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SOME ASPECTS OF PTERIDINE BIOSYNTHESIS IN AMPHIBIANS

by

Hamilton L.^{ee} Stackhouse

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I hereby recommend that this dissertation prepared under my
direction by Hamilton Lee Stackhouse
entitled Some Aspects of Pteridine Biosynthesis in Amphibia
be accepted as fulfilling the dissertation requirement of the
degree of Doctor of Philosophy

Joseph T. Boyers
Dissertation Director

June 5, 1964
Date

After inspection of the dissertation, the following members
of the Final Examination Committee concur in its approval and
recommend its acceptance:*

Mitchell G. Varich
A. R. Kennen
Wayne Serrin
Wm. J. McCarley
Peter E. Pickens

June 15, 1964
June 15, 1964
June 15, '64
June 15, '64
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ABSTRACT

SOME ASPECTS OF PTERIDINE BIOSYNTHESIS IN AMPHIBIANS

by

Hamilton Lee Stackhouse

Bright fluorescing unconjugated pteridines have been extracted from amphibians; yet the biological function of these compounds in vertebrates is not completely understood. There is some evidence, indicating that they can act as pigments, co-factors, and growth-promoting substances. Pteridine biosynthesis is unknown but it appears to have a parallel biosynthesis with the purines. Rana catesbeiana larval integument will convert carbon-14-labeled guanine to at least two pteridines: biopterin and 2-amino-4-hydroxypteridine-6-carboxylic acid. Intermedin will cause pteridine deposition and purine depletion in the dorsal integument of Rana pipiens and Rana sylvatica larvae. Thus, it seems probable that in amphibians the integumental pteridines may be synthesized from the purines. This study was designed to investigate the synthetic pathways of unconjugated pteridines in an in-vivo system.

The hypophyseal placode was removed from early tail bud Rana sylvatica larvae. Normal and hypophysioprivic Rana sylvatica larvae were injected with glycine-(u.l.)- ^{14}C . Some of the hypophysioprivic larvae were injected with a hormone preparation containing large amounts of intermedin. Pteridines and purines were extracted from homogenized

larvae in various ways and purified by paper chromatography. These compounds were identified by matching Rf values of known to unknown compounds, by enzymatic reactions, by photolytic experiments, and by their characteristic absorption spectra. Radioactivity was determined in a liquid scintillation counter.

Five pteridines (sepiapterin, biopterin, Hynobius-blue, isoxanthopterin, and 2-amino-4-hydroxypteridine) and three purines (adenine, hypoxanthine, and guanine) were extracted from Rana sylvatica larvae and identified. When glycine-(u.l.)-¹⁴C was injected into the larvae, it was incorporated into purines and pteridines. This labeled precursor was incorporated into the pteridine ring of isoxanthopterin and 2-amino-4-hydroxypteridine and the radioactive label of the other pteridines was assumed to be located in the pteridine ring. In hypophysioprivic larvae a twofold increase in pteridine deposition occurred after intermedin treatments, while adenine and hypoxanthine showed a concentration decrease. Guanine concentration was essentially unchanged by intermedin treatments. The specific activities of pteridines and purines were determined and from these data a scheme was proposed for biosynthetic interconversions of pteridines in Rana sylvatica larvae. Intermedin may be altering these pathways by some unknown mechanism or mechanisms. Hynobius-blue may have the same chemical structure as tetrahydrosepiapterin or tetrahydrobiopterin. These data also indicate that the free purines in the hypophysioprivic larvae are not converted to pteridines. A scheme indicating the parallel biosynthesis of purines and pteridines has been proposed. Data presented have been discussed in relation to the published results of other authors.

INTRODUCTION

Unconjugated pteridines have been isolated from the skin and eyes of Amphibia (Hama 1953; Gündler 1953, 1954; Nawa et al. 1954; Goto and Hama 1958; Hama and Obika 1959). These bright fluorescing compounds appear to be associated with specific chromatophores, since amphibian integument heavily populated with xanthophores contains large quantities of pteridines, while the melanophore-rich integument possesses minute amounts of integumental pteridines (Bagnara 1961; Obika 1963; Obika and Bagnara 1964). Furthermore, these compounds do not seem to be associated with guanophores (Hama and Obika 1959; Bagnara 1961), which contain purines. Three purines, adenine, hypoxanthine, and guanine (Figure 1), have been reported to be associated with guanophore-laden amphibian integument (Bagnara and Neidleman 1958; Bagnara and Stackhouse 1961).

The biological function of pteridines in vertebrates is not understood. However, they appear to be acting as pigments (Ziegler-Gündler 1956; Kauffman 1959; Ortiz 1962; Obika 1963; Obika and Bagnara 1964), and their ability to act as a co-factor in the oxidation of phenylalanine to tyrosine has been demonstrated (Kaufman 1959; Kaufman and Levenberg 1959; Kaufman 1962, 1964). One unconjugated pteridine, biopterin (for pteridine structures see Figure 2), was first shown to be acting as a growth-promoting substance in the protozoon Crithidia fasciculata (Patterson et al. 1955).

