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SHORT COMMUNICATIONS

Alkaloid inhibition of yeast respiration—Prevention by Ca^{2+}

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SOME alkaloids are strong growth inhibitors of different organisms (including fungi.)[1–4] The biochemical basis of this action is not well understood. We have explored the possibility that some alkaloids may act on cellular energy metabolism by testing their effects on yeast respiration. Nitidine, chelerythrine and sanguinarine (1:2-benzophenanthridine alkaloids found in plants of the Papaveraceae or Rutaceae families)[5,6] were found to be strong inhibitors of ethanol oxidation by yeast (Table 1). Laurifoline and *N*-methylglaucine (aporphine alkaloids) caused the same effect at concentrations 10-fold higher while xanthoplanine (an aporphine alkaloid) and laudanosine (a benzylisoquinoline alkaloid) were much less effective.

TABLE 1. EFFECT OF ALKALOIDS ON YEAST RESPIRATION*

Alkaloid (μM)	Qo_2 (μl/min/mg dry wt)	Inhibition (%)
None	1·11	
Nitidine (30)	0·27	75
Chelerythrine (30)	0·25	77
Sanguinarine (30)	0·25	77
Laurifoline (300)	0·25	77
N-methylglaucine (300)	0·40	64
Xanthoplanine (300)	0·94	14
Laudanosine (300)	0·99	10

* Oxygen uptake was determined manometrically in a Braun Warburg apparatus at 30°. The reaction medium (2·4 ml) was twice distilled water, the substrate was 36 mM ethanol, and 1·7 mg (dry wt) of yeast cells was used per vessel. The alkaloids were added as a dimethylsulfoxide solution. The solvent was without effect at the final concentration used (less than 2 per cent). The Qo_2 values are the average of determinations in duplicate which agreed within 10 per cent. Yeast cells (*Saccharomyces cerevisiae* strain D-261) were grown in a liquid complex medium at 28° with forced aeration. Cells were collected at the end of the logarithmic phase, washed with water and starved for 12 hr at 25° in 50 mM monopotassium phosphate. Then they were washed and suspended in twice distilled water and immediately used.

Other aporphine alkaloids (*O*,*O*'-dimethylmagnoflorine, *N*-methylcoridine, *N*-methylisocoridine and magnoflorine) were without effect up to 0·4 mM.

All the alkaloids used were homogeneous on paper chromatography and were identified by comparing the properties of crystalline derivatives with authentic samples.[6]

As shown in Fig. 1, nearly complete inhibition (95 per cent) of yeast respiration with ethanol as substrate was obtained with 50 μM nitidine (I_{50} was 10 μM). Chelerythrine gave similar results (I_{50} was 6 μM, Fig. 1) except that when oxygen consumption was determined in a Gilson Oxygraph instead of in the Warburg apparatus, it was observed that the inhibition was not instantaneous as with nitidine but had a lag of 3–4 min. This inhibitory effect depended on the amount of yeast present.

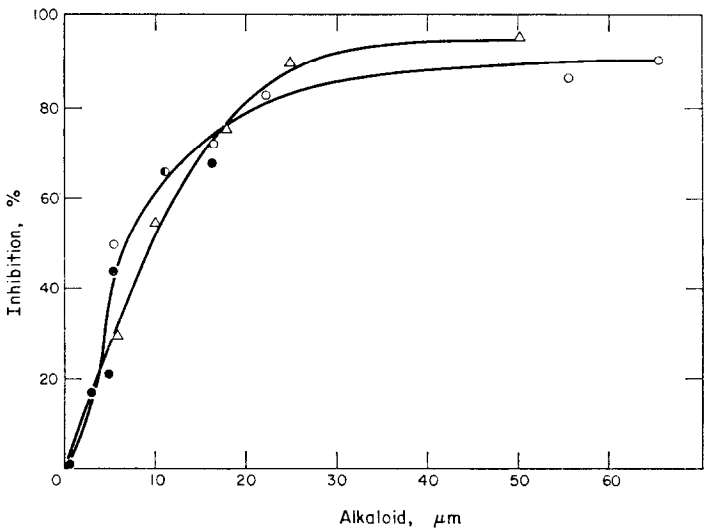

FIG. 1. Inhibition of yeast respiration by nitidine and chelerythrine. The effect of chelerythrine on ethanol oxidation by yeast cells was determined manometrically as described in the legend to Table 1. Starved yeast cells (2·4 mg, dry wt) were used per vessel. Open and closed circles (○, ●) are experimental points obtained with different yeast cultures. The effect of nitidine on oxygen uptake by yeast cells was determined with a Clark electrode in a Gilson Oxygraph at 30°. Reaction medium (1·6 ml) was 56 mM ethanol in twice distilled water. Starved yeast cells (1·48 mg, dry wt) were used. The experimental points (△——△) are the average of determinations in duplicate which agreed within 10 per cent.

