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Nicotianamine, the 'normalizing factor' for the auxotroph tomato mutant *Chloronerva*; a representative of a new class of plant effectors

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Summary. The article surveys our knowledge of the 'normalizing factor', gained from its discovery a quarter of a century ago up to the present time, under the following headings: Discovery; Physiological properties; Isolation and characterization; Structure determination (Identity of the 'normalizing factor' with nicotianamine); Chemical properties; Analysis; Synthesis; Occurrence and physiological role; Related compounds; Prospects.

Discovery

More than 25 years ago genetic and plant-physiological experiments were conducted at the Central Institute for Genetics and Crop Plant Research at Gatersleben (GDR) in the course of which tomato plants (*Lycopersicon esculentum* Mill., var. 'Bonner Beste') were grafted on to tobacco (*Nicotiana tabacum* L.) rootstocks[1]. Among the fruits obtained, 1 contained seeds which gave 67 normal and 22 mutated plants. The mutant was spontaneous, recessive and monogenic, characterized by severely retarded growth and distorted leaves of abnormal shape, and exhibited a pale yellowish chlorosis of intercostal areas of the leaves, which was most distinctly expressed in young leaves and more or less subdued in older ones. Flower buds very rarely developed, did not unfold and eventually died off. The mutant was given the name *chloronerva*[2].

Physiological properties

Normal growth and development could, however, be completely restored by grafts, in which it was irrelevant whether the mutant was used as scion or as rootstock[2] (fig. 1). This normalization of the phenotype also occurred in grafts between the mutant and other species[3]. Scholz and Böhme showed that grafting could be re-

placed by sprays or infiltrations with extracts from the wild type, as well as from other species[3]. When the mutant was cultivated on a medium containing an excess of iron, chlorosis was relieved but the plants were still unable to produce flowers; organic iron chelate (FeEDTA) was more effective than inorganic iron. This indicated the possibility that the effect of iron in these experiments was secondary and that iron was unable to compensate for the defect caused by the mutation. The mutant took up much more iron from the medium than the wild type, irrespective of whether iron sulfate or iron chelate were used. The mutant leaves contained much more iron than the wild type. The accumulation of iron in the mutant reached a maximum in the cotyledons (about 10-fold) and a minimum in the roots, axis and young leaves where iron appeared to be immobilized in the veins[4,5].

The above-mentioned property, i.e. the 'phenotypical normalization' of chloronerva by various methods of administration of extracts from different plant species containing an unknown 'normalizing factor', was used as a basis for a biological test, where sample solutions were 'painted' on to young leaves of mutant seedlings. Normalization is recorded as regreening of the chlorotic leaflets after a few days. Thus, by stepwise dilution of samples and their application on the leaves a semiquantitative test for the 'normalizing factor' was elaborated. Plant extracts are also effective with respect to the growth of isolated roots and to the formation of adventitious roots by isolated leaves[6]. Photosynthesis of the mutant appears to be unimpaired, but the quantitative relation between soluble carbohydrates and starch in the leaves is shifted in favor of the latter. Protein synthesis in the leaves is retarded and soluble amino acids accumulate. Aspartic acid, glutamic acid and arginine are heavily accumulated in leaves, but not in roots, where the most prominent change is the sharp reduction in alanine. Other characteristics are the low amounts of serine, tyrosine and phenylalanine in mutant leaves, while the concentration of the latter is doubled in roots[6,7].

Isolation and characterization of the 'normalizing factor'

When a semiquantitative bioassay for the 'normalizing factor' had been developed, Scholz and Rudolph[8] undertook the isolation of the substance in a pure state from dried alfalfa (*Medicago sativa* L.). Their isolation scheme is given in figure 2. The yield was 0.002% per dry weight. Preliminary experiments showed that the substance was soluble in water, but not in organic solvents. Its ninhydrin-positive reaction and loss of biological activity after treatment with nitrite or acid hydrolysis were indicative of an amino acid or peptide. The pure substance had the following properties: white amorphous powder decomposing above 290 °C, $[\alpha]_D$ −46° (c, 0.28 in water), elemental analysis 44.5% C, 6.87% H, 12.77% N, 350–500 mol.wt (determined by gel chromatography). Sulphur and phosphorus could not be detected. On acid hydrolysis (6 M HCl, 24 h, 100 °C) the substance was converted to a mixture of ninhydrin-positive components. However, none of them could be identified by finger-print paper chromatography or with an amino acid analyzer as any common (coded) amino acid. In addition, the intensity of the color reaction with ninhydrin decreased during hydrolysis in contrast to common peptides or proteins which display the opposite behavior (fig. 3). Since the substance could not be cleaved with trypsin, chymotrypsin and subtilisin it was evident that the 'normalizing factor' is not a normal peptide, but that it could be a peptide-like substance, containing uncommon amino acids or amines, which when liberated by hydrolysis would undergo further reactions with other components (condensation, oxidation, etc.). Spectral measurements were impaired by the non-volatility and insolubility of the substance in organic solvents. Nevertheless, the UV spectrum clearly indicated the absence of any aromatic nucleus or conjugated unsaturated system. The IR spectrum of the substance in KBr indicated the presence of 1 or more carboxyl anions, but no keto or aldehyde group. The peptide bond could not be detected unambiguously. The substance reacted with diazomethane,

