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EFFECTS OF ECONAZOLE NITRATE ON YEAST CELLS AND MITOCHONDRIA

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Abstract—The inhibitory effect of econazole nitrate on the growth of yeast *Saccharomyces cerevisiae* is proportional to the concentration of the product. It depends on the phase of culture and on the number of cells present at the moment of econazole addition into the medium. The most important inhibition is obtained in the exponential phase of growth with a low concentration of cells. It is enhanced with cells which were previously in contact with the product. There is no adaptation of the yeast toward increased concentrations of econazole. The product penetrates the cells and attaches first to particular fractions, later to soluble fractions. The highest concentration of econazole nitrate in cells lies in the mitochondria. No product of econazole metabolism by *S. cerevisiae* was uncovered. Econazole nitrate does not slow down the *in vivo* activities of mitochondrial enzymes (cytochrome *c* oxidase, succinate dehydrogenase and phenylalanyl-tRNA synthetase), but inhibits the biosynthesis of mitochondrial membrane enzymes without affecting that of the synthetase, a matrix enzyme.

Econazole nitrate (1-[2-(2,4)dichlorophenyl)-2-(4-chlorobenzyloxy)-ethyl]-imidazole nitrate) is an antifungal agent. Its broad spectrum activity is demonstrated against most pathogenic agents responsible for human mycosis [1]. Microscopic investigations on bacteria and fungi treated with econazole nitrate have shown alterations on the cellular and subcellular membranes [2].

In previous work, we have shown that the inhibitory effects of this product against the yeast *Saccharomyces cerevisiae* is more important in aerobiosis than in anaerobiosis. But inhibition concentrations effective against cell growth are without effect against some mitochondrial enzyme activities [3].

In this paper, we report on the action of this product on yeast growth, the intracellular distribution of econazole, the search for possible metabolism products from econazole, and some of the latter effects on mitochondrial protein biosynthesis.

MATERIALS AND METHODS

Culture conditions. The yeast strain *Saccharomyces cerevisiae* IL 8-8C ρ^+ was obtained from the Centre de Génétique Moléculaire, Gif-sur-Yvette, France. Culture methods in repression and derepression conditions have been previously described [4]. Econazole nitrate (Laboratoires Cilag-Chemie, Paris, France) in methanolic solution was added to the medium. For the labelled experiments, [^{3}H]econazole nitrate (32 μCi/mg; ^{3}H is fixed to the second carbon of the ethyl residue) was added to 300 ml of derepression medium containing mid-logarithmic growth yeast at a final concentration of 10^{-6} M. Cells were harvested after 1 hr and after 22 hr contact in the stationary phase of growth. The two cell pellets were treated as below.

Obtention and breakage of yeast cells. The cells deposited at 600 *g* were broken by mechanical means and the following fractions were obtained: membranes + nuclei; cytosol + ribosomes; and mitochondria [5]. The mitochondria were purified and the mitochondrial membranes separated in a sucrose gradient [6]. The cytoplasmic ribosomes were obtained by centrifugation (105,000 *g*, 1 hr).

Two-dimensional thin layer chromatography. The silica plates, 10 × 20 cm (DC-Fertigplatten Kieselgel 60 F_{254} Merck) were used. In the first migration (10 cm), the acidic mixture chloroform–methanol–formic acid (85:10:5, v/v/v) was used. The solvent of the second migration (6.5 cm) was alkaline: dioxan–toluene–ammonia (59:40:1, v/v/v). Standards were used in each migration. The plates were examined at 254 nm and again after action of iodine vapour.

Radioactivity assay. An aliquot of each subcellular fraction was assayed for radioactivity in a Packard Tricarb scintillator. The scintillation liquid used was Omnifluor (NEN, Dreieichenhain, F.R.G.) at a concentration of 4 g/l. toluene. Radioactivity assay of the spots after two-dimensional TLC migration was done by dividing the surface migration into 20 cases; the silica gel from each case was scratched off and counted after the addition of Omnifluor.

