00562

POSIBLES ANALOGOS DE CATIONES MONOVALENTES.
ESTUDIOS EN LEVADURAS Y MITOCONDRIAS.

Tesis que presenta la Medico Cirujano Ma. Nancy Carrasco Queijeiro para obtener el grado de Maestria en Ciencias Quimicas (especialidad Bioquimica) en la Division de Estudios de Posgrado de la Facultad de Quimica de la Universidad Nacional Autonoma de Mexico.

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POSIBLES ANALOGOS DE CATIONES MONOVALENTES. ESTUDIOS EN LEVADURAS Y MITOCONDRIAS.

La Bioquimica es una ciencia relativamente joven, cuyo nacimiento podria ubicarse quiza en el ano de 1897 cuando los hermanos Hans y Eduard Buchner prepararon extractos de levaduras con fines medicos y les adicionaron cantidades elevadas de jugo de cana de azucar para protegerlos de infecciones microbianas, descubriendo que el azucar era fermentado rapidamente por el extracto de levaduras. Se inicio asi el estudio de la fermentacion alcoholica en tubo de ensayo en ausencia de celulas, obteniendose la informacion sobre los pasos intermediarios en la fermentacion de la glucosa hasta bioxido de carbono y etanol. Ante este hecho estamos autorizados a decir que la asociacion entre la Bioquimica y las levaduras tiene tantos anos como la Bioquimica misma.

Contemplando en retrospectiva la evolucion de la ciencia que nos ocupa, no podemos experimentar sino sorpresa al descubrir cuanto de lo que sabemos deriva de experimentos realizados en microorganismos tales como levaduras y bacterias, asi como al

verificar en que medida esos conocimientos han encontrado eco y aplicacion en el estudio de celulas humanas.

Uno de los problemas iniciales de toda investigacion biologica consiste en seleccionar una preparacion optima para el estudio en el laboratorio, tanto desde el punto de vista de su manejo como de las caracteristicas intrinsecas de la misma. En lo que a trabajos bioquimicos se refiere, y en particular a experimentos de transporte de iones, las levaduras reunen una serie de ventajas de gran valor:

a. ofrecen una disponibilidad abundante y facil a partir de sus importantes usos industriales en la elaboracion de cerveza, pan, vino, etc.

b. se trata de organismos eucariotes que, sin embargo, pueden manipularse con la misma facilidad que las bacterias, por lo que constituyen un modelo muy atractivo para proyectar a formas superiores el estudio de fenomenos metabolicos, de genetica molecular y de regulacion.

c. a diferencia de otros hongos, las levaduras existen como organismos unicelulares, estado en el que poseen una

tasa metabolica elevada y mayor relacion area/volumen que los hongos micelares.

d. son apropiadas para estudios geneticos debido a su susceptibilidad para la induccion y represion con sustratos adecuados o sus analogos, asi como a su ciclo sexual (de hecho, el efecto de la glucosa como inductor enzimatico fue observado por primera vez en levaduras).

Cuando se trabaja con levaduras es conveniente tener presentes las diferencias y semejanzas que guardan con las bacterias y con otros microorganismos relacionados, tales como hongos, algas, protozoarios, etc. Por ejemplo, las levaduras se parecen a las bacterias en lo que respecta a la funcion de mantener la estabilidad geoquimica de la biosfera, a los metodos que se emplean para aislarlas y cultivarlas, a su capacidad para producir enfermedades infecciosas y a las numerosas aplicaciones industriales de sus fermentaciones; en cambio, difieren de ellas en sus procesos reproductivos, sus caracteristicas de crecimiento, la complejidad anatomica de la arquitectura citoplasmica, tamano celular, actividad metabolica, composicion y ultraestructura de la pared celular, en los agentes quimioterapeuticos a los que son sensibles y el tipo de

enfermedades que producen.

Las levaduras comprenden un numero relativamente pequeno de especies, aproximadamente 350, que corresponden a 39 generos, comparadas con las decenas de miles de especies de algas, bacterias, otros hongos y protozoarios.

El diagrama de la siguiente pagina muestra la ultraestructura de una celula de levadura donde se aprecian, con el detalle que proporciona el microscopio electronico, los elementos senalados al pie de la figura.

En el presente trabajo, que se refiere al sistema de transporte de cationes monovalentes en levaduras, resulta de particular importancia hacer notar algunas propiedades de la pared celular y, naturalmente, de la membrana celular. La pared celular de las levaduras es una estructura rigida, responsable de la forma de las celulas y de su resistencia a los medios hipotonicos (Ref. D:1). Ademas, es una estructura con permeabilidad selectiva; por ejemplo, en Sacharomyces cerevisiae no atraviesan la pared aquellas moleculas de dextrana con peso molecular mayor de 4500 (Ref. D:2). Por otro lado, las levaduras

no son capaces de utilizar moleculas de proteinas presentes en el medio de cultivo (Ref. D:3). En general, la pared celular solamente permite el paso de micromoleculas a los sitios de absorcion en las membranas.

La membrana celular o plasmalema de las levaduras consiste en una estructura trilamelar con un espesor aproximado de 8 nm que muestra invaginaciones numerosas y muy caracteristicas. El analisis de fragmentos aislados de plasmalema ha revelado la presencia de fosfolipidos, proteinas, manosa, y de una ATPasa dependiente de Mg^{++} (Ref. D:4). Se ha demostrado firmemente el papel que la membrana celular desempena en las propiedades osmoticas de las levaduras, debido a su impermeabilidad para ciertos solutos y tambien en los fenomenos de movimiento de diversos compuestos del medio al interior de la celula y viceversa (Ref. D:5).

Algunos de los mecanismos involucrados en el transporte mediado por la membrana ya han sido estudiados con cierto detalle. En terminos generales se ha aceptado que la superficie de la membrana esta ocupada por sitios de union especificos para ciertos sustratos o para grupos de compuestos relacionados estructuralmente. Estos sitios de union pertenecen a sistemas de

transporte que por medio de acarreadores conducen a las moleculas de una a otra superficie del plasmalema. La actividad de dichos acarreadores, transportasas o permeasas, como tambien se les ha denominado, reune atributos caracteristicos de enzimas; asi, exhiben cinetica de saturacion, especificidad de sustrato y estereoespecificidad; la velocidad de transporte depende de la temperatura; los sustratos que utilizan sitios de union en comun compiten entre si de acuerdo a sus respectivas afinidades. Se han reportado modalidades de transporte, tanto activo (con requerimiento energetico) como pasivo (Ref. D:6, D:7).

En nuestro trabajo se ha estudiado con diversos enfoques el sistema de transporte de cationes monovalentes, en levaduras de las especies Sacharomyces cerevisiae y Kluyveromyces lactis con miras a establecer el mecanismo molecular del mismo. En los primeros experimentos se utilizaron como herramientas de estudio los siguientes colorantes cationicos: bromuro de etidio (BE), safranina (SAF), azul de nilo, azul de metileno (AM), violeta de metilo, azul brillante de cresilo, rojo neutro, acriflavina (ACR), verde brillante y verde de metilo, todos ellos sustancias faciles de detectar.

La hipotesis de que los colorantes cationicos interactuan

con el sistema de transporte para cationes en levaduras, se genero a partir de la observacion de los efectos que dichos colorantes ejercen sobre diversas estructuras intracelulares en diferentes tipos de celulas, y de los cambios ocurridos en esos efectos cuando se prueban en presencia de distintos cationes (Ref. A:2,4,6, 8,10,11,13,18,25-28). Para que tengan lugar los efectos de los colorantes sobre moleculas o estructuras intracelulares, aquellos deben establecer contacto primero con la superficie celular para despues ser translocados al interior de la celula.

Los colorantes afectan la movilidad del K+, tanto si este se agrega o no al medio, sin modificar en absoluto el transporte de H+. Cuando hay K+ en el medio los colorantes bloquean su captacion, y cuando no lo hay, favorecen su salida. Con estos antecedentes se inicio un estudio para definir con mayor precision el mecanismo de los efectos mencionados, y la posibilidad de una interaccion de los colorantes cationicos con el sistema mismo del transporte de los cationes monovalentes en la levadura.

Con el objeto de descartar la posibilidad de que los fenomenos mencionados pudieran deberse a la destruccion celular

inducida por los colorantes (fenomeno que puede ocurrir en ciertas condiciones segun Elferink y Booij, ref. A:7), se realizaron experimentos para comprobar la integridad de las celulas: por un lado se incubaron levaduras en presencia de colorantes, se centrifugaron y se midio la absorcion del sobrenadante a 260 nm (longitud de onda a la que absorben los nucleotidos), y por otro lado se midio la absorcion, en las mismas condiciones, del sobrenadante proveniente de celulas de levadura previamente destruidas con acido perclorico. Al comparar las lecturas se encontro que la absorcion de luz del sobrenadante de las celulas incubadas con los colorantes es un decimo o menos de la obtenido con celulas destruidas, resultado que indica claramente que el grado de lisis celular inducida por los colorantes no es significativo ni determinante en los fenomenos registrados. Esta conclusion se vio aun mas apoyada al observar que las celulas cargadas con Ca++ (en presencia de glucosa), exhiben una salida significativa de ese ion y una ulterior recaptacion del mismo, tanto en presencia como en ausencia de colorantes. Si los colorantes provocaran una destruccion celular importante, no seria posible la acumulacion observada de Ca++. Ademas de ello, pudo comprobarse que aun en presencia de los colorantes, las levaduras conservan su capacidad para bombear H+, proceso caracteristico de celulas integras.

Una vez establecido que los colorantes bloquean la captacion de K+, se procedio a medir las velocidades iniciales de captacion de 86Rb+ (isotopo radiactivo con caracteristicas fisicoquimicas muy similares a las del K+) y la inhibicion de esa captacion provocada por los diferentes colorantes, para analizar los efectos sobre las constantes cineticas y tener la posibilidad de mayor informacion. En efecto, los colorantes inhibieron la captacion del Rb+ hasta en un 80% y la determinacion de los parametros cineticos revelo una mayor modificacion de la Km que de la Vmax, indicando que la inhibicion tiende a ser de tipo competitivo; esta tendencia se intensifica a medida que se adicionan concentraciones bajas de Ca++, que por si mismas no ejercen ningun efecto sobre la captacion del Rb+.

Sabiendo que los colorantes inhiben el transporte de K+, se puede suponer que actuan en sitios comunes; con este razonamiento se considero de interes investigar si ocurria el fenomeno inverso, es decir, si el K+ era capaz de interferir con la captacion de los colorantes y mas aun si compartia las mismas propiedades en su transporte. Al estudiar el comportamiento del proceso de captacion de los colorantes por las levaduras, fue posible clasificarlos en tres grupos, de acuerdo con los requerimientos de glucosa para que ocurra la captacion y con la inhibicion de la misma causada por K+ y por Na+. Asi, al primer

grupo pertenece solamente el BE, cuyo requerimiento de glucosa es muy significativo y la inhibicion de la captacion resulta claramente diferenciada entre K+ y Na+, pues es mucho mayor la lograda con el primer cation (K+) que con el ultimo (Na+). Los colorantes del segundo grupo, SAF, ACR, y AM muestran una dependencia parcial de la glucosa y la inhibicion con K+ es menor y mucho menos selectiva cuando se la compara con la lograda por el Na+. El tercer grupo incluye colorantes cuya captacion es independiente de la glucosa, apenas es inhibida por el K+ y practicamente no hay diferencia con la ejercida por el Na+.

Por otro lado, se realizaron experimentos en el espectrofotometro de longitud de onda dual, en busca de mas evidencia en favor de que las levaduras captan los colorantes por medio del mismo sistema de transporte que el K+. Teniendo presente que el espectro de absorcion de la SAF cambia cuando se incorpora a la matriz en virtud del apilamiento de sus moleculas, puede usarse este cambio como indice de la captacion del colorante; por su parte, el AM cambia su espectro al reducirse, y dado que la reduccion ocurre dentro de la celula, el cambio de espectro representa una medida de su captacion; finalmente, la captacion de la ACR pude seguirse tambien por su cambio de fluorescencia puesto que este ocurre cuando se le coloca en un solvente organico o de baja constante dielectrica (por ejemplo,

cuando interactua con la membrana de la celula).

El resultado de estas observaciones puede resumirse diciendo que el cambio de color requiere de la presencia de un sustrato, y que es inhibido por el K+ pero no por el Na+. El mismo fenomeno se registra con los cambios de fluorescencia de la ACR.

Los datos obtenidos sugieren que los colorantes son efectivamente incorporados al interior de las levaduras y no solo adsorbidos a su superficie puesto que la cantidad de colorante tomado es alta, la respiracion celular se inhibe en mas de un 80% y, en el caso del AM el hecho de que se verifique su reduccion ya presupone que ha entrado a la celula.

Los resultados de este trabajo establecen la posibilidad de que moleculas organicas cationicas puedan ser translocadas por acarreadores naturales. Las vias y modalidades de dicha translocacion para los distintos colorantes pueden ser visualizadas en funcion del comportamiento que permitio clasificarlas en tres grupos. Asi, el BE, que pertenece por si mismo a un grupo separado por exhibir la maxima dependencia de la glucosa y la mas alta inhibicion de la captacion de K+ y Rb+,

misma que es de tipo competitivo puro, representa el unico colorante que las celulas captan casi exclusivamente por el sistema de incorporacion de K+. Los colorantes de los dos grupos restantes, que muestran todas las caracteristicas mencionadas solo parcialmente y de un modo menos definido, son quiza captados por las levaduras a traves de algun o algunos otros sistemas ademas del de translocacion de K+.

Los colorantes cationicos son moleculas organicas de manejo sencillo, faciles de detectar y cuantificar, accesibles a determinaciones precisas por metodos fotometricos, sin tener que recurrir a isotopos radiactivos. Nuestro trabajo pone en evidencia el hecho de que tales colorantes cationicos son capaces de actuar como analogos de los cationes inorganicos, al menos en lo que respecta a su captacion en las levaduras. Con base en lo anterior, cabe suponer que, por un lado, otros organismos eucariotes responderian ante los colorantes de un modo parecido, y, por otro, que otros sistemas celulares metabolicos, enzimaticos, geneticos, etc. podrian tambien interactuar con ellos como lo hacen con los cationes inorganicos. Ademas, nuestras observaciones hacen pensar que la carga juega un papel predominante en fenomenos de reconocimiento molecular, dato digno de tomarse en consideracion para dilucidar mecanisticamente la entrada de farmacos y drogas de naturaleza organica tanto a

celulas humanas como a celulas de microorganismos patogenos. Es interesante senalar que nos referimos al papel de la carga en el reconocimiento e incorporacion de sustancias por parte de acarreadores activos, y no solo a su bien conocida caracteristica como factor de permeabilidad a traves de la matriz lipidica.

De los colorantes estudiados el BE pertenece a un grupo separado, ya que posee caracteristicas muy especiales, puesto que es el que parece interactuar en mayor medida con el sistema de transporte de K+. Se sabe desde hace tiempo que tiene propiedades mutagenicas selectivas para el DNA de las mitocondrias (Ref. B:1-4, C:1-9), y que estos organelos son capaces de concentrar el colorante, segun lo revelan estudios de microscopia electronica (Ref. B:11). Con estos antecedentes, se estudiaron las interacciones posibles del BE con la membrana plasmatica y con las mitocondrias de celulas de levadura bajo diferentes condiciones, poniendo especial atencion en el estado de energizacion de la celula. Con este proposito se utilizaron diversos sustratos, grados de oxigenacion, agentes desacoplantes e inhibidores respiratorios.

Los sustratos empleados fueron glucosa o etanol-H2O2, que proporcionan la energia necesaria para cada uno de los tres

fenomenos de membrana estudiados, a saber: captacion de BE, captacion de K+ y salida de K+ inducida por el colorante.

El desacoplante FCCP es capaz de reducir la capatacion de BE y de K+ y la salida de K+ cuando el sustrato empleado es respiratorio (como el etanol), pero no cuando se utiliza glucosa, a menos que se manejen concentraciones mucho mayores de FCCP. Estas observaciones indican que los tres fenomenos se comportan de igual manera en lo que a requerimientos energeticos se refiere.

La interaccion del BE con la levadura, se analizo mas detalladamente mediante el estudio de sus cambios de fluorescencia, teniendo en cuenta que la energizacion de las celulas de levadura es capaz de producir un aumento de la fluorescencia del BE, fenomeno que puede considerarse correspondiente a la captacion del mismo. Con este sistema de monitoreo se obtuvieron trazos que muestran un incremento inicial de fluorescencia, (es decir, de captacion de BE) que despues se estaciona en una meseta, para mas tarde volver a aumentar rapidamente; este ultimo aumento corresponde a una desenergizacion de las mitocondrias causada a su vez por el estado de anaerobiosis alcanzado al agotarse el oxigeno, o bien

por la accion de agentes desacoplantes como el FCCP. Sin embargo, el efecto del FCCP se pierde si se anade a celulas en anaerobiosis.

En suma, los resultados de los cambios de fluorescencia indican que los desacoplantes bloquean la interaccion del BE con las mitocondrias, como lo confirma el hecho de que el FCCP revierte la inhibicion de la respiracion causada por el BE. Es conveniente hacer notar que dicha inhibicion resulta significativa solo cuando tanto la celula como sus mitocondrias se encuentran energizadas; si las mitocondrias no lo estan, no se aprecia la inhibicion.

Cuando se usa etanol como sustrato, la desenergizacion completa de la celula se logra por medio de un desacoplante a bajas concentraciones; ademas, y esto es de mucho interes, se puede alcanzar la desenergizacion selectiva de las mitocondrias utilizando tambien bajas concentraciones de desacoplante, siempre y cuando el sustrato no sea respiratorio, como es el caso de la glucosa.

En lo que se refiere a la interaccion del BE con

mitocondrias aisladas, los experimentos de fluorescencia brindan informacion util. La fluorescencia del colorante aumenta cuando este interacciona con mitocondrias energizadas, lo que se explica por el cambio de polaridad del ambiente en torno a las moleculas de BE unidas a la membrana mitocondrial, en virtud de un proceso dependiente de energia.

Por otro lado, no se dispone de explicacion alguna para la marcada preferencia del BE por el DNA mitocondrial, aunque se sabe que el colorante se intercala entre sus moleculas. Siendo el BE una molecula cationica, el potencial electroquimico de la mitocondria podria actuar como fuerza motriz para su captacion, acumulacion y efectos selectivos en ese organelo. Es posible comparar este fenomeno con la acumulacion de cationes inorganicos en las mitocondrias, donde podemos suponer que intervienen tanto el potencial electroquimico como la operacion de sistemas de transporte mas o menos especificos. Si ninguno de esos factores existiera en otros compartimentos celulares, se explicaria la preferencia del BE por las mitocondrias; y aunque esto aun no aclara el por que de sus propiedaes mutagenicas selectivas para el DNA circular superenrollado de las mitocondrias, si ilustra cual puede ser el mecanismo en cuya virtud la exposicion del mutageno al DNA mitocondrial es alta y prolongada.

En vista de sus efectos mutagenicos, el BE presente en el medio de cultivo es capaz de inhibir el crecimiento de diversas especies de levaduras. Sin embargo, ha sido posible desarrollar cepas de Kluyveromyces lactis que resisten altas concentraciones de BE y continuan creciendo (Ref. C:10), brindando asi un modelo muy interesante para estudiar el mecanismo probable de las interacciones del mutageno con las celulas. Ademas, a la luz de las observaciones realizadas en el sentido de que la captacion del BE guarda relacion con el transporte de K+, las cepas resistentes al colorante constituyen una preparacion excelente para investigar el comportamiento de los movimientos de cationes monovalentes y la posible coincidencia de una sola lesion que afecte a los dos sistemas de transporte.

Se trabajo con tres cepas de Kluyveromyces lactis, a saber: las dos cepas progenitoras, denominadas S1 y S2 (sensibles al BE) y la cepa resistente derivada de aquellas, R.

Las cepas sensibles S1 y S2 captaron el BE mientras que la cepa R resulto incapaz de hacerlo, fenomeno observado tanto al comparar las absorbencias respectivas como al estudiar los cambios de fluorescencia.