Adenine

Hypoxanthine

Guanine

Figure 1. Purines isolated from integument of Rana sylvatica larvae.

Biopterin

AHP-6-COOH

Sepiapterin

2-amino-4-hydroxypteridine

Tetrahydrosepiapterin

(Hynobius-blue)

?

Isoxanthopterin

Figure 2. Pteridine structural formula.

Various investigators have indicated that pteridines are synthesized through a purine precursor. Weygand and Waldschmidt (1955), working with Lepidoptera, Gonepteryx rhamni and Pieris brassicae, P. napi and P. rapae, demonstrated that carbon atoms 4a and 8a and the nitrogen atom 5 of the pteridine ring originate from glycine, as do carbon atoms 4 and 5 and nitrogen atom 7 of the purine ring. Furthermore, the biosynthesis of leucopterin parallels that of the purines in Pieris brassicae (Weygand et al. 1959). Ziegler-Günder et al. (1956) injected guanine-2-¹⁴C into Xenopus larvae and were able to isolate a weakly labeled pteridine, which was converted to 2-amino-4-hydroxypteridine-6-carboxylic acid (AHP-6-COOH). Purines may also serve as a precursor for pteridines in the micrococcus Gaffkya homari (Aaronson and Rodriguiz 1958). Recently, it has been demonstrated that tissue slices of Rana catesbeiana larval integument, when cultured in media containing guanine-2-¹⁴C or guanine-2-4-¹⁴C, were able to convert this purine into at least two pteridines; biopterin and AHP-6-COOH (Levy 1964). Other investigators (Bagnara and Neidleman 1958; Bagnara 1961) have reported that intermedin causes the free purine concentration to decrease and the unconjugated pteridine concentration to increase in the skin of hypophysioprivic Rana pipiens and Rana sylvatica larvae. These results suggest that a close biochemical relationship exists between purines and pteridines. It seems probable that integumental pteridines in amphibians may be synthesized from purines.

In order to understand the biological significance of integumental pteridines, it seems necessary to understand the metabolism of these compounds. Accordingly, this study was undertaken to investigate one

aspect of pteridine metabolism; the synthetic pathways of unconjugated pteridines in an in-vivo system. In addition, the effect of the hypophysis upon the deposition of individual pteridines was studied.

MATERIALS AND METHODS

Rana sylvatica larvae were used throughout this investigation. Eggs were obtained from a collector in Tennessee. Larvae were fed boiled lettuce and reared in charcoal-filtered water.

Glycine-(u.l.)- ^{14}C (Lot # 134-48-35, 36), dissolved in 0.01 N HCl, with a specific activity of 80.0 millicuries / millimole, was obtained from New England Nuclear Corporation.

Hypophysioprivic tadpoles were obtained by removing the hypophyseal placode at the early tail bud stage (Allen 1916; Smith 1916). Operations were considered successful if the larvae became light in color. Some of these larvae received injections of hormones containing chromatophore-stimulating properties. Pitressin was chosen because of its high contamination with intermedin and it was potentiated by treatment with alkali (Bagnara 1957, 1958).

Hormone and isotope injections were made at various developmental stages. The pH of the injected solutions was adjusted between 7 and 8 by adding appropriate amounts of NaOH or HCl. Injections were made with a 31-gauge needle on a Hamilton Microliter Syringe equipped with a mechanical device that allowed repeated injections of 10^{-3} ml. Injections were made through the somites or tail muscles into the body cavity, while the larvae were narcotized with MS 222 dissolved in water at a concentration of 1:10,000.

Pteridines and purines were extracted from the larvae in various ways. In some cases, a slightly modified Awapara's technique was

employed for pteridine and purine extractions (Awapara 1948). Larvae were washed in distilled water, boiled for three minutes to inactivate the enzymes, and homogenized in 20 to 50 parts of 70% ethanol in a glass homogenizer. The homogenates were centrifuged at 3000 rpm for 15 minutes at 0° C. The supernatant plus three parts of chloroform were shaken vigorously for five minutes and the resulting two layers were separated. The epiphasic layer was removed, centrifuged at 15,000 rpm for 15 minutes at 0° C, concentrated by evaporation at 80° C, and chromatographed. In other cases, the larvae were extracted with 70% ethanol or 10% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 15 minutes at 0° C. The 70% ethanol extract was concentrated at 80° C and chromatographed. The 10% TCA extract was washed three times with ether at a volume ration of 1:1, concentrated, and chromatographed. Sometimes fresh starting material was squashed directly on the filter paper (Obika 1963) which was then developed. Both extraction and chromatography were carried out in the dark or in a dark room under a photographic "safe light", in order to prevent photolysis of the pteridines. In certain experiments which utilized photolysis, samples were exposed to normal room light for approximately 24 hours.

