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A quantitative test for superoxide radicals produced in biological systems

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The preparation and properties of a partially succinoylated cytochrome *c*, suited for the detection of superoxide anion radicals in liver microsomes, is reported. By succinoylation of 45% of the primary amino groups of horse heart cytochrome *c* the activity towards solubilized NADPH–cytochrome *P*-450 reductase was diminished by 99% compared with native cytochrome *c*. The capacities of cytochrome b_5 and cytochrome *c* oxidase to reduce the succinoylated ferricytochrome *c* and oxidize succinoylated ferrocytochrome *c* respectively were decreased to a similar extent. However, the bimolecular rate constant for the reduction of the partially succinoylated ferricytochrome *c* by $O_2^{-\cdot}$ was estimated to be one-tenth of the value for the reaction of $O_2^{-\cdot}$ with native ferricytochrome *c* at pH 7.7. On this basis the quantification of $O_2^{-\cdot}$ generated by NADPH-supplemented liver microsomes became possible. The initial rates of succinoylated ferricytochrome *c* reduction determined at various finite concentrations of the cytochrome *c* derivative can be extrapolated to obtain true rates of $O_2^{-\cdot}$ generation in a homogeneous system. The problems encountered in the quantitative determination of $O_2^{-\cdot}$ produced in biological membranes, e.g. microsomes, are discussed.

Biological electron-transfer systems exposed to O_2 tend to undergo autoxidation reactions leading to the formation of H_2O_2 or finally water. It seems that in many cases the superoxide anion radical is the initial intermediate in these reactions, but its detection and quantification in complex biological systems like mitochondria, chloroplasts or microsomes is difficult. Among a variety of established methods (McCord, 1977; Bors *et al.*, 1978) the co-oxidation of adrenaline to adrenochrome has been widely used (Asada *et al.*, 1974; Cadenas *et al.*, 1977; Bartoli *et al.*, 1977). The reliability of this test has been questioned repeatedly, because it is not based on a straightforward reaction (Bors *et al.*, 1975) and superoxide radicals are generated from adrenaline itself (Misra & Fridovich, 1972). Furthermore, strong enhancement of the autoxidation of oxyhaemoglobin (Brunori *et al.*, 1975), reduced cytochrome b_5 (Berman *et al.*, 1976) and reduced cytochrome *P*-450 reductase (Prough & Masters, 1973) has been observed in the presence of adrenaline.

Abbreviations used: TNBS, 2,4,6-trinitrobenzenesulphonic acid; SOD, superoxide dismutase.

Alternatively, ferricytochrome *c*, either native (McCord & Fridovich, 1969) or partially modified by acetylation (Azzi *et al.*, 1975) or succinoylation (Kuthan *et al.*, 1978), was introduced as an indicating scavenger for $O_2^{-\cdot}$. Succinoylated cytochrome *c* has been favoured over acetylated cytochrome *c* because of its lower capability to get reduced by flavin-dependent cytochrome *c* reductases. The inhibition by superoxide dismutase of the reduction of native and modified ferricytochrome *c* provides sufficient evidence for the involvement of $O_2^{-\cdot}$ (McCord, 1977). On this basis also a quantitative determination of superoxide anion radicals became feasible and was applied for the reconstituted and microsomal cytochrome *P*-450 system (Kuthan *et al.*, 1978; Ullrich & Kuthan, 1980).

The objective of this paper is to present (i) selected physicochemical properties of a succinoylated cytochrome *c* derivative suited for the

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detection of $O_2^{-\cdot}$ in biological systems and (ii) to develop an experimental procedure and the underlying theory for the quantitative trapping of superoxide anion radicals by modified ferricytochrome *c*.

The present work was part of the Ph.D. thesis of H.K. at the University of Saarland.

Experimental

Materials

Cytochrome *c* (horse heart; type VI), xanthine oxidase (EC 1.2.3.2), superoxide dismutase (EC 1.15.1.1), TNBS (grade II), AMP and xanthine were purchased from Sigma Chemical Co., catalase (EC 1.11.1.6) was obtained from the Worthington Biochemical Corp., NADPH was from Boehringer-Mannheim, hexobarbitone (sodium salt) was a generous gift of Sterling-Winthrop.

Methods

Liver microsomes from phenobarbital-treated male Sprague–Dawley rats were prepared in 0.25 M-sucrose as described previously (Werringloer *et al.*, 1979).

Partial succinoylation of cytochrome *c* was performed by adding finely grained succinic anhydride (0.42 mmol) to a vigorously stirred ice-cold solution of ferricytochrome *c* (8 μmol) in 40 ml of potassium phosphate buffer (0.03 M) over a period of 30 min. The pH of the solution was kept at 7.6 by titration with 2 M-KOH. After the reaction was complete the solution was stirred for another 20 min, transferred to a dialysis bag and dialysed against double-distilled water, containing 0.1 mM-EDTA, at 4°C, overnight. The modified cytochrome *c* preparations were concentrated about 3-fold and stored at −20°C. The degree of succinoylation was measured by the 'TNBS-method' (Habeeb, 1966).

