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Inhibition of mouse liver respiration by *Chelidonium majus* isoquinoline alkaloids

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Abstract

The alkaloids from *Chelidonium majus* L. which had a significant inhibitory effect in mitochondrial respiration were those which contain a positive charge due to a quaternary nitrogen atom, i.e., chelerythrine, sanguinarine, berberine and coptisine, both with malate + glutamate or with succinate as substrates. When malate + glutamate was used as substrate, chelerythrine and berberine, which contain methoxy groups, were particularly more active, since they had a strong effect even at low concentrations. In submitochondrial particles, berberine and coptisine had a marked inhibitory effect on NADH dehydrogenase activity but practically no effect on succinate dehydrogenase activity, whereas chelerythrine and sanguinarine inhibited more strongly succinate dehydrogenase than NADH dehydrogenase, which is in agreement with the results found for mitochondrial respiration. Protopine and allocryptopine, which did not inhibit mitochondrial respiration, strongly inhibited NADH dehydrogenase in submitochondrial particles, but had no effect on succinate dehydrogenase activity.

Keywords: Protoberberine alkaloids; Benzophenanthridine alkaloids; Structure–activity relationship; Mitochondrial respiration; NADH dehydrogenase; Succinate dehydrogenase

1. Introduction

Chelidonium majus L. is a plant which grows in the wild in Southern and Central Europe, part of Asia, North America and in the Azores archipelago (Kadan et al., 1990; Pavão and Pinto, 1995; Colombo and Bosisio, 1996). Its use as a medicinal plant is very ancient (Paris and Moyse, 1967; Duke, 1985; Xème Pharmacopée Française, 1989; Bézanger-Beauquesne et al., 1990). The medicinal properties mentioned above can be ascribed to the more than 27 alkaloids present in the root and aerial part of the plant, which

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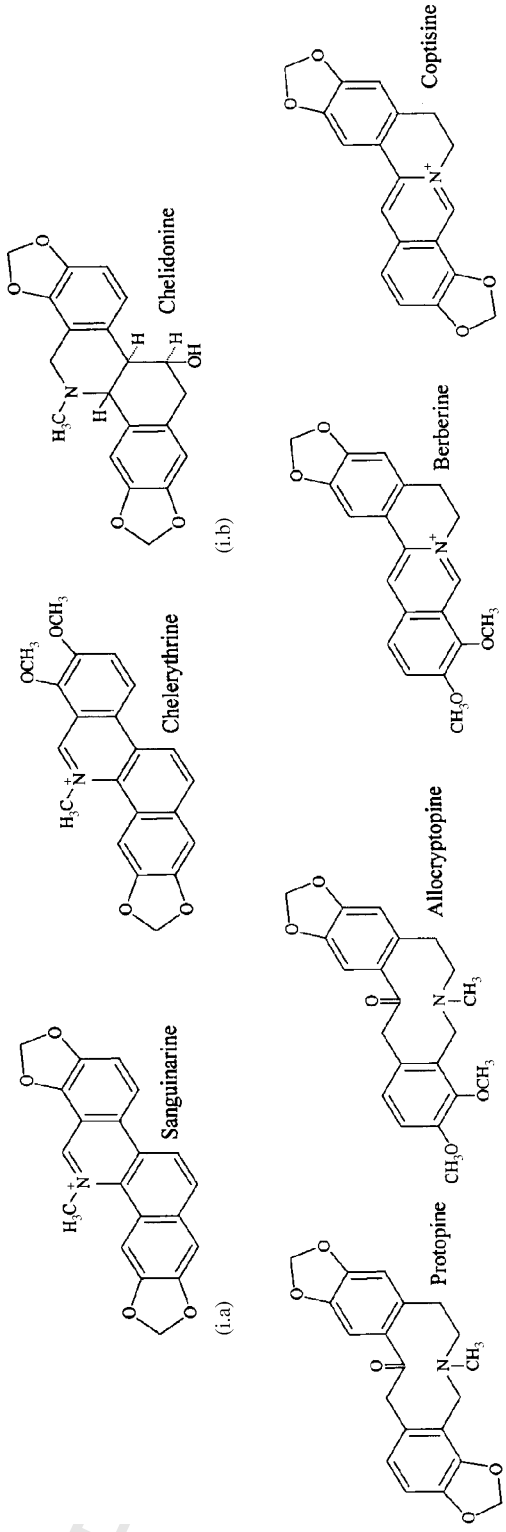

