ISOLATION AND CHARACTERIZATION OF NYSTATIN RESISTANT MUTANTS OF *SACCHAROMYCES CEREVISIAE*

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A THESIS

Submitted to the

AMERICAN UNIVERSITY OF BEIRUT

In partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE IN
BIOLOGY - (GENETICS)

September 1967

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Date Thesis is presented: September 22, 1967.

NYSTATIN RESISTANCE IN

SACCHAROMYCES CEREVISIAE

HASHWA

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Lynn Miller for his encouragement, criticism and constructive suggestions offered during the course of this investigation. I also wish to express my gratitude for the excellent and expert technical assistance of Mrs. V. Athanasiou. I am indebted to Miss V. Madelian for dissecting the yeast asci, to Dr. D.C. Hawthorne (Dept. of Genetics, Univ. of Washington Seattle, Washington) for analyzing my spore germination data, and to the School of Pharmacy for using the Perkin-Elmer Recording Spectrophotometer.

AN ABSTRACT OF THE THESIS OF

Fuad A. Hashwa for M.S. in Biology (Genetics)

Title: Isolation and characterization of Nystatin resistant mutants of Saccharomyces cerevisiae.

Mutants of Saccharomyces cerevisiae resistant to Nystatin (Squibb Mycostatin) were obtained by selective methods. Spontaneous and mutagen induced mutants occurred. Unstable states of resistance appeared but no reversion to the susceptible condition in the stable clones was observed.

One locus for Nystatin resistance was found.

The production of sterols in the highly resistant strains was found to be much less than the non-mutant strains, in some mutants no Ergosterol was detectable.

I believe that these Nystatin resistant mutants will prove to be useful in the study of sterol metabolism.

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I. INTRODUCTION

Objects

This work involves the isolation and genetic and physiological characterization of spontaneous and chemically induced mutants of the yeast Saccharomyces cerevisiae that are resistant to inhibitory and lethal dosages of the antifungal agent Nystatin, also known as Mycostatin.

With these Nystatin resistant mutants, I wish to investigate the following objects:

1. Understand the mode of action of this antibiotic.
2. Study and understand the nature of the resistance to Nystatin. Both of these objects can be approached by comparative studies on the sensitive parental strains and their isolated resistant variants.
3. This work will help to put down a protocol for such an isolation, and as this paper will show, will introduce a new method to obtain Saccharomyces cerevisiae mutants with an altered sterol metabolism. Such mutants may throw more light on the role of sterols in yeasts.

The Chemical Nature of Nystatin

Nystatin is a polyene antibiotic of broad antifungal activity produced by Streptomyces noursei. One of the chemical characteristics that has hindered our work is its slight water solubility at around pH 7. It is somewhat more soluble in the lower aliphatic alcohols, and it is highly soluble in solvents such as Propylene glycol, N, N-dimethylamide or Dimethyl sulfoxide.

Although Nystatin was the first of the so-called polyene antibiotics to be discovered, its chemical structure has not been completely elucidated. It is amphoteric, crystalline with the probable emperical formula $C_{46}H_{75}NO_{18}$. It is known to have both a conjugated diene and conjugated tetraene moiety in the molecule; the infrared spectrum indicates the presence of carboxyl and lactone structures. Because of the extensive unsaturation of the molecule, Nystatin is unstable and hence readily inactivated by heat, light and oxygen, (Hazen and Brown, 1960).

Physiological Effects of Nystatin on Yeasts

Lampen et al. (1957), reported that Nystatin inhibits the growth of most fungi at concentrations between 1 to 10 ug/ml. Studying the effect of Nystatin on the utilization of substrates, they reported that the action of Nystatin on glycolysis, as with oxidation, is characterized by an initial burst of metabolic activity followed quickly by a cessation of glucose utilization. It appears that Nystatin renders the cell incapable of utilizing glucose, but

does not block glucose uptake.

Gottlieb et al. (1958), studying filipin, another polyene antibiotic, noted that the presence of a conjugated polyene structure in filipin suggested that perhaps filipin interfered with the synthesis or the function of carotenoids in the fungi, they add that sterols play a much more important role in the growth process of fungi than has hitherto been suspected, for while a few microorganisms have been shown to require sterols for growth, their studies have indicated that such substances are probably essential metabolites for many fungi. The mechanism by which filipin and other polyenes inhibit fungi, they add, is intriguing. They might either prevent the synthesis of sterols which are necessary for growth or competitively replace the sterol as a cofactor of a reaction vital to the metabolism of the organism. The finding that led to this intriguing hypothesis was that cholesterol or ergosterol protected fungi (yeasts) from toxic effects of filipin, however, that the possibility of an extracellular interaction between sterols and filipin was not eliminated.

Lampen et al. (1959) observed that sterols, bile salts and high concentrations of phosphates have been found to reduce the biological effectiveness of Nystatin or related polyene antibiotics.

Brown and Hazen (1960) found that 1.5 to 13 ug Nystatin/ml prevented growth of a wide variety of yeasts, but that higher levels were fungicidal. They add that the latitude between inhibitory and destructive concentrations was very narrow, however, and dependent on the chemical and physical environment.

Moat et al. (1959) developed a procedure to assess the ability of the antifungal antibiotics (Nystatin, Amphotericin A & B and Filipin) to eliminate wild type cells from a mixed population of mutants (adenine-requiring) of the yeast Saccharomyces cerevisiae. They used Nystatin for selection at the concentration of 2 to 3 ug/ml. They found out that 60 to 95 percent of the Saccharomyces colonies surviving exposure to the antibiotic were respiratory deficient mutants.

Bradely and Jones (1960), studying the mechanism of action of Nystatin on the pathogenic yeast Candida stellatoidea, suggested that possible physiological processes affected by Nystatin are protein synthesis, energy generation or transfer, and permeation.

Lampen et al. (1960) from their results based on spectral growth and respiratory measurements, suggest that purely physico-chemical factors are involved in the action of sterols and that sterols may be effective only in preventing inhibition, rather than in reversing an existing effect of the antifungal agent.

They also observed that a direct interaction can occur between sterols and polyene antibiotics; the structure required for this effect is similar to that needed to prevent the biological action of the polyenes and that the addition of sterols will prevent the antifungal activity of the polyenes. No evidence was obtained that sterols altered an existing inhibition by the polyenes. However their study provided no direct evidence for a metabolic interrelation between sterols and the polyene antifungal agents, although this possibility could not be ruled out.

