Kinetic studies on the reduction of cytochrome c

Reaction with dihydroxy conjugated compounds (catechols and quinols)

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(Received 23 June 1981/Accepted 28 October 1981)

The kinetics of reduction of cytochrome c by catechol(s), quinol(s) and related compounds were investigated by stopped-flow spectrophotometry. Studies on the influence of pH on the rates indicate that only deprotonated forms of these compounds act as reducing agents, with the dianionic forms being the most effective. The pH-independent second-order rate constants are reported. Hammett treatment of the effects of substituents on the aromatic ring structure of the reductants show that for electron transfer to occur the charge on the deprotonated species must not be withdrawn on to the substituents. Possible sites for electron donation to cytochrome c are discussed, and the results presented indicate that the haem edge is a likely candidate.

Eukaryotic cytochrome c is a constituent of the electron-transport chain responsible for transferring electrons from cytochrome c1 to the terminal electron acceptor of the mitochondrion, cytochrome c oxidase (Lemberg & Barrett, 1973). Furthermore, in yeast, cytochrome c also donates electrons to a specific cytochrome c peroxidase (Yonetani, 1976). These electron-transfer processes involve redox changes in the central iron atom of the haem moiety, and the route by which electrons reach and leave this metal atom, enclosed as it is within the hydrophobic crevice of the protein, has been of considerable interest.

The mechanistic studies that have been performed have been greatly helped by the wealth of information available on the structural and dynamic properties of the protein (for review see Dickerson & Timkovich, 1975). Nevertheless, there remains some diversity of opinion with regard to the site of electron entry into the molecule. This site may well depend on the nature of the reductant; thus the Cr2+ ion (and SO42−) is thought to enter the haem crevice and reduce the iron by an inner-sphere mechanism (Sutin & Yandell, 1972; Yandell et al., 1973). The work of Gray and co-workers, (Holwerda & Gray, 1974; Wherland & Gray, 1976), however, strongly suggests that small inorganic reductants and oxidants transfer electrons in an outer-sphere process involving the exposed haem edge. Similar conclusions have been reached by other workers, e.g. Butler et al. (1981) and Ahmed & Millett (1981). Reduction of cytochrome c by ascorbic acid has been extensively studied (Al-Ayash & Wilson, 1979). Al-Ayash & Wilson (1979) suggested that dianionic forms of the reductant donate electrons to the exposed haem edge, but more recent work by Myer et al. (1980) and Pande & Myer (1980) provides evidence that ascorbate binds to the arginine-38 residue and that electron transfer to the haem proceeds via aromatic residues.

In the present investigation we have chosen to study the reduction of mammalian cytochrome c, and some other single-site redox proteins, by a number of dihydroxy conjugated (and related) compounds. These compounds are interesting for two reasons. Firstly, they are analogous in some respects to ascorbic acid, possessing, in the case of catechols, conjugated enediol-like structures. Secondly, they are interesting in their own right, as they are themselves widely distributed in Nature. For example, catechol is a constituent nucleus of 3,4-dihydroxyphenethylamine (dopamine), 3,4-dihydroxyphenylalanine (dopa), adrenaline (epinephrine), vitamin K and some important iron-chelating compounds (enterobactins) found in bacteria. Similarly, the quinones are important constituents of the electron-transport chain of both mitochondria and chloroplasts. Although these compounds are widely found in biological systems, the transient kinetics of their electron-transfer reactions with redox proteins have not been fully investigated [see, however, Kertesz (1968), Miller (1970) and Toppen (1976)]. These compounds have a great advantage in such studies because their properties may be
selectively altered by suitable choice of substituents on the aromatic ring structure, and we have used this feature in the investigations reported below.

Our general conclusions may be summarized as follows. Catechol(s) and quinol(s) act as reductants in either their monoanionic or diamionic forms, the latter being by far the more rapid in donating electrons. This is similar to the results obtained for ascorbic acid (Al-Ayash & Wilson, 1979), although with that compound the monoanion reduces redox proteins very slowly, if at all. Furthermore, our results with substituted compounds lead us to conclude that the charge on the deprotonated forms must reside on the conjugated system, i.e. oxygen atoms and aromatic nucleus, and not be withdrawn on to the substituent if they are to be available for electron transfer. The high rates observed with some of the compounds suggest a rapid outer-sphere mechanism, with the haem edges as the most likely site for electron donation. This may also be considered likely because the anionic species in question would be electrostatically attracted to this region of the molecule by the positive charges known to surround the exposed haem edge (Koppenol et al., 1978).

