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INHIBITION OF NADH OXIDASE AND LACTATE DEHYDROGENASE OF *MYCOPLASMA GALLISEPTICUM* BY COPPER COMPLEXES OF 2,2′-BIPYRIDYL ANALOGUES

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Abstract—In the presence of copper, 2,2′-bipyridyl analogues possess growth-inhibitory activity against *Mycoplasma gallisepticum*. Inhibition of the energy yielding metabolism plays a role in the mechanism of action. We showed that probably inhibition of lactate dehydrogenase and NADH oxidase is involved. Both enzymes were inhibited *in vitro* and *in vivo* by several copper 2,2′-bipyridyl complexes. A two-step mechanism of action is proposed, i.e. first a copper complex enters the cell, then after dissociation of the complex the enzymes are inhibited by free copper.

In our laboratory it was shown that in the presence of copper 2,2′-bipyridyls have antimycoplasmal activity [1–6]. Most of the investigated compounds are as effective as tylosin, a compound in veterinary use against mycoplasma infections, though the most potent compounds are 100 times more active. Smit *et al.* [7, 8] investigated the mode of action on *Mycoplasma gallisepticum* using the copper[I] complex of 2,9-dimethyl-1,10-phenanthroline (DMP) as a model compound. They found selective inhibition of the incorporation of [^{14}C]thymidine into DNA. However, more important for the induction of the toxic effect is the strong inhibition of the energy yielding metabolism due to blockage of the conversion of pyruvate into lactate. Important, because in the case of *Mycoplasma gallisepticum*, glycolysis is the main energy source and respiration is not an "essential to life" process. Further, they found that also a minor route in the energy yielding metabolism, the conversion of pyruvate into acetate, was blocked. Although *in vitro* inhibition of NADH oxidase and lactate dehydrogenase (LDH) by $Cu(DMP)_2NO_3$ was observed, copper sulfate was found to be a stronger inhibitor under such conditions. Smit *et al.* [9] also showed that copper enters the cell, resulting in an inhibition of both enzymes.

Investigations on the influence of 2,2′-bipyridyl analogues on copper uptake by *Mycoplasma gallisepticum* [10] showed that, in the presence of a standard amount of copper at minimal inhibitory concentrations (MIC) of several ligands always the same amount of copper is taken up by mycoplasma cells. Combining this result with the results of Smit *et al.* [7–9] *viz.* that copper sulfate inhibits NADH oxidase and LDH, it seems likely that copper itself is the toxic species.

In order to get more information on what species, copper ions or copper complex, interferes with the enzyme, we established the degree of inhibition of NADH oxidase and LDH in whole cells at MIC condition and quantified *in vitro* the inhibition of both enzymes by various copper complexes and copper sulfate.

The *in vitro* investigations were performed with crude cell extracts [7] in the presence of several substrate concentrations in order to evaluate the enzyme kinetics. The results obtained were used to study the relationship between the stability of the copper complexes and enzyme inhibition.

MATERIALS AND METHODS

Chemicals. 3-(2-Pyridyl)isoquinoline (**I**), 1-amino-3-(2-pyridyl)isoquinoline (**II**), 1-methoxy-3-(2-pyridyl)isoquinoline (**III**), 1-chloro-3-(2-pyridyl)isoquinoline (**IV**), 1-amino-3-(6-methyl-2-pyridyl)-isoquinoline (**V**) and 6,7-dimethoxy-1-amino-3-(2-pyridyl)isoquinoline (**VI**) are from the laboratory stock [4–5]. 2,9-Dimethyl-1,10-phenanthroline (**VII**) was purchased from Aldrich Europe. Water was distilled from an all glass still after deionization over a mixed-bed ion exchanger. All other chemicals were of analytical grade.

Test organism and nutrient medium. The test organism was *Mycoplasma gallisepticum* K514 (Gist-brocades N.V., Delft, The Netherlands). Stock cultures were maintained in Adler medium at −20°. The nutrient medium was a modified Adler medium and consisted of 17.5 g bactopepton (Oxoid), 5.3 g yeast extract (Oxoid), 9.6 g D-glucose, 4.35 g NaCl, 2.1 g $Na_2HPO_4 \cdot 2H_2O$, 25 mg phenol red, 150 ml inactivated horse serum (Flow Laboratories) and 10^6 U penicillin G (Gist-brocades N.V.) per liter final medium. Before adding the filter sterilized horse serum and penicillin, the medium (pH = 8.0) was sterilized for 30 min at 110°.

