POSSIBILITY OF DETERMINATION OF SOME DERIVATIVES OF PTERIN IMPORTANT IN THE DIAGNOSTICS OF MALIGNANT TUMOURS BY USING DIFFERENTIAL PULSE POLAROGRAPHY

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The polarographic behaviour of 6,7-dimethylpterin, xanthopterin, 6-pterinaldehyde, neopterin and 6-hydroxymethylpterin was investigated. These compounds are suitably determined by employing differential pulse polarography; some results suggest the possibility of their determination by cathodic stripping voltammetry with adsorptive accumulation. For all compounds, the effect of pH on polarographic reduction was examined. Under optimal conditions, the calibration curve of all compounds under investigation was linear in the range $2 \cdot 10^{-6} - 1 \cdot 10^{-7}$ mol/l and the detection limit was below $1 \cdot 10^{-7}$ mol/l.

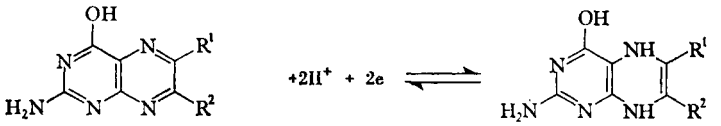
Recently, analytical methods suitable for the determination of derivatives of pterin (2-amino--4-hydroxypteridine) have arisen increasing interest. These products of metabolism are excreted from the organism by urine. Biochemical investigation revealed that their metabolism may be affected by the existence of malignant growth in the organism.

As demonstrated by the results of refs[1-7], the concentration of some pterins in body liquids changes in the case of tumour disease. Thus, *e.g.* refs[1,3] point out that in the uring of patients suffering from malignant tumours the concentration of tetrahydrobiopterin is about 200 time higher than in that of healthy people. Authors of ref.[4] detected 6-pterinaldehyde in the uring of patients treated for cancer: this compound is virtually absent in human urine. Similarly, in ref.[5] it is reported that the concentration of neopterin detected in the urine of patients suffering from the tumour disease was as much as fivefold that detected in the control sample, while the concentration of 6-hydroxymethylpterin, as reported in ref.[6], was 5—10 times higher than the control.

Somewhat different results were obtained by the authors of ref.[7] who did not prove a higher concentration of 6-hydroxymethylpterin in the urine of their patients. In their paper they point out that the quantities of the individual pterins excreted by the organism may be affected by food composition, and recommend that several such compound should be detected in diagnostic tests. Their results suggest that cancer disease causes a rise in the concentration of xanthopterin and neopterin, and at the same time a decrease in the concentration of isoxanthopterin excreted by urine. All these findings lead to a conclusion that detection of pterins in urine may be a suitable diagnostic test for the malignant tumour disease. Usually, these compounds are determined fluorometrically, and their separation is carried out by liquid chromatography[7].

Polarography is another analytical method suitable for the determination of pterin and its derivatives: its recent variants, and particularly differential pulse polarography[8] make possible determination of some compounds in quantities as small as p.p.b.

The polarographic behaviour of pterin and some of its derivatives was investigated by Komenda and coworkers in the early sixties[9-13]. They found that these compounds were reduced at the mercury drop electrode with consumption of two electrons. The reaction takes place on nitrogen atoms in positions 5 and 8.

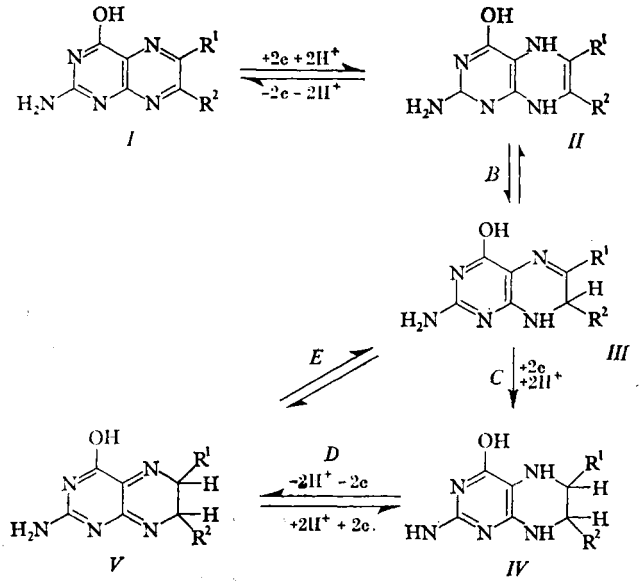


The polarographic reduction is complicated by acidobasic equilibria; in acid medium, the NH_2 group is protonized, in the alkaline medium, the proton is abstracted from the hydroxy group, so that as many as three waves corresponding to the reduction of the individual forms may appear in polarograms depending on pH.

