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ELECTROCHEMICAL CHARACTERISTICS OF NITRO-HETEROCYCLIC COMPOUNDS OF BIOLOGICAL INTEREST. II. NITROSOCHLORAMPHENICOL

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The electrochemical characteristics of nitrosochloramphenicol have been studied in aqueous buffer systems (pH 7.1) using direct current (d.c.) and differential pulse polarography, cyclic voltammetry and coulometric techniques. Up to 4 charge-transfer steps can be identified. The first reduction step is reversible both chemically and electrochemically, the charge-transfer product showing no tendency to undergo further reaction on the electrochemical time-scale. In contrast, the second reduction step is irreversible, with the product undergoing a fast following reaction to yield a redox-active species which was detected by cyclic voltammetry. From the data and by comparison with related systems, two reduction mechanisms are possible and are discussed.

KEY WORDS: Nitrosochloramphenicol, free radicals, reduction, cyclic voltammetry.

INTRODUCTION

It is now well established that the biological action in hypoxia or anoxia of a wide-range of nitro aromatic drugs depends upon the reduction of the nitro group.[1] This is the case whether the drugs are used as radiation sensitizers, in tumour chemotherapy, or as antibacterial agents.

The reduction properties of the NO_2 group are therefore of prime importance, and have received considerable attention, mainly using pulse radiolysis,[2] but also electrochemically.[3] Our attention has focused on the use of a range of electrochemical techniques and bulk synthetic experiments to extract the maximum possible information on the reduction characteristics of these redox-activated compounds.

From coulometry, drugs of the 5-nitroimidazole type are known to require between 3 and 4 electrons for complete reduction, while 2-nitroimidazoles have a 4-electron stoichiometry,[4,5] and chloramphenicol (CAP) is reduced by 6-electrons to the amine.[6,7] The final reduction products of the nitro-heterocycles are not responsible for causing damage at the biological target site, DNA, indicating that the reactive intermediate is a reduction product of 4-electron addition or less. Attention initially was directed to the hydroxylamine and nitroso derivatives, the 4 and 2 electron addition products respectively, as possible candidates.

The hydroxylamine derivatives of the 2-nitroimidazoles have been found to be inactive under anaerobic or hypoxic condition,[4] DNA damage only occurring when

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O_2 can partake in futile cycling[8,9] to form a reactive $OH^{\cdot}$ radical.[10] Nitrosoaryls are known to react rapidly with thiols, particularly glutathione.[11,12] However, reactivity in the absence of oxygen is unknown.

To date there have been no successful attempts to isolate the nitroso-derivative of a heterocyclic drug of biological or medical relevance. In 1978, however, Corbett and Chipko succeeded in chemically reducing chloramphenicol by 2-electrons to yield nitrosochloramphenicol (NO–CAP).[13] Initial studies showed that NO–CAP caused damage to DNA in the presence of O_2 and a transition metal. The effect was inhibited by metal chelating agents and catalase.[14] These observations were indicative that NO–CAP induced DNA damage via superoxide and $OH^{\cdot}$ radical formation in a Fenton type reaction.

To obtain further information on NO–CAP, particularly concerning the charge-tranfer mechanism of the nitroso group reduction, we have conducted a detailed investigation into the electrochemical properties of NO–CAP, which we report here in full. This is of interest not only in its own right, but also as a model compound for nitroso-heterocyclic drugs of biological importance.

MATERIALS AND METHODS

Synthesis of Nitrosochloramphenicol (NO–CAP).