Fig. 1. Structure of the alkaloids used in this study.

belong to three main groups (Fig. 1): (i) benzo[*c*]-phenanthridines, with two subgroups, (i.a) quaternary, like chelerythrine and sanguinarine or (i.b) tertiary, like chelidonine, (ii) protopine and derived thereof, such as protopine and allocryptopine, and (iii) protoberberines such as berberine and coptisine (Lavenir and Paris, 1965; Táborská et al., 1994; Pavão and Pinto, 1995; Tomé and Colombo, 1995; Colombo and Bosisio, 1996). The interest of this plant for medicinal purposes implies the need to know as much as possible about the effects on metabolic processes of the alkaloids it contains. Several alkaloids with the same or related structures have been found to interfere with respiration, either at the level of the electron transport chain (Schewe and Müller, 1976) or as uncouplers (Vallejos and Rizzotto, 1972). Since mitochondrial respiration is the core of metabolic energy, and therefore a process with major importance, in the present work we investigated the effect of some of these alkaloids (Fig. 1) in respiration-linked processes. We selected alkaloids from each of the main groups found in the plants collected in S. Miguel Island, Azores (Pavão and Pinto, 1995). The effects of phenanthrene were also monitored, to allow for effects due only to the aromatic structure of the molecules.

The aim of the present work is (a) to ascertain whether the effects detected follow a similar pattern within each group; (b) if any effect which occurs on oxygen uptake can be explained by events at the level of NADH dehydrogenase (NADH:ubiquinone oxidoreductase, EC 1.6.99.3) or succinate dehydrogenase (succinate:ubiquinone oxidoreductase, EC 1.3.99.1). These two complexes were chosen by their crucial role in the respiratory chain and by evidence from other authors that these systems might be affected by compounds of this type (Schewe and Müller, 1976; McNaught et al., 1995, 1996).

2. Materials and methods

2.1. Animals

The animals used were male albino mice, with approximately 12 weeks of age and an average weight of 20–25 g. The animals were fed ad libitum with a commercial chow and tap water.

2.2. Alkaloids

Chelidonine, berberine chloride and sanguinarine chloride were purchased from Sigma. The other alkaloids were a kind gift from Prof. Slavik (Masaryk University, Brno, Czech Republic). The alkaloids and phenanthrene were used in methanolic solutions. The effect of methanol was tested for all types of experiment, in the range of volumes added to the assay media, and found to be negligible.

2.3. Preparation of mitochondria and submitochondrial particles

Liver mitochondria and submitochondrial particles were isolated according to a published method (Cain and Skilleter, 1987). Protein concentrations were determined using the Bradford Coomassie G250 dye procedure (Bradford, 1976) with bovine serum albumin as standard.

2.4. Oxygen uptake by mitochondria

Oxygen uptake was monitored in a Hansatech Clark-type electrode, model DW1 with a CB1 control box. Oxygen uptake by intact mitochondria was monitored at 30 °C in the presence of either 10 mM malate plus 10 mM glutamate or of 10 mM succinate. The assay medium was 250 mM sucrose, 10 mM Tris–HCl pH 7.4, 5 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$ and 0.2 mM ADP (Cain and Skilleter, 1987). Protein concentration was 0.5 mg/ml assay medium.

2.5. Enzyme activity assays

NADH and succinate dehydrogenase activities were studied on submitochondrial particles, to avoid permeability problems associated with the use of intact mitochondria. Enzyme activities, modified from methods described previously by other authors (Cénas et al., 1991; Liu et al., 1991), were spectrophotometrically monitored using a Shimadzu UV160A split-beam spectrophotometer, at 30 °C in 10 mM Tris–HCl pH 7.4, and with a protein concentration of 0.05 mg/ml. For NADH dehydrogenase the reaction was started by the addition of 0.1 mM NADH and the decrease in absorbance at 340 nm was registered. The basal rate of oxidation of NADH during the time of the

