Leaf Infections: Siderochromes (Natural Polyhydroxamates) Mimic the "Green Island" Effect

Abstract. *Topical application of as little as 5×10^{-12} moles of pure siderochromes (polyhydroxamate iron-transport compounds from bacteria and fungi) onto detached bean leaves causes spots of chlorophyll retention that are surrounded by chlorotic halos. These spots appear similar to "green islands" that are caused by certain fungal infections on leaves and are somewhat similar to zones where senescence is delayed by cytokinin.*

Leaf spots that are caused by parasitic infections contain leaf tissue that is altered by toxins and degradative enzymes from the parasite, as well as by defense responses of the host (*1*). These spots are usually not green. Certain parasitic fungi may, however, produce spots of a different type, called "green islands" (GI's), on rapidly senescing infected leaves (*1, 2*). While the plant as a whole and uninfected portions of the leaves senesce under the combined effects of age and environment, GI's in the form of rings or spots of green leaf tissue centered at the loci of infection somehow remain alive and metabolizing—apparently at the expense of surrounding tissue—under the influence of the fungus. Induction of GI's may thus benefit the parasite nutritionally. Chlorophyll may persist at the sites of infection even after complete drying of the leaves; these green spots are also called GI's.

When cytokinins are applied to leaves, they stimulate metabolism, delay senescence, and maintain green zones. Metabolism is similarly stimulated in both infected and noninfected leaf tissue in GI's (*1, 2*). This implies the presence of causal agents, diffusible metabolites of the fungus, that are not necessarily cytokinins. When compared with senescent tissue or with normal tissue, GI's have an increased respiration, an increased dry weight per unit area, and a net increase of high-molecular-weight products; low-molecular-weight solutes of all kinds are accumulated from tissue surrounding the GI's (*1–4*). Some have called this accumulation a process of "attraction," "mobilization," or active transport, but there is evidence (*2*) that GI's are simply physiological sinks that export less, and by diffusion, import more nutrients than do normal or senescing tissues. GI's remain when the leaves yellow, but sometimes GI's enclosed by chlorotic halos appear on leaves that are still green. Chlorotic halos may be an expression of the metabolic drain toward GI's.

GI's have been observed in conjunction with infections by many different fungi on grasses, legumes, celery, radish, tobacco, rosaceous fruit trees, and pine. They occur most often with infections by the powdery mildew and rust fungi, obligate parasites, notably on cereal grains and rusted beans (*1–4*). GI's with chlorotic borders have been induced experimentally on excised leaves of grains by sterile crude extracts from spores of the rusts and mildews that naturally cause GI's on the same plants (*4*). Assays indicate that simple extracts of spores from bean rust and of certain fungi contain cytokinins (*4, 5*); the activity of cytokinin extracted from nonhydrolyzed bean leaves is increased by rust infection (*3, 5*). Nevertheless, it has not been proved that cytokinins are in any way responsible for naturally occurring GI's, and it is doubtful that the fungal metabolites that induce GI's in vivo could be exclusively cytokinins:

1) Cytokinin-containing fractions from extracts of infected bean leaves are similar to those from uninfected leaves, but are unlike active fractions from bean rust spores or from mycelium (*5*).

2) There is disagreement whether GI's induced by spore extracts (in different systems) accumulate solutes (*1, 2, 4, 5*).

3) Green zones induced by cytokinins do not persist when the leaves are dry nor do they show the clear-cut chlorotic halos of GI's that are induced by infection or by spore extracts [although senescence is accelerated in untreated halves of detached bean leaves wetted with cytokinin (*3*)].

4) High concentrations of cytokinins must be applied in order to induce any effects in intact plants (*3, 4*).

5) *Ustilago nuda*, a smut that infects grains but does not produce GI's, yields extracts which induce GI's on detached grain leaves (*4, 6*).

Species of *Ustilago* produce large amounts of ferrichrome and related siderochromes. [Low-molecular-weight polyhydroxamic acids ($pK_a \sim 9$) and their ferric chelates, collectively called siderochromes, are widely produced by microorganisms, and are important in the solubilization and transport of iron (*7*).] We tested representative siderochromes—ferrichromes typically produced by many higher fungi, rhodotorulic acid from yeastlike heterobasidiomycetes, and ferrioxamines from actinomycetes and some bacteria—and found that all are potent inducers of GI's that mimic, at least visually, the GI's produced naturally on infected leaves. Other chelating agents were inactive. Figure 1 shows typical treated leaves.