Such behaviour was observed for 6-methylpterin[9], 7-methylxanthopterin, 6-methylisoxanthopterin and leucopterin[10].

For a better insight into the mechanism of reduction, in ref.[11] attention was concentrated on pteridine and its derivatives, 4-hydroxypteridine and 2-aminopteridine. 4-Hydroxypteridine behaves similarly to pterin and its derivatives and everything seems to indicate that the mechanism of reduction described above is due to the presence of the hydroxy group in position 4 on the pteridine backbone. Solutions of pteridine and 2-aminopteridine are unstable and undergo chemical changes which can be followed polarographically.

In ref.[12], the dissociation constants of the individual derivatives of pteridin were successfully correlated with half-wave potentials. In the case of some pterins, *e.g.* 6-methylpterin and 7-methylxanthopterin, the oxidized form of these compounds is adsorbed on the surface of the mercury electrode, which is reflected by the formation of a prewave in the d.c. polarogram and by the



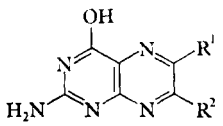
presence of another peak on the polarographic curve recorded by the technique of ac polarography[12].

The mechanism of reduction of pteridine was studied by Mc Alister[14], who used dc polarography, cyclic voltammetry and coulometry. According to this results, the two-electron reduction is complicated by a subsequent chemical reaction leading to the formation of a dimer.

Komenda's work was followed by studies of Kwee and Lund[15] who investigated in greater detail the mechanism of reduction of a number of pterin derivatives and suggested a somewhat different mechanism of their reduction. They came to a conclusion that the product of electrochemical reduction of pterin derivatives is in equilibrium with a further compound which may be further reduced at the mercury drop electrode acording to the mechanism (*B–C–D–E*).

Depending on substituents, the equilibrium of reaction *B* is displaced either towards *II* or towards *III*.

In this study, we have verified the possibility of polarographic determination of the following five derivatives of pterin: 6,7-dimethylpterin (*I*), xanthopterin (*II*), 6--pterinaldehyde (*III*), neopterin (*IV*), and 6-hydroxymethylpterin of the general formula:



where:

Derivative	R^1	R^2
I	CH_3	CH_3
II	OH	H
III	CH=O	H
IV	$CHOHCHOHCH_2OH$	H
V	CH_2OH	H

With respect to the required sensitivity, differential pulse polarography at the static drop electrode was the method chosen for the determination of these compounds. Also, in view of the adsorption phenomena, the possibility was checked of the determination of pterins by the method of fast sweep differential pulse polarography[17] using adsorptive accumulation on the surface of the stationary mercury electrode.

For all compounds, the effect of pH on polarographic reduction was examined, linearity of the calibration curve was checked and the detection limit of determination was determined.

EXPERIMENTAL

Chemicals: 6,7-dimethylpterin, xanthopterin, 6-pterinaldehyde, neopterin, and 6-hydroxymethylpterin were obtained from the Institute of Biophysics and Nuclear Medicine, Faculty of General Medicine, Charles University. The samples were synthesized.

pH dependences were measured in the Britton–Robinson buffer solution prepared according to a procedure described in ref.[16]. The following compounds were used in the buffer preparation: phosphoric acid — Merck, suprapur; boric acid, reagent grade — Lachema Brno; acetic acid, for semicondutors — Lachema Brno. The solutions used were prepared using water, twice distilled in a quartz apparatus and freed from traces of organic compounds by distillation with potassium permanganate. Oxygen was removed from solution by means of argon, purity 99·9%. Traces of oxygen in argon were removed in a washing bottle filled with a 5% solution of chromium dichloride.