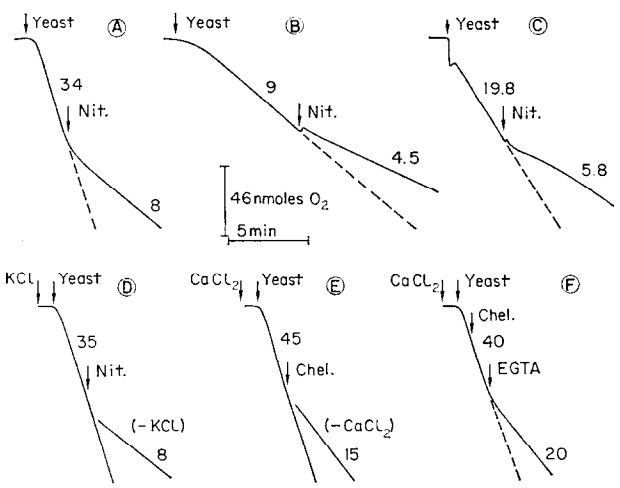

FIG. 2. Inhibition of yeast respiration by nitidine and chelerythrine. Oxygen uptake was determined with a Clark electrode in a Gilson Oxygraph as described in the legend to Fig. 1. Starved yeast cells (1–1·5 mg, dry wt) were used. Substrates were (A) 67 mM glucose; (B) 1 mM sodium pyruvate; (C) endogenous substrates: non-starved yeast cells; (D) (E) and (F) 56 mM ethanol. Nit.; nitidine 24 μM. Chel.: 38 μM chelerythrine. Superimposed curves are shown in (D) and (E) where 46 mM KCl (D) or 0·01 mM $CaCl_2$ (E) were present or absent as stated. In Exp. (F) 0·01 mM $CaCl_2$ was present; EGTA was 1 mM. The numerals on the slopes are nanomoles of O_2 per min per milligram.

The oxidation of glucose, pyruvate and endogenous substrates was also inhibited by nitidine (Fig. 2A, B and C) and chelerythrine. The inhibition of the oxidation of endogenous substrates by non-starved cells (Fig. 2C) suggests that the alkaloid has to enter the cell, and therefore the effect cannot be explained by an alteration of substrate transport. The inhibition of pyruvate and ethanol oxidation suggests that the mitochondria is the site of action of the alkaloid. This is supported by the observation that rat liver mitochondrial respiration and phosphorylation are inhibited by chelerythrine.[7]

We would like to emphasize that these experiments were carried out in twice distilled water. When they were repeated with several buffers at different pH values, no inhibition was observed. Salts also prevented the inhibition. Increasing concentrations of KCl in the reaction medium gradually diminished the inhibitory effect. Figure 2D shows that 46 mM KCl completely prevented the inhibition of yeast respiration by nitidine. NaCl, LiCl and NH_4Cl gave similar results. Divalent cations were far more effective. In the presence of 0·01 mM $CaCl_2$, yeast respiration was not affected by chelerythrine (Fig. 2E). $SrCl_2$, $MnCl_2$ and $MgCl_2$ gave similar results, their effectiveness being $Ca^{2+} \approx Sr^{2+} > Mn^{2+} > Mg^{2+}$. The inhibition by nitidine or chelerythrine of the oxidation of glucose, pyruvate and endogenous substrates by yeast was also prevented by cations.

When $CaCl_2$ was added to nitidine-inhibited cells, it was completely unable to reverse the inhibition. This lack of effect suggests that once nitidine has entered the cell, $CaCl_2$ is no longer effective. The effect of Ca^{2+} can be reversed or prevented by EGTA [ethylene glycol-2-(2-aminoethyl)-tetraacetic acid, a Ca^{2+}chelating agent] as shown in Fig. 2F. The prevention of nitidine or chelerythrine inhibition of yeast respiration by Ca^{2+} cannot be ascribed to an interaction between the cation and the alkaloid since: (a) we have not detected any change in the absortion spectra or in the fluorescence of the alkaloid in water when $CaCl_2$ was added; (b) the intracellular sap has higher ionic strength than that required to avoid the inhibitory effect; and (c) chelerythrine inhibited rat liver mitochondria respiration even in the presence of cations.[7] A possible explanation for this effect of Ca^{2+} and other cations may be that the cations, nitidine or chelerythrine penetrate the cell membrane through a site for cations and that Ca^{2+} (and other cations) competes or interferes with the transport of the alkaloid into the cell.

