EFFECT OF CYCLIC AMP AND ACRIDINE ORANGE ON THE ENZYMATIC REDUCTION OF USNIC ACID IN THE LICHEN *USNEA AURANTIACO-ATRA* (JACQ.) BORY

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(Received: June 27, 1995. Accepted: August 3, 1995)

ABSTRACT

A fluorescence-detection HPLC procedure has been applied to identify and quantify acridine orange in lichen samples. The dye accumulates in thalli of the lichen *Usnea aurantiaco-atra* and, in part, it is recovered from protamine-precipitated nucleic acids extracted from the samples. A supply of exogenous cyclic AMP reverses the uptake of acridine orange by thallus samples and its binding to nucleic acids. The dye impedes neither the loss of endogenously-produced cyclic AMP by thallus samples nor inhibits the uptake of that exogenously supplied. However, part of the endogenously produced cyclic AMP is secreted to the incubation medium in which phosphodiesterase activity has never been detected. Since the synthesis of D-usnic acid: NAD^+(H) oxido-reductase is impeded by acridine orange, oxidative catabolism of usnic acid is inhibited in thalli floated on the dye. Cyclic AMP reverses this effect.

KEY WORDS: Acridine orange, catabolite repression, cyclic AMP, usnic acid, usnic acid: NAD^+(H) oxido-reductase, *Usnea aurantiaco-atra*.

INTRODUCTION

Acridines readily bind to DNA bases that move apart to accomodate acridine molecule and, thus, the dye behaves as an intercalated extra base-pair (Le-Pecq and Paoletti, 1967). Acridine intercalation impedes replication and transcription of bacterial episomes (Sankaran and Pogell, 1973; Slonimsky et al., 1968) probably by reducing DNA superhelicity. This action is reversed by cyclic AMP, but the reversion implies several unexplained facts. A CAP (Catabolite genes Activator Protein)-cyclic AMP complex binds to specific DNA targets near the promoters which are sensitive to glucose repression (Zubay et al., 1970). However, the bind of acridine to DNA is not specific. Thus, the reversion by cyclic AMP of the acridine action must be studied. In eukaryota, a different mechanism produces similar effects, since the catalytic subunit of cyclic AMP-dependent protein kinase induces expression of several sensitive genes (Riabowol et al., 1988).

Several catabolite-sensitive promoters which are inhibited by glucose also occur in lichens (Vicente and Legaz, 1985, 1988). The synthesis of both evernic and usnic acids in *Evernia prunastri* seems to be regulated by the promoters inhibited by glucose (Herrero et al., 1989; Legaz and Vicente, 1991). Inhibition is reversed by cyclic AMP. Atranorin, another phenolic produced by the lichen *Pseudevernia furfuracea*, also seems to be sensitive to the catabolite (García-Junceda et al., 1987). In spite of the unspecific binding of acridine to DNA, the dye produces similar effects. *Himantormia lugubris* produces barbatolic acid through a process which is independent on glucose or acridine supply (Mateos et al., 1991a). However, heat-shocked thalli of *H. lugubris* produce a plasmid of about 0.5 kb (Mateos et al., unpublished) which shows to be sensitive to acridine orange, inhibiting barbatolic acid synthesis. The ability to produce this phenolic is recovered by adding cyclic AMP to heat-shocked thalli.

Since acridine action is dependent on the level of endogenous cyclic AMP in organisms having catabolite-sensitive promoters (Vicente et al., 1993), we attempt to study, in this paper, the relationship between acridine addition to the lichen *Usnea aurantiaco-atra* and the production and accumulation of cyclic AMP in lichen thalli, by considering this last metabolic event as the first step in the regulation of the activity of some of these promoters related to the production or mobilization of usnic acid.

Abbreviations:
cAMP: cyclic adenosine-3',5' monophosphate;
CAP: catabolite genes activator protein;
DEAE-Sephadex: diethyl aminoethyl-Sephadex;
HPLC: high performance liquid chromatography;
Tris-HC1: tris (hydroxymethyl) aminomethane hydrochloride.

MATERIAL AND METHODS

Plant material

Usnea aurantiaco-atra (Jacq.) Bory, growing on soil in King George Island (Antarctica), was used throughout this work. Thalli were air-dried and stored in the dark at 5°C until required.

Incubation conditions and samples preparation

Samples of 0.5 g of air-dried thalli were sterilized by soaking for 5 min in 10% (w/v) sodium hypochlorite. After extensive washing with sterile, distilled water, thallus samples were floated on 25 ml of 0.1 M sodium acetate-acetic acid buffer, pH 6.8, for 3h at 26°C in the dark. When indicated, 0.1 mM acridine orange and 0.5 mM cyclic AMP were added to the buffer. After incubation media were lyophilized, whereas thallus samples were repeatedly washed with abundant sterile, distilled water, gently dried with filter paper, disrupted with liquid nitrogen and macerated in a mortar with 25 ml chloroform: acetonitrile (60:40 v/v) for 15 min at room temperature to extract lichen phenolics and free acridine (Legaz et al., 1992). Homogenates were filtered through Whatman No 3 paper and solid debris were air-dried and stored at -34°C. Filtrates were dried in air flow and residues were redissolved in 1.0 ml acetonitrile (HPLC grade) and filtered through Millipore GS filters (0.22 µm pore diameter) to be chromatographed for quantifying acridine orange.

