The effect of silver ions on the respiratory chain of *Escherichia coli*[1]

P. D. Bragg and D. J. Rainnie

Department of Biochemistry, University of British Columbia, Vancouver, British Columbia

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Silver ions inhibited the oxidation of glucose, glycerol, fumarate, succinate, D- and L-lactate, and endogenous substrates by intact cell suspensions of *Escherichia coli*. Silver ions reacted with the respiratory chain at two levels. The site most sensitive to inhibition was located between the b-cytochromes and cytochrome a_2. The second level of inhibition was in the NADH and succinate dehydrogenase regions of the respiratory chain, and was situated on the substrate side of the flavin components.

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Les ions d'argent inhibent l'oxidation du glucose, du glycérol, du fumarate, du succinate, du D- et L-lactate et les substrats endogènes par des suspensions d'*Echerichia coli*. Les ions d'argent réagissent avec la chaîne respiratoire à deux niveaux. Le point le plus sensible à l'inhibition est localisé entre les cytochromes-b et le cytochrome a_2. Le deuxième niveau d'inhibition est dans la région de la chaîne respiratoire de la déhydrogénase succinate et NADH, et est situé sur le côté du substrat des composants flavines.   [Traduit par le journal]

Introduction

In previous publications (13, 14) we noted that, after an initial brief phase of stimulation, the respiration of *Escherichia coli* metabolizing glucose was inhibited by silver ions. This agreed with the work of Yudkin (17) who, some years ago, found that 10 μM silver sulfate inhibited by 50% the reduction of methylene blue by *E. coli* cells oxidizing glucose, succinate, or lactate. Yudkin also observed that with glucose, dye reduction was initially stimulated by the metal ions before inhibition occurred.

Chappell and Greville (4) found that low concentrations (5–10 μM) of $AgNO_3$ stimulated both the respiration and adenosinetriphosphatase (ATPase) activity of rabbit brain mitochondria, and Brierley (3) reported that silver ions inhibited oxidative phosphorylation in beef heart mitochondria. Therefore, it was considered possible that this latter process might have been affected in *E. coli*, and that the initial stimulatory phase in dye reduction and glucose oxidation might represent an uncoupling action of the silver ions. We subsequently confirmed that hypothesis by correlating the magnitude of respiratory stimulation by silver ions with the extent of energy coupling in the cells (14). However, the site(s) of the inhibitory action of silver ions on substrate oxidation was not determined either by Yudkin or by us.

Although silver ions are known to inhibit transport of succinate into membrane vesicles of *E. coli* (15), there is no information on their action directly on the respiratory chain of this or other microorganisms. There is some indirect evidence as to possible sites of action suggested by experiments with other metal ions. Thus, in heart muscle particles zinc ions inhibited at two sites, one between cytochromes b and c_1, and the other at the flavoprotein level (12). Kleiner and von Jagow (11) using rat liver mitochondria re-examined the site of inhibition of zinc ions and found that they inhibited the respiratory chain at a site between ubiquinone and cytochrome b. In *E. coli* respiratory particles zinc ions inhibited the succinate oxidase system at the level of succinate dehydrogenase (7).

In the present paper we show that silver ions inhibit the respiratory chain of *E. coli*. The most sensitive site of inhibition was between the b-cytochromes and cytochrome a_2. A further site of inhibition was located between the site of substrate interaction with the respiratory chain and flavoprotein.

Materials and Methods

Chemicals

Chloride-free glycylglycine was obtained from Nutritional Biochemicals Corporation. Lithium D- and L-lactate, 3-phosphoglyceraldehyde, and nicotinamide adenine dinucleotide (NAD^+) were purchased from Calbiochem. Silver nitrate was Fisher certified ACS grade. Tris(hydroxymethyl)aminomethane (Tris) (free

base) and ammonium sulfate were of ultrapure-grade obtained from Schwarz-Mann Division of Becton, Dickinson and Company. All other chemicals were reagent-grade.