Sutton et al. (1961), studying the effect of high concentration of Nystatin upon glycolysis and cellular permeability in yeasts, found that a graded series of effects upon yeast permeability to cell ions could be obtained by addition of increasing concentrations of Nystatin. At the lowest levels of Nystatin (1 to 5 ug/ml), the only alteration they observed was rapid loss of Potassium (K^+) ions, which correlated well with inhibition of glycolysis. Increasing level of Nystatin (10 ug/ml) initiated leakage of UV-absorbing nucleotides and caused sufficient change in the membrane that rendered permeability to pyruvate and measurement of decarboxylation possible. At highest Nystatin levels (30 ug/ml), there was some loss of protein, but the bulk of the glycolytic enzymes remained within the cell and the membrane did not appear to be open to the entrance of cell ions by simple diffusion.

Marini et al. (1961) , found that K^+ and NH_4^+ did not prevent the fungicidal or fungistatic actions of Nystatin even under conditions where the protection of glycolysis by these ions was essentially complete. The original yeast cells were not dependent upon added K^+. After a brief treatment with Nystatin at pH 7.0, the cells showed an absolute requirement for K^+ or NH_4^+, it was shown that K^+ was rapidly lost by cells following contact with Nystatin. They concluded that Nystatin directly damages the cell membrane and thus produces a rapid increase in permeability to small ions. The resulting depletion of cellular K^+ halts glycolysis. The addition of K^+ or NH_4^+ restores glycolysis but does not reverse the membrane alterations.

Lampen et al. (1962) asserted that binding by the membrane appears to be the critical event in cell damage. They found out that bound radioactive Nystatin was not displaced during incubation of exponentially growing yeast cells with a large excess of unlabeled Nystatin. They proposed that binding by the cell membrane represents a combination of the polyene and the sterol, probably the free (sic) Ergosterol. They supported this hypothesis by the following evidence:-

a) Organisms sensitive to the polyenes contain substantial amounts of sterols, whereas bacteria, which are insensitive, do not contain any sterols.

b) Sterols can complex with polyenes in vitro, preventing binding of the polyene to the cell, and protect against its inhibitory action.

c) Polyenes were bound rapidly by protoplasts. All of the polyene taken up by the protoplasts was found in the membranes, which is also where the sterol is located.

d) Preliminary experiments showed a rough correlation between the sterol content of protoplasts and their Nystatin-binding capacity.

e) Sterol and Nystatin were released in parallel during rupture of cells.

f) Incubation of Nystatin-treated protoplasts with the sterol-complexing agent digitonin released Ca 80% of the bound Nystatin.

They also emphasized that it is not necessary to propose any metabolic interaction between polyenes and sterols. The polyene is assumed to reach the cell membrane and combine with ergosterol, possibly damaging the membrane in the binding process and producing lethal changes in cell permeability. Concluding that once a binding site is reached, adsorption with no requirement for metabolic energy occurs. Nevertheless, they add that the sites are not readily accessible to polyenes and that temperature (and energy) dependent reaction(s) are essential either to alter the accessibility or reactivity of the binding site or to move the polyene to it.

Stachiewiez and Quastel (1963) discussed that to produce its effect, Nystatin must first be taken up by the cell. As the absorption of Nystatin is specific to organisms that are sensitive to this antibiotic, it appears that only in such cells are these sites present on the membrane that are involved in uptake of Nystatin. The cell membrane and its lipid phase are the structures responsible for preventing the efflux of various small molecules concentrated within the cell.

Demel et al. (1965) presented similar evidence to that listed by Lampen and coworkers (1962), and pointed out that selective toxicity of polyene antibiotics is due to interaction with a compound present only in the membrane of sensitive organisms. They concluded that a) Polyene antibiotics (Filipin and Nystatin) interact specifically with sterols, b) Penetration of only a few antibiotic molecules into the cell membranes of sensitive organisms causes a "reorientation" of the sterol molecules in the membrane and a

consequent alteration in cell permeability.

Zygmuut and Tarovmina (1966) reported that of the numerous steroids tested, ergosterol was the only one which effectively antagonized the antifungal activity of five polyene antibiotics including Nystatin. This finding is consistent with the observation (Lampen et al. 1962) that binding of polyenes with ergosterol is stronger than that with other steroids.

Previous Attempts at Obtaining Nystatin Resistant Mutants

Lampen et al. (1957) reported that Nystatin inhibits the growth of most fungi at concentrations between 1 to 10 ug/ml, but that the development of organisms resistant to high concentration of Nystatin was not observed in their laboratory.

Hebeka and Solotorovsky (1965) isolated strains of Candida albicans, a pathogenic yeast, that are resistant to the polyenes Candidin, Amphotericin B, but not to Nystatin. These were obtained by subculturing the organisms in gradually increasing concentrations of the antibiotics in both a shaker or by repeated transfer on gradient plates. Development of resistance to Nystatin was not shown by any of the strains used or any of the techniques employed. Only a 2 to 3 fold increase in resistance of C. albicans to Nystatin in both liquid media and gradient plates, which they thought might be insignificant, was encountered. They obtained a 150-fold and 60-fold resistance respectively to Candidin and Amphotericin B. They have also noticed that their polyene resistant isolates of C. albicans

have a lower rate of metabolism than their parental sensitive strains and exhibited a slower rate of growth.

Other investigators, Donovick et al. (1955), Littman et al. (1958), Stout and Pagano (1956), and Axelard (1960) were unable to demonstrate the development of resistance of C. albicans to Nystatin.

Previous Attempts at Obtaining Sterol Mutants

Sterols are necessary for growth of yeasts and all other eucaryotic cells. Their synthesis is under the control of a number of enzymes and is dependent on molecular oxygen. Since some yeasts can grow anaerobically in the presence of added sterols, i.e., when sterol synthesis is prevented, (e.g. Andreason found (1953) that the strain Sc-1 of S. cerevisiae requires sterols when grown anaerobically), it seems logical that yeast mutants lacking one or more sterol enzymes could be obtained, and that such mutants would require sterols for aerobic growth.

