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Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria

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Submitochondrial particles from bovine heart in which NADH dehydrogenase is reduced by either addition of NADH and rotenone or by reversed electron transfer generate 0.9 ± 0.1 nmol of O_2^-/min per mg of protein at pH 7.4 and at 30°C. When NADH is used as substrate, rotenone, antimycin and cyanide increase O_2^- production. In NADH- and antimycin-supplemented submitochondrial particles, rotenone has a biphasic effect: it increases O_2^- production at the NADH dehydrogenase and it inhibits O_2^- production at the ubiquinone–cytochrome *b* site. The generation of O_2^- by the NADH dehydrogenase supported by reversed electron transfer was inhibited by rotenone, the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and oligomycin at concentrations similar to those required to inhibit energy-dependent succinate–NAD reductase. Cyanide did not affect O_2^- generation at the NADH dehydrogenase, but inhibited O_2^- production at the ubiquinone–cytochrome *b* site. Production of O_2^- at the NADH dehydrogenase is about 50% of the O_2^- generation at the ubiquinone–cytochrome *b* area at pH 7.4. Additivity of the two mitochondrial sites of O_2^- generation was observed over the pH range from 7.0 to 8.8. An O_2^--dependent autocatalytic process that requires NADH, submitochondrial particles and adrenaline is described.

Isolated mitochondria in State 4 (Chance & Williams, 1956) generate 0.6–1.0 nmol of H_2O_2/min per mg of protein (Loschen *et al.*, 1971; Boveris *et al.*, 1972; Boveris & Chance, 1973), which is estimated to account for about 2% of the oxygen uptake under physiological conditions (Chance *et al.*, 1973, 1979). The effect of specific mitochondrial inhibitors on H_2O_2 production, i.e. the enhancement by antimycin with both succinate and NAD-linked substrates and the inhibition by rotenone with NAD-linked substrates (Loschen *et al.*, 1971; Boveris & Chance, 1973), indicates a reduced member of the respiratory chain located between the rotenone- and antimycin-sensitive sites as the main autoxidizable species generating H_2O_2 in a side reaction to the main electron flow.

Production of O_2^- by mitochondrial membranes in the presence of succinate and antimycin was first reported by Loschen *et al.* (1971); thereafter, Boveris & Cadenas (1975) and Dionisi *et al.* (1975) showed that, under the same conditions, O_2^- can be considered as a stoichiometric precursor of mitochondrial H_2O_2, after determining ratios of O_2^-/H_2O_2 generation rates close to 2.0. Subsequently, Boveris *et al.* (1976) and Cadenas *et al.* (1977) proposed ubisemiquinone as the main univalent reductant of oxygen in mitochondrial membranes; this was reaffirmed after considering the production of O_2^- by isolated succinate–cytochrome *c* reductase (Trumpower & Simmons, 1979) and the effect of protophores and ionophores on H_2O_2 production by coupled mitochondria (Cadenas & Boveris, 1980).

In a study aimed at locating the NADH dehydrogenase in relation to the first phosphorylation site, Hinkle *et al.* (1967) (cf. Racker, 1976) reported the generation of H_2O_2 by the autoxidation of the NADH dehydrogenase flavoprotein. More recently it has been found that the NADH dehydrogenase of submitochondrial particles prepared from potato (*Solanum tuberosum*) (Rich & Bonner, 1978) and from bovine heart (Takeshige & Minakami, 1979) is a source of O_2^-.

In the present paper we offer more evidence of the NADH dehydrogenase-supported production of O_2^- in mitochondrial membranes. Preliminary results have been reported elsewhere (Boveris *et al.*, 1979).