Por lo que respecta a la captacion de K+ se encontro que la cepa R la tenia disminuida en comparacion con las cepas ordinarias (S1 y S2). Sin embargo, es interesante hacer notar que el contenido intracelular de K+ y otros cationes no es diferente en la mutante comparado con las cepas progenitoras. Al medir la captacion de 86Rb+ en las tres cepas, pudo comprobarse que dicho proceso es significativamente mayor en las cepas sensibles al BE que en la mutante. Tambien se midio la salida de K+ inducida por el BE, encontrandose mas alta en las cepas S1 y S2 que en la R.

En apoyo a los hallazgos anteriores, se registro que el efecto estimulante de la respiracion que poseen tanto el BE como el K+ estuvo practicamente ausente en la cepa R. Ademas, los paramentros cineticos Km y Vmax mostraron grandes diferencias entre las cepas sensibles y la resistente, en el sentido de que esta ultima exhibe una alteracion importante de su sistema para transportar cationes monovalentes, aunque no una supresion completa. Esto ultimo ayuda a explicar que el contenido de iones en las celulas sea igual en todas las cepas.

Los colorantes cationicos, siendo moleculas organicas complejas, parecen ser incorporados a las celulas de levadura a traves del mismo sistema de captacion del K+. Este hallazgo

central, idea generadora de la presente tesis, esta muy lejos de significar solamente una mera sorpresa fisicoquimica mas o menos inesperada. Representa, en realidad, un pequeno eslabon en la cadena de datos que integran nuestra idea molecular y dinamica del comportamiento de las membranas biologicas, minusculas barreras que separan a la materia viva del medio circundante.

BIBLIOGRAFIA.

Las referencias precedidas por las letras A, B y C corresponden respectivamente a los siguientes trabajos:

i. Pena, A., Mora, M. A. and Carrasco, N. (1978). Uptake and Effects of Several Cationic Dyes on Yeast. J. Membrane Biol. 47, 261-284.

ii. Pena, A., Clemente, S. M., Borbolla, M., Carrasco, N. and Uribe, S. (1980). Multiple Interactions of Ethidium Bromide with Yeast Cells. Arch. Biochem. Biophys. 201, 420-428.

iii. Brunner, A., Carrasco, N. and Pena, A. (1981). Correlation between Resistance to Ethidium Bromide and Changes in Monovalent Cation Uptake in Yeast. (Enviado a publicacion).

Las referencias precedidas por la letra D corresponden a la

siguiente lista:

1. Nickerson, W. J. (1963). Bact. Rev. 27, 305-324.

2. Gerhardt, P. and Judge, J. A. (1964). J. Bact. 87, 945-951.

3. Matile, P. (1968). Proc. 2nd. Symposium on Yeasts, Bratislava.

4. Matile, P., Moor, H. and Muhlethaler. K. (1967). Arch. Mikrobiol. 58, 201-211.

5. Suomalainen, H. (1968). In "Aspects of Yeast Metabolism" (A. K. Mills, ed.), pp. 1-29. Blackwell Scientific Publications, Oxford and Edinburgh.

6. Cirillo, V. P. (1961). A. Rev. Microbiol. 15, 197-218.

7. Rothstein, A. (1961). In "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.), pp. 270-284. Academic Press, New York.

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Uptake and Effects of Several Cationic Dyes on Yeast

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Summary. Several cationic dyes were found to behave as inhibitors of K^+ uptake in yeast. When added at high concentrations or in a K^+-free medium, dyes can also produce an efflux of K^+. The dyes are taken up by the cells in a process that, in different degrees, for several cations requires glucose and is inhibited to a higher degree by K^+ than by Na^+.

The inhibition of cation uptake is of the competitive type with EB and close to this type with other dyes. Ca^{2+} inhibits the uptake and effects of dyes and in some cases also seems to change the inhibition kinetics on Rb^+ uptake closer to a pure competitive type.

According to preliminary experiments, the efflux of K^+ seems to be of the electrogenic type, and not due to the disruption of the cells. The data indicate that, independently of the existence of other types of interaction (which do exist), dyes seem to interact with the system for monovalent cation uptake of yeast in different degrees of specificity and energy requirement. This interaction can be followed by fluorescence or metachromatic changes or reduction of the dyes as observed in the dual wavelength spectrophotometer and can be inhibited specifically by K^+, but not by Na^+.

Cationic dyes have shown several kinds of effects on living cells of all types. Special attention has been focussed on studies with cationic dyes (the so-called basic dyes) for which, among others, actions have been described on intracellular enzymes [8, 10], on oxidative phosphorylation mechanisms [11, 13, 18, 28], and on the genetic machinery of the cell [25–27] as a consequence of the interaction of these molecules with different molecules or structures within the cell. In yeast cells in particular, studies on the effects of dyes and other cationic molecules have revealed this same kind of interactions [2, 6, 18]. Armstrong [2] had already pointed out that dyes, in order to interact with other components of the cell, had to make contact first with the surface and possibly required to be transported into the yeast cell. With ethidium bromide, it seems to be clear that a mechanism exists for its translocation into

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the cell [20], apparently through the natural transport system for monovalent cations. Chaix and Roncoli [4] reported that K^+, but not Na^+, could decrease the rate of methylene blue reduction by yeast; this could be due to a lower rate of uptake of the dye by the cell in the presence of K^+, but not of Na^+. These antecedents generated the hypothesis that many cationic dyes or molecules might show an interaction with the system for monovalent cation transport in yeast, in spite of the fact that recent data by Elferink and Booij indicated that some triphenylmethane dyes seem to act by inducing the destruction of the cell membrane in yeast [7]. The work presented here consisted in studying the relationship of several cationic dyes to K^+ transport, as well as the effects that several factors could have on the uptake of the dyes themselves, including those of K^+, as compared with Na^+.

Materials and Methods

Yeast cells obtained commercially (La Azteca, S.A.) were prepared as described elsewhere [19] and used within the same day of preparation by keeping them in ice under continuous bubbling of air.

Potassium and H^+ movements were followed with a cationic or a combination electrode and the uptake of the dyes by centrifugation, according to techniques described before [20]. After incubation, the concentrations of the different dyes used were measured by determining the absorbance of the supernatants obtained by centrifugation of the cells in the microfuge. The absorbance was measured for each dye at its absorption maximum and the values were compared with those of calibration curves prepared with known concentrations of the respective dye. Measurement of the uptake of $^{86}Rb^+$ was also described previously [20]. In experiments with dyes, these were added simultaneously to $^{86}Rb^+$, to avoid their effects previous to the addition of the monovalent cation.

To measure the displacement of $^{45}Ca^{2+}$ by dyes, the cells were first incubated for 3 min with 10 μM $^{45}CaCl_2$ in the presence of a buffer and without a substrate. After this incubation the cells were filtered through a Millipore filter (0.45 μm) and washed once with water. The cells were then suspended in water and kept in ice. The preparation obtained had a considerable amount of radioactivity bound, of which at most 10% could be removed by 6 washings with water, but more than 95% could be eliminated by washing with cold $CaCl_2$. These cells were incubated in the presence of the dyes and then separated by filtration and washed with water. After resuspending and plating the cells, the remaining radioactivity was measured in a gas flow counter.

Absorbance changes of the dyes at two wavelengths were followed in an Aminco DW2a spectrophotometer.

The material absorbing at 260 nm was measured essentially as described by Elferink and Booij [7], except that the supernatant obtained after centrifuging the cells was washed twice with approximately 3 vol dichloromethane and once with the same amount of petroleum ether, with the addition of 0.1 vol 20% NaOH.

Respiration was measured with a Clark electrode and a suitable recording system.

Results

Figure 1 shows the effects of the cationic dyes studied on both K^+ uptake and H^+ production by yeast. It is shown that, with all of them, marked effects can be produced on K^+ uptake without modifying the proton production, except for the case of nile blue at 40 μM, which produces not only an inhibition of K^+ uptake, but also an actual efflux of the monovalent cation of the cells. The results obtained with brilliant green and with methyl green are not presented, but none of them altered either K^+ or H^+ movements if compared with the controls.

The experiments presented in Fig. 1 were carried out by measuring the movements of K^+ when the cation was added to the incubation mixture at a concentration of 2.0 mM. When the experiments were carried out in the absence of K^+ added to the medium, i.e., by following the movements of the K^+ of the yeast cells, the results shown in Figs. 2

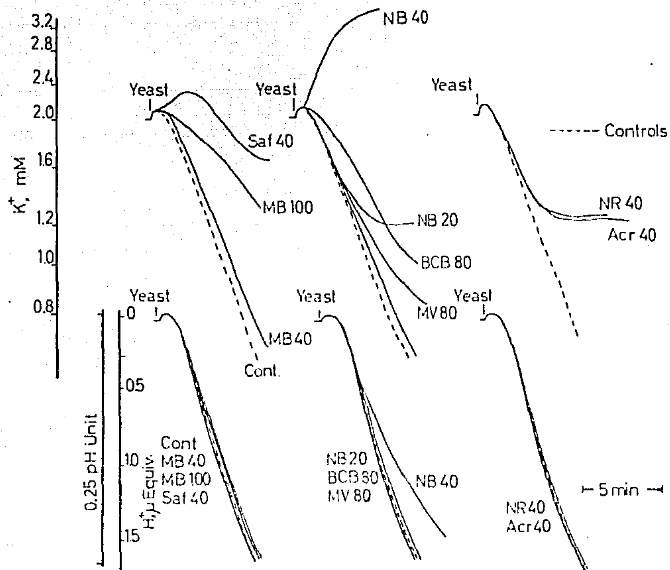


Fig. 1. Effects of several cationic dyes on the uptake of K^+ and the extrusion of H^+ by yeast. The cells (250 mg. wet wt), were added to a medium containing the indicated concentrations of the dyes and the following composition: 20 mM maleate-TEA buffer, pH 6.0; 50 mM glucose, and 2.0 mM KCl, in a final volume of 5.0 ml. Temperature was 30 °C. Figures beside the tracings indicate the concentrations of the dyes used

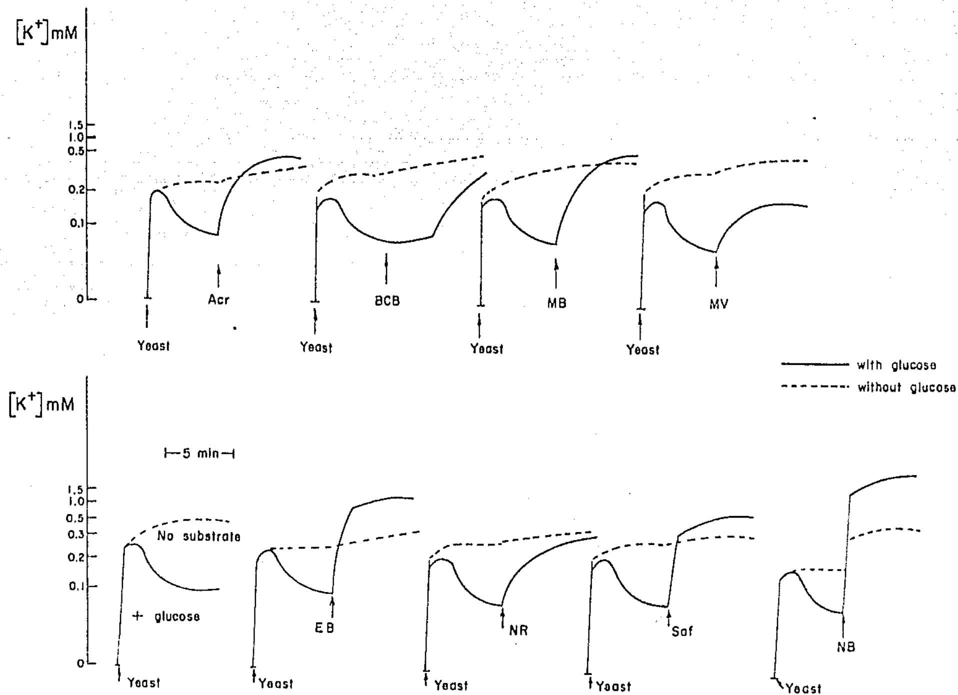


Fig. 2. Effects of dyes at a 50 μM concentration on K^+ movements in yeast added to a K^+-free medium. The experimental conditions were the same as for Fig. 1, but the final volume was 10.0 ml, and the medium did not contain KCl

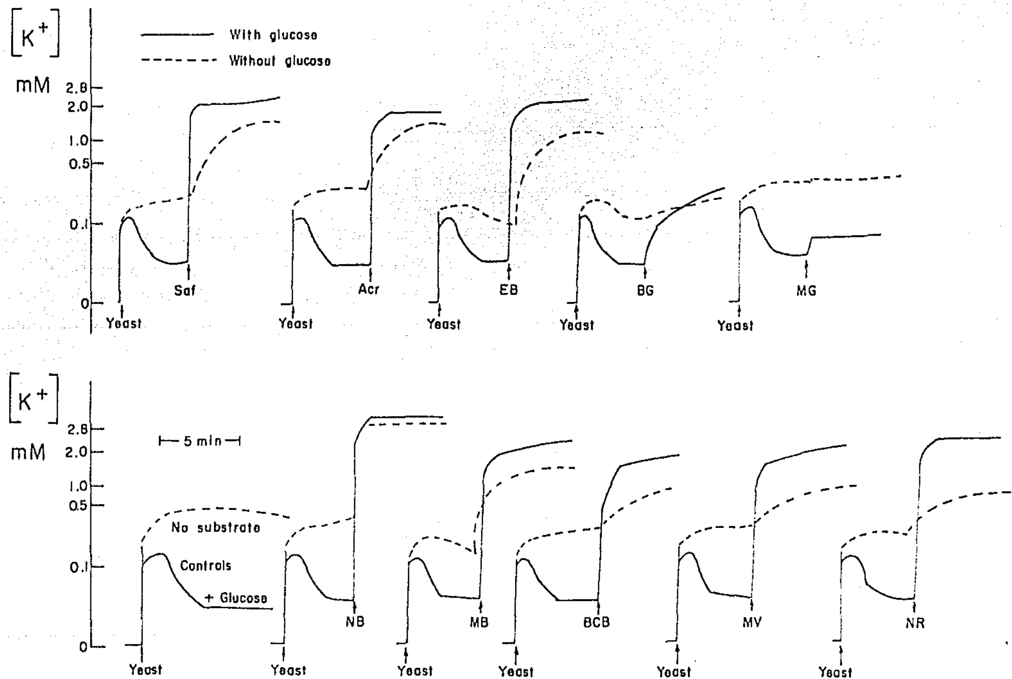


Fig. 3. Effect of dyes at 100 μM concentration on K^+ movements in yeast added to a K^+-free medium. The experimental conditions were the same as for Fig. 2

Table 1. Effect of cationic dyes (80 μM) on the release of material absorbing light at 260 nm, by yeast

	Absorbance of washed supernatant	% of value for the PCA extract
Control	0.237 ± 0.030	2.05
EB	0.754 ± 0.24	6.50
Saf	1.330 ± 0.15	11.53
NB	1.45 ± 0.25	12.5
MB	0.68 ± 0.06	5.8
MV	0.54 ± 0.06	4.6
BCB	0.85 ± 0.05	7.4
NR	1.00 ± 0.37	8.7
Acr	1.27 ± 0.21	11.03
BG	0.89 ± 0.89	7.7
MG	0.65 ± 0.38	5.6
Perchloric acid extract	11.53 ± 1.60	100.0

Cells were incubated for 8 min in the following medium: 10 mM succinate-TEA buffer, pH 6.0; 100 mM glucose; yeast cells, 125 mg, wet wt; final volume, 5.0 ml. After incubation the cells were centrifuged for 5 min in a clinical centrifuge, and the supernatant was decanted. The measurement of absorbance was performed as described under *Methods*.

and 3 were obtained. For these experiments, two concentrations of the dyes were employed, 50 and 100 μM. At the low concentration (Fig. 2), in the absence of substrate, except for nile blue which produced an efflux of K^+, none of the dyes produced a significant effect on the K^+ movements. When added in the presence of glucose, however, all the dyes, except for brilliant green and methyl green (not shown) produced a rapid efflux of K^+ from the cells. When the dyes were added at a higher concentration (100 μM) (Fig. 3), the phenomenon was exaggerated, and an efflux of K^+ was produced even in the absence of glucose, but, nonetheless, in all cases the efflux was more pronounced and rapid in the presence of glucose than when no substrate was added. The weakest action was shown again by methyl green and brilliant green, which produced a slight K^+ efflux, but only when glucose was present.

In view of the report of Elferink and Booij [7], who found that triphenylmethane dyes, among them brilliant green and methyl green, can produce the disruption of yeast cells, the cells were incubated with the dyes and then were centrifuged. The dye was washed from the supernatant and the material remaining that could absorb light at 260 nm measured, as a test of the disruption of the cells. As shown in Table 1, some of the dyes produce an increased appearance of absorbance at

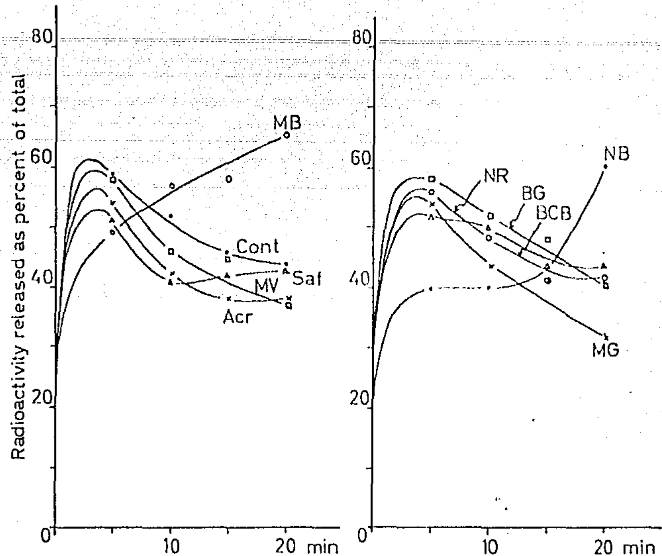


Fig. 4. Effect of cationic dyes on the efflux of $^{45}Ca^{2+}$ in yeast cells previously loaded with the isotope. Cells were loaded previously by incubating them for 30 min with 100 μM $^{45}CaCl_2$, 20 mM MES-TEA buffer, pH 6.0; 400 mM glucose, and 1.5 g yeast, in a final volume of 25 ml, and filtering and washing with 100 μM cold $CaCl_2$. After resuspending in water, the cells were incubated in the following mixture: 20 mM maleate-triethanolamine, pH 6.0; 50 mM glucose; 100 mg yeast cells; final volume, 2.0 ml; temperature 25°. At the indicated times, aliquots were removed and centrifuged for 10 sec in the microfuge. An aliquot of the supernatant was plated and counted. The data are the means of four experiments carried out on different days

260 nm in the medium of incubation. However, the highest values obtained were of only about 12% of that obtained by the complete disruption of the cells with perchloric acid. Further test of the cell integrity was provided by the experiment presented in Fig. 4; yeast cells were loaded with $^{45}Ca^{2+}$ by incubation with the isotopes in the presence of glucose for 30 min. The cells were filtered and washed with 100 μM $CaCl_2$ and resuspended in water. Afterwards, the cells were incubated in the absence or in the presence of the dyes, and the efflux of radioactivity was measured by counting the supernatant obtained after centrifuging the cells in the microfuge at different times. After an important initial efflux of the divalent cation, the cells again took up the radioactivity, essentially in a similar way, except for those incubated in the presence

Table 2. Effects of several cationic dyes on the $^{86}Rb^+$ uptake by yeast

	No. of experiments	% inhibition of uptake	
		0.8 mM Rb^+	4.0 mM Rb^+
Control	22	—	—
Safranine	3	76±6	43 ±12
Acr	4	73±5	49 ±3
MB	3	33±15	4.6±20
NR	3	65±13	40 ±5
BCB	3	64±4	16 ±9
MV	3	48±11	11 ±12
NB	3	86±2	63 ±10

Incubation medium: 20 mM maleate-TEA buffer, pH 6.0; 100 mM glucose. After equilibrating the temperature in a water bath at 30 °C, 100 mg of yeast cells, wet wt, were added. After exactly 2 min, $^{86}Rb^+$ was added at a concentration of 4.0, 2.0, 1.33 or 0.8 mM, followed immediately by the dye at a final concentration of 100 μM. The final volume was 2.0 ml. After 2 min an aliquot was taken and filtered through a Millipore type filter, 0.45 μm pore size, washing once with water, twice with 5 mM RbCl, and twice more with water. The cells were then resuspended in water, and an aliquot was plated and counted. One control was always run with each dye. The values for the Lineweaver-Burk equation were obtained by the least squares method.

of MB[1] or NB, in which the pattern of movement of Ca^{2+} was different from the rest of the cases.