Thalline powders were macerated with 8.0 ml distilled water and centrifuged at 38.000 x g for 30 min at 2°C. Supernatants were separated in two aliquots of approximately 3.5 ml. One of them was brought up to 5% (w/v) protamine sulfate and stored for 20 min with ice-cold protection. Then, this was spun at 43.000 x g for 30 min at 0°C. The pellet was washed with 2.0 ml diluted protamine sulfate solution (0.05% w/v), acridine orange extracted with 2.0 ml acetonitrile (HPLC grade) and the extract filtered through Millipore GS filters as above. The filtrate was used to quantify acridine orange. The other aliquot was precipitated with acetonitrile (v/v), centrifuged at 38.000 x g for 30 min at 2°C to remove precipitated protein and the supernatant was dried in air flow. Residue was redissolved in 10 ml distilled water, filtered through a column (8 cm x 2 cm) of active neutral alumina and eluted with distilled water (Spiteri et al., 1989). Fractions showing absorbance at 260 nm were collected and lyophilized. Lyophilized media were processed in the same way. Residues from both media and thalli were redissolved in 2.0 ml methanol (HPLC grade), filtered through Millipore GS filters and used to quantify cyclic AMP.

Alternatively, thalline powder, after removing lichen phenolics, was extracted with 10 ml 30 mM Tris-HCL buffer, pH 7.8, centrifuged at 20.000 x g for 30 min at 2°C and 5'-nucleotidase activity was assayed in the cell-free extract. Media were dialyzed overnight against 5.0 l of the same buffer, at 4°C, and then lyophilized. Dry residues were redissolved in 2.0 ml Tris buffer and assayed for phosphodiesterase activity.

HPLC analysis of acridine orange and usnic acid

HPLC separation and quantization of acridine orange was performed on a Varian Model 5060 liquid chromatograph equipped with a Fluorichrom TM detector, and a Vista CDS 401 computer, according to Legaz et al. (1992). The chromatographic conditions were as follows: column, Nucleosil 5 C8 (125 mm x 4 mm I.D.) from Varian; sample loading, 10 µl; mobile phase, acetonitrile: water (80:20 v/v) isocratically; flow rate, 1.2 ml min^{-1}; pressure, 102 bars; temperature, 20°C; detector, fluorescence (excitation wavelengths from 340 to 380 nm using filters No 7-54 and 7-60 from Varian, and emission wavelength, 460 nm, using filters No. 3-71 and 4-76, Varian): absorbance units at full scale, 0.002; attenuation, 64; internal standard, 1.0 mg ml^{-1} norstictic acid. The same procedure was used to quantify usnic acid, but an UV set at 254 nm was used as detector and the flux rate was 0.7 ml min^{-1}.

HPLC analysis of cyclic AMP

Samples were chromatographed by using a Varian 5000 liquid chromatograph equipped with a Vista CDS 401 computer, according to the method of Vicente and Mateos (1992). Chromatographic conditions were as follows: reverse phase column (300 mm x 4 mm i.d.) packed with MicroPak MCH-10; loading, 10 µl; mobile phase, methanol : acetic acid : water (80 : 0.5 : 19.5 v/v) isocratically; pressure, 88 bars; temperature, 20°C; flow rate, 1.0 ml min^{-1}; absorbance units at full scale, 0.005; detector, UV set at 254 nm; internal standard, 0.1 mg ml^{-1} 5'-AMP; external standards, 0.1 mg ml^{-1} cyclic AMP, 3'- AMP and adenosine. Retention time for cyclic AMP was 2.38 min.

Purification of cyclic AMP phosphodiesterase and 5'-nucleotidase

About 20.0 g of *U. aurantiaco-atra* thalli were disrupted with liquid nitrogen and homogenized with 50 ml acetone to remove lichen phenolics. Acetone was removed by filtration and dry residue was homogenized with 10 mM Tris-HCl buffer, pH 7.8. Homogenate was centrifuged at 20.000 x g for 20 min at 2°C. The pellet was discarded and the supernatant was brought up to 80% saturation with ammoniun sulfate. After 1 h at 2°C, the mixture was centrifuged at 27.000 x g for 30 min at 2°C. The supernatant was discarded, the pellet was resuspended in 10 ml 10 mM Tris-HCl buffer, pH 7.8, and dialyzed overnight against the same buffer to eliminate ammonia.

Protein in dialysate was adsorbed on calcium phosphate gel (75 mg dry gel per mg protein) and desorbed by increasing the concentration of the buffer. The fraction desorbed with 30 mM Tris-HCl was loaded onto a bed (2.5 cm x 1 cm) of DE-AE-Sephadex, equilibrated with the same buffer, and protein was eluted with increasing concentrations of sodium chloride in the buffer. Alternatively, media after thalli incubation were recovered, dialyzed overnight against 5.0 l 30 mM Tris-HCl buffer, pH 7.8, lyophilized and used to measure phosphodiesterase activity.

Phosphodiesterase activity was assayed in reaction mixtures containing 6.0 µmol cyclic AMP, 1.2 µmol magnesium chloride, 10 µg protein and 30 µmol Tris-HCl, pH 7.8, in a final volume of 3.0 ml. After 30 min at 30°C, reaction was stopped by adding 3.0 ml pure acetonitrile. For 5'-nucleotidase assay, AMP was used instead of the cyclic nucleotide. Cyclic AMP, AMP or adenosine was recovered as above and analyzed by HPLC as described. Protein was estimated by the method of Lowry et al. (1951), modified by Potty (1969). One unit of phosphodiesterase activity was 1.0 µmol of AMP produced per mg protein and minute, whereas that of 5'-nucleotidase activity was 1.0 µmol of adenosine produced per mg protein and minute.

