# Stability of Oxidation Products of Dimethyl-*p*-phenylene-diamine

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The stability of the oxidation products of dimethyl-*p*-phenylenediamine has been studied spectrophotometrically. In contrast to previous reports only the second oxidation product was found to decompose at a significant rate. Velocity constants for its disappearance were determined over a limited range of pH-values (pH 5—8).

Wurster's red (DPD$^+$) is formed as an intermediate product in the oxidation of dimethyl-*p*-phenylenediamine (DPD), a reaction which may be formally represented as [1-3]

$$DPD \rightleftharpoons DPD^+ + e^- \rightleftharpoons DPD^{2+} + 2e^- \qquad (1)$$

Curzon reported [2] that the colour of Wurster's red slowly fades by apparent first-order kinetics, and suggested that hydrolysis of DPD$^+$ by hydroxyl ions is responsible for the fading. It seems, however, equally likely that the second oxidation product DPD$^{2+}$ might be the actual compound submitted to decomposition, the fading of DPD$^+$ being due to a corresponding shift of the existing [3] rapid dismutation equilibrium inherent in reaction (1):

$$\frac{[DPD][DPD^{2+}]}{[DPD^+]^2} = K \qquad (2)$$

Walaas has presented evidence for the formation of a compound (which will be called "compound D") showing UV-absorption maximum at 248 m$\mu$, as a final stable product in the ceruloplasmin catalyzed oxidation of DPD.[4] Preliminary experiments at this laboratory established that compound D is ultimately formed also in non-enzymatic oxidations, and compound D can be considered as a probable transformation product of either DPD$^+$ or DPD$^{2+}$.

For reliable evaluation of kinetic data obtained by enzymatical oxidation of DPD it has become of importance to determine the actual kinetic expression for the formation of compound D. The purpose of the present investigation is to establish the immediate origin of compound D, and to determine velocity constants for its formation.

## EXPERIMENTAL

*Extinction coefficients.* For determination of the extinction coefficient of DPD ($N,N$-dimethyl-$p$-phenylenediamine dihydrochloride was obtained from Eastman-Kodak, N.Y., USA), the extinction of freshly prepared solutions of DPD in acetate buffer, pH 5.5, was recorded using concentrations in the range $10-100$ $\mu$M ($248$ m$\mu$) and $50-500$ $\mu$M ($285$ m$\mu$). Within these ranges the extinction was proportional to the concentration of DPD.

Extinction coefficients of compound D were similarly determined; DPD was assumed to be quantitatively converted into equimolar amounts of compound D 3 h after the addition of ceruloplasmin ($5-10$ $\mu$M) to solutions of DPD ($50-500$ $\mu$M) in 0.05 M acetate buffer, pH 5.5.[5]

Experiments in which DPD ($c_A = 60-100$ $\mu$M) was oxidized by titration with iodine ($c_{ox} = 0-1.6$ $c_A$) were carried out as described previously.[3] Due to the decomposition of DPD$^{2+}$ the extinction of reaction mixtures had to be recorded at 30 sec intervals, and was for determination of extinction coefficients extrapolated to zero time.

*Enzymatic assays.* Ceruloplasmin was obtained by the courtesy of Dr. Björling, AB Kabi, Stockholm. The preparation used was about 65 % pure; the $E_{610}/E_{280}$ ratio was 0.028.[5] Ceruloplasmin concentrations were calculated from the extinction at 610 m$\mu$.[5]

Ceruloplasmin catalyzed oxidations of DPD ($60-200$ $\mu$M) were carried out at 25° in 0.05 M sodium acetate buffer, usually at pH 5.5. For determination of the time-course of the reaction the ceruloplasmin concentration was about 1 $\mu$M.

For determination of velocity constants at pH $5.0-5.5$ the ceruloplasmin concentration was $10-20$ $\mu$M; under these conditions the enzymatic oxidation of DPD into DPD$^{2+}$ was completed within 2 min. The decomposition of DPD$^{2+}$ was then followed spectrophotometrically at 285 m$\mu$.

For determination of velocity constants at other pH-values DPD was enzymatically converted into DPD$^{2+}$ at pH 5.5 as described above. The reaction solution containing DPD$^{2+}$ was then added to 0.2 M acetate (pH $4-5$) or phosphate (pH $5.5-9$) buffer solutions, and when needed the pH was adjusted to the desired value by addition of sodium hydroxide or hydrogen chloride. The decomposition of DPD$^{2+}$ was then followed spectrophotometrically at 285 m$\mu$.

All spectrophotometric measurements were made in a Zeiss PM Q II spectrophotometer.

