Biochemical and Environmental Influence on the Synthesis and Accumulation of Lichen Phenolics

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Abstract

In this review, the action of several environmental factors on the synthesis and accumulation of lichen phenolics is analyzed. Whereas nutrient availability and environmental temperature regulate production of depsides, depsidones, and usnic acids, light has been considered as the main environmental factor affecting phenol accumulation. Its role is analyzed in two ways: as a phytochrome-mediated effector or as a trophic factor, which modifies the pattern of phenolic synthesis, through photosynthesis, by controlling the size of the glucose pool.

Keywords: lichen, lichen phenolics, light, nutrients, temperature

1. Biosynthesis of lichen phenols

Depsides biosynthesis

Thirty years ago, Mosbach (1964) started the studies on lichen phenols biosynthesis by using radioactive precursors supplied to lichen thalli maintained under laboratory conditions. When malonate-2-^{14}C was supplied to *Umbilicaria pustulata* thalli, gyrophoric acid incorporated radioactivity into the 2 and 4 positions of the phenolic rings, as well as into the carboxyl substituent at 1 position. However, when the free carboxyl function of malonyl-CoA was labelled, $^{14}CO_2$ was evolved from lichen thalli whereas gyrophoric

acid did not retain any radioactivity. This pattern of label was in agreement with the mechanism proposed by Gaucher and Shepherd (1968) for an orsellinic acid synthase from *Penicillium madriti*, in which three molecules of malonyl-CoA were decarboxylated to condense with acetyl-CoA bounded to an acyl carrier protein. This mechanism did not require any dehydrogenating reaction in contrast to that found for 6-methylsalicylate-forming aromatic acid synthase, characterized from other species of free-living fungi (Packter, 1980). However, orsellinic acid synthase catalyzes a final dehydration to cyclize the polyketide chain to give orsellinic acid (Fig. 1).

Methyl-3-orsellinate, used to produce-orcinol depsides, could be synthesized by lichens using acetate-1-^{14}C or formate-^{14}C. In this last case, atranorin, a depside from β-orcinol series, incorporated radioactivity into the extra-CHO and -CH$_3$ groups (Yamazaki et al., 1965). The mechanism effecting the transfer of C1 unit did not use the cyclized polyketide chain (Fig. 1) since tritium-labelled methyl-3-orsellinate was incorporated into atranorin whereas tritiated orsellinic acid was not (Yamazaki and Shibata, 1966).

The origin of depsidones

The origin of depsidones remains uncertain but the existence of depside-depsidone pairs in the same lichen species, such as olivetoric-physodic acids in *Cetraria ciliaris* (Culberson, 1964) indicates the possibility that the deepsidone was produced from its depside through a dehydrogenase-catalyzing reaction forming an ether bond between two phenolic units. However, this hypothesis has two main problems. Firstly, it is very rare for the depside-depsidone pairs to occur in the same lichen species (Elix et al., 1984; Rogers, 1989). Secondly, several attempts to cleave the ether linkage by the action of a hypothetical oxido-reductase, coupled by NADH, NADPH or FADH$_2$ failed (Garcia-Junceda, 1990). Alternatively, Sala et al. (1981) proposed that this ether bond could be produced by de-halogenation of a phtalic anhydride derivative, followed by its coupling to a phenol and intramolecular rearrangements. This proposal is explained on the basis of several studies on organic synthesis of depsidones isolated from *Buellia canescens*.