Given the fact that dyes can produce an inhibition of the uptake of K^+, and also an efflux of the monovalent cation present within the cells, it was important to determine the effects of the dyes on the initial rates of uptake of $^{86}Rb^+$, to ascertain their action on the *influx* of monovalent cations. To avoid the effect of K^+ extruded from the cells by the addition of the dye in the absence of external cations, both dyes and $^{86}Rb^+$ were added simultaneously in each case; besides, different concentrations of Rb^+ were employed, in order to get more information about the inhibition produced by each dye.

The results obtained are presented in Table 2; there is an actual inhibition of the uptake of the cation that can be measured on a percentual basis. By measuring this inhibition at the highest and the lowest Rb^+ concentrations employed, it can be seen that at high concentrations of the alkali cation, the inhibition decreases. Besides, there are changes

[1] *Abbreviations:* CTAB, cetyltrimethylammonium bromide; TEA, triethanolamine; FCCP, *p*-trifluoromethoxycarbonylcyanide-phenylhydrazone; EB, ethidium bromide; Saf, safranine; NB, nile blue; MB, methylene blue; MV, methyl violet; BCB, brilliant cresyl blue; NR, neutral red; BG, brilliant green; MG, Methyl green; Acr, acriflavin.

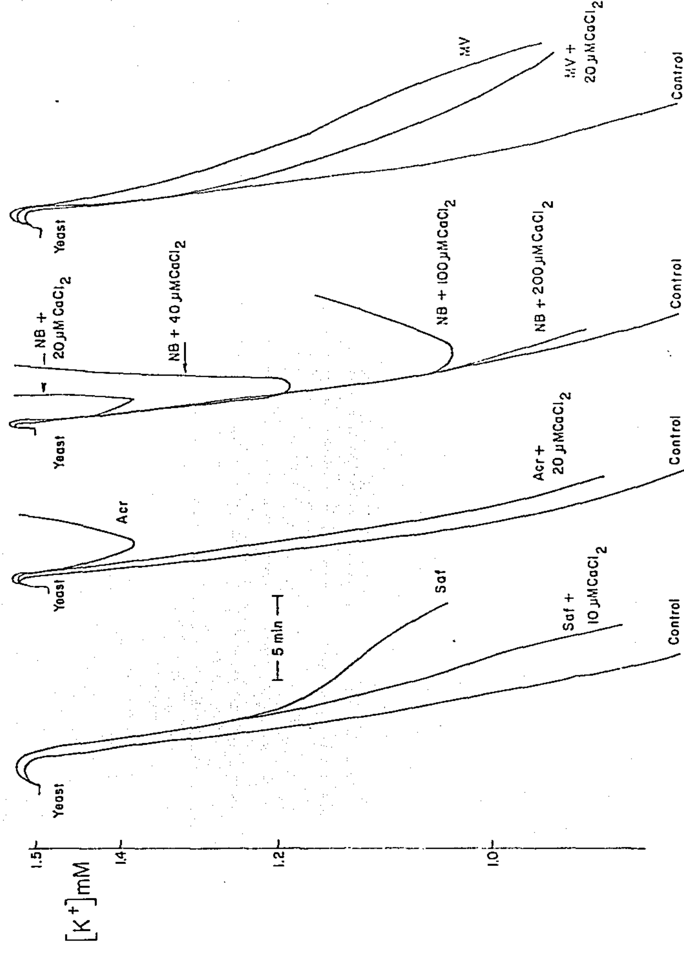


Fig. 5. Reversion by Ca^{2+} of the effects of some dyes on the uptake of K^+ by yeast. The experimental conditions were the same as for Fig. 1, but the indicated concentrations of $CaCl_2$ were included

in the kinetic parameters that in general are more significant for the K_m than for the V_{max} (*see also* Fig. 5); in other words, by these criteria, the inhibition resembles the competitive type, without being purely so, which is somewhat different than what was reported before for ethidium bro-

Table 3. Uptake of different dyes by yeast. Substrate requirement and effects of K^+ and Na^+

	Control no substrate	nmol dye taken up in 3 min by 125 mg yeast				
		Glucose	No substrate +KCl	Glucose +KCl	No substrate +NaCl	Glucose +NaCl
EB	19 ± 14	69 ± 27[a]	27 ± 4	25 ± 9[c]	32 ± 16	51 ± 21
Saf	108 ± 15	144 ± 23[b]	80 ± 10	90 ± 18[d]	87 ± 17	108 ± 23
Acr	147 ± 11	175 ± 12[a]	112 ± 7	130 ± 12[d]	122 ± 10	143 ± 26
NB	187 ± 8	184 ± 23	193 ± 3	188 ± 4	193 ± 2	190 ± 2
MV	190 ± 3	190 ± 7	180 ± 11	186 ± 5	191 ± 2	191 ± 3
MB	130 ± 27	164 ± 12[b]	100 ± 13	91 ± 32[d]	103 ± 29	133 ± 5[e]
BCB	149 ± 11	148 ± 16	141 ± 6	125 ± 31	140 ± 10	142 ± 11
NR	186 ± 5	183 ± 7	174 ± 21	168 ± 7	180 ±	177 ± 7

Incubation conditions: 10 mM maleate TEA buffer, pH 6.0, 50 mM glucose (where indicated); 5 mM KCl or NaCl (where indicated); dyes 40 μM; yeast, 125 mg wet wt; final volume, 5.0 ml; time; 3 min. Dye uptake was measured as described under *Methods*. Data are given as the mean values of 5 experiments with the SD of each value.

[a] P 0.01 and [b] P 0.05 with respect to controls without substrate.

[c] P 0.01 with respect to controls with glucose.

[d] P 0.01, and [e] P 0.02 with respect to controls with glucose.

Statistic significance was calculated by means of the student t test.

mide [20]. Data with methyl green and brilliant green, which do not appreciably inhibit the uptake of K^+, are not presented.

If dyes produce an effect on K^+ uptake by yeast, it is important to define if they are taken up by the cells and, if so, to investigate some characteristics of their uptake. The parameters studied were the requirement for glucose and the effects of the monovalent cations K^+ and Na^+ on the uptake of the dyes; Table 3 shows the results of experiments in which this exploration was made. From the point of view of the substrate requirement, it can be seen that, unlike ethidium bromide which shows a significant requirement for glucose, other dyes are taken up in more than 90% of the amount present without any differences in the presence or absence of glucose. There is, on the other hand, an intermediate group, represented essentially by safranine, acriflavine, and methylene blue, for which the uptake is increased by 12 to 33% in the presence of glucose.

As to the effects of K^+ or Na^+ on the uptake of the dyes, there are also three groups of dyes: the first is again represented by ethidium bromide: its uptake is inhibited 64% by K^+ and only 26% by Na^+ at a 5 mM concentration. The second group shows a smaller inhibition

of uptake by monovalent cations, with a less selective inhibition if K^+ and Na^+ are compared; it includes safranine, acriflavin, and methylene blue. To only a minor extent, brilliant cresyl blue also shows this selective inhibition by K^+. With the rest of the dyes, the inhibition by Na^+ or K^+ was either very low or also nonselective between cations.

Up to this point, there seemed to be some correlation between the effects of some of the dyes and their penetration. The effect of ethidium has been dealt with somewhere else [20], but some of the other inhibited Rb^+ uptake with kinetic characteristics close to the competitive type showed a partial requirement of glucose to be taken up by the cells, and K^+, to a greater extent than Na^+, inhibited its uptake.

It is possible that, as postulated for ethidium bromide [20], these dyes might interact with the K^+ carrier, but presented some other kind of interaction and/or penetration besides. They could interact with the membrane of the cell in nonspecific sites, different from those of K^+ transport, by means of the negative charge that the cell surface has been shown to have [9]. Divalent cations, which can bind to this surface [24] and block the effects of some cationic detergents on yeast [3], might also block the action of dyes by forming a positive layer on the surface of the cell. The possibility was tested with only four of the dyes: safranine, acriflavine, nile blue, and methyl violet. As shown in Fig. 5, Ca^{2+} is capable of reverting the inhibition of K^+ uptake produced by the dyes. With nile blue, perhaps the most active of the dyes tested, higher concentrations of Ca^{2+} were required to produce full reversion of the inhibition produced on K^+ uptake.

Another way of testing the characteristics of the interaction of the dyes with the cells was by determining the inhibition kinetics produced by dyes in the presence and absence of low concentrations of Ca^{2+} that do not produce any effect on the uptake of Rb^+ *per se*. As shown in Fig. 6 for acriflavin, safranine, and methylene blue, the inhibition of Rb^+ uptake produced by the dyes, that in the absence of Ca^{2+} is of a noncompetitive type, diminishes and tends to acquire this form when the divalent ion is present in the incubation mixture. Although results are not shown, at the low concentrations tested for Ca^{2+} (60 μM) and in agreement with the data of Theuvenet and Borst-Pauwels [24], no effect was found for this cation on the uptake of Rb^+ in the control experiments.

By incubating the cells with $^{45}Ca^{2+}$ it should be possible to "label" the negative sites of the surface and afterwards to test the ability of the dyes to displace $^{45}Ca^{2+}$, as described in Table 4. The addition of

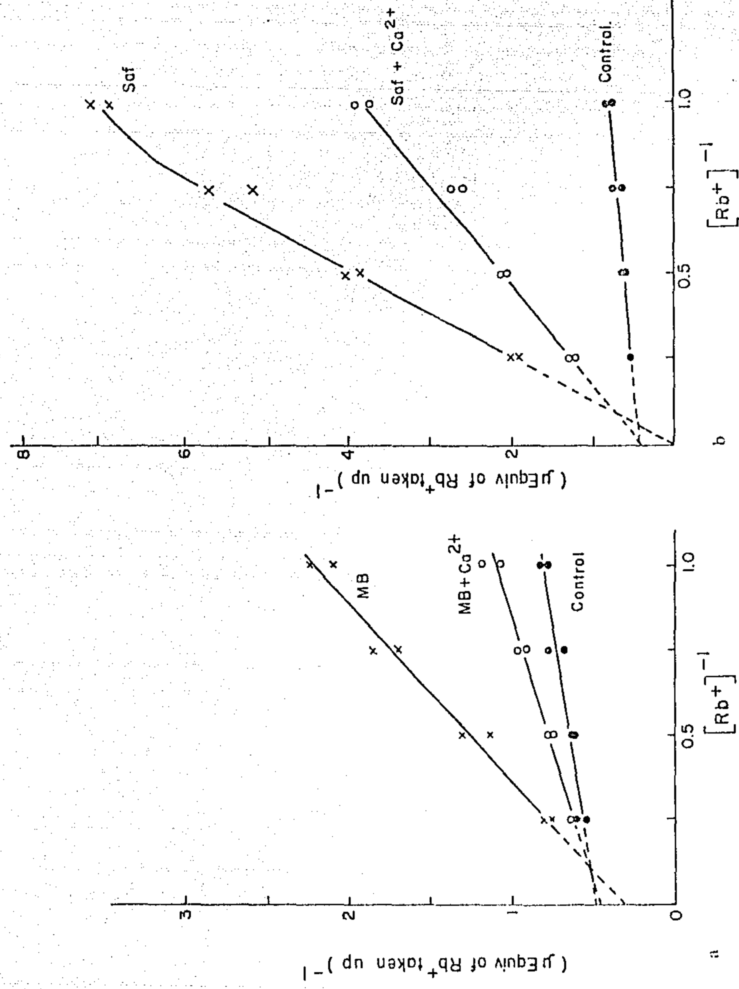
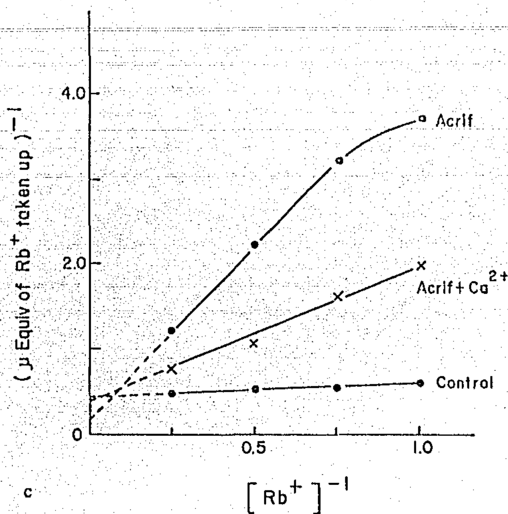


Fig. 6. Changes produced by Ca^{2+} on the inhibition kinetics of $^{86}Rb^{+}$ uptake. Experimental conditions were the same as for Table 2, but where indicated, 60 μM $CaCl_2$ was included in the incubation mixture. The dye concentration was 150 μM

the dyes, if they interact with the negative surface of the cells, should displace at least part of the radioactivity bound. Since the uptake of the dyes, their effect on K^{+} movements and possibly their interaction with the membrane are favored by glucose; the experiment was performed both in the presence and in the absence of glucose. In the absence



of glucose most of the dyes showed an almost negligible effect of displacement of $^{45}Ca^{2+}$; only nile blue and methyl green produced a noticeable lowering of the radioactivity upon incubation. In the presence of glucose, surprisingly, some of the dyes not only do not decrease the radioactivity present in the cells, but actually increase it. This is the case of MB, MV, Acr, EB and Saf. With some of the dyes, the effect has been further studied and it seems to involve the actual penetration of the divalent cation into the cell, since a significant part of the radioactivity is no longer exchangeable by cold $CaCl_2$ [17].

Under the assumption that Ca^{2+} might decrease the interaction of the dyes with nonspecific sites of the membrane, it could be expected that the uptake of dyes occurring in the presence of the divalent cation took place in a larger proportion through more specific sites and, therefore, were more sensitive to the competitive presence of monovalent cations, particularly K^+. The data of Table 5 show the results of the experiment that was carried out with four dyes. Although 40 μM $CaCl_2$ produced a decrease of the dye uptake, the addition of K^+ under these conditions did not produce a larger inhibition of the uptake than when added in the absence of Ca^{2+}.

Safranine has been studied from the point of view of its interaction with membrane systems of mitochondria [5] or liposomes [1]. When

Table 4. Effect of cationic dyes on $^{45}Ca^{2+}$ binding to yeast cells in the presence or absence of glucose

	Cpm remaining in yeast (100 mg) after incubation with or without the dyes, filtering, and washing	
	No substrate	Plus glucose
Controls	3018	3697
	3018	3713
MB	2971	3961
NB	2798	3594
BCB	2915	3512
MV	3360	3941
Acr	2850	4151
EB	3066	4410
Saf	3103	4197
NR	3078	3707
BG	2972	3450
MG	2708	2994

3 g yeast were incubated for 3 min at 30° with 10 μM $^{45}Ca^{2+}$ (293,000 cpm). After filtering and washing twice with water; the cells (100 mg) were resuspended in water and incubated for 2 min in the following mixture: 20 mM maleate-TEA buffer, pH 6.0; 100 mM glucose where indicated, and 50 μM of the respective dye in a final volume of 2.0 ml. After 2 min at 30°, an aliquot of the mixture was filtered and washed 3 times with water. The cells were then resuspended in water, plated, and counted.

Table 5. Effect of K^+ on dye uptake by yeast in the presence or absence of 40 μM $CaCl_2$

	nmol dye taken up in 3 min by 125 mg yeast			
	Without $CaCl_2$		Plus 40 μM $CaCl_2$	
	Control	5 mM KCl	Control	5 mM KCl
Acr	144 ± 7	102 ± 4(30)	116 ± 2	88 ± 13(25)
Saf	113 ± 4	62 ± 5(46)	79 ± 8	61 ± 17(73)
BCB	131 ± 17	123 ± 9(7)	135 ± 2	116 ± 7(6)
MB	122 ± 14	69 ± 11(44)	88 ± 12	59 ± 20(33)

Experimental conditions: 20 mM maleate-TEA buffer, pH 6.0; 50 mM glucose, plus the indicated concentrations of KCl or $CaCl_2$. After equilibrating the temperature in a water bath at 30°, 125 mg yeast were added. Two min later, the dyes at a final concentration of 50 μM were added; after 3 min an aliquot was taken and centrifuged in a microfuge for 10 sec. The dye remaining in the supernatant was measured by the absorbance at the appropriate wavelength. Final volume was 5.0 ml. Data are the means of 4 experiments. ±SD. Figures in parentheses are the percent inhibition produced by K^+ in relation to the respective control.