Assay of D-usnic acid : $NAD^+(H)$ oxido-reductase activity

Thalline powders, prepared as above, were ground in a mortar with sufficient volume of 10 mM Tris-HCl buffer (pH 8.0) to give a final concentration of 0.1 mg protein ml^{-1}. Crude extracts were centrifuged at 21.000 x g for 20 min at 2°C and the supernatants were used for enzyme assay.

Oxido-reductase activity was estimated according to Avalos and Vicente (1987) by following, at 30°C, the rate of enzyme-catalyzed oxidation of NADH by D-usnic acid in a reaction mixture containing 0.1 mg protein, 106 µmol Tris-HCl (pH 8.0), 2.0 µmol D-unsic acid and 4.0 µmol NADH in a final volume of 3.0 ml. A unit of specific activity was defined a 1.0 µmol NADH oxidized per mg protein per min. Controls

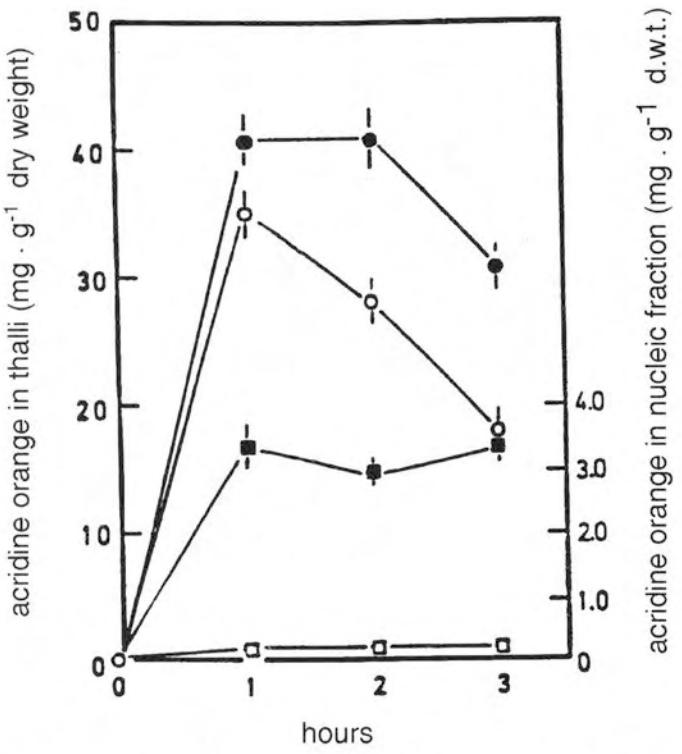

Fig. 1. Time-course of acridine accumulation by thalli (circles) and extracted from protamine-precipitated nucleic acids obtained from thalli (squares) of *U. aurantiaco-atra* floated on 0.1 mM acridine orange (filled symbols) or 0.1 mM acridine orange and 0.5 mM cyclic AMP (empty symbols) in 0.1 M acetate buffer. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

without usnic acid were performed to know the rate of spontaneous oxidation of NADH by cell-free extracts. Protein was estimated by the method of Lowry et al. (1951), modified by Potty (1969), using bovine serum albumin as a standard.

RESULTS

Quantitation of acridine orange uptake and binding

By using chloroform-acetonitrile extracts from lichen thalli and norstictic acid as internal standard, acridine orange was separated as a well-defined peak with a retention time of 11.42 min. This peak quantitatively increased after loading the samples with 10 µg acridine orange. Response of the detector was linear for a range of mass injected, varying from 0 to 5.0 µg acridine orange.

The time-course of acridine orange accumulation in *U. aurantiaco-atra* thalli is shown in Fig. 1. Thalli floated on the dye accumulated acridine orange for the first hour of incubation, and then the amount of the dye in thalli remained constant. When thalli were incubated with 0.5 mM cyclic AMP in the media, lower amounts of acridine orange than those obtained without cyclic AMP were always recovered and, even, accumulation of acridine orange was clearly reversed from the second hour of thalli incubation. Binding of acridine orange to protamine-preciptated nucleic acids rapidly increased for the first one hour of thall incubation, then remained constant (Fig. 1). However, an exogenous supply of cyclic AMP strongly inhibited this binding.

Productiton of cyclic AMP and adenosine

Quantitation of cyclic AMP was performed after filtering aqueous cell-free extracts through a column of alumina. Eluates showing absorbance at 260 nm ranged from 35 ml to 55

