

Determination of the Equilibrium Constant for Dismutation of Intermediates Appearing in Redox Reactions

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The paper concerns reaction mechanisms where a compound is in immediate redox equilibrium with its first and second oxidation products, *e.g.* redox processes involving intermediate formation of a rapidly dismutable free radical. The functional relationship between the concentration of the first oxidation product and the amount of oxidizing agent added to the system is described, and methods are given for determination of the equilibrium constant for dismutation of the intermediate product. The methods have been used for estimation of extinction coefficients and dismutation equilibrium constants of free radicals appearing on oxidation of derivatives of *p*-phenylenediamine.

The oxidation of a large number of compounds has been found to proceed in subsequent steps of one-electron transfer with formation of a more or less stable intermediate product, which, in general, must be assumed to be in immediate redox equilibrium with both the non-oxidized starting material and the final oxidation product. Knowledge of the corresponding equilibrium constant for dismutation of the intermediate product is essential for the purpose of reliable quantitative treatment of the system, and also gives valuable information on the thermodynamics of the redox reaction. The present paper describes the general functional relationship between the concentration of a dismutable intermediate product and the amount of oxidizing agent added to the system, and shows how this relationship can be used for determination of the dismutation equilibrium constant.

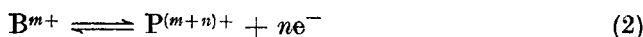
Intermediate oxidation products are often free radicals, which can be detected and estimated by ESR spectrometry, and in the case of organic compounds usually by ordinary spectrophotometry. Methods are, therefore, given for spectrometric determination of the dismutation equilibrium constant, including the common case that the extinction coefficient of the intermediate product (radical) is unknown.

The applicability of the methods described has been established by determination of the extinction coefficient and dismutation equilibrium constant

of Wurster's red, a free radical which is formed on oxidation of *N,N*-dimethyl-*p*-phenylenediamine (DPD).¹ The oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TPD) has also been studied.

THEORETICAL

Consider the sequential redox reactions (1) and (2)



where A, B^{m+}, and P^{(m+n)+} are in immediate redox equilibrium:



With introduction of the convenient notations $A=[A]$, $B=[B^{m+}]$ and $P=[P^{(m+n)+}]$ the equilibrium constant for the dismutation of B^{m+} according to reaction (3) is given by

$$K = A^n P^m / B^{m+n} \quad (4)$$

If c_{ox} concentration equivalents of a sufficiently strong oxidant (*i.e.* an oxidant which can be assumed to be quantitatively reduced when added to the system) are added to a solution of A with the initial concentration c_A we get the stoichiometric relationships

$$c_{ox} = mB + (m+n)P \quad (5)$$

$$c_A = A + B + P \quad (6)$$

Substitution of eqns. (5) and (6) in eqn. (4) gives

$$\left(c_A - \frac{c_{ox} + nB}{m+n} \right)^n \left(\frac{c_{ox} - mB}{m+n} \right)^m = KB_{m+n} \quad (7)$$

whence on differentiation and rearrangement

$$\frac{dB}{dc_{ox}} = \frac{B(c_{ox} - mc_A)}{c_{ox}^2 - (m+n)c_A c_{ox} + mnc_A B} \quad (8)$$

It follows from eqns. (7) and (8) that B increases with c_{ox} from zero to a maximum value B_m , which is obtained for $c_{ox} = mc_A$, and then decreases and becomes zero for $c_{ox} = (m+n)c_A$. The coefficient m can thus be experimentally determined as the number of oxidant equivalents required to obtain a maximum concentration of B^{m+}, and n equals the number of further oxidant equivalents required for complete oxidation of B^{m+}.

Insertion of $c_{ox} = mc_A$ into eqn. (7) gives

$$B_m = \frac{c_A}{1 + [(m+n)/nm](Km^n n^m)_{1/(m+n)}} \quad (9)$$

showing that the value of B_m is dependent on the equilibrium constant K ; the variation of the B/c_{ox} -curves with K is indicated in Fig. 1 for the case $m=1$ and $n=3$. It follows from eqn. (9) that

$$K = m^m n^n \left(\frac{c_A/B_m - 1}{m+n} \right)^{m+n} \quad (10)$$

and eqn. (10) can directly be used for estimation of K from experimental determinations of the maximum concentration of B^{m+} .

