STUDIES IN THE PTERIDINE SERIES

A thesis submitted to the

University of Glasgow

for the degree of

Doctor of Philosophy

by

John A. Blair.

Glasgow University August 1958.

ProQuest Number: 13850352

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 13850352

Published by ProQuest LLC (2019). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGMENTS

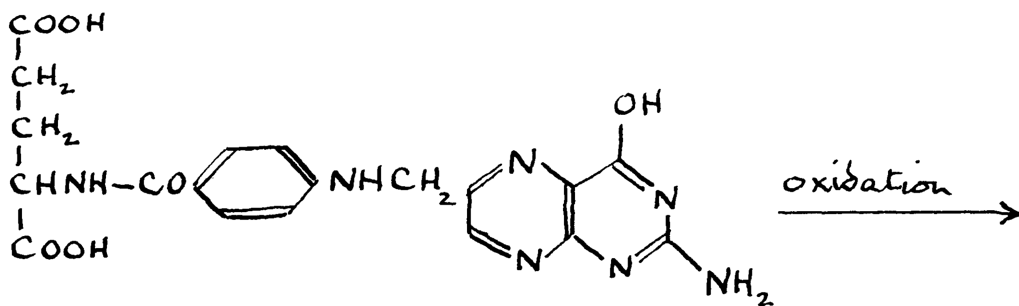
The author wishes to thank the following people for their assistance and advice.

Professor A. Albert.

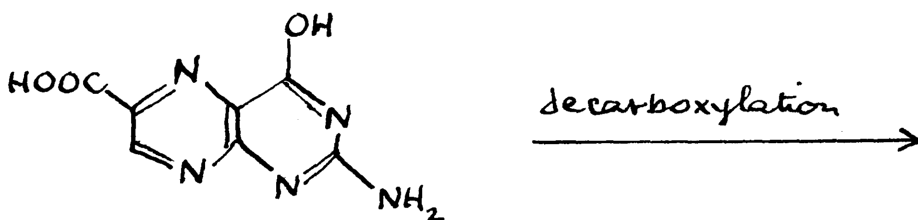
Professor J. Graham.

Professor A. Haddow.

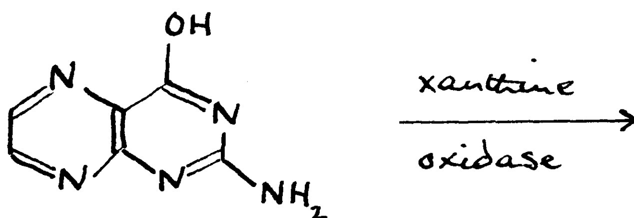
Professor R. Tschesche.



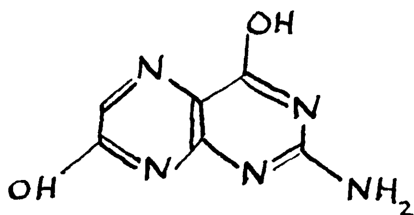
pteroyl-L-monoglutamic acid



2-amino-4-hydroxypteridine-6-carboxylic acid



2-amino-4-hydroxypteridine



<u>iso</u>xanthopterin

Summary

It has been found that the green colour of certain snakes is caused by a structural blue colour together with two yellow pigments. One of these yellow pigments has been identified as riboflavin. Other colourless fluorescent compounds were also isolated. In one species these were isoxanthopterin and 2-amino-4-hydroxypteridine-6-carboxylic acid and in two other species the previous two compounds and 2-amino-4-hydroxypteridine.

A metabolic scheme linking these substances and giving a possible origin for them has been suggested (see facing page).

Further evidence has been obtained for this scheme. There were indications from the literature that folic acid was oxidisable by xanthine oxidase. It has been found that xanthine oxidase is without effect on pteroyl-L-monoglutamic acid but that in the presence of methylene blue and xanthine oxidase pteroyl-L-monoglutamic acid is oxidised to 2-amino-4-hydroxy pteridine-6-carboxylic acid. Isoxanthopterin but not 2-amino-4-hydroxypteridine-6-carboxylic acid has been isolated from normal human urine. It is now suggested that folic acid is catabolised in the mammalian body to the inert end-product isoxanthopterin.

Recent work by other investigators has supported the existence of a catabolic pathway for folic acid.

Albert has suggested that pteridines (e.g. xanthopterin) would be found in higher concentration in tissues undergoing excessive rate of growth than in normal ones.

To test this hypothesis normal hamster kidneys and tumours arising from them (primary and transplanted) were analysed for their pterin content. No xanthopterin could be found. It was found that a blue fluorescent substance, tentatively identified as 2-amino-4-hydroxypteridine-6-carboxylic acid was present in normal kidney tissue in much higher concentration than in the neoplastic issue. Previous surveys of neoplastic tissues have shown that their folic acid content is the same or higher than normal tissues. The reduced content of 2-amino-4-hydroxy pteridine-6-carboxylic acid would therefore suggest that the neoplastic cells degraded folic acid to a much lesser extent than did normal cells. The failure of neoplastic cells to degrade folic acid might explain why neoplasms have a greater growth rate than their normal tissue of origin.

It is known that if golden hamsters are injected with certain pteridines and a variety of other substances their kidneys hypertrophy. When the hypertrophied kidneys were analysed for pterin content neither xanthopterin nor

2-amino-4-hydroxy pteridine-6-carboxylic acid could be isolated. This would suggest that an increased rate of cell division is accompanied by the failure of the cell to degrade folic acid.

It is now suggested that the catabolism of folic acid is a means of controlling cell division; normal cell hyperplasia being due to a local high concentration of folic acid caused by a temporary inhibition of folic acid catabolism; neoplasia being accompanied by a permanent inhibition of folic acid catabolism.

Contents

Part 1. Page

Pigment and Pterins in Snake Skins 1

Part II.

Metabolism of Folic Acid 13

Experimental 40

References 63

Part I

The Pigments and Pterins present in Snake Skins

Identification of Riboflavin as a pigment in P. semivariegatus

It has been known for some time that when whole snakes or their skins are preserved by immersion in 40% formalin solution, a greenish yellow colour is imparted to the solution. This effect is particularly marked with green snakes. Krukenberg, in 1882 (1), described the extraction with alcohol of a yellow pigment from snake skins but he did not isolate it. No further work on this pigment has been described in the literature.

Three species of green snakes were available locally to us.

1. Philothamnus semivariegatus, which occurs only as the green coloured variety.

2. Dispholidus typus, which occurs locally only as the green coloured variety, although black, brown, yellow and olive varieties are found elsewhere e.g. South Africa.

3. Dendrosaspis viridis, which occurs locally as the green variety although a black variety is found in South Aftica.

D. viridis is uncommon, so experiments were started on P. semivariegatus and D. typus.

Specimens of P. semivariegatus were killed with chloroform, decapitated and skinned. The skin was thoroughly cleaned, all traces of subcutaneous tissue being removed by thorough scrubbing. The skin was then cut into pieces and extracted with absolute ethanol in the dark in a Soxhlet apparatus. After cooling and filtering, the ethanolic solution was yellow in colour and had a green fluorescence. On exposure to sunlight both the colour and the fluorescent vanished. The colour and fluorescence also disappeared on the addition of acid, alkali or sodium hydrogen sulphite. These properties suggested that the yellow pigment was riboflavin. However, as each skin contained only a small quantity of the pigment and as it was difficult to obtain more than a few snakes per year, it was impossible to identify the pigment by a rigorous chemical method.

Two micro methods, however, were available. The first method used was paper chromatography; the second was ultraviolet spectroscopy. Paper chromatography has proved a powerful tool in the resolution of complex biological mixtures and in the identification of minute amounts of substances, by comparing their chromatographic behaviour with that of known substances in several

different solvent systems. Qualitative ultraviolet spectroscopy will not definitely identify a compound but an inspection of the absorption curve will, in many cases, indicate the type and probable identity of the compound under investigation.

A satisfactory combination of these two techniques is, firstly, to separate the mixture by paper chromatography and determine the ultraviolet absorption of each substance; and, secondly, having determined the type and probable identity of each substance, to confirm its identity by its similar chromatographic behaviour with a known substance in several different solvent systems.

After the mixture has been separated by paper chromatography the ultraviolet absorption spectrum of the compounds may be determined in two ways. In the first method, which is the more accurate, the substance is eluted from the paper by a suitable solvent (2), e.g. water, $\frac{N}{10}$ ammonium hydroxide solution. This method, however, requires relatively large amounts of substance; it is always necessary to prepare the blank solution by eluting a similar piece of chromatographic paper (developed with the same solvent) with the same eluting solvent, (3), and it is often difficult to elute from the paper (4). In the second method the spectrum is determined directly on the paper (4), a piece of chromatogram paper treated

similarly being used as a blank. This method allows the determination of the spectrum on the minimum amount of material but as the absorption of the blank is high, maxima, especially broad ones, cannot be determined accurately and peaks can only be placed within 5 mu to 10 mu. Spectra obtained by this method are not strictly comparable with spectra obtained in solution, but only with spectra obtained in a similar way on paper. The accuracy of this method might be improved by soaking the paper strips used in one of the oils now commercially available for photometric densitometers.

Two other micro methods could have been used, but were not available. It is possible to determine infra red spectra directly on paper (5) or on eluates from paper (6). The first method does not give a full infra red absorption spectrum because of the strong -OH band absorption of the cellulose. The second method gives a complete curve but requires further study.

Paper electrophoresis is used to separate complex mixtures and to identify individual components, and has been used to separate mixtures of flavine and related compounds (7).

It was found possible to extract the yellow pigment from the skin of P. semivariegatus using 0.5 N ammonium

hydroxide solution, 80% aqueous ethanol and pyridine-propanol-water (3:1:1 v/v). Paper chromatographic analysis of these solutions showed that they all contained substances which fluoresced strongly in ultraviolet light. One fluoresced yellow and could be detected in large quantities as a yellow spot in daylight; one fluoresced purple and the other bright blue.

After paper chromatography of a pyridine-propanol-water (3:1:1 v/v) extract of P. semivariegatus in the same solvent the ultraviolet absorption spectrum of the yellow fluorescing spot was determined using the method of Bradfield and Flood (4). This gave two peaks, 260mu and 370 mu. The spectrum of riboflavin determined under the same conditions had two peaks, 260 mu and 360 mu. The identity of the yellow fluorescent substance as riboflavin was confirmed by its identical chromatographic behaviour with a known sample in three different solvent systems: pyridine-propanol water (3:1:1 v/v) (8), butanol-acetic acid-water (4:1:5 v/v) (9), (10), and tert.-butanol-pyridine-water (50:15)35 v/v) (8).

Identity of Pterins in P. Semivariegatus

The two other fluorescent substances were classified as pteridines by their solubility in ammonium hydroxide, their strong fluorescence in ultraviolet light and their absorption spectra.

The spectra were determined by the method of Bradfield and Flood (4) and showed the typical pterin pattern of two maxima between 200 mu and 400 mu with one peak between 200 mu and 300 mu. The purple fluorescing substance had two peaks, at 255 mu and 335 mu and the bright blue fluorescing substance had two peaks at 250 mu and 355 mu. Their spectra, characteristic fluorescence and R_F values in 3% aqueous ammonium chloride (11) suggested that these substances were isoxanthopterin and 2-amino-4-hydroxy pteridine-6-carboxylic acid. As pterins are infusible, difficultly crystallisable substances, paper chromatography is a convenient method of identification (12) and identity is considered established if an unknown substance shows identical chromatographic behaviour with a known substance in three different solvent systems (12).

The purple fluorescing substance was identified as isoxanthopterin by its identical chromatographic behaviour with a known sample in six different solvent systems viz: 3% aqueous ammonium chloride (13) 5% aqueous acetic acid (14), butanol-acetic acid-water (4:1:5 v/v) (9), (10), (15), pyridine-propanol-water (3:1:1 v/v) (8), tertiary butanol-pyridine-water (50:15:35 v/v) (8), tertiary butanol-pyridine-water (60:15:25 v/v) (8). The bright blue fluorescing substance was similarly identified as 2-amino-4-hydroxy-pteridine-6-carboxylic acid by its identical chromatographic behaviour with a known sample in the same six solvent

systems and in propanol-5% aqueous acetic acid (2:1 v/v) (14) and in dimethyl-formamide-formic acid-water (8:1:1 v/v).

Identity of yellow pigments and Pterins in D. typus

When the skin of D. typus was extracted with pyridine-propanol-water (3:1:1 v/v), 0.5 N ammonium hydroxide, absolute ethanol or 80% aqueous ethanol the solution was coloured yellow but had only a weak green fluorescence. Paper chromatographic analysis as for the extracts of P. semi-variegatus showed the presence of riboflavin, isoxanthopterin-2-amino-4-hydroxy-pteridine-6-carboxylic acid and another yellow, non-fluorescent substance. As judged visually by the spot size and intensity of fluorescence, the amount of riboflavin present in the skin of D. typus was less than that in the skin of P. semivariegatus. Attempts to classify the second yellow pigment failed. Neither the use of suitable spot tests nor its ultraviolet absorption spectrum indicated its possible structure. It may belong to the ill-defined and little-known class of ommatines (16).

When the ethanolic extracts of D. typus were evaporated to dryness and the residue taken up in a small amount of 0.5 N ammonium hydroxide, chromatographic analysis showed two more blue fluorescent substances. One of these has been identified as 2-amino-4-hydroxy-pteridine by its identical behaviour with an authentic sample in the eight

different solvent systems given before. The other blue fluorescent substance has not been identified.

When the combined ethanolic extracts of P. semivariegatus were similarly treated, the second blue fluorescent substance was detected but not 2-amino-4-hydroxy-pteridine. Failure to identify 2-amino-4-hydroxy-pteridine in this extract eliminated the possibility that it arose in the D. typus extract by decarboxylation of 2-amino-4-hydroxy-pteridine-6-carboxylic acid during the working-up process. This concentrated extract of P. semivariegatus also revealed, on chromatographic analysis, traces of the unidentified, yellow, non-fluorescent substance.

Identity of yellow pigments and Pterins in D. viridis

After this investigation had been completed a single specimen of the green mamba, Dendroaspis viridis, became available to us. The anterior and middle portions of the body were leaf green, the posterior portion was dirty yellow. The skin was cut into the green and yellow portions and each separately extracted with 80% aqueous ethanol.

The extract from the yellow skin was evaporated to dryness and the residue taken up in a small amount of 0.5 N ammonium hydroxide solution. Riboflavin and isoxanthopterin were detected in both extracts. 2-Amino-4-hydroxy-pteridine-6-carboxylic acid and 2-amino-4-hydroxy pteridine were

detected in the extract of the yellow skin. No attempt was made to find them in the ethanolic extract. A yellow non-fluorescent pigment was present in both extracts.

When compared with the specimens of P. semivariegatus and D. typus examined, D. viridis had much less riboflavin than either, more yellow non-fluorescent pigment and more 2-amino-4-hydroxy-pteridine. In none of the specimens examined was it possible to detect any flavin-adenine-mono-nucleotide nor flavin adenine-dinucleotide. These nucleotides might have been destroyed during the extraction procedures but later experience using similar methods of analysis on liver and kidney has shown that it is possible to identify flavin-adenine-dinucleotide and flavin-adenine mononucleotide by this method.

The Blue Colouration of the Snakes P. Semivariegatus, D. typus and D. viridis

When the skins of P. semivariegatus, D. typus and D. viridis were extracted with 80% aqueous ethanol until the extracts were colourless, the residual skin was coloured blue. It is commonly found that animals with green skin colouration obtain this effect by superimposing a yellow pigment on a structural blue (16) and it seemed desirable to find whether this blue colour was a structural one or due to a pigment.

Refluxing the skins of P. semivariegatus and D. typus with petroleum ether, benzene, chloroform, ether and ethyl acetate, failed to remove any colouring matter. When the blue skins were warmed with dilute acids or alkalis the skin became black and the solvents yellow.

The extract obtained from the blue skins by warming with 0.5 N ammonium hydroxide was examined by paper chromatography and was shown to contain only the yellow, non-fluorescent pigment, isoxanthopterin and 2-amino-4-hydroxy-pteridine-6-carboxylic acid.

When the skin was immersed in saturated bromine water for 30 minutes it was bleached.

These observations suggested that the blue colour was a structural one. Attempts to confirm this by the usual method of bleaching the melanin and restoring the blue colour by replacing the melanin by an artificial black background (16) such as carbon ink, failed, however. This failure was attributed to the destruction of the colloid system responsible for the light scattering, by the acidity of the bromine solution. The blue skins failed to bleach when left in extensive contact with 30% hydrogen peroxide. As all other available bleaching agents were acid in reaction and would have caused destruction of the colloid system no further attempts were made to bleach the skins.

The presence of a system capable of giving a structural blue was finally elucidated by an histological technique.

Portions of the skins were fixed in 10% neutral formaldehyde and transverse and horizontal sections taken. These were examined, unstained, on slides on which half the field was blackened with carbon black. Examination with transmitted light showed the clear part of the section yellow. When examined with reflected light the portion of the section lying over the blackened half of the slide appeared blue.

Similar technique applied to the blue skin from Dendroaspis viridis showed that it too was a structural blue.

It thus appears that the green colour of the skins of P. semivariegatus, D. typus and D. viridis is due to the presence of a Tyndall blue in association with two yellow pigments, one of which is riboflavin.

In P. semivariegatus, riboflavin is present in much greater amount than the yellow non-fluorescent pigment while in D. viridis the unknown pigment is present in greater amount than riboflavin. D. typus occupies an intermediate position. The skins of all three species also contain isoxanthopterin and 2-amino-4-hydroxy-pteridine-6-carboxylic acid. D. typus and D. viridis also contain

2-amino-4-hydroxy-pteridine while D. typus and P. semivariegatus contain another unidentified blue fluorescent substance. This substance was not looked for in the D. viridis extract.

It may be noted that as the riboflavin content decreases, that of 2-amino-4-hydroxypteridine increases. Correlations between riboflavin content and pterin content of tissues have been noted by previous workers. Koschara and Haug (17) found a parallel relationship between the riboflavin and xanthopterin contents of animal tissues. In the later stages of development of the grasshopper egg the pterin content increases as the riboflavin content decreases (18) (19). From genetic studies it appears there is a close relationship between riboflavin, uric acid and pteridines (20), (21). It has been suggested that there is a common intermediate between riboflavin, folic acid and biopterin (22).

Part II

Metabolism of Folic Acid

Suggested Catabolic Scheme

These three pterins (isoxanthopterin, 2-amino-4-hydroxypteridine-6-carboxylic acid, and 2-amino-4-hydroxypteridine) and riboflavin are widely distributed.

Forrest, Van Baalen and Myers [23] found 2-amino-4-hydroxypteridine and 2-amino-4-hydroxypteridine-6-carboxylic acid in the blue-green alga, Anacystis nidulans, after cessation of photosynthesis. Xanthopterin, isoxanthopterin and icythopterin have been isolated from the scales of Indonesian fresh-water fishes[24]. Xanthopterin, isoxanthopterin and unidentified fluorescent compounds have been found in the eyes of Lepidoptera[25]. Viscontini et al [26, 27] have reported the presence of riboflavin, biopterin, isoxanthopterin, 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxypteridine in Drosophila melanogaster.

