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The uptake of silver ions by *Escherichia coli* K12: toxic effects and interaction with copper ions

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Summary. Growth of *Escherichia coli* in chloride-free medium in batch culture is inhibited completely at concentrations of $AgNO_3$ greater than 2.5×10^{-6} M. Incubation of non-growing cells in HEPES buffer (pH 7.4) at increasing levels of Ag^+ results in the progressive saturation of two types of binding site. At one site, the Ag^+ is not released by washing with 0.1 M nitric acid, and is probably intracellular. Silver bound to the second site is released by acid-washing, but not by buffer washing, and is assumed to be surface-bound. The amounts of Ag^+ taken up from solution at the two sites is 1.6×10^{-7} and 4.6×10^{-7} mol (mg dry weight)$^{-1}$, respectively. Total accumulation of silver is 67 mg (g dry weight)$^{-1}$, similar to literature values found for silver-resistant bacteria. Binding of Ag^+ at intracellular sites (observed at low $[Ag^+]$) appears to be independent of pH. Addition of $AgNO_3$ to growing cells in mid-exponential phase of growth in concentrations that will inhibit growth results in substantially decreased accumulation of silver. Growth yield in chemostat culture is diminished in the presence of added Ag^+, but this effect is moderated by added Cu^{2+}, which may protect copper sites from Ag^+ or compete with Ag^+ for other sites at which Ag^+ exerts toxic effects. Very small amounts of Cu^{2+} are found in cell samples from the chemostat compared to the substantial amounts of Ag^+ taken up, but uptake of Cu^{2+} is decreased at higher $[Ag^+]/[Cu^{2+}]$ ratios.

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Introduction

The toxicity of silver compounds towards bacteria is well known, and has been exploited in the treatment of bacterial infections. The Ag^+ cation is a chemically 'soft' species like mercury and lead, and binds strongly to proteins and nucleic acids, particularly to cysteine $-SH$ residues and also to amino, imidazole, carboxyl and phosphate groups. Silver ions form insoluble complexes with RNA and DNA, and will also be precipitated by a number of biologically important anions. Furthermore, Ag^+ may displace native metal cations from their usual binding sites in enzymes. A range of inhibitory effects result from these interactions, so that Ag^+ is probably the most toxic of the metal cations (Foye 1977; Friberg et al. 1979).

In *Escherichia coli,* silver inhibits several oxidative enzymes (Yudkin 1937), the uptake of succinate by membrane vesicles (Rayman et al. 1972) and accumulation of phosphate (Schreurs and Rosenberg 1982). However, some of the effects on the respiratory chain of *E. coli* previously attributed to Ag^+ by Bragg and Rainnie (1974) are now thought to be due to the nitrate counter ion (Hubbard et al. 1983). Little detailed information is available on the mechanisms underlying these toxic effects or of silver uptake by cells.

Silver-resistant bacteria have been isolated from silver-contaminated environments (Belly and Kydd 1982; Bridges et al. 1979; Pümpel and Schinner 1986), while silver-resistance in *Klebsiella pneumoniae* develops by adaptation to increasing concentrations of $AgNO_3$ (Kaur and Vadehra 1986). Plasmids encoding silver resistance have been found in *Pseudomonas stutzeri* isolated from a silver mine (Haefeli et al. 1984), and *E. coli* (Summers et al. 1978). In the latter case, resistance to silver was dependent on halide

concentration (Silver 1983). In the absence of chloride, cells with or without plasmids showed little difference in their resistance to silver, and both cell types bound silver tightly on the cell surface. Difference in sensitivity of Ag^+ was shown in the presence of chloride, apparently because only the silver-sensitive cells were able to remove Ag^+ from AgCl.

Certain silver-resistant micoorganisms accumulate silver to quite high levels (e.g., Charley and Bull 1979 and Pümpel and Schinner 1986), although it is not always known whether this process involves surface binding of Ag^+ or intracellular uptake. The metal-leaching organisms *Thiobacillus ferrooxidans* and *T. thiooxidans*, growing on sulfide minerals containing silver, bind silver as the sulfide on their cell surfaces (Pooley 1982).

