

PTERIDINES, PURINES AND CAROTENIDS  
IN AMPHIBIAN PIGMENTATION

by

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A Thesis Submitted to the Faculty of the  
DEPARTMENT OF ZOOLOGY  
In Partial Fulfillment of the Requirements  
For the Degree of  
MASTER OF SCIENCE  
In the Graduate College  
THE UNIVERSITY OF ARIZONA

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## ACKNOWLEDGEMENT

Gratitude is expressed by the author to Dr. Joseph T. Bagnara for his critical advice and encouragement during the preparation of this thesis.

## TABLE OF CONTENTS

	Page
INTRODUCTION . . . . .	1
MATERIALS AND METHODS . . . . .	5
EXPERIMENTS . . . . .	9
Pteridines, Dorsal and Ventral Distribution . . . . .	9
Pteridines and CTH Relationships . . . . .	12
<u>Rana pipiens</u> and <u>Hyla arenicolor</u> . . . . .	14
<u>Rana sylvatica</u> . . . . .	15
<u>Ambystoma tigrinum</u> . . . . .	20
Background effects on Pteridines and Purines . . . . .	22
Anurans . . . . .	22
Urodeles . . . . .	23
Purines and CTH Relationship . . . . .	25
Carotenoids, Dorsal and Ventral Distribution . . . . .	29
Carotenoid and CTH Relationship . . . . .	34
DISCUSSION . . . . .	36
SUMMARY . . . . .	43
LITERATURE CITED . . . . .	45

## LIST OF FIGURES

		Page
1	Schematic chromatogram comparing dorsal and ventral skin extracts of <u>Rana pipiens</u> , <u>Rana catesbeiana</u> and <u>Hyla arenicolor</u> . . . . .	10
2	Schematic chromatogram comparing extracts of adult normal and albino <u>Rana sphenoccephala</u> . . . . .	11
3	Schematic chromatogram comparing extracts of <u>Rana pipiens</u> dorsal black spots to the surrounding lighter areas . . . . .	13
4	Schematic chromatogram comparing dorsal skin extracts of normal, CTH-treated-hypophysioprivic and hypophysioprivic <u>Rana pipiens</u> and <u>Hyla arenicolor</u> larvae . . . . .	16
5	Schematic chromatogram comparing dorsal skin extracts of normal CTH-treated and untreated normal <u>Rana pipiens</u> larvae . . . . .	17
6	Schematic chromatogram comparing dorsal skin extracts of normal, CTH-treated hypophysioprivic and hypophysioprivic <u>Rana sylvatica</u> larvae . . . . .	19
7	Schematic chromatogram comparing dorsal skin extracts of normal and hypophysioprivic <u>Ambystoma tigrinum</u> larvae . . . . .	21
8	Schematic chromatogram comparing dorsal skin pteridine extracts of various Anuran larvae that developed on black or white backgrounds . . . . .	24
9	Molecular structures of some purines and pteridines showing structural similarities of the two groups . . . . .	26
10	Comparison of relative total integumental pteridine and purine concentrations in normal and hypophysioprivic <u>Rana pipiens</u> , <u>Hyla arenicolor</u> and <u>Rana sylvatica</u> larvae . . . . .	27
11	Schematic chromatogram comparing dorsal skin purine extracts of normal, CTH-treated-hypophysioprivic and hypophysioprivic anuran larvae . . . . .	28

12	Spectrum of $\beta$ -carotene extract in hexane . . . . .	31
13	Total carotenoid comparisons of dorsal, ventral and yellow of legs of <u>Rana pipiens</u> , <u>Rana catesbeiana</u> and <u>Hyla arenicolor</u> . . . . .	32
14	Total carotenoid concentrations of normal, CTH- treated hypophysioprivic and hypophysioprivic <u>Rana pipiens</u> larvae . . . . .	35

## INTRODUCTION

Smith and Allen, working independently in 1916, showed that an agent responsible for color changes in amphibians is elaborated by the pituitary gland. Various investigators have since shown that this hormonal agent is associated with the intermediate lobe of the hypophysis (Atwell '19; Swingle '21; Smith and Smith '23; Allen '30; Atwell '36). As a result the hormone was named "intermedin" (Zondek and Krohn '32) and this term has prevailed over the years. For many years investigations concerning the function of this hormone have been largely restricted to melanophores and for this reason it has frequently been called melanocyte or melanophore stimulating hormone (MSH). Actually, this principle (MSH or "intermedin") has a much broader function for it also acts on other pigmentary systems. Bagnara ('58) showed that this hormone causes guanophore contraction in anuran larvae. Later, it was shown by Bagnara and Neidleman ('58) that the hormone markedly inhibits the synthesis of guanine in Rana sylvatica larvae. In the course of their investigations of integumental guanine content, these authors showed that this pigmentary hormone is active in stimulating the formation of

pteridines in the skin of Rana pipiens larvae. Therefore, this hormone causes melanophore expansion, stimulates melanin synthesis (Bagnara '58), causes guanophore contraction, inhibits guanine synthesis and stimulates the formation of pteridines. Consistent with these discoveries the hormone has been given the broader designation of chromatotrophic hormone (CTH) (Bagnara '58). The term CTH seems preferable and will be used in this paper.

Pteridines are present in amphibian skin (Busnel '42; Hama '53; Günder '53 and '54; and Bagnara '61), and are generally more prevalent in the darkly pigmented dorsal integument than in the lightly pigmented ventral skin. As a result of these facts, several investigators (Busnel '42; Ziegler-Günder '56; Obika and Hama '60) have suggested that pteridines are somehow involved with dark pigmentation, especially with melanin. This observation, plus the fact that CTH influences pteridine deposition, indicates that a relationship probably exists between pteridines, the hypophysis and pigmentation (Bagnara '61).

In urodeles, the black pigmented integument appears to be void of pteridines; instead, in both anurans and urodeles pteridines appear to be associated with yellow pigmentation. Bagnara ('61) has shown that dorsal yellow spots of Ambystoma maculatum abound in pteridines while adjacent black integument contains only small quantities or none. Bagnara ('61) also demonstrated that pteridines are associated with the yellow pigmented flanks and ventral leg surfaces of Hyla arenicolor



and Rana sylvatica. These areas are pigmented almost exclusively by xanthophores. Pteridines have also been shown to prevail in yellow pigmented areas of goldfish (Hama, Matsumoto, and Obika '60).

The observations of Bagnara ('61) on natural Rana pipiens albino larvae indicate that pteridines are not associated with black pigmentation. Albino tadpoles have expanded melanophores but little or no melanin is present within them. The same pteridine concentrations are found in albino larvae as in normal ones. Moreover, the skin of albino larvae is yellowish.

Although the chromatotrophic hormone has a pronounced effect on melanophores, guanophores and pteridines, it has little or no apparent effect on xanthophores. Neither xanthophores nor the yellow pigments, presumably carotenoids (Parker '48 and Fox '53), seem to be affected markedly by the hypophysis (Bagnara '59). These results, considered in the light of the association between pteridines and yellow pigmentation, make it difficult to understand the relationship between pteridines, pigmentation and the hypophysis. The fact that the role of pteridines in the integument of vertebrates is still unknown (Bagnara '61) adds to the difficulty.

Before any clarification of the function of pteridines in amphibian skin is possible, some of the problems concerning the relationship between pteridines and the pigments should be investigated further. For instance, although it is known that

CTH influences integumental pteridines and purines in anurans, this relationship has not been examined in urodeles. Another problem involves the amount of pteridine deposition occurring after CTH injections. Previous workers have treated hypophysioprivic anuran larvae with CTH over short periods of time. During this period the restoration of pteridines never reached the normal level (Bagnara and Neidleman '58; Bagnara '61). It would be interesting to know whether the level of pteridines would reach normal levels if CTH injections were continued over a longer period. Since carotenoids are yellow pigments and pteridines appear to be associated with this type of pigmentation, it would be of interest to re-examine the relationship between carotenoids and CTH using better quantitative techniques. Moreover, similar precision should apply to the quantitation of pteridines and carotenoids in skin where both appear together.

Although pteridines do not seem to be associated with guanophores and purines (Bagnara '61), it may be possible that a reciprocal relationship exists, in the integument, between pteridines and purines. In hypophysioprivic larvae guanophores are expanded and the guanine content is high while the pteridine concentration is low. This may imply a mechanism of inter-conversion between these compounds.

The purpose of this investigation is to clarify some of the above problems with the hope of adding to our understanding of the relationship between pteridines, pituitary gland, and pigmentation.

## MATERIALS AND METHODS

Various larval and adult amphibians were used in this investigation. Adult Rana sylvatica, Rana sphenoccephala, Ambystoma tigrinum and Ambystoma maculatum were supplied by collectors in the eastern part of the United States. Adult Rana catesbeiana and some of the adult Rana pipiens were acquired from a biological supply house in Wisconsin. The rest of the Rana pipiens, along with Hyla arenicolor, were collected in the vicinity of Tucson, Arizona. Adult Amphiuma tridactylum were collected near New Orleans, Louisiana. Taricha torosa were collected in the vicinity of Stanford University, California. Pleurodeles waltlii larvae were obtained from a breeding colony maintained in the laboratory of the University of Arizona.

Eggs from some of the above mentioned forms were obtained either by induced ovulation or were collected from their natural environment.

Boiled lettuce was the usual diet of the tadpoles and the salamander larvae were fed brine shrimp, white worms (Enchytraeus sp.) or small earth worms.

Hypophysioprivic tadpoles and salamander larvae were obtained by removing the hypophyseal placode, in the early

tailbud stage (Allen '16; Smith '16). Operations were considered successful if the larvae became light in color.

Some of these hypophysioprivic larvae received injections of hormones containing chromatophore stimulating properties. A commercial CTH<sup>1</sup> preparation and pitressin were selected for injection. The latter was chosen because of its high contamination with CTH. These hormones were potentiated by treatment with alkali (Bagnara '57; '58). Hormone injections were made with a fine needled (B.D. 26) hypodermic syringe. All injections were made by inserting the needle through the large tail muscles into the body cavity. The injected volume was .05 ml. in all experiments.

At the end of each experiment the animals were sacrificed and skin from the dorsal surface was removed with watchmaker's forceps. The skin was dried in a vacuum desiccator for approximately 24 hours and subsequently weighed.

Tissue samples to be analyzed for pteridines and purines were extracted with 1 N NH<sub>4</sub>OH for approximately 24 hours. The tissue was separated from the extract by either centrifugation or filtration. The extract was evaporated to dryness at a temperature of 30° C - 35° C. After drying, the extract was diluted to a known volume and chromatographed descendingly on Whatman #1 filter paper in a system of n-butyl alcohol, water and glacial acetic acid (4; 5; 1).

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<sup>1</sup>CTH--A Commercial Armour MSH preparation.

Upon exposure to ultra-violet light, the pteridines on the chromatograms fluoresced brightly while the purines appeared as dark absorption spots. An ultra-violet light at approximately  $360m\mu$  was used to disclose purine absorption. The quantity of each pteridine was estimated visually from the degree of fluorescence induced by ultra-violet light at approximately  $350m\mu$ . A grading system of fluorescence was arbitrarily chosen as follows: (++++), indicating very strong fluorescence; (+++), strong fluorescence; (++) , intermediate; (+), dim; (+), barely identifiable. The validity of this system has been reported previously (Bagnara '61).