NO–CAP, D-(−)-threo-1-(p-nitrosophenyl)-2-dichloro-acetamido-1,3-propane diol, was prepared using the general method employed by Carbett and Chipko,[13] where CAP was reduced with Zn and NH_4Cl to the hydroxylamine, followed by back oxidation with $FeCl_3$ to the nitroso. The temperature of the reaction mixture was kept below 30°C and the total reaction time was less than 10 minutes, as suggested by Eyer and Schneller,[11] to minimize dechlorination of the dichloroacetamido side-chain. In addition, after reaction with $FeCl_3$, the blue-green solution was immediately freeze-dried, then stored under N_2 at 4°C, for subsequent characterization and experimentation. Chloramphenicol was obtained from Sigma Chemical Co., and used without further purification. *Analysis:* found C = 43,14%, H = 4.1%, N = 9.09% (calculated for $C_{11}H_{12}Cl_2N_2O_4$ C = 43%, H = 3.94%, N = 9.12%) *Electronic spectrum* (from 500 to 200 nm in C_2H_5OH using a Pye Unicam SP-800 series B spectrophotometer) 314 nm (logϵ = 4.17) 287 nm (4.11) 214 to 222 nm, pronounced shoulder. *HPLC,* single peak, confirming purity in excess of 97%.

Electrochemical Techniques

Voltammetric measurements were carried out in 0.2 mol/dm^3 Na_2HPO_4, NaH_2PO_4 or 1.5×10^{-2}mol/dm^{3}NaCl, 1.5×10^{-3}mol/dm^3 trisodium citrate (0.1 ssc) buffers, pH = 7.1, previously degassed with water-saturated N_2. Cell solutions ranged from 2 to 60 $\times$ 10^{-5}mol/dm^3 in complex, but routine drug concentrations were 2×10^{-4}mol/dm^3.

A PAR 264A polarographic analyzer, interfaced with a PAR 303E cell-stand with 3-electrode cell configuration and a Bausch and Lomb RE 0088 x–y recorder were used. The electrode arrangement consisted of an aqueous Ag/AgCl reference electrode and a Pt wire auxilliary electrode in a 5–10 ml glass cell. For polarography, the working electrode was a dropping mercury electrode (dme) with an electronically

controlled drop-time (t_d) of 1 second, and a routine scan rate of $5\,mVs^{-1}$. Cyclic voltammetry (CV) used a hanging drop mercury electrode (hdme) with scan rates from 10 to $500\,mVs^{-1}$.

NO–CAP was reduced electrolytically at a constant voltage of either −0.25 or −0.80 Volts in 0.1 ssc under an N_2 atmosphere. Coulometry was carried out by automatic integration of current and time using an integrating multivoltmeter (Time Electronics) connected across a 1 K ohm resistance in the reduction circuit.[4,15] A mercury pool was used as the cathode, and Ag/AgCl as the anode.

RESULTS

The electrochemistry of nitrosochloramphenicol (NO–CAP) was studied in 0.2 mol/dm^3 NaH_2PO_4, Na_2HPO_4 or 0.1 ssc buffers. This was found to be markedly more complex than that found for the parent chloramphenicol (CAP) which, in common with a range of nitro-compounds, is reduced in aqueous media via a single irreversible 4-electron addition.[3]

Both d.c. and differential pulse polarography of NO–CAP showed one clearly defined reduction, with up to two further reductions at more negative potentials (see Table I), but unfortunately, measurements were restricted by poor resolution. However, from the d.c. polarogram, a logarithmic analysis of the first reduction step confirmed Nernstian behaviour, with a gradient of 55 to 60 mV. The differential pulse polarogram of the first reduction showed a symmetrical peak of the same dimensions irrespective of scan direction, with the peak potential differing by 25 mV (the same as the pulse amplitude). The data is therefore in line with a reversible diffusion-controlled electron transfer.

Our primary source of information was cyclic voltammetry (CV), a technique which, by reversing the direction of the potential sweep, direct details concerning the chemical stability of an electrochemically generated species can be obtained. Furthermore, CV allows the detection of redox-active products following the charge-transfer step. Using CV, the full complexity of the NO–CAP reduction scheme was seen, with up to 4 reduction steps being identified (Figure 1).

