THE POLAROGRAPHIC AND VOLTAMMETRIC DETERMINATION OF SEMITRYPANE BLUE*

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The polarographic reduction of the azodye semitrypane blue has been studied, a mechanism has been proposed and optimal conditions have been found for the determination of this substance by TAST polarography (to $2 . 10^{-6}$ mol l^{-1}) and differential pulse polarography (to $1 . 10^{-7}$ mol. $. l^{-1}$) at a classical dropping mercury electrode and by fast scan differential pulse voltammetry (to $1 . 10^{-8}$ mol l^{-1}) and linear scan voltammetry (to $1 . 10^{-8}$ mol l^{-1}) at a hanging mercury drop electrode. The detection limit was decreased to $1 . 10^{-9}$ mol l^{-1} for fast scan differential pulse voltammetry and $1 . 10^{-10}$ mol l^{-1} for linear scan voltammetry by using adsorptive accumulation of the determined substance at the hanging mercury drop electrode.

Azodyes are one of the most widely produced substances in the chemical industry and thus have recently been studied extensively to determine their genotoxic and ecotoxic effects[1]. The dyes suspected of genotoxicity include semitrypane blue (8-amino-1-hydroxy-2-(2-methylphenylazo)-3,6-naphthalenedisulfonic acid, *I*). There are two possible mechanisms through which this substance can be dangerous to health, that are basically analogous to those for structurally analogous trypane blue[2,3]. Its metabolic oxidation can yield the arenediazonium ion, which readily reacts with nucleophilic sites on the genetic material in the cell[2]; metabolic reduction through the action of azoreductase[3] can yield *o*-toluidine, which has been shown to be carcinogenic in tests on laboratory animals[4,5] and which is suspected of human urocarcinogenity[5].

A study of the metabolism of semitrypane blue and the determination of trace amounts of this substance in the working or living environment requires sensitive and selective methods. Thus, this work was carried out to determine this substance using modern polarographic and voltammetric methods, i.e. TAST polarography and differential pulse polarography (DPP) at a classical dropping mercury electrode

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(DME) and fast scan differential pulse voltammetry (FSDPV)[6] and linear scan voltammetry (LSV)[7] at a hanging mercury drop electrode (HMDE). The sensitivily of the latter two methods was increased through adsorptive accumulation of the studied substance at the surface of the hanging mercury drop[8]. All these methods have been found useful for the determination of low concentrations of a number of genotoxic mono-azodyes[9,10] and bis-azodyes[11,12].

EXPERIMENTAL

Reagents

The stock solution of semitrypane blue ($c = 1 . 10^{-3}$ mol l^{-1}) was prepared by dissolving an exactly weighed amount of the pure substance (Research Institute for Organic Synthesis, Pardubice-Rybitví) in redistilled water. This stock solution and more dilute solutions prepared by precise dilution were stored in the dark. The purity of the substance was controlled by thin-layer chromatography[13] using Silufol UV 254 plates (Kavalier, Czechoslovakia) with a 1-propanol–ammonia mobile phase (2 : 1) and the concentration of the stock solutions was controlled titanometrically[14]. The remaining chemicals (phosphoric acid, sodium hydroxide, acetic acid, boric acid) were of p.a. purity (Lachema, Brno); Triton X-100 (alkylphenylpolyethyleneglycol, Serva, Heidelberg) was used as a 0·5% solution in methanol. The water used was doubly distilled in a quartz apparatus.

