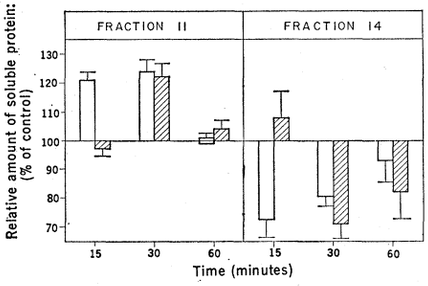

Fig. 3. Quantitative effects of ACTH and cylic AMP on fractions 11 and 14. Total area of the stained protein was fractionated by acrylamide-gel electrophoresis and the area under each peak was determined by planimetry (*13*). The change in the relative contribution of each fraction is shown for cells harvested 15, 30, and 60 minutes after exposure to ACTH (open bars) and cyclic AMP (closed bars). Standard errors are indicated by the line extending from each bar; there were at least five electropherograms in each group. The statistical significance of differences was estimated with Student's *t*-test for independent means (*18*).

of stimulatory amounts of either hormone or nucleotide in the tissue culture medium, both suggest that protein synthesis is involved in the induction of, rather than the maintenance of, increased steroidogenesis. Since there was no effect on fractions 11 and 14 when steroid production was increased by addition of reduced nicotinamide-adenine dinucleotide phosphate or Δ^5-pregnenolone to the culture medium, the possibility that changes in intracellular steroid concentrations were responsible for the effects on protein synthesis can be ruled out.

There is already evidence that cyclic AMP can selectively affect protein synthesis in its mediation of glucagon and catecholamine effects on liver (*15*) and in the synthesis of galactosidase in *Escherichia coli* (*16*). Our findings show that the action of cyclic AMP in steroidogenic cells also includes specific effects on protein synthesis. The discovery of rapid positive and negative changes of only a few protein fractions, before any effect on amount or labeling of total cytosol proteins or total cell proteins, is consistent with our observations of the effect of ACTH on guinea pig adrenal cortex in vivo (*17*) and with Farese's concept of two labile adrenocortical cytosol proteins, one a promoter of cholesterol side-chain cleavage and the other an inhibitor (*5*). The proteins of fractions 11 and 14 may have such effects on an adrenocortical mitochondrial system.

Discovery that the principal intracellular mediator of ACTH action and the hormone itself have similar effects on protein synthesis (when the lag in the steroidogenic effect of the nucleotide is accounted for) encourages the suspicion that these two protein fractions play a role in regulating the induction of increases in steroidogenesis. Perhaps target cells for other polypeptide hormones will also show such changes when similarly examined. Evidence that labile proteins are important in the control of steroid biosynthesis is still, however, no more than circumstantial.

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Colcemid Sensitivity of Fission Yeast and the Isolation of Colcemid-Resistant Mutants

Abstract. *Cell division of the fission yeast,* Schizosaccharomyces pombe, *is reversibly inhibited by the antimitotic agent Colcemid* (N-*deacetyl*-N-*methylcolchicine) in nutrient medium. Cell growth continues until all cells become nonseparating cell doublets in a V configuration. Mutants have been isolated capable of uninhibited growth in the presence of concentrations of Colcemid mycostatic for the parent strain.*

Biochemical and physiological investigations on cell division and mitosis have benefited from the use of agents which interact with cellular components needed for normal chromosome segregation. The colchicine family of alkaloids has wide use in such studies as a mitotic poison for animal and plant cells (*1*). Use of radioactive colchicine has allowed identification and partial purification of a protein component of the microtubules of the mitotic apparatus which binds the drug (*2*). It seemed reasonable to try to apply these drugs to a study of the mitotic apparatus of a microbial eucaryotic cell. The species desired should combine features of chromosome segregation of higher cells with the genetic flexibility of microbes. Initially, several microbial eucaryotes appeared to offer equivalent advantages of ease of cultivation and genetic analysis.

