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ELECTROCHEMICAL PROPERTIES AS A FUNCTION OF pH FOR THE BENZOTRIAZINE DI-*N*-OXIDES

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The electrochemistry of five benzotriazine di-*N*-oxides has been examined by cyclic voltammetry and differential pulse and dc polarographies as a function of pH. Between the pH range 8.5 and 2 the trend to less negative potentials with lowering of pH can be described by an equation of the type $Ep = -apH + b$. Comparison has been made with the mono- and zero-*N*-oxides which were found to show virtually identical trends in electron affinity with pH. The general electrochemical characteristics for the di- and mono-*N*-oxides under acidic conditions were found to be comparable with the zero-*N*-oxide. This was particularly the case on repeat scanning in the cyclic voltammetric mode. The redox mechanism involved reduction by a 4-electron addition step and subsequent loss of the *N*-oxide group(s) yielding the intact benzotriazine heterocycle. The heterocycle was also redox active, involving a reversible 2-electron reduction. For the di-*N*-oxides these two stages could be identified as separate processes at alkaline pH, but ony a single step at acidic values. The mono-*N*-oxide in which the electrochemical behaviour was dominated by the triazine, showed only a single reduction step, although the single *N*-oxide group was redox active.

KEY WORDS: Benzotriazine-*N*-oxides, redox mechanisms, pH effects, free radicals.

INTRODUCTION

The benzotriazine di-*N*-oxides are currently attracting considerable attention as new, non-nitro bio-reductive agents, activated in hypoxia. The lead compound in the series, SR4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide) is reported to show a higher selectivity for hypoxic cells than both quinone alkylating agents and the nitro-imidazole hypoxic cell radiation sensitizers.[1] The cell killing ability of SR4233 has been demonstrated under hypoxia using mammalian cell lines grown *in vitro* and in mouse SCC VIII tumours *in vivo* in combination with an X-ray dose of 20 Gy.[2] Pre- and post-irradiation treatment with SR4233 under hypoxia has also been demonstrated to radiosensitize aerobic Chinese hamster ovary cells.[3] This selective activation in hypoxia implies reductive activation of the drug which is O_2 sensitive. The most likely damage-causing species is the 1-electron addition product as the acceptance of 2 or 4 electrons to give the major reduction products, SR4317 (3-amino-1,2,4-benzotriazine-1-oxide) and SR4330 (3-amino-1,2,4-benzotriazine) respectively show no toxicity towards hypoxic cells *in vitro*.[1]

Investigations into the mechanism of action of these drugs are at an early stage, but given the importance of the reductive activation the charge-transfer properties have

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obviously received some attention. Pulse radiolysis, electrochemistry and enzymatic reduction methods[4,5] and *in vitro* metabolism have examined the distribution of the electron addition products SR4317 and SR4330 with time for SR4233. Voltammetric experiments on a range of di-*N*-oxides have shown two separate electron addition processes, which were assigned to the reduction of the *N*-oxide groups (and their subsequent loss) and the benzotriazine heterocycle at progressively more negative potentials.[6] Structure-activity relationships have been undertaken, where both hypoxic cytotoxicity and stimulation of O_2 consumption by respiration-inhibited cells were found to correlate with the polarographic half-wave reduction potential[7]. By making use of an electrolytic reduction technique in conjunction with a biological assay measuring DNA damage, the required reductive activation of the *N*-oxide function has been demonstrated.[6]

The influence of pH has been examined for SR4233.[8] A general shift towards less negative potentials and a simplification of the reduction process to a single step was observed as the acidity of the medium was increased. This was accompanied by the increased DNA damaging capability of the drug with a fall in the R_{37} value (defined as the % drug reduction required to give 37% cell survival) from 12 to 2.3% as the pH was lowered from 7 to 4. To gain further insight into the biologically important reductive activation mechanism of this series of drugs, we have examined and compared the effect of pH on the electrochemical properties of five di-*N*-oxides using three investigation techniques, cyclic voltammetry, differential pulse polarography and dc polarography. Comparison was also made with the mono- and zero-*N*-oxides as these provided further understanding into the reduction pathway. The results of these studies are presented here in full.

