THE POLAROGRAPHIC AND VOLTAMMETRIC DETERMINATION OF CONGO RED*

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The polarographic reduction of the bisazodye congo red has been studied, a mechanism was proposed for this process and optimal conditions were found for determination of this substance by TAST polarography and differential pulse polarography at a dropping mercury electrode and using fast scan differential pulse voltammetry and voltammetry with linearly increasing voltage at a hanging mercury drop electrode. The detection limit for the latter two techniques is about 10^{-8} mol l^{-1}; a further decrease in the value can be attained by prior accumulation of the determined substance by adsorption on the surface of the working electrode.

Congo red (*I*) is one of the substances suspected of chemical carcinogenity, requiring two-step metabolic activation[1]. *In vitro*[2-4] and *in vivo*[5] experiments have demonstrated that it is metabolically transformed into benzidine, from which it is derived. The different organ specificity of congo red and benzidine, however, indicates that the carcinogenic activity of this bisazodye is not dependent on its metabolites alone. This fact is confirmed by the discovery that congo red is not directly mutagenic in the Ames test[4], but only after prior aerobic activation, while enzymatic anaerobic reduction does not increase its mutagenicity[6].

Very little attention has so far been devoted to the electrochemical behaviour of bisazocompounds. The polarographic reduction of *o*-, *m*,- and *p*-bisazobenzene[7,8] and some more complex bisazodyes[9-13] has been studied. Polarography has been employed to study the interactions of congo red with surface-active substances[14] or with DNA[15] and its aggregation in aqueous solutions[16]. Its oscillopolarographic behaviour has also been studied[17].

This work was devoted to a detailed study of the polarographic reduction of congo red in order to determine optimal conditions for the most sensitive determination using TAST polarography and differential pulse polarography (DPP) at a dropping mercury electrode (DME), fast scan differential pulse voltammetry (FS DPV) and voltammetry with linearly increasing voltage (linear scan voltammetry, LSV) at a hanging mercury drop electrode (HMDE).

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EXPERIMENTAL

Reagents

The stock solution of congo red (0·001 mol l^{-1}) was prepared by dissolving 0·6527 g of the substance (Merck, Darmstadt) in 1 l of redistilled water. The stock solution and solutions with lower concentration prepared by exact dilution were stored in the dark. The purity of the substance was controlled by paper[18], column[19], and thin-layer[20] chromatography and the concentration of the stock solution was controlled titrimetrically[21] and spectrophotometrically[22,23]. Britton–Robinson buffers were prepared in the usual manner[24]. All the chemicals employed were of *p.a.* purity and water was doubly redistilled from a quartz apparatus.

Apparatus

The PA3 polarographic analyzer with 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 OV 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), used as an HMDE, had a capillary diameter of 0·136 mm. Maximal drop size was employed (the valve was opened for 160 ms). In both cases, a saturated calomel reference electrode and auxiliary platinum electrode were used; the latter had a surface area of about 1 cm^2. Where not stated otherwise, work with the classical DME was carried out at a constant 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. Measurements at the HMDE were carried out at polarization rates of 20 mV s^{-1}. Oxygen was removed from the polarographed solutions by bubbling for ten minutes with nitrogen that had previously been purified by passing through an alkaline solution of sodium anthraquinone-2-sulphonate and a solution of chromium(II) ions in hydrochloric acid (both over zinc amalgam).

The OH 404 coulometric analyzer (Radelkis, Budapest) was used for coulometry at constant potential, with automatic compensation of the residual current and electronic integration of the charge passed. 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 mercury pool acted as a cathode and the platinum electrode was the anode, with a saturated calomel reference electrode. During the analysis, the solution was stirred with a magnetic stirrer and bubbled with nitrogen. pH measurements were carried out using a PHM 62 instrument (Radiometer, Copenhagen) with a combined glass and saturated calomel electrode. Spectra were measured on a PU 8800 spectrophotometer (Pye Unicam, England) in quartz cuvettes with a specific width of 1 cm. All measurements were carried out at laboratory temperature.

