PTERIDINES IN BLUE GREEN ALGAE[1, 2]

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The presence of a remarkably high concentration of pteridines in the blue green alga, *Anacystis nidulans*, was demonstrated by Forrest, van Baalen, and Myers in 1957 (2). A number of different pteridines were then isolated and identified. These included 2-amino-4-hydroxypteridine, 2,6-diamino-4-hydroxypteridine (10), a yellow pteridine assigned the structure 2-amino-4-hydroxy-6-propionyl-5,8 or 7,8-dihydropteridine (4), and a glucoside of 2-amino-4-hydroxy-6-(dihydroxypropyl)pteridine (biopterin) (fig 1) (3). Subsequent work has shown that probably all of these compounds are artifacts of isolation. This is based on the fact that Anacystis can be treated in a number of different ways to give varying amounts of these compounds. Thus if the algal cells are simply placed on the origin of a paper chromatogram, and the chromatogram is developed with an ammoniacal solvent, the pteridines which can be identified after development include 2-amino-4-hydroxypteridine, 2,6-diamino-4-hydroxypteridine, and the yellow pteridine accompanied by a light decomposition product. On the other hand, when cells are allowed to stand in the growth medium at 4° for 24 to 48 hours, the principal compound released in the medium is the yellow pteridine. Finally if the cells are acidified with acetic acid and then treated with solid manganese dioxide, the sole pteridine obtained from Anacystis is the glucoside of biopterin (Compound C) (6). These experiments, along with the chemical evidence on the variety of products arising during the reoxidation of a reduced pteridine ring (10) lead to the concept that there is a single compound present in the intact cells which readily breaks down, when the cells are manipulated, to give the products indicated above. This compound probably contains the carbon skeleton of the glucoside of biopterin, but it may be a tetrahydro or dihydro derivative with possibly an exocyclic double bond. Attempts to delineate its structure more rigorously have not been successful.

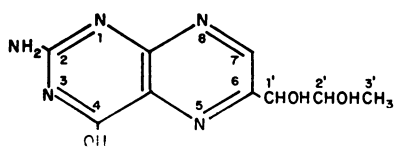

FIG. 1. Structural formula for biopterin. In the glucoside (compound C), the glucose is attached either at the 1′ or at the 2′ carbon.

In preliminary experiments, it was noted that other blue-green algae behaved somewhat similarly to Anacystis when treated under the conditions mentioned above. However, when the acetic acid/manganese dioxide technique, which gives only one pteridine, was discovered and applied to these other algae, it was found that they gave different but closely related blue fluorescent pteridines. A systematic study then revealed that although the compounds produced by this treatment could readily be distinguished, the only difference between them (except in one case to be discussed later) lay in the sugar which was attached to a common molecule, 2-amino-4-hydroxy-6-dihydroxypropylpteridine (biopterin). This was shown by hydrolyzing the compounds with acid after which biopterin could be identified by comparison with an authentic specimen. The acid hydrolysate in any particular case also contained a sugar, which has been identified or tentatively identified by paper chromatography. Since only small quantities of these were available, it has not been possible to apply more rigorous methods of identification to the glycoside components of the molecule.

In the case of one alga, a Synechocystis, acid hydrolysis of the product obtained in the usual way, produced a sugar (glucose) and a pteridine, which was identified by comparison with an authentic sample (5) as 2-amino-4-hydroxy-6-hydroxymethylpteridine. So far as we are aware, this compound has not been previously isolated from natural sources.

MATERIALS & METHODS

Nine strains of blue green algae were examined. With the exception of a marine form and one other, these were obtained through the courtesy of Dr. J.

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Myers from cultures maintained in the Laboratory of Algal Physiology, University of Texas. Their original source is also listed: *Anabaena variabilis* (Kützing), obtained from R. C. Hecker as Cylindrospermum; revised identification by E. G. Pringsheim. *Nostoc muscorum* (Kützing), (Gerloff's strain), obtained from G. C. Gerloff. *Anabaena cylindrica* (Lemmermann), obtained from M. B. Allen. *Anacystis nidulans* (Richter) tentatively identified by F. Drouet. TX27 unidentified strain, however, it is a blue green alga containing phycoerythrin. Synechocystis (Gonzalez strain), obtained from Dr. W. A. Kratz. Synechocystis, high temperature strain, tentatively identified by H. D. Cobb. Synechococcus, obtained from M. B. Allen. Synechocystis, obtained as a lyophilized powder by courtesy of Dr. T. S. Bannister from the Photosynthesis Project, University of Illinois. Apparently, a live culture of this alga is no longer available. Nostoc sp. (Kützing), marine form, isolated by one of us (C. V. B.) from Long Island Sound, N. Y.

