THE UNIVERSITY OF MANITOBA

THE RELATIONSHIP OF PURINE AND PTERIDINE DEPOSITION IN THE SKIN OF RAINBOW TROUT, *SALMO GAIRDNERI* RICHARDSON

by

Francis Herman Premdas

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ABSTRACT

Chromatographic and spectrophotometric methods were used to identify the integumental purines and pteridines in parr of rainbow trout and brook trout. In rainbow trout, quantitative changes in purine and pteridine levels were determined after the administration of TSH and ACTH, that could be expected to induce purine and pteridine synthesis respectively. The incorporation of ^{14}C-glycine into skin purines and pteridines was also followed.

Rainbow trout skin contained guanine, hypoxanthine, GMP, isoxanthopterin (IXP), biopterin, 2-amino-4-hydroxypteridine (AHP), ichthyopterin, and trace amounts of adenine. Brook trout skin contained guanine, hypoxanthine, IXP, AHP-6-COOH, and trace amounts of adenine, GMP, ichthyopterin, and xanthopterin.

In rainbow trout, the purines and pteridines in order of abundance were guanine > hypoxanthine > IXP > biopterin, AHP, GMP > ichthyopterin. Guanine was three to four times more concentrated than hypoxanthine and six to eight times more concentrated than IXP. Isoxanthopterin was twice as concentrated as biopterin and four to five times more concentrated than ichthyopterin. Total purine levels were four to seven times higher than total pteridine levels.

In rainbow trout, TSH significantly increased skin guanine and hypoxanthine but did not affect pteridine levels. Adrenocorticotropin almost doubled pteridine levels but did not influence purine levels.

The labelled precursor, ^{14}C-glycine, was readily incorporated into skin purines and pteridines. In TSH-treated fish, the incorporation of the isotope into purines was accelerated. In ACTH-treated fish, incorporation of the isotope into pteridines was accelerated.

From a consideration of the specific activities and levels of the various purines and pteridines under different treatments, it appeared unlikely that skin GMP was an intermediate in the *de novo* synthesis of guanine.

Despite the possibility of a link in the biosynthetic pathways of purines and pteridines, there was no clear evidence of a reciprocal relationship in purine and pteridine biosyntheses.

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INTRODUCTION

Purines and pteridines are among the principal pigments in the skin of amphibians and fishes (Bagnara, 1966). Studies with amphibians (Bagnara, 1961; Stackhouse, 1966) have shown that when the synthesis of pteridines is favoured there is a decrease in purine synthesis and when purine synthesis is favoured there is reduction in pteridine levels. Since pteridines are probably synthesized from a purine precursor, these studies have been interpreted (Bagnara, 1966) as suggesting that in organisms actively synthesizing both groups of pigments a reciprocal relationship in their syntheses will exist due to some sort of competition for common precursors.

Purine and pteridine metabolic pathways in fish are poorly understood but observations indicate that they too may be closely linked and interdependent. For example, in certain anadromous salmonids a pronounced silvering of the integument occurs at the time of parr to smolt transformation. It is generally held that the silvering is due to free purines (Markert and Vanstone, 1966; Johnston and Eales, 1967; Eales, 1969; Denton, 1971). The source of these free purines remains unknown (Svard, 1958; Keilin, 1959; Barrington, 1961), but interestingly enough, certain UV-fluorescing compounds (presumably pteridines) present in the skin of parr are considerably decreased in quantity or even absent from the skin of smolts (Markert and Vanstone, 1966). Also, in fish (*Girella nigricans*)

held on a dark background there was an increase in melanin formation and a decrease in purine content (Sumner, 1944). It now appears that melanogenesis is accompanied by an increase in pteridine levels (Matsumoto, 1965a).

The present study was an attempt to investigate the relationship of purine and pteridine deposition in the skin of a fish. It was necessary first to establish the identities of the principal purines and pteridines since little information is available on the occurrence of both groups of compounds in any given species. Quantitative changes in these compounds under conditions favouring purine and pteridine synthesis were next determined. The incorporation of a labelled precursor (^{14}C-glycine) was also followed in the hope that some insight into the biosynthetic pathways might be gained.

Rainbow trout, *Salmo gairdneri* Richardson, were used as the test animal because they were readily available and because they become silvery, presumably on account of purine deposition, at smoltification. It was noted during the study that rainbow trout parr contained a number of pteridines that were absent from fry of coho salmon, *Onchorynchus kisutch*, the only salmonid whose pteridines have been reported (Lee *et al.*, 1969). Brook trout, *Salvelinus fontinalis* Mitchill, were readily available, thus for purposes of comparison, some of the parr were examined.

Hormones were used to stimulate either purine or pteridine synthesis. Thyroid stimulating hormone (TSH) was chosen for inducing

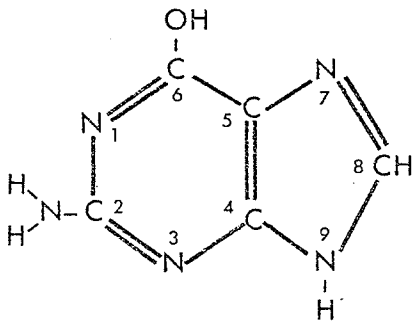
purine deposition because this hormone is known to induce silvering in rainbow trout (Robertson, 1949) and it was used successfully in this laboratory (Chua and Eales, 1971) to induce purine deposition in brook trout. Adrenocorticotropin (ACTH) was used for inducing pteridine synthesis because it has been reported (Matsumoto, 1965a) to induce pteridine synthesis in goldfish.

LITERATURE REVIEW

Purines in the skin of fish

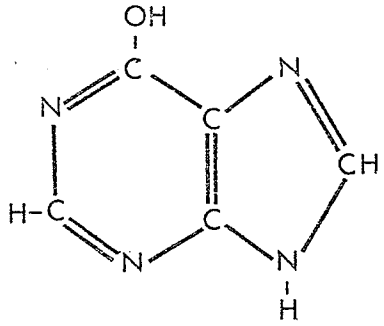
Ehrenberg in 1833 and Wittich in 1854 were among the first naturalists who observed platelets or crystals on the scales and in the underlying layer of skin of fish and considered them responsible for the silvery iridescent appearance of these structures (Keilin, 1959). Barreswil (1861) recognized the silvery material to be composed of guanine (Fig. 1). The occurrence of this purine in the skin of several species of fish has been reported by many investigators (Millot, 1923; Peschen, 1939; Sumner, 1944; Hitchings and Falco, 1944; Robertson, 1948; Neckel, 1954; Nicol, 1963; Nicol and Van Baalen, 1968; Denton, 1971).

Ziegler-Gunder (1956) identified not only guanine but also the closely related compound hypoxanthine (Fig. 1) in the skin of the European chub (*Squalius cephalus*) and the bleak (*Alburnus lucidus*). Greenstein (1966) reported that crystals from the skin of herring (*Clupea harengus*) were composed of both guanine and hypoxanthine. The ratio of guanine to hypoxanthine seemed to vary with the form of the crystals, broad plate-like crystals containing relatively more hypoxanthine than needle-like crystals. Johnston and Eales (1967) reported that the silvery skin and scales of Atlantic salmon parr and smolt contained guanine and lesser amounts of hypoxanthine. Eales (1969) found guanine and lesser quantities of



GUANINE

(2-AMINO-6-HYDROXYPURINE)



HYPOXANTHINE

(6-HYDROXYPURINE)

Fig. 1. Molecular structures of guanine and hypoxanthine, the principal purines in fish skin (from Davidson, J.N., 1965).

hypoxanthine in the silvery layers of the skin of channel catfish (*Ictalurus punctatus*), burbot (*Lota lota*), brook stickleback (*Culaea inconstans*), pike (*Esox lucius*), walleye (*Stizostedion vitreum*), sauger (*S. canadense*), goldfish (*Carassius auratus*), brook trout (*Salvelinus fontinalis*), and trout perch (*Percopsis omiscomaycus*).

In addition to these purines small amounts of other purine compounds have been encountered in the skin of fishes. Markert and Vanstone (1966) reported a UV-absorbing compound, probably guanylic acid (GMP), in the belly skin of parr and smolt of coho salmon; Hayashi and Saito (1968) found inosinic acid (IMP) in the dermis of carp, rainbow trout and chum salmon; Matty and Sheltawy (1967) reported IMP and GMP in the skin of rainbow trout; Nicol and Van Baalen (1968) found uric acid in the skin of cutlassfish (*Trichiurus lepturus*). Nevertheless, studies of several freshwater (Eales, 1969) and marine (Nicol and Van Baalen, 1968) species indicate that guanine and hypoxanthine are the principal purines in the skin of most fish.

Pteridines in the skin of fish

The earliest reports of the occurrence of pteridines in fish are those of Fontaine and Busnel (1939) and Goodrich *et al.* (1941). Fontaine and Busnel found blue and violet fluorescent substances, which they believed to be pteridines, in the skin extracts from twenty-seven species of fish including two cyprinids, the carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*). Goodrich and

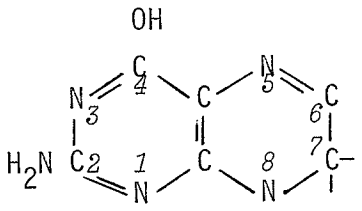
his colleagues reported one pteridine, which they suspected to be erythropterin, in the skin of several species of freshwater tropical species including two cyprinodonts, the swordtail (*Xiphophorus helleri*) and platyfish (*Platypoecilus maculatus*). Much of the attention of later investigators has been directed to the identification of the various pteridines in certain of the cyprinids and cyprinodonts, especially members of the families Cyprinidae, Cyprinodontidae and Poecilidae. The chemical structures and trivial names of some of the pteridines found in the skin of these fishes are summarized in Table I.

In the cyprinids the amounts of violet fluorescent pteridines are especially large and the quantities of blue fluorescent pteridines are rather small (Hama and Fukuda, 1964). Of the violet fluorescent pteridines in carp (*C. carpio*) and goldfish (*C. auratus*) ichthyopterin, isoxanthopterin (IXP) and isoxanthopterin-6-carboxylic acid (IXP-6-COOH) have been identified (Mori *et al.*, 1960). At least two other violet fluorescent pteridines of unknown structure are present and these have been named Cyprino-purple C1 and C2 (Mori *et al.*, 1960). Hama (1963) and Matsumoto (1965a) have reported that in these two species during the early larval stages a blue fluorescent pteridine, biopterin, is the first to be detected. In the later stages the pteridines appear in the sequence ichthyopterin, sepiapterin, Cyprino-purple C (C1 and C2), Hynobius blue, IXP and IXP-6-COOH. In the skin of adults, however, biopterin, Cyprino-purple C and Hynobius blue are no longer encountered.

TABLE I. Chemical structures of some of the pteridines reported in fish skin.

Compound	Pteridine structure* with substituents at C_6	C_7	C_7-N_8 bond	N_8	Synonym
2-Amino-4-hydroxypteridine (AHP)	-H	-H	double	nil	Pterin
2-Amino-4-hydroxy-6-carboxy-pteridine (AHP-6-COOH)	-COOH	-H	"	"	6-Carboxypteridine Ranachrome 5
Xanthopterin	-OH	-H	"	"	----------
Isoxanthopterin (IXP)	-H	-OH	"	"	Ranachrome 4 Cyprino-purple A1
Biopterin	$-CHOH.CHOH.CH_3$	-H	"	"	Ranachrome 1
Ichthyopterin	$-CHOH.CHOH.CH_3$	-OH	"	"	Cyprino-purple A2 7-Hydroxybiopterin
Hynobius-blue	unknown				----------
Ranachrome 3	$-OH.CH_3$	-H	"	"	----------
Cyprino-purple C	unknown				----------
Sepiapterin	$-CO.CHOH.CH_3$	$-H_2$	single	-H	Rhacophoro-jaune
Isosepiapterin	$-CO.CH_2.CH_3$	$-H_2$	"	-H	----------
Drosopterin	unknown				----------

* Pteridine structure:



In two other cyprinids, a kind of rudd (*Tribolodon hakuensis*) and the loach (*Misgurnus anguillicaudatus*), a very similar pattern of pteridine appearance occurs. In these two species, however, biopterin and Cyprino-purple C continue to occur in adult skin (Hama, 1963; Hama and Fukuda, 1964; Matsumoto, 1965a).

Cyprinodonts appear to have mainly blue fluorescent pteridines (Hama and Fukuda, 1964). The pteridines found in the larval stages of the Japanese medaka (*Oryzias latipes*) and swordtail (*X. helleri*) are biopterin, 2-amino-4-hydroxypteridine (AHP), 2-amino-4-hydroxypteridine-6-carboxylic acid (AHP-6-COOH), sepiapterin, IXP, Hynobius blue and drosopterins. In the skin of adults, sepiapterin, Hynobius blue and drosopterins disappear and Ranachrome-3, a blue fluorescent pteridine, appears (Hama, 1963; Matsumoto, 1965a).

The identification of pteridines in other species is less complete. The dorsal skin of eels (*Anguilla anguilla*) contains a number of pteridines including biopterin, ichthyopterin, IXP-6-COOH, and Ranachrome-5 (AHP-6-COOH) (Fontaine *et al.*, 1963). The skin of adults of five species of *Crenilabrus* contains Xanthopterin as the main pteridine (Ziegler, 1963). The belly skin of fry of coho salmon (*O. kisutch*) contains ichthyopterin, AHP-6-COOH, and 7,8-dihydroxanthopterin (Lee *et al.*, 1969).

Biosynthesis of purines

Nucleic acids in the diet are digested and their constituent

purines absorbed, but most of the tissue purines are derived from synthetic or endogenous sources rather than from preformed exogenous sources of the diet (Ganong, 1971; Harper, 1971). Studies on the biosynthesis of purines from simple precursors in bacteria, yeasts, birds and mammals suggest a common pathway involving the formation of purine nucleotides (purine base + sugar + phosphate group) followed by the liberation of the free base (Moat and Friedman, 1960; White *et al.*, 1968; Hartman, 1970; Harper, 1971). There is no evidence that free purines are synthesized in the tissues (West *et al.*, 1966; Harper, 1971).

A possible pathway for the biosynthesis of purines is shown in Fig. 2. The initial steps involve the formation of glycinamide ribotide, a nucleotide structure with glycine. Glycine is utilized in the intact form and contributes C-4, C-5, and N-7 of the evolving purine structure. Transformylation (step 4) followed by amidization (step 5) and ring closure (step 6) result in the formation of an imidazole ring. This imidazole progresses (step 7) to 5-amino-4-imidazole-N-succinyl carboxamide ribotide from which fumaric acid is split off (step 8) to give 5-amino-4-imidazole carboxamide ribotide. This latter compound is formylated (step 9) and ring closure (step 10) results in inosinic acid (Buchanan *et al.*, 1957; Greenberg and Jaenicke, 1957; Buchanan and Hartman, 1959; Hartman and Buchanan, 1959; Buchanan, 1960).

The synthetic pathway which has just been outlined leads to the production of hypoxanthine in the form of its nucleotide. The

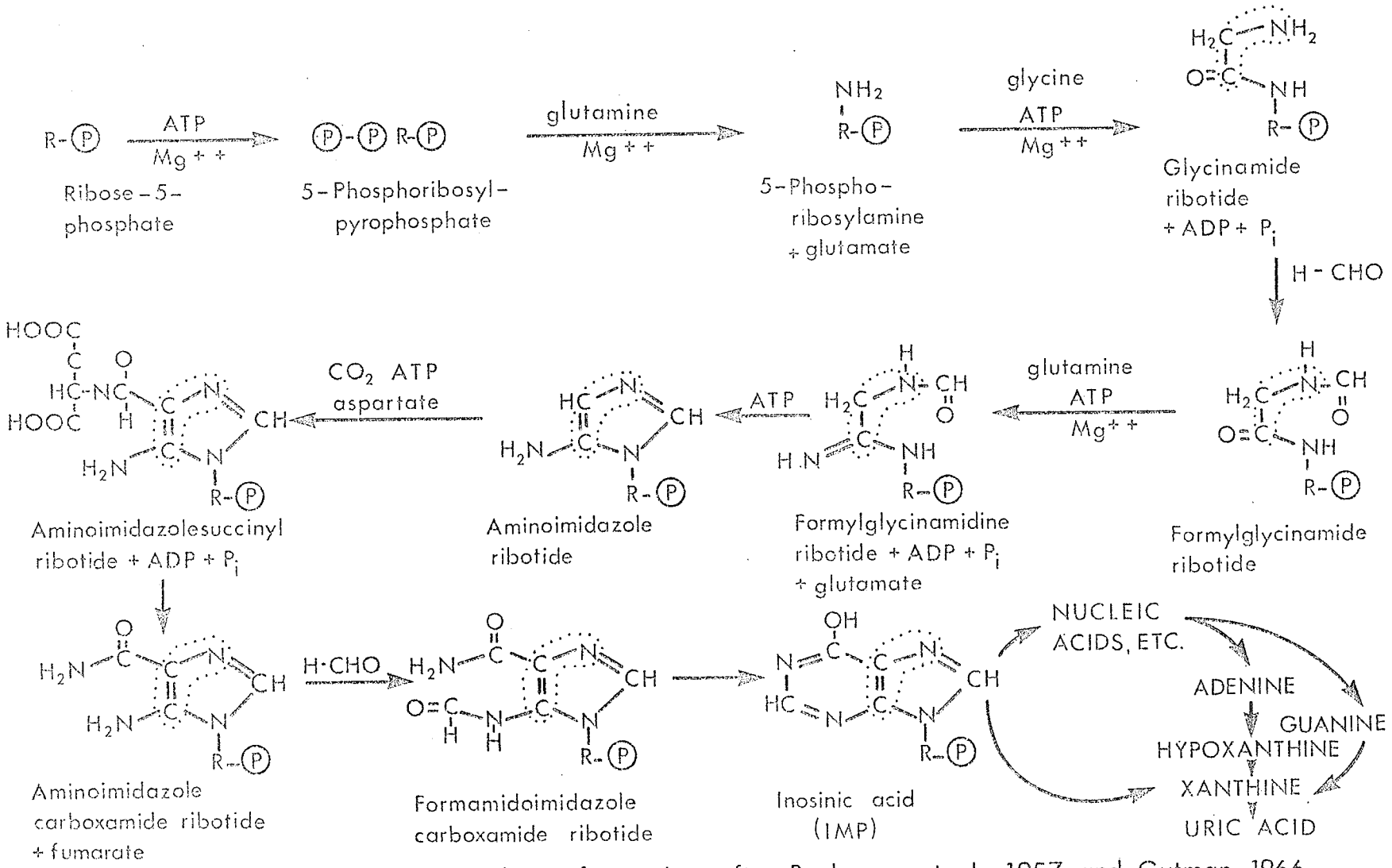


Fig. 2. The biosynthetic pathway for purines after Buchanan et al., 1957, and Gutman, 1966. Glycine is utilized in the intact form and contributes atoms 4, 5 and 7 of the purine ring. Inosinic acid the first purine to be formed may be converted by two general routes to free purine bases.

hypoxanthine nucleotide (IMP) may be transformed by two general routes to free purines (Fig. 2). The first leads chiefly to the formation of adenosine monophosphate (AMP) and guanosine monophosphate (GMP) which are then utilized for the synthesis of nucleic acids and important nucleotides such as ATP, GTP, ADP, the nicotinamide nucleotides, flavin-adenine dinucleotide and coenzyme A. These compounds are eventually degraded to free bases. Alternatively IMP may be converted directly to free purine bases. For example, it appears that many terrestrial animals (most reptiles, birds, insects and land snails) convert excess ammonia (from protein or amino acid metabolism) to IMP which is then degraded to hypoxanthine, xanthine and uric acid (Gutman, 1965; Baldwin, 1967).

