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Effects of β-Pinene on Yeast Membrane Functions

SALVADOR URIBE, JORGE RAMIREZ, AND ANTONIO PEÑA*

Departamento de Microbiología, Centro de Fisiología Celular, and Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México, D.F. México

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The effects of β-pinene on yeast cells were studied. This terpene inhibited respiration with glucose or ethanol as the substrate. The inhibition depended on the ratio of the terpene to the amount of yeast cells; for a fixed concentration of pinene, inhibition decreased as the amount of yeast cells increased. Pinene also inhibited the pumping of protons and K^+ transport, but this inhibition was more marked with ethanol than with glucose as the substrate, indicating the mitochondrial localization of the inhibition. The studies on isolated mitochondria showed a series of effects, starting with the disappearance of the respiratory control and deenergization of the organelles and followed by an inhibition of respiration at higher concentrations of the terpene. The effect on respiration could be localized to the cytochrome *b* region of the electron transport chain. No effect could be detected on the activity of ATPase. The effects can be ascribed to a localization of pinene on membranes which was also accompanied by a decrease in the fluorescence polarization of diphenyl hexatriene, probably meaning an increase in the fluidity of the membrane, localized preferentially to the mitochondria.

The bacteriostatic and bactericidal effects of terpenes have been previously documented (8, 10, 15). A study on the effects of α-pinene was also reported (1), using several microorganisms; it was found that this terpene produces an inhibition of growth, the release of 260-nm absorbing materials from the cells of *Bacillus thuringiensis* and *Saccharomyces cerevisiae*, a slight inhibition of the respiratory control of yeast mitochondria, and the decrease of the viability of spores of *B. thuringiensis*.

The aforementioned studies indicated the need for a more detailed study of a series of effects apparently localized in the membranes of microorganisms. Besides, in a screening study of several allelopathic agents on respiration patterns in yeast cells, we found that one of the most potent of these agents was β-pinene. This paper presents the results of studies performed with a commercial strain of *S. cerevisiae*, taking also into consideration previous effects reported for β-pinene on rat liver mitochondria (16).

MATERIALS AND METHODS

Preparation of yeasts and yeast mitochondria. Cells of a commercial strain of *S. cerevisiae* (La Azteca, S.A.) were incubated for 8 h in a culture medium (3) and then starved overnight. Mitochondria from the yeast cells were prepared as previously described (12). To prepare mitochondria, instead of using a Ribi cell disintegrator, cells were broken during 15 s in a Nossal cell homogeneizer (Braun), with 0.45- to 0.50-mm glass beads, at a speed of 3,500 rpm. Both the suspensions of starved cells and mitochondria were used by 4 h after preparation.

Oxygen consumption. Oxygen consumption was measured by means of a Clark electrode in a variable volume chamber, with an appropriate polarization and recording system. The media used are indicated below for each experiment.

Yeast plasma membranes. Yeast plasma membranes were prepared as described by Fuhrmann et al. (6).

NADH dehydrogenase activity. This mitochondrial activity was measured by following the reduction of 2,6-dichloroindophenol in medium containing 50 mM potassium phosphate buffer (pH 7.6), 100 μM KCN, 25 μM NADH, and variable concentrations of pinene. The final volume was 2.0 ml at room temperature. Mitochondria (2 mg of protein) were added; 2 min later, 80 μM 2,6-dichloroindophenol was added, and the absorbance change was followed in a dual-wavelength spectrophotometer at 600 versus 590 nm. The activity was calculated from the molar extinction coefficient of the dye and the absorbance change against time.

Succinate dehydrogenase activity. The same general procedure was employed by following the reduction of 2,6-dichloroindophenol (with succinate as the substrate) in medium containing 50 mM potassium phosphate buffer (pH 7.6), 100 μM KCN, 275 μM phenazine methosulfate, 5 mM sodium succinate (pH 7.6), 5 μg of rotenone, and variable concentrations of pinene in a final volume of 2.0 ml at room temperature. Mitochondrial protein (2 mg) was added, and after 2 min, 80 μM 2,6-dichloroindophenol was added. The absorbance changes at 600 versus 590 nm were followed, and the activity was calculated as described for NADH dehydrogenase.

NADH:cytochrome *c* reductase activity. To measure this activity, the reduction of cytochrome *c* was followed in an incubation medium containing 50 mM potassium phosphate buffer (pH 7.6), 100 μM KCN, and 5 μM NADH (plus the indicated concentrations of pinene in a final volume of 2.0 ml) at room temperature. Two minutes after the addition of 2 mg of mitochondrial protein to the incubation mixture, 1.5 mg of cytochrome *c* was added, and the absorbance changes were followed at 550 versus 540 nm in a dual-wavelength spectrophotometer. The amount of reduced cytochrome *c* was calculated from the extinction coefficient at the indicated wavelengths.

