Riboflavin 3’- and 5’-Sulfate, Two Novel Flavins Accumulating in the Roots of Iron-deficient Sugar Beet (Beta vulgaris)*

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Roots from iron-deficient sugar beet grown in the presence of calcium carbonate exhibit a yellow color and autofluorescence typical of flavin-like compounds, whereas roots of control, iron-sufficient plants exhibited no yellow color and extremely low autofluorescence. The two major flavins whose accumulation is induced by iron deficiency have been shown to be different from riboflavin, FMN, and FAD by reversed-phase high performance liquid chromatography. These flavins, accounting for 82 and 15% of the total flavin concentration in deficient roots, have been shown unequivocally to be riboflavin 3’-sulfate and riboflavin 5’-sulfate, respectively, by electrospray-mass spectrometry, inductively coupled plasma emission spectroscopy, infrared magnetic resonance, and 1H magnetic resonance. These flavin sulfates have not been found previously in biological systems. The localization of riboflavin sulfates in deficient roots is similar, but not identical, to that of high iron reductase activity. The concentration of riboflavin sulfates has been estimated from root extracts to be at least 1 mM. We hypothesize, based on the similar localization of flavin and that of iron reduction, that the accumulation of riboflavin sulfates induced by iron deficiency may be an integral part of the turbo iron-reducing system in sugar beet roots.

Plants grown under limited iron supply may develop several different iron-acquisition mechanisms that are not expressed or underexpressed when iron supply is high. Two major strategies have been proposed in the recent literature (1, 2). One of these mechanisms, Strategy II, has been found in plant species within the Poaceae and is based mainly in the excretion of small molecules called siderophores. Once excreted to the nutrient solution, the yellow color of the roots of iron-deficient plants was reported to be Rbfl and 5’-sulfate, while flavin excretion and staining, typical of flavin-like compounds, were detected in sunflower. Simultaneous excitation spectroscopy that the yellow color of the subapical root portions in iron-deficient plants is due to the presence of flavins. Two major flavin compounds, accounting for more than 97% of the total flavin content, are shown by HPLC to be different from Rbfl, FMN, and FAD. Furthermore, we show unequivocally by several analytical techniques (electrospray-MS, ICP, IR-spectrometry, and 1H NMR) that the flavins induced by iron deficiency in sugar beet are Rbfl 3’-sulfate and Rbfl 5’-sulfate, two flavins not previously found in biological systems. We hypothesize, based on the similar localization of flavins and that of iron reduction, that the accumulation of flavin compound(s) induced by iron deficiency...
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MATERIALS AND METHODS

Plant Culture—Sugar beet (Beta vulgaris L. cvs. Monohil and F58-554 H1) and spinach (Spinacia oleracea L. cv. Matador) were grown in a growth chamber. Seeds were germinated and grown in vermiculite for 2 weeks. Seedlings were grown for 2 more weeks in nutrient solution (5/5-Hoagland nutrient solution with 22.4 μM iron) and then transplanted (4 plants per bucket) to 20-liter buckets containing half-Hoagland solution with 0 or 44.8 μM iron. Spinach was grown with 0.5 μM iron because at 0 μM the iron roots exhibited necrosis and died quickly. Iron was added in the chelated commercial form Sequestrene 138 from Ciba-Geigy. The pH of the nutrient solution was raised with 1 mM NaOH and 1 g/liter CaCO3 to simulate conditions usually found in the field that lead to iron deficiency; this treatment led to a constant pH of 7.5 throughout the 2-week growth period. Plants were grown with a photosynthetic photon flux density of 400 μE·m-2·s-1 at 25 °C, 80% relative humidity, and a photoperiod of 16 h light/8 h dark.

Extraction of Yellow Pigments from Roots—Root extracts were obtained from plants grown without iron for 10–12 days. Root tips exhibiting the yellow color were excised and ground, in dim light (less than 1 μE·m-2·s-1) and at 4 °C, in a Teflon pestle glass homogenizer with ice-cold 0.1 M ammonium acetate, pH 6.1, 10% trichloroacetic acid, or 50 mM Tris-HCl, pH 6.8. All treatments extracted essentially all of the yellow pigment from the roots. The extract was concentrated to 10 ml at 12,000 × g. The trichloroacetic acid extracts were neutralized with 2 M phosphate buffer to pH 6.1 (15, 16). A ratio of approximately 7 μl of extract/g of fresh root was used.