Apparatus

The PA 4 polarographic analyzer was employed in combination with an XY 4 103 x–y recorder (both from Laboratorní přístroje, Prague). A three-electrode system was employed with a saturated calomel electrode and platinum foil auxiliary electrode. The potential values given are related to the saturated calomel electrode. TAST and differential pulse polarography were carried out using a classical mercury dropping electrode with the following parameters: mercury reservoir height $h = 49$ cm, drop time $t = 6{\cdot}5$ s and flow rate $m = 1{\cdot}113$ mg s^{-1} (in 0·1M-KCl with an applied potential of 0 V). Where not stated otherwise, a polarization rate of 5 mV s^{-1}, electronically controlled drop time of 1 s, mercury reservoir height of 49 cm and pulse height in differential pulse polarography of −100 mV were employed. The hanging mercury drop electrode employed in fast scan differential pulse voltammetry and linear scan voltammetry was the SMDE 1 static mercury drop electrode (Laboratorní přístroje, Prague) with a capillary diameter of 0·163 mm. Where not stated otherwise, a polarization rate of 20 mV s^{-1}, maximal drop size determined by opening the valve for 160 ms and a pulse height in the pulse technique of −100 mV were employed. Oxygen was removed from the polarographed solutions by bubbling with nitrogen, which was purified by passing through a solution of chromium(II) ions in dilute (1 : 1) hydrochloric acid over zinc amalgam.

Coulometry at constant potential was carried out using an OH 404 coulometric analyzer (Radelkis, Budapest) with automatic residual current compensation and electronic charge integration. An all-glass coulometric vessel with a volume of 200 ml and cathode and anode spaces separated by a frit was employed. The anode was a mercury pool, and a saturated calomel electrode and platinum foil auxiliary electrode completed the circuit. The solution was stirred during reduction by a magnetic stirrer and bubbled with nitrogen. The pH was measured using a PHM 62 instrument (Radiometer, Copenhagen) with a glass indicator and saturated calomel

reference electrode. The spectra were measured on a PU 8 800 instrument (Pye Unicam, England) in 1 cm quartz cuvettes. All measurements were carried out at laboratory temperature.

Procedures

The calibration curves were measured three times and evaluated by the method of linear regression. The determination limit was calculated by the method of Skogerboe and Grant[15] as the value ts/a, where s is the standard deviation of the experimental points from the calibration straight line calculated by the method of linear regression, a is the slope of this line and t is the Student coefficient for 99% probability.

In the coulometric determination of the number of electrons exchanged, 90 ml of the base electrolyte were measured into the coulometric vessel and bubbled with nitrogen, with simultaneous initiation of pre-electrolysis at the selected constant potential. After about 20 minutes, when the residual current value decreased below 0·2 mA and no longer changed, the circuit parameters were adjusted for automatic residual current compensation. Then 10·00 ml of a solution of semitrypane blue (c = 1 mmol l^{-1}) prebubbled with nitrogen were added and reduction was carried out at a constant potential with constant stirring and bubbling with nitrogen. Completion of the reduction was indicated by a decrease in the electrolytic current to the residual value (after 40–50 minutes) and the charge passed was found by electronic integration. The reduction was also followed by visible and UV spectrometry and by TAST polarography by removing 10 ml of a solution from the coulometric vessel at preselected intervals and measuring the TAST polarographic curve or UV and visible spectrum. The samples were taken prior to commencing reduction and after 25, 50, 75, and 100% reduction of semitrypane blue (calculated assuming a 4-electron reduction). Because of the fast reoxidation of the products of the coulometric reduction of semitrypane blue, especially in alkaline medium, the samples were taken in an inert atmosphere and the cuvette or polarographic vessel to which the sample was transferred was also rinsed with a stream of nitrogen.

RESULTS AND DISCUSSION

The stability of the stock solutions of semitrypane blue in distilled water was verified spectrophotometrically by measuring the absorbance at 536 nm. A concentration decrease of 0·5, 1, and 2·7% was found for the $1 . 10^{-3}$ mol l^{-1} solution after 15, 30, and 60 days, respectively; the decrease in concentration of the $1 . 10^{-5}$ mol l^{-1} solution over the same time was 1·8, 2·3, and 2·8%, respectively.

TAST *Polarography and Differential Pulse Polarography at a Dropping Mercury Electrode*

Table I and Fig. 1 document the effect of the pH on the TAST and DP polarograms of semitrypane blue. The best developed curves were obtained in acid medium (pH ~ 2). At higher pH values (3–10), the TAST polarograms are very drawn out with clear maxima and the corresponding DP polarograms are sometimes even split into several peaks. In the alkaline region (pH ~ 11–13) the polarograms contain two waves or peaks.

