REACTION OF NUCLEIC ACID BASES WITH THE MERCURY ELECTRODE: DETERMINATION OF SUBMICROMOLAR CONCENTRATIONS OF PYRIMIDINE BASES BY MEANS OF CATHODIC STRIPPING VOLTAMMETRY

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Pyrimidine bases commonly occurring in nucleic acids *i.e.* cytosine, uracil and thymine and further pyrimidine derivatives such as 5-methylcytosine, 5-hydroxymethylcytosine, isocytosine, uracil-6-carboxylic acid (orotic acid), 5-amino-2,4-dioxypyrimidine, 2-amino-4,6-dioxypyrimidine and 4-amino-2,6-dioxypyrimidine and thio derivatives and halogen derivatives of uracil and cytosine were analyzed by means of cathodic stripping voltammetry (CSV). As compared with most of the sulphur-containing substances the deposition potential optima of the pyrimidine derivatives (not containing sulphur) were substantially narrower and shifted to more positive potentials. Cytosine, thymine and uracil can be determined by means of differential pulse CSV at concentrations of the order of magnitude 10^{-7}—10^{-8} mol/l. Nucleosides and nucleotides derived from the pyrimidine bases are inactive and do not substantially interfere with the determination of bases. Bases can be determined even in an excess of DNA and proteins.

We have shown in our preceding paper[1] that pyrimidine bases commonly occurring in nucleic acids and a number of other pyrimidine derivatives yield anodic differential pulse-polarographic (DPP) peaks, normal pulse-polarographic (NPP) and d.c. polarographic waves conditioned by the formation of sparingly soluble compounds with mercury, when a dropping mercury electrode is used. With the aid of a hanging mercury drop electrode (HMDE) we have demonstrated that these substances can be stripped out by scanning in a cathodic direction; we thus formed the basis for an elaboration of a highly sensitive method of determination of these bases by means of the cathodic stripping voltammetry (CSV)[2-4]. In our preceding paper[1] we have worked with concentrations of bases not lower then $5 \cdot 10^{-6}$ mol/l. It will be shown in this paper that pyrimidine bases can be determined by means of CSV at concentrations of the order of magnitude $10^{-7}-10^{-8}$ mol/l.

EXPERIMENTAL

2-Thiouracil was from Fluka and 5-fluorouracil from Calbiochem (Switzerland), 4-thiouracil. 2-thio-6-aminocytosine, 5-chlorouracil, 5-iodouracil, 5-bromocytosine, 5-bromouracil and 5-iodo-

cytosine were purchased from Lachema (Czechoslovakia), 4-thiouridine was kindly donated by Dr M. Wrona (University of Warszaw). Other chemicals and apparatus were the same as in our previous paper[1].

Cathodic Stripping Voltammetry

The use of CSV in the analysis of organic substances is based on their ability to react with the mercury electrode and to form sparingly soluble compounds with mercury[2-4].

The solution to be analyzed was first dearated using oxygen-free nitrogen for 10 min. Experiments were performed with a hanging mercury drop electrode (HMDE) using mostly 6- or 4-division drop (with a surface of 2·9 mm^2 or 1·8 mm^2). A fresh drop of mercury was dialled out and dislodged. This was repeated and the next drop used for the experiment. The initial (deposition) potential was set close to 0 V prior to deposition mostly for 2—6 min. During the deposition the solution was stirred by a stream of nitrogen, controlled by a manometer. With deposition potential still being applied to the cell the stirring was stopped and the solution allowed to come to rest for another 20 s (quiescent period). To observe the stripping phase DPP or linear potential sweep voltammetry (LPSV) was used. Setting for DPCSV: scan rate 5 mV . s^{-1}, pulse amplitude 25 mV, scan range 0·75 V; pulses were applied at 0·5 s intervals. LPCSV: scan rate 200 mV s^{-1}, scan range 0·75 V, deposition time 30 s, without stirring. In experiments where LPCSV and DPCSV methods were compared stirring and deposition time in LPCSV were the same as in DPCSV.