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Materials and methods

Bovine heart submitochondrial particles

Bovine heart mitochondria were isolated by the procedure of Blair (1967) as described by Boveris *et al.* (1976). Mitochondria were suspended in 250mM-sucrose/1mM-EDTA/5mM-Tris/HCl buffer, pH 7.4, and frozen. Submitochondrial particles were prepared by sonicating the thawed mitochondria (20mg of protein/ml) in 250mM-sucrose/15mM-$MgCl_2$/5mM-succinate/40mM-Tris/HCl buffer, pH 7.5, twice for 20s with a 2min interval in a model W185 sonifier cell disruptor (Heat Systems Ultrasonic, Plainview, NY, U.S.A.). Submitochondrial particles were twice washed and suspended in 230mM-mannitol/70mM-sucrose/1mM-EDTA/5mM-Tris/HCl buffer, pH 7.4. All the operations were performed at 0–4°C. Protein determinations were made by the biuret method (Gornall *et al.*, 1949) in the presence of 0.1% sodium deoxycholate.

Determination of enzymic activities

Production of O_2^- was measured by the superoxide dismutase-sensitive oxidation of adrenaline to adrenochrome (Misra & Fridovich, 1972) and the superoxide dismutase-sensitive reduction of acetylated ferricytochrome *c* (Azzi *et al.*, 1975). Adrenochrome formation and cytochrome *c* reduction were monitored in an Aminco–Chance double-beam spectrophotometer (American Instruments Co., Silver Springs, MD, U.S.A.) determining $\varepsilon_{485-575}$ ($\Delta\varepsilon = 2.96$ litre·mmol^{-1}·cm; Green *et al.*, 1956) and $\varepsilon_{550-540}$ ($\Delta\varepsilon = 19$ litre·mmol^{-1}·cm; Azzi *et al.*, 1975). The reaction medium consisted of 230mM-mannitol/70mM-sucrose/30mM-Tris/HCl buffer, pH 7.4. For the pH profile, mannitol/sucrose/Tris solutions of the indicated concentrations were adjusted to the final pH value by addition of HCl.

Energy-dependent succinate–NAD reductase was measured at 340nm ($\varepsilon = 6.2$ litre·mmol^{-1}·cm) in a Gilford model 2000 single-beam spectrophotometer (Gilford Instruments Laboratories, Oberlin, OH, U.S.A.). The reaction medium consisted of 230mM-mannitol/70mM-sucrose/1mM-ATP/3mM-$MgCl_2$/7mM-succinate/1mM-NAD/30mM-Tris/HCl buffer, pH 7.4.

All the enzymic assays were carried out at 30°C.

Preparation of Mn-containing superoxide dismutase

Mn-containing superoxide dismutase was isolated from rat liver mitochondria as described by Salin *et al.* (1978), up to stage 4 [90% $(NH_4)_2SO_4$]. The preparation had an activity of 200 units (McCord & Fridovich, 1969)/mg of protein.

Chemicals

Adrenaline bitartrate, antimycin, ATP, catalase C-100, cytochrome *c* (type VI), NAD, NADH, oligomycin, rotenone and superoxide dismutase [the (Cu + Zn)-containing enzyme] were purchased from Sigma Chemical Co. (Saint Louis, MO, U.S.A.). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was provided by Dr. P. G. Heytler (E.I. du Pont de Nemours Co., Wilmington, DE, U.S.A.). Other reagents were of Analytical grade.

Results

NADH-supported production of superoxide anion

Addition of NADH to submitochondrial particles supplemented with adrenaline and respiratory inhibitors such as rotenone, antimycin, and cyanide brought about adrenochrome formation at rates of 0.4–1.8nmol/min per mg of protein over the first 4–5min after the reaction started [Fig. 1, traces