Midpoint potentials of native and succinoylated cytochrome *c* were determined by titration of the ferricytochromes *c* with potassium hexacyanoferrate(II) as described by Margalit & Schejter (1973).

The reduction of native and modified ferricytochrome *c* was monitored in an Aminco DW-2 spectrophotometer using the wavelength-pair 550 minus 557 nm with the cuvette compartment maintained at 25°C. The calculations were based on an absorption coefficient of 21 $mM^{-1} \cdot cm^{-1}$ (Van Gelder & Slater, 1962).

Maximal rates (V_M) and half-saturation concentrations of native or succinoylated ferricytochrome *c* ($K_{0.5}$) for the trapping of $O_2^{-\cdot}$ were estimated using a computer program for the direct linear plot by the method of Eisenthal & Cornish-Bowden (1974).

H_2O_2 and protein were measured by the ferrithiocyanate method (Hildebrandt & Roots, 1975) and the biuret method (Gornall *et al.*, 1949) respectively.

Results

Characterization of succinoylated cytochrome c

It was noticed in our earlier experiments that succinoylation of all lysine residues of cytochrome *c* blocked its reduction by NADPH–cytochrome *P*-450 reductase but also completely abolished its reactivity with $O_2^{-\cdot}$. Therefore, horse-heart ferricytochrome *c* was succinoylated only partially under controlled conditions of pH, temperature and ratio of succinic anhydride to cytochrome *c*, such that the reduction by detergent-solubilized cytochrome *P*-450 reductase was lowered by about 99% and the reactivity towards superoxide anion radicals kept as high as possible. The succinoylation resulted in the modification of 44.9 ± 0.8% (five preparations) of the TNBS-reactive groups of the cytochrome *c* molecule. In contrast with what has been reported for excessively succinoylated cytochrome *c* (Ilan *et al.*, 1979) this derivative proved to be spectrally identical with the native protein, especially with the 695 nm band, which is a sensitive indicator of the intactness of the cytochrome, maintained. Furthermore, the derivative did not bind CO and hardly showed autoxidation in the reduced state. Electrophoresis on cellulose acetate strips equilibrated in 0.25 M-citrate buffer of various pH values revealed that the modified cytochrome *c* preparations were virtually homogeneous with an apparent isoelectric point of about 4.3 compared with about 10.8 of native cytochrome *c* (Tint & Reiss, 1950). Thus, the succinoylated cytochrome *c* molecule carries a net negative charge at neutral pH, whereas the native protein is positively charged. Furthermore, the midpoint potential for 0.1 M-phosphate buffer, pH 7.0, at 25°C was determined as 227 mV for the partially succinoylated cytochrome *c* and 261 mV for the native molecule compared with −120 mV for excessively succinoylated cytochrome *c* (Ilan *et al.*, 1979).

Reduction of native and succinoylated ferricytochrome c by $O_2^{-\cdot}$

The xanthine oxidase/xanthine system can be used as a source of $O_2^{-\cdot}$ (McCord & Fridovich, 1968). The trapping by ferricytochrome *c* of the $O_2^{-\cdot}$ generated in this system is adequately described by a hyperbolic relationship of initial rates of $O_2^{-\cdot}$-mediated cytochrome *c* reduction and cytochrome *c* concentrations (see the Appendix, eqn. 11). If plotted in the double-reciprocal manner analogous to the familiar Lineweaver–Burk plot, the intersections with the *y*- and *x*-axis have the co-ordinates (0; $1/V_M$) and ($-1/K_{0.5}$; 0) respectively. As shown in Fig. 1, the reduction by $O_2^{-\cdot}$ of succinoylated ferricytochrome *c* compared with native ferricytochrome *c* is diminished as a consequence of the change in net charge and midpoint

potential. Hence, for the trapping of 50% of the $O_2^{-\bullet}$ produced under steady-state conditions by the xanthine oxidase/xanthine system a higher concentration (i.e. $K_{0.5}$ value) of succinoylated cytochrome *c* is required. However, identical maximal rates of trapping will be obtained by extrapolation to infinitely high concentrations of the cytochrome *c* species. This finding is consistent with our assumption that the succinoylated cytochrome *c* is reduced by the superoxide anion radical in a straightforward second-order reaction as has been demonstrated for the reaction of native cytochrome *c* with $O_2^{-\bullet}$ (Simic *et al.*, 1975; Koppenol *et al.*, 1976).

