REACTIONS OF BASES OF NUCLEIC ACIDS WITH MERCURY ELECTRODE. ANODIC PULSE-POLAROGRAPHIC CURRENTS OF PYRIMIDINE BASES AND THEIR DERIVATIVES

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Anodic currents of pyrimidine bases were studied by using differential and normal pulse polarography, cyclic voltammetry, and classical polarography in alkaline medium. All pyrimidine bases occurring usually in nucleic acids (cytosine, uracil, thymine, 5-methylcytosine, and hydroxymethylcytosine) gave anodic DPP peaks, NPP and d.c. polarographic waves at potentials close to 0 V due to the formation of sparingly soluble salts with mercury. Nucleosides and nucleotides derived from cytosine, uracil, and thymine were inactive. The mentioned bases and other pyrimidine derivatives (isocytosine, uracil-5-carboxylic acid, uracil-6-carboxylic acid (orotic acid), 2-amino-4,6-dioxypyrimidine, 2-amino-4,6-dioxy-5-methylpyrimidine, 4-amino-2,6-dioxy-pyrimidine, and 5-amino-2,4-dioxypyrimidine) gave anodic peaks and cathodic stripping peaks during reversal of the potential sweep on a hanging drop electrode, whereas 2-aminopyrimidine and 2-hydroxypyrimidine were inactive; the concentrations used were $5 \cdot 10^{-6}$—$5 \cdot 10^{-5}$M. The behaviour of cytosine, uracil, and thymine in DPP and NPP was studied at various concentrations of the bases, pH values, pulse amplitudes, *etc.* Differences between the behaviour of uracil and thymine were large at higher concentrations at which the electrode surface was fully covered, and they were explained on the assumption of a different adsorption behaviour of these compounds and a large tendency of uracil to form a film on the electrode.

More than twenty years ago, we showed[1] that uracil and thymine give anodic d.c. polarographic waves in an alkaline medium and "reversible" incisions on the oscillopolarographic $dE/dt = f(E)$ curves[2,3]. The latter were similar with other pyrimidine bases of nucleic acids, cytosine[2,3] and 5-methylcytosine[3]. This behaviour was attributed to the formation of sparingly soluble salts with mercury[1,3] and its importance was not recognized for a long time mainly because the sensitivity and accuracy of d.c. polarographic and oscillopolarographic analyses could not satisfy the demands of analytical chemistry. The modern methods of electroanalysis, mainly the derivative (differential) pulse polarography[4] (DPP) make it possible to utilize even the polarographic behaviour of compounds giving ill-defined d.c. polarographic waves or oscillopolarographic incisions. Compounds reacting with mercury can be determined by cathodic stripping voltammetry[4,5], which in combination with DPP, so-called differential pulse cathodic stripping voltammetry (DPCSV), enables to determine compounds in concentrations down to 10^{-7} to 10^{-8}M. The latter method has found recently important applications especially in determining pesticides and other trace impurities in biological material[4-7].

In view of the high sensitivity of DPP and DPCSV, we began anew to study the anodic polarographic currents of nucleic acid bases due to formation of sparingly soluble compounds with mercury. We also started a systematic study of pyrimidine derivatives having a close relation to nucleic acid bases. The object of the present work is to show that all pyrimidine bases of nucleic acids and some of their derivatives can be determined by DPP with a detection limit of the order of 10^{-6}M; and this sensitivity can be significantly enhanced by combining DPP with cathodic stripping voltammetry.