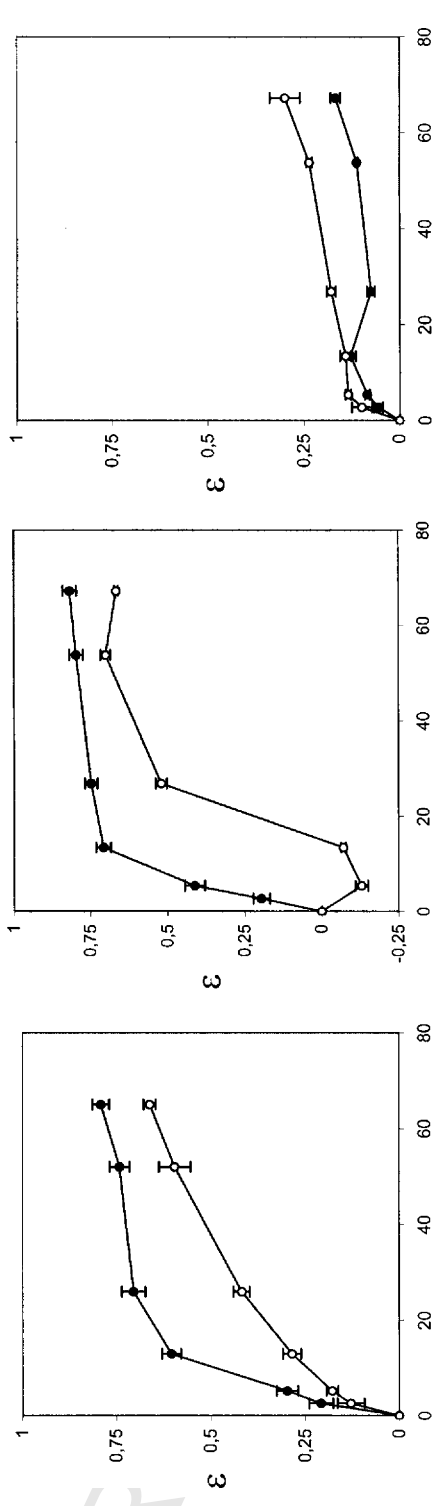

Fig. 2. Inhibition fractions (ε) of oxygen uptake by intact mitochondria in the presence of group (i.a) and (i.b) alkaloids. (○) Malate + glutamate (M + G), (●) succinate (SUC) as substrates. Oxygen uptake was followed in a Clark-type electrode. Concentration of mitochondria was 0.5 mg protein/ml of assay medium. Results are presented as mean ± S.D. (control values: chelerythrine, M + G 16.6 ± 1.9 nmol O_2/min mg, SUC 33.3 ± 2.6; sanguinarine, M + G 17.4 ± 2.3, SUC 27.5 ± 2.6; chelidonine, M + G 15.5 ± 2.0, SUC 32.2 ± 3.6).

assay period was negligible. The medium for succinate dehydrogenase contained 0.001% dichlorophenolindophenol; the reaction was started by the addition of 10 mM succinate and the decrease in absorbance at 600 nm was followed.

2.6. Treatment and presentation of results

Results (media of at least three independent experiments) are presented as relative inhibitions or inhibition degrees (ε), to minimize variability between different mitochondrial extractions. ε was calculated as $(v - v_i)/v$; v is defined as the rate of oxygen uptake or the rate of absorbance decrease at 340 or 600 nm, in the absence of inhibitor and v_i the oxygen uptake or enzyme activity in the presence of an i concentration of inhibitor.

3. Results

3.1. Oxygen uptake by intact mitochondria

The effects of the several groups of alkaloids on oxygen uptake in mitochondria showed different patterns. Chelerythrine and sanguinarine, both containing a quaternary nitrogen atom with a methyl group, strongly inhibited succinate-dependent respiration and, to a lesser extent, malate–glutamate respiration, while chelidonine, an uncharged phenanthridine derivative, had virtually no effect (Fig. 2). Protopine and allocryptopine, both uncharged and with a C=O group, also had no apparent effect (Fig. 3). Berberine and coptisine, both with an unsubstituted quaternary nitrogen atom, had a marked inhibitory effect on malate–glutamate respiration and a smaller, although significant, effect on succinate respiration (Fig. 4). Chelerythrine and berberine, which contain a quaternary nitrogen atom and methoxy substituents, showed a stronger inhibitory effect of malate + glutamate respiration at low concentrations, when compared, with sanguinarine and coptisine, respectively (Figs. 2 and 4). Phenanthrene had a very low effect on oxygen uptake (Fig. 5).

