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UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

FACULTAD DE MEDICINA

EFECTO DEL BETA-PINENO SOBRE LAS MEMBRANAS BIOLOGICAS

TESIS

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS BIOMEDICAS: BIOLOGIA MOLECULAR

PRESENTA

SALVADOR URIBE CARVAJAL

FALLA DE ORIGEN

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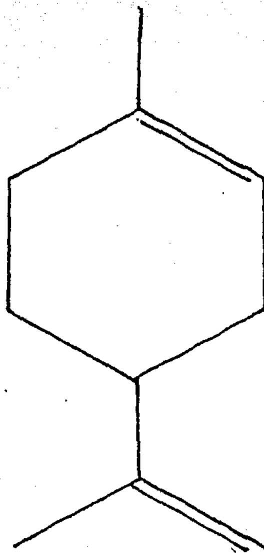
INTRODUCCION

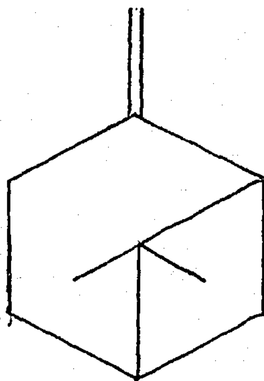
Está bien establecido que muchas plantas producen sustancias tóxicas para otras especies (1). Estas sustancias son liberadas para impedir la proliferación de organismos competidores, lo cual favorece el crecimiento y la proliferación de la especie productora de la sustancia. El fenómeno se conoce como alelopatía y los compuestos liberados al medio se denominan sustancias alelopáticas. La estricnina, la cafeína, la quinina y muchos antibióticos son algunos ejemplos de compuestos alelopáticos utilizados por el hombre (1).

Los monoterpenos son sustancias alelopáticas producidas por algunas plantas superiores (1-10). Alcanzan grandes concentraciones adsorbidos a las partículas coloidales del suelo, donde permanecen estables por meses (2). Una vez en el medio, los monoterpenos inhiben el crecimiento de plántulas y semillas (1-4), así como el metabolismo de bacterias y levaduras (5), en ocasiones permeabilizando las membranas celulares (5). Hay, además, reportes de toxicidad de monoterpenos en animales (6-8) y aún en el hombre (9); sin embargo, son relativamente pocos los estudios realizados sobre los mecanismos de acción de las sustancias alelopáticas. En mitocondrias aisladas de avena, los monoterpenos inhiben el consumo de oxigeno (4). En plantas integras se ha demostrado que inhiben el crecimiento de la raiz

Fig. 1.   ESTRUCTURA QUIMICA DEL BETA-PINENO Y DEL LIMONENO.



limoneno



β-pineno

más que el del tallo (Anaya, no publicado). Una diferencia entre la raíz y el tallo es que la primera depende para su metabolismo exclusivamente de las mitocondrias.

Algunos de los datos mencionados sugieren una interacción de los derivados monoterpénicos con las membranas biológicas, por lo que se decidió estudiar los efectos de estas sustancias sobre el metabolismo celular. Como modelo biológico, se utilizaron levaduras _Saccharomyces cerevisiae_ que tienen un metabolismo celular similar al de los hongos que se hallan en el suelo, y pudieran dar idea sobre el efecto de los terpenos alelopáticos sobre este tipo de organismos.

El consumo de oxigeno es un indicador del estado metabólico celular, así como del estado de acoplamiento mitocondrial; este parámetro fue utilizado para probar la capacidad de diversos compuestos alelopáticos para alterar el metabolismo de la levadura. El consumo de oxigeno se vio inhibido por varios derivados monoterpénicos entre los que el (-)-beta-pineno y el (-)-limoneno fueron los más activos. Ambos compuestos son derivados hidrocarbonados del ciclohexano con una doble ligadura en la cadena lateral (Figura 1), y en las plantas se obtienen como productos del metabolismo del geranil-pirofosfato, del neril-pirofosfato y del linalil-pirofosfato, todos monoterpenos alifáticos (10,11). El proceso de ciclización de estos compuestos parece depender de la presencia de luz (12), y, en _Salvia officinalis_, depende de dos enzimas diferentes, las cuales

catalizan la ciclización estereoespecífica de los monoterpenos alifáticos. La geranil-pirofosfato:pineno ciclasa I (PM 96000) genera principalmente (+)-alfa-pineno, (+)-canfeno y (+)-limoneno, mientras que la geranil-pirofosfato:pineno ciclasa II (PM 55000) transforma el precursor acíclico en (-)-beta-pineno, (-)-alfa pineno y (-)-limoneno (10). Al parecer no hay interconversión de los isómeros (10).

RESUMEN DE LOS RESULTADOS

Debido a que en una exploración inicial con varios alelopáticos se encontró una mayor efectividad del beta-pineno y del limoneno con respecto a las otras substancias, la mayoría de nuestros experimentos se llevaron a cabo con estos dos compuestos. En resultados preliminares se observó una inhibición del consumo de oxígeno proporcional a la concentración del monoterpeno.