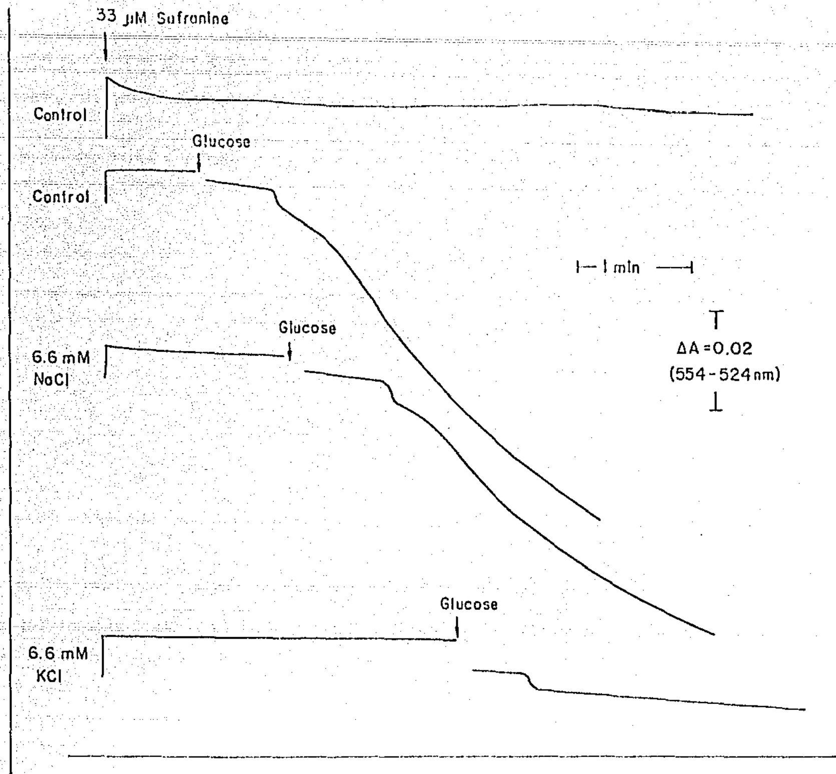


Fig. 7. Effects of substrate, K^+ and Na^+ on the absorbance changes (554-524 nm) of safranine produced by yeast. Incubation mixture: 20 mM maleate TEA buffer, pH 6.0; yeast, 150 mg, wet wt; final volume, 3.0 ml. The experiment was carried out at room temperature. Safranine was added at a 33 µM concentration and glucose at 16.6 mM

it interacts, apparently by being transported into the matrix space, by virtue of the stacking of its molecules, its absorbance spectrum changes, and this can be followed in a dual wavelength spectrophotometer. With the same instrument, the reduction of methylene blue can be followed. In this latter case, it can be assumed that its penetration into the cell is a prerequisite to its reduction, so that the change can be taken as an index of the uptake of the dye. Acriflavine, on the other hand, shows a change in its spectrum when placed in an organic solvent of low dielectric constant, like dioxane or butanol, and this change can also

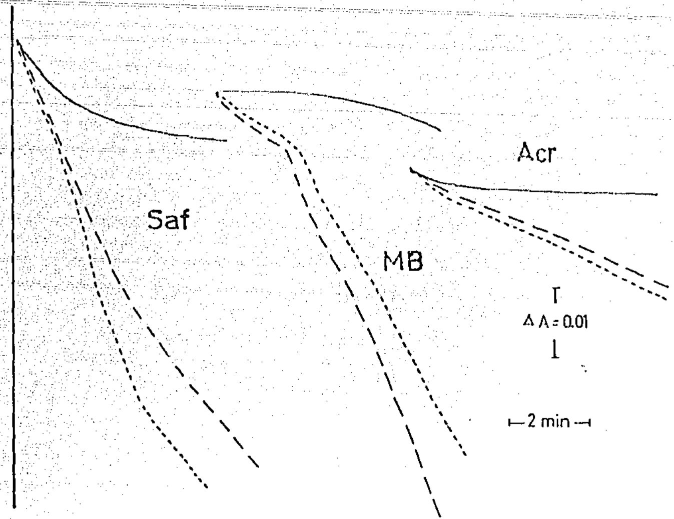


Fig. 8. Effects of Na^+ and K^+ on the absorbance changes of safranine (554-524 nm), acriflavine (475-465 nm), and methylene blue (640-650 nm) during their interaction with yeast cells. The incubation medium was the same as for Fig. 6, but the buffer concentration was 13.3 mM and the yeast amount 100 mg, wet wt. The concentrations of the dyes were 33.3 µM. Key: ---, control; -----, 6.6 mM NaCl; ----, 6.6 mM KCl

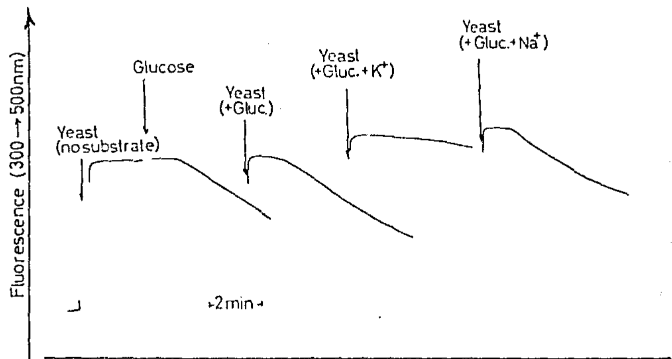


Fig. 9. Effects of substrate, Na^+ and K^+ on the fluorescence of acriflavine upon its interaction with yeast. The experimental conditions were the same as in Fig. 7, but the yeast concentration was 50 mg, wet wt. and fluorescence at 300 → 500 nm was registered

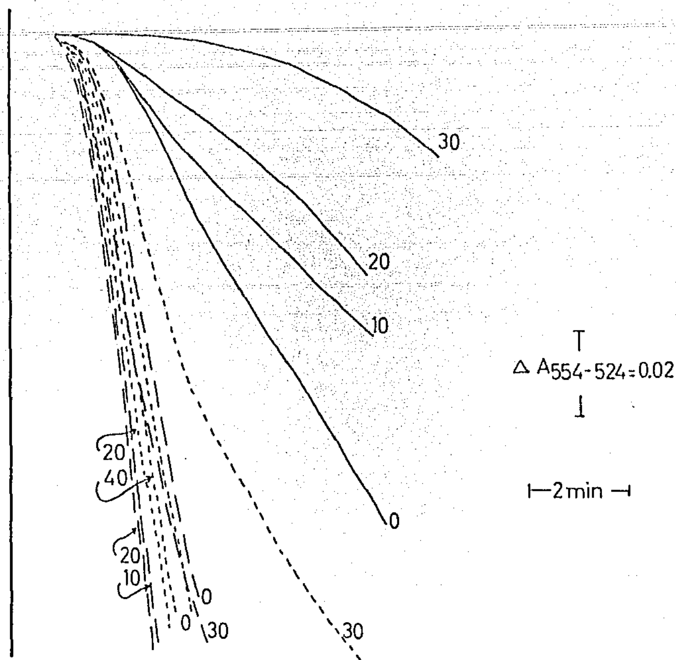


Fig. 10. Effect of different concentrations of Ca^{2+} and Na^+ and K^+ on the metachromatic changes of safranine produced by its interaction with yeast. The experimental conditions were the same as for Fig. 7, except that the indicated concentrations (μM) of $CaCl_2$ were included. The key to the figure is the same as for Fig. 7

be followed as an indication of its interaction with the membrane(s) of the cell. With this in mind, the color changes of the three dyes were followed upon their interactions with the yeast cells. The idea was to investigate the energy (substrate) requirements of the interactions, as well as the effects that both K^+ or Na^+ could have, using the color changes as an index.

The experiment of Fig. 7 shows the detected changes with safranine; the color change requires a substrate, and, besides, it can be inhibited by K^+ but not by Na^+ at 6.6 mM concentration. The complete results are not presented, but similar tracings were obtained by using methylene blue or acriflavine at the adequate wavelengths, under similar conditions. In Fig. 8 it can be seen that with the three dyes the changes observed

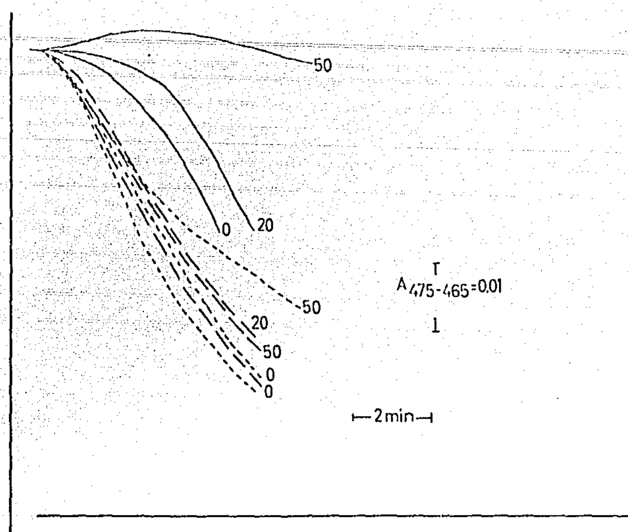


Fig. 11. Effect of different concentrations of Ca^{2+} on the inhibition of the metachromatic changes of acriflavine produced by either Na^+ or K^+ upon its interaction with yeast. The experimental conditions were as those of Fig. 9

in the presence of a substrate can be prevented by the addition of K^+, but not by the addition of an equal concentration of Na^+.

In the case of acriflavin, in Fig. 9, the results of one experiment are presented, in which the interaction of the dye was monitored by recording the fluorescence changes of the dye at 300 → 500 nm. Also in this case, the changes observed require a substrate, and are reverted by K^+, but not by Na^+.

The metachromatic changes observed with the aforementioned dyes can be prevented specifically by K^+ and not by Na^+, depending on the dye concentration used; as the concentration is increased, both the energy requirement and the specificity of the reversion by K^+ tend to disappear. Besides, the phenomenon is not absolutely constant, and with some yeast preparations there is not a clear reversion of the color changes by K^+. In these cases, however, it was found that, at least with safranine and acriflavine, the addition of Ca^{2+} can produce the recovery of the cells to the state in which the metachromatic changes can be inhibited by K^+ and not by Na^+ (Figs. 10 and 11).

Discussion

The K^+ transport system of yeast seems to be sensitive to an already large variety of cationic molecules. Guanidine, and particularly alkylguanidines, can inhibit the uptake of monovalent cations by yeast [15] and the same seems to be true for ethidium bromide [20]. The data presented in this paper show the ability of most of the molecules tested to interact with this same transport system, though in different degrees of effectiveness and specificity. As tools in the study of transport mechanisms, dyes offer the advantage of being easy to detect and to measure their concentration changes and uptake by the cells. A good example of this is given by the experiments of Figs. 7 to 10.

The present studies allowed us to find out that dyes, besides inhibiting K^+ transport and promoting its efflux under certain conditions, can be taken up by the cells in a process that, in different degrees for each one, requires the presence of a substrate, and can be inhibited by monovalent cations, more so by K^+ than by Na^+. These results pose an interesting possibility, i.e., that cationic organic molecules in general can be translocated by the natural carriers of the cells and also interact with sites that "normally" should probably be occupied by divalent cations. In much the same way that there are competitive inhibitors of enzymatic reactions, more or less selective inhibitors can be found for the transport mechanisms of cations, as postulated by Hille [12].

Although the data of Figs. 2 and 3 could indicate that the inhibition of K^+ uptake could be due to the production of an efflux of the cation, the data obtained on the effects on $^{86}Rb^+$ uptake at short incubation periods show clearly that there is an actual inhibition of the influx of cations. It has to be pointed out that the dyes and $^{86}Rb^+$ were added simultaneously to avoid the extrusion of K^+ that is produced if the dye is added in the absence of cations in the medium. The percent inhibition of Rb^+ uptake by the dyes decreases upon increasing the concentration of the monovalent cation, and this is what one would expect if some kind of competition existed between Rb^+ and the dyes.

Elferink and Booij [7] have suggested that the effect of some triphenylmethane dyes may affect yeast by disruption of the cell structure. Although this may be so at high concentrations of the dyes, at the levels used throughout the work, this action is minimal. The measurement of the material absorbing at 260 nm present in the incubation medium showed that this effect is low. The experiments represented in Fig. 1, besides, show that the cells maintain their ability to pump out protons,

a property that would be difficult to carry out by broken cells. Another proof of the integrity of the cells is the increase that most of the dyes produce in the uptake of Ca^{2+} (see Table 4, and Reference 17); it would be impossible for disrupted cells to take up Ca^{2+}. The experiments represented in Fig. 4 also indicate that yeast cells incubated in the presence of dyes maintain their ability to keep and recover Ca^{2+} also when loaded previously with this ion. Even if methylene blue and nile blue produce some efflux of Ca^{2+}, the general behavior of cells treated with dyes is similar to that of the control cells.

Passow *et al.* [14] showed that methylene blue can produce the complete destruction of some cells, while leaving part of them intact. This possibility can not be definitely ruled out; however, our data can be explained in a more simple way than by assuming the existence of two kinds of cells. If this were the case, it would be necessary to postulate that the dyes selectively break some kind of cells, but also in some way produce the stimulation of Ca^{2+} uptake or binding by the remaining cells, and that the breakage of part of them does not show any changes in the proton pumping activity.

If it is accepted that K^+ and dye uptake are related, according to the data obtained, three kinds of dyes could be considered; the first is represented by EB only and is characterized by an important requirement of glucose for the uptake and a substantial inhibition of the uptake by K^+, which is not equaled by Na^+. In previous work [20] it was found that although the dye can bind to the cells in the absence of glucose, its fluorescence at 530 → 600 nm is strictly dependent on the presence of glucose at low concentrations. Perhaps the most important characteristic of EB is that it produces an inhibition of $^{86}Rb^+$ transport which is of a pure competitive type [20] within low concentration ranges of the dye.

The second group is represented by those dyes that fulfill the three aforementioned criteria, but only partially, i.e., they show a relative substrate requirement for uptake; their uptake is also inhibited partially by K^+, but to a smaller extent by Na^+, and finally, the inhibition kinetics of $^{86}Rb^+$ uptake is close to but not clearly of the competitive type. To this group belong acriflavin, safranine, and methylene blue. An interesting proof of the interaction with a K^+ specific site was provided by the blocking of absorbance changes of Saf, MB, and Acr by K^+ but not by Na^+, and the strict requirement for glucose of these changes. It seems that the spectrophotometric measurement of metachromatic changes can be used to monitor more specific interactions. The

measurement of the absolute uptake of the dye by centrifugation, on the other hand, gives the sum of both specific and nonspecific interactions of the dye with the cell.

The third group is represented by those dyes that do not fulfill either one or more of the mentioned criteria. A clear limitation between this and the second group does not exist, and probably very few of the dyes belong absolutely to this third group.

The data, in a very simple way, could be visualized trying to correlate the behavior of the dyes as to their uptake with their effects on K^+ or Rb^+ uptake. It seems that dyes of the first group (EB only) can be taken up by the cells only, or almost only, through the K^+ transport system [20]. Dyes of the second group would seem to be taken up by this transport system also, but not exclusively. The uptake by other route(s) could be substrate independent and not sensitive towards K^+. Dyes of this group would be only partial and noncompetitive inhibitors of monovalent ion transport, and K^+ would only to a minor degree inhibit their transport. The localization of unspecific uptake sites would be different from that of the K^+ transport system, since Ca^{2+} at low concentrations can inhibit dye uptake, but not K^+ transport [24]. The experiment of Table 5 would disagree with this model; as mentioned before, it would be expected that, if in the presence of Ca^{2+} only or mostly nonspecific sites are blocked, there should be a more pronounced inhibition by K^+ on dye uptake percentally when the divalent cation was present. The same can be said about the data on $^{45}Ca^{2+}$ displacement by dyes of the second group. These latter data, however, could be explained in terms of the relative values of the affinity constants of Ca^{2+} and dyes for the sites involved and/or by other interaction sites without the ability to bind Ca^{2+}.

As to the third group, it was already said that perhaps very few dyes fully belong to it. Some of them show some substrate requirement; for some, uptake is inhibited by K^+ and others show a slight tendency to the competitive inhibition of Rb^+ uptake. Some of them displaced $^{45}Ca^{2+}$ from the cells when incubated in the absence of glucose.

The model imagined may be an oversimplification of the facts, and it is possible that more than one extra route for the entrance of dyes is involved besides the K^+ transport system. However, several of the dyes tested seem to be taken up, at least partially, through the K^+ transport system.

Another effect of the dyes consists in the efflux of K^+ they produce from the cells. The measurement of the amount of material absorbing

light at 260 nm and other tests lead us to conclude that the integrity of the cell is maintained. Besides, as mentioned before, the cells in the presence of EB, for instance, even at 250 μM, are able to take up $^{45}Ca^{2+}$ at an increased rate [17], which would not be possible with broken cells. Perhaps the most plausible explanation is an interaction of the dyes with the K^+ carrier which now allows the efflux of K^+; this transport system normally seems to work in such a way that K^+ is kept within the cell once it is taken up. The mechanisms of K^+ efflux are subject to influences and conditions that have been studied with certain detail by Ryan and Ryan [23] and by Rothstein [22]. The efflux of potassium ions depends very much on the presence of other ions in the medium and in the cell, and particularly on the internal pH of the yeast cell. The efflux, on the other hand, is also determined largely by the relative affinities of the monovalent cations, including H^+, for the transport system. Besides K^+ that has been taken up by yeast can be made to leave the cells by the addition of uncouplers [16, 17]. At certain dye concentrations, the efflux of K^+ they produce requires the presence of a substrate. The substrate, however, does not seem to be required for the production of the effect because it causes the uptake of the dye. Many of the dyes tested can be taken up in the absence of a substrate and still do not produce the K^+ efflux unless the substrate is added. It appears that the energy source is necessary for the interaction of the dye with the K^+ carrier. In agreement with this assumption are the results of the spectral changes of absorbance of safranine, acriflavin, and methylene blue (Figs. 7–11) which, in contrast with their uptake, require a substrate and are highly sensitive to K^+, and much less to Na^+. The dyes might represent a way to study changes of the carrier state produced by an efficient energy source in the cell. Cases like this have been described in bacteria [21]; energization changes the state of the galactose carrier. The interaction of the dyes with the carrier seems to be based on their cationic nature, since the effects cannot be reproduced with anionic dyes.

Finally, although it is difficult to decide if the uptake of the dyes represents the actual penetration of the molecules into the cell or only their binding to the surface, there are indications that the substances actually penetrate. The first is the amount taken up by the cells; although it might so happen, it is difficult for such amounts of dye to remain bound exclusively to the surface. The second proof may be more conclusive; the data are not presented, but most of the dyes used produce some inhibition of respiration except for BG and MG, which do not

inhibit. At the 100 μM concentration, the lowest inhibition of respiration was produced by neutral red, and it was of 80% as the mean value of two experiments. Although some of the dye may remain bound to the surface, it is evident that at least part of it penetrates into the cell. Finally, in the case of methylene blue, the data of Fig. 8 show that it is reduced by the cells, and, most probably, this requires that the molecules are taken inside.

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References

1. Akerman, K.E., Saris, N.-E.L. 1976. Stacking of safranine in liposomes during valinomycin-induced efflux of potassium ions. *Biochim. Biophys. Acta* 426:624
2. Armstrong, W.McD. 1958. The effect of some dyestuffs on the metabolism of baker's yeast. *Arch. Biochem. Biophys.* 73:153
3. Armstrong, W.McD. 1963. Surface active agents and cellular metabolism, 3. The effect of metal ions on the inhibitions by dodecyltrimethylammonium bromide of aerobic CO_2 production by baker's yeast. *Arch. Biochem. Biophys.* 102:210
4. Chaix, P., Roncoli, G. 1976. Influences différentes exercées par les ions sodium et potassium sur la vitesse deréduction du blue méthylene par la levure de boulangerie en absence ou en presence de glucose. *Biochim. Biophys. Acta* 9:223
5. Colonna, R., Massari, S., Azzone, G.F. 1973. The problem of cation binding sites in the energized membrane of intact mitochondria. *Eur. J. Biochem.* 34:577
6. De Nobrega Bastos, R., Mahler, H.R. 1974. Molecular mechanisms of mitochondrial genetic activity. Effects of ethidium bromide on deoxyribonucleic acid and energetics of isolated mitochondria. *J. Biol. Chem.* 249:6617
7. Elferink, J.G.R., Booij, H.L. 1975. The action of some triphenylmethane dyes on yeast and erythrocyte membranes. *Arzneim. Forsch.* 25:1248
8. Fry, B.A. 1957. Basic triphenylmethane dyes and the inhibition of glutamine synthesis by *Staphylococcus aureus. J. Gen. Microbiol.* 16:341
9. Furhmann, G.F., Boehm, C., Theuvenet, A.P.R. 1976. Sugar transport and potassium permeability in yeast plasma membrane vesicles. *Biochim. Biophys. Acta* 433:583
10. Gale, E.F., Mitchell, P.D. 1947. The assimilation of aminoacids by bacteria. 4. The action of triphenylmethane dyes on glutamic acid assimilation. *J. Gen. Microbiol.* 1:299
11. Grimwood, B.G., Wagner, R.P. 1976. Direct action of ethidium bromide upon mitochondrial oxidative phosphorylation and morphology. *Arch. Biochem. Biophys.* 176:43
12. Hille, B. 1971. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* 58:599
13. Miko, M., Chance, B. 1975. Ethidium bromide as an uncoupler of oxidative phosphorylation. *FEBS Lett.* 54:347
14. Passow, H., Rothstein, A., Lowenstein, B. 1959. An all or none response in the release of potassium by yeast cells treated with methylene blue and other basic redox dyes. *J. Gen. Physiol.* 43:97
15. Peña, A. 1973. Studies with guanidines on the mechanism of K^+ transport in yeast: *FEBS Lett.* 34:117

16. Peña, A. 1975. Studies on the mechanism of K^+ transport by yeast. *Arch. Biochem. Biophys.* 167:397
17. Peña, A. 1978. Effect of ethidium bromide on Ca^{2+} uptake by yeast. *J. Membrane Biol.* 42:199
18. Peña, A., Chávez, E., Cárabez, A., Tuena de Gómez Puyou, M. 1977. The metabolic effects and uptake of ethidium bromide by rat liver mitochondria. *Arch. Biochem. Biophys.* 180:522
19. Peña, A., Piña, M.Z., Escamilla, E., Piña, E. 1977. A novel method for the rapid preparation of coupled yeast mitochondria. *FEBS Lett.* 80:209
20. Peña, A., Ramírez, G. 1976. Interaction of ethidium bromide with the transport system for monovalent cations in yeast. *J. Membrane Biol.* 22:369
21. Reeves, J.P., Schechter, E., Weil, R., Kaback, H.R. 1973. Dansylgalactoside, a fluorescent probe of active transport in bacterial membrane vesicles. *Proc. Nat. Acad. Sci. USA* 70:2722
22. Rothstein, A. 1974. Relationship of cation influxes and effluxes in yeast. *J. Gen. Physiol.* 64:608
23. Ryan, J.P., Ryan, H. 1972. The role of intracellular pH in the regulation of cation exchanges in yeast. *Biochem. J.* 128:139
24. Theuvenet, A.P.R., Borst Pauwels, G.W.H. 1976. Kinetics of ion translocation across charged membranes mediated by a two site transport mechanism. Effects of polyvalent cations upon rubidium uptake into yeast cells. *Biochim. Biophys. Acta* 426:746
25. Waring, M.J. 1964. Complex formation with DNA and inhibition of *Escherichia coli* RNA polymerase by ethidium bromide. *Biochim. Biophys. Acta* 87:358
26. Waring, M.J. 1965. Complex formation between ethidium bromide and nucleic acids. *J. Mol. Biol.* 13:269
27. Weth, G. 1967. Biologische Wirkungen von Malachitgrün. 1. Mitteilung: Übersicht über bisher bekannte Wirkungen. *Arzneim. Forsch.* 17:1
28. Weth, G., Boiteux, A. 1967. Zur biologischen Wirkung von malachitgrün. 5. Mitteilung: Die Wirkung von Triphenylmethanfarbstoffen auf die oxidative Phosphorylierung in isolierten Rattlebermitochondrien. *Arzneim. Forsch.* 17:1231

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Multiple Interactions of Ethidium Bromide with Yeast Cells

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Experiments were carried out to determine the relationship between different energy states of the yeast cell and the uptake of ethidium bromide (EB). By varying the substrate, oxygenation, and by the use of uncouplers or respiratory inhibitors, it is possible to have energization or not of the whole cell, and also to deenergize specifically the mitochondria. The energy state of the whole cell can be determined by several means. With this system, three kinds of interactions of EB with the cell can be detected. The first one is a binding to the cell that does not seem to require energy. A second interaction is represented by the uptake of the dye into the cell, which does require energy, and is accompanied by an increase of the fluorescence of EB. The third interaction that can be monitored seems to be the uptake or binding of the dye by the mitochondria of the yeast cell; it requires specifically of the energization of this organelle, and manifests itself as a quenching of the fluorescence. The results are consistent with the hypothesis that the selectivity of EB for mitochondrial DNA can be partially explained by the ability of this organelle to concentrate the dye.