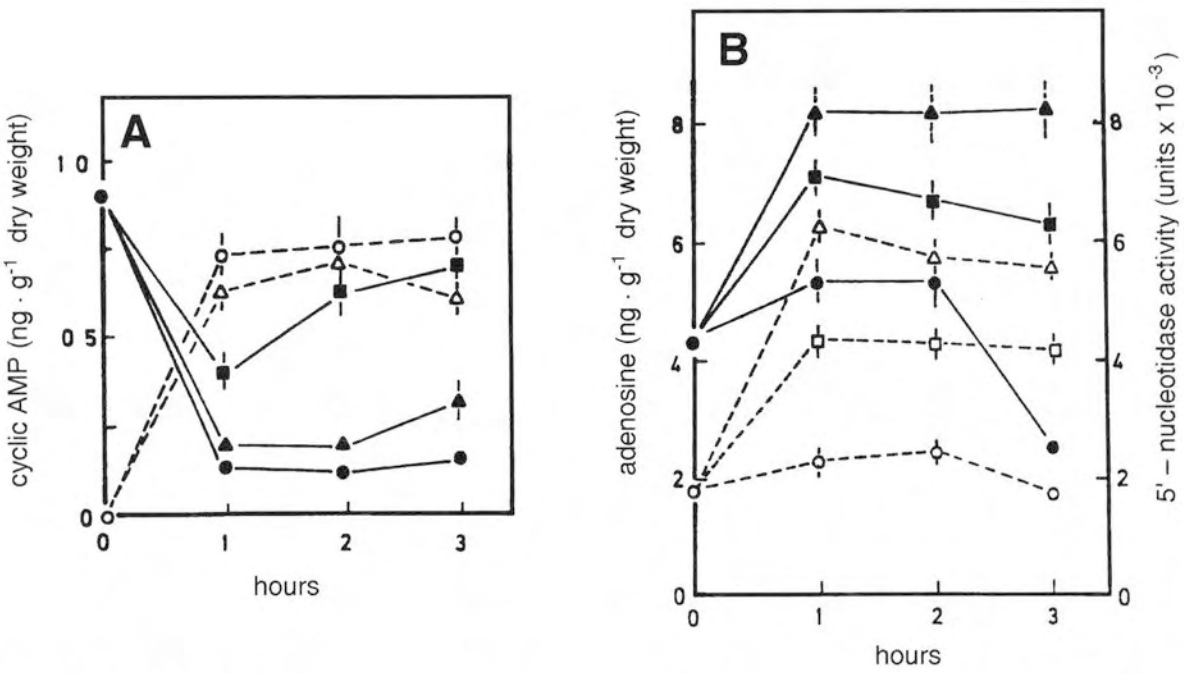

Fig. 2. A) Time-course of cyclic AMP accumulation (cotinuous line) or secretion to the mediun (dashed line) by thalli floated on 0.1 M acetate (●), 0.1 mM acridine orange (▲) and 0.1 mM acridine and 0.5 mM cyclic AMP (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

B) Time-course of adenosine accumulation (continuous line) and 5'-nucleotidase activity (dashed line) by thalli floated on 0.1 M acetate buffer (●), 0.1 mM acridine orange (▲) and 0.1 mM acridine and 0.5 mM cyclic AMP (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

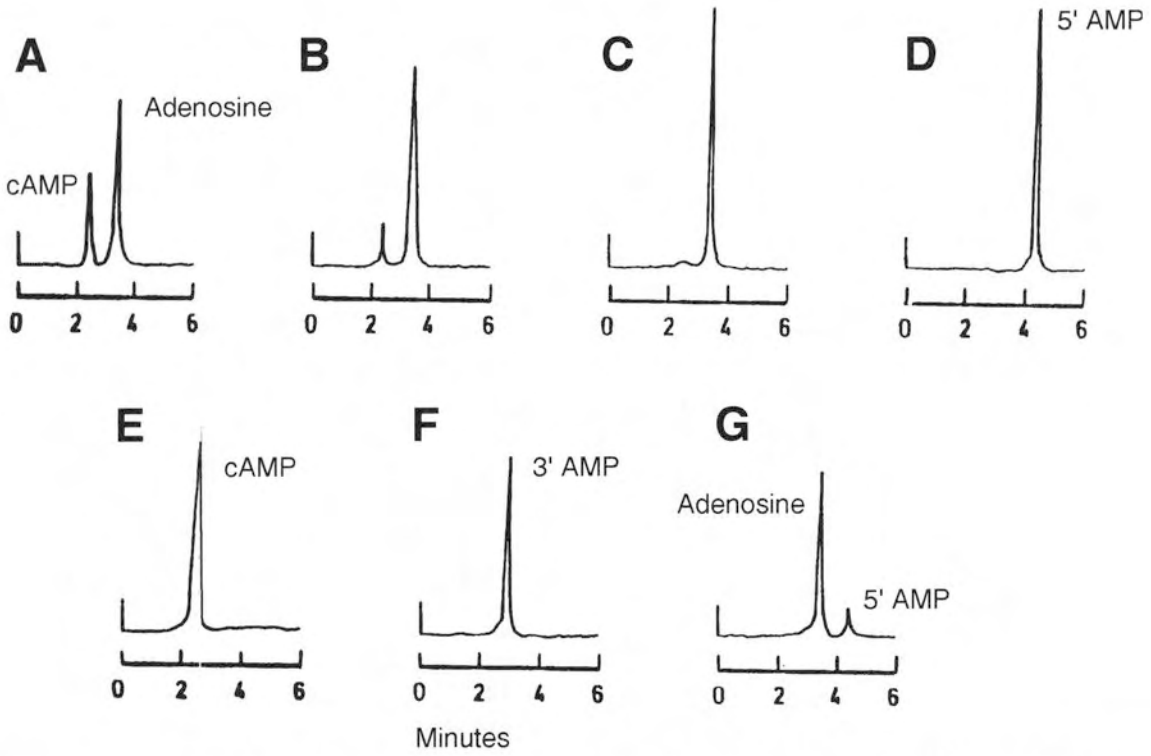

Fig. 3. Chromatographic traces obtained from reaction mixtures containing cyclic AMP as a substrate and A) cell-free extract, B) dialyzed pellet from ammonium sulfate precipitation, and C) desorbed fraction from calcium phosphate gel as a source of enzyme. In D) 5 mM NaCl fraction from DEAE- Sephadex was assayed against cyclic AMP, whereas in E), F) and G), fraction eluted with 25 mM NaCl was assayed against cyclic AMP, 3'-AMP and 5'-AMP, respectively.

ml filtrate. This elution pattern was coincident to that observed by filtering a solution of standard cyclic AMP containing 0.1 mg cyclic nucleotide ml^{-1}. HPLC analysis of both filtrates revealed that the amount of cyclic AMP in the filtered cell-free extract was about 3 times lower than that detected in non-filtered extract. However, no significant differences in the amount of cyclic AMP were found for standards solutions, filtered or not through alumina column. Consequently, filtration was always performed before cyclic AMP analysis.