EXPERIMENTAL

Chemicals

Uracil, thymine, cytosine, 5-hydroxymethylcytosine, 2-amino-4,6-dioxypyrimidine, cytidine, uridine, thymidine, cytidylic acid, uridylic acid, and thymidylic acid were supplied by the firm Calbiochem, 4-amino-2,6-dioxypyrimidine and uracil-5-carboxylic acid were from Edward Willsone Laboratories, 5-amino-2,4-dioxypyrimidine from the firm Light, 5-methylcytosine, 2-amino-4,6-dioxy-5-methylpyrimidine, and orotic acid from Lachema. Isocytosine was kindly furnished by Dr A. Holý, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. Other chemicals were of reagent grade (Lachema) and were used without further purification. Pyrimidine bases and their derivatives were dissolved in triply distilled water and their about 1—5 mM stock solutions were stored in a refrigerator. Their concentration was determined spectrophotometrically at the conditions (pH, μ, λ_{max}, ε_{max}) given by the manufacturers. A borate buffer solution was prepared by mixing 0·05M-H_3BO_3 with 0·05M-KOH.

Apparatus

All polarographic measurements were done on a PAR Model 174 Polarographic Analyser with a three-electrode system. A saturated calomel electrode with a salt bridge filled with supporting electrolyte served as reference and a platinum wire of a large surface area as a counter electrode. Polarograms were recorded on an Omnigraphic Model 9002A X-Y recorder. The dropping mercury electrode had a rate of flow 1·82 mg/s at a height of mercury column 48 cm. The drop time was kept constant by a drop time controller PAR Model 172A. The dependence of the polarographic wave height on the height of mercury column was measured with the use of another capillary with a rate of flow 2·5 mg/s at a height of mercury column 49 cm. Voltammetric and some NPP and DPP measurements were made with a Metrohm E 410 hanging mercury drop electrode. Spectrophotometric measurements were done on a VSU 2-P type apparatus, the pH was measured on Metrohm Compensator E 388. The polarographic cell was a commercial conical cell supplied by the firm PAR with 5—10 ml holding capacity. The measured solutions were deaerated by bubbling nitrogen for 10 min, the temperature of measurement was 25°C. In d.c. polarography, NPP, and DPP, anodic currents were recorded at a potential sweep rate of 1 mV/s. Details about cathodic stripping voltammetry will be given in our next communication[8].

RESULTS

Measurement with a Dropping Mercury Electrode

Pyrimidine Bases of Nucleic Acids

Uracil, thymine, cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine gave anodic DPP peaks, NPP and d.c. polarographic waves at potentials close to 0 V (Figs 1 and 2). Since DPP is considered most suitable for analytical purposes, we preferred this method.

The dependence on pH was followed at a concentration of $5 \cdot 10^{-5}$M in borate buffer in the case of thymine, uracil, and cytosine. The height of the DPP peak for

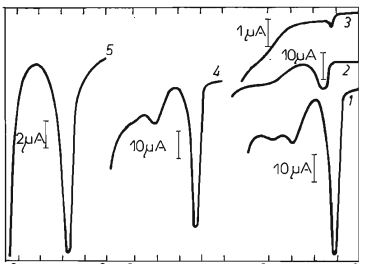

FIG. 1

DP, NP and d.c. Polarograms of Uracil

Uracil concentration: *1*—*3* $1 \cdot 10^{-3}$M; *4* $5 \cdot 10^{-4}$M; *5* $1{\cdot}25 \cdot 10^{-4}$M; *1*, *4*, *5* DPP; *2* NPP; *3* d.c. polarogram. Borate buffer, pH 10·5.

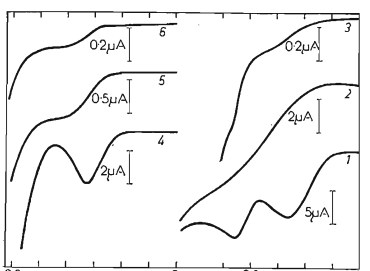

FIG. 2

DP, NP and d.c. Polarograms of Thymine

Thymine concentration: *1*—*3* $1 \cdot 10^{-3}$M; *4*—*6* $6{\cdot}25 \cdot 10^{-4}$M; *1*, *4* DPP; *2*, *5* NPP; *3*, *6* d.c. polarogram. Borate buffer, pH 7·8.

