Mode of Action of the Copper(I) Complex of 2,9-Dimethyl-1,10-Phenanthroline on *Mycoplasma gallisepticum*

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Various physiological important activities of *Mycoplasma gallisepticum* were inhibited by the copper(I) complex of 2,9-dimethyl-1,10-phenanthroline [Cu(DMP)₂NO₃]. The energy-yielding metabolism was inhibited because the conversion of pyruvate into lactate was found to be blocked by Cu(DMP)₂NO₃, indicating a selective inhibition of lactate dehydrogenase. Also, the production rate of acetate and the rate of oxygen uptake by whole cells of *M. gallisepticum* appeared to be strongly decreased. Experiments with crude cell extracts showed an inhibition of reduced nicotinamide adenine dinucleotide (NADH) oxidase by Cu(DMP)₂NO₃ and an even stronger inhibition of NADH oxidase and lactate dehydrogenase by CuSO₄. No preferential inhibition of adenosine 5′-triphosphatase and pyruvate kinase was found. Investigations on the influence of Cu(DMP)₂NO₃ on deoxyribonucleic acid, ribonucleic acid, and protein synthesis with growing cells of *M. gallisepticum* showed a selective inhibition of the incorporation of [³⁵S]thymidine into deoxyribonucleic acid. Cu(DMP)₂NO₃ induced a decrease in the total amount of accessible sulfhydryl groups of whole cells of *M. gallisepticum*, indicating that the observed diverse toxicity of Cu(DMP)₂NO₃ may be associated with the interaction of copper ions with protein sulfhydryl groups.

Investigations on the mode of action of the copper(I) complex of 2,9-dimethyl-1,10-phenanthroline [Cu(DMP)₂NO₃] on *Paracoccus denitrificans* have been described previously (25). Interference with the cytoplasmic membrane, resulting in inhibition of respiratory electron transport, was found to constitute the main mode of action of this copper complex. Cu(DMP)₂NO₃ and related copper complexes are also known to have a strong growth inhibitory effect on *Mycoplasma gallisepticum* (1, 2). This organism is pathogenic for fowl, in which it attacks the epithelium of the respiratory tract.

There are many differences in morphology, ultrastructure, and physiology between *P. denitrificans* and *M. gallisepticum*. *P. denitrificans* is a nonfermentative, gram-negative bacterium. It is dependent on respiratory electron transport for the supply of energy (4) and is able to grow in minimal synthetic media. *M. gallisepticum* is one of the fermentative mycoplasmas in which adenine 5′-triphosphate (ATP) is formed during glycolysis (23). In this organism, respiration is not accompanied by oxidative phosphorylation; however, some additional ATP is generated aerobically from acetyl phosphate and adenosine 5′-diphosphate (ADP) (14). Other features of *M. gallisepticum* are the lack of a cell wall and the requirement of a complex growth medium.

In this paper, we present the results of our investigations with *M. gallisepticum* concerning the effects of Cu(DMP)₂NO₃ on: (i) the energy-yielding metabolism of growing cells in continuous culture; (ii) various enzyme activities in crude cell extracts; (iii) macromolecular synthesis in growing cells; and (iv) the number of accessible sulfhydryl groups of whole cells.

**MATERIALS AND METHODS**

Test organism and nutrient media. The test organism was *M. gallisepticum* strain K514, which was obtained from Gist Brocades N.V., Delft, The Netherlands. Stock cultures were maintained in Adler medium as described previously (1) at −20°C. The nutrient medium used for pH-auxostat experiments consisted of 14.8 g of Bactopepton (Oxoid Ltd.), 5.0 g of yeast extract (Difco Laboratories), 7.4 g of d-glucose, 4.5 g of NaCl, 0.2 g of Na₂HPO₄·2H₂O, 150 ml of inactivated horse serum (Flow Laboratories) and 10⁶ of penicillin G (Mycofarm Delft) per liter of final
medium. Before the addition of the filter-sterilized horse serum and penicillin, the medium was sterilized for 3.5 h at 100°C.