Forrest and Mitchell[28, 29] have described biopterin, isoxanthopterin, 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxypteridine and a yellow fluorescent compound formulated as 2-amino-4-hydroxy-7:8-dihydro-8-lactylpteridine-6-carboxylic acid in the Drosophila mutant sepia. The formulation of the last compound

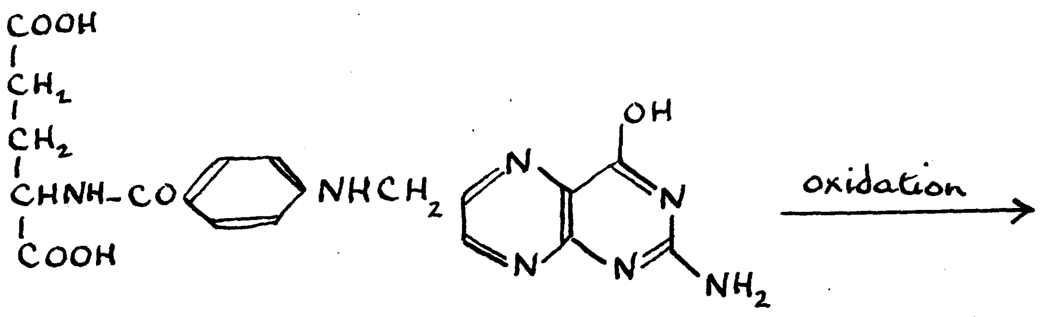
has been challenged[30]. Viscontini et al have found isoxanthopterin, 2-amino-4-hydroxypteridine and biopterin in Astacus fluviatilis[31] and riboflavin, xanthopterin, isoxanthopterin, 2-amino-4-hydroxypteridine and biopterin in Ephestia kühniella[32].

Isoxanthopterin has been detected in the eltrya of lady beetles[33], butterfly wings[34], the skin of Rana nigromaculata[35], the larvae of Bombyx mori[35, 36], Drosophila melanogaster[37] and the silkworm[37].

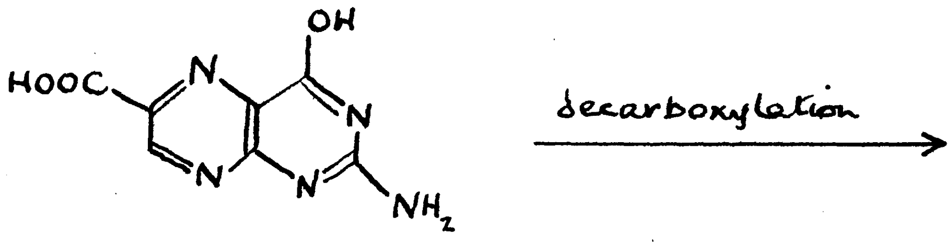
It has also been identified with mesopterin[38] and recognised as a component of fluorescyanin[36]. 2-Amino-4-hydroxypteridine-6-carboxylic acid has been found in frog skins[39], silkworms[37] and Drosophila[37].

Gunder has reported the presence of riboflavin in reptile skins[40]. She also found other fluorescing substances, one of which was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid[41].

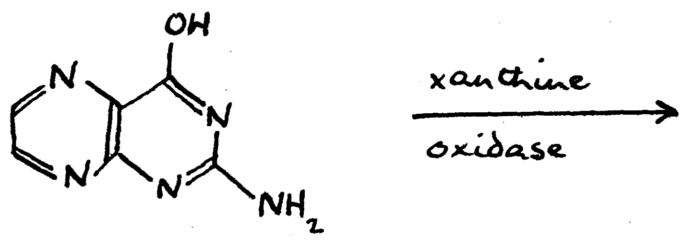
In describing the properties of the alleged 2-amino-4-hydroxy-7:8-dihydro-8-lactylpteridine, Forrest and Mitchell said that it closely resembled riboflavin in colour, fluorescence and chromatographic behaviour but that it could be distinguished from riboflavin by its behaviour in paper chromatography using butanol-acetic acid-water (4:1:5 v/v) as developing solvent, and in its



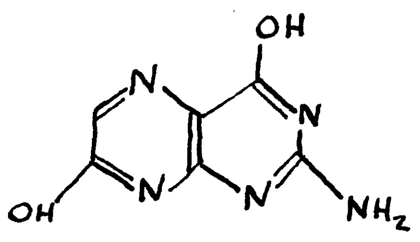
pteroyl-L-monoglutamic acid



2-amino-4-hydroxy pteridine-6-carboxylic acid



2-amino-4-hydroxy pteridine



*iso*xanthopterin

SUGGESTED CATABOLIC SCHEME

ultraviolet spectra, which did not have a maxima in the region 300-400 mu[42].

Danneel and Eschrich[43] have recently reported that the eyes of Drosophila melanogaster contain a yellow fluorescent pigment which has a spectrum similar to that of riboflavin and shows similar chromatographic behaviour to riboflavin. They found that this yellow pigment was confined to the eyes and that the yellow pigment found in the Malpighian tubules of the same insect was riboflavin. Goodwin and Srisukh[44] also have described the occurrence of a yellow fluorescent pigment (not riboflavin) in locust eyes. Forrest et al have reported the occurrence of a yellow fluorescent pigment in algae[23, 45].

The three pterins, isoxanthopterin, 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxy-pteridine are of widespread occurrence and it seems possible they represent the pathway whereby folic acid is catabolised in the animal body. A suggested catabolic scheme is given on the facing page.

The flavoprotein enzyme xanthine oxidase, is already known to oxidise 2-amino-4-hydroxypteridine to isoxanthopterin [47, 48]. Since xanthine oxidase contains riboflavin it is plausible to assume that the riboflavin content of a tissue is a crúde indication of its content of xanthine oxidase, and it is suggestive that the amount of 2-amino-4-hydroxy-

pteridine in the snake skins increases as the amount of riboflavin decreases. Forrest, Glassmann and Mitchell[49] showed that extracts of Drosophila melanogaster oxidised 2-amino-4-hydroxypteridine to isoxanthopterin. As this extract also oxidised xanthopterin to leucopterin and xanthine to uric acid they assumed that the active entity was xanthine oxidase.

Folic acid is known to be readily oxidised by oxygen in alkaline solution to 2-amino-4-hydroxypteridine-6-carboxylic acid[50] and by sulphurous acid and light to dihydro-2-amino-4-hydroxypteridine-6-aldehyde[51, 52, 53]. The latter compound can be readily oxidised to 2-amino-4-hydroxypteridine-6-aldehyde by iodine in sodium iodide[49]. The aldehyde can then be oxidised to the corresponding acid by hydrogen peroxide[54] or enzymatically by xanthine oxidase[47,55] or liver aldehyde oxidase[56]. 2-Amino-4-hydroxypteridine-6-carboxylic acid may be decarboxylated by heat[57] or by ultra-violet light[58,59]. On irradiating folic acid in solution with ultra-violet light it is converted into a mixture of 2-amino-4-hydroxypteridine-6-aldehyde and the corresponding acid[58].

Some time after the publication of this scheme[60] a similar scheme was suggested[33]. Forrest and Mitchell[29] suggested that the Drosophila pterins and uric acid were linked together in the following way.

Uric acid ⟶ isoxanthopterin ⟶ fluorescyanine ⟶ 2-amino-4-hydroxypteridine-6-carboxylic acid ⟶ 2-amino-4-hydroxypteridine. The interconversion uric acid ⟶ isoxanthopterin was based on the known conversion of some purines to pteridines[61,62]. In a later paper[48], however, they showed that an extract of Drosophila melanogaster oxidised 2-amino-4-hydroxypteridine to isoxanthopterin and concluded that this was the origin of isoxanthopterin in Drosophila. This extract also oxidised xanthine to uric acid and this common enzymatic origin explains the parallel occurrence of isoxanthopterin and uric acid in Drosophila mutants[21].

Action of Xanthine oxidase on Pteroyl L glutamic acid

A survey of the published literature on xanthine oxidase showed three reports claiming that xanthine oxidase could oxidise pteroyl glutamic acid. Williams and Elvehjem [63] suggested that xanthine oxidase could oxidise pteroyl glutamic acid. Jacobson and Good[64] claimed that on incubating xanthine oxidase and 2:6 dichlorophenolindophenol with pteroyl glutamic acid under anaerobic conditions, the colour of the dyestuff was discharged within 16 minutes. The end product of this reaction had increased haemopoietic properties compared with the original pteroyl glutamic acid. Norris and Majnarich[65] incubated xanthine oxidase with

pteroyl glutamic acid for 18 hours and obtained a product having similar properties to that of Jacobson and Good. None of these workers identified the end product of the enzymatic oxidation.

Unlike most enzymes xanthine oxidase is not specific in its reactions. It catalyses the re-oxidation of reduced diphosphopyridine nucleotide, all aldehydes, xanthopterin, 2-amino-4-hydroxy-pteridine, xanthine and hypoxanthine. As there is little resemblance between pteroyl glutamic acid and any of these known substrates it seemed unlikely that xanthine oxidase could oxidise it. Support for this view was found by Kalckar et al[56,66,67] who could not detect any change in the growth promoting properties of pteroyl glutamic acid before and after incubation with xanthine oxidase. Slavik[68] failed to observe any action of xanthine oxidase on pteroyl-L-mono-glutamic acid. Rauen et al[69,70] and Yamamota[59] found that when pig liver homogenate was incubated with pteroyl glutamic acid a bright, pale blue fluorescent compound was obtained. Rauen[71] later identified it as 10-formyl pteroyl-L-monoglutamic acid.

As liver is rich in xanthine oxidase it seemed worth while to repeat these experiments on the oxidation of pteroyl-L-monoglutamic acid using milk xanthine oxidase

at stage M5 as prepared by Avis, Bergel and Bray[72]. At our request Professor Haddow kindly sent a solution of xanthine oxidase at stage M5.

The pteroyl-L-monoglutamic acid which was used in these experiments contained a small quantity of 2-amino-4-hydroxy-pteridine-6-aldehyde and a trace of 2-amino-4-hydroxypteridine-6-carboxylic acid. Attempts to remove the former impurity failed, so it was decided to estimate it quantitatively and to allow for its presence in all experiments. The aldehyde was estimated by paper chromatography using butanol-acetic acid-water (4 : 1 : 5 v/v) as developing solvent, and the spot area technique[73,74]. The amount of aldehyde was estimated to be about 3% of the weight of pteroyl glutamic acid.

Milk xanthine oxidase can be directly oxidised by oxygen without the intervention of a hydrogen carrier, so in the earlier experiments xanthine oxidase was incubated alone with pteroyl glutamic acid in a phosphate buffer at a pH near neutrality and at 37°C. A small amount of 2-amino-4-hydroxypteridine-6-carboxylic acid was formed in these experiments but this represented a yield of less than 10% from the pteroyl glutamic acid and did not increase after the first hour of incubation. It was therefore concluded that this small amount of 2-amino-4-hydroxy-pteridine-6-carboxylic acid was formed by the oxidation

of the small amount of 2-amino-4-hydroxy-pteridine-6-aldehyde present[47,55] and that xanthine oxidase does not oxidise pteroyl glutamic acid.

To exclude any possibility that a hydrogen carrier might play a part in the oxidation of pteroyl glutamic acid by xanthine oxidase, a mixture of xanthine oxidase, pteroyl glutamic acid and methylene blue were incubated in a phosphate buffer at a pH near neutrality at 37°C. After eighteen hours incubation the incubated solution was examined by paper chromatography in butanol-acetic acid-water (4 : 1 : 5 v/v). A large bright blue spot with R_F close to that of 2-amino-4-hydroxy-pteridine-6-carboxylic acid was seen. When the incubated solution was examined by paper chromatography in other solvent systems the R_F value of the bright blue spot was quite different from that of 2-amino-4-hydroxy-pteridine-6-carboxylic acid. The possibility that this difference was due to protein present in the extract was checked by adding 2-amino-4-hydroxy-pteridine-6-carboxylic acid to the extract and observing its behaviour in paper chromatography. In each case the 2-amino-4-hydroxy-pteridine-6-carboxylic acid added to the extract showed the same behaviour as that of the bright blue fluorescent substance.

The bright blue fluorescent substance was purified by chromatography on a column of Whatman standard grade (1"

diam. and 14½" high) with 5% by vol. aqueous acetic acid. The eluate containing the bright blue fluorescent substance was evaporated to dryness and the residue taken up in 0.5 N ammonium hydroxide. Determination of the ultraviolet spectrum of this substance in 0.1 N sodium hydroxide showed two maxima, at 261 mu and 361 mu. Further purification of this substance by chromatographing part of the ammoniacal extract on a No. 1 paper using butanol-acetic acid water (4 : 1 : 5 v/v) and eluting the bright blue fluorescent band with 0.1 N sodium hydroxide gave an ultraviolet spectrum with two maxima, at 262 mu and 363 mu. 2-Amino-4-hydroxy-pteridine-6-carboxylic acid has two maxima at 262 mu and 365 mu[49]. The identity of the bright blue fluorescent substance as 2-amino-4-hydroxy-pteridine-6-carboxylic acid was confirmed by comparison of its chromatographic behaviour with that of an authentic sample of the acid in six different solvent systems. (Table I).

The R_F value of the bright blue spot in the incubated extract was sufficiently close to that of 2-amino-4-hydroxy-pteridine-6-carboxylic acid to allow a semi-quantitative determination by the spot area technique[73,74]. As thus determined, the yield of 2-amino-4-hydroxy-pteridine-6-carboxylic acid was about 60% of the theoretical yield

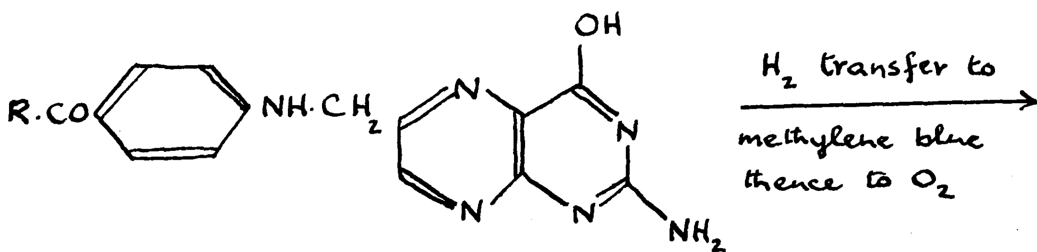
from pteroyl glutamic acid. There is thus no doubt that in the presence of xanthine oxidase and methylene blue pteroyl-L-monoglutamic acid is oxidised.

This oxidation could have been brought about in two ways. Either the oxidation of pteroyl glutamic acid by xanthine oxidase took place at an active centre of the enzyme which was not auto-oxidisable or the methylene blue dehydrogenated the pteroyl glutamic acid to the corresponding anil, which then hydrolysed to the amine and aldehyde.

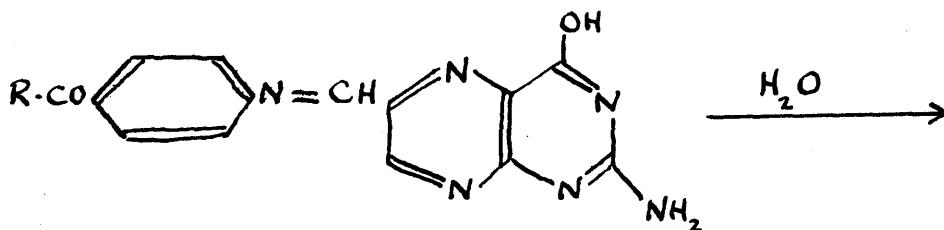
The latter reaction path seemed possible as pteroyl glutamic acid readily transfers two hydrogen stems to form the corresponding anil; and this anil, and model compounds related to it (e.g. I) are readily hydrolysed by water[50].

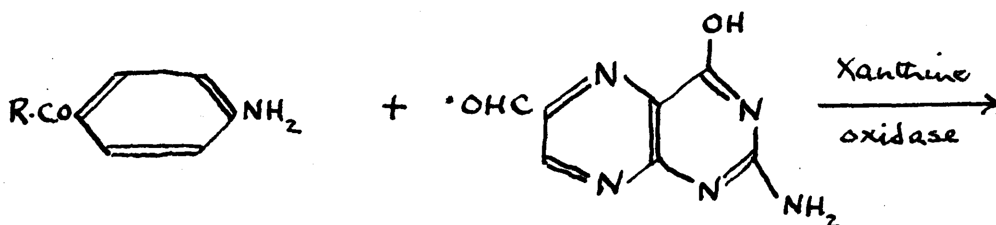
Pteroyl glutamic acid may transfer its two hydrogen atoms to oxygen or an inorganic oxidising agent, or to the pyrazine ring of the pteridine nucleus.

When a mixture of methylene blue and pteroyl glutamic acid was incubated in a phosphate buffer near neutrality at 37°C. for eighteen hours, and the resulting solution analysed by paper chromatography in butanol-acetic acid-water (4 : 1 : 5 v/v) a large blue fluorescent spot was seen, whose R_F value corresponded to that of 2-amino-4-hydroxy-pteridine-6-aldehyde. There was only a small spot corresponding

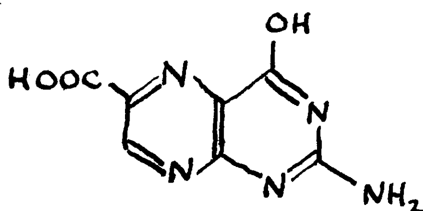


Pteroyl-L-monoglutamic acid

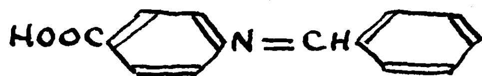




2-amino-4-hydroxypteridine
6-aldehyde



2-amino-4-hydroxypteridine-6-carboxylic acid



(1)

R represents $HOOC \cdot [CH_2]_2 \cdot CH(COOH) \cdot NH$

OXIDATION OF PTEROYL-L-MONOGLUTAMIC ACID

to that of 2-amino-4-hydroxy-pteridine-6-carboxylic acid.

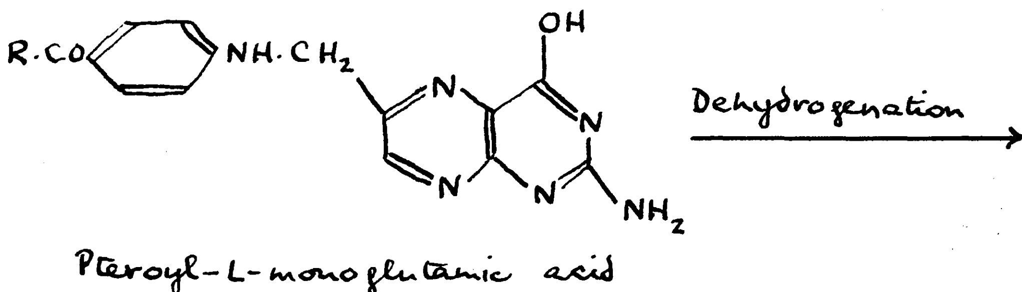
The major blue fluorescent spot was identified as 2-amino-4-hydroxy-pteridine-6-aldehyde by its identical chromatographic behaviour in seven different solvent systems with a known specimen of the aldehyde (Table I). The amount of aldehyde in the digest was quantitatively estimated as before. The yield of aldehyde was about 65% of the theoretical yield from pteroyl glutamic acid.

