Determination of Pterins in Biological Samples by Liquid Chromatography/Electrochemistry with a Dual-Electrode Detector

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The pterins are a family of compounds that are currently of great interest in medicine and biology. Biopterin, in its reduced form, serves as the cofactor to the enzymes which catalyze the rate-limiting reactions in the biosynthesis of the catecholamines and serotonin. As such, it may serve a role in the regulation of these neurotransmitters. Abnormal pterin concentrations have been observed in the urine and serum of patients with several diseases. No currently available analytical method is totally satisfactory for the determination of pterins in biological samples. They lack either specificity or the ability to detect both the oxidized and reduced forms of the pterins. Liquid chromatography/electrochemistry (LCEC) using a dual-electrode detector can overcome both of these problems. A method has been developed that is capable of determining several pterin species and their various oxidation states in biological samples. The dual-electrode detector used in a parallel-adjacent configuration is also capable of enhancing peak identity assignments and selectively determining easily oxidized compounds in the presence of harder to oxidize compounds.

Liquid chromatography/electrochemistry (LC/EC) has become a powerful technique in the determination of trace biological compounds (*1*). The technique offers high selectivity and low detection limits at a low cost. Dual-electrode transducers, in a variety of configurations, have been gaining in popularity due to improved selectivity, detection limits, and peak identification (*2*). The majority of reports describing dual-electrode detection have concerned the series configuration (*3–9*). However, the use of a parallel configuration can also provide many improvements in electrochemical detection. Voltammetric data gathered with a parallel-adjacent dual-electrode detector is especially useful in peak identification (*10, 11*). This configuration can also be used to selectively detect compounds with a wide range of oxidation potentials. In this report, several applications of the parallel-adjacent dual-electrode configuration are demonstrated for the determination of pterins in biological samples.

The pterins are a family of heterocyclics (Figure 1) that are cofactors to several hydroxylases. Pterins occur in three oxidation states, all of which are of interest; however, no current method can directly quantitate all of them. We have recently reviewed earlier methods and described an approach to determining several oxidized pterins in biological samples by using LC/EC (*12*). Bräutigam and Dreesen have reported the determination of tetrahydrobiopterin, the most important reduced pterin, in tissue (*13*). The use of a dual-electrode detector is demonstrated to extend the capability of LC/EC to the simultaneous direct determination of the individual oxidation states of these important redox cofactors. The availability of this approach will greatly aid studies of the role of pterins in the biochemistry of the nervous system.

EXPERIMENTAL SECTION

Apparatus. The liquid chromatographic system employed was a Bioanalytical Systems LC-154 (Bioanalytical Systems Inc., West Lafayette, IN) with an LC-22 temperature controller and an LC-23 column heater. A Biophase 5-μm C-18 column (25 cm × 4.6 mm) was used. Detection was by a Bioanalytical Systems LC-4B dual-electrode amperometric detector using glassy carbon electrodes.

Reagents. Biopterin was purchased from Calbiochem-Behring (La Jolla, CA). Pterin, xanthopterin, and pterin-6-carboxylic acid were obtained from Sigma (St. Louis, MO). *erythro*-Neopterin was purchased from Fluka (Basle, Switzerland). 5,6,7,8-Tetrahydrobiopterin was a gracious gift from A. Neiderweiser (Zürich). 6-Hydroxymethylpterin was prepared by the method of Thijssen (*14*).

Procedures. *Chromatography.* A reverse-phase "ion-pair" chromatographic method was employed for the separation of the various pterin species. A mobile phase of 3 mM octyl sodium sulfate buffered to pH 2.5 with 0.1 M sodium phosphate buffer and 5% methanol was found to give a good separation of the pterins (Figure 2). The column was maintained at a temperature of 30 °C. The mobile phase was prepared with distilled, deionized water and filtered through a 0.22-μm filter (Millipore, Milford, MA) prior to use. Oxygen was removed by continuous purging of the mobile phase with nitrogen and maintaining the mobile phase reservoir at a temperature of 40 °C. A flow rate of 1.0 mL/min was used in all experiments. The injection loop volume was 20 μL.

