DETERMINATION OF CARCINOGENIC AZOBENZENE DERIVATIVES BY CONSTANT-POTENTIAL COULOMETRY

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The reduction of azobenzene, N,N-dimethyl-4-aminoazobenzene, N,N-dimethyl-4-amino-4'--hydroxyazobenzene, and N,N-dimethyl-4-amino-4'-aminoazobenzene was studied by constant--potential coulometry, and conditions were found for the determination of milligramme quantities of these substances in their aqueous–methanolic solutions.

The electrochemical behaviour of azo compounds has recently attracted increased interest, also in connection with the carcinogenic effect of a number of substances of this class[1]. As a part of a systematic study of redox reactions of carcinogenic azobenzene derivatives, methods of their polarographic analysis suitable for their determination in low concentrations have been worked out[2,3]. Suited for higher concentrations, constant-potential coulometry is a method of choice for the determination of the major components of samples and for the accurate determination of the concentration of stock solutions used for the calibration curve plotting for more sensitive methods. The azo group has been determined by coulometric titrations with sufficiently strong reductants such as trivalent titanium[4-8], divalent chromium[9], or dithionite[10], and by constant-potential coulometry employing usually a mercury pool cathode[11-15]. Constant-potential coulometry has also been used for the study of the mechanism of reduction of the azo group in aqueous[16] and nonaqueous[17] solutions. A method for the determination of 4,4'-dihydroxyazobenzene and 4-nitro-4'-hydroxyazobenzene by constant-potential coulometry in buffered aqueous solutions has been developed by us previously[18]. In view of the low solubility of many azobenzene derivatives in aqueous solutions, the determination in mixed aqueous–methanolic solutions is studied in this work for azobenzene (*I*), N,N-dimethyl-4-aminoazobenzene (*II*), N,N-dimethyl-4-amino-4'-hydroxyazobenzene (*III*), and N,N-dimethyl-4-amino--4'-aminoazobenzene (*IV*). Attention is paid not only to the analytical aspects, but also to the course of the reduction, which may be of interest from the point of view of the carcinogenesis of the substances under study.

EXPERIMENTAL

Apparatus

The coulometric determination was carried out with an OH-404 automatic coulometric analyzer (Radelkis, Budapest) in a 200 ml all-glass vessel. A mercury pool on the bottom served as the cathode, and an OH-935 platinum electrode and an OH-933 saturated calomel electrode (Radelkis, Budapest) served as the anode and the reference electrode, respectively. The cathode and anode compartments were separated by a sintered glass frit. The solution was stirred during the analysis using an OP-912 magnetic stirrer (Radelkis, Budapest). Inert atmosphere was provided by a stream of nitrogen, which was purified by passage through an alkaline solution of sodium anthraquinone- -2-sulphonate and solutions of chromium(II) ions in dilute hydrochloric acid in contact with a zinc amalgam; before entering the solution measured, the nitrogen was passed through a metha- nol-containing trap.

The polarographic and voltammetric measurements were performed on a PA 3 polarographic analyzer interfaced to an XY 4103 plotter (both Laboratorní přístroje, Prague). The three- -electrode connexion involving a drop electrode was used for the TAST polarography; controlled drop time 1 s, reservoir height 64 cm, polarization rate 10 mV s^{-1}. The voltammetric measure- ments were carried out with an RDE rotating disc electrode from glassy carbon (Laboratorní přístroje, Prague) 1·5 mm in diameter; the three-electrode connexion was used at a polarization rate of 5 mV s^{-1}. The cyclic voltammograms were obtained using an SMDE 1 hanging mercury drop electrode (Laboratorní přístroje, Prague). A platinum wire auxiliary electrode and a satu- rated calomel reference electrode were invariably used. The spectrophotometric measurements were carried out on a Specord UV VIS spectrophotometer (Carl Zeiss, Jena) in 1 cm quartz cells.