Procedures

The polarographed solution was prepared by measuring 5·00 ml of the given base electrolyte into a volumetric flask, addition of the required amount of depolarizer and diluting 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[25] in the manner described previously[26].

Coulometric measurement of the number of exchanged electrons was carried out by pipetting 50 ml of the base electrolyte into the coulometric vessel along with 45 ml of water, bubbling with nitrogen and simultaneous commencement of pre-electrolysis at the required constant potential.

After about 20 minutes, the residual current value decreased to a value below 0·2 mA and no longer changed. Then the parameters were set on the circuit for automatic compensation of the residual current and 5·00 ml of congo red was added with constant bubbling with nitrogen (0·001 mol l^{-1} congo red). Completion of reduction was indicated by a decrease in the current to the residual value, requiring 45—60 minutes and the charge consumed was determined by digital integration of the current passed. The course of the reduction was studied spectrophotometrically and polarographically by taking 5 ml of solution from the coulometric vessel and measuring the polarographic curve or ultraviolet and visible spectrum. Sampling was carried out before commensing coulometric reduction and at times when 25, 50, 75, and 100% of the congo red was reduced.

The formation of benzidine in the electrochemical reduction of congo red was confirmed by comparison of the UV spectra of $5 . 10^{-5}$ mol l^{-1} congo red in 0·1 mol l^{-1} phosphoric acid reduced by coulometry at constant potential of −300 mV and by 1 g of zinc powder. The formation of benzidine was also demonstrated using thin-layer chromatography, where the pH of the solution obtained from 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 benzene and the combined extracts were evaporated to dryness under decreased pressure. The residue was dissolved in 0·5 ml acetone and 5 µl of solution was applied to the start, along with 5 µl of a standard solution (10 mg benzidine in 10 ml acetone). Ascending chromatography was carried out on Silufol UV 254 thin layers using a benzene–methanol mixture (4 : 1). Detection was carried out by spraying with a 1% solution of *p*-dimethylaminobenzaldehyde (1 g substance in 5 ml concentrated hydrochloric acid diluted with ethanol to 100 ml).

RESULTS AND DISCUSSION

First the stability of the stock solution of congo red in distilled water was measured spectrophotometrically (0·001 mol l^{-1} congo red). Measurement of the absorbance at 482 nm after dilution of the stock solution to $5 . 10^{-5}$ mol l^{-1} indicated that its concentration does not change over the first three days, decreases by 1·6% over 5 days and by 4·4% over 10 days. Thus, a fresh stock solution should be prepared every week and it should be stored in the dark; more dilute solutions must be prepared fresh daily.

TAST *Polarography at a Dropping Mercury Electrode*

The dependence of $E_{1/2}$, I_{lim} and the slope of the logarithmic analysis on the pH is given in Table I. The method of linear regression yielded a value of $\Delta E_{1/2}/\Delta pH =$ = 66·8 mV for the slope of the dependence of $E_{1/2}$ on the pH. This shift of $E_{1/2}$ to more negative values with increasing pH can be explained by prior protonation of the azogroup, leading to a decrease in the electron density in the region of the double bond between the nitrogen atoms, leading to easier electron transfer. A useful polarographic wave cannot be obtained at pH < 4 and pH > 10 because of the marked shortening of the drop time and drop breakage. The observed dependence of the limiting current on the pH in the region pH 6—10 can be explained in terms of the varying number of exchanged electrons (see coulometry at constant potential).