The absorption spectra of the various pteridines and purines were obtained either by measuring the absorption on the chromatogram directly or on eluates of the chromatogram. All U. V. absorption spectra were determined with a Beckman D. U. spectrophotometer. Purines were eluted from the chromatogram with 1 N HCl while 1 N  $NH_4OH$  was used to elute the pteridines. In order to obtain acidic or basic spectra of the various purines and pteridines, from the paper itself, the chromatogram was sprayed with either .1 N HCl or .1 N NaOH. For spectral analysis of eluates, the pH was adjusted by addition of either acid or base.

In order to determine U. V. spectra from the chromatogram directly, the pteridine or purine spots were cut out and placed in a modified Beckman D. U. cuvette holder (Bradfield and Flood '52; Steelink '56).

Dried tissues to be extracted for carotenoids were weighed and treated with 10 ml. of 20% alcoholic KOH for 24 hours or until the tissue dissolved. During this time the tissues were kept in a dark cool place. After the tissues dissolved, 10 ml. of distilled water (an amount equal to the volume of KOH used) and 15 ml. of petroleum ether were added and the mixture was shaken for five minutes. The carotenoids are soluble in the epiphasic petroleum ether layer. After shaking the mixture, a 10 ml. aliquot of the epiphasic layer was removed and the optical density read at a wave length of 440  $m\mu$  on a Coleman Universal Spectrophotometer (Morten and Rosen '49).

## EXPERIMENTS

A. Pteridines, Dorsal and Ventral Distribution. In the course of several experiments, determinations of both the dorsal and ventral distribution of pteridines were made for Rana pipiens, Rana catesbeiana, Hyla arenicolor and Rana sphenoccephala. The experimental results of the latter are new while those of the former three confirm the observations of previous investigators. In all of these anuran species, large quantities of pteridines were present in the dorsal skin, while their ventral integuments contained significantly smaller quantities of pteridines (Figure 1 and 2) (Bagnara '61).

Pteridine nomenclature adopted in this investigation is that of Hama and his collaborators. The following pteridines were found: Biopterin, Ranachrome 3, Isoxanthropterin and AHP-6-carboxylic acid (Goto and Hama '58; Odate, Tatabe, Obika, and Hama '59; Hama, Matsumoto and Obika '60). The latter two were generally present in greater quantities than the former two compounds.

The darkly pigmented dorsal integument of all the anurans tested contains considerably greater amounts of pteridines than the light colored ventral surface. This disparity in distribution of pteridines does not appear to be due to

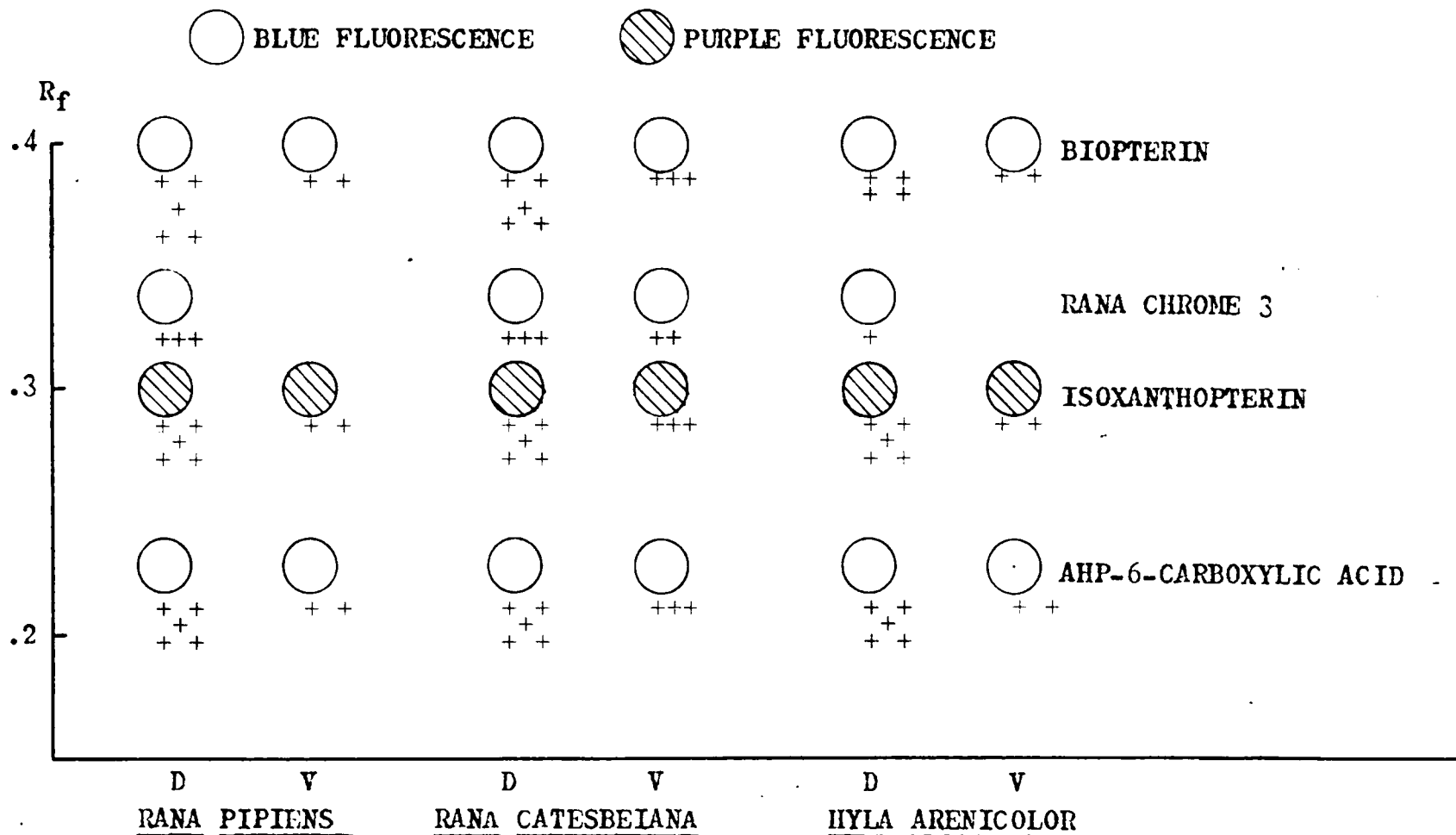


Figure 1 - Schematic chromatogram comparing dorsal (D) and ventral (V) skin extracts of Rana pipiens, Rana catesbeiana and Hyla arenicolor. The fluorescent degree of each spot is graded as follows: +++++, very strong; +++, strong; ++, intermediate; ++, dim; +, barely identifiable.



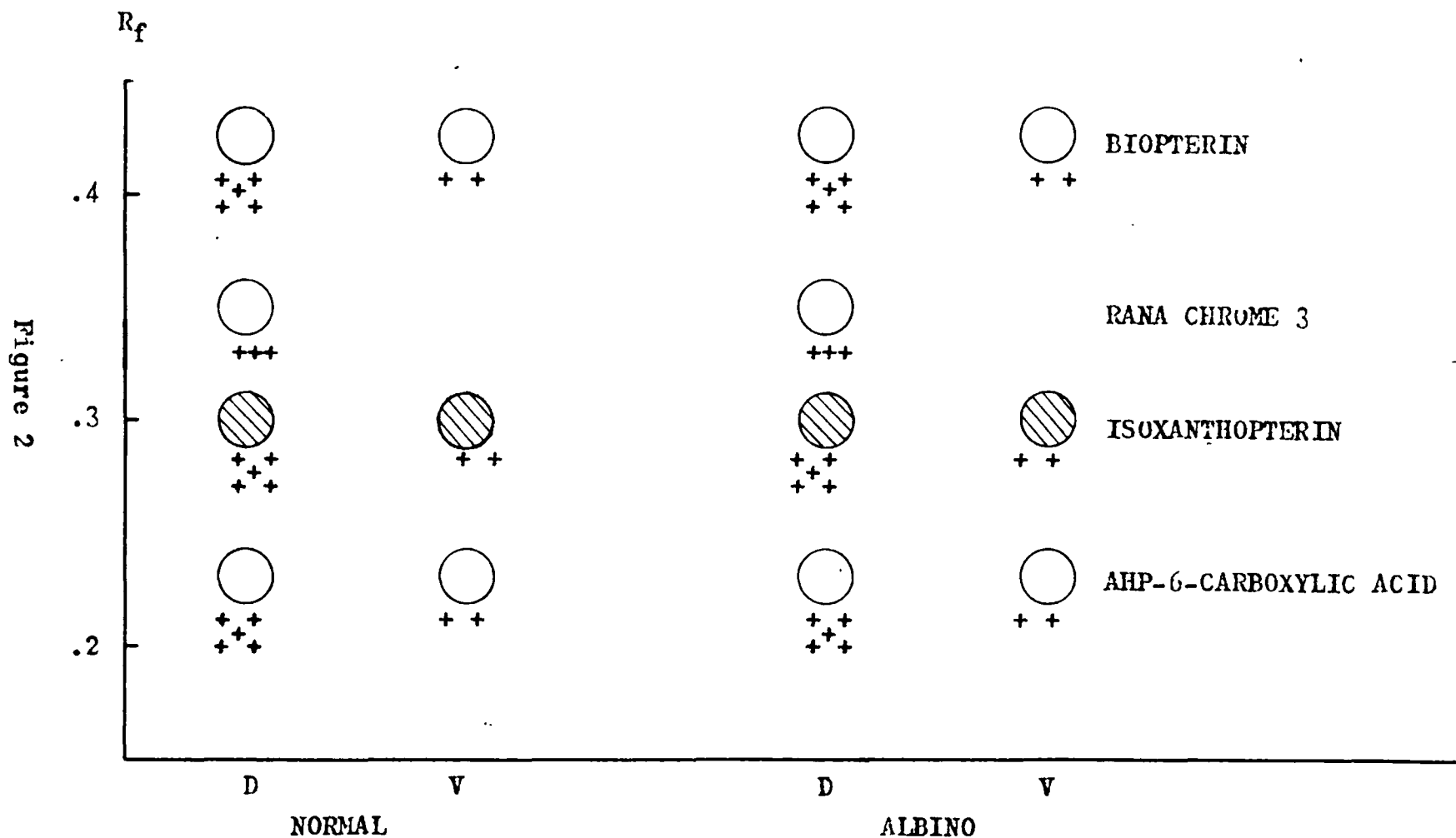


Figure 2 - Schematic chromatogram comparing extracts of adult normal and albino Rana sphenoccephala. See figure 1 for designations.