Apparatus. Polarographic analyzer PA 4, Laboratory Instruments Works, Prague; coordinate recorder XY 4103, Laboratory Instruments Works, Prague; static drop electrode SMDE 1, Laboratory Instruments Works, Prague. The polarographic curves were recorded in a three-electrode connection with the calomel electrode as the reference electrode and the platinum electrode as the auxiliary one under the following conditions.

a) Differential pulse polarography: drop time 2 s; opening time of valve of static drop electrode 160 ms; rate of change of polarization voltage 2 mV/s; pulse amplitude +50 mV; time constant of analog memories 100 ms; pulse width 100 ms; time of taking current sample 20 ms.

b) Fast sweep differential pulse polarography: opening time of valve of static drop electrode 160 ms; rate of change of polarization voltage 10 mV/s; pulse amplitude +50 mV; time constant of analog memories 100 ms; time of taking current sample 20 ms; interval between pulses 0·2 s; waiting time between records of polarographic curve 5 s. The flow rate of the polarographic

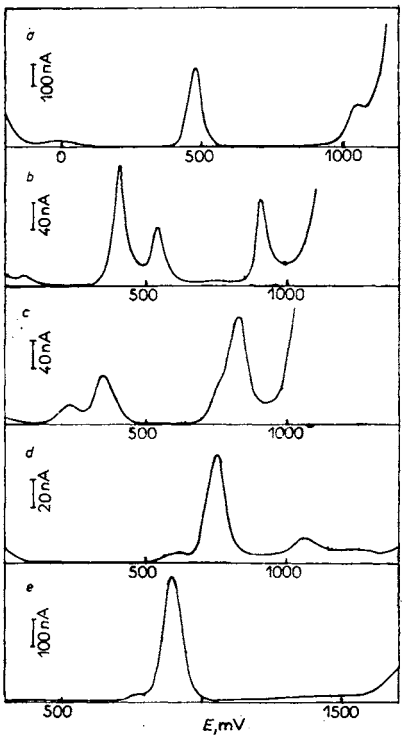


FIG. 1

Polarographic curves of individual pterins: differential pulse polarography at static drop electrode; *a* 6,7-dimethylpterin, $1{\cdot}9 \cdot 10^{-5}$ mol/l, pH 2; *b* xanthopterin, $2{\cdot}94 \cdot 10^{-5}$ mol/l, pH 2; *c* 6-pterinaldehyde, $3{\cdot}31 \cdot 10^{-5}$ mol/l, pH 2; *d* neopterin, $1{\cdot}76 \cdot 10^{-5}$ mol/l, pH 8. *e* Fast sweep differential pulse polarography at the stationary mercury drop electrode; 6-hydroxymethylpterin, $3{\cdot}53 \cdot 10^{-5}$ mol/l, pH 10·4

capillary used was 27·9 mg/s. Within the opening time of the valve, in the Britton–Robinson buffer with pH 2 and potential 0 mV (S.C.E.) the weight of the stationary mercury drop was 3·18 mg, which corresponds to the electrode area $1{\cdot}84 \cdot 10^{-2}$ cm^2. The detection limit was determined from the standard deviation of peak height at the lowest measured concentration. It may be calculated from

$$c_{\min} = t_N s/k ,$$

where t_N is the coefficient of Student's distribution for the given number of measurements and the given probability level (95%), s is the standard deviation and k is the slope of the calibration straight line. The detection limit thus determined gives the minimal difference in concentrations which may be detected with the given probability[18].

RESULTS AND DISCUSSION

6,7-*Dimethylpterin*

In polarograms recorded by the method of differential pulse polarography, a single peak was observed in the pH range 4·1 to 10·4. At pH 2, another peak coinciding with that of the reduction of H^+ appears in the record (Fig. 1*a*). The peak corresponding to the two-electron reduction of 6,7-dimethylpterin is shifted with increasing pH from −475 mV at pH 2 to −950 mV (Fig. 2*a*), and its height decreases with increasing pH (Fig. 3*a*).

A similar character has been observed for polarographic curves recorded by the method of fast sweep differential pulse polarography which have two peaks at pH 2 and a single peak at the other pH values. Potentials of the peaks on polarographic curves recorded by both methods correspond to each other. In the case of fast sweep differential pulse polarography, however, the height of the main peak depends on the direction of polarization of the working electrode; for polarization from positive potential values to negative ones, the peak of reduction of 6,7-dimethylpterin is several times higher that that recorded with the electrode with a renewed surface. When polarization of the electrode proceeded in an opposite direction, this effect was not observed.

