

Production of Superoxide Radicals during Bacterial Respiration

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When sucrose or acetate was added to washed suspensions of a number of aerobic bacteria, exogenous ferricytochrome c^{3+} was reduced, luminol chemiluminescence was enhanced and the rate of adrenaline auto-oxidation was increased. Superoxide dismutase from bovine erythrocytes inhibited all of these reactions, suggesting that superoxide radicals (O_2^-) are formed during aerobic bacterial metabolism. Superoxide radicals were probably generated during respiratory chain activity because O_2^- production by intact bacteria was stimulated by antimycin A and the highest O_2^- -producing activity was observed with membrane fractions with a high concentration of cytochromes. Exogenous superoxide dismutase partially inhibited NADH-dependent oxygen consumption by membrane particles of *Brevibacterium flavum* 22LD.

INTRODUCTION

Superoxide radicals (O_2^-) are generated biologically when a single electron is passed by auto-oxidation from reduced flavins, quinones, haemoproteins and certain other enzymes to molecular oxygen (Fridovich, 1972, 1974, 1975; Merzliak & Sobolev, 1975). Free radicals such as O_2^- and its derivatives are believed to be the cause of oxygen toxicity. Damage to *Azotobacter chroococcum* and *Escherichia coli* by O_2^- has been demonstrated (Buchanan, 1977; Van Hemmen & Meuling, 1977). All aerobic organisms as well as many oxygen-tolerant anaerobes synthesize superoxide dismutase (SOD; superoxide:superoxide-oxidoreductase; EC 1.15.1.1.) which protects them from oxygen toxicity. The resistance of a strain to oxygen toxicity is directly proportional to the activity of SOD and, furthermore, exogenously added SOD protects *E. coli* from hyperbaric oxygen (Gregory & Fridovich, 1973).

Mammalian mitochondria generate O_2^- during the initial reactions of the electron transfer chain before the site of inhibition by antimycin (Loschen *et al.*, 1974; Boveris & Cadenas, 1975). No O_2^- is generated during electron transfer to molecular oxygen by cytochrome oxidase (Chance *et al.*, 1975). Our aims were to determine whether bacteria also generate O_2^- during normal metabolic processes and, if so, whether it is produced as a by-product of initial electron transfer reactions.

METHODS

Organisms and growth. *Escherichia coli* C, *Bacillus brevis*, *Micrococcus glutamicus* P-451, *Mycobacterium lacticola* 49, *Chromobacterium flavum* 54, *Brevibacterium flavum* 22 and its mutant *Brevibacterium flavum* 22LD, which contains five to six times more carotenoid than its parent, were obtained from the culture collection of August Kirchenstein Institute of Microbiology, Academy of Sciences, Latvian SSR, U.S.S.R. All except *E. coli* were grown in medium which contained (per litre) 30 g sucrose, 20 g sodium acetate, 20 g $(NH_4)_2SO_4$, 20 g corn-steep liquor, 0.5 g KH_2PO_4 and 0.5 g K_2HPO_4 . The medium for *E. coli* C contained (per litre) 100 ml aminopeptone, 2 g glucose, 10 μ g thiamin, 5.0 g K_2HPO_4 , 0.65 g KH_2PO_4 and 0.6 g NaCl.

Bacteria were grown in 3 l laboratory fermenters, model FS-5A (Kristapsons & Viesturs, 1973), at 30°C (37°C for *E. coli* C) and pH 7.2 ± 0.05 and foam levels were controlled automatically. The dissolved oxygen tension was measured with a Clark-type oxygen electrode (produced by The Special Design Bureau, Puschino, U.S.S.R.). Growth at a constant dissolved oxygen tension of 10 ± 5 % of the air saturation value was achieved by regulating the air supply while the fermenter was stirred at 500 rev. min⁻¹.

Preparation of bacterial suspensions and subcellular fractions. Bacteria were harvested by centrifugation during the early-stationary phase of growth and immediately washed twice with cold 50 mM-KH₂PO₄/Na₂HPO₄, pH 7.2 (phosphate buffer). Bacteria were resuspended in phosphate buffer at 4°C and broken by grinding with abrasive for 3 min (disintegrator model L-17; Experimental Plant of Medical Equipment, Kiev, U.S.S.R.). The procedure disrupted 90 to 95 % of the bacteria. Subcellular fractions were pellets obtained after centrifugation at 4°C at the following *g* values: fraction I, 2500 *g* for 30 min; II, 5000 *g* for 30 min; III, 11000 *g* for 30 min; IV, 22000 *g* for 30 min; V, 100000 *g* for 60 min; VI, 156000 *g* for 60 min. Fraction VII was the final supernatant. Each pellet was washed twice with phosphate buffer. Details of the preparation and characterization of subcellular fractions obtained from mechanically disrupted cells and protoplasts have been published earlier (Shvinka *et al.*, 1978).

