Pterins in Human Urine*

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SUMMARY

Qualitative and quantitative analyses for pterins (derivatives of 2-amino-4-hydroxypteridine) in human urine were carried out by an improved procedure which involved the use of two new weak ion exchangers, ECTEOLA-Sephadex and phospho-Sephadex.

The predominant pterins found in urine were biopterin \(6(\text{o-erythro-trihydroxypropyl})\)pterin and \(\text{D-erythronopterin} \: 6(\text{o-erythro-trihydroxypropyl})\)pterin, and daily individual outputs of these pterins were 980 \(\mu\)g and 380 \(\mu\)g, respectively. Crithidia fasciculata assays of urine revealed that 1.5 mg of biopterin equivalents were excreted daily. Oral administration of a large amount of folic acid did not affect the amount of these pterins. Furthermore, evidence was obtained which indicated that these pterins occur as the dihydro form. Additional pterins found in urine are \(\text{l-threonopterin} \: 6(\text{l-threotrihydroxypropyl})\)pterin, \(\text{sepiapterin} \: 6(\text{lacticyl-7,8-dihydropterin})\), pterin, and isoxanthopterin (7-hydroxypterin).

The unconjugated pterins \(\text{xanthopterin} \: (1)\), biopterin (2), isoxanthopterin (3), and neopterin (4) have been found in human urine. Only relatively recently, the role of pteridines in higher animals was established by Kaufman (5). He demonstrated that reduced biopterin is the natural cofactor in the hydroxylation of phenylalanine in rats. Hydroxylation of other aromatic amino acids, tyrosine and tryptophan, also require reduced pterins as cofactor (6-8).

Although the presence and the cofactor role of pteridines in higher animals have been described to some extent, the origin of pterins, whether they are synthesized or arise from diet, is not yet clear.

The published data on pterins in human urine (1-4) have been qualitative, so quantitative analysis of urinary pterins may give some information on the origin of pterins and on additional possible metabolic roles of pteridines in man.

In this paper, an improved method of quantitation of urinary pterins is described. By the use of the method, the effect of dietary high folate on urinary pterins was examined. Pterins which had not been detected previously in human urine have been isolated and identified. Applicability of the method and the possible origin of pterins in human urine are discussed.

MATERIALS AND METHODS

Chemicals—Biopterin was isolated from bullfrog skin (9). \(\text{D-Erythroopterin} \: \text{threonopterins}, \text{and neopterin were synthesized from 2,3,4}\)-triamino-6-hydroxypyrimidine sulfate and the appropriate sugars and were subsequently purified (10). Dihydrobiopterin and \(\text{D-erythro}\)-dihydroopterin were prepared and purified by the procedure of Fukushima and Akino (9). Sepiapterin was extracted from the \(\text{sepia} \: \text{mutant of Drosophila melanogaster} \) and purified (9, 11).

Isosepiapterin was made from dihydrobiopterin by a modified method of Katoh and Akino (12). One micromole of dihydrobiopterin was dissolved in 5 ml of distilled water and the solution was applied to a phospho-Sephadex column (11', 1.5 \(\times\) 5 cm). After the development with a few milliliters of distilled water, the column was allowed to stand overnight in the dark at room temperature. The development of the column with water was resumed the next day, and two fluorescent peak fractions, yellow and blue, were obtained. The yellow fluorescent compound moved as a single band or spot when subjected to column or paper chromatography. \(R_f\) values of the compound were similar to those of isosepiapterin in five solvent systems. The yield of isosepiapterin was about 20%. Almost all of the remaining dihydrobiopterin was recovered as biopterin.

Pterin, 6-hydroxymethylpterin, 6-carboxypterin, xanthopterin, and isoxanthopterin were obtained from commercial sources. Lumazine was kindly supplied by Dr. C. M. Baugh. Sephadex G-25 and G-10 were obtained from Pharmacia. ECTEOLA-cellulose was prepared from Sigma and prepared for use by treating with NaCl-HCl solutions (9). Other chemicals were obtained from commercial sources.