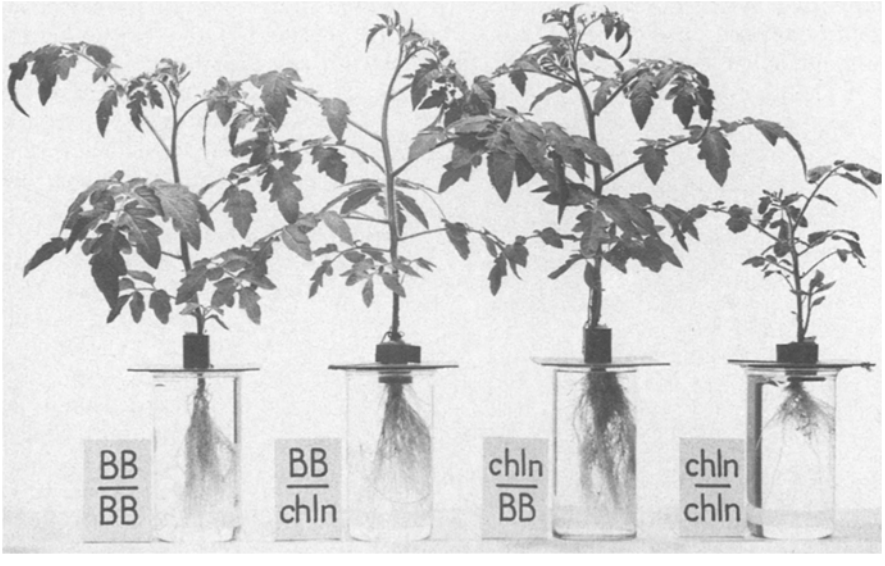


Figure 1. Restoration of the phenotype of the '*chloronerva*' mutant of tomato after mutual grafting with the wild type 'Bonner Beste'. (chln = *chloronerva*, BB = Bonner Beste), according to Scholz and Böhme[7].

Dried alfalfa	1000 g
↓ water extraction, heating, filtration	
crude extract	250 g
↓ decolorization, purification, Wofatit EZ	
extract	140 g
↓ ion exchange chromatography, Dowex 50 × 4, buffer pH 3–5	
amino fraction	26 g
↓ gel filtration, Sephadex G-25, 0.25 N acetic acid	
active fraction I	
↓ ion exchange chromatography, Dowex 50 × 2, pH gradient 3–4	
active fraction II	90 mg
↓ gel filtration, Sephadex G-10, 0.05 N acetic acid	
'normalizing factor'	20 mg

Figure 2. Isolation of the 'normalizing factor' from dried alfalfa shoots, according to Scholz and Rudolph[8].

methanolic hydrogen chloride and acetic anhydride, but no pure crystalline derivative could be isolated for further investigation.

For further work much larger amounts of substance were needed. Therefore collaboration was organized between the Central Institute for Genetics and Crop Plant Research, Gatersleben, the Institute of Plant Biochemistry, Halle, both of the same Academy of Sciences of the GDR, and the Institute of Organic Chemistry and Biochemistry, Prague, of the Czechoslovak Academy of Sciences. At the latter institute a slightly modified large scale isolation scheme was elaborated[9,10] and in the course of the collaboration several tons of fresh alfalfa were worked up and eventually about 6 g of the 'normalizing factor' were isolated. When crystallized from hot water the product contained water of crystallization which could only be removed by thorough drying at elevated temperatures.