Preparation of Reduced Pterins. The dihydropterins were prepared by the method of Kaufman (*15*). Approximately 1 mg of pterin was suspended in 0.4 mL of water and dissolved by the addition of 0.2 mL of 2 M KOH. About 10 mg of zinc dust was added to the solution and swirled. The solution was stored in the dark at room temperature for 5 min and then heated to 55 °C for 1 min. The mixture was cooled and the zinc dust removed by filtration. The dihydropterin solution was acidified by the addition of 0.1 M sodium phosphate buffer, pH 2.

Tetrahydropterins were prepared by a modification of the method of Bobst and Viscontini (*16*). One to five milligrams of pterin was dissolved in 3 mL of trifluoroacetic acid to which 2 mg of PtO_2 was added. Hydrogen was bubbled through this solution for 1 h. The trifluoroacetic acid was removed under a stream of nitrogen. The resulting residue was reconstituted by the addition of 0.1 M sodium phosphate buffer, pH 2. The PtO_2 was removed by filtration.

Sample Preparation. The reduced pterins are labile to oxidation by air; therefore, all sample preparation is carried out under a nitrogen atmosphere with deoxygenated solutions. Reduced pterins were found to bind too strongly to polystyrene based resins to achieve reasonable recoveries while the oxidized pterins are not sufficiently retained by Sephadex type resins. For this reason, a series of cleanup columns must be employed. The reduced pterins are first retained on an SP-Sephadex column and the oxidized pterins are then retained on a Dowex 50 column. The pterins are then eluted in reverse order.

For urine samples, 5 mL was acidified to pH 2 with phosphoric acid and applied to a 0.5-mL SP-Sephadex C-25 column. The eluent of this column was applied to a 0.5-mL Dowex 50-X8 (H^+, 200–400 mesh) column. Both columns were washed with 10 mL of water. The Dowex 50 column was then developed with 5 mL of 0.1 M NaOH which was 5 mM in ascorbic acid. This eluent was applied to the SP-Sephadex column and the eluent collected and acidified to pH 2 with phosphoric acid. This sample was then applied to the analytical column.

The recoveries of the pterins are listed in Table I. Good recoveries were achieved for all of the pterin species using this

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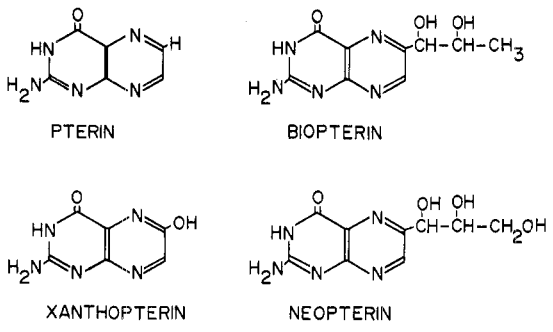

Figure 1. The structures of some oxidized pterins.