3.2. Enzyme activities in submitochondrial particles

In submitochondrial particles, chelerythrine and sanguinarine inhibited succinate dehydrogenase activity to a greater extent than NADH dehydrogenase (Fig. 6). This is a type of pattern similar to the one found on oxygen uptake (Fig. 2). Therefore, the ef-

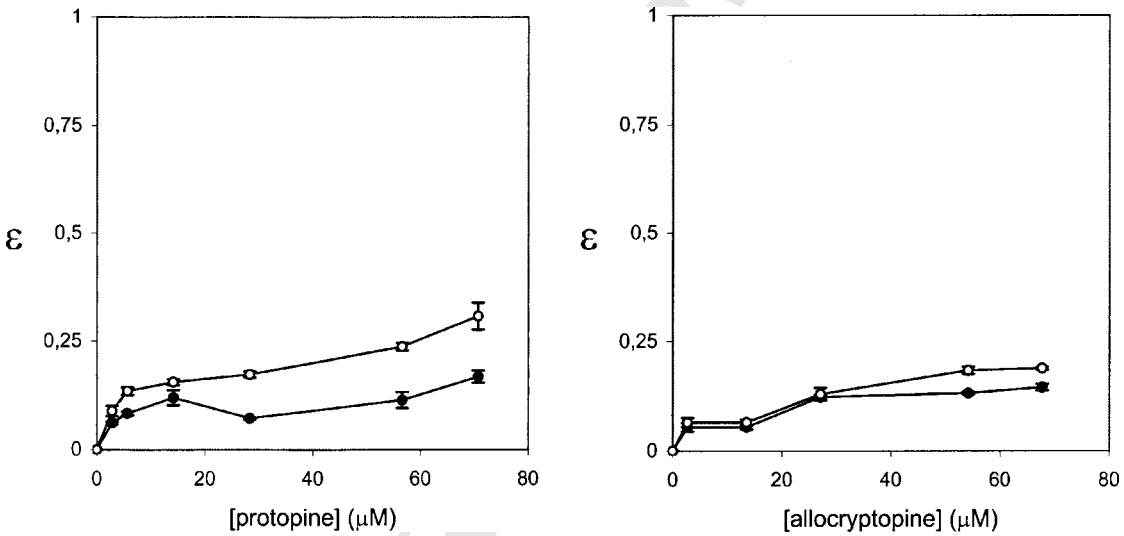

Fig. 3. Inhibition fractions (ε) of oxygen uptake by intact mitochondria in the presence of group (ii) alkaloids. (○) Malate + glutamate (M + G), (●) succinate (SUC), as substrates. Assay conditions were as described above. Results are presented as mean ± S.D. (control values: protopine, M + G 14.4 ± 0.7 nmol O_2/min mg, SUC 26.4 ± 2.2; allocryptopine, M + G 15.2 ± 2.0, SUC 27.9 ± 1.6).