Oxygen uptake [7]. The rate of oxygen uptake (QO_2) of 0.3–0.6 mg mitochondrial proteins [freshly prepared and washed in 10 mM Tris–maleate buffer, pH 6.8, containing 0.6 M mannitol and 0.2% (w/v) bovine serum albumin (BSA)] was measured with a Clark oxygen electrode (Oxygraph Gilson) at 30° in 1.6 ml of 10 mM Tris–maleate buffer, pH 6.5, containing 0.65 M mannitol, 10 mM KCl, 1.3 mM KH_2PO_4 and 0.3% BSA. Other additions are specified.

Determination of mitochondrial enzymatic activities. Succinate dehydrogenase activity was measured by the method of King [8], and cytochrome *c* oxidase

activity according to Wharton and Tzagoloff [9]. The mitochondrial phenylalanyl-tRNA synthetase extraction and its activity determination were performed according to [10] using yeast cytoplasmic tRNA as substrate.

Protein determination. Estimation of proteins was performed by the method of Lowry *et al.* in the presence of 0.4% (w/v) sodium deoxycholate as described in [11]. Bovine serum albumin was used as standard.

RESULTS

Effects of econazole nitrate on yeast cell growth

Econazole nitrate (10^{-6} M) inhibited yeast cell growth in aerobiosis for 36 hr (Table 1) if the inoculation was small (absorbance at 600 nm lower than 0.05). The control culture was already in the stationary phase at 24 hr (Fig. 1). A reversion of the inhibition appeared if the culture was prolonged over 50 hr. Anyway the growth did not reach the usual level and the weight gain of the humid cells was only 16 g/l instead of 26 g/l. The inhibition by econazole nitrate depended not only on the econazole concentration but also on the state of culture when econazole was added to the medium. The nearer to the stationary phase when econazole was added, then the lower the inhibition effect.

With low econazole concentrations (10^{-8} and 10^{-7} M), a long period of latency appeared if a small number of cells were inoculated in a large volume of medium. This period of latency disappeared if the number of cells increased (not shown) and if econazole nitrate was added to the medium at the exponential state of growth (Fig. 1). The cells did not adapt themselves to grow in medium with increasing econazole nitrate concentrations (Fig. 2). At 10^{-5} M, damage was irreversible and there was no more growth when the concentration reached 10^{-4} M. Here again, the number of cells present at the moment of econazole addition into the medium interfered with the response to econazole. If econazole nitrate was added at concentrations of 10^{-6} M and 10^{-5} M, the plateau reached in the stationary phase depended on the number of cells initially present. The inhibition was less, the greater the quantity of cells at the moment of econazole addition. If a high amount (absorbance 600 nm = 0.35) of cells, which previously have never been in contact with econazole, was cultivated in 10^{-4} M econazole, growth was not completely inhibited but greatly slowed down (Figs. 1 and 2). A small quantity of cells, previously cultured in econazole (10^{-5} M), stopped growing in a medium containing 10^{-4} M econazole nitrate.

Table 1. 50% (I_{50}) and 100% (I_{100}) growth inhibition obtained after 36 hr of culture in the presence of econazole nitrate (concentrations in M)

Culture conditions	I_{50}	I_{100}
Aerobiosis	4.0×10^{-8}	1.2×10^{-7}
Anaerobiosis	3.0×10^{-7}	1.2×10^{-6}

Humid cells (100 mg) were incubated in 100 ml medium (starting absorbance at 600 nm × 20 = 0.8).