Succinate:cytochrome *c* reductase activity. This activity was followed by measuring spectrophotometrically the reduction of cytochrome *c* in incubation mixture containing 50 mM potassium phosphate buffer (pH 7.6), 100 μM KCN, 500 μM sodium succinate (pH 7.6), 5 μg of rotenone, plus β-pinene at the indicated concentrations and 2.0 mg of mitochondrial protein. The final volume was 2.0 ml at room temperature. Two minutes after the addition of mitochondria to the incubation medium, the reaction was started by the

* Corresponding author.

addition of 1.5 mg of cytochrome *c*. The absorbance was followed at 550 versus 540 nm in a dual-wavelength spectrophotometer. The reduction of cytochrome *c* was calculated from the molar extinction coeficient at these wavelengths.

Cytochrome *c* oxidase activity. This mitochondrial activity was measured by following the oxygen consumption in incubation medium containing 50 mM potassium phosphate buffer (pH 7.6), 5 mM sodium ascorbate (pH 7.6), 50 μM tetramethyl-*p*-phenylenediamine, 1 μg of antimycin A, and variable concentrations of pinene at a final volume of 3.0 ml. The measurement was performed at room temperature; it was started by the addition of mitochondria (3 mg of protein).

ATPase activity. This enzymatic activity was measured by following the amount of inorganic phosphate liberated by incubating yeast mitochondria with ATP in medium containing 20 mM Tris-HCl (pH 8.5), 2 mM ATP-Tris (pH 8.5), and 5 mM $MgCl_2$. To measure the enzyme activity, 500 μg of mitochondrial protein was mixed with 300 μl of water to disrupt the permeability barriers, with or without variable amounts of pinene. The rest of the incubation mixture was then added to complete 1.0 ml of final volume. After an incubation of 10 min, the reaction was stopped by the addition of 100 μl of 30% cold trichloroacetic acid. The mixture was centrifuged for 5 min at 2,500 rpm. Inorganic phosphate was measured in the supernatant as described by Fiske and Subbarrow (5).

Estimation of the mitochondrial membrane potential. The estimation of mitochondrial membrane potential was carried out by following the fluorescence of *N*,*N'*-dipropyl thiacarbocyanine at 540 versus 590 nm under various conditions as previously indicated (13). An increase in the fluorescence seems to indicate a decrease in the membrane potential, and vice versa (13, 14).

K^+ and H^+ movements. K^+ and H^+ movements were followed by means of a monovalent cation electrode (Corning no. 476220; Corning Glass Works, Corning, N.Y.) and a pH combination electrode, respectively, with a pH meter and a recorder attached.

Fluorescence polarization measurements. Yeast cells, plasma membranes, or yeast mitochondria were incubated in the indicated media with 1 μM 1,6-diphenyl-1,3,5-hexatriene for 10 min at room temperature. The terpene was then added, and the polarization of fluorescence was measured in a spectrofluorometer with two photomultipliers and calcite polarizers at 340 nm excitation wavelength and two 418-nm cutoff Schott filters. This method seems to give good indications of membrane fluidity (2, 9).

Protein determination. Protein determination was carried out by the biuret method.

Reagents. All substances were of the highest grade available. β-Pinene was used as obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and prepared as a 0.2 M solution in dimethylformamide; adequate volumes to obtain the desired concentrations were added. In all cases, controls were prepared by adding the amount of dimethylformamide corresponding to the highest concentrations of pinene. They showed no effect on any activity, except on the respiration of isolated mitochondria or cytochrome *c* oxidase, which was inhibited by ca. 15 to 20%.

RESULTS

Effects of pinene on intact yeast cells. (i) Respiration. Previous studies have shown that β-pinene can inhibit the respiration as well as other functions of rat liver mitochondria (16); it was found also that some effects can be traced to the membranes of microorganisms (1). Figure 1 shows that β-pinene also could inhibit yeast cell respiration, both with glucose and ethanol as substrates. Besides, the inhibition depended on the amount of yeast cells present in the incubation mixture; with 10 mg of yeast cells, a clear inhibition was found with a concentration of 500 μM. When the amount of yeast cells was increased to 50 mg/ml, the same concentration of β-pinene produced only a small inhibition. This already indicated that the terpene was strongly taken up by the cells, most probably at the level of the membranes.