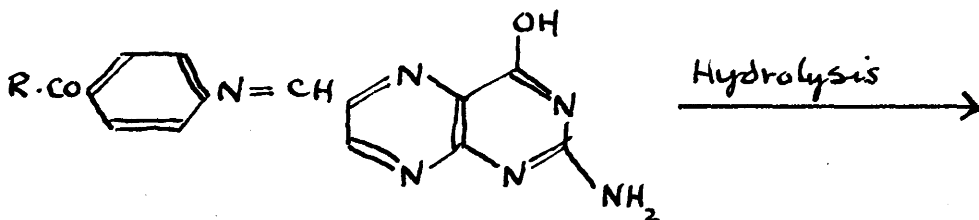
A reaction scheme for the oxidation of pteroyl glutamic acid by methylene blue and xanthine oxidase is given on the facing page.

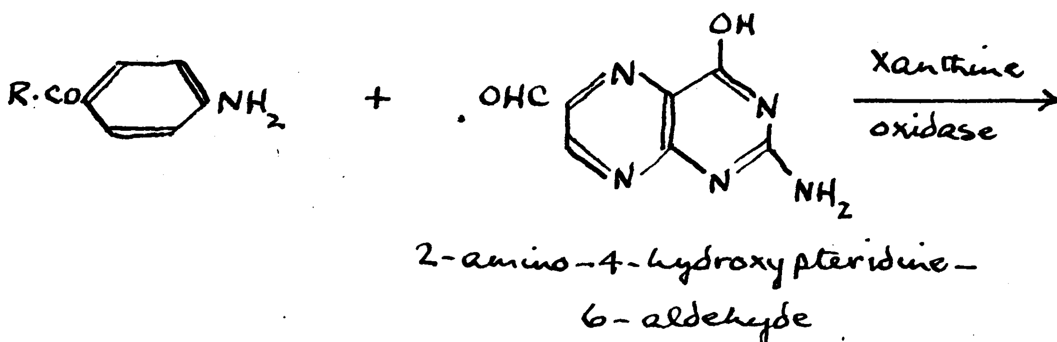
In this scheme xanthine oxidase may be replaced by liver aldehyde oxidase.

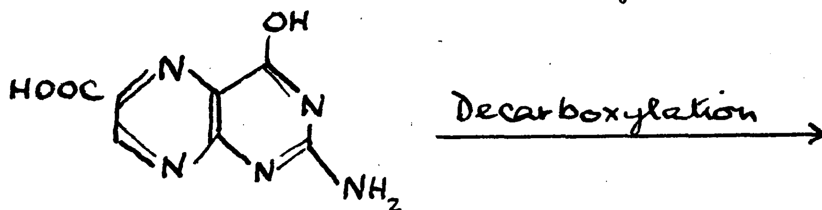
Isolation of isoXanthopterin from Human Urine

The pterins xanthopterin[75] and biopterin[76] have been previously isolated from human urine. As a method of testing the validity of our hypotheses on the catabolism of pteroyl-L-glutamic acid, we examined normal adult male urine for the presence of the pterins isoxanthopterin, 2-amino-4-hydroxypteridine and 2-amino-4-hydroxypteridine-6-carboxylic acid. Analysis of urine as described in the experimental section resulted in the isolation of a small quantity of purple fluorescent substance. On further purification this substance was shown to be isoxanthopterin

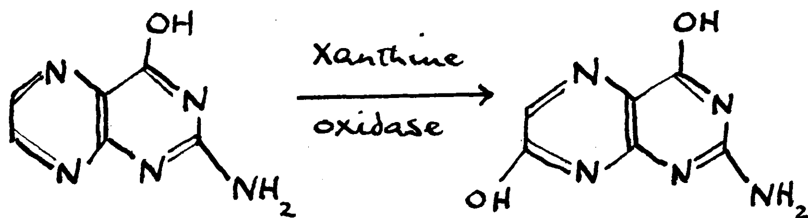








2-amino-4-hydroxypteridine-6-carboxylic acid



2-amino-4-hydroxy-
pteridine

*iso*xanthopterin

R represents $HOOC \cdot (CH_2)_2 \cdot CH(COOH) \cdot NH$

CATABOLISM OF PTEROYL-L-MONOGLUTAMIC ACID

by its similar behaviour to an authentic sample in six solvent systems.

No 2-amino-4-hydroxypteridine-6-carboxylic acid could be isolated from freshly voided human urine. If however, samples were allowed to stand, or if the analysis was unduly protracted a second bright blue fluorescent substance could be isolated. As this substance appeared to be an artefact no attempts were made to purify it further. It was not separable from added 2-amino-4-hydroxypteridine-6-carboxylic acid in five different solvent systems. Biopterin in aqueous solution, in light, readily decomposes to give this acid. It was therefore assumed that it originated from the biopterin present in the urine[76]. The initial chromatograms used to separate the pterins were very badly streaked after the band due to riboflavin and this made it impossible to use this method for the isolation of 2-amino-4-hydroxypteridine. It has been suggested by some authors[77,78] that folic acid is catabolized in the mammalian body by some yet unknown route. It is now suggested that the catabolism of folic acid proceeds by the route shown. Such a metabolic scheme is in harmony with the known facts and is supported by the isolation of isoxanthopterin.

Slavik et al[80] fed rats with tetrahydrofolic acid and isolated a blue fluorescent pteridine metabolite from their urine. This was not identified but it was not identical with isoxanthopterin, xanthopterin, biopterin, urothione; 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxypteridine-6-aldehyde 2-amino-4-hydroxy-6-methyl pteridine, xanthopterin carboxylic acid, isoxanthopterin carboxylic acid or 10-formyl-folic acid[81].

We suggested to Slavik that this compound might be 2-amino-4-hydroxypteridine. An authentic sample was sent for comparison but the results of this are not yet known.

Other workers have supplied evidence for the catabolism of pteroyl-L-monoglutamic acid. Rauen[82] has shown that 2-amino-4-hydroxy pteridine-6-aldehyde is produced when folic acid is incubated with liver mitochondria. His suggested reaction scheme is however very elaborate. Braganca and her coworkers[83,84,85] have demonstrated the presence of an enzyme system in liver and blood cells capable of degrading folic acid to 2-amino-4-hydroxy pteridine-6-aldehyde.

Xanthopterin was the first pterin to be found in human urine and it has been assumed by some workers to be an end-product of folic acid catabolism. Kalcker et al[86,87] found that xanthopterin was not present in freshly voided

urine, but that a colourless non-fluorescent substance was present, which was converted to xanthopterin by air oxidation. Futterman and Silverman[88] found a labile substance in human urine the properties of which resembled an unformylated reduced folic acid and Blakley[89] has demonstrated that di- and tetra-hydrofolic acids are very readily oxidised by molecular oxygen at room temperature to xanthopterin.

Slavik et al[80] found xanthopterin in the urine of rats fed tetrahydrofolic acid but showed that the xanthopterin was formed during the process of isolation by the non-enzymic oxidation of tetrahydrofolic acid.

If, however, any xanthopterin was formed within the body by an enzymic reaction, it might be expected that it would be oxidised by xanthine oxidase to leucopterin[90,91]. This expectation is confirmed by the occurrence of leucopterin in the kidney tubules of golden hamsters given injections of xanthopterin[92]. No leucopterin could be isolated from human urine. It thus appears that xanthopterin has no role in mammalian metabolism.

It has been suggested by Albert that xanthopterin could be made in vivo from guanine, but apart from the conversion of guanine to xanthopterin in vitro in very small yield[61,62],

no other evidence has yet been given. The only known in vivo conversion of purines to pteridines is the conversion of adenine to riboflavin[93] and also to an unknown pteridine[94] (apparently with unusual 2:4-dihydroxy pteridine structure[95]).

Nathan, Hutner and Levin[22] demonstrated that pteroyl-L-monoglutamic acid can be converted into biopterin and suggested that the conversion proceeded via a simple substituted 2-amino-4-hydroxypteridine.

Analysis of Tumours and Hypertrophied Kidneys

It has recently been observed that when golden hamsters are injected with xanthopterin and certain other compounds their kidneys hypertrophy[96]. This effect is due to an increase in the rate of cell division. Normally when adult kidney sections are examined microscopically it is difficult to find a cell in division, but when sections of a kidney which had hypertropied under the influence of pteridines is examined, many cells are seen undergoing division. It has been suggested by Haddow that this hypertrophy is due to the inhibition of the oxidation of xanthine, by the enzyme, xanthine oxidase, to uric acid[96,97]. Failure to oxidise this purine would presumably lead to an excessive cellular concentration which would cause an excessive rate of cell division.

Support for this theory has been obtained by comparing the hypertrophing action of pteridines and other compounds with their inhibiting effect on the oxidation of xanthine by xanthine oxidase. An approximate parallelism has been obtained[96]. It was also thought possible that xanthine oxidase inhibition, or a reduced level of activity, was a characteristic feature of neoplastic cells as compared with normal cells. This effect has been

demonstrated with mouse breast tumours[98]. Richert, Bloom and Westerfeldt[99] have shown that the xanthine oxidase content of rat liver is very much greater than that required for the maintenance of the pathway xanthine ⟶ uric acid and so the significance of altered levels of tissue xanthine oxidase is difficult to assess. If this inhibition of xanthine oxidase activity was a characteristic feature of neoplastic cells and the proposed catabolism of folic acid did take place, then it might be expected that tumour cells would differ in their pterin content from normal ones.

The methods of paper chromatography which had been developed for the study of the snake skin pterins could be used to detect pterins such as xanthopterin and isoxanthopterin in quantities as small as 0.1 ug., so it seemed possible to apply these methods of analysis to a neoplastic growth and its normal counterpart, to see, firstly, if there was a qualitative difference between the pterins present in normal and malignant cells and, secondly, if there was a quantitative difference.

With this end in view we approached professor Haddow, F.R.S., for his advice and cooperation. He very kindly supplied us with normal golden hamsters and

golden hamsters with induced kidney tumours. These tumours were induced by implanting a pellet of stilboestrol under the skin of a young male hamster. After an induction period of nine months the treated animals develop large nodular tumours on the kidneys and shortly afterwards die from renal failure[100]. These tumours arise from the kidney cortex and show all the characteristics of malignancy. They are admirably suited for chemical analysis as they are homogeneous, do not vary in type of neoplasm, are free from cell débris, clearly separated from normal tissue and are easily dissected out[101].

After conducting preliminary experiments on these tumours we used flank transplants of these tumours.[102] These can be produced more quickly and possess the same characteristics of easy dissection as the primary growths. In the analysis of the kidneys of the normal male hamsters the animals were killed with chloroform, the kidneys dissected out and ground with quartz sand. The homogenate was extracted first with ether and then with 0.5 N ammonium hydroxide. The ammoniacal extract was saturated with ammonium sulphate to remove protein, the protein centrifuged off and any pterin-like substances extracted with phenol. The phenol was washed several times with water or dilute acid and then the pterin-like substances were transferred

to solution in water by shaking the phenol solution with water and a large excess of ether[10]. The water solution was evaporated to dryness and the residue taken up in a small volume of 0.5 N ammonium hydroxide. This solution was examined by paper chromatography using propanol-5% aq. acetic acid (2 : 1 v/v) as developing solvent.

The analysis of normal kidney (c. 1g. wet weight) normally gave moving outwards from the origin (1) a bright yellow fluorescent (in U.V.) spot, sometimes visible as a yellow spot in deylight and identified as flavine mononucleotide (2) a faint blue fluorescent spot, provisionally identified as 2-amino-4-hydroxypteridine-6-carboxylic acid (3) a yellow fluorescent spot, identified as riboflavin (4) an unidentified blue fluorescent spot and (5) a faint fluorescent spot.

The flavinemononucleotide and riboflavin were probably formed by the decomposition of flavine adenine dinucleotide present in the tissue. Occasionally a small yellow fluorescent spot whose position corresponded to flavine adenine dinucleotide was found on the chromatogram.

The spot identified as 2-amino-4-hydroxypteridine-6-carboxylic acid was generally present only in small amounts (judged from fluorescence intensity ca. 0.01 ug.) and it

so far has proved impossible to obtain more than one chromatographic run for identification. The solvent used for this purpose was butanol-acetic acid-water (4 : 1 : 5 v/v upper phase), a solvent which clearly separates 2-amino-4-hydroxypteridine-6-carboxylic acid from all other common pterins.

When these analyses were performed on neoplastic tissues a similar chromatographic pattern was obtained with the exception that 2-amino-4-hydroxypteridine-6-carboxylic acid could not be detected in amounts of neoplastic tissue less than 8 grams (i.e. about 8 times the weight of normal tissues).

Xanthopterin could not be detected in either normal tissue or neoplastic tissue. The smallest amount of xanthopterin which could be detected by the paper chromatographic technique used was 0.1 ug.

Surveys of the folic acid content of normal and neoplastic tissues[103,104] has shown that the folic acid content of neoplastic tissues is the same as, or higher than, that of normal tissue. The reduced content of 2-amino-4-hydroxypteridine-6-carboxylic acid in the neoplastic tissues examined would therefore suggest that these neoplastic tissues degraded folic acid to a lesser amount than did the corresponding normal tissues.

To test a hypothesis that the increased rate of cell growths was due to a reduced degradation of folic acid, an identical analysis was carried out with 4 g. of hamster kidneys which had hypertrophied after an injection of 2,4,5-triamino-6-styryl pyrimidine. The analysis showed the presence of flavine adenine dinucleotide, riboflavin and several other unidentified spots. No xanthopterin or 2-amino-4-hydroxypteridine-6-carboxylic acid was found. From this preliminary work it would thus appear that an increased rate of cell division is accompanied by a decreased folic acid catabolism.

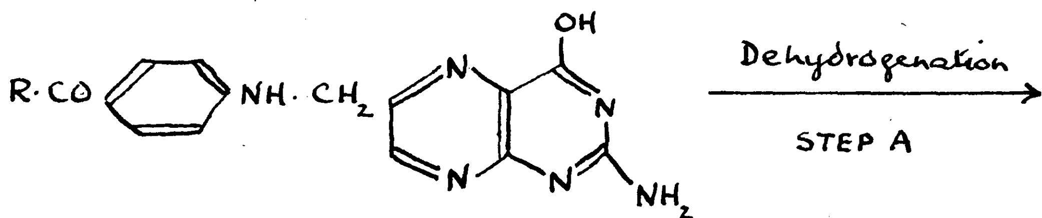
Metabolism of Pteroyl-L-glutamic acid

Pteroyl-L-glutamic acid is an important biological compound. It has been shown to have a curative effect on various forms of anaemia[105,106]. Mammalian liver converts it by reduction and formylation to 5-formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid[107] (i.e. citrovorum factor or leucovorin). Citrovorum factor is essential for cell division[108,109,110]. Pteroyl glutamic acid is also converted to co-enzymes which function in the interconversion of serine and glycine [111,112,113,114,115] and in the biosynthesis and interconversion of purines[116,117,118].

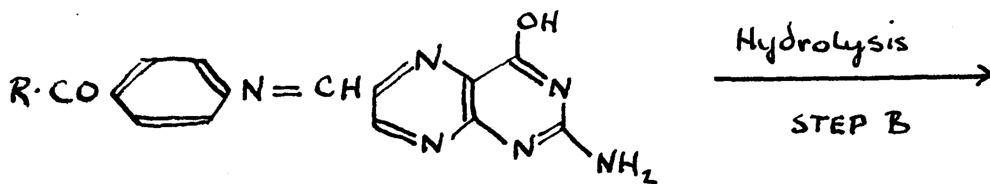
Despite the important biological status of pteroyl glutamic acid, little work has been done on the metabolic fate of the pteroyl glutamic acid which is not used in these activities.

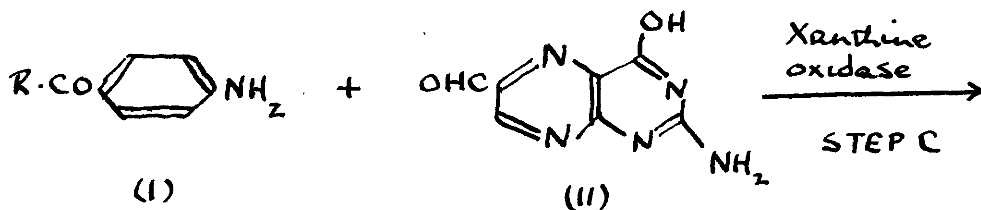
Of the normal human intake of 0.5-1 mg. per day, only about 1% is excreted in the urine. Considerable quantities are excreted in the faeces but this is due to synthesis by the intestinal flora and fauna. When rats are injected with massive doses of pteroyl glutamic acid only a small percentage is recovered in the urine[119].

If, however, large doses of ascorbic acid are fed as well, then the amount of pteroyl glutamic acid recovered

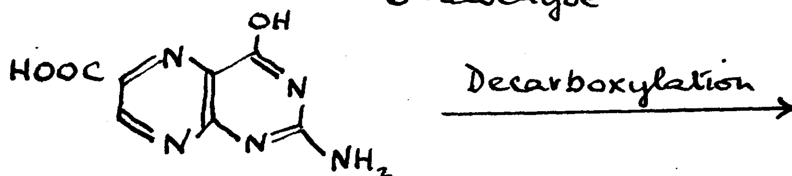


Pteroyl-L-monoglutamic acid

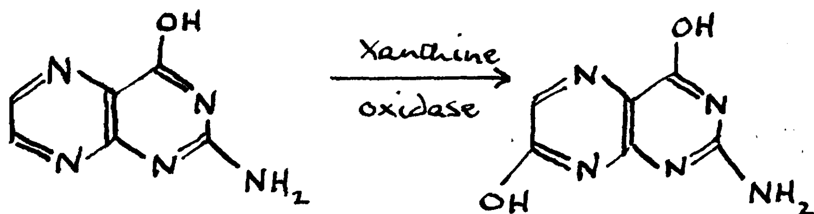




2-amino-4-hydroxypteridine-6-aldehyde



2-amino-4-hydroxypteridine-6-carboxylic acid



2-amino-4-hydroxypteridine

*iso*xanthopterin

R represents HOOC.$[CH_2]_2$.CH(COOH).NH

CATABOLISM OF PTEROYL-L-MONOGLUTAMIC ACID

rises to 90% of the total injected[119]. The authors suggested that this effect was caused by ascorbic acid preventing the oxidation of pteroyl glutamic acid. It has also been reported that ascorbic acid increases the amount of citrovorum factor synthesised by liver slices[80,107,120]. As a similar effect can be obtained by using other powerful reducing agents[82,83,121,122,123] the effect appears to be due to the reducing power of ascorbic acid.