Cultivation in pH-auxostat culture. All pH-auxostat cultures were performed in a 2-liter fermentor with a working volume of 1 liter. About 900 ml of fresh medium was brought into the fermentor vessel and was subsequently inoculated with 100 ml of stock culture. The temperature was held constant at 38°C, and the agitation speed was 285 rpm. A pH electrode was inserted into the culture. A control unit consisting of a pH meter and an electronic on-off regulator for mA systems automatically controlled the pH of the growth medium and the inflow of fresh medium. When the pH of the growth medium fell below the predetermined value of 7.0, two peristaltic metering pumps were switched on, one for the addition of fresh medium and culture discharge, the other for the supply of 0.5 N NaOH. A more detailed description of the apparatus is given by Oltmann et al. (21). Gas was led through the culture and the fresh medium at a constant rate of 15 liters/h.

Analytical determinations. Glucose was determined by the method of Werner et al. (29). Lactate was measured by the method of Hohorst (10). Acetate was determined by the enzymatic method of Rose et al. (24), which is based on the colorimetric determination of acetyl phosphate as described by Lipmann and Tuttle (16). Pyruvate was measured enzymatically with lactate dehydrogenase as described by Van Gent-Ruijters et al. (28).

Determination of mycoplasmal dry weight. Dry weight was determined by taking samples of 200 ml of the culture fluid and of the fresh medium. Both samples were centrifuged for 30 min at 17,000 x g at 4°C. The pellets were suspended in exactly 5.0 ml of PBS buffer (0.06 M sodium phosphate + 0.85% NaCl, pH 7.4) and subsequently dried by heating at 100°C for 24 h. The difference in weight between culture and medium sample gave the mycoplasma dry weight.

Test substances. 2,9-Dimethyl-1,10-phenanthroline (DMP) was commercially available (Merek & Co., Inc.). The copper(I) complex of DMP, Cu(DMP)2NO3, was obtained from the laboratory stock (11).

Preparation of crude cell extracts. Cells of M. gallisepticum were harvested in the exponential phase of growth (optical density at 600 nm = 0.2). The cells were washed twice at 4°C in 10 mM sodium phosphate buffer at pH 7.4 and resuspended in the same buffer in a volume of 1/5 of the original culture. Extracts were prepared by sonic disruption of the cells, using an MSE ultrasonic power unit for three 1-min periods. The resulting material was centrifugated at 40,000 x g for 30 min, and the supernatant was taken as the crude cell extract. Protein was measured by the method of Lowry et al. (17), using bovine serum albumin as a standard.

Incorporation measurements of 14C-labeled substrates. Experiments on the incorporation of thymidine, uracil, or phenylalanine into mycoplasmal deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, respectively, were carried out with exponentially growing cultures of M. gallisepticum. To obtain optimal uptake of labeled precursors, cells of M. gallisepticum were cultivated for about 10 generations in Adler medium supplemented with 5 μg of thymidine, 20 μg of uracil, 20 μg of phenylalanine, and 15 μg of deoxyadenosine per ml. The growing culture was diluted in 4 volumes of Adler medium supplemented with 2 μg of thymidine, 20 μg of uracil, 20 μg of phenylalanine, and 30 μg of deoxyadenosine per ml and various amounts of Cu(DMP)2NO3. Experiments were carried out with 0.1 μCi/ml of [14C]thymidine, [14C]uracil, and [14C]phenylalanine for incorporation in DNA, RNA, and protein, respectively. The incorporation of radioactive labeled material into acid-insoluble material was followed during 8 h of incubation. At intervals, 0.5-ml samples were removed from the culture and added to 4.5 ml of 5% trichloroacetic acid, supplemented with 20 μg of unlabeled substrate per ml. After standing for 60 min at 0°C, the mixtures were filtered on membrane filters (standard grade: Oxoid Ltd.) and washed twice with 5 ml of the same trichloroacetic acid solution. After drying, radioactivity on the filters was determined by scintillation counting.

RESULTS

Effects of Cu(DMP)2NO3 on the energy-yielding metabolism of growing cells of M. gallisepticum in continuous culture. To study the effects of Cu(DMP)2NO3 on the energy-yielding metabolism of M. gallisepticum, we determined the various parameters related to growth and carbohydrate metabolism under standardized conditions (Table 1). M. gallisepticum was cultivated in a continuous culture, using the pH-auxostat method (21). This method has some advantages, compared with the traditional batch culture method. Oxygen supply and pH remain constant throughout the growth period, and an accurate determination of mycoplasmal growth rate is possible. Oxygen strongly affected the energy-yielding metabolism. When air (about 20% O2) was bubbled into the culture medium, mycoplasmal growth stopped completely within 20 h. The loss of viability under these conditions was probably caused by the accumulation of toxic amounts of H2O2, as described by Razin (22).