Enzyme assays. Oxygen consumption (Q_{O_2}) was measured manometrically as described by Shvinka & Toma (1977).

Rates of O₂⁻ formation were determined from the rates of reduction of cytochrome *c*³⁺ or oxidation of adrenaline by O₂⁻ (McCord & Fridovich, 1969; Misra & Fridovich, 1972) or from the enhancement of luminol chemiluminescence (Michelson, 1973; Hodgson & Fridovich, 1973). Substrates for bacterial suspensions were 30 mM-acetate or 30 mM-sucrose, and substrates for cell-free extracts were 6 μM-NADH or 60 μM-succinate. A double-beam spectrophotometer (Specord UV/VIS; Carl Zeiss, Jena, GDR) with thermostatted cuvettes at 30°C was used; substrate was omitted from the reference cuvette.

The reaction mixture for cytochrome *c* reduction contained 0.04 μmol cytochrome *c*³⁺ and 0.2 μmol EDTA in 1.8 ml 0.1 M-Tris/HCl, pH 8.6, and that for adrenochrome formation contained 0.6 μmol L-adrenaline and 0.2 μmol EDTA in 1.8 ml 0.1 M-Tris/HCl, pH 8.6; bacterial suspension (0.2 to 0.5 mg dry wt) or extract (20 to 200 μg protein) was added to start the reaction. Each superoxide radical reduces one molecule of cytochrome *c*³⁺ or oxidizes one molecule of adrenaline (Cadenas *et al.*, 1977); rates of O₂⁻ formation were calculated assuming that ϵ_{550} for cytochrome *c*³⁺ was 19 l mmol⁻¹ cm⁻¹ and ϵ_{480} for adrenochrome was 4.02 l mmol⁻¹ cm⁻¹ (Boveris *et al.*, 1976; Misra & Fridovich, 1972). The initial reaction velocity was constant with either method; the rate of adrenochrome formation was greater than the corresponding rate of cytochrome *c* reduction, but both were consistent with previously reported data (Boveris *et al.*, 1976).

The reaction mixture for luminescence measurements contained 2 mg luminol and bacterial suspension (40 to 60 mg dry wt) in 2 ml 0.5M-Na₂CO₃/NaOH, pH 13.2. Bacteria were preincubated for 20 min at 30°C with 30 mM-sucrose in phosphate buffer, pH 7.2.

Superoxide dismutase activity was measured as described by Misra & Fridovich (1972). The reaction mixture contained 1.8 ml 0.1 M-Na₂CO₃/NaOH (pH 10.2), 0.2 μmol EDTA, 0.6 μmol L-adrenaline and cell-free extract. One unit of SOD activity is that required to inhibit the auto-oxidation of adrenaline by 50 %.

Other assays. Cytochrome *c* concentrations were determined from reduced minus oxidized difference spectra of subcellular fractions obtained with a Specord UV/VIS double-beam spectrophotometer. The samples in the test or reference cuvettes were reduced with a few grains of sodium dithionite or oxidized with a crystal of K₃Fe(CN)₆, respectively. The ϵ_{550} for cytochrome *c* was assumed to be 21 l mmol⁻¹ cm⁻¹ (Sugijama *et al.*, 1973).

Bacteria were extracted twice with methanol at 60°C, and the concentration of carotenoids in the combined extracts was determined assuming that $A_{439\text{nm}}^{1\%_{1\text{cm}}}$ is 2500.

Bacterial dry weight was determined turbidimetrically by measuring A_{660} . Absorbance units were converted to bacterial dry weight according to a calibration curve obtained gravimetrically. Protein was determined by the method of Lowry.

Chemicals. Superoxide dismutase was purified from bovine erythrocytes according to McCord & Fridovich (1969) and Weser *et al.* (1971). The final activity of the suspension was 1260 units mg⁻¹. L-Adrenaline, antimycin A and amytal were from Serva (Heidelberg, F.R.G.), rotenone from Sigma, bovine heart ferri-cytochrome *c* and luminol from Chemapol (Prague, Czechoslovakia) and NADH and succinate from Reanal (Budapest, Hungary). Corn-steep liquor was supplied by Beslan starch factory (Beslan, U.S.S.R.) and aminopeptone was from Plant of Medical Preparations (Leningrad, U.S.S.R.). Other chemicals were supplied by Reachim (Moscow, U.S.S.R.).