The uptake and physiological effects of Ag^+ on *E. coli* growing in batch and continuous culture are described in this paper. This work has been carried out at several copper concentrations to examine the possibility of interaction between copper and silver ions. Silver binds at copper sites in several copper proteins, for example at the blue, type 1 site in plastocyanin (Bohner et al. 1981).

Materials and methods

Organism and growth media. *Escherichia coli* K12 (strain EMG2) was used in this work. A modified medium was designed in which components that might precipitate as silver salts were either removed or lowered in concentration. Chlorides were replaced by nitrates or sulfates, while phosphates and sulfates were lowered in concentration. Phosphate buffers were replaced by Tris buffer, while succinate was maintained as the carbon source. The 'chloride-free' medium contained (mM): Tris (40), $K_2HPO_4 \cdot H_2O$ (28), $KH_2PO_4 \cdot 3H_2O$ (2.2), NH_4NO_3 (18.7), succinic acid (9.9), $CaSO_4$ (0.001), K_2SO_4 (2.0), $MgSO_4 \cdot 7H_2O$ (1.0) and chloride-free trace element solution (10 ml per litre). The final pH was adjusted to 7.4 with sulfuric acid. Chloride-free trace element solution (pH 7 to 8) contained ($g\,l^{-1}$): $Na_2EDTA \cdot 2H_2O$ (5.0), $Fe_2(SO_4)_3$ (0.37), ZnO (0.05), $CuSO_4 \cdot 5H_2O$ (0.015), $Co(NO_3)_2 \cdot 6H_2O$ (0.01), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.01), H_3BO_3 (0.01). Copper sulfate was omitted from copper-free, chloride-free trace element solution. Chemicals were AnalaR or AristaR grades, as available. Ag^+ was added as silver nitrate.

Culture conditions. For batch growth, 100 ml of chloride-free medium was shaken in 1 l conical flasks at 200 rev min^{-1} and 37° C. An all-glass chemostat vessel, with silicone tubing, was used in continuous culture experiments. This had a working volume of 135 ml and was water-jacketed to maintain a temperature of 37° C. Aeration relied on diffusion from the gas phase at the deep vortex surface created by vigorous magnetic stirring. The dilution and air-flow rates were 0.18 h^{-1} and 0.8 l min^{-1}, respectively.

Growth of cultures was monitored by OD_{600} measurements in 1 cm cuvettes in Pye-Unicam SP600 or SP550-600 spectrophotometers. In addition, total biovolume (cell number density X mean cell volume) was calculated by Coulter Counter measurements (Poole 1977).

The chemostat vessel was sampled in steady state conditions. Samples (40 ml) were collected on ice from the effluent and centrifuged at 12000 *g* for 10 min at 4° C. The cell pellet was resuspended in 20 ml buffer (100 mM HEPES-NaOH, pH 7.4) to remove any silver or copper loosely bound to the cells, and then recentrifuged and suspended in 20 ml 0.1 M HNO_3 to remove silver or copper more tightly bound to the cells. The cell pellets (originally cream, but white after acid-washing), supernatant, buffer and acid washes were kept frozen until needed for analysis for metals.

Binding experiments. These were carried out on cells suspended in buffer and on growing cultures at mid-exponential phase. In the former case, cells were grown to stationary phase in 50 ml of chloride-free medium (with 15 mM succinate) at 37° C, harvested at 5000 *g* for 10 min, and washed with 100 mM HEPES buffer (pH 7.4 or 3.0). The cells were suspended in 140 ml of the buffer solution, and 20 ml of this suspension added to each of a series of conical flasks containing appropriate amounts of $AgNO_3$. After shaking for 1 h at 37° C, the cells were harvested at 4000 *g* for 10 min, washed with HEPES buffer of the appropriate pH, and then with 0.1 M nitric acid. Supernatant, both washings and cells were analysed for silver. Alternatively, silver nitrate solution was added to growing cultures at mid-exponential phase. The cells were shaken at 37° C for a further hour before harvesting as described above.

Analysis for metals. Cell pellets were digested in 200–400 µl concentrated nitric acid in a sealed ampoule at 105° C for at least 12 h or until a clear solution was obtained. This was made up to a standard volume (10 or 20 ml) with distilled water.