Whatman # 1 and Whatman 3 mm filter paper were utilized for chromatography and n-propanol-1% ammonia (2:1), n-butanol-acetic acid-water (4:1:1), isopropanol-2% ammonium acetate (1:1), 5% acetic acid, 3% ammonium chloride, and 5% urea were employed as solvent systems.

Chromatograms were developed ascendingly in either one or two dimensions. When the chromatograms were exposed to ultra-violet light, pteridines on them fluoresced brightly while the purines appeared-as

dark absorption spots. An ultra-violet light, equipped with a filter which allowed peak emission at approximately 365 m μ , was used to induce pteridine fluorescence; and an ultra-violet light, equipped with a filter which allowed peak emission at approximately 254 m μ , was used to disclose purine absorption. Pteridines and purines were removed from the filter paper by cutting the paper into narrow strips (approximately one-half inch wide) and eluting descendingly with glass-distilled water in an atmosphere saturated with water vapor.

Purines and pteridines were identified by their characteristic ultraviolet absorption spectra and by matching R_f values of unknown compounds to authentic compounds.¹ R_f values were compared for the six solvent systems. Absorption spectra were determined with a Beckman D. U. Spectrophotometer in 0.1 N HCl or 0.1 N NaOH.

The use of xanthine dehydrogenase (Nutritional Biochemicals Corporation), which converts 2-amino-4-hydroxypteridine to isoxanthopterin (Forrest et al. 1956), was utilized as an additional identification procedure.

Purine concentrations were calculated by the use of the Lambert-Beer Law. Pteridine concentrations were determined by measuring the intensity of fluorescence of pteridines dissolved in McIlvaine's buffer, at a pH of 5. These fluorescence intensities were then compared to those of standard pteridine curves, which were prepared by dissolving known amounts of pteridines in the above buffer system and then reading

¹ Authentic pteridines were kindly supplied by Professor T. Hama, Biological Laboratory, Keio University, Yokohama-Hiyoshi, and by Dr. H. S. Forrest, Department of Zoology, University of Texas, Austin, Texas.

the degree of fluorescence at various concentrations. Standard curves of three pteridines, AHP-6-COOH, isoxanthopterin, and 2-amino-4-hydroxypteridine, were prepared (Figures 3, 4 and 5). The degree of fluorescence was determined with a Beckman D. U. Spectrophotometer equipped with a fluorescence attachment. Fluorescence was read at 450 mμ.

Carbon-14-labeled purines and pteridines were dissolved in 0.1 N HCl and water respectively. Aliquots of the various compounds were placed in a scintillation solvent consisting of naphthalene (60g), 2,5-diphenyloxazole (4g), 1,4-bis-2-(5-phenyloxazolyl)-benzene (0.2g), methanol (100 ml.), ethylene glycol (20 ml.), and p-dioxane (to make one liter) (Bray 1960) and counted in a liquid scintillation counter (Packard Tri-Carb, Model 314 EX). Quenching was determined by use of benzoic acid-¹⁴C as an internal standard (Kerr et al. 1957).

Figure 3. Standard curve of AHP-6-COOH.

Figure 4. Standard curve of isoxanthopterin.

Figure 5. Standard curve of 2-amino-4-hydroxypteridine.

EXPERIMENTS AND RESULTS

Identification of pteridines

Five pteridines were extracted and identified from the integument of Rana sylvatica larvae. Isoxanthopterin, biopterin, sepiapterin, and 2-amino-4-hydroxypteridine were identified by matching Rf values of unknown pteridines to authentic pteridines. A typical pteridine chromatograph pattern is illustrated in Figure 6. The highly photolabile pteridine, Hynobius-blue, when exposed to U. V. light, does not fluoresce, but is rapidly converted to 2-amino-4-hydroxypteridine-6-carboxylic acid (AHP-6-COOH) (Obika 1963), which fluoresces a bright blue when exposed to U. V. light. Hynobius-blue was identified by observing the rapid appearance of the blue color, and then the decomposition product was identified by matching the Rf value to AHP-6-COOH. 2-amino-4-hydroxypteridine was identified by conversion to isoxanthopterin with xanthine dehydrogenase dissolved in a phosphate buffer of pH 7.48, (Forrest et al. 1956). Two pteridines, isoxanthopterin and AHP-6-COOH, were identified by their characteristic absorption spectra. The former compound had absorption maxima at 254 and 339 m μ in 0.1 N NaOH and the latter compound had absorption maxima at 263 and 362 m μ in 0.1 N NaOH. These absorption maxima are in close agreement with those of known compounds. Biopterin and sepiapterin were also identified by the fact that when exposed to light they decompose to AHP-6-COOH.

Figure 6. Schematic two dimensional chromatogram showing typical pteridine pattern of pteridines extracted from Rana sylvatica larvae. Abbreviations: SP = sepiapterin; HB = Hynobius-blue; BP = biopterin; AHP = 2 amino-4-hydroxy-pteridine; IXP = isoxanthopterin.