Separation of Root Yellow Pigments by HPLC—Flavins were separated by HPLC on a 100 × 8-mm Waters Novapak C18 radial compression column (4-μm particle size). Samples were injected with a 20-μl loop, and mobile phases were pumped by a Waters M45 high pressure pump at a flow of 1 ml/min. Detection was made at 375 or 445 nm (absorption maxima of flavins) with a Shimadzu SPD-6AV detector. The column was equilibrated with 0.1 M ammonium acetate buffer (pH 6.0) (95:5, by volume) for 10 min. The sample was injected into the column, and mobile phase A was pumped for another 2 min. A mixture of water:methanol (70:30, by volume) was pumped for 23 min. A wash of 10 min with pure methanol was carried out before re-equilibrating the column as described above. RBfl, FMN, and FAD standards were obtained from Sigma (Madrid, Spain).

Isolation of Yellow Pigments—For the isolation of flavins, neutralized trichloroacetic acid extracts were separated by HPLC as indicated above, and the two major flavin peaks were collected at the detector outlet. To concentrate the flavins, pooled fractions from several runs of chromatograms were diluted with 10 volumes of water and passed through Sep-Pak C18 cartridges (Waters) pre-equilibrated with methanol. Flavins retained in the cartridges were eluted with pure methanol and dried under N2. After isolation both flavins were rechromatographed by HPLC, and their retention times were found to be identical to those of the two major peaks present in the sugar beet crude root extracts.

Molecular Absorption and Infrared and Fluorescence Spectra—Electronic absorption spectra were measured in water at room temperature from 200 to 600 nm with a Shimadzu 2101 PC computer-controlled spectrophotometer, using a 1-nm slit. Flavin concentrations were estimated from absorbances at 445 nm by assuming ε = 12.2 μM-1·cm-1. Infrared spectra (from 4000 to 400 cm-1) were obtained with a Bomem MB-120 spectrometer after 256 scans, from a pellet obtained from a mixture of 50 ng of sample and 5 μg of α-hydroxy KBr (infrared grade) pressed under vacuum at 15 tons. Emission and excitation fluorescence was measured from intact roots with a Perkin-Elmer (Beaconsfield, England) LS-50 computer-controlled spectrofluorometer equipped with a cuvette suitable for solid samples. Emission and excitation fluorescence from root extracts and isolated compounds dissolved in water were measured at room temperature using a Hitachi F-4050 computer-controlled spectrofluorometer. In all cases emission spectra were measured between 400 and 700 nm with an excitation beam of 450 nm (2.5-nm slt), and excitation spectra were measured between 250 and 600 nm for emission at 526 nm (5-nm slit). All spectra were acquired with approximately the same sensitivity or fluorescence by adjusting the vertical gain and integration of the different compounds. Spectra presented in the figures were obtained by multiplying each spectra by a different factor to facilitate direct comparison.

Electrospray-MS—Electrospray-MS spectra were obtained by using a Finnigan (San José, CA) TSQ 700 triple quadrupole mass spectrometer equipped with an Analystics of Branford (Branford, CT) electrospray interface. The HPLC fractions to be submitted to electrospray-MS analysis were evaporated, redissolved in MeOH, and then introduced into the electrospray source at a rate of 1 μl/min using a Harvard Apparatus (Southnatick, MA) perfusion pump. Operating conditions were: electrospray voltage, 3500 V (positive ions) and 2800 V (negative ions); drying nitrogen flow, 5 ml/min; scan range, 200–1000 units; scan rate, 2 s. Spectra presented here are the average signal for 5-min acquisition. Rbfl, FMN, and FAD standards from Sigma were used for electrospray tuning and calibration.