When 5 or 1% O2 was used, growth under steady-state conditions was possible. Therefore, most experiments were carried out with 5 or 1% O2. Unless otherwise stated, no important differences were observed between these two growth conditions.

The effects of Cu(DMP)2NO3 on growth rate and dilution rate under conditions of 5% O2 in the atmosphere are shown in Fig. 1. It should be noted that a rather high concentration (2.8 μM) of Cu(DMP)2NO3 was necessary to avoid adaptation of the cells during prolonged incubation.

We also investigated the influence of Cu(DMP)2NO3 on growth rate and dilution rate under anaerobic conditions. Although anaerobic
growth was possible in pH-auxostat culture for several days with a maximal growth rate of 0.062 h⁻¹, no real steady state could be maintained.

We observed a gradual decrease in growth rate which started about 2 days after inoculation. This might be caused by a small deficiency of some essential growth factor, but the precise reason remains obscure.

In the absence of Cu(DMP)₂NO₃, growth rate decreased from 0.060 h⁻¹ at 50 h after inoculation to 0.042 h⁻¹ at 75 h after inoculation. When 2.8 µM Cu(DMP)₂NO₃ was added at 50 h after inoculation, growth rate decreased much faster to 0.005 h⁻¹ at 75 h after inoculation. The dilution rate paralleled the growth rate in the absence of Cu(DMP)₂NO₃ but decreased more slowly than the growth rate after addition of Cu(DMP)₂NO₃ to 0.016 h⁻¹ at 75 h after inoculation.

Since respiratory electron transport of P. denitrificans was strongly inhibited by Cu(DMP)₂NO₃, the influence of Cu(DMP)₂NO₃ on mycoplasmal oxygen uptake was investigated. Respiratory activity appeared to be inhibited up to 55% (Fig. 2).

The specific rate of consumption of glucose (q glucose) and the specific rate of production of acetate, lactate, and pyruvate changed markedly after the addition of Cu(DMP)₂NO₃ (Fig. 3). Shortly after the Cu(DMP)₂NO₃ was added, a strong decrease of q glucose, q lactate, and q acetate and a remarkable increase of q pyruvate was observed. After prolonged incubation acetate production recovered, whereas the amount of pyruvate produced per mole of glucose slightly decreased. When 1% O₂ was used, comparable results were obtained during the first hour after the addition of Cu(DMP)₂NO₃. Later, q lactate increased from 2 to 4 mmol g⁻¹ h⁻¹, whereas q acetate remained very low (<0.2 mmol g⁻¹ h⁻¹).

The results presented in Fig. 2 and 3 demonstrate that Cu(DMP)₂NO₃ induces important changes in the energy-yielding metabolism of M. gallisepticum.

Effects of Cu(DMP)₂NO₃ on various enzyme activities in crude cell extracts of M. gallisepticum. Most enzymes which function in the energy-yielding metabolism of M. gallisepticum are located in the cytoplasm (22). Crude cell extracts were used to investigate the influence of Cu(DMP)₂NO₃, CuSO₄, and DMP on reduced nicotinamide adenine dinucleotide (NADH) oxidase, lactate dehydrogenase (LDH),

**Table 1. Growth parameters from pH-auxostat cultures of M. gallisepticum with different oxygen concentrations in the atmosphere**

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Generation time (h)</th>
<th>Growth rate (h⁻¹)</th>
<th>Yglycoo</th>
<th>Amt produced (mol/mol of glucose)</th>
<th>YATP</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lactate</td>
<td>Acetate</td>
</tr>
<tr>
<td>5% O₂</td>
<td>6.6</td>
<td>0.105</td>
<td>11.2</td>
<td>1.70</td>
<td>0.24</td>
</tr>
<tr>
<td>1% O₂</td>
<td>7.3</td>
<td>0.095</td>
<td>9.2</td>
<td>1.75</td>
<td>0.17</td>
</tr>
<tr>
<td>0% O₂</td>
<td>11.2</td>
<td>0.062</td>
<td>7.6</td>
<td>1.93</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Growth rate μ was calculated from the observed dilution rate D (D = inflow of fresh medium (liters per hour/culture volume (l))); the relation between growth rate and dilution rate is as follows.