E. Adelberg et al. (1955) made extensive unsuccessful attempts to induce requirement for steroid growth factors in E. Coli and in Saccharomyces cerevisiae . They subjected some 512,800 survivors of ultraviolet irradiation to penecillin selection looking for mutants requiring steroid growth factors. They found none. In another attempt to produce cells with an ergosterol requirement in S. cerevisiae, they used the delayed enrichment procedure of Lederberg and Tatum (1946), with ergosterol being used for the enrichment at a final concentration of 10 to 20 ug/ml, in some cases they added Sodium Desoxycholate or "Tween 80" to promote sterol emulsification and cell

penetration. They obtained a few yeast colonies that exhibited a temporary aerobic requirement for ergosterol but none were permanently deficient.

Starr and Parks (1962) reported that sterol synthesis in S. cerevisiae is maximal at 30°C and progressively decreases as the incubation temperature is increased. Addition of ergosterol to defined media permitted growth at elevated temperatures, thus showing that the inability to grow at higher temperatures is due in part, to inhibition of sterol synthesis. Parks and Starr (1963) tested a number of respiratory deficient mutants of S. cerevisiae for their ability to synthesize ergosterol at 30°C. They noted considerable variations in the amounts of sterols formed, some clones produced more sterols than the wild-type cultures.

Resnick and Mortimer (1966) also used UV irradiation to attempt to induce sterol requiring mutants of S. cerevisiae. They plated surviving cells on Yeast Extract peptone-dextrose (YEPD) medium supplemented with Oleic acid, "Tween 80" and Ergosterol. They discovered two new types of lipid nutritional mutants. One type requires an unsaturated fatty acid; this type of mutant grows with many different unsaturated fatty acids. The other mutant needs either an unsaturated fatty acid or ergosterol for growth.

Prospects of Obtaining Sterol Mutant Among Nystatin-Resistant S. Cerevisiae

The review of the physiological effects of Nystatin on yeasts presented earlier, comprise an attempt to answer the question: "Why

do we think that Nystatin resistant cells of S. cerevisiae might have altered sterol metabolism?".

We predict that Nystatin resistant cells might have an altered sterol metabolism. These mutant cells might have one or more of the following altered possibilities:

1. Mutant cells that produce excessive amounts of sterols; such cells would be protected from the lethal effects of Nystatin as the excess sterols produced would leave a large amount unbound by Nystatin.
2. Mutant cells that produce low amounts of sterols; these cells would have sufficient amounts of sterols for growth but would not be inhibited by Nystatin.
3. Resistant mutant cells having traces or no sterols at all; the prevention or extreme reduction of sterol synthesis in this case would allow such mutants to grow normally in the presence of Nystatin. This situation would result from a lack of one or more enzymes catalyzing the synthesis of sterols. If such cells would grow without any requirements for sterols, then the hypothesis that sterols are necessary for aerobic growth of all eucaryotic organisms would be questioned.

Having such predictions in mind, we used the chemical mutagens Nitrosoguanidine and Ethyl Methane Sulfonate followed by Nystatin selection to isolate Nystatin resistant mutants that might have an altered sterol metabolism.

(Note added in final week: We have learned that an article on "A genetic analysis of resistance to Nystatin in Saccharomyces cerevisiae" by K.A. Ahmed and R.A. Woods can be found in the journal Genetical Research, Vol. 9(2), April 1967. Unfortunately we do not receive this journal and, because of the "six days war", we have not received a reprint).

II. GENERAL MATERIALS AND METHODS

Yeast Strains

The following haploid strains (Table 1) of Saccharomyces cerevisiae were used for the different experiments.

Table 1. Genetic markers of haploid strains.

Strain	Mating type	Nutritional deficiencies	Others
198-2B	"a"	ad_2	gal 3-3, i^-, non-clumpy
419-3C	" "	try_1	non-clumpy
108-3C	" "	try_1, ur	clumpy

Miller (unpublished data) discovered the non-clumpy strains 198-2B and 419-3C which are particularly suited to mutation hunts. The strain 198-2B is a constitutive non-galactose fermenting mutant that also requires adenine for growth. 419-3C is a tryptophane requiring mutant while 108-3C is a double mutant that requires both tryptophane and uracil for growth. We chose 198-2B and 419-3C to be of opposite mating types in order to mate them and their Nystatin resistant isolates for genetic analysis.

198-2B and 108-3C were kindly provided by Prof. H.C. Douglas,

from the Dept. of Microbiology, Univ. of Washington, Seattle. The strain 419-3C is an ascospore isolate obtained by Prof. L. Miller, Biology Dept., American University of Beirut, Beirut, Lebanon.

Media

The complex media were FYAD, yeast extract 1% (Fleischmann Yeast Hydrolysate T4018), Dextrose 1% and agar oxoid No. 2 1%, and liquid FY2 2D having 2% Dextrose. Wickerhams yeast nitrogen base was the synthetic medium, referred to as minimal, (Wickerham 1949). This was prepared and supplemented when necessary with suitable amino acids, bases, Ergosterol and "Tween 80". The amino acids and bases were autoclaved separately and were added to the medium, containing 1% oxoid agar before use. Sporulation medium consisted of 1% Potassium acetate, 0.25% FY extract, 0.1% Dextrose and 0.9% Agar. Tap water was used for the preparation of the above mentioned media.

The Ergosterol/Ethanol and "Tween 80" (Sigma 35B-2480) stock solutions were prepared by the method of Andreason and Stier (1953). Fifty ml stock solution was prepared by combining 0.1094 g of Ergosterol, 11 ml of Tween 80 (these values were half the amount of Ergosterol and Tween 80 that were used by Resnick and Mortimer (1966) with about 40 ml of Ethanol, 0.8 ml per 100 ml media of the stock solution were used, and hence the media contained 3.5 mg Ergosterol per 100 ml and 3.5 volume Tween 80 per 100 ml.

Stock cultures of all strains were streaked on FYAD slants (0.3% FYD and 1% Agar) in 1 dram screw cap vials and incubated 24 hours at 28°C. They were subcultured at infrequent intervals.

Nystatin Preparation and Application

Nystatin (Squibb 5915) 500,000 units per vial was purchased from E.R. Squibb and Sons, New York. Two stock solutions of Nystatin were used. One an aqueous suspension (NH_2O) prepared by adding aseptically 10 ml of sterile distilled water to the vial with a syringe. The second stock preparation is a Nystatin solution (NPG) prepared by adding 20 ml of 1,2 Propylene glycol (Merk's 7478) to vial. I prepared less concentrated suspensions of both stock solutions by diluting a known volume from the stock vial with either sterile distilled water or complex liquid medium. In case of NPG the Nystatin immediately came out of solution upon dilution and appeared as a wavy colloidal cloud of very small but evenly distributed particles. In NH_2O Nystatin's particles (size ranging from 1.97 to 12.9 microns) sedimented at a fast rate. Vials of both stock solutions were stored at $4^{o}C$ and were discarded after a month if not used.

