Investigation into the mechanism of copper uptake by Mycoplasma gallisepticum in the presence of 2,9-dimethyl-1,10-phenanthroline

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Introduction
In several papers from our group the anti-mycoplasmal activity of compounds containing a 2,2'-bipyridyl moiety is discussed.1-3 These compounds are active only in the presence of a substantial, but in itself nontoxic amount of copper. In search for the role of copper in the case of anti-mycoplasmal activity of 2,2'-bipyridyls, Antic et al. suggest that the copper(I) complex is the active species.6,7 This suggestion is supported by a qualitative structure-activity relationship study by Pijper.8 Investigations into the mode of action of the copper(I) complex of 2,9-dimethyl-1,10-phenanthroline (DMP) on Mycoplasma gallisepticum by Smit et al.9 show a strong inhibition of the energy yielding metabolism due to blockage of the conversion of pyruvate into lactate. Additionally an inhibition of the incorporation of 14C-thymidine into DNA has been found.

Interestingly, experiments with crude cell extracts have shown a strong inhibition of NADH oxidase and lactate dehydrogenase (LDH) by Cu(DMP)2NO3, but an even stronger inhibition by CuSO4. In these experiments DMP reduces the inhibitory effects of CuSO4 on both NADH oxidase and LDH. The fact that in crude cell extract copper ions are stronger inhibitors than the copper–DMP complex induced Smith et al. to investigate the copper uptake by Mycoplasma gallisepticum from a medium which contained, besides the normal nutrients, CuSO4 with or without certain concentrations of DMP.10 It was found that in the presence of DMP more copper was taken up, but also that in the cases when copper and DMP were added in a molar ratio 1:2 more copper than DMP was taken up. Copper and DMP are to be expected to be taken up in at least equimolar amounts in case a complex as such is taken up. Additionally, experiments replacing Adler medium by phosphate-buffered saline (PBS) showed that in the absence of copper-binding ligands from the medium, copper is taken up more efficiently.

In a more detailed study we reported on copper uptake by Mycoplasma gallisepticum in the presence of certain 2,2'-bipyridyls and its dependence on the concentration of the ligand present.11 It was established that at the minimal inhibitory concentration (MIC) of all investigated ligands the same amount of copper had been taken up and we suggested that copper itself might be the toxic species.

The results of these studies prompted us to investigate the mechanism of copper uptake. The following rather common mechanisms of metal ion transport across membranes have been described:12-16

Key words
Membrane potentials
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Abstract
In the presence of copper certain 2,2'-bipyridyls show anti-mycoplasmal activity, whereas copper itself causes a toxic effect. In this paper results are presented to elucidate the mechanism of copper uptake in the presence of 2,9-dimethyl-1,10-phenanthroline. The time course of copper and/or ligand uptake under the applied conditions is consistent with a carrier transport mechanism in which 2,9-dimethyl-1,10-phenanthroline operates as a carrier for copper ions. The influence of valinomycin on copper uptake indicates that the transmembrane potential is not the driving force in the carrier process.
- passive diffusion of a metal complex through the cell membrane;
- diffusion of a metal ion through a pore or channel in the cell membrane;
- carrier-mediated metal-ion transport across the cell membrane.

Attempts to elucidate the mechanism of copper uptake by *Mycoplasma gallisepticum* in the presence of DMP are complicated by the fact that these organisms only grow in very complex growth media containing small peptides and amino acids, both of which are known to chelate copper ions. Previously Eriks *et al.* determined the apparent formation constants of copper(II) complexes of Adler medium components: it has been found that in Adler medium only a small amount of the added copper is present as free ions. Considering this low free-copper(II)-ion concentration we decided to elucidate the time course of copper and ligand (DMP) uptake in Adler medium and additionally in PBS, in which only weak chelation of free copper ions takes place and therefore almost complete complex formation between copper and DMP might be expected.

The uptake was followed by measuring the amount of $^{64}$Cu and/or $^{14}$C-DMP bound to mycoplasma cells after a certain period of time. From the uptake versus time curves obtained under the different conditions employed we were able to conclude on the mechanism of uptake. As the uptake of charged particles might be governed by the membrane potential, we carried out some experiments in which the membrane potential was changed by the addition of valinomycin at various K$^+$ concentrations.