During the isolation the purity was also checked by thin-layer chromatography on Silufol plates (Kavalier, Votice, Czechoslovakia), using an n-propanol-water mixture (6:7). On Silufol 254 even the purest samples gave 2 spots. Apart from the main spot at R_f 0.26, reacting violet with ninhydrin, a second one, orange-red colored, appeared with considerable tailing at R_f about 0.65. This behavior was very typical and could be used as a rather specific means of identification. It may be tentatively explained by the binding of silicate ions from the fluorescent indicator (consisting of Zn, Mn, etc. silicates) to the 'normalizing factor' molecule. This view is supported by the observation that the orange-red spot did not appear on silica gel G layers where free silicate ions might be insolubilized by precipitation with calcium sulphate[11]. The large scale isolation procedure was applied to *Cardaria draba* L. (0.00005% yield)[9], *Beta vulgaria* L. (sugar best leaves; 0.00025% yield), *Convallaria majalis* L.[30] and *Equisetum sylvaticum*[30]. In all instances the isolated substances were identical.

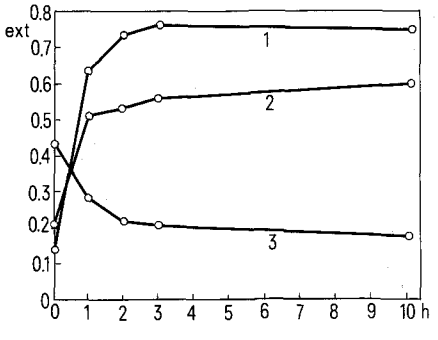


Figure 3. Colour intensity with ninhydrin-hydrindantin of 2 oligopeptides and the 'normalizing factor' after different times of hydrolysis, according to Scholz and Rudolph[8]. Hydrolysis with 6 N HCl, 100 °C; 1, penta-glycine: 2, leucylglycylglycine: 3, 'normalizing factor'.

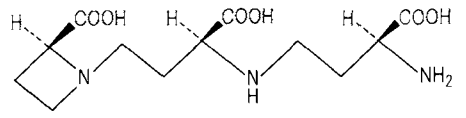
Structure determination[10,12]

The availability of sufficient amounts of pure 'normalizing factor' permitted resumption of the studies of its structure along several lines; chemical degradation, preparation of derivatives and spectral studies. Treatment with 6 N HCl under the conditions of total hydrolysis, mentioned above, seemed to show that the structure of the 'normalizing factor' is of peptidic character; however, this assumption proved later to be wrong (see below). Oxidation with performic acid gave aspartic acid and other, so far unidentified, compounds.

The high resolution mass spectrum of the tetra-(trimethylsilyl) derivative of the 'normalizing factor' showed the molecular peak at M^+ 591 and the composition $C_{24}H_{63}N_3O_6Si_4$, which led – after subtraction of the functional groups – to the composition $C_{12}H_{21}N_3O_6$ for the 'normalizing factor' itself. This result was in complete agreement with the elemental analysis of a pure and well-dried sample (at 180 °C) of the 'normalizing factor', indicating the same composition. This was corroborated by potentiometric titrations, which indicated a molecular weight of 307–310 and also 1 primary amino group in the molecule (on titration in the presence of formaldehyde) and at least 2 carboxyl groups. Both 4-bromo-benzoylation and acetylation of the 'normalizing factor' and subsequent esterification with diazomethane gave mixtures of compounds the isolation and mass spectrographic analysis of which were difficult; nonetheless, they corroborated the proposed composition and later the structure for the 'normalizing factor'.

However, the most important and interesting results were obtained with NMR spectroscopy[10,12]. The ^{13}C-NMR spectrum elucidated the nature of the carbon atoms in the 'normalizing factor'. It indicated 6 CH_2 groups, 3 CH groups, and 3 COOH groups. Hence, the substance does not contain methyl groups. The ^1H NMR spectrum in D_2O indicated the presence of 15 hydrogen atoms. Since the substance contains 21 hydrogen atoms in the molecule, it must contain 6 exchangeable hydrogens. If 3 of them are carboxylic hydrogens and 2 belong to the primary amino group, then the last exchangeable hydrogen must be that of a secondary

amino group. A more detailed analysis of the ^1H NMR spectrum showed that the substance also contains 3 CH_2-CH_2-CH groupings, mutually isolated by hetero atoms, evidently nitrogen atoms. The proton signals of 1 of these groupings were shifted to a relatively lower field, so that it could be considered to be a part of a cycle in the substance. Therefore the hypothesis had to be envisaged that the compound contains a 4-membered, azetidine ring. Thus, on the basis of the composition, the presence of an azetidine cycle, the aspartic acid fragment, 3 CH_2CH_2CH fragments, the presence of a primary amino group, 3 carboxyls, etc, partial and then the total structural formulae for the 'normalizing factor' could be proposed. The structure of N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid (**I**) seemed the most probable. The determination of the probable structure by Buděšínský et al.[10,12] and a literature search led to the conclusion that the amino acid nicotianamine, isolated from natural material for the first time in 1971, was probably identical with the 'normalizing factor', since they had identical physical and chemical properties. (See Noma et al.[13]; Kristensen and Larsen[14]; Noma and Noguchi[15]). A direct comparison of the 'normalizing factor' with an authentic sample of nicotianamine showed that the two were identical.