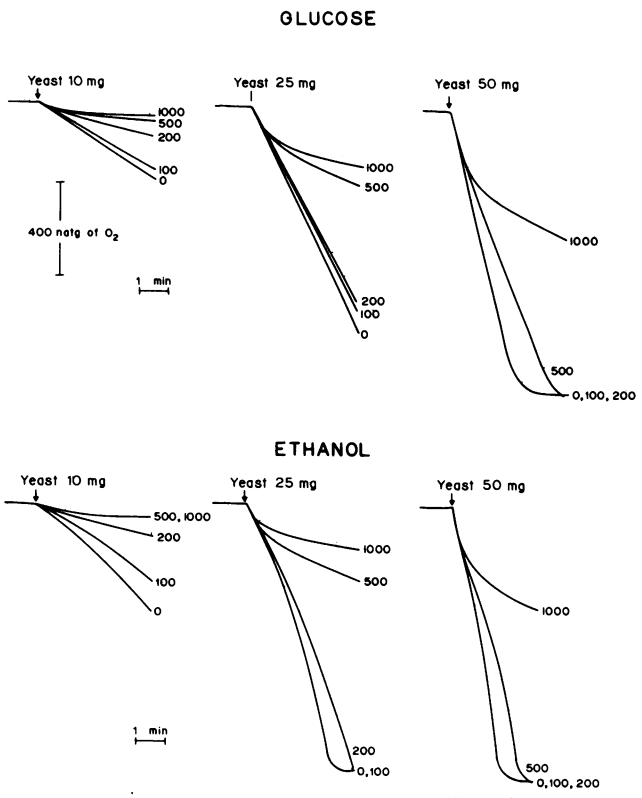

FIG. 1. Effects of β-pinene on the respiration of variable amounts of intact yeast cells with ethanol or glucose as the substrate. The incubation medium was 20 mM morpholinoethanesulfonic acid adjusted to pH 6.0 with triethanolamine; 25 mM glucose or 53 mM ethanol was used as the substrate. Yeast cells of 10, 25, or 50 mg (wet weight) were used as indicated. Final volume was 3.0 ml. Figures to the right of curves indicate the micromolar concentration of pinene present in the medium before the addition of cells.

(ii) Extrusion of H^+. This function was studied because it is catalyzed by an ATPase localized in the plasma membrane of the cell (7, 11), and requires also an effective source of ATP, that can be either fermentation or respiration. Figure 2 shows that, with glucose as the substrate, the inhibition of H^+ pumping by the cells required rather high concentrations of β-pinene. With ethanol, lower concentrations were required, similar to those that produced the inhibition of respiration.

(iii) K^+ transport. This is another function of the plasma membrane of the yeast cell; it seems to be separated from, but dependent upon, H^+ pumping (7, 11). Like H^+ pumping, K^+ transport required higher concentrations of β-pinene to be inhibited with glucose than with ethanol as the substrate (Fig. 3).

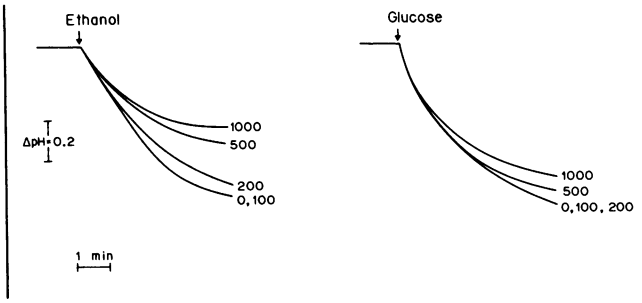

FIG. 2. Effects of variable concentrations of β-pinene on the ability of yeast cells to pump protons into the medium. The incubation medium was the same as described in the legend to Fig. 1, but buffer was omitted; the initial pH was 6.4. Final volume was 5.0 ml, and 42 mg of yeast cells (wet weight) were used. The cells were added ca. 3 min before the substrate.

Effects on yeast mitochondria. (i) Respiration. As expected from the experiments performed with intact yeast cells, respiration of yeast mitochondria was sensitive to rather low concentrations of β-pinene. However, it is difficult to extrapolate strictly the ratios of terpene concentration to the amount of either yeast cells or yeast mitochondria in terms of the possible partition of pinene between the medium and either of the two biological materials. The inhibition of respiration showed several interesting characteristics (Fig. 4): respiration in the absence of ADP (state 4 respiration) was affected only by rather high concentrations of the terpene. At low concentrations (50 or 100 μM), what was more striking was an inhibition of respiration in the presence of ADP (state 3 respiration); at 200 μM pinene and 1 mg of mitochondrial protein per ml, there was practically no response to ADP. However, the uncoupler FCCP added after ADP could produce a significant stimulation of respiration. At higher concentrations of the terpene, there was an inhibition of state 3, state 4, and uncoupled respiration.