ECTEOLA-Sephadex and Phospho-Sephadex—ECTEOLA-Sephadex and phospho-Sephadex (P-Sephadex) were prepared from Sephadex G-25 (line) by the method of Peterson and Sober (13) for the corresponding celluloses. Capacities of ECTEOLA-Sephadex and phospho-Sephadex are 0.46 meq per g (dry weight)

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Another advantage is that P-Sephadex is superior to phospho-
are similar to those obtained with corresponding cellulose ex-
changers (14, 15). The merits of these Sephadexes over the cor-
responding cellulose types are translucency and homogeneity.

Most of these pteridines can be separated by washing with 0.1 N HCl and water.

ECTEOLA-Sephadex thus treated are called "pH 8, 7, or 6", respectively. P-Sephadex in the Hf form was prepared for the separation of pteridines, the absorptivity was reduced with HCl as was the OH form of ECTEOLA-Sephadex. Moistened ECTEOLA-Sephadex (OH) was suspended in 5 volumes of 5 mM NaCl solution. The pH of the suspension was approximately 10.5. To ensure the elution of xanthopterin and isoxanthopterin with the Hf form, the use of pH 5 ECTEOLA, Sephadex is recommended. The results of unknown pterins were compared with those of authentic pterins.

Quantitative Measurement of Pteridines—The amounts of pterins were determined by use of the extinction coefficient for each pterin (9, 17, 18). A fluorescence method was applied when the amount of pterin was small. Pterins were determined by use of the extinction coefficient for each pterin (9, 17, 18). A fluorescence method was applied when the amount of pterin was small. A Turner model 110 fluorescence apparatus was used in order to determine the optical configuration of isolated pterins. The bioassay was performed according to the procedure of Dewey and Kidder (16). The results of unknown pterins were compared with those of authentic pterins.

Column chromatography of pteridines and pterines on ECTEOLA-Sephadex and P-Sephadex columns

A mixture of two to five compounds was applied to a column (1.1 x 20 cm) and developed with water. The fractions (2 or 5 ml) were collected at a rate of 0.5 ml per min and analyzed by ultraviolet absorption or fluorescence.

Table I

Table I

| Compounds            | pH 7 ECTEOLA-Sephadex | P-Sephadex |
|----------------------|-----------------------|------------|
| Bioppterin           | 100                    | 100        |
| Dihydrobioppterin    | 35                     | 35         |
| Erythromeopteron     | 101                    | 95         |
| Thromoneopteron      | 100                    | 97         |
| Erythrodihydroneopteron | 35                  | 35         |
| 7-Neopterin          | 86                     | 111        |
| Sepiapterin          | 41                     | 21         |
| Isosepiapterin       | 45                     | 96         |
| 6-Hydroxymethylpterin | 111                  | 117        |
| 6-Carboxypterin      | e                      | 4.9        |
| Xanthopterin         | e                      | 48         |
| Isoxanthopterin      | e                      | 9.3        |
| Pterin               | 120                    | 113        |
| Lumazine             | 112                    | 7.2        |
| Riboflavin           | 30                     | 8.7        |
| Adenine              | 45                     | e          |
| Adenosine            | 33                     | e          |
| Guanine              | 45                     | e          |
| Guanosine            | 39                     | 72         |
| Xanthanine           | 243                    | 13         |

* For For the separation of dihydropterins from their oxidized forms, the use of pH 8 ECTEOLA-Sephadex is recommended. Also, if the elution of xanthopterin and iso xanthopterin with water is required, the use of pH 6 ECTEOLA-Sephadex is recommended.