Materials and methods

Horse heart cytochrome c (type III; Sigma Chemical Co.) was used without further purification. Pseudomonas aeruginosa cytochrome c-551 and azurin were gifts from Professor M. Brunori (University of Rome, Rome, Italy). Protein concentrations were determined by using the following absorption coefficients: horse ferrocytochrome c, $e_{551} = 27,600\text{M}^{-1}\cdot\text{cm}^{-1}$ (Schejter et al., 1963); ferrocytochrome c-551, $e_{551} = 28,300\text{M}^{-1}\cdot\text{cm}^{-1}$ (Horio et al., 1960); azurin, $e_{625} = 3500\text{M}^{-1}\cdot\text{cm}^{-1}$ (Brill et al., 1968).

Sodium ascorbate, quinol (hydroquinone), tetrachloroquinoI, 3,4-dihydroxyphenethylamine, noradrenaline, 4-methylcatechol, 4-acetylcatechol, 4-t-butylcatechol, protocatechuic acid, protocatechualdehyde and 4-nitrocatechol were purchased from Sigma Chemical Co. as the highest purity grade. Resorcinol, catechol, tetrabromocatechol, 3,4-dihydroxyphenethylamine, hydrocaffeic acid, caffeic acid, 3,5-di-t-butylcatechol and pyrogallol were purchased from Aldrich Chemical Co. o-Methoxyphenol and 2-hydroxybenzyl alcohol were purchased from Fluka Co.

Experiments were performed in 0.2 M-Bistris {2-[bis-(2-hydroxyethyl)aminol-2-(hydroxymethyl)-propane-1,3-diol]/HCl buffer at 25°C unless otherwise stated.

Catechol was recrystallized from benzene three times, and 4-methylcatechol and 4-t-butylcatechol were recrystallized at least twice from water. Recrystallizations were performed under N$_2$.

Crude tetrabromocatechol and 3,5-di-t-butylcatechol were purified by recrystallization from carbon tetrachloride, under N$_2$, followed by recrystallization (three times) under alkaline conditions (pH 11) from ag. 20% (v/v) methanol under N$_2$, to remove oxidation products. The yield was approx. 15% (w/w) of the starting material.

The concentrations of 3,5-di-t-butylcatechol, tetrabromocatechol and tetrachlorocatechol were measured just before use by using the absorption coefficients at 500 nm of $7.5 \times 10^2\text{M}^{-1}\cdot\text{cm}^{-1}$, $9.5 \times 10^2\text{M}^{-1}\cdot\text{cm}^{-1}$ and $1.2 \times 10^3\text{M}^{-1}\cdot\text{cm}^{-1}$ respectively (Arnów, 1937; Waite & Tanzer, 1981).

Tetrachlorocatechol was a gift from Dr. J. Silver (University of Essex, Colchester, U.K.), and 4-amino catechol was a gift from Dr. J. Tillett (University of Essex, Colchester, U.K.). Other compounds used were of the highest purity available.

Stopped-flow experiments were performed in a Durrum-Gibson instrument with 2 cm light-path and a dead-time of 3 ms.

All experiments were performed under anaerobic conditions unless stated otherwise.

Results

Rapid kinetic experiments in which ferricytochrome c was mixed anaerobically with catechol under pseudo-first-order conditions lead to the reduction of the haemoprotein in a single process. In some experiments a small residual portion of the ferric form remained. This was due, however, to the presence of polymers of cytochrome c, which could not be reduced by catechol or indeed by any of the reductants employed in the present study. Polymer-free cytochrome c, prepared by gel-filtration chromatography (Margoliash & Lustgarten, 1962), was completely reduced by the reagents.

Analysis of the progress curves, such as those depicted in Fig. 1, showed that, at pH values of 8 or lower, reduction conformed closely to an exponential process, the pseudo-first-order rate constant for which depended linearly on the catechol concentration (Fig. 2a), with no evidence for a limiting rate over the concentration range employed (up to 0.1 M catechol). Fig. 2(a) yields a value of $1.4 \times 10^2\text{M}^{-1}\cdot\text{s}^{-1}$ for the apparent second-order rate constant ($k_{app}$) at pH 8. This constant was strongly pH-dependent, and Fig. 3 shows that a plot of $\log k_{app}$ was linear with pH over the range from pH 6 to pH 9, yielding a slope of unity (see also Table 1).

It was technically difficult to extend the observations to higher pH values as, with horse heart
cytochrome c, the known alkaline isomerization of the protein intervened, so that only part of the protein was reduced directly, the remainder being reduced at a rate limited by the interconversion from the redox-inactive to redox-active forms (Wilson & Greenwood, 1971). Fig. 1(b) shows the progress curve for the reduction of ferricytochrome c by catechol at pH 9. It is clear that, in keeping with the known pK for isomerization, approximately half the protein is in the 'non-reducible' form.