Preparation of cell extracts. Cells of *Mycoplasma gallisepticum* cultivated at 37° were harvested in the exponential phase of growth (E_{660}: 0.20–0.25) by centrifugation at 8000 *g*, 4° for 10 min. The cells were resuspended in a volume of 10% of the original

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culture in PBS buffer (10 mM Na–phosphate + 0.85% NaCl, pH 7.2) and centrifuged again. After two washings the cells were resuspended in a volume of 1% of the original culture in the same buffer and cooled to 0°. Cell extracts were prepared by ultrasonic disruption of the cells using a MSE ultrasonic power unit (microtip, amplitude 12 μm, low power) for six 30-sec periods. The resulting suspension was centrifuged at 40,000 *g*, 4° for 30 min and the supernatant was taken as the crude cell extract. Protein content was measured by the method of Lowry *et al.* [11] using bovine serum albumin as the protein standard. The cell extract was divided into small portions and stored in liquid nitrogen; no significant decrease in enzyme activity was found during three weeks.

Determination of NADH oxidase activity in the presence or absence of (a) copper (complex). Cell extract was diluted with PBS buffer to a protein concentration of about 75 μg/ml and stored as a stock solution on ice. Prior to the determination of the enzyme activity 2.9 ml of the diluted cell extract was preincubated at 25° for 5 min in the presence of copper sulfate or copper complex (final concentration in the reaction mixture between 0.5 and 30 μM) or in the absence of copper. Then the reaction was started by addition of 0.1 ml of an aqueous NADH solution giving a final concentration in the cuvette between 47.0 and 375.0 μM. The oxidation of NADH was followed spectrophotometrically at E = 340 nm.

Determination of lactate dehydrogenase (LDH) activity in the presence or absence of (a) copper (complex). Cell extract was diluted with PBS buffer to a protein concentration of 150 μg/ml and stored as a stock solution on ice. 2.9 ml of this solution containing copper sulfate or copper complex (final concentration in the reaction mixture between 0.5 and 30 μM) or no extra copper were placed in a Thunberg cuvette; 0.1 ml of a NADH solution (conc. 7 mM) were placed in one loop of the cuvettetop above the solution containing the enzyme; oxygen was removed by bubbling nitrogen through both solutions, then the cuvette was closed gastight after placing 0.1 ml of a pyruvate solution (giving a final concentration between 11.36 and 378.8 μM in the reaction mixture) above the cell extract solution in the other loop of the cuvettetop. Then NADH was added to the enzyme solution and the absorption at E = 340 nm was recorded for at least 5 min in order to test for NADH oxidase activity. If no decrease in absorption was found the reaction was started anaerobically by adding the pyruvate solution and the oxidation of NADH was followed spectrophotometrically at E = 340 nm.

Determination of NADH oxidase and LDH inhibition at MIC in whole cells. Samples of 75 ml Adler medium containing 104 μM copper and ligand at the minimal inhibiting concentration (taken from [5, 6]) were inoculated with 25 ml of a fresh *Mycoplasma gallisepticum* culture being in the exponential phase of growth (E_{660}: $\pm 0.22 \approx \pm 200$ μg dry weight/ml) resulting in a pH of 7.4. This culture was then incubated at 37° for 24 hr. Absence of growth was checked by pH measurement (no pH change; growing cells of *Mycoplasma gallisepticum* produce considerable amounts of acid). When no growth was detected, cell extracts were prepared as described above and enzyme activities were determined at 375.0 μM NADH and respectively 233 μM NADH plus 378.8 μM LDH as described; controls consisted of *Mycoplasma gallisepticum* from the same culture and medium, however, without copper and ligand.

RESULTS

NADH oxidase

In order to analyse the kinetics of the oxidation of NADH 1/V was plotted versus 1/[S]. The good linearity ($r > 0.96$, $12 < N < 20$) indicates that oxidation of NADH obeys Michaelis–Menten kinetics. In addition the plots show that, depending on the copper complex used, in certain cases mixed-type enzyme inhibition is present. A typical example is shown in Fig. 1.

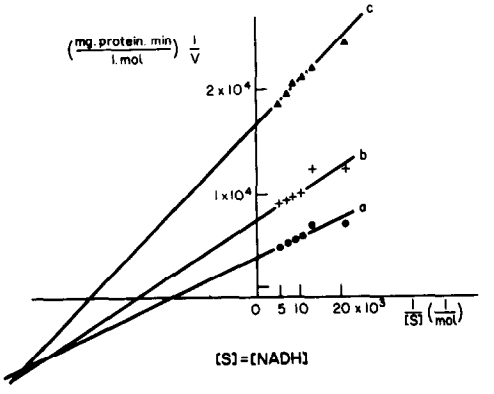


Fig. 1. Lineweaver–Burk plot of NADH oxidase kinetic data in the presence of $CuSO_4$.

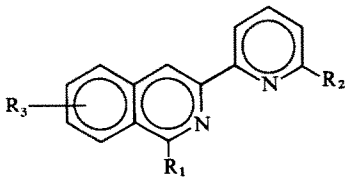
In order to examine whether different batches of cell extracts yield comparable enzyme activities the reaction rates of the control experiments without copper complexes were compared. The results (N = 4, V_{max} = 425 (±35) × 10^{-6} [mol/mg protein · min]) show that in general the method is useful. The variation, however, makes a comparison with the respective controls necessary.