Table II

| Compound                      | Rf Values |
|-------------------------------|-----------|
|                               | A         | B         | C         | D         | E         |
| Bioppterin                    | 0.60      | 0.28      | 0.48      | 0.42      | 0.36      |
| Dihydrobioppterin             | 0.41      | 0.15      | 0.36      | 0.46      | 0.33      |
| Erythromeopteron              | 0.38      | 0.14      | 0.33      | 0.28      | 0.22      |
| Thromoneopteron               | 0.53      | 0.13      | 0.25      | 0.28      | 0.22      |
| Erythrodihydroneopteron       | 0.41      | 0.07      | 0.28      | 0.26      | 0.22      |
| Sepiapterin                   | 0.30      | 0.28      | 0.43      | 0.43      | 0.44      |
| Isosepiapterin                | 0.19      | 0.41      | 0.44      | 0.56      | 0.65      |
| 6-Carboxypterin               | 0.53      | 0.04      | 0.27      | 0.11      | 0.26      |
| Pterin                        | 0.45      | 0.26      | 0.43      | 0.40      | 0.38      |
| 6-Hydroxymethylpterin         | 0.47      | 0.25      | 0.41      | 0.35      | 0.32      |
| Xanthopterin                  | 0.35      | 0.06      | 0.34      | 0.20      | 0.40      |
| Isoxanthopterin               | 0.31      | 0.11      | 0.33      | 0.90      | 0.31      |

Table III

| Compound                      | Amount to show 10 arbitrary units |
|-------------------------------|-----------------------------------|
| Bioppterin                    | 3.3                               |
| Neopterin                     | 3.5                               |
| Schmitzine                    | 3.2                               |
| 6-Carboxypterin               | 4.2                               |
| Isoxanthopterin               | 0.9                               |
| Sepiapterin                   | 90                                |
| Isosepiapterin                | 62                                |

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ter equipped with a primary filter exciting at 360 nm and a secondary filter emitting wave lengths greater than 415 nm was used. The fluorescence properties are shown in Table III.

Human Urine—Urine samples for quantitative analysis were kindly supplied by Dr. Robert M. Herman of the United States Army Medical Research and Nutritional Laboratory. "Normal urine" was collected from four individuals who had been receiving 100 μg of folic acid with a defined diet daily. "High folic urine" was collected from the same individuals after consuming 15 mg of folic acid per day for 3 days. Urine for qualitative analysis was collected at this laboratory. Freshly voided urine was collected from four individuals who had been receiving folic acid per day for 3 days. Kriue11 and stored at -20° until use.

RESULTS

All experiments were carried out in a darkened room, and samples and fractions were stored at -20° between purification steps and analyses, or both.

Experiment 1

Quantitative Analysis of Pterins in Urine from Subjects Receiving a Normal Level of Folate

The 24-hour output from four individuals receiving 100 μg of folic acid per day was pooled, and 1/4 (57 ml) was prepared for quantitative pterin analyses. The mixture was acidified with the addition of 2.5 ml of 6 x HCl (pH ~ 1), and the solution was applied to a Dowex 50 column (H+, 2.5 x 6 cm) (19). After the column was washed with about 60 ml of water, the pteridines which were retained on the resin were recovered by elution with about 60 ml of 1 N NH₄OH. The eluate was concentrated to about 5 ml by a rotary evaporator and the concentrated solution was applied to a pH 7 ECTEOLA-Sephadex column (2.5 x 11 cm). Two fluorescent peaks were obtained by developing the column with water. The column was further developed, first with 0.5 N acetic acid and then with 0.1 N HCl. The elution profile is presented in Fig. 1. Peak I showed yellowish-blue fluorescence; Peak II, blue; Peak III, purple; Peak IV, whitish-blue. Fractions of each peak were combined, concentrated, and subjected to further purification.

