THE POLAROGRAPHIC AND VOLTAMMETRIC DETERMINATION OF TRYPANE BLUE*

Jiří BAREK[a], Janina BALSIENE[b], Barbara TIETZOVÁ[a] and Jiří ZIMA[a]

[a] *Department of Analytical Chemistry, Charles University, 128 40 Prague 2, Czechoslovakia and*
[b] *Department of Organic Chemistry, V. Kapsukas Vilnius State University, U.S.S.R.*

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The reduction of mutagenic bisazodye trypane blue has been studied at a mercury electrode, a mechanism has been proposed and optimal conditions found for its analytical application. The use of a dropping mercury electrode with a renewable surface yielded a detection limit of $8 . 10^{-7}$ mol l^{-1} for TAST polarography, and $8 . 10^{-8}$ mol l^{-1} for differential pulse polarography; the use of a hanging mercury drop electrode with an unrenewed surface during the measurement yielded detection limit of $6 . 10^{-8}$ mol l^{-1} for fast scan differential pulse voltammetry and $4 . 10^{-8}$ mol l^{-1} for linear scan voltammetry. Preliminary adsorptive accumulation of the determined substance on the surface of the hanging mercury drop electrode led to a decrease in the detection limit to $5 . 10^{-9}$ mol l^{-1} for linear scan voltammetry.

Trypane blue can be classified among substances with genotoxic effects (mutagenic, carcinogenic, and teratogenic)[1]. The action of azoreductase transforms it into the carcinogen 3,3'-dimethylbenzidine[2], whose formation has been demonstrated in both in vitro[3] and in vivo[4] metabolic studies. Oxidative activation of this substance leads to the formation of the arenediazonium cation[1], which can also be an ultimate mutagen. Mutagenic substances are also formed by two-step metabolic activation (reduction followed by oxidation) of trypane blue[5]. The genotoxic effects of azodyes derived from derivatives of benzidine are discussed from both a toxicological and ecotoxicological point of view in reviews[6,7]. The increased genotoxic activity of commercial trypane blue has been explained in terms of the presence of impurities of the semitrypane blue type formed by decomposition of one diazotized group of 3,3'-dimethylbenzidine, possibly with replacement by hydrogen or a hydroxyl group[8].

The genotoxic activity of trypane blue makes it desirable that a number of independent methods be available to determine low concentrations of this substance in biological and ecotoxicological studies. A number of simple[9,10] and more complex[11-13] bisazocompounds can be readily reduced polarographically at a mercury electrode. No mention has, however, been made in the literature of the reduction

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of trypane blue. Thus this work was devoted to a detailed study of the polarographic behaviour of this substance and especially to the determination of low concentrations using modern polarographic methods that have already been found useful for the determination of traces of structurally similar bisazodyes[14].

Studies were carried out of the use of the dropping mercury electrode (DME) with TAST polarography or differential pulse polarography (DPP) and of the hanging mercury drop electrode (HMDE) with fast scan differential pulse voltammetry (FSDPV) or linear scan voltammetry (LSV).

EXPERIMENTAL

Reagents

The stock solution of trypane blue (0·001 mol l^{-1}) was prepared by dissolving a precisely weighed amount of the substance as the tetrasodium salt (Research Institute for Organic Syntheses, Pardubice-Rybitví) in redistilled water. The stock solution and solutions with lower concentrations prepared by dilution of the stock solution were stored in the dark. The purity of the substance was controlled by column[8] and thin-layer[15] chromatography. The concentration of the stock solution was controlled titanometrically[16].

Britton–Robinson buffers were prepared in the usual manner[17]. All the chemicals employed were of p.a. purity, and water was doubly distilled in a quartz apparatus.