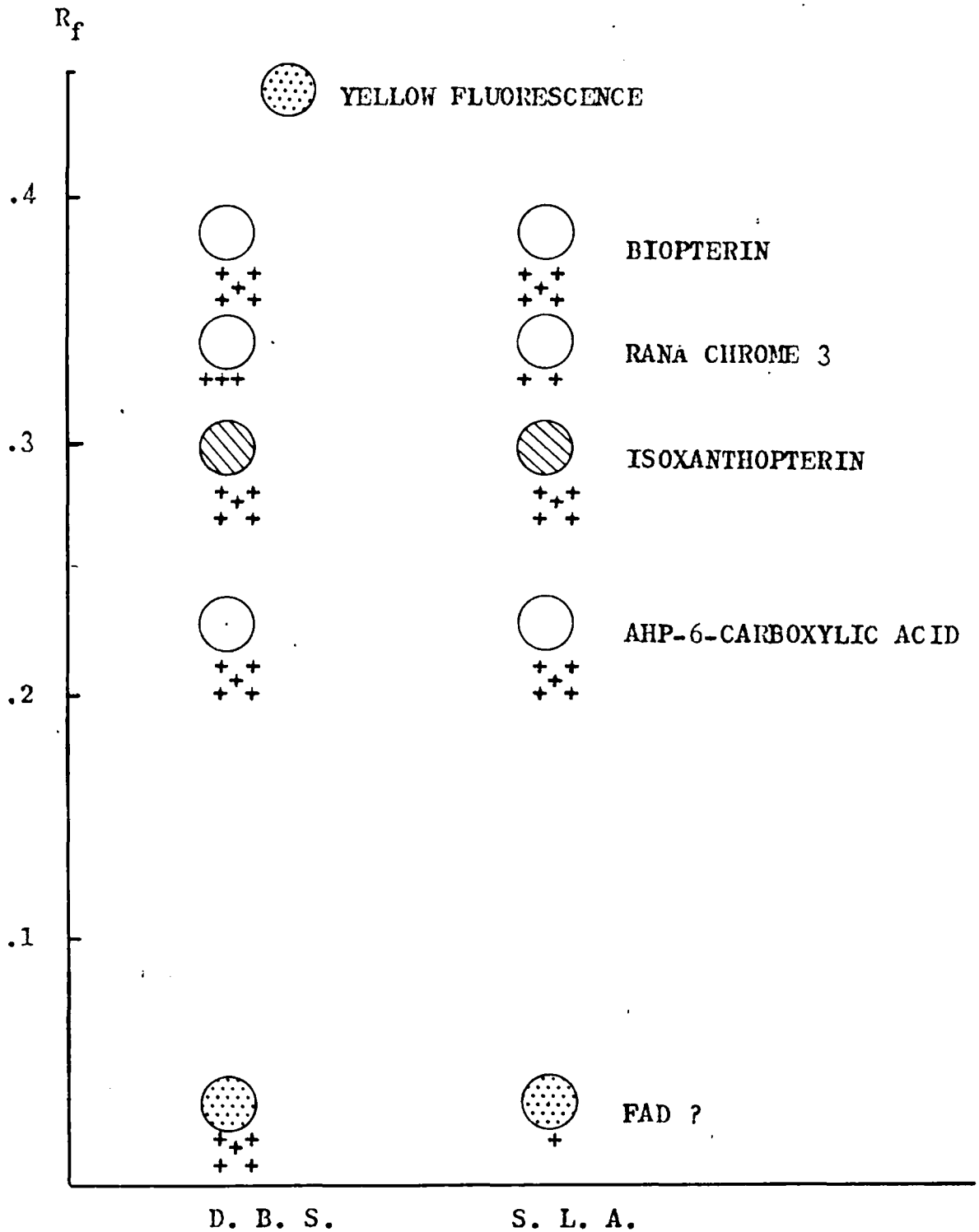
the presence of melanin in the dorsal surface; for as is shown in figure 2, light colored skin from the dorsal surface of an adult albino Rana sphenocephala<sup>1</sup> contains significantly greater quantities of pteridines than skin from its ventral surface and, moreover, contains as much pteridine material as the dorsal integument of normal darkly pigmented individuals. This finding is consistent with the observations of Bagnara ('61) who found that the integumental pteridine concentrations are approximately the same for both albino and normal Rana pipiens larvae.

Although the fluorescence in skin extracts does not seem to be associated generally with melanophores, one yellow fluorescing compound prevails in the dorsal black spots of adult Rana pipiens. These black spots contain more melanin than the surrounding lighter areas, which are usually green in color (Baker '53). Only minute amounts of this yellow compound are extracted from the light areas while large amounts are extracted from the black spots (figure 3). This yellow fluorescing compound has an Rf value of approximately .04 and is probably a flavin that has also been found by Hama ('53) in several amphibians. This Rf value (.04) probably corresponds to that of flavinadenine dinucleotide (FAD) found by Hama and Obika ('58) in Bufo vulgaris formosus.

B. Pteridines and CTH Relationships. It has been

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<sup>1</sup>Both the albino and normal Rana sphenocephala were collected in the same locality.



Schematic chromatogram comparing extracts of Rana pipiens dorsal black spots (D. B. S.) to the surrounding lighter areas (S. L. A.) See figure 1 for designations.

shown previously that integumental pteridines are under the influence of CTH (Bagnara and Neidleman '58 and Bagnara '61). These authors injected hypophysioprivic larvae with various CTH preparations; however, their injections were performed over a relatively short period of time. The integumental pteridine concentrations of the injected larvae increased, but these amounts were somewhat below those found in normal tadpoles. In order to determine whether CTH treatments, if given over a longer period of time, would increase the amount of integumental pteridines of hypophysioprivic larvae to normal levels, the following experiments were performed.

1. Rana pipiens and Hyla arenicolor

Thirty-six hypophysioprivic Rana pipiens and twelve hypophysioprivic Hyla arenicolor larvae were used in the course of four experiments. These larvae were divided into two groups; a control group and a group injected with potentiated CTH preparations. The control group received injections of Holtfreter's solution. The Armour MSH concentration was 25  $\mu$ g/.05 ml. and the concentration of pitressin was .1 pressor units/.05 ml. Approximately six hypophysioprivic hormone-treated larvae were used in each experiment. Normal tadpoles used as controls were not injected.

In these experiments, hormone treatments were given every thirty-six hours for a total of ten injections. The hypophysioprivic control groups, injected with Holtfreter's solution, showed no signs of melanin dispersion or guanophore

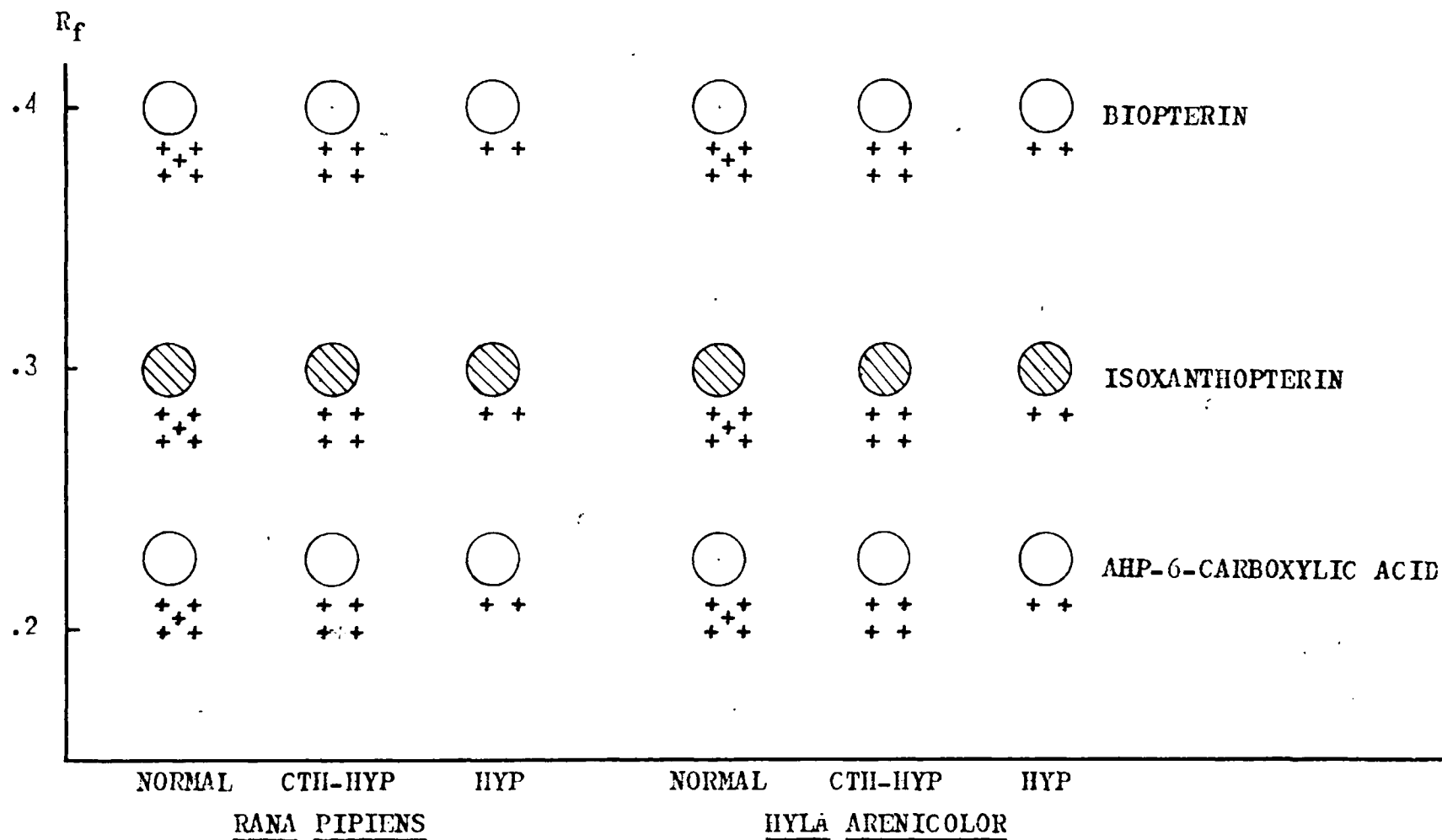
contraction. Due to the response of both chromatophore types, the hormone-treated groups became as dark as, if not darker than, the normal control groups. At the end of the treatment, larvae were sacrificed and skinned. Extracts of their skin were chromatographed. Visual observations of the chromatogram exposed to U. V. light, showed that the normal control group possessed greater fluorescence than the hypophysioprivic control group (figure 4). Fluorescence in the hypophysioprivic hormone-injected groups were intermediate; however, it usually approached that of the normal control groups.

In another experiment, pitressin treatments were given to six normal Rana pipiens larvae to determine whether their integumental pteridine concentrations could be elevated above normal. Hormone injections were made every other day and after a total of eight treatments, the amounts of integumental pteridine concentrations of both the injected and the control group were compared. Normal, CTH-treated larvae had slightly more integumental pteridines than untreated normal larvae (figure 5).