Cell digests, washings and media were analysed for silver and copper as appropriate by atomic absorption techniques at 338.3 nm (Ag) and 324.7 nm (Cu) on a Perkin-Elmer 2380 instrument with a HGA-400 carbon furnace. Standards were prepared by dilution of a commercial copper standard solution or a stock solution of AnalaR silver nitrate into acid, buffer, medium or water as appropriate. Addition of silver nitrate up to the maximum level used (0.9 µM) had no effect on the determination of copper. Copper standards made up in medium gave slightly higher values than expected due to the presence of copper as a contaminant in the reagents used and as a component of the trace element solution.

Difficulties were experienced in the analysis of silver due to the binding of some Ag^+ to glass surfaces.

Results

Effect of chloride-depleted medium on growth yield

Table 1 summarises the differences in composition of the chloride-supplemented and low chloride media. Growth of *E. coli* in chloride-free medium, in the absence of added silver ions, resulted in lower growth yields than those obtained in a medium with normal chloride levels. Total cell volumes obtained in a range of experiments using

Table 1. Differences between Cl^--containing and Cl^--free growth media*

Ion	Cl^--containing medium	Cl^--free medium
Chloride	21	very low, →0
Sulphate	15	2
Phosphate	91	5
Tris	0	40

* Concentrations in mM

normal medium were in the range 10 to 11×10^{-8} μm^3 ml^{-1}, while in chloride-free medium biovolumes were around 4.2×10^{-8} μm^3 ml^{-1}. Addition of KCl to the chloride-free medium (to give a concentration of 1 mM) did not result in an increase in growth yield, suggesting that growth of the cells was not chloride-limited. Decrease in sulfate concentration to 0.5 mM caused a small decrease in growth yield, but increase in sulfate beyond 2 mM did not affect the growth yield, suggesting that 2 mM sulfate was adequate. Increase in phosphate from 5.0 to 12.25 mM resulted in a small rise in growth yield, but the availability of phosphorus in the medium is ample to support a much greater dry weight of cells than that obtained in these experiments (Pirt 1975). The low growth yield in chloride-free medium may result from the presence of Tris buffer, but this possibility was not checked directly.

Effect of silver on growth in batch culture

Addition of silver nitrate to chloride-free medium (to give a final concentration of 10^{-7} M) had no effect on the growth of *E. coli*. At 10^{-6} M $AgNO_3$, the culture had a slightly longer lag phase, but grew to the same extent as in the control experiment. However, cell growth was inhibited completely at concentrations of $AgNO_3$ greater than 2.5×10^{-6} M.

Binding experiments in batch culture

Table 2 gives the silver content of supernatant solution, buffer wash, acid wash and cell pellet for non-growing cells suspended for one hour in a HEPES-NaOH buffer, pH 7.4, in the presence of silver concentrations in the range 10^{-6} to 1.6×10^{-3} M (Column 1). Column 7 gives the total Ag^+ found by analysis (the sum of columns 3–6). In general the total silver recovered agreed quite well with the amount added (column 2), except, that in some cases the experimentally determined total Ag^+ was slightly higher than that added.

Column 6 in Table 2 lists the amount of silver found in the cell pellet. This is the metal not removed by acid washing of the cells and is assumed to be intracellular. In the concentration range up to 0.016 mM Ag^+, almost all the added silver (85% upwards, column 9) is associated with the cell pellet, with up to 2% found in the acid wash (column 8). As the silver concentration is raised above 0.016 mM, the amount of silver in the cell approaches a limiting value, while the silver in the acid wash (surface-bound Ag^+) increases (column 5). Figure 1 shows the amount of Ag^+ found in the cell and on the surface at various silver concentrations. The binding capacity of the cell surface for Ag^+ appears to be about three times greater than that of the intracellular sites at $\sim 46 \times 10^{-8}$ and 15.9×10^{-8} mol (mg dry weight)$^{-1}$ respectively, although the latter sites have higher affinity.