RESULTS

In CSV, the voltammetric peak height and thus also the sensitivity of the determination are dependent on several parameters, as are the potential and time of deposition, the mercury electrode size, the scan rate, *etc.*[2-4]. Therefore the influence of these parameters was tested with the aim of finding optimal conditions for the CSV analysis of pyrimidine bases. Most of the CSV measurements in the present study (and thus also the seeking of the optimal conditions of the analysis) were carried out in connection with differential pulse polarography (DPP), which served as a method for observing the stripping phase.

Deposition

Dependence on the deposition potential was investigated for cytosine, uracil, thymine and 2-thiouracil. As compared with studies using CSV for analyzing sulfphur-containing substances[5-9], a more pronounced dependence of the peak height on the deposition potential was observed in the analysis of uracil, cytosine and thymine (Fig. 1). For 2-thiouracil, which was used as a representative of the sulphur-containing substances, the peak height was independent on the deposition potential in the region from 0·0 V up to +0·15 V. The optimum of the deposition potential for uracil and cytosine was at about +0·1 V. Whereas the curve of uracil had only one, rather broad peak, for cytosine two peaks were observed. The deposition potential suitable for determining the two substances varied about +0·1 V. The use of the

deposition potential corresponding to the more positive peak of the cytosine dependence (Fig. 1), *i.e.* approximately +0·15 V, did not lead to an increase of the detection limit of this substance. The optimum of the deposition potential of thymine was at about +0·23 V at pH 7·8. The peak of the bases increased in dependence on the size of the mercury drop surface. For this reason the measurements were carried out at largest possible drop, which exhibited good stability and reproducibility. In dependence on the deposition time the base peak increased under the given conditions nearly linearly up to 6–8 min; the following growth was less steep. The majority of measurements was therefore performed under conditions of 6 min deposition.

Stripping

The peak height grew with increasing scan rate in the limits 1–5 mV/s; an increase of the scan rate to 10 mV/s led to a lesser increase of the peak for cytosine, while remaining without effect for uracil. In accord with results of our preceding study a pulse amplitude of 25 mV proved to be the most suitable one. For some bases an increase of amplitude to 50 mV (*e.g.* for thymine) led to a decrease of the peak; for others (*e.g.* for cytosine) the peak height increased with increasing amplitude. At the amplitude of 100 mV for all bases the peaks were lower (or even deformed) compared with amplitude of 50 mV.

Both uracil and cytosine yielded well developed DPCSV peaks at pH 10·5 (Fig. 2). The heights of these peaks depended linearly on the base concentration in a rather narrow concentration range: under the conditions given in Fig. 3 the range was approximately from $5 \cdot 10^{-8}$ to $4 \cdot 10^{-7}$ mol/l. At higher concentrations the peak height grew with increasing concentration less steeply. The peak of thymine increased, under the given conditions, in the range $1 \cdot 10^{-7}$–$4 \cdot 10^{-4}$ mol/l considerably less steeply as compared with other bases. A somewhat more steep increase of the thymine peak was observed at pH 7·8; however, the peak remained considerably

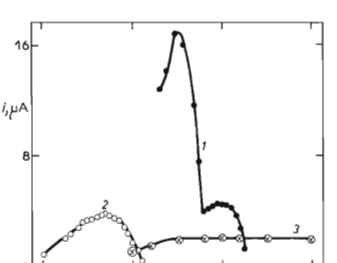

FIG. 1

Dependence of DPCSV Peak Heights of Cytosine, Thymine and 2-Thiouracil on Deposition Potentials

1 $5 \cdot 10^{-7}$M Cytosine; *2* $4 \cdot 10^{-7}$M thymine; *3* $4 \cdot 10^{-7}$M 2-thiouracil. Borate buffer pH 10·5 (pH 7·8, for thymine), HMDE surface 2·9 mm^2, deposition time 2 min.

lower than those of uracil and cytosine at pH 10·5 (Fig. 3). E_s of the uracil peak was even 40 mV more negative than E_s of the cytosine peak and for both compounds was shifted to more negative values with the base concentration. $W_{1/2}$ was approxi-

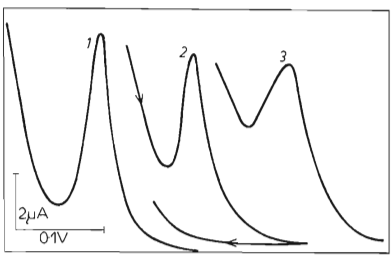

FIG. 2

DPCSV Peaks of Uracil, Cytosine and Thymine

1 Uracil; *2* cytosine; *3* thymine. *1*, *2* borate buffer pH 10·5; *3* borate buffer pH 7·8. Bases at a concentration of $4 . 10^{-7}$M, HMDE surface 2·9 mm^2, deposition potential +0·1 V (+0·025 V for thymine), deposition time 6 min.