Reagents

Stock solutions of substances *I—III* (5 mmol l^{-1}) and *IV* (1 mmol l^{-1}) were obtained by dis- solving the substances (Research Institute for Organic Syntheses, Pardubice - Rybitví) in a known volume of redistilled methanol of reagent grade purity. Their titre was determined titanometrical- ly[19]. Solutions of lower concentrations were made up by diluting the stock solutions with metha- nol. The purity of the chemicals was checked by thin layer chromatography[20]. All solutions were kept in darkness. Britton–Robinson buffer solutions were prepared conventionally[21] and their pH was checked on a PHM 62 digital pH-meter (Radiometer, Copenhagen) equipped with a glass indicator electrode and a calomel reference electrode. Commercial Silufol UV 254 thin layers (Kavalier, Sázava) were employed. All the other chemicals used were of reagent grade purity.

Procedures

Constant-potential coulometry. To the coulometric vessel was added 90 ml of supporting electrolyte (buffer solution, phosphoric or acetic acid) and nitrogen-purged while starting the constant-potential preelectrolysis. Within approximately 15 min the residual current decreased to less than 0·2 mA and then remained constant. The parameters of the circuit for the automatic compensation of the residual current were then set, and 10·00 ml of methanolic solution of sample containing 0·9 — 6·0 mg of *I, II,* or *III,* or 0·5 — 2·4 mg of *IV,* prior freed from oxygen by 10 min nitrogen purging, was added. The finished electrolysis was indicated by a decrease in the electro- lysis current to the value of the residual current before the addition of analyte. The electrolysis was quantitative in approximately 40 min; the charge passed was determined by digital integra- tion.

Spectrophotometric monitoring of the reduction course. Sample aliquots of 5 ml were taken from the coulometric vessel at the beginning of the reduction and after the reduction of 25, 50, 75, and 100% of analyte, and their spectra were scanned over the ultraviolet-visible region.

Polarographic and voltammetric monitoring of the reduction course. In intervals as above, 10 ml or 20 ml aliquots were taken from the coulometric vessel, transferred into the polarographic or voltammetric vessel and nitrogen purged, and their TAST polarograms using a mercury drop electrode or DC voltammograms using a glassy carbon disc electrode were scanned. For *I*, the disc electrode was rotated at 2 000 rpm, whereas for the remaining substances the stationary electrode mode giving better developed curves for them was employed. Voltammograms of solutions containing the expected reduction products in the appropriate concentrations were also measured under identical conditions.

Cyclic voltammetry measurements. The cyclic voltammograms of the azo compounds were measured on a hanging mercury drop electrode in $0.1M$-H_3PO_4–methanol 9 : 1 solutions at pH 2.41 and polarization rates of 10 to 100 $mV\,s^{-1}$.

Thin layer chromatography of the reaction products. The solution after the coulometric reduction was neutralized with 10M-NaOH to pH 7 and extracted with 2 × 10 ml of chloroform. The extract was concentrated to 1 ml in a rotary vacuum evaporator, and 2 μl of the concentrate was applied to a Silufol UV 254 thin layer. The ascending chromatography mode in saturated vapours of the mobile phase was used. The mobile phase was benzene or a benzene–acetone 2 : 1 mixture for *I*, a benzene–acetone 2 : 1 mixture or a benzene–ethyl acetate 4 : 1 mixture for *II*, and a benzene–acetone 1 : 1 or 2 : 1 mixture or a chloroform–acetone 1 : 1 or 1 : 2 mixture for *III* and *IV*. The spots were detected under a UV lamp.

RESULTS

Constant-Potential Coulometry

The effect of the solution composition and of the applied potential on the course of the coulometric reduction was examined first on compound *III*. For 1.206 mg of *III*, the following values (averages of triplicate measurements) were obtained in dependence on pH at a constant potential of −750 mV in Britton–Robinson buffer–methanol 9 : 1 mixtures:

pH	5	4	3	2
III found, mg	1.116	1.133	1.159	1.200

In dependence on the applied potential, the values obtained at pH 2 were as follows:

E, mV	−250	−500	−750	−900
III found, mg	1.194	1.210	1.200	1.307

It can be deduced that the optimum conditions are pH 2 and potentials of −500 to −750 mV. At more negative potentials, an error appears which is probably as-

sociated with the higher residual current which is more difficult to compensate automatically. An advantage of the determination at more positive potentials is its higher selectivity resulting from the fact that interferences from substances reducing at more negative potentials are eliminated.