When the reaction is followed by observation of a quantity E (which will be referred to as the extinction) that is proportional to B we have

$$E = \varepsilon B \quad (11)$$

and B may be substituted for E/ε in the above equations. The proportionality constant ε (extinction coefficient) can be estimated from the experimentally determined E/c_{ox} -curve as follows. For $c_{\text{ox}} \approx 0$ one may approximate eqn. (7) by the linear relationship

$$B = c_{\text{ox}}/m \quad (12)$$

whence

$$E = \varepsilon c_{\text{ox}}/m \quad (13)$$

Similarly, the approximation

$$B = [1 + (m/n)]c_A - (c_{\text{ox}}/n) \quad (14)$$

is valid for $c_{\text{ox}} \approx (m+n)c_A$, and it follows that the slope of the initial (final) part of the E/c_{ox} -curve equals ε/m ($-\varepsilon/n$).

When K is very small the linear relationships (12) and (14) are valid for the larger part of the E/c_{ox} -curves (cf. Figs. 1–2), and good estimates of ε (and hence of K) can be obtained by the above methods. In the general case, however, these estimates can be considerably improved by iterative fitting of eqns. (7) and (11) to the experimental data. This will be shown in some detail for the common case that the formation and disappearance of the intermediate oxidation product take place in two subsequent steps of one-electron transfer.

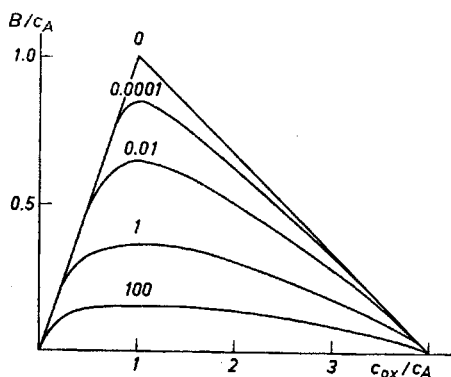


Fig. 1. B/c_{ox} -curves for the case $m=1$ and $n=3$, calculated from eqn. (7) using the value of K indicated at each curve.

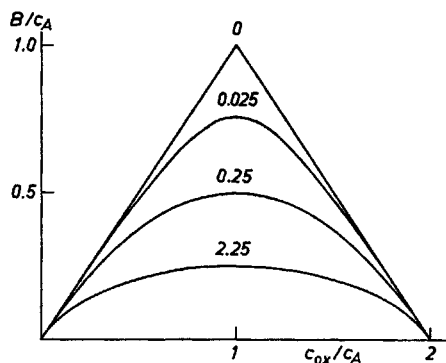


Fig. 2. B/c_{ox} -curves for the case $m=n=1$, calculated from eqn. (15) using the value of K indicated at each curve.

For $m=n=1$ eqn. (7) may be solved for B to give

$$B(K, c_{\text{ox}}) = \frac{c_A - \sqrt{c_A^2 - (1-4K)c_{\text{ox}}(2c_A - c_{\text{ox}})}}{1-4K} \quad (15)$$

The corresponding B/c_{ox} -curves are shown in Fig. 2. Derivation of eqn. (15) with respect to K yields after some mathematical manipulation

$$B' = \frac{\partial B(K, c_{\text{ox}})}{\partial K} = \frac{2 B^2(K, c_{\text{ox}})}{(1-4K)B(K, c_{\text{ox}}) - c_A} \quad (16)$$

If K_1 is a preliminary estimate of K , a series expansion of B in eqn. (15) with retention of linear terms gives the approximate relationship

$$B(K, c_{\text{ox}}) = B(K_1, c_{\text{ox}}) + \Delta K_1 B'(K_1, c_{\text{ox}}) \quad (17)$$

where ΔK_1 is a correction term for the preliminary estimate of the equilibrium constant, *i.e.* $K \approx K_1 + \Delta K_1$. Determination of the linear regression of B on the variable $B'(c_{\text{ox}})$, using the experimental paired values of B and c_{ox} , gives ΔK_1 as the corresponding regression coefficient,² and $K_2 = K_1 + \Delta K_1$ is a considerably refined estimate of K . If necessary, K_2 may be analogously used for calculation of a second correction term ΔK_2 , and the process can be iterated until the correction term becomes arbitrarily small.