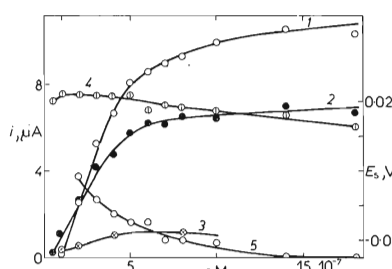

FIG. 3

Dependence of Heights (*i*) and Potentials (*Es*) of DPCSV Peaks on Uracil, Cytosine and Thymine Concentrations

1, *4* Uracil; *2*, *5* cytosine; *3* thymine; *1*, *2*, *3* i; *4*, *5* E_s. Borate buffer pH 10·5. HMDE surface 2·9 mm^2, deposition potential +0·1 V; deposition time 6 min.

mately 30 mV for both peaks and was nearly independent of the base concentration in the concentration range studied. The reproducibility of the base peaks was very good under the given conditions. Cytosine in a concentration of $2 \cdot 10^{-7}$ mol/l could be determined with a mean error of ±2%. Stripping of the uracil deposit in the course of the first potential scan to more negative values reached approximately 90%.

In the preceding study[1] several pyrimidines were tested by means of voltammetry with linear potential sweep (LPS) CSV at a concentration of $5 \cdot 10^{-5}$ mol/l, *i.e.* at a concentration rather high for this method. In the present study we again tested these substances by means of DPCSV in a concentration of $1 \cdot 10^{-6}$ mol/l (the deposition time 6 min, the deposition potential +0·1 V). In accord with the preceding results 2-hydroxypyrimidine and 2-aminopyrimidine were inactive (Table I), isocytosine, 5-methylcytosine and 5-hydroxymethylcytosine yielded very small peaks or inflextions, whereas, uracil, cytosine, thymine, uracil-6-carboxylic acid (orotic acid), 5-amino-2,4-dioxypyrimidine, 2-amino-4,6-dioxypyrimidine, and 4-amino-2,6-dioxypyrimidine yielded well developed peaks (Fig. 4). The following thioderivatives and halogen derivatives of uracil and cytosine were also tested: 2-thiouracil, 4-thiouracil, 4-thiouridine, 2-thio-6-aminocytosine, 5-chlorouracil, 5-bromouracil, 5-iodouracil, 5-fluorouracil, 5-bromocytosine, and 5-iodocytosine. All these substances in a concentration of $5 \cdot 10^{-5}$ mol/l yielded on the hanging drop mercury electrode (HMDE) anodic DPP peaks and LPCSV peaks. More detailed information about the behaviour of these substances will be published elsewhere.

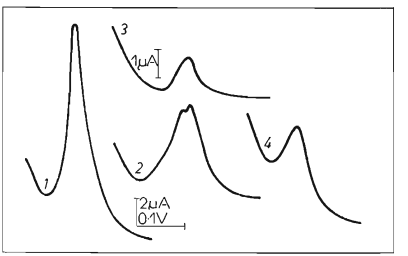

FIG. 4

DPCSV Peaks of Pyrimidine Derivatives

1 $1 \cdot 10^{-6}$M 5-Amino-2,4-dioxypyrimidine; *2* $1 \cdot 10^{-6}$M orotic acid; *3* $2{\cdot}5 \cdot 10^{-7}$M orotic acid; *4* $1 \cdot 10^{-6}$M 2-amino-4,6-dioxypyrimidine. Borate buffer pH 10·5. HMDE surface: *1*, *2*, *4* 2·9 mm^2; *3* 1·8 mm^2. Deposition time: *1*, *2*, *4* 6 min; *3* 2 min. Deposition potential +0·1 V.