Habiendo observado la inhibición del consumo de oxígeno en la levadura, decidimos estudiar, como indicadores de la energización celular, la habilidad para captar potasio y para expulsar protones de la célula. Con estos experimentos, además, fue posible alternar sustratos para energizar selectivamente a la célula a través de la glucólisis o la cadena respiratoria mitocondrial. Tanto la captación de potasio como la expulsión de protones fueron inhibidas por el beta pineno, pero la inhibición se obtuvo a menores concentraciones cuando la energización celular dependía de la mitocondria con etanol como substrato. Por esto se decidió hacer estudios en mitocondrias aisladas.

En mitocondrias aisladas de levadura se estudió el efecto del beta-pineno en diferentes niveles de la cadena respiratoria

mitocondrial, en la actividad de ATPasa mitocondrial y en el potencial transmembranal. El consumo de oxigeno y la generación del potencial transmembranal fueron afectados por el beta-pineno, no así la actividad de ATPasa, que permaneció constante a todas las concentraciones de beta-pineno utilizadas. Estos efectos son compatibles con una interacción del beta-pineno con la membrana mitocondrial. El efecto del terpeno se pudo explicar como una inhibición de la cadena respiratoria a nivel del citocromo b, pero fue interesante observar que otros segmentos de la cadena no fueron afectados o incluso se encontraron estimulados.

Dado que en animales superiores también se ha observado toxicidad de los monoterpenos (7-9), decidimos llevar a cabo estudios en mitocondrias de hígado de rata. Se observaron efectos parecidos a los descritos en mitocondrias de levadura, es decir, pérdida del potencial transmembranal e inhibición del consumo de oxigeno. La única diferencia fue que en las de hígado se requirieron mayores concentraciones de beta-pineno para inhibir la cadena respiratoria mitocondrial. En ambos tipos de mitocondrias aumentó la permeabilidad de la membrana a la salida de potasio al agregar bajas concentraciones de beta-pineno. Al aumentar la concentración de beta-pineno, sin embargo, las mitocondrias de cada especie fueron afectadas de manera diferente, pues las de hígado de rata recuperaron la impermeabilidad de la membrana cuando el beta-pineno alcanzó una concentración de 600 nmoles/mg de proteína mitocondrial. Las mitocondrias de levadura no recuperaron la capacidad de generar un potencial transmembranal dependiente de ATPasa, a pesar de que

la actividad de esta enzima permaneció constante con todas las concentraciones de beta-pineno usadas. El beta-pineno no afectó la actividad de ATPasa mitocondrial en ninguna de las dos especies de mitocondria analizadas.

No nos es posible concluir si hubo un desacoplamiento de la mitocondria de levadura, debido a que a muy bajas concentraciones de beta pineno la cadena respiratoria ya se encontraba inhibida.

Se hicieron algunos estudios de microscopia electrónica en mitocondrias de hígado de rata en ausencia y presencia de diferentes concentraciones de beta-pineno (Figuras 2 a 4). En los controles se observaron las mitocondrias en estado condensado, con las crestas de la membrana visibles (Figura 2). En presencia de las concentraciones de beta pineno a las que se observó desacoplamiento (100 a 200 uM), las mitocondrias aparecieron hinchadas y sin crestas (Figura 3). Al llevar el beta pineno a las concentraciones en que la presencia de potencial transmembranal dependiente de ATPasa volvía a observarse (600 uM) se observó que las mitocondrias recuperaron su apariencia original, con la excepción de la presencia de unos agregados esféricos de aproximadamente 0.5 um de diámetro asociados con la membrana interna mitocondrial (Figura 4). Es probable que estas esferas estén formadas por agregados de beta pineno, y que al agregarse éste, se revierta su efecto desacoplante en la membrana.

Fig. 2. EFECTO DEL BETA-PINENO SOBRE LA ESTRUCTURA DE MITOCONDRIAS DE HIGADO DE RATA AISLADAS, I: CONTROL.

Condiciones Experimentales: Las mitocondrias de higado de rata fueron obtenidas como se describe en el articulo acompañante (Uribe, S., R. Alvarez y A. Peña, Pestic. Biochem. Physiol., (1984) 22: 43-50). El medio utilizado consistió en sacarosa 125 mM, EDTA-Tris 500 uM, pH 7.4; buffer Tris-HCl 10 mM, pH 7.4; succinato-Tris 30 mM pH 7.4; Rotenona: 1 ug/mg proteína mitocondrial; Mitocondrias, 1mg prot./ml. Las mitocondrias fueron incubadas a 30ºC durante dos minutos en un volumen final de 1 ml y luego se procedió a fijarlas con tetróxido de osmio para observarlas al microscopio electrónico. Amplificación 50000 x.

