

COLCEMID INHIBITION OF CELL GROWTH AND THE CHARACTERIZATION OF A COLCEMID-BINDING ACTIVITY IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Under restricted culture conditions, the growth and division of *Saccharomyces cerevisiae* was inhibited by the antimitotic drug Colcemid; in contrast, the related drug colchicine had no effect. The difference in the sensitivity of yeast to these two agents was not dependent on their ability to permeate the cell but rather reflected an inherent difference in the affinity of the two drugs for a cellular-binding site. The binding moiety was characterized by gel filtration as a macromolecule of approximately 110,000 mol wt with an affinity constant for Colcemid of 0.5×10^4 liters per mole; in addition, this macromolecule was retained by diethylaminoethyl (DEAE) ion exchangers. On the basis of these properties, the Colcemid-binding substance in *S. cerevisiae* cells was provisionally identified as microtubule subunits.

INTRODUCTION

The study of mitosis and nuclear division in *Saccharomyces cerevisiae* has been hindered by the lack of an agent suitable for the specific disruption of the mitotic apparatus. Recently, Lederberg and Stetten (1) showed that very high concentrations of the antimitotic drug Colcemid (*N*-methyl *N*-deacetyl colchicine) were effective in arresting the growth of the fission yeast, *Schizosaccharomyces pombe*. These investigators further noted that colchicine failed to inhibit the growth of *S. pombe*, and that neither Colcemid nor colchicine arrested the growth of a number of other species of yeast, including *S. cerevisiae*. The current study was undertaken to examine the basis of the insensitivity of *S. cerevisiae* to Colcemid and colchicine, with the hope: (a) that conditions could be established in which mitosis was inhibited; and (b) that the colchicine alkaloids might serve as

affinity labels for microtubule subunits in further studies on the mechanism of regulation of the mitotic process in a simple eukaryote organism. To determine whether the insensitivity of *S. cerevisiae* to these antimitotic agents was due to impermeability of the cell, and whether binding sites for these agents existed within the cell, tritium-labeled colchicine and Colcemid were used. While tritiated colchicine was already available, a method for the preparation of tritiated Colcemid had to be developed. Studies using the labeled compounds indicated that exponentially growing cultures of *S. cerevisiae* were not freely permeable to Colcemid or colchicine, but that restricted conditions could be found in which Colcemid was effective in preventing vegetative growth. A drug-binding activity was assayed and characterized in crude

extracts, and the binding constants for Colcemid and colchicine were found to be consistent with the concentrations required to inhibit growth *in vivo*.

MATERIALS AND METHODS

Yeast Strains

S288C, a haploid strain of *S. cerevisiae*, and S41, a homothallic diploid strain requiring arginine for growth, were used in these experiments.

Growth Conditions

A supplemented minimal medium consisting of 2% dextrose, 0.67% yeast nitrogen bases without amino acids (Difco Labs, Detroit, Mich.), and vitamins (2) was used routinely. When S41 was used, the medium was supplemented with 20 mg/liter L-arginine. A rich medium (YEP) composed of 2% dextrose, 2% bacto-peptone, and 1% yeast extract (Difco Labs) was used where indicated. Cells were grown at 30°C on a rotary shaker in sterile flasks. Growth was monitored by counting cells in a hemocytometer or by measuring cell density at 600 nm with a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Colcemid and Colchicine

Colcemid was obtained from Ciba Pharmaceutical Co. (Summit, N. J.) and colchicine from Sigma Chemical Co. (St. Louis, Mo.). Tritiated colchicine (5.7 Ci/mmmole) was purchased from New England Nuclear Corp. (Boston, Mass.). Tritiated Colcemid

(5.0 Ci/mmmole) was also produced by New England Nuclear Corp. according to the procedure described below.

Preparation of Tritium-Labeled Colcemid

Tritiated Colcemid was prepared by methylation of *N*-methyl, trimethylcolchicinic acid (NMCA) with tritiated diazomethane in a manner similar to that described for the preparation of tritium-labeled colchicine (3, 4). The reaction products were a mixture of Colcemid and iso-Colcemid, the positional isomer being obtained by interchanging the methyl ether and carbonyl groups of the tropolone ring (Fig. 1). The experimental conditions for the synthesis and resolution of the two tritiated isomers are given below.

Hydrolysis of Colcemid to NMCA

NMCA was prepared according to a modified method for the production of colchicine (5). A 1.0 g sample of Colcemid was dissolved in 40 ml 0.5 N HCl and refluxed at 100°C for 1 hr. The cooled reaction mixture was neutralized to pH 7.0–7.5 by the addition of 1.7 g NaHCO₃. The yellow solution was extracted with 100 ml CHCl₃ and filtered through MgSO₄. The chloroform was then taken off *in vacuo*. The residue was recrystallized from 20 ml methanol and yielded 0.85 g of pale yellow needles (m. p. 135–137°C). Thin-layer chromatography with 5% methanol in chloroform yielded a single spot, *R_f* = 0.2. All thin-layer chromatography was done on silicic acid impregnated paper (Gelman ITLC SAF; Gelman Instrument Co., Ann Arbor, Mich.) and the spots were visualized by fluorescence quenching.