TABLE I
Electrochemical data for Nitrosochloramphenicol and chloramphenicol

		polarography		cyclic	
drug	buffer	differential pulse, Ep(V)	d.c. $E_{1/2}$(V)	voltammetry $E_{1/2}$(V)	ΔEp mV
NO–CAP	phosphate	−0.06	−0.04	−0.045	30
		−0.665	−0.70[b]	−0.600[a]	-
		-	-	−1.1[a,b]	-
	ssc	−0.135	−0.115	−0.129	60
		−0.505, −0.655	−0.70[b]	−0.665[a]	-
		-	-	−1.2[a,b]	-
CAP	Phosphate	−0.410	−0.355	−0.450[a]	-
	ssc	−0.605	−0.535	−0.615[a]	-

[a] irreversible, forward-wave peak potential quoted
[b] very poorly resolved, estimated only

The first reduction step ("a" in Figure 1), was fully reversible, in line with d.c. and differential pulse polarographic data. The reduced product was stable on the electrochemical time-scale, giving return-to-forward peak current ratios (ip_r/ip_f) of unity at all scan rates. The peak-to-peak separation (ΔEp) in 0.1 sec was approximately 60 mV in line with a one-electron transfer ($\Delta Ep = 60/n$ mV, where n = the number of electrons involved in the charge-transfer step), thus confirming the d.c. polarographic data. The couple can thus be represented as in equation (1)

$$R\text{-}NO + 1e^- \Leftrightarrow R\text{-}NO^{\cdot} \quad (1)$$

However, in 0.2 mol/dm^3 phosphate buffer, $\Delta Ep = 30$ mV, indicative of a 2-electron reduction step, *i.e.* the couple illustrated in equation (2)

$$R\text{-}NO + 2e^- + 2H^+ \Leftrightarrow R\text{-}NHOH \quad (2)$$

The second reduction step (labelled "b" in Figure 1) was irreversible, showing the characteristic shift in peak potential to more negative values with increasing scan rate, with no evidence under any conditions for a return wave on the reverse scan. The current response initially increased linearly with drug concentration, but then reached a constant value at a drug concentration of 1×10^{-4} mol/dm^3, irrespective of buffer system. This is in contrast to the first reduction, which showed a linear relationship between current response and drug concentration between 2 and 60×10^{-5} mol/dm^3. This current limiting behaviour most likely indicates that the redox-active species was adsorbed onto the mercury drop working electrode surface and was therefore restric-