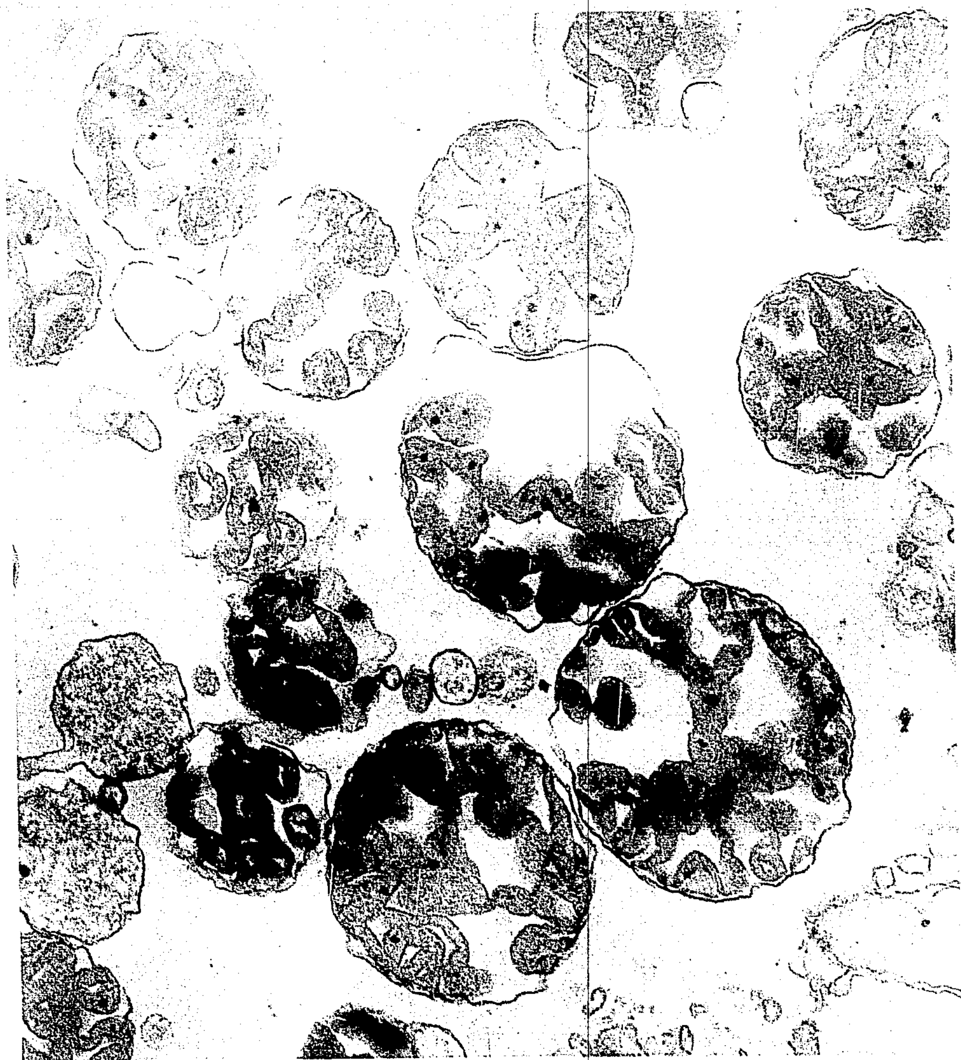


Fig. 3. EFECTO DEL BETA-PINENO SOBRE LA ESTRUCTURA DE MITOCONDRIAS DE HIGADO DE RATA AISLADAS, II: 200 uM DE BETA-PINENO.

Condiciones experimentales: Similares a las descritas en la figura 2, excepto que se agregaron 200 nmoles/mg proteína mitocondrial de beta-pineno a la mezcla de reacción. Amplificación: 31000 x.