## 2. Rana sylvatica

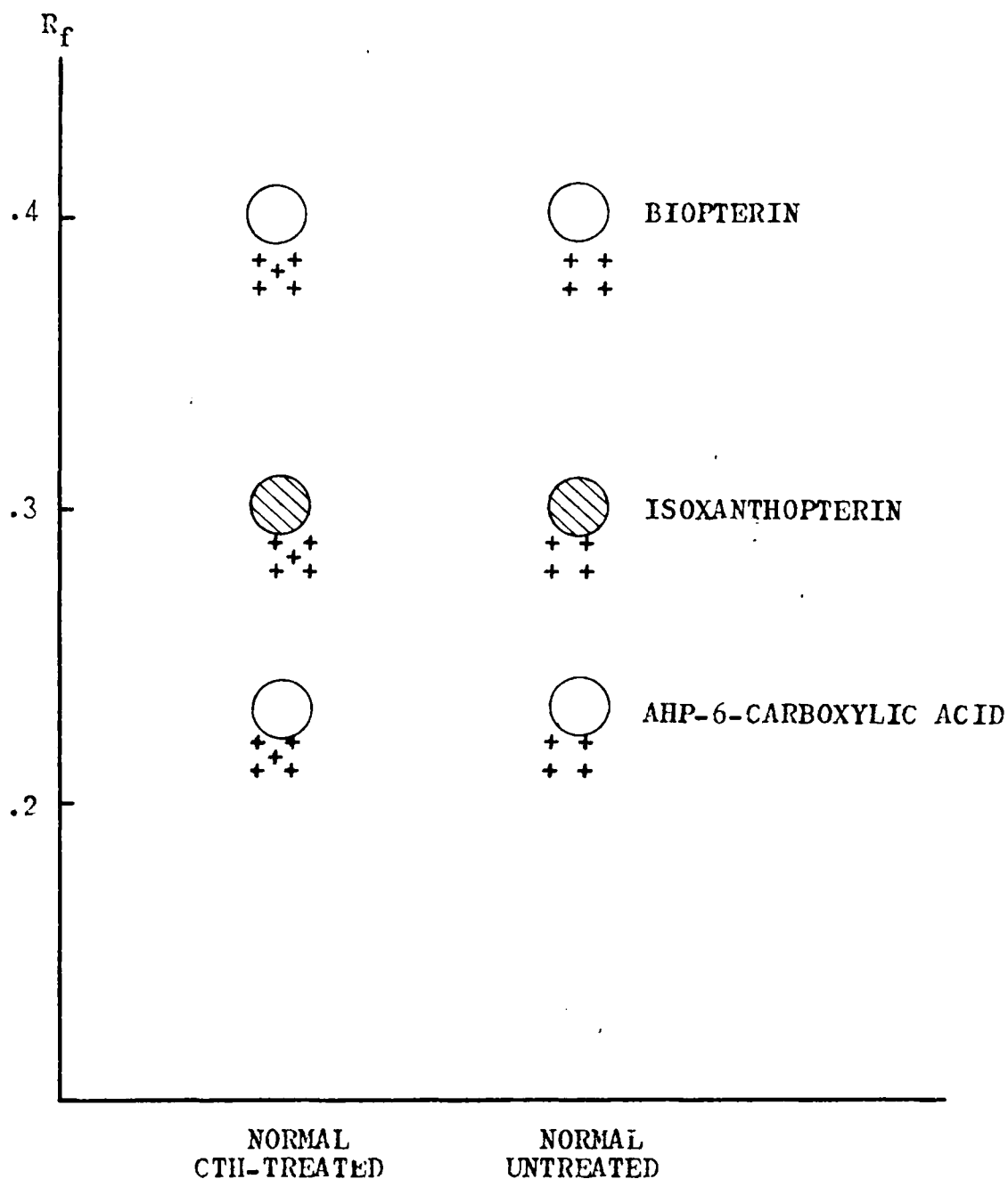
Consistent with the above experiments, hypophysioprivic Rana sylvatica larvae have considerably smaller amounts of integumental pteridines than normal larvae. It has been demonstrated previously that CTH almost completely depletes integumental guanine in hypophysioprivic Rana sylvatica larvae as opposed to only a slight decrease in hypophysioprivic Rana

Figure 4



Schematic chromatogram comparing dorsal skin extracts of normal, CTH-treated-hypophysioprivic (CTH-HYP), and hypophysioprivic (HYP) Rana pipiens and Hyla arenicolor larvae. See Figure 1 for other designations.

Figure 5



Schematic chromatogram comparing dorsal skin extracts of normal CTH-treated and untreated normal Rana pipiens larvae. See Figure 1 for other designations.

pipiens larvae (Bagnara and Neidleman '58). Since integumental guanine of Rana sylvatica is inhibited so markedly by CTH, it was of interest to determine what influence this hormone had on integumental pteridine concentrations of this species.

With this view in mind, twenty-four hypophysioprivic Rana sylvatica larvae were divided into two experimental groups, six larvae in each were injected with potentiated pitressin (at a concentration of .1 pressor unit/.05 ml.). The other six in each group served as controls. To one group hormone treatments were given every day for seven days, while the other group of larvae were given hormone treatments every other day for a total of fifteen treatments. Normal larvae were used as additional controls in both experiments. At the termination of the injection period, the integumental pteridine concentrations were determined for both hormone treated groups and controls.

Larvae that received hormone treatments every day for seven days contained approximately the same integumental pteridine concentrations as the hypophysioprivic control larvae. Both groups had considerably smaller amounts of pteridines present than the normal control larvae.

Hypophysioprivic larvae that received pitressin treatments every other day for fifteen treatments had greater integumental pteridine concentrations than hypophysioprivic control larvae; however, these amounts were not as great as



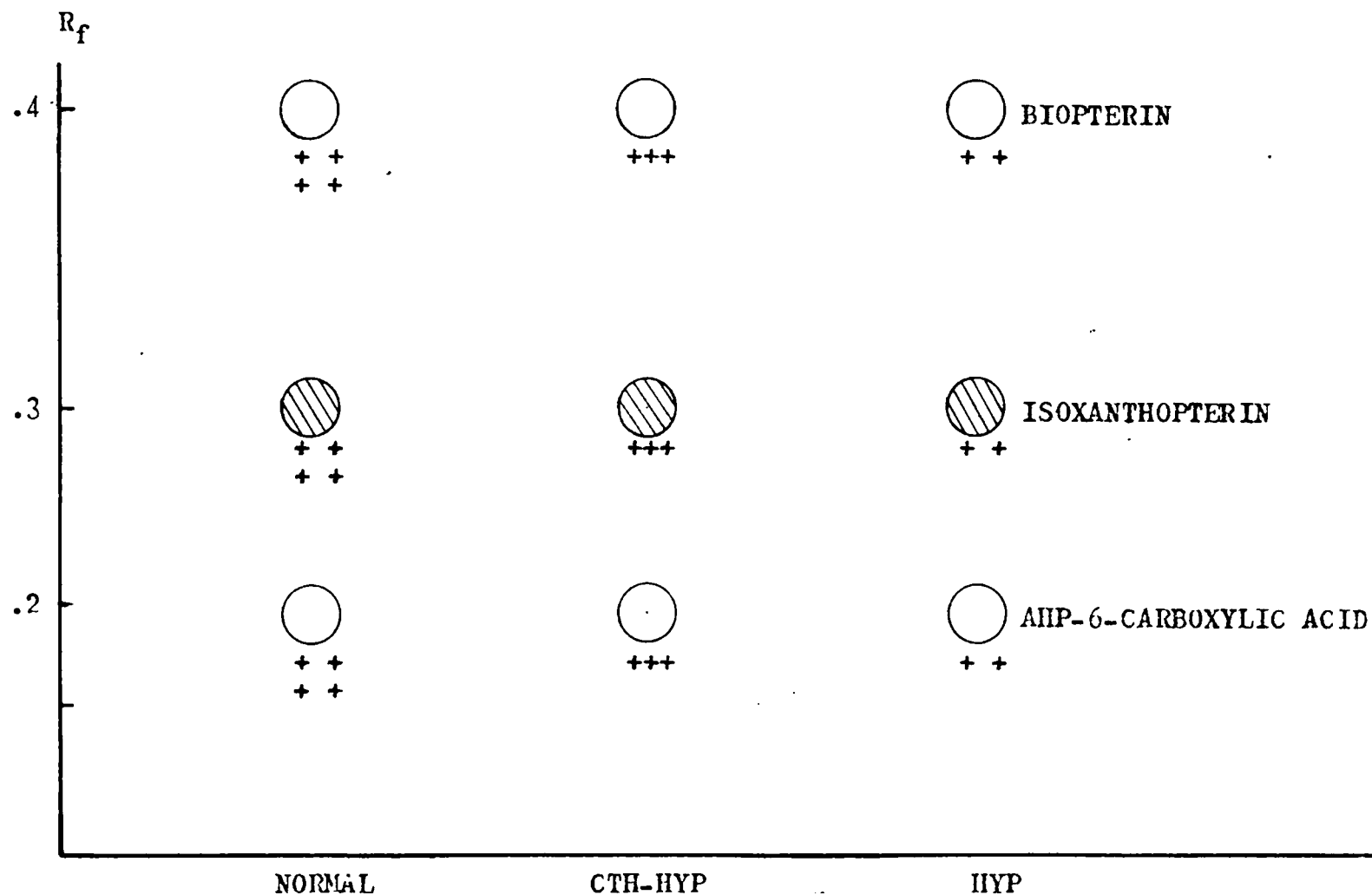


Figure 6

Schematic chromatogram comparing dorsal skin extracts of normal, CTH-treated (every other day for a total of fifteen treatments) hypophysioprivic (CTH-HYP), and hypophysioprivic (HYP) Rana sylvatica larvae. See Figure 1 for other designations.

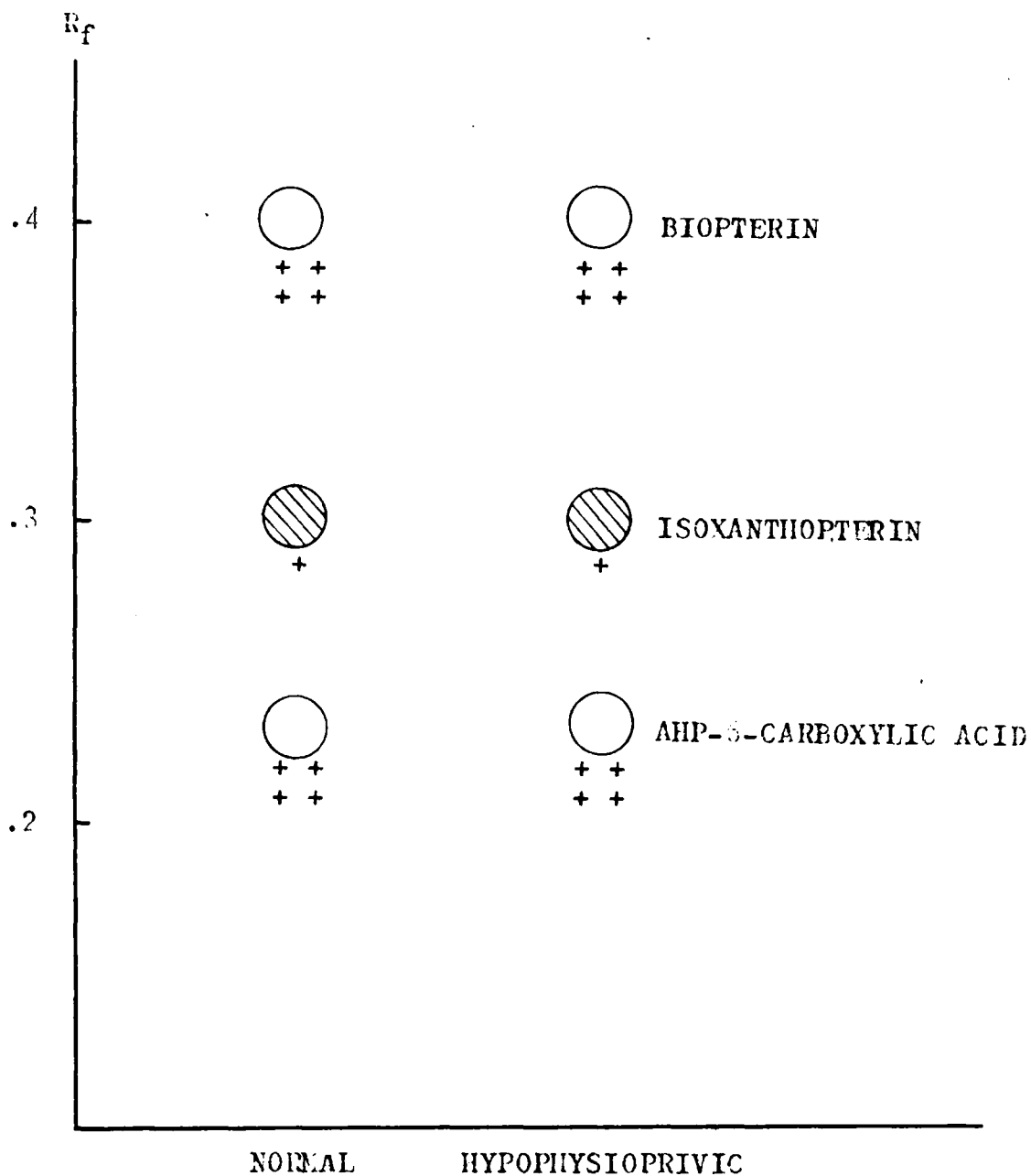
those seen in the skin of normal control larvae. Thus, prolonged CTH treatments stimulate integumental pteridine synthesis in hypophysioprivic Rana sylvatica larvae (figure 6), just as they do in Rana pipiens and in Hyla arenicolor larvae.