Peak I: Sepiapterin, Isolepiapterin, Xanthopterin, and Biopterin—Materials from Peak I were applied to a P-Sephadex column (1.1 x 19 cm), and the column was developed with water. Two yellow fluorescent peaks and one blue fluorescent peak were obtained. The first yellow fluorescent fraction contained riboflavin as determined by P-Sephadex and paper chromatography. Since a clear separation of the second peak (yellow fluorescent) and the third peak (blue fluorescent) was not obtained, all of the fractions containing these two peaks were combined and rechromatographed on a longer P-Sephadex column (0.9 x 40 cm). A bluish-green fluorescent substance was eluted between 20 and 25 ml of water; yellow fluorescent substances between 55 and 55 ml; blue to green fluorescent substances between 55 and 75 ml; blue fluorescent substance between 110 and 140 ml. Each fraction was concentrated and analyzed by paper chromatography. The fluorescent substance in the first peak fraction (20 to 25 ml) did not appear to be a pteridine because of its high Rf values in several solvent systems. Paper chromatography in five solvent systems showed that the second fraction (35 to 55 ml) contained sepiapterin, isosepiapterin, and other yellow fluorescent substances, and the third fraction (55 to 75 ml) contained xanthopterin. The blue fluorescent substance in the fourth fraction (110 to 140 ml) was pure as determined by paper chromatography and the mobility was the same as that of biopterin. The amount was estimated to be 5.5 μg.

Peak II: Pterin, Erythroenceopterin, Bioppterin, and Theononopterin—Material from Peak II was applied to a pH 7 ECTEOLA-Sephadex column (0.9 x 41 cm), and, by development with water, the blue fluorescent peak was resolved into two zones. A major fluorescent fraction was eluted between 105 and 135 ml and a minor fluorescent fraction was obtained between 140 and 165 ml. Each peak fraction was concentrated. The minor blue fluorescent fraction was applied to a P-Sephadex column (1.1 x 19 cm) and was eluted as a single peak from the column. Behavior of this substance on ECTEOLA-Sephadex, P-Sephadex, and paper (five solvent systems) was similar to that for pterin. The amount of the substance was 2.9 μg.

The major fluorescent substance was then applied to a long P-Sephadex column (1.2 x 110 cm). The column was developed with water and 10-ml fractions were collected. As shown by the solid circles in Fig. 2, the major fluorescent substance was resolved into two peaks. The elution volumes of the first peak and the second peak are the same as that obtained with known erythroenceopterin and bioppterin, respectively.

These results were confirmed by paper chromatography of each peak in five solvent systems. The amount of erythroenceopterin found was 15.4 μg and bioppterin 33.5 μg. In the second peak which contained bioppterin, another minor compound was detected. The compound was separated from bioppterin by paper chromatography (Solvent B). The isolated minor compound then migrated as a single spot, similar to a theononopterin on paper in each of five solvent systems. The amount was approximately 1 μg.

The occurrence of bioppterin in Peaks I and II was unexpected. The ammonium eluate from Dowex 50 chromatography must have contained dihydrobiopterin and bioppterin. Fractionation by ECTEOLA-Sephadex separates these two pterins with dihydrobiopterin being contained in Peak I and bioppterin in Peak II.
Subsequent chromatography by P-Sephadex alters any dihydrobiopterin present to biopterin or isosepiapterin. Additional observations regarding dihydropterins are presented in Experiment 5.

Peak III: Isoxanthopterin—the pooled fractions of Peak III from the first ECTEOLA-Sephadex column (acetic acid eluate) were dried by a rotary evaporator, and the residue was dissolved with a small amount of water. The solution was fractionated on a P-Sephadex column (1.1 x 25 cm). A purple fluorescent fraction (20 to 50 ml) was eluted first followed by blue fluorescent fractions. The purple fluorescent fraction was concentrated and applied to a pH 6 ECTEOLASephadex column (0.8 x 11 cm). The column was developed with water and yielded four fluorescent zones. The first zone, eluted between 40 and 50 ml, showed blue fluorescence, and the second, eluted between 60 and 75 ml, showed purple fluorescence. The second fraction was identified as isoxanthopterin (paper chromatography). The amount was 0.9 \( \mu g \). The substances in the first zone and in the other two zones still remaining in the upper part of the column were not identified.

Peak IV: 6-Carboxypterin—Peak IV, the HCl eluate, was dried, and the residue was dissolved in water and applied to a P-Sephadex column (2.5 x 12 cm). All of the blue fluorescent fractions which were eluted first were combined, concentrated, and streaked on Whatman No. 20 paper (35 x 35 cm). The paper was developed in Solvent D. The area corresponding to 6-carboxypterin was cut out, eluted with water, and the eluate was rechromatographed in Solvent E. The fluorescent band was again eluted, and the eluate from the paper was passed through a short P-Sephadex column (0.8 x 5 cm). The 6-carboxypterin thus purified was estimated to be 0.5 \( \mu g \).