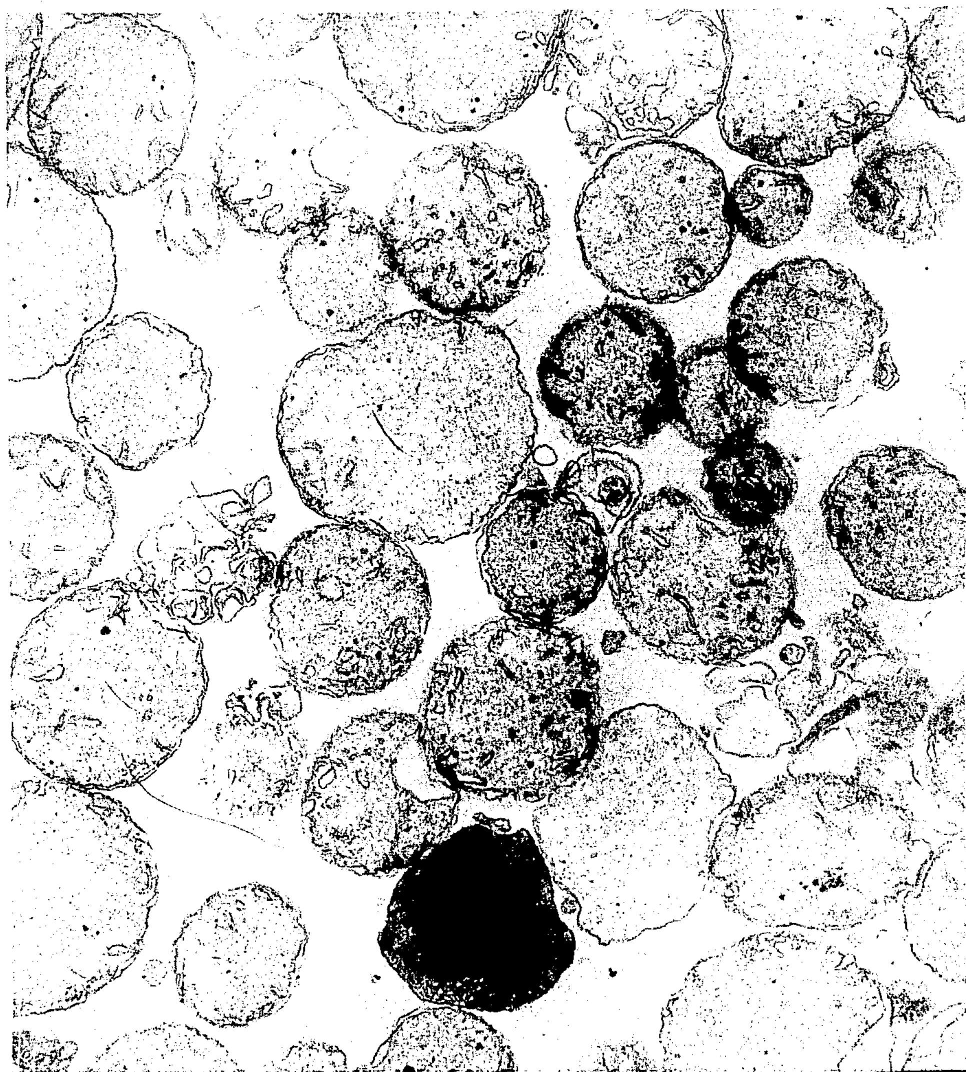
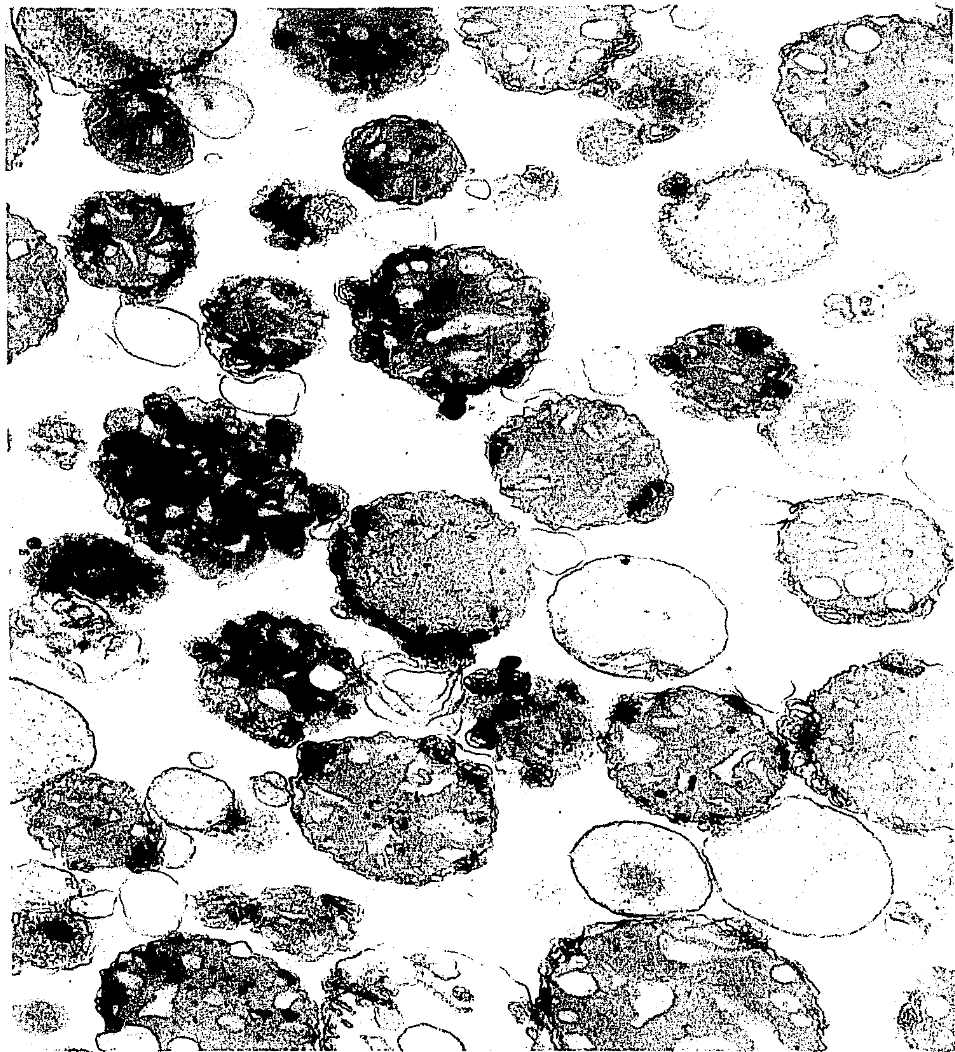


FIGURA 4: EFECTO DEL BETA-PINENO SOBRE LA ESTRUCTURA DE MITOCONDRIAS DE HIGADO DE RATA, III: 600 uM de BETA-PINENO. Condiciones experimentales similares a las descritas en la figura 2, excepto que se agregaron 600 nmoles/ mg prot. mit. de beta-pineno al medio de incubación. Amplificación de 41333 x.



