The Structures of the Reoxidation Products of 7,8-Dihydroneopterin

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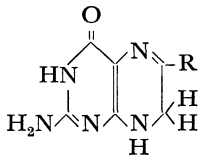
On the reduction of D-*erythro*-neopterin with sodium dithionite, after which it was let stand in neutral pH, three colorless compounds, I, II, and III, and two yellow compounds, I and II, were obtained. On the reduction of D-*erythro*-neopterin with Fe dust in 25% acetic acid, after which it was let stand at a neutral pH, two yellow compounds, II and III, were obtained. From UV and NMR spectral data as well as from the chemical reactions, the structures for these compounds were identified, Colorless Compound I is 2-amino-4-hydroxy-6-(1′,3′-dihydroxypropyl)pteridine; Colorless Compound II, 2-amino-4-hydroxy-6-(D-*erythro*-1′,2′,3′-trihydroxypropyl)-pteridine; Colorless Compound III, 2-amino-4-hydroxy-6-(D-*threo*-1′,2′,3′-trihydroxypropyl)pteridine; Yellow Compound I, 2-amino-4-hydroxy-6-(1′-oxo-3′-sulfopropyl)-7,8-dihydropteridine; Yellow Compound II, 2-amino-4-hydroxy-6-(1′-oxo-3′-hydroxypropyl)-7,8-dihydropteridine, and Yellow Compound III, 2-amino-4-hydroxy-6-(1′-oxo-2′,3′-dihydroxypropyl)-7,8-dihydropteridine.

The biological function of a phosphate of 2-amino-4-hydroxy-6-(D-*erythro*-1′,2′,3′-trihydroxypropyl)-7,8-dihydropteridine (7,8-dihydro-D-*erythro*-neopterin) was proved to serve as a key compound for the biosynthesis of folic acid and the biopterin group of co-factors.[1-3] Recently, the enzymic transformation of 7,8-dihydro-D-*erythro*-neopterin to D-*threo*-neopterin has been demonstrated.[4] Kaufman has reported that 7,8-dihydrobiopterin is obtained from biopterin by treatment with Fe dust in an acidic solution, and the synthetic 7,8-dihydrobiopterin has been demonstrated by using purified rat liver extract to be the phenylalanine-hydroxylation co-factor.[5] Fukushima and Akino have reported that neopterin and biopterin give 7,8-dihydroneopterin and 7,8-dihydrobiopterin respectively by treatment with sodium dithionite.[6]

For the study of the biosynthesis of pteridines, we prepared 7,8-dihydro-D-*erythro*-neopterin by treating it with sodium dithionite or Fe dust in 25% acetic acid, as has been described by the above authors. On the reduction of D-*erythro*-neopterin, followed by its purification on a Sephadex column, we did not get pure 7,8-dihydro-D-*erythro*-neopterin, but some unknown compounds were always produced together with 7,8-dihydro-D-*erythro*-neopterin.[2] The yields of these unknown compounds were increased when the reduced solutions were kept standing in a neutral pH for a longer time. The present paper will report on the structures of these compounds.

On the reduction of D-*erythro*-neopterin by treatment with sodium dithionite, after which it was let stand in a nuetral pH, three colorless and two yellow substances were obtained, *i.e.*, colorless Compounds I, II, and III, and Yellow Compounds I and II. On the reduction of D-*erythro*-neopterin by treatment with Fe dust in 25% acetic acid, after which it was let stand in a neutral pH, the products were two yellow substances, Yellow Compounds II and III.