Table 2. Silver content of buffer, washings and cells in binding studies carried out in HEPES buffer at pH 7.4

1 Added silver	2	3	4	5	6	7	8	9
		Amount in supernatant	Amount in buffer wash	Amount in acid wash	Amount in cells	Total Ag^+	% recovered Ag^+ on	
Conc. mM*	Amount mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	Surface	Cell
0.001	2	0.204	0.0764	0.0347	1.85	2.16	1.61	85.7
0.008	16	1.39	0.0694	0.0903	13	14.5	0.62	89.7
0.016	32	3.82	0.204	0.671	26	30.7	2.18	84.6
0.080	160	1.38	0.287	57.9	88	148	39.1	59.4
0.160	320	3.70	1.29	231	139	375	61.6	37.1
0.800	1600	926	85.6	532	185	1729	30.8	10.7
1.600	3200	2870	125	579	208	3782	15.3	5.5

* 20 ml original volume, 0.654 mg dry weight of cells ml^{-1}

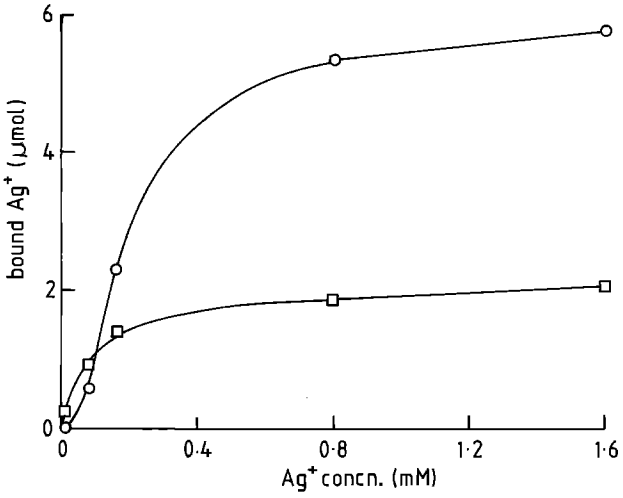

Fig. 1. Dependence of binding of silver to *E. coli* on Ag^+ concentration at pH 7.4. Stationary-phase, non-growing cells were resuspended in 20 ml HEPES-NaOH buffer (pH 7.4, dry weight of cells 0.65 mg ml^{-1}) at various silver concentrations for 1 h. O—O: surface bound silver (acid wash); □—□ intracellular silver

The data in Table 2 show that added silver up until 0.16 mM is accounted for by the sum of intracellular plus surface Ag^+, but that at higher concentrations the amount of silver present in the supernatant solution increases rapidly. The double reciprocal plot of [cell Ag^+] against [total Ag^+] at pH 7.4 is an excellent straight line, suggesting the presence of only one type of binding site for Ag^+ in the cell.

Table 3 gives the results of more detailed binding studies carried out on non-growing cells suspended in HEPES buffer at pH values 3.0 and 7.4 at concentrations of silver (1 to 16 μM) where the metal ion is largely taken up by the cell pellet and is not removed by acid-washing. The results at both pH values are very similar, and shows excellent agreement with the relevant data in Table 2. Figure 2 shows the uptake of Ag^+ at various concentrations of silver and demonstrates the reproducibility of the accumulation process. Table 3 and Fig. 2 also show data obtained for experiments in which silver was added to growing cells in the mid-exponential phase of growth, at silver concentrations above those found to inhibit growth completely. The results show that accumulation of Ag^+ by the silver-inhibited cells is much smaller than accumulation by the non-growing cells in buffer, even when allowance is made for the lower concentration of cells in the former

Table 3. Silver content of buffer, washings and cells in binding studies at low Ag^+ carried out in HEPES buffer at pH 7.4 and pH 3.0 and for cells in the mid-exponential phase of growth