La hidrofobicidad del beta-pineno y su efecto en la levadura y en la mitocondria indican que se localiza en las membranas celulares. Por ello se decidió estudiar la fluidez de la membrana con el indicador fluorescente 1,6-difenil-1,3,5-hexatrieno (DPH) en ausencia y presencia de beta-pineno. Se observó que la polarización de la fluorescencia del DPH era mucho mayor en las membranas mitocondriales que en la membrana plasmática, con un valor intermedio para las células integras. Al agregar concentraciones cada vez mayores de beta pineno, esta polarización disminuyó hasta la cuarta parte en la mitocondria y casi no se modificó en una preparación de membrana plasmática. Esto indica que la fluidez de la membrana mitocondrial era mucho menor que la de la membrana plasmática en ausencia de beta-pineno, pero aumentó marcadamente al agregarse beta pineno, quedando a un nivel similar en ambas membranas en presencia de 1 mM de beta-pineno.

DISCUSION Y CONCLUSIONES

Se observó que el beta-pineno interactúa con la levadura, modificando varios procesos metabólicos localizados en las membranas celulares. El efecto, sin embargo, mostró características diferentes en la membrana plasmática y en la membrana mitocondrial. En la membrana plasmática se requirieron concentraciones muy altas de beta pineno para inhibir los procesos de transporte y permeabilizar la membrana y la fluidez de membrana casi no se vió modificada.

En la levadura integra fue posible demostrar la inhibición del metabolismo mitocondrial _in situ_, a concentraciones de beta-pineno menores que las necesarias para inhibir los procesos dependientes de la glucólisis. Al analizar los efectos del terpeno sobre mitocondrias aisladas de levadura, se observó inhibición de la respiración con disminución del control respiratorio, así como una disminución del potencial transmembranal dependiente de la cadena respiratoria o de la ATPasa. Estos efectos fueron proporcionales a la concentración de beta-pineno.

En las mitocondrias de hígado de rata, la concentración de beta-pineno necesaria para afectar las funciones mitocondriales fue similar a la efectiva en mitocondrias de levadura; sin embargo, la secuencia con que ocurrieron los efectos al aumentar

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la concentración, fue diferente. En las mitocondrias de hígado de rata los primeros efectos observados reflejaron un desacoplamiento de la fosforilación oxidativa, y sólo al aumentar la concentración de beta-pineno se inhibió la cadena respiratoria. En las mitocondrias de levadura se observó una inhibición de la cadena respiratoria desde las concentraciones más bajas utilizadas. Se encontró también otra diferencia: En las mitocondrias de hígado de rata, y no en las de levadura, el aumento en la concentración de beta-pineno resultó en la recuperación de la impermeabilidad de la membrana y restitución del potencial transmembranal generado por la ATPasa. Bajo las mismas condiciones, en la mitocondria de levadura no pudo restablecerse el potencial transmembranal al aumentar la concentración de beta-pineno como se había observado en las mitocondrias de hígado. Por otro lado, ni en la mitocondria de hígado ni en la de levadura hubo efectos sobre la ATPasa mitocondrial.

En mitocondrias de levadura el beta-pineno aumentó la fluidez de la membrana, mientras que en la membrana plasmática los efectos fueron casi nulos. Es imposible en esta etapa determinar la razón por la que el beta-pineno pudo afectar a la membrana mitocondrial más profundamente y a menores concentraciones que a la membrana plasmática. Esto puede ser resultado de una diferente distribución del monoterpeno en ambas membranas o a una mayor susceptibilidad de la membrana mitocondrial a los efectos del beta-pineno.

En las mitocondrias de hígado de rata, la reversión del desacoplamiento parece haber sido causada por un agregamiento de las moléculas del monoterpeno, una vez que éste alcanzó una concentración critica en la membrana. En el microscopio electrónico (Figuras 2 a 4), se observó que en presencia de concentraciones desacoplantes de beta-pineno, las mitocondrias sufrieron hinchamiento y se borraron las crestas de la membrana interna. Al aumentar la concentración hasta un nivel similar al que permite el restablecimiento del potencial transmembranal dependiente de ATPasa y restitución de la impermeabilidad de la membrana al potasio (600 uM), las mitocondrias recuperaron su aspecto original, excepto que se observaron agregados esféricos asociados con la membrana interna mitocondrial; estas esferas pueden ser agregados de beta-pineno, el cual al ser secuestrado de esta manera, puede disminuir su concentración efectiva en la mitocondria. Así se explicaría la recuperación de la impermeabilidad de la membrana.