Identification of purines

Three purines, adenine, hypoxanthine, and guanine, were extracted from the larval integument and identified by matching their Rf values to those of known purines. A typical purine chromatograph pattern is illustrated in Figure 7. These purines were also identified by their characteristic absorption spectra (Figure 8).

Incorporation of glycine-(u.l.)-¹⁴C into pteridines and purines

Normal larvae were injected with 2×10^{-3} ml. of glycine-(u.l.)-¹⁴C. The amount of activity injected was 5×10^4 cpm / 10^{-3} ml. Injections were started at stage 23 - 24 (Pollister and Moore 1937) and repeated twice. A total of three injections was administered at four-day intervals.

Twenty-five days after the first injection, pteridines and purines were extracted from 100 tadpoles according to Awapara's technique. The residue was then extracted with 10% TCA. Extracts were chromatographed in a solvent system of n-propanol-1% ammonia (2:1). The compounds were marked on the chromatograms and eluted. The TCA extracts contained only one pteridine in significant quantities, isoxanthopterin, which was pooled with the isoxanthopterin from the original extract. Purines from the TCA extract were not pooled with those from the first extract.

Pteridines were exposed to light in order to decompose sepiapterin, biopterin, and Hynobius-blue to AHP-6-COOH. Photolysis was performed because AHP-6-COOH can readily be separated from the purines and because it is difficult to prevent photolysis from occurring during purification procedures. 2-amino-4-hydroxypteridine was converted to isoxanthopterin by treating it with xanthine dehydrogenase. All pteridines were purified

Figure 7. Schematic two dimensional chromatogram showing typical purine pattern of purines extracted from Rana sylvatica larvae. Abbreviations: GA = guanine; HY = hypoxanthine; AD = adenine.

Figure 8. Spectra of adenine, hypoxanthine and guanine in 0.1 N HCl.

Compounds were extracted from Rana sylvatica larvae.

Key: hypoxanthine; _____ adenine; -----
guanine.

as AHP-6-COOH or isoxanthopterin by rechromatographing at least twice. The decomposition product (AHP-6-COOH) from biopterin and Hynobius-blue photolysis was pooled because complete separation of these two compounds was difficult to obtain. AHP-6-COOH formed from sepiapterin photolysis was kept separate. Purines were purified by eluting and rechromatographing at least twice.

Purity of purines was determined by spectral data (Figure 8). Spectra of pteridines were not obtained because they were not concentrated enough for determination of absorption spectra. Radioactivity of compounds was determined with the liquid scintillation counter, and radioactivity was found located in the purines and pteridines.

The data (illustrated in Table 1) clearly show that glycine-(u.l.)⁻¹⁴C was incorporated, in significant quantities, into three purines (adenine, hypoxanthine, and guanine) and into at least four pteridines (biopterin and/or Hynobius-blue, sepiapterin, isoxanthopterin, and 2-amino-4-hydroxypteridine). In this experiment the decomposition product (AHP-6-COOH) from biopterin and Hynobius-blue photolysis was not separated. Therefore it was not possible to determine whether either one or both had incorporated the labeled precursor.

AHP-6-COOH, formed from biopterin and Hynobius-blue photolysis, had a specific activity of 6.67×10^4 cpm / μ mole, while the specific activities of isoxanthopterin and sepiapterin were lower. Sepiapterin had the lowest and 2-amino-4-hydroxypteridine had the highest specific activity of all pteridines extracted. Guanine and hypoxanthine had essentially the same specific activity, while the specific activity of adenine was considerably lower (Table 1).

<u>Compound</u>	<u>Micro moles extracted</u>	<u>Specific Activity cpm / μ mole</u>
Guanine	1.60×10^{-1}	1.02×10^4
Adenine	2.36×10^{-1}	3.43×10^3
Hypoxanthine	3.06×10^{-1}	9.80×10^3
Isoxanthopterin	3.20×10^{-2}	4.25×10^4
Biopterin plus Hynobius-blue *	1.80×10^{-2}	6.67×10^4
Seplapterin *	3.20×10^{-2}	6.25×10^3
Unknown purple **	$.34 \times 10^{-2}$	8.82×10^4
2-amino-4-hydroxy- pteridine **	$.32 \times 10^{-2}$	2.68×10^5

Table 1. Data showing purine and pteridine concentrations and specific activities in Rana sylvatica larvae injected with glycine-(u.l.)- ^{14}C .

* Calculations are based on AHP-6-COOH standard curve.

** Calculation is based on isoxanthopterin standard curve.

A purple fluorescing substance was extracted and isolated from the larvae in this experiment. This substance had an Rf value of 0.75 in n-propanol-1% ammonia and it incorporated glycine-(u.l.)- ^{14}C . The calculations of the concentration and specific activity of this compound were based upon the isoxanthopterin standard curve; because its fluorescence appears to be the same color (purple) as the fluorescence of isoxanthopterin. Its specific activity was 8.82×10^4 cpm / μ mole (Table 1).