## RESULTS AND DISCUSSION

The oxidation of DPD was followed spectrophotometrically at 550, 285 and 248 m$\mu$, the wavelengths of maximum absorption of DPD$^+$, DPD$^{2+}$, and compound D, respectively.[4] For determination of extinction coefficients of DPD$^+$ ($\varepsilon_1$) and DPD$^{2+}$ ($\varepsilon_2$) iodine was used as oxidizing agent, and the "initial" extinction of reaction mixtures (the extinction shown before any significant amounts of compound D were formed) was determined as a function of $c_{ox}$, the number of concentration equivalents of iodine added (see Fig. 1). The extinction ($E$) at any wavelength is given by

$$E = \varepsilon_0[\text{DPD}] + \varepsilon_1[\text{DPD}^+] + \varepsilon_2[\text{DPD}^{2+}] \tag{3}$$

where the extinction coefficient of DPD ($\varepsilon_0$) can be determined in the usual way, using pure solutions ($c_{ox} = 0$) of the compound. For $c_{ox} > 2c_A$ ($c_A$ stands for the "total" concentration of DPD) the concentration of DPD and its oxidation products can be expressed in terms of $c_{ox}$, $c_A$, and $K$, the dismutation equilibrium constant ($K = 0.035$):[3]

$$[DPD] = \frac{F+(1-8K)c_A-(1-4K)c_{ox}}{2(1-4K)} \tag{4}$$

$$[DPD^+] = \frac{c_A-F}{1-4K} \tag{5}$$

$$[DPD^{2+}] = \frac{F-c_A+(1-4K)c_{ox}}{2(1-4K)} \tag{6}$$

where

$$F = \sqrt{c_A{}^2-(1-4K)c_{ox}(2c_A-c_{ox})} \tag{7}$$

After determination of $\varepsilon_0$ preliminary estimates of $\varepsilon_1$ and $\varepsilon_2$ were obtained from eqns. (3)—(7) using two (almost) arbitrarily chosen experimental paired values of $E$ and $c_{ox}$. More precise estimates were then calculated by computer programmed iterative statistical fitting [6] of eqns. (3)—(7) to the experimentally determined $E/c_{ox}$ curves shown in Fig. 1; an analogous method has previously been described in detail in relation to determinations of the extinction coefficient of $DPD^+$ at 550 m$\mu$.[3]
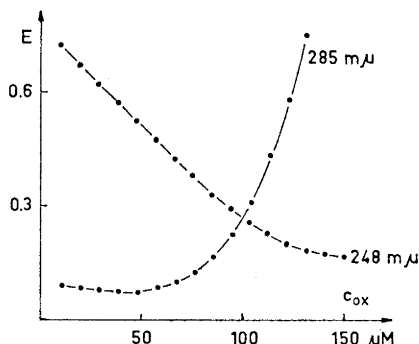


Fig. 1. Initial extinction of reaction mixtures obtained on oxidation of DPD ($c_A = 94$ $\mu$M) by various amounts of iodine.

Extinction coefficients of compound D were determined in the usual way, assuming that DPD is quantitatively converted into compound D 3 h after the addition of fairly large amounts of ceruloplasmin.[4] The extinction coefficients obtained for the different compounds are listed in Table 1.

Table 1. Extinction coefficients ($10^{-4} \times \varepsilon$ M$^{-1}$) of DPD and its oxidation products.

| Compound | 248 m$\mu$ | 285 m$\mu$ | 550 m$\mu$ |
|---|---|---|---|
| DPD | 0.76 | 0.09 | 0 |
| DPD$^+$ | 0.20 | 0.00 | 1.04 |
| DPD$^{2+}$ | 0.39 | 2.3 | 0 |
| Compound D | 1.8 | 0.32 | 0 |

During the above experiments it was observed that the initial rate of formation of compound D (as measured by the slow increase in extinction at 248 m$\mu$) steadily increased with $c_{ox}$, even though the concentration of DPD$^+$ passes through a maximum when $c_{ox}$ equals $c_A$.[3] This observation is not consistent with the formation of compound D by first-order decomposition of DPD$^+$, but indicates that compound D is formed directly from DPD$^{2+}$. More conclusive evidence in this direction was obtained from experiments in which DPD was oxidized by air in the presence of ceruloplasmin. The time-course of this process, as recorded spectrophotometrically at 550, 285, and 248 m$\mu$, is shown in Fig. 2 for a typical experiment. Using the extinction coefficients listed in Table 1, the dismutation equilibrium equation (2), and the stoichiometric relationship

$$c_A = [\text{DPD}] + [\text{DPD}^+] + [\text{DPD}^{2+}] + [\text{compound D}] \qquad (8)$$

it was possible to resolve the recorded extinctions into contributions by each reactant, and thus to determine concentrations of reactants as a function of time. Fig. 3 shows the results of such an analysis of the data given in Fig. 2.
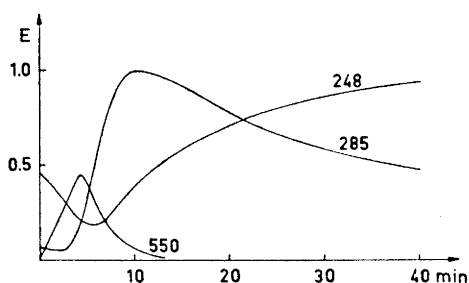


Fig. 2. Variation of extinctions at 550, 285, and 248 m$\mu$ during the ceruloplasmin (0.9 $\mu$M) catalyzed oxidation of DPD (62.2 $\mu$M).
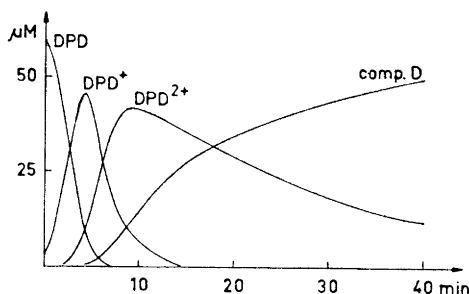
Fig. 3. Variation of concentrations of DPD and its products in the experiment shown in Fig. 2.