thymine decreased with increasing pH from 8 to 11 (Fig. 3) and was highest at pH 7·9 to 7·6 where it was little dependent on pH. Therefore, most measurements were done in this region, where the mean width of the peak ($W_{1/2}$) was about 55 mV. This value increased moderately with pH, then (near pH 9·5) strongly increased until at pH 11·2 it attained nearly 80 mV. The peak potential (E_s) shifted to more negative values with increasing pH and the intersection of both linear parts at pH 9·5 as well as their slopes were in agreement with values found earlier for uracil by d.c. polarography[1]. The shift of the DPP peak potential to more negative values caused by increasing pH was observed also with cytosine and uracil. The peak height for cytosine was nearly independent of pH from 10 to 11·5, but it decreased rapidly below pH 9·5. The peak height of uracil was independent of pH in about the same region; above pH 11 and below pH 9 it decreased, but less abruptly than with cytosine. Further measurements with cytosine and uracil were done mostly near pH 10·5, where both peaks were almost independent of pH.

Dependence of DPP *peaks on the pulse amplitude.* The DPP peak for thymine at a concentration of $6 \cdot 10^{-5}$M increased linearly with the pulse amplitude in the range 5–25 mV (Fig. 4); a deviation form linearity took place at 50 mV and eventually at 100 mV the peak was lower. The value of $W_{1/2}$ was independent of the amplitude from 5 to 25 mV, and above this value it increased. The value of E_s shifted with

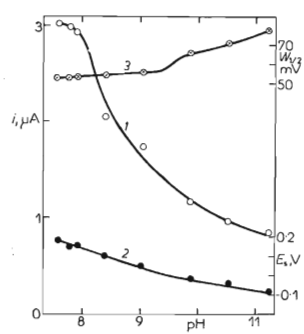

FIG. 3

Dependence of Height (i), Half-Width ($W_{1/2}$) and Potential (E_s) for DPP Peak of Thymine on pH

$6 \cdot 3 \cdot 10^{-5}$M thymine in borate buffer. *1* i; *2* E_s; *3* $W_{1/2}$.

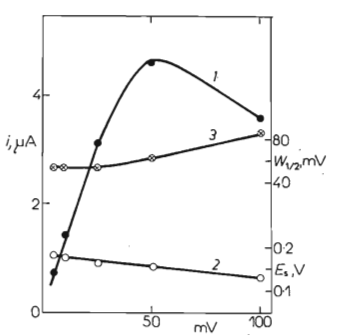

FIG. 4

Dependence of Height (i), Half-Width ($W_{1/2}$) and Potential (E_s) for DPP Peak of Thymine on Pulse Amplitude

$6 \cdot 3 \cdot 10^{-5}$M thymine in borate buffer, pH 7·8. *1* i; *2* E_s; *3* $W_{1/2}$.

increasing amplitude to more positive values in accord with theory. The wave height of uracil and cytosine (at concentrations corresponding to that of thymine) changed with the pulse amplitude (at pH 10·5) analogously. The changes of E_s and $W_{1/2}$ of the uracil peak at a higher concentration (10^{-3}M) with the amplitude were much different (Fig. 5); E_s was independent of the amplitude from 5 to 25 mV and above this value was shifted to more negative values, while $W_{1/2}$ increased markedly in the region 5–50 mV (from 11 to nearly 30 mV).