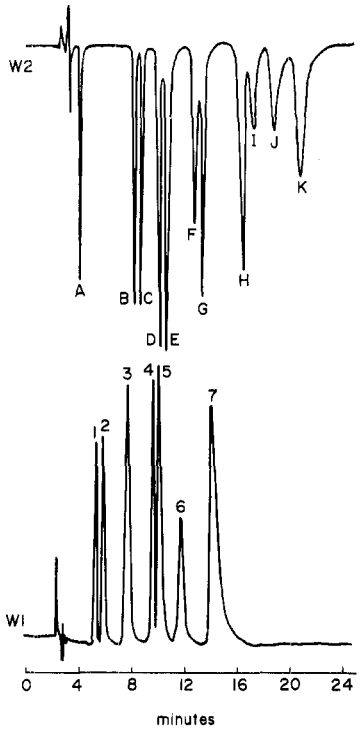

Figure 2. Standard separation of the pterins: mobile phase, 3 mM octyl sodium sulfate, 0.1 M phosphate buffer, pH 2.5; 5% MeOH, 30 °C, W1 = +800 mV, W2 = 700 mV; peak identities, (1) *erythro*-neopterin, (2) *threo*-neopterin, (3) xanthopterin, (4) pterin-6-carboxylic acid, (5) biopterin, (6) 6-hydroxymethylpterin, (7) pterin, (A) tetrahydropterin-6-carboxylic acid, (B) tetrahydroneopterin, (C) dihydroneopterin, (D) dihydroxanthopterin, (E) tetrahydro-6-hydroxymethylpterin, (F) tetrahydropterin, (g) tetrahydrobiopterin, (H) dihydrobiopterin, (I) dihydro-6-carboxylic acid, (J) dihydro-6-hydroxymethylpterin, (K) dihydropterin.

sample preparation method. The reproducibility of the recoveries was excellent for a batch of columns prepared at the same time. To account for any day to day variations in column preparation, a set of standards was subjected to the preparation procedure and recoveries were determined for each new batch of columns.

RESULTS AND DISCUSSION

Electrochemistry of the Pterins. In order to establish the operating conditions for electrochemical detection and to make optimum use of the dual-electrode detector, a knowledge of the electrochemical behavior of the pterins is necessary. This information can be obtained by chromatographically assisted hydrodynamic voltammetry. Voltammetric data can be generated with the assistance of liquid chromatography by repetitively injecting a standard solution and stepping the detector potential between injections. Plotting the normalized current response vs. the applied potential results in the familiar hydrodynamic voltammogram (HDV). This information can be more clearly presented for a number of compounds in a graphical format as shown in Figure 3 for the pterins. The

Table I. Recoveries from Sample Preparation Procedure

	% recovery[a]	% std dev[b]
biopterin	94.5	2.0
dihydrobiopterin	96.6	1.7
tetrahydrobiopterin	90.9	1.9
6-hydroxymethylpterin	96.6	1.6
dihydro-6-hydroxymethylpterin	79.2	1.9
tetrahydro-6-hydroxymethylpterin	93.0	2.3
neopterin	90.1	0.8
dihydroneopterin	78.0	1.7
tetrahydroneopterin	86.0	1.8
pterin	95.4	2.3
dihydropterin	94.1	2.1
tetrahydropterin	96.1	2.4
pterin-6-carboxylic acid	98.2	3.2
dihydropterin-6-carboxylic acid	96.3	1.4
tetrahydropterin-6-carboxylic acid	96.1	2.4
xanthopterin	76.7	4.1
dihydroxanthopterin	87.5	1.2

[a] Determined for concentrations of 5 to 500 μM.
[b] Within a batch of columns prepared together.

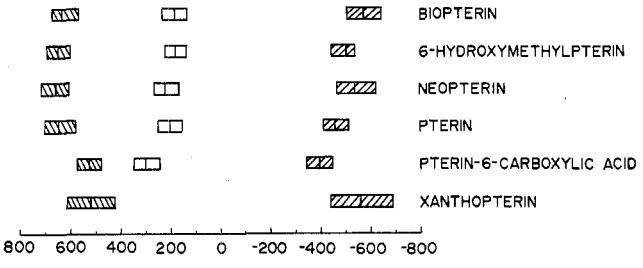

Figure 3. Diagram of the oxidation/reduction potentials of the pterins. The rectangles extend from the $E_{1/4}$ potential to the $E_{3/4}$ potential with the center line indicating the $E_{1/2}$ potential: [lines slanting right] oxidized forms (reductive HDV), [lines slanting left] dihydro forms (oxidative HDV), [open box] tetrahydro forms (oxidative HDV).

rectangles extend from the $E_{1/4}$ potential to the $E_{3/4}$ potential with the center line indicating the $E_{1/2}$ value.