Table 1. Rates of oxygen consumption (Q_{O_2}) and superoxide radical formation ($V_{O_2^-}$) during oxidation of sucrose and acetate by bacterial suspensions

Q_{O_2} was measured manometrically. The reaction medium (2.5 ml) contained bacteria at 4 to 6 mg dry wt ml⁻¹ and 30 mM-sucrose or 30 mM-acetate in 0.05 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.2, at 30 °C. $V_{O_2^-}$ was determined in a parallel experiment from the rate of reduction of cytochrome *c*³⁺ at 30 °C. Only fresh samples were used otherwise the rate of endogenous O₂⁻ formation increased and hindered the detection of substrate-dependent superoxide production.

Organism	Expt no.	Substrate in reaction medium	Q_{O_2} (nmol mg ⁻¹ min ⁻¹)	$V_{O_2^-}$ (nmol mg ⁻¹ min ⁻¹)	$\frac{V_{O_2^-}}{Q_{O_2}}$
<i>Brevibacterium flavum</i> 22LD (pigmented mutant)	1	Acetate	25.4	0.85	0.034
		Sucrose	23.5	0.33	0.014
	2	Acetate	63.9	0.69	0.011
		Sucrose	44.5	1.57	0.035
<i>Brevibacterium flavum</i> 22 (non-pigmented mutant)	1	Acetate	48.4	1.87	0.039
		Sucrose	17.3	0.73	0.042
	2	Acetate	58.4	0.67	0.012
		Sucrose	30.5	1.13	0.037
3	Acetate	47.5	1.21	0.026	
	Sucrose	35.6	2.27	0.064	
<i>Micrococcus glutamicus</i> P-451	1	Acetate	46.6	3.73	0.080
		Sucrose	17.5	1.22	0.070
<i>Bacillus brevis</i>	1	Acetate	64.2	6.22	0.097
		Sucrose	41.0	4.82	0.118
<i>Escherichia coli</i> C	1	Acetate	10.5	1.56	0.148
		Sucrose	14.7	1.85	0.126
<i>Mycobacterium lacticola</i> 49	1	Sucrose	18.0	0.27	0.015
		Acetate	46.0	0.11	0.0024
<i>Chromobacterium flavum</i> 54	1	Sucrose	47.2	0.84	0.018
		Acetate	10.7	0.55	0.051
		Sucrose	9.8	0.72	0.066

RESULTS

Superoxide formation by various bacterial species

When sucrose or acetate was added to a suspension of washed *Brevibacterium flavum* 22LD, cytochrome *c*³⁺ was reduced and adrenaline was auto-oxidized to adrenochrome. Addition of sucrose also enhanced the chemiluminescence of luminol. Exogenous superoxide dismutase inhibited all of these reactions. These results suggest that superoxide radicals are formed during the oxidation of sucrose or acetate by *Brevibacterium flavum* 22LD. Similar results were obtained with various other species of aerobically grown bacteria (Table 1).

The rate of superoxide formation ($V_{O_2^-}$) varied from 0.11 nmol O₂⁻ mg⁻¹ min⁻¹ with *Mycobacterium lacticola* 49 to 6.22 nmol O₂⁻ mg⁻¹ min⁻¹ with *Micrococcus glutamicus* P-451. The ratio of $V_{O_2^-}$ to respiratory rate varied from 0.002 to 0.148 with different strains, but there was no significant difference between $V_{O_2^-}$ values for differently pigmented strains of *B. flavum* (Table 1).

Effect of antimycin A on the rate of superoxide formation

The effect of electron transport inhibitors on $V_{O_2^-}$ in suspensions of *Brevibacterium flavum* 22LD was studied. The ratio $V_{O_2^-}/Q_{O_2}$ was unaffected by 0.1 to 5 mM-NaCN, 0.2 mM-rotenone or 2 mM-amytal, but increased when 130 μM-antimycin A was added. This concentration of antimycin inhibited the rate of oxygen consumption of *Brevibacterium flavum* 22LD by 33%, from 28.4 to 19.0 nmol O₂⁻ mg⁻¹ min⁻¹, but $V_{O_2^-}$ as determined by the rate of adrenochrome formation increased from 0.41 to 0.94 nmol O₂⁻ mg⁻¹ min⁻¹. Thus the