The results are shown in Table IV, Experiment 1. The main pterins detected in the urine were biopterin and erythroneopterin.

### Table IV

| Compound                | Experiment 1 | Experiment 2 | Experiment 3 |
|-------------------------|--------------|--------------|--------------|
| Biopterin               | 38           | 41           | 41           |
| Erythroneopterin        | 15.4         | 36\( ^{b} \) | 14           |
| Threoneopterin          | 1            | 1.5          | 1.9          |
| 6-Carboxypterin         | 0.5          | 0.6          | 1.2          |
| Pterin                  | 2.9          | 2.8          | 1.4          |
| Isoxanthopterin         | 0            | 1.1          | 2.2          |
| Sepiapterin\( ^{a} \)  | +            | +            | +            |
| Xanthopterin\( ^{d} \) | +            | +            | +            |
| Isosepiapterin\( ^{e} \)| +            | +            | +            |

\( ^{a} \) Inasmuch as the 24-hour urinary output of individuals varied and quantities analyzed varied, the amount of pterins found is expressed in terms of micrograms per 4% of daily output. See text for details of preparation of urine samples.

\( ^{b} \) On the basis of the addition of 24.5 \( \mu g \) of erythroneopterin, the recovery was calculated to be 82%.

\( ^{c} \) Compound was identified but not quantitated (+).

\( ^{d} \) Isolation procedure may not be suitable for this compound because of its hydration and dehydration (20).

\( ^{e} \) This pterin was concluded to be an artifact derived from dehydrobiopterin during chromatography on Dowex 50 and P-Sephadex.

A threoneopterin, sepiapterin, isosepiapterin, 6-carboxypterin, xanthopterin, isoxanthopterin, and pterin were also detected, but the amount of these pterins was small. *Crithidia* activity of whole urine revealed that about 60 \( \mu g \) of biopterin equivalents were present in 57 ml of the urine mixture.

### Experiment 2

**Recovery Test**

For the evaluation of the recovery of pterins from urine and the reproducibility of the data in Experiment 1, 24.5 \( \mu g \) of authentic \( \alpha \)-erythroneopterin were added to 57 ml of normal urine and the mixture was analyzed as in Experiment 1. The results from the step using the long P-Sephadex column chromatography which separates erythroneopterin from biopterin are presented in Fig. 2. The increased amount of Peak 1 material found in Experiment 2 versus Experiment 1 is reflective of the added erythroneopterin to the urine sample, whereas the biopterin peak remained unchanged. The quantitation of these pterins and others are summarized in Table IV, Experiment 2. The recovery of the added erythroneopterin was calculated to be 82%, and the amounts found for other pterins was in good agreement to those found in Experiment 1.

### Experiment 3

**Effect of High Folate Diet on Urinary Pterins**

The effect of high folate intake on urinary excretion of pterins was studied. A urine mixture of 1% each of the 24-hour output from four individuals receiving 15 mg of folic acid was prepared. The volume of the pooled urine was 77 ml, and the urine sample was analyzed by the same procedure used in Experiments 1 and 2. Results given in Table IV, Experiment 3, show that dietary folate did not affect the amount of biopterin and neopterins in...
FIG. 3. Elution profiles of pterins from pH 7 ECTEOLAS-cellulose columns. A, a mixture of authentic biopterin (I), 
\( \beta \)-erythroneopterin (II), and \( \alpha \)-threoneopterin (III). B, pterins from urine: biopterin (I), erythroneopterin (II), threoneopterin (III). Three separate chromatograms are put together in B. Chromatographic conditions are essentially the same as was reported by Jackson and Shiota (21). Each sample solution was made 5 mM with respect to borax (pH 9). The solution (0.6 ml) was applied to a pH 7 ECTEOLAS-cellulose column (1.1 x 20 cm), and the column was developed with a linear gradient consisting of 150 ml of 5 mM borax in the mixing chamber and 250 ml of 25 mM borax in the reservoir, and 2.5-ml fractions were collected. The amount of pterins was determined by fluorescence and expressed as arbitrary units. When biopterin was applied to the system, sometimes a small peak appeared in fractions 13 to 15 together with the main peak (24-28).

urine. The slight increase of 6-carboxypterin and isoxanthopterin may be attributed to the folic acid ingested.