The pterins occur in three relatively stable oxidation states. Fully oxidized pterins can be reduced in a two-electron, two-proton step to 7,8-dihydropterins. Dihydropterins can then be further reduced to 5,6,7,8-tetrahydropterins (*17*). An exception to this scheme is xanthopterin, in which an enolic hydroxyl group in the 6 position blocks reduction to the tetrahydro state. The potentials at which the various oxidation states of the pterins can be oxidized or reduced can be seen in Figure 3.

The maximum sensitivity for electrochemical detection is found by operating on the limiting-current plateau of the hydrodynamic voltammogram. However, when at high potentials, the background current and resulting noise can become limiting. Under these conditions, better detection limits can often be achieved by operating at slightly lower potentials. For this reason, a potential of −700 mV vs. the Ag/AgCl electrode was chosen as a compromise between maximizing the current response and minimizing the background current for the detection of the oxidized pterins.

Because the tetrahydropterins are relatively easily oxidized, background considerations are unimportant and a potential on the limiting-current plateau for all species can be employed. A potential of +500 mV vs. the Ag/AgCl electrode can be used to detect the tetrahydropterins. A more positive potential is necessary for the detection of the dihydropterins. Again, background considerations are not significant and a potential of +800 mV vs. the Ag/AgCl electrode can be used; this is on

Table II. Detection Limits

	LOD,[a] pmol
biopterin	0.60
dihydrobiopterin	0.81
tetrahydrobiopterin	0.94
6-hydroxymethylpterin	0.78
dihydro-6-hydroxymethylpterin	0.79
tetrahydro-6-hydroxymethylpterin	0.71
neopterin	0.47
dihydroneopterin	0.66
tetrahydroneopterin	0.67
pterin	0.96
dihydropterin	0.90
tetrahydropterin	0.91
pterin-6-carboxylic acid	0.60
dihydropterin-6-carboxylic acid	0.71
tetrahydropterin-6-carboxylic acid	0.54
xanthopterin	0.57
dihydroxanthopterin	0.50

[a] Limit-of-detection at signal-to-noise ratio of >3.

the limiting-current plateau for all of the reduced pterins studied.

Simultaneous Detection of Oxidized and Reduced Pterin Species. The use of a parallel-adjacent dual-electrode detector makes the simultaneous detection of both oxidizable and reducible species in a sample possible. This is accomplished by poising one electrode at a reducing potential and the other electrode at an oxidizing potential. With operation at +800 mV for the reduced pterins and at −700 mV for the oxidized pterins, all three oxidation states for several pterin species can be detected in a sample with a single chromatographic injection.

One of the advantages of electrochemical detection is the large range of concentrations over which the current response is linear. Operating with this dual-electrode method, the current response was found to be linear over 4 orders of magnitude, from several nanomoles to less than a picomole injected. Detection limits of less than a picomole at signal-to-noise ratios greater than 3 were achieved for all of the pterin species studied (Table II). It is therefore possible to directly analyze most biological samples for pterins by this method with no preconcentration step.

To illustrate the utility of this method to a complex biological sample, the excretion pattern of the pterins in urine was studied. The first morning urine was collected from a volunteer for a 1-week period. Samples were kept in dark bottles and were processed within 1 h of collection. Figure 4 illustrates a typical chromatogram for a urine sample subjected to the sample preparation procedure and analyzed by parallel-adjacent dual-electrode LC/EC. As previously reported (*18*, *19*) the pterins occur predominantly in their reduced states (Table III). Biopterin was found to occur mostly as tetrahydrobiopterin and xanthopterin occurred predominantly as 7,8-dihydroxanthopterin. Neopterin, on the other hand, was found to be approximately half oxidized neopterin and half tetrahydroneopterin.