It should be noted that the evidence for the pathway described above for the biosynthesis of free purines via IMP is derived from various experimental preparations, notably avian liver and microorganisms, and does not necessarily represent the established pathway in any one species (Harper, 1971). One report (Matty and Sheltawy, 1967) has stated that in rainbow trout "the early stages of guanine synthesis were similar to those for mammalian purine biosynthesis. Neither skin IMP or GMP were intermediates in the guanine and hypoxanthine biosynthesis which suggests some differences in the later stages of the synthetic pathway". Unfortunately, details of the study have not been published and are not available.

Biosynthesis of pteridines

Several investigators working with bacteria (McNutt, 1956; Vieria and Shaw, 1961; Reynolds and Brown, 1964; Shiota and Palumbo, 1965), insects (Weygand and Waldschmidt, 1955; Weygand *et al.*, 1959; Watt, 1967) and amphibians (Ziegler-Gunder *et al.*, 1956; Levy, 1964; Sugiura and Goto, 1968; Fukushima, 1970) have indicated that the pteridine ring is derived from the purine ring of a guanosine nucleotide. A plausible biosynthetic scheme is shown in Fig. 3. The first step in the pathway involves the elimination of carbon atom 8 from a guanosine nucleotide, probably guanosine triphosphate (Shiota and Palumbo, 1965; Watt, 1967; Fukushima, 1970). The carbon atom 8 is liberated as formic acid (Shiota and Palumbo, 1965; Burg and Brown, 1968). The other product of the ring-opening reaction is then supposed to undergo rearrangement followed by ring closure to give dihydroneopterin triphosphate (Burg and Brown, 1968; Fukushima, 1970). This initial pteridine is the precursor of both conjugated pteridines (those of the folic acid series that contain a *p*-aminobenzoylglutamine acid residue attached to the pteridine ring) and unconjugated pteridines (all others).

Very little is known about the interrelationships of the various pteridines. In fruit flies (*Drosophila melanogaster*), AHP was probably a precursor of IXP (Hubby and Throckmorton, 1960), sepiapterin was converted to biopterin (Tiara, 1961), and 6-hydroxymethylpteridine and reduced AHP were converted to sepiapterin and biopterin (Sugiura and Goto, 1968). In goldfish (*C. auratus*)

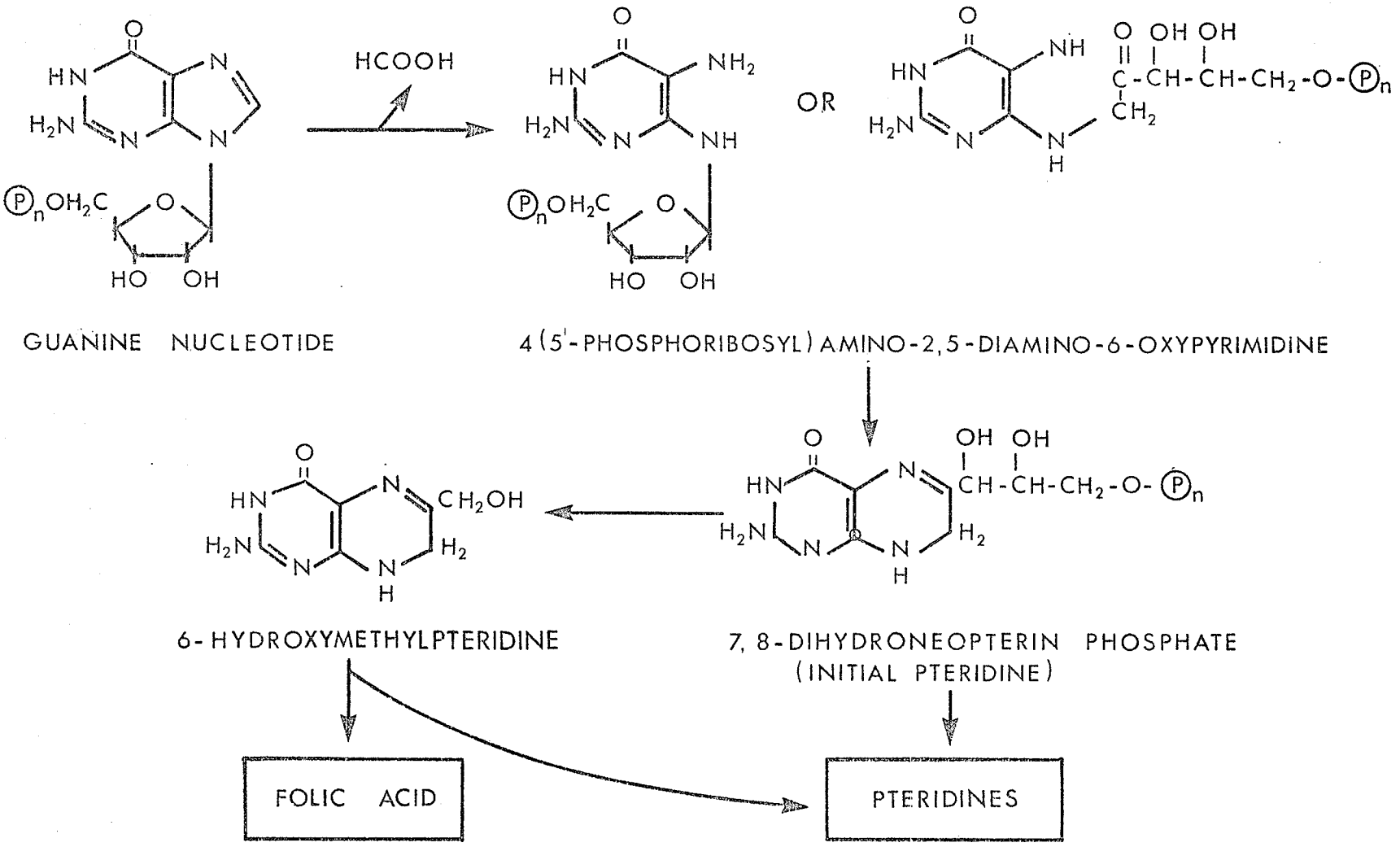


Fig. 3. Scheme for the biosynthesis of pteridines after Sugiura and Goto, 1968, and Forrest and Van Baalen, 1970. n = 1-3.

biopterin was converted to IXP through Ranachrome-3 (Hama *et al.*, 1960) and Cyprino-purple C to ichthyopterin (Matsumoto, 1965a). In the skin of tadpoles of the bull frog (*Rana catesbiana*) the initial pteridine, dihydroneopterin phosphate, was converted to IXP via reduced hydroxymethylpteridine and reduced AHP (Sugiura and Goto, 1968) and sepiapterin was found to be a precursor of biopterin (Fukushima, 1970).

Many of the pteridines found in insects and amphibians are found in fish and the various pathways, schematically represented in Fig. 4, probably represent fundamental steps in pteridine metabolism.

Induction of purine synthesis by hormonal treatment

Crude extracts and purified hormones from pituitary and thyroid glands have been used to induce silvering and purine deposition in certain salmonids. Silvering was induced in parr of Atlantic salmon (Landgrebe, 1941) and rainbow trout (Robertson, 1948) by injections of mammalian thyroid and pituitary extracts; in brown trout by injections of purified mammalian TSH (Smith, 1956); and in rainbow trout by prolonged immersion in thyroxine (Matty and Sheltawy, 1967). Prolonged immersion in thyroxine also increased skin purine synthesis and deposition in rainbow trout (Matty and Sheltawy, 1967). Injections of mammalian TSH and thyroid powder significantly increased the levels of dermal purines in brook trout (Chua and Eales, 1971).

Increased activity of the thyroid gland occurs in certain sal-

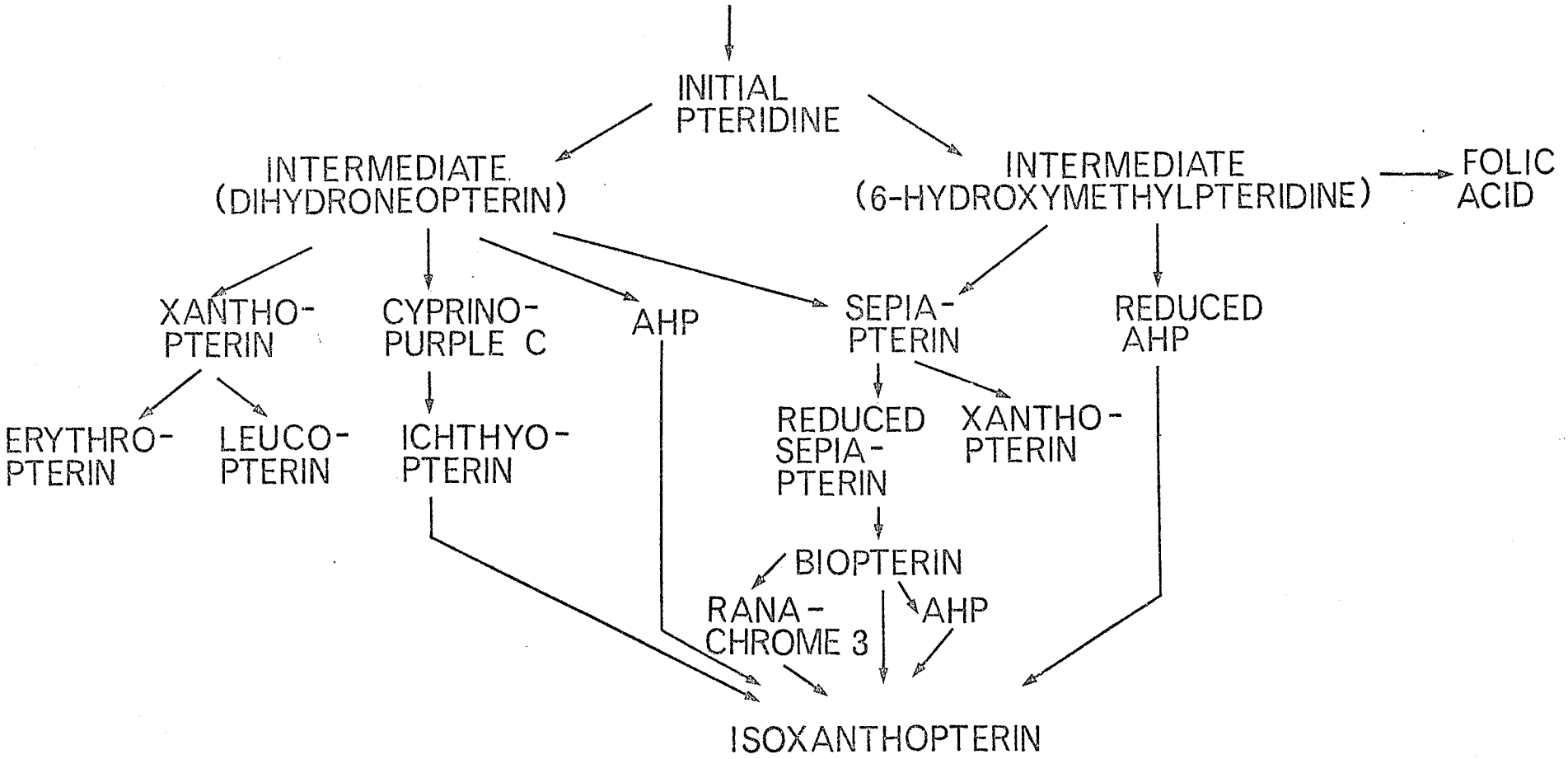


Fig. 4. Possible metabolic relationship among the pteridines after Matsumoto, 1965 a.

monids at the time of transformation from parr to silvery smolt (Hoar, 1939; Fontaine, 1948; Robertson, 1948), but the relationship between thyroid activity and integumentary purine metabolism remains unclear. Thyroid preparations administered to coho salmon sometimes induced marked purine deposition and at other times failed to do so (Hoar *et al.*, 1951). Injections of TSH induced hyperplasia of the thyroid of Atlantic salmon parr but failed to induce smoltification (Hoar *et al.*, 1951). Migrating chum and pink salmon, which become silvery prior to or during migration, have relatively inactive thyroids (Hoar and Bell, 1950).

Induction of pteridine synthesis by hormonal treatment

Crude extracts and purified hormones from the intermediate lobe of the pituitary gland have been used to induce melanogenesis in fish and amphibians (Bagnara and Neidleman, 1958; Bagnara, 1964; Lee and Lee, 1971) and pteridine deposition in amphibians (Bagnara, 1966; Stackhouse, 1966). Effects of the hormones (melanophore stimulating hormones, MSH) on pteridine synthesis in fish, however, have not been investigated.

Adrenocorticotropin has been found to induce melanogenesis (Chavin, 1956) and pteridine synthesis (Matsumoto, 1965a) in goldfish. It appears that ACTH, like MSH, acts directly on the chromatophores since addition of ACTH to pieces of caudal fin of goldfish in tissue culture stimulated melanogenesis (Chavin, 1956). The ability of ACTH

to stimulate chromatophores seems to be due to its partial similarity to MSH. They have in common the heptapeptide, Met-Gly-His-Phe-Arg-Thy-Gly (Lee and Lerner, 1959).

Chromatophores, their associated purines and pteridines, and changes in skin coloration

In amphibians, xanthophores are located immediately below the basal lamella of the dermis. Iridophores are found beneath the xanthophores. Under each iridophore is found a melanophore from which processes extend upward around the iridophore. Finger-like structures project from these processes and occupy fixed spaces between the xanthophores and iridophores (Bagnara *et al.*, 1968; Bagnara and Hadley, 1969). The same type of chromatophore arrangement is probably found in fishes (Kawaguti, 1965; Matsumoto, 1965b; Kawaguti and Kamishima, 1966).

In amphibians and fishes, purines and pteridines are not located within the same chromatophores. Purines occur in iridophores. Pteridines are found within melanophores, xanthophores and erythrophores (Hama, 1963; Hama and Fukuda, 1964; Hama *et al.*, 1965; Matsumoto, 1965a, 1965b; Obika and Matsumoto, 1968).

Changes in skin coloration may be caused by movements of pigment granules within chromatophores. Darkening of the skin may be brought about by the dispersion and upward movement of the melanin from the body of the melanophore to fill the fingers which obscure the under-

lying iridophore with its light-reflecting purine crystals. A paling of the skin is accomplished by the movement of the granules from the fingers (Fingerman, 1965; Bagnara and Hadley, 1969).

The relative state of dispersion or aggregation of the pigments is also associated with variations in the amount of pigments within chromatophores. Prolonged aggregation correlates with a loss of pigment and conversely prolonged dispersion with an increase in the quantity of pigment (Waring, 1963). It has been suggested that in the aggregated state, when pigment granules are quite crowded together fewer synthetic sites are available than in the dispersed state (Waring, 1963; Bagnara, 1966).

In hypophysectomized amphibian tadpoles it was observed that the pigments in iridophores were dispersed and purine synthesis increased while pigments in melanophores (and probably xanthophores) were aggregated and pteridine synthesis decreased. When such hypophysectomized animals were treated with MSH chromatophores reverted to their former state, pteridine synthesis was restored to its former level, and purine synthesis was decreased (Bagnara, 1961, 1966; Stackhouse, 1966). In order to account for the observed purine-pteridine relationship, Bagnara (1966) has suggested that in view of the possible close link in the biosynthetic pathways of purines and pteridines there is some sort of competition for common precursors. When pigment granules in iridophores are aggregated synthetic sites on the granules are less accessible and precursors common to both purine and pteridine synthesis become available to the pteridine biosynthetic system of the other chromatophores.

The result is a decrease in purine synthesis and an increase in pteridine production. On the other hand, when iridophore pigments are dispersed purine synthesis is favoured at the expense of pteridine production.

MATERIALS AND METHODS

Living material

One-year-old rainbow trout (*Salmo gairdneri* Richardson) and brook trout (*Salvelinus fontinalis* Mitchill) from the Province of Manitoba Trout Hatchery, West Hawk Lake, were maintained at the University in dechlorinated Winnipeg City water and fed daily (approx. 1.5% of wet body weight) on Ewos Trout Grower (F141) pellets (Astra-Ewos, A.B., Sodertalje, Sweden).

Identification of purines and pteridines

The fish were killed by a sharp blow to the head, frozen in liquid nitrogen, and skin from directly below the dorsal fin (Fig. 5) removed from the sides of the animals (Chua and Eales, 1971). The skin sections were extracted with 70% ethanol (Obika, 1963; Stackhouse, 1966), 10% trichloroacetic acid (TCA) (Matsumoto, 1965a; Stackhouse, 1966), or 0.5M NH_4OH (Lee *et al.*, 1969). For extraction with alcohol, the skin was ground (mortar and pestle and a little fine sand) in a volume of alcohol twenty times that of the wet weight of the skin. The homogenate was heated in a water bath at 75°C for 20 minutes and centrifuged at 2000 *g* for 15 minutes. The supernatant was mixed with chloroform three times its volume and centrifuged. The aqueous layer, containing the purines and pteridines, was used for chromatography.

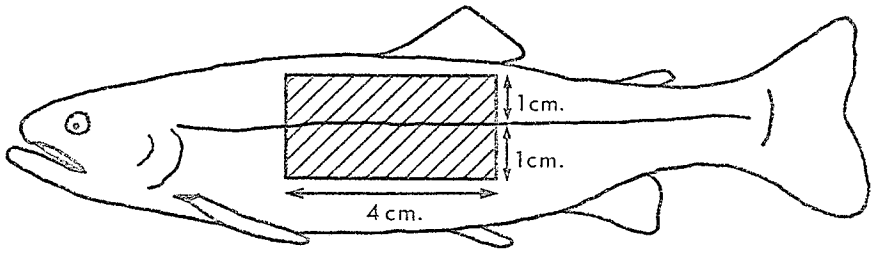


Fig. 5. Sketch of a rainbow trout showing designated area of skin removed for analysis.

For extraction with 10% TCA, the skin was ground in a volume of TCA twenty times that of the wet weight of the skin. The homogenate was heated in a water bath at 70° C for 20 minutes and centrifuged. The supernatant was washed three times with ether at a volume ratio of 1:1. For extraction with 0.5*M* NH_4OH, the skin was cut up into small pieces, placed in NH_4OH so that 1 ml contained approximately 125 mm^2 of skin, and left for 12 hr at room temperature with continual mixing on a Fisher Roto Rack. The extract was centrifuged and the supernatant used for chromatography.

To prevent photolysis of certain pteridines, extraction and chromatography were carried out in a dark room under a photographic safety light (Matsumoto, 1965a; Stackhouse, 1966).

For paper chromatography, a portion of the extract (0.1 to 0.2 ml) was applied along a two-inch line on Whatman No. 3 MM chromatography paper. The sample was applied in 10-µl volumes and was dried between applications to ensure a narrow strip. Five to 100 µl of 5m*M* solutions of adenine, guanine, hypoxanthine, xanthine, adenosine, guanosine, inosine, xanthosine, adenosine-5′-monophosphate, guanosine-5′-monophosphate, inosine-5′-monophosphate, xanthosine-5′-monophosphate, pterin (2-amino-4-hydroxypteridine), pterin-6-carboxylic acid, biopterin, xanthopterin, isoxanthopterin (Sigma Chemical Co., Sigma grade), pterin-7-carboxylic acid and leucopterin (Aldrich Chemical Co.) were spotted as standards.

Chromatograms were developed ascendingly or descendingly in *n*-propanol-1% ammonia (2:1 v/v); upper organic layer, *n*-butanol-acetic acid-water (4:1:5 v/v/v); methanol-concentrated HCl-water (7:2:1 v/v/v)

or 3% ammonium chloride (Matsumoto, 1965a; Stackhouse, 1966; Lee *et al.*, 1969). Chromatographic development was performed in Shandon 500 glass chromatographic tanks (Consolidated Ltd., Weston, Ontario).