Colchicine induces abnormal cell morphology in *Chlamydomonas eugametos* (*3*) and inhibits regeneration of flagella in *C. reinhardi* (*4*). It inhibits and then synchronizes cell division (*5*) and inhibits regeneration of cilia in *Tetrahymena pyriformis* (*6*). We had elected to work with the fission yeast, *Schizosaccharomyces pombe,* because its mode of cell division seemed to favor the detection of aberrancies of this process and because of its suitability for genetic analysis (*7*). We now report the sensitivity of this yeast to colchicine and its *N*-deacetyl-*N*-methyl derivative Colcemid (Ciba), and the isolation of drug-resistant mutants.

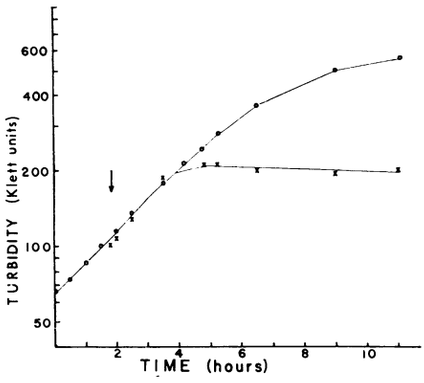

Fig. 1. Turbidity of a culture of *S. pombe*. Cells were growing in YEG medium at 32°C with aeration (open circles), and with $5 \times 10^{-3}M$ Colcemid (crosses) added to a portion at the time indicated by the arrow. Values of turbidity are in Klett-Summerson units obtained with a No. 42 blue filter and a cell suspension of 14-mm path length.

Cultures of *S. pombe* in medium containing 0.5 percent Difco yeast extract and 3 percent glucose (YEG) are inhibited in their growth when exposed to concentrations of Colcemid greater than $3 \times 10^{-3}M$ (Fig. 1). During the initial 3 hours after cultures are exposed to the drug, the turbidity doubles at the same rate as that of cultures of untreated cells and then reaches a plateau. Synthesis of DNA, RNA, and protein generally follows the changes in turbidity (*8*).

Microscopically, the treated cells assume a "V" configuration in which two cells remain attached at a joint terminus off the major axis of the cells (Fig. 2, left); only a small percentage of the untreated cells are in the V configuration (Fig. 2, right). The V configuration of cell doublets is resistant to agitation in a Vortex mixer and appears to be a sensitive indication of Colcemid inhibition since it can be detected at drug concentrations insufficient to inhibit markedly overall increases in optical density. After addition of $5 \times 10^{-3}M$ Colcemid, the number of single cells or cells with normal division appearance decreases, while the number of V doublets increases steadily from a base line of 5 percent of the cells until all the cells have this appearance (Fig. 3). These observations suggest that there is a late stage in the cell division of this yeast, which is critically sensitive to the drug rather than some general interference with macromolecular metabolism.

Although the consequences of long-term incubation in YEG-Colcemid have not been studied fully, the inhibition can be reversed. A Poisson distribution analysis of the viability of cell doublets formed by 3 hours' exposure to Colcemid indicated that about 20 percent of them could form morphologically normal progeny when diluted into fresh YEG medium free of Colcemid.

Inhibition by Colcemid was also observed with cell cultures spread on YEG agar plates to which different concentrations of Colcemid were spotted. Inhibition of growth on solid medium and production of cell doublets by colchicine (Sigma) in liquid medium required higher concentrations (above $5 \times 10^{-2}M$) than Colcemid. For this reason, most of the inhibition studies in vivo were made with Colcemid.

The resistance of *S. pombe* to vinblastine, a different mitotic poison, was studied. In microdroplets of YEG medium, vinblastine at $1 \times 10^{-3}M$ produced cells with a shriveled appearance rather than attached cell doublets.