The decrease in the limiting current at pH < 6 is apparently connected with aggregation of congo red in this medium with formation of micelles[27], leading to visually observable formation of colloidal solutions. The highest and best developed waves were obtained in medium with pH 7, in which all subsequent dependences were measured. The dependence of $\log I_{lim}$ on the log of the flow rate m at constant drop time of 1 s has a slope of 0·65 ($\sim 2/3$) and the dependence of $\log I_{lim}$ on the log of the drop time t has a slope of 0·16 ($\sim 1/6$). It thus holds that $I_{lim} = \text{const.}\ m^{2/3}t^{1/6}$ which, together with the linear dependence of the limiting current on the depolarizer

TABLE I

The effect of the pH on the TAST and DP polarography of congo red ($c = 10^{-4}$ mol l^{-1})

pH	$E_{1/2}$ mV	I_{lim} μA	Slope of log. analysis mV	E_p mV	I_p μA
3·99	−229	0·14	46·0	−220	0·38
5·08	−348	0·70	39·9	−310	1·95
5·99	−421	1·20	27·6	−375	2·93
7·01	−485	1·25	38·9	−440	3·38
7·97	−542	1·25	46·2	−500	2·70
8·99	−588	0·96	44·8	−550	1·60
10·09	−655	0·58	40·5	−605	1·70

TABLE II

The parameters of the calibration straight lines and calculated detection limits for various methods of determining congo red (I)

Method	$c(I)$ μmol l^{-1}	Slope μA mol^{-1} l	Intercept μA	Correlation coefficient	Detection limit mol l^{-1}
TAST	1 — 10	$1{\cdot}52 \cdot 10^4$	$5{\cdot}3 \cdot 10^{-3}$	0·9928	$4{\cdot}0 \cdot 10^{-7}$
DPP	0·1 — 1	$6{\cdot}14 \cdot 10^4$	$9{\cdot}0 \cdot 10^{-4}$	0·9980	$2{\cdot}2 \cdot 10^{-8}$
FS DPV[a]	0·1 — 1	$1{\cdot}05 \cdot 10^5$	$-6{\cdot}8 \cdot 10^{-2}$	0·9834	$4{\cdot}9 \cdot 10^{-8}$
FS DPV[b]	0·1 — 1	$1{\cdot}70 \cdot 10^5$	$2{\cdot}7 \cdot 10^{-3}$	0·9977	$2{\cdot}4 \cdot 10^{-8}$
LSV[a]	0·1 — 1	$7{\cdot}85 \cdot 10^4$	$1{\cdot}6 \cdot 10^{-3}$	0·9973	$1{\cdot}9 \cdot 10^{-8}$
LSV[c]	0·01— 0·1	$6{\cdot}10 \cdot 10^5$	$2{\cdot}2 \cdot 10^{-3}$	0·9975	$2{\cdot}0 \cdot 10^{-9}$

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

concentration indicates the diffusion character of the limiting current. The dependence of the wave height on the depolarizer concentration is linear in the range 100 – – 1 μmol l^{-1}; its parameters and the detection limit are given in Table II.

Coulometry at Constant Potential

The time dependence of the coulometric reduction of congo red at constant potential in media with various pH values is given in Fig. 1, from which it follows that 8 electrons are exchanged in acid and neutral media and 4 electrons in alkaline media. This fact is also confirmed by the dependence of the height of the TAST polarographic wave on the charge Q, which is linear and intercepts the horizontal axis at a value of Q corresponding to exchange of 8 electrons at pH 7 and 4 electrons at pH 10. Spectrophotometric changes during the coulometric reduction at pH 10 are depicted in Fig. 2, from which it can be seen that the absorbance at 500 nm decreases, accompanied by decolouration of the solution and an increase in the absorbance at 245 nm, attributed to the formation of the hydrazo group.

Proposed Reduction Mechanism

The slope of the logarithmic analysis in relationship to the coulometrically determined number of exchanged electrons indicates that the studied process is irreversible.