For further analyses of the data secondary plots [12] were made by plotting the slope of the line of the Lineweaver–Burk plot or the V_{max} of the reaction against the concentration of (a) copper (complex). From the slopes (K_I) and intercepts (K_I') of these lines the inhibition constants (either K_I of K_I') were calculated. The good linear behaviour of the obtained secondary plots ($r > 0.97$) and the fact that the intercept of the calculated line is very close to the value of the slope (or $1/V_{max}$) of the uninhibited reaction indicates that a simple reaction mechanism is involved. The results, calculated from these plots and the IC_{50}-values (obtained graphically from a V_{max} vs concentration inhibitor plot taking V_{max} equal to $1/2V_{max}$ of the uninhibited reaction), are shown in Table 1.

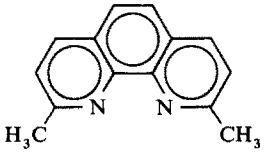
Lactate dehydrogenase

The conversion of pyruvate into lactate by LDH is an extensively studied reaction. Several authors [13–17] demonstrated that the coenzyme (NADH)

Table 1. Kinetic data of NADH oxidase inhibition by (a) copper (complex)



	R_1	R_2	R_3	K_I*	K_I'†	IC_{50} (μM)	MIC (μM)
I	H	H	H	1.735×10^{-5}	6.686×10^{-6}	6.74	25.0
III	MeO	H	H	5.381×10^{-6}	1.412×10^{-5}	7.82	0.8
IV	Cl	H	H	1.053×10^{-6}	1.675×10^{-6}	2.09	12.0
V	NH_2	CH_3	H	2.443×10^{-6}	4.384×10^{-6}	2.75	0.1
VI	NH_2	H	6,7-DiMeO	1.289×10^{-5}	1.237×10^{-5}	18.78	39.0



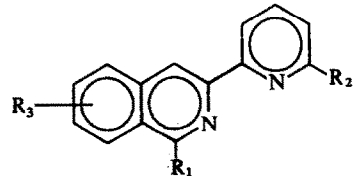
VII	DMP			1.858×10^{-6}	1.153×10^{-5}	7.35	0.1
	$CuSO_4$			5.192×10^{-3}	3.080×10^{-7}	0.41	—

* K_I: I + E $\rightleftharpoons$ EI † K_I': I + ES $\rightleftharpoons$ EIS

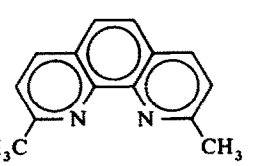
binds first (ordered ternary complex mechanism) and consequently catalyzes the conversion of the substrate (pyruvate).

Normally, in the case of a two-substrate reaction, effects of variation of both enzyme concentration and substrate concentration are investigated. However, in our case the conversion of pyruvate into lactate is inhibited by high pyruvate concentrations. Therefore, only the effect of the inhibitor on the coenzyme (NADH)–enzyme complex and the ternary complex (NADH–pyruvate–enzyme) could be investigated. This was realized by using the maximal NADH concentration without causing inhibition of the LDH. Since, under the applied condition, the free enzyme concentration is very low (as a consequence of the high NADH concentration), the data were evaluated by assuming that a one-substrate mechanism is involved as described for NADH oxi-

Table 2. Kinetic data of LDH inhibition by (a) copper (complex)



	R_1	R_2	R_3	K_I*	K_I'†	IC_{50} (μM)	MIC (μM)
I	H	H	H	2.306×10^{-5}	2.294×10^{-5}	21.40	25.0
II	NH_2	H	H	5.060×10^{-6}	3.835×10^{-5}	11.07	1.6
III	MeO	H	H	9.259×10^{-6}	4.849×10^{-6}	8.32	0.8
IV	Cl	H	H	1.786×10^{-6}	7.178×10^{-6}	3.53	12.0
V	NH_2	CH_3	H	3.391×10^{-6}	7.012×10^{-6}	6.24	0.1
VI	NH_2	H	6,7-DiMeO	4.090×10^{-6}	4.160×10^{-6}	2.93	39.0