The above experiments confirm the findings reported by Toppen (1976) that catechol rapidly reduced cytochrome c in a pH-dependent process. Catechol, like ascorbic acid (Wilson & Greenwood, 1971), can reduce only the form of the protein that predominates around neutral pH values.

In order to elucidate the mechanism of reduction of cytochrome c by dihydroxy conjugated compounds, of which catechol is an example, and also to facilitate comparison with the mechanism repor-
ted for ascorbic acid (Al-Ayash & Wilson, 1979), we have extended this study to include the reactions of substituted catechols and some quinols with this haemoprotein. Also, to investigate the specific role of the protein in determining the rate and mechanism of the redox reactions, we have conducted some experiments with cytochrome c-551 and the blue copper protein azurin isolated from the bacterium *Pseudomonas aeruginosa*.

Catechols substituted at position 4 all acted as reducing agents for cytochrome c, and the rates of reduction under pseudo-first-order conditions are reported as a function of reductant concentration at pH 8.0 in Figs. 2(a) and 2(b). Examination of these Figures indicates that the reaction is second-order in reductant concentration for all the substituted catechols, the apparent second-order rate constant being strongly dependent on the nature of the substituent (Table 1).

The pH-dependences of these apparent second-order rate constants are given in Fig. 3. The substituents –CH₃, –C(CH₃)₃, –CO₂H and –CH₂–CO₂H, like that reported above for unsubstituted catechol, yield linear plots of \( \log k_{\text{app}} \) versus pH, with slopes close to unity. Similar results were obtained with the following substituents: –CH₃–CH₃–CO₂H, –CH(OH)–CO₂H, –CH=CH–CO₂H and –NH₂. The formyl (CHO) and nitro (NO₂) substituents, however, exhibited very little if any pH-dependence over the pH range explored.

Mechanisms to account for the pH-dependence of such redox reaction rates are generally framed in terms of the interconversion of protonated into deprotonated forms of the reducing agent in response to pH changes (Holwerda & Gray, 1974; Weis, 1975; Toppen, 1976; Al-Ayash & Wilson, 1979; Rich & Bendall, 1979, 1980), and include the provision that one of those forms (often a deprotonated form) is the better reducing agent. In principle, however, acid–base equilibria involving groups on the protein that have pK values in the neighbourhood of, or higher than, the pH range explored may also account for such behaviour. The relative pH-independence of the reduction of horse cytochrome c by 4-nitrocatechol compared with catechol itself suggests that it is the reductant and not the protein that is responsible. Nevertheless, to check this we examined the reduction of *Pseudo-
monas aeruginosa cytochrome c-551 and the blue copper protein azurin by catechol (Figs. 4a and 4b), and compared these with the same reaction with horse cytochrome c. It is clear that, although the proteins are very different in overall charge, nature of site and even the metal itself (Adman, 1979), the pH-dependences (Fig. 4b) are similar, suggesting that it is the nature of the reducing agent that is pH-dependent. Similar conclusions have been reached with ascorbic acid as the reductant, and it is noteworthy that the relative magnitudes of the reduction rates of three proteins by catechol are the same as those reported for ascorbate (Al-Ayash & Wilson, 1979).

For cytochrome c, for all the reductants used, apart from 4-acetylcatechol and hydrocaffeic acid, there was no evidence for approach to a first-order rate limit at high reductant concentrations. However, with 4-acetylcatechol and hydrocaffeic acid such an upper rate limit was noticed. Fig. 5 reports the dependence of the observed pseudo-first-order rate constant, measured at pH 8, on 4-acetylcatechol concentration at a number of NaCl concentrations. This clear salt-concentration-dependence is also shown in Fig. 6, in which catechol and 4-acetylcatechol are compared and where it is seen that the rate constant falls linearly with the square root of ionic strength. Such a result

![Graph showing the effect of ionic strength on the pseudo-first-order rate constant for the reduction of mammalian cytochrome c by 4-acetylcatechol at pH 8.0. The buffer was Bistris/HCl and the ionic strength was varied by the addition of NaCl: ○, 0.1; □, 1.0; △, 1.5. For other conditions see Fig. 1 legend and the text.](image)

![Graph showing the reduction of redox proteins by catechol.](image)
was reproduced with hydrocaffeic acid. On the other hand, it was noted that the very low rate of reduction of cytochrome $c$ by positively charged molecules such as 3,4-dihydroxyphenethylamine and noradrenaline was enhanced by salt addition.