Added silver		Amount in supernatant	Amount in buffer wash	Amount in acid wash	Amount in cells	Total Ag^+ found	Ag^+ in cell % of recovered
Final conc. mM	Amount mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	mol × 10^{-8}	
HEPES buffer pH 7.4[b]							
0.001	2	0.176	0.015		1.86[a]	2.05	90.7
0.004	8	0.962	0.053		6.99[a]	8.0	87.4
0.008	16	1.56	0.078		13.4[a]	15.0	89.3
0.012	24	2.76	0.291		17.8[a]	20.9	85.2
0.016	32	6.73	0.475		26.1[a]	33.3	78.4
HEPES buffer pH 3.0[c]							
0.001	2	0.032	0.028	0.014	1.85	1.92	96.4
0.004	8	0.074	0.065	0.155	7.58	7.87	96.3
0.008	16	0.162	0.148	0.431	13.2	13.9	95.0
0.012	24	0.278	0.241	0.463	19.2	20.2	95.0
0.016	32	0.343	0.718	0.787	25.5	27.3	93.4
Silver added to growing cells[d]							
0.001	2	0.489	0.067	0.023	(0.18)	0.762	(24.0)
0.004	8	3.22	0.127	0.042	4.63	8.02	57.7
0.008	16	12.5	0.308	0.066	5.93	18.8	31.5
0.012	24	20.8	0.306	0.051	7.18	28.3	25.4
0.016	32	22.7	0.833	0.163	9.03	32.7	27.6

[a] Acid wash and cell digest were combined before measurement of silver
[b] 20 ml original volume, 0.654 mg dry weight of cells ml^{-1}
[c] 20 ml original volume, 0.689 mg dry weight of cells ml^{-1}
[d] 20 ml original solution, 0.439 mg dry weight of cells ml^{-1}

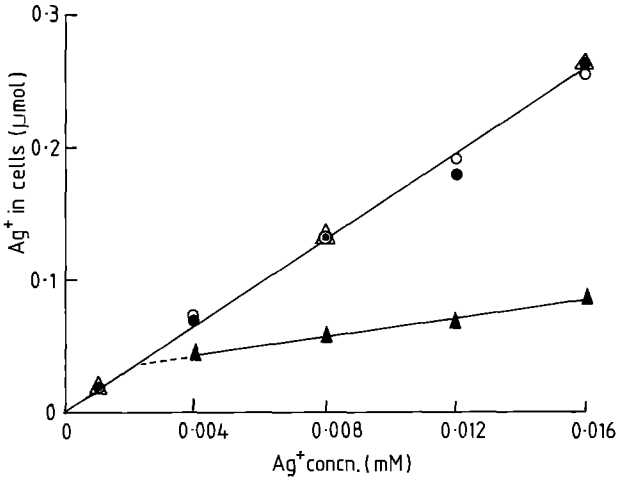

Fig. 2. Binding of silver by growing and non-growing *E. coli* and the effect of pH. Stationary-phase, non-growing cells were resuspended in HEPES-NaOH buffer (pH 7.4, dry weight of cells 0.65 mg ml^{-1}) at various silver concentrations for 1 h (●—●) or in HEPES-H_2SO_4 buffer (pH 3.0, dry weight of cells 0.69 mg ml^{-1}) for 1 h (○—○). Relevant results from Table 2 (pH 7.4) are included (△—△). An experiment was also carried out on growing cells in chloride-free growth medium (▲—▲). At optical density 0.4 (mid-exponential phase, dry weight of cells 0.44 mg ml^{-1}) various silver concentrations were added to the cells. The cells were harvested 1 h after addition of $AgNO_3$

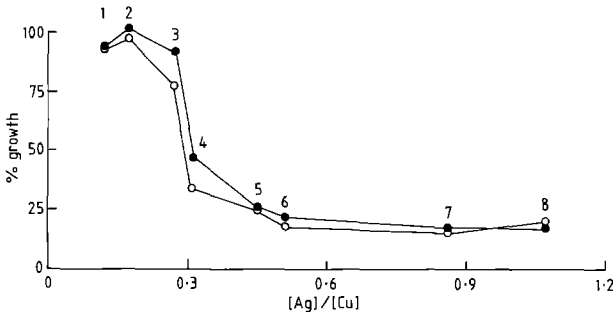

Fig. 3. The effect of silver-copper ratio on the growth of *E. coli* in continuous culture. Silver and copper concentrations (μM) respectively were: point 1; 0.13, 1.1: point 2; 0.36, 2.16: point 3; 0.30, 1.1: point 4; 0.66, 2.16: point 5; 0.60, 1.34: point 6; 0.565, 1.1: point 7; 0.94, 1.1: point 8; 0.60, 0.56. Values of pH were: 1; 7.90: 2; 7.98: 3; 7.91: 4; 7.41: 5; 7.27: 6; 7.42: 7; —: 8; 7.27. Growth is expressed as a percentage of control for biovolume (○) or OD_{600} (●)