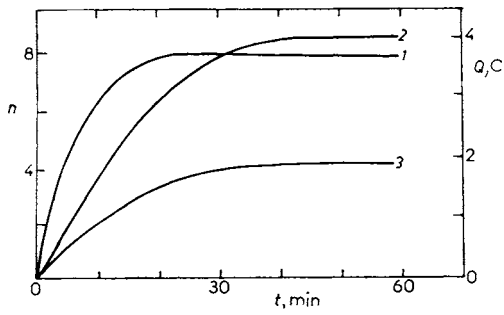


FIG. 1

The dependence of the charge passed Q and the number of exchanged electrons n on time in the coulometric reduction of congo red at constant potential. *1* pH 2, −300 mV; *2* pH 7, −600 mV; *3* pH 10, −1 100 mV

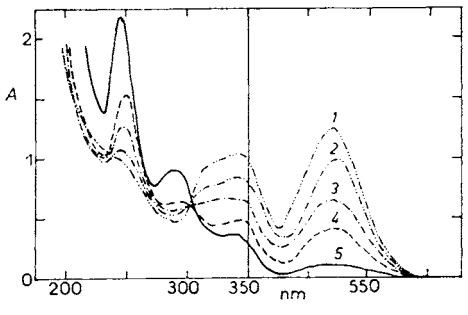


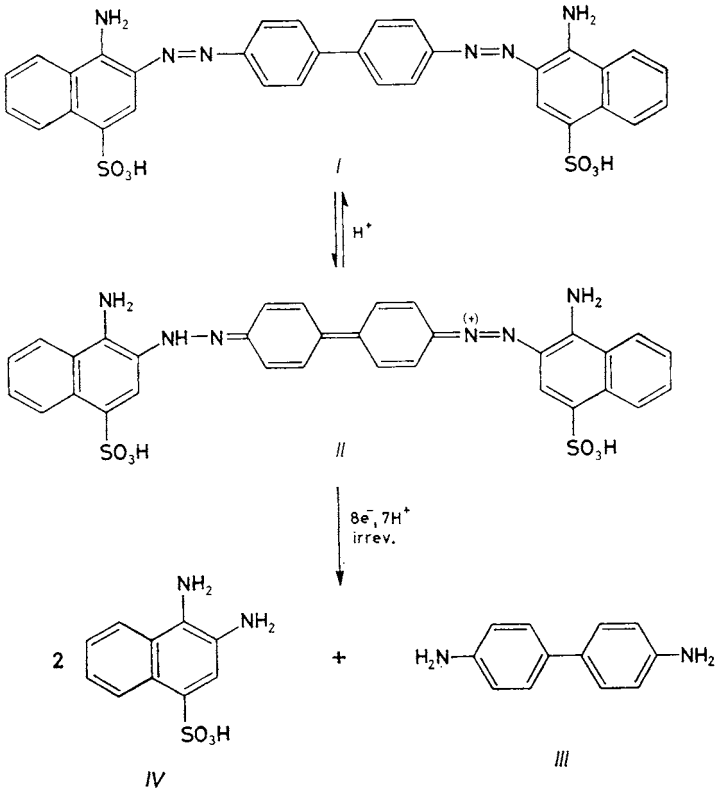
FIG. 2

Spectrophotometric study of the reduction of 5 . 10^{-5} mol l^{-1} congo red by coulometry at constant potential of −1 100 mV at pH 10. Reduced % of the initial substance (calculated on the basis of four-electron reduction): *1* 0; *2* 25; *3* 50; *4* 75; *5* 100

The following dependence of the half-wave potential on the depolarizer concentration at pH 7 is also in agreement with the irreversible character of the process:

c (mol l^{-1})	10^{-4}	$4 . 10^{-5}$	10^{-5}	$4 . 10^{-6}$
$E_{1/2}$ (mV)	−490	−495	−500	−505

The irreversibility of the process was also confirmed by cyclic voltammetry of a congo red solution ($c = 10^{-4}$ mol l^{-1}) at pH 2 and 7 at a HMDE at a polarization rate of 2 − 100 mV s^{-1}: no anodic peak was observed on any of the measured voltammograms. A slight anodic peak was observed at pH 10; this may be connected with the oscillopolarographically determined[17] reversibility of the process in 1M sodium hydroxide.