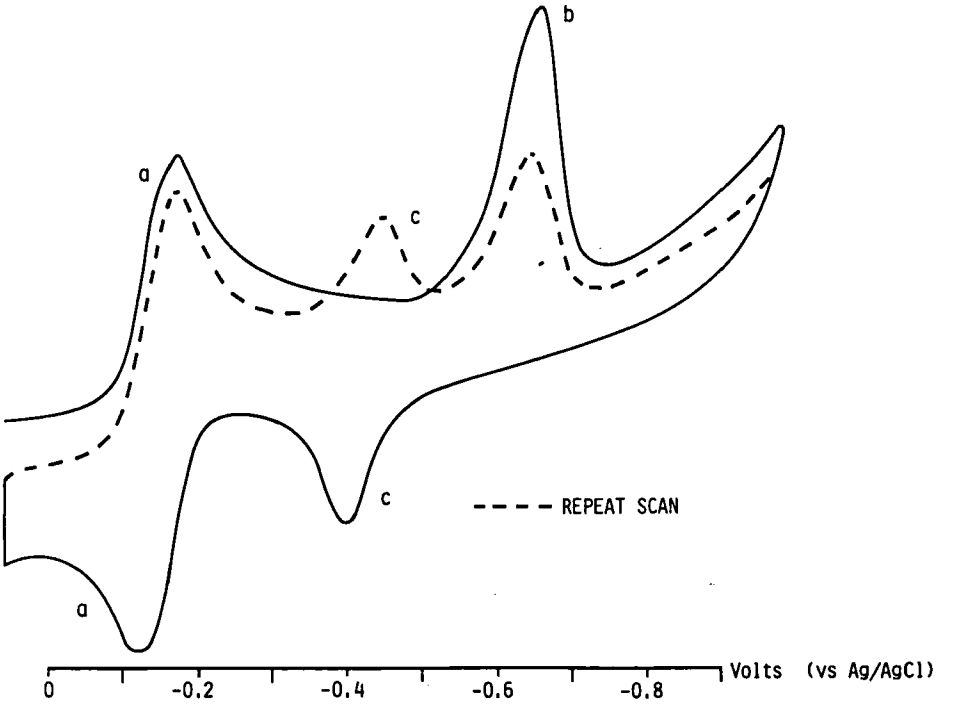


FIGURE 1 The cyclic voltammetry of NO-CAP in 0.1 ssc buffer

ted by the size of the drop. At low concentrations, *i.e.* 2×10^{-5} mol/dm^3, the current response for both first and second reduction steps was comparable.

The third reduction wave (not shown in Figure 1) was highly irreversible, with a very distended appearance from which it was only possible to estimate the peak potential as between $Ep_f = -1.0$ and -1.1 V.

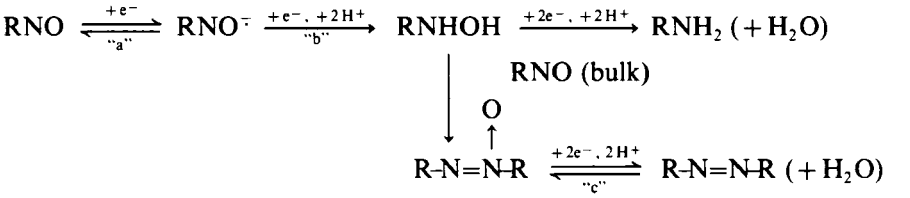
On the reverse scan, a wave was observed at $Ep_r = -0.262$ or -0.40 V in phosphate and ssc buffers respectively, which was due to the formation of a new redox-active species (labelled "c" in Figure 1). This was clearly the result of a chemical reaction following the second reduction step, being totally absent if the scan was reversed prior to the second reduction step (switching potential, $E_\lambda > -0.60$ V). Furthermore, the current response directly mirrored that of the second reduction step with respect to drug concentration. Repeat cycling showed the corresponding reduction wave, only on the second and subsequent negative scans, with $Ep_f = -0.302$ or -0.44 V (thus $E_{1/2} = 0.282$ and -0.420 V, $\Delta Ep = 40$ mV) in phosphate and ssc buffers respectively. A concommitant decline in the current response for process "b" was observed on the second negative scan. The first reduction was unaffected by switching potential.

Coulometry was undertaken at two values corresponding to the first and second reduction steps. At a fixed potential of -0.25 V, an n value of 1 (± 0.01) was found. If the potential was then switched to -0.80 V, the addition of further 3.5 electrons resulted (*i.e.* total n value required for complete reduction was 4.5).