Ethidium bromide (EB)[1] has been known for a long time as a selective mitochondrial mutagen in yeast; the molecule seems to interact specifically with the mitochondrial DNA of the cells, and this appears to be the final basis for its mutagenic effects (1–4). Bastos and Mahler (4) have postulated a mechanism for this selectivity, and very detailed studies have been carried out to determine the characteristics of the EB–DNA interactions (5, 6); however, no reasons have been given for the specificity toward mitochondrial DNA. Gitler *et al.* (7) and Azzi and Santato (8) on different grounds, reported also an interaction of EB with animal mitochondria, which was energy dependent. Afterward, several more papers have appeared, describing further details on the effects and interaction of EB with mitochondria (9–12). Simultaneously, some work has been done on what seems a logical premise to the interaction of the dye with mitochondria, the contact and uptake at the level of the surface of the cell and the plasma membrane (13–15). This latter work has given some indication on the possible mechanisms by which EB is taken up by yeast cells to produce its mutagenic effects; experimental evidence has been provided indicating that EB can be taken up by the cells by means of an active and specific system (13). In these latter studies it was found also that when EB is taken up by intact yeast cells, its fluorescence is enhanced.

Once the dye is taken up by the cells, its selectivity toward mitochondrial DNA could be enhanced if the mitochondria could concentrate the dye, as has been proposed (12). In fact, ultrastructural studies in other systems indicate that there is an actual accumulation of EB in the mitochondria (11). The present paper contains the description of experimental work performed to gain further insight into the possible interactions of ethidium bromide with the yeast cell. The data support previous views that EB is taken up actively by the cells, and concentrated

[1] Abbreviations used: EB, ethidium bromide; TEA, triethanolamine; FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone.

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TABLE I

THE UPTAKE OF EB WITH GLUCOSE OR ETHANOL AS SUBSTRATE: EFFECTS OF OXYGENATION AND UNCOUPLING[a]

Condition	EB uptake (nmol(100 mg 10 min)$^{-1}$)
No substrate	62.4 ± 27
No substrate + H_2O_2	57.6 ± 21
No substrate + FCCP	48.0 ± 24
No substrate + H_2O_2 + FCCP	52.8 ± 31
Glucose	225.6 ± 21
Glucose + H_2O_2	230.4 ± 13
Glucose + FCCP	211.2 ± 31
Glucose + H_2O_2	220.8 ± 10
Ethanol	53.0 ± 26
Ethanol + H_2O_2	202.0 ± 13
Ethanol + FCCP	30.0 ± 21
Ethanol + H_2O_2 + FCCP	62.0 ± 13

[a] Experimental conditions were the following: 13.3 mM maleate-TEA, pH 6.0; 166 μM EB; yeast cells, 300 mg wet wt. Where indicated, one or more of the following were included: 66.7 μM glucose, 166 μM ethanol; 0.02% hydrogen peroxide; or 8 μM FCCP. Final volume was 3.0 ml; temperature, 30°C. The incubation was started by the addition of yeast cells to the incubation mixture previously equilibrated to 30°C. After 10 min, an aliquot was taken and centrifuged for 10 s in a microfuge. The supernatant was diluted 15-fold, and its fluorescence measured at 530 → 590 nm. The EB concentrations were calculated from readings obtained with a standard curve. Values are given as the means of five experiments performed in different days ± the standard deviation from the mean.

afterward by the mitochondria, also by an energy-dependent mechanism.

EXPERIMENTAL

Yeast was obtained commercially, and prepared as described previously (16), by incubating in a culture medium and aerating after to starve the cells. Ethidium bromide uptake and fluorescence changes were performed as described before (13). K^+ movements were recorded also as described (14) by means of a cationic electrode. Yeast mitochondria were prepared by the method developed by Peña *et al.* (16). Incubation conditions and procedures are described in each experiment. Oxygen consumption was measured in a temperature-controlled chamber, by means of a Clark oxygen electrode and an adequate recording system.

RESULTS

Since many of the results of this work depend on the energy state of the cell, dependent itself on either fermentation or respiration, several simple experiments were performed to confirm the effects of different conditions on three energy-requiring parameters, the uptake of ethidium bromide, the uptake of K^+, and the efflux of K^+ produced by EB. In the results presented in Table I, it can be seen that energy for the uptake of EB can be provided by glucose or ethanol, but the latter requires of an adequate supply of oxygen. The uncoupler FCCP[2] can reduce the uptake of EB, but only when ethanol, a respiratory substrate is provided. The concentrations employed of FCCP (8 μM) are of the order required to uncouple mitochondrial oxidative phosphorylation in isolated yeast mitochondria (16) but are ineffective to block the uptake of the dye when glucose is used as the substrate. There is one more fact that requires mention; the cells are incubated in the presence of the dye, and the uptake is measured by its disappearance; there is a small amount which is taken from the medium, insensitive to the energy state of the cell, which is probably due to binding of EB to the exterior of the cells.

It has been described before that yeast cells can be energized to take up K^+ from the medium by several substrates (17). We used again glucose and ethanol to energize the uptake of K^+, and the results were similar to those of EB uptake (Fig. 1). Both glucose and ethanol plus H_2O_2 can be used as substrates, and the latter can be blocked by the addition of low concentrations of an uncoupler, while with glucose, much higher concentrations of uncoupler are required to block the uptake. Antimycin A has no effect with glucose at concentrations up to 20 μg/ml, but blocks K^+ uptake with ethanol–H_2O_2 as substrate at concentrations of 2 μg/ml (data not shown).

Ethidium bromide and other cationic dyes, seem to interact with the system for the uptake of monovalent cations in yeast

[2] We appreciate the kind gift of FCCP by Dr. P. G. Heytler, Dupont de Nemours, Wilmington, Del.

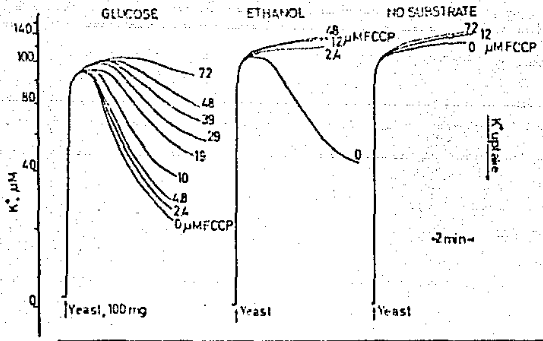

FIG. 1. Use of ethanol and glucose as substrates, and effect of FCCP on K^+ uptake by yeast. Incubation conditions: 16 mM maleate-TEA, pH 6.0, 60 mM glucose or 166 mM ethanol; with ethanol, 0.02% hydrogen peroxide was also included; yeast, 100 mg wet wt. The indicated concentrations of FCCP were added as small volumes of a 6 mM solution in dimethyl formamide. No exogenous K^+ was added. Final volume was 5.0 ml, and temperature, 25°C.

(13–15); besides, when added in a medium in which no K^+ has been added, the dye can produce the efflux of K^+ (15), in a process that appears to be electrogenic and energy dependent (14). The energy requirements of this same process were tested also with the two previously used substrates, and the addition of an uncoupler. The results of Fig. 2 show that also in the case of the efflux of K^+ from yeast, energized by ethanol plus H_2O_2, low concentrations of FCCP can block the phenomenon. When glucose was the substrate, the concentrations of FCCP required to block the efflux were much higher.

In summary, it seems that the requirements of energy are of a similar type for the uptake of EB, the uptake of K^+ and the efflux of K^+ produced by EB. Finally, it could be shown, using ethanol as substrate, and the uncoupling effect of FCCP, that energy is required to produce the efflux of K^+ by EB during the whole process, and not only to initiate it. The results of Fig. 3 show that deenergization of the cells by FCCP can stop immediately the efflux of K^+ produced by EB.

In previous work (13), it was found that the energization of yeast cells can produce an increase in the fluorescence of EB, which may be coincident with its penetration into the cells. By modifying the concentrations of the dye and that of yeast cells, a value can be found in which, as shown in Fig. 4, there is an initial increase of fluorescence that reaches a plateau after some time, but then starts again to increase rapidly, as

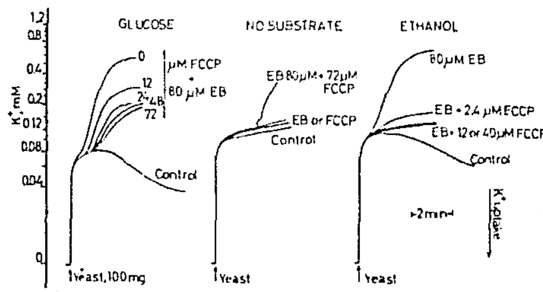

FIG. 2. Effects of EB (80 μM) on K^+ movements by yeast with two different substrates. Experimental conditions were as for Fig. 1, but 80 μM EB was included in the indicated tracings.

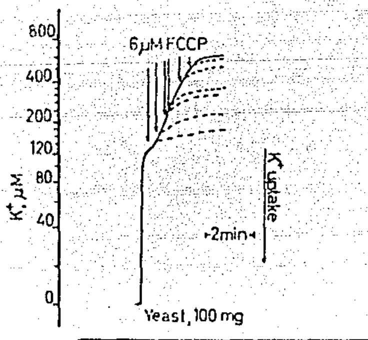

FIG. 3. Effect of the addition of FCCP at different times, upon the efflux of K^+ produced by EB with ethanol as substrate. The experimental conditions were similar to those of Fig. 2, with ethanol–H_2O_2 as substrate, 80 μM EB, and 6 μM FCCP.

shown in tracings A, B, C, G, H, and I of Fig. 4. This second phase of fluoresence increase is coincident with the depletion of oxygen from the incubation medium. The importance of oxygen can be demonstrated as in tracings B and H of the same figure, by the addition of H_2O_2, that produces a decrease of the fluorescence if added after anaerobiosis is reached. However, the sole addition of hydrogen peroxide does not seem to be enough: if it is added after FCCP, an uncoupler, it has practically no effect (tracings C, I, and E). The presence of H_2O_2 during the whole incubation prevents the installation of anaerobiosis, and so, the second phase of fluorescence increase does not appear unless FCCP is added (tracings D and J). To summarize, the first increase of fluorescence seems to depend on the energization of the cell, whether by respiration or fermentation; the second increase, on the other hand, seems to depend on deenergization of the mitochondria, that can be attained either by anaerobiosis or by uncoupling.

The experiments presented in Figs. 5 and 6 show clearly that FCCP can produce an increase of EB fluorescence in yeast only in aerobiosis, either with glucose or ethanol as substrates. This was tested in two ways; the first one was by the addition of FCCP at different times, before or after the anaerobic state was reached. Both with ethanol and glucose as substrate, the addition of FCCP before anaerobiosis was attained, produced an increase of the fluorescence. If the uncoupler was added after anaerobiosis, with ethanol as substrate, no change was produced, but with glucose a further increase of fluorescence was observed upon the addition of the uncoupler. If, however, both with ethanol and glucose, anaerobiosis was avoided by the inclusion of H_2O_2, the

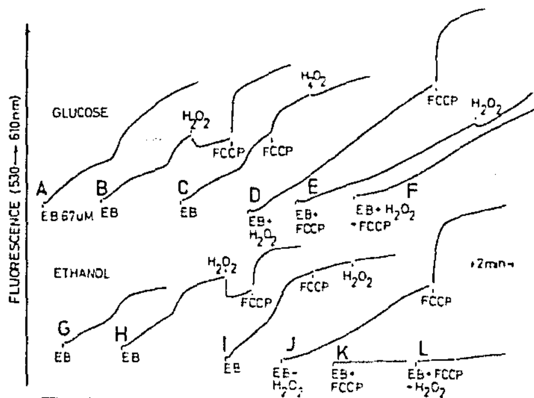

FIG. 4. Fluorescence changes of EB (530 → 610 nm) upon its interaction with yeast cells under different conditions. Incubation: 16 mM maleate-TEA, pH 6.0; 67 mM glucose, or 166 mM ethanol; 0.02% H_2O_2; EB was 67 μM; FCCP was 4 μM, yeast cells 25 mg wet wt. Final volume was 3.0 ml, and temperature, approximately 20°C. EB was added 2 min after yeast cells.

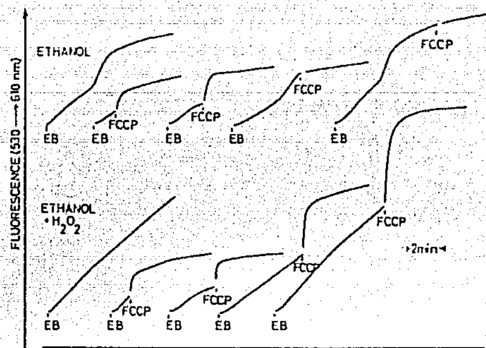

FIG. 5. Effect of the addition of FCCP at different times upon the fluorescence changes of EB to yeast cells with ethanol as substrate, with or without H_2O_2. Incubation conditions were as for Fig. 4, but only ethanol was used as substrate.

addition of FCCP produced the fluorescence increase at any time of the tracing. In other words, when added to aerobic cells, FCCP always produces an increase of the fluorescence of EB. When the uncoupler is added to anaerobic cells, no change is seen if ethanol is the substrate, and a small increase can be observed when glucose is being consumed. This is not illogical; fermentation of glucose can be expected to support mitochondrial energization under anaerobic conditions.

Since Gitler *et al.* (7) and Azzi and Santato (8) have found that with energized liver mitochondria, the interaction of EB produces an increase of fluorescence, the results here reported seem to be somewhat unexpected; increased fluorescence seems to be observed upon deenergization of mitochondria. However, unpublished results from our laboratory, and from Estrada (also unpublished),[3] show that with liver mito-

[3] Estrado-O., S., personal communication.

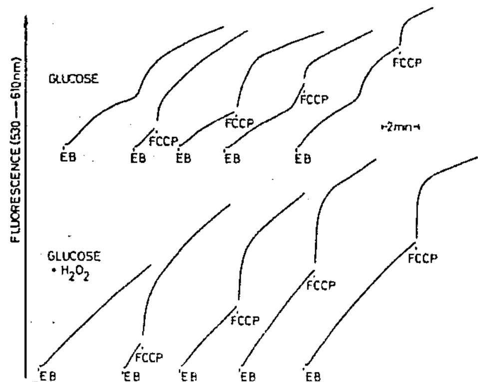

FIG. 6. Effect of the addition of FCCP at different times upon the fluorescence changes of EB with yeast cells, with glucose as substrate, with or without H_2O_2. Incubation conditions were as for Fig. 4, but only glucose was used as substrate.

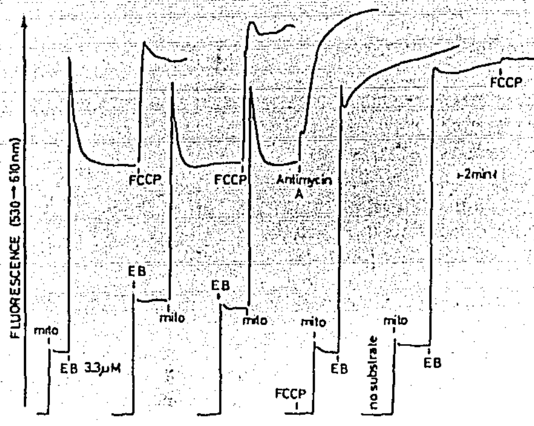

FIG. 7. Fluorescence changes of EB upon interacting with yeast mitochondria. Incubation conditions: 0.6 M mannitol, 0.1% defatted albumin, 10 mM phosphate-TEA, pH 6.5, 160 mM ethanol, except where indicated; EB was 3.3 μM, FCCP 4 μM, and 3 μg of antimycin A was added. 426 μg of mitochondrial protein were used per trace. Final volume was 3.0 ml; temperature was approximately 20°C.

chondria, when low concentrations of EB are used (around 3 μM), the findings of literature (7, 8) are confirmed; there is an increased fluorescence upon energization, and vice versa. If concentrations of 30 μM or more are used, the contrary is observ[ed]: energization of mitochondria produces a [de-]crease, and deenergization an increa[se in] fluorescence.

To test this possibility, first the experiment shown in Fig. 7 was performed using isolated yeast mitochondria. At 3.3 μM concentration, upon the addition of EB to mitochondria with a substrate, a large increase of fluorescence is observed, followed by a decrease. Deenergization of the mitochondria afterward, produces an increase, instead of a decrease in the fluorescence. Deenergization could be produced either by uncoupling or by inhibition of respiration. If FCCP was added before mitochondria, or if substrate was omitted, the decrease of fluorescence was not observed. The concentration dependence of the mentioned fluorescence changes can be easily observed in Fig. 8; opposite changes upon energization and deenergization are produced when ethidium bromide interacts with mitochondria at 0.66 or at 3.3 μM concentrations.

There may be a very simple explanation for the concentration dependence of the fluorescence changes of EB upon its interaction with mitochondria in different energy states. With liver mitochondria, it is accepted that upon energization, EB is bound to the mitochondrial membrane (7, 8) and this tends to increase its fluorescence. However, the fluorescence of the dye depends very much on its concentration. The data presented in Fig. 9 show clearly that it does not matter if EB is in a medium of low or high dielectric constant, fluorescence is quenched at high concentrations of the dye.

The results of Table I could be interpreted either as binding or as uptake of EB by the cells at the level of the cell membrane. The fluorescence changes indicate that the molecule actually reaches the mitochondria of the cell; however, further proof of this was obtained by determining the effects of EB on respiration by intact cells. Again, if respiration is inhibited by the dye, this is most probably because it reaches the mitochondria. The results of Fig. 10 show that, in fact, EB can inhibit respiration, as expected from our hypothesis. Also in agreement with the fluorescence results, which indicate that uncouplers block the interac-

tion of EB with mitochondria, the addition of FCCP reverses the inhibition of respiration produced by several concentrations of the dye.

