Oxidation through the Cytochrome System of Substituted Phenylenediamines

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The oxidation of dimethyl-*p*-phenylenediamine through the cytochrome system is only very slightly increased by additions of cytochrome *c* (Borei & Renvall, 1949). It can be questioned whether the endogenous cytochrome *c* of the oxidase preparation, in mediating between the oxidase and the substrate, is sufficient to account for the whole oxidation, or if the reductant is directly attacked by the oxidase.

In this investigation the question of the participation of cytochrome *c* has been studied for a number of reductants of the phenylenediamine type. The influence of additions of external cytochrome *c* has been recorded. The endogenous cytochrome *c* has been inactivated by low-temperature treatment. The toxicity of the reductants was investigated.

MATERIAL AND METHODS

Test system. The oxidation of the reductant, in the presence of a cytochrome oxidase preparation and cytochrome *c*, was followed at 39° in differential manometers; shaking frequency 120 round trips/min.; total fluid volume 3 ml. The reductant (final concn. 0·03 M) was tipped at the beginning of the experiments from a dangling cup into the main compartment. The velocity was obtained from the difference between the readings at 0 and 13 min. Phosphate buffer of pH 7·3 and of final optimun concentration 0·083 M (cf. Borei, 1950) was used. In the left-hand flask a similar sample was placed, the only difference being that the oxidase preparation was boiled. By this means the autoxidation of the substrate under the conditions of the experiment was largely compensated for. KOH in the centre cup was omitted, since the CO_2 output was found to be negligible.

The amount of cytochrome *c* was varied in the different experiments. The amount of oxidase was always adjusted to correspond to an O_2 uptake at 39° of 150 μl. during the first 13 min. in a 3 ml. test system composed as follows: phosphate buffer, pH 7·3, of final concn. 0·083 M, cytochrome *c* $40{\cdot}6 \times 10^{-6}$ M, Cl^- 0·044 M, and *p*-phenylenediamine (added from a dangling cup) 0·03 M.

In some experiments the unneutralized hydrochloride of the reducing substance was used. In this case the pH of the test system was lowered, but the only observed influence on the outcome of the experiment was a decreased oxidation rate. In all experiments the final chloride ion concentration was adjusted to 0·044 M, except in the experiments with amine dihydrochlorides, where it was 0·074 M.

Cytochrome oxidase preparation. The procedure of Keilin & Hartree (1947) with the modifications of Keilin & Hartree (1949) was followed, the only deviation being that the milling with quartz sand in a mechanical mortar was replaced by a mincing of the washed-out muscle mass for 2 min. at 4° on the Waring Blendor. The source was heart muscle from freshly slaughtered pigs. The preparation was preserved at 4° and used during the first 4 days after preparation.

Cytochrome c of appropriate concentration, was prepared according to Keilin & Hartree (1945). It contained 0·34% Fe, and was used dissolved in a 0·5% (w/v) NaCl solution.

Reducing agents. Quinol, *p*-aminophenol hydrochloride and *p*-phenylenediamine: Schering-Kahlbaum, 'reinst'. *N*-Methyl-*p*-phenylenediamine.2HCl: Eastman-Kodak. *NN*-Dimethyl-*p*-phenylenediamine.2HCl: Merck, twice recrystallized under nitrogen in the cold from a solution in glacial acetic acid. *NNN'N'*-Tetramethyl-*p*-phenylenediamine.HCl: British Drug Houses. *NN*-Diethyl-*p*-phenylenediamine.HCl: Eastman-Kodak.

EXPERIMENTS AND RESULTS

Participation of cytochrome c

The influence of increasing amounts of added cytochrome *c* on the oxidation of a number of reductants through the cytochrome system is represented in Fig. 1. All activities are referred to the oxygen uptake obtained when the final concentration of added cytochrome *c* is $45{\cdot}7 \times 10^{-6}$ M. With increasing complexity of the reductant added, the cytochrome *c* is less and less able to increase the rate of oxygen uptake already obtained with the oxidase preparation alone. In fact, for tetramethyl-*p*-phenylenediamine, which stands at the end of the substitution series, the oxidation rate is slightly lowered if cytochrome *c* is added. The decreasing susceptibility to cytochrome *c* addition can mean, either (1) that the endogenous cytochrome *c* of the oxidase preparation becomes sufficient to handle an increasing amount of substrate oxidation, or (2) that the substrate with increasing complexity becomes more and more capable of reacting directly with the oxidase, thus rendering the cytochrome *c* unnecessary for the catalysis.