Culture Conditions and Preparation of Cell Suspensions

Escherichia coli strain 482 of the culture collection of the National Research Council of Canada was used in all investigations reported here. The cells were cultured in two ways as described below.

Culture Method A

The cells were grown on a minimal salts medium of the following composition: 0.7% K_2HPO_4; 0.3% KH_2PO_4; 0.1% $(NH_4)_2SO_4$, and 0.02% $MgSO_4$. Carbon sources and the concentrations used were glucose 0.4%, glycerol 0.4%, DL-lactic acid 0.8%, sodium succinate 0.4%, and potassium fumarate 0.4%. The medium was adjusted to pH 7.0 before autoclaving. Growth was initiated by inoculating 600 ml of sterile medium with a 10% (v/v) overnight culture grown on the appropriate carbon source. The culture was vigorously aerated via a sintered glass sparger (Kimax 12C) at an air flow rate of 6.7 liters/min. Growth at 37° was followed by measuring the absorbance at 420 nm of an aliquot of the culture in a cuvette with 1 cm path length. The cells were harvested in the late exponential phase of growth by centrifugation at 5900 × g for 10 min at 4°. The cells were washed twice by centrifugation from 0.85% NaCl at 4300 × g for 10 min and were resuspended at a dilution of 1:10 (w/v) in the buffer indicated.

Culture Method B

The cells were grown on the minimal medium used in method A but with 0.2% $(NH_4)_2SO_4$ and with the addition of 6 μM ferric citrate. The carbon source was 1.4% (w/v) sodium succinate·$6H_2O$. Growth was initiated by inoculating 350 ml of the sterile medium in a 1-liter erlenmeyer flask with a 10% (v/v) culture grown for 9 h on the same medium. The culture was vigorously shaken (120 strokes/min) at 37° for 15 h on a reciprocating shaker (New Brunswick Scientific Co.). The cells were harvested by centrifugation at 4400 × g for 15 min; washed with 0.01 M Tris-HCl buffer, pH 7.3, containing 0.01 M $MgCl_2$ (350 ml), and then with 0.1 M glycylglycine buffer, pH 8.0 (40 ml); and recovered by centrifugation.

For experiments with whole cells the final washing step with glycylglycine buffer was repeated, and the cells suspended in a volume of the same buffer equivalent to 15 times the wet weight of the cells. The cell suspension was kept at room temperature during the duration (about 3 h) of the experiment.

For the preparation of respiratory particles the whole cells were suspended in a volume of glycylglycine buffer equivalent to 10 times the wet weight of the cells.

Preparation of Respiratory Particles

A few crystals of deoxyribonuclease (DNase) (Worthington Biochemical Corp.) and M $MgCl_2$ (final concentration, 4 mM) were added to the cell suspension which was then disrupted at 0° in a French pressure cell (Aminco) operated at 20 000 psi. The extract was centrifuged at 27 000 × g for 15 min. The supernatant fraction was recentrifuged at 176 000 × g for 2 h and the well-packed pellet of respiratory particles suspended in a volume of

0.1 M glycylglycine buffer, pH 8.0, equivalent to 5 times the wet weight of cells from which the particles were derived. The suspension was stored at 0° for the duration of the experiment.

Assays

Oxygen uptake was measured with an oxygen monitor (Yellow Springs Instrument Co. model 55) equipped with a Clark type oxygen electrode (Yellow Springs Instrument Co. model 5533) and a linear chart recorder. Reduced minus oxidized difference spectra were recorded using a Cary model 15 spectrophotometer equipped with a 0.1-absorbance slide-wire. The redox state of a respiratory chain component was measured by comparing the absorbance increment caused by the reduced component at a selected wavelength in the reduced minus oxidized difference spectrum to the absorbance increment obtained in the presence of sodium dithionite which fully reduced the components of the respiratory chain. The selected wavelengths for flavoprotein and cytochromes b_1, a_1, and a_2 were 465 nm, 559 nm, 590 nm, and 628 nm, respectively. The components in the reference cuvette were maintained in the oxidized form with 0.05% H_2O_2.