Entre las conclusiones que consideramos interesantes en esta etapa, está el que los efectos del beta-pineno sean diversos, dependiendo de las membranas utilizadas. Se encontraron diferencias claras en las alteraciones del funcionamiento y la conformación de las membrana plasmática de levadura, o en la mitocondrial, de levadura o de hígado.

También es importante mencionar que aún en la membrana en la que se llevaron a cabo más estudios, que fue la de mitocondria de

levadura, hay enzimas que se inhiben con menores concentraciones que otras, algunas cuya función no fue alterada y, finalmente, una que se estimuló por la presencia del monoterpeno.

Toda esta fenomenología abre posibilidades en cuanto a los mecanismos, ya sean resultado de la interacción más o menos específica del terpeno con las enzimas, o de los componentes lipídicos de la membrana, que, al alterarse por la presencia del pineno, pueden modificar de diferente forma a las enzimas membranales.

PERSPECTIVAS

En el futuro, esperamos continuar nuestros estudios sobre la toxicidad de los monoterpenos en diferentes sistemas biológicos. Hay datos muy interesantes en la literatura que indican que estas substancias generan un aumento en la concentración del citocromo P-450 en hepatocitos de rata; éstos tienen una capacidad más grande que los controles para metabolizar monoterpenos. Por otro lado, si se induce el sistema de oxidasas inespecificas con beta-naftoflavona, fenobarbital o 3-metil colantreno, el aumento en citocromo P-450 que resulta, no se traduce en un aumento en la capacidad de la célula para detoxificar monoterpenos (7). Esto sugiere que el sistema de oxidasas generado por una u otra sustancia parece tener una cierta especificidad. Nos parece importante estudiar este sistema porque las oxidasas inespecificas de la célula participan en múltiples procesos de detoxificación que en ocasiones generan sustancias con propiedades tóxicas. El benceno, por ejemplo, es metabolizado para formar mutágenos y cancerígenos. Es posible que algunos monoterpenos modifiquen a las oxidasas específicas para regular su interacción con dichas substancias mutágenas. Para explorar esta posibilidad es necesario empezar por definir el mecanismo por el cual cada sustancia genera un aumento de oxidasas inespecíficas y determinar su toxicidad en la célula.

En relación con los estudios de toxicidad que se pudieran llevar a cabo, está el caso de varios monoterpenos que contienen oxigeno, como por ejemplo, el pulegón (3), que inhibe el crecimiento celular con mayor efectividad que el beta pineno o el limoneno, pero no mostró efectos sobre el consumo de oxigeno ni la integridad de la membrana cuando fue agregado a las levaduras (no publicado). Sería interesante determinar el mecanismo de toxicidad de estos compuestos.

La mezcla de derivados alelopáticos entre si o con sales y otros compuestos orgánicos resulta en una potenciación de los efectos tóxicos de cada compuesto para ciertos organismos (13), y es así como se encuentran estos compuestos en la naturaleza, sea en las resinas de los árboles o en las suspensiones coloidales en que se encuentran en el suelo. En la industria se están estudiando diferentes mezclas de alelopáticos como posibles preservadores de alimentos (13). Al conocer el mecanismo de acción de cada una de estas sustancias, es posible que pueda comprenderse mejor el mecanismo por el cual la presencia de sales y la combinación de dos o más alelopáticos lleva a la potenciación de los efectos tóxicos.

Hay varios grupos interesados en el estudio de estas substancias como herbicidas y desparasitantes (1), ya que su toxicidad en muchos casos parece ser selectiva para alguna especie (1). Otro tema interesante en el estudio de los monoterpenos es el que los enfoca como precursores de feromonas

en los insectos, cuya liberación es controlada neuralmente, (7) con especificad que depende del sexo y la especie que se observen (14). Esto es especialmente interesante para la agricultura, donde estas sustancias son utilizadas para atrapar insectos parásitos de cultivos (14).

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Effects of β-Pinene, a Nonsubstituted Monoterpene, on Rat Liver Mitochondria

S. URIBE, R. ALVAREZ, AND A. PEÑA

Departamento de Microbiología, Centro de Investigaciones en Fisiología Celular, and Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado postal 70-600, 04510 México, D.F., México

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β-Pinene uncouples oxidative phosphorylation and inhibits respiration in isolated rat liver mitochondria. The uncoupling effects are observed at lower concentrations (100 to 200 μ*M*) than the inhibition of respiration (400 μ*M*). At low concentrations, the effects observed could be explained by an increase of the passive permeability of the mitochondrial membrane produced by the terpene. Higher concentrations seemed to inhibit respiration through an effect on the electron transport chain. At the highest concentrations tested (600 to 1200 μ*M*), β-pinene seemed to produce a partial resealing of the mitochondrial membrane. All effects can be explained by the interaction of β-pinene with the mitochondrial membrane. Other hydrophobic molecules tested do not show the effects of β-pinene or limonene on mitochondria.