### 3. Ambystoma tigrinum

Considerable work has been done which demonstrates the effectiveness of CTH on anuran pigmentation; however, relatively little work has been done on the effects of this hormone on urodele pigmentation. Chavin ('56) reported that CTH does not influence melanogenesis in hypophysioprivic or decapitated Ambystoma tigrinum larvae. However, since CTH influences chromatophores as well as the synthesis of pteridines in anurans, it would be of interest to determine what effect CTH has on the integumental pteridines in urodeles.

To determine these effects, if any, the integumental pteridine concentrations of normal and hypophysioprivic Ambystoma tigrinum larvae were compared. As shown by figure 7, both groups were found to have approximately the same concentrations of integumental pteridines. Apparently, the hypophysis has no marked affect on integumental pteridines of Ambystoma tigrinum. This may be true for urodeles in general.

Hypophysioprivic Ambystoma tigrinum larvae are golden yellow in color. Observations of skin mounts showed that this color is due, in large part, to loss of melanin. Contrary to the observations of Chavin, it seems that the pituitary gland does indeed influence melanogenesis in such larvae.



Schematic chromatogram comparing dorsal skin extracts of normal and hypophysioprivic *Ambystoma tigrinum* larvae. See figure 1 for other designations.

To determine what effect CTH has on larval chromatophores of this urodele, hypophysioprivic larvae were injected with CTH. After injections were made, direct observations showed that the melanophores expanded and the guanophores contracted.

C. Background Effects on Pteridines and Purines. It is generally known that amphibians become dark upon long exposure to dark backgrounds and pale upon long exposure to light backgrounds (for literature see Parker '48). Presumably, this is due to an increased release of CTH under darkened conditions and a decreased release of the hormone under lighted conditions (Bagnara, unpublished). If this is the case, hormonally controlled pigmentary phenomena might be studied simply by altering endogenous CTH production by the use of different backgrounds.

Accordingly, various amphibian eggs were allowed to develop in aquaria painted either white or black. The following amphibians were used: Rana pipiens, Rana sylvatica, Rana sphenocephala, Ambystoma tigrinum, Ambystoma maculatum and Pleurodeles waltlii. At least twelve larvae were used in each experiment. Each experiment was repeated at least once and was terminated shortly before the larvae reached metamorphosis.

#### 1. Anurans

The anurans that developed on the black background consistently became dark while those on the white background became somewhat pale in color. White background adapted larvae

had contracted melanophores and expanded guanophores while the chromatophores on the black background adapted larvae reacted in the opposite way. These observations indicate that CTH levels were modified by differences in background. Chromatographic analysis of skin extracts indicated that anuran larvae on the black background consistently had larger quantities of integumental pteridines present than those raised on a white background (figure 8). This result, which was undoubtedly due to increased CTH levels in dark background adapted animals, supports the concept that CTH influences synthesis and deposition of pteridines in anuran larvae. Since CTH affects guanophores (Bagnara '58), large quantities of integumental purines were found in larvae that developed on a white background and relatively small amounts of purines were found in larvae raised on a black background.

## 2. Urodeles

In general, urodeles that developed on black backgrounds became dark while those on white backgrounds became light; however, the marked differences in coloration seen among anurans raised on white backgrounds as opposed to those reared on black backgrounds were not seen in urodele larvae. Although the urodele larvae changed color according to background differences, approximately the same quantity of pteridines and purines were found in larvae raised on either light or dark backgrounds. These results, which indicate that the pituitary gland does not appear to be involved in pteridine synthesis in urodeles,

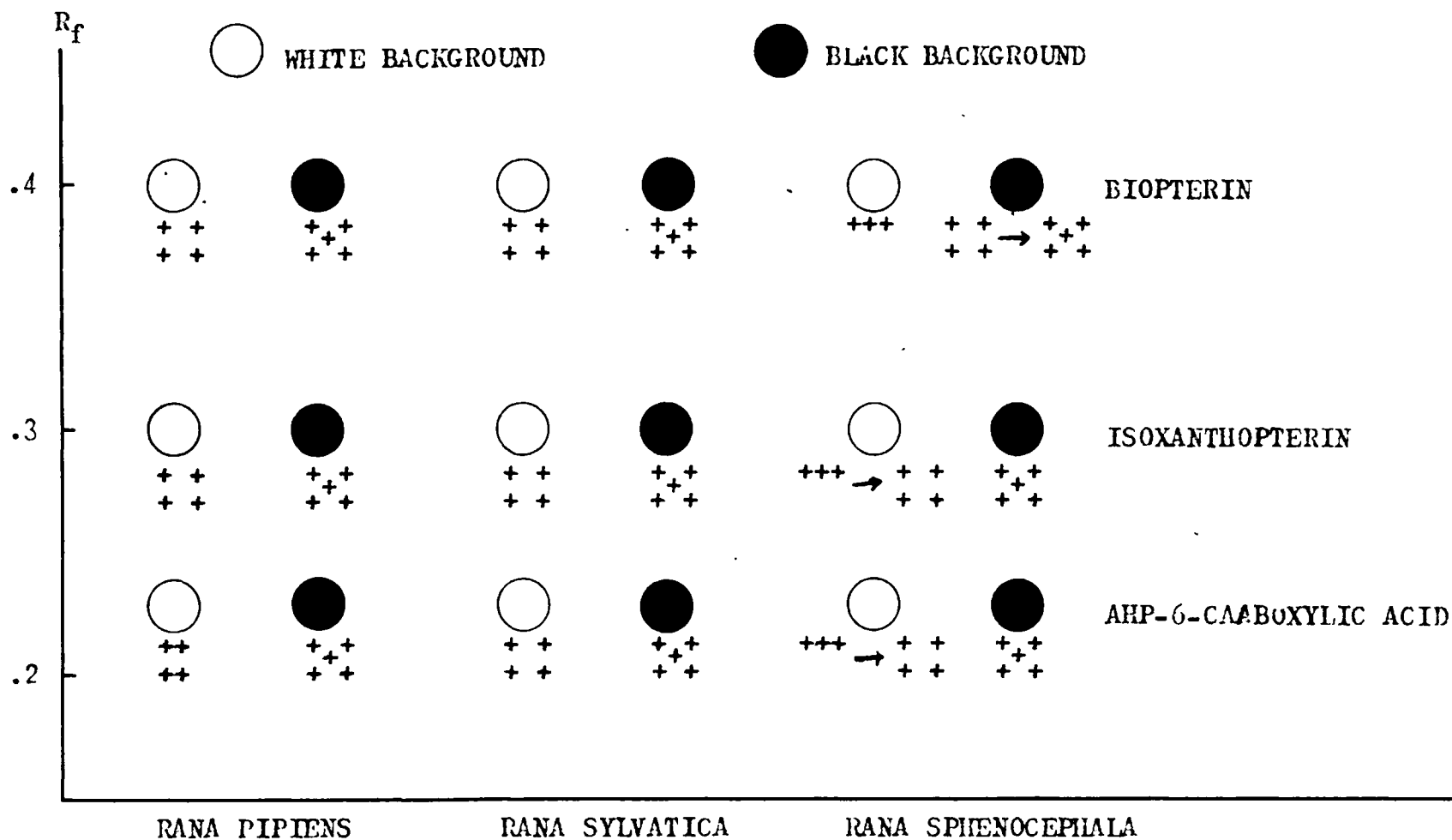


Figure 8

Schematic chromatogram comparing dorsal skin pteridine extracts of various Anuran larvae that developed on black or white backgrounds. See Figure 1 for other designations.

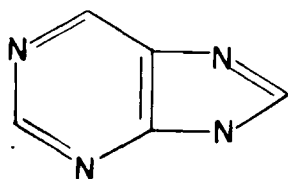
are consistent with those obtained in experiments which compared the integumental pteridines of normal Ambystoma tigrinum larvae with those of hypophysioprivic larvae.

D. Purines and CTH Relationship. Due to the structural similarity between pteridines and purines (figure 9), because both are found in skin of most amphibians, and because both compound groups are affected by CTH, it was of interest to examine more closely the purine content of amphibian skin.

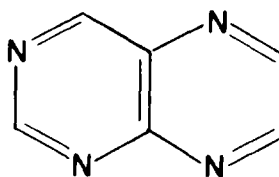
That guanine is indeed a major component of amphibian guanophores was shown by Bagnara and Neidleman ('58). When these investigators treated hypophysioprivic Rana pipiens larvae with CTH, very little decrease in integumental guanine was observed. Other workers (Bagnara and Stackhouse '61) have indicated that hypoxanthine and adenine are also constituents of guanophore laden skin. In order to examine the purine-pteridine relationship and in order to determine whether CTH affects adenine and hypoxanthine as it does guanine, the following observations were made on the same tadpoles used in the pteridine experiments discussed previously.

Purines (guanine and hypoxanthine) were found in larger concentrations in hypophysioprivic Rana pipiens, Hyla arenicolor and Rana sylvatica than in normal larvae. The hypophysioprivic CTH-treated groups, Rana pipiens and Hyla arenicolor, had intermediate amounts of integumental purines (figure 10). The normal groups contained the smallest amount of integumental purines. This result is just the reverse of that which

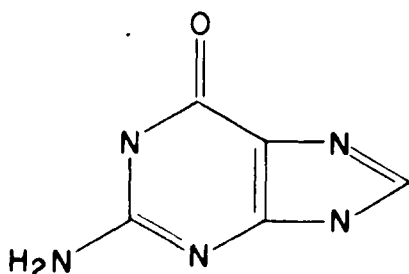
Figure 9

PURINES

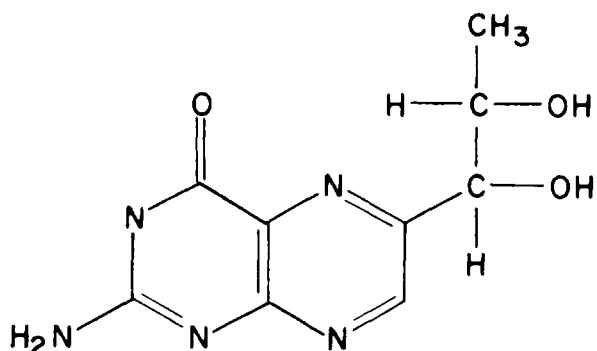
purine

PTERIDINES

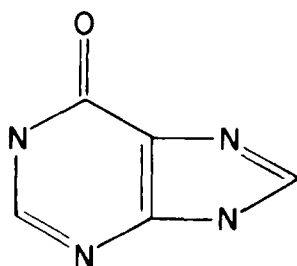
pteridine



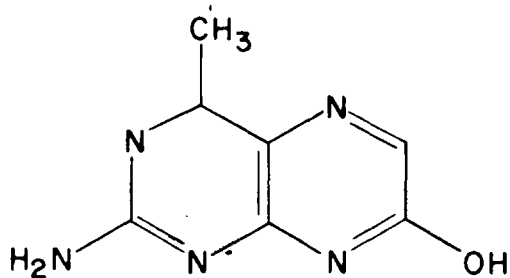
guanine



biopterin



hypoxanthine



isoxanthropterin

Molecular structures of some purines and pteridines showing structural similarities of the two groups.



