three times; yield 40 to 55 mg. on different runs, dried 16 hours at 115°, $[\alpha]^{26}D$ −52° (0.1 *N* HCl, *c*, .54); solubility: in mg./ml. water, 90°, 3; 4°, 0.5; 1 *N* HCl and 1 *N* NaOH, more than 25 at 25°; ether, acetone and alcohol less than 0.1; biological activity, 0.05 millimicrogram per ml. required for one-half maximum growth of *C. fasciculata* in 144 hours.

Anal. Calcd. for $C_9H_{11}N_5O_3$: C, 45.6; H, 4.64; N, 29.5. Found: C, 46.0; H, 4.85; N, 28.4; C, 45.9; H, 4.83; N, 30.3.

Acknowledgment.—We wish to thank Dr. R. C. Gore, Stamford Laboratories, American Cyanamid Co., for the infrared analysis and Mr. L. Brancone and staff for the microanalytical determinations.

PEARL RIVER, NEW YORK

[CONTRIBUTION FROM THE NUTRITION AND PHYSIOLOGY SECTION, RESEARCH DIVISION, AMERICAN CYANAMID CO., LEDERLE LABORATORIES]

The Isolation and Characterization of a Pteridine Required for the Growth of *Crithidia fasciculata*[1]

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Procedures are described for the isolation from human urine of a compound named biopterin required for the growth of the protozoön *Crithidia fasciculata*. These involve adsorption on charcoal, counter-current solvent distribution and partition chromatography. Pure biopterin crystallized from water in pale yellow spheres. Biopterin was tentatively characterized as a 2-amino-4-hydroxyalkylpteridine from its ultraviolet absorption spectra. Alkyl substituents of $C_3H_7O_2$ were indicated from the elemental analyses, and 1.7 periodate oxidation equivalents per mole were consumed at *p*H 2. The pteridine fragment was 2-amino-4-hydroxy-6-formylpteridine identified by ultraviolet absorption. No formaldehyde, formic acid or ammonia were in the reaction mixtures. The presence of a methyl group in biopterin was established by infrared absorption. From these data it was concluded that biopterin is 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine.

Previous observations indicated that the protozoön *Crithidia fasciculata* required an unknown factor for growth which was present in a number of natural materials.[2] A preliminary report was made on the isolation of this factor which was named biopterin and was identified as 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine.[3] Forrest and Mitchell[4] have since reported the isolation of 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine from Drosophila. It was identified from degradation studies and by comparison with a synthetic specimen. However, the optical configuration was not determined. Some of the properties of one of the pteridines isolated from Drosophila by Viscontini, *et al.*,[5] are similar to those of biopterin, but these authors have not yet established beyond doubt the structure of their compound.[6,7] This paper describes the isolation and characterization of biopterin in detail.

Isolation.—For the isolation of biopterin, human adult male urine was chosen as a starting material because of its availability and relatively constant potency. The bioassay previously described[2] was used to develop the isolation procedures. Batches of 200 liters of normal adult male urine were purified by the procedures outlined in Table I.

Adsorption on charcoal was used as a first step to separate biopterin from the large volumes of urine.

TABLE I

THE SCHEME FOR THE ISOLATION OF BIOPTERIN FROM HUMAN ADULT MALE URINE

Procedure	Bioactivity, mg. Biopterin	Solids, g.
1. Five 200-liter batches of urine	314	11,850
2. 1 kilo charcoal/100 liters urine. Eluate, NH_4OH:ethanol:water, 10:50:50	113	1,715
3. Counter-current solvent distribution *n*-butyl alc./water, *p*H 5; 40 tubes, 40 transfers, one liter per phase	115	360
4. Partition chromatography *n*-butyl alc./0.05 *M* phosphate, *p*H 4.5	80	
5. Partition chromatography on composite of 5 batches		
(a) *n*-butyl alc./0.01 *M* phosphate, *p*H 8.2	46	
(b) *n*-butyl alc./0.01 *M* sulfate, *p*H 2.3	39	
(c) *n*-butyl alc.:ethyl acetate 15:85/0.01 *M* sulfate, *p*H 2.2	64	1.5
(d) *n*-butyl alc.:ethyl acetate 25:75/0.01 *M* phosphate, *p*H 5	26	
6. Chromatographic adsorption on Magnesol[8] and developed with 0.05 *N* aqueous ammonia; fractions collected by fluorescence and evaporated to dryness		
7. Residue extracted with 5 ml. 0.5 *M* HCl; soln. was adjusted to *p*H 6, and the ppt. crystallized from 12 and 7 ml. hot water; washed twice with alcohol, ether and dried at 115° over P_2O_5 for 16 hr.; weight 13 mg.		