INTRODUCTION

Monoterpenes are widely found in nature; they are expelled into the environment by many higher plants (For a review see (1)). Reports show that they are toxic to organisms widely distributed in the phylogenetic scale, including gram-positive and gram-negative bacteria, yeast (2), plants (3–5), insects (2, 6–8), mammals (8, 9), and even man (10). All these studies seem to show a general pattern of toxicity consisting of an alteration of many membrane-related processes, such as oxygen consumption and ion permeability. Sometimes, the membrane is damaged to such an extent that it releases cytoplasmic macromolecules into the environment (2).

Repeated intraperitoneal injection of α-pinene results in an increased level of cytochrome *P*-450 in rat liver microsomes and a consequent increase in the detoxification rate of α-pinene by the unspecific microsomal oxidase system (8).

Monoterpenes dissolve gallstones; therefore, low doses have been administered orally to cholelithiasic patients without noticeable side effects (11).

With this information, a study of the toxicity of the monoterpenes on isolated mammalian systems seemed justified. Here we report the results of studies on the effects of β-pinene, a nonsubstituted monoterpene of medium toxicity (1, 4), on isolated rat liver mitochondria. Rat liver mitochondria were used because this is a very well-defined system; most of their metabolic processes are tightly linked to membranes, and there is evidence of monoterpene toxicity to mitochondria from other sources (1–6).

MATERIALS AND METHODS

Mitochondria. Male or female Wistar rats, weighing between 150 and 200 g, were obtained from our local colony and sacrificed by cervical luxation and decapitation (12). The liver was extracted, washed, and minced in the preparation medium (0.25 *M* Sucrose, 1 m*M* EDTA, adjusted with Tris, pH 7.4), and subsequently homogenized in 150 ml of the same medium in a glass–Teflon homogenizer. Mitochondria were isolated from the homogenate by differential centrifugation as described elsewhere (12); the above operations were carried out

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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.

matrix to be used, but these characteristics are shared also by ATP and succinate, which do not show this protective property. On the other hand, we have obtained a very similar pattern of effects with ethidium bromide (21), a cationic fluorescent dye. We have explained this by relating this curious phenomenon to a possible inhibition of the NADH-ubiquinone segment of the respiratory chain by the dye (21).

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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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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.

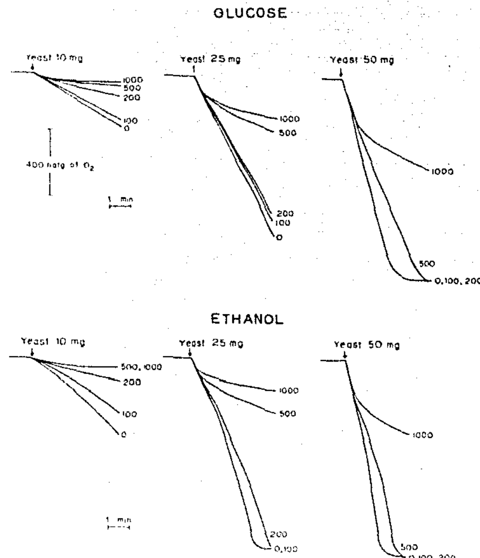

FIG. 1. Effects of β-pinene on the respiration of variable amounts of intact yeast cells with ethanol or glucose as the substrate. The incubation medium was 20 mM morpholinoethanesulfonic acid adjusted to pH 6.0 with triethanolamine; 25 mM glucose or 53 mM ethanol was used as the substrate. Yeast cells 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).

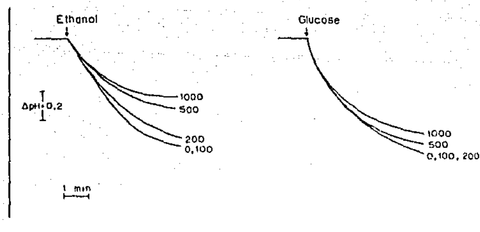

FIG. 2. Effects of variable concentrations of β-pinene on the ability of yeast cells to pump protons into the medium. The incubation medium was the same as described in the legend to Fig. 1, but buffer was omitted; the initial pH was 6.4. Final volume was 5.0 ml, and 42 mg of yeast cells (wet weight)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

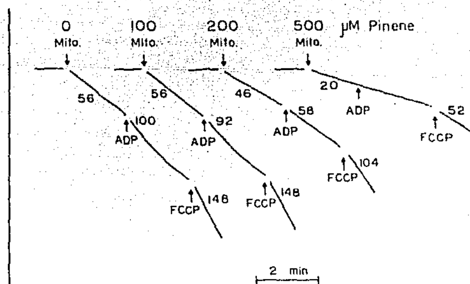

FIG. 4. Effects of β-pinene on the respiration of yeast mitochondria. The incubation mixture contained 0.6 M mannitol, 10 mM NaH_2PO_4 (pH 6.5), and 1.0% defatted bovine serum albumin. Also used was 15 mM succinate, with pH adjusted to 6.5 with NaOH, and 1 mg of mitochondria protein per ml. Pinene was 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.