The time-course of cyclic AMP content of *U. aurantiaco-atra* thalli is shown in Fig. 2A. The amount of cyclic AMP naturally decreased after incubation of lichen thalli on the acetate buffer in the dark, and acridine orange did not significantly affect this pattern of disappearance. A part of this cyclic AMP was recovered from the medium, mainly for the first hour of thalli incubation. However, a small increase in the amount of endogenous cyclic AMP was found at the third hour of incubation. When 0.5 mM cyclic AHP was added to acridine orange-containing media, larger amounts of the cyclic AMP than those without addtion of exogenous cyclic AMP were recovered from incubated thalli with an initial decrease. This initial decrease, common for the three incubations, could be related to the large amount of cyclic AMP secreted from the thallus to the medium (Fig. 2A). The time-course of cyclic AMP secretion was almost identical for thalli floated on acetate or acridine orange.

Adenosine production slightly increased in thalli floated on acetate in the dark for the first hour of incubation, and then dramatically decreased (Fig. 2B). A supply of acridine orange significantly increased the amount of adenosine accumulated in lichen thalli but enhancement of adenosine production from cyclic AMP exogenously supplied was not found (Fig. 2B). Anyway, amounts of adenosine produced were always larger than those that could be expected from cyclic AMP disappearance. The time-course of adenosine content was related to the time-course of 5'-nucleotidase activity found for the lichen thalli (Fig. 2B). Neither AMP nor adenosine were found in the incubation medium.

To study the origin of adenosine, cyclic AMP phosphodiesterase was purified by ammonium sulfate precipitation, adsorption chromatography on calcium phosphate gel, and ion exchange chromatography on DEAE-Sephadex. Adenosine was always produced as the unique reaction product from cyclic AMP used as a substrate but only 5'-AMP was produced by using fraction eluted with 5 mM NaCl, from DEAE-Sephadex. That, eluted with 30 mM NaCl, was unable to use cyclic AMP as a substrate, but adenosine was produced by supplying 5'-AMP to the reaction mixtures (Fig. 3). Thus, *U. aurantiaco-atra* thalli possessed both enzymes, cyclic AMP phosphodiesterase and 5'-nucleotidase, which co-purified to be separated only after ion-exchange chromatography. In addition, phosphodiesterase activity was never found in the incubation medium when thalli were floated on acetate, acridine orange or on a mixture of the dye and cyclic AMP (data not found).

Effect of cyclic AMP and acridine orange on the production of usnic acid

In parallel to these changes, the content of usnic acid in thalli floated on acetate decreased for the first hour of incubation, but this decrease was completely impeded by acridine orange. Addition of cyclic AMP to dye-containing media restored the ability of lichen thalli to remobilize usnic acid (Fig. 4A). Very low amounts of this phenolic were recovered from

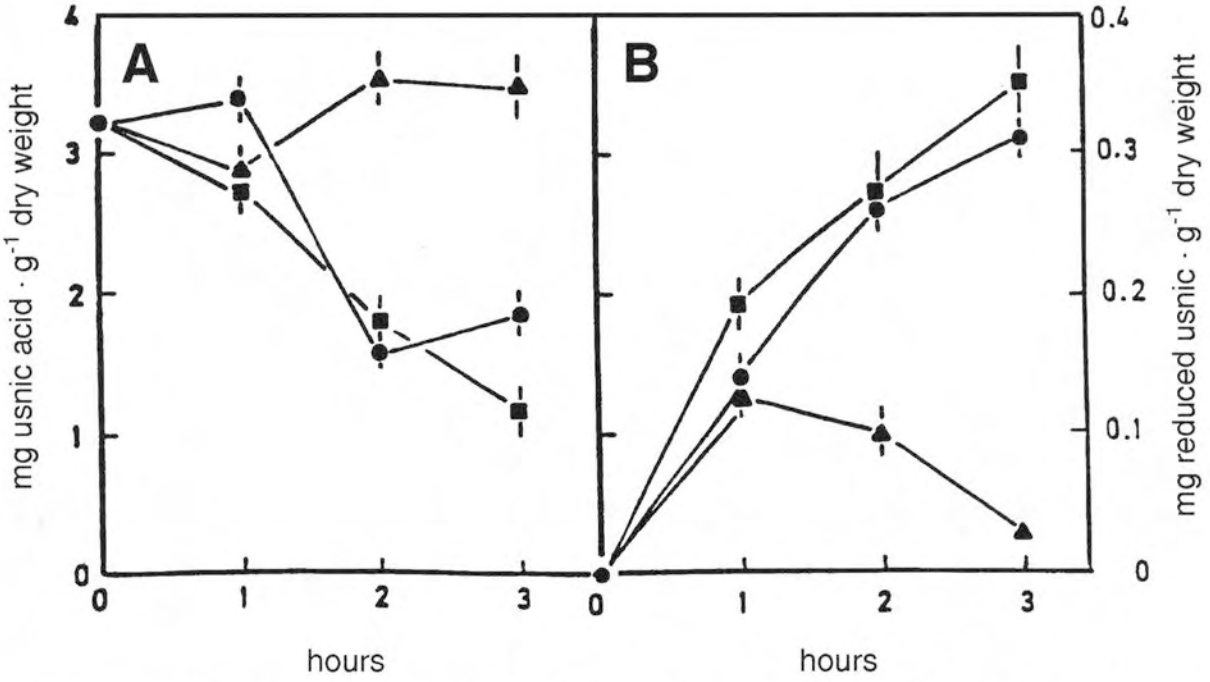

Fig. 4. A) Time-course of usnic acid accumulation by thalli floated on 0.1 M acetate buffer (●), 0.1 mM acridine orange (▲) and 0.1 mM acridine and 0.5 mM cyclic AMP (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

B) Time-course of reduced usnic acid production by thalli floated on 0.1 M acetate buffer (●), 0.1 mM acridine orange (▲) and 0.1 mM acridine and 0.5 mM cyclic AMP (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

incubation media, but a product of the usnic acid reduction showed a time-course of accumulation completely inverse to that found for usnic acid remobilization (Fig. 4B).