Inspection of Fig. 3 clearly shows that the rate of formation of compound D is directly dependent upon the concentration of DPD$^{2+}$, and that there is a quantitative correspondence between the amount of DPD$^{2+}$ disappearing and the amount of compound D formed in the late phase of the reaction. It may be concluded that compound D is formed directly from DPD$^{2+}$.

When fairly large amounts of ceruloplasmin were used the enzymatic conversion of DPD into DPD$^{2+}$ was completed within some minutes. After that time the decrease in extinction at 285 m$\mu$ (and the increase in extinction at 248 m$\mu$) merely reflects the spontaneous[4] reaction

$$\text{DPD}^{2+} \xrightarrow{\ k\ } \text{compound D}$$
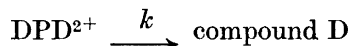
Assuming that this reaction is governed by first-order kinetics DPD$^{2+}$ would decrease exponentially with time

$$[DPD^{2+}] = c_0 e^{-kt} \tag{9}$$

Such a separate treatment of the spontaneous process is justified by the results of a previous theoretical study of mechanisms in which enzymatic products are submitted to non-enzymatic first-order decomposition.[7] According to eqn. (8) and (9) we then have

$$[\text{compound D}] = c_A - c_0 - e^{-kt} \tag{10}$$

The extinction of the reaction solution is given by

$$E = \varepsilon_2[DPD^{2+}] + \varepsilon_3 [\text{compound D}] \tag{11}$$

or with insertion of eqns. (9)—(10)

$$E = c_0 (\varepsilon_2 - \varepsilon_3) e^{-kt} + \varepsilon_3 c_A \tag{12}$$

where $\varepsilon_3 c_A$ equals $E_\infty$, the final extinction of the solution. Eqn. (12) may be written in the form

$$\ln (E - E_\infty) = \ln c_0(\varepsilon_2 - \varepsilon_3) - kt \tag{13}$$

and a preliminary estimate of $k$ can be obtained in the usual way by plotting $\log (E - E_\infty)$ against time. As shown in Fig. 4 such plots give straight lines, confirming that the reaction follows first-order kinetics.
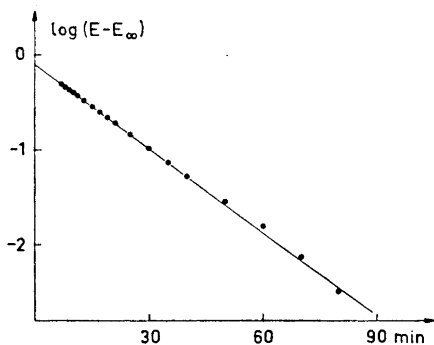


Fig. 4. First-order decomposition of DPD$^{2+}$ as revealed by plotting $\log (E - E_\infty)$ against time for a typical experiment ($c_A = 80 \mu M$; pH 5.5).
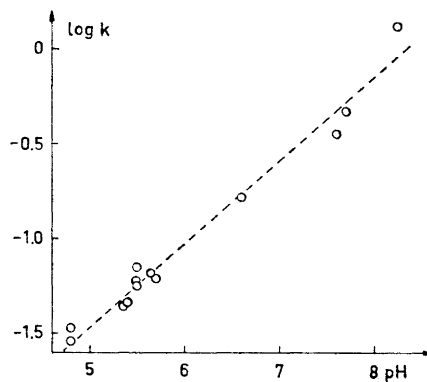
Fig. 5. Variation of the velocity constant for decomposition of DPD$^{2+}$ with pH.

More precise estimates of $k$ were determined statistically[6] by fitting eqn. (13) to the experimental data using a computer programmed iterative method of regression analysis with estimation of $k$, $E_\infty$, and $\ln c_0(\varepsilon_2 - \varepsilon_3)$. Such determinations were carried out at different pH-values, and the results obtained are shown in Fig. 5, where $\log k$ is plotted against pH.

It appears that $\log k$ is approximately linearly related to pH over the pH-range studied, indicating that the pH-dependence of $k$ may be given as

$$k = k'[OH^-]^n \tag{14}$$

or

$$\log k = \log k' + n(pH - pK_w) \tag{15}$$

Calculation of the regression of $\log k$ on pH using the data shown in Fig. 5 gave ($k$ being given in $min^{-1}$)

$$\log k = 0.44\ pH - 3.68$$

The experimental conditions under which the apparent decomposition of $DPD^+$ was studied by Curzon[2] were not sufficiently precisely stated to allow a detailed comparison with the present results to be made. It appears, however, that the larger part of the observed fading of $DPD^+$ could be attributed to the dismutation equilibrium shift caused by decomposition of $DPD^{2+}$.

## REFERENCES

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