type) until a smooth homogenate was obtained. Cells were sheared in a precooled Gaulin press at 10,000 psi, cooled to 10°C or less, and sheared again. Cellular debris was pelleted by centrifugation at 10,000Xg for 90 minutes. The resulting supernatant was fractionated with saturated ammonium sulfate. The pellet obtained between 35 and 50% saturation was retained and redissolved by dialysis against 4 l of buffer A with one change of buffer. This material was loaded onto a 1500 ml Biogel A-0.5m, 100-200 mesh column and eluted with buffer A. Fifteen

ml fractions were collected and alternate fractions assayed for cyclohydrolase I activity. Fractions containing activity were pooled and applied to a 20-25 ml GTP Sepharose column which had been equilibrated in 10 mM Potassium phosphate, 2.5 mM EDTA, pH 7.0 (buffer B). The column was washed with 1.1 buffer B made 0.3 M in KCl, rinsed with 5-6 column volumes of buffer B, and cyclohydrolase I eluted with 3-4 column volumes of buffer B made 0.25 mg/ml in GTP. Five ml fractions were collected and alternate fractions assayed for cyclohydrolase activity. Fractions containing activity were pooled and concentrated in an Amicon ultrafiltration cell using a UM 10 or PM 30 filter, and dialyzed to remove GTP. Cyclohydrolase was stored at -20°C.

RESULTS

Initial preparations of GTP cyclohydrolase I failed to yield appreciable amounts of activity. GTP binding to the activated Sepharose was poor. A comparison of cyclohydrolase yields in these initial preparations, in which column material being activated and/or coupled was stirred with a magnetic stirrer, and in subsequent preparations, in which column material was stirred manually during activation and tumbled on a rotary stirrer during coupling, illustrates the need for avoiding magnetic stirrers when working with Sepharose (Table 5). Microscopic examination of Sepharose beads has shows that they are fractured by the action of the magnetic stir bar (Neame et al. 1975).

Although reasonable cyclohydrolase yields could be obtained after the use of a magnetic stir bar was discontinued, these yields were still not reproducibly good. In an effort to sort out other parameters affecting cyclohydrolase yields, experiments were conducted

Table 5. Effect of Magnetic Stirrer on GTP Sepharose Column Preparation

Magnetic Stirrer Used	µmol GTP Bound Per ml Column	Cyclohydrolase Activity
yes	0.23	none
yes	0.28	none
yes	0.55	none
no	2.68	yes
no	3.88	yes

to optimize the preparation of the GTP affinity column. The GTP:$NaIO_4$ ratio was altered to determine if a decrease in this ratio would have a deleterious effect on the GTP being activated. The standard laboratory method was used with GTP:$NaIO_4$ ratios of 1:1, 1:2, and 1:3. Table 6 shows that the amount of GTP bound to the matrix did not decrease significantly with decreasing GTP:$NaIO_4$ ratios and that the cyclohydrolase binding capacity of the columns was unaffected.

Three methods for preparing GTP Sepharose were evaluated for their efficiency in binding GTP and for their cyclohydrolase I binding capacity. Differences in these methods are summarized in Table 7. Table 8 gives the results of two separate experiments. No one method showed better GTP binding efficiency than any other. GTP was bound quantitatively; within the concentration limits tested there was no saturation of the binding sites on the Sepharose. There were no differences in cyclohydrolase I binding capacity due to the method used. Binding was an average of 15 times better in the first experiment. There was no obvious explanation for this difference.

The effect of the rate of application of enzyme to the GTP Sepharose column on the column binding efficiency and recovery from the column was investigated. Data from all cyclohydrolase I preparations was summarized and divided into two groups: one group was composed of experiments in which enzyme was applied to the column at the maximum permissible flow rate for the column dimensions used, the second group consisted of experiments in which enzyme was loaded onto the column at 30 to 60% of the maximum rate. As indicate in Table 9, the rate of sample application did not have a clear effect on column binding