To eliminate effects related to the control of respiration by ADP, the experiment shown in Fig. 5 was performed. It

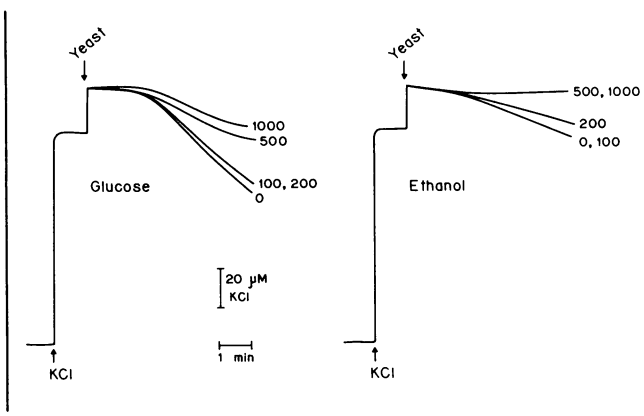

FIG. 3. Effects of various concentrations of β-pinene on K^+ transport by yeast cells with glucose or ethanol as the substrate. Experimental conditions were as described in the legend to Fig. 1, but 100 μM KCl was included in the incubation medium, and the final volume was 8.0 ml. Also, 67 mg of yeast cells was added where indicated.

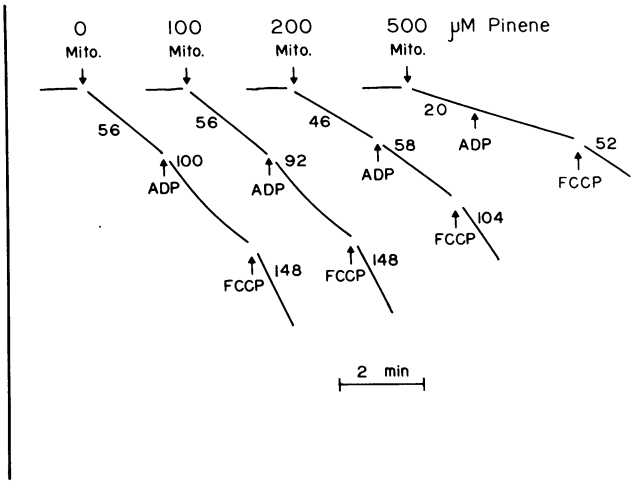

FIG. 4. Effects of β-pinene on the respiration of yeast mitochondria. The incubation mixture contained 0.6 M mannitol, 10 mM NaH_2PO_4 (pH 6.5), and 1.0% defatted bovine serum albumin. Also used was 15 mM succinate, with pH adjusted to 6.5 with NaOH, and 1 mg of mitochondria protein per ml. Pinene was present at the indicated concentrations. Final volume was 3.0 ml. Temperature was 30°C. ADP was added at a concentration of 80 μM, and FCCP was added at 3 μM. Figures aside the curves indicate the oxygen consumption rates in nanogram-atoms of O_2 per minute per milligram.

shows the effects of pinene on the respiration of mitochondria incubated in a hypotonic medium that had lost the ability to respond to either ADP or FCCP. Under these conditions, pinene inhibited respiration within a concentration range similar to that required to inhibit O_2 consumption by coupled mitochondria.

(ii) ATPase activity. In mitochondria isolated from yeast cells, no activity of ATPase could be detected, even in the presence of uncouplers, unless hypotonic media were used. This fact did not allow us to study this mitochondrial activity under the same conditions as coupled respiration. However, even at very high concentrations of the terpene, no inhibi-

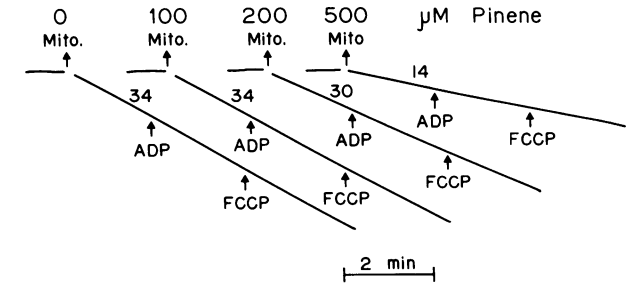

FIG. 5. Effects of β-pinene on the respiration of isolated yeast mitochondria incubated in a hypotonic medium. The incubation conditions were similar to those described in the legend to Fig. 4, but only 100 mM mannitol, 2 mM sodium phosphate buffer, and 0.25% defatted albumin were used. Figures beside the curves are also as described in the legend to Fig. 4. For these experiments, the same mitochondria as described in the legend to Fig. 4 were used.