The purpose of this experiment was to determine if radioactive glycine would be incorporated into purines and pteridines by Rana sylvatica larvae, and the data (Table 1) clearly show this to be the case. Thus it seemed natural, as a next step, to test the hypothesis that purines are converted to pteridines.

Conversion of purines to pteridines

Seventy-five well-developed and completely hypophysioprivic larvae were injected with 2×10^{-3} ml. of glycine-(u.l.)- ^{14}C . The amount of activity injected was 5×10^4 cpm / 10^{-3} ml. Injections were started at stage 23 - 24 (Pollister and Moore 1937) and repeated twice at four-day intervals. A total of three injections was administered.

Two weeks after the first isotope injection, these larvae were divided into two equal groups. The larvae were approximately 2 cm. in length at this time. One group received injections of intermedin every other day for a total of ten treatments. The volume injected each time was 6×10^{-3} ml.

The untreated hypophysioprivic larvae were silvery in color; and microscopic observation revealed that the guanophores were expanded, while the melanophores were contracted and few in number. The reverse situation was found in the hormone-treated hypophysioprivic larvae, which looked like normal tadpoles with expanded melanophores and contracted guanophores. Complete disappearance of guanophores, as reported by Bagnara (1958), was never obtained. Xanthophores were expanded and appeared in equal numbers in both groups.

Two days after the last hormone treatment, the two groups were adjusted to equal wet weights and homogenized in 70% ethanol, which extracted the pteridines and purines at the same time. Extracts were chromatographed in a solvent system of n-propanol-1% ammonia (2:1). Excellent separation of the various compounds was obtained in this experiment by developing full sheets of 3-mm filter paper ascendingly for 24 hours. The compounds were marked on the chromatogram and eluted.

Equal volumes of the above extract from both groups were placed on separate sheets of filter paper, which were developed two-dimensionally using 5% acetic acid in one direction and n-propanol-1% ammonia in the other. A schematic chromatogram of this type is presented in Figure 6. When these chromatograms were exposed to U. V. light, the difference in pteridine concentrations was visually detected. Photographs of these chromatograms, exposed to U. V. light, were taken to illustrate pteridine deposition after intermedin treatments (Figure 9). In order to make these photographs, ultra-violet light of the activating system had to be eliminated. This was accomplished by the use of an

Eastman Kodak 2 B Filter. Pteridine concentration is proportional to the degree of fluorescence.

By comparing the control group (photograph A, Figure 9) to the intermedin-treated group (Photograph B, Figure 9), it is obvious that intermedin treatments have increased the concentration of all five pteridines (sepiapterin, Hynobius-blue, biopterin, isoxanthopterin, and 2-amino-4-hydroxypteridine).

Pteridines were exposed to light in order to decompose sepiapterin, biopterin, and Hynobius-blue to AHP-6-COOH. The decomposition product (AHP-6-COOH) from each of these three pteridines was kept separate so that the concentration and amount of isotope incorporation of each compound could be determined. 2-amino-4-hydroxypteridine was not converted to isoxanthopterin in this experiment. Because of the excellent separation, it was isolated without this step. All pteridines were purified as isoxanthopterin, 2-amino-4-hydroxypteridine, or AHP-6-COOH by eluting and rechromatographing twice. Purines were purified by eluting and rechromatographing twice.

Purity and concentration of the purines were determined from spectral data. Concentration of the pteridines was determined from standard curves. Radioactivity of the compounds was then determined in the liquid scintillation counter.

Figure 9. Photographs of two-dimensional chromatograms, exposed to ultra-violet light, demonstrating relative pteridine concentrations. A, hypophysioprivic Rana sylvatica larvae used as controls. B, intermedin-treated hypophysioprivic Rana sylvatica larvae; note greater fluorescence in intermedin-treated group.

Abbreviations: I = isoxanthopterin; S = sepiapterin; H = Hynobius-blue; A = 2-amino-4-hydroxypteridine; B = bipterin.

A

B

Data from this experiment (illustrated in Table 2) demonstrate that intermedin treatments caused a twofold increase in pteridine concentrations in hypophysioprivic Rana sylvatica larvae. It is interesting that all five pteridines (sepiapterin, Hynobius-blue, biopterin, isoxanthopterin, and 2-amino-4-hydroxypteridine) have this twofold concentration increase. Thus, intermedin appears to be affecting the deposition of the five pteridines in the same way. Intermedin treatments caused a decrease in the concentrations of adenine and hypoxanthine, while the concentration of guanine remained essentially the same. This differs from the results reported by Bagnara and Neidleman (1958), in that these authors found a decrease in guanine after intermedin treatments in hypophysioprivic Rana sylvatica larvae.

The specific activities of four pteridines (sepiapterin, Hynobius-blue, biopterin, and isoxanthopterin) decreased after hypophysioprivic larvae were treated with intermedin, while one pteridine (2-amino-4-hydroxypteridine) had a slight increase in specific activity. Intermedin caused a small increase in the specific activities of two purines (adenine and guanine), while one purine (hypoxanthine) was essentially unchanged. Data are tabulated in Table 2.