Table 6. Effect of Varying GTP:$NaIO_4$ Ratio in GTP Sepharose Preparation

GTP:$NaIO_4$ Ratio	GTP Added to Sepharose µmol	GTP Bound to Sepharose µmol	Attachment %	Total GTP Bound to Column (µmol)	Units Bound to Column	Units/Total µmol Bound GTP
1:1	5.88	4.25	72.24	1.58	1.47	0.93
1:2	5.88	4.08	69.34	1.52	1.64	1.08
1:3	5.88	2.68	68.42	1.55	1.87	1.21

Table 7. Summary of Differences in the Methods Used to Prepare GTP Cyclohydrolase I

	Standard Lab Method	Lamed et al. (1973)	Jackson et al. (1973) Modified
1. Coupling of CNBr-activated Sepharose to adipate dyhydrazide	pH 9.5	pH 9.5	pH 9.0
2. µmol GTP added per ml Sepharose	2.0	4.0	2.37
3. Incubation conditions for GTP periodation	30 minutes room temperature 0.1M NaAc pH 5.0	1 hour 4°C 0.1M NaAc pH 5.0	30 minutes room temperature 0.1M citrate/phosphate buffer pH 5.0
4. Incubation conditions for coupling of activated GTP to Sepharose hydrazide	2 hours room temperature	1.5 hours 4°C	2 hours room temperature at pH 3.0

Table 8. Effect of Method of Preparation of GTP Sepharose on Attachment of GTP and on Cyclohydrolase Binding

Method	GTP Added to Sepharose µmol	GTP Bound to Sepharose µmol	Attachment %	Total GTP Bound to Column µmol	Units Bound to Column µmol	Units/Total µmol Bound GTP	% Binding (Relative to Method 1)
Experiment 1							
1.	5.9	4.3	72	1.6	1.5	0.9	100
2.	11.8	9.4	80	3.4	4.3	1.3	136
3.	69.7	7.0	10[a]	2.8	2.7	1.0	103
Experiment 2							
1.	17.8	17.9	101	29.9	2.1	0.1	100
2.	35.4	34.2	96	57.0	4.0	0.1	99
3.	21.0	21.4	102	33.6	2.3	0.1	95

[a]The GTP:$NaIO_4$ ratio was 10:1 because of an error. Thus only 10% of the GTP could be activated by the periodate reaction and subsequently coupled to the activated Sepharose-hydrazide.

Table 9. Effects of Flow Rate on: (1) Activity Lost in the Flow-through, (2) Recovery in GTP Elution, and (3) Total Recovery

Flow Rate	Number of Experiments	GTP Cyclohydrolase Activity[a]		
		In Flow-through	In GTP Elution	Total Recovery
Fast (1.77 ml/min.)[b]	2	14-66%	1-13%	33-68%
Slow (0.54-1.0 ml/min.)	9	39-76%	0- 9%	49-83%

[a]Given here as percent of total activity loaded onto the column.

[b]Maximum flow rate was defined as 1 ml/minute/cm^2 cross sectional area of the column used, and was used as a guide for determining a reasonable rate of application to all columns. BioRad columns, 0.7 cm diameter, were used in these experiments; maximum flow rate for this size column was calculated to be 1.77 ml/minute.

(as measured by the activity recovered in the flow-through volume), on activity eluted from the column with GTP, or on the total recovery of units loaded.

In an experiment done specifically to evaluate the effect of applying sample at 50% and 10% of the maximum flow rate, more activity was recovered from the slower column. These was no appreciable difference in the amount of activity lost in the flow-through volume (Table 10). The rate of sample application had little or no effect on the amount of enzyme bound per ml column volume nor did it affect the amount recovered in the GTP elution.

The amount of purification achieved using the GTP-Sepharose column was evaluated and compared with the values given in Yim and Brown (1976) (Table 11). The amount of purification in the present study averaged 121-fold, as compared with Yim's 350-fold purification. Total activity was higher before the GTP-Sepharose column step in the earlier work and the percent recovery from the GTP-Sepharose was markedly higher (69.5% vs. 6.5% in the present study). Only 0.42 µmol GTP were bound per ml column, as compared to 2.77 µmol GTP bound per ml column in the present study, however, the binding efficiency expressed as units bound per µmol GTP was 925 times greater than that of the present study.

