Short Communications

The Cytochrome *c*-Catalysed Oxidation of Ascorbate in the Presence of Sodium Dodecyl Sulphate and *NNN'*-Tetramethyl-*p*-phenylenediamine

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Jacobs (1960) introduced TMPD* as a reductant of cytochrome *c* in intact mitochondria. This reagent is often used as an electron carrier in the polarographic determination of cytochrome oxidase activity with ascorbate as the terminal electron donor. During experiments to determine cytochrome oxidase activity in plant extracts I found that traces of sodium dodecyl sulphate greatly increased the oxygen uptake in reaction mixtures containing TMPD, cytochrome *c* and ascorbate. The plant extracts were not necessary and the oxygen uptakes were not significantly affected when these were omitted. The present results show that sodium dodecyl sulphate and some other anionic surface-active agents modify cytochrome *c* to give a complex that can catalyse the rapid oxidation of ascorbate only when TMPD is also present.

Table 1 shows that at pH 6.0 oxygen was taken up rapidly by reaction mixtures containing the four components cytochrome *c*, TMPD, sodium dodecyl sulphate and ascorbate; the rate was much lower when any one of these compounds was omitted. The optimum pH was 6.0-6.2; below pH 6.0 oxygen uptake was slower, and above pH 6.2 the oxygen uptake of controls containing ascorbate and TMPD was no longer negligible and increased as rapidly as that of the complete reaction mixtures. EDTA did not inhibit the oxygen uptake and was therefore added as a routine to diminish any oxidation of ascorbate catalysed by free metal ions. When catalase was incorporated into the reaction mixtures the oxygen uptake was halved; when it was added after the start of the reaction there was an immediate output of oxygen, equal to or less than half the total oxygen absorbed, followed by a halving of the initial rate of oxygen uptake. These results suggest that hydrogen peroxide is a product of this reaction. In the presence of catalase, 1 g-atom of oxygen was absorbed/mol of ascorbate oxidized. Without catalase, more than 1 but usually less than 2 g-atoms of oxygen were used/mol of ascorbate originally present in the reaction mixtures; the erratic results without catalase probably come from secondary reactions between ascorbate and hydrogen peroxide.

The optimum concentration for sodium dodecyl

* Abbreviation: TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

sulphate was 2 mM; at lower concentrations much less oxygen was absorbed, and at concentrations greater than 2 mM sodium dodecyl sulphate inhibited the oxygen uptake. The maximum rate of oxygen uptake with 2 mM-sodium dodecyl sulphate in reaction mixtures containing 100 μ g of cytochrome *c*, 10 μ mol of EDTA and 25 μ g of catalase in 3 ml of 66 mM-sodium orthophosphate buffer, pH 6.0, was with 2 μ mol of TMPD and 6 μ mol of ascorbate. Extra ascorbate or TMPD did not affect this maximum rate of oxygen uptake.

Cytochrome *c* could not be replaced by haemin, the haemoproteins haemoglobin, horseradish peroxidase or catalase, or cysteine or GSH. TMPD was also specific for this reaction and could not be replaced by the related *NN*-dimethyl-*p*-phenylenediamine, *NN*-dimethyl-*p*-nitroaniline, *o*-, *m*- or *p*-phenylenediamine, *o*-, *m*- or *p*-dihydroxybenzene (catechol, resorcinol and quinol), bis-(*p*-dimethylaminophenyl)methanol [4,4'-bis(dimethylamino)-benzhydrol], 1,1'-dimethyl-4,4'-bipyridylium chloride (paraquat), 1,1'-ethylene-2,2'-bipyridylium

Table 1. *Effects of TMPD, sodium dodecyl sulphate and cytochrome c on the oxidation of ascorbate*

Reaction mixtures contained EDTA (10 μ mol), catalase [from bovine liver, 2 $\times$ recrystallized; from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] (25 μ g), TMPD (2 μ mol), ascorbate (10 μ mol), sodium dodecyl sulphate (5 μ mol) and cytochrome *c* [from Boehringer Corp. (London) Ltd., London W.5., U.K.] (100 μ g) in 66 mM-sodium orthophosphate buffer, pH 6.0. Sodium dodecyl sulphate, TMPD, ascorbate or cytochrome *c* was omitted as indicated. The rates of oxygen uptake were determined polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) by measuring the maximum slope recorded after addition of the reagents. All the reaction mixtures were saturated with air and were maintained at 30°C.