Results

Effect of Silver Ions on Substrate Oxidation

The effect of 86 μM $AgNO_3$ on the oxidation of glucose, glycerol, fumarate, and D-lactate by whole cells is shown in Fig. 1. Inhibition of oxygen uptake occurred rapidly with all of these substrates after the addition of $AgNO_3$. That the inhibition was due to silver ions was shown in control experiments where no inhibition was obtained by addition of KNO_3. The effect of silver ions on the oxidation of succinate and L-lactate was similar to that on fumarate and D-lactate. The cells used in these experiments were grown on the same substrate as was subsequently used in the oxygen-uptake experiment. However, the same pattern of results were obtained if the effect of silver ions on substrate oxidation was measured using cells grown on different carbon sources.

To avoid the uncertainties introduced by a possible effect of silver ions on the transport of substrate, the effect of this inhibitor on the oxidation of endogenous substrates was examined. This experiment was carried out in a different way to those shown in Fig. 1. An aliquot of a cell suspension was added to buffer containing $AgNO_3$ and the uptake of oxygen then measured. Even in the absence of inhibitor nonlinear rates of oxygen uptake were obtained. For this reason rates were measured at two particular time intervals (4 and 8 min) after addition of $AgNO_3$ to the cell suspension. The rates were then compared
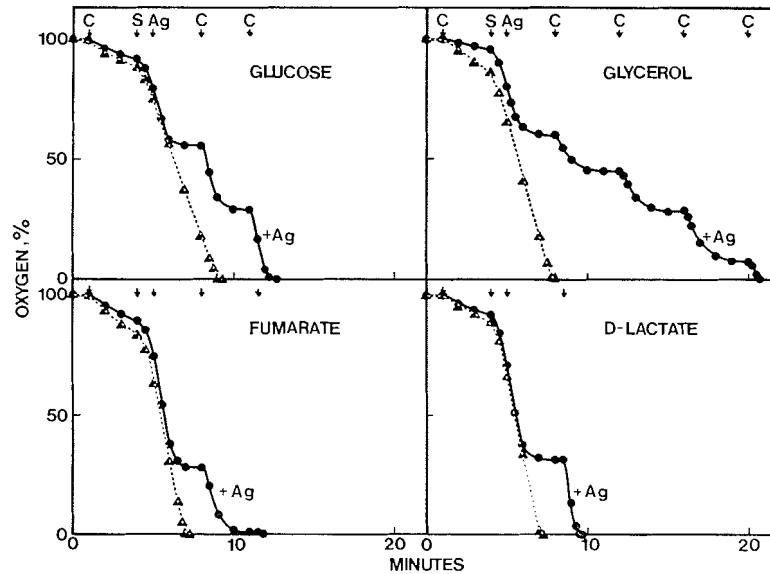
FIG. 1. Effect of silver ions on oxidation of substrates by intact cells of *E. coli*. Oxygen uptake was measured at 37° in suspensions of cells grown (culture method A) on, and assayed with the same carbon source. The assay system (5.1 ml) contained 288 mM glycylglycine-KOH buffer, pH 7.0, and 0.4 mM KCl. At the indicated time (top of figure) 0.05 ml of cell suspension (C), 0.05 ml M substrate (S), and 0.03 ml 15 mM AgNO₃ (Ag) were added. In the control (broken line) only the initial addition of cell suspension and substrate were made. Oxygen level is expressed as % saturation.

with those obtained at these two time intervals in the absence of inhibitor to give "% initial value." As shown in Fig. 2A oxidation of endogenous substrates was inhibited by silver ions and the extent of inhibition increased with time.