The concentration dependence was measured in both 0·1M-H_3PO_4 (pH 1·74) and 0·1M-NaOH (pH 13·1) media.

TABLE I

The effect of the pH on the TAST and DP polarograms of semitrypane blue; $c = 2 . 10^{-4}$ mol l^{-1}

pH	$E_{1/2}$ mV	I_{lim} μA	Slope[a] mV	E_p mV	I_p μA
1·74[b]	−77	0·835	35·5	−40	1·89
2·05	−110	0·845	45·4	−75	1·89
3·04	−195	0·860	47·4	−170	1·42
4·04[c]	−310	0·915	102·4	−260	0·87
4·04	—	—	—	−425	0·52
5·09[c]	−480	0·950	154·2	−280	0·19
5·09	—	—	—	−425	0·46
5·09	—	—	—	−550	1·14
6·16	−634	0·900	106·1	−635	1·93
7·05	−714	0·920	54·5	−680	2·17
8·00	−766	0·920	36·9	−725	2·11
9·01	−813	0·890	30·4	−775	2·20
9·97	−867	0·915	47·5	−840	1·26
11·01[c]	−896	0·500	55·2	−860	0·57
11·01	−1 093	0·365	75·4	−1 045	0·47
12·02[c]	−1 001	0·900	151·4	−875	0·43
12·02	—	—	—	−1 055	0·53
13·10[c,d]	−1 002	0·895	148·9	−875	0·43
13·10	—	—	—	−1 045	0·53

[a] Slope of the logarithmic analysis; [b] using 0·1M-H_3PO_4; [c] the polarogram consists of more than one wave or peak; [d] using 0·1M-NaOH.

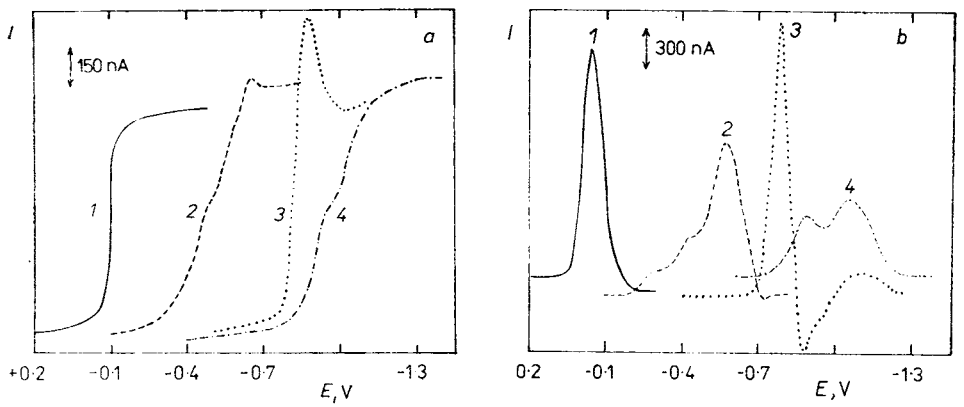


FIG. 1

The effect of the pH on the TAST (*a*) and DP (*b*) polarograms of semitrypane blue ($c = 2 . . 10^{-4}$ mol l^{-1}). pH 1·74 (*1*); 5·0 (*2*); 9·0 (*3*) and 13·1 (*4*)