Further support for the conclusion that cations interfere with the transport of these alkaloids was provided by experiments in which yeast cells were incubated with chelerythrine (67 nmoles/mg dry wt) for 10 min at 30°, and then washed with twice distilled water. Respiration remained inhibited after the washing. The concentration of alkaloid in the supernatant solution was determined spectrophotometrically at 268 nm. The chelerythrine concentration in the incubation mixture was reduced by 72 per cent in the absence of $CaCl_2$, but only by 10 per cent when the latter (50 μM) was present. Thus, $CaCl_2$ prevented not only the inhibition of respiration, but also the uptake of the alkaloid by the cell.

Observations similar to those reported here for nitidine and chelerythrine were also made with laurifoline. An important structural similarity in all these alkaloids is the presence of a positively charged nitrogen atom. Chelidonine and homochelidonine, benzophenanthridine alkaloids that lack the positive charge in their nitrogen atom, did not inhibit yeast respiration.

The effects described here are not restricted to *S. cerevisiae* since we have obtained qualitatively similar results with other species, namely *Saccharomyces carlsbergensis* and *Torula utilis*.

The strong inhibition of yeast respiration by these alkaloids at low concentrations may have some bearing on the physiological role of the alkaloids in the plant, and suggests a biochemical basis for their toxic and pharmacological properties. The striking interference of cations, specially divalent cations, with the inhibition may be worth studying in further detail since it seems to be closely related to the transport of alkaloids and perhaps other cationic substances into the yeast cell.

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Acceleration of protein synthesis by angiotensin—Correlation with angiotensin's effect on catecholamine biosynthesis

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ANGIOTENSIN-II has a potent effect in accelerating noradrenaline (NA) biosynthesis in a number of sympathetically innervated tissues.[1–3] This action may have some importance in relation to the homeostatic control mechanisms *in vivo* within the sympathetic neuron, for maintenance of constant levels of endogenous NA, and may possibly be involved in the relationship between the renin–angiotensin system and the development of certain hypertensive states. In a further investigation of the possible mechanism of action of angiotensin in accelerating the biosynthesis of NA, we have found that this peptide has marked effects on protein synthesis. The following results may help to explain not only why angiotensin increases the synthesis of NA, but may also have wider implications in respect to the possible physiological and pathological roles of the renin–angiotensin system.

Guinea pig atria were dissected out from male animals killed by cervical dislocation. The tissues were incubated at 37° for 1 hr in Krebs–Henseleit solution containing either tyrosine-^{14}C (10 mCi/m-mole, 5×10^{-5} M) or tyrosine-^{14}C and leucine-^{3}H (250 mCi/m-mole, 2×10^{-5} M). Where indicated, angiotensin-5-valine amide II (Ciba) was added immediately prior to beginning the incubation. At the end of the incubation period, the atria were blotted and frozen on solid carbon dioxide. The atria were weighed and then homogenized in 15% trichloroacetic acid containing 0·1% disodium edetate. The homogenate was centrifuged for 15 min at 20,000 *g* and the supernatant taken for the isolation of catechols by the alumina column procedure.[4] The tissue and supernatant were maintained at 4° throughout all these procedures.

The residual tissue pellet was extracted with 2 ml of cold 0·4 N perchloric acid and allowed to stand for 1 hr at 4°; the sample was then centrifuged at 20,000 *g* for 10 min and the supernatant discarded. The washed pellet was then re-extracted with 2 ml of 0·4 N perchloric acid at 95° for 20 min to hydrolyze and extract nucleic acids. The sample was cooled and recentrifuged; the hot perchloric acid extract plus a 1-ml wash (0·4 N perchloric acid) of the pellet was kept for pentose analyses by the diphenylamine spectrophotometric method using deoxyadenosine as the standard.[5] Amino acid incorporation into protein was determined by dissolving the final protein pellet in 3 ml of Soluene (Packard). The sample was then counted in 20 ml of a dioxane–toluene–ethanol scintillation fluid[6] in a Packard scintillation counter. Standard procedures for quench and overspill correction were used in experiments with both ^{14}C- and ^{3}H-labeled amino acids. Amino acid incorporation into protein was expressed in terms of dis./min/g wet wt of tissue or in dis./min/nmole of DNA deoxyribose.

Since the actions of many hormones have been shown to be associated with effects on protein synthesis, the possibility that the previous observed actions of angiotensin on NA biosynthesis might be explained by an effect on protein synthesis seemed plausible. When atria were incubated with 5×10^{-7} M angiotensin as previously reported,[1–3] the biosynthesis of NA was 40–100 per cent greater than that of the control tissues. At the same time, it was found that tyrosine incorporation into protein was also elevated to about the same extent by angiotensin (Fig. 1). This observation was suggestive that angiotensin at a concentration (5×10^{-7} M) that was producing a maximal increase in NA synthesis was also increasing protein synthesis. Previous experiments have indicated that angiotensin at concentrations of 10^{-9} to 5×10^{-7} M has no significant effect on the uptake of tyrosine into the guinea pig atria[1,3]. Angiotensin at a higher concentration (10^{-5} M), which produced