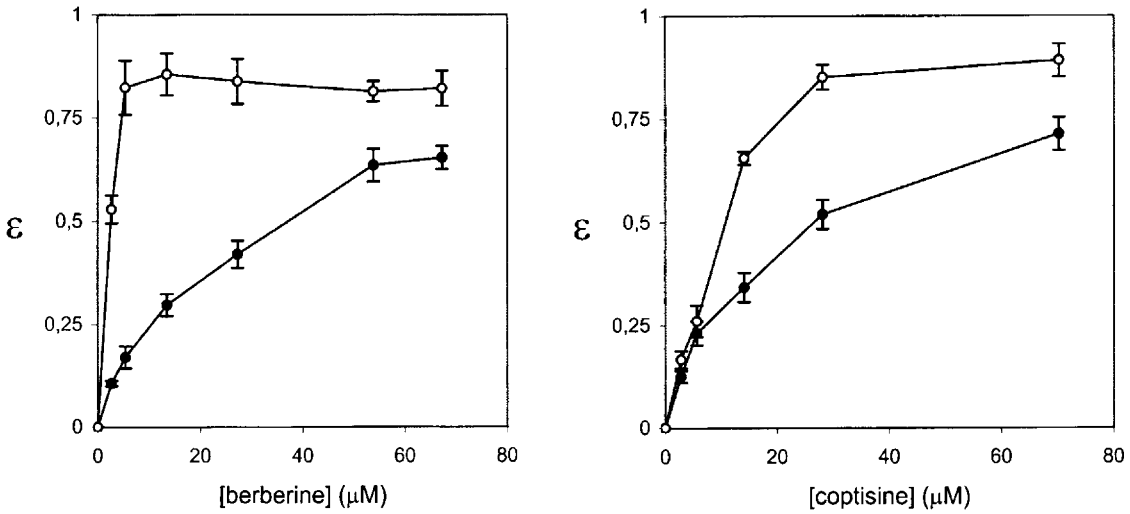

Fig. 4. Inhibition fractions (ε) of oxygen uptake by intact mitochondria in the presence of group (iii) alkaloids. (○) Malate + glutamate (M + G), (●) succinate (SUC) as substrates. Assay conditions were as described above. Results are presented as mean ± S.D. (control values: berberine, M + G 17.5 ± 1.9 nmol O_2/min mg, SUC 27.5 ± 4.2; coptisine, M + G 14.7 ± 1.7, SUC 28.5 ± 3.5).

fect on mitochondrial respiration is essentially in the agreement with the effect on the two enzymes. Chelidonine caused a slight decrease on NADH dehydrogenase but not on succinate dehydrogenase activity (Fig. 6). Protopine and allocryptopine had a very strong inhibitory effect on NADH dehydrogenase activity and did not affect succinate dehydrogenase (Fig. 7). Berberine and coptisine did not inhibit NADH dehydrogenase so strongly as would be expected by their effect on oxygen uptake, and had no effect on succinate dehydrogenase (Fig. 8). Phenanthrene, although it did not affect oxygen uptake to a great extent, had a marked inhibitory effect on NADH dehydrogenase in submitochondrial particles but not on succinate dehydrogenase (Fig. 9).

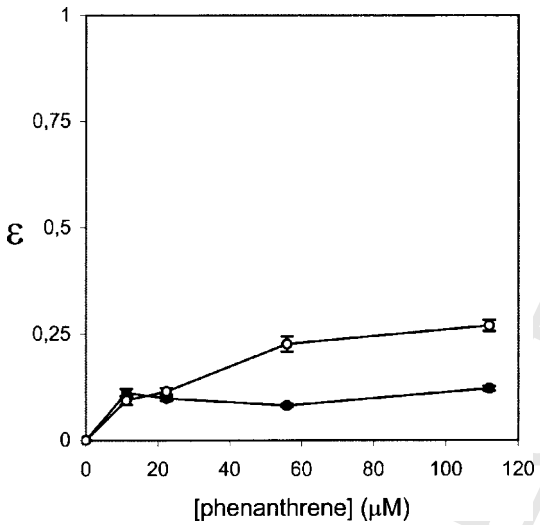

Fig. 5. Inhibition fractions (ε) of oxygen uptake by intact mitochondria in the presence of phenanthrene. (○) Malate + glutamate (M + G), (●) succinate (SUC) as substrates. Assay conditions were as described above. Results are presented as mean ± S.D. (control values: M + G 15.7 ± 1.9 nmol O_2/min mg, SUC 27.8 ± 1.6).

4. Discussion

The alkaloids with a charge due to a quaternary nitrogen atom presented a high inhibitory activity on oxygen uptake (Figs. 2 and 4). Some authors have already observed that alkaloids containing a quaternary nitrogen atom are the ones with the highest biological activity (Ulrichová et al., 1984; Dostál and Potácek, 1990; McNaught et al., 1995, 1996).

The alkaloids which contain a methyl group linked to the quaternary nitrogen atom seemed to have a more significant effect on succinate-dependent processes (Figs. 2 and 6). Berberine and coptisine had practically no effect on succinate dehydrogenase