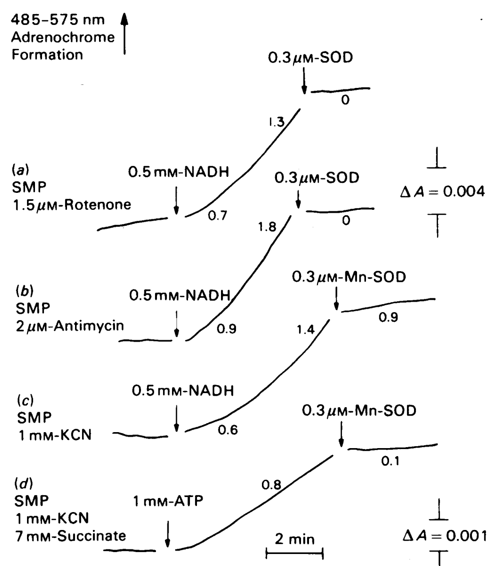


Fig. 1. *Production of superoxide anion by submitochondrial particles*

Reaction mixture was as described in the Materials and methods section for the measurement of O_2^- production [traces (*a*), (*b*) and (*c*)]; 0.2µM-catalase haem and 1mM-adrenaline were added. Bovine heart submitochondrial particles equivalent to 0.72mg of protein/ml were used for traces (*a*), (*b*) and (*c*) and equivalent to 0.28mg of protein/ml were used for trace (*d*). For trace (*d*) the experimental conditions were as described in the Materials and methods section for energy-dependent succinate–NAD reductase. Numbers adjacent to the traces indicate nmol of O_2^-/min per mg of protein. Abbreviations used: SMP, submitochondrial particles; SOD, superoxide dismutase (unless otherwise specified, it refers to the copper–zinc enzyme; Mn-SOD means the manganese-containing enzyme).

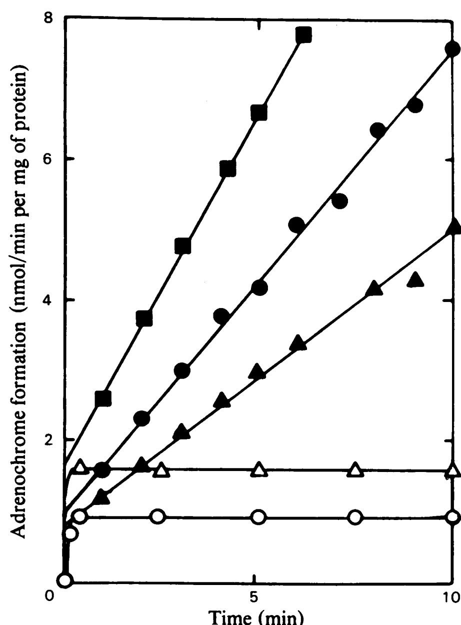


Fig. 2. *Rates of adrenochrome formation by submitochondrial particles*

Experimental conditions were as described in the legend to Fig. 1. Bovine heart submitochondrial particles equivalent to 0.55mg of protein/ml were used. ●, 0.5mM-NADH and 1.5μM-rotenone, no catalase was added; ▲, 0.5mM-NADH, 1.5μM-rotenone and 0.2μM-catalase haem; ■, same as ●, except that 0.82mg of protein/ml were used; △, 7mM-succinate and 1.5μM-antimycin; and ○, adrenochrome formation by reversed electron transfer; experimental conditions were as for trace (*d*) in Fig. 1, 0.62mg of protein/ml were used.

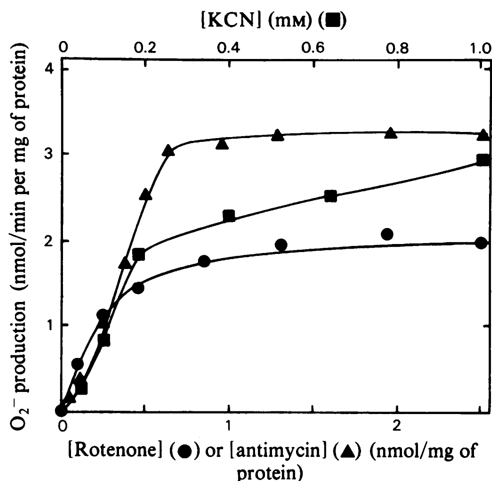


Fig. 3. *Effect of respiratory inhibitors on superoxide-anion production*

Experimental conditions were as described in the legend to Fig. 1 [traces (*a*), (*b*) and (*c*)]. Bovine heart submitochondrial particles equivalent to 0.60mg of protein/ml were used.