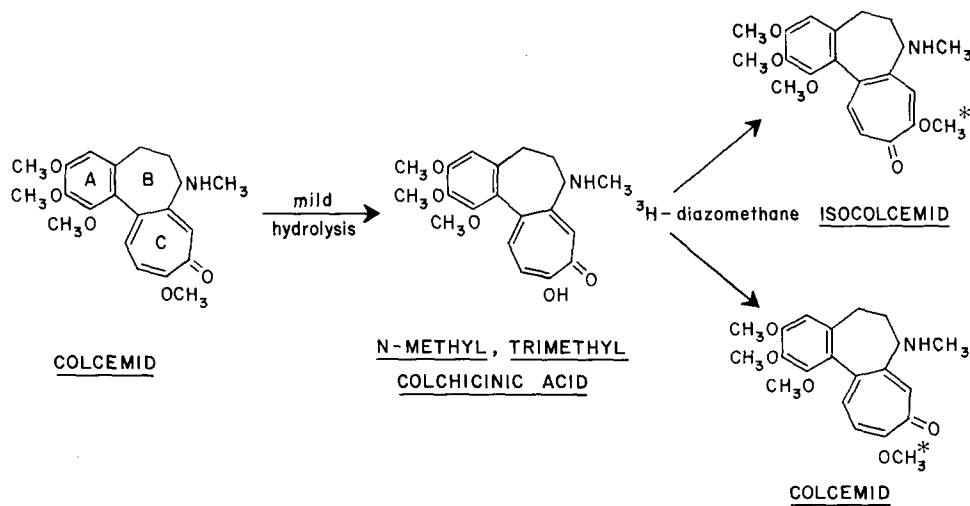


FIGURE 1 Diagram showing reaction pathway for the synthesis of Colcemid-³H.

Preparation of Diazomethane

Diazomethane was generated by adding 4.3 g *N,N'*-dimethyl-*N,N'*-dinitrosoterephthalamide (6) (70% in mineral oil; Aldrich Chemical Co., Inc., Milwaukee, Wis.) to 10 ml 2-ethoxyethanol, 15 ml 30% sodium hydroxide, and 60 ml dichloromethane in a distilling flask. Distillation into 30 ml dichloromethane in a receiving flask produced a final volume of 70 ml diazomethane in dichloromethane solution. The concentration of diazomethane was approximately 0.25 M as determined by benzoic acid titration. A dichloromethane solution of diazomethane was preferred to an ether solution because of the greater solubility of NMCA in dichloromethane.

Methylation of NMCA to Colcemid

A 0.2 g sample of NMCA dissolved in 5 ml dichloromethane was mixed with 20 ml of the dichloromethane solution of diazomethane at room temperature. The reaction mixture was allowed to stand 30 min after which time a ferric chloride test (7) for the presence of NMCA was negative. Thin-layer chromatography with 100% methanol yielded two spots, the slower of which ($R_f = 0.59$) was coincident with Colcemid. The faster spot ($R_f = 0.67$) was presumed to be iso-Colcemid. The methylation reaction favored the production of Colcemid to iso-Colcemid in approximately a 4:1 ratio. This was in contrast to the methylation of colchicine to colchicine and isocolchicine in which the isocompound was favored by a 3:1 ratio (3).

Analysis of the New England Nuclear Corporation Product

Crystallized NMCA was prepared as described above and sent to New England Nuclear Corp. for methylation with tritiated diazomethane and separation of the isomers by silica gel chromatography. The tritiated product was returned as a 75 $\mu\text{g}/\text{ml}$ benzene:ethanol (9:1) solution with a specific activity of 5.0 Ci/mmole, and was stored in the dark under nitrogen at 4°C. A 5 μl sample of the tritiated product was added to 100 μl of a 10 mM solution of Colcemid in methanol, and 10 μl of this mixture was chromatographed on Gelman ITLC SAF paper strips using 100% methanol as a solvent. The chromatograms were cut transversely into 1 cm sections and eluted individually with 1 ml methanol. The eluates were examined at 350 nm for absorption, and 0.5 ml of the eluates were withdrawn for scintillation counting in Bray's solution (8). Fig. 2 shows that the radioactivity peak superimposed on the absorption peak, indicating that the radioactive product chromatographed identically with Colcemid. Since the chromatographic system resolved Colcemid ($R_f = 0.59$) from iso-Colcemid

($R_f = 0.67$) and colchicine ($R_f = 0.70$), it was concluded that the tritiated product was not contaminated with these compounds. The tritiated material had an ultraviolet spectrum identical to that of Colcemid and, in addition, bound to microtubule subunits prepared from porcine brain tissue (unpublished results). We therefore regarded the product to be pure Colcemid- ^3H .

Measurement of Uptake of Colcemid and Colchicine

The degree to which yeast grown under different physiological restrictions was permeable to colchicine or Colcemid was determined by measuring the extent to which the yeast cells excluded the drug. Cells were harvested from cultures by centrifugation and 1.0 volume of packed cells was resuspended in 0.5–1.0 volume of media to obtain a slurry. 0.05 ml of 25 mM Colcemid was added to 0.8 ml of cell slurry and the mixture was incubated at 37°C for 1.5 hr with occasional agitation. Samples of the incubated slurry were taken up in 100- μl capillary tubes (Scientific Products, Evanston, Ill.); the capillary tubes were then sealed at one end with plasticine (J. L. Hammett Co., Cambridge, Mass.), placed in 15-ml conical, glass centrifuge tubes (for support), and centrifuged at 1450 *g* for 30 min in an IEC swinging bucket clinical centrifuge (International Equipment Co., Needham Heights, Mass.). The volume fraction of packed cells was defined as the ratio: length of packed cells/length of total suspension. From parallel centrifugations of the bulk slurry, samples of the supernatant were measured at 350 nm with a Beckman DB spectrophotometer in order to determine the concentration of Colcemid or colchicine. These concentrations were compared with the values obtained for samples from tubes to which no cells had been added. If the yeast cells were completely impermeable to Colcemid or colchicine, then the concentration of these drugs in the supernatant of the cell suspensions would be significantly higher than that in those samples to which no cells had been added. If, on the other hand, the cells were freely permeable, the concentration of Colcemid or colchicine would nearly equal that of the control, but would be slightly greater because of the volume occupied by structural elements of the cells. To establish these relative changes in the drug concentration in the supernatant, Blue Dextran (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) was used as a measure for the substances which were completely excluded from the cell and tritiated water (2.5×10^6 dpm/g, Packard Instrument Co., Inc., Downers Grove, Ill.) was used as a measure for freely permeable molecules. Blue Dextran was assayed spectrophotometrically at 600 nm and tritiated water was measured by liquid scintillation counting.

