Metal complex formation by nicotianamine, a possible phytosiderophore[1]

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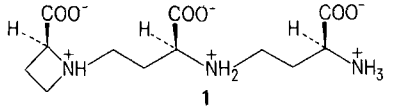
Summary. The acid dissociation constants of nicotianamine (**1**) ($pK_1 = 6.97$, $pK_2 = 9.13$, $pK_3 = 9.75$; 0.1 M $KClO_4$, 25 °C) and the stability constants for its 1:1 complexes with bivalent metal ions ($\log K_{Cu} = 18.6$, $\log K_{Ni} = 16.1$, $\log K_{Co} = 14.8$, $\log K_{Zn} = 14.7$, $\log K_{Fe} = 12.1$, $\log K_{Mn} = 8.8$, $\log K_{Mg} \approx 4.5$; 0.1 M $KClO_4$, 25 °C) were determined using potentiometric titrations in aqueous solution. Fe(III)-nicotianamine complexes were not detected under the same experimental conditions.

In 1960 the recessive, semi-lethal mutant *chloronerva* of the tomato, *Lycopersicon esculentum* Mill. cv Bonner Beste, was described[5]. Biochemical experiments revealed a disturbed iron metabolism of the mutant, leading to an excessive iron absorption by the roots, on the one hand, and an irregular distribution within young leaves on the other[6,7]. Further investigations[8] led to the isolation and identification of nicotianamine, (2*S*:3′*S*:3″*S*)-*N*-[3-(3-amino-3-carboxyproylamino)-3-carboxypropyl]-azetidine-2-carboxylic acid (**1**), a unique nonprotein amino acid. It has been shown[8] that **1** leads to a phenotypical normalization of the tomato mutant. Screening experiments[9] indicated its wide distribution in the plant kingdom. It has been suggested that nicotianamine (**1**) plays an essential role in cellular iron transport and/or metabolism[8]. The complex formation between **1** and Fe(III) (but not Fe(II)) has been considered in order to explain the appearance of a positive Cotton effect at about 250 nm in ORD experiments[9,10]. According to Scholz[10] this coordination might be relevant for the iron transport in pants. The ESR-spectrum and the cyclic voltammogram of a ferric nicotianamine complex have been described recently by Sugiura et al.[11], but experimental data have not been given in detail. On the other hand Fushiya et al.[12] mentioned that **1** did not have any ability to chelate iron. The present paper deals with the complex formation between **1** and iron as well as some bivalent metal ions in aqueous solutions.

Methods and materials. The measurements were carried out by means of potentiometric titrations of nicotianamine (**1**) solutions, acidified solutions of **1** (0.2 M $HClO_4$) and **1**-metal perchlorate solutions, respectively, with carbonate-free 0.01 M KOH at 25 ± 0.2 °C under CO_2- and O_2-free N_2. All solutions were 0.1 M in $KClO_4$. The initial concentration of **1** was 5.10^{-4} M, the initial metal perchlorate concentrations were 5.10^{-4} M and $7.5 \cdot 10^{-3}$ M. The pH of the solutions was read from a Präcitronic Labor Model MV 88 pH Meter (VEB Präcitronic, Dresden, GDR) fitted with a combined glass electrode (Einstabmesskette Ingold, type 405, Dr W. Ingold KG, pH-Messtechnik, Frankfurt a.M., FRG)[13] under continuous stirring at 200 rpm with a magnetic stirrer. The pH meter-electrode system was calibrated with NBS standard buffers. All chemicals used were analytically pure. $Fe(ClO_4)_3$ was prepared by dissolving ferric hydroxide in aqueous $HClO_4$[14]. The given data are average values of 3 measurements.



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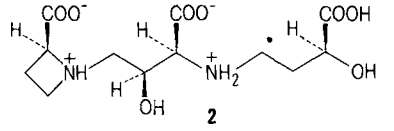

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Results. The acid dissociation constants K_n of nicotianamine (H_3NA), defined by $K_1 = [H^+]\,[H_2NA^-]/[H_3NA]$, $K_2 = [H^+]\,[HNA^{2-}]/[H_2NA^-]$ and $K_3 = [H^+]\,[NA^{3-}]/[HNA^{2-}]$, were determined using the graphic method of Schwarzenbach et al.[15]. Nicotianamine (**1**) is in zwitterionic form. Under the experimental conditions only the dissociation constants of the 3 'ammonium groups' could be obtained: $pK_1 = 6.97 \pm 0.02$, $pK_2 = 9.13 \pm 0.11$ and $pK_3 = 9.75 \pm 0.04$, where $pK_n = -\log K_n$. Our results are in approximate agreement with those published for analogous groups in mugineic acid (**2**): $pK_4 = 7.78$ and $pK_5 = 9.55$ ($\mu = 0.1$, KNO_3, 20 °C)[11]. The formation constants of the metal-nicotianamine complexes, defined by $K = [MNA^-]/[M^{2+}]\,[NA^{3-}]$, were estimated according to Ackermann and Schwarzenbach[16]. The resultant values are listed in the table. Only 1:1 metal-nicotianamine complexes were observed by application of the chosen method. The same log K-values were obtained, independent of the metal ion concentrations used. Nicotianamine reacts in the zwitterionic form **1**; only in the case of the formation of the Mg(II) complex is the reacting species the anion H_2NA^-. H_3NA (**1**) and H_2NA^- release their protons in the course of the coordination. The initial course of the titration curve of the $Fe(ClO_4)_3$-nicotianamine system (1:1) was identical with that of pure $Fe(ClO_4)_3$ solution of the same concentration. After approximately 3 equivalents of KOH had been added (pH range 5–6), ferric hydroxide had precipitated quantitatively. Further addition of KOH gave a titration curve as obtained with a pure nicotianamine solution. These facts indicate that under the experimental conditions used a significant complex formation between Fe(III) and **1** could not be detected.