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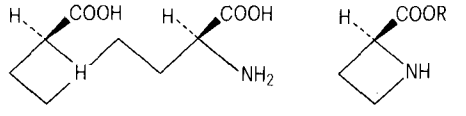
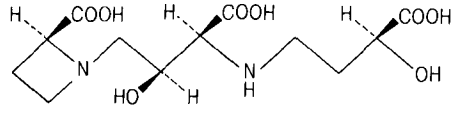


III II a R=H b R=CH_3



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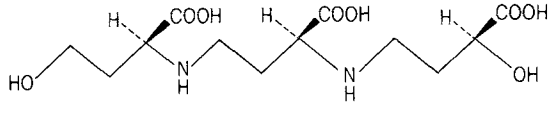

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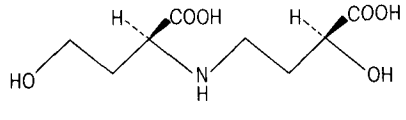

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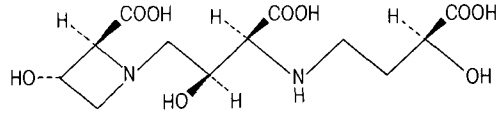

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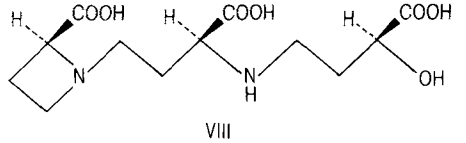


VIII

Nicotianamine was isolated by Noma et al.[13] from the tobacco plant *(Nicotiana tabacum)*; hence the name. The same authors also proposed a structure for it, which, however, was not quite correct, i.e. with a carboxyl group in a wrong position. Their structure was based mainly on the NMR and the mass spectra of nicotianamine itself and of its trimethyl ester, respectively. Oxidative degradation of nicotianamine with permanganate gave aspartic acid. In 1974 Kristensen and Larsen[14] isolated nicotianamine from beechnuts and assigned it the correct structure, (**I**), including stereostructure mainly on the basis of spectral evidence (NMR, MS). From the formula of nicotianamine it is evident that it is a derivative of 2-azetidine carboxylic acid and 2,4-diaminobutyric acid. They also managed to synthesize nicotianamine from L-azetidine-2-carboxylic acid in 1 step. Probably assuming that nicotianamine was formed in plant tissue from azetidine-2-carboxylic acid (**IIa**) by trimerization, they condensed it by boiling in a 0.1 M NaOH solution (with half an equivalent of the base) for 24 h and isolated 6% of nicotianamine and 20% of a dimer of structure (**III**) from the reaction mixture, proving thus the structure of nicotianamine by synthesis. Hence, the full chemical name of nicotianamine is: (2S:3'S:3"S)-N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid.

Recently, the azetidine ring was also detected and proved in mugineic acid (**IV**) and related substances (Nomoto et al.[16], Fushiya et al.[17,18]) which may be considered derivatives of nicotianamine. The structure of nicotianamine has been further corroborated by total synthesis[19].

Chemical properties

The majority of the chemical properties of nicotianamine have already been mentioned in the preceding sections. There is, however, a very important property which has not yet been mentioned; i.e. its ability to form complexes with metal ions. Scholz[20] proved the complex formation between iron and nicotianamine and copper and nicotianamine by ORD spectroscopy. In the case of iron it seemed to be limited to its trivalent state. The complex formation can be prevented with citric acid. The complexes are formed in a 1:1 ratio, but not very readily. The copper-nicotianamine complex could be chromatographed on silica gel G plates in phenol –water 3:1 without decomposition.