Apparatus

The PA3 polarographic analyzer with the XY-4105 recorder (Laboratorní přístroje, Prague) was used in a three-electrode arrangement. The classical DME employed has a drop time of 3·00 s at 0 V in 0·1M-H_3PO_4 and a flow rate of 4·63 mg s^{-1} at a mercury reservoir height of 25 cm. The static mercury drop electrode SMDE 1 (Laboratorní přístroje, Prague) that was used as an HMDE had a capillary diameter of 0·136 mm. The maximum drop size was employed (the valve was left open for 160 ms). In both cases, a saturated calomel reference electrode and auxiliary platinum electrode with an area of about 1 cm^2 were used. Where not stated otherwise, work with the classical DME was carried out using a polarization rate of 5 mV s^{-1}, electronically controlled drop time of 1 s, mercury reservoir height of 25 cm, and DPP modulation amplitude of −100 mV. A polarization rate of 20 mV s^{-1} was used in work with the HMDE. Oxygen was removed from the polarographed solution by bubbling for ten minutes with nitrogen, that was previously purified by passing through an alkaline solution of sodium anthraquinone-2-sulfonate and a chromium(II) ions solution in hydrochloric acid (both 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 was employed and the cathode and anode spaces were separated by a frit. The cathode was a mercury pool, the anode consisted of a platinum foil and the reference electrode was a saturated calomel electrode. The solution was stirred with a magnetic stirrer during the determination and continuously bubbled with nitrogen. The pH was measured using a PHM 62 instrument (Radiometer, Copenhagen) with a combined glass and saturated calomel electrode. Spectra were measured on a PU 8 800 spectrophotometer (Pye Unicam, England) in quartz cuvettes with a specific thickness of 1 cm. All measurements were carried out at laboratory temperature.

Procedures

Polarographic solutions were prepared by measuring 5·00 ml of the given base electrolyte, addition of the required amount of depolarizer and dilution with water to 10·00 ml.

Calibration curves were measured in triplicate and evaluated by the method of linear regression. The detection limit was calculated by the method of Skogerboe and Grant[18] as the $t \,.\, s/a$ value, where s is the standard deviation of the measured points from the calculated straight line, a is the slope of this line and t is the Student coefficient for 99% reliability dependent on the number of points employed in construction of the calibration straight line.

Coulometric determination of the number of electrons exchanged was carried out by measuring 50 ml of the base electrolyte and 45 ml of water, the solution was bubbled with nitrogen and pre-electrolysis was commenced at the required constant potential. After about 20 minutes, the residual current value decreased below 0·2 mA and no longer changed. Parameters were then adjusted in the circuit for automatic residual current compensation and 5·00 ml of trypane blue ($5 \,.\, 10^{-4}$ mol l^{-1}) were added with constant nitrogen bubbling. Completion of the reduction was indicated by a decrease in the current to the residual value, the time required was 45—60 min and the charge consumed was found by digital current integration. The course of the reduction was studied spectrophotometrically and polarographically by regularly removing 5 ml of solution from the vessel and measuring the polarographic curve or ultraviolet and visible spectrum. Sampling was carried out prior to commencing the coulometric reduction and at time when 25, 50, 75, and 100% of the trypane blue was reduced.

The presence of 3,3′-dimethylbenzidine formed in the electrochemical reduction of trypane blue was demonstrated by comparison of the UV spectra of $5 \,.\, 10^{-5}$ mol l^{-1} of the substance in 0·1M phosphoric acid reduced both by constant potential coulometry at −300 mV and by 1 g of powdered zinc. The formation of 3,3′-dimethylbenzidine was also demonstrated by thin-layer chromatography; the pH of the solution obtained by coulometric reduction at −300 mV was adjusted to a value of 7 by addition of 2 mol l^{-1} sodium hydroxide (controlled using universal pH paper), the solution was extracted with 3 × 30 ml of benzene and the combined extracts were evaporated to dryness in a vacuum. The residue was dissolved in 1 ml of acetone and 30 µl of solution was applied to the start. Simultaneously, 30 µl of a standard solution (10 mg of 3,3′-dimethylbenzidine in 10 ml of acetone) was also applied. Chromatography was carried out in the ascending arrangement on Silufol UV 254 thin layers using a benzene–methanol (4 : 1) mixture. Detection was carried out by spraying with a 1% solution of *p*-dimethylaminobenzaldehyde (1 g of the substance dissolved in 5 ml of concentrated hydrochloric acid was diluted to 100 ml with ethanol).