To explain the occurrence of iso-structural depside-depsidone pairs, Elix et al. (1987) and Culberson and Elix (1989) suggested that C-hydroxylation of a *para*-depside in the 5′-position would be followed by acyl migration and subsequent Smiles rearrangement of the *meta*-depside formed, to lead to the corresponding orcinol-depsidones (Fig. 2).
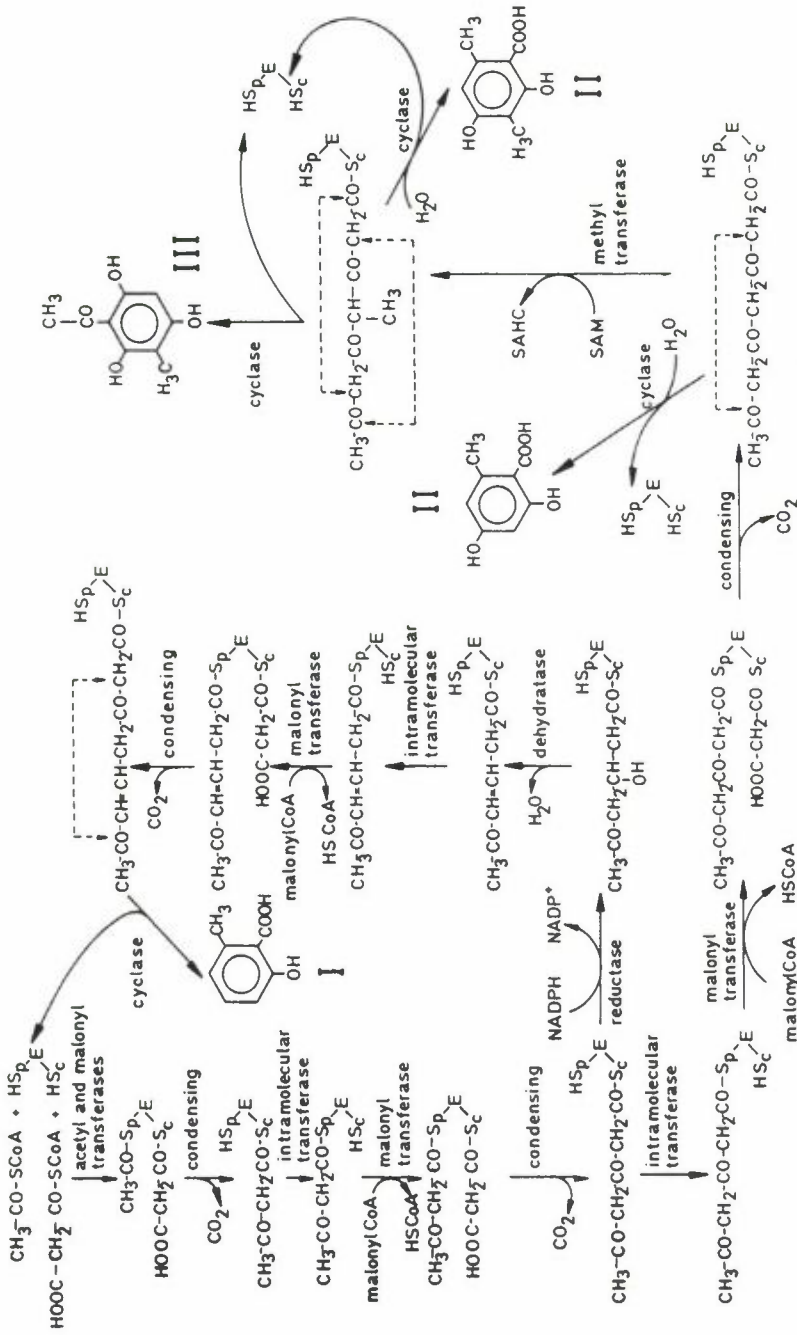
Figure 1. Proposed mechanism of aromatic acid synthase in which S_p and S_c represent active sites formed by phosphopanteteine and cystein residues, respectively. When the multienzyme complex includes dehydrogenating and dehydrating subunits, the enzyme synthesizes 6-methylsalicylate (I). The lack of these subunits defines an orsellinic acid synthase (II). Formation of derivatives such as methyl-3-orsellinate requires that a methylation reaction, possibly using S-adenosylmethionine (SAM), was effected on the polyketide chain rather than on orsellinate. This would define a methyl-3-orsellinate synthase (III). An identical situation is shown for synthesizing methylphloroacetophenone from a methylpolyketide by using a methylphloroacetophenone synthase (IV).
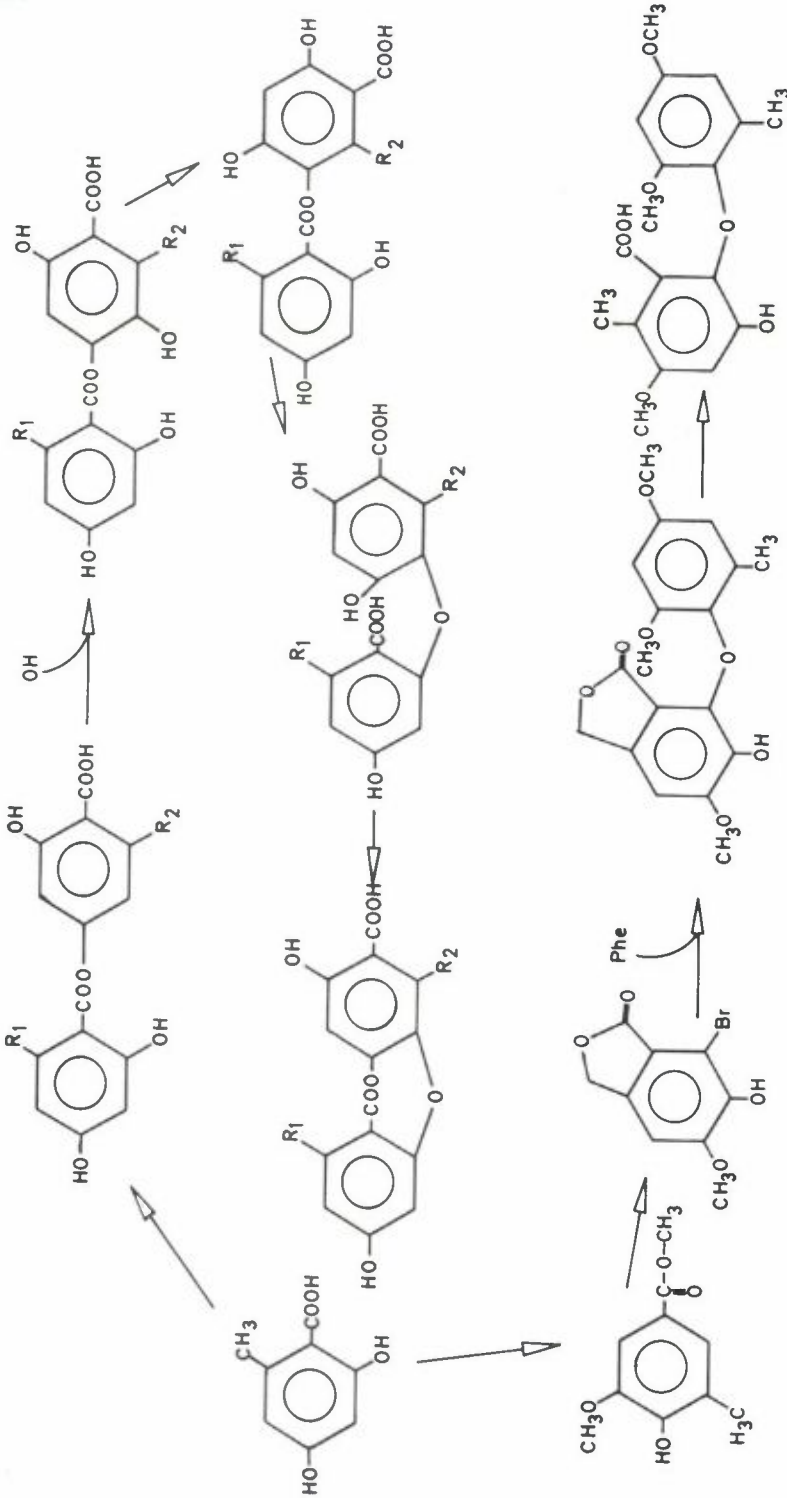
Figure 2. Proposed mechanism for organic synthesis of depsidones that mimics their biogenesis from a depside precursor or, alternatively, through a phtalic anhydride.