(*a*), (*b*) and (*c*)]. Superoxide dismutase inhibited adrenochrome formation, indicating O_2^- involvement; the Mn-enzyme was used when cyanide was present in the reaction mixture and the (Cu+Zn)-enzyme was utilized in the other cases. The rates of adrenochrome formation increased as the reaction proceeded during the approx. 10min monitoring time (Figs. 1 and 2). This increase was sensitive to catalase (Fig. 2), thus identifying a participation of H_2O_2 in the autocatalytic reaction. Protein concentration was also found to have a role in the autocatalytic process; higher contents of submitochondrial particles in the reaction mixture gave steeper slopes in the plots of specific activities versus time (Fig. 2). However, the rate of adrenochrome formation kept constant and linear with protein concentration when O_2^- formation was supported by succinate in antimycin-supplemented membranes and when reversed electron transfer was utilized to reduce oxygen to O_2^- (Fig. 2). Consequently, when NADH was present in the reaction mixture, initial rates (at about 1min, not different from the values obtained from zero-time extrapolation of the plots of adenochrome-formation rates versus time) were considered to represent true rates of O_2^- generation.

The amounts of rotenone and antimycin required to produce a maximal effect on O_2^- production (0.36 and 0.60nmol/mg of protein respectively; Fig. 3) indicate that their action was exerted through an inhibition of the main electron-transfer system. The rates of O_2^- generation were higher in the presence of antimycin than in the presence of rotenone (Fig. 3). The profile of cyanide effect was biphasic; more than one site was involved in cyanide action and residual (Cu+Zn)-superoxide dismutase trapped inside the submitochondrial vesicles is probably one of the targets.

The effect of rotenone on O_2^- production supported by NADH in antimycin-supplemented mitochondrial membranes was tested by using the acetylated cytochrome *c* assay (Azzi *et al.*, 1975). This assay was found to be less influenced by the autocatalytic reaction already described than was the adrenochrome method. However, it cannot be used in the presence of cyanide, since submitochondrial particles react with cytochrome *c* very rapidly. The effect of rotenone was biphasic: we observed a slight increase in O_2^- production below 0.3nmol of rotenone/mg of protein and a clear inhibition at higher concentrations (Fig. 4). A similar biphasic effect of rotenone was described in pigeon

heart mitochondria in the presence of antimycin, when assayed for H_2O_2 generation supported by NAD-linked substrates (Boveris & Chance, 1973).

Generation of superoxide anion supported by reversed electron flow

Submitochondrial particles in the presence of cyanide, succinate, and adrenaline showed, upon addition of ATP, adrenochrome formation at a rate of 0.8nmol/min per mg of protein, which was inhibitable by Mn-superoxide dismutase [Fig. 1, trace (*d*)]. An initial lag phase of about 30s, presumably due to membrane energization, was followed by a linear rate of adrenochrome (O_2^-) formation (cf. Fig. 2). No autocatalytic reaction was observed; no NADH was present in the reaction mixture. The ATP-dependent production of O_2^- was inhibited by rotenone, the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and oligomycin, with titres similar to those required to inhibit the energy-dependent succinate–NAD reductase (rotenone, 0.28 and 0.20nmol/mg of protein; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 0.11 and 0.22μM; and oligomycin, 0.77 and 0.86nmol/mg of protein respectively; Figs. 5*a*, 5*b* and 5*c*).

Effect of cyanide on the generation of superoxide anion

Cyanide had markedly different effects on O_2^- production supported by either NADH and rotenone or succinate and antimycin; a slight stimulation was observed in the former case and a marked inhibition in the latter one (Fig. 6). The slight increase observed in the presence of NADH and rotenone seems to indicate both a basic insensitivity of the system to cyanide and a small contamination by the cyanide-sensitive (Cu + Zn)-superoxide dismutase. On the contrary, cyanide inhibited the production of O_2^- up to 98% in the presence of

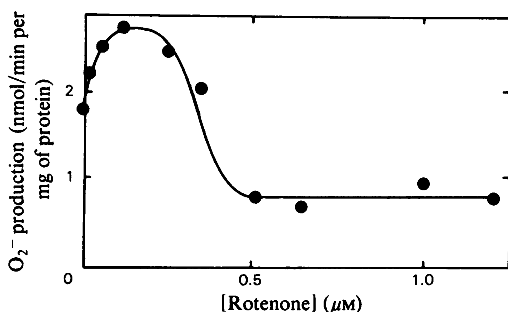


Fig. 4. *Effect of rotenone on superoxide-anion production by antimycin-supplemented bovine heart submitochondrial particles*

Experimental conditions were as described in the legend to Fig. 3. NADH (0.5mM) and catalase haem (0.2μM) were added. Acetylated ferricytochrome *c* (10μM) was used to measure O_2^- production.