The reductive condensation of amines and aldehydes to give secondary amines is a well established chemical reaction[124] and it would seem that steps A and B could be readily reversed. The anil has been shown to be easily reduced by anhydrous formic acid to N-10-formylpteroyl-L-glutamic acid[125].

The reductive condensation of I and II to yield pteroyl glutamic acid in poor yield by catalytic hydrogenation has been described by Weygand[126]. The low yield was due to accompanying hydrogenation of the pyrazine ring.

Condensation of p-amino-benzoyl-L-glutamic acid and N^2-acetyl-2-amino-4-hydroxy-pteridine-6-aldehyde with thiocresol or formic acid at 70°C as the reducing agents, has been described[127]. We tried reacting III with IV directly in the presence of thiocresol, but failed. We have tried to condense III and IV in aqueous solution

in the presence of ascorbic acid but failed due either to the high insolubility of III or to the inhibition of the condensation of III and IV by water.

Since steps A and B are chemically, and probably biologically, reversible, step C i.e. the enzymic oxidation of the aldehyde, is the step which determines the rate at which pteroyl glutamic acid is removed.

Direct evidence for this metabolic cycle is as yet limited to the demonstration of the oxidation of pteroyl glutamic acid by xanthine oxidase and methylene blue to 2-amino-4-hydroxy-pteridine-6-carboxylic acid; the isolation of what probably is this acid in small quantities from hamster kidneys; its occurrence in other animal tissues and the isolation of isoxanthopterin from human urine. But to assume this metabolic cycle, allows an explanation of a number of previously unrelated facts.

2-Amino-4-hydroxy-pteridine-6-aldehyde is a most powerful inhibitor of the oxidation of xanthine by xanthine oxidase; yet repeated experiments to show depression of liver xanthine oxidase activity by injecting test animals with massive doses of 2-amino-4-hydroxy-pteridine-6-aldehyde have always failed[128,129,130,131,132]. Haddow failed to produce kidney hypertrophy in hamsters using 2-amino-4-hydroxy-pteridine-6-aldehyde[96] despite

the successful use of other related pteridines.

On this assumed metabolic cycle 2-amino-4-hydroxy-pteridine-6-aldehyde could be transformed into pteroyl glutamic acid, provided adequate amounts of p-amino-benzoyl-L-glutamic acid and reducing agents were available; and it need not necessarily be effected by xanthine oxidase.

Analyses of rat liver show that only 6% of the folic acid and its derivatives is present as folic acid, and that the remaining approximate 94% occurs as the citrovorum factor. It would thus appear likely that little or no degradation of pteroyl glutamic acid, by any route, takes place in the liver. Injected 2-amino-4-hydroxy-pteridine-6-aldehyde would therefore be quickly transformed into pteroyl glutamic acid. It may also be remembered that liver aldehyde oxidase can carry out the required oxidation.

The protective action of ascorbic acid on injected pteroyl glutamic acid can readily be accommodated, as it could provide hydrogen atoms for the reduction of the anil to pteroyl glutamic acid.

Xanthine oxidase has two active centres, one used for the oxidation of reduced diphosphopyridine nucleotide and the other used for the oxidation of aldehydes and

xanthine. It has been known for some time that xanthopterin and other pteridines will competitively inhibit the oxidation of xanthine by xanthine oxidase[96]. As the same active centre[133] is used for the oxidation of aldehydes and xanthine, inhibitors of the oxidation of xanthine will also inhibit the oxidation of 2-amino-4-hydroxy-pteridine-6-aldehyde.

The injection of xanthopterin and other pteridines into test animals should thus prevent the degradation of pteroyl glutamic acid and increase its cellular concentration. This inhibition of the catabolism of pteroyl glutamic acid thus explains the anti-anaemic effect of xanthopterin when given to anaemic test animals[134,135,136,137,138]. Xanthopterin, however, is ineffective in human pernicious anaemia and in chicks[139,140,106].

Pteridines have also been shown to have a beneficial effect on the growth of chicks fed sub-optimal amounts of folic acid[139].

It has been suggested[141] that the kidney hypertrophy of hamsters, when injected with pteridines, was due to the inhibition of the oxidation of pteroyl glutamic acid by xanthine oxidase. This would seem likely as the approximate parallelism observed between the kidney hypertrophy and the inhibition of xanthine oxidase

oxidation of xanthine would also hold for the inhibition of pteroyl glutamic acid catabolism.

Inhibition of xanthine oxidase would thus not only give an increased concentration of xanthine due to failure to oxidise it, but an increased concentration of citrovorum factor, an increased rate of synthesis of purines from 4-amino-5-imidazole-carboxyamide ribotide, and an increased rate of synthesis of serine and other amino acids from glycine.

Thus, inhibition of the oxidative functions of xanthine oxidase would give rise to a series of anabolic reactions conducive to tissue hypertrophy.

Miller[142,143] has shown that a variety of rapidly growing tissues (e.g. embryonic tissues, transplantable malignant tumours) utilise 4-amino-5-imidazole carboxamide at a much higher rate than do the corresponding normal tissues. As a folic acid coenzyme is necessary to supply the one carbon fragment to convert 4-amino-5-imidazole carboxamide to purine these results would imply an enhanced utilisation of folic acid.

Experimental

Part I

Riboflavin as a pigment of Philothamnus semivariegatus and Dispholidus typus

The organ-free skin of a specimen of P. semivariegatus was extracted in the dark, with absolute ethanol in a soxhlet extractor; the solution was then cooled and filtered from insoluble matter. Although no pigment could be isolated from this solution, its yellow colour, strong, green fluorescence in daylight and the ready loss of both colour and fluorescence on irradiation in sunlight or on addition of dilute acid, dilute alkali or $NaHSO_3$ suggested that the pigment present was riboflavin.

The organ-free skin of a specimen of P. semivariegatus was extracted in the dark with a mixture of water: pyridine: n-propanol (1 : 3 : 1 v/v throughout) (50 ml.) on a steam-bath (45 min.), cooled, and filtered from insoluble material. The solution was yellow with a green fluorescence in daylight.

Similar extraction of the skin of a specimen of D. typus gave a yellow solution with only a faint green fluorescence in daylight. Paper chromatography of these extracts and of a saturated solution of riboflavin in the

water-pyridine-n-propanol solvent on descending chromatograms of Whatman No. 1 paper irrigated with this solvent showed that each extract gave a number of colourless spots which fluoresced in ultraviolet light, and a yellow spot which travelled at the same speed as the riboflavin spot.

To show that none of the other substances found in the snake-skin extracts was a decomposition product of riboflavin due to irradiation or chemical treatment of the extract, solutions of authentic riboflavin solutions were treated as follows:

A saturated solution of riboflavin in water: pyridine: n-propanol was irradiated in tropical sunlight (4.5 hours). Half of this solution was retained (solution 1); the remainder was warmed on a steam bath in the dark (45 min.) (solution 2). A saturated solution of riboflavin in distilled water was irradiated in tropical sunlight (4.5 hours). Pyridine (6 ml.) and n-propanol (2 ml.) were added to this solution (2 ml.). Half of it was kept (solution 7); the remainder was warmed on a steam bath in the dark (45 min.) (solution 6).

Paper chromatography of these four solutions, the two snake-skin extracts, and a saturated solution of riboflavin in water : pyridine : n-propanol (0.02 ml.

of each solution) by the descending method on Whatman No. 1 paper gave a chromatogram which showed that none of the substances obtained from the snake-skin extracts was derived from riboflavin by decomposition, and that both s snake-skin extracts contained a substance, the behaviour of which in the solvent system, was identical with that of riboflavin.

It was noticed that the yellow spot in the extract of D. typus did not lose its colour on exposure to light, as did the riboflavin spot. Use of other solvent systems showed that this spot could be resolved into riboflavin and a second, non-fluorescent, yellow pigment.

Paper chromatography of the snake-skin extracts and a saturated solution of riboflavin in water : pyridine : n-propanol (0.02 ml. of each solution spotted on paper and air-dried) by the descending method on Whatman No. 1 paper using n-butanol : acetic acid : water (50 : 15 : 35 v/v) as developing solvents gave chromatograms which showed that the extract of P. semivariegatus contained only one yellow pigment which was identical with riboflavin, and that the extract of D. typus contained two yellow pigments, one of which was identical with riboflavin.

A paper chromatogram of the extract of P. semivariegatus and a saturated solution of riboflavin in water : pyridine : n-propanol was developed with this solvent and the paper was

air-dried (1 m.) and then dried at 100°C (45 min.). The riboflavin and yellow-pigment spots were cut off the paper and their ultraviolet absorption spectra were determined on the paper by the method of Bradfield and Flood. The absorption maxima were for the yellow pigment 260 and 370 mu ; and for riboflavin 260 and 360 mu.

Pterins in Philothamnus semivariegatus and Dispholidus typus

Immediately after killing each specimen, it was skinned and the skin freed from any subcutaneous tissue. The whole skin was then extracted on a steam bath with successive 50 ml. portions of 80% aqueous ethanol until a colourless extract was obtained (8 to 10 portions). The ethanolic extracts were combined. What remained of the tissue was warmed with 50 ml. 0.5 N ammonium hydroxide for 30 minutes.

The ethanolic extracts and the ammoniacal extracts were used without any further preparation for the paper chromatograms.

Whatman No. 1 filter paper was used, all solvents were redistilled and solvent systems were allowed to stand for three days at room temperature (25-28°C(before use. Spots of 5 ul. size were used.

Chromatograms were developed at room temperature using the ascending technique. The criteria of identity were

(I) the parallel behaviour of a known substance and the substance in the extract, and

(II) the failure of the known substance to separate from a mixed chromatogram of the known and extract substance.

All experimental procedures were undertaken in the dark.

Both the ethanolic and ammoniacal extracts of the skins contained two colourless fluorescing compounds when chromatographed and viewed in ulteaviolet light at 3,600 Å. One of these fluoresced purple and the other bright blue.

The substances in the ammoniacal extract were shown to be the same as those in the ethanolic extract by their identical behaviour in paper chromatography using six different solvent systems, viz: 3% aqueous ammonium chloride; 5% aqueous acetic acid; butanol-acetic acid-water (4 : 1 : 5 v/v upper phase); pyridine-propanol-water (3 : 1 : 1 v/v); tertiary butanol-pyridine-water (50 : 15 : 35 v/v); tertiary butanol-pyridine-water (60 : 15 : 25 v/v).

The two fluorescing substances extracted with pyridine-propanol-water (3 : 1 : 1 v/v) were also shown to be identical with the above by the same method.

These substances were identified as pterins by their solubility in ammonium hydroxide, by their strong fluorescence in ultraviolet light and by their ultraviolet spectra. These were determined by the method of Bradfield

and Flood after paper chromatography using butanol-acetic acid-water (4 : 1 : 5 v/v). The spectra showed the typical pterin pattern of two or more maxima in the range 2,000-4,000 Å, with one peak between 3,000-4,000 Å. The purple fluorescing compound gave peaks at 2,550Å and 3,350 Å and the bright blue fluorescing compound gave peaks at 2,500 Å and 3,550 Å.

Individual pterins may be identified by comparing their chromatographic behaviour with that of a known pterin using at least three different solvent systems.

The purple fluorescing substance was thus identified as isoxanthopterin by its identical chromatographic behaviour with that of an authentic sample using the previous six different solvent systems.

The bright blue fluorescing substance was similarly identified as 2-amino-4-hydroxy-pteridine-6-carboxylic acid, by its identical chromatographic behaviour with that of an authentic sample in the same six solvent systems and in propanol-5% acetic acid (2 : 1 v/v) and in dimethyl formamide-formic acid-water (8: 1 : 1 v/v).

When the combined ethanolic extracts from D. typus were evaporated to dryness and the residue taken up in 10 ml. 0.5 N ammonium hydroxide, and paper chromatogram showed two new spots fluorescing bright blue in ultraviolet light.

One of these substances has been identified as 2-amino-4-hydroxy-pteridine by its identical behaviour with that of an authentic sample in the eight solvent systems quoted above. The other blue fluorescing substances has not been identified. When the combined ethanolic extracts from P. semivariegatus were similarly treated no 2-amino-4-hydroxy-pteridine could be detected. The second blue fluorescing substance was present.

This concentrated extract of P. semivariegatus also showed a yellow non-fluorescent spot in butanol-acetic acid-water. Similar to the yellow pigment in D. typus.

Yellow Pigment and Pterins of Dendroaspis viridis

A male specimen of the green mamba, Dendroaspis viridis, was killed and skinned. The skin was freed from any subcutaneous tissue and divided into two portions, a green anterior portion and a dirty yellow posterior portion. Each was extracted separately.

The Yellow Portion. This was extracted with 10 portions of 50 ml. each of 80% aqueous ethanol, in the dark. The final extract was colourless. The combined extracts were evaporated to dryness and taken up in 50 ml. 0.25 N ammonium hydroxide solution. The insoluble residue was filtered off.

This solution was used for the paper chromatographic

analysis. Conditions were the same as those used for the pterins of P. semivariegatus and D. typus.

On paper chromatography of this solution four fluorescent spots could be seen; one yellow, one purple, one bright blue and one blue.

The yellow fluorescent substance was identified as riboflavin by its identical chromatographic behaviour with that of a specimen in six solvent systems, viz: 3% aqueous ammonium chloride; 5% aqueous acetic acid; butanol-acetic acid-water (4 : 1 : 5 v/v), pyridine-propanol-water (3 : 1 : 1 v/v); tertiary-butanol-pyridine-water (60 : 15 : 25 v/v); and tertiary-butanol-pyridine-water (50 : 15 : 35 v/v). The amount of riboflavin present was much smaller than that in P. semivariegatus and D. typus.

The purple fluorescent spot was similarly identified as isoxanthopterin; the bright blue fluorescent spot as 2-amino-4-hydroxy-pteridine-6-carboxylic acid; and the blue fluorescent spot as 2-amino-4-hydroxy-pteridine.

This extract also contained a large amount of a yellow non-fluorescent pigment which on paper chromatography in butanol-acetic acid-water (4 : 1 : 5 v/v) behaved similarly to the unknown yellow pigment of D. typus.

The Green Portion. The green skin was extracted with six portions of 80% aqueous ethanol. The final extract

was colourless. The extracts were combined and used for paper chromatography.

On paper chromatography the extract was shown to contain three fluorescent substances; one yellow, one purple and one blue. The yellow fluorescent substance was identified, as previously, as riboflavin and the purple one as isoxanthopterin.

Oxidation of Pteroyl-L-Glutamic Acid

Incubation of PGA with xanthine oxidase

As PGA is readily decomposed by light, this and all other incubations were performed in darkness. Blank incubations were done where necessary to eliminate any possible side-effects.

PGA (0.3 ml. of a solution of 0.3 mg./ml. in 0.15 M. phosphate buffer pH 7.7) and xanthine oxidase solution 0.1 ml.) were heated at 36-37° under aerobic conditions for 18 hr.

Paper chromatography of 10 ul of the digest with butanol-acetic acid water (4:1:5 by vol.) as solvent showed a faint pale-blue spot with R_F value close to that of 2-amino-4-hydroxy-pteridine-6-carboxylic-acid. As judged by the size of the spot formed on paper chromatograms with butanol-acetic acid water (4:1:5 by vol.) the amount of acid formed did not appreciably increase after the first hour of incubation and represented less than 10% of the theoretical yield of carboxylic acid from PGA.

Incubation of PGA with xanthine oxidase and methylene blue

A solution (3 ml.) of 0.15 M phosphate buffer (pH 7.7) containing 1.56 mg. PGA and 0.54 mg. methylene blue was mixed with xanthine oxidase solution (1 ml.) and

incubated at 36-37° under aerobic conditions for 18 hr.

Paper chromatography of 10 ul of this digest on No. 1 Whatman paper with butanol acetic acid water (4:1:5 by vol.) showed on examination in ultraviolet light a large, bright, pale-blue spot with an R_F value close to that of 2-amino-4-hydroxy-pteridine-6-carboxylic acid, and a small, faint, blue spot corresponding to that of 2-amino-4-hydroxy-pteridine-6-aldehyde. The protein present in the digest seriously inter interfered with the chromatographic behaviour of the 2-amino-4-hydroxy-pteridine-6-carboxylic acid in other solvents. The bright blue fluorescent substance was purified by chromatography on a column of cellulose powder (Whatman standard grade, column 1 in diam. x 14½ in. high) with 5% by vol. aqueous acetic acid. The eluate containing the bright blue fluorescent substance was evaporated to dryness and the residue taken up in 1 ml. of 0.5 N NH_4OH Determination of the ultraviolet spectrum of this fluorescent substance in 0.1 N NaOH showed two maxima, at 261 mu and 361 mu. 2-Amino-4-hydroxy-pteridine-6-carboxylic acid has two maxima, at 262 mu and 365 mu (Mowat et al 1948). The identity of the bright blue fluorescent substance as 2-amino-4-hydroxy-pteridine-6-carboxylic acid was confirmed by comparison of its

Table 1

	R_F Bright light blue fluorescent substance
Butanol acetic acid water (4:1:5 by vol.)	0.12
Propanol (5% aq. acetic acid (2:1 by vol.)	0.23
5% aq. acetic acid	0.49
3% aq. ammonium chloride	0.59
tert-Butanol pyridine water (60:15:25 by vol.)	0.09
tert-Butanol pyridine water (50:15:35 by vol.)	0.22
Pyridine propanol water (3:1:1 by vol.)	0.26

R_F 2-amino-4-hydroxy pteridine-6-carboxylic acid	R_F Blue fluorescent substance	R_F 2-amino-4-hydroxy pteridine-6-aldehyde
0.12	0.33	0.33
0.23	0.46	0.46
0.49	0.52	0.52
0.59	0.43	0.43
0.09	0.27[1]	0.28[1]
0.22	0.54	0.54
0.26	0.55	0.55

1. Blue spot in digest coincided with methylene blue spot.

chromatographic behaviour with that of an authentic sample of the acid (Table 1).

Quantitative estimation of the acid was carried out as follows. 10 ul of the digest and 10 ul of a solution of 2-amino-4-hydroxy-pteridine-6-carboxylic acid (0.1 mg./ml. of 0.5 N NH_4OH) were placed on Whatman No. 1 paper, air-dried and developed with butanol-acetic acid water (4:1:5 by vol.) (ascending). The paper was air-dried, viewed in ultraviolet light and the bright, pale-blue spots cut out and weighed. The yield of acid was about 60% of the theoretical yield from PGA.

Incubation of methylene blue and PGA

A solution (4 ml.) of 0.15 M phosphate buffer (pH 7.4) containing 1.84 mg. PGA and 1.04 mg. methylene blue was incubated at 36-37° under aerobic conditiond for 18 hr. Paper chromatographic analysis of 10 ul of the digest using butanol-acetic acid water (4:1:5 by vol.) showed only a small spot corresponding to 2-amino-4-hydroxy-pteridine-6-carboxylic acid (R_F value 0.12) but a large blue fluorescent spot of R_F 0.33. This blue fluorescent substance was identified as 2-amino-4-hydroxy-pteridine-6-aldehyde by its identical chromatographic behaviour in seven different solvent systems with a known specimen of the aldehyde (Table I).