It was verified that the Britton-Robinson buffer can be replaced with 0·1M phosphoric acid. The accuracy and reproducibility of the determination along with the established numbers of exchanged electrons are given in Table I.

Spectrophotometric Monitoring of the Reduction Course

The spectral changes for the solutions of compounds *I* and *III* during the electrolysis are shown in Fig. 1. For *I*, the peak at 31 000 cm^{-1} belonging to the starting substance decreases and that at 40 500 cm^{-1}, which corresponds to hydrazobenzene as verified by a comparison with an authentic sample, increases. The latter peak decreases with time, which can be accounted for by a number of conceivable rearrangements of hydrazobenzene in the solutions used, as confirmed by a subsequent thin layer chromatography examination. For *III*, as well as for *II* and *IV*, a monotonic decrease in absorbance is observed, which is consistent with the fact that aromatic

TABLE I

Results of determination of azobenzene derivatives by constant-potential coulometry at −750 mV in 0·1M-H$_3$PO$_4$–methanol 9 : 1 solutions

Substance	n	Added mg	Found[a] mg	Relative standard deviation %
I	2	0·911	0·907	2·41
		1·822	1·810	0·77
		4·555	4·517	0·22
II	4	1·127	1·111	0·44
		2·254	2·225	0·49
		5·635	5·562	0·23
III	4	1·206	1·200	1·49
		2·412	2·363	0·41
		6·030	6·015	0·23
IV	4	0·481	0·469	0·62
		1·202	1·187	1·50
		2·403	2·380	0·54

[a] Averages of 7 replicate determinations, from which the standard deviation was also calculated.

amines formed by the four-electron reduction of these azo compounds do not absorb within the spectral region used.

The dependence of the absorbance on the charge passed, transformed to the number of electrons per molecule of depolarizer (n), is shown in Fig. 2 for compounds I
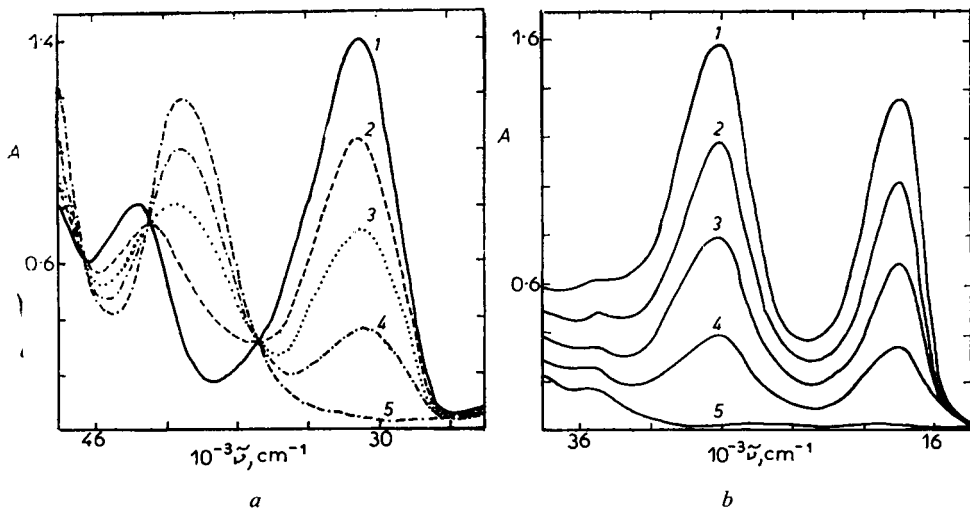