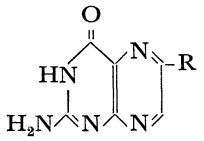
On the basis of UV and NMR spectral data as well as the chemical reactions, the structures for these compounds were deduced to be as follows:



R; $-CO-CH_2-CH_2SO_3H$
Yellow Compound I

R; $-CO-CH_2-CH_2OH$
Yellow Compound II

R; $-CO-\underset{OH}{CH}-CH_2OH$
Yellow Compound III



R; $-CHOH-CH_2-CH_2OH$
Colorless Compound I

R; $-\underset{OH}{CH}-\underset{OH}{CH}-CH_2OH$
Colorless Compound II

R; $-\underset{OH}{CH}-\underset{OH}{CH}-CH_2OH$
Colorless Compound III

Experimental

Reduction of D-erythro-*Neopterin by Treatment with Sodium Dithionite and the Isolation of the Products.* D-*erythro*-Neopterin (140 mg) was suspended in 2 ml of water, and then 2M sodium hydroxide was added until a clear solution was obtained. After the addition of sodium dithionite (400 mg), the solution was left at 90—95°C for 15 min, then, after the addition of 6M hydrochloric acid (1 ml), the solution was heated at 90—95°C. The solution was neutralized with ammonia, and the excess sodium dithionite was removed by means of a Sephadex G-25 (fine) column (3×25 cm, developer; water) and the eluate was concentrated to 200 ml by means of a rotary evaporator below 40°C. The solution was left standing for 48 hr at room temperature in the dark. The solution was acidified with acetic acid, and then it was placed on a 3×25 cm column of Florisil; the column was subsequently washed with 0.001M acetic acid, and 20% aqueous acetone successively. Colorless matters were eluted by the first solvent, and yellow matters by the second solvent. The colorless substances were separated into two fluoresent bands by means of a cellulose column (4×25 cm, developer; 2-propanol—1% ammonia: 2 : 1). The eluate of the

1) K. Sugiura and M. Goto, *Biochem. Biophys. Res. Commun.*, **28**, 687 (1967).
2) K. Sugiura and M. Goto, *J. Biochem.* (Tokyo), **64**, 657 (1968).
3) J. B. Mathis and G. M. Brown, *J. Biol. Chem.*, **245**, 3015 (1970).
4) G. M. Brown, "Chemistry and Biology of Pteridines," ed. by K. Iwai, M. Akino, M. Goto and Y. Iwanami, International Academic Printing Co., Tokyo (1970), p. 243.
5) S. Kaufman, *Proc. Natl. Acad. Sci. U. S.*, **50**, 1085 (1963).
6) T. Fukushima and M. Akino, *Arch. Biochem. Biophys.*, **128**, 1 (1968).

first was concentrated and the residue was recrystallized from water to give colorless needles; 12 mg (8.6%); this was Colorless Compound I. The eluate of the second was purified by chromatographic method using six sheets of paper (40×40 cm, Toyo Roshi Co. Ltd., No. 54, developer; 2-propanol—1% ammonia: 2 : 1). The fluorescent bands were then cut out, and the materials were eluted from it with water. The eluates were subsequently evaporated to dryness. There was a trace of the (R_f, 0.21) fraction; this was Colorless Compound III. The residue of the (R_f, 0.31) fraction was recrystallized from water to give colorless needles; 29 mg (21%); this was Colorless Compound II. The yellow substances were separated into two fluorescent bands by means of a cellulose column (3×20 cm, developer; 0.05% ammonia). The eluate of the first was concentrated, the residue was purified by means of a Sephadex G-25 (fine) column (3×25 cm, developer; water), the eluate was concentrated to dryness *in vacuo*, and the residue was recrystallized from 50% ethanol to give yellow needles; 48 mg (25%); this was Yellow Compound I. The eluate of the second such substance was concentrated, the residue was purified by means of a DEAE-cellulose column (3×25 cm, developer; water), the eluate was concentrated to dryness *in vacuo*, and the residue was recrystallized from water to give yellow needles; 25 mg (18%); this was Yellow Compound II. The samples were dried at 70—80°C/0.01 mmHg for 12 hr over P_2O_5 for analysis.

Colorless Compound I:

Found: C, 44.02; H, 4.50; N, 29.55%. Calcd for $C_9H_{11}O_3N_5 \cdot 0.5H_2O$: C, 43.90; H, 4.88; N, 28.46%.