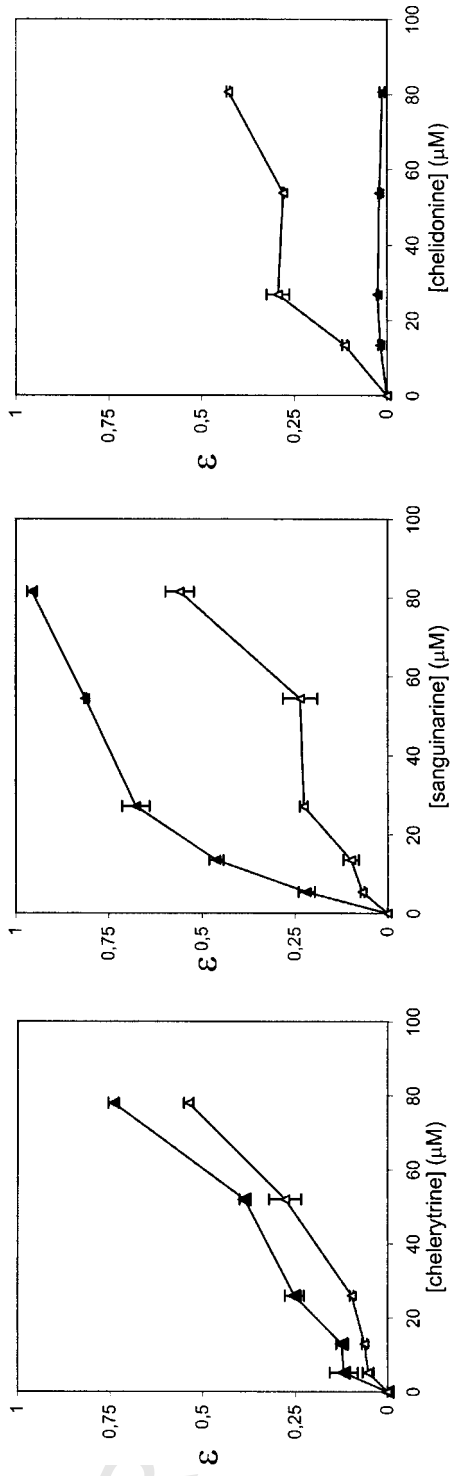

Fig. 6. Inhibition fractions (ε) of NADH dehydrogenase, NADH DH (△) and succinate dehydrogenase, SDH (▲), in the presence of group (i) alkaloids. Enzyme activities were spectrophotometrically monitored at 340 nm (NADH DH) and at 600 nm (SDH). Concentration of SMPs was 0.05 mg protein/ml of assay medium. Results are presented as mean ± S.D. (control values: chelerythrine, NADH DH 0.317 ± 0.014 μmol NADH/min mg, SDH 0.075 ± 0.002 μmol succinate/min mg; sanguinarine, NADH DH 0.235 ± 0.016, SDH 0.073 ± 0.002; chelidonine, NADH DH 0.352 ± 0.001, SDH 0.095 ± 0.001).

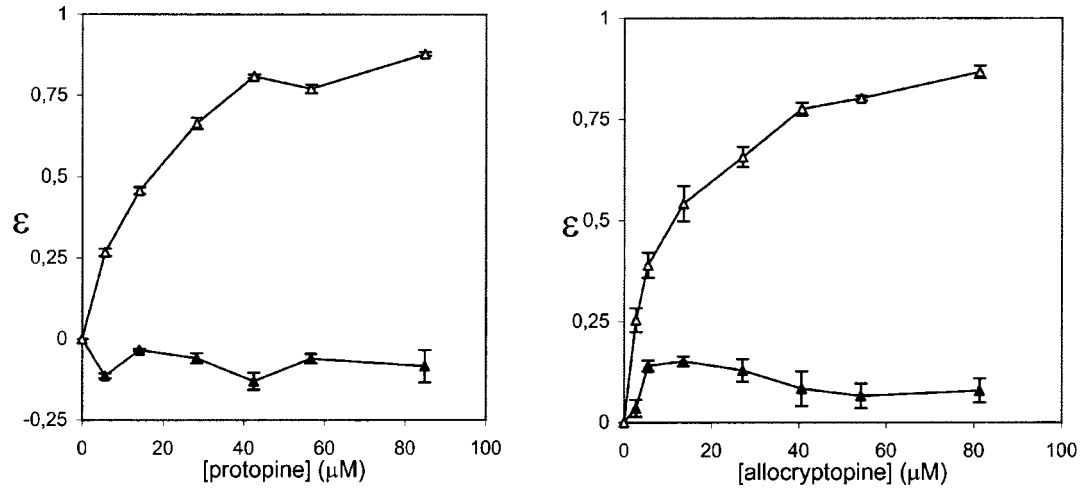

Fig. 7. Inhibition fractions (ε) of NADH dehydrogenase, NADH DH (△) and succinate dehydrogenase, SDH (▲), in the presence of group (ii) alkaloids. Assay conditions were as described above. Results are presented as mean ± S.D. (control values: protopine, NADH DH 0.293 ± 0.018 μmol NADH/min mg, SDH 0.071 ± 0.005 μmol succinate/min mg; allocryptopine, NADH DH 0.286 ± 0.023, SDH 0.074 ± 0.005).