FIG. 1

Spectrophotometric monitoring of the reduction of I (a) and III (b) by coulometry at a constant potential of -750 mV in $0 \cdot 1$M-H_3PO_4–methanol $9 : 1$ solutions. Initial concentrations $8 . 10^{-5}$ mol l^{-1} and $1 . 10^{-4}$ mol l^{-1} for I and III, respectively. Optical path length 1 cm. Fraction of reduced initial substance (%): *1* 0, *2* 25, *3* 50, *4* 75, *5* 100
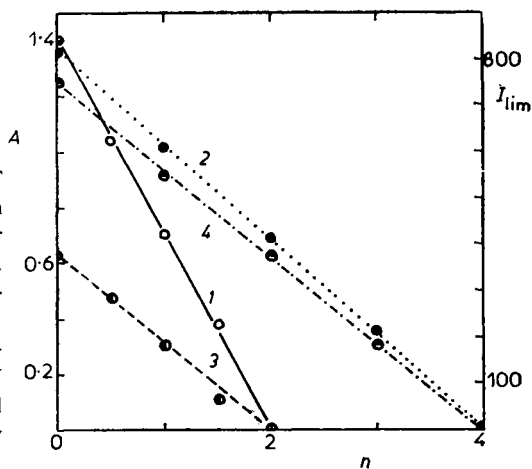

FIG. 2

Dependences of absorbance (A) and TAST polarographic limiting current $(I_{lim},$ nA) on the passed charge converted to the number of electrons per depolarizer molecule (n). Wavenumbers for the absorbance measurements 31 000 cm^{-1} for I *1* and 18 000 cm^{-1} for III *2*. Potentials for the TAST measurements -430 mV for I *3* and -540 mV for III *4*. Initial concentrations $8 . 10^{-5}$ and $1 . 10^{-4}$ mol l^{-1} for I and III, respectively

and *III*; two and four electrons are exchanged for the two substances, respectively. For substances *II* and *IV*, four-electron exchange occurs.

Polarographic Monitoring of the Reduction Course

The exchange of two electrons for compound *I* and four electrons for substances *II−IV* is evidenced also by the dependence of the TAST polarographic limiting current on the value of *n* (Fig. 2). Fig. 3 shows the changes in the TAST polarogram of compound *III* during the electrolysis. The records also enable us to estimate the usable potential region for the quantitative reduction of this substance by constant--potential coulometry.

Voltammetric Monitoring of the Reduction Course

The voltammograms of the four compounds under study, obtained before the reduction, after the reduction of 50% of the substances, and after the complete reduction are shown in Fig. 4. Voltammograms are also shown for the assumed reduction products, namely, hydrazobenzene for *I*, an equimolar mixture of aniline and N,N-di-methyl-*p*-phenylenediamine for *II*, an equimolar mixture of *p*-aminophenol and N,N--dimethyl-*p*-phenylenediamine for *III*, and an equimolar mixture of *p*-phenylene-diamine and N,N-dimethyl-*p*-phenylenediamine for *IV*. In all cases, the wave corresponding to the cathodic reduction of the azo compound decreased and that in the anodic region corresponding to the oxidation of the amines formed increased. The voltammograms after the reduction and those of solutions containing the appropriate amounts of the expected reaction products matched each other closely. ·
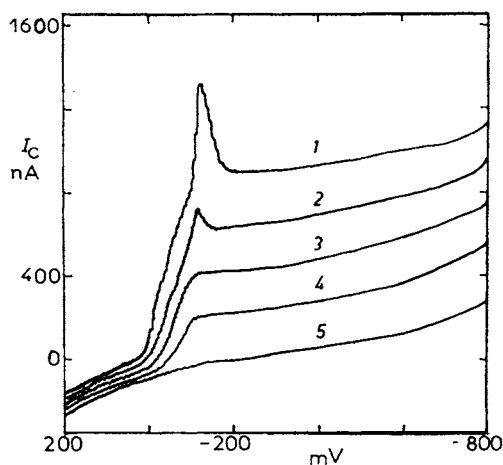