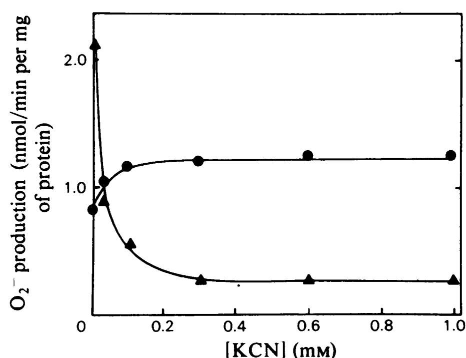


Fig. 6. *Effect of cyanide on superoxide-anion production by submitochondrial particles*

▲, 7mM-succinate and 2μM-antimycin; ●, 0.5mM-NADH and 2μM-rotenone. Other experimental conditions were as described in the legend to Fig. 1.

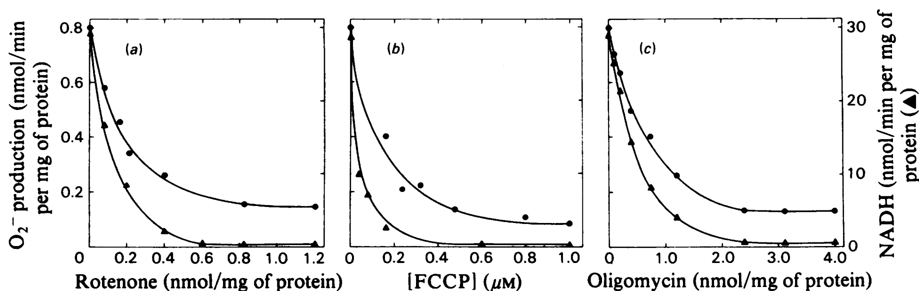


Fig. 5. *Effect of rotenone, carbonyl cyanide-p-fluoromethoxyphenylhydrazone (FCCP) and oligomycin on superoxide-anion production by reversed electron transfer*

Experimental conditions were as described in the legend to Fig. 1 [trace (*d*)].

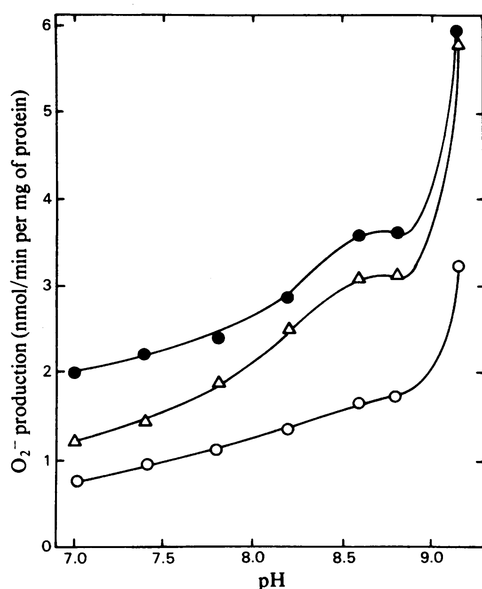


Fig. 7. *pH-dependence of the production of superoxide anion in bovine heart submitochondrial particles*

Experimental conditions were as described in the Materials and methods section. Acetylated ferricytochrome *c* (10μM) was used to measure O_2^- production. ●, 0.3mM-NADH and 2μM-antimycin; △, 7mM-succinate and 2μM-antimycin; and ○, 0.3mM-NADH and 2μM-rotenone. Submitochondrial particles equivalent to 0.2–0.8mg of protein/ml were used.

succinate and antimycin, with a half-maximal effect at about 30μM (Cadenas & Boveris, 1980). We took advantage of this effect of cyanide to assay O_2^- production by the NADH dehydrogenase reduced by reversed electron transfer and minimized background succinate-supported production of O_2^- (see Fig. 1*d*).