activity, although the inhibition of succinate-dependent oxygen uptake was quite marked. The pattern we observed in submitochondrial particles agreed with the results reported in another study (Schewe and Müller, 1976), which reports the effect of berberine on NADH oxidase and succinate–cytochrome *c* oxidoreductase in beef heart submitochondrial particles. The authors found that berberine had a strong inhibitory effect on NADH oxidase and a much lower effect on succinate dehydrogenase activity.

In the present work, the effects of the group i and ii alkaloids tested on NADH dehydrogenase were very

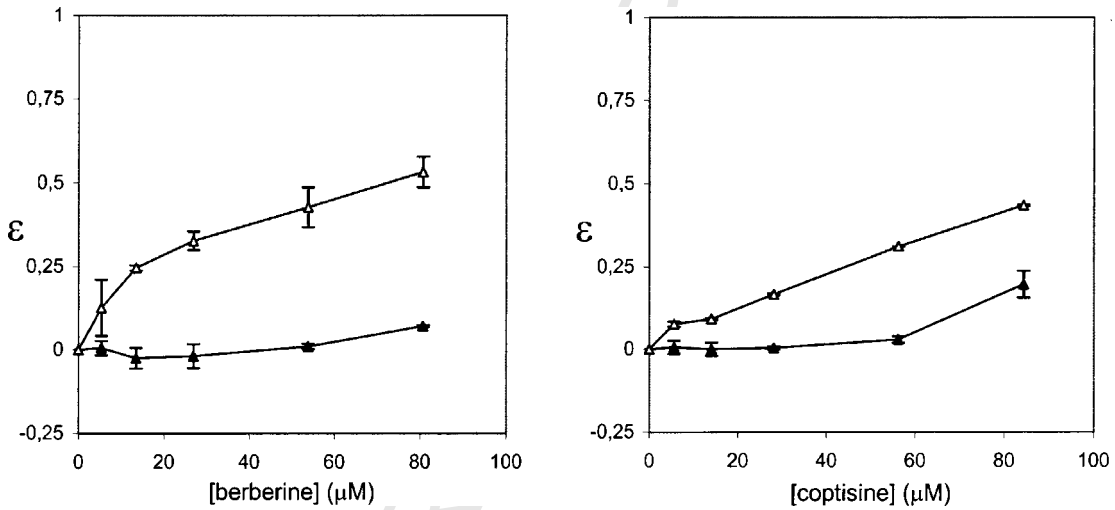

Fig. 8. Inhibition fractions (ε) of NADH dehydrogenase, NADH DH (△) and succinate dehydrogenase, SDH (▲), in the presence of group (iii) alkaloids. Assay conditions were as described above. Results are presented as mean ± S.D. (control values: berberine, NADH DH 0.338 ± 0.016 μmol NADH/min mg, SDH 0.116 ± 0.004 μmol succinate/min mg; coptisine, NADH DH 0.337 ± 0.009, SDH 0.087 ± 0.002).

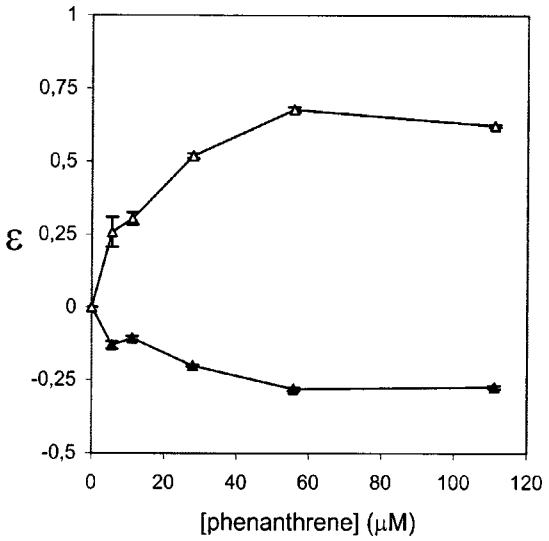

Fig. 9. Inhibition fractions (ε) of NADH dehydrogenase, NADH DH (△) and succinate dehydrogenase, SDH (▲), in the presence of phenanthrene. Assay conditions were as described above. Results are presented as mean ± S.D. (control values: NADH DH 0.286 ± 0.024 μmol NADH/min mg, SDH 0.075 ± 0.003 μmol succinate/min mg).