<u>Compound</u>	<u>Animal Group</u>	<u>Micro moles extracted</u>	<u>Specific activity (cpm/μ mole)</u>
Isoxanthopterin	Intermedin	2.64×10^{-2}	1.62×10^4
	Control	1.44×10^{-2}	2.50×10^4
Biopterin *	Intermedin	2.68×10^{-2}	1.72×10^4
	Control	1.56×10^{-2}	3.28×10^4
2-amino-4-hydroxypteridine	Intermedin	1.25×10^{-2}	2.76×10^4
	Control	$.66 \times 10^{-2}$	2.55×10^4
Hynobius-blue *	Intermedin	2.80×10^{-2}	1.74×10^4
	Control	1.44×10^{-2}	2.72×10^4
Sepiapterin *	Intermedin	$.84 \times 10^{-2}$	1.48×10^4
	Control	$.36 \times 10^{-2}$	2.44×10^4
Adenine	Intermedin	.63	1.76×10^3
	Control	1.06	1.31×10^3
Guanine	Intermedin	.23	7.97×10^3
	Control	.22	6.65×10^3
Hypoxanthine	Intermedin	.44	5.00×10^3
	Control	.60	5.04×10^3

Table 2. Data showing the relationships between concentration and specific activity of pteridines and purines extracted from hypophysioprivic Rana sylvatica larvae (control group) and intermedin injected hypophysioprivic Rana sylvatica larvae (intermedin group).

* Calculations are based on AHP-6-COOH standard curve.

DISCUSSION

In order to understand the metabolic pathways of pteridine metabolism in a given organism, the identity of all prominent pteridines present in this organism must first be determined. In this investigation it has been demonstrated that the pteridine complement of Rana sylvatica larvae consists of at least five pteridines: biopterin, Hynobius-blue, sepiapterin, isoxanthopterin, and 2-amino-4-hydroxypteridine. This pattern resembles that of the closely related species, Rana japonica. The only exception is that the latter contains 2-amino-4-hydroxypteridine-6-carboxylic acid (Hama 1963). Since this compound is a decomposition product of sepiapterin (Viscontini and Möhlmann 1959), Hynobius-blue (Obika 1963), and biopterin (Hama 1953), its presence in Rana japonica larvae could be due to photolysis of labile pteridines during isolation and identification procedures. However, this difference can be explained by another hypothesis, which will be discussed later.

Amphibian integument heavily populated with guanophores contains large concentrations of free purines, while integument slightly populated with these pigment cells has only small amounts of free purines. Three purines, adenine, guanine, and hypoxanthine, have been identified from various amphibians (Bagnara and Neidleman 1958; Bagnara and Stackhouse 1961). These three purines were also isolated from Rana sylvatica larvae.

Glycine-2-¹⁴C is known to be incorporated into the pteridine frame of leucopterin in Pieris (Weygand and Waldschmidt 1955; Weygand et al. 1961). Other pteridines, drosopterin, isoxanthopterin, sepiapterin, and the high blues (probably 2-amino-4-hydroxypteridine and biopterin), have been labeled by the feeding of glycine-2-¹⁴C to Drosophila larvae (Brenner-Holzach and Leuthardt 1961). Results presented in the present investigation show that glycine-(u.l.)-¹⁴C is also incorporated into the five pteridines isolated and identified from Rana sylvatica larvae. Because isoxanthopterin and 2-amino-4-hydroxypteridine do not possess a side chain, it was assumed that the labeling occurred in the pteridine nucleus. When sepiapterin, Hynobius-blue, and biopterin were decomposed to AHP-6-COOH, some activity could have been lost, but since the decomposition product still contained significant amounts of radioactivity, the labeling must have been on the pteridine frame.

A bright fluorescing purple substance, which had an Rf value of 0.75 in n-propanol-1% ammonia, was found in one experiment. It is not known whether this compound is a pteridine. However, radioactive glycine was incorporated into it in significant amounts. Experiments to characterize this compound are anticipated.

Glycine is known to be incorporated into uric acid of pigeon excreta (Buchanan et al. 1948), and the biosynthetic pathways of purines have been well established (Buchanan and Hartman 1959). The free purines, adenine, guanine and hypoxanthine, which were isolated and identified from Rana sylvatica larvae, also incorporate glycine-(u.l.)-¹⁴C.

Once it was established that the purines and pteridines incorporated the radioactive glycine, the next step was to test the hypothesis that purines are converted to pteridines. In this experiment intermedin was used as a trigger to initiate pteridine synthesis in hypophysioprivic Rana sylvatica larvae. The results demonstrate the biosynthetic relationship of purines to pteridines as well as the concentration change of the individual pteridines and purines.