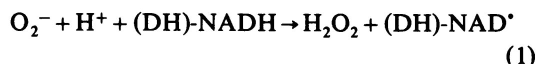
pH-dependence

In order to assay the pH-dependence of O_2^- production by submitochondrial particles, we used acetylated cytochrome *c* assay to avoid the adrenaline autoxidation that takes place at alkaline pH values (Misra & Fridovich, 1972; Bors *et al.*, 1978). Increasing rates of O_2^- production were observed upon increasing pH values over the whole range assayed (Fig. 7). The antimycin-supplemented mitochondrial membranes with NADH or succinate as substrate showed a second maximum at pH 8.6–8.8, which seems close to the optimal pH values previously reported, about 8.0–8.2 (Loschen *et al.*, 1971; Boveris & Cadenas, 1975). In the presence of NADH and rotenone, the rise in activity at pH 8.6–8.8 was not found, in agreement with the findings of Takeshige & Minakami (1979). Production of O_2^- in the presence of NADH and antimycin is close to the sum of the O_2^- production in the presence of NADH and rotenone plus the one supported by succinate in the presence of antimycin, in the pH range from 7.0 to 8.8 (Fig. 7). Since the rates of production of both O_2^- and its disproportionation product (H_2O_2) show similar pH-dependences (Boveris & Cadenas, 1975), it is apparent that the pH effects reflect a property of the reaction generating O_2^- and are not related to the increasing lifetimes of O_2^- at high pH values (Fridovich, 1974).

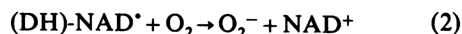
Discussion

Production of O_2^- by the NADH dehydrogenase of the respiratory chain was reported by Rich & Bonner (1978) in potato mitochondria, and by Takeshige & Minakami (1979) in bovine heart mitochondria. The present results agree with those of the latter authors on the capacity of the NADH dehydrogenase of mammalian mitochondria for generating O_2^-. However, we differ in that: (*a*) our quantitative values are far lower; (*b*) we distinguish two sites of O_2^- production in the mitochondrial respiratory chain; and (*c*) generation of O_2^- at the ubiquinone–cytochrome *b* area is higher than at the NADH dehydrogenase.

The capability of NADH and dehydrogenase-bound NADH [(DH)-NADH] to react with O_2^- (eqn. 1 below; Chan & Bielski, 1974; Bielski & Chan, 1977) places difficulties in measuring the actual rates of O_2^- generation in the presence of NADH as substrate; the reduced nicotinamide nucleotide will compete with the O_2^--detecting system and, more important, it is able to initiate an O_2^--dependent autocatalytic process by which further O_2^- is generated, as is outlined by the following equations:

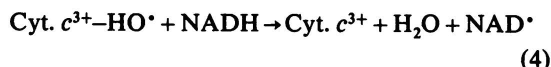


$$O_2^- + H^+ + (DH)\text{-}NADH \rightarrow H_2O_2 + (DH)\text{-}NAD^{\bullet} \quad (1)$$



$$(DH)\text{-}NAD^{\bullet} + O_2 \rightarrow O_2^- + NAD^+ \quad (2)$$

$$H_2O_2 + \text{Cyt. } c^{3+} \rightarrow \text{Cyt. } c^{3+}\text{–}HO^{\bullet} + HO^{\bullet} \quad (3)$$



$$\text{Cyt. } c^{3+}\text{–}HO^{\bullet} + NADH \rightarrow \text{Cyt. } c^{3+} + H_2O + NAD^{\bullet} \quad (4)$$

$$NAD^{\bullet} + \text{adrenaline} \rightarrow ASQ^{\bullet} + NAD^+ \quad (5)$$

$$ASQ^{\bullet} + O_2 \rightarrow O_2^- + \text{adrenochrome} \quad (6)$$

where Cyt. *c* is cytochrome *c* and $ASQ^{\bullet}$ is adrenaline semiquinone.