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The appearance on reductant addition of the α bands of the cytochrome components a and c in cytochrome-c-complemented oxidase preparations with addition of potassium cyanide, was observed in the Zeiss microspectroscope. With quinol both bands are strong and appear readily. With both p-phenylenediamine and dimethyl-p-phenylenediamine the c band is only slightly weaker. This indicates that cytochrome c is not by-passed even in the oxidation of the substituted phenylenediamine. The strong coloration, which occurs with tetramethyl-p-phenylenediamine, prevents observations using this particular reductant.

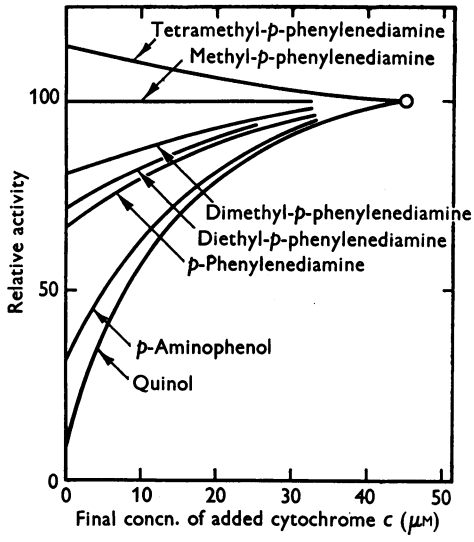

Fig. 1. Influence of added cytochrome c on the oxidation of different reductants through the cytochrome system (heart-muscle cytochrome oxidase preparation). For all reductants the activities are referred to the oxygen uptake obtained when the final concentration of added cytochrome c is $45{\cdot}7 \times 10^{-6}$ M (relative activity = 100).

In order to inactivate the endogenous cytochrome c, the oxidase preparation was subsequently frozen three times in liquid air and thawed at room temperature. Such a treatment was found by Keilin & Hartree (1938, 1940) to impair seriously the oxidation of p-phenylenediamine; cytochrome c addition showed that though the activity of the oxidase itself was practically unaffected, the endogenous cytochrome c had been largely eliminated.

Liquid-air treatment of the oxidase leads for all reductant types to a considerable decrease in the oxidation rate. It will be noted (Fig. 2, pecked curves) that the relative activities increase with the complexity of the reductant. This effect is, however, much less pronounced with the frozen oxidase than with the untreated one. Thus the depression obtained by freezing is more pronounced with the complex reductants than with the simpler ones. It looks, however, as if freezing does not completely eliminate the endogenous cytochrome c, and as if the trace left could mediate the oxidation with an increasing efficiency the more complex the reductant is.

When cytochrome c is added to the frozen oxidase (Fig. 2, □—□) a considerable increase in oxidation