RESULTS AND DISCUSSION

Study of the Mechanism of the Electrochemical Reduction of Trypane Blue

First the effect of the pH on the behaviour of trypane blue in TAST polarography at a dropping mercury electrode was determined. Table I lists the determined $E_{1/2}$, I_{lim} and logarithmic analysis slope values for TAST polarography and peak potential (E_p) and current (I_p) values for DPP in dependence on the pH. The observed shift of $E_{1/2}$ and E_p to more negative values with increasing pH can be explained in terms of prior protonation of the azogroup, leading to a decrease in the electron density in the region of the double bond between the nitrogen atoms, facilitating electron

transfer. As the pH increased, the wave became more drawn out or even split. At pH > 6, the TAST polarograms were abnormal in character as the part corresponding to the limiting current decreased slightly as the potential increased to more negative potentials; this phenomenon can be explained in terms of repulsion of the negatively charged trypane blue species from the electrode surface. The highest and best developed waves and peaks were obtained at pH 2. It was also found that 0·1M-H_3PO_4 can be used in place of Britton–Robinson buffer with this pH; all subsequent measurements were thus carried out in this medium.

The dependence of $\log I_{lim}$ on the log of the flow-rate m during TAST polarography at a constant drop time has a slope of 2/3 and the dependence of $\log I_{lim}$ on the log of the drop time at a constant reservoir height has a slope of 1/6. It thus holds that $I_{lim} = \text{const.}\ m^{2/3}t^{1/6}$. This fact and the determined linear dependence of the limiting current on the square root of the reservoir height in DC polarography and on the depolarizer concentration in DC and TAST polarography reflect the diffusion character of the process.

The time dependence of the coulometric reduction of trypane blue at a constant potential of −300 mV in 0·1M-H_3PO_4 medium is depicted in Fig. 1, from which it follows that a total of 8 electrons are exchanged. This fact is also confirmed by the

TABLE I

The effect of the pH on the polarographic behaviour of $5 . 10^{-5}$ mol l^{-1} trypane blue

pH	$E_{1/2}$ mV	I_{lim} μA	Slope[a] mV	E_p mV	I_p μA
2·04	−135	0·235	41·3	−115	1·59
2·99	−207	0·205	50·3	−175	1·38
3·99	—[b]	0·235	—[b]	−270[b]	0·66[b]
3·99	—[b]	—	—	−425[b]	0·58[b]
5·08	—[b]	0·275	—[b]	−275[b]	0·18[b]
5·08	—[b]	—	—	−550[b]	1·26[b]
5·99	−642	0·238	77·2	−625	1·44
7·01	−739	0·225	54·5	−650	1·56
7·97	−779	0·225	49·9	−740	1·53
8·99	−824	0·203	48·8	−770	1·51
10·09	−878	0·208	47·2	−835	1·52
10·99	−905	0·200	48·9	−860	1·58
12·05	−945	0·195	53·3	−890	1·47

[a] Slope of the logarithmic analysis; [b] the TAST wave is very drawn-out and the DPP peak is split and logarithmic analysis cannot be carried out.

dependence of the height of the TAST polarographic waves on the charge passed, Q, which is linear and intercepts the abscissa at a Q value corresponding to exchange of 8 electrons. It can be seen from the spectrophotometric study of coulometry at constant potential (Fig. 2) that it involves reduction of the chromophore and decolourization of the solution.

The slope of the logarithmic analysis with respect to the number of exchanged electrons indicates that the studied process is irreversible. This irreversibility also explains the following dependence of the half-wave potential on the depolarizer concentration.

c, mol l^{-1}:	$10 . 10^{-5}$	$5 . 10^{-5}$	$1 . 10^{-6}$	$5 . 10^{-6}$
$E_{1/2}$, mV:	−110	−115	−120	−125

The irreversible character of the reduction of trypane blue was also confirmed by cyclic voltammetry at the HMDE of a $5 . 10^{-5}$ mol l^{-1} solution with pH 2·7 and 10 and polarization rates of 2−100 mV s^{-1}. No anodic peak was observed.