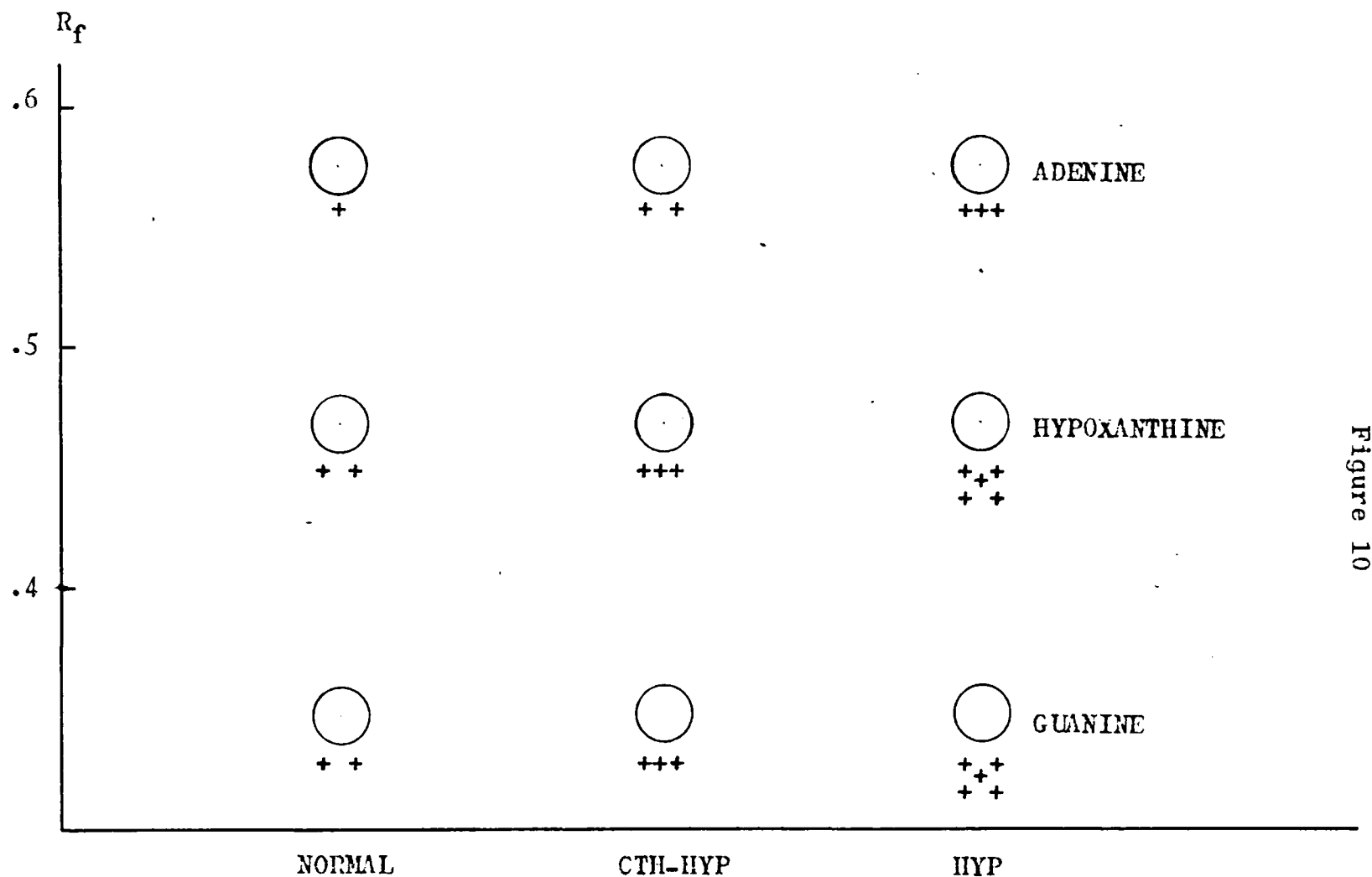


Figure 10

Schematic chromatogram comparing dorsal skin purine extracts of normal, CTH treated hypophysioprivic (CTH-HYP), and hypophysioprivic (HYP) Anuran larvae. Degree of U. V. absorption of each purine is graded as follows: +++++, very strong; +++++, strong; +++, intermediate; ++, dim; and +, not identifiable.

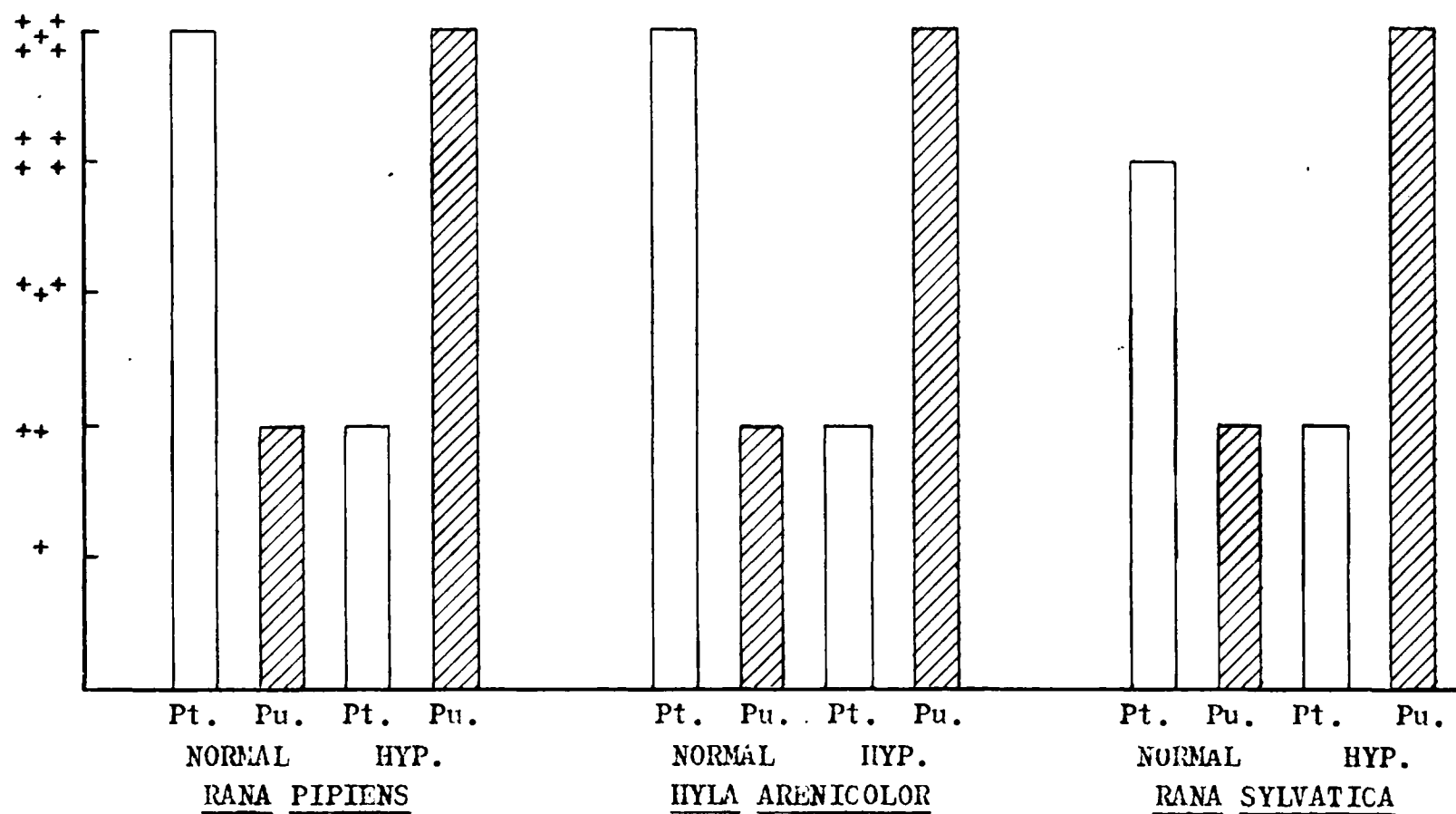


Figure 11

Comparisons of relative total integumental pteridine (pt.) and purine (pu) concentrations in normal and hypophysioprivic Rana pipiens, Hyla arenicolor and Rana sylvatica larvae. For designations of concentrations see Figures 1 and 12.

occurs with integumental pteridien concentrations (figure 11). As the integumental pteridine concentrations increase, the integumental purine concentrations decrease and in hypophysioprivic Rana sylvatica larvae treated with CTH, guanine decreases markedly. CTH-treated hypophysioprivic Rana pipiens larvae also show a decrease in integumental guanine but not as much as that seen in Rana sylvatica tadpoles. In general, CTH stimulated all integumental purines to decrease proportionally in hypophysioprivic Rana pipiens and Hyla arenicolor larvae.

Rana sylvatica larvae respond to CTH treatments in a somewhat different pattern than the above mentioned larvae. The guanine in hypophysioprivic larvae, treated with CTH for a prolonged period, completely disappeared and a compound that is probably adenine appeared in large quantities. Hypophysioprivic larvae that received CTH treatments at twenty-four hour intervals had approximately the same quantity of integumental guanine and adenine present as the control group. There is a possibility that guanine is being converted to adenine. Hypoxanthine and another probable purine (not yet identified) appeared to decrease very little, if any, in the CTH-treated animals.