Bagnara and Neidleman (1958) demonstrated clearly that the guanine concentration decreased when intermedin was injected into hypophysioprivic Rana sylvatica larvae. This was also shown to be partially true for Rana pipiens larvae. The present investigation demonstrates that, after ten intermedin treatments administered every other day, concentrations of hypoxanthine and adenine decrease, while that of guanine remains essentially the same. This is in contrast with the results of Bagnara and Neidleman (1958). However, it should be recalled that these authors extracted purines from only the dorsal skin. Moreover, with the northern race of Rana sylvatica, which they used, intermedin injections caused complete disappearance of guanophores. In the present experiments, purines were extracted from homogenates of entire larvae of a southern race or subspecies of Rana sylvatica. It should be pointed out also that, after intermedin injections, complete disappearance of guanophores was not obtained. Possibly the absence of guanine reduction could be due to utilization of a different race of Rana sylvatica, or it could be the result of extracting whole larvae. The fact that guanine did not decrease in this investigation,

as it did in the experiment of Bagnara and Neidleman, leads one to postulate that guanine leaves the skin as a result of hormone treatments and that it is pooled somewhere else in the animal.

The fact that intermedin causes pteridine deposition has been demonstrated previously for hypophysioprivic larvae of Rana sylvatica and Rana pipiens (Bagnara and Neidleman 1958; Bagnara 1961). Evidence presented in this investigation supports the observations for the former species. Moreover, it also shows clearly that intermedin causes approximately a twofold increase in the concentration of each of the five pteridines present.

Considering the specific activities of the pteridines extracted from hypophysioprivic Rana sylvatica larvae (control group, Table 2), the following scheme for the biosynthetic interconversion of the pteridines has been proposed (Figure 10).

Since biopterin had a specific activity considerably higher than the other pteridines in the control group, it is considered to be the first pteridine synthesized from the labeled glycine precursor. This is in agreement with the studies of Obika (1963), in that this author demonstrated biopterin to be the first pteridine to appear during ontogenetic development.

Observations from studies concerning the general distribution of pteridines in amphibians (Hama 1963; Obika 1963) will help demonstrate the feasibility of this scheme. For example, when large concentrations of sepiapterin are present, isoxanthopterin is usually found in small quantities; and the latter pteridine is found in small

Figure 10. Proposed scheme for biosynthesis of pteridines in hypophysioprivic *Rana sylvatica* larvae.

amounts when 2-amino-4-hydroxypteridine is present in large concentrations (Obika and Hama 1960). Sepiapterin has been found to occur only in the presence of Hynobius-blue, and biopterin seems to be associated with these two compounds. The proposed scheme readily accounts for these generalizations, in that when the synthesis of sepiapterin is favored, the pathways leading to the synthesis of isoxanthopterin would be slowed down because Hynobius-blue, a key intermediate, would be converted to sepiapterin, thus reducing the substrate for the synthesis of 2-amino-4-hydroxypteridine, which gives rise to isoxanthopterin. 2-amino-4-hydroxypteridine is converted to isoxanthopterin by xanthine dehydrogenase (Forrest et al. 1956). Thus, when xanthine dehydrogenase is present in significant amounts, 2-amino-4-hydroxypteridine would be rapidly converted to isoxanthopterin. Therefore, isoxanthopterin would be accumulated at the expense of a decrease in 2-amino-4-hydroxypteridine concentration. If the enzyme was not present or present in an insignificant amount, the converse would be true. It is also obvious from the proposed scheme that sepiapterin would be produced only when the precursors, biopterin and Hynobius-blue, are present.

Next to biopterin, Hynobius-blue has the highest specific activity. Thus it is considered to be derived from biopterin in the proposed scheme (Figure 10). If this is the case, the structure of Hynobius-blue could be the same as tetrahydrobiopterin or tetrahydrosepiapterin. There are supporting reasons for this reaction from *in vitro* studies. Taira (1961) demonstrated that dihydro-folic acid reductase from chicken liver converted sepiapterin to tetrahydrosepiapterin, which upon auto-oxidation was converted to a completely

dehydrated form having a blue fluorescence, and the latter compound was converted to AHP-6-COOH by photo-decomposition or by oxidation with potassium permanganate.

Taira (1961) also observed that the enzymatic mixture contained a significant quantity of isoxanthopterin. He demonstrated that the chicken liver extract was converting 2-amino-4-hydroxypteridine to isoxanthopterin. This fact led the above author to assume that 2-amino-4-hydroxypteridine was derived from tetrahydrosepiapterin and converted enzymatically to isoxanthopterin.

Therefore, the metabolic sequence for pteridines proposed (Figure 10) appears to be occurring in in-vitro systems as well as in hypophysioprivic Rana sylvatica larvae. These biosynthetic relationships may be true for amphibians in general.