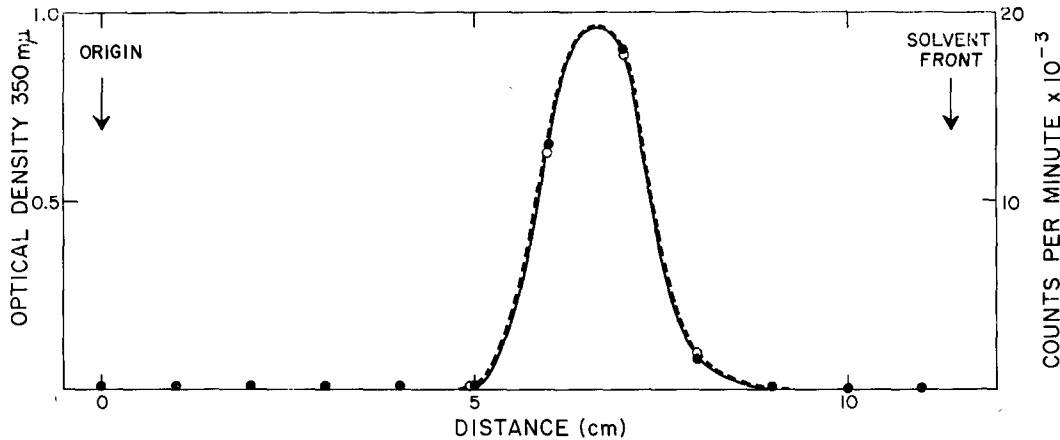


FIGURE 2 Thin-layer chromatography of Colcemid- ^3H and carrier Colcemid. (●) absorption at $350\text{ m}\mu$, (○) radioactivity in cpm. The plots were adjusted to coincide at the height of the peak; see text for details.

The per cent permeability, P , of the cells to the drugs was determined from the expression
$$P = \frac{R_{\text{H}_2\text{O}}}{R} \frac{(R_{\text{BD}} - R)}{(R_{\text{BD}} - R_{\text{H}_2\text{O}})} \times 100$$
 (see Appendix for derivation). R was defined as the ratio of the concentration of drug in the supernatant of the cell slurry to the concentration of the same amount of drug added to an equivalent volume of buffer. R_{BD} and $R_{\text{H}_2\text{O}}$ denoted similar ratios for Blue Dextran and tritiated water.

Preparation of Yeast Extracts

Exponentially growing yeast cells were harvested from YEP by centrifugation at 3000 g for 15 min and all subsequent procedures were carried out at $0\text{--}4^\circ\text{C}$. The pelleted cells were washed twice in cold water, and resuspended in 15 ml of 0.05 M potassium phosphate buffer, pH 6.8, per 2 g packed cells. The suspension was passed twice through a French Press at 20,000 psi to rupture the cells, and the solution was then made to 0.1 mM guanosine triphosphate (GTP). The broken cells were centrifuged at $15,000\text{ g}$ for 20 min and the resultant supernatant was centrifuged at $100,000\text{ g}$ for 2 hr. The high-speed supernatant was used immediately for binding studies. Protein concentration was determined by the method of Lowry as modified by Layne (9).

Binding of Colcemid or Colchicine to Yeast Extracts

Small samples of tritiated Colcemid or colchicine stock solutions in benzene:ethanol (9:1) were evaporated to dryness with a stream of nitrogen and the dried compounds were dissolved directly in yeast extract. A range of drug concentrations was obtained by

varying the volume of tritiated stock solution evaporated. Samples were incubated for 2 hr at 37°C in sealed vials protected from the light. The binding reaction was quenched by chilling the vials to 4°C .

Two methods of measuring Colcemid or colchicine binding were used. Generally, 0.1 ml of an incubated sample was loaded on a Sephadex G-100 (Pharmacia Fine Chemicals Inc.) column ($0.9 \times 20\text{ cm}$). The column was eluted with 0.067 M potassium phosphate buffer, pH 6.8, containing 0.1 M KCl (PK solution) at 4°C and fractions of 0.25 ml were collected. The void volume was determined with a Blue Dextran marker. The fractions were counted in Bray's solution in a Packard Tri-Carb scintillation counter (Packard Instrument Co.). Counting efficiency was determined by the addition of toluene- ^3H as an internal standard and was found to be 34%.

A second method of measuring binding was also used. In this case, 0.1 ml of each incubation mixture was filtered through four thicknesses of DEAE filter paper (Whatman DE-81 Chromedia paper; H. Reeve Angel & Co., Inc., Clifton, N. J.) according to the assay procedure for porcine brain microtubule protein.¹ The filters were washed five times with 10 ml PK solution and the filters were counted in Bray's solution.

A procedure using DEAE-cellulose columns (Whatman DE52) was also employed to assay binding. A 20 ml column was made in a 30 ml disposable syringe using DE52 equilibrated in PK buffer. A 0.5 ml sample of yeast extract was applied to the column and eluted at 4°C with a linear gradient of $0.1\text{--}1.0\text{ M}$ KCl.

¹ Borisy, G. G. 1972. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. *Anal. Biochem.* In press.

Electron Microscopy

Isolated nuclei were prepared from exponentially growing yeast harvested from YEP medium, and were the gift of Dr. M. M. Bhargava. They were fixed, embedded, and sectioned in the laboratory of Dr. H. Ris according to previously published methods (10). Sections were examined and micrographs taken with a Philips 300.