Individual leaves from mature, trifoliolate bean leaves (*Phaseolus vulgaris* variety pinto "Topcrop") were cut off at the petiole, were spotted with 5-μl portions of various compounds in distilled water and 0.1 percent Tween 20 (a nonphytotoxic surface-active agent to wet the leaf), were placed separately in closed petri dishes lined with filter paper and moistened with a wet ball of cotton, and were incubated at 24° to 27°C (*4*). After 3- to 4-days of storage in the dark or under room lighting (no direct sunlight),

Table 1. Green island response of detached mature pinto bean leaves to siderochromes and other iron-binding agents, all applied in 5-μl portions as solutions in aqueous 0.1 percent Tween 20 at neutral *p*H; +, consistently produced green islands; ±, occasionally produced green islands; —, no response; no symbol, not tested.

Agent	Response of leaf to solution of molarity			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Ferrichrome	+	+	+	±
Ferrichrome A, potassium salt	+			
Deferrichrome A, potassium salt	+			
Ferrioxamine B	+			
Deferrioxamine B mesylate (Desferal)	+			
Ferrioxamine E	+			
Deferrioxamine E (Nocardamin)	+			
Ferric rhodotorulate	+	+	±	
Rhodotorulic acid	+	+	±	
Ferric ethylenediaminetetraacetate	—			
Ethylenediaminetetraacetate, sodium salt	—			
Ferric citrate	—			
Ferric 2,3-dihydroxybenzoate, sodium salt	—			
2,3-Dihydroxybenzoate, sodium salt	—			

chlorotic halos began to delineate GI's at the sites of application. These halos always appeared when we applied siderochrome that was sufficient to cause chlorophyll retention. Chlorosis then spread outward from the halos and inward from edges of the leaves; after 7 to 12 days the leaves were completely yellow except where siderochromes had been applied. The GI's persisted when the chlorotic leaves were dried. Injury at the sites of application had no effect, but slightly larger GI's were obtained by spotting the siderochrome solutions on the under, rather than on the upper, surfaces of the leaf. There were occasional small brown necrotic spots within the GI's, and there was also some necrosis along veins within GI's when siderochromes were applied to the undersides. The necrosis did not appear to involve the growth of fungi, but we did not exclude the possibility of growth of bacteria. The GI's, at 4 days or longer, were otherwise dark green and of healthy appearance under low-power magnification. With leaves essentially as green as GI's, disks cut from the inside and the outside of chlorotic halos had about the same rate of O_2 uptake in the dark, as measured by the O_2 electrode. Ferrioxamines tended to produce tight clusters of many small GI's rather than the solid patches of green obtained with other siderochromes.

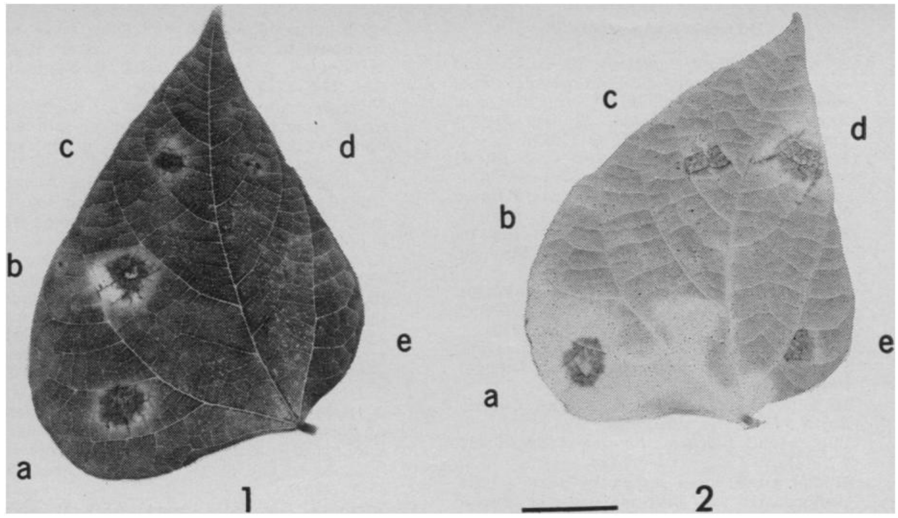

Fig. 1. Green islands induced by spotting 5-μl portions of solutions of various siderochromes onto mature pinto bean leaves (spotted surfaces at 6 days). The scattered faint spots are water droplets. Sites on upper surface of leaf 1: *a*, $10^{-3}M$ rhodotorulic acid; *b, c,* and *d*, $10^{-3}M$, $10^{-4}M$, and $10^{-5}M$ ferric rhodotorulate, respectively; *e*, 0.1 percent aqueous Tween 20, solvent control. Sites on undersurface of leaf 2: *a*, $10^{-3}M$ rhodotorulic acid; *b*, solvent control; *c*, $10^{-3}M$ potassium salt of deferrichrome A; *d*, $10^{-3}M$ deferrioxamine B mesylate (Desferal); *e*, $10^{-3}M$ deferrioxamine E (Nocardamin). Scale, 2 cm.