The amount of aldehyde in the digest was quantitatively estimated as before. The yield of aldehyde was about 65% of the theoretical yield from PGA.

Isolation of Isoxanthopterin from Human Urine

Conc. HCl (25 ml.) was added to adult male urine (250 ml.) which had been freshly voided into a flask containing a trace of phenol. The urine was then extracted with liquid phenol (50 ml.) and the phenol layer shaken up with water (25 ml.) and ether (100 ml.). The aqueous layer was washed twice with ether (100 ml. portions) and then evaporated to dryness under reduced pressure.

The residue was dissolved in water (5 ml.) and a few drops of aq. NH_3 were added until the solution was alkaline. The solution was filtered and evaporated to dryness under reduced pressure and the residue dissolved in aq. 0.5 N-NH_3 (0.5 ml.).

The dark-brown, syrupy liquid was spotted on Whatman No. 3 MM paper so that it filled a rectangle 3 in. x 1 in. The paper was then developed (descending, overnight, in darkness) with propanol -5% acetic acid (2:1, v/v), air-dried and viewed in 365 mu light.

Three fluorescent areas could be seen: nearest the origin, a well-defined purple-blue band (1); next, a bright-yellow band (2); finally, a smaller yellow band (3).

Table 2

	R_F unknown	R_F isoxanthopterin
Butanol-acetic acid-water (4:1:5 vol. by vol.)	0.23	0.23
Propanol-5% aq. acetic acid (2:1 by vol.)	0.33	0.33
tert-Butanol-pyridine-water (50:15:35 vol. by vol.)	0.35	0.35
Propanol-1% aq. NH_3 (2:1 by vol.)	0.19	0.19
Propanol-1$\overline{N}$ HCl (2:1 by vol.)	0.33	0.33

After the third yellow band the chromatogram was badly streaked. The first band was cut out and eluted with aq. 0.5N-NH_3. Further purification by chromatography, with No. 3MM paper and _tert_.-butyl alcohol-pyridine-water (50:15:35, by vol.), resolved this into three subsidiary bands. The eluate of the middle band (R_F 0.3-0.4) was chromatographed on No. 1 Whatman paper with propanol-aq. 1% NH_3 (2:1, v/v). Finally the eluate from this last chromatogram was evaporated to dryness under reduced pressure and the residue dissolved in aq. 0.5N-NH_3 (0.05 ml.). This solution was used for identification.

The purple fluorescent substance showed identical chromatographic behaviour on Whatman No. 1 paper with that of an authentic sample of _iso_xanthopterin, in butanol-acetic acid-water (4:1:5, by vol.); propanol-5% acetic acid (2:1 v/v); propanol-1% aq. NH_3 (2:1, v/v); propanol-N-HCl (2:1, v/v); and tert.-butyl alcohol-pyridine-water (50:15:35 by vol.); (Table 2).

With tert.-butyl alcohol-pyridine-water (60:15:25, by vol.) a difference of 0.06 was found in the R_F values of the unknown and _iso_xanthopterin. A mixture of the unknown and _iso_xanthopterin, however, did not separate into two spots and had a similar R_F to the unknown. This difference in R_F values of the unknown and _iso_xanthopterin was therefore due to the presence of impurities.

An additional amount of *iso*xanthopterin could be obtained by eluting band (2) and purifying it as before. In one experiment band (1) did not appear and *iso*xanthopterin was isolated from band (2).

For each identification 5 ul. of solution was used and the dried papers were viewed in 365 mu light. Papers were also viewed in 254 mu light. This was less satisfactory as these pterins do not fluoresce so brilliantly as in 365 mu light.

If the samples of urine were allowed to stand, or if the preliminary stages of the analysis were protracted, an additional bright blue band was found between band (1) and the origin. This band was eluted with aq. 0.5 N-NH_3. It was not separable from authentic 2-amino-4-hydroxy-pteridine-6-carboxylic acid in five solvent systems. It was probably, therefore, 2-amino-4-hydroxypteridine-6-carboxylic acid formed from the decomposition of biopterin. No leucopterin could be obtained. If present it would have been seen between the origin and *iso*xanthopterin.

Analysis of Normal Golden Hamster Kidney, Neoplastic tissue derived from it and Hypertrophied Kidney

The analytical methods used were based on that of Crammer[10].

Analysis of Normal Kidney.

The animal was killed with chloroform, kidneys dissected out, weighed (ca. 1 g. per animal) and ground to a fine paste with the minimum amount of quartz powder. The mass was then extracted with refluxing peroxide-free anhydrous ether (100 ml. : 1 hr in total darkness). The ether was decanted off and the solid mass extracted with $\frac{N}{2}$ aqueous ammonia (20 ml. : 1 hr. : in total darkness on a boiling water-bath). The solid and liquid mixture was then transferred to a centrifuge tube, the extraction flask rinsed out with $\frac{N}{2}$ aqueous ammonia (5 ml.), the washings added to the contents of the centrifuge tube and this solution was saturated with excess solid ammonium sulphate. The protein precipate was centrifuged off and the clear supernatant liquid was filtered off. The protein residue was washed with $\frac{N}{2}$ aqueous ammonia (10 ml.), this solution saturated with excess solid ammonium sulphate and the protein precipitate centrifuged off. The clear supernatant liquid was filtered off and added to the first solution. The pH of the combined solutions was

adjusted to about 3 by the cautious addition of dilute hydrochloric acid. The acidified solution was extracted with liquid phenol (10 ml.) and the phenol washed six times with $\frac{N}{10}$ hydrochloric acid (30 ml. portions). Small quantities of phenol were added from time to time to keep the volume of phenol constant at 10 ml. The phenol solution was then extracted with anhydrous peroxide - free ether (50 ml.) and water (1-2 ml.). The aqueous layer was washed once with ether (50 ml.) and evaporated to dryness in vacuo at room temperature. The minute quantity of solid obtained was dissolved in $\frac{N}{2}$ aqueous ammonia and transferred to No. 1 Whatman filter paper by alternate spotting and drying so as to fill a spot of approximately 10 ul size. The paper was then developed with propanol - 5% aqueous acetic acid (2:1 by volume) (ascending; overnight; total darkness), air-dried and viewed in 365 mu ultraviolet light. A number of fluorescent spots were seen.

(1) nearest the origin, a large bright-yellow fluorescent spot, identified as flavine - adenine - mononucleotide

(2) next a faint blue fluorescent spot, provisionally identified as 2-amino-4-hydroxy pteridine-6-carboxylic acid

(3) next a yellow fluorescent spot, identified as riboflavin

(4) next a blue fluorescent spot, unidentified

(5) finally a faint fluorescent spot, unidentified.

Analyses were carried out on pairs of normal kidneys taken from male animals (10 animals) older than sixteen weeks. The smallest weight of kidney used was 0.5 g.; most pairs weighed 0.9 - 1.0 g. A bulk experiment in which 5 pairs of kidneys (total weight 4.35 g.) was also done.

<u>Identification of Substances on Chromatograms</u>

1. The bright yellow spot was eluted from the chromatogram with $\frac{N}{2}$ aqueous ammonia and identified as flavine - adenine - mononucleotide by its similar behaviour to a known sample in 3 solvent systems [butanol-acetic acid-water (4:1:5 vol. by vol); <u>tert</u>.-butanol-pyridine-water (60:15:25 vol. by vol.) <u>tert</u>.-butanol-pyridine-water (50:15:35 vol. by vol.)].

2. The blue fluorescent spot was eluted with $\frac{N}{2}$ aqueous ammonia. The amount of material was too small to allow comparison in more than one solvent system. When chromatographed in butanol-acetic acid-water (4:1:5 vol. by vol.) it showed similar behaviour to a known sample of 2-amino-4-hydroxypteridine-6-carboxylic acid. The solvent system clearly distinguishes between 2-amino-4-hydroxy pteridine-6-carboxylic acid and the other known pterins.

3. The yellow fluorescent spot was eluted with $\frac{N}{2}$ aqueous ammonia. It was identified as riboflavin by paper chromatography with a known sample in 3 solvent systems [tert.-butanol-pyridine-water (60:15:25 vol. by vol.); tert.-butanol-pyridine-water (50:15:35 vol. by vol.); propanol-1% aqueous ammonia (2:1 by vol)]. Xanthopterin and riboflavin are not separated in the initial propanol-5% aqueous acetic acid (2:1 vol. by vol.) used. Riboflavin and xanthopterin are readily distinguished by chromatography in propanol-1% aqueous NH_3 (2:1 vol. by vol.). Their R_F values differ by ca. 0.2 units and xanthopterin fluoresces bright blue while riboflavin fluoresces bright yellow. Paper chromatography of the second yellow spot after elution showed no spot corresponding to xanthopterin.

Analyses of Neoplastic Tissues

Preliminary analyses were done on primary neoplasms but most of the analyses were carried out on transplanted kidney tumours.

The animal was killed with chloroform. the neoplastic tissues dissected out and carefully separated from normal tissue and necrotic tissue. The analysis was then carried out as for normal tissue. The results were similar, in that flavine-adenine-mononucleotide and

riboflavin were isolated and identified as for normal tissues and xanthopterin was found to be absent. The blue spot corresponding to 2-amino-4-hydroxypteridine-6-carboxylic, however, was not visible on the chromatograms from tissue masses of less than 8 grams .

Results. (With transplanted neoplasms).

	No. of animals :	Wt. of tumour :	Results
Experiment 1.	2	6.08 g	No 2-amino-4-hydroxypteridine-6-carboxylic acid present.
2.	1	2.5 g.	"
3.	1	2.7 g.	"
4.	3	8.6 g.	2-Amino-4-hydroxypteridine-6-carboxylic acid present.
5.	1	1.2 g.	No acid present.
6.	1	3.6 g.	"
7.	1	3.2 g.	"
8.	1	3.0 g.	"
9.	1	4.5 g.	"
10.	1	5.1 g.	"

Analysis of Hypertrophied Kidney

3 Adult male hamsters were injected with 2:4:5-triamino-6-styryl pyrmidine (20 mg. in 0.5 ml. arachis

oil each) 55 hours later they were killed with chloroform and the kidneys dissected out. Total weight of kidneys was 4.3 g. (approx. 30-40% increase in weight over controls). The analysis was conducted as for normal kidneys but the final chromatogram was run on Whatman No. 3 MM paper using the same solvent system as before. On air-drying and viewing the chromatogram in 365 mu light the following spots were seen.

(1) Bright yellow fluorescent spot, shown by subsequent elution and paper chromatography to be flavine-adenine-mononucleotide.

(2) A blue fluorescent spot; this could not be eluted from the paper chromatogram by large amounts of $\frac{N}{2}$ aqueous ammonia and was therefore not identical with 2-amino-4-hydroxypteridine-6-carboxylic acid which is readily eluted.

(3) A yellow fluorescent spot which could not be eluted with $\frac{N}{2}$ aqueous ammonium hydroxide.

(4) A yellow fluorescent spot, which was shown to be riboflavin by elution and subsequent paper chromatography. No xanthopterin was present. (differentiation between xanthopterin and riboflavin in propanol 1% aqueous ammonia (2:1 vol. by vol.)).

(5) An unidentified blue fluorescent spot.

(6) An unidentified yellow fluorescent spot.

(7) A large yellow fluorescent spot identical (paper chromatography) with the product of warming 2:4:5-triamino-6-styryl pyrimidine with $\frac{N}{2}$ aqueous ammonia solution.

Since it was possible that spots 2 and 3 could have concealed a 2-amino-4-hydroxypteridine-6-carboxylic acid spot the eluates were carefully examined by paper chromatography for traces of this acid. None could be detected.

Experimental Animals

The normal golden hamsters used were bred from a stock given by the Chester Beatty Research Institute. They were kept in an air-conditioned room (22°-24° C.) and were free from external and internal parasites. Diet consisted of milk powder, Bemax, rat nut-cake, grain (whole rice, maize and sorghum) green vegetables and carrot. Animals with tumours (primary and transplanted) were supplied from the Chester Beatty Research Institute. They were kept under the same conditions as the normal animals.

References

1. Krukenberg, Vergleichend-physiologische Studien, 1882 series 2 part 2 p. 92.
2. Brimley and Barrett, Practical Chromatography, London. Chapman and Hall 1953.
3. Albert, J. Chem. Soc., 1955, 2690.
4. Bradfield and Flood, J. Chem. Soc., 1952, 4740.
5. Bellamy, J. Appl. Chem. 1953, 3, 421.
6. Toribara and Stefano, Anal. Chem., 1954, 26, 1519.
7. Cerletti and Siliprandi, Biochem. J., 1955, 61, 324.
8. Forrest and Todd, J. Chem. Soc., 1950, 3295.
9. Partridge, S.M., Biochem. J., 1948, 42, 238.
10. Crammer, Nature, 1948, 161, 349.
11. Tschesche and Korte, Chem. Ber., 84, 641, 801 (1951).
12. Albert, Quart. Rev., 1952, 6, 199.
13. Renfrew and Platt, J. Amer. Pharm. Assoc., 1950, 39, 657.
14. Forrest and Mitchell, J. Amer. Chem. Soc., 1954, 76, 5656.
15. Good and Johnston, Nature, 1949, 163. 31.
16. Fox, Animal Biochromes, 1954, Cambridge University Press.
17. Koschara and Haug, Z. Physioll Chem. 1939, 259, 97.
18. Bodine and Fitzgerald, Physiol. Zoology, 1948, 21, 93.
19. Burgess, Arch. Biochem., 1949, 20, 347.
20. Kikkawa, Adv. in Genetics, 1953, 5, 130.
21. Daneel and Zimmermann, Zeit. für Naturforschung, 1957, 12b, 730.

22. Nathan, Hutner, Levin, Nature, 1956, 178, 741.

23. Forrest, Van Baalen and Myers, Science, 1957, 125, 699.

24. Dupont, Naturwiss., 1958, 11, 268.

25. Grossbach, Zeit. für Naturforschung, 1957, 12b, 462.

26. Viscontini, Schoeller, Loeser, Karrer and Hadorn, Helv. Chim. Acta, 1955, 38, 397.

27. Viscontini, Loeser, Karrer and Hadorn, Helv. Chim. Acta, 1955, 38, 1222.

28. Forrest and Mitchell, J. Amer. Chem. Soc., 1954, 76, 5656.

29. Forrest and Mitchell, J. Amer. Chem. Soc., 1955, 77, 4865.

30. Fidler and Wood, J. Chem. Soc., 1957, 4157.

31. Viscontini, Schmid and Hadorn, Experientia, 1955, 11, 390.

32. Viscontini, Kuhn and Egelhaaf, Zeit. für Naturforschung, 1956, 11b, 501.

33. Oshima, Seki and Ishizaki, Genetics, 1956, 41, 4.

34. Schopf and Becker, Ann., 1936, 524, 49.

35. Nawa, Gota, Matsuura, Kakizawa and Hirata, J. Biochem. Japan, 1954, 41, 657.

36. Tschesche and Korte, Chem. Ber., 1954, 87, 1713.

37. Nawa and Taira, Proc. imp. Acad. Japan, 1954, 30, 632.

38. Tschesche and Korte, Chem. Ber., 1951, 84, 641.

39. Hama, Experientia, 1953, 9, 299.

40. Gunder, Zeit. vergleich Physiologie, 1954, 36, 78.

41. Ziegler and Ziegler-Gunder, Zeit. für Naturforschung, 1955, 10b, 642.

42. Forrest and Mitchell, Chemistry and Biology of Pteridines, 1954, p. 143. J. & A. Churchill, London.

43. Danneel and Eschrich, Zeit. für Naturforschung, 1956, 11 b, 103.

44. Goodwin and Srisukh, Biochem. J., 1951, 49, 84.

45. Baalen, Myers and Forrest, Proc. Nat. Acad. Sci., U.S.A. 1957, 43, 701.

46. Baalen, Myers and Forrest, Proc. Nat. Acad. Sci. U.S.A., 1958, 44, 239.

47. Lowry, Bessel and Crawford, J. Biol. Chem., 1949, 180, 399.

48. Petering and Schmidt, J. Amer. Chem. Soc., 1950, 72, 2995.

49. Forrest, Glassmann and Mitchell, Science, 1956, 124, 725.

50. Mowat, Boothe, Hutchings, Stokstad, Waller, Angier, Semb, Cosulich and Subbarow, J. Amer. Chem. Soc., 1948, 70, 14.

51. Weygand, Schmied-Kowarzik, Wacker and Rupp, Chem. Ber., 1950, 83, 460.

52. Waller, Goldman, Angier, Boothe, Hutchings, Mowat and Semb, J. Amer. Chem. Soc., 1950, 72, 4630.

53. Blair, Nature, 1957, 179, 489.

54. Forrest and Walker, J. Chem. Soc., 1949, 83.

55. Klenow, Biol. Medd., Koh., 1951, 18, 14.

56. Kalckar, Kjeldgaard and Klenow, Biochem. Biophys. Acta, 1950, 5, 586.

57. Forrest and Mitchell, J. Amer. Chem. Soc., 1954, 76, 5658.

58. Lowry, Bessey and Crawford, J. biol. Chem., 1949, 180, 389.

59. Viscontini, Loeser and Egelhaaf, Naturwissenschaften, 1956, 43, 379.

60. Blair, Chem. and Ind., 1955, 1158.

61. Albert, Biochem. J., 1954, 57, X.

62. Albert, Biochem. J., 1957, 65, 124.

63. Williams and Elvejehem, Proc. Soc. Exper. Biol., 1949, 71, 303.

64. Jacobson and Good, Quart. J. Med., 1952, 21, 1.

65. Norris and Majnarich, Science, 1949, 109, 32.

66. Kalckar and Klenow, J. biol. Chem., 1948, 172, 351.

67. Kalckar, Kjeldgaard and Klenow, J. biol. Chem., 1948, 174, 771.

68. Slavik and Matoulkova, Chem. Listy, 1953, 47, 1516.

69. Rauen and Waldman, Experientia, 1950, 6, 387.

70. Rauen, Waldman and Buchka, Hoppe-Seyl. Zeit., 1951, 288, 10.

71. Rauen, Biochem. Z., 1957, 328, 562.

72. Avis, Bergel and Bray, J., 1956, 1100.

73. Fisher, Parsons and Holmes, Nature, 1948, 161, 764.

74. Fisher, Parsons and Morrison, Nature, 1949, 164, 183.

75. Koschara, Hoppe-Seyl, Z., 1936, 240, 127.

76. Patterson, Saltza and Stokstad, J. Amer. Chem. Soc., 1956, 78, 5871.

77. Williams, Eakin, Beerstecher and Shive, The Biochemistry of B Vitamins, 1950, p. 574. New York. Rheinold Publishing Corporation.

