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# Neopterin: Isolation from Human Urine

By Atsushi Sakurai and Miki Goto

(*From the Department of Chemistry, Gakushuin University, Toshima-ku, Tokyo*)

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A variety of 2-amino-4-hydroxypteridines with a polyhydroxyalkyl side chain on the 6-position have been isolated. These are 2-amino-4-hydroxy-6-(L-*erythro*-1′, 2′-dihydroxypropyl) pteridine (biopterin) (*1*, *2*), (2-amino-4-hydroxy-6-pteridinyl) glycerol phosphate (*3*), 2-amino-4-hydroxy-6-(D-*erythro*-1′, 2′, 3′-trihydroxypropyl) pteridine (neopterin) (*4*, *5*), 2-amino-4-hydroxy-6-(D- or L-*threo*-1′, 2′, 3′-trihydroxypropyl pteridine (monapterin) (*6*, *7*), 2-amino-4-hydroxy-6-(D- or L-*erythro*-1′, 2′, 3′-trihydroxypropyl) pteridine (bufochrome) (*8*) and a 2-amino-4-hydroxy-6-1′, 2′, 3′, 4′, 5′-pentahydroxypentylpteridine derivative (*9*). Recently, the enzymatic formation of neopterin and its phosphate from guanosine triphosphate was demonstrated (*10*), and the biochemical significance of biopterin was clarified (*11—13*). We wish to report on the isolation from human urine in a small amount of a compound* whose properties are consistent with those of 2-amino-4-hydroxy-6-(D-*erythro*-1′, 2′, 3′-trihydroxypropyl) pteridine, *i.e.*, neopterin.

*Isolation of Pteridine I*—The human adult male urine was collected at the Defense Forces in Tokyo and at the Defense Academy in Yokosuka.

500 liters of the urine was acidified with hydrochloric acid (pH 3), passed through a Florisil column (10 kg. Florisil) to remove coloring materials. The pteridines in the effluent were absorbed on charcoal (1.2 kg.), which was then washed with water. The compounds were then eluted with 2% ammonium hydroxide-ethanol (3: 1) (8 liters), and the eluate was evaporated to dryness *in vacuo*. I was separated from biopterin by the use of a Dowex 1×8 column (9×15 cm., $HCO_3^-$, 200—400 mesh; developer 0.03 *M* formic acid/ammonia, the pH value being changed continuously from 9.0 to 7.5). The effluent containing I (25 liters) was subjected to the purification procedures using charcoal (15 g.) and a Dowex 1×8 column (5.5×20 cm., $HCO_3^-$), as described above. The effluent containing I (7.5 liters) was made alkaline with ammonia (pH 9—10) and placed on a Dowex 1×8 column (5.5×10 cm., $HCO_3^-$); the compound was adsorbed in a 4.5 cm. band. The column was washed with water (5 liters) and then with dilute formic acid (pH 2—3). The eluted solution was neutralized with ammonia and evaporated to dryness *in vacuo*. The repeated crystallization of the residue from water gave faint yellow needles (25 mg.). 50 mg. of the pure substance was obtained from 1,000 liters of urine in repetition of the procedures. $[\alpha]_D^{22°} = +38°$ (c=0.186, in 0.1 *N* hydrochloric acid).

Calcd. for $C_9H_{11}N_5O_4$: C: 42.69, H: 4.38, N: 27.67%
Found: C: 42.82, H: 4.58, N: 27.37%

The ultraviolet and infrared absorption spectra are given in Figs. 1 and 2.

*Permanganate Oxidation of I*—One mg. of I was dissolved in 0.1 *N* sodium hydroxide. Saturated potassium permanganate (*ca.* 0.02 ml.) was added until a permanent purple color persisted. After heating at 90°C for 30 minutes, excess permanganate was destroyed by addition of a little alcohol, and the mixture was filtered through Hyflo-Supercel. The absorption spectrum of the filtrate corresponded exactly to that of 2-amino-4-hydroxy-6-car-

* Abbreviated as I in this paper.

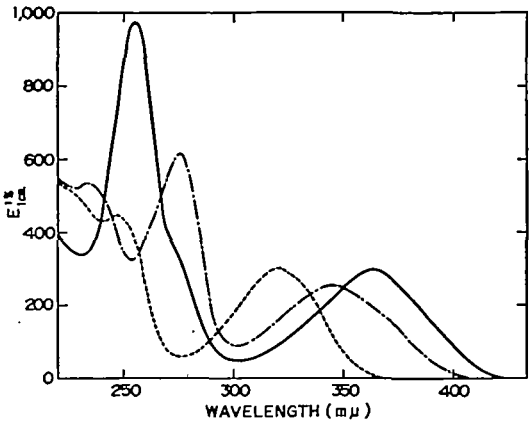


FIG. 1. Ultraviolet absorption spectrum of I.
——: In 0.1 *N* NaOH
–·–·–: In water
·····: In 0.1 *N* HCl

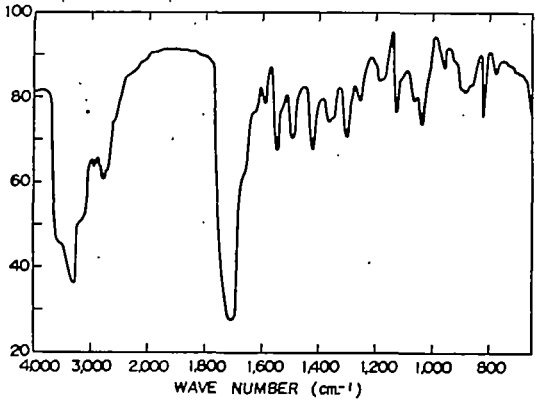


FIG. 2. Infrared absorption spectrum of I.

boxypteridine (*14*) and the identity of the oxidation product was confirmed by paper chomatographic comparison with an authentic sample of the acid.