RESULTS

Effect of Colcemid and Colchicine on the Growth of *S. cerevisiae*

Concentrations of Colcemid and colchicine sufficient to prevent mitosis in mammalian cells (3) were found not to inhibit the growth of yeast. However, under restricted growth conditions, and at higher concentrations, Colcemid was inhibitory; in contrast, under the same conditions, colchicine was without effect. The inhibition by Colcemid was significantly affected by the physiological state of the cells. When yeasts which had been growing exponentially in minimal medium were transferred to fresh minimal medium containing Colcemid, growth was retarded (Fig. 3 a). Even with 10 mM Colcemid, however,

growth was not completely stopped. A more pronounced inhibition was found when cells from early stationary phase were used (Fig. 3 b). In this case, 10 mM Colcemid completely prevented growth. Sensitivity to Colcemid was greatest in cells which had been in stationary phase for 8 hr (Fig. 3 c). When stationary phase cells were resuspended in fresh minimal media, they reinitiated growth after a short lag. Colcemid concentrations of 1 mM in the resuspension media significantly retarded growth, and concentrations above 2 mM were completely inhibitory. However, if yeast were grown in YEP, Colcemid was no longer inhibitory, even in stationary phase cultures. When similar experiments were carried out using concentrations of up to 10 mM colchicine, no significant inhibition was found in cell cultures at any phase of growth.

The effect of Colcemid on the growth of *S. cerevisiae* was reversible. If stationary phase cells were incubated with 5 mM Colcemid for a period of several hours, washed twice in minimal medium without dextrose, and resuspended in fresh minimal medium without Colcemid, growth began after an additional lag period (Fig. 4). Recovery was observed after incubations of up to 7 hr

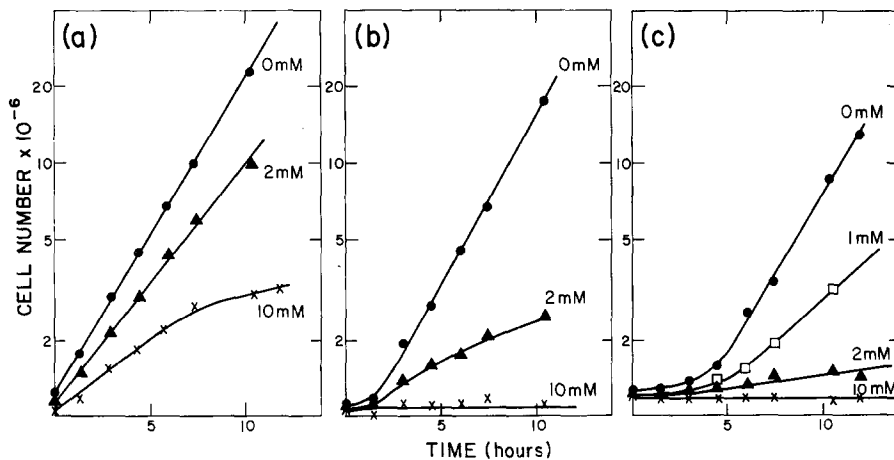


FIGURE 3 Effect of Colcemid on the growth of *S. cerevisiae* at different physiological stages. S41 was grown in minimal medium containing arginine and tested for Colcemid sensitivity at three stages of growth. For each stage, cells were resuspended at a cell density of 1×10^6 cells/ml in fresh minimal medium containing varying amounts of Colcemid. (a) Cells were harvested in logarithmic phase (1×10^7 cells/ml) and resuspended in media containing 0 mM (●), 2 mM (▲), or 10 mM (X) Colcemid. (b) Cells were harvested at the end of logarithmic growth (8×10^7 cells/ml) and resuspended in media containing 0 mM (●), 2 mM (▲), or 10 mM (X) Colcemid. (c) Cells were harvested after 8 hr in stationary phase (8.5×10^7 cells/ml) and resuspended in media containing 0 mM (●), 1 mM (□), 2 mM (▲), or 10 mM (X) Colcemid.

with 5 mM Colcemid. As shown in Table I, the lag period after removal from Colcemid and before growth increased with longer times of incubation. The significance of the additional lag is not clear, but the lag might be partially due to the time required for Colcemid to dissociate from cellular-binding sites (3).

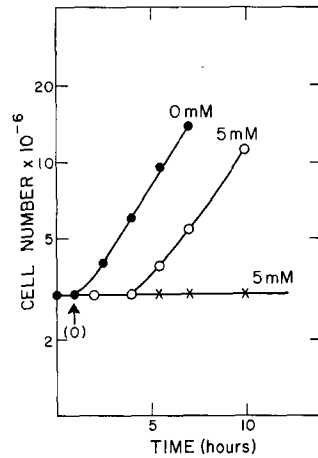


FIGURE 4 Reversibility of Colcemid inhibition. S288C was grown to late stationary phase in minimal medium and resuspended at a cell density of 3×10^6 cells/ml in fresh minimal media containing no Colcemid (●), or 5 mM Colcemid (X). After 1 hr, a sample of the cells incubated in 5 mM Colcemid was washed and returned at the same cell density to fresh minimal medium containing no Colcemid (○). The arrow marks the time at which the sample of cells was washed to remove Colcemid.

TABLE I
Recovery of *S. cerevisiae* from Colcemid Inhibition

Time of incubation in 5 mM Colcemid	Lag time before the initiation of growth
hr	hr
0	1.0
1	2.5
4	4.0
7	6.5

Stationary phase cells of S288C were washed and resuspended in minimal medium containing 5 mM Colcemid at a density of 1×10^6 cells/ml. At intervals, samples were removed, washed twice with minimal medium, and resuspended at the same concentration of cells in fresh minimal medium without drug. The interval before the initiation of exponential growth was measured as in Fig. 4.