When the formation of an intermediate product is followed spectrometrically, the regression analysis may include simultaneous determination of the extinction coefficient. Substitution of eqn. (11) in eqn. (15) gives

$$E = \varepsilon B(K, c_{\text{ox}}) \quad (18)$$

which on series expansion becomes

$$E = \varepsilon_1 B(K_1, c_{\text{ox}}) + \Delta \varepsilon_1 B(K_1, c_{\text{ox}}) + \Delta K_1 \varepsilon_1 B'(K_1, c_{\text{ox}}) \quad (19)$$

where ε_1 and K_1 are preliminary estimates, and $\Delta \varepsilon_1$ and ΔK_1 the corresponding correction terms. Arbitrarily precise estimates can then be obtained by iterative determination of the bilinear regression of E on the variables $B(c_{\text{ox}})$ and $B'(c_{\text{ox}})$, using experimental paired values of E and c_{ox} . The statistical method also gives standard deviations of the estimates;² detailed calculation schemes referring to eqn. (19) are given in the appendix.

EXPERIMENTAL

Assays. The E/c_{ox} -curves for DPD and TPD (*N,N*-dimethyl-*p*-phenylenediamine dihydrochloride and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride were obtained from Eastman-Kodak, Rochester, N.Y., USA) were determined spectrophotometrically using iodine as oxidizing agent. Iodine fulfills the requirement of a sufficiently strong oxidant,³ and may be assumed to be quantitatively reduced when added to solutions of DPD and TPD; c_{ox} thus equals the concentration of iodide ions in the reaction mixtures (or the equivalent amount of added iodine). Reactions were carried out at 25° in 10 mM acetate or phosphate buffer solutions, pH 5.5, containing 10 μ M EDTA. The choice of buffer had no influence on the results obtained. Buffer solutions of DPD and TPD were prepared immediately before use. Various known amounts ($c_{\text{ox}} = 0.0 - 0.5$ mM) of iodine in aqueous solution (standardized with thiosulphate) were added last to the

reaction mixtures, which contained a fix known amount (c_A about 0.1 mM) of DPD or TPD in a final volume of 50 ml. Colour development was rapid and the extinction of the solution was determined at 550 $m\mu$ and 563 $m\mu$, respectively, in a Zeiss PM Q II spectrophotometer; these were found to be the wavelengths of maximum absorption.^{1,3}

Stability of colours. The extinction of reaction solutions was found to decrease slowly (about 0.3 % per min), confirming the observations made by Curzon.³ Correction for the instability in extinction was made by observation at 30 sec intervals and extrapolation to zero time; the correction terms were, in general, insignificant for the extinction recorded after 30 sec.

Reversibility of reactions. When iodine was added in excess solutions of DPD immediately turned red, but the colour rapidly faded due to oxidation of the intermediately formed radical (Wurster's red). Subsequent addition of reducing agents (*e.g.* DPD itself) immediately restored the red colour, confirming that a reversible equilibrium exists between DPD and its first and second oxidation products.^{1,3}

The oxidation of TPD appears to be more complex. A blue radical was immediately formed on addition of excess of iodine, but the subsequent oxidation of this radical was fairly slow. When c_{ox} exceeded $3c_A$ determinations of the final extinction became very difficult due to the above instability in extinction, and the corresponding part of the E/c_{ox} -curve could not be reliably determined; similar difficulties were encountered with DPD when c_{ox} exceeded 1.7–1.9 c_A .

On addition of reducing agents to a solution of TPD which had been decolourized by treatment with iodine in excess, the solution turned red. The absorption spectrum of this solution was indistinguishable from that of a solution of Wurster's red. Consequently, it appears that the complete oxidation of TPD includes irreversible steps of (possibly oxidative) demethylation.

RESULTS

The experimentally determined E/c_{ox} -curve for the oxidation of DPD by iodine is shown in Fig. 3. The curve exhibits a maximum when c_{ox} equals c_A ($m=1$), confirming that Wurster's red may be denoted DPD^+ .^{1,3} The symmetry of the curve shows that n equals m ; the final oxidation product will, consequently, be denoted DPD^{2+} . A preliminary estimate $\epsilon_1=10^4$ M⁻¹ for the extinction coefficient of DPD^+ was calculated from the initial slope of the E/c_{ox} -curve using eqn. (13). Hence the maximum concentration of DPD^+