It can readily be seen from eqn. 14 of the Appendix that in the absence of catalysts for the $O_2^{-\bullet}$ dismutation the ratio of the $K_{0.5}$ values for the trapping with native and modified ferricytochrome *c* equals the inverse ratio of the corresponding second-order rate constants for the reduction by $O_2^{-\bullet}$. The calculated $K_{0.5}$ values derived from the experiment shown in Fig. 1 were 2.1 μM and 23.4 μM respectively. Consequently, the reduction of the succinoylated derivative of cytochrome *c* by $O_2^{-\bullet}$ proceeds slower by a factor of about 10 at pH 7.7. Fortunately, the diminished electron-transfer rate facilitated the determination of initial rates of reduction by $O_2^{-\bullet}$ in dependence on the concentration of the $O_2^{-\bullet}$ scavenger. Moreover, if employed in superoxide dismutase assays a 10-fold increase in sensitivity is gained compared with the classical assay using native ferricytochrome *c* as indicating scavenger (see Misra & Fridovich, 1977).

In full accord with results using native cytochrome *c* as detector of $O_2^{-\bullet}$ (McCord & Fridovich, 1968) a linear relationship between the maximal rates of ferricytochrome *c* reduction by superoxide anion radicals and the xanthine oxidase concentration was established (Fig. 2).

This finding supports our assumption that the maximal rates of cytochrome *c* reduction truly reflect the rates of $O_2^{-\bullet}$ generation in a homogeneous system. Furthermore, the non-linear relationship between the concentration of modified ferricytochrome *c*, necessary to trap 50% of the generated $O_2^{-\bullet}$, and the concentration of xanthine oxidase, confirms previous findings with native ferricytochrome *c* (McCord & Fridovich, 1968). The experimental data are in excellent agreement with the theoretical considerations (see eqn. 14 of the Appendix), which predict that an *n*-fold increase in the rate of $O_2^{-\bullet}$ production will be reflected by an $n^{\frac{1}{2}}$-fold increase in the corresponding $K_{0.5}$ value. This correlation,of course, is valid only in the absence of superoxide dismutase activities or other $O_2^{-\bullet}$-consuming reactions (see the Appendix).

As shown in Fig. 3 the initial rates of reduction of succinoylated ferricytochrome *c* were decreased on addition of 5 nM-SOD but virtually equal V_M values

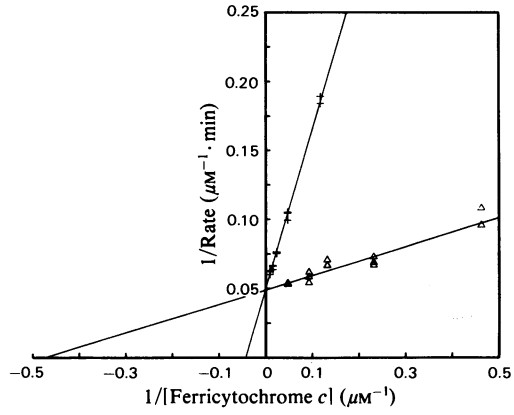

Fig. 1. *Initial rates of reduction of native* (△) *and succinoylated* (+) *ferricytochrome c by* $O_2^{-\bullet}$ *(double-reciprocal plot)*

The reaction mixtures contained 20 μg of xanthine oxidase/ml, 0.2 mM-xanthine, 1200 units of catalase/ml, 0.1 mM-EDTA and the indicated concentrations of native or succinoylated cytochrome *c* buffered at pH 7.7 by 0.1 M-Tris/HCl buffer. All reactions were performed at 25°C in a total volume of 1 ml. For further details see under 'Methods'.

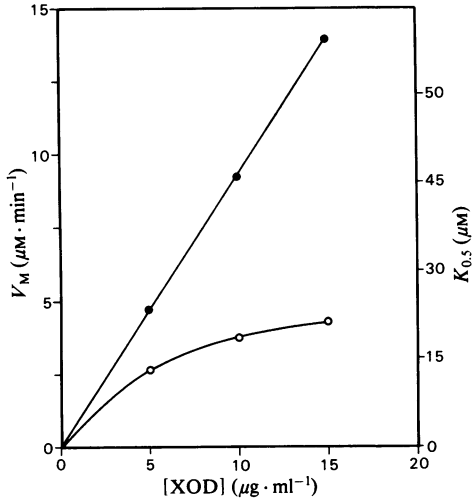

Fig. 2. *Dependence of the maximal rate,* V_M (●), *and the half saturation concentration of succinoylated ferricytochrome c,* $K_{0.5}$ (○), *of* $O_2^{-\bullet}$ *trapping on the concentration of xanthine oxidase (XOD)*

Initial rates of reduction of succinoylated ferricytochrome *c* were measured as a function of the concentration of succinoylated ferricytochrome *c* at the indicated concentrations of xanthine oxidase. Assay conditions were as described in Fig. 1. The individual values of V_M and $K_{0.5}$ were computed by using the direct linear plot by the method of Eisenthal & Cornish-Bowden (1974).

of ferricytochrome *c* reduction were obtained in the presence and absence of SOD. Moreover, the Figure shows that SOD or compounds reacting with superoxide anion radicals by oxidation or reduction act as competitors with respect to the indicating scavenger and thus increase the value of $K_{0.5}$ (see also eqn. 10 of the Appendix).