Intracellular distribution of econazole nitrate

After 1 hr contact of the cells with [^{3}H]econazole nitrate, we found 27% of the initial radioactivity in the total cells. It is approximately half the 55% found in the cells after 22 hr contact (Table 2). At first, econazole nitrate attaches to membranes, nuclei,

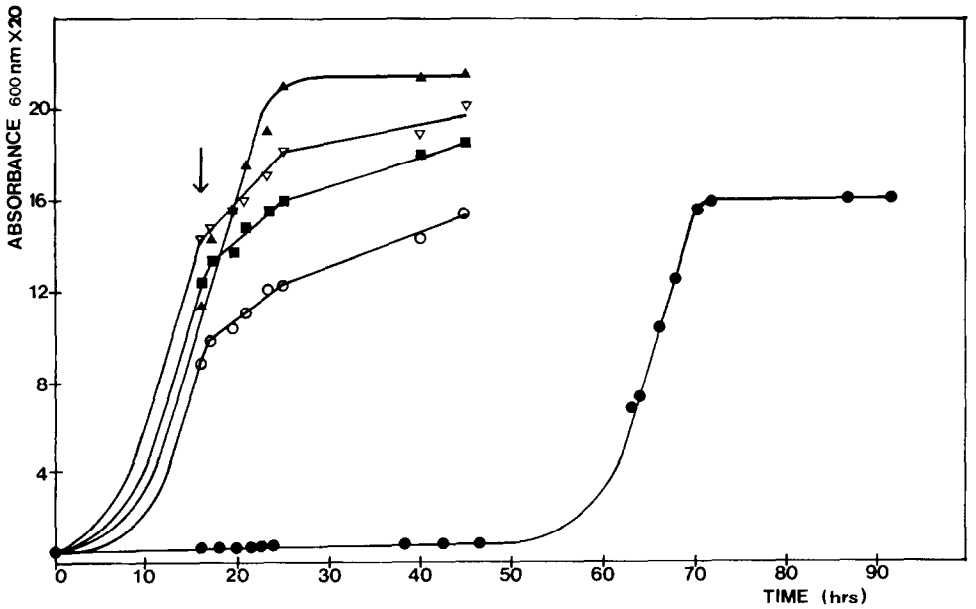

Fig. 1. Effect of econazole nitrate on the aerobic growth of *S. cerevisiae*. (▲) Growth of control cells without econazole; (▽, ○, ■) 10^{-4} M econazole nitrate was added to the medium after 16 hr of growth without econazole but at increasing cell concentrations; (●) 10^{-6} M econazole nitrate was added to the medium at zero time.