To rule out a possible action on other metabolic pathways the effect of AgNO₃ on the succinate and reduced NAD (NADH) oxidase activity of respiratory particles was examined (Fig. 2B). The particles were preincubated for 5 min with
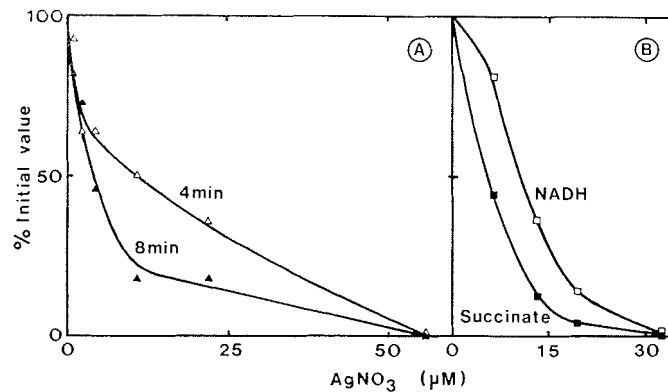

FIG. 2. Effect of concentration of AgNO₃ on the rate of oxidation of endogenous substrates by whole cells (A), and on the oxidation of NADH and succinate by respiratory particles (B). In A the oxygen consumption was measured at 37° following the addition of 1.0 ml cell suspension to 3.5 ml 0.1 M glycylglycine buffer, pH 8.0, containing the indicated concentration of AgNO₃. The rates of oxygen uptake at 4 and 8 min after the cell suspension was added to the AgNO₃ are plotted. Initial values for rate of oxygen uptake in the absence of inhibitor: 4 min, 2.45 μg atoms O/min per gram cells; 8 min, 1.93 μg atoms O/min per gram cells. In B 1.5 ml respiratory particle suspension (6.2 mg protein) was preincubated at 21° for 5 min with AgNO₃. NADH (2.8 μmol) or disodium succinate (5 μmol) was then added and the rate of oxygen uptake measured by the spectrophotometric technique (Ref. 1). Initial value: NADH oxidase, 0.37 μg atoms O/min per milligram protein; succinate oxidase, 0.15 μg atoms O/min per milligram protein.

the inhibitor before the substrate was added. Although the extent of inhibition of the two systems at any particular concentration of AgNO$_3$ depended on the substrate used, the effective concentrations of AgNO$_3$ were similar to those found for inhibition of the oxidation of endogenous substrate by whole cells (Fig. 2A). Although this suggests that the effect observed on the endogenous respiration of intact cells might be due to the action of silver ions on the respiratory chain it does not rule out sites of inhibition elsewhere.

Effect of Silver Ions on Cytochrome Reduction and Oxidation

The sites of action of silver ions in the respiratory chain were examined in the following experiments. The components of the respiratory chain in whole cells were permitted to become fully reduced by endogenous substrates. This was confirmed in control experiments using sodium dithionite to reduce the respiratory chain components. Various concentrations of AgNO$_3$ were then added and the reduction state of these components was followed by repeatedly scanning the difference spectrum. At 13 µM AgNO$_3$ (Fig. 3A) oxidation of cytochrome a_2 was induced, while there were much smaller changes in the redox state of flavoprotein and cytochrome b_1. Addition of NaNO$_3$ did not cause any such changes. Although the changes observed at 465 nm will be referred to as "flavoprotein" it is likely that nonheme iron makes a major contribution to the absorbancy at this wavelength (1). At higher concentrations of AgNO$_3$ (26 and 66 µM) (Fig. 3b, c) oxidation of flavoprotein and cytochrome b_1 was induced by addition of the inhibitor. It is presumed that oxidation of these components occurs because of a slow rate of diffusion of oxygen into the open cuvette and because rereduction of these components by endogenous substrates has been prevented by the silver ions.