The difference in pteridine sets found in Rana japonica larvae and Rana sylvatica larvae can possibly be explained from the proposed scheme for pteridine interconversions (Figure 10). AHP-6-COOH may be an intermediate in the conversion of Hynobius-blue to 2-amino-4-hydroxypteridine. Thus Rana sylvatica larvae could have the ability rapidly to convert this intermediate to 2-amino-4-hydroxypteridine and subsequently to isoxanthopterin, while Rana japonica larvae could accumulate the intermediate because of the lack of necessary enzymatic activity, such as that of xanthine dehydrogenase.

However, when the data (Table 2) are considered for the intermedin-injected hypophysioprivic Rana sylvatica larvae (intermedin group), it is not understood why 2-amino-4-hydroxypteridine showed an increase in specific activity, while the other four pteridines showed

decreased specific activities. This difference in specific activities indicates the existence of pathways other than those proposed here (Figure 10). Intermedin appears to be initiating these alternate pathways by some unknown mechanism or mechanisms. Therefore the following additional pathways have been postulated (Figure 11) in an attempt to account for the differences in the specific activities of the pteridines. In this scheme, 2-amino-4-hydroxypteridine and biopterin would be synthesized from a precursor that has a high specific activity. The specific activity of the latter compound would be decreased by dilution, while the activity of the former compound would remain high because its conversion to isoxanthopterin appears to be blocked by intermedin treatments. Isoxanthopterin would be synthesized from another precursor, such as Hynobius-blue.

Guanylic acid has been indicated as a precursor of pteridines (Weygand et al. 1961), and labeled guanine is converted to at least two pteridines in the skin of Rana catesbeiana larvae (biopterin and AHP-6-COOH) (Levy 1964). The latter compound is probably a decomposition product of the photolabile pteridines present. It is apparent from the work of other investigators (Ziegler-Günder 1956; Aaronson and Rodriguiz 1958; Brenner-Holzack and Leuthardt 1961; Weygand et al. 1961; Levy 1964) that the biosynthesis of purines and pteridines is closely related. This investigation demonstrates (Table 2) that the concentration of two purines (adenine and hypoxanthine) decreases and one purine (guanine) concentration remains unchanged in hypophysioprivic Rana sylvatica larvae after treatments with intermedin, while the pteridine concentrations increase. Therefore, in order to indicate the

Figure 11. Proposed scheme for pteridine biosynthesis in intermedin treated hypophysioprivic Rana sylvatica larvae.

relationship between purines and pteridines extracted from Rana sylvatica larvae, the following scheme (Figure 12) is proposed.

In this scheme the purines are synthesized via known pathways (Buchanan and Hartman 1959) and they may be interconverted (for literature see Moat and Friedman 1960). The pteridines are synthesized from the key intermediate, guanylic acid, as indicated by Weygand et al. (1961). The lack of intermedin in hypophysioprivic larvae inhibits pteridine synthesis after guanylic acid; thus increasing the synthesis of purines. However, once intermedin was replenished, pteridine synthesis was increased; thus probably depleting the concentration of precursors for purine synthesis and subsequently causing the concentration of purines to decrease. Considering the specific activities of the purines (Table 2), it does not appear that they are being converted to pteridines in Rana sylvatica larvae because their specific activities are much lower than those of the pteridines.

Figure 12. Scheme postulated for the biosynthesis of pteridines and their relationship to purines in hypophysioprivic Rana sylvatica larvae.

SUMMARY

1. Five pteridines (sepiapterin, biopterin, Hynobius-blue, isoxanthopterin, and 2-amino-4-hydroxypteridine) were extracted from homogenized Rana sylvatica larvae and identified by matching Rf values of unknown to known compounds, by photolytic experiments, by enzymatic reactions, and by their absorption spectra.

2. Three purines (adenine, hypoxanthine, and guanine) were extracted from homogenized Rana sylvatica larvae and identified by their absorption spectra and by the matching of Rf values to known compounds.

3. 2-amino-4-hydroxypteridine-6 carboxylic acid was identified as a decomposition product of three pteridines extracted from Rana sylvatica larvae: sepiapterin, biopterin, and Hynobius-blue.

4. Glycine-(u.l.)¹⁴C, when injected into Rana sylvatica larvae, was incorporated into the five pteridines and three purines. The labeled carbons were located in the pteridine frame of at least isoxanthopterin and 2-amino-4-hydroxypteridine and they were probably located in the pteridine frame of sepiapterin, biopterin, and Hynobius-blue.

5. Hypophysioprivic Rana sylvatica larvae showed a twofold concentration increase of all five pteridines after intermedin treatments. After hormone treatments, adenine and hypoxanthine concentrations decreased, while guanine remained at essentially the same concentration.

6. A scheme for the biosynthetic interconversion of the pteridines in hypophysioprivic Rana sylvatica larvae has been proposed.

These pathways appear to be altered after intermedin treatments, and these alternate pathways have been indicated.

7. Hynobius-blue is proposed to have the same structure as tetrahydrobiopterin or tetrahydrosepiapteridine.

8. The free purines do not appear to be converted to integumental pteridines in hypophysioprivic Rana sylvatica larvae.

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