Developed chromatograms were air-dried at room temperature and scanned under ultraviolet radiation at 254 mμ and 365 mμ (UV chromatogram Viewer, Fisher Scientific Co.) for UV-absorbing and -fluorescing spots. The substances in the spots from the extract were tentatively identified by comparing (i) their colour of fluorescence or absorbance and (ii) their relative rates of flow (R_f) with those of the standards.

Two-dimensional thin-layer chromatography (TLC) was also employed in the analysis of the extracts. Two to 10 μl of the extract or standards were applied in the form of a single spot to Eastman 6064 cellulose thin-layer sheets. The material was applied in 0.5-μl volumes, with drying in between applications to ensure a small spot. The chromatograms were developed ascendingly at room temperature in TLC glass jars or in glass sandwich chambers (Eastman Chromatogram Developing Apparatus 6071). Development in the first direction was with *n*-propanol -1% ammonia (2:1) and in the second direction with *n*-butanol-acetic acid-water (4:1:5).

To confirm the identities of the extracted compounds, UV-absorption spectra of those that were sufficiently concentrated were compared with the UV-absorption spectra of authentic compounds. For this purpose, extracts and solutions of authentic compounds were spotted in narrow streaks and developed twice on the same paper with *n*-propanol-1% ammonia (2:1) by one-dimensional ascending rechromatography (Matsumoto, 1965a). Substances were removed from the filter paper by cutting the paper into

narrow strips and eluting descendingly with glass-distilled water in an atmosphere saturated with water vapour (Matsumoto, 1965a; Stackhouse, 1966). Each eluate was concentrated and rechromatographed with *n*-butanol-acetic acid-water (4:1:1) or methanol-concentrated HCl-water (7:2:1). Each spot, with a reference blank (an area equal in size and corresponding to the same R_f as the spot), was cut from the chromatogram, eluted in 0.1*N* HCl or 0.1*N* NaOH and the UV-absorption spectrum between 360 and 220 mμ determined with a SP 800 Spectrophotometer (Unicam Instruments Ltd.).

Estimation of skin purine and pteridine concentrations

Sections of skin directly below the dorsal fin, 1 cm above and 1 cm below the lateral line and 4 cm in length, were removed (Fig. 5). The skin was weighed, cut up into small pieces, placed into 12-ml centrifuge tubes containing 5 ml of 10% TCA and left for 12 hr at room temperature with continual mixing on a Fisher Roto Rack. The tubes were centrifuged, the supernatant saved and the tissue extracted a second time with 5 ml of TCA for 3 hr. The supernatants were combined and washed three times with equal volumes of ether, the ether layer being discarded at each washing. The resulting aqueous extract was transferred to a graduated cylinder and the volume recorded.

Aliquots of the extract were applied as narrow streaks and developed twice on the same paper with *n*-propanol-1% ammonia (2:1) by one-dimensional ascending rechromatography. Purine compounds were

eluted in 0.1*N* HCl and the concentration of the eluate determined by comparing the extent of absorbance to standard curves (Fig. 6). Pteridines were eluted with glass-distilled water. To each eluate an equal volume of citric acid-phosphate buffer, pH 5, was added (Matsumoto, 1965a; Stackhouse, 1966) and its concentration estimated by comparing fluorescence intensity at 410 mμ to standard curves (Fig. 7, 8 and 9). The degree of fluorescence was determined with a Unicam SP 800 Spectrophotometer equipped with fluorescence attachment.

The concentration of purines and pteridines in the skin was estimated by substituting the appropriate values in the following equation:

$$\text{concentration } (\text{mg/cm}^2 \text{ skin}) = \frac{\text{conc. of eluate (mg/ml)} \times \text{vol. of eluate (ml)} \times \text{vol. of extract (ml)}}{\text{vol. of extract used (ml)} \times \text{area of skin (cm}^2\text{)}}$$

Concentrations were expressed as mg/cm^2 as this form of reference is not affected by any change in skin thickness which might be the result of hormonal treatment (Sembrat, 1956).

For comparison with values obtained by other investigators, the concentrations of purines were expressed as mg/g wet tissue. These values were readily calculated since the wet weight of the tissue used was known. In the case of the pteridines, it was necessary to express the concentrations on the basis of dry weight of tissue. In order to do this, measured areas of skin adjacent to the portion of skin that was used for analysis were removed and the dry weight (mg/cm^2) determined. This value was used to estimate the dry weight of the skin analyzed.

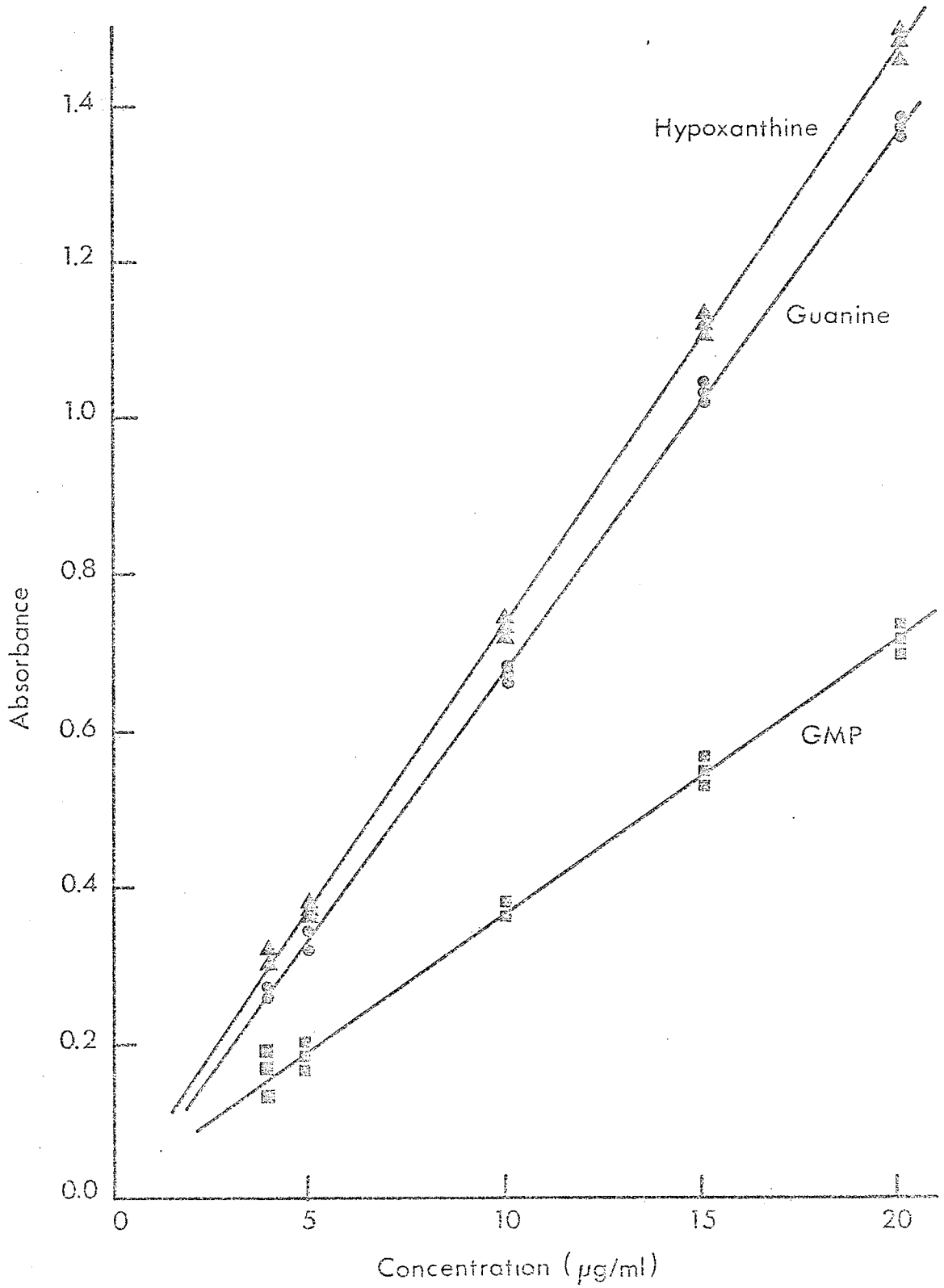


Fig. 6. Relation between concentration and absorbance of hypoxanthine (at 246 nm), guanine (at 248 nm) and GMP (at 255 nm) in 0.1 N HCl.

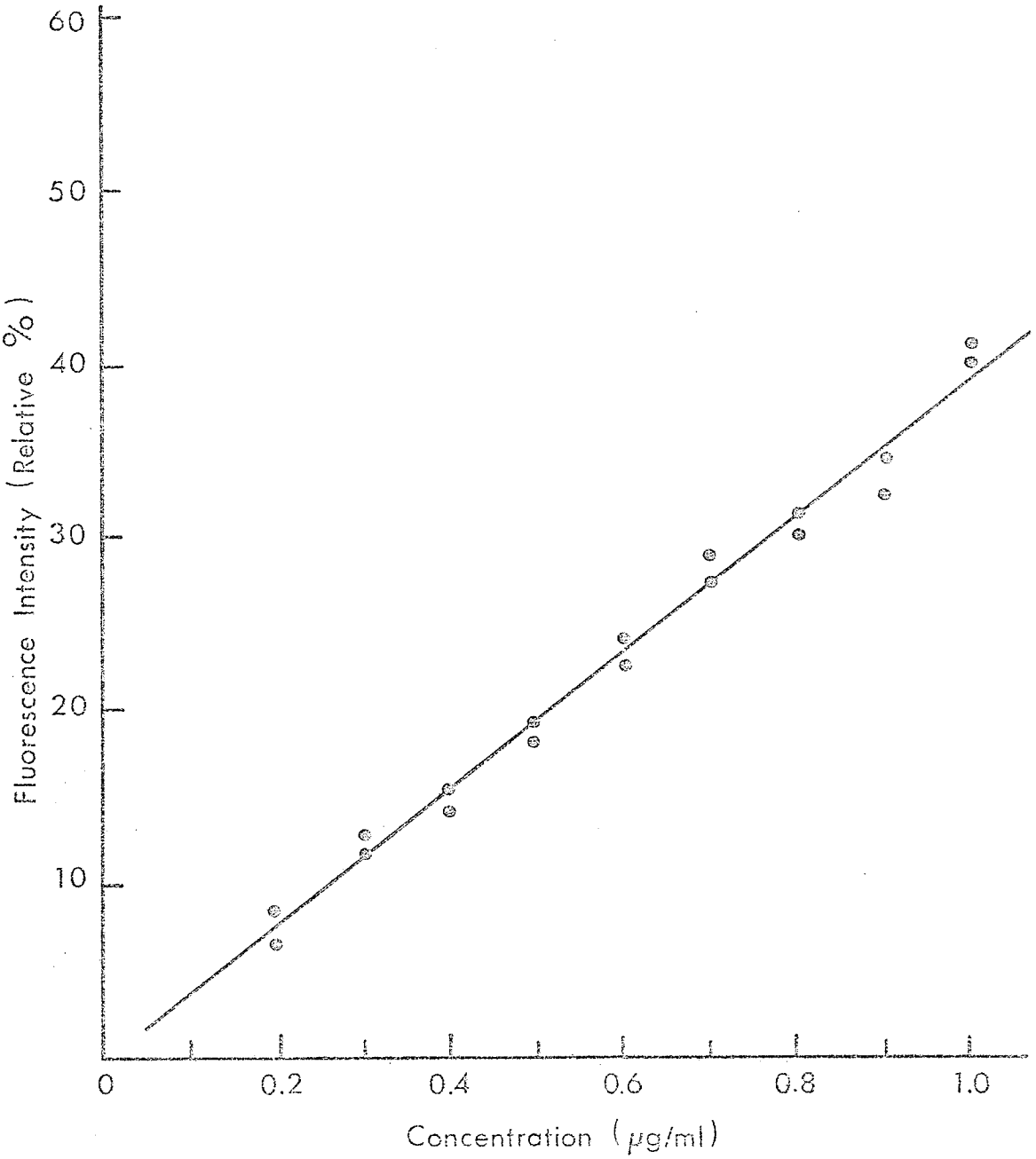


Fig. 7. Standard curve of AHP at 410 nm in citric acid–phosphate buffer, pH 5.

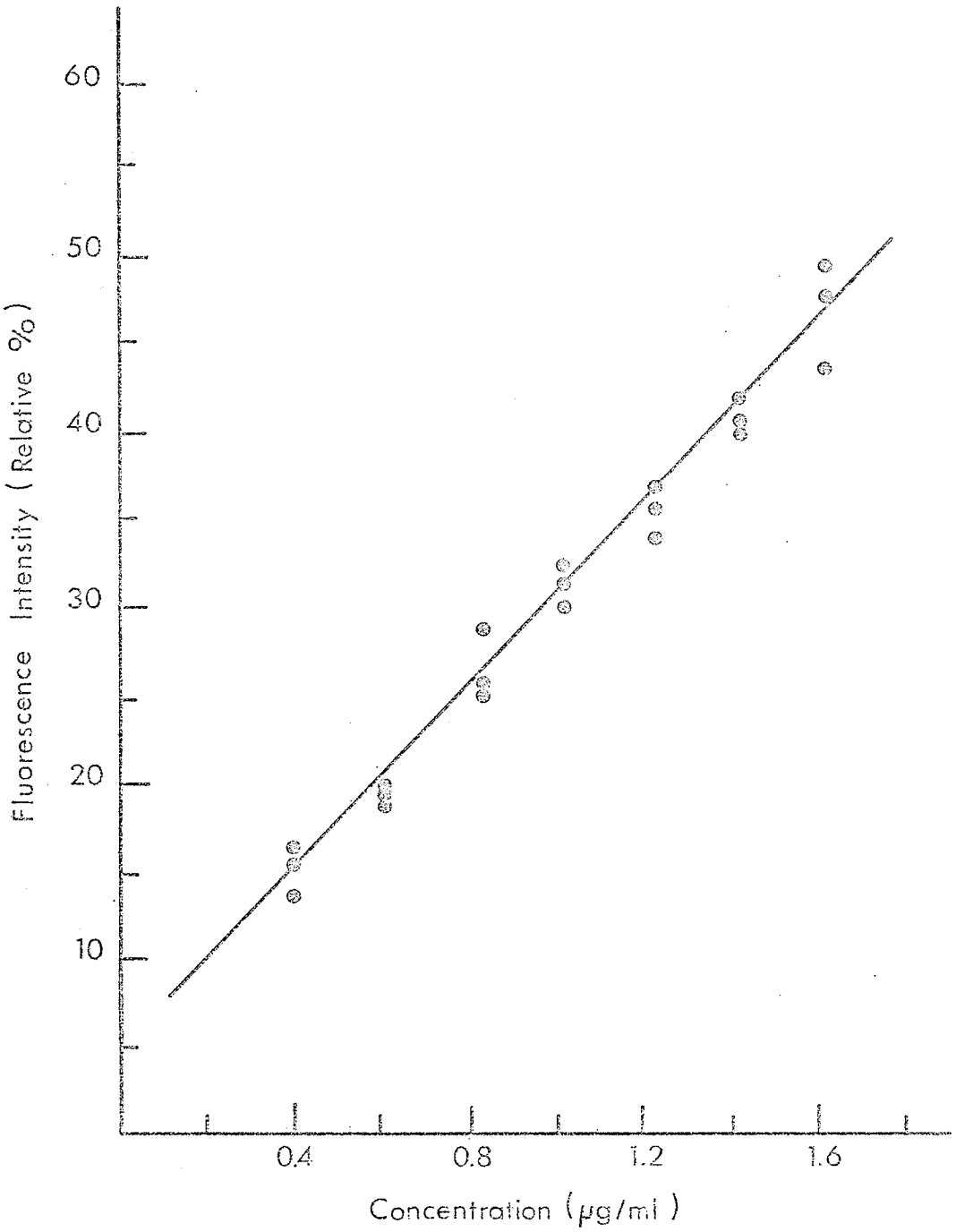


Fig. 8. Standard curve of AHP-6-COOH at 410 nm in citric acid – phosphate buffer, pH 5.

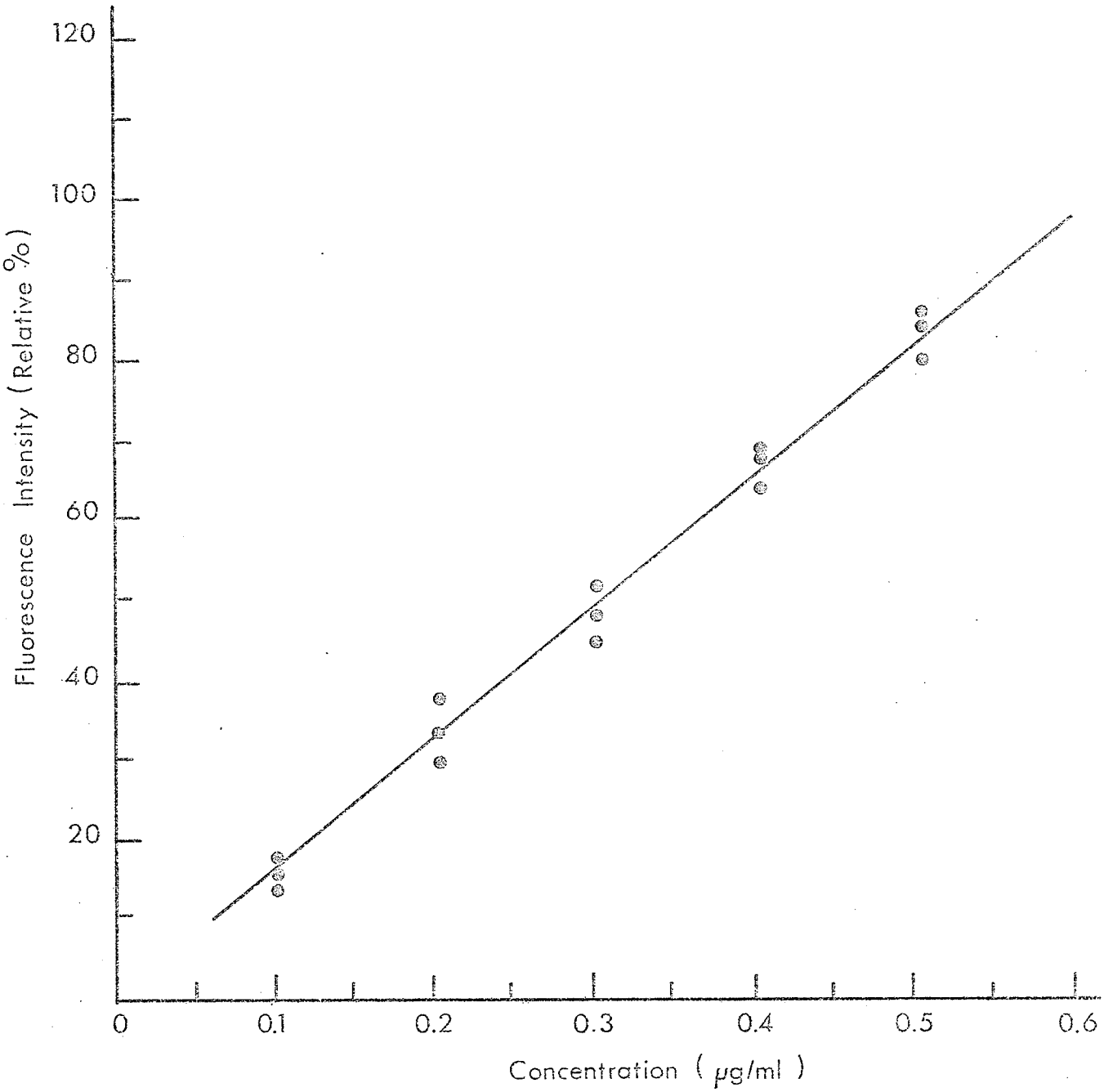


Fig. 9. Standard curve of IXP at 410 nm in citric acid–phosphate buffer, pH 5.