DISCUSSION

Investigation of several parameters thought to affect the preparation of GTP cyclohydrolase I has lead to some improvement in the isolation of the enzyme. One set of variables examined involved the preparation of the enzyme. The specific activity of the present

Table 10. Effects of 50% vs. 10% Maximum Flow Rate on: (1) Activity Lost in the Flow-through, and (2) Recovery in the GTP Elution

	GTP Cyclohydrolase I Activity	
Flow Rate	In Flow-through	Recovered in GTP Elution
10% of maximum (0.17 ml/min.)	81%	2%
50% of maximum (0.91 ml/min.)	76%	0%

Table 11. Comparative Summary of Purification Using GTP Sepharose Column

Enzyme	Total Activity Units	Specific Activity Units/mg	Relative Specific Activity
1. Data from Yim and Brown, 1976			
Crude Extract	6150	0.18	1.0
Ammonium Sulfate Fraction	5020	0.61	3.4
Eluate from Sephadex G25	4190	2.0	11.1
Eluate from GTP Sepharose	2910	700	3890
2. Data from Experiment of April 28, 1981			
Crude Extract	No aliquot tested		
Ammonium Sulfate Fraction	No aliquot tested		
Eluate from Ultrogel	2588	0.28	1.0
Eluate from GTP Sepharose 1)	43	37	131
2)	80	34	118
3)	45	32	114

preparation was only 14% that of Yim and Brown (1976) at the point at which it was loaded onto the GTP affinity column. The specific activity at earlier steps in the preparation was not determined; this will have to be done in order to determine the steps where significant loss of activity occurs. Two suggestions can be made at this point: First, the second pass through the Gaulin press results in a relatively small increase in the percent of cells broken; each pass through the Gaulin heats the suspension 16°C. Although E. coli cyclohydrolase I is relatively heat stable (Yim and Brown, 1976) it may be advisable to accept a decrease in the total units obtained per gram of E. coli and pass the cells through the press only once. Secondly, it has been observed that enzyme activity was coeluted with the clear yellow or straw colored fractions from the Biogel sizing column. Substantial losses in activity may occur in the interval between the elution of these fractions and their application to GTP Sepharose. This interval could be shortened significantly if the fractions were not assayed for activity but rather were pooled according to their color.

A significant percent of the total enzyme activity loaded was often found in the flow through volume. No significant increase in yield or decrease in the activity found in the flow through volume was obtained by applying enzyme to the GTP Sepharose column at less than the maximum rate of 1.77 ml/minute.

The second set of parameters investigated were those pertaining to the preparation of the GTP Sepharose affinity column. Sepharose bead structure was damaged by the action of magnetic stir bars (Neame et al., 1973); GTP binding and overall yield was markedly increased

when a rotary stirrer was used. The effect of $NaIO_4$ on GTP was examined at three concentrations and was found to have no adverse effects on GTP binding to the column and enzyme binding to the GTP. Three protocols for the preparation of GTP Sepharose were compared. No significant differences in GTP binding or enzyme binding capacity were observed. It is frustrating to note that the enzyme binding was some 15 times better in the first of the two trials, as this experiment was done under less than optimal conditions.

In the present preparations, over six times more GTP was bound per ml Sepharose than the amount reported by Yim and Brown (1976), yet enzyme binding efficiency was considerably less. Two possible explanations may be offered. The cyanogen bromide-activated Sepharose will react with primary amino groups (Ahrgren et al., 1972). If not all of the activated Sepharose has been coupled to adipate dihydrazide, GTP may become bound to the matrix directly via its 2-amino group. GTP bound in this way may not be recognized by the enzyme. This type of binding is unlikely, however, as the cyanogen bromide-Sepharose should be quantitatively coupled to the adipate dihydrazide.

Another way to explain poor enzyme binding despite good GTP binding would be to postulate that the GTP has lost its terminal phosphate during coupling and/or storage. Both acid and alkaline hydrolase of the terminal phosphate can occur, although the extremes of pH necessary were not found in these preparations. This possibility, however unlikely, could be investigated by preparing a [γ^{32}-P] labeled GTP column and comparing the estimate of percent binding with this label to that obtained with an [8-^{3}H] label.