DISCUSSION

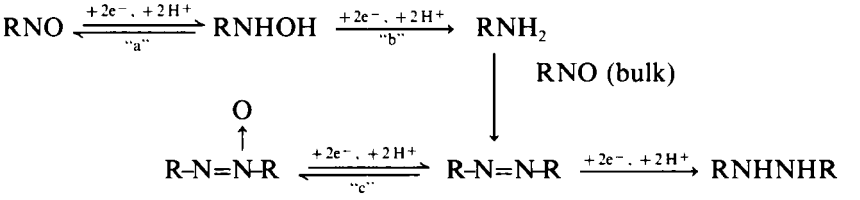
The first reduction of NO–CAP (process "a" Figure 1) gives rise to a product with no apparent tendency to undergo further chemical reactions on the time-scale of the voltammetric experiment. In the cyclic voltammetric mode, ΔEp for a reversible diffusion controlled process is 60 mV/n. The results in 0.1 ssc therefore indicate n = 1. This was confirmed by d.c. polarography in both 0.1 ssc and 0.2 mol/dm^3 phosphate buffer systems, where a logarithmic analysis confirmed Nernstian behaviour, with gradients of 58 and 54 mV respectively. These results are indicative that the first reduction step involves the reversible transfer of one electron – thus assigned to a NO–CAP/$NO^{\cdot}$–CAP^{-} couple, as in equation (1). The second reduction "b" can consequently be assigned to the one-electron addition (plus protonation) to form the hydroxylamine. The presence of the new redox-active species formed as a consequence of the second reduction step can be explained by the reaction of the hydroxylamine (the 2nd reduction product) with unreduced NO–CAP present in the bulk solution. This results in the formation of the azoxy derivative, itself redox active, the presence of which is detected by the new electrode response, process "c". We have previously observed a highly irreversible reduction with a very distended forward wave, at potentials in the range -1.0 to -1.1 V in the CVs of a range of nitro-aromatic compounds. In those cases the reduction was assigned to the hydroxylamine to amine conversion, the two-electron $RNHOH/RNH_2$ couple. A similar assignment would be in line with our present observations on the NO–CAP system. Scheme 1 represents the redox mechanism discussed above

Scheme 1



An alternative interpretation of the data is, however, possible. In the literature, nitrosobenzene is reported as undergoing a 2-electron reduction to the hydroxylamine (equation 2)[16]. This would be in line with our CV data in 0.2 mol/dm^3 phosphate buffer, where $\Delta Ep = 30$ mV. In view of this a reassignment of the electrochemistry is in order, which can be illustrated in scheme 2

Scheme 2



The reversible first electron transfer now reprsents a RNO/RNHOH couple and the irreversible 2nd reduction due to amine formation. The 3rd reduction and process "c" are both now assignable to the reduction and oxidation respectively of a new redox-active species, the azo derivative, the result of reaction of the amine with unreduced NO–CAP in the bulk solution. Process "c" in both schemes is thus assigned to an azo/azoxy couple.

Two possible reduction schemes are therefore possible, but closer examination of the data would seem to favour scheme 1.

The only voltammetric evidence for NO–CAP suggesting scheme 2 is the CV data in 0.2 mol/dm^3 phosphate buffer (the d.c. polarographic data is in line with the ssc results favouring n = 1). The literature reports the tendency of the 2-electron reduction product of nitrosobenzenes to react with unreduced material to form the azoxy derivative[17] and non-integral values for n were obtained between 1 and 2 by coulometry. However, we observed no tendency in the NO–CAP system for the first reduction product to participate in any chemical reaction following the charge transfer step, ip_r/ip_f from the CV being unity under all experimental conditions. We would also expect to see the redox response for the azoxy derivative on the first negative scan; instead, however, it is only after the second reduction that we note formation of a new redox-active species. In scheme 1 we have identified the new electrode response as being assigned to azoxy formation, due to reaction between HOHN–CAP and NO–CAP but taking place only as a consequence of process "b" (representing HOHN–CAP formation). The classic reversibility displayed by process "a" might not be expected to be associated with a NO–CAP/HOHN–CAP couple, which involves an overall 2 electron and 2 proton addition, requiring a considerable re-organisation of the molecular orbitals. A quasi-reversible reduction would be more in line with these

requirements. There thus appears to be a number of unfavourable factors for scheme 2.

The evidence against scheme 1 amounts to the reported literature behaviour for nitrosobenzene and the ΔEp value from the CV in 0.2 mol/dm^3 phosphate buffer. In favour of scheme 1, the similarity was noted between the electrode response for the irreversible third reduction and the reduction found for a range of nitro-aromatic compounds, assigned to $RNHOH/RNH_2$. Nitroso compounds are widely used as spin-trapping agents for short-lived free-radicals, attesting the stability of the $RNO^{\cdot-}$ under certain conditions. The chemical reversibility of the first redox-couple in scheme 1 would not therefore seem so surprising.