It was also decided to investigate the reaction of other dihydroxy aromatic compounds with the cytochromes, and resorcinol and quinol were therefore examined. The former compound reduced neither mammalian nor bacterial cytochrome $c$ under the conditions explored (i.e. up to 10 mM reductant and between pH 6 and pH 9). Quinol, however, as expected from the work of Greenwood & Palmer (1965), Castro et al. (1975) and Rich & Bendall (1980), reduced cytochrome $c$ rapidly and completely in a second-order process (Fig. 7). This Figure also gives the comparable results for tetrachloroquinol. The second-order rate constant for tetrachloroquinol at pH 8 is $1.78 \times 10^4$, in good agreement with other reports (Greenwood & Palmer, 1965; Castro et al., 1975), and about 10000-fold greater than the value for the unsubstituted compound.

As was found for catechol and most of its derivatives, the logarithm of the apparent rate constant for the reduction of cytochrome $c$ by quinol and tetrachloroquinol was strongly pH-dependent. Fig. 8(a) illustrates this dependence when mammalian ferricytochrome $c$ was the oxidant. However, for reasons given above, one is restricted in the pH range when employing this protein, and to test any postulated mechanism it was desirable to extend our observations to higher pH values. We therefore performed similar experiments with *Pseudomonas* cytochrome $c$-551. This protein was chosen because its haem-linked alkaline isomerization has $pK > 11$. 

![Fig. 6. Effect of ionic strength ($I$) on the pseudo-first-order rate constant for the reduction of mammalian cytochrome $c$ by catechol and 4-acetylcatechol.](image)

Also shown are Brønsted plots of the effect of the ionic strength on the logarithm of the rate constants of cytochrome $c$ reduction by catechol and 4-acetylcatechol. Cytochrome $c$ concentration was constant throughout the experiment (10 $\mu$M), and the concentration of the reductant was always 10 mM at pH 8.0, Bistris/HCl buffer being used. Ionic strength was varied by the addition of NaCl, as indicated in Fig. 5 legend. For full experimental details see the text.
Reduction of cytochrome c by organic compounds

(Vinogradov, 1970). Thus it is possible to explore pH values up to pH 10.5 without encountering kinetic complications resulting from the presence of interconverting forms of the protein (Fig. 8b).

The continuous lines in Figs. 8(a) and 8(b) were generated from a model together with the rate constants given in Table 1, which are discussed below.

Discussion

Nature of the reducing species

Fig. 3 shows that the logarithm of the apparent second-order rate constant for reduction of cytochrome c by catechol and its methyl, t-butyl and acetyl derivatives is linearly dependent on pH, with slope close to unity. As the pH range explored is significantly below that of the first pK of these compounds (see Table 1), the linear behaviour depicted is most easily rationalized on the basis that the monoanion is the reducing agent. Under these circumstances it can be shown that the relationship between the apparent second-order rate constant and pH is given by eqn. (1):

$$\log k_{\text{app}} = \log k_+ + \pH - pK_1$$

(1)

where $k_+$ is the true second-order rate constant and

Fig. 7. Concentration-dependence of the pseudo-first-order rate for the reduction of mammalian cytochrome c by quinol (O) and tetrachloroquinol (□) at pH 8.0

For conditions see Fig. 1 legend and the text, except that cytochrome c concentration was 2μM when tetrachloroquinol was the reductant and the reaction was monitored at 425 nm.

Fig. 8. Effect of pH on $\log k_{\text{app}}$ for the reduction of cytochromes c by quinols

(a) Reduction of mammalian cytochrome c by quinol and tetrachloroquinol. (b) Reduction of bacterial cytochrome c-551 by quinol and tetrachloroquinol. For conditions see Fig. 7 legend and the text. The points are experimental values, and the continuous lines are computed from the model indicated in the text.
pK_i refers to the first ionization constant of catechol. Eqn. (1) and the data in Fig. 3 therefore allow the determination of the pH-independent rate constant for reduction (Table 1). A similar argument applies to quinol, the monoanion of which also seems to be a competent reducing agent (see Figs. 8a and 8b). From Figs. 8 and 9 it is clear that the dianions are also reducing agents, as expected from earlier studies with ascorbic acid (Al-Ayash & Wilson, 1979). Indeed, the dianions of the catechols and quinol reduce cytochromes more rapidly than do the monoanions.