In acid medium, the reversibility of the polarographic reduction of semitrypane blue decreased with decreasing concentration, resulting in drawing out of the TAST polarographic wave or a decrease in height, broadening and overall deformation of the DP polarographic peaks (see Fig. 2*b*). Addition of 0·1 ml of a 0·5% solution of Triton X-100 in methanol to 25 ml of the polarographed solution considerably increased the reversibility of the process (see Fig. 2*a*), resulting in an improvement in the shape of the DP polarographic peaks, and permitted the determination of much lower concentrations of semitrypane blue. It is noteworthy that addition of the assumed product of the electrochemical reduction of the studied substance has a similar effect on the shape of the TAST wave and DP peak. This product was obtained by completely reducing 100 ml of solution of semitrypane blue in 50% (v/v) acetic acid, i.e. to discoloration, by the addition of 100 mg of powdered zinc with intense stirring. Addition of 0·25 ml of the solution formed to 25 ml of the polarographed solution had practically the same effect as the addition of Triton X-100. It can thus be assumed that well developed TAST waves or DP peaks are obtained at higher concentrations of semitrypane blue (above $6 \cdot 10^{-5}$ mol l^{-1}) as a result of the positive effect of the products of the electrochemical reduction on the reversibility of the process. At lower depolarizer concentrations, the concentration of the product formed on the electrode surface also decreases, as does its positive effect on the shape of the measured curves. Thus, a linear calibration curve can be obtained in 0·1M-H_3PO_4 in the presence of Triton X-100 in the concentration range 10^{-4} to 10^{-6} mol l^{-1} for TAST polarography and in the range 10^{-4} to 10^{-7} mol l^{-1} for DP

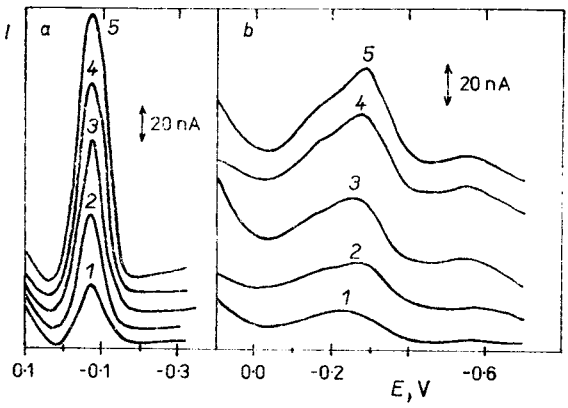


FIG. 2

The DP polarograms of semitrypane blue (*I*) in 0·1M-H_3PO_4 in the presence (*a*) and absence (*b*) of Triton X-100, *c*(*I*) (μmol l^{-1}): *1* 2; *2* 4; *3* 6; *4* 8; *5* 10

polarography. The parameters of these straight lines calculated by the method of linear regression and the calculated determination limits are given in Table II.

Two waves or peaks were obtained in alkaline media (0·1M-NaOH, pH 13·1); the peak at a potential of about −875 mV whose height is a linear function of the concentration in the range 10^{-4} to 10^{-7} mol l^{-1} is analytically more useful, while the second peak lies at −1 045 mV and this dependence is linear only in the range 10^{-4} to 10^{-5} mol l^{-1}. It should be noted that, as the concentration of semitrypane blue decreases, the height of the second peak decreases much faster than that of the first peak and the second peak disappears completely at concentrations below 10^{-6} mol l^{-1}.

The Mechanism of the Polarographic Reduction

It was confirmed by measuring the dependence of the peak height on the square root of the mercury reservoir height in 0·1M-H_3PO_4 and 0·1M-NaOH media that this is a diffusion-controlled process.

It follows from the slope of the semilogarithmic analysis (see Table I) and the number of exchanged electrons found by constant potential coulometry (see below) that the process is irreversible. This fact was also confirmed by measuring the cyclic

TABLE II

Parameters of the calibration curves and determination limits for the various methods of determining semitrypane blue (*I*)

Method	Concentration of *I* μmol l^{-1}	Slope mA mol^{-1} l	Intercept nA	Correlation coefficient	Determination limit μmol l^{-1}
TAST[a]	100—10	5·5	6·5	0·9991	—
TAST[a]	10—1	5·2	2·7	0·9942	1·8
DPP[a]	1—0·1	12·6	0·85	0·9962	0·15
DPP[b]	1—0·1	4·3	0·08	0·9987	0·08
FSDPV[c]	0·1—0·01	26·5	0·07	0·9994	0·005
FSDPV[d]	0·01—0·001	970	0·24	0·9980	0·001
LSV[c]	0·1—0·01	48	0·02	0·9997	0·003
LSV[e]	0·01—0·001	680	0·12	0·9993	0·0006
LSV[f]	0·001—0·0001	4 825	−0·21	0·9987	0·00008