MATERIALS AND METHODS

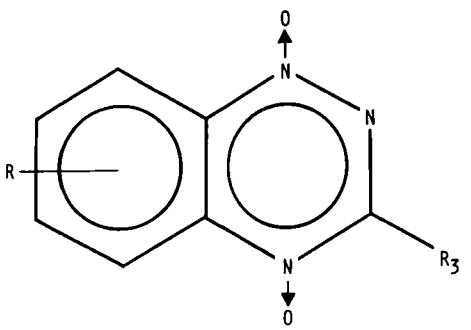
All the SR compounds were obtained from Professor J.M. Brown (University of Stanford, USA) and used as received without purification.

Voltammetric studies employed a PAR 264A polarographic analyzer interfaced with a PAR 303 cell stand with 3-electrode configuration and a Bausch and Lomb RE0088 x-y recorder. An aqueous Ag/AgCl reference electrode and a Pt wire auxilliary electrode were used in a 5–10 ml glass cell protected from light. Differential pulse and dc polarographies used an electronically controlled dropping mercury electrode (dme) with drop time of 1 second. The routine scan rate was $5\,mV\,s^{-1}$. Cyclic voltammetry used a stationary hanging drop mercury electrode with scan rates from 10 to $500\,mV\,s^{-1}$, but the typical value was $100\,mV\,s^{-1}$.

All voltammetric measurements were carried out in 1.5×10^{-1} mol dm^{-3} NaCl and 1.5×10^{-2} mol dm^{-3} trisodium citrate buffer (1.0 SSC) purged with H_2O-saturated N_2. This buffer concentration was found to be more effective over the pH range than the more usual 0.1 SSC. The pH of the solution was varied over the range 2 to 10.5, being monitored by a Whatman PHA 250 pH probe. Voltammetric measurements were taken every 0.5 to 1.0 pH unit by all three techniques. Cell solutions were 1×10^{-4} mol dm^{-3} with respect to drug concentraton, which was maintained throughout.

RESULTS

The structures of the drugs examined in this study are shown in Figure 1. The electrochemistry at approximately neutral pH has been previously reported.[6] This general behaviour was not found to change at alkaline pH above 8.5 with the di-*N*-oxides SR4233, SR4308 and SR4286 showing two reduction steps. By cyclic voltammetry, CV, only the second or more negative step was seen to give a product which had stability on the electrochemical time-scale, by the clear oxidation wave on the reverse scan with peak-to-peak separation, ΔEp of 40 to 80 mV. The return-to-forward peak current ratio was difficult to determine due to the overlapping nature of the forward current response, but a good estimate would be ip_r/ip_f of 0.7 to 0.9, increasing with scan rate, v. The repeat scan CV showed a decrease in the peak current of the first reduction and a relative increase in the more negative on the second and subsequent forward scans. The differential pulse polarography (dpp) confirmed the two step process, but due to their closely overlapping nature a full analysis of the individual steps was not possible; however, peak potentials could be easily measured. The dc polarography showed two distinct waves, both showing linear relationships from a logarithmic analysis, illustrating diffusion control. The first reduction always had the greater current response, but smaller gradient, by at least a ratio of 2:1. Reduction potentials showed only a slight negative shift with increasing pH, together with some separation between the reduction steps. SR4318 showed only a single



SR No.	R	R3	Oxides	pH dependence [a]	Ep [b]
4233	H	NH_2	2	Ep=-0.086pH+0.11	-0.49,-0.52
4286	6(7)-OMe	NH_2	2	Ep=-0.089pH+0.105	-0.52,-0.59
4308	6(7)-Cl	NH_2	2	-	-0.425,-0.51
4317	H	NH_2	1	Ep=-0.097pH+0.145	-0.535,
4330	H	NH_2	0	Ep=-0.099pH+0.145	-0.545
4318	7-Cl	ONH_4	2	Ep=-0.088pH+0.155	-0.46
4355	H	ONa	2	Ep=-0.094pH+0.095	-0.56

FIGURE 1 Structure and dependence of reduction potential on pH of the benzotriazine-*N*-oxides. [a]pH-dependence relationship holds between pH 8.5 and 2 determined from the differential pulse polarography data, but the same behaviour is found if CV or dc polarography is used. [b]Reduction potentials (volts) for the drugs at pH 7 determined using the relevant pH dependence equation or graphically as appropriate.

reduction step but the response was sufficiently broadened that it might easily consist of overlapping processes. A very minor response was noted as a weak anodic shoulder on the main reduction by both differential pulse and on the forward scan in the CV. A clear and well-defined return wave was present on the return scan with ΔEp of 150 mV. SR4355 showed similar behaviour but the electrode response in all techniques was very distended in appearance. Although a return wave was observed in the CV mode it was impossible to establish whether this was associated with the first or second reduction steps. The ΔEp was 140 to 260 mV between first and second forward waves respectively. Repeat cycles provided no further information due to the generally poor resolution.