UV: $\lambda_{max}^{0.1M\ NaOH}$ mμ (ε), 255 (23.1×10³), 368 (7.77×10³); $\lambda_{min}^{0.1M\ NaOH}$ mμ (ε), 232 (9.28×10³), 302 (1.4×10³); $\lambda_{max}^{H_2O}$ mμ (ε), 236 (13.2×10³), 275 (14.8×10³), 348 (6.8×10³); $\lambda_{min}^{H_2O}$ mμ (ε), 254 (8.0×10³), 296 (2.36×10³); $\lambda_{max}^{0.1M\ HCl}$ mμ (ε), 246 (1.15×10³), 324 (8.47×10³); $\lambda_{min}^{0.1M\ HCl}$ mμ (ε), 275 (2.1×10³).

Yellow Compound I:

Found: C, 32.08; H, 3.95; N, 20.45; S, 8.94%. Calcd for $C_9H_{11}O_5N_5S \cdot 2H_2O$: C, 32.05; H, 4.48; N, 20.77; S, 9.50%.

UV: $\lambda_{max}^{0.1M\ NaOH}$ mμ (ε), 268 (1.74×10³), 438 (14.2×10³); $\lambda_{min}^{0.1M\ NaOH}$ mμ (ε), 234 (4.6×10³), 340 (1.8×10³); $\lambda_{max}^{H_2O}$ mμ (ε), 267(17.6×10³), 418 (11.3×10³); $\lambda_{min}^{H_2O}$ mμ (ε), 238 (7.0×10³), 325 (1.2×10³); $\lambda_{max}^{0.1M\ HCl}$ mμ (ε), 248 (11.0×10³), 406 (9.2×10³), $\lambda_{min}^{0.1M\ HCl}$ mμ (ε), 256 (6.4×10³), 316 (1.2×10³).

NMR (CF_3COOD) : δ3.56 (bs, 4H), 4.61 (s, 2H).

Yellow Compound II:

Found: C, 44.21; H, 4.61; N, 28.56%. Calcd for $C_9H_{11}O_3N_5 \cdot 0.5H_2O$: C, 43.90; H, 4.88; N, 28.46%.

UV: $\lambda_{max}^{0.1M\ NaOH}$ mμ (ε), 269 (17.3×10³), 440 (12.3×10³), $\lambda_{min}^{0.1M\ NaOH}$ mμ (ε), 237 (5.0×10³), 340 (1.3×10³); $\lambda_{max}^{H_2O}$ mμ (ε), 267 (18.1×10³), 417 (10.4×10³); $\lambda_{min}^{H_2O}$ mμ (ε), 240 (6.9×10³), 340 (1.5×10³); $\lambda_{max}^{0.1M\ HCl}$ mμ (ε), 282 (11.8×10³), 408 (8.0×10³); $\lambda_{min}^{0.1M\ HCl}$ mμ (ε), 254 (7.1×10³), 340 (1.7×10³).

NMR (CF_3COOD): δ 3.33 (t, J=5.8 Hz, 2H), 4.14 (t, J=5.8 Hz, 2H), 4.56 (s, 2H).

Bromine Oxidation of Yellow Compound I. A suspension of Yellow Compound I (40 mg) in 2M hydrochloric acid (20 ml) was treated with bromine (*ca.* 1 g) for a few minutes at room temperature. The mixture was then concentrated to 10 ml by means of a rotary evaporator and the solution was treated with charcoal (1 g). The adsorbed fluorescent compound was eluted with a mixture of 3% aqueous ammonia and ethanol (3 : 1). The eluate was purified by the chromatographic method, using a cellulose column (3×25 cm, developer; 50% ethanol). The eluate was evaporated to dryness, and the residue was crystallized from 50% ethanol (yellow powder, 36 mg, 74%; this was Yellow Compound I·Br_2). The sample was dried at 70—80°C/0.01 mmHg for 3 hr over P_2O_5 for analysis.