FIG. 3

Polarographic monitoring of the reduction of *III* by coulometry at a constant potential of -750 mV in $0·1\text{M-H}_3\text{PO}_4$–methanol 9 : 1 solutions. Initial concentration of *III* 1 . $. 10^{-4}$ mol l^{-1}. Fraction of *III* reduced (%): *1* 0, *2* 25, *3* 50, *4* 75, *5* 100
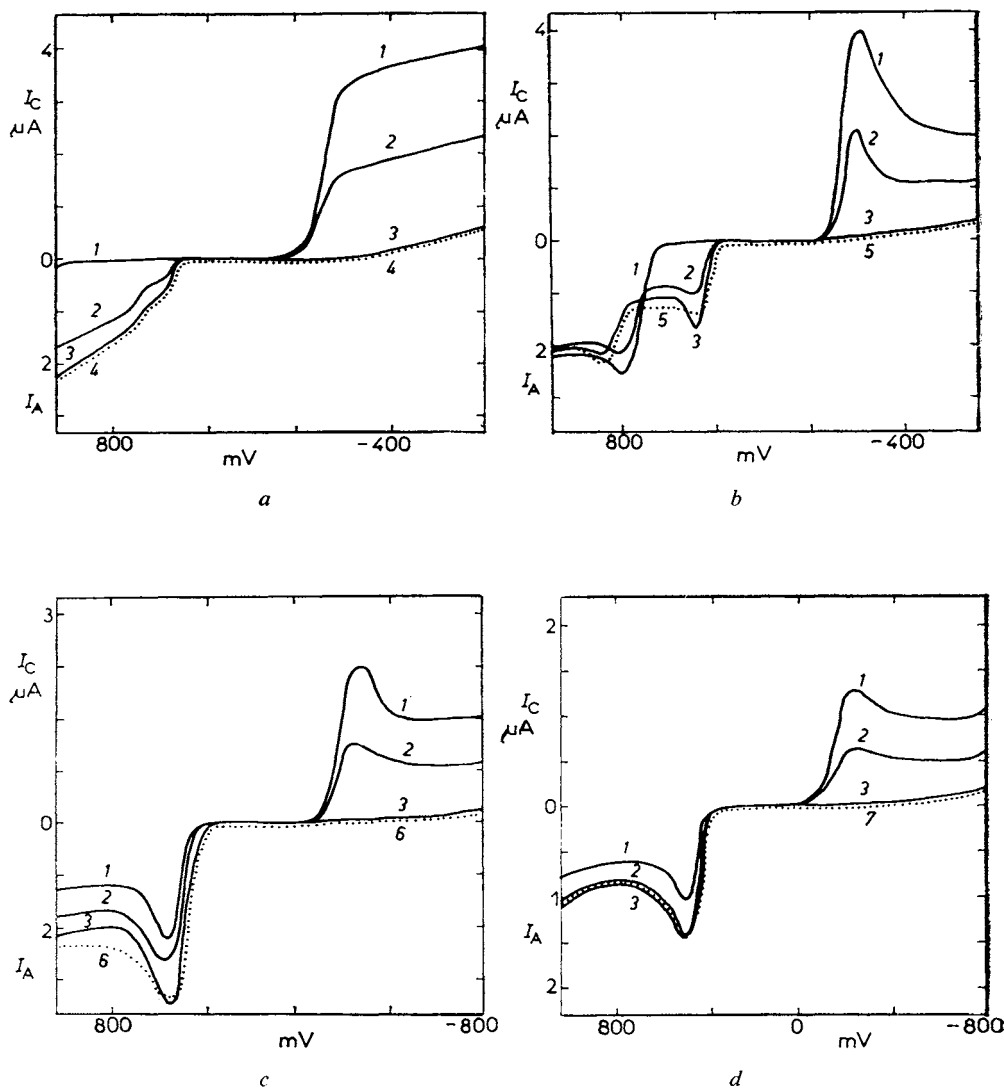
FIG. 4

Voltammetric monitoring of the reduction of I (a), II (b), III (c), and IV (d) by coulometry at a constant potential of -750 mV in 0.1M-H_3PO_4–methanol $9:1$ solutions. Initial concentrations (mol l^{-1}): I $5 . 10^{-5}$, II $2.5 . 10^{-4}$, III $2.5 . 10^{-4}$, IV $1 . 10^{-4}$. Fraction of substance reduced (%): 1 0, 2 50, 3 100. The dotted lines are voltammograms of the mixtures of the expected reduction products, *viz.* hydrazobenzene ($5 . 10^{-5}$ mol l^{-1}) for I 4, aniline and N,N--dimethyl-p-phenylenediamine ($2.5 . 10^{-4}$ mol l^{-1} each) for II 5, p-aminophenol and N,N-dimethyl-p-phenylenediamine ($2.5 . 10^{-4}$ mol l^{-1} each) for III 6, and p-phenylenediamine and N,N-dimethyl-p-phenylenediamine ($1 . 10^{-4}$ mol l^{-1} each) for IV 7

Cyclic Voltammetry Measurements

A cyclic voltammogram of *III* on a hanging mercury drop electrode is shown in Fig. 5, documenting the irreversible course of the reduction of this azo compound. The traces for the remaining azo compounds under study had the same character.