The mono- and zero-*N*-oxides, SR4317 and SR4330, showed only a single reduction step which was highly resolved by all techniques. In the CV mode, ΔEp ranged from 50 to 80 mV, but SR4330 had consistently the greater ip_r/ip_f ratio, typically 0.90 as compared to 0.4–0.5 for SR4317. Differential pulse polarography showed a single symmetrical peak with a 5–10% decrease in peak current on changing scan direction from negative to positive. The dc polarogram also showed a single wave, which gave a linear logarithmic analysis, with gradients of 40–50 mV. Again no significant shift in reduction potential was observed with increasing pH.

Major changes were found in the electrochemistry at acid pH. In all cases a shift towards less negative potentials *i.e.* increased electron affinity, was observed as the acidity of the medium was increased. Over a limited pH range, 7 to 6.5, the voltammetry was distinctly different from that obtained at either more acid or alkaline conditions. This, in the di-*N*-oxides, generally had the form of a marked broadening of the current response in all investigation modes, but which particularly effected the appearance of the first reduction step. There was evidence that this broadening was due, at least in part, to the combination of overlapping processes, for example, SR4233 showed a weak anodic shoulder on the first reduction wave at pH 6.7. There was also a marked shift, but only of the first reduction, to less negative potentials. Care must be taken, however, as due to the diffuse nature of the voltammetry, peak measurements, particularly in the CV mode, could be difficult. Between these pH values only, both SR4317 and SR4330 showed a two stage reduction (Figure 2 illustrates the comparative behaviour of SR4330 at pH 6.6 and 3.8).

On further decreasing the pH, the separation between the two reductions rapidly disappeared, so that only a single step was observed. The appearance of the voltammetry was very similar for all the di-*N*-oxides and the mono-*N*-oxide. By differential pulse polarography the single peak was highly symmetrical, with peak potential differences between negative and positive scan directions of 30–50 mV and a 10% difference in peak current. The dc polarography showed a single wave, which gave a linear logarithmic analysis, with gradients of 45–70 mV for the di-*N*-oxides and SR4317. In the CV mode the forward wave was very noticeably greater than the return wave and was frequently sharp or pointed in appearance. The ip_r/ip_f ratios reflected this with typical values of 0.3 to 0.5 for the di-*N*-oxides and 0.45 for the mono-*N*-oxide and ΔEp between 20 and 50 mV and 40 mV respectively. Repeat cycling in the CV showed an increase in ip_r/ip_f, with SR4317 giving a value of unity, and the response losing its sharpness and attaining a more normal appearance.

The exceptions were SR4355 and SR4318 where the ΔEp was 100 mV. We noted that the minor process observed at alkaline pH for SR4318 was more clearly resolved between pH 6 and 4. Repeat cycle CV showed distinct growth of this process on second and subsequent scans, but a decline in the main reduction step. The return

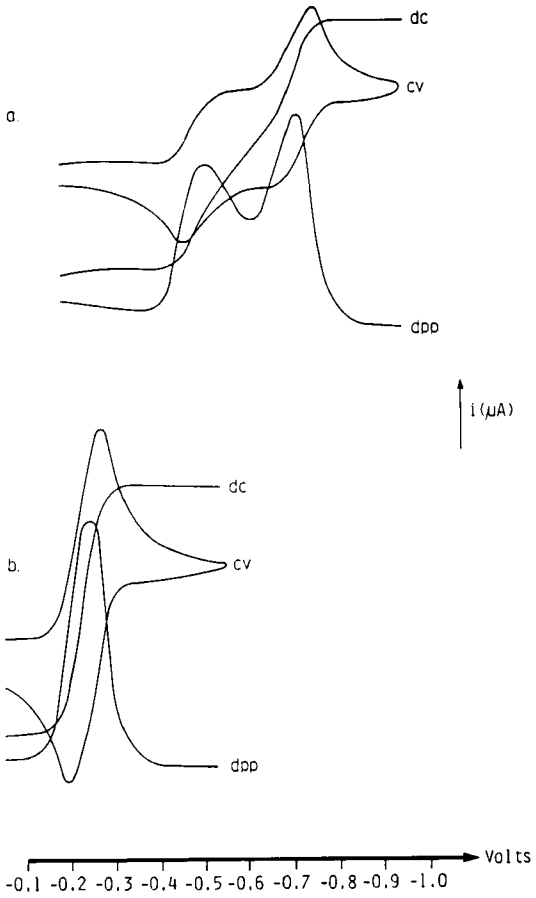


FIGURE 2 Voltammetric behaviour or SR4330 a) pH 6.6; b) pH 3.8.