Enzymic oxidation and reduction of succinoylated cytochrome c

Microsomes prepared by the standard procedure are usually contaminated with components of the mitochondrial respiratory chain, i.e. ferricytochrome *c* reductase and ferrocytochrome *c* oxidase activities (Dallner, 1974).

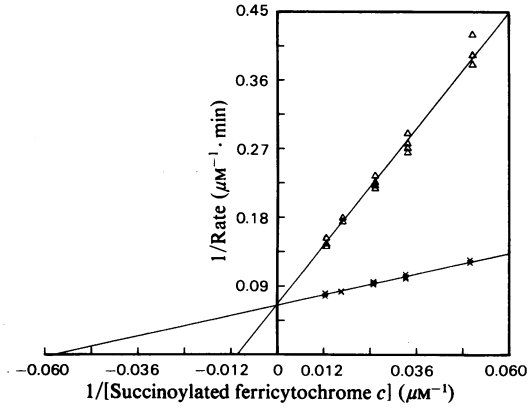

Fig. 3. *Trapping of* $O_2^{-\cdot}$ *by succinoylated ferricytochrome c in the presence* (△) *and the absence* (+) *of SOD*
The reaction mixtures were prepared as described in Fig. 1. SOD was present at a concentration of 5 nM. The lines were fitted to the corresponding data sets as outlined under 'Methods'.

Fig. 4 shows the reduction and subsequent reoxidation of native and partially succinoylated cytochrome *c* in the presence of microsomes and limiting amounts of NADPH.

Native ferricytochrome *c* is efficiently reduced by NADPH–cytochrome *P*-450 reductase and reoxidized in a fast reaction. The complete inhibition by potassium cyanide of the reoxidation points to the involvement of cytochrome *c* oxidase. In contrast, succinoylated ferricytochrome *c* was reduced at a rate about two orders of magnitude lower and displayed negligible reoxidation under the experimental conditions. A fast reoxidation process could be initiated by the addition of catalytic amounts of native ferricytochrome *c*. Probably succinoylated ferrocytochrome *c* is able to reduce native ferricytochrome *c*, which is then re-oxidized by cytochrome *c* oxidase.

These findings are consistent with previous reports that partial succinoylation or acetylation of cytochrome *c* drastically reduce its capability to mediate the electron flow of the respiratory chain (Takemori *et al.*, 1962; Wada & Okunuki, 1968).

Finally, cytochrome b_5, an intrinsic haemoprotein of the endoplasmic reticulum, lost 99% of its capacity to mediate the reduction of succinoylated ferricytochrome *c* by NADH and NADH–cytochrome b_5 reductase. This is of importance, since cytochrome b_5 is also reduced by NADPH–cytochrome *P*-450 reductase in NADPH-supplemented liver microsomes (Cohen & Estabrook, 1971).

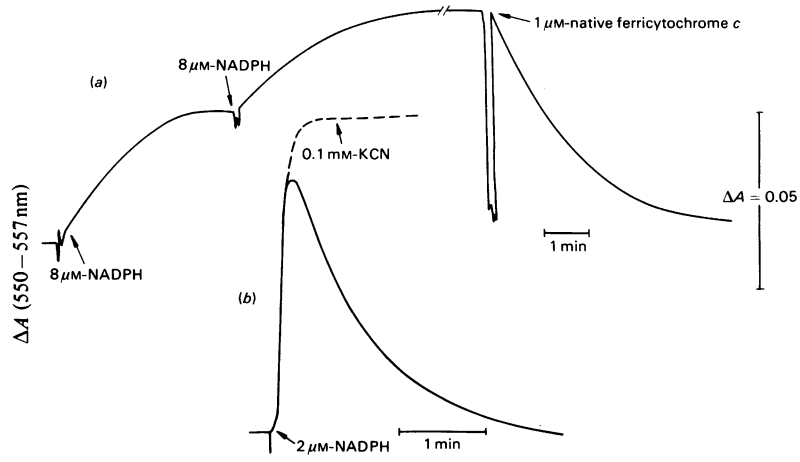

Fig. 4. *Reduction and reoxidation of native* (*b*) *and succinoylated* (*a*) *cytochrome c by liver microsomes*
Rat liver microsomes were diluted to a concentration of 0.3 mg/ml in 50 mM-potassium phosphate buffer, pH 7.6, containing 0.1 mM-EDTA, 10.7 μM-native ferricytochrome *c* and 8 μM-succinoylated ferricytochrome *c* respectively. The reactions were initiated by adding NADPH of the indicated final concentrations.