In aqueous solution the UV spectrum of nicotianamine displays a hardly-discernible maximum at 260 nm. On addition of $FeNH_4(SO_4)_2 \cdot 12H_2O$ the maximum increases above the value expected for an additive effect[20]. It was shown by ORD measurements that this was due to complex formation. A fresh solution of nicotianamine (3 mg) and $FeSO_4$ (12 µmol) in 3 ml of 0.1 M acetate buffer of pH 4.5 gave no absorption maximum in the UV region. The extremes of the ORD curve were very weak, probably owing to traces of Fe^{3+}. After 24 h standing the solution gave a UV-spectrum where a clear shoulder could be observed. The maximum formed was optically active and produced a distinct Cotton effect. This shows that iron was bound to an asymmetric centre. The Cotton effect can also be detected immediately after the mixing of the components if $Fe_2(SO_4)_3$ is used. Hydroxylamine prevents the complex formation completely, showing that the complex is formed with Fe^{3+} only. Since the addition of an equimolar amount of citric acid to the iron solution prevents the formation of a complex with nicotianamine, it may be assumed that citric acid gives stronger complexes.

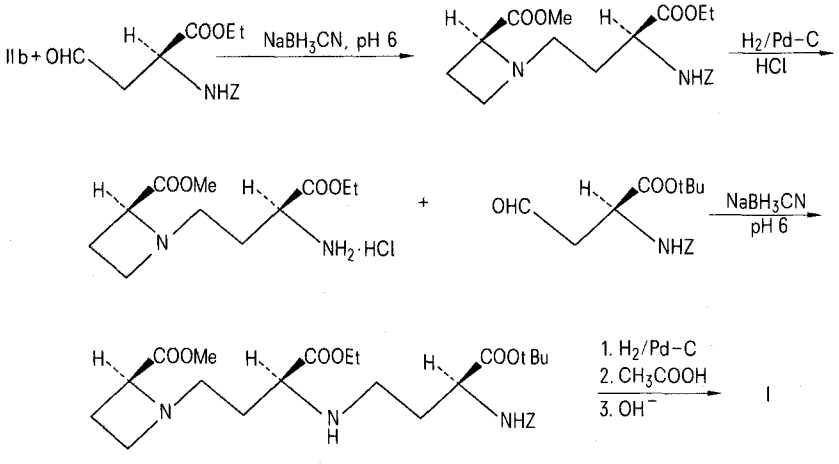
A solution of $CuSO_4$ turns darker blue on addition of nicotianamine. The absorption spectrum of the complex has a maximum at 290 nm and the ORD curve displays a strong Cotton effect. Ripperger[10] demonstrated that the molar ratio of the components in the complex is 1:1. These data were completed recently by the investigation of the complexing ability of nicotianamine with bivalent cations of Fe, Cu, Co, Ni, Mn, and Mg, as well as with Fe (**III**) carried out by Beneš et al.[24]. Their results show that bivalent cations form complexes with nicotianamine but a significant complex formation between Fe (**III**) and nicotianamine could not be detected. Thus, their results are to a certain extent contradictory to the preceding results. Figure 4 shows the Dreiding model of an iron complex with nicotianamine[10]. Nicotianamine is soluble in water. Its solubility in cold water is poor, in boiling water it is in the 1:10 range. It is insoluble in organic solvents. However, the addition of small amounts of inorganic acids, for example HCl, increases its solubility in methanol or ethanol enormously, since nicotianamine is converted to a positively charged species. The peculiar behavior of nicotianamine in hot 6 M HCl (fig. 3), which simulated the behavior of a peptide on the one hand, but contradicted it on the other, can now be explained by the cleavage of the azetidine ring with HCl (addition) and condensation of the chloro derivatives formed with the amino groups of nicotianamine and its degradation products.

Analysis

Paper chromatographic and thin-layer chromatographic analyses have already been mentioned above. Rudolph and Scholz[21] have elaborated a sensitive method of analysis of the 'normalizing factor' (nicotianamine) in plants, using amino acid analyzer. The peak of nicotianamine could be resolved from the peaks of serine and homoserine, if the pH of the buffer was kept strictly at pH 3.16. Noma and Noguchi[15] also determined nicotianamine in plants using an amino acid analyzer. The conditions of analysis were strikingly similar to those of Rudolph and Scholz[21]. Preliminary experiments with HPLC on silica gel or hydrophobic phases show that this method is also feasible. However, so far no other method of detection as sensitive as that with ninhydrin has been found or devised for amino acid analyzer or HPLC assays. The newest, so far unpublished, results[34] show that carefully selected conditions of amino acid analysis permit the separation of nicotianamine even from very complicated natural mixtures and its discrimination from heavily interfering natural amino acids.