Since reduced usnic acid is produced by the action of an usnic acid : NADH oxido-reductase, the effect of acridine orange on this enzyme activity was tested. There was no reductase activity in stored, untreated thalli, but it developed after thalli incubation on acetate. Only D-usnic acid, not its L-isomer, was reduced by cell-free extracts supplied with NADH. Acridine orange impeded the appearance of reductase activity, whereas the addition of cyclic AMP to acridine-containing media produced an increase of this activity, similar to that found for thalli floated on acetate alone (Fig. 5). Neither activation nor inhibition of reductase activity by acridine orange or cyclic AMP were found in vitro, using cell-free extacts from lichen thalli floated for 3h on acetate as a source of enzyme.

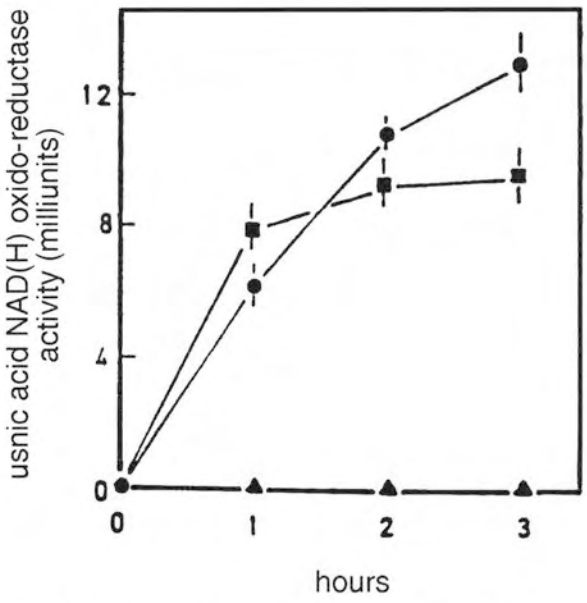

Fig. 5. Time-course of D-usnic acid : $NAD^+(H)$ oxido-reductase activity of thalli floated on 0.1 M acetate buffer (●), 0.1 mM acridine orange (▲) and 0.1 mM acridine and 0.5 mM cyclic AMP (■). Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

DISCUSSION

HPLC measurement of acridine orange reveals that acridine orange readily binds to polynucleotides of *U. aurantiaco-atra*, although a substantial part of the dye remains as a free molecular species in the lichen tissue (Fig. 1). In addition, formation of complexes between acridine orange and cyclic AMP has not been detected. Since fluorimetric detector was chosen to estimate acridine orange in HPLC, extraction of the dye from nucleic acids was required to avoid the quenching of acridine fluorescence after binding, reported by Gale et al. (1972).

Exogenously-supplied cyclic AMP seems to act not only by impeding the binding, but also inhibiting acridine orange uptake by plant materials, as shown in Fig. 1. Since uptake of acridine orange is probably a diffusive process, the apparent antagonism between the dye and cyclic AMP would be explained through changes in membrane potential induced by the binding of the cyclic nucleotide to some membrane components (Chung et al., 1988). In relation to this hypothesis, Ransom and Francko (1993) found that membrane polarity of planctonic algae became less negative with increasing doses of cyclic AMP. According to this, acridine orange, which behaves as a cation in solution, can even be removed from the lichen cell walls, if adhered, by electrostatic repulsion.

This fact does not exclude the supposed action of cyclic AMP for impeding the binding of acridines to nucleic acids (Zubay et al., 1970). The inhibition of the binding of dye to polynucleotides in thalli floated on acridine and cyclic AMP is clearly related to the increase in the concentration of endogenous nucleotide following thalli incubation (Figs 1 and 2A). This is in agreement with the reported increase in the amount of cyclic nucleotide at the third hour of thalli incubation on acridine (Fig. 2A). On the other hand, the initial decrease of the amount of cyclic AMP recovered from lichen thalli can be, in part, explained on the basis of the large amount of cyclic AMP recovered from the culture medium. Extracellular release of ths cyclic nucleotide has been described for cyanobacteria and green algae (Francko and Wetzel, 1980) as well as for *Neurospora crassa* (Sahw and Harding, 1987). Increase in cyclic AMP concentration after acridine treatment of lichen thalli has also been reported for other Antarctic *Usneacea*, such as *Himantormia lugubris* (Mateos et al., 1991b) but, in this case, the process was always followed by an accelerated catabolism of cyclic AMP, catalyzed by a 5'-nucleotidase, which produced adenosine. This process has also been described for many species of higher plants (Brown and Newton, 1981, 1992). However, nucleotidase activity is not enhanced by exogenous cyclic AMP when acridne is supplied to lichen thalli together with cyclic nucleotide, as deduced from Fig. 2B. Adenosine can be produced by the sequenced action of cyclic AMP phosphodiesterase and 5'-nucleotidase, the occurrence of which has been found in *U. aurantiaco-aura* (Figs 2B and 4), but, since adenosine concentration surpasses that expected from the catabolism of cyclic AMP (Fig. 2A and B), it is necessary to think that there is a lot of other adenosine nucleotides, being much more abundant intracellularly, which are able to release adenosine into the cell. Acridine probably acts as an inhibitor of lichen nucleotidase in a such way that the level of adenosine does not increase after the nucleotide uptake (Fig. 2A). Cyclic AMP uptake is not affected by a release of phosphodiesterase from the lichen cells to the incubation medium, since the secretion of this enzyme has not been found for *U. aurantiaco-aura*, in contrast to that described for other plant species (Akazawa and Hara-Nishimura, 1985; Wink 1984).