H NMR—One and two-dimensional spectra were recorded on a Varian Unity 300 apparatus. Proton chemical shifts were referenced relative to tetramethylsilane standards. Normal H spectra were collected by using a HOD presaturation sequence. The sample (0.4 mg) was dissolved in CDOD (0.7 ml), and the spectra were recorded at 30 °C after 1024 scans. The phase-sensitive two-dimensional proton double quantum-filtered COSY (DQCOXY) spectra were obtained with the usual sequence and phase cycle with full quadrature detection in the two dimensions by using the same sample as above with a 1.5-s repetition rate and a spectral window of 1000 Hz covering the region between 1.55 and 5.20 ppm. The spectra from the root extract was obtained with a Varian Unity 300 apparatus. Proton chemical shifts were referenced relative to tetramethylsilane standards. Normal H spectra were collected by using a HOD presaturation sequence. The sample (0.4 mg) was dissolved in CDOD (0.7 ml), and the spectra were recorded at 30 °C after 1024 scans. The phase-sensitive two-dimensional proton double quantum-filtered COSY (DQCOXY) spectra were obtained with the usual sequence and phase cycle with full quadrature detection in the two dimensions by using the same sample as above with a 1.5 s repetition rate and a spectral window of 1000 Hz covering the region between 1.55 and 5.20 ppm. The spectra from the root extract was obtained with a Varian Unity 300 apparatus.

RESULTS

Yellow Areas of Roots from Iron-deficient Sugar Beet Grown in the Presence of Calcium Carbonate Exhibit Fluorescence Characteristics Typical of Flavins—When grown in nutrient solution with no iron added and buffered at high pH by calcium carbonate, the subapical portions of sugar beet roots developed a distinct yellow color. A complete anatomical and cytochemical study of these roots is being completed and will be presented in a separate study. Under these conditions the nutrient solution did not develop a yellow color. Conversely, when sugar beet was grown in unbuffered nutrient solutions, the medium developed a yellow color within a few days, and the only roots that exhibited yellowing were those situated above the solution level.

The yellow areas of iron-deficient sugar beet roots growing in the presence of calcium carbonate exhibited strong green fluorescence when illuminated with blue or UV light. The characteristics of this fluorescence from intact roots, which exhibited an emission peak at 523 nm (Fig. 1A) and excitation peaks at 380 and 460 nm (Fig. 1B), suggest the presence of a flavin-type compound (18). On the other hand, control, iron-sufficient roots exhibited only extremely low fluorescence. The presence of compounds containing the isoalloxazine ring in iron-deficient roots was confirmed from the absorption (not shown), fluorescence emission (Fig. 1A), and fluorescence excitation (Fig. 1B) spectra of the root extracts.

Flavins Present in Roots of Iron-deficient Plants Are Free Flavins—The possible presence of flavins linked noncovalently to proteins in iron-deficient sugar beet roots was investigated. Roots were extracted with 50 mM Tris-HCl, pH 8.0, and the extract was passed through a DEAE-cellulose column equilibrated with extraction buffer. A yellow band was weakly retained and then eluted with extraction buffer plus 0.1 M NaCl. A single peak exhibiting an absorbance maximum at 445 nm was recovered and concentrated by lyophilization. This peak was applied to a calibrated Sephadex G-75 superfine column pre-equilibrated with extraction buffer plus 20 mM NaCl and eluted using the same buffer. Again a single peak absorbing at 445 nm was found, with an elution volume indicating a molecular mass below 3000 Da. Both the ex-
The Major Flavins Present in Roots of Iron-deficient Plants Are Different from Rbfl, FMN, and FAD—Rbfl, FMN, and FAD standards can be easily separated by reverse-phase HPLC (Fig. 2A). Retention times for these compounds were 19.5, 14.6, and 13.5 min, respectively. When a root extract was separated by the same method, three compounds with absorption spectra characteristic of flavins, with absorption maxima around 270, 375, and 445 nm, were found (Fig. 2B). The first and second peak (thereafter referred to as compounds X1 and X2, respectively) from root extracts exhibited retention times of 14.9 and 15.2 min. Therefore, their chromatographic mobility was intermediate between those of FMN and Rbfl. The mobility of flavin standards was found not to change when loaded along with root extracts. Compounds X1 and X3 from root extracts accounted for approximately 82 ± 3% and 15 ± 3% of the total absorbance at 445 nm (mean ± S.D. of four different extracts, each from a different batch of plants). A small peak with a mobility identical to that of Rbfl (retention time of approximately 19.5 min) was also found in the extracts, accounting for 3 ± 1% of the total absorbance at 445 nm. Similar ratios between compounds X1 and X3 were found when the extracts were made with 10% trichloroacetic acid (Fig. 2B), 0.1 M ammonium acetate, pH 6.1, or 50 mM Tris-HCl, pH 6.8 (not shown). Extracts from control plants exhibited very low amounts (less than 1% of the total flavin present in iron-deficient plants) of Rbfl and FMN and traces of other unidentified compounds absorbing at 445 nm (not shown). Compounds X1 and X2 never appeared in extracts from control plants.