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 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-

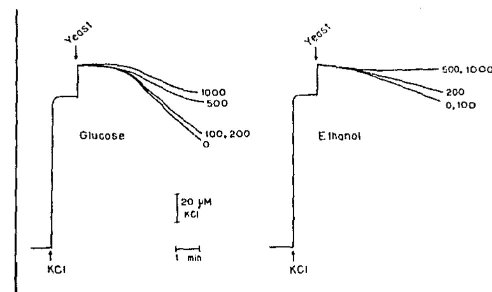

FIG. 3. Effects of various concentrations of β-pinene on K^+ transport by yeast cells with glucose or ethanol as the substrate. Experimental conditions were as described in the legend to Fig. 1, but 100 μM KCl was included in the incubation medium, and the final volume was 8.0 ml. Also, 67 mg of yeast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

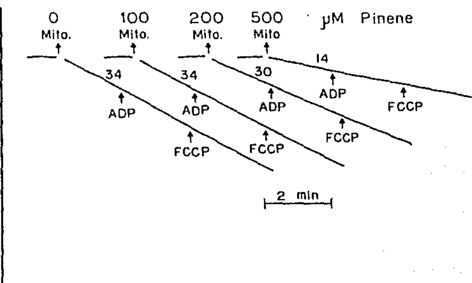

FIG. 5. Effects of β-pinene on the respiration of isolated yeast mitochondria incubated in a hypotonic medium. The incubation conditions were similar to those described in the legend to Fig. 4, but only 100 mM mannitol, 2 mM sodium phosphate buffer, and 0.25% defatted albumin were used. Figures beside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

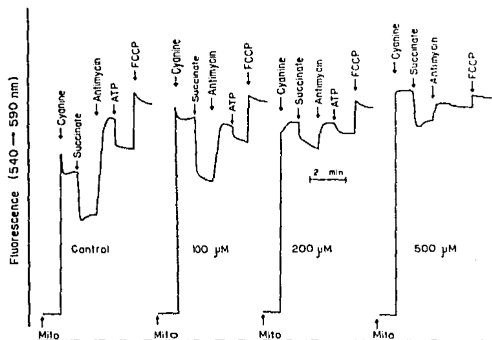

FIG. 6. Effects of β-pinene on the membrane potential of yeast mitochondria. The potential was measured as described in the text. Incubation mixture was the same as described in the legend to Fig. 4, with 15 mM succinate or 2 mM ATP-Na (pH 6.5). Mitochondria were added at 1.0 mg/ml. Final volume was 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.

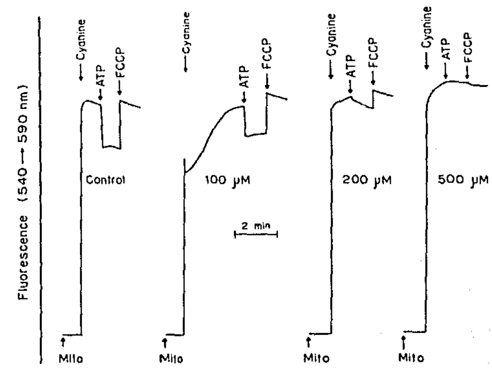

FIG. 7. Effects of β-pinene on membrane potential of yeast mitochondria with ATP in the presence of antimycin A. The experiment was carried out as described in the legend to Fig. 5, but with 5 mM ATP-Na adjusted to pH 6.5 as the substrate. A concentration of 50 nM antimycin A was present in the medium.

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.

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).

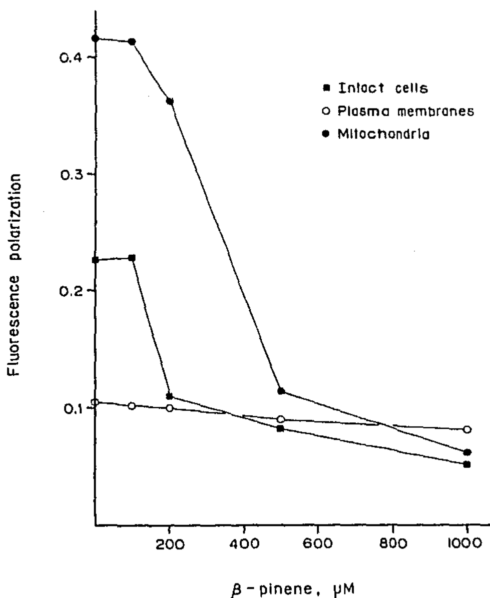

FIG. 8. Effects of β-pinene on the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in intact yeast cells, yeast plasma membranes, and yeast mitochondria. Incubation medium for yeast cells and plasma membranes was 20 mM morpholinoethanesulfonic acid-triethanolamine (pH 6.5). A sample of 1 uM 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.

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.

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