*Periodate Oxidation of I* (*9*) (*15*) (*16*)—**Method I.** A solution of I (3.275 mg.) in water (10 ml., to effect solution a drop of ammonia was added, pH 7.8) was treated with 10 ml. potassium metaperiodate solution (6.0 mg./ml. water) at 0°C. At intervals 2 ml. aliquots were withdrawn and the undestroyed periodate was estimated by titration of the iodine liberated in boric acid by potassium iodide. With a molecular weight of 253, the consumption of periodate=2.9 mole per mole (Fig. 3). The

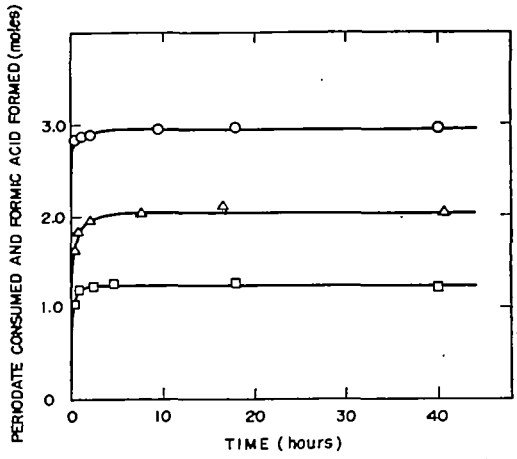


FIG. 3. The periodate consumption of I. pH 7.8, ○; 0.1 *N* HCl, △; and the formation of formic acid: □

resulting product was a mixture of 2-amino-4-hydroxy-6-carboxypteridine and 2-amino-4-hydroxy-6-formylpteridine as identified by chromatography and electrophoresis.

**Method II.** A solution of I (0.906 mg.) in 0.1 *N* hydrochloric acid (6 ml.) was treated with a solution of potassium metaperiodate (9.01 mg.) in water (6 ml.) at 0°C. At intervals 2 ml. aliquots were withdrawn and the periodate consumptions were determined; the pteridine consumed 2.0 mole per mole (Fig. 3). The resulting product was only 2-amino-4-hydroxy-6-formylpteridine as identified by chromatography and electrophoresis (Table I) (*18*).

Formaldehyde was determined using the procedure of McFadyen (*16*). From a standard curve obtained by oxidation of known amounts of D, L-serine, a sample of the material (352.4 μg.) was estimated to give 40 μg. formaldehyde: this represents 0.96 mole per mole.

Formic acid was determined as follows: a solution of I (1.261 mg.) in water (10 ml.) was treated with 10 ml. potassium metaperiodate solution (1.502 mg./ml. water) at 0°C. At intervals 2 ml. of aliquots were withdrawn, and 1 ml. ethylene glycol (0.1%) was added. After 15 minutes formic acid was titrated with 0.997 $\times 10^{-2}$ *N* sodium hydroxide (phenolphthalein was used as an indicator); 1.2 mole formic acid per mole was estimated (Fig. 3).

Acetaldehyde was determined by the procedure reported by Block and Bolling (*17*), using the color developed with *p*-hydroxydiphenyl in sulfuric acid. A solution containing *ca.* 500 μg. of the substance gave no color with this reagent.

*Microhydrogenation of I*—The compound (0.5 mg.) in sodium hydroxide solution (0.5 ml., 0.1 *N*) was hydrogenated over Adams' catalyst (1 mg.). Hydrogenation was continued for 32 hours. The catalyst was removed and the mixture was allowed to stand overnight in air. Chromatography of the resulting solution showed that the product is 2-amino-4-hydroxypteridine.

*Synthesis of 2-Amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxypropyl)pteridine*—2, 4, 5-Triamino-6-hydroxypyrimidine sulfate (1.6 g.) was condensed with D-ribose (1 g.) by the procedure described by Weygand *et al.* (*18*). The product was once crystallized from water, then purified by chromatographic method using a cellulose column (15×30 cm.; developer: *n*-butyl alcohol-ethyl alcohol-water=4:1:1). The blue-fluorescent bands were eluted and the eluate was evaporated to dryness *in vacuo*. The residue was purified by the use of a Dowex 1×8 column (5.5×30 cm., $HCO_3^-$, 200—400 mesh) as described before (*19*). The compoundwas obtained in faint yellow needles by crystallization from water (60 mg.). $[\alpha]_D^{22°} = +38°$ (c=0.250, in 0.1 *N* hydrochloric acid).