The two major flavins (compounds X1 and X2) present in root extracts from iron-deficient plants were isolated by re-covering fractions from several chromatographic runs. The pooled fractions corresponding to these flavins were concentrated with Sep-Pak cartridges as described under "Materials and Methods." When rechromatographed, compounds X1 and X2 gave distinct peaks of chromatographic mobilities identical to those present in crude root extracts (Fig. 2C). Each of the purified compounds was slightly contaminated with the other one, but this contamination was always below 0.5 and 3% for compounds X1 and X2, respectively, as judged from the HPLC chromatograms.

Spectral Characteristics of Iron Deficiency-induced Flavins—The electronic absorption spectra of compounds X1 and X2 were very similar to those of Rbfl and FMN (Fig. 3A). All flavins exhibited absorption peaks at 222, 267, 373, and 445 nm. Some differences can be appreciated between flavins in the relative intensity of the two UV absorption peaks (Fig. 3A). The fluorescence emission spectra of all flavins were also remarkably similar, exhibiting a single peak at 526 nm (Fig. 3B). Different flavins also exhibited similar fluorescence excitation spectra, although some differences were apparent in the UV region of the spectra (Fig. 3C). These results illustrate how difficult it is to identify flavins based only on the electronic absorption and/or fluorescence spectral characteristics. Identification of Flavins Present in Roots of Iron-deficient Sugar Beet—The two flavins separated from root extracts by HPLC were studied by electron impact-, thermospray-, and electrospray-MS (only electrospray-MS results are presented here). No molecular or structural information could be obtained from electron impact-MS using a solid probe introduction method, probably because of the thermolability of the
compounds being analyzed. In agreement with the spectrophotometric data, initial results obtained by thermospray-MS indicated that these fractions contained compounds with a flavin-like structure, but clear molecular information could not be obtained. However, electrospray-MS, a soft ionization technique that uses no high temperature sources for ionization, are presented in Fig. 4. In the positive ion mode, Rbfl showed characteristic signals at m/z 377 ([M + H]+), 479 ([M + Na]+), and 775 ([2M + Na]+) (Fig. 4A) but exhibited no response in the negative ion mode (not shown). The FMN standard exhibited a poor response in the positive ion mode (not shown) with signals at m/z 501 ([M + 2 Na – H]+), 479 ([M + Na]+), 413, and 301. In the negative ion mode, FMN showed intense signals at m/z 455 ([M – H]−) and 227 ([M – 2H]−2) due to the diacidic phosphate group (Fig. 4B). Compounds X1 and X2 showed a poor response in the positive ion mode; major ions were found, in both cases, at m/z 501, 413, 399, and 377 (not shown). In the negative ion mode both fractions gave identical spectra characterized by an intense signal at m/z 455 (similar to that corresponding to [M – H]− in FMN) and weak signals at m/z 911 and 933 (similar to that corresponding to [2M – H]− and [2M + Na – 2H]−, respectively, in FMN) (Fig. 4, C and D). These data indicated for both problem compounds a molecular weight of 456, the same as that for FMN. However, no signal at 227 ([M – 2H]−2 for FMN) could be detected, indicating the presence of only one acidic proton in these compounds (Fig. 4, C and D).