Essentially similar results to those with whole cells were obtained at comparable concentrations of AgNO$_3$ when respiratory particles were used with either NADH (data not shown) or succinate (Fig. 3d–f) as substrates. In additon it was observed that the behavior of cytochrome a_1 measured in respiratory particles followed that of cytochrome b_1 and flavoprotein. This is of interest since nothing is known about the site or role of this cytochrome in the respiratory chain of E. coli.
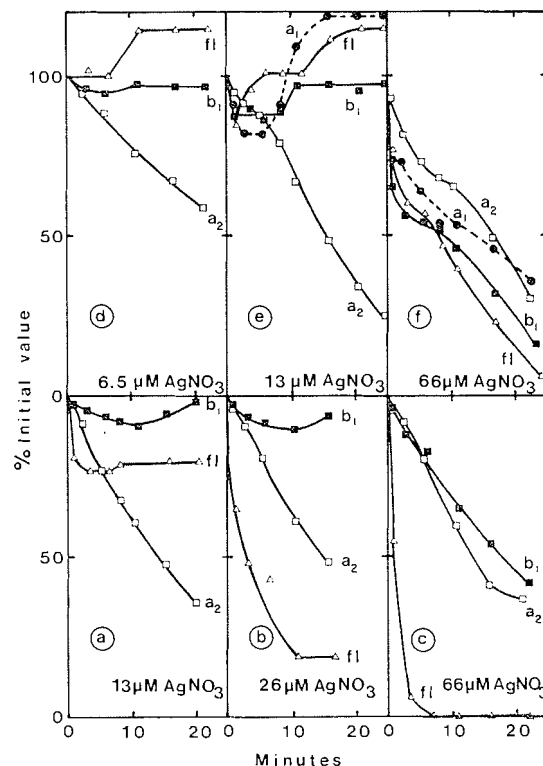

FIG. 3. Effect of addition of AgNO$_3$ on the redox states of flavoprotein, and cytochromes b_1, a_1, and a_2, in whole cells (a–c) and respiratory particles (d–f). In a–c 1.5 ml cell suspension (culture method B) in a cuvette was allowed to become anaerobic to reduce the components of the respiratory chain. At 0 min the indicated concentration of AgNO$_3$ (2–10 µl) was added and the difference spectrum scanned at frequent intervals versus a cuvette containing the cell suspension maintained aerobic by the presence of 0.05% H$_2$O$_2$. The redox states of the respiratory chain components determined as described in Materials and Methods are expressed relative to their initial value before AgNO$_3$ was added. Experiments d–f were carried out in the same way except that the respiratory particles (5.9 mg protein) were reduced by the addition of 5 µmol Na$_2$ succinate before the addition of AgNO$_3$. Temperature, 28°. The initial values of the absorbance increments for flavoprotein and cytochromes b_1 and a_2 in whole cells (a–c) were 0.110, 0.095, and 0.042 absorbance units, respectively, and for flavoprotein and cytochromes b_1, a_1, and a_2 in respiratory particles (d–f) were 0.042, 0.063, 0.005, and 0.031 absorbance units, respectively.

Results are not given for the behavior of cytochrome a_1 in whole cells since the peak was small and it was not possible to correct satisfactorily for baseline irregularities at this wavelength.

The absorbance attributed to cytochrome a_2 in the reduced *minus* oxidized difference spectrum consists of a peak at 628 nm and a trough at 650 nm. The peak is a measure of the reduced

cytochrome in the sample cuvette and the trough a measure of the oxidized cytochrome in the reference cuvette (8). When 159 µM AgNO₃ was added to respiratory particles reduced by succinate, the absorbance peak resulting from reduced cytochrome a_2 disappeared more rapidly than the peak for the oxidized cytochrome that was formed. Thus, at 20 min essentially all of the peak at 628 nm had disappeared while the trough at 650 nm was still prominent (Fig. 4). If the reduced cytochrome had been converted to the oxidized form this trough would not have been apparent. This result suggests that in the presence of silver ions reduced cytochrome a_2 is converted to a form which does not absorb light at 628 nm.