\[ μ = D + \frac{1}{x} \frac{dx}{dt} \]

in which \( x = \) cell density in grams (dry weight) per liter and \( t = \) time in hours. Under steady-state conditions, \( μ = D \). \( Y_{\text{glycoo}} = \) grams (dry weight) produced per mole of glucose consumed. \( Y_{\text{ATP}} = \) grams (dry weight) produced per mole of ATP produced (26). The moles of ATP produced per mole glucose were calculated from glucose, lactate, acetate, and pyruvate concentrations, assuming a glycolytic pathway and the formation of 1 additional mol of ATP per mol of acetate produced. The rates of metabolism of glucose, acetate, lactate, and pyruvate were measured in the continuous culture. Therefore, culture samples were taken at the indicated times and were immediately centrifuged (15 min; 14,000 × g, 4°C). The supernatant was stored at −20°C until analytical determinations were carried out. Calculation of \( q \) values was based on the following equation:

\[ q = \frac{D(S_i - S) - \frac{ds}{dt}}{x} \text{ (mmol g}^{-1} \text{h}^{-1}) \]

in which \( q = \) specific rate of consumption or production, \( D = \) dilution rate (per hour), \( x = \) cell density (grams per liter), \( t = \) time (hours), \( S_i = \) substrate or product concentration in fresh medium (millimoles per liter), and \( S = \) substrate or product concentration in culture vessel at time \( t \) (millimoles per liter).

* Because, under anaerobic conditions, no real steady state was observed, values corresponding to maximal growth rate are shown.
Fig. 1. Influence of Cu(DMP)$_2$NO$_3$ on growth rate and dilution rate of M. gallisepticum, cultivated in pH-auxostat culture. At the indicated time (arrow), Cu(DMP)$_2$NO$_3$ was added to the culture vessel and the medium vessel to a final concentration of 2.8 μM. Gas containing 5% O$_2$ and 95% N$_2$ was led into the culture and the fresh medium. Initial cell density was 0.15 g (dry weight) per liter.

Fig. 2. Influence of Cu(DMP)$_2$NO$_3$ on respiratory activity of M. gallisepticum cultivated in pH-auxostat culture. Growth conditions are described in the legend to Fig. 1. At intervals, 50 ml of the culture overflow was collected. Cells were harvested (15 min; 14,000 × g; 4°C) and resuspended in 10 mM sodium phosphate buffer (pH 7.4) containing 0.85% NaCl and 1 mg of yeast extract per ml (PBSY buffer) to a cell density of 15 mg (dry weight) per ml. The rate of oxygen uptake was determined polarographically, using the oxygen pulse method as described previously (25) in PBSY buffer supplemented with 8 mM sodium lactate. The final cell density was 3 mg (dry weight) per ml. The 100% respiratory activity corresponds to an oxygen uptake of 6.7 nmol of O$_2$/min per mg (dry weight).

Fig. 3. Influence of Cu(DMP)$_2$NO$_3$ on specific rate of consumption of glucose and specific rates of production of lactic acid, acetic acid, and pyruvic acid of M. gallisepticum cultivated in pH-auxostat culture. Growth conditions are described in the legend to Fig. 1.

and pyruvate kinase (Table 2). NADH oxidase activity was inhibited by Cu(DMP)$_2$NO$_3$ at a rather low concentration, and a strong inhibition of both NADH oxidase and LDH activities by CuSO$_4$ was observed. Pyruvate kinase was only affected by a rather high concentration of CuSO$_4$. The free ligand (DMP) had no measurable effect on these enzymes.