Biosynthesis of usnic acids

The biosynthetic pattern of usnic acids was studied by Taguchi et al. (1966) using several species of lichens and four labelled precursors, such as acetate-1-^{14}C, malonate-2-^{14}C, ^{14}CH$_3$-CO-phloroacetophenone, and ^{14}CH$_3$-CO-methyl-phloroacetophenone. These metabolites were predicted from the phloroglucinolic cyclisation that produces usnic acids. When acetate-1-^{14}C was used, the units of usnic acid incorporated radioactivity at the 1, 3, 5, and 7 positions whereas position 1 did not appear as a labelled atom if malonate-2-^{14}C was used as precursor. This fact implies that the -CO-CH$_3$ group of each phenolic unit derives from the acetate molecule.

Condensation of two phenolic units to produce usnic acid is carried out from two methylphloroacetophenone molecules, as revealed by using labelled precursors. Usnic acid retained radioactivity in the acetyl substituent when ^{14}CH$_3$-CO-methyl-phloroacetophenone was supplied to lichen thalli, but no radioactivity was retained in this group when ^{14}CH$_3$-CO-phloroacetophenone was used (Fig. 1, Taguchi et al., 1966).

2. Roles of Lichens Phenols

Lichens produce unique chemical substances, phenols, which are very different from those synthesized by other plant species (Culberson et al., 1977). The major biosynthetic steps for the production of lichen phenols are those of the fungus (Shibata, 1976), but the algal partner seems to be able to change the pattern of chemical substitution of some of those components (Hamada and Ueno, 1987).