wave remained unchanged. The ΔEp between the return wave and the minor process was 35 mV. On the second forward scan for SR4355, a new reduction wave was seen to develop, approximately 100 mV to positive potentials of the original reduction, with concommitant 50% decline in current. This new reduction step was clearly associated with the return wave, having a similar current response, $ip_r/ip_f = 0.60$, $\Delta Ep = 20$ mV, and being present if the switching potential was shifted to positive potentials of the original reduction on the second scan. No further changes were observed on subsequent scans.

SR4330 showed a higher degree of chemical reversibility than found at alkaline pH (see Figure 2) with $ip_r/ip_f = 0.9$ to 1.0 and $\Delta Ep = 50$–70 mV in the CV, a symmetrical peak in the differential pulse polarography and diffusion control from the dc polarography with gradients of 40–60 mV.

All the compounds showed an increase in electron affinity by a shift in the reduction potentials to less negative values with increased acidity. By considering the less negative process when two reductions were seen, then the trend can be described by the equation

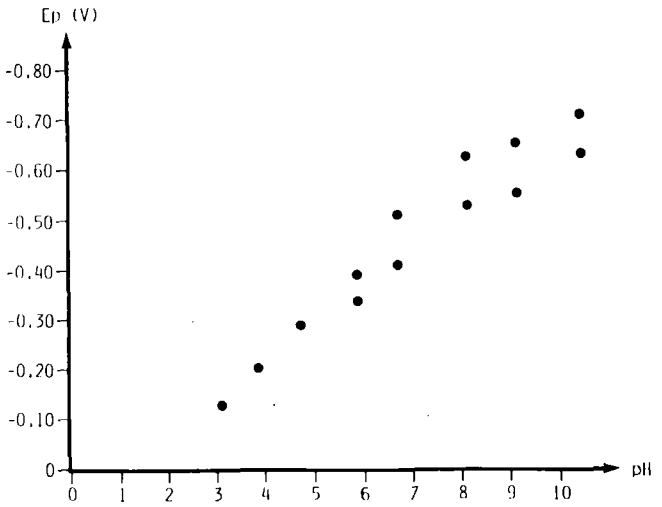


FIGURE 3 Variation of reduction potentials with pH for SR4308.

$$Ep = -apH + b$$

This relationship holds between pH 2 and 8.5. Figure 1 lists the details found for all the compounds. The reduction potentials at pH 7 are also included for comparison. Measurements have been made from the differential pulse polarographic mode, as peak potentials were easier to determine by this technique when the response was distended or overlapping. Essentially the same values were obtained, however, irrespective of the technique due to the high reversibility of the charge-transfer step at acid conditions. The exception was SR4308, where although the same overall shift in potential was found, no single relationship could describe the changes observed, falling into two categories between approximately 8.5 and 6 and 5 and 2 (Figure 3).

DISCUSSION

The influence of pH on the reduction characteristics of the di-, mono- and zero-benzotriazine-*N*-oxides illustrated not only a shift in electron affinity, but also gave information on the general redox mechanism. Overall, little change was observed above pH 8.5, with 2 reduction steps being observed for the di-*N*-oxides and 1 for the mono- and zero-*N*-oxides. As the pH was decreased, the two reduction steps were seen to coalesce so that only a single step was observed below pH 5 by all techniques. This single step contained both *N*-oxide and triazine reduction features, hence the return wave on the reverse scan in the CV mode, associated with the latter feature. The chemical reversibility was also found to improve at lowered pH as observed by a general sharpening of the electrode response with ΔEp decreasing. This was also supported by differential pulse polarography which showed a decrease in peak width at half height and in peak current variation with scan direction, and by the gradient found by a logarithmic analysis of the dc polarography which also decreased. The complexity of the voltammetry found between pH 7 and 6.5 was clearly associated

with the redox properties of the benzotriazine heterocycle, as SR4330 also showed two reduction processes between these restricted pH values (Figure 2). This was possibly due to protonation of the triazine ring, yielding a more easily reducible species.