The dependence of the Colcemid sensitivity of yeast on the physiological state of the cells and the richness of the medium suggested that the effect of Colcemid and colchicine might be limited by the ability of the agents to enter the cell. It seemed possible that under conditions of starvation (stationary phase cells in minimal medium), the cell might not be able to maintain a fully impermeable membrane; Colcemid or colchicine might therefore penetrate the cell more readily. A similar suggestion was supported by observations made on the effects of inhibitors of macromolecular synthesis in yeast (11). The extent of uptake of Colcemid and colchicine was therefore measured under different physiological conditions to determine whether changes in sensitivity to the drugs were primarily dependent on uptake. Thick suspensions of both exponentially growing and stationary phase cells were equilibrated with colchicine and Colcemid, and the amount of drug present in the cells was determined as described in Materials and Methods. (The amount of drug present in the cells is a sum of both the free compound and the compound complexed to cellular sites; however, the experiments described below on binding of Colcemid-³H in vitro indicated that the concentration of cellular-binding sites was small compared to intracellular concentrations of free drug. Therefore, the uptake data primarily measured permeability.) The results of these measurements, given in Table II, indicated that both Colcemid and colchicine could enter the stationary phase cell almost as freely as water. On the other hand, entry of Colcemid into exponential phase cells was restricted. After 1.5 hr, only 34% as much Colcemid had entered the cell as compared to a fully permeable molecule. However, the permeability measurement for exponentially growing cells of 34% should be regarded as an upper limit. Since incubation with Colcemid was carried out in suboptimal conditions for exponential growth (thick cell slurry), the cells used in this part of the study may resemble cells at early stationary phase. Since stationary phase cells are more permeable, the 34% permeability observed in the pseudo-exponential phase cells may be partially attributed to nonideal growth conditions. However, it does appear that the ability of Colcemid to enter cells during active growth is substantially reduced compared with the permeability of stationary phase cells; this difference

TABLE II
Measurement of the Uptake of Colcemid and Colchicine by *S. cerevisiae* in
Different Physiological States

	Volume fraction of packed cells (v_p)	Ratio of supernatant concentrations (R)	Per cent permeability* (P)
A. Stationary phase cells incubated in minimal medium without dextrose			
Blue Dextran	0.437	1.58	0
Tritiated water	0.429	1.05	100
Colcemid	0.428	1.11	90
Colchicine	0.438	1.10	91
B. Logarithmic phase cells incubated in mini- mal medium			
Blue Dextran	0.325	1.41	0
Tritiated water	0.320	1.03	100
Colcemid	0.322	1.28	34

The relative permeability of Colcemid and colchicine was determined as described in Materials and Methods. Each determination included the volume fraction of packed cells and the ratio of concentration of the supernatant as compared with a control of equal volume but without cells. Blue Dextran was used as a standard for a substance completely excluded from the interior of yeast, and tritiated water was used as a standard for a substance which was freely permeable.

$$*P = \frac{R_{H_2O} (R_{BD} - R)}{R (R_{BD} - R_{H_2O})} \times 100.$$

in permeability may account for the difference in the inhibition of growth by Colcemid under varying physiological conditions.

Binding of Colcemid and Colchicine to Yeast Extracts

While the measurements of drug uptake presented above may account for the changes in sensitivity of *S. cerevisiae* to Colcemid under various growth conditions, the results do not provide any explanation for the lack of inhibition by colchicine. Colchicine appeared to be as permeable to stationary phase cells as Colcemid, yet colchicine was without effect even at a concentration of 10 mM. To determine whether this difference in growth inhibition reflected an inherent difference in the affinity of these two agents for a cellular-binding site, measurements of Colcemid and colchicine binding in crude extracts were made.

The binding of Colcemid and colchicine to freshly prepared yeast extracts over a range of drug concentrations from 0.01 to 0.1 mM was carried out as described in Materials and Methods.

The amount of each drug bound by the extract was assayed by Sephadex G-100 gel filtration. The peak of bound radioactivity eluted from the column at a volume approximately equal to 1.1 times the void volume, indicating that the molecular weight of the Colcemid-binding moiety was approximately 110,000 daltons. As seen in Fig. 5, the peak of activity was symmetrical, suggesting a single-binding species. The tailing of counts in the fractions after the main peak was probably a result of dissociation of Colcemid from the bound complex during the elution of the column.

The results from a set of equilibrium-binding measurements for both Colcemid and colchicine are presented in the form of a double reciprocal plot in Fig. 6. Since the slope of a double reciprocal plot is proportional to the reciprocal of the affinity constant, it is evident from Fig. 6 that the binding of Colcemid was approximately an order of magnitude greater than the binding of colchicine. From the Colcemid-binding data, an affinity constant of approximately 0.5×10^4 liters per mole was determined. The binding constant for colchicine, after normalizing the colchicine specific activity to that of Colcemid,

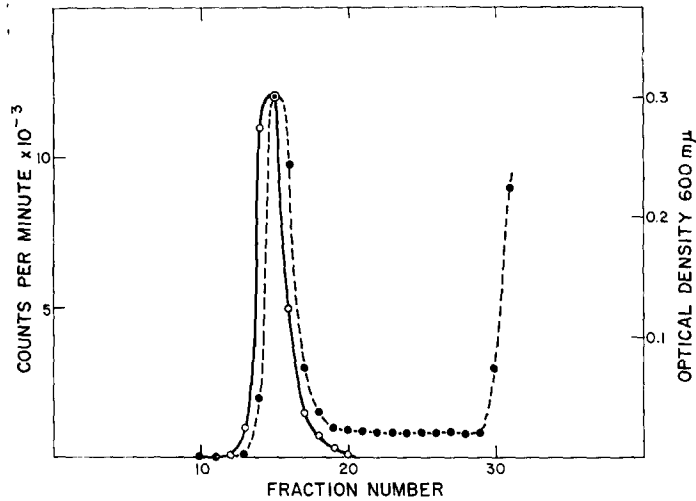


FIGURE 5 Sephadex G-100 assay of Colcemid-binding protein. Freshly prepared yeast extract of S41 at 22.7 mg/ml protein was incubated with 0.05 mM Colcemid- ^3H as described in Materials and Methods and 0.1 ml of the incubation mixture loaded on a G-100 column. A peak of bound tritiated Colcemid (●) emerges from the column just after a peak of Blue Dextran (○).