The equilibrated growth of the symbionts

There has been much speculation on the function of lichen phenols. According to Culberson and Ahmadjian (1980) hypothesis, lichen phenols may form because the alga protects itself by preventing the fungus from synthesizing toxic compounds. It has been postulated that the fungal partner regulates the rate of growth and proliferation of algal cells in the thallus (Honegger, 1987) by translocating substances which act as phycocidal agents (Kinraide and Ahmadjian, 1970). In relation to this, algal production of some esterases (Vicente and Legaz, 1988) and dehydrogenases (Avalos and Vicente, 1987a), which catabolize lichen phenolics, can be seen as a defense mechanism against the fungal attack on the alga (Ahmadjian, 1987). On the other hand, some of the phenolic products move from the mycobiont, where they have been produced, to the photobiont cells, appearing as crystalline deposits on their

cell walls (Ahmadjian and Jacobs, 1985; Honegger, 1986) or even as soluble cytosolic components (Avalos and Vicente, 1987b).

Equilibrated growth of bacterial population

Lichen phenols, like antibiotics (Vicente, 1989), can be used to stabilize bacterial populations on the thalli. When Ahmadjian (1989) achieved resynthesis of *Peltigera canina*, he reported that bacterial contamination was common, both on spore discharge plates and in culture tubes that contain the photobiont of this lichen species. However, bacterial cells were not observed in the gelatinous sheaths of the lichenized *Nostoc* of three *Peltigera* species, but they were in the sheaths of the cultured photobiont of *P. spuria* (Koriem and Ahmadjian, 1986). The general absence of bacteria in the *Nostoc* sheaths suggested that the cyanobiont may produce toxic compounds which suppressed bacterial growth.

Related to this finding, Zook (1983) reported that he isolated mainly actinobacteria from the thallus of *P. canina*, a species with few lichen phenols, and proposed that actinobacteria found in this species provide a protective role by producing antibiotics.

The role of bacteria during lichenization is not clear. Remmer et al. (1986) hypothesized that hormones (auxin, cytokinine) required for correct thallus differentiation, may be produced by the alga, the fungus, or by bacteria within the thallus.

Lichen phenols as light screening agents

Lichen phenols have often been described as light screening agents (Rundel, 1978; Lawrey, 1984). A good correlation between usnic acid concentration in the cortex of specimens of *Cladonia subtenuis* (Rundel, 1969) or parietin in the cortex of *Xanthoria parietina* (Hill and Woolhouse, 1966; Richardson, 1967) and the light intensity of the habitat has been reported. Even the colour of the thalli in nature indicates higher metabolic activity in the light than that expected in the dark. We have studied thallus samples of *Cladonia verticillaris* collected in an open ground receiving full sunlight and the same species growing under a canopy formed by several shrubs that filters about a half of the incident light intensity on its surface. Analysis by HPLC of the methanolic extracts of both samples revealed that there were no significant differences in the concentration of atranorin, but sunny *Cladonia* contained about 1.4 times more fumarprotocetraric acid and 2.55 times more orcinol than those found in shaded specimens (Legaz et al., 1986).

Sometimes, a role as accessory photosynthetic pigments for lichen phenols has been suggested. Atranorin, for example, can absorb light of shorter wavelengths and re-emit fluorescence at longer wavelengths, which would be used by chlorophylls (Rao and Leblanc, 1965; Redon, 1985), but this function has been questioned (Lawrey, 1984).

Hemiparasitical action of epiphytic lichens

Epiphytic lichens play a secondary role as pathological organisms against the phytophores on which they grow (Asahina and Kurokawa, 1952; Hale, 1983; Ascaso and Rapsch, 1985). *Quercus pyrenaica* branches, as well as those from other deciduous species, supporting the epiphytic lichens *Evernia prunastri* and *Ramalina calicaris* contained both evernic and everninic acids which were acropetally translocated, via xylem, to reach the leaves (Avalos et al., 1986). However, lichen phenolics were not found in the mesophyllic cells and, thus, the photosynthetic ability of leaves was not affected (Table 1, Avalos et al., 1986). In the earlier spring, evernic acid accumulated in foliar buds (Vicente, 1988), where it inhibited respiration and development of leaf blades (Legaz et al., 1988).

In contrast to that found for deciduous species, epiphytic lichens on the evergreen, *Q. rotundifolia*, injected usnic acid, instead of depsides, into xylem vessels that reached leaves and enhanced the respiratory uptake of oxygen (Gimenez and Vicente, 1989a). This phenol preferentially accumulated in leaves beneath the implantation point of the lichen on oak branches (Gimenez and Vicente, 1987b) in which it inhibited photosynthetic electron transport (Gimenez and Vicente, 1989c).

Other functions of lichen phenolics

Lichen phenolics are well known compounds producing chemical alteration and neogenesis of rock material (Ascaso, 1985) and are also responsible for adaptation and selection patterns of some herbivores (Lawrey, 1984, 1986).