The site of silver action between NADH or succinate and cytochrome b_1 which was observed at higher concentrations of inhibitor was further examined in respiratory particles by measuring the effect of AgNO₃ on the rate of reduction of

cytochrome b_1 by substrate which occurred when all the oxygen in the reaction medium had been consumed (Fig. 5). Succinate reduced cytochrome b_1 in a single phase of reduction whereas the reduction of this cytochrome observed using NADH as substrate was markedly biphasic. This suggested that part of the cytochrome b_1 pool or another b-type cytochrome was reduced less readily by NADH than by succinate. We have obtained evidence previously by use of inhibitors for two b-cytochromes in *E. coli* (9), and this subsequently has been supported by the data of Shipp (16). Recently, Haddock and Schairer (6) have suggested that the two different b-type cytochromes (cytochromes b_{556} and b_{558}) are associated with different respiratory pathways one of which terminates in cytochrome o and the other in cytochrome a_2.

The results shown in Fig. 5 indicate that silver ions will inhibit the reduction of both species or both pools of cytochrome b_1.

Prevention by Glutathione of Inhibition by Silver Ions

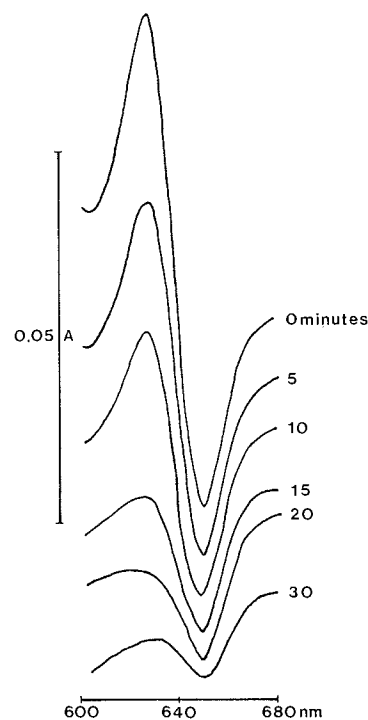We showed earlier that glutathione could

FIG. 4. Effect of addition of AgNO₃ on the redox state of cytochrome a_2 in respiratory particles. The experiment was performed as described in Fig. 4, *d–f*. The results are shown as a series of reduced *minus* oxidized difference spectra recorded at the indicated times following addition of 159 µM AgNO₃ to the membrane particles (5.9 mg protein) reduced by Na₂ succinate.
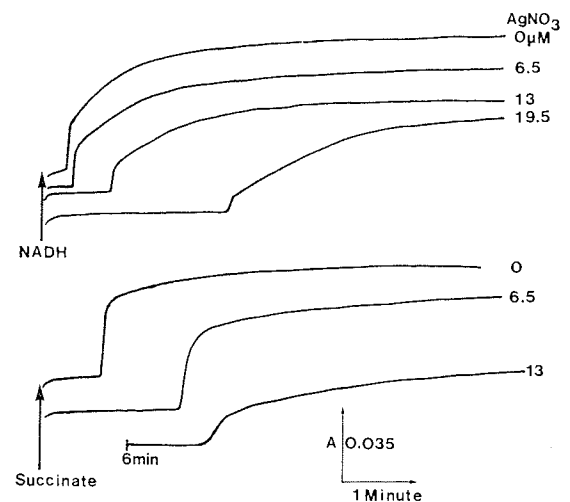