Preliminary work to test the completeness of extraction of purines and pteridines indicated that extracting twice with 5 ml of 10% TCA was sufficient for removing the purines and pteridines from the skin, since extraction for a third time with TCA did not reveal any more purines or pteridines. However, at the end of the first experiment (with TSH-treated fish) the amounts of purines recovered were low when compared with extraction with 1*N* HCl. Further tests showed that if skin already twice-treated with 10% TCA were further treated with 1*N* HCl additional small amounts of guanine and hypoxanthine could be extracted. In the later experiments, extraction with 10% TCA was followed by extraction with 1*N* HCl.

It was reasonable to expect that some of the compounds extracted from the skin would be lost during separation procedures, especially in view of Matsumoto's findings (1965a) that when pteridines were added to fish tissue (boiled liver) and recovered by TCA extraction and paper chromatography, the recovery averaged approximately 70 per cent. To determine the extent of loss, solutions with known amounts of purines (0.1 to 1.0 mg) and pteridines (0.025 to 0.1 mg) were added to boiled fish liver and the per cent recovery determined. Boiled liver rather than skin was used as boiled liver contained no detectable (by paper chromatography) purines or pteridines but boiled skin still contained guanine and hypoxanthine. The per cent recovery was 91.8 ± 1.6 for guanine, 89.1 ± 1.3 for hypoxanthine, 88.8 ± 1.3 for GMP, 72.4 ± 1.5 for AHP, 72.2 ± 1.9 for IXP, and 69.3 ± 1.6 for biopterin recovered as AHP-6-COOH (see Appendix III).

Induction of purine and pteridine synthesis

Three experiments were performed with rainbow trout to determine changes in the levels of skin purines and pteridines under conditions favouring either purine or pteridine synthesis. In each experiment, twenty rainbow trout, in groups of ten each, were kept in covered 20-litre green polyethylene pails with running water at 12 ± 1°C. Injections of hormone (treated group) or of solvent only (control group) were administered every other day. The preparations were injected into the abdominal cavity, through the ventral belly surface anterior (2 to 3 mm) to the pelvic fins, in volumes that never exceeded 0.05 ml with disposable 1-ml tuberculin syringes. For each injection the fish were anaesthetized in a solution of tricaine methanesulfonate (1:20,000 w/v). The fish were fed daily (Ewos pellets, approx. 1.5% wet body weight) throughout the experiments.

To induce purine deposition, 2 mIU bovine TSH/g body wt was administered on alternate days. This dose of TSH was tried for although minimum levels of TSH effective in inducing purine deposition are not known, it was known that this low level of hormone was capable of stimulating the thyroid of rainbow trout (Hickman, 1962; McNichol, unpublished data). In one experiment (March 10 to April 1, 1971) a total of 11 injections of TSH (Sigma Chemical Co.) suspended in solvent (10 ml solution containing 0.03 g phenol and 0.15 g dextrose) was administered. In another experiment (June 14 to July 10, 1971) a total of 14 injections of TSH (Nordic Biochemicals) was administered. Controls received a corresponding volume (0.04 ml) of solvent at appropriate times.

For the induction of pteridine synthesis, fish were injected with ACTH (Nutritional Biochemicals). The dose, 2 IU/injection every other day for three weeks (June 19 to July 6, 1971), was based on that used for induction of pteridine synthesis in goldfish (Matsumoto, 1965a). Controls received 0.05 ml of 0.6% saline (prepared with sterile water) at appropriate times (Chavin, 1956).

Incorporation of ^{14}C-glycine into skin purines and pteridines

The experiments to follow changes in purine and pteridine levels under different treatments were coupled with experiments to follow the incorporation of a labelled precursor, ^{14}C-glycine, into these compounds. Twenty-four hours after the last hormone or sham treatment, each fish was injected intraperitoneally with 2.5 µCi of ^{14}C-glycine (Amersham Radiochemicals). Six hours later the fish were killed and the skin analyzed as previously described. Preliminary tests had confirmed the finding of Sheltawy (1967) that the radioisotope was extensively incorporated into skin purines within two to six hours. These tests also showed that pteridines rapidly incorporated the radioisotope.

Radioactivity measurements were made with a Packard Tri-carb Scintillation Spectrometer, Model 3200. Up to 0.4 ml of eluate (with purine or pteridine compound) was added to 10 ml of scintillation fluor, consisting of naphthalene (60 g), POP (4 g), POPOP (0.2 g), methanol (100 ml), ethylene glycol (20 ml) and *p*-dioxane (to make 1 litre) (Bray, 1960). Optimal counter settings were at a pulse height of 10-1000

divisions and 15% gain (see Appendix IV). Efficiency, as determined with standard *n*-hexadecane-^{14}C, was 83% (see Appendix V). Quenching was determined by the external standard method and was found to be negligible.

Statistical Analysis

Comparisons between control and experimental groups were made using Student's *t* test (Snedecor, 1956).

OBSERVATIONS AND RESULTS

Identification of skin purines and pteridines

Paper chromatography of rainbow trout skin extracts in four solvent systems revealed three UV-absorbing and four UV-fluorescing spots (Table II). The spots on chromatograms of 0.5*M* NH_4OH and 70% alcohol extracts were fewer in number and smaller in size than spots on chromatograms of 10% TCA extracts. Typical chromatograms in one solvent system are shown in Fig. 10. Absorbance or fluorescence of spots on chromatograms of 0.5*M* NH_4OH and 70% alcohol extracts were less intense than corresponding spots on chromatograms of 10% TCA extracts.

The color of fluorescence or absorbance and R_f values (Table II) indicated that the UV-absorbing substances were guanine, hypoxanthine and GMP and that three of the UV-fluorescing substances were biopterin, AHP and IXP. The fourth UV-fluorescing spot had R_f values similar to those reported (Hama *et al.*, 1960; Mori *et al.*, 1960) for ichthyopterin.

Two-dimensional thin-layer chromatography of the extracts revealed, in addition to the seven substances mentioned above, traces of others (Fig. 11). All extracts (70% alcohol, 0.5*M* NH_4OH and 10% TCA) contained trace amounts of two substances with R_f values corresponding to those for adenine and AHP-6-COOH. Traces of two other UV-absorbing substances whose R_f values did not correspond to any of the authentic purine compounds were found in 10% TCA extracts (Fig. 11).

TABLE II. R_f values and color of fluorescence (f) or absorbance (q) of standard compounds and substances from the skin of trout.

Method of separation: Ascending paper chromatography in *n*-propanol-1% ammonia (2:1)

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			0.5*M* NH_4OH extract			10% TCA extract			10% TCA extract		
	R_f	f	q	R_f	f	q	R_f	f	q	R_f	f	q	R_f	f	q
Adenine	.63		v	.55		v	.55		v	.55		v	.55		v
Adenosine	.63		v	.50	b		.50	b		.50	b		.43	b	
Hypoxanthine	.55		v												
Xanthine	.51		v	.43	b		.38		v	.43	b		.38		v
AHP	.50	b		.38		v	.25	v		.38		v	.25	v	
Biopterin	.43	b													
Xanthosine	.39		v	.31	v					.32	v		.18	b	
Guanine	.38		v	.25	v					.25	v				
Guanosine	.38		v												
Uric acid	.31		v							.14		v			
Isoxanthopterin	.25	v													
AMP	.23		v												
Xanthopterin	.21	y													
Leucopterin	.21	b													
AHP-7-COOH	.19	y													
AHP-6-COOH	.18	b													
IMP	.16		v												
GMP	.14		v												
XMP	.12		v												

Color of fluorescence or absorbance indicated by b = blue, v = violet, y = yellow-green.

TABLE II cont'd

Method of separation: TLC in *n*-propanol-1% ammonia

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			0.5*M* NH_4OH extract			10% TCA extract			10% TCA extract		
	Rf	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q
Adenine	.68		v	.68		v	.68		v	.68		v	.68		v
Inosine	.67		v	.66		v	.66		v	.66		v	.66		v
Hypoxanthine	.66		v												
Xanthosine	.59		v	.48	b		.48	b		.48	b		.45	b	
Guanosine	.55		v	.45	b		.45	b		.45	b		.43		v
AHP	.48	b													
Uric acid	.45		v	.43	v	v	.43	v	v	.43	v	v	.34		v
Biopterin	.45	b		.34	v	v	.34	v	v	.34	v	v	.29	v	
Guanine	.43		v												
AMP	.40		v	.29	v		.29	v		.29	v		.21	v	
Xanthine	.39		v	.21	v		.21	v		.21	v		.20	b	
IMP	.36		v												
GMP	.34		v										.18	y	
XMP	.33		v												
Isoxanthopterin	.21	v													
Leucopterin	.21	b													
AHP-6-COOH	.20	b													
Xanthopterin	.18	y													
AHP-7-COOH	.16	y													

Color of fluorescence or absorbance indicated by b = blue, v = violet, y = yellow-green.

TABLE II cont'd

Method of separation: Ascending paper chromatography in *n*-butanol-acetic acid-water (4:1:5)

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			0.5*M* NH_4OH extract			10% TCA extract			10% TCA extract		
	Rf	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q
Adenine	.56		v	.50	b		.50	b		.50	b		.50	b	
Biopterin	.50	b		.40		v	.40		v	.40		v	.40		v
Adenosine	.41		v												
Hypoxanthine	.40		v	.38	b		.38	b		.38	b		.27	v	v
AHP	.38	b		.27	v	v	.27	v	v	.27	v	v	.22	b	
Xanthopterin	.37	y													
Xanthine	.29		v												
Guanine	.27		v												
Isoxanthopterin	.27	v													
AHP-6-COOH	.22	b													
Inosine	.22		v												
Guanosine	.21		v												
Uric acid	.18		v												
Xanthosine	.18		v												
AHP-7-COOH	.17	y													
Leucopterin	.13	b													
AMP	.06		v												
GMP	.04		v												
IMP	.03		v												
XMP	.02		v												

Color of fluorescence or absorbance indicated by b = blue, v = violet, y = yellow-green.

TABLE II cont'd

Method of separation: TLC in *n*-butanol-acetic acid-water (4:1:5)

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			0.5*M* NH_4OH extract			10%TCA extract			10% TCA extract		
	Rf	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q
Adenine	.54		v	.54		v	.54		v	.54		v	.54		v
Biopterin	.52	b		.52	b		.52	b		.52	b		.52	b	
AHP	.50	b													
Xanthopterin	.46	y		.50	b		.50	b		.50	b		.46	y	
Hypoxanthine	.44		v	.44		v	.44		v	.44		v	.44		v
Adenosine	.42		v												
Xanthine	.32		v	.30	v		.30	v		.30	v		.31	b	
AHP-6-COOH	.31	b		.25		v	.25		v	.25		v	.30	v	
Isoxanthopterin	.30	v													
Guanine	.26		v	.06		v	.06		v	.06		v	.25		v
Leucopterin	.26	b											.06		v
Inosine	.25		v												
Guanine	.25		v												
AHP-7-COOH	.22	y													
Xanthosine	.19		v												
Uric acid	.16		v												
GMP	.06		v												
AMP	.05		v												
IMP	.04		v												
XMP	.02		v												

Color of fluorescence or absorbance indicated by b= blue, v= violet, y= yellow-green.

TABLE II cont'd

Method of separation: Descending paper chromatography in methanol-conc. HCl-water (7:2:1)

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			0.5*M* NH_4OH extract			10% TCA extract			10% TCA extract		
	Rf	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q
Biopterin	.55	b		.55	b		.55	b		.55	b				
AHP	.45	b		.45	b		.45	b		.45	b				
AMP	.45		v	.33		v	.33		v	.42		v			
GMP	.42		v	.25	v		.25	v		.33		v			
Adenine	.34		v	.20		v	.20		v	.25	v				
Hypoxanthine	.33		v							.20		v			
AHP-6-COOH	.32	b													
IMP	.31		v												
Xanthine	.29		v												
XMP	.29		v												
Xanthopterin	.29	y													
Isoxanthopterin	.25	v													
Guanine	.20		v												

Color of fluorescence or absorbance indicated by b = blue, v = violet, y = yellow-green.

TABLE II cont'd

Method of separation: Ascending paper chromatography in 3% ammonium chloride

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			0.5*M* NH_4OH extract			10% TCA extract			10% TCA extract		
	Rf	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q	*Rf*	f	q
AHP-7-COOH	.55	y								.54		v			
Adenine	.54		v							.48		v			
Adenosine	.51		v							.44	b				
Inosine	.49		v							.38		v			
Hypoxanthine	.48		v							.35	v				
AHP-6-COOH	.46	b								.31	b				
AHP	.44	b								.25		v			
Xanthopterin	.44	y													
Xanthine	.42		v												
Guanine	.38		v												
Uric acid	.36		v												
AMP	.36		v												
Xanthosine	.35		v												
Isoxanthopterin	.35	v													
IMP	.34		v												
Guanosine	.32		v												
Biopterin	.31	b													
GMP	.25		v												

Color of fluorescence or absorbance indicated by b = blue, v = violet, y = yellow-green.

TABLE II cont'd

Method of separation: TLC in 3% ammonium chloride

Standard compounds				Rainbow trout									Brook trout		
				70% alcohol extract			$0.5M$ NH_4OH extract			10% TCA extract			10% TCA extract		
	Rf	f	q	Rf	f	q	Rf	f	q	Rf	f	q	Rf	f	q
AHP-7-COOH	.50	y								.47		v			
AHP-6-COOH	.48	b								.41	b	v			
Adenine	.47		v							.38	v	v			
Adenosine	.47		v							.35	b				
Inosine	.45		v							.21		v			
IMP	.45		v												
Hypoxanthine	.41		v												
AHP	.41	b													
Xanthopterin	.41	y													
Isoxanthopterin	.39	v													
Xanthosine	.38		v												
Guanine	.38		v												
Biopterin	.36	b													
Guanosine	.35		v												
AMP	.30		v												
Xanthine	.27		v												
GMP	.21		v												

Color of fluorescence or absorbance indicated by b = blue, v = violet, y = yellow-green.

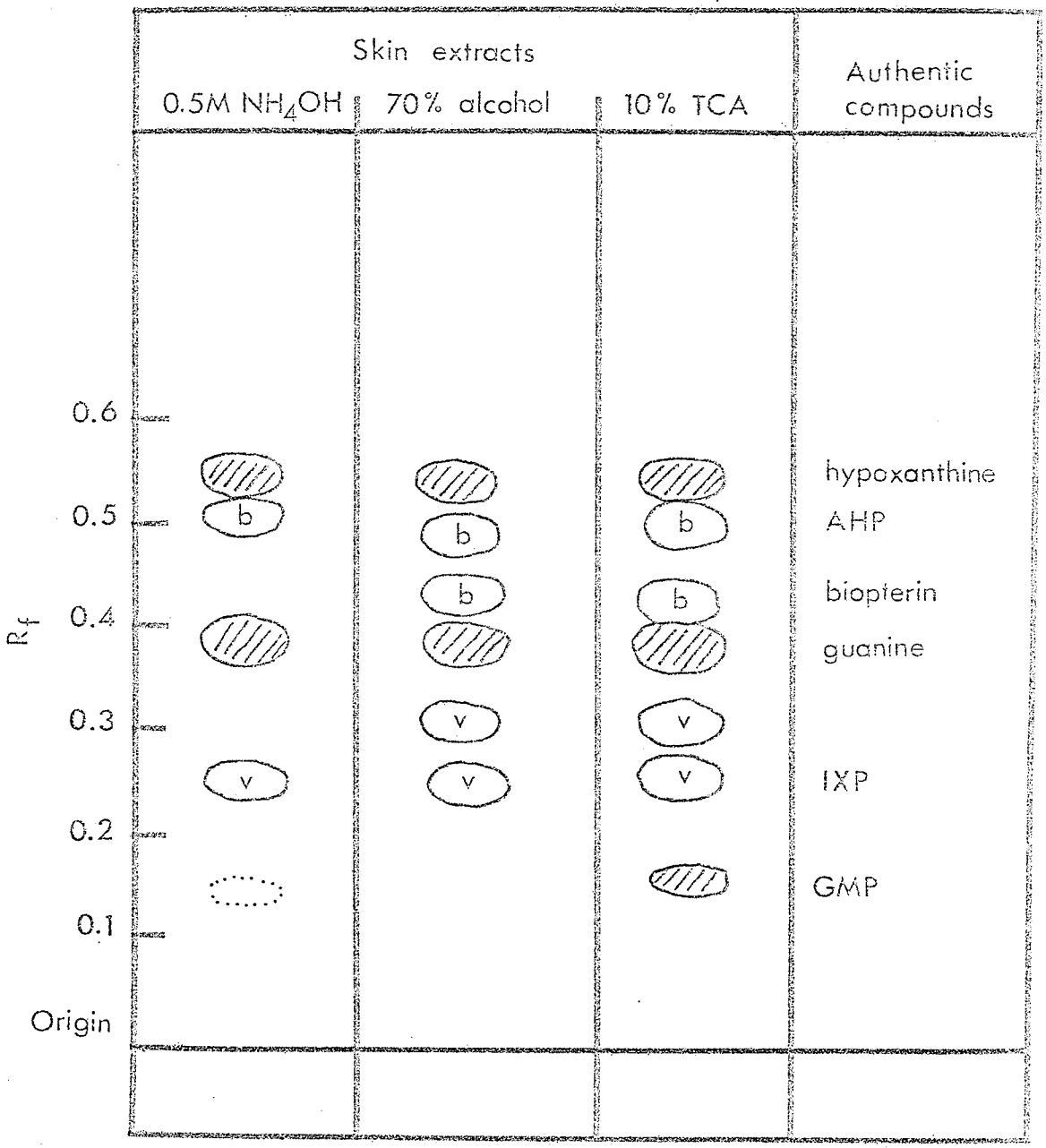


Fig. 10. Paper chromatograms of rainbow trout skin extracts developed ascendingly in solvent system n-propanol-1% ammonia (2:1). UV-absorbing substances shown in hatched enclosures; UV-fluorescing substances in unhatched enclosures. Color of fluorescence, at 310 nm, is indicated by b (blue) and v (violet).