Adenosine 5′-triphosphatase (ATPase) is located at the inner site of the cytoplasmic membrane (20). ATPase activity was determined in isolated membranes. The specific activity was 40 nmol of P$_i$ per mg of protein per min. Also, in the crude cell extract, ATPase activity could be demonstrated with a specific activity that differed only slightly from the activity in the membrane fraction. Investigation of the effect of Cu(DMP)$_2$NO$_3$ on the ATPase activity was not possible. For an accurate determination of ATPase activity, a high concentration of protein and Cu(DMP)$_2$NO$_3$ had to be used, which resulted in precipitation of Cu(DMP)$_2$NO$_3$. Therefore, only the effect of CuSO$_4$ on ATPase activity was studied. To compare the effect of CuSO$_4$ on
at room temperature. LDH activity was determined by the decrease in extinction of NADH at 340 nm. With NADH oxidase, the reaction was started by the addition of 0.25 mM NADH. LDH activity was determined under anaerobic conditions, because under aerobic conditions, NADH oxidase competes for the common substrate NADH. With LDH, NADH was added to the anaerobic reaction mixture. After incubation with (or without) Cu(DMP)$_2$NO$_3$, the reaction was started by the addition of 4 mM pyruvate. With pyruvate kinase, 1.6 mM phosphoenolpyruvate was added to the reaction mixture. After incubation, the reaction was started by the addition of 1.6 mM ADP and incubation was continued for 100 min, after which trichloroacetic acid was added up to 2.5%. After precipitation and centrifugation, the pH of the supernatant was adjusted to 7.3, and pyruvate was assayed as described in the text. Specific activity of NADH oxidase was 770 nmol of NADH/mg of protein per min. Specific activity of LDH was 630 nmol of NADH/mg of protein per min. Specific activity of pyruvate kinase was 55 nmol of pyruvate/mg of protein per min. Concentrations higher than 40 μM Cu(DMP)$_2$NO$_3$ could not be tested accurately because of precipitation of the complex. ID$_{50}$, 50% inhibitory dose.

ATPase with that on NADH oxidase, we determined both activities in crude cell extracts with high protein concentration. CuSO$_4$ inhibited NADH oxidase more strongly than ATPase (Fig. 4).

Effects of Cu(DMP)$_2$NO$_3$ on macromolecular synthesis of growing cells of M. gallisepticum. Figure 5 shows the combined results of incorporation experiments with $^{14}$C-labeled precursors of protein, DNA, and RNA. It appeared that after addition of 0.74 μM Cu(DMP)$_2$NO$_3$, the rates of incorporation of $[^{14}\text{C}]$phenylalanine and $[^{14}\text{C}]$uracil decreased to nearly the same extent but the incorporation rate of $[^{14}\text{C}]$thymidine decreased more strongly. It was calculated that incorporation of labeled precursors in protein, RNA, and DNA was inhibited 40, 47, and 78%, respectively, after 8 h of incubation. Inhibition of growth was estimated from acid production, determined from h 4 to 8 after addition of Cu(DMP)$_2$NO$_3$. Acid production decreased 46% in the presence of 0.74 μM Cu(DMP)$_2$NO$_3$. The results indicate a possible selective inhibition of DNA synthesis. Comparable results were obtained when concentrations of 0.19 and 2.96 μM Cu(DMP)$_2$NO$_3$ were used.

Effect of Cu(DMP)$_2$NO$_3$ on the number of accessible sulfhydryl groups of whole cells of M. gallisepticum. Various metal complexes, including copper-1,10-phenanthroline (9), are able to interact with free sulfhydryl groups of physiologically essential proteins (11) or cofactors (13). Also, compounds not structurally related to Cu(DMP)$_2$NO$_3$, but having a similar mode of antimicrobial action, are known to affect the number of sulfhydryl groups (19). The influence of Cu(DMP)$_2$NO$_3$ on free thiol content of whole cells of M. gallisepticum is shown in Table 3. A significant reduction of the total number of accessible sulfhydryl groups was observed after incubation of M. gallisepticum in the presence of Cu(DMP)$_2$NO$_3$.

![Figure 4](http://aac.asm.org/)

**FIG. 4.** Influence of CuSO$_4$ on ATPase and NADH oxidase activities in crude cell extracts of M. gallisepticum. The crude cell extract was isolated as described in the text except that the phosphate buffer was replaced by a 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid buffer (HEPES buffer), pH 7.5, supplemented with 145 mM NaCl. ATPase activity was determined as described by Tsuchiya and Rosen (method II) (27). NADH oxidase activity was determined as described in Table 2, footnote a. Protein concentration was 3 mg/ml. Incubation was at room temperature for 30 min. Specific activity of ATPase: 41 nmol of P$_i$/mg of protein per min. Specific activity of NADH oxidase: 770 nmol of NADH/mg of protein per min.
FIG. 5. Influence of Cu(DMP)\(_2\)NO\(_3\) on macromolecular synthesis of \(M.\) gallisepticum. At zero time, 0.74 \(\mu\)M Cu(DMP)\(_2\)NO\(_3\) and one of the \(14\)C labeled precursors were added to a growing culture of \(M.\) gallisepticum. The amount of incorporation of \(14\)C-labeled precursors into acid-insoluble material is presented in relative units compared with the data obtained with a reference culture with no Cu(DMP)\(_2\)NO\(_3\). The initial cell density was 85 \(\mu\)g/ml.