Figure 11 presents also the results of an experiment in which glucose or ethanol were used to test the inhibition of respiration by 320 μM EB. The dye inhibits respiration significantly only under conditions in which mitochondria and the cell are energized. It does not inhibit if the mitochondria are deenergized, even if the rest of the cell has an adequate energy level, as is the case when glucose in the presence of 3.6 μM FCCP is the substrate. As reported before (13), before inhibition appears, EB produces a stimulation of respiration, both with glucose or ethanol as substrates.

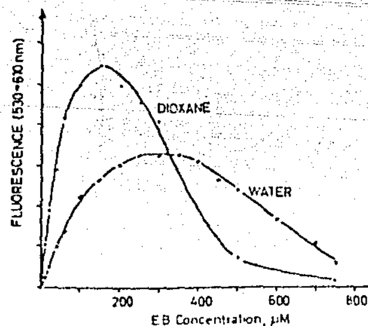

FIG. 9. Fluorescence of EB at different concentrations in media of different dielectric constants. Fluorescence was measured at 530 → 610 nm in 10 mM phosphate-TEA, pH 6.5, or in dioxane. The maximal volume of 20 mM EB added was 112.5 μl, to a final volume of 3.0 ml.

DISCUSSION

The first part of the work was designed to test different energy sources for what may be considered as plasma membrane phenomena, K^+ uptake, EB uptake, and the efflux of K^+ that EB can produce in yeast cells. The results agree with previous reports (17) indicating that ethanol-O_2 can be used also as an effective substrate to energize the cells. The effects of FCCP are interesting, in the sense that, as previously found (18), higher concentrations are required to inhibit the uptake of K^+ or other plasma membrane phenomena, than to uncouple mitochondria (16). The experiments provide a system in which cells can be deenergized by the use of uncouplers at low concentrations when ethanol is the substrate, but besides, with glucose as substrate, FCCP at low concentrations produces the selective deenergization of the mitochondria, while the cell itself maintains its energy supplies. Besides, yeast can be used to determine the energy requirements of several systems, as well as the relevance that mitochondrial function may have for them. From this point of view, the three parameters studied show the same characteristics; they require energy that can be provided by mitochondrial oxidative phosphorylation, but if energy can be obtained from fermentation, blocking of mitochondrial function by uncoupling or anaerobiosis does not alter the studied phenomena that occur in the plasma membrane.

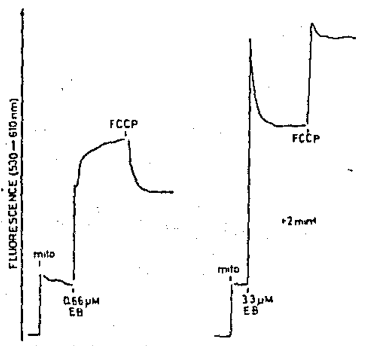

FIG. 8. Dependence of the fluorescence changes of EB with yeast mitochondria on the concentration of the fluorescent probe. Incubation conditions were as for Fig. 7, but as indicated, 0.66 or 3.3 μM concentrations of EB were used; ethanol was the substrate.

Although it may be difficult to decide if the disappearance of EB from the medium represents uptake or simply binding, there are several indications that the dye is actually getting inside the cells: (a) the changes of fluorescence observed, that are related to the energy state of the mitochondria are more easily explained by a direct interaction of the dye with these organelles; (b) EB inhibits respiration, and the inhibition

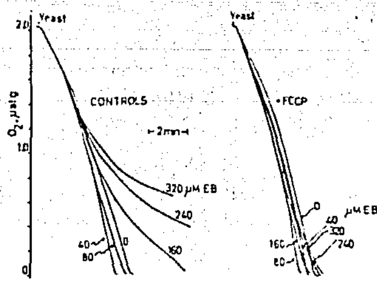

FIG. 10. Effect of several concentrations of EB on respiration of intact yeast cells, both in the presence and in the absence of FCCP. Incubation conditions: 16 mM maleate-TEA buffer, 80 mM glucose, yeast cells, 25 mg wet wt; final volume 5.0 ml: temperature, 250°C. EB was added at the indicated concentrations (μM). FCCP was 3.6 μM.

is also related to the energy state of the mitochondria; it would be complicated to explain this inhibition by a mechanism that did not involve the direct contact of EB with mitochondria, (c) in other cells, EB has been found to accumulate in the mitochondria (11), and (d) if EB produces effects on mitochondrial DNA, it must be because it reaches it, and to this purpose it has to be taken up by the mitochondria. It is obvious of course that to interact with the mitochondria, EB has to be taken up before with the cells, as it was suggested some time ago by Armstrong, for other dyes (19). However, it is difficult to know how much of the amount that is taken up actually gets into the cell or mitochondria, and how much remains bound to the cell. It is possible that the amount of bound EB that does not get into the cells is that taken up in the absence of any substrate.

Both from the experiments with intact cells, those with isolated yeast mitochondria, those performed with liver mitochondria (12), as well as from data in other systems (1, 11) it has to be accepted that EB shows a real interaction with mitochondria. At least in one respect, both in intact cells and with isolated mitochondria, the fluorescence studies indicate that this interaction depends on the energy state of the cell. The assumption is supported by the fact that the dye produces a much lower inhibition of respiration when the mitochondria are deenergized more or less selectively by low uncoupler concentrations.

The data on EB fluorescence obtained with isolated mitochondria can be explained in many ways. The fluorescence of this dye is sensitive to the polarity of the environment of the molecules (7, 8); data are not presented, but the fluorescence spectrum and intensity of EB are also modified by pH and viscosity of the medium, as well as by the concentration of the dye in solution. Reported fluorescence enhancement upon the interaction of the dye with energized mitochondria (7, 8) was explained by the change of polarity of the environment of the EB molecules bound, by an energy-dependent process, to the mitochondrial membrane.

The changes of fluorescence of EB at higher concentrations upon interaction with yeast mitochondria seem to be opposite to those already reported (7, 8), but data that are not published, similar to those of this paper have been obtained with liver mitochondria both by us and by Estrada.[3] Several explanations can be offered, as the interaction of the dye with membrane or matrix components that might quench its fluorescence, changes of polarity, viscosity or pH of the microenvironment of the molecules, or its simple concentration, either in the membrane or in the matrix, or in both mitochondrial components. The data of Fig. 9 provide support to this latter idea; the con-

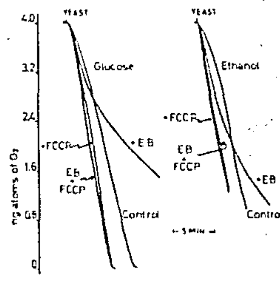

FIG. 11. Effect of 320 μM EB on respiration of intact yeast cells, with ethanol or glucose as substrates. Experimental conditions were as in Fig. 10, but only one concentration (320 μM) of EB was employed. Ethanol concentration was 133 mM.

centration of the dye can quench its fluorescence, whether it is in a hydrophobic or hydrophilic environment. Actually, any of the other mechanisms mentioned to quench fluorescence would require the concentration of the dye, because it would be necessary that a large fraction of the added molecules of EB were subject to any of those changes to observe the quenching. Given the small volume of the mitochondria under the incubation conditions, that represents at most a few microliters, any mechanism postulated requires the accumulation of the dye by the mitochondria. In support of this, data have been obtained with liver mitochondria that show that EB requires lower concentrations to inhibit respiration in the coupled (state 3) that in the uncoupled state or with inverted submitochondrial particles (12), in agreement with the data of Figs. 10 and 11. Besides, also liver mitochondria are able to take up significant amounts of EB in an energy-dependent process (12). It may be pertinent to mention, besides, that binding of EB to specific molecules, like DNA produces an increase, and not a decrease of fluorescence (20).

The active uptake of EB by the mitochondrion provides an attractive hypothesis to explain the selectivity of the dye to intercalate with mitochondrial DNA. It is true that EB is an intercalating drug (3, 5, 6), but there is no explanation for its preference for mitochondrial DNA. Since EB is a cationic organic molecule, the electrochemical potential of the organelle could be the driving force for its uptake, accumulation, and selective action.

Studies are being carried out to determine the effects that EB could have on the energetics of yeast mitochondria, since in liver mitochondria it has been reported that the dye inhibits oxidations and ATPase (12), behaves as an uncoupler (9, 12), and inhibits adenine nucleotide translocation (10). These antecedents, with high probability allow us to expect a series of effects of EB on yeast mitochondrial functions.

REFERENCES

1. Slonimski, P., Perrodin, G., and Croft, (1968) *Biochem. Biophys. Res. Commun.* 30, 232–239.
2. Mahler, H. R., and Perlman, P. S. (1972) *J. Supramol. Structure* 1, 105–123.
3. Waring, M. J. (1965) *J. Mol. Biol.* 13, 269–282.
4. Bastos, R. N., and Mahler, H. R. (1974) *J. Biol. Chem.* 249, 6617–6627.
5. Tsai, C. C., Jain, S. C., and Sobell, H. M. (1977) *J. Mol. Biol.* 114, 301–315.
6. Jain, S. C., Tsai, C. C., and Sobell, H. M. (1977) *J. Mol. Biol.* 114, 317–331.
7. Gitler, C., Rubalcava, B., and Caswell, A. (1969) *Biochim. Biophys. Acta* 193, 479–481.
8. Azzi, A., and Santato, M. (1971) *Biochem. Biophys. Res. Commun.* 44, 211–217.
9. Miko, M., and Chance, B. (1975) *FEBS Lett.* 54, 347–352.
10. Grimwood, B. G., and Wagner, R. P. (1976) *Arch. Biochem. Biophys.* 176, 46–52.
11. McGill, M., Baur, P. S., and Hsu, T. C. (1976) *Exp. Cell. Res.* 99, 7–14.
12. Peña, A., Chávez, E., Cárabez, A., and Tuena de Gómez-Puyou, M. (1977) *Arch. Biochem. Biophys.* 180, 522–529.
13. Peña, A., and Ramírez, G. (1975) *J. Membrane Biol.* 22, 369–384.
14. Peña, A. (1978) *J. Membrane Biol.* 42, 199–213.
15. Peña, A., Mora, M. A., and Carrasco, N. (1978) *N. Membrane Biol.*
16. Peña, A., Piña, M. Z., Escamilla, E., and Piña, E. (1977) *FEBS Lett.* 80, 209–213.
17. Ryan, H., Ryan, J. P., and O'Connor, W. H. (1971) *Biochem. J.* 125, 1081–1089.
18. Peña, A. (1975) *Arch. Biochem. Biophys.* 167, 397–409.
19. Armstrong, W. McD. (1958) *Arch. Biochem. Biophys.* 73, 153–160.
20. Olmsted, J., III, and Rearns, D. R. (1977) *Biochemistry* 16, 3647–3654.

PRIVILEGED COMMUNICATION

Title: Correlation between Resistance to Ethidium Bromide and Changes in Monovalent Cation Uptake in Yeast[1]

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Running title:

Ethidium resistance and cation uptake

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Index listing entries:

- Ethidium bromide
- Mitochondria
- Yeast
- Ion uptake
- Fluorescent monitors
- Cationic dyes

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FOOTNOTES

1. This work was partially supported by grants No. 1646, PCCBNAL-790256 and 1669 of the Consejo Nacional de Ciencia y Tecnología of Mexico.

2. Abbreviations:

   EB, ethidium bromide; CTAB, cetyltrimethylammonium bromide; TEA, triethanolamine; MES, 2-(N-Morpholino) ethanesulfonic acid

Correlation between Resistance to Ethidium Bromide and Changes in Monovalent Cation Uptake in Yeast.

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The mutagenic properties of ethidium bromide (EB)[2] and other intercalating drugs (1) have been studied widely, particularly because of the selectivity of these agents in impairing mitochondrial functions (1-3). The basis for the action of EB seems to be its ability to intercalate with the DNA molecules (4-6), but additional factors may be involved which have to do with particular characteristics of the interaction of EB with mitochondria (7-9). Mutants of Kluyveromyces lactis that are resistant to higher concentrations of EB in the culture medium than are the wild type strains have been described (10). These mutants, in many cases, show cross resistance to decamethylene biguanidine and to octylguanidine (10-12), molecules that have both shown effects on K^+ uptake by yeast (13). Also, a brief report has been published (12) on what seems to be a difficulty of the mutants to take up the mutagen. Since a series of interactions of the mutagen with the cell have to take place in order to obtain the final effect on mitochondrial DNA (9), several explanations can be given for the resistance to the drug. Besides

this, the uptake of EB in yeast cells has been found to show interesting characteristics; it seems that this transport has some relationship to the system for monovalent cation uptake (14). With all these antecedents, it was considered of interest to study some of the interactions of EB with a K. lactis EB-resistant mutant as compared to its parental strains, including the measurement of the dye uptake. Data were found that indicate some relationship between resistance to the drug and an alteration in the ability of the cells to take up monovalent cations.

MATERIALS AND METHODS

Three strains of K. lactis were employed throughout this work. The two sensitive ones were W600B, designated in the paper as S_1, and WM37, designated as S_2. These haploid strains were kindly supplied by Dr. James R. Mattoon. The strain resistant to EB was KA6-8A, and was obtained from the other two strains. The general characteristics of this type of strains have been described before (10,11). Strain KA6-8A was purified and a resistant clone selected before initiating the work.

Cells were grown in the medium described by De Kloet et al. (15) during approximately 24 hours in a girotory shaker at 250 rpm, using a loopful of cells from a slant as inoculum. This was used as the seed, either immediately or after up to one week around 25 ml (approximately 0.24 ml of cytocrit value) of this was used to inoculate 1 l of medium, which was incubated in the shaker at 30° for 24 hours.

After growth, the cells were collected and washed twice with water by centrifugation, and resuspended in water to give a ratio of 0.5 g of wet weight per ml of the suspension. The cation content of the cells was measured by extraction with 30 percent perchloric acid. The cation concentrations were then measured in the extracts by flame photometry.

K^+ movements were monitored by means of a cationic electrode (Beckman 39047) connected to an electrometer, amplifier and recording system. This same instrument was used to measure H^+ concentrations, using a pH combination electrode.

Fluorescence changes of EB were measured in a Mark I Farrand spectrofluorometer with a recorder attached.

Respiration was followed by means of a Clark electrode and an adequate power source and recording device.

Ethidium bromide uptake was measured by adding the cells to the incubation mixture containing the dye, previously equilibrated to the water bath temperature. Aliquots were then taken to separate the cells in a Microfuge. The concentration of the remaining dye in the supernatant was measured by comparing the absorbance of the supernatant (475 nm) with that of a standard curve of the dye.

The uptake of $^{86}Rb^+$ was measured by incubating the cells with the isotope, filtering and washing with cold 5 mM RbCl to eliminate trapped and bound $^{86}Rb^+$. The radioactivity taken up by the cells was measured by means of an end window-gas flow detector or by scintillation counting. To get the values of Km and Vmax, the chi square method was used.

RESULTS

As previously mentioned a significant difference can be consistently observed between the parental strains and the resistant mutant, with respect to the ability to grow at higher EB concentrations in the medium. The mutant used throughout this work, KA6-8A, carries either a single nuclear or several closely linked nuclear mutations that confer resistance to ethidium bromide. The mutant was derived from a cross between W600BR: α, Ade_1, ade_2, leu, EB^R, and WM37: a, his, (10,11), and can grow in glycerol medium containing 10uM EB, whereas sensitive strains can only tolerate 5 uM EB.

EB uptake by the wild type and the mutant strains

Since the mutant resistant to EB used in this work (KA6-8A) is different to those employed before (12), it had to be verified if it has also an impairment to take up EB. In fact, this was found to be the case, as shown by Fig. 1. Along the time studied, EB was taken up normally by the wild type strains (S_1 and S_2). It should be mentioned also that the experimental conditions are somewhat different to those employed before (12).

Comparative fluorescence changes of EB

Fig. 2 shows that, under the same conditions used to measure the uptake of EB, again, both wild type strains showed an increase of fluorescence when the monitor was added. The resistant mutant, on the other hand, did not show any increase of fluorescence beside the initial one that can be observed in the absence of the cells. This is in full

agreement with the data reported for other mutants (12) studied before.

Uptake of monovalent cations and effects of EB.

It was reported before that EB seems to show a rather specific interaction with the system for monovalent cation uptake in yeast (14). This fact suggested the possibility that the EB resistant mutant, that has an impaired ability to take the mutagen, might have also an impairment of the uptake of this monovalent cation.

The measurement of the uptake of K^+ (Fig. 3) revealed an important impairment in the case of the resistant mutant of K. lactis, as compared with the wild type strains. The uptake of K^+ was studied with the endogenous K^+ or with 1 mM cation added, with similar results. It is important that, as shown in the last three tracings of the figure, by disrupting the cells with CTAB, similar amounts of monovalent cations are released from both the wild type and the resistant strain.

Measurements were made also of the content of several cations, including K^+ in the three strains used. Table I shows that there was no significant difference in the content of the four cations determined.

Fig. 4 shows also the ability of both the sensitive and the resistant strains to take up $^{86}Rb^+$. This experiment may give more information about the influx of the monovalent cation. The initial rates of uptake are much higher for the wild type that for the EB resistant strains.

EB and other cationic dyes (16), when added to yeast can produce not only the inhibition of K^+ uptake, but also the efflux of the internal cation from the cells, in a process that requires of energy (16,17,18).

Since this effect may be due to an interaction of the dyes with the monovalent transport system of yeast, the effect of EB was tested. Fig. 5 shows that especially at the lower EB: yeast ratios, the dye can produce a large efflux of K^+ in the wild type strains, and not in the resistant one. As the amount of cells was decreased, and the amount of dye per cell increased, EB could produce also the efflux of monovalent cation in the resistant strain.

Finally, experiments were carried out to determine the kinetic constants of the three strains for the uptake of Rb^+. As expected, (Fig. 6) there was a large change, both in the Km and the Vm values of the R strain. A large number of experiments had to be accumulated to obtain statistical significance, due to the variability of the results in different experiments.

The proton pumping capacity of the strains.

It has been postulated that yeast cells can transport monovalent cations because of the existence of a proton pump, driven by ATP (19-22) that at low pH can function only if K^+ is added to the medium, but at high values of pH can function in the absence of cations. It seems that at high pH values, proton pumping can become "uncoupled" from monovalent cation transport, and be coupled, perhaps to anion extrusion by the cells. (19). This circumstance offers the possibility of assaying the proton pumping activity of the cells, free from the ion uptake capacity. Although the ability to pump protons is the deriving force for monovalent cation uptake, it can work independently. It was decided to study the proton pumping capacity of the cells under conditions under which it is

uncoupled from monovalent cation uptake, i.e., at pH 7.5 and using ethanol as substrate, to avoid the extensive formation of CO_2 that is produced when glucose is fermented. The results of Fig. 7 show clearly that there are only small differences in the ability of the three strains studied to pump protons.

Effects of EB on respiration.

Another similarity between EB and K^+ is revealed in the stimulation that both agents can produce on respiration. This stimulation was also studied in all strains. The results of Table II show that neither EB nor K^+ can stimulate respiration in the R mutant. On the other hand, as found with Saccharomyces cerevisiae (14), in both wild type strains used in this work, respiration was stimulated by the addition of EB or K^+.

DISCUSSION

The data presented seem to be rather consistent to indicate a close relationship between the transport of monovalent cations and that of EB by yeast cells, that was suggested before (14). The data of this paper pointing in that direction are: a) The low capacity of a mutant resistant to EB to take up the dye, coincident with a low capacity to take up monovalent cations; b) the absence of fluorescence changes observed for EB in the resistant mutant, as compared with the two wild type strains; c) the incapability of EB to produce the efflux of monovalent cations in the resistant mutant within moderate concentration ranges, and d) the absence of stimulation of respiration produced both by EB and K^+ in the

mutant, as compared with the normal effect observed in the wild type strains.