SCHEME 1

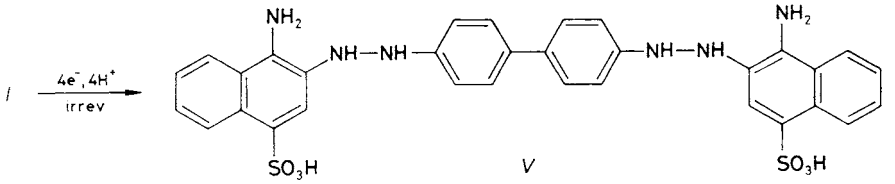
Although spectral changes occur at pH < 6 (Fig. 3), connected with structural changes in the transition from the red to the blue form, exchange of 8 electrons is

retained. In the acid region, the UV spectra of solutions after reduction by coulometry at constant potential and after chemical reduction with powdered zinc are identical, confirming the complete electrochemical reduction of both azo groups to the corresponding amines. The presence of benzidine formed was also demonstrated by thin-layer chromatography (an R_F value of 0·28 was found for the reduction product and for standard benzidine and their identity was also confirmed by mixed chromatography).

$$I \xrightarrow[\text{irrev.}]{8e^-,H^+} IV + III + IV$$

SCHEME 2

It can thus be assumed that, at pH $\leqq$ 3, where the red form (*I*) is converted to the blue form, assigned structure (*II*) (ref.[20]), congo red is irreversibly reduced with exchange of 8 electrons according to Scheme 1. At pH from 6 to 8, direct 8-electron reduction of the red form occurs according to Scheme 2; at higher pH values, four-electron irreversible reduction occurs according to Scheme 3. The increased reversi-



SCHEME 3

bility of the reduction in the strongly alkaline region may be connected with the assumed[17] conversion of the red form (*I*) to form (*VI*) in strongly alkaline medium, according to Scheme 4.

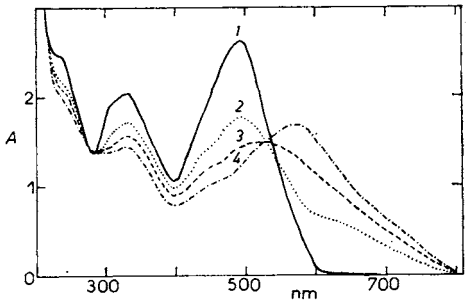
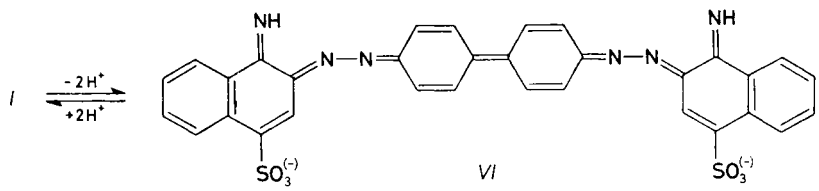


FIG. 3

The effect of the pH on the spectrum of congo red ($c = 1 . 10^{-4}$ mol l^{-1}). Specific cuvette width 1 cm. *1* pH 6·03; *2* pH 5·01; *3* pH 4·00; *4* pH 2·99



SCHEME 4

The proposed mechanism is thus analogous to the mechanism of reduction of *o*- or *p*-bisazobenzene[7,8] or of some other bisazodyes[9-13].

Differential Pulse Polarography

The dependence of the peak potential E_p and peak current I_p on the pH is given in Table I. The observed dependence of E_p on the pH has the same character as the dependence of $E_{1/2}$ on the pH and can be explained analogously. The method of linear regression yielded a value for the slope of this dependence of $\Delta E_p/\Delta pH = 64$ mV. The observed dependence of I_p on the pH is a result of combination of the effect of the pH on the height of the limiting current and on the reversibility of the studied process. The maximal I_p value was obtained in medium with pH 7, in which all the other dependences were measured.