3. Environmental conditions of phenol biosynthesis and accumulation

Nutrients

There are no records, to my knowledge, about the influence of nutrients on lichen acid metabolism. It is possible that the secondary compounds of lichens can be recycled and used as respiratory substrates when a depletion

in photosynthesis or exogenous supply of organic nutrients occurs in a way similar to that described for flavonoids (Barz and Hosel, 1975) and alkaloids (Waller and Nowacki, 1978) in higher plants.

Ammonia supplied to *Cladonia sandstedei* thalli changed the quantitative composition of its phenolic fraction (Vicente et al., 1984). Both fumarproto-cetraric acid and atranorin disappeared after 5 hr when thalli were incubated on 20 mM ammonium sulfate, and a new substance, as revealed by HPLC with a retention time similar to that of norstictic or salazinic acids, appeared (Vicente et al., 1984).

Quantitative changes in the composition of the phenolic fraction of several lichen species are also possible. Brown and Legaz (personal communication) found that phosphate increased usnic acid and decreased evernic acid concentrations in *E. prunastri* thalli.

Temperature

Hamada (1982) studied the content of salazinic acid in *Ramalina siliquosa* thalli and demonstrated that this content was higher when the annual main temperature was higher. On the other hand, the salazinic acid concentration in lichens growing on dark-coloured rocks (basic rocks) was higher than that found in lichens growing on acidic, light-coloured rocks. This difference can be due to different nutritional conditions but it is also probable that the temperature near the dark surface was higher than that near the light surface. Similar results were obtained for both divaricatic and salazinic acids from *R. subbreviuscula* (Hamada, 1983), and for protocetraric acid in *R. siliquosa* (Hamada, 1985).

Culberson et al. (1983) have found that barbatic acid and its derivatives increased at low temperature values for four cloned lines of *C. cristatella*.

Archer (1981) postulated that the action of temperature on the accumulation of phenolic products in lichens was a secondary effect of light, since the highest concentration of secondary compounds by heavily lighted *Cladonia* species was due to the establishment of very saturated solutions of lichen phenols in the tips of thalli as a consequence of water evaporation.

Light

Light has been considered as the main environmental factor affecting the synthesis of lichen phenolics. Usnic acid, expressed as percent of thallus dry weight, linearly increased as a response to sunlight intensity received on the surface of podetial apex of *C. subtenuis* (Rundel, 1969). Atranorin seems to

be a compound that does not change its concentration under different light intensities. Fahselt (1981) found that atranorin concentration was not affected by four different values of light intensity, whereas fumarprotocetraric acid concentration increased as light intensity increased. Measurements were performed by densitometry of thin-layer chromatograms. A similar relationship has been found by Stephenson and Rundel (1979) for vulpinic acid from *Letharia vulpina*.

In any case, there are differences between **actual concentration** of lichen phenols and actual new synthesis of these compounds. When ^{14}C-urea was supplied to *E. prunastri* thalli floating on phosphate buffer, analysis of retained radioactivity in the thallus showed that four times more label was incorporated under white light than in darkness (Blanco et al., 1984). This indicates that light accelerated the new synthesis of lichen phenolics and that the rate does not change in lichen thalli maintained in the dark (Fig. 3).
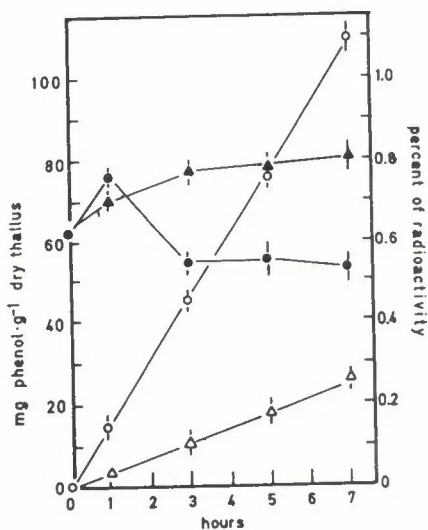

Figure 3. Time-course of total phenol accumulation (filled symbols) or newly synthesized phenols (empty symbols) in *Evernia prunastri* thalli floated on ^{14}C-urea in the dark (triangles) or in white light (circles) with a photon flux density of 150 μmol m^{-2}s^{-1}. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

4. Hypothesis and results about the action of light on phenolic production

Light increases phenolic concentration through two general mechanisms: by increasing the size of the hexose pool derived from photosynthesis and by

regulating the level of orsellinate derivatives by tee action of Pfr, the far-red absorbing form of phytochrome (Avalos and Vicente, 1986a). Phytochrome is affecting several enzymes of the lichen phenol metabolism, such as orsellinate depside hydrolase (Avalos and Vicente, 1986b), and usnic acid:NAD^+ (H) oxido-reductase (Avalos and Vicente, 1987a). Activation or induction of these enzymes involve the increase of the level of cyclic AMP (Vicente and Avalos, 1989), but the system escapes from photoreversibility when part of this cyclic AMP is used by a protein kinase to phosphorylate, possibly, Pfr (Vicente and Avalos, 1990).