Although plant cells are generally more resistant than animal cells to the colchicine alkaloids and require concentrations greater than $10^{-4}M$ to delay the metaphase stage of mitosis (*9*), we wished to be sure that the high concentrations of Colcemid needed to inhibit *S. pombe* did not harbor a different inhibitory impurity. Ascending chromatography of Colcemid preparations on thin-layer silica-gel sheets (Eastman chromagram No. 6060) revealed one fluorescent major component with an R_F of 0.72 in solvent system A and with an R_F of 0.78 in solvent system B (*10*). A minor component of about 1 percent or less of the preparation stayed near the origins. Inhibitory activity was found only with material eluted from the main component region and at concentrations similar to the original Colcemid preparations. Inhibitory activity was absent from the remainder of the chromatogram. Since the two solvent systems used in these separations were not used in the original commercial purification of Colcemid, we feel that the growth-inhibiting agent in our preparations is indeed Colcemid.

The sensitivity of *S. pombe* to Colcemid was not increased by use of Glusulase-induced protoplasts (*11*), by prior treatment of the cells with $10^{-2}M$ ethylenediaminetetraacetate, or by concomitant exposure to 1 percent dimethylsulfoxide. No useful difference was seen over a *pH* range of 4 to 6.

Other yeast strains were examined for their sensitivity to Colcemid in anticipation of a direct applicability of this approach. Surprisingly, although different isolates of *S. pombe* of both mating types were sensitive to Colcemid, identical treatments were ineffective with *Saccharomyces carlsbergensis*, *S. cerevisiae*, *S. chevalieri*, *S. italicus*, *S. lactis*, *S. marxianus*, *Hansenula wingei*, and *Torula monosa* (*12*). The characteristic budding form of cell division of these other yeasts obscured determination of V configurations of cell doublets, although abnormal doublets were clearly not formed in increased numbers.

When YEG agar plates supplemented with $8 \times 10^{-3}M$ Colcemid are inoculated with a few hundred cells of *S. pombe* and incubated at 32°C, microcolonies containing a few to a few hundred progeny cells form. Variable proportions of these microcolonies develop to about 0.2 to 0.5 mm in diameter in the course of incubation for a week. When inocula of the order of 10^8 cells are used, about ten colonies are readily distinguishable from the background of

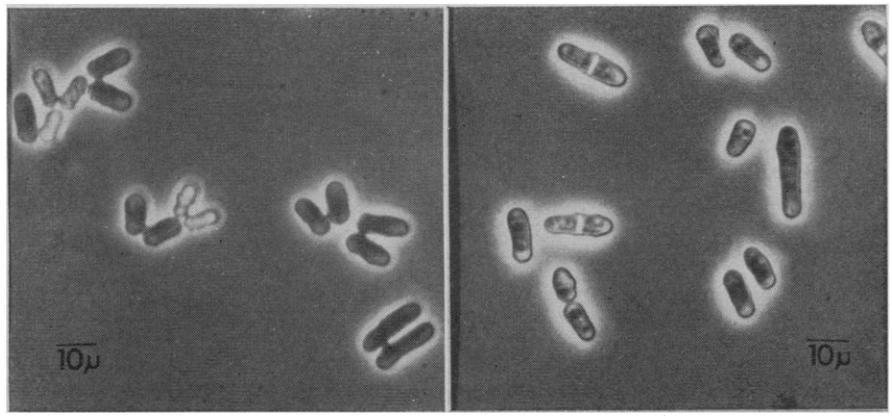

Fig. 2. Cells of *Schizosaccharomyces pombe* treated as in Fig. 1. (Left) Cells 3 hours after exposure to $5 \times 10^{-3}M$ Colcemid in growth medium; (right) cells in growth medium without Colcemid.

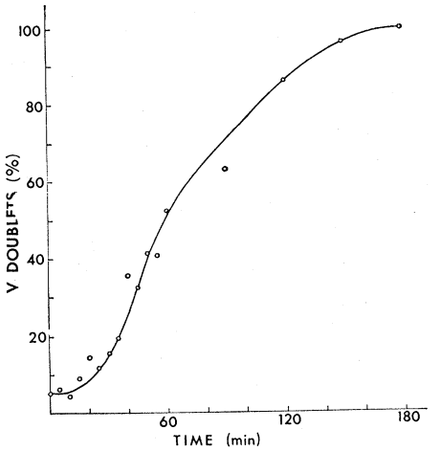

Fig. 3. Percentage of V-shaped doublets in cells of a culture of *S. pombe* exposed to Colcemid as in Fig. 1.