Dependence on the base concentration. $1 . 10^{-3}$M uracil gives a well-defined main DPP peak with $E_s = -0{\cdot}04$ V (Fig. 1) and two smaller ones ($E_s = +0{\cdot}04$ and $+0{\cdot}09$ V). In the potential range of the main peak, a peak can be observed also on the NP polarogram and, in addition, a peak on the limiting d.c. polarographic current, whereas in the potential range of the more positive DPP peaks the NP and d.c. polarographic waves have the usual form. In the range from $1 . 10^{-3}$M to $5 . 10^{-4}$M, the heights of the main and middle peaks are little dependent on concentration (Fig. 6), whereas the most positive peak at a concentration of $5 . 10^{-4}$M almost disappears. On decreasing the concentration further, both remaining peaks decrease until at $1{\cdot}25 . 10^{-4}$M only the main peak remains. Its height is in the range $3 . 10^{-4}$ to $5 . 10^{-5}$M linearly dependent on the uracil concentration, and the E_s value is shifted to more positive potentials with decreasing concentration; this shift is less marked in the region where the peak height depends only little on the concentration. The peak half-width $W_{1/2}$ increases with decreasing concentration. The behaviour of cytosine in DPP is similar to that of uracil. The E_s value of the main DPP peak of cytosine is more positive (Fig. 6), the peak height is independent of the concentration in a broader range and its maximum value is roughly one half that of uracil; both peak heights

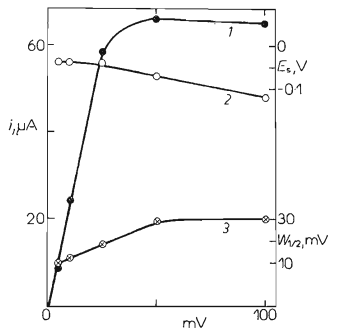

FIG. 5

Dependence of Height (*i*), Half-Width ($W_{1/2}$) and Potential (E_s) for DPP Peak of Uracil on Pulse Amplitude

$1 . 10^{-3}$M uracil in borate buffer, pH 10·5. *1 i*; *2* E_s; *3* $W_{1/2}$.

are almost equal in the region of linear concentration dependence. The detection limit of cytosine is about $5 \cdot 10^{-6}$M. The NPP curve of cytosine at higher concentrations has the form of a peak, at lower concentrations (where the DPP peak height depends linearly on concentration) the form of a wave. The d.c. polarographic wave height of cytosine at $1 \cdot 10^{-3}$M concentration is directly proportional to the height of mercury column, whereas at lower concentrations (in the region of linear concentration dependence of the peak height) it is directly proportional to the square root of this height. These facts are in accord with the behaviour of uracil reported earlier[1]. The behaviour of thymine is much different; its maximum DPP peak height is about one tenth of that of uracil (Fig. 6) and moreover the peak is much wider ($W_{1/2}$ = 75 mV in the region of concentration independence of the peak height). At lower concentrations, the value of $W_{1/2}$ drops with decreasing concentration, hence its concentration dependence is inverted with respect to that in the case of uracil and cytosine (Fig. 6). The value of E_s for thymine is more positive than for cytosine and is shifted to more negative values with increasing concentration similarly to uracil and cytosine.

Nucleosides and Nucleotides

Uridine, uridylic acid, cytidine, cytidylic acid, thymidine, and thymidylic acid in concentrations $6 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$M gave at pH 10·5 either inflexions on the DPP curves (in the potential range where the bases gave well-defined peaks) or were quite inactive in accord with earlier oscillopolarographic measurements[3].

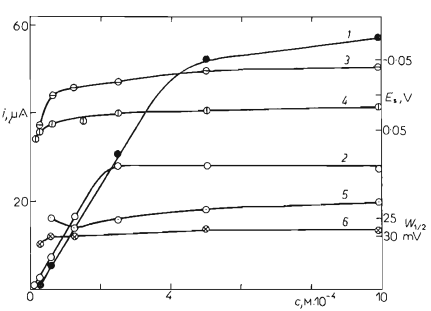

FIG. 6

Dependence of Height (i), Half-Width ($W_{1/2}$) and Potential (E_s) for DPP Peaks of Uracil and Cytosine on Concentration

1, *3*, *5* uracil; *2*, *4*, *6* cytosine; *1*, *2* i; *3*, *4* E_s; *5*, *6* $W_{1/2}$. Borate buffer, pH 10·5.