TABLE 1. Effects of β-pinene on several activities of isolated yeast mitochondria[a]

Sample	ATPase	% Inhibition	NADH: DCPIP	% Inhibition	Succ: DCPIP	% Inhibition	NADH: cyt *c*	% Inhibition	Succ: cyt *c*	% Inhibition	Cyt *c*: oxidase	% Inhibition
Pinene (μM)												
0	189	0	57	0	26	0	53	0	108	0	127	0
100	197	−4	57	0	15	38	37	28	42	61	122	4
200	197	−4	89	−56	15	38	25	53	17	84	115	10
500	180	5	82	−43	15	38	7	85	10	91	108	16
1,000	177	6	101	−75	15	38	7	86	2	98	100	21
Dimethylformamide (10 μl/ml)	189	0	57	0	20	22	53	0	84	22	103	19
Oligomycin	12	94										
DCCD	26	86										
NaN_3	42	78										

[a] The measurements were made as described in the text. Results are expressed as follows: ATPase, nanomoles of inorganic phosphate hydrolyzed per minute per milligram; NADH:2,6-dichloroindophenol (DCPIP) and succinate (succ):DCPIP, nanomoles of DCPIP reduced per minute per milligram; NADH:cytochrome *c* (cyt *c*) and succ:cyt *c*, nanomoles of cytochrome *c* reduced per minute per milligram; and cyt *c*:oxidase, nanogram-atoms of O_2 consumed per minute per milligram. The inhibitors were added at the following concentrations: oligomycin, 10 μg/ml; dicyclohexyl carbodiimide (DCCD), 10 μM; and NaN_3, 1 mM.

tion of ATPase could be detected. Both the control mitochondria and those incubated in the presence of up to 1 mM pinene showed an activity of 180 to 190 nmol of inorganic phosphate hydrolyzed per mg of mitochondrial protein per min. (Table 1). Several inhibitors, especially oligomycin, which produced an inhibition close to 100%, showed that this was, in fact, a mitochondrial ATPase.

(iii) Mitochondrial transmembrane potential. The fluorescence of *N-N'*-dipropyl thiacarbocyanine should show a quenching when a membrane potential that is negative inside is generated in the mitochondria (13, 14). The results of Fig. 6 show that the fluorescence of the cyanine was quenched even before the addition of a substrate, probably because of the oxidation of endogenous substrates. Upon the addition of succinate, a further quenching was observed; this quenching could be reverted by the addition of antimycin A and produced again, although to a smaller extent, by the addition of ATP, and reverted once more by the addition of an uncoupler. When pinene was present, the initial fluorescence of the cyanine was higher, and the quenching of the fluorescence generated with succinate or ATP was smaller, depending on the concentration of the terpene. At 1 mM β-pinene, these changes were no longer observed. Similar results were obtained by energization only with 5 mM ATP (Fig. 7) in mitochondria incubated in the presence of antimycin A. In these experiments, the initial levels of fluorescence were higher, with some delay at 100 μM pinene, probably because of the inhibition of the oxidation of endogenous substrates.

Effects of pinene on the respiratory chain. These effects were measured by following the effects of the terpene (at various concentrations on several segments of the respiratory chain in isolated mitochondria incubated in hypotonic media) to eliminate the permeability barriers and effects on respiratory control. Stimulation of NADH dehydrogenase was observed when the effects of the terpene were studied

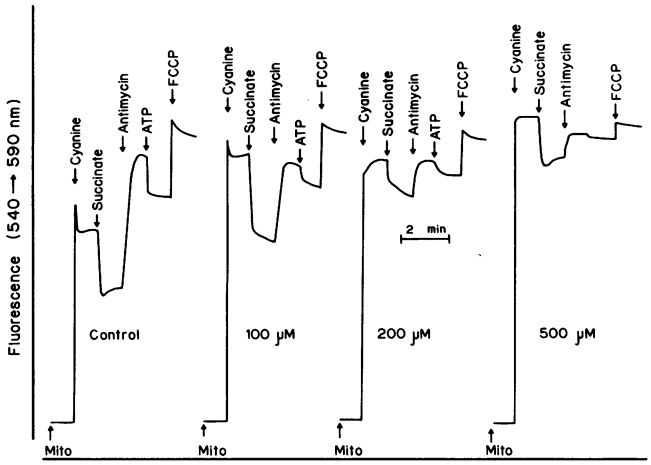

FIG. 6. Effects of β-pinene on the membrane potential of yeast mitochondria. The potential was measured as described in the text. Incubation mixture was the same as described in the legend to Fig. 4, with 15 mM succinate or 2 mM ATP-Na (pH 6.5). Mitochondria were added at 1.0 mg/ml. Final volume was 2.0 ml, and the experiment was carried out at room temperature. Where indicated, 50 nM antimycin A or 3 μM FCCP was added. The concentration of *N-N'*-dipropyl thiacarbocyanine was 0.5 μM.