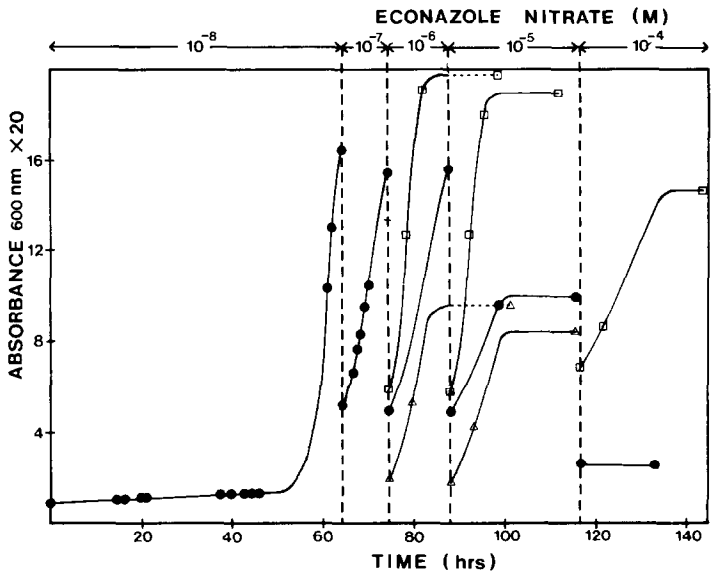

Fig. 2. Search for an adaptation of the growth of *S. cerevisiae* toward econazole nitrate. (●) Cells grown in medium containing 10^{-8} M econazole were harvested at 64 hr and a quarter of the cells was re-incubated in fresh medium containing 10^{-7} M econazole; at 74 hr, a quarter of the harvested cells was re-incubated in medium containing 10^{-6} M econazole; at 87 hr, a quarter of the harvested cells was re-incubated in 10^{-5} M econazole; at 116 hr, a quarter of the harvested cells was reincubated in 10^{-4} M econazole. (□) Fresh cells were incubated, respectively, in 10^{-6}, 10^{-5} and 10^{-4} M econazole nitrate. Initial absorbances at 600 nm × 20 were, respectively, 5.8, 5.6 and 6.6. (△) Fresh cells were incubated, respectively, in 10^{-6} and 10^{-5} M econazole nitrate. Initial absorbances at 600 nm × 20 were, respectively, 2.0 and 1.8.

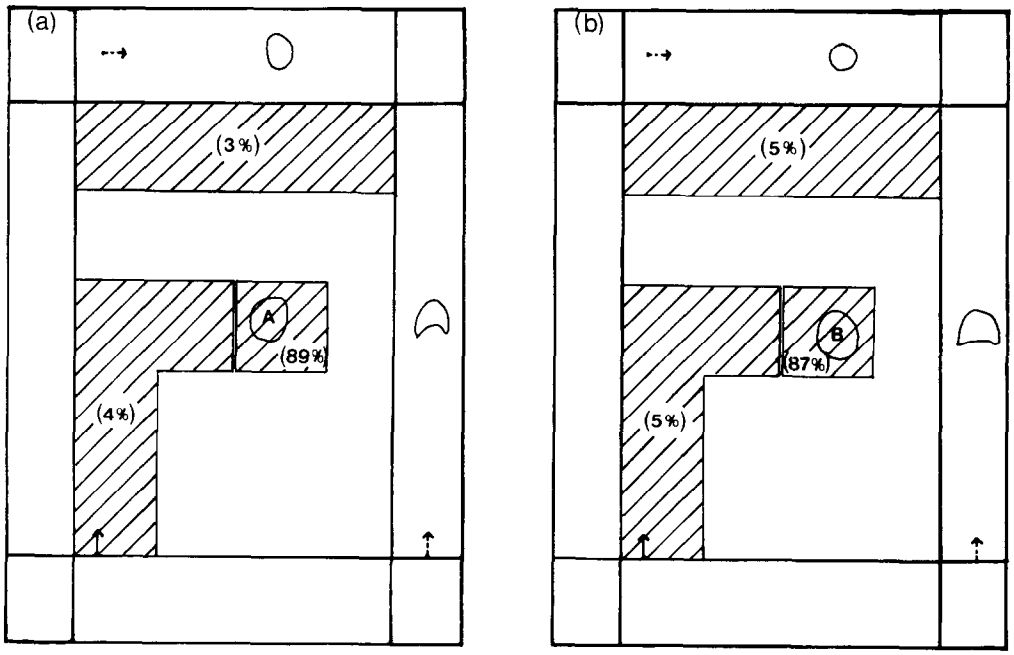

Fig. 3. Two-dimensional thin layer chromatography of (a) pure [^{3}H]econazole nitrate and (b) chloroform extract of cells grown in the presence of [^{3}H]econazole nitrate. (→): Deposit and sense of migration; (- - →): deposit and sense of migration of standard econazole nitrate; (////) radioactive areas; (*x*%): percentage of radioactivity found; A and B: econazole nitrate spots after two-dimensional migration.