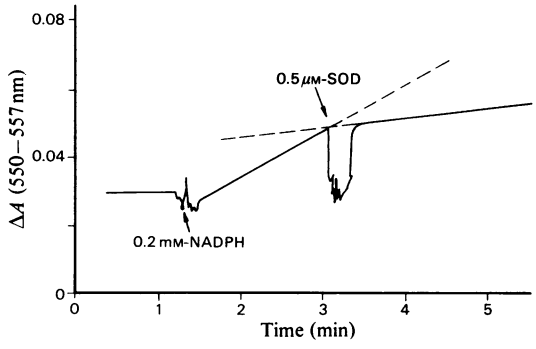

Fig. 5. *Detection of $O_2^{-\bullet}$ by succinoylated cytochrome c in NADPH-supplemented liver microsomes*

Rat liver microsomes were diluted to a final concentration of 0.4 mg/ml in 0.1 M-Tris/HCl buffer, pH 7.7, containing 0.1 mM-EDTA and 30.3 μM-succinoylated ferricytochrome *c*. NADPH and SOD were added as indicated. The reactions were performed at 25°C in semi-microcuvettes of 10 mm light path.

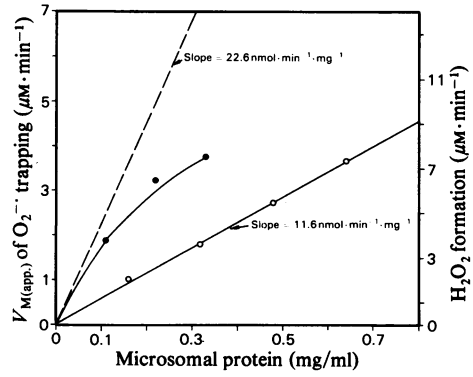

Fig. 6. *Apparent maximal rates of $O_2^{-\bullet}$-mediated reduction of partially succinoylated ferricytochrome c (●) and H_2O_2 formation (○) at various concentrations of liver microsomes*

●, Initial rates of $O_2^{-\bullet}$-mediated reduction of succinoylated ferricytochrome *c* at various concentrations of the modified ferricytochrome *c* and the indicated concentration of microsomes. Maximal rates were computed by the method of Eisenthal & Cornish-Bowden (1974). The curve and the slope of the tangent at its origin were computed by assuming a hyperbolic relationship for the dependence of the maximal rates of $O_2^{-\bullet}$ trapping on the microsomal protein concentration. ○, results for an assay mixture as described above, except that an NADPH-regenerating system (3.75 mM-isocitrate, 0.2 units of isocitrate dehydrogenase/ml) was present instead of succinoylated cytochrome *c* and EDTA. All reactions were initiated by addition of 0.2 mM-NADPH at 25°C.

As a consequence of the low residual activities of the NADPH–cytochrome *P*-450 reductase and of cytochrome b_5 towards the modified cytochrome *c* only minimal interference should be expected with the cytochrome *P*-450-supported mono-oxygenation reactions in the presence of succinoylated ferricytochrome *c*. In fact, the *N*-demethylation of (+)-benzphethamine was virtually uninhibited by 25 μM-succinoylated cytochrome *c* or by 0.3 μM-SOD.

Obviously, neither the modified ferricytochrome *c* nor SOD (Strobel & Coon, 1971) interfere with the formation of the activated oxygen complexes of microsomal cytochrome *P*-450.

Detection of $O_2^{-\bullet}$ in rat liver microsomes

Liver microsomes generate H_2O_2 in the presence of NADPH and O_2 (Gillette *et al.*, 1957; Thurman *et al.*, 1972; Hildebrandt & Roots, 1975). The formation of superoxide anion radicals as precursors of H_2O_2 has been proposed (Werringloer, 1977; Kuthan *et al.*, 1978; Estabrook *et al.*, 1979). To detect $O_2^{-\bullet}$, microsomes were incubated with NADPH and succinoylated cytochrome *c* (Fig. 5).

The addition of an excess of SOD allowed us to differentiate between $O_2^{-\bullet}$-mediated and direct enzymic reduction. Apparent maximal rates of $O_2^{-\bullet}$ generation could be determined by the procedure outlined above. Fig. 6 shows the extrapolated rates of $O_2^{-\bullet}$-dependent reduction of modified cytochrome *c* as a function of the microsomal protein concentration.

In contrast with the findings with the xanthine oxidase/xanthine system as the source of $O_2^{-\bullet}$, a non-linear relationship was found. This deviation from linearity still requires explanation, but it is not due to changes in the absorbance of the cytochrome *c* derivative on addition of increasing amounts of microsomal protein. Furthermore, the formation of H_2O_2 is linearly dependent on the concentration of microsomes (Fig. 6). Compounds known to stimulate cytochrome *P*-450-dependent H_2O_2 formation in liver microsomes also enhanced the rate of $O_2^{-\bullet}$ production as judged from the apparent maximal rate of $O_2^{-\bullet}$-dependent reduction of succinoylated cytochrome *c* (Table 1).