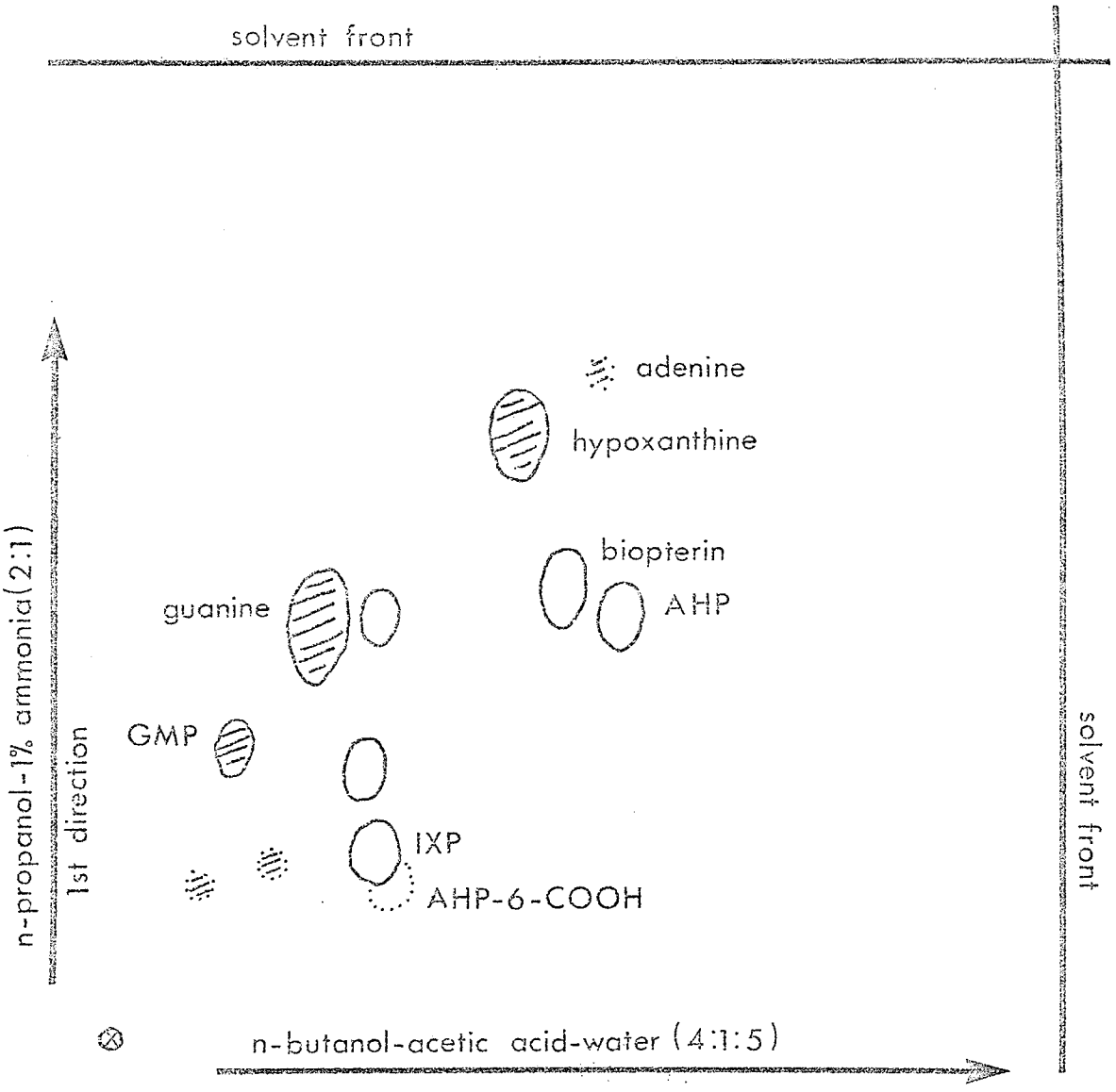


Fig. 11. Two-dimensional thin-layer chromatogram of TCA skin extract of rainbow trout. UV-absorbing substances shown in hatched enclosures; UV-fluorescing substances in unhatched enclosures. Trace compounds indicated by broken lines. Also indicated are authentic compounds with R_f values that correspond to particular spots.

Paper chromatography of 10% TCA skin extracts of brook trout revealed two UV-absorbing and three UV-fluorescing spots with R_f values corresponding to guanine, hypoxanthine, biopterin, IXP and AHP-6-COOH (Table II).

Thin-layer chromatography of 10% TCA extracts revealed in addition to the substances mentioned above the presence of four others (Fig. 12). These had R_f values corresponding to adenine, GMP, xanthopterin and ichthyopterin (Table II). 2-Amino-4-hydroxypteridine (AHP) was not detected.

The identities of guanine, hypoxanthine, GMP, IXP and AHP were supported by UV-absorption spectra (Fig. 13, 14, 15, 16 and 17). The identity of biopterin was supported by its decomposition to AHP-6-COOH when exposed to light (Hama *et al.*, 1965; Stackhouse 1966). Absorption spectra of authentic AHP-6-COOH and the degradation product of the extract (biopterin) are shown in Fig. 18. The UV-absorption spectrum of the fourth UV-fluorescing compound is shown in Fig. 19. Exposure of the compound to UV-irradiation caused its conversion to IXP as is the case with ichthyopterin (Hama *et al.*, 1960; Mori *et al.*,1960). In the absence of authentic ichthyopterin, however, the identity of this compound is only tentative.

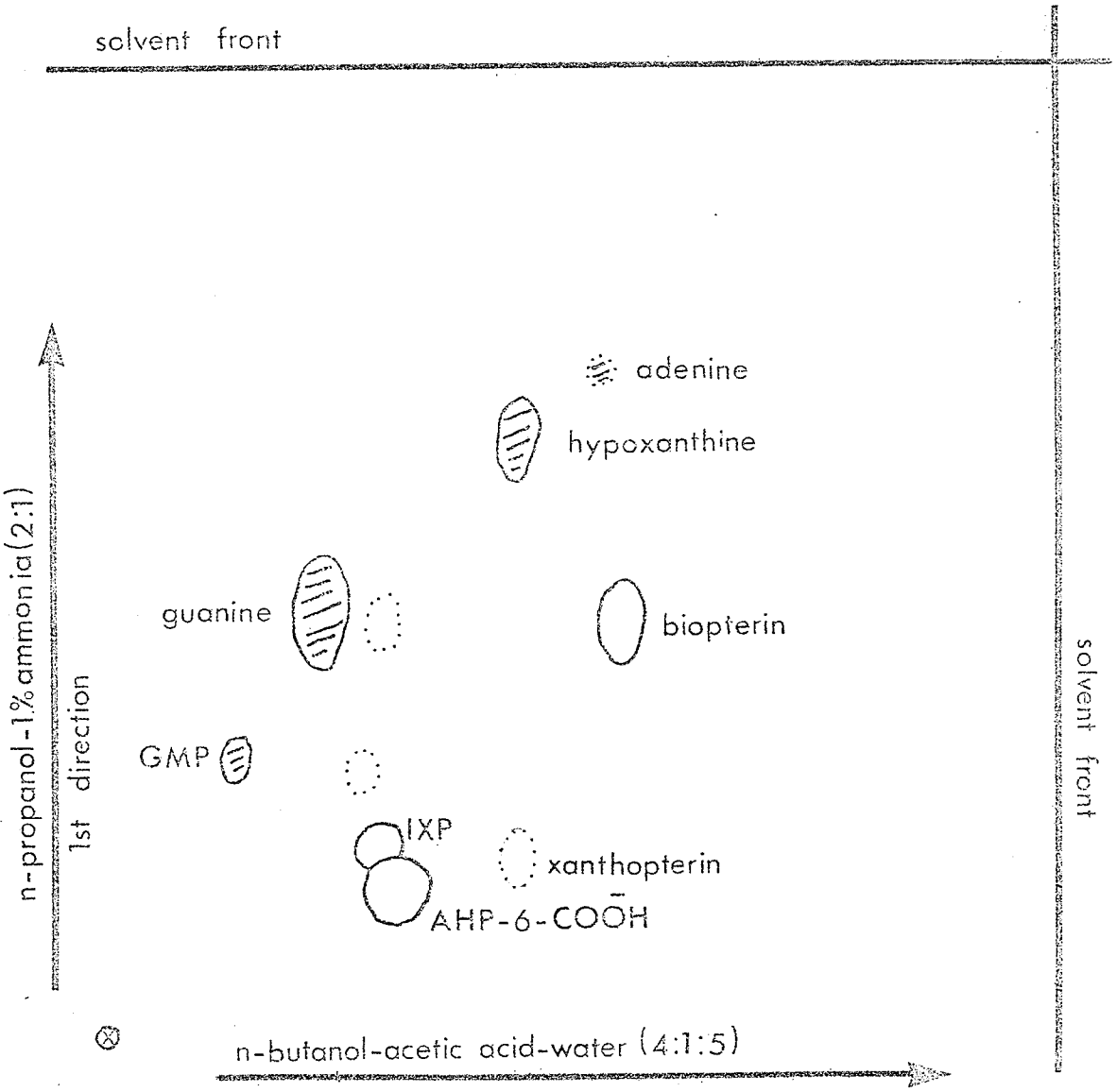


Fig 12. Two-dimensional thin-layer chromatogram of TCA skin extract of brook trout. UV-absorbing substances shown in hatched enclosures; UV-fluorescing substances in unhatched enclosures. Trace compounds indicated by broken lines. Also indicated are authentic compounds with R_f values that correspond to particular spots.

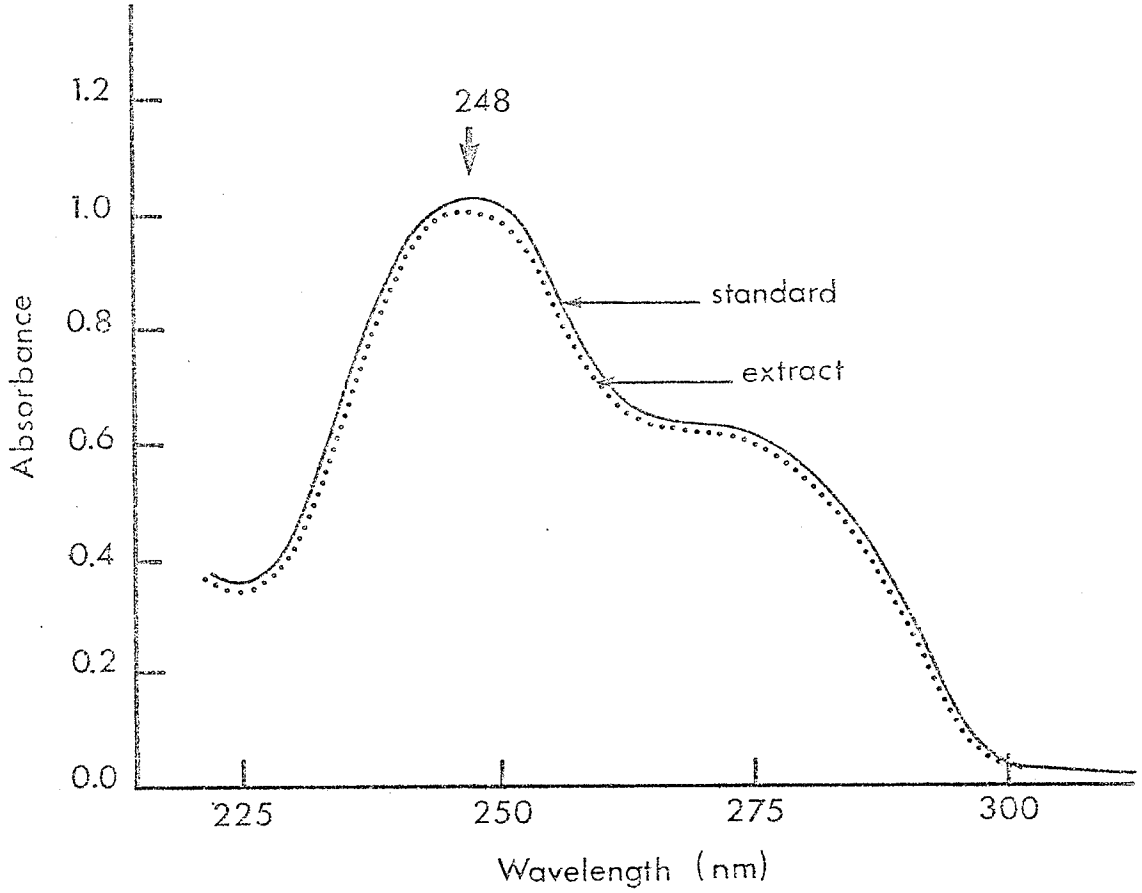


Fig. 13. Ultraviolet absorption spectra of guanine and extract from the skin of rainbow trout in 0.1N HCl.

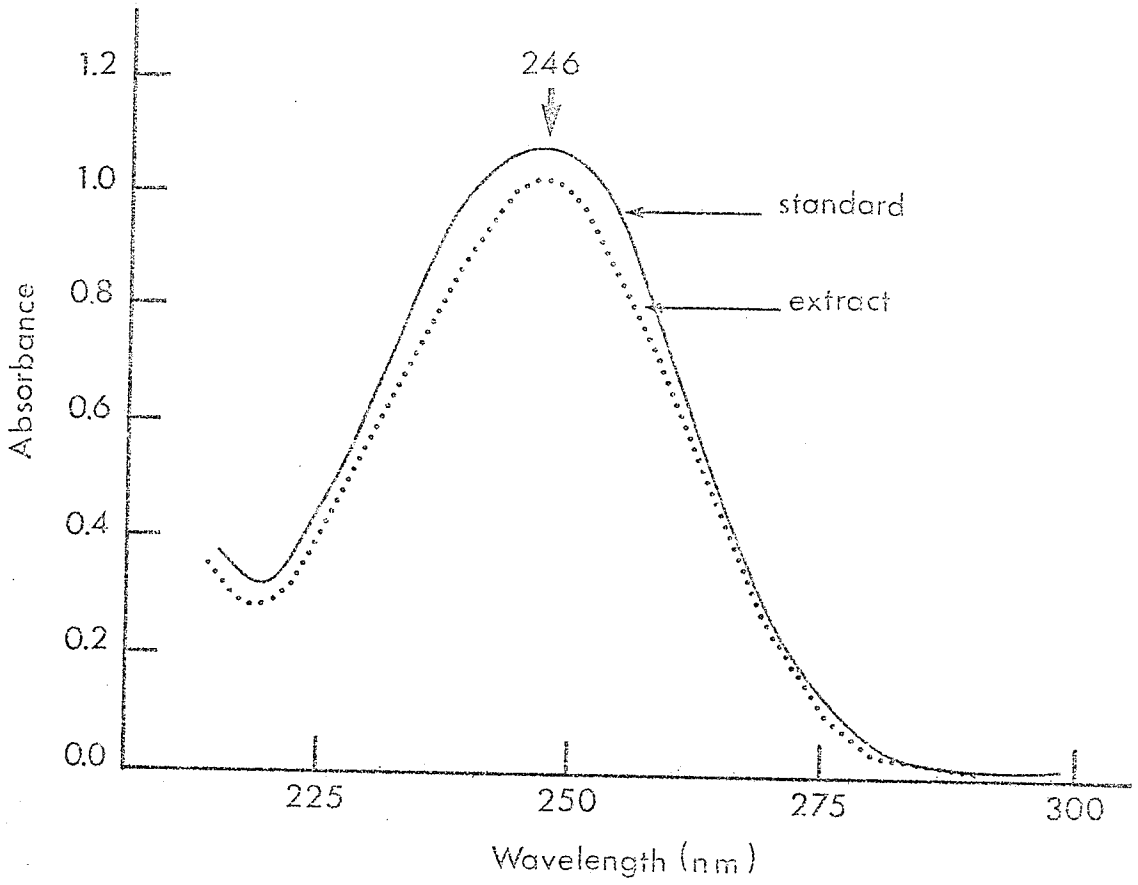


Fig. 14. Absorption spectra of hypoxanthine and extract from the skin of rainbow trout in 0.1 N HCl.

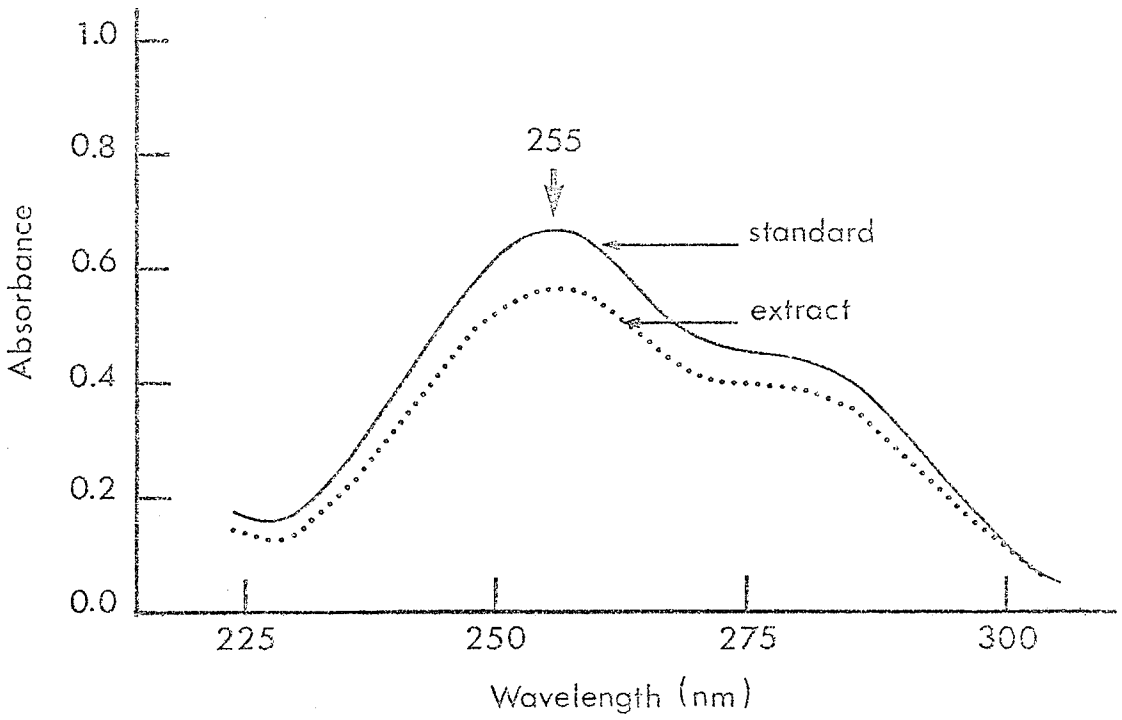


Fig. 15. Absorption spectra of GMP and extract from the skin of rainbow trout in 0.1N HCl.

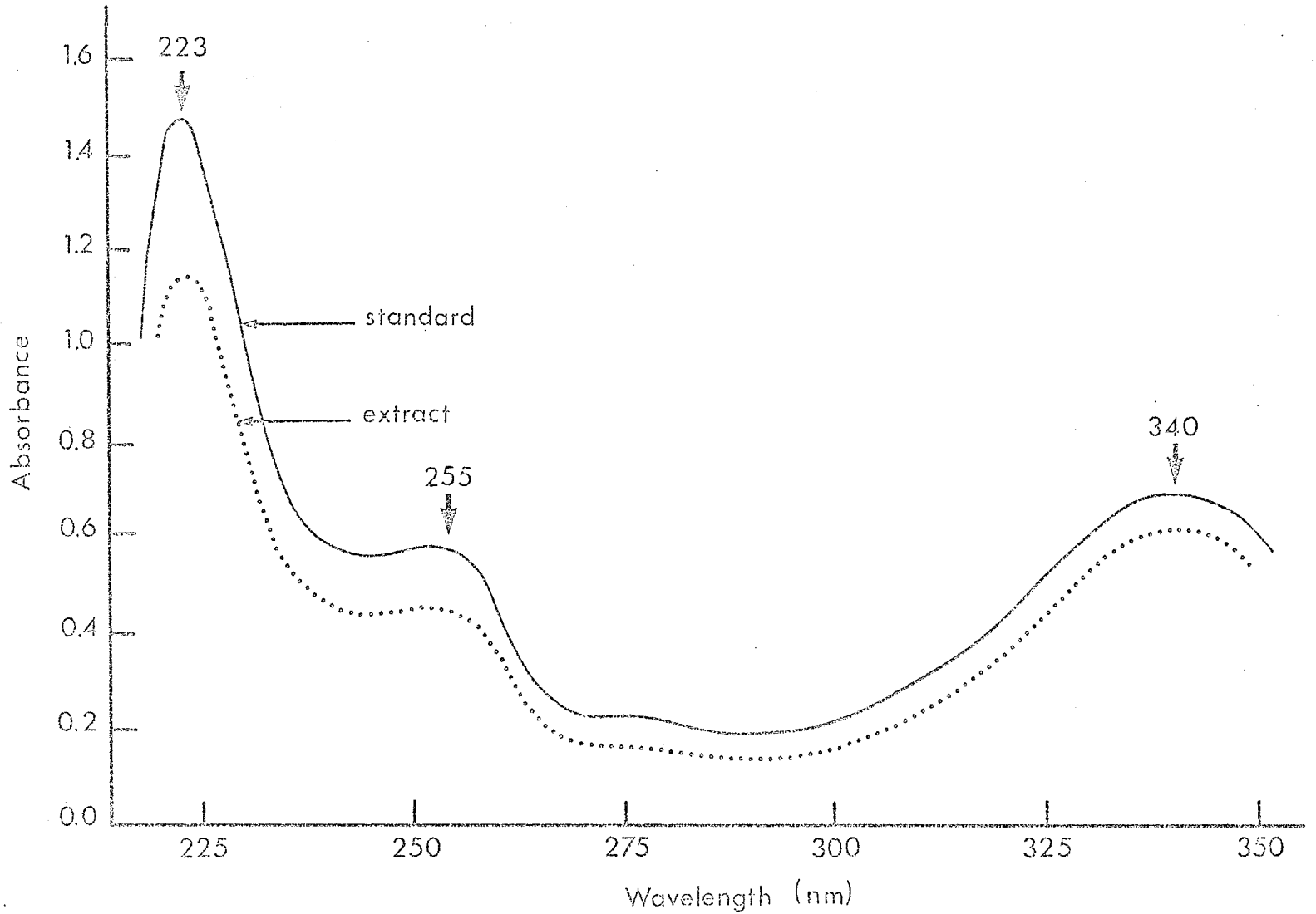


Fig. 16. Ultraviolet absorption spectra of isoxanthopterin and extract from the skin of rainbow trout in 0.1 N NaOH.