Table 2. Radioactivity (cpm) of [^{3}H]econazole nitrate found in the culture medium, in the cells, and in subcellular fractions after 1 and 22 hr incubation

Fractions	1 hr incubation of cells with [^{3}H]econazole nitrate in 100 ml of medium				22 hr incubation of cells with [^{3}H]econazole nitrate in 200 ml of medium			
	Total [^{3}H] radio-activity	% Initial radioactivity	Proteins (mg)	Radioactivity per mg of proteins	Total [^{3}H] radio-activity	% Initial radioactivity	Proteins (mg)	Radioactivity per mg of proteins
Culture medium	687,000	100.0	—	—	1,374,000	100.0	—	—
Cells	187,950	27.4	226.9	828	756,200	55.0	505.4	1496
Cell membranes and nuclei	69,200	10.1	51.7	1339	154,980	11.3	118.5	1308
Mitochondria	15,093	2.2	7.6	1996	54,960	4.0	17.8	3083
Ribosomes	37,177	5.4	19.4	1921	111,596	8.1	56.2	1986
Cytosol	46,000	6.7	148.3	310	296,454	21.6	298.8	992

mitochondria and ribosomes. It is also found in soluble fractions (cytosol). In the mitochondria, the specific radioactivity increased from 1996 cpm/mg after 1 hr incubation to 3083 after 22 hr incubation. It was the highest ratio observed. The ratio in the cytosol was very low after 1 hr contact (310) but increased after 22 hr contact (992). After long incubation, the radioactivity concentrated into mitochondria and also increased in the cytosol.

Search for metabolites from econazole nitrate

The radioactivity found could be supported by [^{3}H]econazole nitrate or by products of its metabolism by yeast. To answer this question, aliquots from the following fractions, medium cleared of cells (i), cells (ii) and inner membranes of mitochondria (iii), were extracted with chloroform. Each extract was deposited on a separate TLC plate and subjected to two-dimensional chromatography. Unlabelled econazole nitrate was used to overload each deposit. On a check plate it can be seen that 89% of the total radioactivity was found around the econazole nitrate spot (Fig. 3a). For the three fractions (i, ii, iii) we found, respectively, 81, 87 and 97% (Table 3). No significant radioactivity was found in any other 254 nm absorbing spot (Fig. 3b).

Effects of econazole nitrate on the activity of mitochondrial enzymes

The effects on the biosynthesis of succinate dehydrogenase and cytochrome *c* oxidase are shown in Fig. 4. Econazole nitrate at 10^{-8} M induces a 50% reduction of the cytochrome *c* oxidase content. The econazole concentration inducing 50% reduction of succinate dehydrogenase is two-fold higher. The reduction can be due either to an inhibition of enzyme biosynthesis or to the increase of enzyme degradation.

No significant effect was noticed on respiration with low concentrations (10^{-8} M) of econazole nitrate present during incubation in the stationary phase of the cells (Table 4). Higher concentrations depressed the respirations significantly with the three substrates used: 10^{-4} M econazole nitrate induced 59% inhibition of respiration on ethanol, 40% inhibition on succinate and 27% inhibition on succinate with bypass on the second energy coupling site. This means that 10^{-8} M econazole nitrate is not sufficient to produce a significant degradation of the mitochrondrial enzymes, especially succinate dehydrogenase and cytochrome *c* oxidase. Consequently, it

Table 3. Radioactivity found in the econazole nitrate spot after two-dimensional thin layer chromatography of different fractions on silica gel plates

Fractions	% Initial radioactivity in the econazole nitrate spot
Medium after cells sedimentation	81
Cells	87
Inner mitochondrial membranes	97

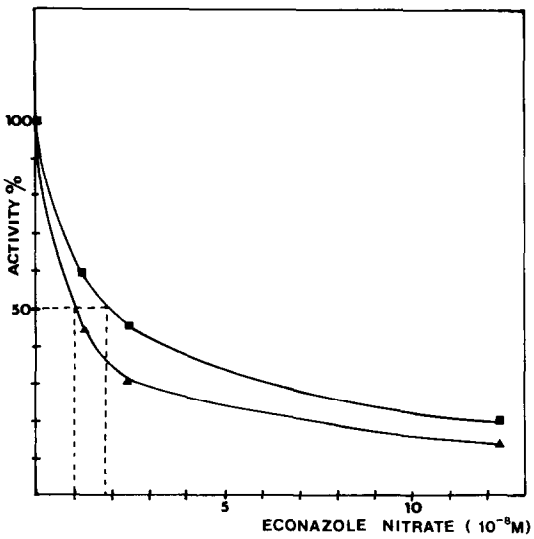

Fig. 4. Econazole nitrate action on the succinate dehydrogenase (■) and cytochrome *c* oxidase (▲) activities. The activities are expressed in percentage of specific activity (enzyme units/mg protein) of extracted enzymes compared to that from control cells.