Table 3. Effect of Cu(DMP)\(_2\)NO\(_3\) on the total number of accessible sulfhydryl groups of whole cells of \(M.\) gallisepticum

<table>
<thead>
<tr>
<th>Cu(DMP)(_2)NO(_3) concn ((\mu)M)</th>
<th>SH ((\mu)mol/g [dry wt])</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.3</td>
</tr>
<tr>
<td>1</td>
<td>9.7</td>
</tr>
<tr>
<td>2</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>3.1</td>
</tr>
<tr>
<td>8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\(M.\) gallisepticum was cultivated in batch cultures until the cell density reached 0.09 mg (dry weight) per ml. To a culture volume of 800 ml, 200 ml of fresh Adler medium was added, the pH was adjusted to 7.0, and the culture was divided into five portions of 200 ml with an initial cell density of 0.07 mg (dry weight) per ml. Cu(DMP)\(_2\)NO\(_3\) was added in a concentration of 0, 1, 2, 4, and 8 \(\mu\)M, respectively, and the cultures were incubated for 4 h. After incubation, cells were harvested and washed twice in PBS buffer and resuspended to a cell density of 3.5 mg (dry weight) per ml in PBS buffer. The total number of accessible sulfhydryl groups was determined spectrophotometrically at 324 nm, using 4,4′-dithiopyridine as described previously (8). SH. Accessible sulfhydryl groups.

**DISCUSSION**

In this paper, previous studies on the mode of action of copper complexes of some 2,2′-bipyridyl analogs on \(P.\) denitrificans are extended with an investigation of the mode of action of Cu(DMP)\(_2\)NO\(_3\) on \(M.\) gallisepticum.

The presence of Cu(DMP)\(_2\)NO\(_3\) induced a number of effects on the energy-yielding metabolism of \(M.\) gallisepticum. Under all cultivation conditions, mycoplasmal growth rate decreased immediately after addition of Cu(DMP)\(_2\)NO\(_3\) (Fig. 1), and oxygen consumption was inhibited (Fig. 2). However, in contrast with \(P.\) denitrificans, oxygen consumption of \(M.\) gallisepticum seemed not to be essential for growth (Table 1), and consequently, inhibition of oxygen consumption is not likely to be the main effect of this copper complex on \(M.\) gallisepticum.

The product balance of glucose was changed drastically and quickly by Cu(DMP)\(_2\)NO\(_3\), especially the accumulation of pyruvate indicates that the energy-yielding metabolism is selectively inhibited (Fig. 3). Presumably, pyruvate is accumulated because LDH, which catalyzes the conversion of pyruvate into lactate, is inhibited, since lactate production was also strongly decreased. The minor degradation route of pyruvate to acetate was blocked too, since acetate production ceased shortly after addition of Cu(DMP)\(_2\)NO\(_3\). The consequence of the inhibition of pyruvate degradation accompanied by the inhibition of oxygen uptake would be a deficiency of oxidized nicotinamide adenine dinucleotide, resulting in the observed decrease of consumption of glucose (Fig. 3).

Under anaerobic conditions, no acetate production took place (Table 1), and only the conversion of pyruvate into lactate could be blocked, probably as the result of a selective inhibition of LDH.

From experiments with crude cell extracts, it appeared that NADH oxidase was strongly inhibited by Cu(DMP)\(_2\)NO\(_3\), and CuSO\(_4\) (Table 2). This is in agreement with the observed inhibition of NADH oxidase of \(P.\) denitrificans (25). In the conversion of pyruvate into acetate, pyruvate dehydrogenase and NADH oxidase are involved. Pyruvate dehydrogenase activity could not be determined accurately since LDH had a much higher specific activity in cell extracts of \(M.\) gallisepticum. NADH, which might be produced by the pyruvate dehydrogenase reaction, would be used immediately for the reduction of pyruvate into lactate by LDH. Thus, it is not clear whether the observed strong inhibition of NAD- oxidase completely accounts for the inhibition of acetate production.