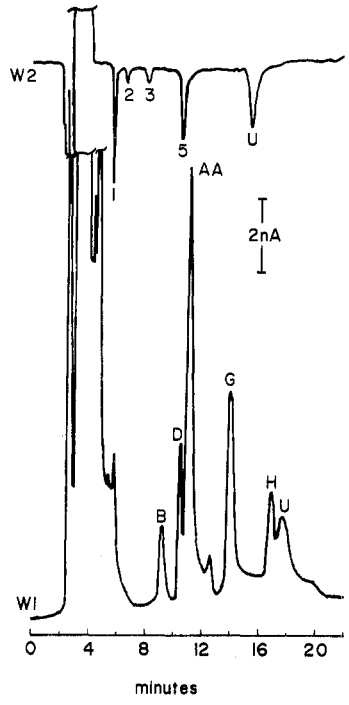

Figure 4. Dual-electrode detection of reduced and oxidized pterins in urine: W1 = +800 mV, W2 = −700 mV. Peak identities are the same as those in Figure 2 except AA is ascorbic acid and U indicates an unidentified peak.

To ensure that all of the pterins were being detected by this dual-electrode method, aliquots of each urine sample were also analyzed by reductive LC/EC following oxidation of the pterins by iodine solution (*12*). The concentration of the pterins found after oxidation compare well with the sum of the concentrations of all the oxidation states for each pterin species (Table IV). The total concentrations of the pterins also compare favorably with previously reported values (*20*, *21*).

Peak Identification by Voltammetric Characterization. Typically, chromatographic peaks are identified by comparing their retention times to those of standards. Voltammetric information can also be used in assigning peak identities by comparing the HDV of an unknown peak to that of a standard compound. If the compounds are the same, then the HDV's will coincide. The combination of voltammetric characterization with retention data provides peak identity assignments with a high degree of certainty even in quite complex samples.

It has been shown that it is not necessary to obtain the entire voltammogram of an analyte but only to compare the current response over the potential region where the response is changing most rapidly (*10*). In this method, the current response at each potential is compared to the current response

Table III. Urinary Pterin Excretion Pattern[a]

day	H_4-BIOP	H_2-BIOP	BIOP	H_4-NEOP	NEOP	H_2-XANTH	XANTH
1	598	132	94	137	148	197	46
2	669	161	123	170	182	261	88
3	584	100	107	152	163	186	36
4	425	281	88	120	130	164	41
5	455	52	81	109	118	132	27
6	592	135	96	122	130	201	52
7	564	128	84	131	143	188	29
av	555	141	96	134	145	190	46

[a] Concentration expressed in μmol/mol of creatinine.

Table IV. Comparison of Oxidized and Unoxidized Samples

	biopterin		neopterin		xanthopterin	
day	ox.[a]	unox.	ox.[a]	unox.	ox.[a]	unox.
1	801	824	291	285	246	243
2	912	953	360	352	334	349
3	799	791	307	315	207	222
4	790	794	247	250	214	205
5	603	588	218	227	151	159
6	811	823	271	252	264	253
7	770	776	272	274	215	217

[a] Sum of the concentrations of the tetrahydro, dihydro, and oxidized forms.

at the highest potential applied. If the sample component and the standard are the same compound, then the current ratios will be equal. The voltammetric characterization data for the pterins in urine is summarized in Tables V and VI.

With a single electrode detector this voltammetric characterization can become a tedious and time-consuming process, especially if the separation time is long. This is because only a single data point can be obtained per injection. For routine determination of peak purity the parallel-adjacent dual-electrode can overcome this handicap (*11*). By poising the electrodes at potentials on the slope of the HDV, a good estimation of peak purity can be made with a single chromatographic injection. Figure 5 shows the chromatograms obtained when the electrodes are poised at +500 mV and +300 mV vs. the Ag/AgCl electrode. Current ratios of 0.57 for tetrahydrobiopterin and 0.55 for tetrahydroneopterin were calculated in this manner. These values compare well with the current ratios found by the more involved single electrode procedure. In this manner a dual-electrode detector can be used to assure peak purity during routine analysis while the longer single electrode procedure can be used to determine peak identity during methods development.