yeast microcolonies by their more rapid growth. The drug concentration used is not fully inhibitory, so that the spontaneous mutation rate per cell division need not be unusually high for this frequency of appearance. Other isolates were made by first enriching for growth of resistant cells in liquid YEG medium containing $5 \times 10^{-3}M$ Colcemid. When the presumptive mutant colonies are picked and streaked on YEG-Colcemid plates, colonies are readily visible in 1 to 2 days and are 2 mm in diameter by 3 days. A similar tolerance to Colcemid in YEG agar is shown by mutants previously grown on YEG agar alone, and hence the resistance is an inherited trait. The residual sensitivity to Colcemid of different mutants is readily measured by streaking them along YEG plates containing a lateral gradient of drug concentrations (*13*). Concentrations tolerated by the mutants are estimated by the range of the gradient which permits yeast growth. Resistance among mutants varies (up to $10^{-2}M$ Colcemid) by this definition.

Unlike colonies of sensitive cells, colonies of mutants on YEG agar contain single cells, numerous cell doublets, and cohesive aggregates of cells. When the mutants are grown on YEG agar with Colcemid, elongated cells with several division plates are frequent. Fortunately, the mutants have retained normal characteristics of fertility and ascospore formation when crossed with wild-type cells, and they appear to be suitable for further genetic analysis.

Our observations with *S. pombe* suggest that it may be suitable for a study of mitosis in which antimitotic agents are used. Its choice on the basis of cell division characteristics proved fortuitous since a priori there was no reason to believe it would be more sensitive than other Ascomycetes. The inertness of other yeast species to Colcemid is unexplained, and in a formal sense they behave as Colcemid-resistant strains.

Cytological examinations of *S. pombe* and of other yeasts have revealed microtubular elements within the nucleus (*14*). A presumptive mitotic target for the colchicine alkaloids appears to be present, although it remains to be isolated and more directly demonstrated. The main argument for a mitotic apparatus in yeast species still rests on the need for a mechanism to carry out the observed equipartite distribution of a number of genetic linkage groups. The extent to which the responsible structure in yeast resembles that of higher cells remains to be determined. The immediate question that we are led to ask is whether Colcemid inhibits cell division in *S. pombe* by interacting with a mitotic apparatus, or whether the observed inhibition is secondary to some other property of the drug.

Caution is warranted on the interpretation of the resistant mutants. The explanations that must be considered include permeability changes and detoxification mechanisms as well as structural changes in the mitotic apparatus which might affect either the binding of Colcemid or the functioning of a possible complex between Colcemid and the mitotic apparatus. There is a possible analogy with streptomycin resistance in bacteria, where there are separate mechanisms involving either a detoxifying modification of the drug or a change in a protein subunit of the bacterial 30*S* ribosome, so that protein synthesis is no longer inhibited by the drug (*15*). Until we can assign appropriate mechanisms of resistance, we propose the designation *cid* for mutants which differ from wild-type in *N*-deacetyl-*N*-methylcolchicine *i*nhibition of *d*ivision.

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Induction and Isolation of Auxotrophic Mutants in Somatic Cell Cultures of Nicotiana tabacum

Abstract. *A method for the selection of auxotrophic mutants from somatic cell cultures of* Nicotiana tabacum *depends upon the incorporation of 5-bromodeoxyuridine into the DNA of wild-type cells and upon its lack of incorporation into the DNA of auxotrophic cells. There may be more than one functional copy of essential genes in the haploid genome of* Nicotiana tabacum.

The present work was undertaken to develop methods for the isolation of auxotrophic mutants from somatic cell cultures of higher plants. Recent developments in plant tissue culture research indicate that haploid tissue is easily obtained from anther culture of several solanaceous species (*1*) and that single tobacco cells in culture can be treated as experimental organisms (*2*). A previous paper describes a technique for the isolation of auxotrophic mutants in the haploid generation of ferns (*3*). This report describes the successful utilization of the mutant isolation technique on single haploid tobacco cells in culture.

The selective technique employed is