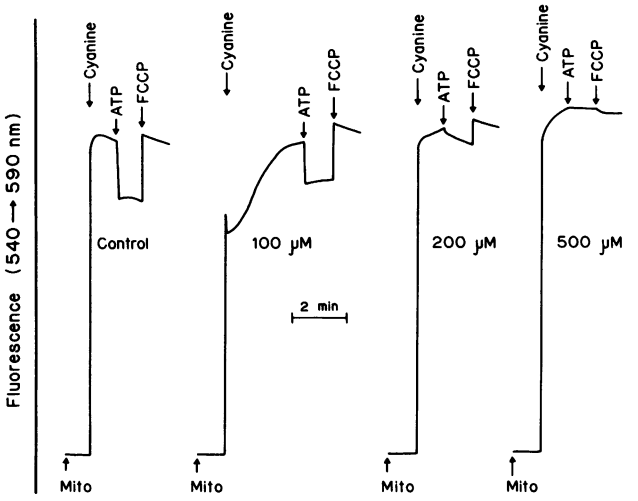

FIG. 7. Effects of β-pinene on membrane potential of yeast mitochondria with ATP in the presence of antimycin A. The experiment was carried out as described in the legend to Fig. 5, but with 5 mM ATP-Na adjusted to pH 6.5 as the substrate. A concentration of 50 nM antimycin A was present in the medium.

on this isolated activity (Table 1). No effect could be demonstrated on the cytochrome *c* oxidase activity, except for an inhibition of ca. 10%, that could be observed also with dimethylformamide (used for dissolving pinene). Succinate dehydrogenase was already inhibited up to a maximum of ca. 40% with the lower concentrations of the terpene. However, the most striking inhibition was detected in the reduction of cytochrome *c*, both with NADH or succinate as substrates; this inhibition was almost complete.

Fluorescence polarization changes. (i) Intact cells. Since the effects of the terpene could be traced to functions localized in the membranes of yeast cells, this general interaction might be evidenced by the measurement of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene as an indicator of possible changes of the fluidity of the membranes. In experiments carried out with intact cells (Fig. 7), the addition of β-pinene produced a large decrease in the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene which was compatible with an increase of the fluidity of the membrane(s) when pinene was present.

(ii) Plasma membranes. The same experiments, carried out with a preparation of plasma membranes from yeast cells (6), also showed a decrease in the fluorescence polarization in the presence of β-pinene. This decrease was, however, very small, when compared with that observed in intact cells (Fig. 8). Besides, the values of polarization in the absence of pinene were already much lower than those of intact cells or mitochondria under similar conditions.

(iii) Mitochondria. When the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene was measured with isolated mitochondria, a rather large decrease was observed in the presence of β-pinene (Fig. 8), similar to that observed with intact cells.