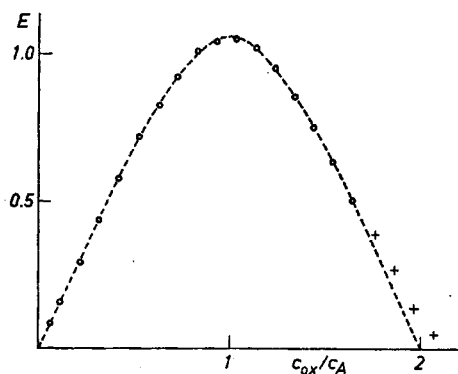


Fig. 3. E/c_{ox} -curve for the oxidation of DPD. Experimental points used for regression analysis are indicated by circles. The dashed curve has been calculated from eqn. (15) for $\epsilon=1.04 \times 10^4$ M⁻¹ and $K=0.0348$.

Table 1. Fitting of eqn. (15) to the data shown in Fig. 3 by iterative regression analysis.

Iteration number	Preliminary estimates		Correction terms	
	K	$\varepsilon \times 10^{-4}$	ΔK	$\Delta \varepsilon \times 10^{-4}$
0	0.02780	1.00000	+0.00678	+0.03874
1	0.03458	1.03874	+0.00023	+0.00092
2	0.03481	1.03966	-0.00004	-0.00015
3	0.03477	1.03951	+0.00001	+0.00006
Result:	0.03478	1.03956		

was estimated as $B_m = 0.75c_A$. Insertion of this into eqn. (10) gave $K_1 = 0.0278$ as a preliminary estimate of the equilibrium constant for the rapid reaction



The results of the subsequent iterative regression analysis of the data given in Fig. 3 are shown in Table 1. Three iterations were required to obtain a self-consistent solution at the 0.1 % level of precision, and calculation of the corresponding residual variance showed that eqn. (15) could be excellently fitted to the experimental data. The dashed curve in Fig. 3 indicates the theoretical E/c_{ox} -curve calculated from eqn. (15) using the final estimates $\varepsilon_{550} = 1.04 \times 10^4 \text{ M}^{-1}$ (0.01×10^4) and $K = 0.035$ (0.002). Figures within brackets stand for the respective standard deviations.

As shown in Table 2 the quotient B_m/c_A was found to be constant (0.73) when c_A was varied within the range 0.03–0.14 mM, confirming that the equilibrium constant K is independent of c_A (*cf.* eqn. (10)).

The E/c_{ox} -curve obtained by oxidation of TPD is shown in Fig. 4. Maximum extinction occurs when c_{ox} equals c_A ($m=1$), confirming that a radical TPD^+ is formed.¹ The curve is asymmetrical, and the slope of the curve for $c_A > c_{\text{ox}} > 2c_A$ indicates a value of n approximately equal to 3. Consequently, the oxidation of TPD^+ involves several steps of electron transfer, at least some of which appear to be irreversible (see experimental section), and the required assumptions for determination of a dismutation equilibrium constant are not fulfilled.

Table 2. Effect of variation of the initial concentration of DPD (c_A) on the maximum radical concentration (B_m).

c_A (μM)	E_m	B_m (μM)	B_m/c_A
140	1.06	101.9	0.728
104	0.780	75.0	0.721
70	0.535	51.4	0.734
52	0.392	37.7	0.725
35	0.268	25.8	0.737
		Average:	0.729

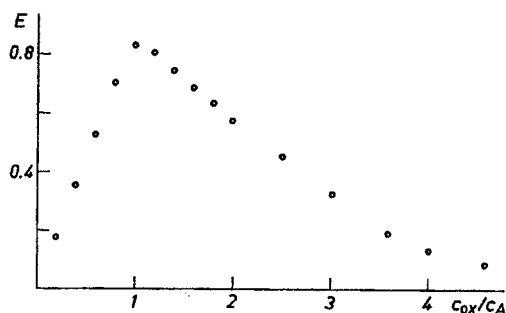
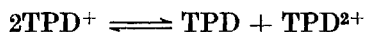


Fig. 4. Experimentally determined E/c_{ox} -curve for the oxidation of TPD.