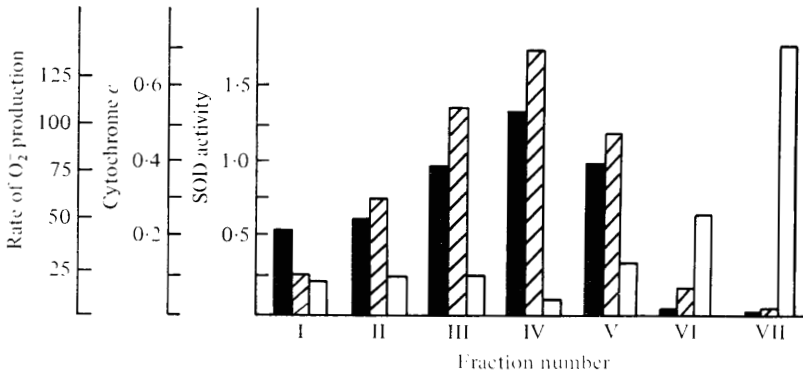


Fig. 1. Activity of the superoxide-generating system in subcellular fractions of *Brevibacterium flavum* 22LD. Bacteria were mechanically disrupted with an abrasive. Fractions I to VI were pellets obtained by centrifugation at different g values (see Methods); fraction VII was the final supernatant. ■, Rate of O_2^- production [$\text{nmol (mg protein)}^{-1} \text{min}^{-1}$], determined from NADH-dependent cytochrome c reduction; ▨, cytochrome c [$\text{nmol (mg protein)}^{-1}$]; □, SOD activity [$\text{units (mg protein)}^{-1}$].

$V_{O_2^-}/Q_{O_2}$ ratio increased 3.4 times relative to the control. Antimycin A also stimulated luminol chemiluminescence; after 2 min, the emission intensity had increased by 30%.

Localization of the superoxide-producing system in subcellular fractions of Brevibacterium flavum 22LD

The distribution of the superoxide-generating system between soluble proteins and six types of particulate material was determined (Fig. 1). The most active fraction, IV, also contained the highest concentration of cytochrome c_{555} per mg protein. The distribution of other cytochromes (a_{604} , b_{567}) and carotenoids was similar to cytochrome c . $V_{O_2^-}$ in fraction IV was $107 \text{ nmol mg}^{-1} \text{min}^{-1}$ during NADH oxidation and $45 \text{ nmol mg}^{-1} \text{min}^{-1}$ during succinate oxidation, but SOD was relatively inactive.

These NADH- and succinate-dependent activities were completely inhibited by exogenous superoxide dismutase, and disappeared after the membranes were boiled or the suspension was bubbled with nitrogen. The pH optimum for O_2^- production was 8.6 in both test systems, and the temperature optimum was 37°C .

The NADH oxidase activity of fraction IV was inhibited 26% by $0.1 \text{ mg SOD ml}^{-1}$ but was unaffected by boiled SOD or albumin.

DISCUSSION

The generation of superoxide radicals during sucrose or acetate oxidation by various bacterial species was readily detected. $V_{O_2^-}$ for bacterial suspensions varied over a 60-fold range, but was far lower than $V_{O_2^-}$ for membranes which had been washed free from SOD. Thus, it is possible that O_2^- is generated intracellularly and is only partially released into the external medium. Alternatively, because exogenous NADH stimulated the rate of O_2^- formation by washed bacteria, the O_2^- -generating site might, as in leucocytes, be located at the cell surface (Goldstein *et al.*, 1977).

The correlation between $V_{O_2^-}$ and the cytochrome content of membrane extracts, together with the dependence of O_2^- formation on substrate oxidation clearly implicates the electron transfer chain as the site of O_2^- formation. Furthermore, as in mitochondria, O_2^- is generated during the initial stages of electron transfer from substrates to oxygen, before the site of inhibition by antimycin A. Superoxide is formed more rapidly by bacteria than by mito-

chondria, however: $V_{O_2^-}$ during NADH oxidation was $107 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ for membrane fraction IV from *B. flavum* 22LD compared with 4.5 to $6.9 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ for mammalian mitochondria (Loschen *et al.*, 1974; Boveris & Cadenas, 1975).

Superoxide is produced by mammalian mitochondria as an intermediate in the formation of peroxides (Loschen *et al.*, 1974; Boveris & Cadenas, 1975; Cadenas *et al.*, 1977). Some micro-organisms release substantial quantities of H_2O_2 into the medium, so O_2^- is probably formed as an intermediate in this process (Low *et al.*, 1968; Jones *et al.*, 1970). It has been suggested that O_2^- released by leucocytes provides an antibacterial defence mechanism (Babior *et al.*, 1973; Devlin *et al.*, 1977), so it is possible that O_2^- and H_2O_2 formation at the bacterial cell surface is also a defence mechanism against other bacterial species.

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