Synthesis

In addition to the 'trimerization' synthesis of Kristensen and Larsen[14], Fushiya et al.[19] have published a synthesis based on the condensation of the methyl ester of L-azetidine-2-carboxylic acid (**IIb**) with the ethyl ester of L-aspartic acid β-semialdehyde derivative (N-acylated with a $COOCH_2C_6H_5$ group = Z, for example) in the presence of $NaBH_3CN$, hydrogenolysis on palladium, and the repetition of the sequence with tert-butyl ester of L-aspartic acid β-semialdehyde, followed by final saponification of the protecting ester groups[37] (scheme). Using similar synthons and procedures the Japanese authors[19] also synthesized the related mugineic acid (**IV**) and avenic acids A (**V**) and B (**VI**). Recently, Ripperger et al. published[22] a synthesis of the optical antipode of



the 'normalizing factor', i.e. (+)- or (R,R,R)-nicotianamine, making use of (R)-azetidine-2-carboxylic acid and the trimerization procedure of Kristensen and Larsen[14], and a synthesis of the proline analogue of nicotianamine[35].

Occurrence and physiological role

Nicotianamine has been quantitatively determined or biologically assayed in a number of different plants[9,10,14,15,21,30] (table 1). These data demonstrated that all spermatophyta hitherto investigated gave positive, and all sporophyta (mosses, fungi, algae) negative results. However, newer results indicate the presence of nicotianamine even in lower plants[34]. The highest concentrations were observed in young leaves, the lowest in seeds. Its presence was independent of the chlorophyll content; chlorophyll-less organs and tissues, chlorophyll-deficient mutants and etiolated leaves revealed normal amounts of nicotianamine. Since the mutant *chloronerva,* the only vascular plant where no trace of nicotianamine has been detected, exhibits severe defects of growth and development as well as its specific type of chlorosis, this substance obviously represents a newly-discovered type of effector, essential for the growth and development of higher plants. It increases root elongation after application to the leaves or to the roots of mutant seedlings[32]. Furthermore, since the biological activity of nicotianamine is very high (re-greening of leaves at 10^{-6}M concentration; 10^{-9} moles per seedling)[7], its activity approaches that of phytohormones.

The assumption that nicotianamine plays a role in the iron metabolism of higher plants is supported by a number of observations. In addition to those already described it should be mentioned that according to the Dreiding model in figure 4 nicotianamine fulfils the steric requirements for hexadentate chelate formation with iron. Azetidine-2-carboxylic acid (**IIa**) and N-(3-amino-3-carboxypropyl)-azetidine-2-carboxylic acid (**III**), although components of the nicotianamine molecule, are biologically inactive[7,10]. Biological activity is not only exerted by the natural (−)-nicotianamine, but also by the synthetic antipode, (+)-nicotianamine[22], which has the same complexing capacity with iron as the natural isomer, but should behave differently if it is assumed that it might possibly be bound to a macromolecule, e.g. at cell surface. The proline analogue of Faust et al.[35] was also found to be active.

Summarizing these observations, nicotianamine was tentatively designated a 'phytosiderophore'[10,12], in analogy to the siderophores of microbial origin[23] (cf. Scholz[36]). Whereas microbial siderophores are powerful chelators of Fe^{3+}, which are produced and excreted under the influence of iron deficiency and are reabsorbed as Fe^{3+}-chelate by specific sites of the cell membrane, this property or similar functions still have to be