(1) Presented in part at the 128th Meeting of the American Chemical Society, Minneapolis, Minn., September 11–16, 1955, Abstracts p. 65C.

(2) H. P. Broquist and A. M. Albrecht, *Proc. Soc. Exptl. Biol. Med.*, **89**, 178 (1955).

(3) E. L. Patterson, H. P. Broquist, A. M. Albrecht, M. H. von Saltza and E. L. R. Stokstad, THIS JOURNAL, **77**, 3167 (1955).

(4) H. S. Forrest and H. K. Mitchell, *ibid.*, **77**, 4865 (1955).

(5) M. Viscontini, M. Schoeller, E. Loeser, P. Karrer and E. Hadorn, *Helv. Chim. Acta*, **38**, 397 (1955).

(6) M. Viscontini, E. Loeser, P. Karrer and E. Hadorn, *ibid.*, **38**, 1222 (1955).

(7) M. Viscontini, E. Loeser, P. Karrer and E. Hadorn, *ibid.*, **38**, 2034 (1955).

(8) Westvaco Chemical Division, South Charleston, West Virginia.

There was a considerable loss in activity, but the results were reproducible from batch to batch and gave a twofold to threefold purification of biological activity per unit of solids which was not found to be the case with other adsorbents. The charcoal eluates after counter-current solvent distribution were nearly free of salts and highly solvent soluble materials and were thick brown sirups.

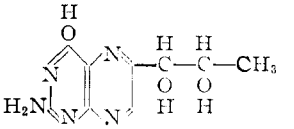
The material was then purified by partition chromatography. Celite 545[9] supporting the immobile aqueous phase (50 ml./100 g. of support) was packed into a 4 × 36 inch column. The sample was introduced in the aqueous phase on the top 3-inch segment of the column. The mobile phase was run through under 2 p.s.i. at a flow rate of one liter per hour. The distribution coefficient of biopterin between *n*-butyl alcohol and water was 0.25 over the range of *p*H 2 to 8. Advantage was taken of this by running two partition columns using the same solvent system at different *p*H values which further separated biopterin from the impurities in the sample. Examination of the columns with an ultraviolet light revealed the presence of several fluorescent bands, and the biological activity was closely correlated with one of these. This relationship was used in the later stages to select the desired fractions rather than waiting for the biological assay which took 5 to 7 days. Ultimately, it was shown that this band was a mixture of fluorescent materials but that pure biopterin itself exhibited a very strong blue fluorescence. For quantitative measurements the fluorescence of the fractions was read with a Pfaltz and Bauer fluorophotometer. The samples were diluted in 0.1 *M* borate, *p*H 9, for reading since the fluorescence of biopterin showed a change with *p*H and a maximum at *p*H 9.

The sample from the last partition column was adsorbed on a 3 × 12 inch column of Magnesol[8]–Celite 545[9] 1:2 and developed with 0.05 *N* aqueous ammonia. Biopterin was extracted into 0.5 *N* HCl from the residue obtained on evaporation of the developer. The solution was neutralized, and the pale yellow precipitate of biopterin crystallized from hot water as small spheres. From 5 to 15 mg. of biopterin were finally isolated from 1000 l. of fresh urine with a recovery of 3 to 5% based on bioassay. The biological activity was not destroyed by acid and alkali or during storage in solution. The activity was lost during treatment with oxidizing agents and upon exposure to strong sunlight.

Structure.—The ultraviolet absorption curves of biopterin were very similar to those of 2-amino-4-hydroxyalkylpteridines with maxima at 253 and 363 mμ in 0.1 *N* NaOH and at 322 and 247 mμ in 0.1 *N* HCl. The $E^{1.0\%}_{1.0cm}$ at 253 mμ in 0.1 *N* NaOH was 935. These data suggested biopterin was a 2-amino-4-hydroxypteridine bearing simple substituents. This was substantiated by the observation that the alkaline permanganate oxidation product of biopterin was 2-amino-4-hydroxy-6-carboxypteridine as identified by its ultraviolet absorption spectra and chromatographic behavior on paper.