78. Welch, Nicol, Ann. Rev. Biochem., 1952, 21, 633.

80. Slavik, Dvorakova, and Slavikova, Chem. Listy, 1957, 51, 1536.

81. Slavik, personal communication, 1958.

82. Rauen, Zeit. für Natürforschung, 1958, 13b, 45.

83. Braganca, Aravindakshan and Ghanekar, Biochem. Biophys. Acta, 1957, 25, 623.

84. Aravindakshan and Braganca, Biochim. Biophys. Acta, 1958, 27, 345.

85. Kenkare and Braganca, Nature, 1958, 181, 548.

86. Kalckar, Floystrup and Schou., Proc. 1st. Internat. Congr. Biochem., 1949, 75.

87. Floystrup, Schou, Kalckar, Acta Chem. Scand. 1949, 3, 985.

88. Futterman and Silverman, J. Biol. Chem., 1957, 224, 31.

89. Blakley, Biochem. J., 1957, 65, 331.

90. Wieland and Liebig, Ann., 1944, 555, 146.

91. Kalckar, Kjeldgaard and Klenow, Biochim. Biophys. Acta, 1950, 5, 575.

92. Haddow, Chemistry and Biology of Pteridines, 1954, 101. J. and A. Churchill, London.

93. McNutt, J. Biol. Chem., 1956, 219, 365.

94. McNutt and Forrest, J. Amer. Chem. Soc., 1958, 80, 951.

95. Forrest and McNutt, J. Amer. Chem. Soc., 1958, 80, 739.

96. Bergel, Lectures on the Scientific Basis of Medicine, 1954-1955, IV, p. 62, University of London, London.

97. Quoted by Avis, Bergel and Bray, J. Chem. Soc., 1955, 1100.

98. Lewin, Lewin and Bray, Nature, 1957, 180, 763.

99. Richert, Bloom and Westerfeld, J. Biol. Chem., 1957, 227, 1957.

100. Kirkman and Bacon, Cancer Research, 1950, 10, 122.

101. Easty and Ambrose, Nature, 1955, 176, 1256.

102. Horning, Zeit. Krebsforschung, 1956, 61, 1.

103. Pollack, Taylor, Williams, Univ. of Texas Publications, 1942, No. 4237, p. 56.

104. Swendseid, Bethell and Bird, Cancer Research, 1951, 11, 864.

105. Ungley and Thompson, Brit. Med. J., 1950, 1, 919.

106. Watson and Witts, Brit. Med. J., 1952, 1, 13.

107. Nicol and Welch, Proc. Soc. Exp. Biology, 1950, 74, 52, 403.

108. Jacobson, J. Path. Bact., 1952, 64, 245.

109. Jacobson, International Congr. Cell Biology, New Haven, U.S.A., 1950.

110. Jacobson, Trans. 13th Conference on Problems of Ageing, New York, 1951.

111. Plant, Betheil and Lardy, J. Biol. Chem., 1950, 184, 795.

112. Kisliuk and Sakami, J. Amer. Chem. Soc., 1954, 76, 1456.

113. Kisliuk and Sakami, J. Biol. Chem., 1955, 214, 47.

114. Blakley, Biochem. J., 1954, 58, 448.

115. Alexander and Greenberg, J. Biol. Chem., 1955, 214, 821.

116. Buchanan and Schulman, J. Biol. Chem., 1953, 202, 241.

117. Greenberg, Fed. Proc., 1954, 73, 745.

118. Wilkinson and Davies, Nature, 1958, 181, 1070.

119. Guggenheim, Halevy, Neumann, Usieli, Biochem. J., 1956, 62, 281.

120. Broquist, Stokstad and Jukes, J., Lab. clin. Med., 1951, 38, 95.

121. Welch, Nichol, Anker, Boehne, J., Pharmacol., 1951, 103, 403.

122. Guggenheim, Leibowitz and Rakower, Biochem., J., 1953, 55, 388.

123. Doctor and Trunnel, Proc. Soc., exp. Biol., N.Y., 1954, 87, 498.

124. Emerson, Organic Reactions, 1948, IV, 174.

125. Lindlar and Klaeui, 1950, U.S. Patent, 2,520,156.

126. Weygand, Wacker and Schmied-Kowarzik, Chem. Ber., 1949, 82, 25.

127. Sletzinger, Rheinhold, Grier, Beachem. and Tishler, J. Amer. Chem. Soc., 1955, 77, 6365.

128. Dietrich, Monson, Williams and Elvehjem, J. Biol. Chem., 1952, 197, 37.

129. Remy and Westerfeldt, J. Biol. Chem., 1951, 193, 659.

130. Keith, Broach, Warren, Day and Totter, J. Biol. Chem., 1948, 176, 1095.

131. Byers, J. Amer. Pharm. Assoc., 1952, 41, 611.

132. Westerfeldt and Richert, J. Biol. Chem., 1950, 184, 163.

133. Fridovitch and Handler, J. Biol. Chem., 1958, 231, 899.

134. Tschesche and Wolf, Z. physiol. Chem., 1936, 244, I.

135. Simmons and Norris, J. Biol. Chem., 1941, 140, 679.

136. Totter, Shukers, Kolson, Mims and Day, J. Biol. Chem., 1944, 152, 147.

137. Totter and Day, J. Biol. Chem., 1943, 147, 257.

138. Norris and Majnarich, Science, 1949, 109, 32.

139. Daniel, Scott, Norris and Heuser, J. Biol. Chem., 1948, 173, 123.

140. Spies and Stone, Southern Med. J., 1947, 40, 46.

141. Jacobson, Chemistry and Biology of Pteridines, 1954, p. 103 J. and A. Churchill, London.

142. Miller and Warren, 1953, 205, 331.

143. Miller, J. Biol. Chem., 1957, 225, 715.

(*Reprinted from Nature, Vol.* 179, *pp.* 489–490, *March* 2, 1957)

Photo-reduction of Pteridines

THE recent communication by Prof. A. Albert[1] on the photo-reduction of pteridines prompts me to record another example. In the preparation of 2 - amino - 4-hydroxy - pteridine-6 - aldehyde by the method of Waller *et al.*[2] it was found that if the initial decomposition of pteroyl-L-glutamic acid to dihydro-2-amino-4-hydroxy-pteridine-6-aldehyde was done in the dark only a poor yield was obtained (as judged by the amount of 2-amino-4-hydroxy-pteridine-6-aldehyde formed on subsequent oxidation). If the reacting solution was irradiated with light from a 100-W. bulb then a good yield was obtained. It thus seems that the decomposition of pteroyl-L-glutamic acid to the dihydro aldehyde is a light-catalysed intramolecular hydrogen transfer from the 9 : 10 position followed by hydrolysis of the resulting anil. It has been previously reported that pteroyl-L-glutamic acid is decomposed by ultra-violet light to 2-amino - 4-hydroxy - pteridine - 6-aldehyde[3]. The position of the hydrogen atoms of the dihydro 2-amino-4-hydroxy-pteridine-6-aldehyde in the pyrazine ring has not been established ; but this aldehyde would seem to be a possible intermediate in the biological synthesis of the yellow fluorescent eye pigment[4] of *Drosophila* and possibly also of the red pteridine pigments[4]. The primary step in the degradation of pteroyl-L-glutamic acid would seem to be hydrogen transfer intramolecularly to the pyrazine ring or intermolecularly to a suitable hydrogen acceptor (for example, oxygen[5] or methylene blue[6]).

J. A. BLAIR

University College of the Gold Coast,
Achimota, Gold Coast.

Dec. 12.

[1] Albert, A., *Nature*, **178**, 1072 (1956).

[2] Waller, C. W., Goldman, A. A., Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., and Semb, J., *J. Amer. Chem. Soc.*, **72**, 4630 (1950).

[3] Lowry, O., Bessel, O., and Crawford, E., *J. Biol. Chem.*, **180**, 389 (1949).

[4] Forrest, H. S., and Mitchell, H. K., *J. Amer. Chem. Soc.*, **76**, 5656 (1954).

[5] Mowat, J. H., Boothe, J. H., Hutchings, B. L., Stokstad, E. L. R., Waller, C. W., Angier, R. B., Semb, J., Cosulich, D. B., and Subbarow, J., *J. Amer. Chem. Soc.*, **70**, 14 (1948).

[6] Blair, J. A., *Biochem. J.*, **65**, 209 (1957).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

(*Reprinted from Nature, Vol.* 180, *p.* 1371 *only, Dec.* 14, 1957)

Pigments and Pterins in the Skin of the Green Mamba, *Dendroaspis viridis*

IT has previously been reported that the skins of the green snakes *Philothamnus semivariegatus* and *Dispholidus typus* contain riboflavin, *iso*xanthopterin and 2-amino-4-hydroxpteridine-6-carboxylic acid[1,2]. *D. typus* also contains 2-amino-4-hydroxypteridine[2] and an unknown yellow pigment[1].

After this investigation had been completed, a single male specimen of the green mamba, *Dendroaspis viridis*, became available to us. The anterior and middle portions were leaf green, and the posterior portion dull yellow.

The animal was skinned and the skin cut into the green and yellow portions.

The yellow skin was extracted with 50 ml. portions of boiling 80 per cent aqueous ethanol, in the dark, until the extract was colourless. The combined extracts were evaporated to dryness, the residue dissolved in 50 ml. of $N/4$ aqueous ammonia and the solution filtered from insoluble material. Analysis of this solution by paper chromatography showed the presence of a yellow non-fluorescent pigment; and, on viewing in 365 mμ light, an additional yellow fluorescent substance, a purple fluorescent substance, a bright blue fluorescent substance and a blue fluorescent substance.

The yellow fluorescent substance was identified as riboflavin by its similar behaviour to an authentic sample in three solvent systems; pyridine/propanol/water (3 : 1 : 1 by vol.); tert-butanol/pyridine/water (50 : 15 : 35 by vol.); and tert-butanol/pyridine/water (60 : 15 : 25 by vol.).

The purple fluorescent substance was identified as *iso*xanthopterin, the bright blue fluorescent substance as 2-amino-4-hydroxypteridine-6-carboxylic acid, and the blue fluorescent substance as 2-amino-4-hydroxpteridine. This was done by comparing their behaviour with that of authentic samples in the three previous solvent systems and in butanol/acetic acid/water (4 : 1 : 5 by vol.); 5 per cent aqueous acetic acid (by vol.); and 3 per cent aqueous ammonium chloride.

The yellow non-fluorescent substance could not be identified. Its chromatographic behaviour in butanol/acetic acid/water (4 : 1 : 5 by vol.) was similar to that of the unknown yellow pigment in *D. typus*[1].

The green portion of the skin was extracted with 50 ml. portions of boiling 80 per cent aqueous ethanol until the extract was colourless. The extracts were combined, and the presence of riboflavin and *iso*-xanthopterin was shown by paper chromatography, as before. No attempt was made to identify 2-amino-4-hydroxypteridine-6-carboxylic acid nor 2-amino-4-hydroxypteridine in the ethanolic extract. The yellow non-fluorescent substance was present.

I am grateful to Mr. A. H. Booth of the Zoology Department of this College for supplying the specimen of *D. viridis* ; and to the Chemical Society for a grant in aid of this investigation.

J. A. BLAIR

University College of Ghana,
Achimota, Ghana, West Africa.

[1] Blair, J. A., and Graham, J., *Biochem. J.*, **56**, 286 (1954).
[2] Blair, J. A., and Graham, J., *Chem. and Ind.*, 1158 (1955).

Printed in Great Britain by Fisher, Knight & Co., Ltd.. St. Albans.

(*Reprinted from Nature, Vol.* 181, *p.* 996 *only, April* 5, 1958)

Metabolic Role of Xanthopterin

ALBERT[1] has suggested that xanthopterin would be found in higher amounts in neoplastic tissues than in normal. In anticipation of confirming this by direct experiment, we have analysed for their pterin content, normal kidney tissue of the golden hamster and transplantable neoplasms in the golden hamster, derived from stilbœstrol-induced renal tumours[2]. No xanthopterin could be found. The largest amount of tissue used was 1 gm. of normal tissue and 8 gm. of neoplastic tissue, and the smallest amount of xanthopterin that could be detected by the paper chromatographic technique used was 0·1 μgm.

Xanthopterin has been isolated from human urine[3]. If, however, xanthopterin is present as a tissue metabolite, some of it should be oxidized to leucopterin by xanthine oxidase[4]. This expectation is borne out by the occurrence of leucopterin in the kidney tubules of golden hamsters given massive doses of xanthopterin[5]. An analysis of human urine failed to reveal leucopterin (which would have been found between the origin and 2-amino-4-hydroxypteridine-6-carboxylic acid in the propanol–5 per cent aqueous acetic acid (2:1 v/v) solvent system used)[6].

Kalckar *et al.*[7] found that xanthopterin was not present in freshly voided urine, but that a colourless non-fluorescent substance was present which was converted to xanthopterin by air oxidation. Futterman and Silverman[8] found a labile substance in human urine the properties of which resembled an unformylated reduced folic acid, and Blakley[9] has demonstrated that di- and tetra-hydrofolic acids are readily oxidized to xanthopterin.

Slavik *et al.*[10] found xanthopterin in the urine of rats fed tetrahydrofolic acid but showed that the xanthopterin was formed during isolation by the non-enzymic oxidation of tetrahydrofolic acid.

The present burden of evidence is therefore that xanthopterin is absent from mammalian tissues. The biological effects of xanthopterin in mammals should therefore be attributed only to the disturbance of a normal metabolic pathway by an artificially introduced substance.

A fuller account of this work will be published elsewhere in due course.

We wish to thank the Chemical Society for a grant in aid of this investigation, and Prof. A. C. Haddow

and the Chester Beatty Research Institute for the supply of normal hamsters and hamsters with tumours.

J. A. BLAIR

University College of Ghana,
Achimota, Ghana.

[1] Albert, A., *Biochem. J.*, **65**, 124 (1957); **57**, x (1954).

[2] Horning, E. S., *Z. Krebsforschung*, **61**, 1 (1956).

[3] Koschara, W., *Hoppe Seylers Z.*, **240**, 127 (1936).

[4] Wieland, H., and Liebig, R., *Ann.*, **555**, 146 (1944). Kalckar, H. M., Kjeldgaard, N. O., and Klenow, H., *Biochim. Biophys. Acta*, **5**, 575 (1950).

[5] Haddow, A. C., "Chemistry and Biology of Pteridines", 101 (J. and A. Churchill, London, 1954).

[6] Blair, J. A., *Biochem. J.* (in the press).

[7] Kalckar, H. M., Floystrup, T., and Schou, M., Proc. 1st Internat. Congr. Biochem., 75 (1949). Floystrup, T., Schou, M., and Kalckar, H. M., *Acta Chem. Scand.*, **3**, 985 (1949).

[8] Futterman, S., and Silverman, M., *J. Biol. Chem.*, **224**, 31 (1957).

[9] Blakley, R. L., *Biochem. J.*, **65**, 331 (1957).

[10] Slavik, K., Dvorakova, A., and Slavikova, V., *Chem. listy*, **51**, 1536 (1957).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

[FROM THE BIOCHEMICAL JOURNAL, VOL. 65, No. 2, pp. 209–211, 1957]
[*All rights reserved*] PRINTED IN GREAT BRITAIN

Some Observations on the Oxidative Degradation of Pteroyl-L-glutamic Acid

BY J. A. BLAIR
University College of the Gold Coast

(*Received* 14 *May* 1956)

Several workers have studied the action of xanthine oxidase on pteroyl-L-glutamic acid (PGA). Jacobson & Good (1952) claimed that xanthine oxidase oxidized PGA and that the product had increased haemopoietic properties. Norris & Majnarich (1949) reported that incubation of xanthine oxidase with PGA gave material of increased haemopoietic power. On investigating a claim that PGA inhibited the oxidation of xanthine by xanthine oxidase, Kalckar & Klenow (1948) and Kalckar, Kjeldgaard & Klenow (1948, 1950) found that this inhibition was due to traces of 2-amino-4-hydroxypteridine-6-aldehyde usually present in PGA, and that this effect could be eliminated by incubating the PGA with xanthine oxidase. This was shown to be due to the oxidation of the aldehyde to 2-amino-4-hydroxypteridine-6-carboxylic acid by the enzyme. They found no change in the growth-promoting properties of PGA before and after incubation with xanthine oxidase. Rauen & Waldman (1950), Rauen, Waldman & Buchka (1951) and Yamamoto (1954) found that on incubating pig-liver homogenate with PGA, a bright, pale-blue fluorescent compound was formed. This was not identified.

It seemed desirable to repeat this work on the oxidation of PGA, with milk xanthine oxidase at stage M 5 as prepared by Avis, Bergel & Bray (1955). It has been confirmed that xanthine oxidase has no action on PGA; but when xanthine oxidase, methylene blue and PGA are incubated at 36–37° at a pH near neutrality, PGA is oxidized to 2-amino-4-hydroxypteridine-6-carboxylic acid. This oxidation proceeds by the intermediate formation of 2-amino-4-hydroxypteridine-6-aldehyde, which is then oxidized by the xanthine oxidase to 2-amino-4-hydroxypteridine-6-carboxylic acid (Lowry, Bessel & Crawford, 1949; Klenow, 1951). This is shown by the isolation of the aldehyde as the major product of the incubation of PGA with methylene blue.

MATERIALS

Pteroyl-L-*glutamic acid*. This was obtained commercially. By paper chromatography, with butanol–acetic acid–water (4:1:5, by vol.) as solvent, it was shown to be contaminated with 2-amino-4-hydroxypteridine-6-aldehyde and a trace of 2-amino-4-hydroxypteridine-6-carboxylic acid. The quantitative estimation of the aldehyde was made by the spot-area technique of Fisher, Parsons & Morrison (1948) and Fisher, Parsons & Holmes (1949). A portion (10 μl.) of a standard solution of the aldehyde (0·116 mg. in 1 ml. of 0·5 N-NH_3 soln.) and 10 μl. of a solution of PGA (3·73 mg. in 1 ml. of 0·5 N-NH_3 soln.) were placed on no. 1 Whatman paper, air-dried and developed with butanol–acetic acid–water (4:1:5, by vol.; ascending). After air-drying, the paper was viewed in ultraviolet light (365 mμ.) and the blue fluorescent spots of aldehyde were cut out and weighed. The amount of aldehyde present was estimated to be about 3% of the weight of PGA.

Xanthine oxidase. This was provided by the Chester Beatty Research Institute at stage M5 of purification (Avis *et al.* 1955), in a solution of M phosphate, pH 6, containing 4 mg. of xanthine oxidase/ml. It was used without further purification.

2-*Amino*-4-*hydroxypteridine*-6-*aldehyde*. This was prepared by the method of Waller *et al.* (1950) with the modification that it was found necessary to irradiate the reacting solution with light from a 100 w lamp.

EXPERIMENTAL AND RESULTS

Incubation of PGA with xanthine oxidase

As PGA is readily decomposed by light, this and all other incubations were performed in darkness. Blank incubations were done where necessary to eliminate any possible side effects.

PGA (0·3 ml. of a solution of 0·3 mg./ml. in 0·15 M phosphate buffer, pH 7·7) and xanthine oxidase solution (0·1 ml.) were heated at 36–37° under aerobic conditions for 18 hr.