When mature leaves were spotted with siderochromes, they consistently produced GI's. When very young leaves were spotted, however, they rotted before yellowing, and very old leaves became chlorotic in several days and produced few, if any, GI's. Leaves of any age on healthy, intact plants showed no response to spotting with siderochromes.

Results of experiments on detached mature pinto bean levels are summarized in Table 1. In this system ferrichrome was 10 to 1000 times more potent than were cytokinins at the lowest concentrations reported to produce green zones on detached wheat leaves (*4*), and much more potent than the cytokinins used in repeated applications to delay senescence of detached bean leaves (*3*). The diameter of GI's was roughly proportional to the logarithm of the siderochrome concentration; a similar relation has been noted on detached wheat leaves spotted with cytokinin (*4*).

We do not know if siderochromes stimulate metabolism and cause accumulation of nutrients in GI's. The siderochromes listed in Table 1 lack aromatic groups, and they vary in size, shape, and charge: structurally, they are completely unlike cytokinins. Cytokinins delay senescence whether they are spotted or are widely applied on leaves, or when portions of the leaf are floated on cytokinin solutions (*1–3, 5*); bean leaves floated on siderochrome solutions yellowed rapidly, completely, and unexplainedly. We therefore expect that siderochromes will not show cytokinin activity in assays other than in tests of senescence of leaves by spotting (*8*). Siderochromes have not been isolated and characterized from the fungi that cause GI's by infection. A bacterial growth factor assay showed that two GI-producing fungi in pure culture produced siderochromes in concentrations sufficient to cause GI's on detached bean leaves—but so did two fungi that do not cause GI spotting (*9*), and so do *Ustilago* and many other fungi. The failure of externally applied siderochromes or cytokinins to produce GI's bordered by chlorosis on intact plants also suggests that neither class of compounds is sufficient to account for naturally occurring GI's. However, we hypothesize that siderochromes are necessary for chlorotic halos and for persistence of GI's on dried leaves. We further hypothesize that siderochromes in GI's affect the function and the metabolism of fungal cytokinins and endogenous plant hormones, or that they directly affect the breakdown of chlorophyll. How might such interaction occur?

Properties of siderochromes include (*7*): (i) chelation of transition metal ions by the hydroxamate groups, with very strong binding of ferric iron; (ii) solubility in water and lipid of many of the metal-free and the metal-complex forms; (iii) in microbial systems, innocuousness or growth-factor activity related to iron uptake (except sideromycin antibiotics not tested for GI activity); (iv) in hydroponic plants, ready utilization of siderochrome iron for growth. We conclude, first, that the chelating and membrane permeability properties of siderochromes are responsible for GI's. Fixation of iron from siderochromes into nondissociable forms (such as iron porphyrins) in leaves would yield metal-free siderochromes for further binding and diffusion of metal ions; siderochromes gave the same results with or without chelated iron (Table 1). Second, the potency of the GI effect shows that siderochromes must function at a catalytic level. Last, sequestration and translocation of metals by siderochromes could poison sensitive enzymes [certain metal salts leave green spots on leaves (*4*)] and greatly alter the rates of enzymes activated by divalent transition metals. This might be the case with such enzymes as indoleacetic acid (auxin) oxidase (*10*) and others involved with metabolism of plant hormones or of chlorophyll.

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6. Similar green zones caused by yeast extract, peptone, and so forth (*4*), may be due to cytokinins from degraded nucleic acids.
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8. The cytokinin activity claimed for 8-hydroxyquinoline because of chlorophyll retention in detached leaves [S. E. Chua, *Nature* **225**, 101 (1970)] was disproved by callus assay and attributed to the antibacterial nature of the chelator [A. Rameshwar and P. L. Steponkus, *ibid.* **228**, 1224 (1970)].
9. *Heterosporium iridis* (from R. D. Raabe), on irises, primarily *Iris germanica*, produces brown-bordered spots with dark green outer rings; these annular GI's persist while the surrounding tissue yellows. *Septoria lycopersici* (Raabe) causes similar GI's on the tomato plant, *Lycopersicon esculentum*. *Helminthosporium* sp. (strain 1150 of C. I. Kado) produces brown spots, not GI's, on leaves of oats, *Avena sativa*. *Septoria* sp. (Kado strain 1326) causes reddish necrotic spots on *Phlox drummondii*. Small malt agar blocks of all four fungi gave bacterial growth zones several centimeters in diameter on a lawn of *Arthrobacter* Jg-9 (ATCC 25091) in terregens assay medium–agar [A. D. Antoine, N. E. Morrison, J. H. Hanks, *J. Bacteriol.* **88**, 1672 (1964)]; this assay is sensitive to $\geqq 10^{-10}M$ to $10^{-9}M$ siderochromes.
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Solute Concentration Gradients in Frog Muscles at 0° C: Active Transport or Adsorption?