CHAPTER IV

STUDIES ON THE INTERACTION OF SEPIAPTERIN SYNTHASE WITH CIBACRON BLUE F3GA

INTRODUCTION

Studies on the interaction of sepiapterin synthase with Cibacron blue F3GA were initiated in the hope of being able to purify the enzyme by affinity chromatography. Cibacron blue can function as an NADPH analogue (Dean and Watson, 1979); binding of sepiapterin synthase by its NADPH-requiring activity might be expected to occur. This principle had previously been exploited by Krivi and Brown (1979), who reported an 8-fold purification of sepiapterin synthase with 50% recovery of the total enzyme units applied.

MATERIALS AND METHODS

Materials

G25 Sephadex was purchased from Pharmacia. Cibacron blue Sepharose (Cibacron blue F3GA covalently attached to Sepharose CL 6B), Pipes (Ultrol), and NADPH (tetrasodium salt, type I) were obtained from Sigma Chemical Company. NADH (sodium salt) was from Pabst Laboratories. Ammonium sulfate (ultrapure) was from Schwarz/Mann. All other chemicals were reagent grade.

Preparation of Sepiapterin Synthase

Crude extracts of Drosophila heads were prepared according to Krivi and Brown (1979) and were stored at -20°C.

Assay for Sepiapterin Synthase Activity

Sepiapterin synthase activity was assayed by monitoring the production of sepiapterin fluorometrically. Details of this assay were given in an earlier section. Units of activity were defined as nmoles sepiapterin produced per minute at 42°C. Krivi and Brown (1979) have defined enzyme units as nmoles sepiapterin produced per hour at 30°C. These values were adjusted for comparison with the present study by assuming that the reaction rate would double with the 12 degree rise in temperature.

Assay for NADPH Concentration in Eluate

The concentration of NADPH at which enzyme was eluted from the Cibacron blue Sepharose was determined spectrophotometrically. The millimolar extinction coefficient for NADPH is 6.2 l/mmole cm at 340 nm.

RESULTS

Enzyme was routinely eluted from the blue Sepharose column with 3 mM NADPH-containing buffer (Krivi and Brown, 1979). On occasion, enzyme activity was found in fractions in which the NADPH concentration was only 0.7-1.0 mM. Attempts were made to elute the enzyme with buffer containing less NADPH. Elution at lower NADPH concentrations was not consistent or reproducible, therefore the molarity of NADPH in the elution buffer could not be reduced.

Elution was attempted with buffer containing NADH. No enzyme activity was eluted at concentrations of up to 7 mM NADH. This

confirmed the data of Krivi and Brown (1979) showing the specificity of the enzyme for NADPH.

Sepiapterin synthase activity could be eluted from the column with buffer containing 1 M NaCl. The presence of NaCl in the reaction mixture significantly inhibited enzyme activity (Figure 12); fractions containing NaCl were therefore desalted over G25 Sephadex before being assayed for activity. Elution with buffer containing 1 M NaCl was able to remove significant amounts of enzyme from the blue Sepharose after elution with NADPH-containing buffer (Table 12).

Further purification of sepiapterin synthase over Cibacron blue Sepharose followed the conditions outlined in Krivi and Brown (1979). Briefly, enzyme, at 2.69 units per ml column volume, was loaded onto the column and unbound proteins washed off with 5-6 column volumes of 50 mM Pipes, 10 mM β-mercaptoethanol made 10% in glycerol. Enzyme was then eluted with four column volumes of this buffer made 3 mM in NADPH. Additional enzyme was eluted in buffer made 1 M in NaCl. Recovery at these steps is shown in Table 13. Fractions eluted with NADPH were concentrated by ultrafiltration and the specific activity of the enzyme before and after chromatography on covalently bound blue Sepharose was determined (Table 14). While Krivi and Brown (1979) have reported a 7.8-fold increase in specific activity for this step, a 3.85-fold decrease in specific activity was observed in this experiment. The specific reasons for this decrease were not investigated.