Measurements on a Hanging Mercury Drop Electrode

Our aim was to elaborate a sensitive analytical method for pyrimidine bases by using DPCSV with a stationary mercury electrode. We therefore studied the behaviour of these compounds also on a hanging mercury drop electrode by using cyclic voltammetry, DPCSV, and DPP. Cyclic voltammograms of uracil, cytosine, thymine, and 5-methylcytosine are shown in Fig. 7. With uracil and cytosine, we used also DPP, and with uracil also DPCSV at concentrations $5 . 10^{-5}$ and $5 . 10^{-6}$M, which are suitable for DPP in combination with a dropping mercury electrode (Fig. 7). Both compounds give well-developed DPP peaks on the hanging mercury drop and their detection limit is close to that with the use of the dropping electrode. The results obtained with the latter electrode can thus serve both in interpreting the DPP measurements with the dropping electrode and in proposing a DPCSV analysis of pyrimidines. Some results are given in Table I. It is seen that most of the studied derivatives (besides nucleic acid bases), *i.e.*, isocytosine, uracil-5-carboxylic acid, uracil-6-carboxylic acid (orotic acid), 5-amino-2,4-dioxypyrimidine, 4-amino-2,6-dioxypyrimidine, 2-amino-4,6-dioxypyrimidine, and 2-amino-4,6-dioxy-5-methylpyrimidine in a concentration of $5 . 10^{-5}$M give cathodic and anodic peaks due to the formation of salts with mercury. In contrast, 2-aminopyrimidine and 2-hydropypyrimidine do not give under the same conditions any anodic peak, but only tiny cathodic peaks.

DISCUSSION

Out results show that all pyrimidine bases usually present in nucleic acids (uracil, thymine, cytosine, *etc.*) give anodic polarographic currents due to the formation of salts with mercury. The anodic current obtained with pyrimidine derivatives

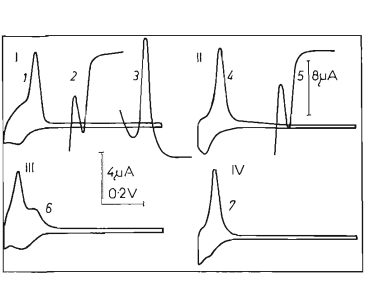

Fig. 7

Polarograms and Voltammograms of Uracil (I), Cytosine (II), Thymine (III), and 5-Methylcytosine (IV)

1, *4*, *6*, *7* Cyclic voltammograms, time of deposition 30 s, sweep rate of potential 0·2 V/s, without stirring, deposition potential +0·1 V. *2*, *5* DPP, 2 mV/s, pulse amplitude 25 mV, pulses at 0·5 s intervals, initial potential −0·1 V, anodic curves. *3* DPCSV, time of deposition 2 min, time of equilibration 20 s, 5 mV/s, pulses at 0·5 s intervals, cathodic curves. Concentration $5 . 10^{-5}$M, borate buffer, pH 10·5. Hanging mercury drop of 1·8 mm^2 surface area.

containing no sulphur is not conditioned by the presence of the —NH—CONH— group as assumed by certain authors[1,9,10], since the anodic peak was obtained with isocytosine, 2-amino-4,6-dioxypyrimidine, and 2-amino-4,6-dioxy-5-methylpyrimidine (Table I), which do not contain this group, whereas 2-oxypyrimidine, in which this group is present, is inactive. According to our results, all compounds that gave anodic currents contained either the group —HN—C(=O)—NH— or —HN—C(=NH)—NH_2, the pyrimidine nucleus was substituted at least in two positions (at least in 2 and 4), one of the substituents being OH and the other NH_2 or OH, and the N−1 atom was free. Substitution in position 5 or 6 influenced, but did not eliminate the resulting effect. The inactive compounds have either only one substituent on the pyrimidine nucleus or substituted N−1 position (nucleosides and nucleotides).