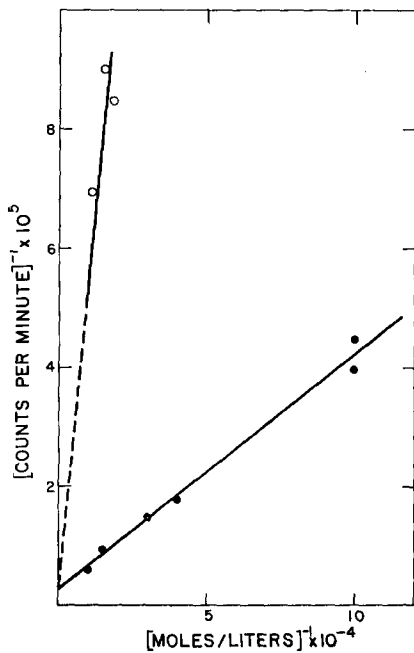


FIGURE 6 Double-reciprocal plot of Colcemid and colchicine binding in yeast extract. S41 extract at 20.8 mg/ml was prepared as described in Materials and Methods, incubated with varying concentrations of colchicine- ^3H , and assayed for binding by Sephadex G-100 chromatography. The results for Colcemid (●) and colchicine (○) are both normalized to the specific activity of Colcemid- ^3H .

was estimated at 0.04×10^4 liters per mole. Therefore, it was concluded that *S. cerevisiae* crude extracts contained a binding site (or sites) which exhibited a relatively low affinity for Colcemid and, more importantly, an even lower affinity for colchicine. These results are consistent with the *in vivo* observations on Colcemid and colchicine sensitivity.

The yeast Colcemid-binding activity was further characterized by chromatography on DEAE ion exchangers. Because of the large number of aspartic and glutamic residues found in amino acid analyses of microtubule protein, it has been determined that tubulin proteins are quite acidic (13-19). The acidity is the basis on which tubulin is retained by DEAE ion exchangers, and this property has been utilized as the basis of a method for assaying colchicine binding¹ (12, 17, 19, 20).

Samples of a yeast extract incubated with Colcemid- ^3H were assayed by the DEAE filter method and the Sephadex G-100 column method. A comparison of the results is presented in Table III. Approximately 25-30% of the counts observed in the G-100 determinations of Colcemid binding were retained by the DEAE filters. This efficiency was considerably lower than that obtained for colchicine binding (65%) in extracts of porcine brain tissue;¹ this difference was probably due to a greater dissociation constant of the yeast-binding moiety for Colcemid, result-

ing in loss of counts from the filter stack during the washing procedure.

The Colcemid-bound complex was retained more completely on a DEAE column than on the filters, and could be preferentially eluted with 0.45 M KCl. A mixture of yeast extract at 20.8 mg/ml protein and 0.1 mM tritiated Colcemid was incubated at 37°C for 2 hr and 0.5 ml was then loaded on a DEAE column equilibrated at

pH 6.8 with PK buffer. From a parallel Sephadex G-100 filtration of the same preparation, it was determined that the DEAE column had been loaded with approximately 75,000 cpm of Colcemid-bound complex. The DEAE column was then eluted with a 400 ml linear gradient of 0.1–1.0 M KCl. In parallel experiments, it was found that unbound Colcemid counts were washed from the DEAE column with 0.1 M KCl. As shown in Fig. 7, the bound tritiated Colcemid was retained on the DEAE column and was eluted primarily as a single peak with 0.45 M KCl. Approximately 65,000 cpm were recovered in this peak. This chromatographic behavior was similar to that which has been described for porcine brain (12) and chick embryo brain (17, 19) microtubule protein.

TABLE III
Comparison of DEAE Filter Paper and Sephadex G-100 Assays for Colcemid-Binding Protein

Assay	Bound cpm	% bound
Sephadex G-100	28,500	100
DEAE filter	7,700	27

Yeast extract was incubated for 2 hr at 37°C with 0.1 mM tritiated Colcemid and the incubation mixture was used for measuring binding. The assays were carried out as described in Materials and Methods. The DEAE paper assay represents the average of four sets of filters.

DISCUSSION

Colcemid, but not colchicine, appeared to exert a significant effect on the growth of *S. cerevisiae*. This result was consistent with the observations of Lederberg and Stetten on the drug-specific in-