Where both monoanion and dianion act as reducing agents, eqn. (2) predicts the observed rate constant as a function of proton concentration:

\[
  k_{\text{app}} = \frac{K_1 (k_{+1} [H^+] + k_{+2} K_2)}{([H^+]^2 + K_1 [H^+] + K_1 K_2)}
  \tag{2}
\]

where \( K_1 \) and \( K_2 \) are the ionization constants of the phenolic hydroxy groups and \( k_{+1} \) and \( k_{+2} \) are the pH-independent second-order rate constants for reduction by the monoanion and dianion respectively. This equation collapses to eqn. (1) where \([H^+]|\gg K_1\).

Eqn. (2), together with the literature pK values given in Table 1, has been used to fit the experimental data for the reduction of both mammalian cytochrome c and bacterial cytochrome c by some catechols and quinols. The values of \( k_{+1} \) and \( k_{+2} \) used are reported in Table 1, and the experimental points and the computed curve are shown in Figs. 8 and 9. Over the pH range of the experiments depicted in Fig. 8(a) quinol operates predominantly via the monoanion, whereas tetrachloroquinol operates via the dianion. The use of cytochrome c-551 (Fig. 8b) allows a higher pH region to be used in which the dianion of quinol becomes significantly populated. The same considerations apply to Figs. 9(a) and 9(b), where the comparable data for catechol and its tetrahalogenated derivatives are given.

The pH-invariance of the rate of reduction by the formyl and nitro derivatives of catechol given in Fig. 3 seems, at first sight, to be in contradiction to the foregoing discussion. For example, the first pK of the nitro derivative is low (pK_i = 6.8) (Table 1), and thus the monoanion predominates over the pH range explored, with the dianion making significant contributions at the higher pH values. By comparison with catechol one would therefore expect both a higher rate constant at a given pH value and a strong pH-dependence. Neither of these is observed. Similar discrepancy appears for the formyl derivative. In order to understand these results, it is necessary to consider the effects of the substituent in more detail.