VII	DMP			9.071×10^{-6}	2.209×10^{-5}	7.26	0.1
	$CuSO_4$			1.215×10^{-6}	1.731×10^{-5}	1.38	—

* K_I: I + E $\rightleftharpoons$ EI † K_I': I + ES $\rightleftharpoons$ EIS

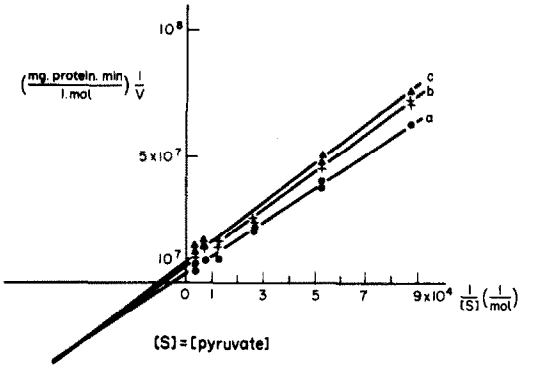


Fig. 2. Lineweaver–Burk plot of LDH kinetic data in the presence of $CuSO_4$.

dase. A typical Lineweaver–Burk plot, demonstrating mixed-type inhibition, is shown in Fig. 2. The values for K_I, K_I' and IC_{50} for LDH are listed in Table 2.

Inhibition of NADH oxidase and LDH in whole cells of Mycoplasma gallisepticum

Experiments performed on whole mycoplasma cells with five ligands (**II**, **IV**, **V**, **VI** and **VII**) at MIC conditions as described in Materials and Methods showed complete inhibition of NADH oxidase and LDH activity, whereas *Mycoplasma gallisepticum* cultures grown for reference use in the absence of copper and ligand had the expected enzyme activity. These results demonstrate that *in vivo* at MIC the energy yielding metabolism is blocked due to inhibition of both enzymes.

DISCUSSION

In general, the IC_{50}-values are in good agreement with the inhibition constants calculated from Michaelis–Menten kinetics. The IC_{50}-value is close to the lowest K_I-value, indicating that one type of interaction between inhibitor and enzyme or enzyme–substrate complex is mainly responsible for the inhibition.

The copper complexes of ligand **III**, **IV**, **V** and **VII** inhibited NADH oxidase predominantly in a competitive way. The copper complex of ligand **VI** shows pure non-competitive inhibition and the copper complex of ligand **I** as well as copper sulfate predominantly uncompetitive inhibition.

Since copper sulfate is a stronger inhibitor of NADH oxidase than the copper complexes (see Table 1), inhibition of NADH oxidase may be caused by free copper ions after dissociation of the copper complexes. However, the K_I-, K_I', or IC_{50}-values did not correlate with the stability constants of the complexes [18] indicating that the found inhibition should be caused by the complex and the copper ions. This is also in accordance with the different types of inhibition caused by the investigated complexes, as well as the large difference between the effect of the complex of **VI** on LDH and NADH oxidase.

Also, in the case of LDH, a reasonably good agreement was found between IC_{50}-values and inhibition constants, indicating that a one-substrate mechanism underlies the reaction.

Copper and the complexes of the ligands **II**, **IV**, **V** and **VII** appeared to be predominantly competitive inhibitors; the complexes of ligands **I** and **VI** inhibited LDH purely non-competitively and that of **III** predominantly uncompetitively.

As with NADH oxidase, copper sulfate is the strongest inhibitor and again the K_I-, K_I'- or IC_{50}-values do not correlate with the stability constants.

Remarkably, the complex of **VI** inhibited both enzymes in a purely non-competitive way, it is also the only complex which differs very strongly in its inhibition potency on both enzymes. On NADH oxidase the complex of **VI** is the weakest inhibitor. In the case of LDH, however, it is the most potent copper complex; this last observation is especially remarkable because of the fact that ligand **VI** shows the highest MIC value (the least toxic compound) for *Mycoplasma gallisepticum*. This relatively high potency of the complex of **VI** on LDH shows that most probably this particular copper complex does not enter the cytosol without dissociation or does not reach the cytosol except when high concentrations are used.

In an earlier investigation [10] under MIC condition a copper uptake to the same level by *Mycoplasma gallisepticum* was found under the influence of **VI** and other ligands, which may indicate that dissociation of the complexes takes place indeed. In addition, the results of the experiments with whole cells seem to support the dissociation model, as both enzymes are completely inhibited at MIC condition. None of the tested copper complexes inhibits *in vitro* the enzymes to a higher degree than copper sulfate which has *in vivo* no influence at all at the used concentration. These observations also suggest that in whole cells the copper complexes dissociate before inhibiting the enzymes.

In conclusion, the results clearly indicate that in cell extracts both enzymes are inhibited by free copper ions at a lower concentration than the tested copper complexes, which by themselves also inhibit the enzymes directly without dissociation. No relationship between the inhibition constants and the MIC values exists, which is in accordance with the conclusion that for certain complexes, e.g. **VI**, no significant complex concentration is present in the cytosol after adding the complex to whole cells.

In whole cells the inhibition of growth is caused by two sequential events, *viz.* the entrance of copper in the mycoplasma cell via a carrier mechanism followed by inhibition of both NADH oxidase and LDH by free copper, ultimately resulting in inhibition of the glycolysis [7].

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