The Nystatin Overlay Technique

The Nystatin overlay technique was especially designed by L. Miller for the selection of Nystatin resistant cells of S. cerevisiae. To a warm (liquid) 5 ml FYAD cotton plugged test tube placed in a waterbath at $45^{o}C$, add the appropriate concentration of Nystatin and quickly but gently mix the contents of the tube (to avoid bubble formation) and pour immediately on top of a shallow FYAD plate (containing 20 ml FYAD), making sure that contents of the test tube

are spread all over plate. The use of a flat surface is quite essential here in order to obtain an evenly distributed Nystatin concentration in the overlay. Nystatin overlays using an NH_2O solution were not effective for Nystatin resistance selection because a) Nystatin is insoluble in water and b) Nystatin has different particle sizes. Both of these factors make an even distribution of the Nystatin concentration in the overlay very difficult. We overcome this difficulty by the use of Nystatin dissolved in Propylene glycol. (For further details on conc(s) of overlays see mutation hunts).

Mutagens

I have used the chemical mutagens Ethyl Methane Sulfonate (EMS) and N-methyl-N^1-Nitro--N-Nitrosoguanidine (NGu) for the chemical induction of Nystatin resistant mutants of S. cerevisiae, while I used Methylproflavin for the induction of Respiratory deficient cells.

I used 0.15 ml/5 ml FYe 2D culture of a standard EMS solution (Eastman's 7830). Stock bottle was wrapped in tin foil and stored in a desiccator at 4^oC. Nitrosoguanidine (Aldrich M 6200), stock solution 1 mg per ml is kept frozen and allowed to thaw before use, was used at a final concentration of 20 ug/ml. L. Miller prepared the Methylproflavin, stock solution 1 mg per ml kept at room temperature, by the method of Marcovish (1951) and A. Albert (1951).

Sterol Extraction

We extracted and determined the Ergosterol content in our yeast strains by the method of Breivik and Owades (1957). We used UV spectroscopic n-Heptane (Fluka's 54363), absolute Ethanol (Merck 970), and 24/40 standard taper glassware.

We took absorbance readings on a Model DU Beckman quartz spectrophotometer with a photomultiplier at a slit width of 0.10 mm, while we used a Perkin-Elmer Model 202 recording spectrophotometer for our Ergosterol spectra. I will discuss further details of specific procedures in results section.

LIST OF ABBREVIATIONS

Word or phrase	Abbreviation
Adenine	Ad
Concentration	conc
Dextrose	Dex
Ergosterol	Erg
Ethyl Methane Sulfonate	EMS
Fleischmann Yeast Agar Dextrose	FYAD
" " 2% "	FYe 2D
" " Agar 2% Glycerol	FYA 2G
Minimal medium	Min
Microgram per ml	ug/ml
Millimicron	mu
Nitrosoguanidine	NGu
Nystatin	Nys
" in Propylene glycol	NPG
" in water	NH_2O
" sensitive	Nys^s
" resistant	Nys^r
Propylene glycol	PG
Polyoxyethelene sorbitan monooleate	Tween 80
Respiratory deficient	petite
" sufficient	grande
Tryptophane	try
Uracil	ur
Wickerhams medium	W°hams
Dilution	dil

III. RESULTS AND DISCUSSION

Sterol Mutation Hunt by Replica Plating

Object: The object of this experiment was to isolate Ergosterol requiring mutants of the yeast strain 419-3C. In the previous unsuccessful attempts to isolate such mutants Adelberg (1955), Resnick and Mortimer (1966) irradiated the cells with Ultraviolet light. In this series of experiments I treated the cells with the chemical mutagen EMS.

Method: To a stationary culture of 419-3C I added 0.03 ml of EMS per ml culture along with 0.97 ml FY2 2D per ml culture after I took out samples for control dilution counts. I treated the cells for a period of 40 minutes in a shaking waterbath at 28°C, centrifuged and resuspended them in an equal volume of the medium. Then I diluted and plated about 50 cells per plate on 20 FYAD plates. I incubated the plates for a 72-hours period and then replicated them onto the following plates:

i) W°ham°s Dex minimal (+ Try)
ii) " " " + 15 amino acids
iii) " " " + 5 bases
iv) " " complete (amino acids + bases)
v) " " " + Ergosterol (35 ug/ml)
vi) FYAD

Discussion: This attempt to obtain Ergosterol requiring mutants of the yeast strain 419-3C was not successful. In four series of trials I replicated 32,000 cells that survived mutagen treatment onto appropriate plates, yet less than 0.02% of these treated cells showed a sort of retarded growth on W°ham°s minimal plates lacking Ergosterol, but supplied with needed amino acids and bases. These cells were not "petites" and behaved normally upon a second transfer. I also obtained in the same series, two amino acid mutants and three base requiring clones. The reasons this method failed to give Ergosterol-requiring mutants, I think, are the following:

1. Selection of mutants by replica plating involves the use of a limited number of cells per experiment.
2. EMS (Ethyl methane sulfonate) seems to be an inadequate mutagen in this respect.
3. This method has no selective power.

What is needed is a "method" or "compound" that would interfere or alter sterol metabolism. Such an interference or change might result in a partial or complete sterol requirement; if such a thing is possible.

Isolation of Respiratory Deficient Yeast

Object: I isolated respiratory deficient cells of the strains 198-2B and 419-3C to:

1. Obtain Nystatin resistant respiratory deficient cells "petites" in order to compare them with the Nystatin resistant respiratory sufficient cells "grandes".

2. Check the reports by Moat et al. (1959) and Snow (1966) that petite cells are more resistant to low concentrations of Nystatin than the grandes.
3. Check for any alteration in the sterol metabolism of the Nystatin resistant petites. Parks and Starr (1963) isolated petite cells having more sterols than their parental grandes.