**Substituent effects**

It is apparent from Table 1 that the value of \( k_{+1} \) is strongly dependent on the nature of the substituent. This dependency may be rationalized by the Hammett treatment of the substituent effect (Johnson, 1973; Shorter, 1973). Fig. 10 reports a plot of the logarithm of \( \log k_{+1} \) from Table 1 versus the electronic parameter (\( \sigma \)) for each substituent. For substituents that withdraw electrons form the ring system, the rate decreases linearly with increasing \( \sigma \) value. This is so albeit that the pK_i values also fall (Table 1) with \( \theta \) and, as the previous section shows that deprotonated forms are the reducing agents, one
Table 1. Parameters governing the reduction of horse heart cytochrome c by dihydroxy conjugated compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituent σ value at para position*</th>
<th>pK$_1$ (OH)†</th>
<th>pK$_2$ (OH)†</th>
<th>pK$_1$ (R)†</th>
<th>k$_{app}$ at pH 8 (M$^{-1}$.s$^{-1}$)</th>
<th>k$_{+1}$ for monoanion (M$^{-1}$.s$^{-1}$)</th>
<th>k$_{+2}$ for dianion (M$^{-1}$.s$^{-1}$)</th>
<th>N</th>
<th>Group at 4-position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>0</td>
<td>9.37</td>
<td>13.7</td>
<td>—</td>
<td>1.41 × 10$^2$</td>
<td>3.31 × 10$^3$</td>
<td>7.08 × 10$^0$</td>
<td>1</td>
<td>H</td>
</tr>
<tr>
<td>4-Methyl catechol</td>
<td>−0.17</td>
<td>9.7</td>
<td>12.95</td>
<td>—</td>
<td>3.35 × 10$^2$</td>
<td>1.59 × 10$^4$</td>
<td>—</td>
<td>1</td>
<td>−CH$_3$</td>
</tr>
<tr>
<td>4-t-Butyl catechol</td>
<td>−0.2</td>
<td>9.6</td>
<td>12.5</td>
<td>—</td>
<td>1.78 × 10$^2$</td>
<td>7.08 × 10$^3$</td>
<td>—</td>
<td>1</td>
<td>−C(CH$_3$)$_3$</td>
</tr>
<tr>
<td>3,5-Di-t-butylicatechol</td>
<td>—</td>
<td>9.5‡</td>
<td>13.7‡</td>
<td>—</td>
<td>1.25 × 10$^2$</td>
<td>3.13 × 10$^3$</td>
<td>—</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4-Aminocatechol</td>
<td>−0.66</td>
<td>10.65</td>
<td>12.11</td>
<td>8.12</td>
<td>7.94 × 10$^3$</td>
<td>3.55 × 10$^4$</td>
<td>—</td>
<td>1</td>
<td>−NH$_2$</td>
</tr>
<tr>
<td>4-Acetylecatechol</td>
<td>−0.07</td>
<td>9.44</td>
<td>12.0</td>
<td>4.25</td>
<td>1.19 × 10$^2$</td>
<td>3.28 × 10$^3$</td>
<td>—</td>
<td>1</td>
<td>−CH$_2$−CO$_2$−</td>
</tr>
<tr>
<td>Hydrocaffeic acid</td>
<td>−0.13</td>
<td>9.44§</td>
<td>12.0§</td>
<td>4.25§</td>
<td>1.20 × 10$^2$</td>
<td>3.31 × 10$^3$</td>
<td>—</td>
<td>1</td>
<td>−CH$_2$−CH$_2$−CO$_2$−</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.24</td>
<td>9.2</td>
<td>12.0</td>
<td>4.2</td>
<td>17.8</td>
<td>2.82 × 10$^2$</td>
<td>—</td>
<td>1</td>
<td>−CH=CH−CO$_2$−</td>
</tr>
<tr>
<td>4-Nitrocatechol</td>
<td>1.27</td>
<td>6.78</td>
<td>10.9</td>
<td>—</td>
<td>0.462</td>
<td>0.03</td>
<td>3.98 × 10$^2$</td>
<td>~0</td>
<td>−NO$_2$</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>0.68</td>
<td>8.64</td>
<td>13.1</td>
<td>4.26</td>
<td>3.98</td>
<td>17.38</td>
<td>—</td>
<td>~0.8</td>
<td>−CO$_2$−</td>
</tr>
<tr>
<td>Protocatechualdehyde</td>
<td>1.03</td>
<td>7.27</td>
<td>11.4</td>
<td>—</td>
<td>2.43</td>
<td>0.43</td>
<td>6.31 × 10$^3$</td>
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<td>−CHO</td>
</tr>
<tr>
<td>Tetrachlorocatechol</td>
<td>0.95‡</td>
<td>5.8</td>
<td>10.1</td>
<td>—</td>
<td>5.62 × 10$^4$</td>
<td>3.3 × 10$^5$†</td>
<td>4.0 × 10$^6$†</td>
<td>1</td>
<td>−Cl</td>
</tr>
<tr>
<td>Tetrabromocatechol</td>
<td>0.95‡</td>
<td>5.8‡</td>
<td>10.1‡</td>
<td>—</td>
<td>3.16 × 10$^4$</td>
<td>3.3 × 10$^5$†</td>
<td>4.0 × 10$^6$†</td>
<td>1</td>
<td>−Br</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenyl-ethylamine</td>
<td>Very slow</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>3,4-Dihydroxybenzylamine</td>
<td>Very slow</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Noradrenaline</td>
<td>−0.1</td>
<td>9.4</td>
<td>12.0</td>
<td>4.25</td>
<td>1.1 × 10$^2$</td>
<td>2.75 × 10$^3$</td>
<td>—</td>
<td>1</td>
<td>−CH$_2$−NH$_3$</td>
</tr>
<tr>
<td>3,4-Dihydroxy mandelic acid</td>
<td>Very slow</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
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<tr>
<td>Quinol</td>
<td>—</td>
<td>9.91</td>
<td>11.56</td>
<td>—</td>
<td>4.47 × 10$^2$</td>
<td>2.57 × 10$^4$†</td>
<td>4 × 10$^3$†</td>
<td>1</td>
<td>−OH(=CH)−CH$_2$NH$_3$</td>
</tr>
<tr>
<td>Tetrachloroquinol</td>
<td>0.95‡</td>
<td>~6*</td>
<td>9.5**</td>
<td>—</td>
<td>1.78 × 10$^6$</td>
<td>2.57 × 10$^4$†</td>
<td>4 × 10$^3$†</td>
<td>1</td>
<td>−OH(=CH)−CO$_2$−</td>
</tr>
<tr>
<td>2,3-Dihydroxypyridine</td>
<td>—</td>
<td>4.12</td>
<td>11.51</td>
<td>—</td>
<td>95.8</td>
<td>—</td>
<td>3.1 × 10$^5$</td>
<td>1</td>
<td></td>
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</tbody>
</table>

* Values obtained (i) Johnson (1973), (ii) Shorter (1973) and (iii) Hansch & Leo (1979).
† Values obtained mainly from Serjeant & Dempsey (1979).
‡ Assumed to be the same as for 3,5-dimethylphenol.
§ Assumed to be the same as for 4-acetylicatechol.
¶ Assumed to be the same as for tetrachlorocatechol.
** Measured by spectrophotometric titration.
†† From computer simulations shown in Figs. 8 and 9.
might expect that the rate should increase with this electronic parameter.