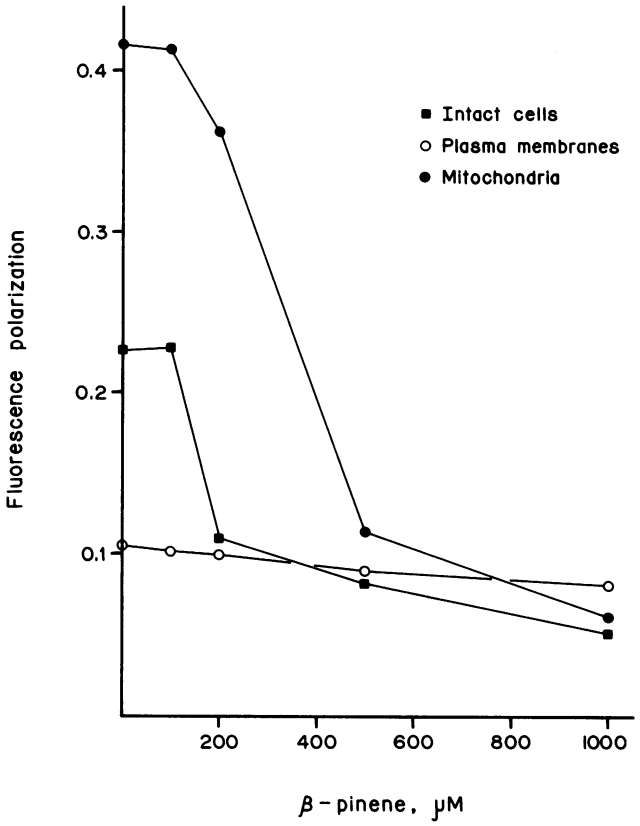

FIG. 8. Effects of β-pinene on the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in intact yeast cells, yeast plasma membranes, and yeast mitochondria. Incubation medium for yeast cells and plasma membranes was 20 mM morpholinoethanesulfonic acid-triethanolamine (pH 6.5). A sample of 1 uM 1,6-diphenyl-1,3,5-hexatriene was added 10 min before β-pinene. The measurement was made as soon as possible after the addition of pinene. For yeast mitochondria, the medium was the same as described in the legend to Fig. 4. A sample of 16.6 mg of yeast cells, 10 mg of plasma membrane protein, or 10 mg of yeast mitochondrial protein was used.

Effects of pinene on the integrity of the mitochondrial membrane. The effects of pinene on the integrity of the mitochondrial membrane were tested by measuring the efflux of K^+ into a K^+-free medium with various concentrations of pinene. It was found that β-pinene, contrary to what was observed with rat liver mitochondria (16), did not produce the increased passive efflux of K^+ from the organelles. The addition of Triton X-100, on the other hand, produced the efflux of ca. 50 neq of K^+ per mg of protein (data not shown).

DISCUSSION

The effects of β-pinene reported here were, in some ways, expected, for the hydrophobic character of this terpene has to favor its partition into the membranous structures of the cell. The work of Andrews et al. (1) already showed effects indicating lesions on yeast membranes and other microorganisms. Also, the work reported before on the effects of β-pinene on liver mitochondria (16) could be explained mostly on the basis of the interaction of the terpene with the membrane of this organelle.

However, the effects do not seem to take place uniformly on all membranes of the yeast cell, and even those observed on isolated yeast mitochondria were different from the ones found with liver mitochondria (16). Starting with the effects of pinene on H^+ and K^+ movements in whole cells, it seemed clear that they were observed at lower concentrations when the substrate was ethanol, which requires the integrity of the mitochondrial functions to provide energy to the cell in the form of ATP. The concentrations required to inhibit the movements of H^+ and K^+ were similar to those required to inhibit respiration, but only when ethanol was the substrate. Both fermentation and the transport systems themselves seem to require higher concentrations of the terpene to be inhibited. It should be pointed out that, in our experiments, at the concentrations of β-pinene used, no leakage of the cell K^+ was observed.

The effects on respiration with intact cells were observed at higher concentrations of pinene than with isolated mitochondria. It is possible that in intact cells, part of the added inhibitor was diluted by being bound to other structures of the cell besides the mitochondria.

Regarding the effects on mitochondrial function, the terpene produced a series of effects related to its localization in the inner membrane. With liver mitochondria, pinene produced an increase in mitochondrial permeability that was probably the basis for the uncoupling action observed (16). In yeast mitochondria, this effect was not simple; no actual uncoupling was observed, and the terpene never stimulated respiration in the absence of ADP (state 4). At rather low concentrations of pinene (ca. 100 μM), the respiration stimulated by ADP (state 3) was inhibited, but that stimulated by FCCP (or that observed in mitochondria that had lost their responsiveness to ADP or FCCP by incubating them in hypotonic media) was inhibited much less at similar concentrations. This effect might be due to an inhibition of the

adenine nucleotide translocator. This suggestion might be supported by the fact that pinene, being neither an uncoupler of oxidative phosphorylation nor an inhibitor of the ATPase, could block the energization of mitochondria by ATP. At somewhat higher concentrations (200 μM or higher), the organelles started to lose their ability to be energized by succinate or ATP; this was probably due to the direct inhibition of respiration or the adenine nucleotide translocator, respectively. The studies performed indicated a direct effect on the cytochrome *b* region of the respiratory chain.

This preferential interaction of pinene with mitochondria also seems to have a counterpart in fluorescence polarization studies. Intact cells showed a large change in this parameter upon the addition of pinene, and this change was found to be equally large with mitochondria. However, when the effect of pinene was measured in the same parameter with isolated plasma membranes, a rather small change was found. This might be due to either a different solubility of the terpene in both membranes or different abilities to produce the changes in membrane fluidity. Our experiments do not allow us to conclude definitely on these two possibilities. It should be pointed out, however, that these results are in agreement with the fact that other substances show similar behavior in yeast cells; uncouplers, which produce their effects at rather low concentrations when tested with yeast mitochondria (12), have to be used at much higher concentrations when used to inhibit or revert, for instance, the proton pumping at the level of the plasma membrane (4, 11).