A possible molecular weight of 456, identical to that FMN, prompted us to investigate the possible presence of P in the flavins isolated from iron-deficient sugar beet roots. However, both 31P NMR and ICP indicated the absence of any significant amount of P in both flavins. Another possibility could have been the presence of a –SO3H group, instead of a –PO3H2 group, that could explain both the presence of a single acidic site and a molecular weight of 456. Differences between P and S include slightly different monoisotopic masses (0.01 units) and isotopic patterns, but these differences cannot be detected by using a low resolution quadrupole mass spectrometer. Indeed, the presence of S in the isolated compounds was confirmed by ICP emission spectroscopy, the results giving molar stoichiometries of 0.93 and 0.89 mol of S per mol of flavins X1 and X2, respectively. Infrared spectra showed a complex pattern of absorptions comparable with the Rbfl or FMN spectra with the exception of bands at 1251, 1226, 1066, and 1012 cm−1 that are fully compatible with the presence of a sulfate ester (results not shown).

The combined use of 1H NMR and DQCOSY (see Figs. 5 and 6) led to the assignment of the chemical shifts and coupling constants of the hydrogen network in the problem compounds X1 and X2 (Tables I and II). The comparison of the chemical shifts and coupling constants of these compounds with those of FMN (19), and N(3)-carboxymethyl-Rbfl (NCMR) (20) permitted elucidation of the structure of these flavins, including the position and nature of the substituent (Tables I and II). The chemical shift of the pair of signals near 8 and 2.5 ppm and their relative integral (1:3) (Fig. 5, A and B) were very similar to those found in Rbfl and Rbfl analogues. These signals correspond to two hydrogens at positions 6 and 9 (signals around 8 ppm) and the two methyl groups at positions 7 and 8 (signals close to 2.5 ppm) (21). Since the position of these signals is known to be very sensitive to ring substitution and redox state (21), it could be concluded that compounds X1 and X2 have a ring system very similar to that of Rbfl in its oxidized state. The signals in the region 3.5–5.5 ppm come from a network of several coupled hydrogens and correspond to the ribityl side chain. The chemical shift characteristics of compound X2 were closer to FMN than to NCMR; however, X2 exhibited increased shifts for 5′(Ha) and 5′(Hb) and decreased shifts for 4′(H) and 3′(H) when compared with FMN (Table I). The coupling constants were also similar to those of FMN (Table II and Fig. 6). These data indicated that compound X2 had a substituent more electron-receptive than a phosphate group in position 5′, thus being in good agreement with the presence of a sulfate suggested by other techniques. In the case of compound X1, the chemical shifts for hydrogens in positions 1′, 4′, and 5′ were very similar to those found in NCMR; a quite large increase in chemical shift appeared in the case of 5′(H) along with a smaller increase for 2′(H). This was indicative of a substituent in 3′. Furthermore, the coupling constants relating 3′(H) with the neighboring hydrogens were lower than in NCMR indicating the presence of a more electron-receptive group than hydroxyl (Table II and Fig. 6). From these data it was concluded that compounds X1 and X2 were Rbfl 3′-sulfate and Rbfl 5′-sulfate, respectively.

Rbfl 3′-Sulfate Does Not Result from an Artificial Trans-esterification During Extraction—No detectable interconversion was found between Rbfl 3′-sulfate and Rbfl 5′-sulfate when the lyophilized flavins were dissolved and stored for up to 24 h in the different media used for extraction (i.e. 10% trichloroacetic acid, 0.1 m ammonium acetate, pH 6.1, 50 mm Tris-HCl, pH 8.0 or 50 mm Tris-HCl, pH 6.8) either on ice or
FIG. 4. Electrospray-MS in the positive ion mode of RbfI (A), in the negative ion mode of FMN (B), compound X₁ (C), and compound X₂ (D).
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plant species are considered as very efficient in iron acquisition (9). Whether or not the accumulation of Rbfl sulfate(s) is a general response of iron-efficient species to iron deficiency is currently being investigated.