It should be noted that a possible interaction between Cu(DMP)\(_2\)NO\(_3\) and dihydrolipoic acid would also induce a blockade in the conversion of pyruvate into acetate. Dihydrolipoic acid is a cofactor of the pyruvate dehydrogenase com-

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plex, which possesses two free sulphydryl groups and is involved with the oxidation of pyruvate to acetyl coenzyme A. The mechanism of action of, for instance, the copper-chelating dithiocarbamates (13) and dibutyltin dichloride (5) is suggested to be the oxidation of dihydrolipoic acid.

It is known that the copper(II) complex of 1,10-phenanthroline and copper(II) ions are able to cause structural changes in the ATPase enzyme (11). Organolead compounds (6) and alkyltin compounds (5), which resemble Cu(DMP)\(_2\)NO\(_3\) in affecting microbial growth and energy metabolism, are also known to inhibit this enzyme. However, we did not observe a strong inhibition of ATPase (Fig. 4) in crude cell extracts. In fact, NADH oxidase and LDH were inhibited much more strongly by Cu(DMP)\(_2\)NO\(_3\) or CuSO\(_4\). The high 50% inhibitory dose value of CuSO\(_4\) for pyruvate kinase (Table 2) indicates that this enzyme is not preferentially inhibited. These results are in agreement with the suggestion, based on experiments with growing cells, that the conversion of pyruvate into acetate and lactate is preferentially inhibited by Cu(DMP)\(_2\)NO\(_3\).

The fact that CuSO\(_4\) is more active in inhibiting enzyme activities in crude cell extracts than is Cu(DMP)\(_2\)NO\(_3\), whereas the opposite is true for the inhibition of cell growth, suggests that dissociation of the copper complex must take place. However, various ways are possible, e.g., formation of Cu(DMP)\(^+\), Cu\(^+\), or, after oxidation, even Cu\(^2+\). The stability constant of Cu(DMP)\(^+\) is reported in the literature (18), with a value of \(\log K_1 + \log K_2 = \log K = 19.1\). Various copper(II)-peptide complexes, however, are known to have comparable or even much higher stability constants (18).

The inactivity of CuSO\(_4\) on growing cells of \textit{M. gallisepticum} may be caused by inactivation of the copper ions by complex formation with medium components (2, 25). Also, the penetration of the lipophilic cytoplasmic membrane may be difficult for the hydrophilic copper ions, whereas penetration of a more lipophilic copper complex may be easier.

We observed a selective inhibition of incorporation of thymidine in DNA (Fig. 5). This is in remarkable contrast to the results of similar experiments with \textit{P. denitrificans}, in which no selective inhibition was found (25). The inhibition of incorporation of a DNA precursor might suggest a preferential blockade of DNA synthesis, although an alternative explanation would be the occurrence of a selective inhibition of thymidine uptake into the mycoplasmal cell.

We conclude that various biological activities can be inhibited by Cu(DMP)\(_2\)NO\(_3\) or a dissociation product of this complex. We also observed that the mode of action of Cu(DMP)\(_2\)NO\(_3\) on \textit{M. gallisepticum} differs from that on \textit{P. denitrificans}, although some similarities have been found. Therefore, it is unlikely that a selective, common mode of drug action exists for Cu(DMP)\(_2\)NO\(_3\).

The molecular mechanism responsible for the observed diverse toxicity of Cu(DMP)\(_2\)NO\(_3\) may be associated with the interaction of copper ions with protein sulphydryl groups which has been described frequently (3, 9, 12). The results presented in Table 3 give support to this idea. However, other possible interactions of copper ions with biological molecules have to be considered. Model studies of the coordination of copper in biological systems showed the imidazole nitrogen (histidine), the terminal amino nitrogen (lysine), and the deprotonated peptide nitrogen to be potential binding sites for copper (15). Still another mode of action has been suggested by Graham et al., who reported superoxide formation induced by 1,10-phenanthroline copper(I) complex to be responsible for the oxygen-dependent cleavage of DNA (7).

The observed inhibition of several physiological processes confirms that copper compounds are able to interact with diverse biological molecules. Further studies on the molecular mechanism of action of Cu(DMP)\(_2\)NO\(_3\) are being pursued in these laboratories. In particular, the possible transport function of the ligand will be investigated, using radiolabeled copper and DMP.

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LITERATURE CITED