First the stability of the polarographed congo red solution was measured. The results obtained are listed in Table III, from which it follows that the stability decreases somewhat with decreasing concentration, but is always sufficient from an analytical point of view.

The following dependence of E_p and I_p on the modulation amplitude at a drop time of 1 s and mercury reservoir height of 25 cm was found:

modulation amplitude (mV)	−100	−50	−25	−12·5
E_p(mV)	−445	−490	−505	−510
I_p (μA)	3·13	1·85	0·88	0·43

and the following dependence of I_p on the square root of the reservoir height h at a drop time of 1 s and modulation amplitude of −100 mV:

$h^{1/2}$ ($cm^{1/2}$)	8	7	6	5
I_p(μA)	6·20	5·15	4·13	3·13

An increase in I_p from 3·13 μA at a drop time of 1 s to 5·93 μA at 2 s was also observed at a modulation amplitude of −100 mV and mercury reservoir height of 25 cm, in agreement with theory.

The dependence of I_p on the depolarizer concentration was measured in the range 100 – 0·1 μmol l^{-1}, where it is linear. Its parameters and the calculated detection limits are listed in Table II.

Fast Scan Differential Pulse Voltammetry at a Hanging Mercury Drop Electrode

Once again, the best developed curves were obtained in medium with pH 7, in which all subsequent dependences were measured. It was found (see Table IV) that the peak height increases with increasing time from the formation of the drop up to the beginning of the measurement, which can be explained by accumulation of congo red by adsorption on the surface of the working electrode. It further follows from Table IV

TABLE III
The stability of congo red (*I*) in Britton–Robinson buffer, pH 7

c (*I*), mol l^{-1}	% c after t, min				
	0	15	30	45	60
10^{-4}	100·0	100·0	100·0	100·0	99·8
10^{-5}	100·0	99·5	99·2	98·5	97·5
10^{-6}	100·0	99·2	98·9	97·6	96·7

TABLE IV
The effect of the accumulation time t on the peak height of congo red I_p, measured by the FS DPV method at a HMDE

t, s	I_p, nA			
	a	*b*	*c*	*d*
5	1 010	95	1 860	156
10	1 130	154	1 900	175
30	1 430	210	1 940	200
60	1 610	245	1 960	270

[a] Accumulation in unstirred solution, depolarizer concentration $2 . 10^{-5}$ mol l^{-1}; [b] accumulation in unstirred solution, depolarizer concentration $9 . 10^{-7}$ mol l^{-1}; [c] accumulation in stirred solution, recorded 10 s after termination of stirring, depolarizer concentration, $2 . 10^{-5}$ mol l^{-1}; [d] accumulation in stirred solution, recorded 10 s after termination of stirring, depolarizer concentration $9 . 10^{-7}$ mol l^{-1}.

that this process can be accelerated by stirring and that, with increasing accumulation time, the peak height approaches a certain limiting value, apparently dependent on maximal drop coverage by the adsorbed substance. Similarly, the accumulation is useful only at low concentrations of congo red, while it has practically no effect on measurements at concentrations of about 10^{-4} mol l^{-1}. The concentration dependence is linear in the range 10 – 0·1 μmol l^{-1} and its parameters are listed in Table II.

Linear Scan Voltammetry at a Hanging Mercury Drop Electrode

Once again, it was found that the peak height increases with time from the formation of the drop up to commencement of the measurement and that this increase is emphasized by stirring. Calibration curves in medium with pH 4, in which the best developed voltammograms were obtained, are linear in the range 10 – 0·01 μmol l^{-1}. Their parameters and the calculated detection limits are listed in Table II, from which it follows that this method is more sensitive than FS DPV. However, a calibration curve or the method of two standard additions must be employed, as the calibration straight lines do not pass through the origin and deviate from linearity at higher concentrations.

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