Light and the size of hexose pool

According to Brown et al. (1987), *E. prunastri* thalli showed high values of net photosynthesis up to 16 hr of incubation in water, as fresh material or after desiccation, when it was measured as carbon dioxide fixation into ethanol-soluble carbohydrates. Rewetted thalli of the same lichen species reached saturated net photosynthesis after 1.5 hr of light when it was measured as carbon dioxide influx by an infra-red gas analyzer (Herrero et al., 1989). Under the latter conditions, the total amount of D-usnic acid in the thalli did not vary but that of evernic acid slightly decreased after 24 hr of light. However, concentration of both phenolics triples after the same time periods when lichen thalli were infiltrated with 10 μM DCMU, 3-3'-(dichlorophenyl)-1, 1-dimethylurea (Herrero et a., 1989). Consequently, DCMU impeded new production of glucose in the light, but mannitol accumulation in these lichen thalli only decreased from 3.0 mg g^{-1} dry thalli h^{-1} to 2.25 mg g^{-1} dry thalli h^{-1} after 3 hr treatment (Fig. 4).

On the other hand, physodic acid production by *Pseudevernia furfuracea* seems not to depend on dark-light cycles, thallus desiccation or acetate supply. In this case, DCMU slightly increased physodic acid accumulation in the light whereas that of its catabolite, 5'-hydroxyolivetoric acid, remained unchanged (Garcia-Junceda et al., 1987). DCMU also increased the concentration of atranorin 10 times (Fig. 5).

When photosynthesis is simulated in the dark by adding glucose to lichen thalli, the amount of both usnic and evernic acids in *E. prunastri* thalli, or physodic acid in *Ps. furfuracea*, was strongly depleted. In contrast, *E. prunastri* thalli loaded with mannitol actively accumulated usnic and evernic acids (Fig. 6).
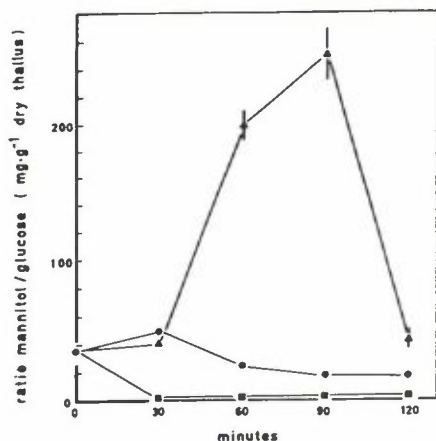
Figure 4. Time-course of the ratio mannitol/glucose in *E. prunastri* thalli floated for 2 hr on distilled water in the dark (squares), in white light (circles) with a photon flux density of 150 μmol m^{-2}s^{-1}, or in white light but infiltrating the thalli with 10 μM DCMU (triangles). Values are the mean of four replicates. Vertical bars give standard error where larger than the symbols.

Equilibria in the ratio mannitol/hexoses

According to the results described above, synthesis of lichen phenols in light seems to be regulated by the value of the ratio of mannitol/glucose, i.e., by the ability of the fungal partner to produce mannitol by decreasing the size of the glucose pool. Accumulation of mannitol in *E. prunastri* thalli was achieved in the dark at a rate 1.7 times higher than in the light (Legaz and de Torres, 1987). This accumulation displaced its maximum from a pH value of 6.0 to 8.0 when thallus samples are floated on MOPS or HEPES buffers (Legaz and de Torres, 1987). Little information about the enzymes of mannitol metabolism in lichens is now available. However, fructose-6-P-producing mannitol-1-P dehydrogenase from *Aerobacter aerogenes* has an optimum pH value of 9.5 (Liss et al., 1962) whereas *Evernia* dehydrogenase acts at pH 6.0 (Mateos and Legaz, personal communication). In addition, acidic pH values, such as 5.0, displace the equilibrium of the reaction catalyzed by mannitol dehydrogenase to mannitol production, whereas basic pH values, near 8.0, enhanced the formation of fructose (Martinez et al., 1963).