It is interesting to note that pinene stimulated NADH dehydrogenase activity and inhibited cytochrome *b* segment activity but altered neither the ATPase activity nor other segments of the respiratory chain. The interaction of the terpene with the mitochondrial membrane did not affect all functions of the inner mitochondrial membrane in the same way. The inhibition was selective, indicating that the interaction of the terpene was not just producing a general alteration of the mitochondrial structure and function.

Our results are in general agreement with those of Andrews et al. (1) in the sense that the effects of these molecules are localized in the membranes of the cell. However, there was some discrepancy in the concentrations required to observe the effects. With mitochondrial protein concentrations of less than 1 mg/ml, for instance, these authors required concentrations of 2.06 mg of α-pinene per ml (ca. 15 mM) to obtain a decrease in respiratory control. It is possible, however, that α-pinene is less effective than its β-isomer. In any case, the results of these authors, as well as those reported here, show that the effects of terpenes were due to alterations produced at the level of the membranes of yeast cells. Within the lower concentration range, β-pinene could produce several alterations of the mitochondrial function; at higher concentrations, it could alter the functions of the plasma membrane.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Andrews, R. E., L. W. Parks, and K. D. Spence.** 1980. Some effects of Douglas fir terpenes on certain microorganisms. Appl. Environ. Microbiol. **40**:301–304.
2. **Andrich, M. P., and J. M. Vanderkooi.** 1976. Temperature dependence of 1,6-diphenyl-1,3,5-hexatriene in phospholipid artificial membranes. Biochemistry **123**:447–453.
3. **De Kloet, S. R., R. K. A. Van Wermeskerken, and V. V. Konigsberger.** 1961. Studies of protein synthesis by protoplasts of *Saccharomyces carlsbergensis*. Biochim. Biophys. Acta **47**: 138–143.
4. **De la Peña, P., F. Barros, S. Gascón, S. Ramos, and P. Sánchez-Lazo.** 1982. The electrochemical proton gradient of *Saccharomyces*. The role of potassium. Eur. J. Biochem. **123**: 447–453.
5. **Fiske, C. A., and Y. Subbarrow.** 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375–400.
6. **Fuhrmann, G. F., C. Boehm, and A. P. R. Theuvenet.** 1976. Sugar transport and potassium permeability in yeast plasma membrane vesicles. Biochim. Biophys. Acta **433**:583–596.
7. **Goffeau, A., and C. W. Slayman.** 1981. The proton translocating ATPase of the fungal plasma membrane. Biochim. Biophys. Acta **639**:197–223.
8. **Kushner, D. J., and G. T. Harvey.** 1962. Antibacterial substances in leaves: their possible role in insect resistance to disease. J. Insect Pathol. **4**:155–184.
9. **Lakowicz, J. R.** 1983. Principles of fluorescence spectroscopy. Plenum Publishing Corp., New York.
10. **Morris, O. N.** 1972. Inhibitory effects of foliage extracts of some forest trees on commercial *Bacillus thuringiensis*. Can. Entomol. **104**:1357–1361.
11. **Peña, A.** 1975. Studies on the mechanism of K^+ transport in yeast. Arch. Biochem. Biophys. **167**:397–409.
12. **Peña, A., M. Z. Piña, E. Escamilla, and E. Piña.** 1977. A novel method for the rapid preparation of coupled yeast mitochondria. FEBS Lett. **80**:209–213.
13. **Peña, A., S. Uribe, J. P. Pardo, and M. Borbolla.** 1984. The use of a cyanine dye in measuring membrane potential in yeast. Arch. Biochem. Biophys. **231**:217–225.
14. **Sims, P. J., A. S. Waggoner, C. H. Wang, and J. F. Hoffman.** 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry **13**:3315–3330.
15. **Smirnoff, W. A., and P. M. Hutchinson.** 1965. Bacteriostatic and bacteriocidal effects of extracts of foliage from various plant species on *Bacillus thuringiensis* var. *thuringiensis* Berliner. J. Invertebr. Pathol. **7**:273–280.
16. **Uribe, S., R. Alvarez, and A. Peña.** 1984. Effects of pinene, a non substituted monoterpene, on rat liver mitochondria. Pest. Biochem. Physiol. **22**:43–50.