TABLE I

Conditions of determination of pterins by differential pulse polarography

Compound	pH	E_p mV	Linearity range mol/l	Detection limit mol/l
6,7-Dimethylpterin	2·0	−475	$2 \cdot 10^{-5}$–$1 \cdot 10^{-7}$	$2{\cdot}5 \cdot 10^{-8}$
Xanthopterin	2·0	−410	$2 \cdot 10^{-6}$–$1 \cdot 10^{-7}$	$4 \cdot 10^{-8}$
6-Pterinaldehyde	2·0	−830	$2 \cdot 10^{-6}$–$1 \cdot 10^{-7}$	$3{\cdot}2 \cdot 10^{-8}$
Neopterin	8·0	−755	$2 \cdot 10^{-5}$–$1 \cdot 10^{-7}$	$8{\cdot}6 \cdot 10^{-8}$
6-Hydroxymethylpterin	10·4	−890	$3 \cdot 10^{-5}$–$1 \cdot 10^{-7}$	$7{\cdot}3 \cdot 10^{-8}$

A rise in sensitivity is caused by the adsorption of 6,7-dimethylpterin on the surface of the mercury electrode which takes place at potentials more positive than that of the peak. As documented by Fig. 4, maximal adsorption takes place in a neutral medium.

A certain disadvantage of fast sweep differential pulse polarography consists in the narrower concentration range in which the calibration curve is linear (Table II). In the case of differential pulse polarography at the static drop electrode the calibration curve is linear within the whole range of measured concentrations.

Xanthopterin

The polarographic behaviour is rather complicated, probably due to a number of acidobasic equilibria. In the acid range, at pH 2 and 4, four peaks may be observed in differential pulse polarograms (Fig. 1*b*); at pH 6, another fifth peak situated between the third and fourth one appears on the polarographic curve. In the alkaline

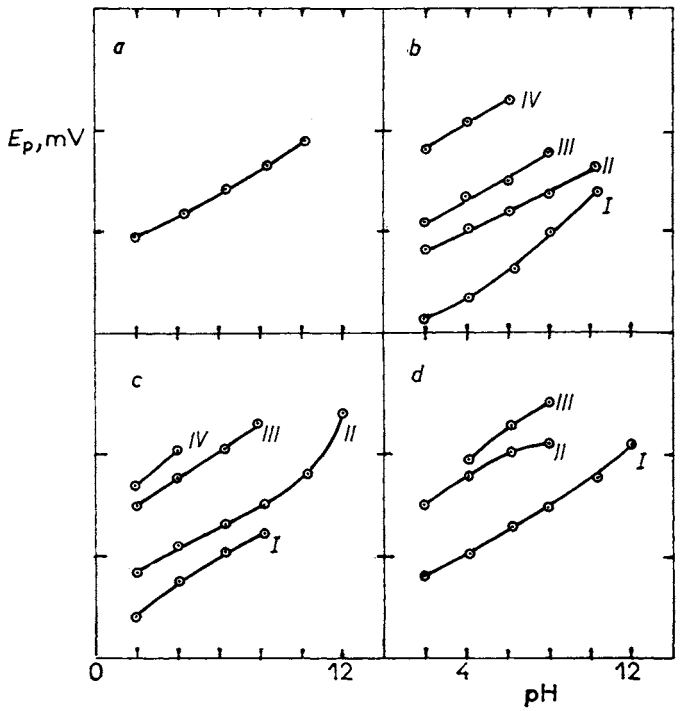


FIG. 2

Effect of pH on peak potentials on the polarographic curve recorded by the method of differential pulse polarography at the static drop electrode. Pulse amplitude +50 mV, drop time 2 s, scan rate 2 mV/s. *a* 6,7-Dimethylpterin $1{\cdot}91 \cdot 10^{-5}$ mol/l; *b* xanthopterin $2{\cdot}94 \cdot 10^{-5}$ mol/l; *c* neopterin $1{\cdot}76 \cdot 10^{-5}$ mol/l; *d* 6-hydroxymethylpterin $3{\cdot}53 \cdot 10^{-5}$ mol/l

range the number of peaks decreases with increasing pH, at pH 8, the fourth and fifth one disappear, at pH 10 also the third, so that only two peaks can be seen on the polarographic curve (Fig. 2*b*).