These data are in agreement with the conclusions reached before (14), based in the following facts: EB can inhibit competitively the transport of monovalent cations (Rb^+); monovalent cations can inhibit the uptake of EB with a selectivity pattern similar to that of the monovalent cation transport; EB, can stimulate the extrusion of H^+, as K^+ does, and both agents can produce a stimulation of respiration. This agreement is also interesting because the experiments reported in this paper were performed with a different yeast, K. lactis, and those reported before (14), were obtained with a strain of S. cerevisiae.

It is difficult to postulate a mechanism for the inhibition of monovalent cation uptake produced by EB. Except for the fact that is a cationic dye, it is difficult to imagine how the competition between the organic and the inorganic cation may take place, especially because of the complicated structure of EB. However, the data of this paper add further support to the idea that the mutagen is taken up by the cells through the same system as monovalent cations. No other lesion could be demonstrated besides the inability to transport EB and K^+ by the mutant. The possibility was excluded that the incapacity were in what has been postulated the driving force for monovalent cation uptake, an ATP driven proton pump (19-22). The results of Fig. 7 show that both the wild type and the mutant strains have a similar ability to pump H^+, and the lesion must be something that causes an impairment of the function of the transport system.

The kinetic data for the uptake of Rb+, comparing the wild type strains with the EB resistant one, may suggest a profound alteration of the transport system. Starting with the curves of Fig. 4, in which 4 mM Rb^+ was used, a concentration well above the Km for the transport of the cation in the wild type strains, a large decrease is observed in the rate of transport of the cation. The changes of the kinetic constants for the EB resistant mutant are large both for the Km and for the Vm.

The content of cations of the mutant, almost normal in comparison with the wild type strains was rather unexpected. However, it must be mentioned that the ability of the cells to transport monovalent cations is diminished, but not absent. It is possible that the wild type strains have an excess capacity to transport monovalent cations and that in the EB resistant mutant, the low rate of uptake of monovalent cations may be enough to maintain a normal content of them, inside the cells. Besides, the normal content of other cations in the cells is in further agreement with the generally normal conditions of the mutant strain.

Experiments are in progress to obtain more information on the lesion (s) involved in the resistance of the mutant to EB and the difficulty to take up monovalent cations. The mutant seems to be promising in this respect; besides, it is important to point out that this is the fourth of this kind. It is possible that the alteration on the uptake of cations, organic and inorganic, is a common feature that may allow the adaptation of yeast to unfavorable conditions. This latter fact in itself is stimulating to further study the EB resistant strains.

REFERENCES

1. Markovich, H. (1951). Ann. Inst. Pasteur, 81, 452-468.
2. Bulber, C.J.E.A. (1964). Antonie Van Leeuwnhoek J. Microbiol. Serol., 30, 1-9.
3. Slonimski, P., Perrodin, G. and Croft, J. (1968). Biochem. Biophys. Res Comm., 30, 232-239.
4. Waring, M.J. (1965) J. Mol. Biol., 13 269-282.
5. Tsai, C.C., Jain, S.C. and Sobell, H.M. (1977) J. Mol. Biol. 114, 301-315.
6. Jain, S.C. Tsai, S.C. and Sobell, H.M. (1977) J. Mol. Biol. 114, 317-331.
7. Bastos, R.N. and Mahler, H.R. (1974). Biol. Chem., 249, 6617-6627.
8. Peña, A., Chávez, E., Cárabez, A. and Tuena de Gómez Puyou, M. (1977). Arch. Biochem. Biophys. 180, 522-529.
9. Peña, A., Clemente, S.M., Borbolla, M., Carrasco, N. and Uribe, S. (1980). Arch. Biochem. Biophys. 201, 420-428.
10. Brunner, A., Mas, J. Célis, E. and Mattoon, J.R. (1973). Biochem. Biophys. Res. Comm., 53, 638-644.
11. Célis, E., Mas, J. and Brunner, A. (1975). Genet. Res. Camb. 25, 59-69.
12. Célis, E., Mas, J. and Brunner, A. (1975) FEBS Letters, 57, 241-244.
13. Peña, A. (1973) FEBS Letters, 34, 117-119.
14. Peña, A. and Ramírez, G. (1975) J. Membrane Biol. 22, 369-384.
15. De Kloet, S.R., Van Wermeskerken, R.K.A. and Koningsberger, V.V. (1961) Biochim. Biophys. Acta, 47, 138-143.
16. Peña, A., Mora, M.A. and Carrasco, N. (1979). J. Membrane Biol. (1979) 47, 261-284.
17. Peña, A. (1978) J. Membrane Biol. 42, 199-213.

18. Borbolla, M. and Peña, A. (1980) J. Membrane Biol. 54, 149-156.

19. Peña, A., Cinco, G., Gómez-Puyou, A. and Tuena, M. (1972) Arch. Biochem. Biophys. 153, 413-425.

20. Peña, A., Cinco, G., Gómez-Puyou, A. and Tuena, M. (1969) Biochim. Biophys. Acta, 180, 1-8.

21. Peña, A., (1975). Arch. Biochem. Biophys. 167, 397-409.

22. Peña, A. (1976) In Mitochondria, Bioenergetics, Biogenesis and Membrane Structure. Packer, L. and Gómez Puyou, A., Editors. Academic Press pp 21-30.

Table I. Content of Several Cations by the Three Strains of *K. lactis* Used in this Work.

Strain	µmoles (g of yeast)$^{-1}$		
	K^+	Na^+	Ca^{2+}
S_1	74.0	10.2	36.0
S_2	72.5	12.4	25.0
R	72.0	8.2	24.4

The cations were extracted with 30% perchloric acid from a suspension containing 250 mg. of yeast. The contents of the cations were measured by flame photometry. Each figure is the mean of two determinations.

Table II. Effects of EB and K^+ on Respiration by Wild Type and EB Resistant Strains of *K. lactis.*

Strain	Ratio to a control without any addition		
	10 mM KCl	200 uM EB	300 uM EB
S_1	1.17-1.17	1.08-1.05	1.03-1.05
S_2	1.28-1.33	1.08-1.23	1.07-1.13
R	1.03-0.98	0.98-0.95	0.95-0.95

Incubation: 20 mM MES-TEA buffer, pH 6.0; 108 mM ethanol; yeast cells, 50 mg; final volume, 5.0 ml; Temperature, 25°. Respiration rates were measured at 1 min after the addition of yeast.

FIGURE LEGENDS

Fig. 1. Uptake of EB by two wild type and one resistant strain of K. lactis to EB. Experimental conditions: 100 mM glucose; 20 mM MES-TEA buffer, pH 6.0; yeast cells, 200 mg; final volume, 12 ml; temperature, 25°. The incubation was started by the addition of the cells. The measurement of EB uptake was carried out as described in the Methods section. EB concentration was 266 uM.

Fig. 2. Fluorescence changes of EB upon its interaction with S and R strains of K. lactis. Incubation conditions were as follows: 100 mM glucose; 20 mM MES-TEA buffer, pH 6.0; yeast cells, 50 mg; final volume, 3.0 ml; room temperature. The incubation was started by the addition of the yeast cells, and after 2 min, EB (266 μM) was added and the fluorescence changes recorded.

Fig. 3. K^+ movements by the S and R strains of K. lactis, in a medium containing 1 mM KCl or with the endogenous content of monovalent cations. Incubation conditions: 20 mM MES-TEA buffer, pH 6.0; 100 mM glucose; 1.0 mM or no added KCl. Final volume was 5.0 ml; temperature, 25°. In the last three tracings, substrate was omitted, and only 10 mg of yeast were added after the inclusion of 400 μM CTAB.

Fig. 4. Transport rate of $^{86}Rb^+$ by the S and R strains of K. lactis. Incubation: 100 mM glucose; 20 mM MES-TEA buffer, pH 6.0; 4.0 mM $^{86}RbCl$;

yeast, 100 mg; final volume, 2.0 ml; temperature, 25°. After equilibrating the temperature of the medium, the incubation was started by the addition of the yeast cells. Then aliquots were taken, filtered and washed several times with 5 mM RbCl. The filters were dried and counted in scintillation vials.

Fig. 5. Effects of EB (200 μM) on the K^+ movements of the three strains of K. lactis in a K^+ free medium. Incubation: 8 mM maleate-TEA buffer, pH 6.0; 40 mM glucose; final volume, 5.0 ml. Temperature, 25°. Yeast cells were added after equilibrating the temperature of the medium. EB and CTAB were added where indicated at 200 μM concentration.

Fig. 6. Kinetic analysis of the transport of $^{86}Rb^+$ by the S and R strains of K. lactis. Incubation conditions were as for Fig. 4, but yeast cells were added to the medium without $^{86}Rb^+$. After two minutes, the isotope was added; an aliquot was taken after two more minutes, filtered and treated as described for Fig. 4.

Fig. 7. Measurement of H^+ extrusion by the S and R strains of K. lactis at pH 7.5. Incubation conditions: 6.5 mM MES-TEA buffer, pH 7.5; 135 mM ethanol; approximately 1.5 mM H_2O_2; yeast, 250 mg; final volume, 6.2 ml; temperature, 25°C. Yeast cells of the different strains were added where indicated and the pH was recorded. A calibration curve was constructed by the addition of HCl to the incubation medium.

SUMMARY

A mutant of _Kluyveromyces lactis_ resistant to ethidium bromide was studied and found to have an impairment to transport the dye. As described for other mutants of this kind, the fluorescence changes of the dye that are observed when the cells transport it, were not observed in the mutant strain.

Simultaneous to this difficulty to take up the mutagen, the cells showed a diminished ability to take up monovalent cations, as compared to the wild type strains.

The defect of the mutant strain does not seem to reside in the capacity to pump out protons, which also indicates that it has no alterations of the general energy conversion systems. This view is also supported by the fact that the growth yields are similar in both the mutant and the wild type strains.

Both ethidium and K^+ fail to stimulate respiration of the mutant yeast when present in the medium, as compared to the wild type strains.

The mutant strain shows a normal cation content, which indicates that the impairment to take up monovalent cations, although much decreased, may still be enough to maintain a normal content of cations within the cells.

According to the investigation carried out, the mutant cells seem to be normal, except for the fact that they are unable to transport both ethidium and K^+ from the medium. The data support the hypothesis that ethidium bromide and K^+ may be transported by the same system in yeast.

PRIVILEGED COMMUNICATION

Title: Correlation between Resistance to Ethidium Bromide and Changes in Monovalent Cation Uptake in Yeast[1]

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- Ion uptake
- Fluorescent monitors
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FOOTNOTES

1. This work was partially supported by grants No. 1646, PCCBNAL-790256 and 1669 of the Consejo Nacional de Ciencia y Tecnología of Mexico.

2. Abbreviations:

   EB, ethidium bromide; CTAB, cetyltrimethylammonium bromide; TEA, triethanolamine; MES, 2-(N-Morpholino) ethanesulfonic acid

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The mutagenic properties of ethidium bromide (EB)[2] and other intercalating drugs (1) have been studied widely, particularly because of the selectivity of these agents in impairing mitochondrial functions (1-3). The basis for the action of EB seems to be its ability to intercalate with the DNA molecules (4-6), but additional factors may be involved which have to do with particular characteristics of the interaction of EB with mitochondria (7-9). Mutants of Kluyveromyces lactis that are resistant to higher concentrations of EB in the culture medium than are the wild type strains have been described (10). These mutants, in many cases, show cross resistance to decamethylene biguanidine and to octylguanidine (10-12), molecules that have both shown effects on K^+ uptake by yeast (13). Also, a brief report has been published (12) on what seems to be a difficulty of the mutants to take up the mutagen. Since a series of interactions of the mutagen with the cell have to take place in order to obtain the final effect on mitochondrial DNA (9), several explanations can be given for the resistance to the drug. Besides

this, the uptake of EB in yeast cells has been found to show interesting characteristics; it seems that this transport has some relationship to the system for monovalent cation uptake (14). With all these antecedents, it was considered of interest to study some of the interactions of EB with a K. lactis EB-resistant mutant as compared to its parental strains, including the measurement of the dye uptake. Data were found that indicate some relationship between resistance to the drug and an alteration in the ability of the cells to take up monovalent cations.

MATERIALS AND METHODS

Three strains of K. lactis were employed throughout this work. The two sensitive ones were W600B, designated in the paper as S_1, and WM37, designated as S_2. These haploid strains were kindly supplied by Dr. James R. Mattoon. The strain resistant to EB was KA6-8A, and was obtained from the other two strains. The general characteristics of this type of strains have been described before (10,11). Strain KA6-8A was purified and a resistant clone selected before initiating the work.

Cells were grown in the medium described by De Kloet et al. (15) during approximately 24 hours in a girotory shaker at 250 rpm, using a loopful of cells from a slant as inoculum. This was used as the seed, either immediately or after up to one week around 25 ml (approximately 0.24 ml of cytocrit value) of this was used to inoculate 1 l of medium, which was incubated in the shaker at 30° for 24 hours.

After growth, the cells were collected and washed twice with water by centrifugation, and resuspended in water to give a ratio of 0.5 g of wet weight per ml of the suspension. The cation content of the cells was measured by extraction with 30 percent perchloric acid. The cation concentrations were then measured in the extracts by flame photometry.

K^+ movements were monitored by means of a cationic electrode (Beckman 39047) connected to an electrometer, amplifier and recording system. This same instrument was used to measure H^+ concentrations, using a pH combination electrode.

Fluorescence changes of EB were measured in a Mark I Farrand spectrofluorometer with a recorder attached.

Respiration was followed by means of a Clark electrode and an adequate power source and recording device.

Ethidium bromide uptake was measured by adding the cells to the incubation mixture containing the dye, previously equilibrated to the water bath temperature. Aliquots were then taken to separate the cells in a Microfuge. The concentration of the remaining dye in the supernatant was measured by comparing the absorbance of the supernatant (475 nm) with that of a standard curve of the dye.

The uptake of $^{86}Rb^+$ was measured by incubating the cells with the isotope, filtering and washing with cold 5 mM RbCl to eliminate trapped and bound $^{86}Rb^+$. The radioactivity taken up by the cells was measured by means of an end window-gas flow detector or by scintillation counting. To get the values of Km and Vmax, the chi square method was used.

RESULTS

As previously mentioned a significant difference can be consistently observed between the parental strains and the resistant mutant, with respect to the ability to grow at higher EB concentrations in the medium. The mutant used throughout this work, KA6-8A, carries either a single nuclear or several closely linked nuclear mutations that confer resistance to ethidium bromide. The mutant was derived from a cross between W600BR: α, Ade_1, ade_2, leu, EB^R, and WM37: a, his, (10,11), and can grow in glycerol medium containing 10uM EB, whereas sensitive strains can only tolerate 5 uM EB.

EB uptake by the wild type and the mutant strains

Since the mutant resistant to EB used in this work (KA6-8A) is different to those employed before (12), it had to be verified if it has also an impairment to take up EB. In fact, this was found to be the case, as shown by Fig. 1. Along the time studied, EB was taken up normally by the wild type strains (S_1 and S_2). It should be mentioned also that the experimental conditions are somewhat different to those employed before (12).

Comparative fluorescence changes of EB

Fig. 2 shows that, under the same conditions used to measure the uptake of EB, again, both wild type strains showed an increase of fluorescence when the monitor was added. The resistant mutant, on the other hand, did not show any increase of fluorescence beside the initial one that can be observed in the absence of the cells. This is in full

agreement with the data reported for other mutants (12) studied before.

Uptake of monovalent cations and effects of EB.

It was reported before that EB seems to show a rather specific interaction with the system for monovalent cation uptake in yeast (14). This fact suggested the possibility that the EB resistant mutant, that has an impaired ability to take the mutagen, might have also an impairment of the uptake of this monovalent cation.

The measurement of the uptake of K^+ (Fig. 3) revealed an important impairment in the case of the resistant mutant of K. lactis, as compared with the wild type strains. The uptake of K^+ was studied with the endogenous K^+ or with 1 mM cation added, with similar results. It is important that, as shown in the last three tracings of the figure, by disrupting the cells with CTAB, similar amounts of monovalent cations are released from both the wild type and the resistant strain.

Measurements were made also of the content of several cations, including K^+ in the three strains used. Table I shows that there was no significant difference in the content of the four cations determined.

Fig. 4 shows also the ability of both the sensitive and the resistant strains to take up $^{86}Rb^+$. This experiment may give more information about the influx of the monovalent cation. The initial rates of uptake are much higher for the wild type that for the EB resistant strains.

EB and other cationic dyes (16), when added to yeast can produce not only the inhibition of K^+ uptake, but also the efflux of the internal cation from the cells, in a process that requires of energy (16,17,18).

Since this effect may be due to an interaction of the dyes with the monovalent transport system of yeast, the effect of EB was tested. Fig. 5 shows that especially at the lower EB: yeast ratios, the dye can produce a large efflux of K^+ in the wild type strains, and not in the resistant one. As the amount of cells was decreased, and the amount of dye per cell increased, EB could produce also the efflux of monovalent cation in the resistant strain.

Finally, experiments were carried out to determine the kinetic constants of the three strains for the uptake of Rb^+. As expected, (Fig. 6) there was a large change, both in the Km and the Vm values of the R strain. A large number of experiments had to be accumulated to obtain statistical significance, due to the variability of the results in different experiments.

The proton pumping capacity of the strains.

It has been postulated that yeast cells can transport monovalent cations because of the existence of a proton pump, driven by ATP (19-22) that at low pH can function only if K^+ is added to the medium, but at high values of pH can function in the absence of cations. It seems that at high pH values, proton pumping can become "uncoupled" from monovalent cation transport, and be coupled, perhaps to anion extrusion by the cells. (19). This circumstance offers the possibility of assaying the proton pumping activity of the cells, free from the ion uptake capacity. Although the ability to pump protons is the deriving force for monovalent cation uptake, it can work independently. It was decided to study the proton pumping capacity of the cells under conditions under which it is

uncoupled from monovalent cation uptake, i.e., at pH 7.5 and using ethanol as substrate, to avoid the extensive formation of CO_2 that is produced when glucose is fermented. The results of Fig. 7 show clearly that there are only small differences in the ability of the three strains studied to pump protons.

Effects of EB on respiration.

Another similarity between EB and K^+ is revealed in the stimulation that both agents can produce on respiration. This stimulation was also studied in all strains. The results of Table II show that neither EB nor K^+ can stimulate respiration in the R mutant. On the other hand, as found with Saccharomyces cerevisiae (14), in both wild type strains used in this work, respiration was stimulated by the addition of EB or K^+.

DISCUSSION

The data presented seem to be rather consistent to indicate a close relationship between the transport of monovalent cations and that of EB by yeast cells, that was suggested before (14). The data of this paper pointing in that direction are: a) The low capacity of a mutant resistant to EB to take up the dye, coincident with a low capacity to take up monovalent cations; b) the absence of fluorescence changes observed for EB in the resistant mutant, as compared with the two wild type strains; c) the incapability of EB to produce the efflux of monovalent cations in the resistant mutant within moderate concentration ranges, and d) the absence of stimulation of respiration produced both by EB and K^+ in the

mutant, as compared with the normal effect observed in the wild type strains.

These data are in agreement with the conclusions reached before (14), based in the following facts: EB can inhibit competitively the transport of monovalent cations (Rb^+); monovalent cations can inhibit the uptake of EB with a selectivity pattern similar to that of the monovalent cation transport; EB, can stimulate the extrusion of H^+, as K^+ does, and both agents can produce a stimulation of respiration. This agreement is also interesting because the experiments reported in this paper were performed with a different yeast, K. lactis, and those reported before (14), were obtained with a strain of S. cerevisiae.

It is difficult to postulate a mechanism for the inhibition of monovalent cation uptake produced by EB. Except for the fact that is a cationic dye, it is difficult to imagine how the competition between the organic and the inorganic cation may take place, especially because of the complicated structure of EB. However, the data of this paper add further support to the idea that the mutagen is taken up by the cells through the same system as monovalent cations. No other lesion could be demonstrated besides the inability to transport EB and K^+ by the mutant. The possibility was excluded that the incapacity were in what has been postulated the driving force for monovalent cation uptake, an ATP driven proton pump (19-22). The results of Fig. 7 show that both the wild type and the mutant strains have a similar ability to pump H^+, and the lesion must be something that causes an impairment of the function of the transport system.