Experiment 4

Optical Configuration

In Experiment 1, \( \alpha \)-threoneopterin, erythroneopterin, and biopterin were found. In order to determine the structure of the side chain of these pterins, initially an analytical ECTEOLAS-cellulose sodium borate chromatography system (21) was used to confirm the diastereoisomeric forms, then a Crithidia bioassay (16) was used to establish the optical structure. The urinary erythroneopterin and biopterin to be analyzed were those obtained in Experiment 1. However, since the amount of threoneopterin found in Experiment 1 was low, 1 liter of urine was processed by a procedure similar to Experiment 1 to obtain about 10 \( \mu g \) of threoneopterin. Each of these pterins was subjected to ECTEOLAS-cellulose sodium borate column chromatography, and fractions were assayed for Crithidia activity. Fig. 3A illustrates the chromatographic elution profile of authentic pterins and Fig. 3B is the elution profile of purified urinary pterins as biopterin, erythroneopterin, and threoneopterin.

The Peak III fractions of urinary threoneopterin (Fig. 3B) were combined and acidified with addition of 6 N \( \text{HCl} \) to pH 1. The acidified solution was applied to a Dowex 50 (H\(^+\)) column (2.5 x 3 cm). After the column was washed with water, the pterin was recovered by 1 x \( \text{NH}_3\text{OH} \). The eluate was concentrated and purified by a P-Sephadex column (0.8 x 5 cm). Crithidia growth factor activity of threoneopterin purified in this way was examined by the system of Dewey and Kidder (16). Fig. 4 shows that the threoneopterin from urine supported the growth of Crithidia similarly to L-threoneopterin. Hence, these results suggest that the configuration of the side chain is \( \mathrm{L} \)-threo.

Crithidia activities of urinary biopterin and erythroneopterin were also assayed and found to be similar to those of authentic biopterin and \( \beta \)-erythroneopterin, respectively. These are consistent with the data of Patterson et al. (2) and those of Sakurai and Goto (4).

Experiment 5

Dihydropterins in Urine

In Experiment 1, biopterin was isolated from Peaks I and II of the pH 7 ECTEOLAS-Sephadex column chromatography step. According to the elution volumes for the pteridines presented in Table I, dihydrobiopterin should be contained only in Peak I and biopterin in Peak II. Since dihydrobiopterin is converted to isosepiapterin and biopterin on a P-Sephadex column as is shown under “Materials and Methods,” the presence of biopterin and isosepiapterin in Peak I suggests the occurrence of dihydrobiopterin in the urine. Accordingly, an experiment was performed to isolate and identify dihydrobiopterins in urine.

In order to isolate and identify dihydrobiopterins from urine, the procedure was modified by using Sephadex column chromatography which had been shown to be effective (9, 17, 22, 23) instead of Dowex 50 and P-Sephadex chromatography. To minimize spontaneous oxidation of dihydropterins, prolonged storage of samples was avoided even at \(-20^\circ\). Freshly voided urine was frozen at \(-20^\circ\). The frozen urine was thawed and the precipitate was removed by centrifugation at 5\(^\circ\). The supernatant fluid (200 ml) was applied to a Sephadex G-25 column (fine grade, 7 x 25 cm). The column was developed with water, and 20-ml fractions were collected. Salts (\( \text{AgNO}_3 \) precipitable substances) were eluted in fractions 41 to 54. A dark brown material was eluted in fractions 67 to 70. Just after these brown
fractions, most of dihydrobiopterin emerges, and fractions 71 to 84 were collected, combined, and concentrated. During concentration by rotary evaporation, care was taken to avoid drying the sample. A total of 1 liter of urine was subjected to the chromatography.