case. Furthermore, the amount of silver in the acid wash is decreased. A possible explanation of this result is that the speciation of silver in the culture medium is different from that in buffer. However, it is noteworthy in Figure 2 that the plot of (bound Ag^+) against (added Ag^+) for silver-inhibited cells cuts the line for non-growing cells at about 2.2 μM Ag^+, close to the value which produces inhibition of growth of *E. coli*, suggesting that an explanation based upon speciation of silver is over simplified.

Effect of $AgNO_3$ on growth yield in chemostat culture

Figure 3 shows the effect on the growth of *E. coli* of varying the $[Ag^+]/[Cu^{2+}]$ ratio in the medium, growth being expressed as a percentage of the yield (as biovolume and OD) in the absence of silver. Increase in $[Ag^+]$ at a constant $[Cu^{2+}]$ of 1.1 μM (points 1, 3, 6, 7) resulted in decrease in growth yield, which eventually levelled off at about 15–20% of the control value. Data obtained at other copper concentrations (points 2, 4, 5, 8) also fall on the plot, showing that the toxic effects of silver depend on the $[Ag^+]/[Cu^{2+}]$ ratio rather than the concentration of silver. Thus points 4 and 6 in Figure 3 were obtained for silver concentrations of 0.66 and 0.57 μM, respectively, but the higher silver concentration exerts a smaller toxic effect due to the presence of higher copper concentration (2.16 μM).

It was found that copper was neither toxic nor growth-limiting over the range of entering copper concentrations used in this work (results not shown). Microscopic examination of the culture growing at high $[Ag^+]$ showed elongated cells and some long filaments (results not shown).

Addition of silver to the medium entering the chemostat had little effect on growth in chloride-containing medium. Additions of $AgNO_3$ to give 0.60 and 0.30 μM Ag^+ at 1.1 μM Cu^{2+} resulted in decreases in growth yield to 80%–92% and 92%, respectively of that found in the absence of silver. Concentrations of Ag^+ of 0.57 and 0.30 μM in the chloride-deficient medium resulted in biovolumes of 18 and 77% respectively. It appears, therefore, that, under the growth conditions employed in these experiments, the cells do not abstract Ag^+ from AgCl.

Distribution of silver and copper in chemostat samples

The bulk of the added copper was found to be present in the supernatant solution (between 85% and 93% of the total added), with very small amounts present in the acid wash, buffer wash and cell pellet. The recovery of copper was always slightly greater than the copper added due to its presence as an impurity in the medium chemicals.

Medium to which no copper was added still contained 0.3 μM copper from this source. Analysis for silver was unsatisfactory with poor agreement between added silver and total analysed silver (which gave a low answer). However, only a small fraction of the silver was found in the acid wash, with the bulk present in the cell pellet (results not shown), in accord with the conclusions obtained from the binding studies in batch culture. It appears that Ag^+ has a much higher affinity for cells than does Cu^{2+}.

Discussion

Biological methods are being applied increasingly to the recovery of metals from aqueous solutions, including, for example, the recovery of precious metals from the electroprocessing, photographic and jewellery industries (Morper 1986). Metal recovery or removal may involve binding to cell surfaces, intracellular translocation of the metal or the formation of extracellular metal precipitates. A commonly observed mechanism involves rapid surface binding to negatively charged groups on the cell wall, which may be followed by a slower, metabolism-dependent translocation to the cell interior.