Since the apparent maximal rates of $O_2^{-\bullet}$ trapping are dependent on the protein concentration, the stoichiometry of $O_2^{-\bullet}$ to H_2O_2 formation is also a function of protein concentration. A stoichiometry of $O_2^{-\bullet}$ to H_2O_2 formation approaching 2:1 can be calculated by using specific maximal rates of $O_2^{-\bullet}$ trapping determined at low protein concentration (Fig. 6).

Table 1. *Apparent maximal rates of $O_2^{-\cdot}$ trapping by succinoylated cytochrome c and H_2O_2 formation in rat liver microsomes*

Rat liver microsomes (0.35 mg/ml and 1 mg/ml for $O_2^{-\cdot}$ and H_2O_2 determination respectively) were incubated at 25°C in 0.1 M-Tris/HCl buffer, pH 7.7, containing 0.4 mM-NADPH, 1 mM-sodium azide, 2 mM-AMP and 1 mM substrate as indicated. H_2O_2 was determined by the ferrithiocyanate method.

Substrate added	Maximal rate of $O_2^{-\cdot}$ trapping (nmol/min per mg of protein)	H_2O_2 generated (nmol/min per mg of protein)
—	9.6	10.9
Hexobarbitone	17.6	21.3
Coumarin	19.2	22.3

Discussion

Partially succinoylated ferricytochrome *c* was used for the detection and quantification of $O_2^{-\cdot}$ radicals generated by the xanthine oxidase/xanthine system and by liver microsomes incubated with NADPH. Quantification of $O_2^{-\cdot}$ radicals produced in these systems was accomplished by measuring the initial rate of $O_2^{-\cdot}$-mediated reduction of succinoylated ferricytochrome *c* at various concentrations of the cytochrome *c* derivative. Thus, maximal rates of $O_2^{-\cdot}$ trapping could be obtained by extrapolation of rate values obtained at finite concentrations of partially succinoylated ferricytochrome *c*. This procedure seems to be fairly general; neither the cytochrome *c* derivative nor the exogenous superoxide dismutase, necessary for the differentiation of $O_2^{-\cdot}$-mediated and direct enzymic reduction of the succinoylated cytochrome *c*, interfere with the investigated source of superoxide anion radicals.

With NADPH-supplemented liver microsomes only apparent maximal rates of $O_2^{-\cdot}$ trapping were obtained, as judged from the non-linear dependence of the extrapolated rates on the protein concentration (Fig. 6). However, the apparent maximal rates of reduction of succinoylated ferricytochrome *c* by $O_2^{-\cdot}$ radicals were found to reflect adequately changes in the rate of generation of $O_2^{-\cdot}$ by microsomes as judged from the corresponding rates of H_2O_2 formation (Table 1).

Since both succinoylated ferricytochrome *c* and exogenous SOD are likely to be unable to penetrate the microsomal membrane, they could not scavenge $O_2^{-\cdot}$ radicals in the immediate vicinity of the major $O_2^{-\cdot}$ source, i.e. cytochrome *P*-450 (Ullrich & Kuthan, 1980), embedded in the membrane.

As a consequence, perhydroxyl radicals, possibly released within the membrane by autoxidation of the oxygenated ferrous complex of cytochrome *P*-450, could react with unsaturated fatty acids (Lynch & Fridovich, 1978) or thiol groups (Asada & Kanamatsu, 1976) without being competitively quenched by the exogenous scavengers. Furthermore, superoxide anion radicals may partially be released into the lumen of the microsomal vesicles inaccessible to cytochrome *c*. Nonetheless, a ratio close to 2:1 for $O_2^{-\cdot}$ and H_2O_2 generation could be calculated by extrapolating to a microsomal protein concentration of zero, whereas lower ratios were found at higher protein concentrations (Fig. 6). The stoichiometry of about 2:1 for $O_2^{-\cdot}$ to H_2O_2 production confirms our former results with xanthine oxidase/xanthine standards for the calibration of $O_2^{-\cdot}$ generated by the reconstituted (Kuthan *et al.*, 1978) and microsomal (Ullrich & Kuthan, 1980) cytochrome *P*-450 system.