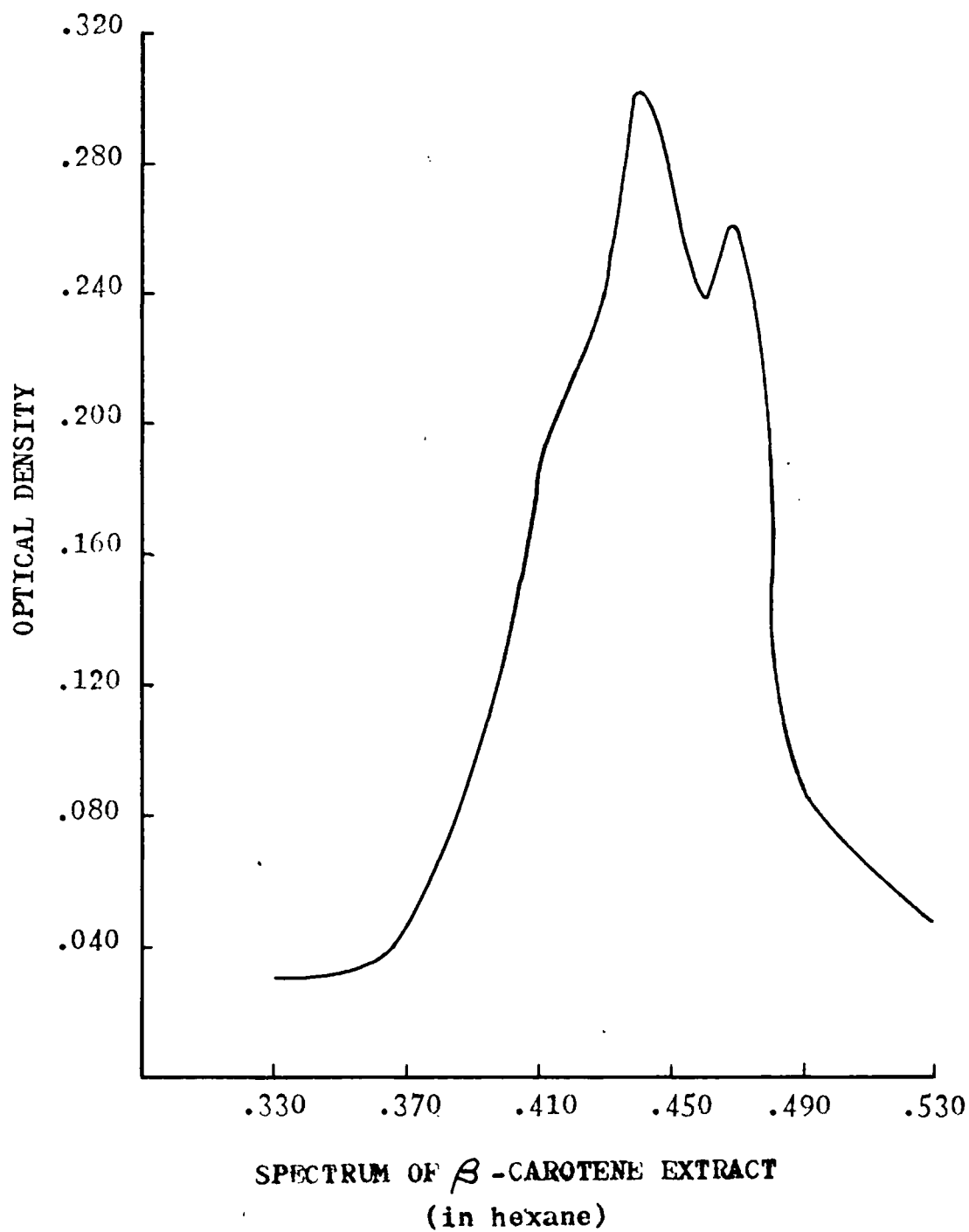
Integumental purine concentrations were found to be higher in the ventral skin than in the dorsal skin of the adult anurans investigated and this seems to argue against the inter-conversion of pteridines to purines.

E. Carotenoids, Dorsal and Ventral Distribution. It

has been suggested by Bagnara ('61) that pteridines are associated with yellow pigmentation. In amphibians, along with many other organisms, this yellow pigmentation is usually due to presence of carotenoids (Fox '53). Thus, if pteridines and yellow pigmentation are related, there should be a parallelism between carotenoid and pteridine distribution. In order to test this, total integumental carotenoid concentrations were determined for the following amphibians: Rana pipiens, Rana catesbeiana, Hyla arenicolor, Amphiuma tridactylum, Taricha torosa, and Ambystoma tigrinum. In addition to total carotenoid determination, the type of carotenoids present was determined according to the methods of Morten and Rosen ('49). These authors reported integumental carotenoids of Rana temporaria to be composed of two major fractions:  $\beta$ -carotene and xanthophylls, with xanthophylls being in larger quantities than  $\beta$ -carotene. In the present experiments  $\beta$ -carotene (figure 12) appeared to be the major carotenoid present in both Rana pipiens and Rana catesbeiana. Xanthophylls are present in somewhat smaller quantities.

These total carotenoid determinations indicate that integumental carotenoids are more concentrated in the heavily pigmented dorsal skin than in the lightly pigmented ventral skin of anuran. This is expected, since xanthophores, the yellow pigment cells, are found in large numbers in the dorsal skin while the ventral integument has few xanthophores. The yellow flanks and ventral rear leg surface of Hyla arenicolor are intermediate in total carotenoid concentrations compared with the dorsal and ventral integument (figure 13). The

Figure 12



# CAROTENOID AND PTERIDINE RELATIONSHIP

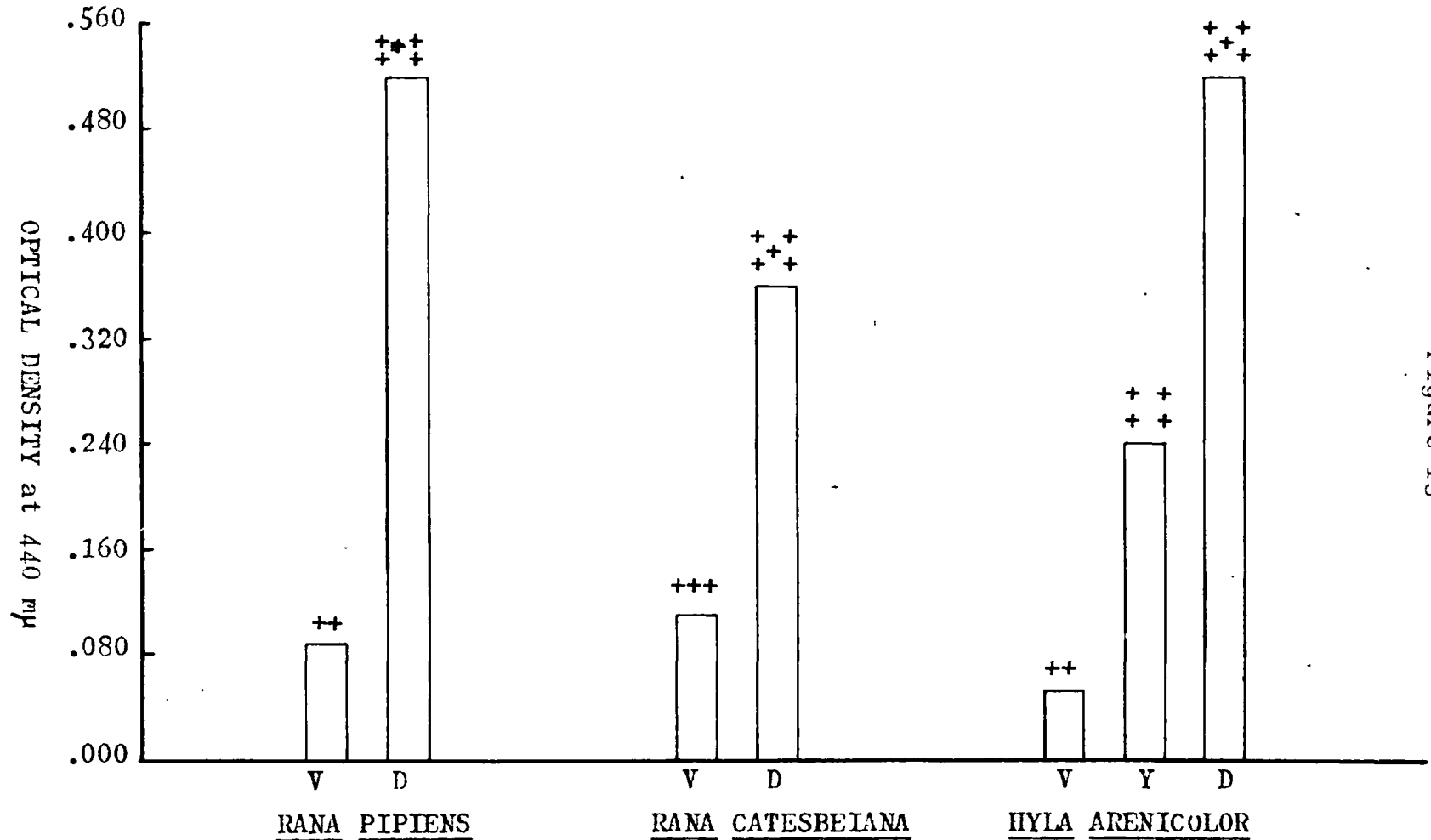


Figure 13

Total carotenoid comparisons of dorsal (D), ventral (V), and yellow of leg (Y) of Rana pipiens, Rana catesbeiana, and Hyla arenicolor. Pteridine carotenoid relationships - indicated by plus marks on top of bars.

ventral skin of the bullfrog looks more yellow than that of Rana pipiens or Hyla arenicolor. Consistent with this, there are large quantities of both carotenoids and pteridines in the ventral surface.

The urodeles investigated presented a characteristic picture of carotenoid distribution. Observations of whole mounts of dorsal and ventral skin of Taricha torosa showed large numbers of xanthophores to be present in each area, and the total carotenoid concentration was found to be approximately the same in both surfaces. However, small quantities of pteridines were found in either surface. Total carotenoid determinations of the yellow ventral skin of Ambystoma tigrinum showed it to have a higher carotenoid content than the dorsal black or yellow areas. The latter two areas contained approximately the same amounts of carotenoids. It is possible that some yellow pigmentation is due to pteridines (Fox and Vevers '60; Ziegler-Günder '56, and Hama personal communication). However, Bagnara ('61) shows by extracting with fat solvents that yellow pigmentation is not due to pteridines in Hyla

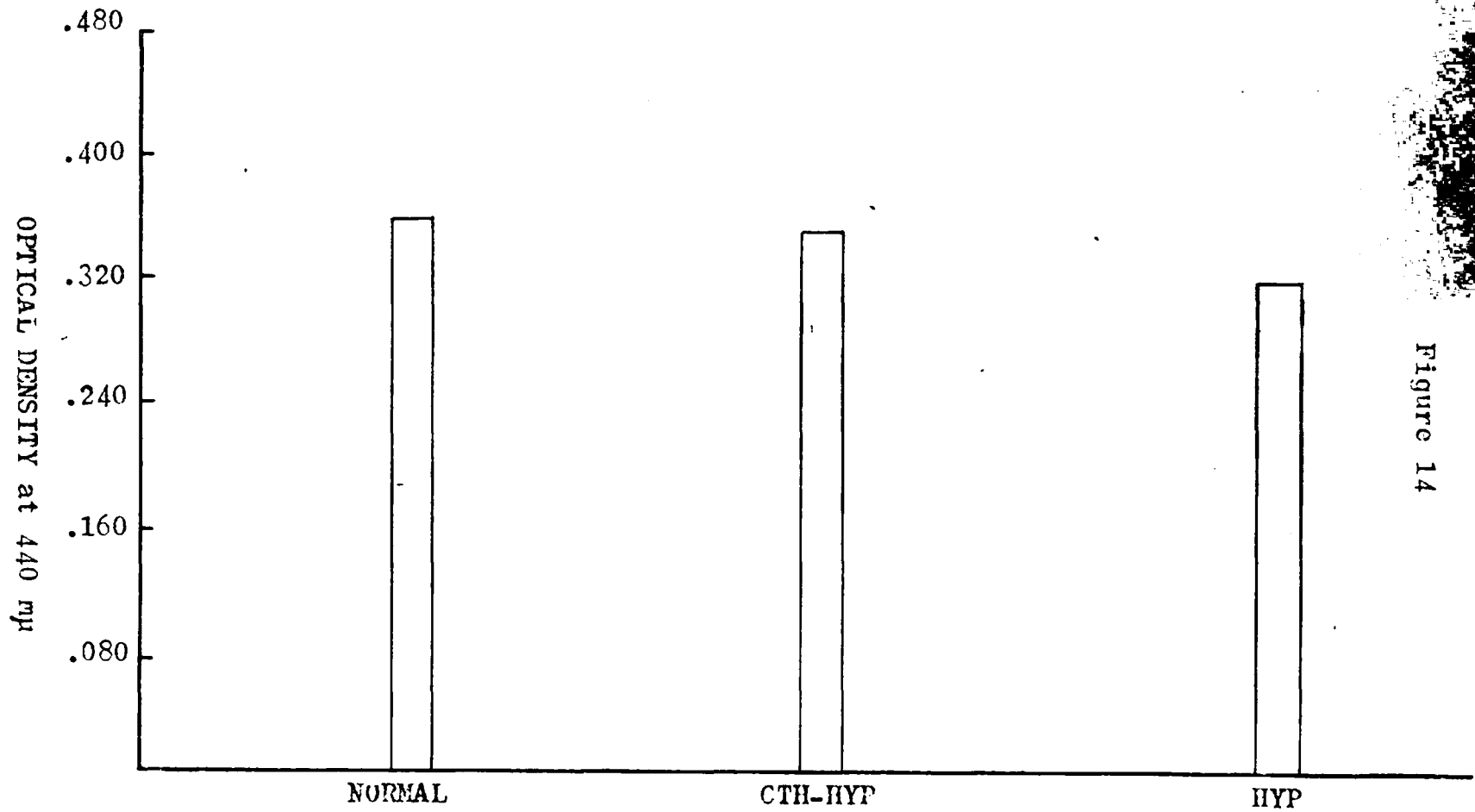
The black dorsal skin and the grey ventral skin of Amphiuma tridactylum contains few if any xanthophores. Both areas are void of carotenoids and only negligible quantities of integumental pteridines were found; thus further supporting the fact that pteridines are not associated with black pigmentation.