The structure of the salts of pyrimidine bases with mercury is open for discussion. The reaction of nucleic acids with Hg(II) in solution was studied by many authors *e.g.*[11]. However, since nucleosides and nucleotides of pyrimidine bases give no anodic currents, it is probable that the mercury salts formed by them and by natural and synthetic polynucleotides are different in nature from those responsible for the anodic currents observed by us.

The dependences of $E_{1/2}$ and E_s on pH suggest that OH^- ions participate in the electrode process with each of the three bases. With respect to pH values of the bases[12] it can be expected that uracil and thymine ($pK = 9{\cdot}5$ and $9{\cdot}9$) will at pH 10·5 form anions whereas cytosine ($pK = 12{\cdot}2$) will be present prevailingly in the undissociated form; thymine will be at pH 7·8 only little dissociated. Our results suggest that the extent of dissociation of the base in solution has no appreciable effect on the formation of its salt with mercury.

A comparison of the dependences of the DPP peak height on the base concentration (Fig. 6) shows that the peak height attains a limiting value which is for each base different, their ratio being uracil : cytosine : thymine = 1 : 0·5 : 0·1; and the limiting values are attained at different concentrations. The peak heights of these bases at a concentration of $6{\cdot}25 \cdot 10^{-5}$M (in the region of linear concentration dependence) are in a quite different ratio (1 : 1·3 : 0·5 for DPP and 1 : 1·7 : 0·5 for d.c. polarography) (Fig. 6). This difference seems to be caused not only by a different consumption of electrons but rather by different areas occupied by the adsorbed complexes of the bases with mercury and by sorption phenomena. The role of the latter is evidenced by the small value of $W_{1/2}$ of the uracil peak at higher concentrations (about 20 mV as compared with 75 mV for thymine, Figs 1 and 2) and by the anomalous dependence of $W_{1/2}$ on the pulse amplitude (Fig. 5). Tensammetric DPP peaks of surfactants have similar properties[13,14]. An increase of faradaic DPP peaks and changes of their form caused by adsorption of reactants were described in the case of simple inorganic depolarizers[15–17]. Very strongly adsorbed reactants can give double peaks[17], which were observed by us at high base concentra-

TABLE I

Peak Heights and Potentials for Pyrimidine Derivatives

CV cyclic voltammetry, K cathodic peak, A anodic peak. Concentration of the derivatives $5 \cdot 10^{-5}$ M, hanging mercury drop electrode. Compare Fig. 7 for other details.

Pyrimidine derivative		Peak potential E_s, V	Peak height i, μA
Uracil			
CV	K	−0·08	4·7
	A	+0·01	0·8
DPCSV		−0·05	8·2
DPP		−0·01	4·3
Cytosine			
CV	K	−0·04	5·5
	A	+0·03	0·9
DPCSV		+0·01	9·0
DPP		+0·01	9·0
Thymine			
CV	K	−0·00	2·8
		−0·08	0·3
	A	−0·04	0·6
5-Methylcytosine			
CV	K	−0·02	4·4
	A	+0·01	0·2
5-Hydroxymethylcytosine			
CV	K	−0·03	4·5
	A	+0·01	0·3
Isocytosine[a]			
CV	K	−0·03	10·2
	A	+0·03	1·0
Orotic acid			
CV	K	−0·13	3·9
	A	−0·13	0·4
Uracil-5-carboxylic acid			
CV	K	−0·07	3·7
	A	−0·04	0·6
2-Amino-4,6-dioxypyrimidine			
CV	K	−0·17	1·44
		−0·56	4·4
	A	[b]	
2-Amino-4,6-dioxy-5-methylpyrimidine			
	K	−0·02	9·36
	A	+0·05	3·5
4-Amino-2,6-dioxypyrimidine			
CV	K	−0·28	0·6
		−0·51	6·0
	A	—	—

TABLE I
(*Continued*)

Pyrimidine derivative		Peak potential E_s, V	Peak height i, μA
5-Amino-2,4-dioxypyrimidine			
CV	K	−0·11	3·8
	A	−0·02	0·2
2-Aminopyrimidine			
CV	K	—	—
	A	—	—
2-Hydroxypyrimidine			
CV	K	—	—
	A	—	—