The combined concentrate (50 ml) was applied to a Sephadex G-10 column (5 × 22 cm). The column was developed with water, and 13-ml fractions were collected. Fractions 40 to 60 were combined and concentrated. This combined fraction should contain any dihydrobiopterin present judging from a chromatography of authentic dihydrobiopterin. The concentrated solution from the G-10 column was applied to a pH 8 ECTEOLA-Sephadex column (2.5 × 28 cm). The column was developed with water, and 5-ml fractions were collected. The elution pattern is shown in Fig. 5. Although a violet fluorescent substance was eluted as a single peak, some other ultraviolet light absorbing peaks were observed. Dihydropterins in the violet fluorescent peak were estimated to be about 0.5 mg from the absorption at 330 nm. This peak appeared to contain two violet fluorescent compounds. Fractions 33 to 42 were combined, concentrated, and streaked on paper (35 × 35 cm). By developing the paper in Solvent B under an atmosphere of argon overnight, three fluorescent bands, one major (I) and two minor ones (II and III), were obtained. Band I (RF 0.14), Band II (RF 0.07), and Band III (RF 0.28) corresponded to the mobility of dihydrobiopterin, erythro-dihydronopterin, and biopterin, respectively (Table II). Each fluorescent band was eluted with water by a descending procedure under argon gas.

The eluate from Band I was then applied to a pH 8 ECTEOLA-Sephadex column (0.8 × 8 cm), and a violet fluorescent fraction was collected. This procedure clearly separates dihydropteridines from the oxidized forms. The ultraviolet absorption spectra of the violet fluorescent fraction were identical with those of 7,8-dihydrobiopterin which showed the characteristic bathochromic shift of 7,8-dihydropteridines (Fig. 6) (24). As is shown in Fig. 7, the violet fluorescent compound migrated similarly to dihydrobiopterin in five solvent systems. These results indicate that the violet fluorescent compound from Band I is 7,8-dihydrobiopterin.

The eluate from Band II was treated similarly to Band I. The ultraviolet absorption spectra of the violet fluorescent fraction from Band II were essentially the same as those shown in Fig. 6. Paper chromatographic behavior of the violet fluorescent compound was identical with that of erythro-7,8-dihydronopterin in five solvent systems. Therefore, the violet fluorescent
compound from Band II was concluded to be erythro-7,8-dihydroopterin.

The fluorescent compound from Band III was identified as biopterin by paper chromatography. This indicates some oxidation of dihydrobiopterin to biopterin during concentration and paper chromatography.

Since sepiapterin and isosepiapterin should be eluted a little later than dihydrobiopterin and dihydronopterin from the Sephadex columns (15), fractions 84 to 100 from the Sephadex G-25 column and fractions 61 to 80 from the Sephadex G-10 column were combined and concentrated. The concentrated solution was applied to a pH 7 ECTEOLA-Sephadex column (2.5 x 10 cm). On elution with water, a yellow fluorescent substance was obtained in fractions where sepiapterin and isosepiapterin were expected to appear. The yellow substance was further purified by a P-Sephadex column, and the eluate was concentrated and applied to paper (35 x 35 cm). After development in Solvent B, two yellow fluorescent bands were obtained. The RF of the upper band was similar to that of sepiapterin and the RF of the lower band was about half that of the upper one.

The yellow fluorescent compound of the upper band was eluted with 0.1 N acetic acid, and the acid in the eluate was removed by passing it through a pH 7 ECTEOLA-Sephadex column (2.5 x 5 cm). The yellow fluorescent compound in the final eluate was pure as judged by paper chromatography; it migrated as sepiapterin in each of the five solvent systems. The ultraviolet absorption spectrum in water was also similar to that of sepiapterin. Therefore, the yellow fluorescent compound was concluded to be sepiapterin. The amount was 3 μg.