The present results show that silver cations are accumulated by non-growing *E. coli* cells. At silver concentrations less than 16 μM, uptake is independent of pH, and the accumulated silver cannot be removed by subsequent acid washing of the cells. Furthermore, the Ag^+-inhibited cells have less uptake capacity than non-growing cells in buffer. Since surface-binding phenomena are usually independent of the viability of the cells, this suggests that accumulation is ultimately intracellular. It is probable that Ag^+ is taken up in a metabolism-independent process, and is then bound at specific sites inside the cell. Saturation of these intracellular sites then allows binding of Ag^+ on the cell surface. When both types of site are saturated, the total amount of Ag^+ accumulated from solution by non-growing cells in 67 mg (g dry weight)$^{-1}$. The silver accumulation capacity of various silver-tolerant microorganisms has been given by Pümpel and Schinner (1986). Accumulation by 19 tolerant bacterial strains lay between 7.3 and 44 mg (g dry weight)$^{-1}$, with a mean value of 23. It is noteworthy that our result with a laboratory strain of *E. coli* is higher, demonstrating that resistant organisms may not necessarily bind metals to a greater extent than non-resistant strains.

Silver ions exert toxic effects on growing cells. One indirect cause could be chloride-limitation. Thus, although the use of a nominally chloride-free medium does not lead to chloride-limitation of growth of *E. coli*, this may occur in the presence of Ag^+ as any chloride adventitiously present may be precipitated out of solution as the silver salt. This possibility is not easy to demonstrate. A more direct cause of toxicity is the binding of Ag^+ to essential sites such as proteins and enzymes. For example, inhibition of succinate dehydrogenase may result from the binding of Ag^+ to an iron-sulphur cluster with precipitation of silver sulphide. However, the evidence presented in this paper for the moderation of silver toxicity by added copper, as shown by the correlation of the toxic effects of silver on the growth of *E. coli* with the silver:copper ratio rather than with the concentration of silver alone, suggests that silver and copper are competing for sites at which Ag^+ exerts its toxic effects and that high copper concentrations protect cells by virtue of this competition. Alternatively, silver may compete for copper sites associated with the transport and function of copper so that the presence of silver leads, in effect, to copper deficiency. Indeed recent studies by Hubbard et al. (unpublished work) have demonstrated a growth requirement for copper. The function of copper is not yet established beyond doubt, but it appears to be associated with cytochrome *o* (Kita et al. 1984). The cells used in the present work will utilise cytochrome *o* as a major oxidase, as they were grown under conditions of adequate aeration. It would be of interest to determine levels of cytochrome *o* and other cytochromes in cells grown under various silver concentrations.

Figure 4 shows the cell metal content per unit of volume plotted against the silver-copper ratio in the medium. As this ratio is increased (to

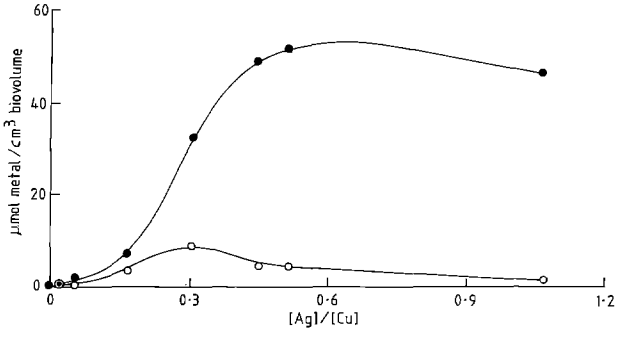

Fig. 4. Dependence of cellular concentrations of silver (●) and copper (○) on the $[Ag^+]/[Cu^{2+}]$ ratio in the medium

higher Ag^+) so the amount of silver in the cell rises to a maximum at $[Ag^+]/[Cu^{2+}]=0.5$. The concentration of copper in the cell also appears to increase until $[Ag^+]/[Cu^{2+}]=0.3$ but then progressively decreases. There have been no reported studies of the mechanism of copper transport by *E. coli* but the uptake of Ag^+ may allow some co-transport of Cu^{2+}. As the concentration of silver is increased so binding of Cu^{2+} cannot compete with that of Ag^+ and uptake of copper is diminished. If the toxicity of Ag^+ is linked to competition with Cu^{2+}, then toxic effects of Ag^+ should be enhanced at $[Ag^+]/[Cu^{2+}]>0.3$. It is noteworthy that repeated observation of cell yield and growth confirms that growth of cultures is very sensitive to changes in this ratio and becomes very poor as the ratio is increased above 0.3.

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