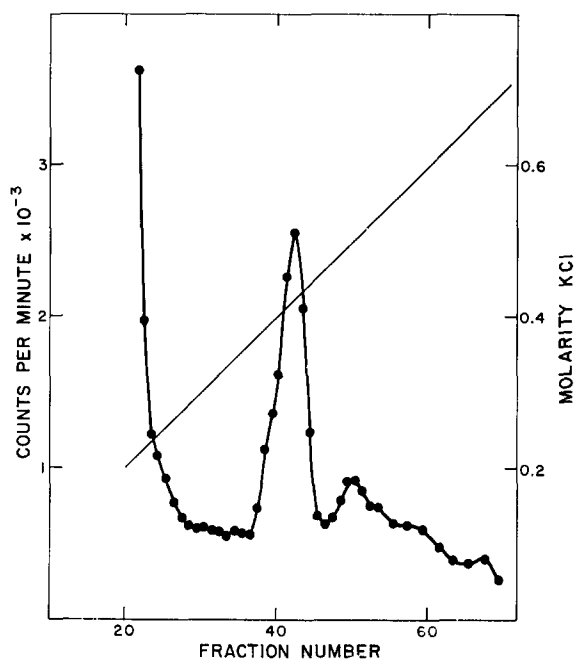


FIGURE 7 DEAE-cellulose column chromatography of yeast extract incubated with Colcemid-³H. A 30 ml column of Whatman DE-52 was equilibrated with PK buffer, pH 6.8. The column was loaded with 0.4 ml yeast extract at 20.8 mg/ml protein containing approximately 0.02 mM Colcemid-³H. The extract contained approximately 75,000 cpm of bound Colcemid as measured by Sephadex G-100 chromatography. The column was eluted with a linear gradient from 0.1 to 1.0 M KCl, and 5 ml fractions collected. The column was assayed by measuring the radioactivity in 0.5 ml of each fraction.

hibition of growth in *S. pombe* (1). However, even in the case of Colcemid, inhibition of growth occurred only at very high concentrations (1–10 mM). This was in contrast to the human cell lines, strain HeLa or KB, in which a concentration of 10^{-7} M colchicine was sufficient to block division (3). The Colcemid sensitivity of yeast cells appeared to be dependent on their growth phase; cells which were growing exponentially were far more insensitive than cells which had reached a limit for one or more essential components in the medium. These observations suggested that the stationary phase cells were unable to maintain a completely impermeable barrier to Colcemid and colchicine. Evidence supporting the hypothesis that a significant change in permeability accompanied the transition from active growth to stationary phase was provided by direct measurements of Colcemid and colchicine uptake; these results demonstrated that the stationary phase cells were more permeable to the drugs than the exponential phase cells. However, it is likely that the limited uptake of Colcemid seen in exponential phase cells was an overestimate since the cells no longer grew optimally in the thick suspension used for assaying permeability. Nevertheless, the change in permeability does seem to account for the change in Colcemid sensitivity *in vivo*.

In stationary phase cells, a concentration of 1 mM Colcemid was found to inhibit the growth rate by 50%. Measurements of the Colcemid binding of cell extracts and analysis of the data by a double-reciprocal plot gave an estimate of 0.2 mM for the dissociation constant of the binding protein. This compares to the value of 0.25 μ M for the dissociation constant of the colchicine-binding protein in strain KB cells (3), and is consistent with the relative concentrations of Colcemid or colchicine required to inhibit growth in yeast and human cells. If one assumes that the action of Colcemid involves the binding of one Colcemid molecule per binding-protein molecule, then 50% of the protein would have bound Colcemid at a concentration of 0.2 mM. Thus, the measurements on stationary phase cells of Colcemid binding *in vitro* and of Colcemid inhibition *in vivo* are in reasonable agreement (factor of five difference). The fact that inhibition of growth occurred with higher concentrations of Colcemid than estimated from the *in vitro* binding studies may indicate that the effective concentration of Colcemid in the yeast cells was

somewhat lower than the concentration measured. This observation may be accounted for by the compartmentalization of karyokinesis in yeast cells. The mitotic apparatus in yeast is entirely intranuclear (21), and the nuclear envelope does not break down during division. It is not known whether the site of action of Colcemid in yeast cells is in the cytoplasm, nucleus, or both, but one might suggest that the effective concentration of Colcemid in yeast cells is only the intranuclear amount. Our data cannot discriminate between Colcemid in the cytoplasm and that in the nucleus. However, if one assumes that the nucleus is the site of action of Colcemid, the measured permeability of 90% is consistent with the hypothesis that the cell membrane is wholly permeable while the nuclear envelope is only partially permeable; this difference in permeability would account for the intranuclear concentration of Colcemid being lower than that measured for the whole cell. An alternative possibility is that more than 50% of the microtubule subunits must be bound with Colcemid in order for growth to be inhibited. The lack of colchicine inhibition of growth can be attributed to the much lower affinity of the binding protein for colchicine.

It is believed that colchicine (and Colcemid) are specific affinity labels for microtubule protein (22, 23). It is therefore reasonable to tentatively assume that the Colcemid-binding macromolecule in yeast is microtubule protein. The stronger binding affinity of Colcemid relative to colchicine in the yeast system is consistent with the reports of others on the more potent inhibitory effect of Colcemid on cell division in *S. pombe* (1) and sea urchin eggs (24), and on ciliary regeneration in *Tetrahymena* (25). The Colcemid-binding moiety in yeast has a molecular weight of approximately 110,000 daltons and is acidic in nature as judged by DEAE ion exchange chromatography. These properties are characteristic of the colchicine-binding protein in human cells, strain KB (22), sea urchin eggs (26), brain tissue (12, 23), neuroblastoma cells (20), and sea urchin sperm tails (27).

Another line of argument which supports the identification of the Colcemid-binding macromolecules in yeast as microtubule subunits is the rough correlation between the number of microtubules observed in *S. cerevisiae* nuclei and the amount of Colcemid-binding activity. Fig. 8 shows electron micrographs of sections through three nuclei isolated by Dr. M. M. Bhargava (10). Microtubule profiles in both longitudinal and