Until now, the flavin enrichment in iron-deficient roots has been generally ascribed in the scientific literature to Rbfl. This was accepted by analogy to the idea, accepted so far, that the flavin compound excreted to the growing medium of several plant species grown in hydroponics under iron deficiency was Rbfl. Flavin accumulation in the roots has been mentioned for iron-efficient cultivars of a few plant species grown under low iron (9–13). In all cases the identification of the flavin as Rbfl was based on the absorption and/or the fluorescence spectra of the flavin excreted to the nutrient solution. However, both the absorption and the fluorescence spectra are practically identical for Rbfl and Rbfl sulfates (as well as for FMN and many other flavins, all of them containing an isoalloxazine ring) (see Fig. 3). Therefore, to ascertain whether or not the accumulation of Rbfl sulfate(s) is a ubiquitous response of plants to iron deficiency, a new re-evaluation of each case is necessary. HPLC has not been used previously to study the nature of the flavins accumulated in the roots of iron-deficient plants but was used in one instance to study the nature of flavins released to the medium by iron-deficient plants (22). In that study one of the main components absorbing in the blue region of the spectra was suggested to be Rbfl on the basis of the similarity of its retention time with that of pure Rbfl (22). In this context, it should be noted that when nutrient solutions containing any of the two flavins isolated in this work were aged for several days in the buckets used to grow the plants significant amounts of Rbfl were formed at the expense of the original compounds (not shown).

This is the first time, to our knowledge, for a sulfated flavin to be reported in plants. Rbfl sulfates have been used as Rbfl analogs in flavin chemistry (23, 24) but have never been found in biological systems. A large number of sulfated flavonoids, however, have been reported to date (25–27). Speculation suggests better solubility of the sulfated products, but very little information concerning the specific roles of sulfate esters is available (28). Other proposed functions for sulfated compounds include an alternative excretory mechanism or a storage role (27).

Our finding that Rbfl sulfates are located preferentially at the subapical root zone provides an explanation for the observation that some areas of roots from iron-deficient plants exhibit strong fluorescence. The fluorescence of iron-deficient roots has been described before in pepper (29) and cotton (30). In both cases the fluorescence was attributed to endogenous iron chelators of the phenolic acid type. Only recently Welkie et al. (13) have indicated that Rbfl accumulated in adventitious pepper roots not submerged in the nutrient solution and that this accumulation occurred in root zones similar to those reported previously to be fluorescent in the same species (29). In sugar beet roots flavin fluorescence was almost nonexistent in the root tips and very strong in the subapical root portion that is thicker and has root hairs (not shown).

To assess the possible physiological significance of the flavin accumulation in the roots of iron-deficient plants, it would be necessary to estimate the actual flavin concentrations in the root. The only reports of Rbfl concentrations in root tissues of iron-deficient plants, to our knowledge, refer to roots of sunflower (10), tobacco (6, 14), and tomato (6). The flavin concentration in sunflower roots was reported to be 8–9 μM in iron-sufficient plants and 23–79 μM in iron-deficient plants, the maximum values being obtained by keeping the pH high (10). In iron-deficient tobacco the total Rbfl...
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Fig. 6. DQ COSY of a mixture of compounds X1 and X2 showing the coupling correlations (X1, dotted lines; X2, solid lines).

Table I

| Proton   | FMN*  | NCMR* | X1 | X2 |
|----------|-------|-------|----|----|
| 6        | 7.96  | ND    | 7.96| 7.96|
| 9        | 8.08  | ND    | 8.04| 8.05|
| CH3(T)   | 2.58  | ND    | 2.47| 2.47|
| CH3(G)   | 2.61  | ND    | 2.60| 2.58|
| 1'(Ha)   | ND    | 4.99  | 5.10| 5.20|
| 1'(Hb)   | ND    | 4.77  | 5.05| 4.90|
| 2'(H)    | 4.47  | 4.37  | 4.62| 4.46|
| 3'(H)    | 3.95  | 3.93  | 4.58| 3.86|
| 4'(H)    | 4.14  | 2.97  | 4.04| 4.05|
| 5'(Ha)   | 4.15  | 3.88  | 3.96| 4.33|
| 5'(Hb)   | 3.92  | 3.74  | 3.75| 4.18|

*Data were obtained from Refs. 19 and 20 for FMN and NCMR, respectively.
†ND, no data reported.