Omission	O ₂ uptake (μ l/min)
None	1.50
TMPD	0.04
Sodium dodecyl sulphate	0.08
Ascorbate	0.00
Cytochrome <i>c</i>	0.08

chloride (diquat), 2,6-dichlorophenol-indophenol, bis-(*p*-dimethylaminophenyl)methane or bis-(*p*-dimethylaminophenyl)phenylmethane (Leuco Malachite Green).

Sodium dodecyl sulphate could be replaced by some anionic surface-active agents, including cyclohexylammonium dodecyl sulphate, Mannoxol OT (sodium dioctyl sulphosuccinate), sodium octadecyl sulphate, Igepon A (sodium oleylethanesulphonate) and Igepon T (sodium *N*-methyl-*N*-oleoyltaurate), but not by sodium octyl sulphate, sodium *n*-butyl sulphate, sodium cholate, sodium deoxycholate or sodium taurocholate. In the presence of cyclohexylammonium dodecyl sulphate, sodium octadecyl sulphate, Igepon A and Igepon T, each at a concentration of 2 mM, oxygen was absorbed at 87, 72, 80 and 48% respectively of the rate found with 2 mM-sodium dodecyl sulphate. All non-ionic wetting agents tested, including Triton X-100, Triton X-145, Tween 20 and Lissapol N, were ineffective and neither inhibited nor could replace sodium dodecyl sulphate. The cationic surface-active agents cetyltrimethylammonium bromide and cetylpyridinium chloride not only did not replace sodium dodecyl sulphate but also inhibited its action; this inhibition could be overcome by increasing the concentration of sodium dodecyl sulphate to more than that of the inhibitor. The chelating agents EDTA, azide, *o*-phenanthroline, salicylaldehyde and bipyridyl did not inhibit; cyanide was not tried, as this interfered with the polarographic method of determination, but the results suggest that free metal ions are not involved in these oxidations.

Benzoquinone reacts with cytochrome *c* to form a complex that catalyses the oxidation of ascorbate (Morrison, Steele & Danner, 1969). Complex-formation of this type was not detected between cytochrome *c* and TMPD with or without sodium dodecyl sulphate. When mixtures of cytochrome *c* with either or both sodium dodecyl sulphate and TMPD were chromatographed on Sephadex G-25 and eluted with 0.1 M-sodium orthophosphate buffer, pH 6.0, the starting materials were resolved in the eluate. The cytochrome *c* that was recovered, in contrast with cytochrome *c* still in the presence of sodium dodecyl sulphate, showed the characteristic peak of the reduced form at 550 nm when treated with dithionite. The recovered cytochrome *c* would not catalyse the oxidation of ascorbate unless both TMPD and sodium dodecyl sulphate were also

added; this suggests that it had been completely separated from these compounds. These results are similar to those of Keilin & Hartree (1940), who suggested that the complex formed between sodium dodecyl sulphate and cytochrome *c* was easily dissociable, and contrast with those of Tsushima & Miyajima (1956), who claimed this formation to be irreversible.

Spectrophotometry showed that the reaction of sodium dodecyl sulphate (330 μ M) with reduced cytochrome *c* (4.6 μ M) under anaerobic conditions shifted the Soret band from 418 to 396–398 nm and was accompanied by an intensification of the extinction from 0.725 to 0.840 cm^{-1} . A slight shoulder appeared at 480 nm and, in contrast with reduced cytochrome *c*, this sodium dodecyl sulphate complex had no apparent absorption bands between 500 and 600 nm. Oxidized cytochrome *c* reacted with sodium dodecyl sulphate to give a spectrum identical with that of this complex formed with reduced cytochrome *c*. The 396–398 nm absorption band was stable in anaerobic conditions and was not significantly altered by adding TMPD (150 μ M) or ascorbate (1.5 mM); it was unstable in aerobic conditions and slowly decreased in intensity. This decrease was faster when TMPD and ascorbate were also present, when the extinction decreased from 0.84 to 0.58 cm^{-1} in 4 h. The decrease in extinction at 396 nm could not be correlated with the uptake of oxygen, but this uptake was directly related to the decrease in extinction at 263 nm, which was attributed to the oxidation of ascorbate.

The present results show that small concentrations of some anionic surface-active agents reversibly modify cytochrome *c* to give a complex that catalyses the rapid aerobic oxidation of ascorbate through the intermediate electron carrier TMPD. They suggest that, when this reagent and ascorbate are used in the polarographic or manometric methods of determining cytochrome oxidase activity, care must be taken in interpreting the results if sodium dodecyl sulphate or similar anionic surface-active agent has been used in the preparation of the enzyme.

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