similar. The fact that chelidonine, protopine and allocryptopine, bearing no charge, inhibited NADH dehydrogenase but had practically no effect in intact mitochondria, may have been due to the abolition of permeability barriers, since NADH dehydrogenase faces the inner side of the membrane in intact mitochondria and the outer side in submitochondrial particles (Harmon et al., 1974).

The comparative analysis between the results of respiration and of enzyme activities suggests that the uncharged compounds we tested had more difficulty in passing across the mitochondrial membrane to gain access to the enzyme molecules. The analysis of results reported by another research group (McNaught et al., 1995, 1996) corroborate this hypothesis. These authors reported that isoquinolinium cations were more active inhibitors of respiration in intact mitochondria than isoquinolines. In mitochondrial fragments, the presence of a quaternary atom was not essential for the inhibition of complex I activity (McNaught et al., 1995, 1996). This is probably correct since we found that protopine and allocryptopine produced a marked effect on NADH dehydrogenase. The differences found between mitochondria and mitochondrial fragments may be explained by a preferential transport and accumulation of the cations as opposed to the uncharged isoquinoline molecules. The high membrane potential in mitochondria may result in a selective attraction of lipophilic cations, leading to their accumulation on the matrix side (Ramsay and Singer, 1986; Ramsay et al., 1987; Murphy, 1997). The concentration of positively charged alkaloids in intact mitochondria may therefore be much higher than the concentration of the other substances tested in the present work.

The presence of a quaternary atom is not enough to confer inhibitory activity to molecules, since ammonium acetate, tetramethylammonium iodide and tetrapropylammonium iodide had no effect on enzyme activity (results not shown). Phenanthrene, with a full aromatic structure and no substituents, caused a decrease on NADH dehydrogenase activity (Fig. 9). NADH dehydrogenase inhibition may be associated with the presence of at least two adjacent aromatic rings, which are present in berberine, coptisine, chelerythrine and sanguinarine structures (Figs. 6 and 8). Inhibition by protopine and allocryptopine is likely due to the carbonyl group, which may react with catalytically important SH groups in the enzyme molecule or perhaps with the iron–sulfur clusters of complex I.

The presence of four consecutive aromatic groups and a positive charge, which exist in chelerythrine and sanguinarine, may be a structure associated with the inhibition of succinate dehydrogenase. The positive charge is probably necessary, since phenanthrene, with the same aromatic rings but with no charge, did not inhibit this enzyme. Many observed biological effects of these two alkaloids involve the formation of a labile covalent bond between SH groups of cell components and the electrophilic C_6 carbon (Sedo et al., 2002). The imminium bond in sanguinarine and chelerythrine is susceptible to a nucleophilic attack and consequently plays a key role in the inhibition of SH proteins. The fact that hepatocytes incubated with these two alkaloids suffered a dose-dependent GSH depletion corroborates the idea that they bind to this SH peptide (Ulrichová et al., 2001).

The presence of methoxy groups also contributes to the difference in the inhibition strength of malate–glutamate-dependent oxygen uptake at low alkaloid concentrations between the positively charged alka-

loids which contain methoxy groups and those where they are absent (chelerythrine versus sanguinarine and berberine versus coptisine, Figs. 2 and 4). This may be due to an easier passage of these alkaloids across the membrane and/or to an increased inhibition of NADH dehydrogenase, in the case of berberine.

Berberine, containing both a quaternary nitrogen atom and methoxy groups, was the most biologically active of all the alkaloids tested, and therefore it should have the highest toxicity.

We suggest that the biological effects of the alkaloids on mitochondria are due to (i) the positive charge of the alkaloids, which causes their accumulation inside the organelle and (ii) inhibition of both NADH and/or succinate dehydrogenase activity and probably also inhibition at the cytochrome level, since in some cases the effects on respiration are not fully explained by the effects on the enzymes. This is corroborated by preliminary results from our laboratory (unpublished data) which show that berberine inhibits cytochrome aa_3 reduction.

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