Found: C, 29.26; H, 3.11; N, 19.86; S, 8.46%. Calcd for $C_9H_9O_5N_5S \cdot 3.5H_2O$: C, 29.83; H, 4.41; N, 19.33; S, 8.83%.

UV: $\lambda_{max}^{0.1M\ NaOH}$ mμ (ε), 277 (16.2×10³), 316 (7.4×10³), 372 (11.5×10³); $\lambda_{min}^{0.1M\ NaOH}$ mμ (ε), 240 (4.58×10³), 308 (7.3×10³), 332 (6.8×10³); $\lambda_{max}^{0.1M\ HCl}$ mμ (ε), 234 (9.72×10³), 270 (11.7×10³), 318 (10.6×10³); $\lambda_{min}^{0.1M\ HCl}$ mμ (ε), 230 (9.70×10³), 250 (8.8×10³), 291 (7.12×10³).

NMR (CF_3COOD): δ 4.00 (m, 4H), 9.16 (s, 1H).

2,4-Dinitrophenylhydrazone of Yellow Compound II. An aqueous solution of Yellow Compound II reacted immediately with 2,4-dinitrophenylhydrazine in 2M hydrochloric acid to give a dark-red precipitate. This was collected, washed with water, and dried at 70—80°C/0.01 mmHg for 12 hr over P_2O_5 for analysis.

Found: C, 42.99; H, 3.48; N, 29.64%. Calcd for $C_{15}H_{15}O_6N_9$: C, 43.17; H, 3.60; N, 30.22%.

The Reduction of D-erythro-*Neopterin by Treatment with Fe Dust in 25% Acetic Acid and the Isolation of the Products.* To a solution of D-*erythro*-neopterin (2 g) in 200 ml of 25% acetic acid, Fe dust was added. The mixture was shaken for 30 min at 50—60°C. The Fe dust was then removed, and the solution was neutralized with ammonia and left standing for 12 hr at room temperature in the dark. The solution was subsequently acidified with acetic acid, and then the solution was placed on a 3×25 cm column of Florisil; the yellow substances were adsorbed at the top of the column. The column was washed well with 0.01% acetic acid, and the yellow substances were eluted with 20% aqueous acetone. The eluate was concentrated. The yellow substances were purified by means of a chromatographic method, using a cellulose column (7.5×35 cm, developer; water) and a DEAE-cellulose column (2.5×25 cm, developer; water). For the exclusion of Yellow Compound II, the eluate was placed on a 7.5×25.0 cm column of cellulose and the column was eluted with *n*-butanol–ethanol–water (2 : 1 : 1). The eluate of the second substance was concentrated to dryness, and the residue was recrystallized from water to give a yellow powder; 453 mg (23%); this was Yellow Compound III. The sample was dried at 70—80°C/0.01 mmHg for 2 hr over P_2O_5 for analysis.

Found: C, 41.23; H, 4.54; N, 27.06%. Calcd for $C_9H_{11}O_4N_5.0 \cdot 5H_2O$: C, 41.22; H, 4.96; N, 26.71%.

UV: $\lambda_{max}^{0.1M\ NaOH}$ mμ (ε), 270 (17.5×10³), 446 (12.8×10³); $\lambda_{min}^{0.1M\ NaOH}$ mμ (ε), 240 (6.2×10³), 350 (1.4×10³); $\lambda_{max}^{H_2O}$ mμ (ε), 268 (17.9×10³), 424 (10.3×10³); $\lambda_{min}^{H_2O}$ mμ (ε), 236 (6.4×10³), 340 (1.4×10³); $\lambda_{max}^{0.1M\ HCl}$ mμ (ε), 273 (12.8×10³), 416 (8.4×10³); $\lambda_{min}^{0.1M\ HCl}$ mμ (ε), 254 (8.2×10³), 340 (1.6×10³).

NMR: (CF_3COOD): δ 4.55 (bs, 2H), 4.73 (s, 2H), 5.70 (bs, 1H). ($(CD_3)_2SO$-D_2O): δ 3.68 (d, J=5.2 Hz, 2H), 4.15 (s, 2H), 5.00 (t, J=5.2 Hz, 1H).