NADH bound to dehydrogenase is oxidized by O_2^- four to five orders of magnitude faster than free

NADH (eqn. 1; Bielski & Chan, 1977). Reactions (2), (3) and (4) have been reported to occur at slightly alkaline, near neutral pH values (Misra & Fridovich, 1973; Chan & Bielski, 1974). We postulate a reaction between adrenaline and NAD˙ (eqn. 5) to involve the catecholamine in the autocatalytic process; however, oxidation of adrenaline by HO˙ (Bors *et al.*, 1978) is equally likely. Adrenaline semiquinone (ASQ˙) produced in reaction (5) reacts with oxygen to yield O_2^- and adrenochrome as final products, after cyclization of the adrenaline quinone formed as intermediate (Bors *et al.*, 1978). The sum of eqns. (1) to (6) shows that two O_2^- anions, one HO˙ radical and one adrenochrome molecule can be generated per one initial O_2^- anion. Since HO˙ is also capable of reacting with adrenaline, giving adrenochrome (Bors *et al.*, 1978), the autocatalytic nature of the process is easily understood. Even in the absence of adrenaline, eqns. (1)–(4) yield two O_2^- anions and one HO˙ radical per initial O_2^- anion. The effects of catalase and of the concentration of submitochondrial particles (Fig. 2) can be explained by eqn. (3), in which catalase utilizes one of the reactants and submitochondrial particles provide the other reactant, endogenous cytochrome *c*.

It seems clear that the mitochondrial respiratory chain generates O_2^- at two different sites: the NADH dehydrogenase and the ubiquinone–cytochrome *b* area.

The production of O_2^- supported by reversed electron transfer indicates that a component of the isopotential group of approx. −280 to 320 mV (Wilson *et al.*, 1974), most likely the flavoprotein NADH dehydrogenase, is responsible for O_2^- production (Figs. 1 and 5). In submitochondrial particles supplemented with NADH as substrate, rotenone is able to stimulate O_2^- production by the NADH dehydrogenase and to inhibit O_2^- generation at the ubiquinone–cytochrome *b* site (Fig. 3). Cyanide distinguishes between the two O_2^- production sites: it inhibits the ubiquinone–cytochrome *b* site but does not affect NADH dehydrogenase (Fig. 6), as pointed out by Rich & Bonner (1978). Additivity of the two sites of O_2^- production was observed in an extended pH range (Fig. 7).

NADH dehydrogenase appears quantitatively less active than the ubiquinone–cytochrome *b* site as O_2^- generator at pH 7.4. Fully reduced NADH dehydrogenase produces 0.90 ± 0.07(S.E.M.)nmol of O_2^-/min per mg of protein (Figs. 2, 3, 4, 6 and 7; Rich & Bonner, 1978; Thayer, 1977), whereas the ubiquinone–cytochrome *b* site generates 1.85 ± 0.20(S.E.M.)nmol of O_2^-/min per mg of protein (Figs. 2, 3, 4 and 6; Loschen *et al.*, 1971; Boveris & Cadenas, 1975; Dionisi *et al.*, 1975; Cadenas & Boveris, 1980). The higher rates of O_2^- production observed by Takeshige & Minakami (1979) might be due to an unresolved participation of the autocatalytic process already described.

Generation of O_2^- by flavoprotein autoxidation is well documented; flavoproteins acting as electron-transferases and dehydrogenases appear likely to act in one-electron steps by formation of a stable semiquinone, and to react with oxygen yielding O_2^- rather than H_2O_2 (Massey *et al.*, 1969; Fridovich, 1974). In any case, O_2^- formation from the iron–sulphur clusters of NADH dehydrogenase is equally possible (Misra & Fridovich, 1972; Ohnishi, 1975). A clear distinction between the flavin semiquinone and the iron–sulphur clusters as the source of O_2^- seems unlikely, since electron transfer rates between intramolecular redox groups (Ruzicka & Crane, 1970) exceed the autoxidation rate by a factor of 10^4.

The ubiquitous mitochondrial membranes with rates of 2–3 nmol of O_2^-/min per mg of protein may be considered the most important physiological source of O_2^- in animal cells.

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