Light produces a net efflux of protons from the thallus to the media whereas this situation is reversed in the dark. So, light, and the alkaline intracellular environment which light produces, are related to both photosynthetical carbon dioxide reduction and an increase of the size of hexose-P/hexose pool by inhibiting mannitol-1-P and mannitol dehydrogenases (Fig. 7). This can ex-
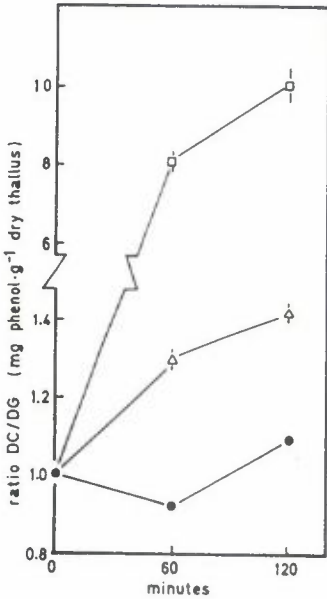
Figure 5. Time-course of atranorin accumulation, expressed as the ratio value of treatment in distilled water (DC) to 10 mM glucose incubation (DG), in *Pseudevernia furfuracea* thalli floated on distilled water in the dark (circles), 10 μM in white light (triangles) with a photon flux rate of 130 μmol m^{-2}s^{-1}, or 10 mM acetate (squares) in the dark. Values are the mean of four replicates. Vertical bars give standard error where larger than the symbols.
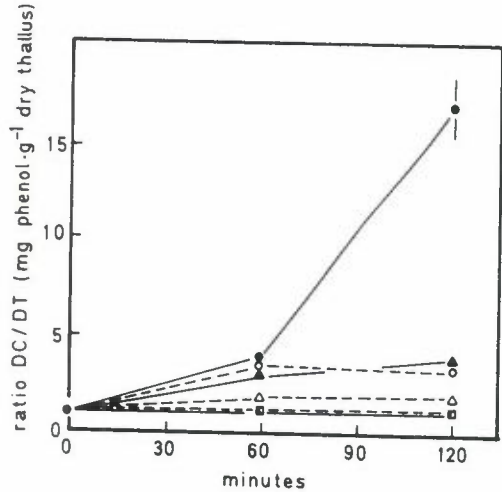
Figure 6. Time-course of evernic acid (filled symbols) and usnic acid (empty symbols) accumulation by *E. prunastri* thalli floated in the dark on 16.7 mM mannitol (squares), 1.7 mM glucose (circles), and 1.7 mM glucose and 0.5 mM cyclic AMP (triangles), expressed as the ratio of phenol accumulated in thallus samples floated on distilled water (DC) to that obtained after different treatments (DT). Values are the mean of four replicates. Vertical bars give standard error where larger than the symbols.

plain why increasing values of photon flux density, varying from 2 to 100 μmol m^{-2}s^{-1} do not enhance mannitol accumulation (Legaz and de Torres, 1987).

This is not different from observations described in the literature about the chemical nature of the translocatable products between the symbionts. Although ribitol was the main translocated compound from the algal to the fungal partner (Smith, 1980; Richardson, 1985), small amounts of glucose are secreted by whole thalli and, so, possibly also transferred (Vicente and Blanco,
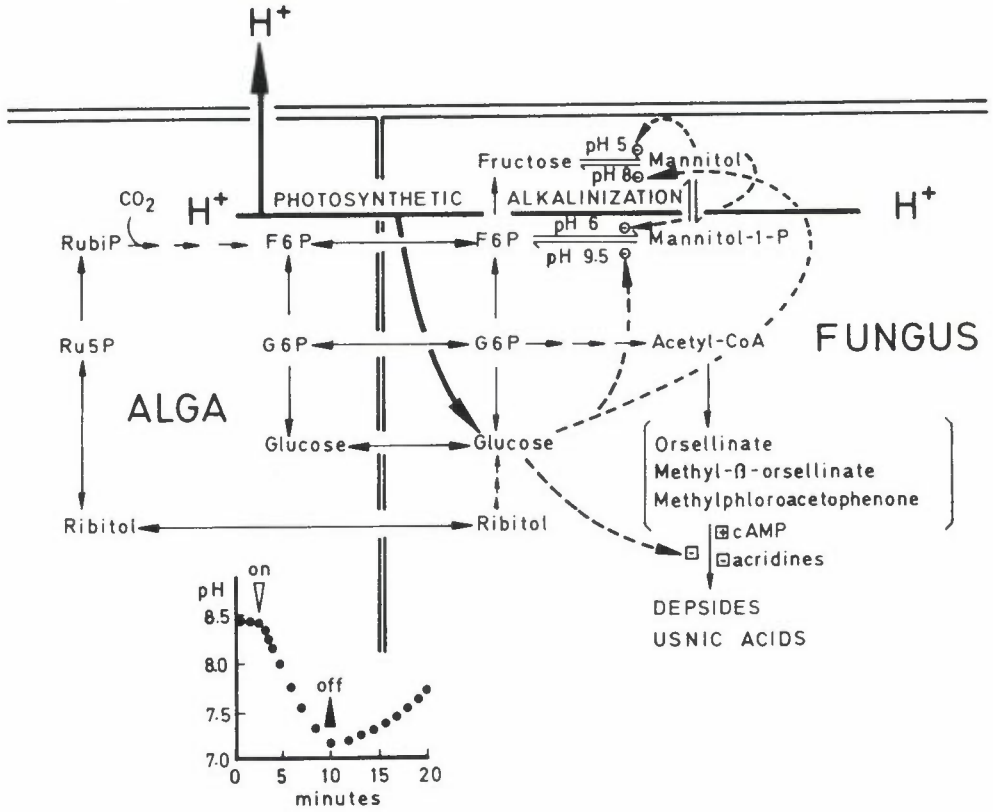
Figure 7. Diagrammatic representation of the catabolite negative control of the accumulation of phenols as a function of the size of glucose pool in the mycobiont. Photosynthesis in the photobiont implies hexose production as well as proton efflux, that promotes mannitol (or mannitol-1-P) production by the fungal partner. Consequently, the internal pH value of the algal component decreases in light (insert in the Figure). Mannitol does not affect phenolic production whereas this last decreases after glucose accumulation when mannitol dehydrogenases produce hexose at basic pH values.