TABLE II

Conditions of determination of pterins by fast sweep differential pulse polarography

Compound	pH	E_p mV	Linearity range mol/l	Detection limit mol/l
6,7-Dimethylpterin	2·0	−455	$2 . 10^{-6} - 1 . 10^{-7}$	$2 . 10^{-8}$
Neopterin	8·9	−745	$2 . 10^{-6} - 1 . 10^{-7}$	$6{\cdot}4 . 10^{-8}$
6-Hydroxymethylpterin	10·4	−850	$3 . 10^{-5} - 1 . 10^{-7}$	$3{\cdot}9 . 10^{-8}$

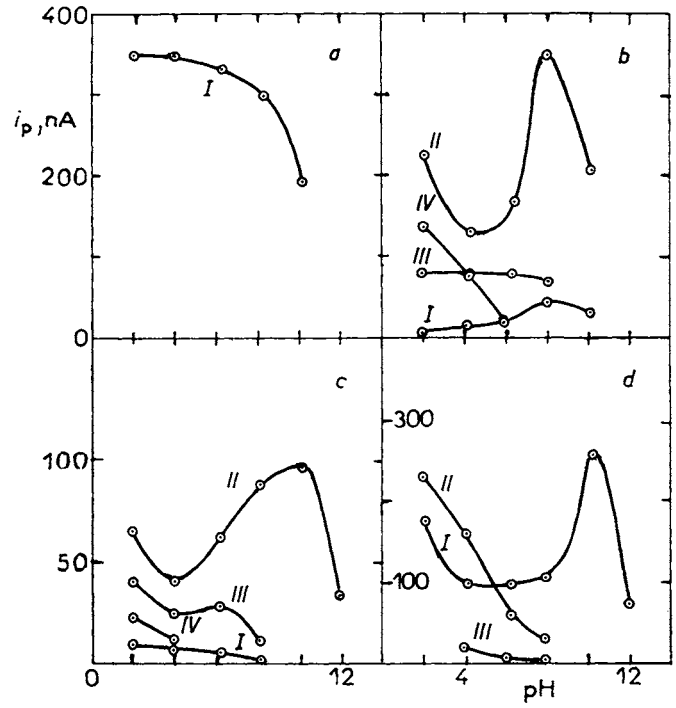


FIG. 3

Effect of pH on the peak heights on the polarographic curve recorded by the method of differential pulse polarography at the static drop electrode. Pulse amplitude +50 mV, drop time 2 s, scan rate 2 mV/s. *a* 6,7-Dimethylpterin $1{\cdot}91 . 10^{-5}$ mol/l; *b* xanthopterin $1{\cdot}91 . 10^{-5}$ mol/l; *c* neopterin $1{\cdot}76 . 10^{-5}$ mol/l; *d* 6-hydroxymethylpterin $3{\cdot}53 . 10^{-5}$ mol/l. Roman numeral denotes the serial number of the peak

As documented by Fig. 3*b*, the most suitable procedure for the analytical determination of xanthopterin is an evaluation of the second peak whose height assumes its maximal value at pH 2 and 8. A certain disadvantage, however, consists in the comparatively narrow concentration range in which the calibration curve is linear (Table I). At pH 2, also the fourth peak lying at the potential −0·9 V may be used for analytical purposes. In this case sensitivity is roughly half the usual value, but the calibration curve is linear within the whole range of concentrations under study.

Fast sweep differential pulse polarography at the stationary mercury drop electrode is not suitable for the determination of xanthopterin. A number of overlapping peaks, the heights of which cannot be exactly evaluated, can be seen on the polarographic curves.

6-Pterinaldehyde

Polarographic curves of this compound vary with time. With a freshly prepared solution, four peaks whose height decreases with time can be observed in the differential pulse polarogram. The stationary state is reached after a fortnight when the third peak disappears completely. All these facts suggest that a slow chemical reaction takes place in solutions of 6-pterinaldehyde in the Britton–Robinson buffer.