Our coulometric evidence strongly suggests scheme 1, where the number of electrons required for the first reduction step was 1. In a less complicated system, this data could be taken as definitive proof for scheme 1 being the redox mechanism followed. In this particular instance, however, if scheme 2 were followed it would be possible to obtain an n value of 1 for the 2-electron reduction "a" but *only* if equation (3) occurred with 100% efficiency. A more likely result would be a non-integral value between 1 and 2 as found for nitrosobenzene. As already discussed, the CV shows no evidence for any chemical reaction following the first charge-transfer step.

$$RNO + RNHOH \longrightarrow R\text{-}N{=}N(\downarrow O)\text{-}R + H_2O \qquad (3)$$

The arguments presented above would strongly indicate scheme 1 as the more likely, although further experiments, employing *in situ* spectro-electrochemistry, particularly using FTIR techniques, should provide clearer information regarding the entire reduction pathway. Work is in progress to assess the biological action of NO–CAP under reduction and non-reducing conditions *vis-a-vis* its ability to damage DNA, and relate this to the behaviour of the parent compound (CAP).

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References

1. Edwards, D.I., *Biochem. Pharmacol.*, **35,** 53 (1986)
2. Wardman, P. and Clarke, E.D., in *"New Chemo and Radiosensitizing Drugs"*, Eds. A. Breccia and J.F. Fowler, (1985) Lo Scarabeo, Bologna, pp 21–38
3. Tocher, J.H. and Edwards, D.I., *Free Rad. Res. Communs.*, **4,** 269 (1988)
4. Knox, R.J., Knight, R.C. and Edwards, D.I., *Biochem. Pharmacol.*, **32,** 2149 (1983)
5. Middlestadt, M.V. and Rauth, A.M., *Int. J. Radiat. Oncol. Biol. Phys.*, **8,** 709 (1983)
6. Skolimowski, I.M., Knight, R.C. and Edwards, D.I., *J. Antimicrob. Chemother.*, **12,** 535 (1983)
7. Skolimowski, I.M., Rowley, D., Knight, R.C. and Edwards, D.I., *J. Antimicrob. Chemother.*, **7,** 593 (1981)
8. Perez-Rayes, E., Kalyanaraman, B. and Mason, R.P., *Molec. Pharmacol.*, **17,** 239 (1980)
9. Sealy, R.C., Swartz, H.M. and Olive, P.L., *Biochem. Biophys. Res. Commun.*, **82,** 68 (1978)
10. Laderoute, K.R., Eryavac, E., McClelland, R.A. and Rauth, A.M., *Int. J. Radiat. Oncol. Biol. Phys.*, **12,** 1215 (1986)
11. Eyer, P. and Schneller, M. *Biochem. Pharmacol.*, **32,** 1029 (1983)
12. Polnaszek, C.F., Peterson, F.J., Holtzman, J.L. and Mason, R.P., *Chem. Biol. Interact.*, **51,** 263 (1984)
13. Corbett, M.D. and Chipko, B.R., *Antimicrob. Ag. Chemother.*, **13,** 193 (1978)
14. Murray, T., Downey, K.M. and Yunis, A.A., *Biochem. Pharmacol.*, **31,** 2291 (1982)

15. Knox, R.J., Edwards, D.I. and Knight, R.C., *Int. J. Radiat. Oncol. Biol. Phys.*, **10,** 1315 (1984)
16. Smith, W.H. and Bard, A.J., *J. Am. Chem. Soc.*, **97,** 5203 (1975)
17. Vijayalakshamma, S.K. and Subrahmanya, R.S., *Electrochim. Acta,* **17,** 471 (1972)

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