Thin Layer Chromatography of the Reduction Products

With benzene as the mobile phase, three spots were observed for *I* with R_F 0, 0·50 and 0·88; under these conditions, R_F of *I* is 0·88 and R_F of hydrazobenzene is 0·50 With a benzene–acetone 2 : 1 mixture, three spots were observed with R_F 0·47, 0·56 and 0·98; R_F of *I* is 0·98, R_F of hydrazobenzene is 0·91. The presence of the spot of *I* can be explained in terms of re-oxidation of hydrazobenzene by oxygen in the air during the isolation of the reaction products, whereas the absence of the spot of hydrazobenzene can be due to its rearrangement to amino derivatives of biphenyl. The presence of a primary aromatic amino group in the remaining spots was also confirmed by its reaction with *p*-dimethylaminobenzaldehyde. The presence of the corresponding aromatic amines in the reaction mixtures was proved also for compounds *II*−*IV*.

DISCUSSION

The results give evidence that the reduction of the four carcinogenic azo compounds studied follows the pathway
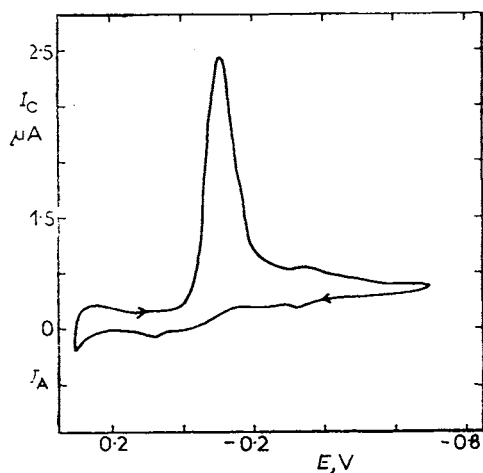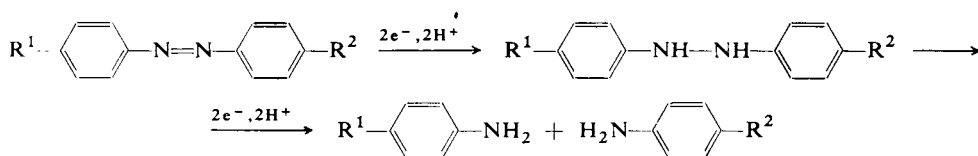

Fig. 5

Cyclic voltammogram of *III* ($1 . 10^{-4}$ mol . . l^{-1}) on a hanging mercury drop electrode in $0·1$M-H_3PO_4–methanol 9 : 1 solution. Polarization rate 100 mV s^{-1}; the potential is measured against a saturated silver chloride electrode

$$R^1-\langle\ \rangle-N{=}N-\langle\ \rangle-R^2 \xrightarrow{2e^-,2H^+} R^1-\langle\ \rangle-NH{-}NH-\langle\ \rangle-R^2 \longrightarrow$$

$$\xrightarrow{2e^-,2H^+} R^1-\langle\ \rangle-NH_2 + H_2N-\langle\ \rangle-R^2$$

with $R^1 = R^2 = H$ for *I*; $R^1 = (CH_3)_2N$, $R^2 = H$ for *II*; $R^1 = (CH_3)_2N$, $R^2 = OH$ for *III*; and $R^1 = (CH_3)_2N$, $R^2 = NH_2$ for *IV*. The reduction of *I* is finished after the exchange of two electrons, and the hydrazobenzene formed can undergo various rearrangements to give the corresponding amino derivatives of biphenyl. For *II−IV*, four electrons are exchanged in acid solutions, the azo group splits up and the corresponding aromatic amines are formed.

It can also be concluded that constant-potential coulometry can be used for the determination of milligramme quantities of these biologically active substances.

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