FIG. 5. Effect of AgNO₃ on the reduction of cytochrome b_1 in respiratory particles. Respiratory particles (1.5 ml, 6.2 mg protein) were preincubated for 5 min at 21° with the indicated concentration of AgNO₃. At 0 min 25 µl NADH (2.8 µmol) or 10 µl Na₂ succinate (5 µmol) was added and the reduction of cytochrome b_1 followed. The extent of reduction of cytochrome b_1 (ordinate) was measured in an Aminco-Chance dual wavelength spectrophotometer as the absorbance increment at 560 nm relative to 540 nm. In the lowest trace the reduction curve is shown only from 6 min after the addition of succinate to the cuvette.
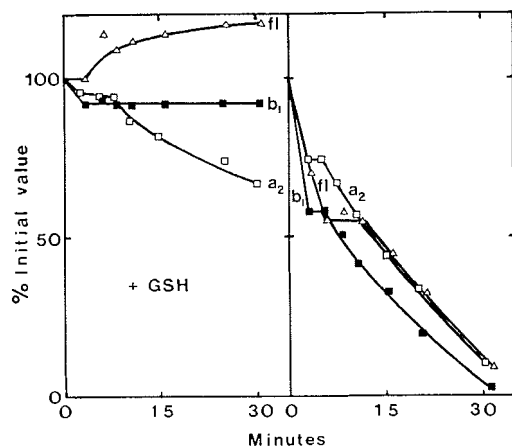
FIG. 6. Effect of glutathione (GSH) on the action of AgNO$_3$ on the redox state of flavoprotein, and cytochromes b_1 and a_2 in respiratory particles. The experiment was performed as described in Fig. 4, d–f, except that in the results shown in the left panel 0.62 mM glutathione was present before the addition of 62 μM AgNO$_3$. Membrane particles, 7.0 mg protein. Temperature, 22°. The initial values of the absorbance increments for flavoprotein and cytochromes b_1 and a_2 were 0.034, 0.059, and 0.030 absorbance units, respectively.

prevent inhibition of glucose oxidation by silver ions in whole cells (13). Similar results can be obtained with respiratory particles (Fig. 6). In the presence of 0.62 mM glutathione silver inhibition at the site between substrate and cytochrome b_1 did not occur, and the extent of inhibition at the site between cytochrome b_1 and cytochrome a_2 was substantially reduced.

Discussion

Inhibition by silver ions of substrate oxidation in whole cells could involve sensitive sites in substrate transport, substrate metabolism before oxidation via the respiratory chain, and effects on the respiratory chain itself. There is insufficient evidence to predict which system determines the sensitivity to silver ions of the oxidation of any particular substrate by whole cells.

Two sites of inhibition by silver ions were detected in the respiratory chain. The site of inhibition located between the b-cytochromes and cytochrome a_2 was more sensitive than that found between NADH or succinate and "flavoprotein" (flavin and nonheme iron; 1). Thus, there was no detectable effect on the latter site while the former was at least partially inhibited by 13 μM AgNO$_3$. This concentration of silver ions gave significant

inhibition (~80%) of respiration in both whole cells and respiratory particles suggesting that most, but not all, of the reducing equivalents from substrate passed to oxygen via this site. That this site might be bypassed, at least in the presence of higher concentrations of silver ions, is suggested by the results shown in Fig. 3b, c, and f, where oxidation of "flavoprotein" and the b-cytochromes can occur at a greater rate than that of cytochrome a_2. However, the electron acceptor may not be oxygen in this case.

The disappearance of the absorption peak of reduced cytochrome a_2 in the presence of silver ions without the immediate appearance of an absorption peak of oxidized cytochrome a_2 suggests that an intermediate form of cytochrome a_2 is formed which does not absorb strongly between 610 and 670 nm. Recently, Kauffman and Van Gelder have also suggested that such an intermediate might exist (8). This result does not prove that silver ions are reacting directly with cytochrome a_2 although this might occur.

The less sensitive site of silver inhibition of the respiratory chain which occurs before "flavoprotein" is probably that which is sensitive to zinc ions (7). Thiols can protect this site with both of these metal ions which suggests that unless the thiol effectively removed the metal ion, the sulfhydryl groups might be involved. Succinate dehydrogenase and NADH-cytochrome b_1 reductase activities in $E.$ $coli$ are also inhibited by sulfhydryl-reacting mercurials (2, 10). The precise location of these groups was not determined but if the bacterial system resembles that of the mammalian mitochondrion it is likely that several sulfhydryl groups with different reactivities are present in the dehydrogenase regions of the respiratory chain (5). Furthermore, the site of action of silver ions may be the same as that of mercurials (5).

Acknowledgments

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