**Methods**

**Test Organism and Nutrient Medium**

The test organism was *Mycoplasma gallisepticum* strain K514 (Gist-brocades NV, Delft, the Netherlands). Stock cultures were maintained in Adler medium as described at $-20\,^\circ$C. The nutrient medium was a modified Adler and consisted of 17.5 g bactopepton (Oxoid, Basingstoke, UK), 5.3 g yeast extract (Oxoid), 9.6 g D-glucose, 4.35 g NaCl, 2.1 g Na$_2$HPO$_4$·2H$_2$O, 25 mg phenol red, 150 ml inactivated horse serum (Flow laboratories, Irvine, UK), and 10$^9$ U penicillin G (Gist-brocades) per liter final medium. Before adding the filter-sterilized horse serum and penicillin, the medium was sterilized during 30 min at 110$^\circ$C.

**Chemicals**

$^{64}$Cu was obtained as aqueous solution of cupric chloride from (Amersham, UK). $^{14}$C-DMP was prepared by Smit *et al.* Valinomycin was obtained from Boehringer Mannheim (Mannheim, FRG) and DMP from Aldrich Europe (Brussels, Belgium). All other chemicals were of analytical grade and were used without further purification. Water was freshly distilled from an all glass still after deionization over a mixed-bed ion exchanger.

**Assay for the Uptake of $^{64}$Cu and $^{14}$C-DMP**

Cells of *Mycoplasma gallisepticum* were grown in Adler medium at 37$^\circ$C, kept at a pH of about 7.0 and a cell density of about 0.2 OD (optical density, measured spectrophotometrically as absorbance at $\lambda = 660$ nm). This culture was used for inoculation in experiments with Adler medium. When phosphate-buffered saline (PBS; $1 \times 10^{-3} M$ Na-phosphate + 0.85% wt/vol NaCl, pH = 7.2) was used, mycoplasma cells from a culture maintained as described were harvested by centrifugation (8,000 g, 4$^\circ$C); the cells were then resuspended in PBS and diluted to the desired dry weight and used for inoculation.

Test tubes containing 5 ml Adler medium or PBS with either $^{64}$Cu, $^{14}$C-DMP or $^{64}$Cu + $^{14}$C-DMP were placed in a water bath maintained at 37$^\circ$C. After inoculation with 1 ml *Mycoplasma gallisepticum* culture (Adler medium or PBS) the samples were filtered at various time intervals through a membrane filter with a pore size of 0.2 $\mu$m (Sartorius, Göttingen, FRG; type SM 25, No. N 11307) and washed twice with 1 ml PBS. Scintillation fluid (5 ml) was added to the filters placed in a vial. $^{64}$Cu was counted by liquid scintillation counting in the $^{32}$P channel on an LKB rackbeta scintillation counter (LKB, Bromma, Sweden). After at least ten times the half-life time of $^{64}$Cu (12.7 h), the samples were counted again and the counts resulting from $^{14}$C-DMP were subtracted from the former value given after correction for decay, efficiency, background and conversion, and the amount of copper. $^{14}$C-DMP was counted by liquid scintillation counting in the $^{14}$C channel after decay of $^{64}$Cu.

**Preparation of the Filters**

The filters were placed in a vacuum filter unit, wetted with 0.5 ml PBS and allowed to swell for 10 min. The top plate was fixed tightly and the filters were washed after washing twice with 1.0 ml PBS. The washing procedure of cells resulted for each test series in a constant amount of activity bound to the filters.

**Determination of Mycoplasmal Dry Weight**

Dry weight was determined according to the following procedure. Samples of 200 ml of culture fluid and of fresh medium were centrifuged for 30 min at 17,000 g at 4$^\circ$C. The pellets were suspended in exactly 5.0 ml PBS and subsequently dried by heating at 100$^\circ$C for 24 h. The difference in weight between culture and medium sample gave the mycoplasma dry weight. In addition, a calibration line for spectrophotometric dry-weight determination was made as follows. A dilution series of the above mycoplasmal culture was made using fresh culture medium. A sample of 2.5 ml and 0.1 ml 1 M PBS buffer was placed in a cuvette and measured at $\lambda = 660$ nm against fresh culture medium. The resulting line ($r = 0.9977$; $n = 9$) was used for rapid determination of dry weight with all experiments.

**Determination of Copper Uptake in the Presence of Valinomycin and K$^+$**

*Mycoplasma gallisepticum* was grown as described above. To determine the copper uptake 2 ml of a mycoplasmal culture were added to 6 ml Adler medium containing 16.7 $\mu$g/ml CuSO$_4$·5H$_2$O, 0.027 $\mu$g/ml DMP and if suitable valinomycin (final concentration $5 \times 10^{-5} M$). In the case of high K$^+$ concentration KCl instead of NaCl was used during the preparation of the medium. The test tubes containing the culture were incubated at 37$^\circ$C for 30 min. After filtration through a membrane filter and washing the