Cautions in order to avoid bacterial contaminations as well as to remove cyclic AMP-co-eluting chemicals by filtratian of extracts through a column of neutral alumina, as recommended by Spiteri et al. (1989), have been revealed as necssary, since unknown substances elute from HPLC column at the same retention time that cyclic AMP (2.4 min) and adenosine (3.4 min). Sodium hypochlorite eliminates bacterial contamination of lichen thalli and, thus, the possible microbial origin of cyclic AMP must be discarded. The hypothetical artifactual formation of cyclic AMP from endogenous ATP during extraction is avoided by precipitating protein with acetonitrile immediately after thallus disruption in water.

Competititon between both acridine orange and cyclic AMP or even between glucose and cyclic AMP is on the basis of a mechanism of catabolite repression for several enzymes of phenolic metabolism in *E. prunastri*, another *Usneaceae species* (Herrero et al., 1989). In this lichen species acridine orange does not affect D-usnic acid oxido-reductase activity (Legaz and Vicente, 1991). From this study it becomes evident, that the accumulation of usnic acid in *U. aurantiaco-atra* is only achieved in the absence of a catabolic oxido-reductase, the synthesis of which is impeded by acridine orange (Figs 4 and 5). The binding of acridine orange to nucleic acids, shown in Fig. 1, could be related to the inhibition of usnic acid oxido-reductase synthesis in a similar way to that found for several bacterial enzymes (Sankaran and Pogell, 1973). In addition, this effect is reversed by adding cyclic AMP to the culture medium (Fig. 5). The dye does not inhibit the synthesized enzyme since no action of acridine orange on in vitro enzyme activity has been found. A supply of exogenous cyclic AMP is not required by *U. aurantiaco-atra* for the production of the oxido-reuctase in the dark (Fig. 5) in opposite to that described for E. prunastri (Avalos and Vicente, 1989), even when the amount of endogenous cyclic AMP strongly decreases after the first hour of thalli culture on acetate (Fig. 2A). This decrease is due to the activation of phosphodiesterase after thalli rehydration, as well as to the release of cyclic AMP to the medium. A supply of exogenous cyclic AMP seems only to be required to reverse the action of acridine orange in order to restore the cellular ability for synthesizing oxido-reductase (Fig. 5). The different behaviour between two lichen species can be explained on the basis of the natural content of this nucleotide. *E. prunastri* contains about 7.0 pmol cyclic AMP g^{-1} dry thallus whereas the concentration of this nucleotide after incubating *Usnea* thalli on acetate for 3h is 0.1 nmol g^{-1} dry weight (Fig. 2A).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Dirección General de Investigación Científica y Tecnológica (Spain) PB90 0048. We wish to thank Raquel Alonso for her technical assistance.

LITERATURE CITED

AKAZAWA T., HARA-NISHIMURA I., 1985. Topographic aspects of biosynthesis, extracellular secretion, and intracellular storage of proteins in plant cells. Ann. Rev. Plant Physiol. 36: 441-472.

AVALOS A., VICENTE C., 1987. Equivalence between P_{fr} and cyclic AMP in the induction of D-usnic acid dehydrogenase in the lichen *Evernia prunastri*. Plant Physiol. 84: 803-807.

AVALOS A., VICENTE C. 1989. Action of filipin on phytochrome-modulated D-usnic acid: $NAD^+(H)$ oxido-reductase activity in the lichen *Evernia prunastri*. J. Plant Physiol. 135: 478-482.

BROWN E.G., NEWTON R.P., 1981. Cyclic AMP and higher plants. Phytochemistry 20: 2453-2463.

BROWN E.G., NEWTON R.P., 1992. Analytical procedures for cyclic nucleotides and their associated enzymes in plant tissues. Phytochem. Anal. 3: 1-13.

CHUNG C.H., LIM S. U., SON P.S., BULIN J.D., GOODIN J.R., 1988. Swelling of etiolated oat protoplasts induced by phytochrome, cyclic AMP and gibberellic acid: a kinetic study. Plant Cell Physiol. 29: 855-860.

FRANCKO D.A., WETZEL R.G., 1980. Cyclic adenosine-3':5'-monophosphate: production and extracellular release from green and blue-green algae. Physiol. Plant. 49: 65-67.

GALE E.F., CUNDLIFFE E., REYNOLDS P.E., RICHMOND M.H., WARING M.J., 1972. The Molecular Basis of Antibiotic Action. John Wiley & Sons, London, pp. 188-213.

GARCIA-JUNCEDA E., GONZALEZ A., VICENTE C., 1987. Photosynthetical and nutritional implications in the accumulation of phenols in the lichen *Pseudevernia furfuracea*. Biochem. System. Ecol. 15: 289-296.

HERRERO P., PEDROSA M.M., NORATO J., VICENTE, C., 1989. Some features about usnic acid accumulation and its movement between the symbionts of the lichen *Evernia prunastri*. J. Plant Physiol. 135: 170-174.

LEGAZ M.E., VICENTE C., 1991. An experimental approach to molecular biology of lichens: regulation of catabolite-sensitive promoters in some *Usneaceae*. J. Hattori Bot. Lab. 70: 167-180.