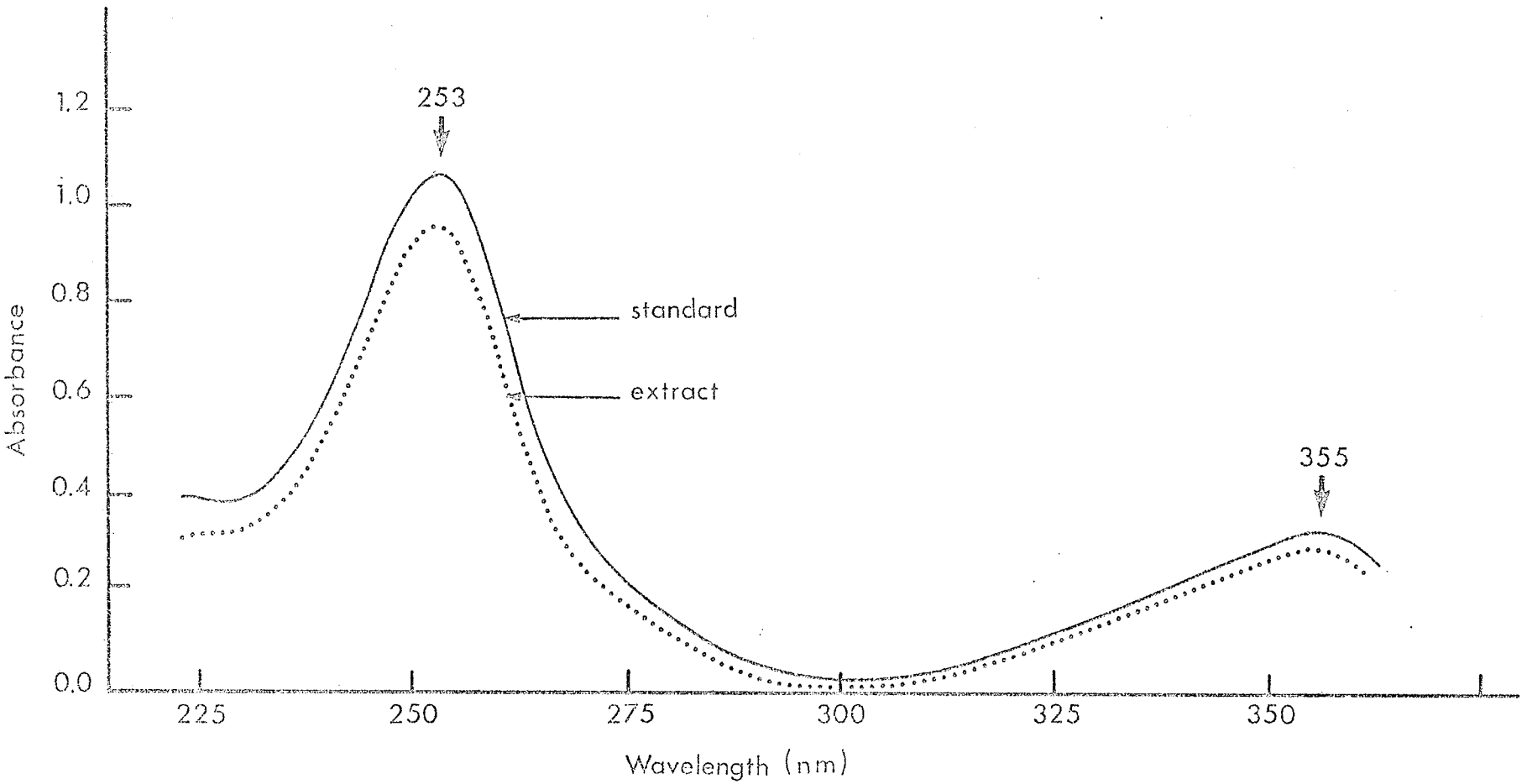


Fig. 17. Ultraviolet absorption spectra of AHP and extract from the skin of rainbow trout in 0.1 N NaOH.

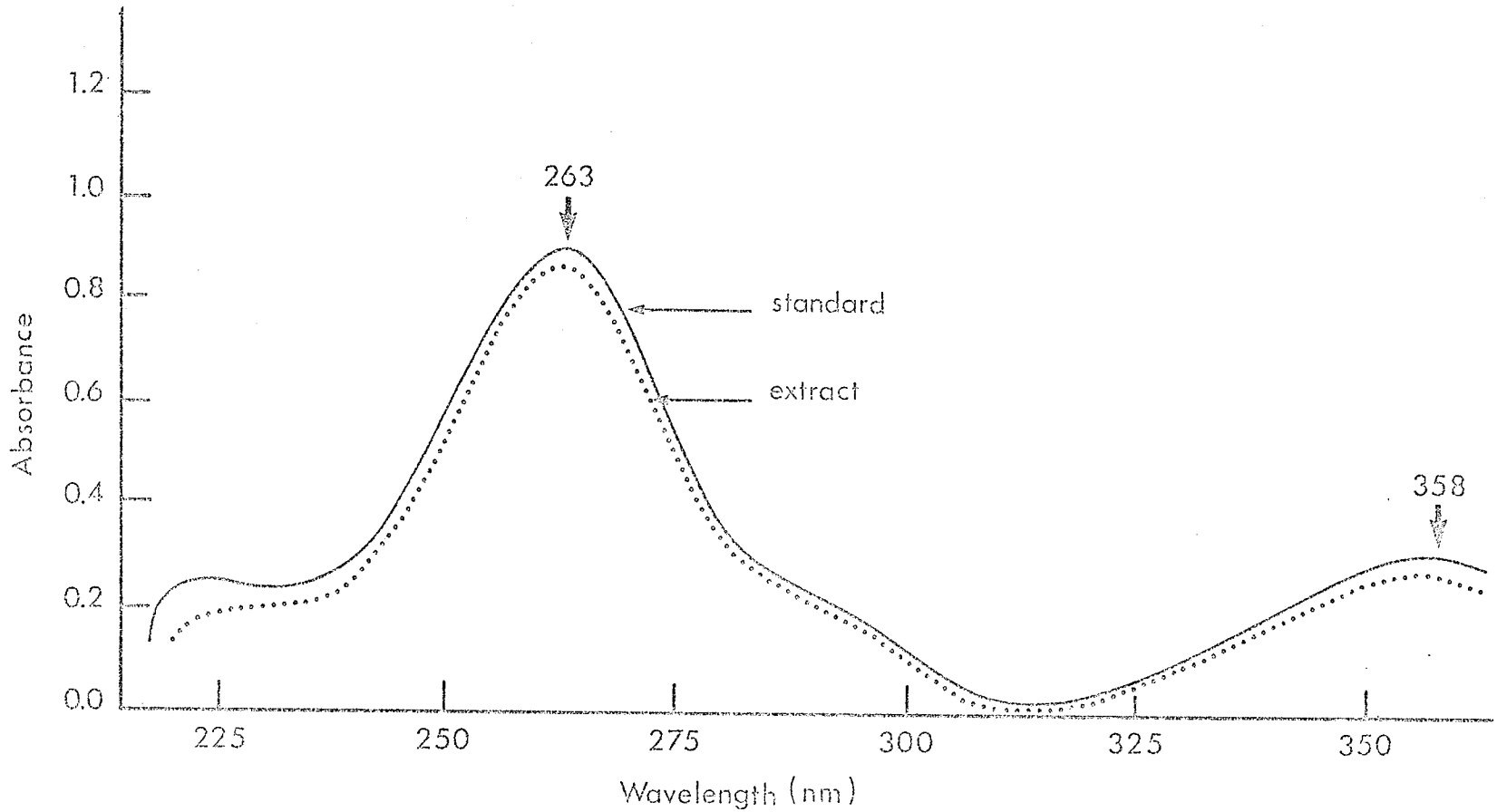


Fig. 18. Ultraviolet absorption spectra of AHP-6-COOH and degradation product from the extract from the skin of rainbow trout in 0.1 N NaOH.

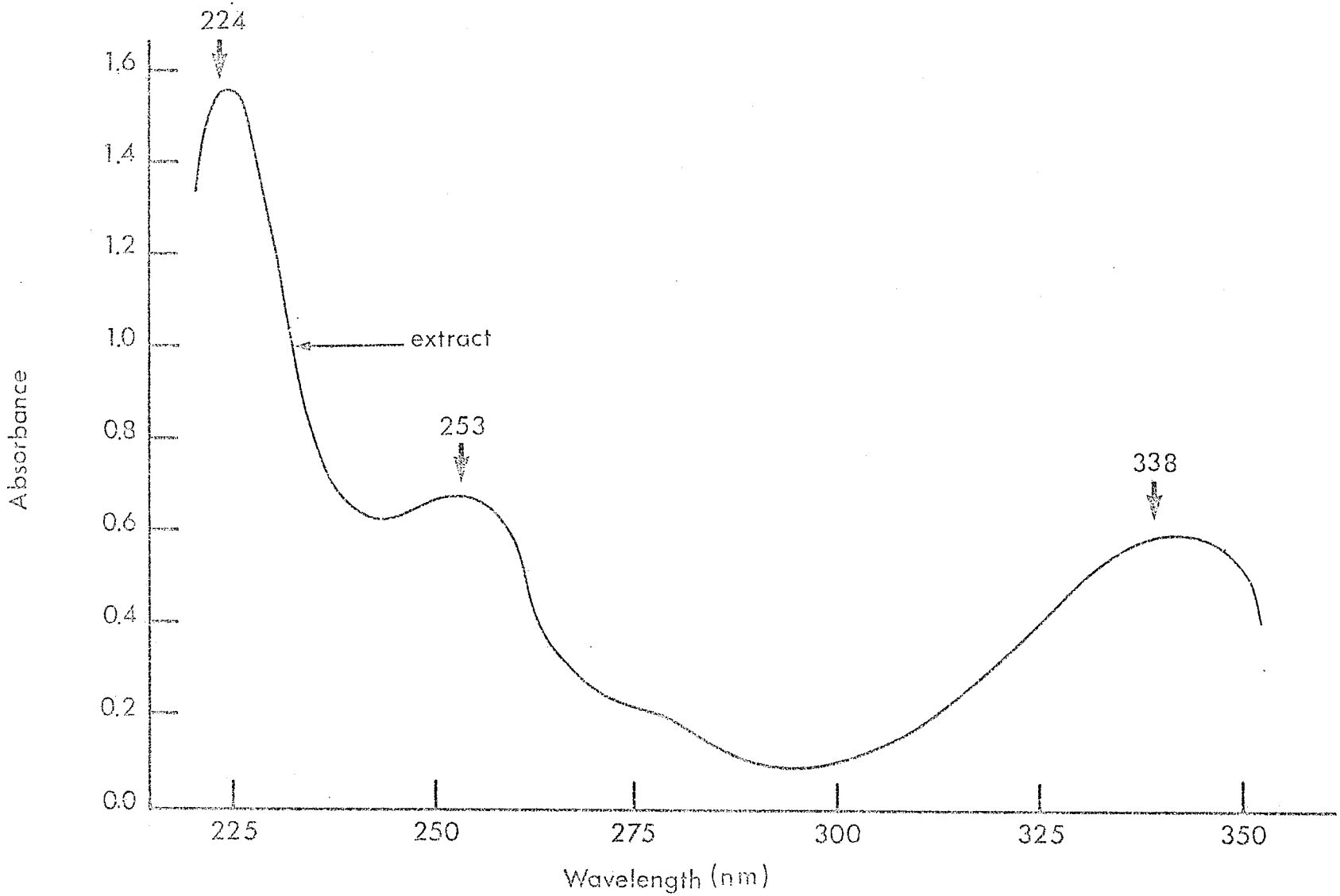


Fig. 19. Ultraviolet absorption spectrum of purple-fluorescing compound from the skin of rainbow trout in 0.1N NaOH. It is similar to but not identical with the absorption spectrum of isoxanthopterin.

Synthesis and deposition of skin purines and pteridines in rainbow trout under conditions (TSH-treatment) favouring purine synthesis

Rainbow trout injected with 2 mIU TSH/g body wt on alternate days for 3 weeks were visually more silvery than controls.

Skin from the lateral surface was analysed for the two purines, guanine and hypoxanthine, that are generally considered responsible for silvering. The results are given in Table III.

Guanine levels of control (0.137 ±0.006 mg/cm^2) and treated 0.145 ± 0.008 mg/cm^2) fish were not significantly different ($p > 0.4$). Hypoxanthine in treated fish (0.053 ± 0.002 mg/cm^2) was significantly higher ($p < 0.01$) than in controls (0.038 ± 0.004 mg/cm^2). The totals of these purines (i.e. guanine plus hypoxanthine) in control and treated fish were not significantly different ($p > 0.05$).

In this experiment the concentrations of the other substances were not determined, however, all substances were radioassayed. In control and treated fish the radioisotope entered into skin purines (guanine, hypoxanthine and GMP) and pteridines (biopterin, AHP, IXP and ichthyopterin). The specific activities of guanine and hypoxanthine were determined. They were significantly higher ($p < 0.001$) in treated than in control fish (Table III).

From the results, it appeared that this experiment to induce purine deposition was terminated too quickly; that it was terminated just when TSH-treatment was beginning to exert its effects on purine synthesis and deposition (see discussion below). Therefore, the experiment was repeated with the duration of treatment extended.

TABLE III. Concentration and specific activity of guanine (G) and hypoxanthine (H) from the skin of control and TSH-treated (2 mIU/g body wt on alternate days for 3 weeks) rainbow trout injected with 14-C-glycine.

	Weight of fish (g)	Concentration (mg/cm^2 skin)			Specific activity (cpm/μmole)	
		G	H	G + H	G	H
Control						
1	39.1	0.144	0.049	0.193	128	89
2	28.5	0.157	0.047	0.204	98	125
3	17.9	0.111	0.017	0.128	92	182
4	19.8	0.146	0.040	0.186	127	103
5	16.5	0.113	0.022	0.135	120	221
6	18.8	0.128	0.031	0.159	105	177
7	23.5	0.166	0.033	0.199	147	200
8	32.5	0.121	0.056	0.177	92	68
9	17.2	0.149	0.036	0.185	125	127
10	34.4	0.134	0.045	0.179	97	112
Mean	24.8	0.137	0.038	0.174	113	140
S.E.	±2.6	±0.006	±0.004	±0.008	±6	±16
Treated						
1	32.7	0.153	0.064	0.217	169	228
2	24.0	0.125	0.049	0.174	221	312
3	27.0	0.172	0.056	0.228	253	371
4	37.4	0.100	0.056	0.156	267	342
5	29.4	0.148	0.059	0.207	213	163
6	18.0	0.138	0.044	0.182	355	496
7	21.1	0.164	0.045	0.209	180	331
8	17.1	0.157	0.053	0.210	271	373
Mean	25.8	0.145	0.053	0.198	241	327
S.E.	±2.5	±0.008	±0.002	±0.009	±21	±35

In the second experiment to induce purine deposition, treatment (2 mIU TSH/g body wt on alternate days) was administered for 4 weeks. Treated fish were distinctly more silvery than controls. From visual examination of the skin, it appeared that more of the melanophores ventral to the lateral line were contracted (pigments aggregated) in treated than in control fish. Pigments in melanophores above the lateral line appeared to be in the same state of dispersion in control and treated fish.

The weights of control and treated fish were not significantly different. The wet weight of control fish before sham treatment was 38.3 ± 2.4 g and at the end of the experiment it was 43.7 ± 2.9 g. Thyrotropin-treated fish were 38.6 ± 2.8 g at the start of the experiment and 43.1 ± 2.5 g after treatment.

The results of analysis of skin from control and treated fish are shown in Table IV. The data on which the table was based are given in Appendix VI.

Guanine and hypoxanthine levels of treated fish were approximately twice those of controls. The levels of GMP in both groups were not significantly different.

For treated fish the specific activity of guanine was approximately four times higher than for control fish; hypoxanthine one and one-half times higher. The specific activities of GMP for control and treated fish were not significantly different. The specific activities of pteridines also were not significantly different, except for IXP which was significantly higher in control (1742 cpm/μmole) than in treated (1245 cpm/μmole) fish ($p < 0.001$).

TABLE IV. Concentration and specific activity of purine and pteridine compounds from the skin of control and TSH-treated (2 mIU/g body wt on alternate days for 4 weeks) rainbow trout injected with 14-C-glycine.

	Concentration (mg/cm^2 skin)			Specific activity (cpm/μmole)		
	Control mean ±S.E.	Treated mean ±S.E	p	Control mean ±S.E.	Treated mean ±S.E.	p
Guanine	0.242 ±0.015	0.440 ±0.026	<0.001	79 ±8	314 ±36	<0.001
Hypoxanthine	0.072 ±0.005	0.150 ±0.004	<0.001	185 ±27	260 ±14	<0.025
GMP	0.015 ±0.001	0.013 ±0.001	>0.2	1464 ±68	1279 ±74	>0.05
Biopterin	0.012 ±0.001	0.012 ±0.001	>0.5	1642 ±77	1864 ±172	>0.2
AHP	0.013 ±0.001	0.011 ±0.001	>0.2	1426 ±80	1380 ±120	>0.5
IXP	0.027 ±0.001	0.029 ±0.001	>0.4	1742 ±82	1245 ±95	<0.001
Ichthyopterin	0.006 ±0.0004	0.007 ±0.0005	>0.05	1026 ±126	818 ±76	>0.1

p= probability that there is no difference between means.

Synthesis and deposition of skin purines and pteridines in rainbow trout under conditions (ACTH-treatment) favouring pteridine deposition

Rainbow trout injected with 2 IU ACTH on alternate days were visually much darker than controls after 3 weeks of treatment. Melanophores ventral to the lateral line appeared more fully expanded (pigments dispersed) in treated than in control fish.