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References

Asada, K. & Kanamatsu, S. (1976) *Agric. Biol. Chem.* **40**, 1891–1897

Asada, K., Kiso, K. & Yoshikawa, K. (1974) *J. Biol. Chem.* **249**, 2175–2181

Azzi, A., Montecucco, C. & Richter, C. (1975) *Biochem. Biophys. Res. Commun.* **65**, 597–603

Bartoli, G. M., Galeotti, T., Palombini, G., Parisi, G. & Azzi, A. (1977) *Arch. Biochem. Biophys.* **184**, 276–281

Berman, M. C., Adams, C. M., Ivanetich, K. M. & Kench, J. E. (1976) *Biochem. J.* **157**, 237–246

Bors, W., Saran, M., Michel, C., Lengfelder, E., Fuchs, C. & Spöttl, K. (1975) *Int. J. Radiat. Biol.* **28**, 353–371

Bors, W., Saran, M., Lengfelder, E., Michel, C., Fuchs, C. & Frenzel, C. (1978) *Photochem. Photobiol.* **28**, 629–638

Brunori, M., Falcioni, G., Fioretti, E., Giordino, B. & Rotilio, G. (1975) *Eur. J. Biochem.* **53**, 99–104

Cadenas, E., Boveris, A., Ragan, C. I. & Stoppani, A. O. M. (1977) *Arch. Biochem. Biophys.* **180**, 248–257

Cohen, B. S. & Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143**, 37–53

Dallner, G. (1974) *Methods Enzymol.* **31A**, 191–201

Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720

Estabrook, R. W., Kawano, S., Werringloer, J., Kuthan, H., Tsuji, H., Graf, H. & Ullrich, V. (1979) *Acta Biol. Med. Ger.* **38**, 423–434

Gillette, J. R., Brodie, B. B. & LaDu, B. N. (1957) *J. Pharmacol. Exp. Ther.* **119**, 532–540

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–762

Habeeb, A. F. S. A. (1966) *Anal. Biochem.* **14**, 328–336

Hildebrandt, A. G. & Roots, J. (1975) *Arch. Biochem. Biophys.* **171**, 385–397

Ilan, Y., Shafferman, A., Feinberg, B. A. & Lau, Y.-K. (1979) *Biochim. Biophys. Acta* **548**, 565–578

Koppenol, W. H., Van Buuren, K. J. H., Butler, J. & Braams, R. (1976) *Biochim. Biophys. Acta* **449**, 157–168

Kuthan, H., Tsuji, H., Graf, H., Ullrich, V., Werringloer, J. & Estabrook, R. W. (1978) *FEBS Lett.* **91**, 343–345
Lynch, R. E. & Fridovich, I. (1978) *J. Biol. Chem.* **253**, 1836–1845
Margalit, R. & Schejter, A. (1973) *Eur. J. Biochem.* **32**, 492–499
McCord, J. M. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M. & Fridovich, I., eds.), pp. 11–17, Academic Press, London, New York and San Francisco
McCord, J. M. & Fridovich, I. (1968) *J. Biol. Chem.* **243**, 5753–5760
McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **244**, 3170–3175
Misra, H. P. & Fridovich, I. (1977) *Anal. Biochem.* **79**, 553–560
Prough, R. A. & Masters, B. S. S. (1973) *Ann. N.Y. Acad. Sci.* **212**, 89–93
Simic, M. G., Traub, I. A., Tocci, J. & Hurwitz, P. A. (1975) *Biochem. Biophys. Res. Commun.* **62**, 161–167
Strobel, H. W. & Coon, M. J. (1971) *J. Biol. Chem.* **246**, 7826–7829
Takemori, S., Wada, K., Ando, K., Hosokawa, M., Sekuzu, J. & Okunuki, K. (1962) *J. Biochem. (Tokyo)* **52**, 28–37
Thurman, R. G., Ley, H. G. & Scholz, R. (1972) *Eur. J. Biochem.* **25**, 420–430
Tint, H. & Reiss, W. (1950) *J. Biol. Chem.* **182**, 385–396
Ullrich, V. & Kuthan, H. (1980) in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Gustafsson, J.-A., Carlstedt-Duke, J., Mode, A. & Rafter, J., eds.), pp. 267–272, Elsevier, Amsterdam
Van Gelder, B. F. & Slater, E. C. (1962) *Biochim. Biophys. Acta* **58**, 593–595
Wada, K. & Okunuki, K. (1968) *J. Biochem. (Tokyo)* **64**, 667–681
Werringloer, J. (1977) in *Microsomes and Drug Oxidations* (Ullrich, V., Roots, J., Hildebrandt, A. G., Estabrook, R. W. & Conney, A. H., eds.), pp. 261–268, Pergamon Press, Oxford
Werringloer, J., Kawano, S. & Estabrook, R. W. (1979) *Acta Biol. Med. Ger.* **38**, 163–175

APPENDIX

Trapping of $O_2^{-\cdot}$ by ferricytochrome *c*

Superoxide anion radicals produced by an appropriate source (e.g. oxidoreductase) react spontaneously with their conjugated acid, i.e. the perhydroxyl radical, with a fast second-order decay rate (Bielski & Allen, 1977):

$$HO_2^{\cdot} + O_2^{-\cdot} \xrightarrow[H_2O]{k_1} O_2 + H_2O_2 + OH^- \quad (1)$$

In the presence of SOD an additional decay occurs, which is first-order with regard to $O_2^{-\cdot}$ and [SOD] (Fielden *et al.*, 1974).