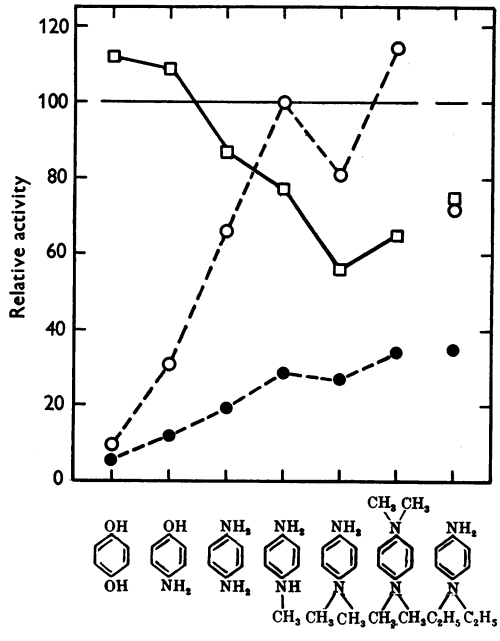

Fig. 2. Catalytic activity, with different reductants, of cytochrome oxidase treated with liquid air. All activities refer to the oxygen uptake obtained with cytochrome-c-complemented untreated oxidase (relative activity = 100). ○--○, Untreated oxidase + reductant; ——, untreated oxidase + cytochrome c + reductant; ●--●, frozen oxidase + reductant; □—□, frozen oxidase + cytochrome c + reductant. Final concentration of cytochrome c in complemented experiments, $40{\cdot}6 \times 10^{-6}$ M. Types of reductant employed indicated below graph.

rate, above the rate obtained with frozen oxidase alone, is observed with all reductants. These experiments clearly demonstrate that cytochrome c is an important part of the carrier chain, and that it mediates the catalytic oxidation of each of the members of the reductant series quinol → tetramethyl-p-phenylenediamine.

It is found, however, that cytochrome c addition to the frozen oxidase will not always bring the oxidation up to the level of oxygen consumption found in the corresponding experiment with untreated oxidase. As shown in Fig. 2 a complete restoration is obtained for the simpler substrates,

Table 1. *Oxidation rate with different reductants*

(Final concentration of added cytochrome *c*, $45{\cdot}7 \times 10^{-6}$ M; of reductant, 0·03 M. Complete oxidation of quinol to quinone requires 1008 μl. O_2.)

Reductant	pH	μl. O_2 consumed after 3 min.	8 min.	18 min.	28 min.
Quinol	7·3	28	74	144	184
p-Aminophenol	6·6	20	53	87	108
p-Phenylenediamine	7·1	38	106	230	328
N-Methyl-*p*-phenylenediamine	6·3	46	99	158	199
NN-Dimethyl-*p*-phenylenediamine	6·5	39	73	92	97
NNN'N'-Tetramethyl-*p*-phenylenediamine	6·4	27	76	132	135
NN-Diethyl-*p*-phenylenediamine	7·2	31	59	70	73

but with increasing reductant complexity this effect is progressively less complete. It might be that the low-temperature treatment has affected the oxidase preparation in such a way that the spatial arrangement of its components has been modified, thus altering the interaction properties, which presumably vary with the molecular structure of the reductant (cf. Discussion).

The time/oxygen uptake curves differ considerably for the different reductants, as can be seen from Table 1. With the simpler reductants the oxidation rate is maintained at a high level for a much longer time than with the more complex ones. Especially with the di- and tetra-alkylated phenylenediamines the oxidation very rapidly comes to a complete standstill. This is due to a toxic effect of the oxidation products.

Toxicity of the reductant

The oxidation of dimethyl-*p*-phenylenediamine is initially very rapid, but soon drops to zero (cf. Table 1). This is due to a toxic effect on the cytochrome system of the oxidation products of dimethyl-*p*-phenylenediamine. As shown in Fig. 3 a subsequent addition of *p*-phenylenediamine or more dimethyl-*p*-phenylenediamine is unable to induce any further oxidation. On the other hand, if *p*-phenylenediamine is the original reductant, further addition of *p*-phenylenediamine is able to increase the uptake, whereas, also in this case, dimethyl-*p*-phenylenediamine addition rapidly brings the reaction to a standstill.

The autoxidation of dimethyl-*p*-phenylenediamine is quite fast. It is thus possible to obtain a certain amount of oxidation products in a dimethyl-*p*-phenylenediamine solution before it is added to the test system. It is found that the higher the concentration of the oxidation product is beforehand, the more toxic will the influence be, i.e. the sooner will the oxygen uptake in the test system stop. Boiled oxidase will practically completely check the autoxidation, and in the test system itself there is no measurable oxygen uptake after the catalysis proper has stopped. The autoxidation is thus probably due to a metal ion catalysis, and suppressed by a formation of metal-protein complexes in the test system. Added 1-naphthol intensifies the autoxidation, and furthers formation of oxidation