Method: The procedure for the isolation of the petite cells is as follows: I grew cultures of the strains 198-2B and 419-3C, in FYe 2D plus 28 ug/ml ergosterol, at 28°C in a shaking waterbath. I plated 0.1 ml samples of dilution 5.5 (expect 50 cells per plate) on FYAD plus Ergosterol and Methylproflavin plates. The concentrations of Methylproflavin used were 10 ug/ml, 5 ug/ml, 2 ug/ml, 1 ug/ml, 0.5 ug/ml, 0.2 ug/ml and 0 ug/ml. I added the Methylproflavin and the Ergosterol to the medium just before pouring the plates. Then I plated another 0.1 ml samples per plate of the same yeast dilution on a similar series of FYAD-Methylproflavin plates but lacking Ergosterol. I incubated all plates at 28°C for a 48 to 72 hours period after which I replicated these plates to FYA 2 Glycerol (FYA 2G) plates, to test for presence of respiratory defficient colonies.

Results: Table 2 shows the results for the "petite" isolates of the two strains 198-2B and 419-3C.

Table 2. Growth of Methylproflavin treated cells on medium containing Glycerol as an energy source.

Conc. of Methyl-proflavin on Master plates	Colonies[x] replicated onto					
	FYAD		FYA2G + Ergosterol		FYA2G+Ergosterol	
	198-2B	419-3C	198-2B	419-3C	198-2B	419-3C
0 ug/ml	+	+	+	+	+	+
0.2 "	+	+	+	+	+	+
0.5 "	+	+	+	+	+	+
1.0 "	+	+	+	+	+	+
2.0 "	+	+	±	±	±	+
5.0 "	+	+	-	-	-	-
10.0 "	+	+	-	-	-	-

As seen from Table 2 the concentration of 5 ug/ml of Methyl-proflavin was sufficient to induce 100% "petite" cell formation. These petite cells were identified by their failure to grow on a medium with glycerol used as the energy source instead of dextrose, because respiratory defficient cells which lack several respiratory enzymes cannot ferment this carbohydrate.

I only picked up small colonies from 5 ug/ml Methylproflavin plates of both strains and kept on FYAD slants (in triplicate) and coded them as 198-2B-P and 419-3C-P. I will use these isolates in later experiment to :

[x] Colonies were first grown on FYAD plates containing Methylproflavin and were replicated to FYA 2 Glycerol plates after 72-hours of growth at 28°C.

1. Isolate Nystatin resistant petite mutants.

2. Determine their sterol contents.

Effects of Propylene Glycol on Growth

Object: A major problem that faced out mutation hunts was that Nystatin is insoluble in water, and as a result, we never obtained an evenly distributed Nystatin concentration in an overlay. This insolubility lead to a sectoring phenomenon, where cells grew in one area because the Nystatin could not be evenly distributed all over the plate. We observed this phenomenon more frequently with plates of low Nystatin concentration, (such as the 500 u and the 1500 u plates). 1, 2 Propylene glycol (PG) which readily dissolves Nystatin, solved the problem of uneven distribution of the Nystatin particles.

Method: We had to test the effect of Propylene glycol alone on yeast cells in order to find out whether this solvent exhibits any inhibitory effect on the growing yeast cells. So we plated out, about 50 cells per plate, of a stationary phase culture of 198-2B on sets of FYAD plates that have different Propylene glycol concentrations. We used five plates of each concentration, and obtained the following values that are listed in Table 3.

Results:

Table 3. Effect of Propylene glycol on growth of yeast cells.

	Propylene glycol concentrations				
	4% v/v	2% v/v	1.2%	0.0%	Notes
Average cells per plate after 72-hours incubation at 28°C	60	64	62	64	All colonies were of same size

These results show that Propylene glycol up to 4% v/v has no inhibitory effect nor does it interfere or hinder the growth of yeast cells. Hence I decided to use Propylene glycol as a solvent for Nystatin in all the mutation hunts, at a concentration of 3.2 to 4% v/v in the overlays and a maximum of 0.8% v/v of PG in liquid media.

Effect of Nystatin on Growth of Yeast Cells

Object: Low concentrations of both aqueous Nystatin and Nystatin dissolved in Propylene glycol exhibit inhibitory effect on growth of the original sensitive strains of Saccharomyces cerevisiae (198-2B and 419-3C) in complex liquid medium (FYe 2D). Nystatin, in an overlay, also inhibits the growth of cells on FYAD plates.

The aim of this experiment was a) to study the inhibitory effect of Nystatin on yeast, b) to find out the minimal inhibitory

concentration, i.e. concentration needed to obtain 100% or less survival after a 48-hours treatment, and c) to determine the lethal concentration of this antibiotic.

Method: I performed these growth tests on the following strains:

a) 198-2B
b) 198-2B-P
c) 419-3C
d) 419-3C-P

For each test. I prepared a series of tubes that contained a gradually increasing concentrations of Nystatin in FYe 2D upto a final volume of 5 ml. I inoculated these tubes with 0.1 ml samples (around 10^7 cells per ml) of a stationary culture and incubated them in a shaking waterbath at $28^{o}C$ for 48 hours. Later, after a visual observation of the turbidity in the culture tubes, I plated 0.1 ml samples of the appropriate dilution of each culture tube onto 3 FYAD plates and incubated for 72 hours at $28^{o}C$.

Results and discussion: Table 4 gives the details of scoring the growth tests for 198-2B in FYe 2D/NPG.

Table 4. Growth test of 198-2B in presence of Nystatin.

Nystatin conc. in units/ml	Visual scoring after 48 hrs.	72 hrs.	No. of cells surviving/ml	Percentage[x] survival
0	+	+	4.9×10^8	100%
1.25	+	+	1.5×10^7	"
2.5	+	+	6.0×10^6	"
5.0	+	+	4.2×10^6	"
7.5	+	+	3.2×10^6	"
10	-	+	3.7×10^5	16.5
12.5	-	+	1.6×10^5	7.0
15	-	+	1.7×10^5	7.5
17.5	-	+	0	-
20	-	+	2×10^4	0.9
25	-	-	0	0
30	-	-	0	0

[x] Cell inoculum was 2.24×10^6

It is important to note here, that in liquid media Nystatin tends to lose its inhibitory activity after 48 hours of incubation at 28°C. The data showing the effect of various concentrations of Nystatin on the growth and survival rate of the yeast strains 198-2B, 198-2B-P, 419-3C, and 419-3C-P in liquid complex medium are in Figures 1 and 2. (Please note that the curves for the Nystatin resistant mutants which are included in these two figures will be discussed in a later section).