The trend to less negative potentials with the lowering of pH could be described by a linear relationship which held between pH 2 and 8.5 if the less negative potential was considered where two reduction steps were observed (Figure 1). The exception was SR4308 (Figure 3), although the general trend toward less negative potentials with a lowering of pH was obeyed.

From previous studies under approximately neutral conditions,[6] the 2 stage reduction of the di-*N*-oxides was assigned to the irreversible 4-electron reduction of the two *N*-oxide groups, followed by the quasi-reversible reduction of the benzotriazine heterocycle. This was reinforced in the present study by repeat cycle CV at more extreme pH values where the voltammetric resolution was often improved. For SR4233, SR4286 and SR4308 at alkaline pH the second and subsquent forward scans showed that the relative current response of the 1st reduction decreased, but the 2nd reduction showed an increase (with resultant increase in the ip_r/ip_f for the second redox couple). This would be in in line with the 1st step leading to reduction and subsequent loss of both *N*-oxide functions (as H_2O) yielding the benzotriazine heterocycle which was also redox active. Due to its chemically reversible redox behaviour, the zero-*N*-oxide was then in greater concentration at the electrode surface and hence an increase in response was seen on repeat cycling. This also explained the marked increase in ip_r/ip_f value on the second cycle at acid pH due to a decrease in the forward wave current *i.e.* removal of some contribution from the *N*-oxide reduction. The behaviour of SR4355 and SR4318 could also be explained in a likewise fashion. Substitution of $-NH_2$ by the electron-withdrawing groups $-ONa$ and $-ONH_4$ respectively (giving the dissociated phenoxide salt in aqueous solution) resulted in the benzotriazine reduction now occurring at potentials less negative than *N*-oxide reduction; hence, the formation of a new reduction step on the 2nd forward scan. The shoulder present on the initial forward scan for SR4318 was due to a small amount of impurity of the parent zero-*N*-oxide present in the original sample. For SR4355 at alkaline pH, the extremely distended nature of the response made association of the return wave with the first or second reduction step difficult, but the smaller ΔEp and small relative increase in the 1st reduction current would suggest that in this instance benzotriazine reduction might occur prior to *N*-oxide reduction.

The mono-*N*-oxide, SR4317, showed only a single reduction despite containing a redox-active *N*-oxide function. This was assigned as being due to the electrochemical behaviour being dominated by the triazine heterocycle so that independent *N*-oxide reduction was not observed. This was further substantiated from the present studies with the virtually identical behaviour found for SR4317 and SR4330 with pH (Figure 1). Repeat scan CV of SR4317 yielded an overall response compatible on the second scan with that for SR4330, having ideal characteristics of a reversible 2-electron reduction process ($ip_r/ip_f = 1$, $\Delta Ep = 30$ mV). This would suggest that the *N*-oxide in SR4317 was not redox inactive but was reduced (and lost) as in the di-*N*-oxides, but could not be identified as an individual process. In addition, it was noted that the single step observed under acid conditions for the di-*N*-oxides was very similar in character ($\Delta Ep = 30$–40 mV, ip_r/ip_f increasing on second scan, symmetrical differential pulse polarography, dc polarography gradients of 45–70 mV) to that of the mono-*N*-oxide, and therefore was also dominated by reduction of the triazine heterocycle.

Linear relationships have also been found for the reduction potentials of the bio-reducible nitroimidazoles with pH,[9] for example, misonidazole under analogous conditions to the present investigation obeyed the relationship

$$Ep = -0.076\,pH + 0.07$$

The DNA damaging capability of the drugs was also found to increase with acidity (as measured by viscosity changes)[10] providing strong evidence that it was the protonated form of the electron addition product which was responsible for biological activity. Similar behaviour has been found for the benzotriazine series, with SR4233 showing a decrease in R_{37} value from 12.6 to 2.3% with lowering of pH from 7 to 4 as measured by a double transfection technique.[6,8] This would indicate that it was the protonated 1-electron addition product which was the active species. More detailed work on the biological effect of pH changes is under current investigation and will be the subject of a separate publication.

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