DISCUSSION

Cibacron blue F3GA affinity chromatography was used in an attempt to duplicate the purification of sepiapterin synthase according

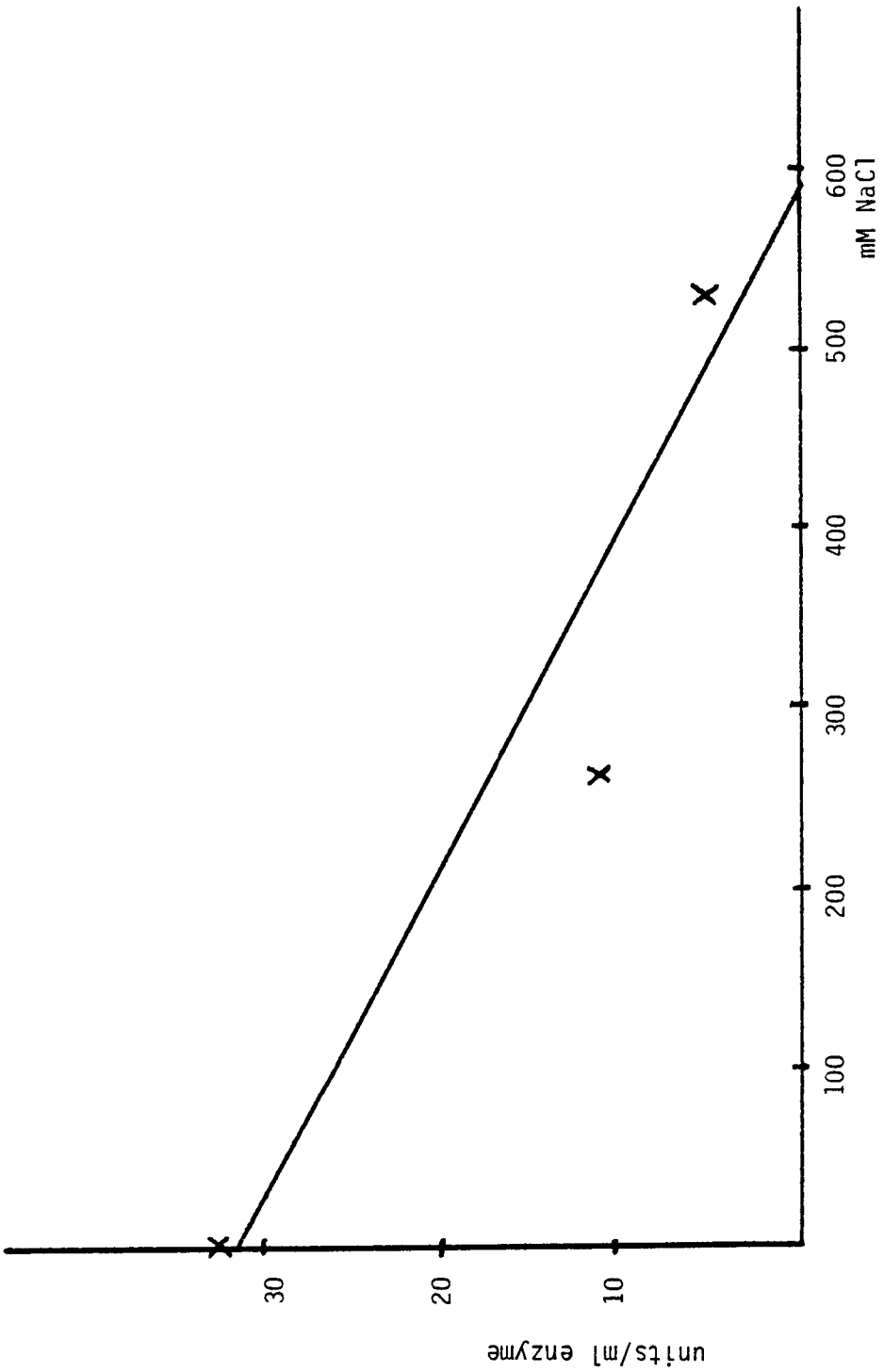

Figure 12. Inhibition of sepiapterin synthase activity with NaCl.