In addition we tested the influence of some substances which are used in separation or purification steps in work with pyrimidine bases, and can thus be contained in the samples taken for analysis. The DPCSV peak yielded by solutions of $3 \cdot 10^{-7}$ mol/l uracil or cytosine was slightly increased (by less than 10%) in the presence of 0·015 mol/l NaCl with 0·0015 mol/l sodium citrate (SSC/10 – used frequently in the work with polynucleotides); however, higher concentrations of chlorides influenced the peak height significantly and should therefore be avoided. A small increase of the peak was induced by the presence of 0·01 mol/l sodium phosphate and 0·1 mol/l $NaClO_4$. Changes in concentration of borate buffer within the range 0·025–0·1 mol/l and the presence of K_2SO_4 in concentrations of 0·05–0·33 mol/l influenced the peak height only slightly. $1 \cdot 10^{-4}$ mol/l EDTA yielded its own peak, which overlapped the base peak. The following substances decreased significantly the peak height: 0·1M Tris, polyethylene glycol (10 μg/ml), lysozyme (10 μg/l).

Greater attention was paid to the influence of proteins and nucleic acids on the DPCSV peak height of the bases, because the nucleic acid bases should often be determined in the presence of these substances. Lower protein concentrations caused only a small decrease of the peak height of uracil; the uracil peak corresponded to 78% of its original height with a nearly tenfold (by weight) protein excess. The influence of the presence of DNA was manifested in a similar way. These results indicate that CSV could be used for investigating changes in concentration of pyrimidine bases in various enzymatic reactions and other processes in the presence of a small excess of nucleic acids and proteins.

Determation of Bases, Nucleosides and Nucleotides in Mixtures

Uracil can be determined in the presence of thymine at pH 10·5; (the peak height of uracil in a concentration of $6 \cdot 10^{-7}$ mol/l remained unchanged after the addition of thymine in a concentration of $2 \cdot 10^{-7}$ mol/l, thymine in the equimolar concentration decreased the uracil peak height by 10%). Even though E_s of the peaks of uracil and cytosine (Fig. 2) are different, it is not possible to distinguish them in a mixture. Addition of cytosine to uracil in the equimolar concentration ($3 \cdot 10^{-7}$ mol/l) led to a broadening (and an increase) of the peak. The influence of the presence of one of the two substances could nearly be eliminated by a suitable change of the deposition potential, the sensitivity of the determination was however, decreased. In accord with results of the preceding study[1] cytidylic acid behaved as an inactive compound (in concentrations up to $1 \cdot 10^{-5}$ mol/l). The equimolar concentration of this substance decreased slightly the peak height of $6 \cdot 10^{-7}$ mol/l cytosine. Higher concentrations of cytidylic acid caused an increase of the cytosine peak ($1{\cdot}1 \cdot 10^{-6}$ by 10%, $1{\cdot}1 \cdot 10^{-5}$ mol/l by about 20%). The presence of an excess of uridine or uridylic acid lead, on the other hand, to a decrease of the uracil peak.

DISCUSSION

It is usually assumed in the literature that the use of CSV in organic analysis is limited mainly to sulphur-containing substances[5-7,9]. The results presented in this paper and other of our works[1,10,11] show that there exist a large group of heterocyclic compounds not containing sulphur, which react with the electrode mercury and can be determined by means of CSV. In the CSV analysis of these substances it is necessary to pay sufficient care to experiments dealing with the influence of the deposition potential on CSV peaks; the range of optimum deposition potentials is usually rather narrow and is located at positive potentials (Fig. 1). On the other hand in sulphur-containing organic substances the regions of the deposition potential optima are considerably wider[9] and stripping peaks appear at more negative potentials, usually around $-0{\cdot}5$ V. If the former substances are tested by means of CSV at one deposition potential only, inactivity of a certain substance does not exclude the possibility of its determination by means of CSV when a more suitable deposition potential is applied. Our statement that 5-methylcytosine, 5-hydroxymethylcytosine and isocytosine (Table I) produced at a concentration of $1 \cdot 10^{-6}$ mol/l only inflections should be considered from this point ov view. Considering the fact that these substances produced anodic DPP peaks[1] it seems to be highly probable that after finding the deposition potential optima it will become possible to determine these substances at low concentrations by means of CSV, similarly to *e.g.* cytosine. In testing a large group of substances it is thus advisable first to find under what conditions they produce anodic d.c. polarographic or NPP, DPP and voltammetric peaks, respectively, and then subject them, under suitable conditions, to detailed CSV analysis at various deposition potentials.