With respect to the small quantity of the sample which was at our disposal, the stability of solutions of 6-pterinaldehyde could not be verified in greater detail, and also it was not possible to prepare a fresh solution each time before the measurement. Thus the results reported below were obtained a fortnight after preparation of the basic solution, when the polarographic behaviour had already reached the stationary state.

Three peaks could be seen in the differential pulse polarograms within the whole pH range in which the test for polarographic activity was performed (Fig. 1*c*). The first two peaks overlap within the whole range of pH values under investigation. The third peak reaches its maximal height at pH 2 and 4. The second peak which attains its maximal height at pH 2 may also be used for the analytical determination. In both cases the calibration curves are linear within the concentration range $2 \cdot 10^{-6} - 1 \cdot 10^{-7}$ mol/l.

Three peaks can again be observed on polarographic curves recorded by the method of fast sweep differential pulse polarography at the stationary drop mercury electrode. The heights of the first and second peaks increase compared to the results obtained with the static drop electrode with renewed surface, due to adsorptive accumulation.

Neopterin

Differential pulse polarograms have four peaks in the acid medium at pH 2–4, three peaks in the neutral range at pH 6–9 (Fig. 1*d*) and only a single peak in the alkaline medium. The dependence of potentials of the peaks on pH is demonstrated in Fig. 2*c*; the effect of pH on the peak height can be seen in Fig. 3*c*.

The analytical determination is suitably performed using the second peak which appears within the whole range of pH values used in the investigation, the height of which reaches its maximal value at pH 8–10.

Similarly to the case of 6,7-dimethylpterin, also with neopterin at potentials more positive than that of the second peak the oxidized form of the depolarizer is adsorbed on the surface of the mercury electrode, so that, when the method of fast sweep differential pulse polarography at the stationary mercury electrode is used, the height of the second peak increases as a result of adsorptive accumulation. As demonstrated by Fig. 4, adsorption is highest at pH 8.

In view of these facts, the possibility of determination of neopterin by cathodic stripping voltammetry was verified. The optimal potential of electrolysis at pH 8 is -100 to -200 mV (S.C.E.) with the time of hydrolysis extended from 5 to 360 s by only 50%.

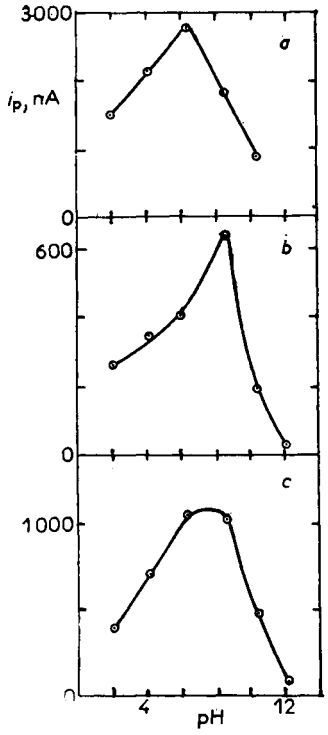


FIG. 4

Effect of pH on the sensitivity of determination by the method of fast sweep differential pulse polarography. Pulse amplitude +50 mV mV, scan rate 10 mV/s. *a* 6,7-Dimethylpterin, $1{\cdot}91 \cdot 10^{-5}$ mol/l; *b* neopterin $1{\cdot}76 \cdot 10^{-5}$ mol/l; *c* 6-hydroxymethylpterin $3{\cdot}53 \cdot 10^{-5}$ mol/l