The kinetic data for the uptake of Rb+, comparing the wild type strains with the EB resistant one, may suggest a profound alteration of the transport system. Starting with the curves of Fig. 4, in which 4 mM Rb^+ was used, a concentration well above the Km for the transport of the cation in the wild type strains, a large decrease is observed in the rate of transport of the cation. The changes of the kinetic constants for the EB resistant mutant are large both for the Km and for the Vm.

The content of cations of the mutant, almost normal in comparison with the wild type strains was rather unexpected. However, it must be mentioned that the ability of the cells to transport monovalent cations is diminished, but not absent. It is possible that the wild type strains have an excess capacity to transport monovalent cations and that in the EB resistant mutant, the low rate of uptake of monovalent cations may be enough to maintain a normal content of them, inside the cells. Besides, the normal content of other cations in the cells is in further agreement with the generally normal conditions of the mutant strain.

Experiments are in progress to obtain more information on the lesion (s) involved in the resistance of the mutant to EB and the difficulty to take up monovalent cations. The mutant seems to be promising in this respect; besides, it is important to point out that this is the fourth of this kind. It is possible that the alteration on the uptake of cations, organic and inorganic, is a common feature that may allow the adaptation of yeast to unfavorable conditions. This latter fact in itself is stimulating to further study the EB resistant strains.

REFERENCES

1. Markovich, H. (1951). Ann. Inst. Pasteur, 81, 452-468.
2. Bulber, C.J.E.A. (1964). Antonie Van Leeuwnhoek J. Microbiol. Serol., 30, 1-9.
3. Slonimski, P., Perrodin, G. and Croft, J. (1968). Biochem. Biophys. Res Comm., 30, 232-239.
4. Waring, M.J. (1965) J. Mol. Biol., 13 269-282.
5. Tsai, C.C., Jain, S.C. and Sobell, H.M. (1977) J. Mol. Biol. 114, 301-315.
6. Jain, S.C. Tsai, S.C. and Sobell, H.M. (1977) J. Mol. Biol. 114, 317-331.
7. Bastos, R.N. and Mahler, H.R. (1974). Biol. Chem., 249, 6617-6627.
8. Peña, A., Chávez, E., Cárabez, A. and Tuena de Gómez Puyou, M. (1977). Arch. Biochem. Biophys. 180, 522-529.
9. Peña, A., Clemente, S.M., Borbolla, M., Carrasco, N. and Uribe, S. (1980). Arch. Biochem. Biophys. 201, 420-428.
10. Brunner, A., Mas, J. Célis, E. and Mattoon, J.R. (1973). Biochem. Biophys. Res. Comm., 53, 638-644.
11. Célis, E., Mas, J. and Brunner, A. (1975). Genet. Res. Camb. 25, 59-69.
12. Célis, E., Mas, J. and Brunner, A. (1975) FEBS Letters, 57, 241-244.
13. Peña, A. (1973) FEBS Letters, 34, 117-119.
14. Peña, A. and Ramírez, G. (1975) J. Membrane Biol. 22, 369-384.
15. De Kloet, S.R., Van Wermeskerken, R.K.A. and Koningsberger, V.V. (1961) Biochim. Biophys. Acta, 47, 138-143.
16. Peña, A., Mora, M.A. and Carrasco, N. (1979). J. Membrane Biol. (1979) 47, 261-284.
17. Peña, A. (1978) J. Membrane Biol. 42, 199-213.

18. Borbolla, M. and Peña, A. (1980) J. Membrane Biol. 54, 149-156.

19. Peña, A., Cinco, G., Gómez-Puyou, A. and Tuena, M. (1972) Arch. Biochem. Biophys. 153, 413-425.

20. Peña, A., Cinco, G., Gómez-Puyou, A. and Tuena, M. (1969) Biochim. Biophys. Acta, 180, 1-8.

21. Peña, A., (1975). Arch. Biochem. Biophys. 167, 397-409.

22. Peña, A. (1976) In Mitochondria, Bioenergetics, Biogenesis and Membrane Structure. Packer, L. and Gómez Puyou, A., Editors. Academic Press pp 21-30.

Table I. Content of Several Cations by the Three Strains of *K. lactis* Used in this Work.

Strain	μmoles (g of yeast)$^{-1}$		
	K^+	Na^+	Ca^{2+}
S_1	74.0	10.2	36.0
S_2	72.5	12.4	25.0
R	72.0	8.2	24.4

The cations were extracted with 30% perchloric acid from a suspension containing 250 mg. of yeast. The contents of the cations were measured by flame photometry. Each figure is the mean of two determinations.

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Table II. Effects of EB and K^+ on Respiration by Wild Type and EB Resistant Strains of K. lactis.

	Ratio to a control without any addition		
Strain	10 mM KCl	200 uM EB	300 uM EB
S_1	1.17-1.17	1.08-1.05	1.03-1.05
S_2	1.28-1.33	1.08-1.23	1.07-1.13
R	1.03-0.98	0.98-0.95	0.95-0.95

Incubation: 20 mM MES-TEA buffer, pH 6.0; 108 mM ethanol; yeast cells, 50 mg; final volume, 5.0 ml; Temperature, 25°. Respiration rates were measured at 1 min after the addition of yeast.

FIGURE LEGENDS

Fig. 1. Uptake of EB by two wild type and one resistant strain of K. lactis to EB. Experimental conditions: 100 mM glucose; 20 mM MES-TEA buffer, pH 6.0; yeast cells, 200 mg; final volume, 12 ml; temperature, 25°. The incubation was started by the addition of the cells. The measurement of EB uptake was carried out as described in the Methods section. EB concentration was 266 uM.

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Fig. 7. Measurement of H^+ extrusion by the S and R strains of K. lactis at pH 7.5. Incubation conditions: 6.5 mM MES-TEA buffer, pH 7.5; 135 mM ethanol; approximately 1.5 mM H_2O_2; yeast, 250 mg; final volume, 6.2 ml; temperature, 25°C. Yeast cells of the different strains were added where indicated and the pH was recorded. A calibration curve was constructed by the addition of HCl to the incubation medium.

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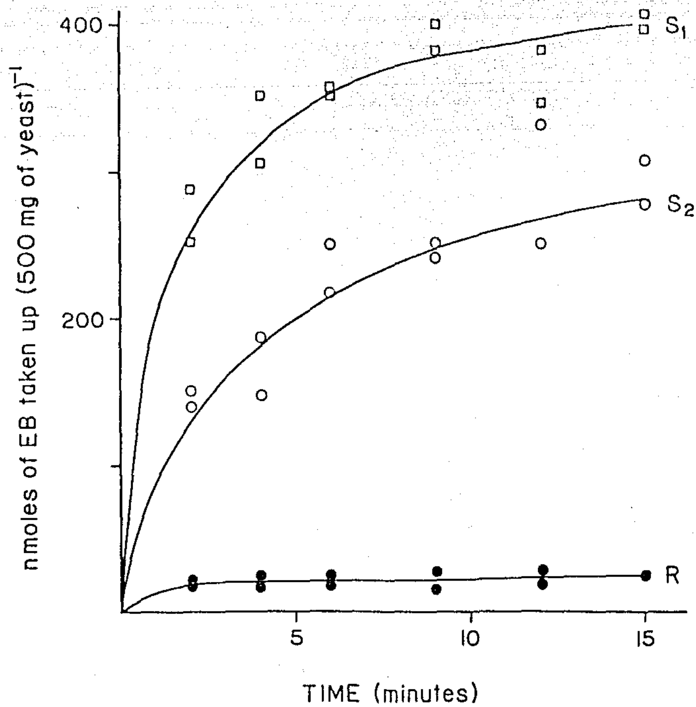
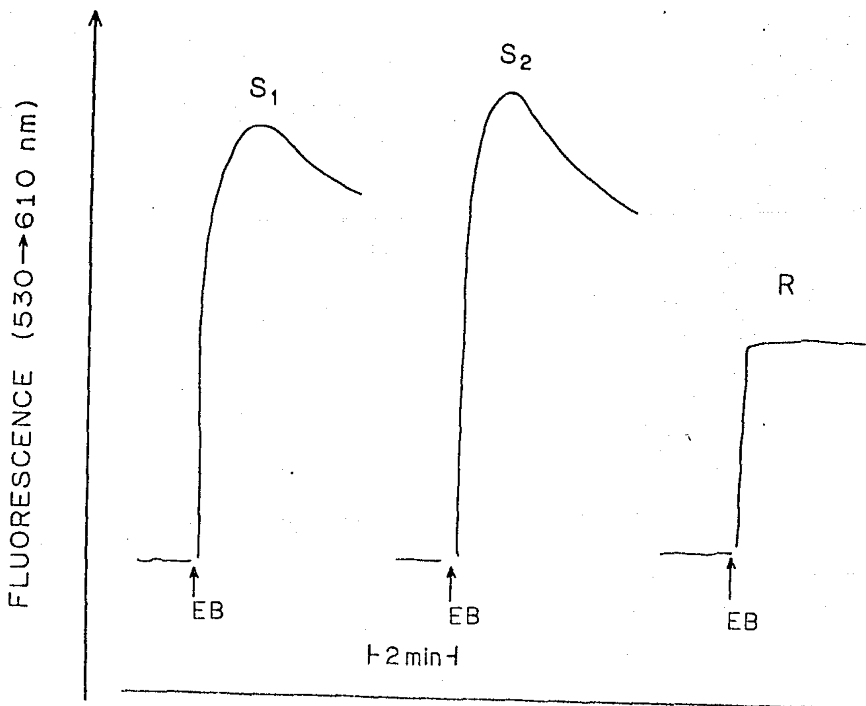
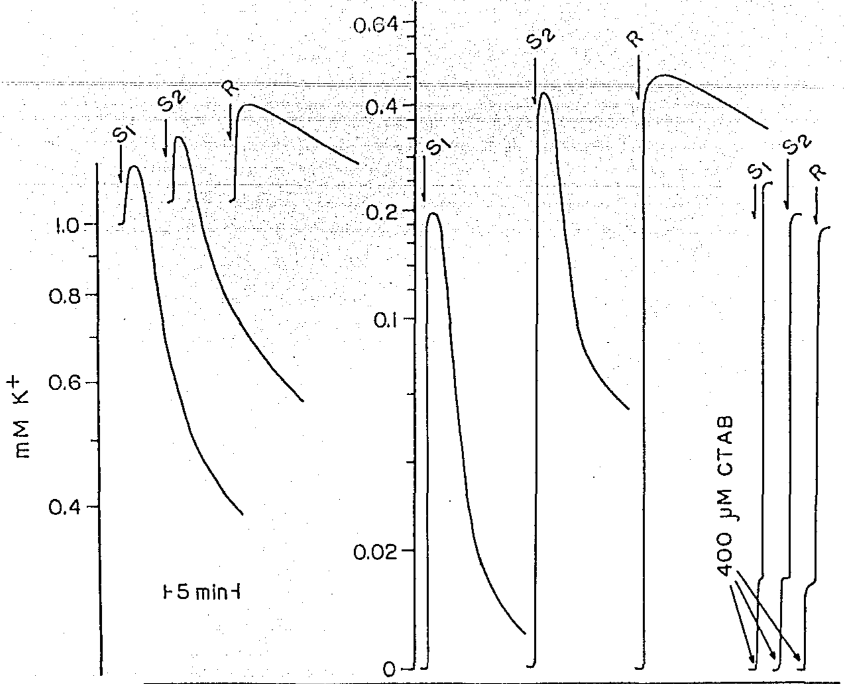
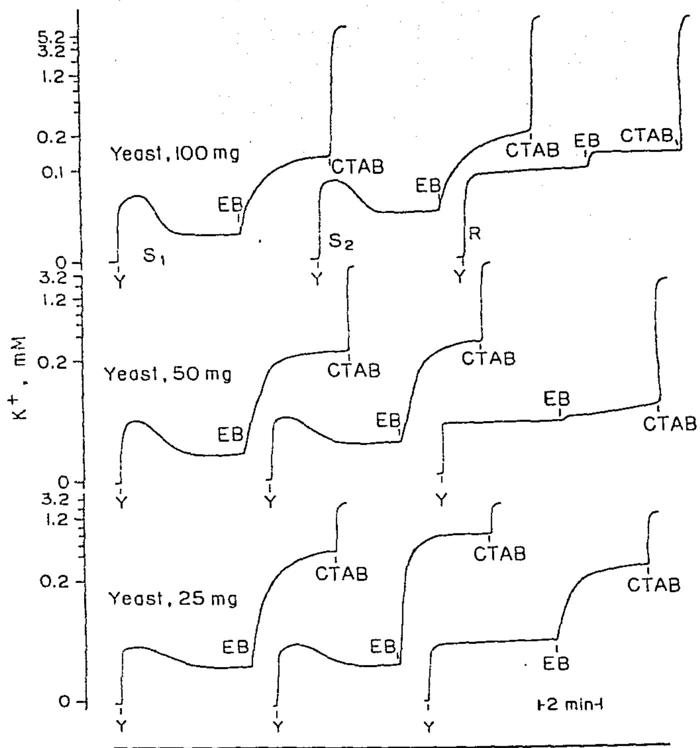
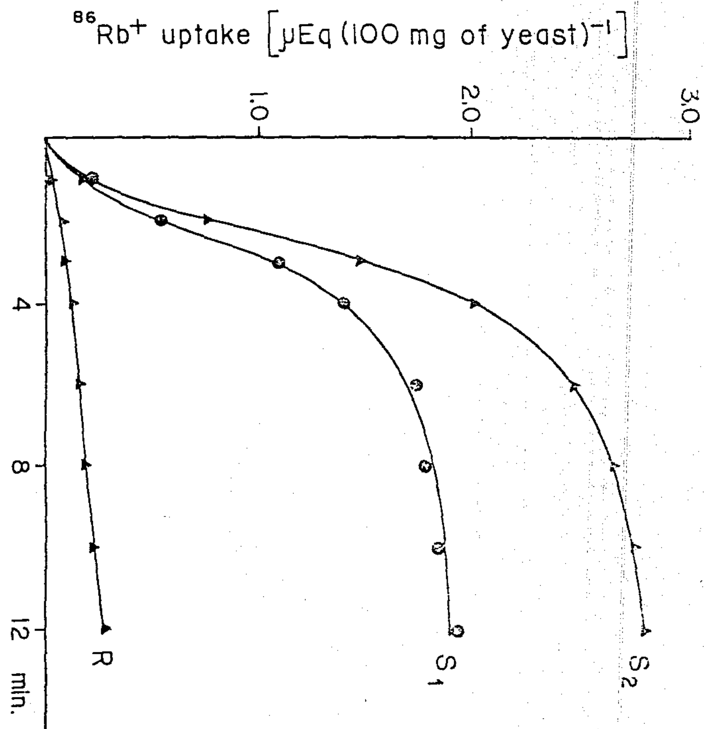
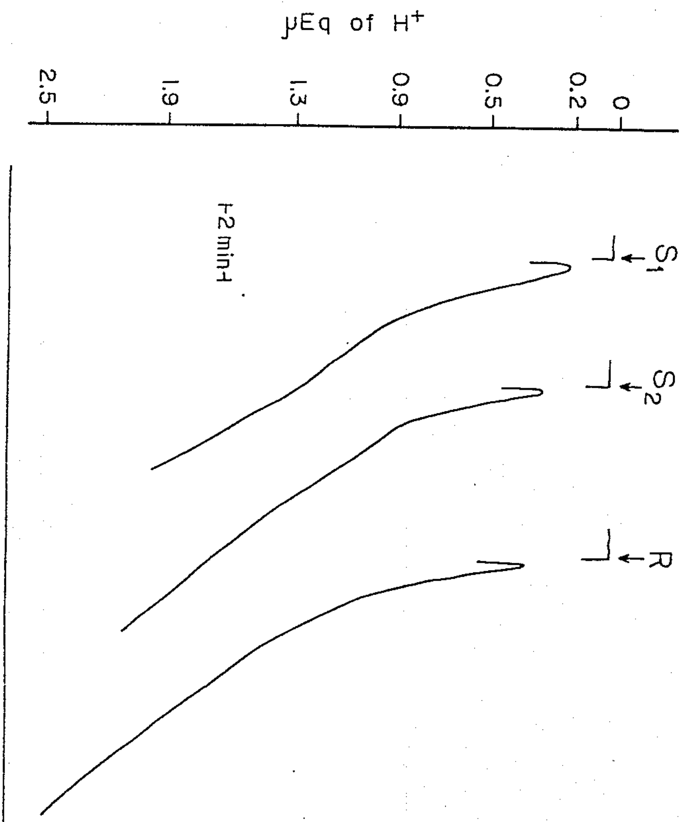

400
200
nmoles of EB taken up (500 mg of yeast)$^{-1}$
S1
S2
R
5
10
15
TIME (minutes)


FLUORESCENCE (530→610 nm)
S1
S2
R
EB
EB
EB
2 min

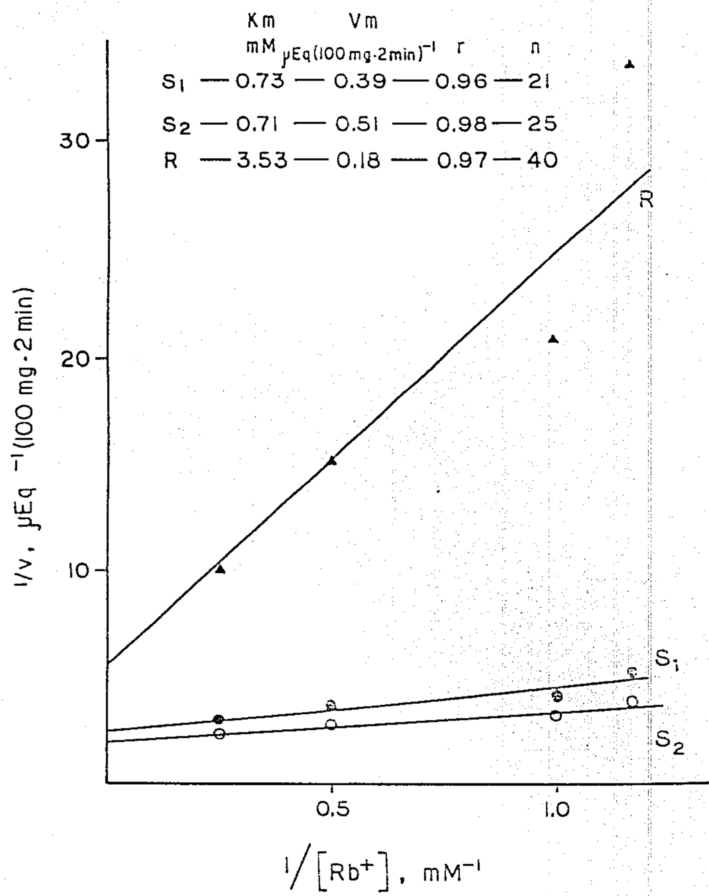

mM K+
1.0
0.8
0.6
0.4
S1
S2
R
5 min
0.64
0.4
0.2
0.1
0.02
0
S1
S2
R
400 μM CTAB
S1
S2
R


K+, mM
5.2
3.2
1.2
0.2
0.1
0
Yeast, 100 mg
Yeast, 50 mg
Yeast, 25 mg
S1
S2
R
Y
EB
CTAB
2 min


86Rb+ uptake [μEq (100 mg of yeast)-1]
1.0
2.0
3.0
4
8
12
min.
R
S1
S2



µEq of H+
2.5
1.9
1.3
0.9
0.5
0.2
0
2 min
S1
S2
R



Km mM
Vm µEq(100 mg·2 min)⁻¹
r
n
S1 — 0.73 — 0.39 — 0.96 — 21
S2 — 0.71 — 0.51 — 0.98 — 25
R — 3.53 — 0.18 — 0.97 — 40
1/v, µEq⁻¹(100 mg·2 min)
30
20
10
R
S1
S2
0.5
1.0
1/[Rb+], mM⁻¹