[a] 0·1M-H_3PO_4 in the presence of Triton X-100; [b] 0·1M-NaOH; [c] Britton–Robinson buffer, pH 8·04 without accumulation; [d] hundred-fold diluted Britton–Robinson buffer, pH 8·04, adsorptive accumulation for 300 s in stirred solution; [e] as *d*, with adsorptive accumulation for 120 s in stirred solution; [f] as *d*, with adsorptive accumulation for 900 s in stirred solution.

voltammograms of solutions of semitrypane blue ($c = 10^{-4}$ mol l^{-1}) at a hanging mercury drop electrode. No anodic peak was observed in medium of pH 2–14 at a polarization rate of 10 to 500 mV s^{-1}. The observed shift in the half-wave potential or the peak potential with increasing pH towards more negative potentials is apparently connected with preliminary protonation of the azo group.

The fact that the overall height of the TAST polarographic waves is practically independent of the pH indicates that the total number of exchanged electrons remains constant over the whole studied pH range. The observed splitting of the waves into two poorly resolved waves of about the same height at certain pH values (e.g. 4·5 or 11) is apparently connected with the possible two-step reduction of the azo dye to the corresponding amines through the hydrazo compound as the reaction intermediate. This is reflected in the formation of two peaks in the DP polarograms, where the heights of these peaks correspond to the steepness of the TAST polarographic waves.

The number of exchanged electrons was found by coulometry at a constant potential of −350 mV in 0·1M-H_3PO_4 medium and −1 500 mV in 0·1M-NaOH medium.

It can be seen from the spectrophotometric study of the reduction in 0·1M-H_3PO_4 medium (see Fig. 3*b*) that the chromophore, i.e the azo group, is reduced. As the absorbance decreased at 536 nm, it increased in the region around 250 nm, apparently as a result of splitting of the azo group into two amino groups. The dependence of the absorbance at 536 nm on the charge transferred recalculated to the

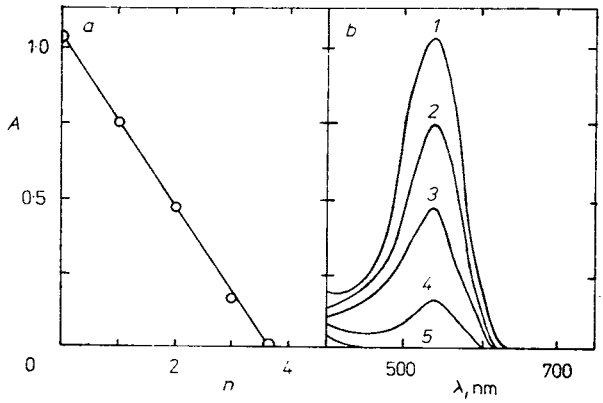


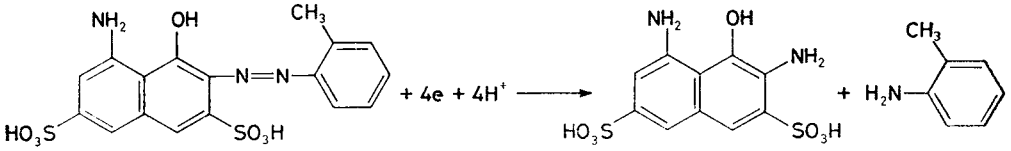
FIG. 3

Spectrophotometric study of the reduction of 10^{-4} mol l^{-1} semitrypane blue by constant potential coulometry at −350 mV in 0·1M-H_3PO_4 medium (pH 1·74). *a* Dependence of the absorbance at 536 nm on the charge passed recalculated to the number of electrons *n* per molecule of semitrypane blue; *b* spectra of the solution after passage of charge corresponding to $n = 0$ (*1*), 1 (*2*), 2 (*3*), 3 (*4*) and 4 (*5*)

number of electrons exchanged per molecule (n) intersects the abscissa at a value corresponding to the exchange of 3·6 electrons (see Fig. 3*a*). This could be explained by the disappearance of the intermediary hydrazo compound by a benzidine type rearrangement, yielding the rather low experimental value of $n = 3{\cdot}6$ compared to the expected value of $n = 4$. Analogous conclusions followed from study of the reduction by TAST polarography (see Fig. 4).