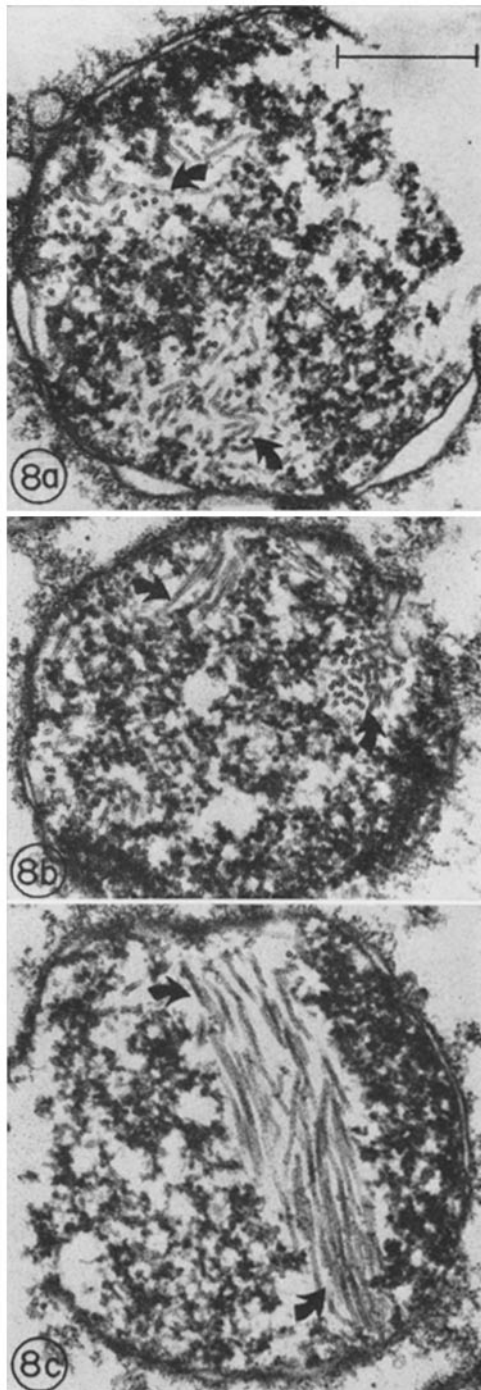


FIGURE 8 Electron micrographs of sections through a pellet of isolated yeast nuclei. *a*, *b*, and *c* are thin sections showing bundles of microtubules in longitudinal and cross-section (arrows). Approximately 40–80 microtubules can be counted in these sections. Scale, $0.5 \mu\text{m}$. $\times 37,000$.

cross-section are commonly observed in random sections; between 40 and 80 microtubules are found per nucleus. Presumably, serial section data would show that the actual number of microtubules per nucleus would be slightly higher than these figures. Assuming that there are sufficient subunits to make 60 microtubules of $1 \mu\text{m}$ length (equal to one-half the nuclear diameter), one can calculate that there would be approximately 0.018 pg of microtubule protein per nucleus; this amount would constitute approximately 2.4% of the nuclear protein and 0.18% of the total cellular protein (10). Assuming that all the microtubule subunits are recovered in the high-speed supernatant of yeast homogenates (which contains approximately 50% of the total cell protein), microtubule protein would comprise 0.36% of the extract protein.

From the intercept of the double-reciprocal plot in Fig. 6, the number of Colcemid-binding sites can be determined. Assuming that the binding sites are dimer subunits of 110,000 mol wt and that they have one molecule of Colcemid bound per dimer (12), one can calculate that Colcemid-binding protein is 0.6% by weight of the total extract protein. This is in good correspondence with the figure of 0.36% calculated from the estimate of the numbers of microtubules per nucleus.

In conclusion, this study has demonstrated the existence of Colcemid-binding activity in *S. cerevisiae*. The affinity of the binding moiety for an analog of colchicine, its molecular weight of 110,000 and its retention properties on DEAE ion exchangers all suggest that the Colcemid-binding moiety may be provisionally identified as microtubule subunits. The correlation between the amount of binding activity and the number of microtubules observed in yeast nuclei also supports this conclusion. Further experiments are in progress to isolate and purify this protein, and to establish its role in the growth and division of yeast.

The authors wish to thank Dr. J. B. Olmsted for a critical reading of the manuscript.

This work was supported in part by the National Institutes of Health Grant A-10610-01 to Dr. Halvorson and the National Science Foundation Grant GB-8735 to Dr. Borisy. Dr. Haber wishes to acknowledge support from a National Science Foundation Postdoctoral Fellowship.

Received for publication 28 April 1972, and in revised form 25 May 1972.

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APPENDIX

Permeability, p , is defined as the ratio C_i/C_e , where C_i is the concentration of compound inside the cell and C_e is the concentration external to the cell. The distribution of a compound external and internal to cells is governed by the following condition for the conservation of mass:

$$CV = C_e V_e + C_i V_i \quad (1)$$

where V is the total suspension volume, V_e is the volume external to cells, and V_i is the volume internal to cells; C_e and C_i are, respectively, the concentrations of compound external and internal to the cells, and C is the concentration of compound that would result if a sample of drug equal to that added to the cell slurry is added to an equivalent volume of media. C_e is experimentally measured as the concentration of drugs in the supernatant of the cell suspension after packing the cells by centrifugation. Defining the ratio of supernatant concentrations as $R = C_e/C$ and dividing

Equation 1 by VC_e , we obtain:

$$1/R = v_e + pv_i, \quad (2)$$

where v_e and v_i are, respectively, the volume fractions external and internal to the cells. For Blue Dextran, $p = 0$ and

$$1/R_{BD} = v_e. \quad (3)$$

For tritiated water, $p = 1$ and

$$1/R_{H_2O} = v_e + v_i. \quad (4)$$

Combining Equations 3 and 4, we obtain

$$v_i = 1/R_{H_2O} - 1/R_{BD}. \quad (5)$$

Hence,

$$1/R = 1/R_{BD} + p \left(\frac{1}{R_{H_2O}} - \frac{1}{R_{BD}} \right) \quad (6)$$

or, per cent permeability, P, is given as

$$P = \frac{R_{H_2O}(R_{BD} - R)}{R(R_{BD} - R_{H_2O})} \times 100. \quad (7)$$