The steep slopes of the curves, which are common to all the strains tested, indicate that the margin between inhibitory (fungistatic) and lethal (fungicidal) concentrations of Nystatin is very narrow. This margin falls within an average range of 4.5 units per ml (i.e. 1.5 ug/ml) for NPG and 10 units per ml (3.3 ug/ml) for NH_2O.

The minimal inhibitory concentration and the lethal concentration of Nystatin for each of the four non-mutant strains are shown in Table 5.

Table 5. Inhibitory and lethal concentrations of Nystatin on sensitive cells.

Strain used	Min. Inhib. conc. units/ml in		Lethal conc. units/ml in	
	NPG	NH_2O	NPG	NH_2O
198-2B	8-10	8-10	30	60
198-2B-P	"	"	"	100
419-3C	5-6	15	30	60
419-3C-P	10-15	20	"	80

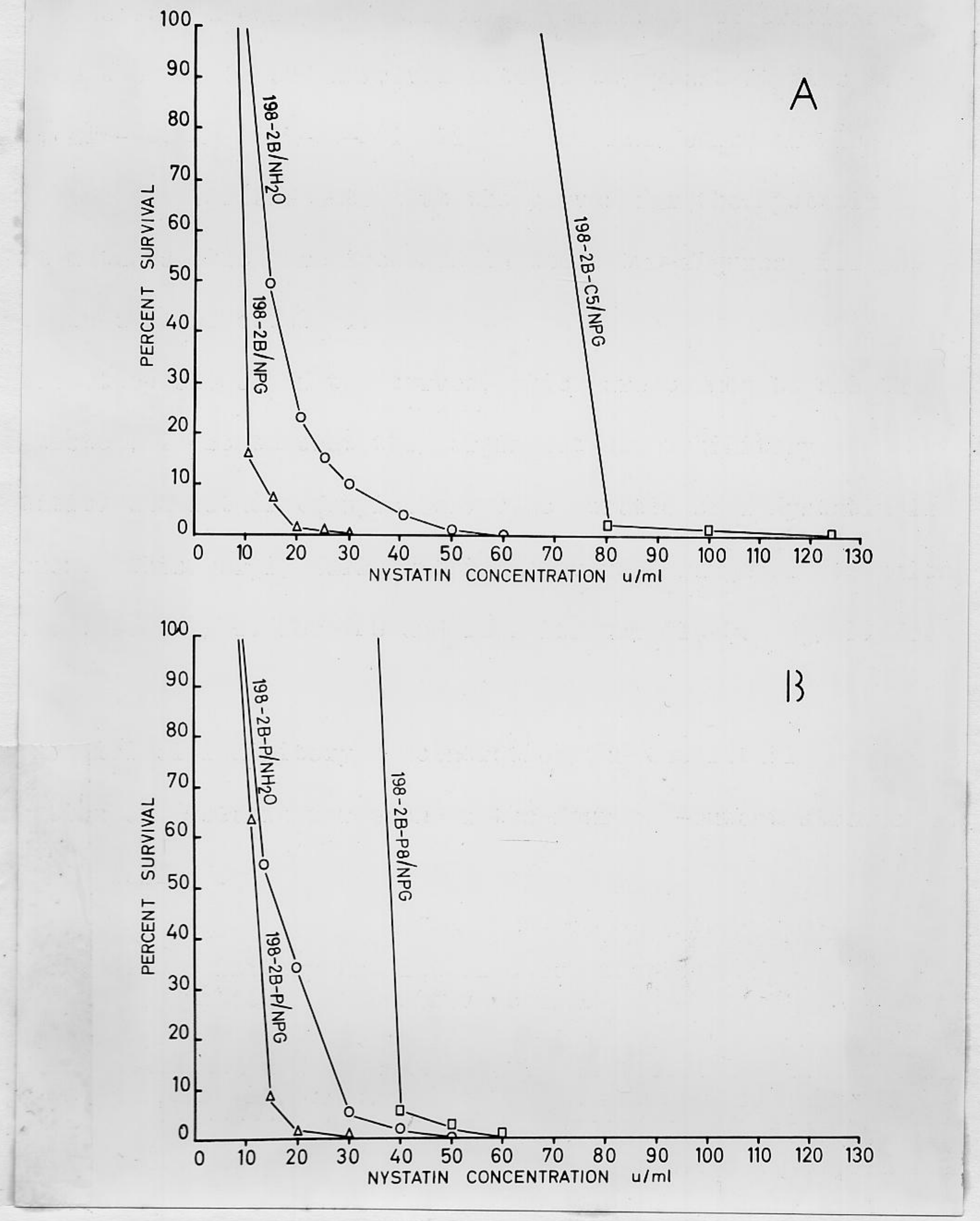

Figure 1. Effect of Nystatin survival of

(A) 198-2B (B) 198-2BP

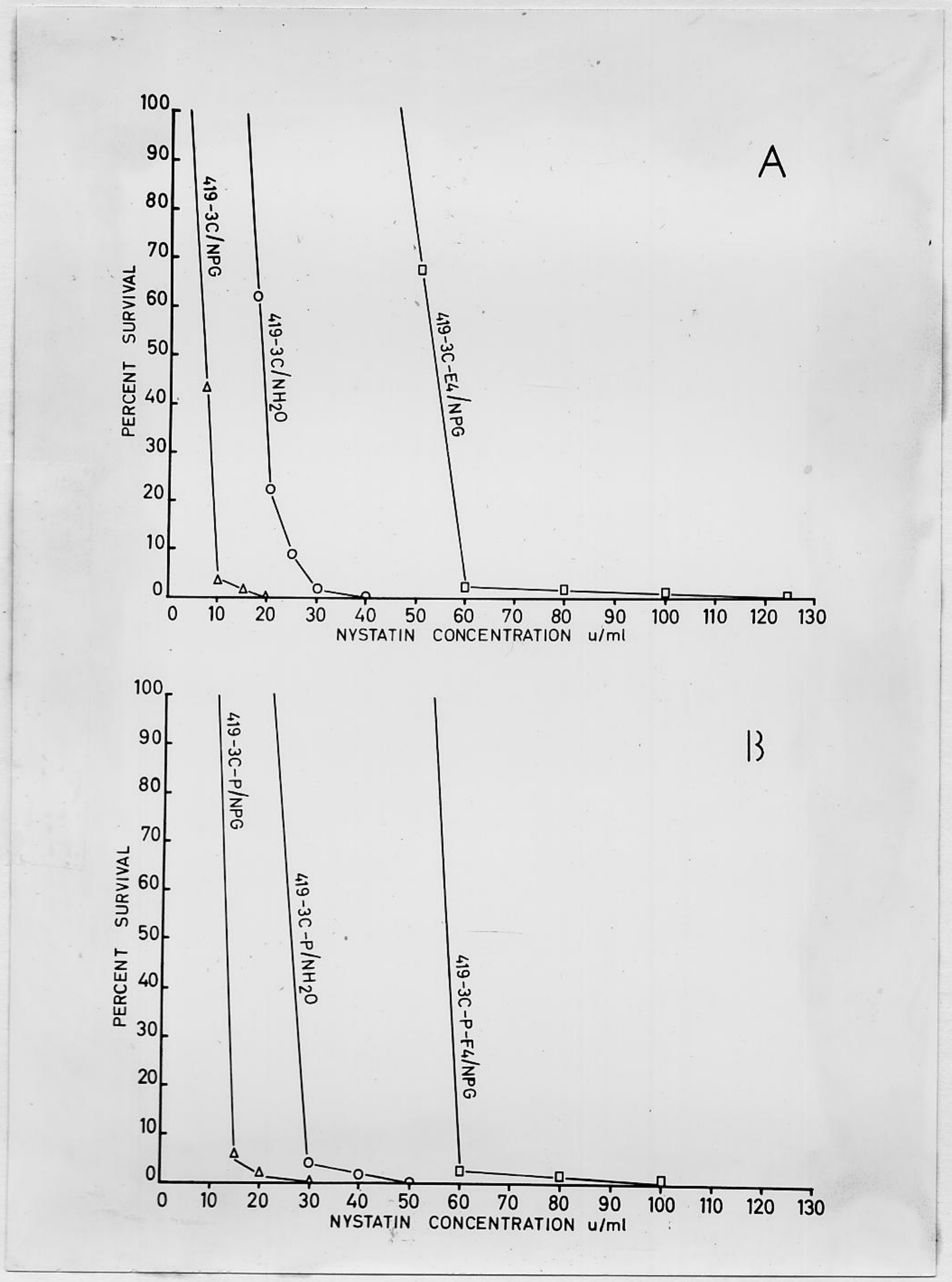

Figure 2. Effect of Nystatin on survival of

(A) 419-3C (B) 419-3CP

Please note in Table 5 that 419-3C is more sensitive to Nystatin than 198-2B; although their petite isolates seem to differ in this respect as 198-2B-P is more sensitive than 419-3C-P. This observation will be re-emphasized in the section on sterol content and sensitivity to Nystatin.

Isolation of Nystatin Resistant Cells

By replica plating:

Object: An essential feature of the replica plating technique is the selection of Nystatin resistant yeast mutants in the absence of Nystatin. By means of a velvet pad (velveteen cloth) 50 to 100 yeast colonies from the original plate, called Master plate, are "printed" onto plates having Nystatin overlays. If one of the replicated colonies is resistant or contains resistant cells, a colony will be formed on the Nystatin plate. By marking the position of the pad, or colonies, in relation to the plates, it is possible to identify the resistant colony on the master plate.

Method: In this experiment I treated a stationary phase 108-3C yeast culture with 20 ug/ml of Nitrosoguanidine for 45 minutes in a shaking waterbath at 28°C. I then plated about 50 cells per plate of the treated cells on 20 FYAD plates and incubated the plates at 28°C for 72 hours - these plates are the Master plates. I replicated the master plates onto FYAD plates with an aqueous Nystatin overlay. The FYAD/NH_2O plates had the following Nystatin concentrations, 500, 1500 and 2500 unit per plate. After incubating the replicas for 48 to 72 hours at 28°C, I counted all colonies that grew on the Nystatin plates