It was found that the number of exchanged electrons approached four in 0·1M--NaOH medium; atmospheric oxygen produces reoxidation of the products formed in the coulometric reduction to yield a coloured, polarographically reducible compound that is different from the original semitrypane blue. These reoxidation products exhibited an absorption maximum at about 370 nm and a half-wave potential of about $-1{\cdot}4$ V. It cannot be excluded that the genotoxic effect of semitrypane blue could be a result of the action of these reoxidation products formed after enzymatic reduction or a rearrangement reaction of the reaction intermediates.

It can thus be assumed that semitrypane blue is reduced irreversibly at a dropping mercury electrode with exchange of four-electrons according to Scheme 1.



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SCHEME 1

The formation of *o*-toluidine has also been demonstrated by thin-layer chromatography after extraction from the reaction mixture into benzene by the same procedure as that employed to demonstrate the formation of 3,3'-dimethylbenzidine in the coulometric reduction of trypane blue[12].

Fast Scan Differential Pulse Voltammetry and Linear Scan Voltammetry at a Hanging Mercury Drop Electrode

The dependence of the peak potential E_p of semitrypane blue on the pH in FSDPV at the HMDE (see Table III) has the same character as the $E_{1/2}$ dependence in TAST polarography on the pH and can be explained analogously. The peak height and number reflect the steepness of the TAST polarographic waves and splitting into two waves where present. The best developed waves in FSDPV at the HMDE were obtained in Britton–Robinson buffer medium at pH 8 which is connected with a change in the peak shape on a decrease in the concentration of semitrypane blue.

TABLE III

The effect of the pH on the FSDPV voltammograms of semitrypane blue (I); $c(I) = 1 \cdot 10^{-5}$ mol . l^{-1}

pH	E_p, mV			I_p, nA		
	1st peak	2nd peak	3rd peak	1st peak	2nd peak	3rd peak
2·05	−0·030	−0·355	−0·430	278	40	35
3·04	−0·125	−0·445	—	168	50	—
4·04	−0·210	−0·460	—	118	55	—
5·01	−0·380	−0·570	—	53	78	—
6·02	−0·350	−0·585	—	25	125	—
7·04	−0·410	−0·655	—	15	270	—
8·04	−0·450	−0·695	—	10	358	—
9·01	−0·475	−0·730	—	10	325	—
9·97	−0·530	−0·760	—	13	308	—
11·01	−0·595	−0·820	—	15	233	—
12·02	—	−0·865	—	—	125	—

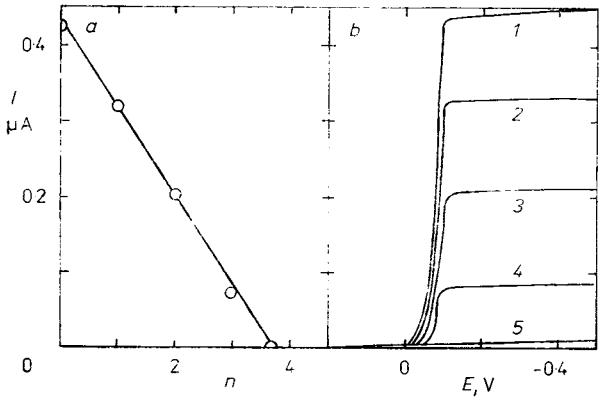

FIG. 4

TAST polarographic study of the reduction of 10^{-4} mol l^{-1} semitrypane blue by constant potential coulometry at −350 mV in 0·1M-H_3PO_4 medium (pH 1·74). *a* Dependence of the limiting current at −300 mV on the charge passed recalculated to the number of electrons n per molecule of semitrypane blue; *b* TAST polarograms after passage of charge corresponding to $n = 0$ (*1*), 1 (*2*), 2 (*3*), 3 (*4*) and 4 (*5*)

Under these conditions, the dependence of the peak height on the depolarizer concentration is linear in the range 10^{-5} to 10^{-8} mol l^{-1} and its parameters are given in Table II. Analogous results can be obtained using LSV at the HMDE (Table II).