The analytical data, which were not entirely satisfactory due to a scarcity of material, suggested an empirical formula of $C_9H_{11}N_5O_3$. By subtracting the empirical formula of the pteridine moiety an alkyl side chain of $C_3H_7O_2$ which was fully saturated was indicated. The side chain contained at least one optical center since biopterin was optically active. When biopterin was subjected to periodate oxidation[10] it consumed 1.7 and 4.5 oxidation equivalents at *p*H 2 and 8.5, respectively, per molecular weight 237. About two equivalents were taken up very rapidly in both titrations. The additional equivalents consumed at *p*H 8.5 were taken up quite slowly (Fig. 1). The pteridine fragments were optically inactive, and from their ultraviolet absorption spectra they were identified as 2-amino-4-hydroxy-6-formylpteridine from the *p*H 2 oxidation and 2-amino-4-hydroxy-6-carboxypteridine from the *p*H 8.5 oxidation (Fig. 2). When synthetic 2-amino-4-hydroxy-6-formylpteridine was titrated with periodate, about 2.5 oxidation equivalents per mole were consumed at *p*H 8.5 and essentially none at *p*H 2 (Fig. 1). The uptake at the higher *p*H was quite slow and similar in rate to that of the second two equivalents by biopterin at this *p*H, and the oxidation product was 2-amino-4-hydroxy-6-carboxypteridine. Synthetic 2-amino-4-hydroxypteridine and 2-amino-4-hydroxy-6-carboxypteridine consumed no periodate at either *p*H. Therefore, the true periodate titration was two oxidation equivalents per molecular weight of 237. An additional two equivalents were slowly consumed at the alkaline *p*H in the oxidation of 2-amino-4-hydroxy-6-formylpteridine to 2-amino-4-hydroxy-6-carboxypteridine. No formaldehyde, formic acid nor ammonia was found in the periodate oxidation reaction mixtures of biopterin.

Conclusions from these data about the structure of biopterin can be summarized as follows. Biopterin is a 2-amino-4-hydroxypteridine bearing only one alkyl group, and this is in the 6-position. The alkyl group has an empirical formula of $C_3H_7O_2$ and at least one optical center. It contains two adjacent hydroxyl groups. Neither alcohol group is primary and one is secondary and adjacent to the pyrazine ring. Biopterin is then 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine.



The presence of the terminal alkyl methyl group was confirmed by infrared analysis which showed characteristic C–CH_3 absorption at a frequency of 2975 cm.$^{-1}$. The configuration about the two asymmetric carbon atoms in the side chain cannot be assigned from these data. A comparison of the properties of biopterin with those of synthetic 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine,[11] indicated they are the same.

Experimental

Properties of Biopterin.—Pale yellow microcrystalline spheres from water, charring without melting at 250–280°; solubility in mg./ml. water: at 90°, 4; 20°, 0.7; 1 *N* NaOH

(9) Johns-Manville Corp., New York, N. Y.

(10) E. L. Jackson, "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

(11) E. L. Patterson, R. Milstrey and E. L. R. Stokstad, THIS JOURNAL, **78**, 5868 (1956).

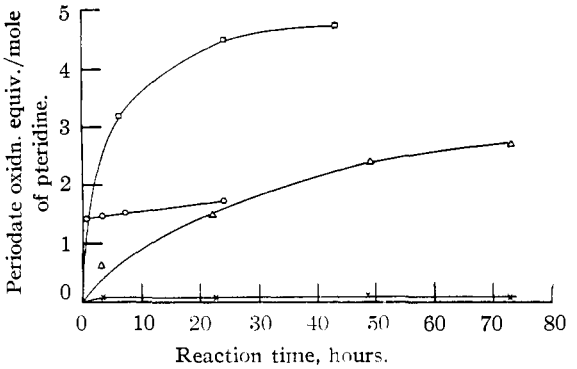


Fig. 1.—The periodate oxidation of biopterin and 2-amino-4-hydroxy-6-formylpteridine: biopterin, *p*H 2, ⊙; *p*H 8.5, ⊡; 2-amino-4-hydroxy-6-formylpteridine, *p*H 2, ×; *p*H 8.5, ◬.

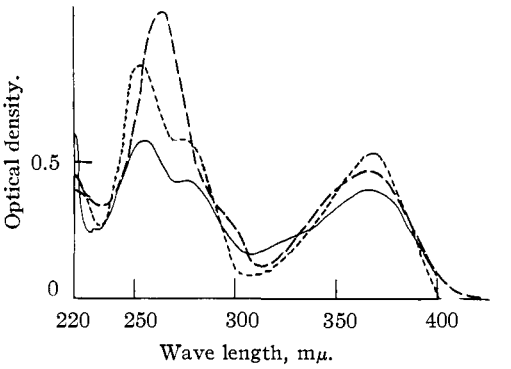


Fig. 2.—The ultraviolet absorption in 0.1 *N* NaOH of 2-amino-4-hydroxy-6-carboxypteridine – – –, 2-amino-4-hydroxy-6-formylpteridine ------; the *p*H 2 periodate oxidation product of biopterin ——.

and 1 *N* HCl, more than 25; alcohol, ether, acetone, benzene, less than 0.1; $[\alpha]^{24}D$ −50° (0.1 *N* HCl, *c* 0.4); biological activity: 0.05 mμg./ml. required for one-half maximum growth of *C. fasciculata* in 144 hours.