LEGAZ M.E., PEDROSA M.M., MATEOS J.L., CAFFARO S.V., VICENTE C., 1992. High-performance liquid chromatography determination of acridine orange in nucleic acids isolated from dye-treated *Himantormia lugubris*. J. Chromatogr. 607: 245-251.

LE-PECQ J.B., PAOLETTI C., 1967. A fluorescent complex between ethidium bromide and nucleic acids. Physical-chemical characterization. J. Mol. Biol. 27: 87-106.

LOWRY O.H., ROSEBROUGH N.H., FARR A.L., RANDALL R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

MATEOS J.L., CONDE E., MIRANDA T., VICENTE C., 1991a. Regulation mechanisms of phenolic production in the lichen *Himantormia lugubris*, as deduced from the analysis of metabolite accumulation. Plant Sci. 77: 1-9.

MATEOS J.L., XAVIER FILHO L., VICENTE C., 1991b. Adenosine accumulation in thalli of the lichen *Himantormia lugubris*, as indicative of phosphodiesterase actvity. Endocyt. Cell Res. 7: 275-282.

POTTY V.H., 1969. Determination of protein in the presence of phenols and pectins. Anal. Biochem. 29: 535-539.

RANSOM W.D., FRANCKO D.A., 1993. Measurement of membrane potentials in natural planktonic assemblages: comparison of methods and influence of dissolved cyclic AMP. In: Plant Signals in Interactions with other Organisms. Schultz J.C., Raskin I. (eds.). American Society of Plant Physiologists, Rockville, pp. 210-212.

RIABOWOL K .T., FINK J. S., GILMAN M. Z., WALSH D. A., GOODMAN R. H., FERAMISCO J. R., 1988. The catalytic subunit of cAMP-dependent protein kinase induces expression of genes containing cAMP-responsive enhancer elements. Nature 336: 83-86.

SANKARAN L., POGELL B.M., 1973. Differential inhibition of catabolite-sensitive enzyme induction by intercalating dyes. Nature New Biol. 245: 257-260.

SAHW N.H., HARDING R.W., 1987. Intracellular and extracellular cyclic nucleotides in wild-type and white collar mutant strains of *Neurospora crassa*. Temperature dependent efflux of cyclic AMP from mycelia. Plant Physiol. 83: 377-383.

SLONIMSKY P.P., PERRODIN G., CROFT J.J., 1968. Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal "petites". Biochem. Biophys. Res. Commun. 30: 232-239.

SPITERI A., VIRATELLE O.M., RAYMOND P., RANCILLAC M., LOBOUSSE J., PRADET A., 1989. Artefactual origins of cyclic AMP in higher plant tissues. Plant Physiol. 91: 624-628.

VICENTE C., LEGAZ M.E., 1985. Repression of arginase and agmatine amidinohydrolase by urea in the lichen *Evernia prunastri*. Phytochemistry 24: 217-219.

VICENTE C., LEGAZ M.E. 1988. Lichen enzymology. In: Handbook of Lichenology. Galun M. (ed.). CRC Press, Boca Raton. Vol. 1, pp. 239-284.

VICENTE C., MATEOS J.L., 1992. A simple assay for adenosine 3', 5'-cyclic monophosphate (cyclic AMP) and its application to the study of cyclic AMP production in the lichen *Himantormia lugubris*. Phytochem. Anal. 3: 14-19.

VICENTE C., PEDROSA M. M., CAFFARO S.V., MATEOS J.L., LEGAZ M.E., 1993. Catabolite sensitive promoters in lichens. In: Endocytobiology V: Endocytobiology and Symbiosis. Sato S., Ishida M., Ishikawa H. (eds.), Tübingen University Pres, Tübingen, pp. 49-58.

WINK M., 1984. Evidence for an extracellular lytic compartment of plant cells suspension cultures: the cell culture medium. Naturwissenschaften 71: 635-638.

ZUBAY G., SCHWARTZ D., BECKWITH J., 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. USA 66: 104-110.

WPŁYW CYKLICZNEGO AMP I ORANŻU AKRYDYNY NA REDUKCJĘ ENZYMATYCZNĄ KWASU USNINOWEGO U POROSTU *USNEA AURANTIACO-ATRA*

STRESZCZENIE

Metodę fluorescencyjno-detekcyjną HPCL zastosowano do identyfikacji i pomiaru ilościowego oranżu akrydyny poroście. Barwnik ten akumuluje się w plechach porostu *Usnea aurantiaco-atra* i częściowo odzyskiwany jest z protaminowych osadów kwasów nukleinowych, ekstrahowanych z próbek. Wprowadzenie egzogennego, cyklicznego AMP obniża pobieranie oranżu akrydyny przez plechy i jego wiązanie z kwasami nukleinowymi. Barwnik nie zmienia poziomu endogennie produkowanego cyklicznego AMP w próbkach plechy, ani nie hamuje jego egzogennego pobierania. Jednak część produkowanego endogennie cyklicznego AMP wydzielana jest do pożywki, w której nigdy nie stwierdzono działania fosfodiesterazy. Ponieważ synteza oksydoreduktazy kwasu D – usninowego: $NAD^+(H)$ obniżana jest przez oranż akrydyny, w plechach zawieszonych w barwniku hamowane jest utlenianie kwasu usinowego. Cykliczny AMP odwraca to działanie.

SŁOWA KLUCZOWE: Oranż akrydynowy, represja kataboliczna, cykliczny AMP, kwas usinowy, oksydoreduktaza kwasu usninowego: $NAD^+(H)$, *Usnea aurantiaco-atra.*