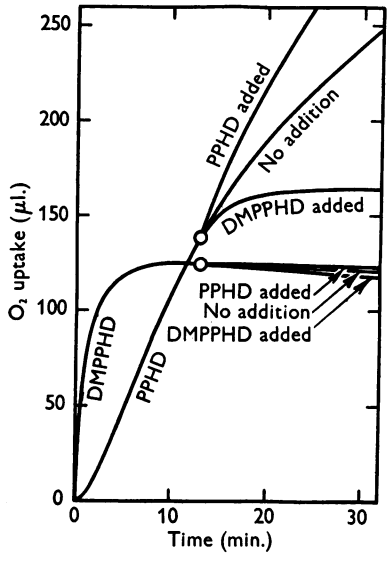

Fig. 3. Toxicity of dimethyl-*p*-phenylenediamine. Oxidation of *p*-phenylenediamine (PPHD) and dimethyl-*p*-phenylenediamine (DMPPHD) through the cytochrome system. Original reductants (0·03 M final concentration) added at start of the experiment. After 13 min. further reductant (0·03 M) is added, as indicated in the figure.

products also in the test system. Accordingly, 1-naphthol addition most strikingly increases the toxic effect of dimethyl-*p*-phenylenediamine on the cytochrome system. Nevertheless, a mixture of 1-naphthol and dimethyl-*p*-phenylenediamine, which constitutes the 'Nadi reagent', has been much used for the biochemical demonstration of cytochrome oxidase activity.

A similar toxic effect has been observed for

Table 2. *Oxidation-reduction potentials*

Reduced member	Potential at pH 7 (V.)	Reference
Quinol	0·285* (0)	Conant & Fieser (1924)
	0·282* (0)	Ball & Chen (1933)
	0·277* (4·65)	Ball & Chen (1933)
	0·289* (0)	Jenny (1949)
	0·273* (7·3)	Slater (1949*a*)
p-Aminophenol	0·311* (0·76)	Conant & Pratt (1926)
	0·316* (7·57)	Fieser (1930)
	0·319* (0)	Jenny (1949)
p-Phenylenediamine	0·387* (0)	Fieser (1930)
N-Methyl derivative	0·337* (0)	Fieser (1930)
NN-Dimethyl derivative	0·335* (1·98)	Clark, Cohen & Gibbs (1926)
	0·343†	Michaelis & Hill (1933)
	0·307* (4·62)	Michaelis & Hill (1933)
NNN'N'-Tetramethyl derivative	0·222* (3·66)	Michaelis & Hill (1933)
	0·263†	Michaelis & Hill (1933)
NN-Diethyl derivative	0·355†	Michaelis & Hill (1933)
Cytochrome *c* (ferro-)	0·253	Wurmser & Filitti-Wurmser (1938)
	0·262	Stotz, Sidwell & Hogness (1938)
	0·279* (7·4)	Barron (1939)
	0·243†	Paul (1947)

* Figure calculated from E'_0 at indicated pH value, applying the correction 0·0592 V./pH unit at 25°.
† Figure taken from curve or calculated from dissociation formula.

diethyl-*p*-phenylenediamine and tetramethyl-*p*-phenylenediamine, but not for quinol, *p*-aminophenol or *p*-phenylenediamine. There is an indication of toxicity with monomethyl-*p*-phenylenediamine. It ought also to be observed that the quinol oxidation stops before the oxygen uptake has reached the value corresponding to total first-step oxidation of the reductant.

DISCUSSION

From experiments with quinol and *p*-phenylenediamine Keilin & Hartree (1938) concluded that the only catalytic property which can be definitely ascribed to the cytochrome oxidase is that of mediating the oxidation of reduced cytochrome *c* (cf. also Slater, 1949*b*). The experiments of the present investigation, especially the ones with liquid-air-treated oxidase, all indicate that this conclusion holds true not only for the reductants used in Keilin & Hartree's original investigations, but also for the whole series quinol → tetramethyl-*p*-phenylenediamine. Thus cytochrome *c* is always concerned in the oxidation of the reductants. There is no indication that cytochrome *c* should be wholly or even partly by-passed in the oxidation of the more highly substituted reductants.