The algae were grown in large test tubes (3.5 × 29.5 cm) containing 125 ml of Medium C (7) supplemented with extra nitrate. Illumination was provided by two 20 w fluorescent lamps placed on each side of the culture tubes, giving about 500 ft-c on each side at the level of the tubes. Air, enriched with 0.5 % carbon dioxide was bubbled continuously through the medium. *Anacystis nidulans* and Synechocystis, the two high temperature strains, were grown at 37°; the rest were grown at 25° C. The marine species was grown in a similar manner using the ASP-2 medium of Provasoli et al (8).

The harvested cells (ca. 0.5 g dry wt/tube) from 6 tubes were resuspended in water (5 ml), and dilute acetic acid (2.5 N; 10-15 ml) and manganese dioxide powder (15 mg) were added. After thorough mixing, the whole was centrifuged and the supernatant collected. The cells were washed with aqueous acetone (20 %; 10 ml), recentrifuged, and the supernatants combined.

After evaporation to small bulk (2–4 ml), the extract was streaked on filter paper (Whatman no. 17; 46 × 57 cm) which was then irrigated with the solvent, 1-propanol : 1 % ammonium hydroxide (2 : 1). The appropriate band was located by its blue fluorescense in ultraviolet light (max. emission at 360 mμ) and the material was eluted from it using 1 % ammonium hydroxide. It was then rechromatographed on heavy filter paper, using butanol : acetic acid : water (4 : 1 : 1) as solvent. Elution of the blue fluorescent band from the second chromatogram gave a solution which was used for determination of the ultraviolet spectrum of the compound; for further paper chromatography to determine its R_f values and to compare it with known or similarly isolated materials (table I); and for acid hydrolysis. (In order to provide a convenient reference point the R_f values are recorded in relation to the R_f for biopterin which is given the value one.)

Acid Hydrolysis of Products From Algae. In order to remove interfering materials from eluates from paper chromatographic purification, the blue fluorescent compounds were adsorbed from solution by the addition of a small amount of charcoal (Darco G60). The charcoal was thoroughly washed with water and the fluorescent materials were then eluted from it with ethanol : ammonium hydroxide (1 : 1; 10 ml). This eluate was evaporated to small bulk and treated with the appropriate amount of sulfuric acid. The extent of hydrolysis was followed by checking samples at intervals by paper chromatogra-

Table I

Paper Chromatographic & Hydrolysis Data on Compounds from Blue Green Algae

	4 % Sodium citrate	1-Propanol, 1 % NH_3 (2 : 1)	1-Butanol, acetic acid, water (4 : 1 : 1)	1-Butanol, methyl ethyl ketone, NH_3, water (5 : 3 : 1 : 1)	2-Butanol, formic acid, water (8 : 2 : 5)	Acid strength & time for hydrolysis	Sugar (tentative)
Anabaena var.	1.23	0.97	0.67	0.50	0.91	2 N for 3 hr	6-deoxy-d-glucose
Nostoc mus.	1.23	0.97	0.67	0.50	0.91	2 N for 3 hr	6-deoxy-d-glucose
Anabaena cyl.	1.40	0.94	0.45	0.30	0.80	2 N for 2 hr	Galactose
Anacystis nid.	1.23	0.80	0.37	0.42	0.67	2 N for 2 hr	Glucose
TX27	1.35	0.61	0.45	0.09	0.87	5 N for 4 hr	Xylouronic acid
Synechocystis	1.13	0.83	0.48	0.60	0.84	2 N for 2 hr	Xylose or ribose
Synechocystis*	1.23	0.80	0.37	0.42	0.67	2 N for 2 hr	Glucose
Synechococcus	1.13	0.83	0.48	0.60	0.84	2 N for 2 hr	Xylose or ribose
Synechocystis**	1.34	0.30	0.15	0.0	0.61	2 N for 1 hr	Glucose
Nostoc***	1.23	0.97	0.67	0.50	0.91	2 N for 3 hr	6-deoxy-d-glucose
Biopterin	1.00	1.00	1.00	1.00	1.00		

* High temperature form
** Lyophilyzed preparation
*** Marine form

phy. After complete hydrolysis had taken place, the sulfuric acid was removed by precipitation with barium hydroxide, and the resulting neutral solution was treated to separate the products in either of two ways: I. Charcoal was again added until the solution was no longer fluorescent. The charcoal was removed by centrifugation and treated as before to recover the fluorescent material. This was then compared spectrophotometrically and chromatographically with authentic specimens. The supernatant was evaporated to small bulk and this solution was used for paper chromatographic comparison with known sugars, using butanol: acetic acid: water (4:1:1) as solvent. II. The hydrolysis mixture was passed through a small column (1 × 3 cm) of Filtrol, previously washed with dilute acetic acid. The fluorescent material was adsorbed at the top of the column. The eluate and water washings were collected, evaporated, and the concentrated solution used for chromatographic comparison with sugars as before. The fluorescent material was washed from the column with 30 % aqueous acetone. Again, the concentrated eluate was used for chromatography, and for ultraviolet absorption spectrophotometry.