Treated fish showed a significant loss in weight. The wet weight of treated fish at the start of the experiment was 23.6 ± 1.1 g and after ACTH-treatment 21.0 ± 1.4 g. Control fish were 23.6 ± 0.8 g at the start of the experiment and 27.2 ± 2.3 g at the end of the experiment.

Table IV shows the results of analysis of skin from control and treated fish. The data on which the table was based are given in Appendix VII.

The concentrations of guanine, hypoxanthine and GMP in both groups of fish were not significantly different. With the exception of AHP, pteridines were significantly increased in treated fish. The levels of AHP in control and treated fish were similar.

The specific activities of two purines, guanine and hypoxanthine, of control and treated fish were not significantly different, but the specific activity of GMP was significantly increased in treated fish. The specific activities of all four pteridines (biopterin, AHP, IXP, ichthyopterin) were significantly increased in treated fish.

TABLE V. Concentration and specific activity of purine and pteridine compounds from the skin of control and ACTH-treated rainbow trout injected with 14-C-glycine.

	Concentration (mg/cm^2 skin)			Specific activity (cpm/μmole)		
	Control mean ±S.E.	Treated mean ±S.E.	p	Control mean ±S.E.	Treated mean ±S.E.	p
Guanine	0.144 ±0.006	0.131 ±0.006	>0.1	96 ±5	120 ±14	>0.1
Hypoxanthine	0.053 ±0.003	0.049 ±0.003	>0.4	243 ±15	328 ±43	>0.05
GMP	0.009 ±0.0006	0.008 ±0.0005	>0.2	2241 ±153	3256 ±144	<0.001
Biopterin	0.009 ±0.0007	0.026 ±0.0012	<0.001	1482 ±157	4716 ±466	<0.001
AHP	0.014 ±0.001	0.013 ±0.001	>0.2	1178 ±134	2613 ±349	<0.005
IXP	0.023 ±0.003	0.034 ±0.002	<0.005	2687 ±356	4210 ±483	<0.025
Ichthyopterin	0.006 ±0.0003	0.018 ±0.0005	<0.001	730 ±52	1477 ±95	<0.001

p = probability that there is no difference between means.

DISCUSSION

Purines and pteridines occurring in the skin of rainbow and brook trout

(a) Purines

Guanine and hypoxanthine were the principal purines in both rainbow and brook trout skin. This finding is in accord with previous observations on these two species (Matty and Sheltawy, 1967; Chua and Eales, 1971) and other teleosts (Markert and Vanstone, 1966; Johnston and Eales, 1967; Nicol and Van Baalen, 1968; Eales, 1969; Lee *et al.*, 1969; Denton, 1971).

Guanosine monophosphate was present in small amounts in rainbow trout and only in trace amounts in brook trout. This purine has been previously reported in rainbow trout (Matty and Sheltawy, 1967) and tentatively identified in coho salmon (Markert and Vanstone, 1966). In studies using extraction and separation procedures similar to the present study, GMP was not observed in parr and smolt of Atlantic salmon (Johnston and Eales, 1967) or in coho salmon fry (Lee *et al.*, 1969).

Adenine occurred in trace amounts in both species. Adenine is one of the principal purines in amphibian skin (Bagnara and Neidleman, 1958; Bagnara and Stackhouse, 1961; Stackhouse, 1966) but has not yet been reported in fish skin. Paper chromatography has usually been the method employed by previous investigators for the separation and characterization of compounds in fish skin extracts. This method was

not sensitive enough to reveal the small amounts of adenine in the skin of rainbow and brook trout. It would be interesting to use thin-layer chromatography to determine whether adenine occurs in the skin of other fishes.

Adenine was present in skin extracts after alcoholic extraction which would not cause hydrolysis of nucleosides and nucleotides. Thus it appeared that adenine was not a degradation product but occurred in the skin as a nitrogen base as is the case with guanine and hypoxanthine (Johnston and Eales, 1967).

Inosine monophosphate, previously reported in the skin of rainbow trout (Matty and Sheltawy, 1967; Hayashi and Saito, 1968), coho salmon (Lee *et al.*, 1969), and chum salmon (Hayashi and Saito, 1968),was not found in either rainbow or brook trout. Since the rainbow trout used in the present study were similar in size and age to those used in the previous studies, the failure to detect IMP is puzzling. It is usually difficult to remove fish skin without taking some of the underlying muscle tissue (Lee *et al.*, 1969; personal observation). Fish muscle is rich in IMP (Jones and Murray, 1960; Dyer *et al.*, 1966; personal observation) and it is possible that muscle rather than skin was the source of IMP in the previous studies. In the present study it was found that freezing the fish in liquid nitrogen immediately after they were killed allowed the removal of skin that was free of underlying muscle layers.

(b) Pteridines

The principal pteridines in rainbow trout were IXP, ichthyopterin (tentative identification), AHP and biopterin. A small amount of AHP-6-COOH occurred on thin-layer chromatograms. AHP-6-COOH may have been a degradation product of biopterin which is photolabile and readily degraded to AHP-6-COOH (Hama *et al.*, 1965; Stackhouse, 1966).

In brook trout, IXP, AHP-6-COOH and biopterin were the principal pteridines. Ichthyopterin and xanthopterin were minor components.

The pteridine patterns of rainbow and brook trout were similar but not identical. This was not surprising as the coloration of the two species is rather different.

The pteridine patterns of brook and rainbow trout bore some resemblance to those reported for adult teleosts (Table VI). However, since pteridine patterns may change during development (see literature review) any further comparisons of the patterns of the parr stage of rainbow and brook trout with adult or larval stages of other species would not be fruitful. It should be mentioned, nevertheless, that the pteridine profiles of both brook and rainbow trout bore little resemblance to the pteridine profile of the belly skin of coho salmon fry (Table VI). This is the only salmonid whose pteridine pattern has so far been reported (Lee *et al.*, 1969). The differences in patterns were due not only to species differences and differences in stages of development (one-year old parr vs 4-month old fry) but also to the type (location) of skin examined, since dorsal skin contains a more complex mixture of pteridines than belly skin (Lee *et al.*, 1969).

TABLE VI. Comparison of pteridines in the skin of rainbow and brook trout with those reported in other species. Color of fluorescence of compounds is indicated by b (blue), v (violet), and g (green to yellow).

	Biopterin (b)	AHP (b)	IXP (v)	AHP-6-COOH (b)	Xanthopterin (g)	Ichthyopterin (v)	Ranachrome 3 (b)	Reference
Order Cypriniformes Suborder Cyprinoidei Family Cyprinidae								
Carassius auratus (goldfish - adult)	+		+	+		+		a,b,c
Cyprinus carpio (carp - adult)	+		+	+		+		a,b,c
Tribolodon hakuensis (rudd - adult)	+		+	+		+		a,b,c
Misgurnus anguillicaudatus (loach - adult)	+		+	+		+		a,b,c
Order Cyprinodontiformes Suborder Cyprinodontoidei Family Cyprinodontidae								
Oryzias latipes (Medaka - adult)	+	+	+	+	+		+	a.b.c
Family Poeciliidae								
Xiphophorus helleri (swordtail - adult)	+	+	+	+	-		+	a,b,c
Order Clupeiformes Suborder Salmonoidei Family Salmonidae								
Oncorhynchus kisutch (Coho salmon - fry	-	-	-	+	+	+		Lee *et al.*, 1969
Salvelinus fontinalis (brook trout - parr)	+	-	+	+	(+) ?	(+) ?		present study
Salmo gairdneri (rainbow trout - parr)	+	+	+	(+)	-	+ ?		present study

Reference: (a) Hama, 1963; (b) Hama and Fukuda, 1964; (c) Hama *et al.*, 1965.

It has been suggested that despite the ubiquitous occurrence of pteridines in fishes and amphibians, there is a certain pattern of their distribution among the various families (Bagnara, 1966). For example, while Cyprinidae have a wide array of pteridines quite typically they possess large amounts of violet-fluorescing pteridines and only rather small amounts of blue-fluorescing ones (Hama, 1963). The few Cyprinodontidae investigated have mainly blue-fluorescing pteridines (Hama and Fukuda, 1964). No distinctive pattern for the salmonids was seen. Violet-fluorescing ichthyopterin was the principal pteridine in belly skin of fry and coho salmon (Lee *et al.*, 1969). In the present study, violet-fluorescing IXP was undoubtedly the principal pteridine in brook and rainbow trout, but the amounts of blue- and violet-fluorescing compounds, judging from the size of spots and intensity of fluorescence on paper chromatograms were approximately the same. This visual impression was confirmed in the case of rainbow trout by spectroanalysis (Tables IV and V). However, whether or not adult salmonids have a characteristic pteridine pattern remains to be investigated.

Differences in size of spots on chromatograms and intensity of absorbance or fluorescence of spots indicated that purines and pteridines were more concentrated in TCA extracts than in the two other extracts. Differences in the concentration of pteridines after extraction with alcohol and with acid have been interpreted as indicating that the substances exist partly in a free and easily extractable form and partly in a bound form associated with protein (Matsumoto, 1965a). Just by analogy with the case of pteridines, it is likely that the

purines exist partly in a bound form and partly in a free and easily extractable form.

In summary, rainbow trout skin contained guanine, hypoxanthine, GMP, 2-amino-4-hydroxypteridine (AHP), biopterin, isoxanthopterin, ichthyopterin and trace amounts of adenine. Brook trout skin contained guanine, hypoxanthine, biopterin, isoxanthopterin, 2-amino-4-hydroxy-6-carboxypteridine (AHP-6-COOH) and trace amounts of adenine, GMP, ichthyopterin and xanthopterin.

Levels of purines and pteridines in rainbow trout skin

In rainbow trout, guanine averaged from 0.137 to 0.242 mg/cm^2, hypoxanthine from 0.035 to 0.072 mg/cm^2, and GMP 0.009 and 0.015 mg/cm^2 (Tables III, IV and V).

The concentrations of guanine and hypoxanthine were within the ranges found in rainbow trout (Sheltawy, 1967), coho salmon smolts (Nicol and Van Baalen, 1968) and brook trout (Chua and Eales, 1971). These findings are summarized in Table VII.

The concentration of GMP corresponded to 1.0 and 0.6 μM/g wet skin. These levels were considerably higher (approximately twenty times higher) than that found in perchloric acid extracts of skin from entire rainbow trout (Sheltawy, 1967). The discrepancy may be due to the degradation of the nucleotide during perchloric acid extraction.

The purine levels of rainbow trout varied with the size of the animal, large fish containing higher concentrations (mg/cm^2) than

TABLE VII. Guanine and hypoxanthine content of the skin of certain salmonids.

	No. of fish	Wt. of fish (g)	Guanine (mg/cm^2)	Hypoxanthine (mg/cm^2)
rainbow trout (present study)	10	24.8 ±2.6	0.137 ±0.006	0.038 ±0.004
	10	27.2 ±2.3	0.144 ±0.006	0.053 ±0.003
	10	43.7 ±2.9	0.242 ±0.015	0.072 ±0.005
rainbow trout (Sheltawy, 1967)	9	44.20	0.218 ±0.041	
	4	58.25	0.240 ±0.015	
brook trout (Chua and Eales, 1971)	15	24.06 ±1.18	0.167 ±0.010	0.059 ±0.004
	15	25.29 ±1.85	0.165 ±0.011	0.054 ±0.003
	15	65.25 ±4.88	0.146 ±0.012	0.059 ±0.004
	15	106.76 ±7.37	0.153 ±0.008	0.070 ±0.004
coho salmon smolt at various stages of silvering (Nicol and Van Baalen, 1968)			0.25 to 0.57	

smaller ones (Table VII). Data from Sheltawy's study (1967) with rainbow trout showed the same trend. Since large fish tend to be more silvery in appearance than small fish, it might be expected that the purine content of larger fish would be higher than that of small ones. However, the data of Chua and Eales (1971) showed that skin purine levels of brook trout were similar in small (24 g) and large (106 g) fish (Table VII). Further work is obviously needed before any generalization can be made.

With regard to the pteridines, levels of two of them, AHP and ichthyopterin, were similar in small (27 g) and large (44 g) rainbow trout. The average concentrations of AHP were 0.014 and 0.013 mg/cm^2 skin respectively. Ichthyopterin averaged 0.006 mg/cm^2 in both groups. The levels of the other two pteridines, biopterin and IXP, were increased in the larger fish. Biopterin averaged 0.009 mg/cm^2 in small fish and 0.012 mg/cm^2 in larger fish. Isoxanthopterin averaged 0.023 mg/cm^2 in small fish and 0.027 mg/cm^2 in the larger fish.

Data on the concentrations of pteridines in fish or amphibia are scarce. Matsumoto (1965a) found that in adult goldfish skin IXP averaged 0.358 µg/mg dry skin and ichthyopterin averaged 0.691 µg/mg dry skin. In rainbow trout the level of IXP was equivalent to approximately 1.7 µg/mg dry skin, almost five times the concentration reported in goldfish. The level of ichthyopterin was approximately 0.4 µg/mg dry skin, 57 per cent of the amount reported in goldfish. Concentrations of other pteridines in goldfish or of pteridines in other species have not been reported.

It is interesting that the ratios of purines to pteridines in small (27 g) and large (44 g) rainbow trout were different. In the small fish purines (0.206 $\mu g/cm^2$) were four times higher than pteridines (0.052 $\mu g/cm^2$), in the larger fish purines (0.429 $\mu g/cm^2$) were seven times higher than pteridines (0.058 $\mu g/cm^2$).

In summary, the purines and pteridines in order of abundance were guanine > hypoxanthine > isoxanthopterin > biopterin, AHP, GMP, > ichthyopterin. Guanine was three to four times more concentrated than hypoxanthine and six to eight times more concentrated than isoxanthopterin. Isoxanthopterin was twice as concentrated as biopterin and four to five times more concentrated than ichthyopterin. Total purine levles were four to seven times higher than total pteridine levels.

Influence of TSH and ACTH on purine and pteridine deposition

Rainbow trout adminstered TSH over a 3-week period were visually more silvery than controls. Skin hypoxanthine was significantly increased. Guanine and the sum of the two purines (i.e. hypoxanthine + guanine) tended to be increased, but the increases were not statistically significant (Table III).

The visually-observed silvering likely involved a redistribution of pigments within chromatophores. Robertson (1949) found that in TSH-treated rainbow trout, the pigments in many of the melanophores ventral to the lateral line were aggregated. Underlying iridophores would therefore be no longer obscured and the sides would appear

silvery. The state of the melanophores in this experiment was not noted, but in the second experiment with TSH it was observed that in treated fish the pigments of many of the melanophores in the flanks were aggregated.

It appears that the usual state of the pigments in melanophores of amphibians and fishes is to be dispersed under the influence of pituitary MSH (Bagnara, 1966). Thyroxine has an antagonistic effect on MSH (Wright and Lerner, 1960). It was assumed that the effect of TSH would be achieved indirectly by stimulating the secretion of thyroid hormones (though the experiments did not exclude the possibility of a direct effect of TSH on the chromatophores), and it would be attractive to attribute the aggregated state of the pigments of the melanophores to some action of thyroid hormones affecting MSH. That the situation is more complex than this was made evident by the observation that the melanophores above the lateral line of TSH-treated fish appeared no different from control fish. That the pigments of melanophores of the flanks may be aggregated while those in other areas are in their usual state of dispersion has been observed in both naturally silvering trout and trout in which silvering was induced by feeding mammalian thyroid extract (Robertson, 1949).

The finding that TSH increased the incorporation of ^{14}C-glycine into guanine and hypoxanthine but that the level of only hypoxanthine was significantly increased (Table IV) suggested that hypoxanthine synthesis must have been influenced earlier than guanine synthesis, and that the duration of the experiment (3 weeks) was too short for the

hormone to exert its effect on skin guanine deposition. If purine synthesis in fish is along classical pathways (e.g. Buchanan and Hartman, 1959; Murray *et al.*, 1970) the reactions from the initial purine nucleotide (IMP) to hypoxanthine would involve two enzyme-catalyzed steps: IMP to inosine to hypoxanthine. The conversion of IMP to guanine would involve at least four enzyme-catalyzed steps: IMP to xanthine monophosphate to GMP to guanosine. Earlier stimulation of the IMP to hypoxanthine pathway than the IMP to guanine pathway could account for hypoxanthine but not guanine being significantly increased. If stimulation of the IMP to guanine pathway had just begun when the isotope was introduced, the net increase in the amount of guanine could have been still too small to be significant but the accelerated incorporation of the isotope would be detected.

In a longer experiment (4 weeks), using larger fish, TSH significantly increased skin guanine and hypoxanthine but did not affect GMP and pteridine levels (Table IV). Previous workers, in general, ascribed silvering induced by hormonal treatment to guanine deposition but did not measure guanine concentrations. The danger of this was illustrated by the results of the first experiment with TSH. It is only recently that Chua and Eales (1971) clearly demonstrated that TSH significantly increased dermal purine (hypoxanthine and guanine) deposition in brook trout. Their findings are now extended to rainbow trout.

It was evident that TSH was capable of influencing pteridine synthesis, for although pteridine levels in control and treated fish were not significantly different the specific activity of the princi-

pal pteridine, IXP, was significantly reduced (Table IV). It is likely that pteridine levels would have been reduced if the experiment were of longer duration but this remains to be investigated.

Administration of ACTH almost doubled the pteridine content of rainbow trout skin, but the four pteridines did not show the same increase in concentration. Isoxanthopterin was increased 50 per cent; biopterin and ichthyopterin were increased three-fold; AHP was not increased. The hormone did not affect purine levels, but incorporation of ^{14}C-glycine into GMP was significantly increased (Table V).

With regard to the pteridines, the findings are in general agreement with those of Matsumoto (1965a) who found that ACTH increased the pteridine content of red-scaled goldfish skin, but while some pteridines were increased the levels of others were unchanged. In amphibian larvae however, when pteridine synthesis was induced by intermedin (MSH) treatment all pteridines showed the same two-fold increase (Stackhouse, 1966). Apart from these two reports, data on the effects of hormones on pteridine deposition are lacking.

Injection of ACTH stimulated melanogenesis in both intact and hypophysectomized goldfish (Chavin, 1959), had some melanogenic effect in the killifish, *Fundulus heteroclitus* (Kosto *et al.*, 1959), and stimulated pteridine synthesis in goldfish (Matsumoto, 1965a). The present study extends these findings to rainbow trout. However, since most preparations of ACTH are highly contaminated with MSH (Lerner, 1966), it should be mentioned that the purity of the hormone in the present study (as in the previous ones) was not known. Also, as Tepperman (1968)

pointed out, there is the possibility that the changes induced were not by the ACTH molecule itself but by an MSH-active peptide split from the longer ACTH peptide.

In summary, TSH significantly increased skin guanine and hypoxanthine but did not influence pteridine levels. Adrenocorticotropin almost doubled the pteridine content of rainbow trout skin but did not affect purine levels.

Purine and pteridine pathways and the relationship between them

Comparison of the three purines from rainbow trout skin showed that the specific activity of GMP was considerably higher than that of hypoxanthine or that of guanine (Tables IV and V). This was consistent with the accepted pathway for purine biosynthesis, according to which GMP is a precursor of guanine (e.g. Buchanan, 1960; Murray *et al.*, 1970).

The findings were also in accord with those of other investigators. Sheltawy (1967) followed the incorporation of ^{14}C-glycine into skin purines of rainbow trout and found that the specific activities of GMP, AMP and IMP were higher than those of guanine and hypoxanthine. Hayashi (1972) followed the incorporation of ^{14}C-formate into skin purines of rainbow trout and though he did not recover any nucleotides from the acid extracts of the skin, he found that the specific activity of the nucleoside, inosine, was considerably higher than that of guanine or hypoxanthine.