Occurrence of nicotianamine ('normalizing factor') in plants

Family	Species	Organ	µmol/g dry wt[a]	Normalizing effect	Ref.
Spermatophyta					
Solanaceae	*Datura metel*	leaves	1.20		15
	Lycium chinense	leaves	6.90		15
	Lycopersicon esculentum	leaves	0.63	+	21
	L. esculentum	leaves	0.50		15
	L. esculentum	fruits	0.14		21
	L. esculentum	seeds	trace		21
	L. esulentum, mut. *chloronerva*	leaves	0.00	−	21
	L. esculentum, mut. *xantha*$_3$	chlorotic leaves		+	21
	Nicotiana tabacum cv. Bright Yellow	leaves	0.50		15
	N. tabacum cv. Xanthi	leaves	1.90		15
	N. glutinosa	leaves	0.70		15
	N. rustica	leaves	1.90		15
	N. arentsii	leaves	1.20		15
	N. alata	leaves	0.3		15
	N. debney	leaves	1.3		15
	Solanum melongena	leaves	0.50		15
	S. tuberosum	pealed tubers	0.10	+	21
Fabaceae	*Medicago sativa*	shoots	0.40	+	21
	M. sativa	seeds	0.04		21
	Pisum sativum	shoots	0.12		21
Brassicaceae	*Cardaria draba*	leaves[b]	0.01	+	9
	Arabidopsis thaliana	shoots	0.43		21
Chenopodiaceae	*Beta vulgaris*	leaves[b]	0.08	+	10
Cuscutaceae	*Cuscuta europaea*	shoots[c]		+	21
Rutaceae	*Citrus sinensis*	fruits (juice)	0.00		30
Convallariaceae	*Rhodea japonica*	leaves	trace		15
	Convallaria majalis	leaves	0.02	+	30
Fagaceae	*Fagus sylvatica*	seeds	5.00		14
Poaceae	*Zea mays*	leaves	0.20		15
	Hordeum vulgare	leaves (etiolated)		+	21
Aspidiaceae	*Dryopteris filix-mas*	leaves	0.05	+	21
Hipolepidaceae	*Pteridium aquilinum*	leaves	0.00	−	30
Equisetaceae	*Equisetum sylvaticum*	leaves		+	30
Sporophyta					
Polytrichaceae	*Polytrichum commune*	gametophyte	0.00	−	21
Sphagnaceae	*Sphagnum* sp.	gametophyte	0.00	−	21
Colestraceae	*Scenedesmus* sp.		0.00	−	21
Tricholomataceae	*Cylocybe georgii*		0.00	−	21
Saccharomycetaceae	*Saccharomyces cerevisiae*		0.00	−	21

[a] In ref.[21] calculated from mg per 100 g dry weight, in ref.[15] calculated from µmol per g fresh weight, under assumption that 10 g fresh weight of leaves corresponds to 1 g of dry weight; [b] The quantity calculated according to the amount of the material isolated from fresh juice; [c] Sponging upon *Euphorbia cyparissias.*

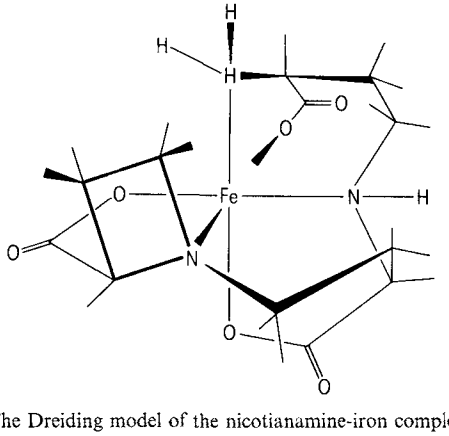


Figure 4. The Dreiding model of the nicotianamine-iron complex[10].

demonstrated in the case of nicotianamine and related compounds. There are arguments that Fe^{2+} rather than Fe^{3+} is complexed by nicotianamine[24]. Thus, nicotianamine may be involved in Fe^{2+} transport within the plant tissues or cell. It cannot be excluded that nicotianamine influences iron metabolism indirectly after conversion to mugineic acid or that the interrelations with iron are side-effects of some more important phenomena. Although for some time the nicotianamine problem seemed to be without parallel in studies of the physiology of higher plants, recent observations by Japanese authors, reported below, gave new support to our original ideas.

Related compounds

Mugineic acid and related substances: The marked susceptibility of rice to iron chlorosis led to the discovery (Takagi[25]) that root-washings of water-cultured oat and rice contained several amphoteric iron-chelating substances, and that the release of the chelators into the washings increased greatly under iron-stress conditions. A group of Japanese authors managed to isolate and to determine the structures of these chelators, which were shown to be new amino acids; mugineic acid (**IV**), isomugineic acid (configuration unknown), 3-hydroxymugineic acid (**VII**) and 2′-dehydroxymugineic acid (**VIII**). All of them may be considered to be derivatives of nicotianamine. The statement that 'mugineic acid is the first compound participating in the iron uptake and transport in higher plants' is exaggerated, since it has been known for some time that nicotianamine (or more correctly: the 'normalizing factor') participates in the iron uptake and transport. Therefore a tentative hypothesis can be expressed, i.e. that nicotianamine is the parent substance of a group of chelators containing an azetidine ring in the molecule. These chelators are probably of great importance for the plants, since some of them, when added to nutrient solutions of water-cultured rice at pH 7, increase the content of chlorophyll[33]. Mino et al.[33] have shown that in contrast to mugineic acid and 2′-dehydroxymugineic acid nicotianamine practically did not increase the chlorophyll content or the[59] iron uptake in the water-cultured rice plant.
Among other chelators tested by them HEDTA was also rather active, while EDTA, desferrioxamine and citric acid were only weakly active. They also proved by X-ray diffraction studies that the mugineic acid-iron complex and also the mugineic acid-cobalt complex have approximately the same structure as the tentative Dreiding model of the nicotianamine-iron complex shown on figure 4, with the difference that the primary amino group nitrogen-ligand in nicotianamine is substituted in mugineic acid by the hydroxyl group oxygen-ligand which evidently imparts to the complex a much greater stability and thus, probably, its activity in tests with Gramineae. Recently, a group of Japanese authors isolated 2 other substances of similar type from oats, i.e. avenic acid A (**V**) and avenic acid B[17,18] (**VI**). Hence, on purely structural evidence one may speculate that all these substances belong to a new class of important, physiologically active chelators in plants, derived from 2-azetidinecarboxylic acid. This amino acid (**IIa**) was discovered simultaneously by Fowden[26] and Virtanen[27] in *Convallaria majalis* and *Polygonatum officinale* and some other plants. It may be assumed that it is biogenetically linked with nicotianamine and other chelators just mentioned, though in which ways is not yet known. It belongs among the amino acids and represents the lower homologue of proline.