Paper chromatography of 10 μl. of the digest with butanol–acetic acid–water (4:1:5, by vol.) as solvent showed a faint pale-blue spot with R_F value close to that of 2-amino-4-hydroxypteridine-6-carboxylic acid. As judged by the size of the spot formed on the paper, the amount of acid formed did not appreciably increase after the first hour of incubation and represented less than 10% of the theoretical yield of carboxylic acid from PGA.

Incubation of PGA with xanthine oxidase and methylene blue

A solution (3 ml.) of 0·15 M phosphate buffer, pH 7·7, containing 1·56 mg. of PGA and 0·54 mg. of methylene blue was mixed with xanthine oxidase solution (1 ml.) and incubated at 36–37° under aerobic conditions for 18 hr.

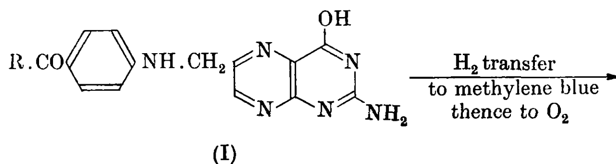
Paper chromatography of 10 μl. of this digest on no. 1 Whatman paper with butanol–acetic acid–water (4:1:5, by vol.) showed on examination in ultraviolet light a large, bright, pale-blue spot with an R_F value close to that of 2-amino-4-hydroxypteridine-6-carboxylic acid, and a small, faint-blue spot corresponding to that of 2-amino-4-hydroxypteridine-6-aldehyde. The protein present in the digest seriously interfered with the chromatographic behaviour of the 2-amino-4-hydroxypteridine-6-carboxylic acid in other solvents. The bright-blue fluorescent substance was purified by chromatography on a column of cellulose powder (Whatman standard grade, column 1 in. diam., 14½ in. high) with 5 % (by vol.) aqueous acetic acid. The eluate containing the bright-blue fluorescent substance was evaporated to dryness and the residue taken up in 1 ml. of 0·5N-NH_3 soln. Determination of the ultraviolet spectrum of this fluorescent substance in 0·1N-NaOH showed two maxima, at 261 and 361 mμ. 2-Amino-4-hydroxypteridine-6-carboxylic acid has two maxima, at 262 and 365 mμ. (Mowat *et al.* 1948). The identity of the bright-blue fluorescent substance as 2-amino-4-hydroxypteridine-6-carboxylic acid was confirmed by comparison of its chromatographic behaviour with that of an authentic sample of the acid (Table 1).

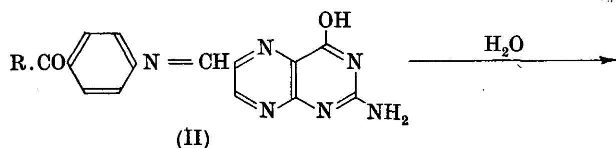
Table 1. *Identification of oxidation products of PGA by means of paper chromatography*

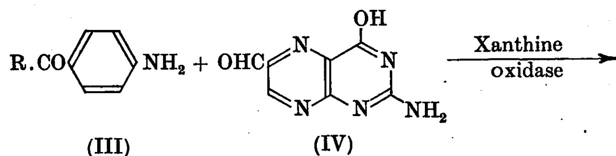
A volume (5 μl.) of the solution of the unknown and of the standard were run on the same chromatogram. Temp. 25–28°. Spots were viewed in ultraviolet light (365 mμ.).

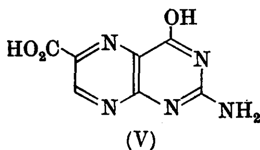
	R_F Bright light-blue fluorescent substance	R_F 2-Amino-4-hydroxy-pteridine-6-carboxylic acid	R_F Blue fluorescent substance	R_F 2-Amino-4-hydroxy-pteridine-6-aldehyde
Butanol–acetic acid–water (4:1:5, by vol.)[a]	0·12*	0·12	0·33	0·33
Propanol–5 % acetic acid (2:1, v/v)[b]	0·23	0·23	0·46	0·46
5 % acetic acid[b]	0·49	0·49	0·52	0·52
3 % ammonium chloride[c]	0·59	0·59	0·43	0·43
tert.-Butanol–pyridine–water (60:15:25, by vol.)[d]	0·09	0·09	0·27†	0·28†
tert.-Butanol–pyridine–water (50:15:35, by vol.)[d]	0·22	0·22	0·54	0·54
Pyridine–propanol–water (3:1:1, by vol.)[d]	0·26	0·26	0·55	0·55

* R_F values in this solvent are much affected by the time of equilibration. The value given is for solvent which has been equilibrated with the aqueous phase for 1 month. An R_F value of 0·22 is obtained if the solvent is equilibrated for 3 days.
† Blue spot in the digest coincided with methylene blue spot.
[a] Good & Johnson (1949) [b] Forrest & Mitchell (1954). [c] Renfrew & Platt (1950) [d] Forrest & Todd (1950).









Quantitative estimation of the acid was carried out as follows. A portion (10 µl.) of the digest and 10 µl. of a solution of 2-amino-4-hydroxypteridine-6-carboxylic acid (0·1 mg./ml. of 0·5N-NH_3 soln.) were placed on Whatman no. 1 paper, air-dried and developed with butanol–acetic acid–water (4:1:5, by vol.; ascending). The paper was air-dried, viewed in ultraviolet light and the bright, pale-blue spots were cut out and weighed (Fisher *et al.* 1948, 1949). The yield of acid was about 60% of the theoretical yield from PGA.

Incubation of methylene blue and PGA

A solution (4 ml.) of 0·15M phosphate buffer, pH 7·4, containing 1·84 mg. of PGA and 1·04 mg. of methylene blue, was incubated at 36–37° under aerobic conditions for 18 hr. Paper chromatographic analysis of 10 µl. of the digest with butanol–acetic acid–water (4:1:5, by vol.) showed only a small spot corresponding to 2-amino-4-hydroxypteridine-6-carboxylic acid (R_F value 0·12), but a large blue fluorescent spot of R_F 0·33. This blue fluorescent substance was identified as 2-amino-4-hydroxypteridine-6-aldehyde by its identical chromatographic behaviour in seven different solvent systems with a known specimen of the aldehyde (Table 1). The yield of aldehyde, estimated as before, was about 65% of the theoretical yield from PGA.

DISCUSSION

A reaction scheme for the oxidation of PGA (I) to 2-amino-4-hydroxypteridine-6-carboxylic acid (V) by xanthine oxidase and methylene blue is given on p. 210.

[R represents $HO_2C.[CH_2]_2.CH(CO_2H).NH-$.]

Compounds of the type (I) readily transfer hydrogen to suitable hydrogen acceptors and form anils [corresponding to (II)] which are readily hydrolysed by water to the corresponding amine and aldehyde (Mowat *et al.* 1948). Such dehydrogenation and hydrolysis explains the aerobic alkaline hydrolysis of PGA to 2-amino-4-hydroxypteridine-6-carboxylic acid (Mowat *et al.* 1948) and the sulphurous acid cleavage of PGA to the dihydro-2-amino-4-hydroxypteridine-6-aldehyde (Waller *et al.* 1950). 2-Amino-4-hydroxypteridine-6-aldehyde (IV) is then oxidized to the corresponding carboxylic acid (V) by xanthine oxidase (Lowry *et al.* 1949; Klenow, 1951). This oxidation may also be effected by liver aldehyde oxidase (Kalckar *et al.* 1950).

As the incubation of PGA with xanthine oxidase shows, the enzyme is without effect on PGA. The small amount of carboxylic acid formed in this experiment was derived from the aldehyde present as an impurity in the PGA.

SUMMARY

When pteroyl-L-glutamic acid is incubated with xanthine oxidase and methylene blue it is oxidized to 2-amino-4-hydroxypteridine-6-carboxylic acid. This reaction proceeds by the dehydrogenation and hydrolysis of pteroyl-L-glutamic acid to 2-amino-4-hydroxypteridine-6-aldehyde which is then oxidized by the enzyme to 2-amino-4-hydroxypteridine-6-carboxylic acid.

We wish to thank Professor A. Haddow and Mr R. C. Bray for the gift of xanthine oxidase, and the Chemical Society for a grant.

REFERENCES

Avis, P. G., Bergel, F. & Bray, R. C. (1955). *J. chem. Soc.* p. 1100.

Fisher, R. B., Parsons, D. S. & Holmes, R. (1949). *Nature, Lond.*, **164**, 183.

Fisher, R. B., Parsons, D. S. & Morrison, C. A. (1948). *Nature, Lond.*, **161**, 764.

Forrest, H. S. & Mitchell, H. K. (1954). *J. Amer. chem. Soc.* **76**, 5656.

Forrest, H. S. & Todd, A. R. (1950). *J. chem. Soc.* p. 3295.

Good, P. M. & Johnson, A. W. (1949). *Nature, Lond.*, **163**, 31.

Jacobson, W. & Good, P. M. (1952). *Quart. J. Med.* **21**, 1.

Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1948). *J. biol. Chem.* **174**, 771.

Kalckar, H. M., Kjeldgaard, N. O. & Klenow, H. (1950). *Biochim. biophys. Acta*, **5**, 586.

Kalckar, H. M. & Klenow, H. (1948). *J. biol. Chem.* **172**, 351.

Klenow, H. (1951). *Biol. Medd., Kbh.*, **18**, 14.

Lowry, O., Bessel, O. & Crawford, E. (1949). *J. biol. Chem.* **180**, 399.

Mowat, J. H., Boothe, J. H., Hutchings, B. L., Stokstad, E. L. R., Waller, C. W., Angier, R. B., Semb, J., Cosulich, D. B. & Subbarow, Y. (1948). *J. Amer. chem. Soc.* **70**, 14.

Norris, E. R. & Majnarich, J. J. (1949). *Science*, **109**, 32.

Rauen, H. M. & Waldman, H. (1950). *Experientia*, **6**, 387.

Rauen, H. M., Waldman, H. & Buchka, M. (1951). *Hoppe-Seyl. Z.* **288**, 10.

Renfrew, A. G. & Platt, P. C. (1950). *J. Amer. pharm. Ass.* **39**, 657.

Waller, C. W., Goldman, A. A., Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H. & Semb, J. (1950). *J. Amer. chem. Soc.* **72**, 4630.

Yamamoto, K. (1954). *Vitamins (Japan)*, **7**, 398.

[FROM THE BIOCHEMICAL JOURNAL, VOL. 68, No. 3, pp. 385–387, 1958]
[*All rights reserved*] PRINTED IN GREAT BRITAIN

Isolation of *iso*Xanthopterin from Human Urine

BY J. A. BLAIR
University College of Ghana, Ghana, West Africa

(*Received* 20 *August* 1957)

The pterins xanthopterin (2-amino-4:6-dihydroxypteridine) (Koschara, 1936) and biopterin (2-amino-4-hydroxy-6-[1:2-dihydroxypropyl-(L-*erythro*)]-pteridine) (Patterson, Saltza & Stokstad, 1956) have been isolated from human urine. It has been suggested that the pterins 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxypteridine and *iso*xanthopterin (2-amino-4:7-dihydroxypteridine) are products of catabolism of pteroyl-L-glutamic acid (Blair & Graham, 1955). It has also been shown that pteroyl-L-glutamic acid is converted by methylene blue and xanthine oxidase into 2-amino-4-hydroxypteridine-6-carboxylic acid (Blair, 1957). It seemed desirable, therefore, to examine human urine further.

When freshly voided adult male urine was analysed, as described below, a small quantity of a purple fluorescent substance could be isolated. On further purification this substance was shown to be *iso*xanthopterin, by its similar behaviour to an authentic sample in six solvent systems.

No 2-amino-4-hydroxypteridine-6-carboxylic acid could be isolated from freshly voided human urine. If, however, samples were allowed to stand, or if the analysis was unduly protracted, a bright-blue fluorescent substance could be isolated. This was not separable from 2-amino-4-hydroxypteridine-6-carboxylic acid in five different solvent systems. It was assumed that this acid was derived from the decomposition of biopterin in the urine.

The method used was not suitable for the isolation of 2-amino-4-hydroxypteridine.

EXPERIMENTAL

Conc. HCl (25 ml.) was added to adult male urine (250 ml.) which had been freshly voided into a flask containing a trace of phenol. The urine was then extracted with liquid phenol (50 ml.; Cranmer, 1948) and the phenol layer shaken up with water (25 ml.) and ether (100 ml.). The aqueous layer was washed twice with ether (100 ml. portions) and then evaporated to dryness under reduced pressure.

The residue was dissolved in water (5 ml.) and a few drops of aq. NH_3 were added until the solution was alkaline. The solution was filtered and evaporated to dryness under reduced pressure and the residue dissolved in aq. 0·5N-NH_3 (0·5 ml.).

The dark-brown, syrupy liquid was spotted on Whatman no. 3MM paper so that it filled a rectangle 3 in. × 1 in. The paper was then developed (descending, overnight, in darkness) with propanol–5% acetic acid (2:1, v/v), air-dried and viewed in 365 mμ light.

Three fluorescent areas could be seen: nearest the origin, a well-defined purple–blue band (1); next, a bright-yellow band (2); finally, a smaller yellow band (3). After the third yellow band the chromatogram was badly streaked. The first band was cut out and eluted with aq. 0·5N-NH_3. Further purification by chromatography, with no. 3MM paper and *tert.*-butyl alcohol–pyridine–water (50:15:35, by vol.), resolved this into three subsidiary bands. The eluate of the middle band (R_F 0·3–0·4) was chromatographed on no. 1 Whatman paper with propanol–aq. 1% NH_3 (2:1, v/v). Finally the eluate from this last chromatogram was evaporated to dryness under reduced pressure and the residue dissolved in aq. 0·5N-NH_3 (0·05 ml.). This solution was used for identification.

The purple fluorescent substance showed identical chromatographic behaviour on Whatman no. 1 paper with that of an authentic sample of *iso*xanthopterin, in butanol–acetic acid–water (4:1:5, by vol.; Good & Johnson, 1949), propanol–5% acetic acid (2:1, v/v; Forrest & Mitchell, 1954*a*), propanol–1% aq. NH_3 (2:1, v/v; Hadorn & Mitchell, 1951), propanol–N-HCl (2:1, v/v; Forrest & Mitchell, 1954*b*), and *tert.*-butyl alcohol–pyridine–water (50:15:35, by vol.; Forrest & Todd, 1950).

With *tert.*-butyl alcohol–pyridine–water (60:15:25, by vol.; Forrest & Todd, 1950) a difference of 0·06 was found in the R_F values of the unknown and *iso*xanthopterin. A mixture of the unknown and *iso*xanthopterin, however, did not separate into two spots and had a similar R_F to the unknown. This difference in R_F values of the unknown and *iso*xanthopterin is therefore due to the presence of impurities.

An additional amount of *iso*xanthopterin could be obtained by eluting band (2) and purifying it as before. In one experiment band (1) did not appear and *iso*xanthopterin was isolated from band (2).

For each identification 5μl. of solution was used and the dried papers were viewed in 365 mμ light. Papers were also viewed in 254 mμ light. This was less satisfactory as these pterins do not fluoresce so brilliantly as in 365 mμ light.

If the samples of urine were allowed to stand, or if the preliminary stages of the analysis were protracted, an additional bright blue band was found between band (1) and the origin. This band was eluted with aq. 0·5N-NH_3. It was not separable from authentic 2-amino-4-hydroxypteridine-6-carboxylic acid in five solvent systems. It was probably, therefore, 2-amino-4-hydroxypteridine-6-carboxylic acid formed from the decomposition of biopterin.

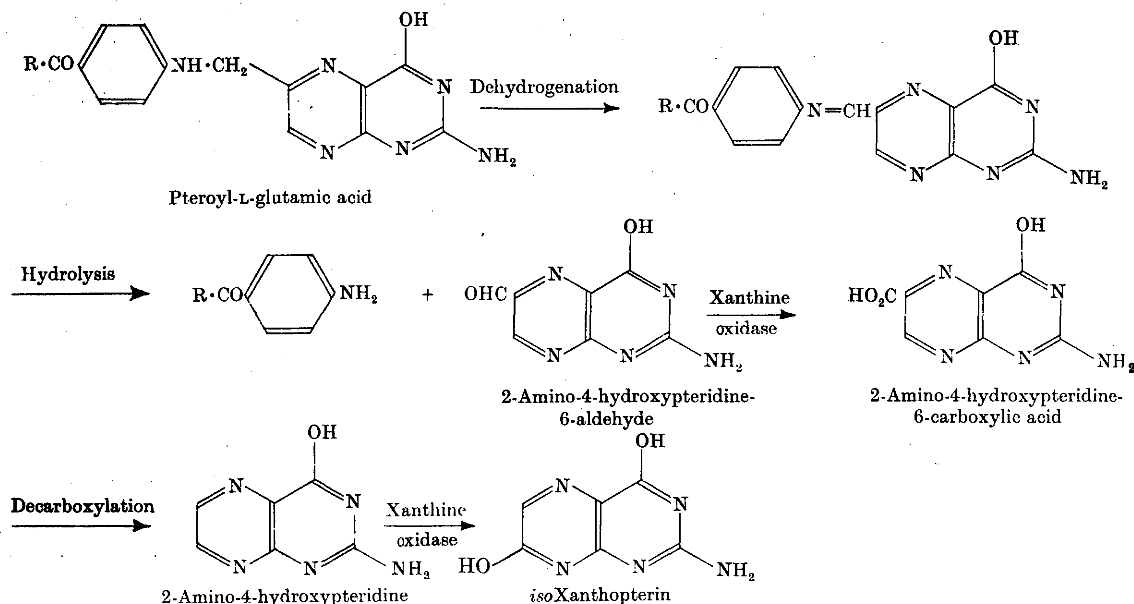


Fig. 1. Catabolism of pteroyl-L-glutamic acid. R represents $HO_2C \cdot [CH_2]_2 \cdot CH(CO_2H) \cdot NH \cdot$.

DISCUSSION

It has been suggested that pteroyl-L-glutamic acid is catabolized in the body by some yet unknown route (Williams, Eakin, Beerstecher & Shive, 1950). It is now suggested that the catabolism of pteroyl-L-glutamic acid proceeds as in Fig. 1.

Evidence for the conversion of pteroyl-L-glutamic acid into 2-amino-4-hydroxypteridine-6-carboxylic acid by processes of dehydrogenation, hydrolysis and enzymic oxidation has been described in a previous paper (Blair, 1957).

The pterins 2-amino-4-hydroxypteridine-6-carboxylic acid, 2-amino-4-hydroxypteridine and *iso*xanthopterin are widely distributed in Nature (Forrest, Van Baalen & Myers, 1957; Viscontini, Kuhn & Egelhaaf, 1956; Forrest & Mitchell, 1955; Viscontini, Schmid & Hadorn, 1955; Viscontini, Loeser, Karrer & Hadorn, 1955; Nawa & Taira, 1954; Hama, 1953; Busnel & Drilhon, 1949; for earlier references see Blair & Graham, 1955).