and then subcultured (on FYAD slants) only one resistant colony from each master plate (i.e. not containing Nystatin). Unfortunately all the seemingly resistant mutants were false positives (i.e. Nystatin sensitive) upon retesting on Nystatin plates.

Discussion: This method gave no encouraging results and proved to be inadequate for isolating Nystatin resistant mutants due to the following limitations:

1. The small number of cells replicated per experiment. In three trials I replicated about 4,800 of both survivals of Nitrosoguanidine treated and untreated cells, using about 120 master plates.
2. The uneven distribution of the aqueous Nystatin concentration in the overlay. Here a few colonies which fail to grow in one marked region of the 500 units plate would grow in the same region of the 1500 units plate.

Because of these limitations I decided to stop using this method.

Mass mutation hunt:

Object: Mutation hunts of Nystatin resistant mutants by Mass selection were the means with which I isolated a number of Nystatin resistant clones. This method was more practical than selection of mutants by replica plating, as it provided the following advantages over replica plating:

1. Each experiment takes a maximum of 4 days, while the replica plating method requires a minimum of 7 days to get results.

2. Mass selection involves much less work, both in preparations and manipulation.
3. Here one can expose at least 10^7 cells per plate to Nystatin in comparison to 50 to 100 cells per plate in replica plating. Thus the chances of selecting resistant mutants are much higher.
4. The use of Propylene glycol as a solvent for Nystatin (NPG) at this stage was a crucial turning point in this method giving an even distribution of Nystatin in the overlays and avoiding the growth of non-resistant clones.

Method: In this series of mutation hunts I used the following yeast strains:

198-2B and its "petite" isolate 198-2B-P

419-3C " " " " 419-3C-P.

The detailed protocol for these trials is as follows:

1. Grow overnight culture of the desired yeast strain in 6 ml FYe 2D. Use heavy inoculum of a recent slant culture.
2. Dilution series a) Haemocytometer count of a 1/10 dilution, count 200 to 300 cells.
   b) Plate 4 x 0.1 ml dil. 5×10^{-6} (about 50 cells) of overnight culture on FYAD.
   Plate 4 x 0.1 ml dil. 2.5×10^{-6} (about 25 cells) of overnight culture on FYAD.

The cultures always contain $1\text{-}2 \times 10^8$ cells per milliliter.