Table 12. Elution of Enzyme Activity from Blue Sepharose[a]

	Percent of Total Units Loaded	
	Experiment 1	Experiment 2
1. Activity eluted with 3 mM NADPH	28%	31%
2. Activity eluted with 1M NaCl	11%	20%

[a]Enzyme was allowed to bind to the column and unbound proteins were washed through. The enzyme was then specifically eluted with buffer containing 3 mM NADPH. Following this elution, additional activity was recovered from the column by eluting with buffer containing 1M NaCl.

Table 13. Recovery of Sepiapterin Synthase Activity during Purification on Cibacron Blue Sepharose

Enzyme	Total Activity Units	Recovery as Percent of Units Loaded	Recovery as Percent of Units Bound
Ammonium Sulfate Fraction	973	100%	n.a.[a]
Flow-through	213	22%	n.a.[a]
Eluted with 3 mM NADPH	273	28%	36%
Eluted with 1M NaCl[b]	71	11%	14%

[a] n.a. = not applicable.

[b] Enzyme was desalted over G25 Sephadex. Recovery is expressed as percent of units loaded as measured with a sample of enzyme which had also been subjected to chromatography over G25 Sephadex.

Table 14. Specific Activity of Sepiapterin Synthase

Enzyme	Total Activity[a] Units	Specific Activity Units/mg Protein[b]
Ammonium Sulfate fraction	1.49	0.21
Fraction from Cibacron blue Sepharose	0.65	0.05

[a]Activity was determined using the fluorometric assay described earlier.

[b]Total protein concentration was evaluated with a Lowry assay (Lowry et al., 1951) using a bovine serum albumin standard.

to Krivi and Brown (1979). No purification of the enzyme was achieved using this procedure. Overall recovery from the column was also poor; approximately 36% of the total activity bound was recovered in the NADPH elution as opposed to about 54% in Krivi and Brown's purification (1979).

Activity was not eluted from the blue Sepharose with NADH, demonstrating the specificity of the enzyme for NADPH. Elution with NADPH was occasionally observed at concentrations as low as 0.7 mM, however 3 mM NADPH was used to ensure elution. Even at this high concentration, elution was not quantitative. Additional activity could be recovered from the column by elution with 1 M NaCl. This suggests that there was, in addition to a biospecific adsorption of the enzyme to the dye as an NADPH analogue (Dean and Watson, 1979), an ionic component to the dye-protein interaction which was not broken by elution with the NADPH cofactor. The use of salt gradients for elution of proteins from dye affinity columns has been reported (Lowe et al., 1980; Ogawara and Horikawa, 1979). Hydrophobic interactions may also contribute to binding. Glycerol or ethylene glycol have been used to reduce hydrophobic interactions and facilitate elution (Seelig and Colman, 1977; Lowe et al. 1979). Other parameters which have been shown to affect binding to Cibacron blue and other anionic dyes include rate of application, temperature, and pH. In general, increased binding efficienty is observed when the rate of application is reduced. Higher temperatures may increase binding as well; conversely, decreasing the temperature may increase recovery (Qadri and Dean, 1980). Binding to anionic dyes is generally tighter at low pH; pH gradients may be

used to separate proteins which bind to the dye with different affinities, or pH may be stepped up to facilitate elution (Dean and Watson, 1979).

An additional consideration in the design of dye affinity columns is the choice of dye. Procion Brilliant Blue (Beissner and Rudolph, 1978), Procion Red HE 3B (Dean et al., 1979), Direct blue 14 (C. I. 23850), and Reactive black 1 (C. I. 17916) (Stellwagen, 1979) have all been shown to bind NADPH-requiring enzymes. Dyes can be screened for their potential as ligands by enzyme inhibition or inactivation studies (Clonis and Lowe, 1980; Seelig and Colman, 1977; Qadri and Dean, 1980), then evaluated in terms of the ease of recovery of enzyme.

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