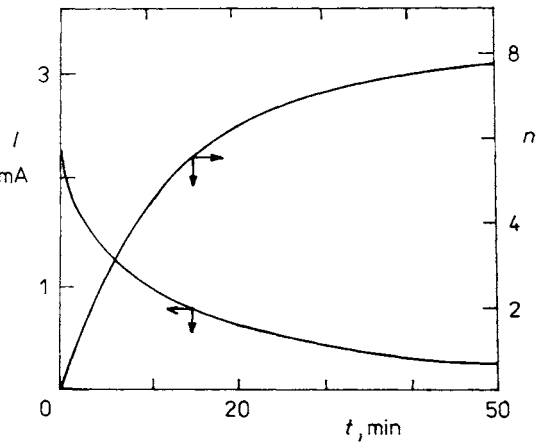


FIG. 1

The time course of the coulometric reduction of trypane blue at a constant potential of −300 mV in 0·1M-H_3PO_4 medium. *I* current passing; *n* charge passed recalculated to the number of electrons per molecule. Initial depolarizer concentration $2·5 . . 10^{-5}$ mol l^{-1}

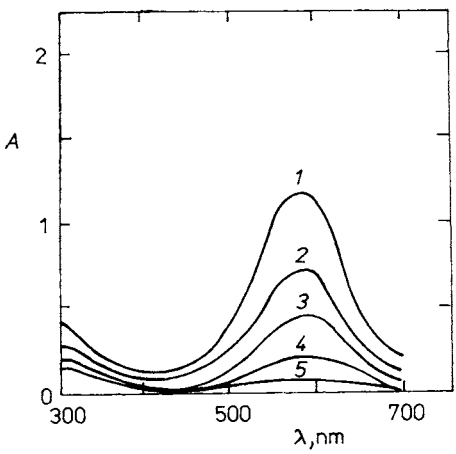
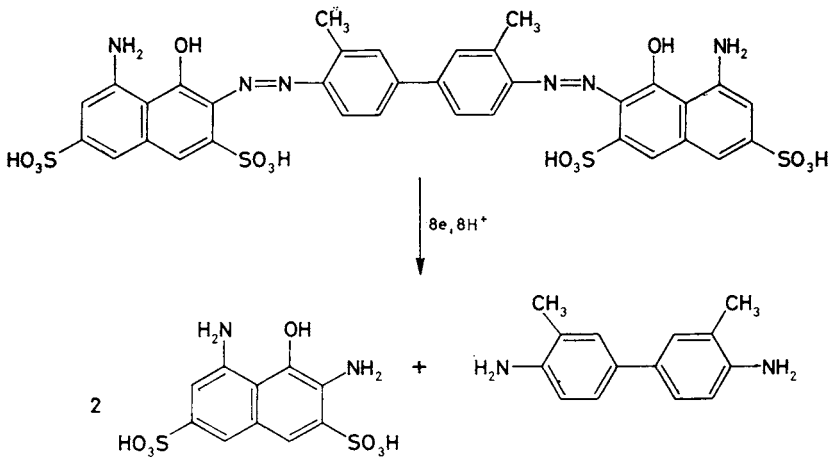


FIG. 2

Spectrophotometric study of the reduction of trypane blue by coulometry at constant potential of −300 mV in 0·1M-H_3PO_4 medium. Specific cuvette thickness 1 cm; initial depolarizer concentration $2·5 . 10^{-5}$ mol . $. l^{-1}$. Reduced % of the initial substance (calculated on the basis of an eight-electron reduction): *1* 0, *2* 25, *3* 50, *4* 75, *5* 100

It can thus be assumed that trypane blue undergoes irreversible, eight-electron reduction in 0·1M-H_3PO_4 according to the Scheme 1.



SCHEME 1

This scheme also is in agreement with the fact that the UV spectra of solutions after coulometric reduction at constant potential and chemical reduction with powdered zinc are identical, confirming the complete electrochemical reduction of both azogroups to the corresponding amines. The 3,3'-dimethylbenzidine formed was identified using thin-layer chromatography (a value of $R_F = 0{\cdot}37$ was found for both the reduction product and authentic 3,3'-dimethylbenzidine and mixed chromatography confirmed that the two substances are identical).

Analytical Utilization of the Electrochemical Reduction of Trypane Blue

First the stability of the stock solution of trypane blue after dilution to $1 \cdot 10^{-5}$ mol . $. l^{-1}$ was studied spectrophotometrically. It was found that its concentration does not change over a period of 30 days.