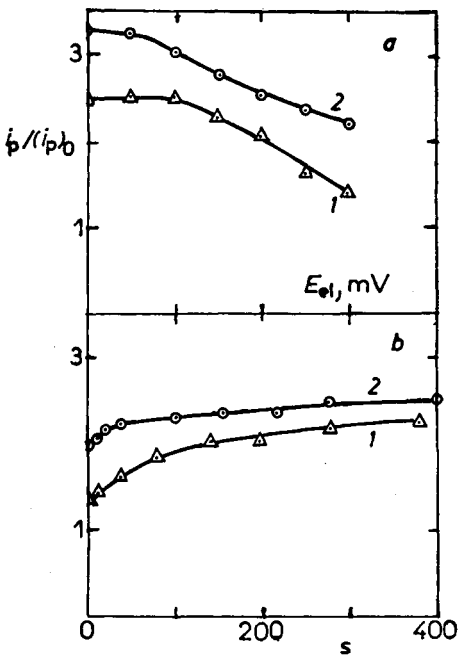


FIG. 5

Effect of the potential *a* and time *b* of electrolysis on the sensitivity of determination of pterins by cathodic dissolution voltammetry at the stationary drop electrode. Fast sweep dpp, pulse amplitude +50 mV, scan rate 10 mV/s; concentration: *1* neopterin $5{\cdot}6 \cdot 10^{-7}$ mol/l, *2* 6-hydroxymethylpterin $5{\cdot}64 \cdot 10^{-7}$ mol/l

All these results indicate that neopterin is best determined at pH 8. A higher sensitivity may be reached by using the method of fast sweep differential pulse polarography, but the concentration range in which the calibration curve is linear is comparatively narrow (Table II). In the case of differential pulse polarography at the static drop electrode the calibration curve is linear within the whole range of concentration used.

6-Hydroxymethylpterin

The number of peaks that can be observed in differential pulse polarograms at pH 2 is two, in weakly acid and neutral region there are three peaks and in the alkaline medium, only one (Fig. 2*d*). Analytical determination is suitably carried out using the first peak in series; this peak appears in the whole range of pH values in which the test for polarographic activity was carried out. Its height is maximal at pH 10 (Fig. 3*d*).

Polarographic curves recorded by the method of fast sweep differential pulse polarography have a similar character; in acid and neutral medium they have three peaks, the latter two overlapping each other, while in the alkaline medium there is only a single peak. Compared with the differential pulse polarography at the static drop electrode with renewed surface the first peak is approximately five times higher, due to the adsorptive accumulation of oxidized form of the depolarizer which takes place at potentials more positive than that of the first peak.

We verified also the possibility of determination by cathodic voltammetry using adsorptive accumulation. As documented by Fig. 5, the optimal potential of electrolysis lies in the range of potentials between 0 and 100 mV, but with the time of hydrolysis extended from 5 to 500 the peak height increases by mere 50%.

The calibration curves are linear for both methods, *i.e.*, differential pulse polarography at the static drop electrode and fast sweep differential pulse polarography at the stationary mercury drop electrode, within the whole range of investigated concentrations (Tables I, II).

CONCLUSION

The results reported in this study show that differential pulse polarography is a suitable analytical method for the determination of derivatives of pterin. Of the five pterins investigated, the method can be used in the determination of 6,7-dimethylpterin, xanthopterin, neopterin, and 6-hydroxymethylpterin. All the calibration curves are linear in the concentration range $2 . 10^{-6} - 1 . 10^{-7}$. With 6-pterinaldehyde, chemical changes occur in solution which impede its analytical determination, if detailed methodics are to be worked out, attention must therefore be concentrated on the problem of sample storage.

6,7-Dimethylpterin, neopterin, and 6-hydroxymethylpterin can also be determined by the method of fast sweep differential pulse polarography. The sensitivity is higher, but the concentration range in which the calibration curve is linear is usually narrower.

The two methods are compared in Tables I and II. In all cases the sensitivity of both methods lies below 0·02 ug/ml, which completely satisfies the requirements of analysis. Thus, differential pulse polarography is an adequate alternative of other analytical methods used in the detection of pterins.

The occurrence of adsorption observed for three of the pterins under study, *viz.*, 6,7-dimethylpterin, neopterin, and 6-hydroxymethylpterin, suggests the possibility of their determination by cathodic stripping voltammetry with adsorptive accumulation. In the Britton–Robinson buffer, however, the increase in sensitivity with extended time of electrolysis (Fig. 5) is rather small. When working out the respective methodics, attention should be devoted to the choice of a suitable basic electrolyte.

In view of mutual interferences, several pterins cannot be determined simultaneously. Practical analyses of the pterin content in urine call for a suitable separation method. Probably the most adequate analytical method for simultaneous determination of all the five pterins is liquid chromatography with electrochemical detection; the method will be examined in detail in the forthcoming part of this study.

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Note added in proof: In Fig 5*a*. the middle point on the abscissa corresponds to the −400 mV.