The R_f values of the pteridines are given in Table 1.

Results and Discussion

Yellow Compound II gives ultraviolet spectra similar to those of isosepiapterin.[7] The analytical data indicate that the empirical formula for the compound is

7) H. S. Forrest, C. Van Baalen and J. Myers, *Arch. Biochem. Biophys.*, **83**, 508 (1959).

$C_9H_{11}O_5N_5$. The yellow compound contains a keto group on the side chain, since the compound forms a 2,4-dinitrophenylhydrazone. The NMR spectrum of the compound in CF_3COOD has two triplets, at 3.33 ppm and 4.14 ppm, and a singlet at 4.56 ppm, the proton ratio of the signals being 2 : 2 : 2. The two triplet signals (4.56 ppm and 3.33 ppm) showed that this compound has a $-CH_2-CH_2-$group. The signal at 4.56 ppm was assigned to the methylene protons at the 7 position. The triplet signal at 3.33 ppm was assigned to the methylene protons adjacent to a keto group, and the triplet signal at 4.14 ppm, to the methylene protons adjacent to a hydroxyl group; this couples with the signal (3.33 ppm) of the neighboring methylene protons (J=5.8 Hz), and *vice versa* (J= 5.8 Hz). Therefore, the structure of Yellow Compound II was determined to be 2-amino-4-hydroxy-6-(1′-oxo-3′-hydroxypropyl)-7,8-dihydropteridine.

When Yellow Compound II was reduced with sodium borohydride after treatment with bromine in an acidic solution, Colorless Compound I was obtained in an almost quantitative yield. Thus, Colorless Compound I is 2-amino-4-hydroxy-6-(1′,3′-dihydroxypropyl)pteridine.

Colorless Compound II was indistinguishable from authentic *erythro*-neopterin,[8] and Colorless Compound III was identical with authentic *threo*-neopterin,[8] as determined by a study of the ultraviolet spectra and R_f values. It remains to be decided which configurations the products have, although it is most probable that Colorless Compound II and III are D-isomers.

Yellow Compound I gives ultraviolet spectra similar to those of isosepiapterin, and it has an acidic group. The permanganate oxidation to 2-amino-4-hydroxy-6-carboxypteridine shows that the compound is a 6-substituted pteridine. On treatment with bromine in acidic solution, Yellow Compound I yielded a new compound, Yellow Compound I·Br_2, which gives ultraviolet spectra similar to those of 2-amino-4-hydroxy-6-acetyl-7-methylpteridine.[9] Accordingly, Yellow Compound I has the keto group on carbon 1′ of the side chain, and its ring system has the 7,8-dihydro structure. Yellow Compound I contains a $-CH_2SO_3H$ group, but does not contain a $-CH_2OSO_3H$ group, since the compound remained unchanged by the acidic hydrolysis. The NMR spectrum of Yellow Compound I in CF_3COOD has a broad singlet at 3.56 ppm and a singlet at 4.61 ppm; the proton ratio of the signals is 4 : 2. The NMR spectrum of Yellow Compound I·Br_2 in CF_3COOD has a broad singlet at 3.5—4.0 ppm and a singlet at 9.16 ppm; the proton ratio of the signals is 4 : 1. The signal of Yellow Compound I at 4.16 ppm was assigned to the methylene protons at the 7 position, while in the spectrum of Yellow Compound I·Br_2 its signal (4.16 ppm) was transferred to 9.16 ppm as a vinyl proton. The signal of Yellow Compound I at 3.56 ppm and that of Yellow Compound I·Br_2 at 3.5—4.0 ppm were assigned to the protons of a $-CH_2-CH_2-$ group on the side chain, because their spectra could be explained by the A_2B_2 spin system and the slight chemical-shift difference between the methylene adjacent to a keto group and the methylene adjacent to a sulfonic acid group. These findings suggest that Yellow Compound I is 2-amino-4-hydroxy-6-(1′-oxo-3′-sulfopropyl)-7,8-dihydropteridine.