The oxidation-reduction potentials of cytochrome *c* and of the reducing substances employed are compiled in Table 2. It will be observed that substituting amino groups for hydroxyl groups raises the potential, whereas an increasing methyl substitution of amino hydrogen continuously lowers the potential of the diamine (cf. Fieser, 1930). (Concerning the significance in the physiological pH range of the figure given for phenylenediamine, see Slater, 1949*a*.)

It is obvious that the oxidation-reduction potential of cytochrome *c* is lower than that of any of the reductants used. However, this does not necessarily exclude cytochrome *c* from acting as an effective carrier in the oxidation of the reductants, since, as was already pointed out by Ball & Chen (1933), a substance with a higher oxidation-reduction potential can continue to reduce one with a lower, provided the oxidized state of the former is unstable. As the oxidized form rapidly decomposes, a steady state of continuous oxidation of the reductant results. In the present case it is obvious that the reaction does not stop at the primary oxidation product of the reductant. Instead, in some instances, a further oxidation takes place, and in addition, all the diamines form strongly coloured polymerization products.

The results of the experiments indicate that the more chemically complex the reductant is, the less cytochrome *c* is required for a given relative oxidation velocity. Thus the endogenous cytochrome *c* of the oxidase preparation itself is sufficient to mediate the oxidation with a maximum or almost maximum velocity if reductants at the end of the series are employed, i.e. di- and tetra-substituted diamines. This must mean that the turnover rate of cytochrome *c* is continually increasing in the series

quinol → tetramethyl-*p*-phenylenediamine. As seen from Table 2, merely the differences in oxidation-reduction potential between reductant and cytochrome *c* do not give a clue to the observed, continually increasing efficiency of the carrier. An instability of the oxidation products of the reductants may completely change the picture, but as the extent of instability and tendency to polymerization are quantitatively unknown factors, it is impossible to draw any definite conclusions from such considerations. Furthermore, as cytochrome oxidase and cytochrome *c* function together as a colloidal unit, it is also necessary to consider the ability of the different reductants to penetrate to the carrier, i.e. cytochrome *c*, in the immediate neighbourhood of the cytochrome oxidase. This factor was already touched upon by Slater (1949*a*) in a comparison between the efficiencies of low concentrations of cytochrome *c* to mediate to the cytochrome oxidase in the oxidation of *p*-phenylenediamine and ascorbic acid.

Dimethyl-*p*-phenylenediamine has been found (Runnström, 1930, 1932; Örström, 1932) to augment the oxygen consumption of unfertilized and fertilized sea-urchin eggs. It has also been found (Krahl, Keltch, Neubeck & Clowes, 1941; Borei, 1945) that the oxidases of the echinoderm egg and of the mammalian tissue resemble each other very closely in all properties investigated. Their prosthetic groups are probably identical. From the experiments in this investigation it is thus justifiable to conclude that cytochrome *c* is a part of the echinoderm oxidase system. Cytochrome *c* has, however, never been demonstrated in the sea-urchin egg, but Borei (1951) thinks there is strong circumstantial evidence for its presence. It occurs in the sperm (Rothschild, 1948), but the amount introduced in the egg at fertilization is of minor importance, as is indicated by the similarities in type of the respiration before and after fertilization (Robbie, 1946, 1948), and demonstrated by the fact that parthenogenetically activated eggs raise their oxygen consumption in the same manner as do fertilized ones (Warburg, 1910; Loeb & Wasteneys, 1913; Borei, 1948). Furthermore, as dimethyl-*p*-phenylenediamine induces the same respiratory intensity in unfertilized as in fertilized eggs (Runnström, 1932), a release at fertilization of previously inactive cytochrome *c*, as suggested by Korr (1937), is not likely as an explanation of the respiratory changes initiated by the sperm entrance. For a full review of the respiratory events at fertilization see Borei & Renvall (1949), Runnström (1949), and Borei (1951).