can be concluded that 10^{-8} M econazole nitrate inhibits the synthesis of the two enzymes rather than increasing their proteolysis or their leakage from the cell.

The mitochondrial phenylalanyl-tRNA synthetase from yeast is different from its cytoplasmic counterpart [4]. Econazole (5.9×10^{-8} M) did not influence its activity or its cellular content. When extracted from yeast inoculated without econazole its specific activity was 269 pmoles/min per mg protein (Table 5). When econazole was present, the specific activity was almost unchanged: 263 pmole/min per mg protein. The econazole concentration which induced a 50% inhibition of cell growth did not decrease the amount of extracted enzyme activity. The K_m value of the enzyme from cells incubated with econazole did not increase significantly.

Table 5. Comparison of the mitochondrial Phe-tRNA synthetase activities in cells grown in the derepression medium containing 5.9×10^{-8} M econazole nitrate and in cells grown in the absence of econazole nitrate

	Medium without econazole	Medium with econazole
Weight (g) of humid cells harvested after 16 hr in 5.5 l. culture medium	112.2	100.7
Specific activity of mitochondrial enzyme (pmole of $tRNA^{Phe}$/l min per mg of protein)	268.9	263.0
K_m of Phe-tRNA synthetase (nmole of $tRNA^{Phe}$/l.)	3.12	3.57

DISCUSSION

Effects of econazole nitrate on the cell growth

Econazole nitrate (10^{-8} M) effects a 50% inhibition of *S. cerevisiae* growth in aerobiosis. But this effect depends on the state of culture at the moment of econazole addition into the medium. The most important effect is obtained in the exponential phase when the biosynthetic processes are at their maximum level and need large amounts of ATP from mitochondrial origin. The degree of inhibition decreases when the culture reaches the stationary phase, where biosynthetic processes are low. The same results were brought out on *Candida albicans* by Cope [12]. Inhibition of the cell growth by econazole can be explained either by metabolism of econazole or by sequestration of this product by cell constituents like proteins or lipids.

There is no adaptation of the yeast towards increased concentrations of econazole. The inhibitory effect, proportional to econazole concentration, is greater on cells which were previously in contact with the product than on cells which were undergoing their first contact. The quantity of cells intervenes in the answer to the inhibitor; the effect on growth is greater the smaller the number of cells. This reveals

Table 4. Influence on mitochondrial respiration of econazole nitrate during incubation of cells in the stationary phase*

	QO_2			
Substrates	Control	Econazole nitrate (10^{-8} M)	(10^{-6} M)	(10^{-4} M)
Ethanol: NADH→O_2	39.68	42.16	28.23	16.28
Succinate: succinate→O_2	94.25	84.33	56.45	56.42
Succinate + antimycin A + TMPD: succinate→O_2, second energy complex site by-passed	249.26	238.10	170.36	182.29

* Cells were grown in derepression medium until the stationary phase was attained. Then econazole nitrate was added at the indicated concentration and culture was continued for 5.5 hr. QO_2 values are expressed in nmole O_2 consumed/min per mg mitochondrial protein. Details of the respiration medium are given in Materials and Methods. The following additions were made: 10 µl of ethanol or 2.5 µmole of succinate or 2.5 µmole of succinate + 13 µmole of $MgCl_2$ + 260 ng of antimycin A + 480 nmole of *N,N,N',N'*,-tetramethyl-*p*-phenylendiamine-dihydrochloride (TMPD).