Anal. Calcd. for $C_9H_{11}N_5O_3$: C, 45.5; H, 4.64; N, 29.5. Found: C, 43.7; H, 4.86; N, 29.3; S, neg. qual. test.

Permanganate Oxidation.—Two mg. of biopterin was dissolved in 0.1 ml. of 1 *N* NaOH. Saturated potassium permanganate (*ca.* 0.03 ml.) was added until a permanent purple color persisted. The sample was heated on the steam-bath for 30 minutes. The excess permanganate was destroyed with ethanol (*ca.* 0.005 ml.), and the manganese dioxide was removed by centrifugation and washed twice with 0.10 ml. of water. The combined centrifugates were acidified with sulfuric acid to *ca. p*H 2. The buff colored precipitate was centrifuged down and washed once with water. A portion of the precipitate was dissolved in cyclohexylamine:water 50:50, and a volume of this solution containing *ca.* 5γ of pteridine was put on a 0.5″ wide No. 1 Whatman paper strip and developed by the descending technique with 5% cyclohexylamine in *n*-butyl alcohol saturated with water. The R_f of the one fluorescent spot was 0.15 compared to 0.14 and 0.07 for authentic samples of 2-amino-4-hydroxy-6-carboxy- and 7-carboxypteridines, respectively, run as controls.

Periodate Oxidation.—Three- to four-mg. samples were dissolved in either 0.05 *M* sodium bisulfate, *p*H 2, or 0.1 *M* sodium carbonate, *p*H 8.5, at concentrations of 0.85 mg./ml. Standard sodium metaperiodate (0.176 *N*) was added to a concentration of 25 to 30 microöxidation equivalents per mg. of pteridine. A precipitate formed within a minute after the periodate was added in all the samples. These and appropriate blanks were kept at room temperature and protected from sunlight. At intervals 0.5-ml. aliquots were withdrawn and quenched in 2.0 ml. of 0.1 *M* sodium bicarbonate containing 20.0 microöxidation equivalents of standard sodium arsenite (0.1000 *N*) and 10 mg. of potassium iodide. After 15 minutes the samples and blanks were titrated with standard potassium iodide–iodine (0.0900 *N*) to a starch end-point. The oxidation equivalents per mole were then calculated as

$$\text{ox. eq./mole} = (\text{eq. } I_2 \text{ sample} - \text{eq. } I_2 \text{ blank}) \times \frac{\text{g. of pteridine in sample}}{\text{mol. wt. of pteridine}}$$

The periodate oxidation of the other pteridines was carried out by the same procedure modifying the amount of periodate so that there was 50 to 100% excess.

Determination of Formic Acid, Formaldehyde and Ammonia in the Periodate Oxidation Mixtures.—After the final aliquot for periodate titration was removed the precipitated pteridines were recovered by centrifugation, and the excess periodate was destroyed with the calculated amount of sodium arsenite. The *p*H 2 reaction mixtures were adjusted to *p*H 9 with sodium carbonate. Each reaction mixture was then sublimed by the procedure of Grant.[12] Aliquots of the distillates were then used for the determination of formaldehyde using chromatropic acid[13] and for the determination of ammonia with Nessler reagent. The residues from the sublimations were acidified with sulfuric acid and again sublimed as before. Aliquots of the distillates were used for the determination of formic acid by the procedure of Grant.[14] Since it was difficult to obtain reproducible results with this procedure, the determinations were repeated on all the test samples.

Acknowledgment.—We wish to thank Dr. R. C. Gore, Stamford Laboratories, American Cyanamid Co., for the infrared analysis and Mr. L. Brancone and staff for the microanalytical determinations.

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(12) W. M. Grant, *Ind. Eng. Chem., Anal. Ed.*, **18**, 729 (1946).
(13) C. E. Bricker and W. A. Vail, *Anal. Chem.*, **22**, 720 (1950).
(14) W. M. Grant, *Ind. Eng. Chem., Anal. Ed.*, **19**, 206 (1947).