Limits of detection of uracil, cytosine and thymine ($\sim 5 \cdot 10^{-8}$ mol/l) estimated in this paper do not represent definite values. A significant increase in sensitivity might be reached by increasing the electrode surface. This can be achieved by using mercury film electrodes; work with them is, however, somewhat more complicated than with the HMDE, and their use is not dictated by the need to limit the deposit diffusion into the electrode mercury as is the case with metal deposits in anodic stripping voltammetry[2-4]. The deposit which is formed in CSV is poorly soluble and cannot therefore diffuse into the mercury. Thus the use of larger mercury pool electrodes in CSV looks promising. It will, however first be necessary to find out whether the lower stability of the surface of these electrodes will not cause an undesirable tearing of the deposited insoluble films and other phenomena which could unfavourably influence the sensitivity and accuracy of the analysis.

Limits of detection of the bases can also be decreased by increasing the deposition time. In this case, however, the disadvantage due to time-consuming procedure will not in most cases be compensated by the gain in sensitivity. The detection limits estimated in this paper by means of DPCSV and LPCSV differed only slightly

CSV determination of the pyrimidine bases can be thus performed even in laboratories where no pulse polarograph is available.

So far the determination of unlabelled pyrimidine bases at concentrations below the detection limit of UV spectrophotometry has been limited to postlabelling with a radioactive tracer[12,13]. Thus the CSV analysis of pyrimidine bases represents the most sensitive method of determination of these bases which does not require radioactive tracers. Combination of CSV with the high pressure liquid chromatography might result in greater sensitivity and resolution of the chromatographic analysis. Chromatographic electrochemical detectors which have recently been designed[14,15] are not constructed for the CSV analysis; our preliminary results[11] suggest that electrochemical detection based on principles of CSV will be possible. CSV in combination with various separation techniques can even now replace the usual UV-spectrophotometric method, with a gain in sensitivity of at least one order of magnitude. In some cases it will be possible to take advantage of the fact that nucleosides and nucleotides do not significantly interfere with the determination of bases for the elimination or shortening of certain separation steps. The fact that bases can be determined in an excess of polynucleotides is of great importance

TABLE I

CSV of Pyrimidine Derivatives

Pyrimidine derivatives at a concentration of $1 \cdot 10^{-6}$ mol/l, borate buffer pH 10·5 (pH 7·8 for thymine). Deposition time 6 min, deposition potential +0·1 V. HMDE surface 2·9 mm^2. +, Stripping peak; I, inflection; —, neither peak nor inflection appeared under the given conditions.

Substance	CSV response
Uracil	+
Cytosine	+
Thymine	+
5-Methylcytosine	I
5-Hydroxymethylcytosine	I
Orotic acid	+
2-Amino-4,6-dioxypyrimidine	+
4-Amino-2,6-dioxypyrimidine	+
5-Amino-2,4-dioxypyrimidine	+
2-Aminopyrimidine	—
2-Hydroxypyrimidine	—
Isocytosine	I

in biochemical analysis. Our recent results show that besides pyrimidine derivatives purine bases can be determined by means of CSV with about the same sensitivity. It can be thus expected that CSV will soon become an important tool in nucleic acid research.