Skin GMP, however, may not have been a precursor to the newly synthesized guanine. Under TSH treatment skin guanine level and specific activity increased relative to controls but neither the level nor specific activity of GMP was different from that of controls (Table IV). Similar results were obtained by Sheltawy (1967) who found that prolonged immersion in thyroxine significantly increased the levels and specific activities of skin guanine and hypoxanthine in rainbow trout but did not significantly alter the levels or specific activities of skin GMP and IMP.

Although purines are abundant in skin iridophores, there is no direct evidence to indicate the site of purine synthesis in fishes. It is quite possible that purines synthesized elsewhere (e.g. in the liver) are transported to the skin. However, judging from the finding of Matty and Sheltawy (1967) that labelled glycine was rapidly incorporated into purines in skin *in vitro*, it may be assumed that the iridophores play an important role in purine metabolism as a site not merely of accumulation but also of purine formation. If this is so, it appears that guanine biosynthesis in the skin of fish may not be along "classical" pathways.

With regard to the pteridines, IXP had the highest specific activity (control fish, Tables IV and V). Proposed pathways for pteridine synthesis indicate that biopterin, AHP and ichthyopterin are precursors to IXP. If this is so, it is not understood why the specific activity of IXP should be higher than the specific activities of biopterin, AHP or ichthyopterin unless IXP was synthesized through

other intermediates. That this might have been the case is suggested by the fact that TSH did not affect the synthesis (when judged from specific activities) of biopterin, AHP or ichthyopterin, yet IXP biosynthesis was slowed down (Table IV).

The level and specific activity of AHP in ACTH-treated fish (Table V) indicated that it was being converted to some other compound. In fruit flies, AHP was probably an intermediate in the formation of IXP (Hubby and Throckmorton, 1960). However, specific activities indicated that in rainbow trout AHP was not an intermediate in the formation of IXP.

From a consideration of specific activities, it appears that three routes may have been involved in pteridine formation: one route leading (from the initial pteridine) to biopterin and AHP, another to ichthyopterin, and the third to IXP. This would be in accord with metabolic pathways proposed for pteridines (see Fig. 4).

With regard to the biosynthetic interrelationship of purines and pteridines, it was readily seen from specific activities (Tables IV and V) that the free purines, guanine and hypoxanthine, were not precursors to the pteridines. It also appeared unlikely that skin GMP was an intermediate in the biosynthesis of the pteridines. Various investigators have indicated that the pteridines are synthesized through a purine precursor and several hypothesized pathways (e.g. Stackhouse, 1966) have been based on the assumption that GMP was the precursor molecule. Recent investigators (Burg and Brown, 1968; Fukushima, 1970) indicate that the precursor molecule is the triphosphate nucleotide, GTP.

On account of the possibility of a link in the biosynthetic pathways of purines and pteridines, it has been hypothesized that in organisms synthesizing both types of pigments some sort of competition for substrates will exist, and that under conditions favouring purine synthesis pteridines will be decreased and under conditions favouring pteridine synthesis purines will be decreased.(Bagnara, 1966). In the present study, there was no clear evidence for a reciprocal relationship between purine and pteridine syntheses in rainbow trout. Increase in purines was not accompanied by a decrease in pteridines, and increase in pteridines was not accompanied by a decrease in purines.

SUMMARY

The principal purines and pteridines in rainbow trout skin were guanine, hypoxanthine, guanylic acid, isoxanthopterin, biopterin, 2-amino-4-hydroxypteridine and ichthyopterin. Adenine occurred in trace amounts. Brook trout skin contained guanine, hypoxanthine, isoxanthopterin, biopterin, 2-amino-4-hydroxy-6-carboxypteridine and trace amounts of guanylic acid, adenine and xanthopterin.

In rainbow trout, TSH significantly increased skin guanine and hypoxanthine but the levels of the other compounds were not significantly altered. Three pteridines, biopterin, isoxanthopterin and ichthyopterin, were significantly increased by ACTH-treatment; the levels of the other compounds were not significantly altered.

In rainbow trout, ^{14}C-glycine was rapidly incorporated into skin purines and pteridines. From a consideration of the concentrations and specific activities of the compounds under the different treatments, it appeared that skin GMP was not a precursor in the *de novo* synthesis of guanine. It also appeared that the purine compounds isolated (guanine, hypoxanthine and GMP) were not intermediates in the synthesis of pteridines.

Despite the possibility that the biosynthetic pathways of purines and pteridines may be linked, no reciprocal ralationship between purine and pteridine syntheses was observed.

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APPENDIX I

RELATION BETWEEN CONCENTRATION AND ABSORBANCE. (Data for Fig. 6)

Concentration (µg/ml)	Absorbance		
	Guanine	Hypo-xanthine	GMP
4	0.26	0.30	0.14
4	0.27	0.32	0.16
5	0.34	0.36	0.18
5	0.32	0.38	0.20
5	0.36	0.37	0.19
10	0.68	0.74	0.38
10	0.66	0.74	0.37
10	0.67	0.72	0.38
15	1.02	1.10	0.55
15	1.01	1.12	0.56
15	1.00	1.10	0.54
20	1.36	1.49	0.70
20	1.38	1.46	0.72
20	1.37	1.48	0.73

Guanine	$Y = 0.069X - 0.011$
Hypoxanthine	$Y = 0.073X + 0.006$
GMP	$Y = 0.035X + 0.022$

APPENDIX II

RELATION BETWEEN CONCENTRATION AND FLUORESCENCE INTENSITY. (Data for Fig. 7, 8 and 9.)

Compound	Concentration (μg/ml)	Fluorescence Intensity (relative %)		
IXP	0.1	15	17	12
	0.2	33	38	30
	0.3	48	42	40
	0.4	72	76	68
	0.5	80	84	74
AHP-6-COOH	0.4	12	15	18
	0.6	20	18	18
	0.8	25	32	36
	1.0	30	33	35
	1.2	32	34	37
	1.4	42	40	40
	1.5	46	50	42
AHP	0.2	8	6	
	0.3	13	14	
	0.4	14	15	
	0.5	18	17	
	0.6	21	20	
	0.7	27	30	
	0.8	31	32	
	0.9	35	30	
	1.0	42	40	

IXP: $Y = 16.9X - 1.97$
AHP-6-COOH: $Y = 26.36X + 5.21$
AHP: $Y = 39.67X - 0.86$

APPENDIX III

RECOVERY OF COMPOUNDS ADDED TO 0.5 GRAM BOILED FISH LIVER HOMOGENATES.

Compound	µg added	percent recovery			mean ± S.E.
Guanine	1,000	94	98	88	
	500	92	82	94	
	100	92	90	96	91.8 ± 1.6
Hypoxanthine	1,000	90	84	92	
	500	88	82	90	
	100	90	92	94	98.1 ± 1.3
GMP	1,000	92	94	82	
	500	90	90	91	
	100	90	84	86	88.8 ± 1.3
AHP	100	70	72	78	
	50	74	64	76	
	25	77	70	71	72.4 ± 1.5
IXP	100	76	80	76	
	50	72	64	70	
	25	66	68	80	72.2 ± 1.9
Biopterin (as AHP-6-COOH)	100	77	69	71	
	50	76	63	64	
	25	70	67	64	69.3 ± 1.6

APPENDIX IV

CARBON-14 COUNT RATE AT A PULSE HEIGHT OF 10 - 1000 DIVISIONS.
(Data for counter settings, page 34.)

% Gain	CPM	% Gain	CPM
1	1,097	11	18,300
2	7,075	12	18,504
3	10,794	13	18,861
4	12,978	14	19,425
5	14,601	14.5	19,621
6	15,542	15	19,894
7	16,309	15.5	19,514
8	16,831	16	19,384
9	17,415	17	18,755
10	17,932	18	18,464

APPENDIX V

RADIOACTIVITY COUNTS OF KNOWN AMOUNTS (4,842 DPM) OF *n*-HEXADECANE-^{14}C. (Data used for determining counting efficiency, page 34.)

Sample	CMP
1	4,319
2	4,127
3	4,058
4	3,943
5	3,816
6	4,081
7	4,143
8	4,227
9	3,656
Mean ± S.E.	4,041 ± 69

APPENDIX VI

CONCENTRATION OF COMPOUNDS FROM THE SKIN OF CONTROL RAINBOW TROUT.
(Uncorrected data for Table IV.)

Fish No.	Concentration (μg/cm² skin)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	191	81	9	7.9	13.3	18.5	3.5
	219	82	8	6.1	11.0	19.3	3.2
2	259	103	15	8.8	5.5	25.5	5.0
	207	75	15	10.8	7.0	24.5	5.3
3	240	57	16	7.3	12.6	20.4	2.7
	233	54	20	5.0	10.6	19.3	3.1
4	308	42	11	10.9	6.2	13.7	4.2
	240	50	9	9.7	8.2	14.8	4.3
5	252	74	13	12.0	11.4	20.6	5.8
	268	60	15	10.3	9.8	14.6	5.4
6	278	55	11	5.9	7.4	24.1	4.9
	273	57	8	4.7	9.0	23.3	5.1
7	155	44	13	5.0	14.1	21.5	3.6
	146	43	11	6.7	12.6	18.9	3.4
8	170	70	13	9.1	4.7	15.2	3.9
	179	82	9	12.1	6.2	15.9	4.4
9	230	63	16	5.6	9.0	21.9	4.4
	226	74	14	4.4	8.2	17.4	4.3
10	143	54	20	12.7	9.8	20.8	4.0
	167	58	17	10.9	11.4	18.5	4.5
Mean	221.7	63.8	13.2	8.3	9.4	18.7	4.3
S.E.	±13.8	±4.8	±1.1	±0.9	±0.8	±1.0	±0.3

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VI cont'd

CONCENTRATION OF COMPOUNDS FROM THE SKIN OF TSH-TREATED RAINBOW TROUT. (Uncorrected data for Table IV.)

Fish No.	Concentration (μg/cm² skin)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	372	156	14	6.5	5.1	18.9	4.1
	386	148	14	7.0	5.9	19.6	4.3
2	466	113	16	8.8	7.4	18.1	5.0
	423	148	17	9.7	5.9	21.1	5.3
3	475	138	7	11.5	11.4	22.2	4.7
	484	143	9	10.3	9.4	21.5	5.4
4	466	117	9	7.6	12.1	20.4	5.5
	405	143	8	7.0	12.6	20.0	4.9
5	400	110	14	10.3	7.4	23.3	5.2
	367	117	14	8.2	9.0	24.2	4.8
6	508	134	7	6.0	5.9	24.5	4.9
	554	143	9	7.9	7.4	23.3	5.3
7	292	152	12	4.0	7.9	17.4	4.8
	273	136	10	5.0	6.2	18.5	4.3
8	339	154	10	8.8	6.6	19.6	4.5
	330	141	10	10.3	8.6	20.0	4.2
9	451	127	12	7.3	6.2	20.8	6.0
	423	123	11	9.7	7.4	21.9	5.8
10	334	119	12	5.9	10.6	19.6	6.6
	325	127	10	7.4	9.4	17.7	6.3
Mean	403.9	134.5	11.3	8.0	8.1	20.6	5.1
S.E.	±24.1	±3.8	±0.4	±0.7	±0.6	±0.6	±0.3

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VI cont'd

SPECIFIC ACTIVITY OF COMPOUNDS FROM THE SKIN OF CONTROL RAINBOW TROUT. (Uncorrected data for Table IV.)

Fish No.	Specific activity (cpm/μmole)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	84	131	925	1061	1211	1818	555
	65	104	1032	1265	1395	1500	862
2	126	152	1316	1218	1571	1579	786
	128	181	1478	1258	1214	1697	531
3	56	112	1426	1356	1122	1639	1666
	73	96	1305	1742	1024	1558	1504
4	83	152	1237	1373	875	1400	1074
	72	153	1154	1133	718	1310	961
5	58	115	1030	1600	1136	1648	721
	60	107	1316	1484	1131	1542	624
6	61	272	1175	1055	1000	1767	742
	43	278	1258	1448	971	1634	934
7	42	309	1694	1613	1363	1078	1007
	61	252	1362	1428	1081	1303	1463
8	44	151	1086	1750	1611	1148	719
	40	90	1324	1693	1333	1000	754
9	49	84	1195	1059	1142	1307	503
	54	49	1252	1111	1062	1354	588
10	59	159	837	1410	1578	1351	559
	60	133	1027	1328	1250	1424	552
Mean	66	154	1221	1369	1190	1453	856
S.E.	±8	±22	±65	±67	±57	±68	±105

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VI cont'd

SPECIFIC ACTIVITY OF COMPOUNDS FROM THE SKIN OF TSH-TREATED RAINBOW TROUT. (Uncorrected data for Table IV.)

Fish No.	Specific activity (cpm/μmole)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	191	231	937	2225	874	909	890
	228	236	1148	2295	917	628	1089
2	140	173	1164	1127	1000	939	449
	268	167	798	1383	1135	1078	478
3	151	215	1207	1760	1172	1235	594
	170	214	1147	1640	1195	1025	489
4	209	247	704	1021	1048	1194	798
	182	190	655	1409	1138	1083	979
5	364	246	883	1746	965	1121	595
	330	262	1092	1764	1108	1209	714
6	223	191	1172	1235	786	863	577
	132	167	1351	959	551	829	586
7	236	201	1053	1111	1764	1290	1007
	225	243	1065	1129	2008	1666	976
8	203	221	1133	1654	1192	885	491
	186	185	1125	1703	1246	1028	604
9	445	251	1174	1378	1229	675	465
	397	332	1628	967	1413	561	577
10	514	176	943	2167	1111	1228	592
	447	188	975	2378	1168	1218	692
Mean	262	217	1067	1555	1151	1038	683
S.E.	±36	±12	±62	±143	±100	±80	±63

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VII

CONCENTRATION OF COMPOUNDS FROM THE SKIN OF CONTROL RAINBOW TROUT.
(Uncorrected data for Table V.)

Fish No.	Concentration ($\mu g/cm^2$ skin)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	125	61	14	10.3	11.8	16.3	4.0
	139	59	10	9.5	12.6	14.8	4.3
2	109	57	7	5.3	7.8	20.4	4.0
	101	63	7	3.5	9.4	21.1	4.5
3	148	40	7	6.2	10.2	10.0	4.2
	153	42	11	6.7	8.2	11.1	4.5
4	108	38	8	9.5	7.4	19.2	3.5
	104	33	6	8.5	9.4	20.4	3.7
5	122	44	6	7.7	9.0	10.7	5.0
	143	57	10	7.0	7.4	11.8	4.9
6	118	41	7	5.0	10.6	7.7	5.5
	153	42	7	6.1	9.0	8.1	5.7
7	139	33	9	6.2	14.9	14.1	4.3
	143	34	10	5.3	16.5	17.0	4.2
8	160	63	7	7.3	6.2	17.4	3.0
	141	52	8	7.9	7.8	13.7	3.1
9	155	56	7	8.5	10.2	22.9	4.7
	132	44	9	7.0	9.0	23.8	5.3
10	115	40	7	3.8	11.7	15.5	3.6
	139	43	7	4.4	13.3	15.5	3.7
Mean	132.5	47.1	8.2	6.8	10.1	15.6	4.3
S.E.	±5.2	±3.1	±0.5	±0.6	±0.8	±2.0	±0.3

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VII cont'd

CONCENTRATION OF COMPOUNDS FROM THE SKIN OF ACTH-TREATED RAINBOW TROUT. (Uncorrected data for Table V).

Fish No.	Concentration (μg/cm² skin)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	113	40	6	15.8	11.0	20.0	10.4
	127	45	6	17.7	12.9	21.5	11.1
2	49	50	9	21.9	12.9	27.7	14.1
	94	52	8	24.2	10.6	25.9	12.9
3	137	58	6	21.9	5.5	26.6	13.3
	146	56	8	20.6	7.0	30.5	14.1
4	97	40	10	20.6	5.9	22.2	11.1
	92	50	6	17.7	7.8	17.0	9.6
5	113	33	7	20.6	11.0	30.3	11.5
	134	35	6	21.9	9.4	29.6	12.9
6	105	37	6	13.4	7.4	17.7	14.4
	141	39	6	14.1	8.6	18.5	14.1
7	127	33	11	14.7	10.6	21.5	12.6
	132	36	9	18.3	12.2	20.0	11.5
8	148	55	9	23.0	8.6	26.6	13.3
	130	52	9	20.6	9.8	28.1	11.9
9	143	42	7	18.9	8.6	24.9	12.9
	120	44	7	20.6	7.4	21.5	11.9
10	104	35	6	15.3	9.4	23.8	13.3
	99	38	6	18.9	7.0	24.1	12.9
Mean	120.1	43.6	7.4	19.0	9.2	24.1	12.5
S.E.	±5.4	±2.5	±0.4	±0.9	±0.7	±1.3	±0.4

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VII cont'd

SPECIFIC ACTIVITY OF COMPOUNDS FROM THE SKIN OF CONTROL RAINBOW TROUT. (Uncorrected data for Table V.)

Fish No.	Specific activity (cpm/μmole)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	87	222	1378	1484	444	2137	490
	82	239	2048	1379	650	2222	512
2	65	165	1154	1576	1372	3444	419
	70	158	1052	1500	815	2684	552
3	61	207	1655	1079	1238	3444	604
	68	208	1628	1047	1364	3500	736
4	98	184	2306	621	1413	2790	476
	73	237	2510	453	1060	2166	526
5	91	252	2020	1702	1306	3100	786
	83	237	1819	1613	1728	3619	724
6	76	160	1788	1000	1136	3071	731
	71	182	1272	968	869	2500	820
7	112	186	2038	1368	650	1120	256
	92	131	1730	1225	866	1161	470
8	56	211	1823	755	1311	1032	471
	74	175	1961	503	1176	1200	530
9	79	241	2600	1509	651	1073	591
	94	319	2241	1704	596	1238	638
10	82	165	2206	1826	533	1714	775
	88	178	2147	1407	478	1428	619
Mean	80	202	1869	1236	983	2241	586
S.E.	±4	±12	±131	±112	±130	±297	±43

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.

APPENDIX VII cont'd

SPECIFIC ACTIVITY OF COMPOUNDS FROM THE SKIN OF ACTH-TREATED RAINBOW TROUT. (Uncorrected data for Table V.)

Fish No.	Specific activity (cpm/μmole)						
	Purine compounds			Pteridine compounds			
	G	H	GMP	BP	AHP	IXP	IP
1	71	234	2245	5367	2232	3638	1675
	82	220	2775	4127	2450	2736	1464
2	90	191	2852	4417	1137	3040	1468
	80	228	2790	4853	1829	3717	1250
3	76	509	2918	2656	3666	2125	1092
	77	540	3112	2062	3071	2823	1129
4	93	160	2100	2556	1739	3500	960
	77	179	2510	3800	1709	4419	988
5	134	237	3461	3406	1767	2037	1073
	127	218	3456	2567	1351	1641	1034
6	107	259	2130	5309	3690	5218	1550
	79	284	2108	5613	3147	4788	1468
7	160	218	2418	5511	1951	2210	1155
	173	218	2691	3875	1395	1916	1121
8	64	395	3070	5225	588	5020	1260
	55	431	2734	5453	947	5360	1509
9	79	163	2379	2551	1764	4644	1465
	71	167	2690	1515	2484	5727	1367
10	163	260	3108	4297	3135	3571	840
	152	272	2551	3448	3536	2093	775
Mean	100	269	2715	3930	2179	3511	1232
S.E.	±12	±36	±120	±389	±291	±402	±79

Abbreviations: G = guanine, H = hypoxanthine, BP = biopterin, IP = ichthyopterin, others as in text.