[a] Measured at a concentration of $5 \cdot 10^{-4}$ M. [b] Anodic peak appeared at more positive initial potentials, the compound gave an anodic DPP peak.

tions (Figs 1 and 2) and hence could be possibly also caused by strong adsorption. On the other hand, the presence of more than one peak could be caused by an adsorption postwave known from classical polarography[18] or by the formation of several types of complexes with mercury. The reactant adsorption is evidenced by NP polarograms[19] or uracil and cytosine at high concentrations (Fig. 1), which show a decrease of the limiting current on the plateau of the wave. In contrast, no such decrease appeared on the NP polarogram of thymine (Fig. 2).

Adsorption of pyrimidine bases on the mercury electrode was studied in detail with the use of a.c. polarography[20–27]. The results show that these bases lower by their adsorption the differential capacity of the electrode double layer. Reorientation of the bases can take place on the electrode surface at certain potentials at high concentrations[23]. However, a.c. polarographic measurements do not give sufficient information about adsorption of the bases in the region of positive potential, where formation of their complexes with mercury takes place, since pseudocapacity currents make the study of adsorption difficult[22]. Cummings and Elving[28] studied recently the electrochemical behaviour of uracil in dimethyl sulphoxide and observed also in this case the formation of an insoluble salt with mercury, presumably monovalent, the uracil ring being perpendicular to the electrode surface.

The large difference in DPP behaviour of uracil and thymine at high concentrations (where the peak height is almost independent of the base concentration; Figs 1, 2, and 6) could be explained on the assumption that the uracil rings are in the mercury complex situated perpendicularly to the electrode surface (as in the medium of dimethyl sulphoxide[28]) and form a film. Such an arrangement could be favoured

by an increase of the base concentration on the electrode surface caused by a strong chemisorption of uracil. In such a case, reorientation of the uracil rings would take place in the potential region where the mercury complex is formed, since the free uracil ring is oriented perpendicularly to the electrode surface only close to the potential of zero charge (where it causes the formation of pits on the a.c. polarograms) and at other potential values it is oriented planary, as follows from the studies of its adsorption behaviour[20,26]. Reorientation of the bases and the formation of a film can cause profound changes in the differential capacity of the double layer in a narrow potential region resulting in the formation of a tensammetric peak[14]. The large increase of the DPP peak of uracil at higher concentrations can be hence due to a contribution of a tensammetric maximum (at the same potential as that of the faradaic DPP peak) combined with the influence of the depolarizer adsorption, as mentioned by other authors[15–17]. On the other hand, in the case of thymine, it is probable that prior to the formation of the mercury complex the base is either not adsorbed or adsorbed only weakly, as evidenced by the form of the NPP wave (Fig. 2). After the formation of the mercury complex, the chemisorption bond is weaker than with uracil, so that neither a perpendicular orientation nor the formation of a film takes place. Accordingly, no marked increase of the DPP peak of thymine that could be ascribed to sorption effects is observed.

From the point of view of biochemical analysis, the DPP determination of the bases under study offers new possibilities. The sensitivity and accuracy of the determination is comparable with the commonly used UV spectrophotometry. The values of E_s of the DPP peaks (Figs 1, 2, and 6) are sufficiently different to distinguish the individual bases in their pure solutions. Moreover, DPP enables to distinguish pyrimidine bases from nucleosides and nucleotides. However, there is only little hope that this method will replace spectrophotometric analysis of the bases, which has been well established in biochemical laboratories. For this reason, our work is not analytically oriented. In our opinion, the cathodic stripping voltammetry of the bases, which is derived from the present work and will be discussed in our subsequent communication[8], will find wide application in biochemical analysis mainly thanks to its high sensitivity, which surpasses the methods used nowadays at least by an order of magnitude.

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