The yellow fluorescent compound in the lower band appeared to be somewhat similar to a yellow compound which was formed from dihydroopterin (21), but further analysis was not made. There was no evidence for the occurrence of isosepiapterin. The finding of isosepiapterin in Experiment 1, therefore, was concluded to be an artifact due to dehydrogenation of dihydrobiopterin.

DISCUSSION

From the data in Table IV, the daily outputs of biopterin and erythroopterin are calculated to be 1 mg and 0.4 mg per person (per 1.4 liters of urine), respectively. The Crithidia activity of urine assayed 1.5 mg of biopterin equivalents per 1.4 liters, whereas Patterson et al. (2) found 0.5 mg of biopterin equivalent per 1.4 liters of urine.

At the pH 7 ECTEOLA-Sephadex step in Experiment 5 (Fig. 6), about half of the total pterins in urine (0.5 mg per liter) were detected as dihydropterins. In consideration of the instability of dihydropterins, a much larger portion may be present as reduced derivatives. The occurrence of reduced pterins has been reported in several organisms; dihydrobiopterin in rat (5) and Ascars (23), tetrahydrobipterin derivatives in Anacystis nidulans (25), insects and Amphibia (26), and sepiapterin in insects (27), fish, and Amphibia (28). The coenzyme forms of pterins, both naturally occurring and synthetic, have been demonstrated to participate in hydroxylation reactions as their reduced forms (5, 8). Furthermore, most of their reactions require them to be reduced (18, 29). Hence, it can generally be assumed that pterins are metabolically active in a reduced state.

Since a large portion of pterins is present as labile reduced forms in urine, degradation or conversion of reduced pterins to other pterins may have taken place during isolation. As has been mentioned in the previous section, isosepiapterin in Experiment 1 is an artifact. 6-Carboxypterin may also be an artifact. Blair (3) detected 6-carboxypterin in human urine only when urine was allowed to stand or the preliminary stages of the analysis were protracted. Reduced pterins were converted to anthopterin nonenzymatically as well as enzymatically (30). Therefore, it is not certain whether vanthopterin is present in urine or not. As isoxanthopterin is formed from pterin enzymatically (31) both pterins seem to be present in urine. It has been reported that sepiapterin reductase was detected in several animals (32, 33). Hence, it is probable that a small amount of its substrate, sepiapterin, is excreted in urine. In conclusion, biopterin, n-erythropteron, l-threoneopterin, sepiapterin, pterin, isoxanthopterin, or their reduced forms are present in human urine.

Although biopterin is probably an essential substance for the biosynthesis of adenalin and serotonin, its origin in higher animals is not clear. Biopterin may arise in three ways in higher animals. (a) It is taken in as a vitamin; (b) it is synthesized from other pterins such as folate; (c) it is synthesized from a nonpteridine precursor. Our results on the urinary excretion of pterins indicate that high folic acid intake does not affect the level of biopterin and neopterins. According to Goodfriend and Kaufman (34), a folate deficient diet failed to reduce the hepatic level of cofactor for phenylalanine hydroxylase (presumably reduced biopterin) in rat except when inanition was also induced. Also, in the case of germ-free chicks, the amount of ingested folic acid did not have an effect on the Crithidia activity of serum, and in the case of man, Crithidia activity and the folate level in serum were not correlated with each other (35). These results may exclude a precursor role of folate for biopterin in these animals. Pabst and Rembold (36) proposed a de novo synthesis of biopterin in the rat based on the observations that biopterin output was not altered by the administration of antibiotics or by changing the amount of dietary folic acid or riboflavin, and that biopterin was excreted constantly by rats grown on a biopterin free diet for two generations. As yet, definitive data about biopterin synthesis in man are lacking.

As we have shown, pterins with three carbon side chains in human urine are biopterin, n-erythropteron, l-threoneopterin, and sepiapterin. Recently, Fukushima (15) proposed that the intermediary metabolites of the biosynthetic pathway from GTP to biopterin are n-erythropteron, l-threoneopterin, and sepiapterin. These three pterins which are the constituent pterins in urine may suggest the involvement of these metabolites in the biopterin biosynthetic pathway.

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