Calcd. for $C_9H_{11}N_5O_4$: C: 42.69, H: 4.38, N: 27.67%
Found: C: 42.51, H: 4.70, N: 27.71%

The sample was dried *in vacuo* at 110°C for 2 hours.

On treatment with potassium metaperiodate, this material consumed 2 mole per mole (pH 3), liberated a mole of formic acid and a mole of formaldehyde and gave 2-amino-4-hydroxy-6-formylpteridine, as identified by chromatography and electrophoresis. On oxidation with permanganate only 2-amino-4-hydroxy-6-carboxypteridine was obtained.

*Comparison of I with Synthetic 2-Amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxypropyl)pteridine*—The reactions which led to a direct comparison with synthetic 2-amino-4-hydroxy-6-(D-*erythro*-1', 2', 3'-trihydroxypropyl) pteridine are described above. The ultraviolet and infrared absorption spectra and optical rotation value of I were identical with those of the synthetic material (Figs. 1 and 2). The paper chromatographic comparison of I with

TABLE I

*Paper Chromatography and Electrophoresis of Pteridines*

| Substance | Solvents[1] | | | | | Electrophoresis[2] |
|---|---|---|---|---|---|---|
| | I | II | III | IV | V | |
| The pteridine from urine | 0.15 | 0.25 | 0.42 | 0.68 | 0.64 | 9 |
| $KMnO_4$ oxidation product | 0.18 | 0.15 | 0.40 | 0.47 | 0.44 | 52 |
| $NaIO_4$ oxidation product | 0.36 | 0.24 | 0.30 | 0.44 | 0.32 | 8 |
| 2-Amino-4-hydroxypteridine (P) | 0.36 | 0.34 | 0.49 | 0.53 | 0.46 | 7 |
| P-6-$(CHOH)_2CH_2OH$ from D-ribose | 0.15 | 0.25 | 0.42 | 0.68 | 0.64 | 9 |
| P-6-CHO | 0.36 | 0.24 | 0.30 | 0.44 | 0.32 | 8 |
| P-6-$CO_2H$ | 0.18 | 0.15 | 0.40 | 0.47 | 0.44 | 52 |

1) Solvents: I=*n*-butyl alcohol-acetic acid-water (4:1:1)
II=*iso*-propyl alcohol-1% ammonia (2:1)
III=*sec*-butyl alcohol-formic acid-water (8:2:5)
IV=3% ammonium chloride
V=4% sodium citrate

2) Distance (in mm.) to anode after paper electrophoresis at pH 4.65 (sodium acetate buffer) for 90 minutes at 36 V/cm.

the synthetic material is given in Table I.

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REFERENCES

(*1*) Patterson, E.L., Saltza, M.H.v., and Stokstad, E.L.R., *J. Am. Chem. Soc.*, 78, 5868, 5871 (1956)
(*2*) Forrest, H.S., and Mitchell, H.K., *J. Am. Chem. Soc.*, 77, 4865 (1955)
(*3*) Goto, M., and Forrest, H.S., *Biochem. Biophys. Research Communs.*, 6, 180 (1961)
(*4*) Rembold, H., and Buschmann, L., *Liebigs Ann. Chem.*, 662, 72 (1963)
(*5*) Rembold, H., and Buschmann, L., *Chem. Ber.*, 96, 1406 (1963)
(*6*) Viscontini, M., Pouteau-Thouvenot, M., Bühler-Moor, R., and Schroeder, M., *Helv. Chim. Acta*, 47, 1948 (1964)
(*7*) Schmidt, G.H., and Viscontini, M., *Helv. Chim. Acta*, 49, 344 (1966)
(*8*) Goto, T., *Jap. Jour. Zool.*, 14, 91 (1963)
(*9*) Goto, M., Kobayashi, K., Sato, H., and Korte, F., *Liebigs Ann. Chem.*, 689, 221 (1965)
(*10*) Guroff, G., and Strenkoski, C.A., *J. Biol. Chem.*, 241, 2220 (1966)
(*11*) Kaufman, S., *J. Biol. Chem.*, 237, 2712 (1962)
(*12*) Kaufman, S., *J. Biol. Chem.*, 239, 332 (1964)
(*13*) Brenneman, A.R., and Kaufman, S., *Biochem. Biophys. Research Communs.*, 17, 177 (1964)
(*14*) Weygand, F., and Schäfer, G., *Chem. Ber.*, 85, 307 (1952)
(*15*) Willand, H.H., and Freathouse, L.H., *J. Am. Chem. Soc.*, 60, 2869 (1938)
(*16*) McFadyen, D.A., *J. Biol. Chem.*, 158, 107 (1945)
(*17*) Block, R.J., and Bolling, D., *J. Biol. Chem.*, 130, 365 (1939)
(*18*) Weygand, F., Wacker, F., and Schmied-Kowarzik, V., *Chem. Ber.*, 82, 25 (1949)
(*19*) Goto, M., Ohno, M., Forrest, H.S., and Lagowski, J.M., *Arch. Biochem. Biophys.*, 110, 444 (1965)