The advantage of these methods using the HMDE lies in the higher polarization rates and thus shorter time required to record the voltammogram. There is a danger of passivation of the electrode and of the detrimental effect of surface-active substances in the analyzed solution, as the surface of the working electrode is not renewed during the recording of the voltammetric curve.

TABLE IV

The effect of the accumulation time t on the peak height of semitrypane blue in FSDPV at the HMDE. Accumulation potential −0·4 V, one hundred-fold diluted Britton–Robinson buffer, pH 8·04

t, s	I_p^a, nA	I_p^b, nA
30	0·9	3·3
60	1·2	6·3
120	1·8	10·8
300	3·5	19·8

[a] Without stirring; [b] with stirring, recorded 15 s after stirring stopped.

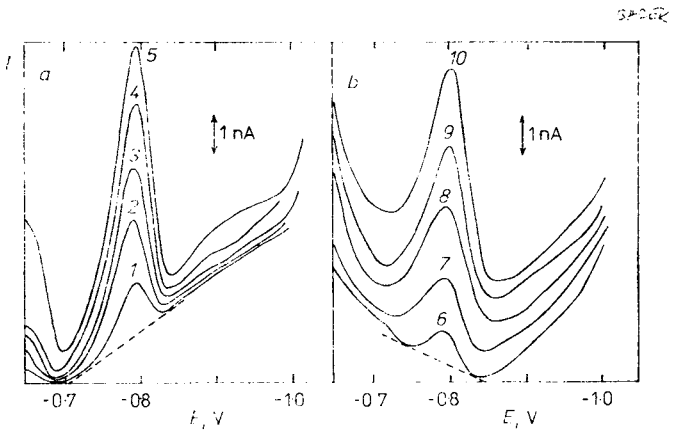

FIG. 5

Determination of semitrypane blue (I) by LSV at the HMDE. *a* Adsorptive accumulation for 2 minutes in stirred solution, $c(I)$ (nmol l^{-1}): *1* 2, *2* 4, *3* 6, *4* 8, *5* 10. *b* Adsorptive accumulation for 15 minutes in stirred solution, $c(I)$ (nmol l^{-1}): *6* 0·2, *7* 0·4, *8* 0·6, *9* 0·8, *10* 1. The dashed line is the baseline from which the peak height was measured.

A further advantage of work with the HMDE is that the sensitivity of the determination can be increased through adsorptive accumulation of the determined substance on the surface of the hanging mercury drop electrode. It has been found in FSDPV and LSV at the HMDE that the peak height for semitrypane blue depends on the time elapsed between formation of the hanging mercury drop and the recording of the voltammogram, and this increase is greater in stirred solutions (see Table IV). It is then preferable to employ Britton–Robinson buffer, pH 8, diluted a hundred-fold as the base electrolyte, to decrease the detrimental effect of trace impurities in the chemicals employed to prepare the buffer on the shape of the curve for the base electrolyte. The working electrode potential during accumulation has no effect on the peak height obtained after adsorptive accumulation. The determination can be carried out in the concentration range 10^{-8} to 10^{-9} mol l^{-1} when FSDPV at the HMDE is carried out with accumulation for 300 s in stirred solution. In LSV at the HMDE, an accumulation period of 120 s in stirred solution is sufficient for determination in the concentration range 10^{-8} to 10^{-9} mol l^{-1}, because of the smaller noise level in the simpler electronic circuitry. When the accumulation time in stirred solution is increased to 900 s, a linear concentration dependence is obtained in the range 10^{-9} to 10^{-10} mol l^{-1}. The voltammograms obtained in the lowest concentration range and the evaluation method are depicted in Fig. 5.

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