Results

Paper chromatography of the algal extracts showed that essentially they contained only one blue fluorescent compound when the cells were treated with acetic acid and manganese dioxide. However, in some cases, several minor, very faint fluorescent bands could be detected. These were not further investigated. The ultraviolet absorption spectra of the various, purified compounds and biopterin were identical with respect to positions of maxima and minima. On hydrolysis, they gave biopterin identified by its ultraviolet absorption spectrum and by paper chromatographic comparison with an authentic specimen. The conditions for hydrolysis and the tentative identification of the sugars thus produced are listed in table I. Several of the compounds yielded biopterin and glucose. The original compounds were then co-chromatographed and shown to be identical. The compound isolated from TX27 was acidic (paper electrophoresis) but, on hydrolysis, it gave biopterin and the acidic portion of the molecule thus resided in the glycosidic moiety. This reacted with the spray reagent to give the characteristic red color of a pentose. It has therefore been tentatively identified as a xylouronic acid.

The one exception to the above statements was provided from the study of the compound obtained from the lyophilized sample of Synechocystis. On acid hydrolysis, as with the other compounds, the ultraviolet absorption spectrum of the hydrolysate was unchanged. However the compound responsible for this ultraviolet absorption spectrum was shown by paper chromatography in a number of solvents to be 2-amino-4-hydroxy-6-hydroxymethylpteridine (table II).

Table II

R_f Values of Pteridine Products After Hydrolysis

	Synechocystis*	Other algae	-6-CH_2OH	Biopterin
4 % Sodium citrate	0.40	0.63	0.40	0.63
1-Propanol, 1 % NH_3 (2:1)	0.55	0.56	0.55	0.56
1-Butanol, acetic acid, water (4:1:1)	0.42	0.33	0.42	0.33
1-Butanol, methyl ethyl ketone, NH_3, water (5:3:1:1)	0.14	0.12	0.14	0.12
2-Butanol, formic acid, water (8:2:5)	0.64	0.58	0.64	0.58

* Lyophilyzed

Discussion

Since the function of biopterin glucoside (or the natural glucoside from which it is derived) in Anacystis is unknown, it is difficult to assess the biochemical implications of the discovery that one of a number of different sugars may be attached to biopterin in different blue-green algae. The distinct compounds which result may prove to be useful in taxonomic studies within this phylum, since they may provide a simple means for demonstrating a distinction or a similarity between species. A much more extensive study than the one reported herein would have to be made to test the validity of this approach.

Perhaps the most interesting compound described in this paper is the glucoside of 2-amino-4-hydroxy-6-hydroxymethylpteridine isolated from a Synechocystis. 2-Amino-4-hydroxy-6-hydroxymethylpteridine has been implicated, in the form of its tetrahydro derivative, in the biosynthesis of folic acid-like compounds (1, 9).

It is tempting to speculate that the compound isolated from Synechocystis represents an oxidation product from an intermediate in folic acid biosynthesis in this organism, possibly accumulating due to some metabolic disharmony. The other compounds described in this paper would then perform a like function in the specific algae from which they have been isolated. From a theoretical point of view, a similar function for a 6-hydroxymethyl compound and a 6-dihydroxypropyl compound is not at all improbable, since it has been shown that in the reduced form, the side chain in the 6-position of the pteridine ring is

quite labile (10). The three carbon dihydroxypropyl side chain could therefore be replaced by a one carbon fragment, the new molecule then condensing with *p*–aminobenzoylglutamic acid according to the scheme proposed by both Shiota and Brown. In fact, the important intermediate in folic acid biosynthesis may well be 2–amino–4–hydroxy–5,6,7,8–tetrahydropteridine (or a dihydro compound), since it has been shown that this compound, with formaldehyde and *p*–aminobenzoylglutamic acid will condense in the test tube in low yield to give folic acid (unpub. data). Thus the compounds described in this paper would represent end-products derived from compounds capable of supplying the organism with its requirement of reduced 2–amino–4–hydroxypteridine for folic acid biosynthesis. Unfortunately, the available evidence from bacteria is that biopterin and folic acid are not interconvertible (11). However, it would seem worthwhile to repeat such interconversion experiments using blue green algae instead of bacteria.

Summary

A number of new, related compounds have been isolated from a variety of species of blue green algae, using a standard technique. With one exception, these compounds have been shown to be glycosides of biopterin (2–amino–4–hydroxy–6–[1′,2′–dihydroxypropyl] pteridine), differing in the nature of the sugar attached to the side chain. The glucoside of 2–amino–4–hydroxy–6–hydroxymethylpteridine has been isolated from one species. From the method of isolation and other evidence, it seems likely that all of these compounds exist in the intact cell as tetrahydro or dihydro derivatives. The possible significance of these compounds is discussed.

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