Selective Determination of Compounds with Widely Varying Oxidation Potentials. Parallel-adjacent dual-electrode detection can also make simultaneous determination of several components of widely varying oxidation (reduction) potential in a sample more feasible. With a single electrode detector, a potential must be applied which is sufficiently positive (negative) to oxidize (reduce) all of the components of interest. It is often necessary to use higher potentials when several compounds are to be determined. Because the complexity of the chromatogram increases at higher potentials, the response from one or more of the desired components can often be obscured by interferences. Parallel-adjacent dual-electrode detection can overcome this problem by using one electrode to detect the hard to oxidize (reduce) components and the other electrode to determine the more easily oxidized (reduced) components with greater selectivity. This is done by setting one electrode at a high potential while the second electrode is set at a lower potential and simultaneous "high" and "low" potential chromatograms are obtained. The high potential chromatogram is used for the determination of compounds which are hard to oxidize (reduce). The low potential chromatogram is used to determine the more easily oxidized (reduced) components, often with the better detection

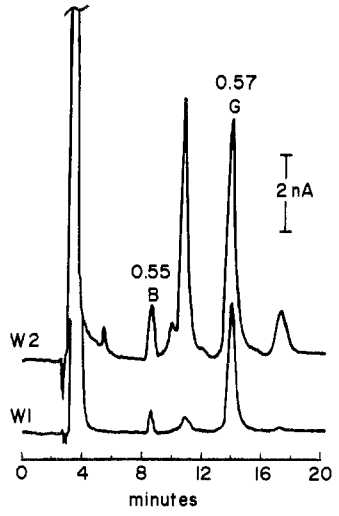

Figure 5. Peak purity assessment of tetrahydropterins in urine by parallel dual-electrode detection: W1 = +300 mV, W2 = +500 mV. The current ratios are listed above their corresponding peaks. Peak identity is the same as those in Figure 2.

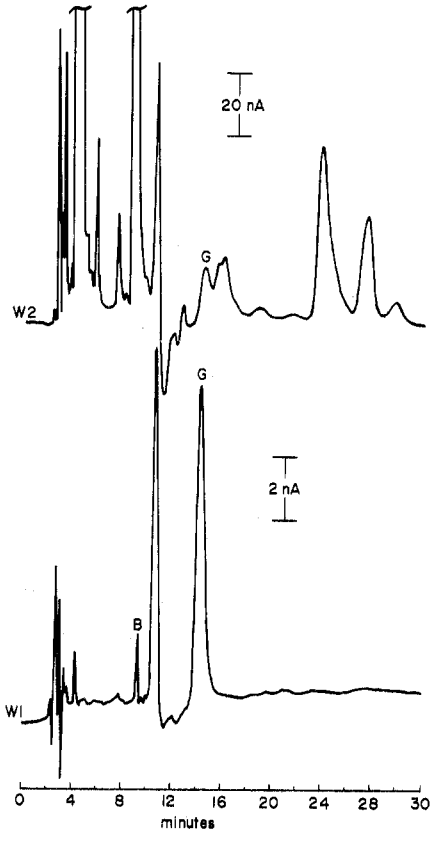

Figure 6. Improved detection of easily oxidized pterins in urine by dual potential monitoring: W1 = +800 mV, W2 = +400 mV. Peak identities are the same as those in Figure 2.

Table V. Voltammetric Characterization—Reduced Pterins in Urine

	H_2-XANTH[a]		H_2-BIOP[a]		H_4-BIOP[b]		H_4-NEOP[b]	
E, mV	std	samp	std	samp	std	samp	std	samp
600	0.82	0.79	0.61	0.59				
500	0.16	0.15	0.21	0.18				
400	0.02	0.02	0.09	0.09	0.82	0.83	0.82	0.82
300					0.61	0.63	0.61	0.59
200					0.17	0.19	0.16	0.18

[a] Currents normalized to that observed at +700 mV. [b] Currents normalized to that observed at +500 mV.