The pH-dependences and the Hammett plot of Fig. 10 taken together may be interpreted by postulating, not only that must the diphenol be deprotonated, but also that the electron must be available for electron transfer from the hydroxy groups and the aromatic nucleus, and not be withdrawn on to the substituent; i.e. the electron density on the oxygen atom of the deprotonated hydroxy group must be high. In the light of this hypothesis, the behaviour of the nitro substituent may be reconsidered. Although the nitro group (also the formyl group) lowers $pK_a$, thus increasing the concentration of the deprotonated forms, the contribution of the hybrid in which the electron may be formally represented as residing on the substituent dominates (structure I). In such ionic hybrids the electron is apparently not available for transfer to the haem group of cytochrome $c$, and thus the observed rates are very low.

Where, however, $pK$ values are lowered but the electron density is not delocalized from the phenol-ate anion, i.e. tetrahalogenated quinol and catechol, the rate is greatly enhanced, as expected if the deprotonated forms act as reducing agents (Rich & Bendall, 1980). This view also provides a basis for an explanation of the pH-independence of the reduction with the nitro and formyl groups; for example, the monoanion of the nitro group represented in structure (I) is comparable with phenol insofar that it has no conjugated hydroxy group. Phenol, either as itself or a phenolate ion, cannot reduce cytochrome $c$ (Burns et al., 1976; Steeken & Neta, 1979) (see also the Results section), and thus by analogy further deprotonation of nitrocatechol monoanion would be predicted to have little effect on the rate of reduction.

\[
\begin{align*}
\text{O}^- & \quad \text{N}^+ \\
\text{O}^- & \quad \text{OH}
\end{align*}
\]

(I)

**Mechanism**

From the above discussions we conclude that the active species are deprotonated forms, either mono- or di-anions, in which the hydroxy groups are negatively charged. It is well established that the catechols and quinols can donate two electrons/molecule (Mihailovic & Čeković, 1971; Becker, 1974; Chambers, 1974), forming eventually quinones as the final product. As cytochrome $c$ and azurin are one-electron acceptors, it follows that the mechanism of reduction must involve radicals. The reduction of single-site redox proteins by ascorbic acid, another dihydroxy conjugated compound, has been discussed by Al-Ayash & Wilson (1979). A similar mechanism involving formation of radicals by electron donation from either the mono- or di-anionic forms followed by radical dismutation would seem to apply to the compounds under consideration here. A parallel mechanism is also possible for the quinols, although in this case the hydroxy groups are *para* to each other, relieving any steric hindrance. For this mechanism to operate it is important to have two conjugated hydroxy groups on the ring system, so that radicals may readily dismute to give the known products of oxidation, the quinones. Where such conjugation is not present, i.e. in phenol and in resorcinol, in which there are two hydroxy groups *meta* to each other, quinone structures are not formed and reduction of cytochrome $c$ does not take place.

The rate of reduction of cytochrome $c$ by tetrahalogenated quinol and catechol is extremely rapid. At pH 8, pseudo-first-order rates of reduction of approx. 150 s$^{-1}$ are easily achieved. Such rates are far higher than reported values for the rate of

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**Fig. 10. Substituent effect on the second-order pH-independent rate for the reduction of cytochrome $c$ by catecholic compounds**
'crevice opening' for ferricytochrome c. The rate for methionine sulphur dissociation from the central iron atom has been measured under a variety of conditions, and values of 8 s⁻¹ (Al-Ayash & Wilson, 1979) and 60 s⁻¹ (Yandell et al., 1973; Creutz & Sutin, 1973) have been reported. As these rates are lower than rates of reduction, one may discount inner-sphere mechanisms of reduction in which these halogenated reducing agents approach the metal atom after dissociation of the intrinsic ligand. As we believe that the rapid rate of reduction by the halogenated compounds merely reflects their low pK values and does not imply a different mechanism for the other compounds under consideration, we suppose that all the reducing agents employed act as outer-sphere reductants. Furthermore, there is no marked difference in the rates of cytochrome c reduction by catechol, 4-methylcatechol, 4-t-butyl-catechol and 3,5-di-t-butylcatechol, suggesting that approach to the central iron on the sterically restricted cavity, as demanded by an inner-sphere mechanism, does not occur. Similar conclusions have been drawn from studies with ascorbic acid, which, as a conjugated dihydroxy compound reducing redox proteins via a dianionic form, is analogous to the quinols and catechols (Al-Ayash & Wilson, 1979).