Table II

| Proton       | FMN*  | NCMR* | X1 | X2 |
|--------------|-------|-------|----|----|
| 1'(Ha)-1'(Hb)| 14.0  | 12.5  | 14.0| 13.5|
| 1'(Ha)-2'(H) | 10.0  | 9.6   | 9.5 | 10.0|
| 1'(Hb)-2'(H) | 2.3   | 2.6   | 3.5 | 3.0 |
| 2'(H)-3'(H)  | 4.5   | 4.8   | 3.5 | 5.0 |
| 3'(H)-4'(H)  | ND    | 6.9   | 4.0 | 8.0 |
| 4'(H)-5'(Ha)| ND    | 2.9   | 3.5 | 3.0 |
| 4'(H)-5'(Hb)| ND    | 6.4   | 6.0 | 6.0 |
| 5'(Ha)-5'(Hb)| ND   | 12.0  | 12.0| 10.5|

*Data were obtained from Refs. 19 and 20 for FMN and NCMR, respectively.
†ND, no data reported.

Concentrations were reported to be in the range of 10–17 μmol g⁻¹ fresh mass, roughly equivalent to 11–20 mM on a root water basis (6, 14), whereas in iron-sufficient tobacco and in iron-deficient and iron-sufficient tomato the concentration was approximately 1–3 mM (6). However, since the maximum solubility of Rbfl in water is approximately 130–850 μM, depending on pH and other factors (18), claims of free Rbfl concentrations above 10 mM in root (and also leaf) tissue should be taken with caution. These values may have been caused by interferences in the fluorometric assay. Alternatively, the flavin in question may have been different from (i.e., more soluble than) Rbfl. In other plant materials, such as legume nodules, the concentration of Rbfl has been recently shown to be in the range of 60–80 nmol g⁻¹ fresh weight (approximately 70–95 μM on a nodule water basis) (31). Our first estimates indicate that the concentration of Rbfl sulfates...
may be at least 450 \( \mu M \) to 1 mM on a root water basis in the root subapical zone. This concentration could be even higher in case there was any flavin compartmentalization in the root. This relatively high concentration led us to examine the solubilities of Rbfl and Rbfl 3'-sulfate in water by measuring the absorbance of saturated flavin solutions. The maximum solubility found for Rbfl was 140 \( \mu M \). However, with the limited amount of Rbfl 3'-sulfate that could be obtained from roots it was not possible to obtain saturated solutions; solutions as concentrated as 20 mM were still not saturated, thus indicating that Rbfl 3'-sulfate is at least 150 times more soluble than Rbfl. This is in good agreement with the high solubility, close to 200 mM, of other Rbfl esters such as FMN (18).

Since the rates of Fe(III) reduction by roots are 10–20 times higher in iron-deficient (high flavin content) than in iron-sufficient (negligible flavin content) sugar beet (results not shown), it is tempting to postulate a possible relationship between the presence of flavin sulfates at high concentrations and reducing capacity. This possibility is supported by the fact that the concentration of flavin is very low in the root tips that have very little reducing activity and high in the subapical root portions that have root hairs and exhibit high reductase activity. However, some areas with high levels of fluorescence but without root hairs lack significant reducing capacity (not shown). Therefore, our data indicate that the presence of Rbfl sulfates is not the sole requirement for Fe(III) reduction. The possible involvement of flavin redox intermediates in iron reduction was suggested by Cakmak et al. (32), and in some speculations has been devoted to the possible roles of flavins in redox processes (1, 7).

It remains to be elucidated whether flavin accumulation in the roots or flavin excretion to the medium is the true response of sugar beet and other iron-efficient plant species to iron deficiency. It is obvious that any possible plant mechanism directed toward acquiring iron, such as Strategy I, must have evolved under conditions leading to iron deficiency in nature. Under these natural conditions, for instance when calcium carbonate is present and external pH is high, flavin is accumulated in the roots and not excreted to the media. Significant flavin excretion takes place only in conditions unrelated to naturally occurring iron deficiencies, for instance when no calcium carbonate is present and the pH of the nutrient solutions decreases due to the functioning of the ATPase (7). These facts suggest that flavin accumulation may be an integral part of the response of dicots to iron deficiency.

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