Prospects

The fact that nicotianamine seems to be linked with the iron transport and thus with the metabolism of chlorophyll is sufficiently important to warrant a thorough study of the role of this and related substances in the physiology of plants. The importance of such a study is stressed by the fact that the tomato mutant *chloronerva* without an exogenous supply of nicotianamine is incapable of reproduction. Therefore the problems, such as the determiantion of the normalizing effect of mugineic acid and its derivatives, biogenesis of nicotianamine and related substance, a more thorough study of the chlorosis elicited by nicotinamine deficiency, appearance of nicotianamine in phylogeny, etc. are of the utmost importance.
The synthesis of nicotianamine and its derivatives and analogues, the study of the complexes of these compounds with metals[24,28,29,33,35], as well as the comparisons of their biological activities in various tests will open the way for further study of its function in plants.

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Full Papers

The regulatory peptide system of the large bowel in equine grass sickness[1]

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Summary. In recent years, distinct changes in regulatory peptides have been found in a number of gastrointestinal diseases. Grass sickness is a fatal disease of horses for which the etiology has yet to be fully ascertained.
In this study, the peptide-containing nerves and ganglionic and mucosal endocrine cells of the ileum, colon and rectum were investigated in horses with sub-acute or chronic grass sickness and compared with normal controls using immunocytochemistry, at both the light and electron microscopical levels, and radioimmunoassay.
A substantial loss of both peptide-containing cells and nerves was found in all of the sick horses, particularly in the ileum. Electron microscopy revealed marked degeneration of nerves in the gut wall. Fibers containing granules immunostained for substance P or VIP, using the immunogold staining technique, underwent extensive degranulation in grass sickness, with the formation of multiple vacuoles.
Radioimmunoassay of peptide content also showed that the most drastic changes occurred in the ileum. For example, VIP content was significantly reduced from 109 ± 19.8 (mean ± SEM) pmoles/g in controls to 6.8 ± 1.4 pmoles/g in grass sickness ($p < 0.001$) and substance P from 65.9 ± 8.1 to 31.3 ± 9.5 ($p < 0.02$). These results may have applications in the diagnosis and treatment of grass sickness.

Introduction

Grass sickness is a fatal disease of young horses which has drastic effects in terms of both the suffering of the animals and the associated financial loss. The etiology and pathogenesis of the disease remain largely unknown, despite extensive research.
The characteristic features of grass sickness have been described as expressions of 'sympathicotonia'[2] and take the form of sweating, muscular tremors and extensive bowel dysfunction. The main gastrointestinal symptoms at the acute and early sub-acute stages are bowel stasis with no bowel sounds and massive inpouring of fluid into the stomach and small intestine, whilst the colon becomes compacted with dry feces coated in dark mucus. In late sub-acute and chronic cases bowel sounds may return. There is no fluid in the stomach and the contents of the small intestine become semi-liquid. There is no known method of prophylaxis or treatment and, at the moment, the prognosis is hopeless in the acute cases and only a very small number of sub-acute and chronic cases recover[3].
The regulatory peptides form a large and heterogeneous group of biologically active substances present throughout the bodies of all animals so far examined. Their discovery has revolutionized all previous concepts of gut physiology[4]. These peptides are found, in the gut, in mucosal endocrine cells, from which they are released to act as circulating or local hormones, or in nerve fibers, where they may function as neurotransmitters or neuromodulators. The peptide containing nerves form a major component of the autonomic nervous system and