The site of electron donation from catechol and quinol to the cytochrome is not known, but many studies now suggest (Ilan & Shafferman, 1979a, b; McCray & Kihara, 1979; Ahmed & Millett, 1981; Butler et al., 1981; Tessie, 1981) that the haem edge may act as the route by which electrons pass to the central metal. The protein surrounding the haem edge provides a number of positively charged residues, to which reductants carrying negative charges, such as those considered in the present work, may be attracted. Fig. 6 shows that the reduction of cytochrome c is strongly ionic-strength-dependent. The logarithmic decrease in rate with 1/ionic-strength is indicative of attractive electrostatic interactions between the negatively charged catechol and a positive charge or charges on the cytochrome c molecule. (Supporting the above idea is the very low rate of positively charged molecules, e.g. 3,4-dihydroxyphenethylamine.)

Studies on the reduction of cytochrome c by ascorbate (Myer et al., 1980; Pande & Myer, 1980) provide evidence for the binding of an ascorbate ion to a positively charged arginine residue, position 38 in the sequence, located at the 'bottom right-hand side' of the molecule. However, we do not favour binding of the molecules considered here to arginine-38 as a prerequisite for electron transfer. The position of this residue, away from the haem edge, implies that electron transfer to the central iron atom must proceed via an aromatic residue, tryptophan-59, a pathway that may be expected (Myer et al., 1980) to be of 'low efficiency'. In fact, the electron-transfer rates reported above indicate a highly efficient process, with second-order rate constants under some conditions approaching the diffusion-controlled limit, lower rates being attributed to the protonation state of the reductant and to the nature of the substituent it bears. In addition, except for 4-acetylcatechol, which is discussed below, we see no evidence from our kinetic studies to suggest specific binding of reductant to residues on the protein, i.e. we see no approach to a rate limit over the concentration ranges explored. However, we do note in some cases a non-zero intercept in the plots of k₀ versus reductant concentration (Figs. 2a, 4a and 7), and this may reflect the dissociation constant of a protein–reductant complex.

We favour a mechanism in which electrons are donated to the haem edge. From an examination of a model of cytochrome c, it is apparent that this molecule carries a number of positively charged domains that circumscribe the haem edge at the top of the molecule. Koppenol et al. (1978) have shown that these domains establish an electrostatic field that directs negatively charged molecules towards the haem edge. Such electrostatic orientation and direction of small molecules adequately accounts for the reactions of cytochrome c with a variety of inorganic redox agents.

The ε-amino group of the lysine-13 residue, located close to the top of the haem crevice, is of particular importance in this regard. Our results, obtained with organic molecules, seem to be consistent with this hypothesis. A noteworthy exception is 4-acetylcatechol, whose rate of reduction of c reaches a rate limit (Fig. 5), and similar results have been observed with hydrocaffeic acid.

4-Acetylcatechol possesses an additional negative charge on the 'backside' of the molecule, opening the possibility for non-productive binding to the positively charged residues in the vicinity of the haem edge. The rate limit, at approx. 1 s⁻¹ at low ionic strength, may now reflect changes in the binding position and/or migration of the 4-acetylcatechol molecule on the surface of the protein before the electron transfer. One would, of course, expect to observe similar limiting kinetics with protocatechuic acid, as this also possesses a similarly placed negative charge. However, for reasons discussed above in relation to the Hammett plot, the rate of electron transfer is so low that the rate limit of approx. 1 s⁻¹ for migration is not approached, and thus no limit is observed.

The overall pattern that emerges, therefore, is that either singly or doubly deprotonated reducing agents, in which the oxygen atom of the dissociated hydroxy group possesses a high electron density, approach the haem edge, under the influence of and directed by the positively charged groups surrounding the haem pocket. Electron transfer to the
central iron by an outer-sphere mechanism, involving the delocalized electrons of the haem moiety, leads to reduction of the iron. Organic radicals formed in this process dismute, to give the final quinone products. Where such dismutation cannot occur without slow polymerization processes intervening, reduction is imperceptibly slow, e.g. with phenol and resorcinol.

We thank Dr. J. G. Tillett and Dr. J. Silver for the gift of some of the compounds used, and Professor M. Brunori for the gift of cytochrome c-551 and azurin. We thank Dr. G. Wallmark, Dr. K. Bowden and Dr. R. C. Hider for valuable and stimulating discussions. M. T. W. acknowledges the support of a Science Research Council research grant (GR/B/5022/5).

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