$$2O_2^- \xrightarrow[2H_2O]{SOD} O_2 + H_2O_2 + 2OH^- \quad (2)$$

Alternatively, superoxide anion radicals can reduce native (Koppenol *et al.*, 1976) or modified ferricytochrome *c* (cyt^{3+}) by univalent electron transfer:

$$O_2^{-\cdot} + cyt^{3+} \xrightarrow{k_3} cyt^{2+} + O_2 \quad (3)$$

Under defined conditions (pH, ionic strength, temperature etc.) reactions (1)–(3) can be described by the following differential equations:

$$v_1 = -\frac{d[O_2^{-\cdot}]}{dt} = k_1[HO_2^{\cdot}][O_2^{-\cdot}] = k_1'[O_2^{-\cdot}]^2 \quad (4)$$

$$v_2 = -\frac{d[O_2^{-\cdot}]}{dt} = k_2[SOD][O_2^{-\cdot}] \quad (5)$$

$$v_3 = -\frac{d[O_2^{-\cdot}]}{dt} = k_3[cyt^{3+}][O_2^{-\cdot}] \quad (6)$$

Under steady state conditions the sum of the rates of dismutation ($v_1 + v_2$) and of trapping (v_3) balance the rate of $O_2^{-\cdot}$ generation (V_M):

$$V_M = v_1 + v_2 + v_3 \quad \text{or} \quad V_M - v_3 = v_1 + v_2 \quad (7)$$

Division of the sum of eqns. (4) and (5) by eqn. (6) leads to:

$$\frac{v_1+v_2}{v_3}=\frac{k'_1[O_2^{-\bullet}]^2+k_2[O_2^{-\bullet}][SOD]}{k_3[cyt^{3+}][O_2^{-\bullet}]}=\frac{k'_1[O_2^{-\bullet}]+k_2[SOD]}{k_3[cyt^{3+}]} \tag{8}$$

Multiplication of eqn. (8) by $v_3[cyt^{3+}]$ yields:

$$(v_1+v_2)[cyt^{3+}]=\{k'_1[O_2^{-\bullet}]+k_2[SOD]\}(v_3/k_3) \tag{9}$$

Substitution of (v_1+v_2) by eqn. (7) and subsequent transformation result in:

$$(V_M-v_3)[cyt^{3+}]=\{k'_1[O_2^{-\bullet}]+k_2[SOD]\}(v_3/k_3)$$

$$V_M[cyt^{3+}]=\{k'_1[O_2^{-\bullet}]+k_2[SOD]\}(v_3/k_3)+v_3[cyt^{3+}]$$

$$v_3=\frac{V_M[cyt^{3+}]}{(k'_1/k_3)[O_2^{-\bullet}]+(k_2/k_3)[SOD]+[cyt^{3+}]} \tag{10}$$

In the absence of SOD, eqn. (10) is simplified to:

$$v_3=\frac{V_M[cyt^{3+}]}{(k'_1/k_3)[O_2^{-\bullet}]+[cyt^{3+}]} \tag{11}$$

Eqn. (11) defines a rectangular hyperbola through the origin formally identical with the Michaelis–Menten equation. The formal analogy is maintained if, in addition to the spontaneous decay of $O_2^{-\bullet}$, metals or superoxide dismutases (eqn. 10) catalyse the disproportionation. By analogy with the K_m value, a parameter ($K_{0.5}$) can be defined as follows:

$$K_{0.5}=[cyt^{3+}]_{0.5}=(k'_1/k_3)[O_2^{-\bullet}]_{0.5}+(k_2/k_3)[SOD]$$

or if SOD is absent:

$$K_{0.5}=[cyt^{3+}]_{0.5}=(k'_1/k_3)[O_2^{-\bullet}]_{0.5} \tag{12}$$

According to eqn. (12), $K_{0.5}$ represents the concentration of ferricytochrome *c* that traps 50% of the generated $O_2^{-\bullet}$ under steady-state conditions.

Thus:

$$v_{3(0.5)}=k_3[O_2^{-\bullet}]_{0.5}[cyt^{3+}]_{0.5}=k_3[O_2^{-\bullet}]_{0.5}K_{0.5}=V_M/2 \tag{13}$$

Combination of eqns. (12) and (13) and rearrangement yields:

$$K_{0.5}=(k'_1/2k_3^2)(V_M)^{\frac{1}{2}} \tag{14}$$

References

Bielski, B. H. J. & Allen, A. O. (1977) *J. Phys. Chem.* **81**, 1048–1050

Fielden, E., Roberts, P. B., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G. & Calabrese, L. (1974) *Biochem. J.* **139**, 49–60

Koppenol, W. H., Van Buuren, K. J. H., Butler, J. & Braams, R. (1976) *Biochim. Biophys. Acta* **449**, 157–168