Estimation of the extinction coefficient of TPD^+ from the initial part of the E/c_{ox} -curve gave $\epsilon_{564} = 1.24 \times 10^4 \text{ M}^{-1}$. Hence one obtains $B_m/c_A = 0.92$, and it may for comparison be noted that this corresponds to a low value ($K = 0.002$) of the equilibrium constant for the hypothetical reaction



It, therefore, appears that the above estimate of ϵ_{563} is reliable; the oxidation of TPD^+ may probably be completely neglected as long as c_A exceeds c_{ox} by a factor of about 1.1 (*cf.* Fig. 1).

DISCUSSION

The above results show that the methods described in the theoretical section successfully can be used for investigation of complex redox processes, where the oxidized compound is in immediate equilibrium with its first and second oxidation products. All calculations required for iterative determination of extinction coefficients and equilibrium constants from experimental data can be carried out on an ordinary desk calculator, and if the preliminary estimates are not too badly chosen a few iterations suffice to give accurate and unbiased estimates.

DPD and TPD are used as substrates in standard determinations of ceruloplasmin (EC 1.12.3) activity by spectrophotometric methods,⁴⁻⁶ and a precise knowledge of the extinction coefficients of DPD^+ and TPD^+ is, evidently, desirable. In previous investigations the radical concentration has been assumed to be proportional to the amount of oxidant added. Such an assumption appears valid in the case of TPD^+ (see above), and the present determination $\epsilon_{563} = 1.24 \times 10^4 \text{ M}^{-1}$ agrees well with the values reported by Curzon³ ($\epsilon_{563} = 1.25 \times 10^4$) and Michaelis¹ ($\epsilon_{560} = 1.22 \times 10^4$). In the case of DPD^+ , however, the previously accepted value^{3,4} ($\epsilon_{550} = 0.98 \times 10^4 \text{ M}^{-1}$) is significantly lower than the one obtained in the present work (1.04×10^4). This can be explained as an effect of the non-linearity of the E/c_{ox} -curve. The above

assumption of proportionality is only valid (on the 1 % level of error) for $c_{\text{ox}} < 0.1c_A$, and the use of larger amounts of oxidant will result in underestimation of the magnitude of the extinction coefficient. Such errors are avoided by application of the present iterative method, which has a further advantage in yielding estimates with a known precision (as measured by the corresponding standard deviations).

The iterative regression method for estimation of dismutation equilibrium constants should also be preferred when the extinction coefficient of the intermediate oxidation product is known, or when the concentration of intermediate products can be reliably determined by non-spectrometric techniques. In the regression analysis information from all parts of the E/c_{ox} -curve can be utilized, whereas determinations of the equilibrium constant from eqn. (10) are based on one single point of the curve.

The present work appears to establish that DPD is in immediate equilibrium with its oxidation products in accordance with reaction (20). The consequences of this fact with respect to the enzymatic oxidation of DPD are under investigation.

APPENDIX

The experimentally determined paired values E_i and c_{ox_i} ($i=1, 2, \dots, n$) are used to compute the following sums

$$\begin{aligned} S_1 &= \sum_{i=1}^n B_i^2 & S_2 &= \sum_{i=1}^n n(B_i')^2 & S_3 &= \sum_{i=1}^n nE_i^2 \\ S_4 &= \sum_{i=1}^n B_i B_i' & S_5 &= \sum_{i=1}^n E_i B_i & S_6 &= \sum_{i=1}^n E_i B_i' \end{aligned}$$

where each $B_i = B(K, c_{\text{ox}_i})$ and $B_i' = B'(K, c_{\text{ox}_i})$ is calculated from eqn. (15) and (16), respectively. The correction terms are given by

$$\begin{aligned} \Delta\varepsilon &= \frac{S_2 S_5 - S_4 S_6}{S_1 S_2 - S_4 S_4} - \varepsilon \\ \Delta K &= \frac{S_1 S_6 - S_4 S_5}{\varepsilon(S_1 S_2 - S_4 S_4)} \end{aligned}$$

For the self-consistent solution the standard deviations are obtained as

$$\begin{aligned} \text{S.D.}(\varepsilon) &= \sqrt{\frac{VS_2}{S_1 S_2 - S_4 S_4}} \\ \text{S.D.}(K) &= \frac{1}{\varepsilon} \sqrt{\frac{VS_1}{S_1 S_2 - S_4 S_4}} \end{aligned}$$

where V stands for the residual variance:

$$V = (S_3 + \varepsilon^2 S_1 - 2\varepsilon S_5)/(n-2)$$

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