F. Carotenoid and CTH Relationship. Bagnara's ('59) observations indicate that carotenoids are not influenced by CTH, however, his experiments were done on relatively few larvae and his data showed considerable variation. It was of interest, therefore, to re-examine this problem more thoroughly.

Ten large hypophysioprivic Rana pipiens larvae were divided into two groups and one of these groups served as controls. Five normal larvae were selected as additional controls. Potentiated pitressin was injected in the larvae of one hypophysioprivic group every other day. The experiment was terminated after seven injections and the total carotenoid concentrations were determined. The results (figure 14) of this experiment indicate that carotenoids are not influenced by CTH, and these observations agree with the previous observations of Bagnara ('59).



# CTH CAROTENOID RELATIONSHIP



Total carotenoid concentrations of normal, CTH treated hypophysioprivic (CTH-HYP) and hypophysioprivic (HYP) Rana pipiens larvae.

Figure 14

## DISCUSSION

Several amphibians have been investigated under various physiological conditions and the presented data indicate a relationship between CTH of the hypophysis and various skin pigments. The specific pigments involved are carotenoids, pteridines and purines.

It is significant that skin of most amphibians which has an abundance of pteridines usually has an abundance of xanthophores. Thus, integumental pteridines appear to be directly associated with yellow pigmentation and yellow pigmentation is, in most cases, due to carotenoids (Parker '48; Fox '53). Carotenoids were reported to be present in the skin of Rana temporaria by Morten and Rosen ('49). However, these authors did not mention the distribution, dorsal or ventral, of these pigments in this frog. Heavily pigmented dorsal skin of most amphibians, especially anurans, contains a greater number of xanthophores than the lightly pigmented ventral integument does. Therefore, it is not surprising to find large carotenoid concentrations in areas of skin rich in xanthophores and small amounts of these compounds where few xanthophores are located. Experiments with Rana pipiens, Rana catesbeiana

and Hyla arenicolor substantiate this direct relationship between carotenoids and pteridines and show that carotenoids are mainly distributed in the dorsal integument. The canyon tree frog, Hyla arenicolor, in another way further emphasizes this direct relationship. The flanks and ventral rear leg surfaces are yellow and these areas are intermediate between the dorsal and ventral skin surfaces in both pteridine and carotenoid concentrations.

The above discussion indicates that pteridines are directly associated with yellow pigmentation. This conclusion is supported by observations on the natural albino Rana sphenoccephala. This albino frog, which is yellowish, has melanophores present, but very little, if any, melanin can be observed; therefore, since pteridines are present in equal amounts in both the normal and the albino frog, a lack of association between melanin and pteridines is indicated. This is in agreement with the observations of Bagnara ('61) on natural albino Rana pipiens larvae and adult Ambystoma maculatum. In the latter animal, pteridines were concentrated in the dorsal yellow spots, while the adjacent black integument was void of these compounds. Bagnara ('61) also reported that little, if any, integumental pteridines are present in the melanophore rich skin of Amphiuma tridactylum. It is striking that in the present experiments few, if any, carotenoids were extracted from the integument of this species.

The only apparent exception to the above can be

observed in adult Ambystoma tigrinum wherein small quantities of pteridines are found in the dorsal integument, while carotenoids are found only in the ventral skin. However, it should be pointed out that some of the experimental animals were kept in captivity for approximately two months before the carotenoid determinations were made. These animals were fed a regular diet of meal worms, but it is difficult to maintain a natural diet which includes the proper amount of carotenoids in the laboratory. It should also be pointed out that the presence of carotenoids does not necessarily mean pteridines will be present, but pteridines are always, with the above exception, associated with yellow pigmentation.

In general, pteridines are associated with yellow pigmentation and the above observations support this; however, a bright yellow fluorescing compound was isolated from the dorsal black spots of Rana pipiens, while the surrounding integument contained extremely small amounts of this compound. This yellow fluorescent compound is probably a flavin, and since it is associated with melanin it may be functioning in melanin synthesis.

In these experiments total pteridine concentrations were relied on more than concentrations of individual pteridines because it is known (Hama, Matsumoto and Mori '60) that pteridine compounds are changed upon exposure to light. For example, Rana-chrome-3 is converted to AHP-6-carboxylic acid in the presence of light (Mori, Matsumoto and Hama '60; Matsumoto,

Kajishima and Hama '60; Hama personal communication).

In the course of this investigation, the role of the hypophysis in relation to pteridines, purines, and carotenoids, was investigated by removal of hypophysis, replacement therapy experiments, or by stimulation of endogenous CTH release from normal tadpoles. In any case, the CTH level was elevated. The anurans investigated generally followed a definite pattern, in that increased CTH levels caused pteridine synthesis and purine inhibition. However, Rana sylvatica larvae are an apparent exception to this generalization.

Hypophysioprivic Rana sylvatica larvae, treated with CTH, completely loose their integumental guanine (Bagnara and Neidleman '58). These investigators reported that CTH-treated hypophysioprivic Rana pipiens larvae did not completely loose their guanine. Both of these observations have been repeated and confirmed in these experiments. However, as the guanine disappears in CTH-treated hypophysioprivic Rana sylvatica larvae, a new compound appeared. This is probably adenine, for both spectral and Rf values of this compound conform to those of adenine. This indicates that CTH influences the depletion of guanine and synthesis of adenine. This could possibly mean a conversion of guanine to adenine. Only guanine is depleted in this animal and the other purines do not appear to be greatly influenced by CTH treatments. It is apparent that the pituitary gland is involved here because normal larvae have large amounts of pteridines and small amounts of

purines present in their integument, while hypophysioprivic larvae have just the reverse.

The fact that purines decrease in concentration as pteridines increase leads one to hypothesize that an interconversion takes place between the two groups of compounds, and that this interconversion is somehow affected by the pituitary gland. This hypothesis is conceivable because of the similarity of structure between the two groups, plus the fact that such an interconversion has been demonstrated in the laboratory (Albert '57).

It is interesting to note that while purine and pteridine concentrations are influenced by CTH, the integumental carotenoids are not. It is paradoxical that pteridines are associated with the yellow pigments and they are the only pigmentary substance that is not influenced by the hypophysis. This observation is in agreement with that of Bagnara ('59).

The integumental purines are assumed to occur in the guanophores in a free state and not incorporated into nucleic acids because the quantity extracted was proportional to the number of guanophores present. For example the ventral surface of anurans, such as adult Rana pipiens and Hyla arenicolor, is pigmented almost exclusively by guanophores and the dorsal surface of these anurans contains few of these pigment cells. The ventral skin contains large concentrations of purines while the dorsal integument has small quantities of these compounds present.

Experiments with urodele larvae indicate that in this group the pituitary gland is not involved in pteridine synthesis. Various urodele larvae reared on black or white backgrounds always contained approximately the same amount of integumental pteridines and purines. These compounds were found in the same concentrations in hypophysioprivic and normal Ambystoma tigrinum larvae. These two facts tend to eliminate the pituitary gland as a purine or pteridine influencing mechanism in the urodele larvae investigated. Also, the pituitary gland may not be involved with integumental purines and pteridines in urodeles in general.

The fact that Ambystoma tigrinum larvae deprived of their hypophysis possess little, if any, melanin implies that the hypophysis controls melanogenesis. Moreover, when CTH was injected into the peritoneal cavity of these larvae, melanogenesis occurred with the expansion of the melanophores. However, it has been reported that the pituitary gland does not influence melanogenesis in hypophysioprivic or decapitated Ambystoma tigrinum larvae (Chavin '56).

The difference between the present observations and those of Chavin ('56) could possibly be due to different experimental procedures employed. In these experiments, CTH was injected into the peritoneal cavity while Chavin immersed the embryos in a solution of CTH.

Thus, CTH causes melanogenesis and melanophore expansion in hypophysioprivic Ambystoma tigrinum larvae, and it also causes melanophore contraction. The melanophore contracting

reaction is a much weaker reaction than melanophore expansion. This is the same as observed in anuran larvae. Although CTH causes xanthophore contraction and melanophore expansion in hypophysioprivic larvae, the reactions in general are much weaker than those observed in anuran larvae.

The fact that anurans are more sensitive to CTH than urodeles suggest that the hormone affects each group in a different way. This may be due to species difference in response to a foreign CTH preparation.



## SUMMARY

1. Pteridines are more prevalent in the darkly pigmented dorsal integument of amphibians than in the lightly pigmented ventral skin.

2. Integumental pteridines were found in equal amounts in either normal or albino adult Rana sphenoccephala, indicating a lack of association between melanin and pteridines.

3. A yellow fluorescing substance probably a flavin, is associated with the dorsal black spots of adult Rana pipiens.

4. Guanine, hypoxanthine and adenine were present in amphibian integument and all are probably located in guanophores.

5. CTH treatments both increase integumental pteridine concentrations and decrease integumental purine concentration in hypophysioprivic larvae of Hyla arenicolor, Rana pipiens and Rana sylvatica. The amount of pteridine increase is proportional to the duration of treatments. This hormone also raised pteridine concentrations of normal larvae higher than normal.

6. Anuran larvae reared on a black background have greater integumental pteridine concentrations than larvae

reared on a white background while the integumental purine concentrations were greater in skin of white background adapted larvae. This is probably due to endogenous CTH increase.

7. CTH treatments given to hypophysioprivic Rana sylvatica larvae over an extended period increased integumental pteridines and caused, by some means, the depletion of guanine and the synthesis of a compound which is probably adenine.

8. Hypophysioprivic Ambystoma tigrinum larvae have the same concentrations of integumental pteridines as do normal larvae. The same is true of either dark or light adapted normal larvae. The pituitary gland is not involved in pteridine synthesis in these larvae and may not be in urodeles in general.

9. CTH when injected interperitoneally into hypophysioprivic Ambystoma tigrinum larvae caused melanophore expansion and xanophore contraction.

10. In anurans, integumental carotenoids are more concentrated in the dorsal skin than in the ventral integument and a relationship exists between integumental carotenoids and pteridines.

11. Integumental carotenoids of hypophysioprivic Rana pipiens larvae are not influenced by CTH treatments.

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