Table VI. Voltammetric Characterization—Oxidized Pterins in Urine

	neopterin		xanthopterin		biopterin	
E, mV	std	samp	std	samp	std	samp
−600	0.77	0.80	0.62	0.60	0.73	0.70
−500	0.35	0.33	0.27	0.26	0.33	0.35
−400	0.11	0.10	0.07	0.06	0.10	0.10

[a] Currents normalized to that observed at −700 mV.

limits because of decreased complexity of the chromatogram.

Increased selectivity for easily oxidized compounds in a complex sample is illustrated by the dual-electrode detection of reduced pterins in urine. Figure 6 shows the simultaneous chromatograms obtained from urine samples with one electrode at +800 mV and the other electrode at +400 mV. This urine sample was acidified and filtered to remove proteins but was not subjected to the sample preparation procedure. It can be seen that the chromatogram obtained at the lower potential is much less complex than the chromatogram obtained at +800 mV. In this manner, tetrahydropterins can be determined with lower detection limits and with less sample preparation.

CONCLUSION

It has been shown that operating a dual-electrode amperometric detector in the parallel-adjacent configuration can offer advantages in versitility, peak identity/purity assessment, and selectivity. In addition, a method has been presented for the determination of pterins in biological samples. Although the determination of pterins in urine was illustrated, this approach is applicable to a wide range of biological samples.

Registry No. Biopterin, 22150-76-1; dihydrobiopterin, 6779-87-9; tetrahydrobiopterin, 17528-72-2; 6-hydroxymethylpterin, 712-29-8; dihydro-6-hydroxymethylpterin, 3672-03-5; tetrahydro-6-hydroxymethylpterin, 31969-10-5; neopterin, 2009-64-5; dihydroneopterin, 1218-98-0; tetrahydroneopterin, 25976-00-5; pterin, 2236-60-4; dihydropterin, 17838-80-1; tetrahydropterin, 1008-35-1; pterin-6-carboxylic acid, 948-60-7; dihydropterin-6-carboxylic acid, 17833-48-6; tetrahydropterin-6-carboxylic acid, 7449-02-7; xanthopterin, 119-44-8; dihydroxanthopterin, 1131-35-7; *erythro*-neopterin, 39923-31-4; *threo*-neopterin, 2277-42-1.

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Poly(perfluoroalkyl ether) Stationary Phase for Gas Chromatography

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Efficient packed columns having between 2000 and 2500 theoretical plates/m can be prepared with the poly(perfluoroalkyl ether) Fomblin YR. Its maximum allowable operating temperature is a function of the stability of the liquid phase film, 255 °C for a 10% (w/w) loaded packing on Chromosorb P, and not by column bleed. The absolute retention of organic compounds on Fomblin YR is generally an order of magnitude, or greater, less than on conventional nonfluorinated liquid phases. Fomblin YR may be used to separate nonpolar and moderately polar organic compounds with good peak shape. Polar compounds such as alcohols, amines, and phenols, etc., produce asymmetric peaks.

Separations in gas–liquid chromatography occur because of selective interactions between the sample and the liquid phase. All components have essentially the same residence time in the gas phase. Thus, many liquids have been evaluated as stationary phases for gas chromatography but only a few of these can be described as having unique separation properties (*1*). In general, there exists a large number of stationary phases having nearly identical separation properties, from which a small number can be identified as preferred phases characteristic of all others. Recently we have described the properties of some novel stationary phases for gas chromatography, namely, organic molten salts (*2*, *3*) and substituted poly(phenyl ethers) (*4*–*6*). The former represent phases having ionic functional groups providing both different and stronger interactions with polar compounds than found for conventional liquid phases and the latter an attempt at standardization by developing thermally stable, nonpolymeric phases having a defined chemical structure reproducible via synthesis. In this paper we wish to introduce a further development, the use of perfluorocarbon stationary phases for the analysis of organic compounds at temperatures lower than those required with conventional nonfluorinated phases. It is hoped that phases of this kind will eventually extend the molecular weight

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