As regards the analysis of pyrimidine derivatives which do not represent the usual nucleic acid components, the CSV determination of orotic acid, which is a precursor in nucleic acid synthesis, might find important place in biochemical analysis. E_s of the CSV peak of this substance is more negative than E_s of the uracil peak and the limit of orotic acid detection is lower. Polarographic behaviour of the thiouracil derivatives has been described in earlier papers[16–18] which showed that these substances form salts with mercury and yield anodic d.c. polarographic waves. So far no attempt has been made to use CSV for their analysis. 2-Thiouracil and 4-thiouracil are contained in some tRNA as minor bases[19] and the possibility of their microdetermination by means of CSV may be important. As compared with uridine, which is CSV inactive, 4-thiouridine produces well-developed CSV and anodic DPP peaks. It is thus not excluded that it will be possible to electrochemically determine thiouracils directly in tRNA without prior hydrolysis. The CSV determination of halogenated bases and further pyrimidine derivatives frequently used in the molecular-biological research and in pharmacy may also become important.

While in the final stages of writing this paper we have read an article recently published by Florence[20], who tested by means of CSV a number of various substances and found that with small exceptions (flavines, flavones, pterines and porphyrines) the appearance of the CSV peaks was limited to the sulphur-containing substances. In his experiments uracil and cytosine did not produce CSV peaks. The tests were performed at the deposition potential $-0{\cdot}1$ V, deposition time 5 min, in sodium borate at a base concentration $1 \cdot 10^{-6}$ mol/l and CSV peaks were measured in the potential range from $-0{\cdot}2$ to $-1{\cdot}2$ V. It follows from this paper as well as from the preceding one[1] that the procedure chosen by Florence[20] could not yield positive results because the deposition potentials and potentials of the stripping peaks of pyrimidine bases are located at more positive values. It is thus possible that further substances not containing sulphur classified in the Florence paper[20] as inactive will appear as CSV active if analysed under a more suitable conditions. It can be concluded that the approach to testing substances for their CSV activity used in this and in our previous paper[1] is more laborious, but it yields more reliable results than the way chosen by Florence[20].

REFERENCES

1. Paleček E., Jelen F., Manoušek O.: This Journal *45*, 3460 (1980).
2. Barendrecht E. in the book: *Electroanalytical Chemistry* (A. J. Bard, Ed.), Vol. 2, p. 53. M. Dekker, New York 1967.
3. Brainina Kh. Z.: Talanta *18*, 513 (1971).

4. Vydra F., Štulík K., Juláková E.: *Roupouštěcí polarografie a voltammetrie.* Published by SNTL, Prague 1977.
5. Davidson I. E., Smyth W. F.: Anal. Chem. *49*, 1195 (1977).
6. Smyth M. R., Osteryoung J. G.: Anal. Chem. *49*, 2310 (1977).
7. Smyth M. R., Smyth W. F.: Analyst (London) *103*, 529 (1978).
8. Kůta J., Paleček E. in the book: *Topics in Bioelectrochemistry and Bioenergetics* (G. Milazzo, Ed.), in press.
9. Smyth W. F., Davidson I. E.: *Proc. Conf. Electroanalysis in Hygiene, Environmental, Clinical and Pharmaceutical Chemistry* (W. F. Smyth, Ed.), p. 271, Elsevier, London 1980.
10. Paleček E.: Anal. Lett. *13*, 331 (1980).
11. Paleček E.: Unpublished results.
12. Randerath K., Randerath E. in the book: *Procedures in Nucleic Acid Research* (G. L. Cantoni, D. R. Davies, Eds), Vol. 2, p. 796. Harper and Rox, New York 1971.
13. Davies P. L., van de Sande J. H., Dixon G. H.: Anal. Biochem. *93*, 26 (1979).
14. Schwartzfager D. G.: Anal. Chem. *48*, 2179 (1976).
15. *Polarographic Instrumentation.* Princeton Applied Research Research T-391-6/78 AO (1978).
16. Dryhurst G.: *Electrochemistry of Biological Molecules*, p. 224. Academic Press, New York 1977.
17. Wrona M., Czochralska B.: J. Electroanal. Chem. Interfacial Electrochem. *48*, 433 (1973).
18. Wrona M., Czochralska B., Shugar D.: J. Electroanal. Chem. Interfacial Electrochem. *68*, 355 (1976).
19. McCloskey F., Nishimura S.: Accounts Chem. Res. *10*, 403 (1977).
20. Florence T. M.: J. Electroanal. Chem. Interfacial Electrochem. *97*, 219 (1979).

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