SUMMARY

1. The oxidation through the cytochrome system (heart-muscle cytochrome oxidase preparation) of quinol, *p*-aminophenol, *p*-phenylenediamine, and mono-, di-, and tetra-methyl-substituted *p*-phenylenediamines, is decreasingly dependent on added cytochrome *c* in the order given. For the most complex reductants the endogenous cytochrome *c* of the oxidase preparation is in itself sufficient for a maximum oxidation rate.

2. If the endogenous cytochrome *c* of the oxidase is inactivated by low-temperature treatment, externally added cytochrome *c* can augment the oxidation of all reductants. Cytochrome *c* is always a part of the carrier chain; in no case does the oxidase act directly on the reductant.

3. The dimethyl-, tetramethyl-, and diethyl-substituted *p*-phenylenediamines form oxidation products which are toxic for the oxidase.

4. The significance of the oxidation-reduction potential of the various members of the reductant series has been considered.

5. The implication of the participation of cytochrome *c* in the oxidation of dimethyl-*p*-phenylenediamine is discussed for respiration experiments on fertilized and unfertilized sea-urchin eggs. The suggestion that cytochrome *c* is invariably a part of the echinoderm oxidase system is very strongly supported.

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Chemistry of Insect Cuticle

1. THE WATER-SOLUBLE PROTEINS

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During the last decade there has been a great increase in knowledge of the insect cuticle, which is now recognized to possess an exceedingly complex structure (see Wigglesworth, 1948, 1949). The cuticle consists of two layers, an inner endocuticle and an outer epicuticle. The endocuticle is composed of chitin and protein; the protein in its outer part is sometimes hardened (tanned) to form a dark exocuticle. The epicuticle is composite, consisting of an inner tanned lipoprotein layer, followed by a polyphenol layer which is overlaid by a wax layer. In some insects, but not all, there is also a cement layer which covers and protects the wax layer.

The most important properties of the cuticle are its flexibility and elasticity (which allow of movement but of only limited extension), its hardness (giving a rigid support for the attachment of muscles as well as a protective covering) and its impermeability, especially to water. This last property has enabled insects and related arthropods, in spite of their small size and relatively enormous surface area, to colonize far drier environments than most other small invertebrates. The epicuticle is mainly responsible for the impermeability to water. It is the cuticle which has first to be penetrated by contact insecticides before their toxic effects can be exerted.

The non-chitinous material in the cuticle is mainly protein (in the insects which have been studied, the protein content varied from 25 to 37 % (Odier, 1823; Lafon, 1943*a*)); the inner parts of the cuticle readily give various protein colour tests, such as the biuret, Millon's and xanthoproteic reactions (Kühnelt, 1928; Campbell, 1929; Wigglesworth, 1933; Pryor, 1940*a*, *b*).

The water-soluble proteins from several insect cuticles have been isolated and examined by Trim (1941*a*, *b*) and by Fraenkel & Rudall (1940, 1947). However, these workers made no mention of the fact that the protein, as extracted from the cuticles, was not homogeneous. It is shown below that the extracted protein can be separated electrophoretically into a number of component proteins; hence it is desirable to reconsider the conclusions of Trim and of Fraenkel & Rudall. The extractable proteins fail to coagulate in hot water and are soluble in hot, but not cold, aqueous trichloroacetic acid. They are precipitated from aqueous solutions by ethanol in concentrations above 45 %, by one-third-saturated ammonium sulphate and by saturated sodium chloride. After precipitation by either trichloroacetic acid or ethanol the proteins readily redissolve in water; even after heating in boiling water they remain in solution. Heating does not alter their reaction with quinones. The cuticular proteins thus show physical properties unlike those of any other known proteins.

Trim (1941*a*, *b*) characterized the cuticular proteins from two insects (*Sarcophaga falculata* and *Sphinx ligustri*) by determining nitrogen distribution, tyrosine, tryptophan, glycine and β-hydroxy-α-amino-acids. He concluded that the protein was quite distinct from collagen in its chemical composition and that it bore some resemblance to the sericin of silk.

Fraenkel & Rudall (1940, 1947), using X-ray analysis methods, have shown that, unlike most vertebrate skeletal proteins, insect cuticular proteins from soft (*Sarcophaga*) cuticles, give β-type diffraction. By X-ray and related studies they