2-Amino-4-hydroxypteridine-6-carboxylic acid can be decarboxylated by heat (Forrest & Mitchell, 1954*b*) or by ultraviolet light (Lowry, Bessey & Crawford, 1949*a*; Viscontini, Loeser & Egelhaaf, 1956) to 2-amino-4-hydroxypteridine; and this can be oxidized by xanthine oxidase to *iso*xanthopterin (Lowry, Bessey & Crawford, 1949*b*).

It is therefore reasonable to suppose that these three pterins are intermediate in the biological degradation of pteroyl-L-glutamic acid. The isolation of *iso*xanthopterin from human urine supports this scheme. Further work is in progress.

SUMMARY

1. *iso*Xanthopterin has been isolated from human urine.

2. 2-Amino-4-hydroxypteridine-6-carboxylic acid could not be isolated from freshly voided human urine.

3. A scheme for the catabolism of pteroyl-L-glutamic acid is suggested.

We wish to thank the Chemical Society for a grant and Professor D. H. R. Barton, F.R.S., for accommodation during the long vacations in 1956 and 1957.

REFERENCES

Blair, J. A. (1957). *Biochem. J.* **65**, 209.
Blair, J. A. & Graham, J. (1955). *Chem. & Ind.* p. 1158.
Busnel, R. G. & Drilhon, A. (1949). *Bull. Soc. zool. Fr.* **74**, 21.
Cranmer, J. L. (1948). *Nature, Lond.*, **161**, 349.
Forrest, H. S. & Mitchell, H. K. (1954*a*). *J. Amer. chem. Soc.* **76**, 5656.
Forrest, H. S. & Mitchell, H. K. (1954*b*). *J. Amer. chem. Soc.* **76**, 5658.
Forrest, H. S. & Mitchell, H. K. (1955). *J. Amer. chem. Soc.* **77**, 4865.
Forrest, H. S. & Todd, A. R. (1950). *J. chem. Soc.* p. 3295.
Forrest, H. S., Van Baalen, C. & Myers, J. (1957). *Science*, **125**, 699.
Good, P. M. & Johnson, A. W. (1949). *Nature, Lond.*, **163**, 31.

Hadorn, E. & Mitchell, H. K. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 650.
Hama, T. (1953). *Experientia*, **9**, 299.
Koschara, W. (1936). *Hoppe-Seyl. Z.* **240**, 127.
Lowry, O. H., Bessey, O. A. & Crawford, E. J. (1949*a*). *J. biol. Chem.* **180**, 389.
Lowry, O. H., Bessey, O. A. & Crawford, E. J. (1949*b*). *J. biol. Chem.* **180**, 399.
Nawa, S. & Taira, T. (1954). *Proc. imp. Acad. Japan*, **30**, 632.
Patterson, E. L., Saltza, M. H. & Stokstad, E. L. R. (1956). *J. Amer. chem. Soc.* **78**, 5871.
Viscontini, M., Kuhn, A. & Egelhaaf, A. (1956). *Z. Naturf.* **11** b, 501.
Viscontini, M., Loeser, E. & Egelhaaf, A. (1956). *Naturwissenschaften*, **43**, 379.
Viscontini, M., Loeser, E., Karrer, P. & Hadorn, E. (1955). *Helv. chim. acta*, **38**, 1222.
Viscontini, M., Schmid, H. & Hadorn, E. (1955). *Experientia*, **11**, 390.
Williams, R. J., Eakin, R. E., Beerstecher, E. & Shive, R. (1950). *The Biochemistry of B Vitamins*, p. 574. New York: Rheinhold Publishing Corporation.

[FROM THE BIOCHEMICAL JOURNAL, VOL. 56, No. 2, pp. 286–287, 1953]
[*All rights reserved*] PRINTED IN GREAT BRITAIN

The Pigments of Snake Skins

1. THE ISOLATION OF RIBOFLAVIN AS A PIGMENT OF THE SKINS OF THE GREEN SNAKES *PHILOTHAMNUS SEMIVARIEGATUS* AND *DISPHOLIDUS TYPUS*

BY J. A. BLAIR AND J. GRAHAM
University College of the Gold Coast

(*Received* 14 *August* 1953)

It is frequently noted that when whole snakes or their skins are preserved by immersion in 40% formalin solution, a greenish yellow colour is imparted to the solution, and that this effect is particularly marked with green snakes. A survey of the literature showed that no previous work had been done on snake-skin pigments.

Extraction with absolute ethanol of the skins, freed from organs, of specimens of the green snakes *Philothamnus semivariegatus* and *Dispholidus typus* gave yellow solutions which had a greenish fluorescence in daylight. The amount of pigment obtained was too little for a rigorous, chemical identification, but the general chemical properties of the extract from *P. semivariegatus* suggested that riboflavin was present. Methods for the identification of riboflavin by paper chromatography have been published (Forrest & Todd, 1950; Crammer, 1948), and use on the snake-skin extracts of the developing solvents described by these authors showed that riboflavin was the only pigment present in the extract from *P. semivariegatus*, and that riboflavin and another, unidentified, yellow pigment were present in the extract from *D. typus*. Comparison of the ultraviolet absorption spectra on paper (Bradfield & Flood, 1952) of the yellow pigment from *P. semivariegatus* and of riboflavin showed that they were identical.

The skin of both specimens after extraction was bluish grey and this residual colouring matter could only be obtained by warming with 0·01 N sodium hydroxide solution, when the extracted skin turned black. Investigations on this pigment and on the yellow pigment from *D. typus* are proceeding.

EXPERIMENTAL

The organ-free skin of a specimen of *P. semivariegatus* was extracted in the dark with absolute ethanol in a Soxhlet extractor, the solution was then cooled and filtered from insoluble matter. Although no pigment could be isolated from this solution, its yellow colour, strong, green fluorescence in daylight, and the ready loss of both colour and fluorescence on irradiation in sunlight or on addition of dilute acid, dilute alkali, or $NaHSO_3$ suggested that the pigment present was riboflavin.

The organ-free skin of a specimen of *P. semivariegatus* was extracted in the dark with a mixture of water:pyridine:*n*-propanol (1:3:1, v/v throughout) (50 ml.) on a steam bath (45 min.), cooled, and filtered from insoluble material. The solution was yellow with a green fluorescence in daylight. Similar extraction of the skin of a specimen of *D. typus* gave a yellow solution with only a faint green fluorescence in daylight. Paper chromatography of these extracts and of a

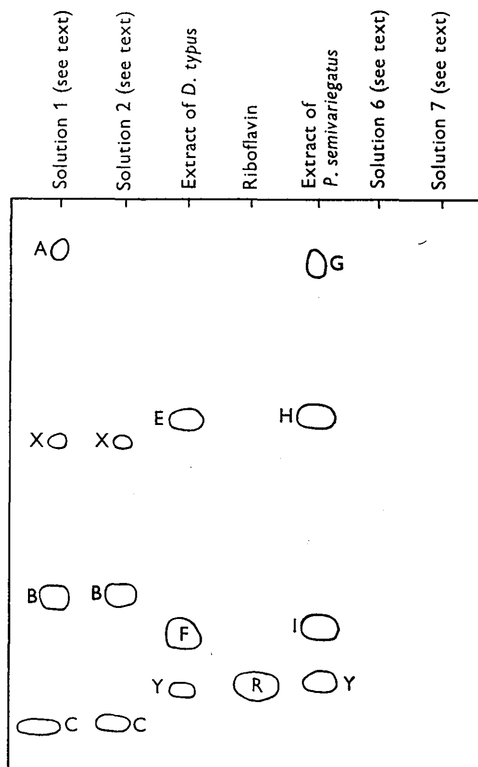


Fig. 1. Paper chromatogram developed with water: pyridine:*n*-propanol (1:3:1, v/v) and solvent allowed to drip off lower edge of paper. Spots A–C, E–I and X, were colourless, showing the following fluorescences in u.v. light: A, G, X, yellow; B, grey; C, bluish grey; E, H, light-blue; F, I, bluish violet. R and Y were yellow in daylight, fluorescing yellow in u.v. light. R and Y (fifth column) faded on exposure to daylight, Y in third column did not. No spots were visible in daylight or in u.v. light in the sixth and seventh columns.

saturated solution of riboflavin in the water:pyridine:*n*-propanol solvent on descending chromatograms of Whatman no. 1 paper irrigated with this solvent (Forrest & Todd, 1950) showed that each extract gave a number of colourless spots which fluoresced in ultraviolet light and a yellow spot which travelled at the same speed as the riboflavin spot.

To show that none of the other substances found in the snake-skin extracts was a decomposition product of riboflavin due to irradiation or chemical treatment of the extract, solutions of authentic riboflavin solutions were treated as follows.

A saturated solution of riboflavin in water:pyridine:*n*-propanol was irradiated in tropical sunlight (4·5 hr.). Half of this solution was retained (solution 1); the remainder was warmed on a steam bath in the dark (45 min.) (solution 2). A saturated solution of riboflavin in distilled water was irradiated in tropical sunshine (4·5 hr.). Pyridine (6 ml.) and *n*-propanol (2 ml.) were added to this solution (2 ml.). Half of it was kept (solution 7); the remainder was warmed on a steam bath in the dark (45 min.) (solution 6).

Paper chromatography of these four solutions, the two snake-skin extracts, and a saturated solution of riboflavin in water:pyridine:*n*-propanol (0·02 ml. of each solution) by the descending method on Whatman no. 1 paper gave the chromatogram in Fig. 1 which shows that none of the substances obtained from the snake-skin extracts was derived from riboflavin by decomposition (spot G appeared only after the extract had been kept for several days), and that both snake-skin extracts contained a substance, the behaviour of which in the solvent system was identical with that of riboflavin. It was noted that the spot Y in column 3 (extract of *D. typus*) did not lose its yellow colour on exposure to light as did the riboflavin spot. Use of other solvent systems showed that this spot could be resolved into riboflavin and a second, non-fluorescent, yellow pigment.

Paper chromatography of the snake-skin extracts and a saturated solution of riboflavin in water:pyridine:*n*-propanol (0·02 ml. of each solution spotted on paper and air-dried) by the descending method on Whatman no. 1 paper using *n*-butanol:acetic acid:water (Crammer, 1948) and *tert.*-butanol:pyridine:water (50:15:35, v/v; Forrest & Todd, 1950) as developing solvents gave chromatograms, Figs. 2 and 3, which show that the extract of *P. semivariegatus* contains only one yellow pigment, which is identical with riboflavin, and that the extract of *D. typus* contains two yellow pigments, one of which is identical with riboflavin.

A paper chromatogram of the extract of *P. semivariegatus* and a saturated solution of riboflavin in water:pyridine:*n*-propanol was developed with this solvent and the paper was air-dried (1 hr.) and then dried at 100° (45 min.). The riboflavin and yellow-pigment spots were cut off the paper and their ultraviolet absorption spectra were determined on the paper by the method of Bradfield & Flood (1952). The absorption maxima were for the yellow pigment, 260 and 370 mμ.; and for riboflavin, 260 and 360 mμ.

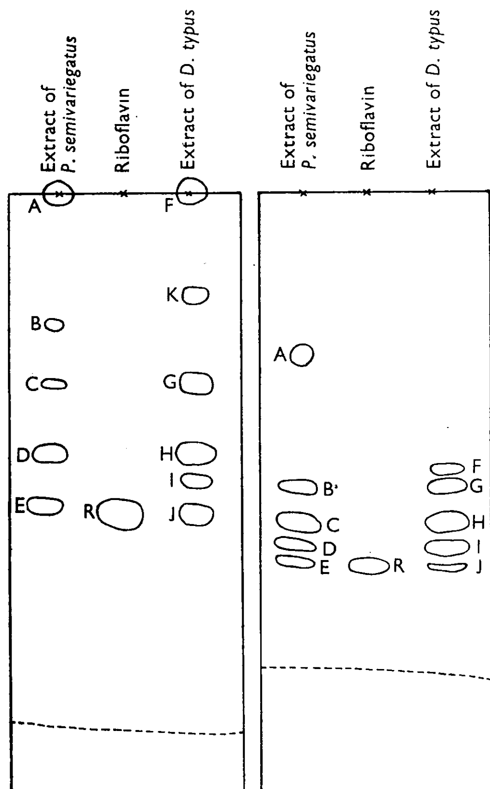


Fig. 2. Fig. 3.

Fig. 2. Paper chromatogram developed with *n*-butanol: acetic acid:water (Crammer, 1948) for 20 hr. Solvent front marked by dotted line. Spots A–J were colourless, showing the following fluorescences in u.v. light: A, D, F, H, bluish violet; B, yellow; C, G, I, light-blue; E, yellow, becoming greyish yellow; J, yellow, becoming greyish, then light-blue. K was yellow, did not fade in daylight, nor did it fluoresce. R was yellow, fluorescing yellow, and becoming greyish yellow on prolonged u.v. irradiation.

Fig. 3. Paper chromatogram developed with *tert.*-butanol: pyridine:water (50:15:35, v/v) for 34 hr. Solvent front marked by dotted line. Spots A–D, G–I were colourless, showing the following fluorescences in u.v. light: A, yellow; B, D, G, I, light-blue; C, H, bluish violet. E, J and R had the properties of R in Fig. 1, F of spot K in Fig. 2.

SUMMARY

1. Riboflavin occurs as a yellow pigment in the skins of the green snakes *Philothamnus semivariegatus* and *Dispholidus typus*.

2. Another yellow pigment occurs in the skin of *D. typus*.

3. Both skins also contain a pigment which is extractable by 0·01 N sodium hydroxide solution.

The authors wish to thank Mr A. H. Booth of the Zoology Department of the University College of the Gold Coast for bringing this problem to their notice, and for assistance in supplying and identifying the snakes.

REFERENCES

Bradfield, A. E. & Flood, A. E. (1952). *J. chem. Soc.* p. 4740.
Crammer, J. L. (1948). *Nature, Lond.*, **161**, 349.
Forrest, H. S. & Todd, A. R. (1950). *J. chem. Soc.* p. 3295.

Reprinted from Chemistry and Industry, 1955, *pp.* 1158–1159

IDENTITY OF PTERINS PRESENT IN SNAKE SKINS

By J. A. Blair and J. Graham

Chemistry Department, University College of the Gold Coast, Achimota, Gold Coast

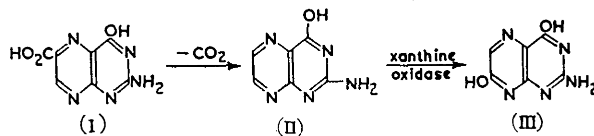
When the organ- and fat-free skins of the green snakes, *Philothamnus semivariegatus* and *Dispholidus typus* (Boomslang), are extracted with portions of 80% aqueous ethanol and the combined extracts chromatographed on Whatman No. 1 Paper, three distinct spots are seen when the chromatogram is viewed in ultraviolet light. One fluoresces yellow, one purple and the third a bright sky-blue. In daylight none of these is visible but when a more concentrated solution is used the spot which fluoresces yellow is visibly yellow. This has previously been identified as riboflavin.[1]

The purple fluorescing substance has now been identified as *iso*xanthopterin by its similarity in chromatographic behaviour in six different solvent systems to an authentic specimen (3% aqueous ammonium chloride,[2] 5% aqueous acetic acid,[3] *n*-butanol-acetic acid-water (4 : 1 : 5 v/v upper phase),[4] pyridine-*n*-propanol-water (3 : 1 : 1 v/v),[5] *tert.*-butanol-pyridine-water (50 : 15 : 35 v/v),[5] *tert.*-butanol-pyridine-water (60 : 15 : 25 v/v)[5] and the bright sky-blue fluorescing substance as 2-amino-4-hydroxypteridine-6-carboxylic acid by comparison with an authentic specimen in the same six solvent systems and in *n*-propanol-5% acetic acid (2 : 1 v/v)[3] and dimethylformamide-formic acid-water (8 : 1 : 1 v/v).

When the combined extracts from *Dispholidus typus* are evaporated to dryness and the residue taken up in 0·5N-NH_4·OH, a paper chromatogram of this solution shows another spot fluorescing light blue in ultra-violet light. This substance has been identified as 2-amino-4-hydroxypteridine by comparison with an authentic sample in the eight solvent systems listed above.

Subsequent to the time when these observations were made, Karrer reported the presence of riboflavin, *iso*xanthopterin, 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxypteridine in *Drosophila.*[6] Previously, *iso*xanthopterin has been detected in butterfly wings,[7] the skin of *Rana nigromaculata* [8] the larvae of *Bombyx mori* [8, 9] identified with mesopterin,[10] and recognised as a component of fluorescyanin.[9]

It is conceivable that 2-amino-4-hydroxy pteridine-6-carboxylic acid (I) and 2-amino-4-hydroxypteridine (II) are intermediates in the biological degradation of pterins, e.g. folic acid, to the relatively inert end-product of metabolism, *iso*xanthopterin (III). Also



since the flavoprotein enzyme xanthine oxidase will convert 2-amino-4-hydroxypteridine to *iso*xanthopterin *in vitro*,[11] it may exercise the same function *in vivo* in addition to its rôle as remover of xanthine and hypoxanthine from the general metabolic pool of purines. If so, inhibition of xanthine oxidase[12] would bring about an abnormal increase in cellular concentration of 2-amino-4-hydroxypteridine, as well as of xanthine and hypoxanthine. Further, since it is known that 2-amino-4-hydroxypteridine stimulates mitosis and cell division in the epitheluim of the renal tubes, it is possible that this substance is concerned in the general process of mitosis and cell division.

We are very grateful to Prof. R. Tschesche for gifts of *iso*xanthopterin and 2-amino-4-hydroxypteridine-6-carboxylic acid and to Prof. A. Albert and Dr. D. Brown for the gift of 2-amino-4-hydroxypteridine.

Received July 18, 1955

References

1 Blair, J. A. & Graham, J., *Biochem. J.*, 1954, **56**, 286
2 Renfrew, A. G. & Platt, P. C., *J. Amer. pharm. Assoc.*, 1950, **39**, 657
3 Forrest, H. S. & Mitchell, H. K., *J. Amer. chem. Soc.*, 1954, **76**, 5656
4 Good, & Johnson, A. W., *Nature Lond.*, 1949, **163**, 31
5 Forrest, H. S. & Todd, A. R., *J. chem., Soc.* 1950, 3295
6 Viscontini, M., Schoeller, M., Loeser, E., Karrer, P. & Hadorn, E., *Helv. Chim. Acta*, 1955, **38**, 397
7 Schöpf, G. & Becker, E., *Liebigs Ann.*, 1936, **524**, 49
8 Nawa, S., Gota, M., Matsuura, S., Kakizawa, H. & Hirata, Y., *J. Biochem. Japan*, 1954, **41**, 657
9 Tschesche, R. & Korte, F., *Chem. Ber.*, 1954, **87**, 1713
10 Tschesche, R. & Korte, F., *ibid.*, 1951, **84**, 641
11 Lowry, O., Bessel, O. & Crawford, E., *J. biol. Chem.*, 1949, **180**, 399
12 Haddow, A., *et al.*, *Brit. Emp. Cancer Camp. Ann. Report*, 1953, **31**, 35

[8535] PRINTED BY HARRISON AND SONS, LTD., ST. MARTIN'S LANE, LONDON