the importance of the following ratio: mass of inhibitor per mass of cells present initially. Therefore, in the first phase of growth, this ratio is high. It is responsible for the long period of growth latency. During the exponential phase, this ratio decreases and is weakened in the stationary phase. Therefore, the inhibitory effect diminishes to zero. Secondly the ratio intervenes on the plateau reached at the stationary phase: the lower the ratio, the higher the plateau. Dependence on the ratio supports the hypothesis of sequestration of econazole nitrate by cell constituents, which could lead to a disorganisation of yeast vital processes and even stop all growth. Shigematsu *et al.* [13] reported specific interaction between ketoconazole and *Candida albicans* cytochrome *c* oxidase with 10^{-4} M imidazole derivative concentration.

Econazole entrance in cells, subcellar distribution and its eventual metabolism

A preliminary study with [^{125}I]econazole nitrate, iodated according to the Greenwood and Hunter method [14] showed penetration of this product into the cells and its presence in soluble fractions (unpublished results). But an important desiodation takes place in yeast. Therefore we could not discover in detail the intracellular distribution.

In the present study with [^{3}H]econazole nitrate, we are able to check the intracellular distribution of the compound. After a short incubation (1 hr), the radioactivity fixes essentially to the particular fractions: membranes, nuclei, mitochondria, ribosomes. With longer incubation (22 hr), the radioactivity is also found in the cytosol. But the highest concentration occurs in the mitochondria. The question arises whether the radioactivity is worn by econazole or by metabolic derivative(s). When all the fractions were submitted to two-dimensional TLC no other product was uncovered. Our results are in agreement with those of Van Den Bossche *et al.* [15] who recovered [^{3}H]miconazole entirely unchanged from *Candida albicans*. If metabolization occurred, the onset of the cultures and the plateau reached in Figs. 1 and 2 would be identical whether the cells previously had or had not been in contact with econazole. But this is seemingly not the case.

Action of econazole on three mitochondrial enzymes

The econazole action is most efficient in aerobiosis and this product inhibits respiration [3]. Therefore we studied the effect on the biosynthesis of two enzymes from the mitochondrial inner membrane: succinate dehydrogenase, which is entirely synthesized on cytoribosomes, and cytochrome *c* oxidase, which is synthesized partially on mitoribosomes and on cytoribosomes [16]. We were also interested in another enzyme, phenylalanyl-tRNA synthetase, present in the matrix but which is synthesized on cytoribosomes and imported into mitochondria [4, 17]. Previous experiments [3] showed that 10^{-8} M econazole nitrate does not influence any of the three enzyme activities *in vitro*. Econazole does not induce an increase of the proteolysis or of the leakage from the cell of succinate dehydrogenase and cytochrome *c* oxidase, as shown in our experiments. But the same concentration of econazole nitrate in the incubation medium brings about a 50% drop in cytochrome c oxidase synthesis; for succinate dehydrogenase a two-fold higher concentration is required. As the cells grow on non-fermentable substrates, respiration is absolutely necessary, and so are succinate dehydrogenase and cytochrome *c* oxidase, two respiratory chain enzymes. Even a three-fold higher concentration does not disturb the biosynthesis and the K_m value toward tRNA of the phenylalanyl-tRNA synthetase. Therefore we can conclude that the enzyme biosynthesis which is realised partially on mitoribosomes is more sensitive than that which is entirely localized on the cytoribosomes. On the other hand, the biosynthesis of inner membrane proteins, like cytochrome *c* oxidase and succinate dehydrogenase, is more susceptible to inhibition than that of matrix enzymes like phenylalanyl-tRNA synthetase. Cell incubation with 5.9×10^{-8} M econazole nitrate apparently does not influence the entrance into the mitochondria of the cytoplasmic made mitochondrial phenylalanyl-tRNA synthetase.

Our research is now concentrated more specifically on the nature of the mitochondrial membrane constituent(s) which may be the target of the econazole nitrate action.

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