Table 1. Paper chromatography and electrophoresis of pteridines

Compounds	R_f in Solvents[a]				
	A	B	C	D	E
2-Amino-4-hydroxy-pteridine (pterin)	0.52	0.36	0.36	0.45	0
Pterin-6-carboxylic acid	0.40	0.16	0.14	0.51	−28
D-*erythro*-Neopterin	0.55	0.31	0.15	0.64	0
Colorless Compound II					
D-*threo*-Neopterin	0.52	0.28	0.15	0.63	0
Colorless Compound III					
Colorless Compound I	0.60	0.40	0.30	0.61	0
Yellow Compound I	0.46	0.26	0.03	0.48	−28
Yellow Compound II	0.43	0.31	0.26	0.28	0
Yellow Compound III	0.41	0.23	0.16	0.31	
Yellow Compound I $-Br_2$	0.47	0.28	0.03	0.62	−32
Isosepiapterin	0.52	0.49	0.58	0.24	0

a) Solvents: A, 2-Propanol–2% ammonium acetate (1:1); B, 2-Propanol–1% ammonia (2:1); C, *n*-Butanol–acetic acid–water (4:1:1); D, 3% Ammonium chloride; E, Distance to anode in electrophoresis; buffer: 0.05 M acetic acid–sodium acetate (pH 4.25); 30 min and 500 V/25 cm.

Yellow Compound III gives ultraviolet spectra similar to those of sepiapterin.[10] The analytical data indicate that the empirical formula for the compound is $C_9H_{11}O_4N_5$. The reduction of the compound with sodium borohydride, followed by oxidation with manganese oxide to *erythro*-neopterin and *threo*-neopterin, shows that Yellow Compound III has a side chain of a $-CO-CHOH-CH_2OH$ or $-CHOH-CO-CH_2OH$ group. The NMR spectrum of Yellow Compound III in $(CD_3)_2SO$–D_2O has a doublet at 3.68 ppm, a singlet at 4.15 ppm, and a triplet at 5.00 ppm, the proton ratio of the signals being 2 : 2 : 1. The signals (a doublet signal at 3.18 ppm (J=5.2 Hz) and a triplet signal at 5.00 ppm (J=5.2 Hz)) show that Yellow Compound III has a $=CH-CH_2-$ group. The signal at 3.68 ppm was assigned to the methylene protons adjacent to a hydroxyl group; the signal at 5.00 ppm, to the methylene group adjacent to a hydroxyl group and a keto group, and the signal at 4.15 ppm, to the methylene protons at the 7 position. Accordingly, the structure of Yellow Compound III is determined to be 2-amino-4-hydroxy-6-(1′-oxo-2′,3′-dihydroxypropyl)-7,8-dihydropteridine.

It may be concluded that these compounds are not produced by the process of the reduction of D-*erythro*-neopterin, but by the process of the reoxidation of 7,8-dihydro-D-*erythro*-neopterin. When the reaction mixture of sepiapterin and the sepiapterin reductase was

8) K. Sugiura, H. Yamashita, and M. Goto, This Bulletin, **45**, 3564 (1972).

9) K. Sugiura and M. Goto, *ibid.*, **42**, 2662 (1969).

10) S. Nawa, *ibid.*, **33**, 1555 (1960).

treated with diluted acid after an almost complete reduction of the pteridine, biopterin was produced together with isosepiapterin.[11] This fact is not inconsistent with the above observation. This type of reaction including epimerization, oxidation, and reduction, is similar to certain reactions of carbohydrates. The relation of the C=N bond at 5 and 6 positions to the polyhydroxyalkyl group at the 6 position of 7,8-dihydroneopterin is similar to that in a keto and polyhydroxyalkyl group in carbohydrates.

11) S. Katoh and M. Akino, *Experientia*, **22**, 793 (1966).