Discussion. The complex formation constants obtained indicate strong coordination properties towards bivalent metal ions. The resultant values (table) are comparable with those of *N,N,N′,N′*-ethylenediaminetetraacetic acid or *N,N′*-ethylenediaminediacetic acid, respectively, and follow the Irving-Williams rule of formation constants, i.e. the sequence of stability is Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II). A (distorted) octahedral configuration of the metal-nicotianamine complexes is highly probable in solution. It is not possible to decide by potentiometric measurements whether the 6 coordination sites of the central ions are occupied by the potentially hexadentate nicotianamine or whether the ligand is only quinquedentate or quadridentate. In such a case water acts as an

Stability constants of the metal(II)-nicotianamine complexes $[MNA]^-$ (25 °C, 0.1 M $KClO_4$)

M	Cu	Ni	Co	Zn	Fe	Mn	Mg
$\log K_{MNA^-}$	18.6	16.1	14.8	14.7	12.1	8.8	≈4.5

additional ligand. Surprisingly, a Fe(III) complex is not observed under our conditions, but cannot be excluded in solutions of other composition[9-11], e.g. the Cotton effect described previously[9] was measured in a solution of **1** and $FeCl_3$ in water (concentration of both $3 \cdot 10^{-3}$ M). The chelating properties of the 2 related amino acids nicotianamine (**1**) and mugineic acid (**2**) show remarkable differences. Apart from the comparable stability of their Cu(II) complexes, the stability constants for the Fe(II)- and Zn(II)-complexes of **2** are much smaller than those for the complexes of **1**. The stability constant for the Fe(III) complex of **2** is relatively high (log K = 18.1)[11]. The different complexing behavior of the amino acids may be due to the presence of a terminal hydroxy group in **2** instead of a primary amino group in **1**.

Because of the different coordination behavior of nicotianamine (**1**) towards Fe(II) and Fe(III) it seems reasonable to assume that the cellular iron transport is mediated in the ferrous and not in the ferric form. On the other hand it could be supposed that **1** is biochemically converted into a compound of the mugineic acid type, which forms a stable complex with Fe(III), but the normalizing effect of the antipode of **1**, (+)-nicotianamine[4], might be an argument against this assumption. Finally in further physiological investigations the idea should not be neglected that perhaps **1** has only an indirect influence on iron transport.

1 Part 13 in the series 'On the 'normalizing factor' for the tomato mutant *chloronerva*', for part 12 see Ripperger et al.[4].
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Photobiological activity of 5,7-dimethoxycoumarin

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Summary. Some biological properties of 5,7-dimethoxycoumarin (DMC) include dark induced frameshift mutagenesis in bacteria, lethal photosensitization and the formation of sister chromatid exchanges in Chinese hamster cells. The number of sister chromatid exchanges per unit of cell lethality produced by DMC is almost the same as observed with 5-methoxypsoralen.

The photobiology of furocoumarins and their biochemical reactions with nucleic acids have been extensively studied in recent years[1-3]. Furocoumarins are known to intercalate within the DNA strands in a dark reaction[4,5], and UV-radiation of this complexed material can lead to monoadduct formation (photocyclization at the 3,4- or 4′,5′-bonds) and to di-adduct formation (interstrand cross-links)[6-8]. The mutagenic and carcinogenic effects of furocoumarins are attributed mainly to interstrand cross-link formation, monoadducts being assumed to be much less active[9,10].

Psoralen use in conjunction with UV-A radiation (PUVA therapy) for treatment of psoriasis and mycosis fungoides has been remarkably successful[11-14], although concern was raised regarding the possibility of skin cancer associated with such treatment[15,16]. Recent results[17,18] indicate that this concern was justified, there being an increased risk of squamous cell carcinoma (2.7-times higher than expected) in patients treated with PUVA who had been previously exposed to other potential carcinogens, with the relative risk increasing with the number of PUVA treatments. In order to reduce the possibility of such side effects, non-cross-linking compounds such as 3-carbethoxypsoralen (3-CPs) and 5,7-dimethoxycoumarin (DMC) have been investigated as potential substitutes in PUVA therapy[9,19-21]; the results of clinical trials with monofunctional reagents are still incomplete. Since no evidence was available for the photobiological effects of DMC, such a study was undertaken.

DMC is found naturally in several citrus oils, including oils of bergamot, lime and lemon[22-24], in concentrations varying from 0.46% (lime) to 0.053% (lemon)[22]. To what extent DMC contributes to the well-known photosensitizing effects of lime oil is not known as this oil contains a substantial number of other coumarins and furocoumarins[23].

This communication reports that DMC is a frameshift mutagen in the dark, that it lethally photosensitizes mammalian cells and induces in them sister chromatid exchanges (SCEs).

Materials and methods. Chemicals. The furocoumarins used in this study were characterized and their purity established as previously described[25]. DMC (Aldrich Chemical Co., USA) was twice recrystallized (85% ethanol) prior to use. 3-CPs was a gift from Dr E. Moustacchi, Paris, France.

Bacterial mutation studies. Frameshift mutagenesis studies in the dark were conducted on *E. coli lac-, z, thiamine* as