TAST *polarography and differential pulse polarography at a dropping mercury electrode.* First, the stability of the polarographed solution of trypane blue in 0·1M-H_3PO_4 was determined using TAST polarography for 10^{-4} and 10^{-5} mol l^{-1} solutions and DPP for 10^{-5} and 10^{-6} mol l^{-1} solutions. No observable change in the concentration occurred during 60 minutes after preparation. The dependence of the wave height on the depolarizer concentration for TAST polarography at a DME is linear in the range $10^{-4}-10^{-6}$ mol l^{-1}. In agreement with the theory, it

was found that the DPP peak height increases with increasing pulse height, accompanied by a shift in E_p to more positive potential values. The peak height also increases with increasing reservoir height and drop time. The concentration dependences are linear in the range $10^{-4}-10^{-7}$ mol l^{-1}. Table II gives the parameters of the calibration straight lines, calculated by linear regression, and detection limits for TAST polarography and DPP at the DME.

Fast scan differential pulse voltammetry and linear scan voltammetry at a hanging mercury drop electrode. It was found in FSDPV at the HMDE that the peak height increases with increasing pulse height and drop size. The peak height in LSV at the HMDE also increases with increasing drop size and increasing polarization rate. However, the charging current also increases with increasing polarization rate, leading to distortion of the curve shape. An optimal compromise polarization rate of 20 mV s^{-1} was selected. The concentration dependences for both methods are linear in the range $10^{-5}-10^{-7}$ mol l^{-1}. The parameters of the calibration straight lines and calculated detection limits are also given in Table II. Methods employing the HMDE can employ a faster polarization rate and thus the time required to record the voltammogram is shorter. A disadvantage lies in the increased danger of electrode passivation and possible effects of surface-active substances in the analyzed solution, as the electrode surface is not renewed during recording of the voltammetric curve.

A further advantage of the HMDE is that the sensitivity of the determination can be increased by adsorptive accumulation of the determined substance on the surface of the working electrode. It has been found that in FSDPV and LSV the peak height

TABLE II

The parameters of the calibration straight lines and detection limits found for trypane blue by various methods

Method	c μmol l^{-1}	Slope μA mol l^{-1}	Intercept μA	Correl. coef.	Detection limit, mol l^{-1}
TAST	2—10	$4{\cdot}0 \cdot 10^3$	$-5 \cdot 10^{-3}$	0·9971	$8 \cdot 10^{-7}$
DPP	0·2—1	$7{\cdot}6 \cdot 10^4$	$1 \cdot 10^{-2}$	0·9876	$8 \cdot 10^{-8}$
FSDPV[a]	0·2—1	$5{\cdot}6 \cdot 10^3$	$-5 \cdot 10^{-4}$	0·9987	$6 \cdot 10^{-8}$
LSV[a]	0·2—1	$1{\cdot}4 \cdot 10^5$	$3 \cdot 10^{-4}$	0·9976	$4 \cdot 10^{-8}$
FSDPV[b]	0·02—0·1	$2{\cdot}2 \cdot 10^4$	$-6 \cdot 10^{-4}$	0·9952	$2 \cdot 10^{-8}$
LSV[c]	0·02—0·1	$6{\cdot}4 \cdot 10^5$	$7 \cdot 10^{-4}$	0·9832	$5 \cdot 10^{-9}$

[a] Without accumulation (recorded 5 s after drop formation); [b] with 10 s accumulation in stirred solution (recorded 10 s after stirring stopped); [c] with 30 s accumulation in stirred solution (recorded 10 s after stirring stopped).

for trypane blue depends on the time elapsed between drop formation and initiation of recording. This effect is increased by stirring of the solution. It followed from preliminary experiments that the optimal accumulation time for FSDPV is 10 s and for LSV, 30 s in stirred solution, at a working electrode potential of +100 mV vs the saturated calomel electrode. In both cases, the recording is carried out 10 s after termination of stirring. Under these conditions, accumulation leads to roughly a five-fold increase in the peak height for the determined substance. A further increase in the accumulation time does not lead to any further substantial increase in the peak height. Under these conditions, the calibration curves for both techniques are linear in the range $10^{-6}-10^{-8}$ mol l^{-1}. Their parameters and calculated detection limits are also listed in Table II. The calibration line becomes curved at concentrations above 10^{-6} mol l^{-1}, probably due to complete coverage of the working electrode surface by adsorbed depolarizer. The lower detection limit of LSV compared to FSDPV is connected with the lower noise in the simpler electronic circuit.

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