Abstract. *In isolated frog muscle that has been incubated for 7 days at 0°C in vitro K+ and Na+ remain at normal concentrations. Amino acids are accumulated against a concentration gradient at this temperature; for example, glycine accumulates in muscle to a concentration ten times that in the external solution. The amount of cycloleucine accumulated is greater at 0°C than at 25°C. These findings, which are difficult to explain on the basis of metabolically linked active transport, are consistent with the view that solute accumulation by cells is the result of adsorption on specific sites.*

The loss of K^+ and gain of Na^+ associated with cooling of many mammalian tissues are usually considered to be due to a decrease in the rate of metabolic reactions linked to active transport mechanisms (*1*). However, the evidence of Reisin *et al.* suggests that this observation may be explained by a reversible change in Na^+ and K^+ preference of the cell, a change that occurs at a critical temperature, which is below the normal body temperature of the animal (*2*). This change should not occur in tissues from species adapted to temperatures near freezing, so that normal concentration gradients of ions and other solutes would be maintained at 0°C. In support of this concept, I have found that muscle tissue from a northern poikilotherm, Wisconsin *Rana pipiens*, maintains its normal K^+ and Na^+ content and is also able to accumulate amino acids against a concentration gradient during a prolonged incubation at 0°C in vitro.

Four small leg muscles, the sartorius, iliofibularis, semitendinosus, and tibialis anticus longus, were dissected, with all fibers intact, from healthy frogs. After being washed for 6 hours in glucose-free Ringer phosphate (RP) (*3*) at 25°C to reduce the concentration of endogenous amino acids and hormones, the muscles were placed in RP in a watertight vial and immersed in a water bath with continuous slow shaking at either 0° ± 0.1°C or 25° ± 0.5°C. For studies of amino acid accumulation the appropriate concentration of ^{14}C-labeled glycine or cycloleucine, with carrier, was added to the incubation medium. After the desired length of time the tendinous ends were removed from the muscles. The muscles were then blotted, weighed, dried overnight (100°C), and weighed again; the difference in weights was taken as the water content of the sample. The K^+ and Na^+ contents were determined by flame photometry of hot $1N$ HCl extracts of the muscles. Labeled free amino acids were extracted with 5 percent trichloroacetic acid. Aliquots of the extracts were counted with Bray's solution in a liquid scintillation counter.

Figure 1A shows the Na^+, K^+, and water content of frog muscles maintained for periods of up to 12 days at 0°C. The water content remained constant at an average value of 80.6 percent. An initial rise in Na^+ and K^+ content was observed; this increase also occurs after the tissue is incubated for 6 hours at 25°C, and is probably the result of transfer of the muscle from plasma to Ringer solution. The concentrations of both Na^+ and K^+ remained constant from day 1 to day 7, after which K^+ began to fall slowly and Na^+ to rise.

These findings are not due to impermeability of the muscle membrane at this well-controlled low temperature because:

1) In potassium-free solution at 0°C, muscles show a net gain of Na^+ and a net loss of K^+ (Fig. 1B), an indication that both ions are free to cross the cell membrane.

2) The slowly exchanging fraction of labeled Na^+ at 0°C has a half-time of 9.5 hours (*4*).

3) Labeled glucose enters frog muscle at 0°C, reaching a steady-state concentration after 15 hours (*5*).

4) Muscles loaded with Na^+ can accumulate K^+ at 0°C (*6*).

5) Amino acids are accumulated against a concentration gradient by frog muscle at 0°C.

The results in Fig. 2 show that glycine and cycloleucine attain concentrations ten and three times that of the external medium, respectively, at 0°C. This process of accumulation is slow, requiring about 3 days to reach a steady-state concentration. Similar gradients have been obtained with lysine and tryptophan. Although the temperature dependence of amino acid accumulation has been described for a number of tissues (*7*), few studies have been carried out at temperatures as low as 0°C. Cohen and Rickenberg (*8*) reported that the steady-state concentration of valine accumulated by *Escherichia coli* was the same at 0°C as at 37°C, although the process required 120 minutes to come to equilibrium at the lower temperature compared to 1 minute at 37°C. On the other hand, Blasberg and Lajtha (*9*) found much lower concentrations of amino acids in slices of the mouse brain at 0°C than at 37°C. One cannot, however, be certain from their data that a steady state had been attained.