1985). Dependence of photosynthetic ability upon pH values can be related to the high rate of growth reported for *Cladonia vulcani* and its photobiont, *Trebouxia excentrica*, at pH 4.0 (Yoshimura et al., 1987).

Regulation of lichen phenolic biosynthesis and the mechanism of catabolite repression

The starting point of our rationale is that an exogenous supply of glucose mimics hexose accumulation in the mycobiont. Transfer of glucose from the photobiont, under natural conditions, is not specifically required. Conversion

of ribitol to mannitol (Smith, 1990) necessarily uses the pentose-P pathway (Bielesky, 1982; Vicente and Legaz, 1988), and then, glucose may be produced by the fungus so that the size of the fungal glucose pool can increase by inhibiting mannitol dehydrogenases. On this basis, it is possible to explain the lack of phenol accumulation by thalli loaded with glucose, because this hexose may inhibit some enzymes of depside and usnic acid synthesis. However, two well-known enzymes of this pathway, orsellinate depside hydrolase and usnic acid oxido-reductase, do not change their activity in the presence of glucose *in vitro* conditions. Urea, another catabolite repressor (Sanzey and Ullman, 1976) does not inhibit orsellinate depside hydrolaase for a wide range of concentrations (Gonzalez, 1984).

The glucose effect (or catabolite repression) defined here, is partially reversed by clclic AMP (Fig. 6). A CAP-cyclic AMP complex in prokaryotes acts at the same point of glucose on DNA, by favouring the formation of the transcription initiation complex (Zubay et al., 1970) and displacement of glucose, or catabolite repressor. In eukaryotes, the catalytic subunit of A kinase is sufficient to induce expression of cyclic AMP-responsive genes (Riabowol et al., 1988). So, the partial reversion of the glucose effect on the accumulation of evernic and usnic acis by cyclic AMP, found in *E. prunastri* thalli, can be taken as evidence of a catabolite repression mechanism (Fig. 6).

In agreement with this proposal, acridine orange also inhibits phenol accumulation in *E. prunastri* thalli. Acridine dyes are known to intercalate between
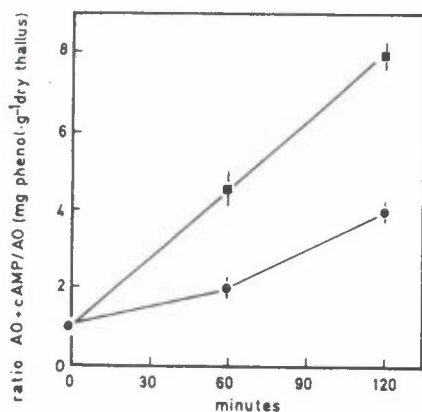

Figure 8. Time-course of evernic acid (squares) or usnic acid (circles) accumulation by *E. prunastri* thalli floated in the dark on 0.1 mM acridine orange or the same concentration of dye supplied with 0.5 mM cyclic AMP. Values are the mean of four replicates. Vertical bars give standard error where larger than the symbols.

the bases of DNA double helix causing an extension and unwinding of the deoxyribose backbone, and this effect is partially reversed by the CAP-cyclic AMP complex (Sankaran and Pogell, 1973). Effectively, a supply of cyclic AMP to acridine-loaded thalli restored the pattern of phenol accumulation (Fig. 8).

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