3. Plate 0.1 ml of original yeast culture (for isolation of spontaneously occurring Nys. resistant mutants) on:

a) 2 x 500 units plate Nys. overlay FYAD

b) 2 x 1500 " " " " "

c) 2 x 2000 " " " " "

d) 2 x 4000 " " " " "

e) 1 x FYAD

4. Add 1.0 ml of diluted Nitrosoguanidine in FYe 2D to remaining overnight culture and incubate with shaking for 45 minutes at 28°C. Nitrosoguanidine was used at a final conc. of 20 ug/ml.

5. Centrifuge the cells in a clinical centrifuge. Pour off supernatant fluid and resuspend the yeast pellet in 5 ml FYe 2D.

6. Dilution series II. Plate 4 x 0.1 ml dil. 5×10^{-6} of treated cells on FYAD.
   4 x 0.1 ml dil. 2.5×10^{-6} of treated cells on FYAD.

7. Plate 0.1 ml of original treated (undiluted) culture on

a) 5 x 500 unit plate Nys. overlay FYAD

b) 5 x 1500 " " " " "

c) 5 x 2500 " " " " "

d) 5 x 4000 " " " " "

e) 1 x FYAD

8. Incubate all plates at 28°C for 72 hours.

9. Count, as Nys^r, all colonies that grow on Nystatin plates, except on the 500 u plate.

10. Pick up one resistant colony (if any) from each Nystatin

plate and subculture on an FYAD slant devoid of Nystatin.

Results and discussion: Table 6 shows a summary of some of the mutation hunts carried on the yeast strains 198-2B, 419-3C and their "petite" forms.

As shown in Table 6 we obtained a large number of Nystatin resistant mutants by using the mutagen Nitrosoguanidine and EMS with both the 198-2B and 419-3C strains. I have spent much time and effort in attempting to find an optimum concentration of each mutagen and an optimum time of treatment of the cultures for induction of Nystatin resistance. Many of the mutation hunts at the beginning of this series of experiments failed to provide us with any resistant mutants. I have listed in the protocol the optimum conditions I believe needed for a successful mutation hunt.

In case of EMS, I have used 0.05 ml EMS per ml FYe 2D yeast culture, while the time and conditions of treatment were the same as with Nitrosoguanidine. Before I discuss the results of the various mutation hunts, I would like to point out that it is necessary to use the Nystatin plates within 24 hours of their preparation, also that I have scored the Nystatin resistant mutants after an incubation period of 72 to 96 hours at 28°C, so I have neglected all colonies that appeared after that period. I have observed these two measures in all my hunts because Nystatin is unstable and is rendered ineffective after 96 hours at 28°C.

Throughout the various Nystatin resistant isolation hunts I repeatedly detected the presence of a few "spontaneously" occurring Nystatin resistant cells with varying levels of resistance. I

Table 6. Frequency of spontaneous and induced mutations to Nystatin resistance.

Trial No.	Strain used	Titer before treatment (cells/ml)	Total No. untreated plated cells	Mutagen	Survival percent (average	Total No treated cells plated	No. spontaneous mutants from Nys. plates with 1500 units	2000 units	2500 units	4000 units	Total No.	Frequency of spont. mutants	No. induced mutants[e] from Nys. plates with 1500 units	2000 units	2500 units	4000 units	Total No.	Frequency of induced mutants
Series I[a]	198-2B	$1.24x10^8$	$3.86x10^8$	N.Gu	38.4	$5.6x10^8$	4	1	1	0	6	$1.64x10^{-8}$	29	4	6	3	42	$7.5x10^{-8}$
" II	198-2B	$1.83x10^8$	$1.46x10^8$	EMS	32.5	$1.01x10^8$	2	0	0	0	2	$1.37x10^{-8}$	2	0	0	0	2	$2x10^{-8}$
" III	198-2B-P	$7.7 x10^7$	$3.08x10^7$	N.Gu	49.3	$1.21x10^8$	39	31	40	-	110	$3.6 x10^{-6}$	83	56	36	-	175	$1.44x10^{-6}$
" IV A	419-3C	$2.23x10^8$	$1.34x10^8$	N.Gu	70.5	$5.03x10^8$	1	1	0	0	2	$6.3 x10^{-9}$	6	1	0	0	7	$1.4 x10^{-8}$
"[b] IV B	419-3C	$2.64x10^8$	$1.3 x10^8$[c]	EMS[d]	18.6	$1.28x10^8$	-	-	-	-	-	-	1	2	0	0	3	$2.3 x10^{-8}$
"[b] V	419-3C-P	$1.6 x10^7$	$1.28x10^7$	EMS[d]	20.2	$1.096x10^7$	49	23	12		84	$6.6 x10^{-6}$	19	3	7	0	29	$2.64x10^{-6}$

[a] Data collected from 7 mutation hunts.

[b] Data collected from 2 mutation hunts.

[c] Spont. data from series IV A.

[d] EMS conc. was 0.05 ml/ml culture .

[e] Mutants were scored and isolated after 72 to 96 hours of incubation at 28°C.

isolated these mutants directly from Mystatin plates, without being exposed to any mutagen. These mutants appeared after 72 hours of incubation at 28°C on the 500 and 1500 unit plates, but in one case I got a mutant on one 2500 unit plate.

For some reasons, which I hope to clarify later when discussing sterol production by the various yeast strains, the 198-2B resistant cells seemed to have a higher rate of spontaneous occurrence (average of eight trials was 1.5×10^{-8}) than those of 419-3C which was 6.3×10^{-9}. This finding is in agreement with the earlier finding that 419-3C is more sensitive to Nystatin than 198-2B. The relatively higher frequencies of spontaneous mutations for the "petite" isolates (3.6×10^{-6} for 198-2B-P and 6.6×10^{-6} for 419-3C-P) are difficult to interpret. I think it is either due to the nature of the respiratory deficiency or perhaps due to the reduced sterol synthesis in some of these strains!?.

It is evident from mutation frequency values in Table 6 that mutagen treatment does induce higher frequencies of Nystatin resistance in the "grande" cells, while such treatment did not increase the frequency of mutation in the "petite" cell.

Growth, Stability and Level of Resistance of Nystatin Resistant Mutants

Object: In this section I will present data obtained by retesting all my Nystatin resistant mutants on solid media containing Nystatin to check for:

a) The stability of the mutation.

b) The level of resistance of the mutants in complex medium containing Nystatin.

c) Any other observed changes in the behavior of the mutant cells.

Methods: I have kept all my isolated Nystatin resistant mutants on non-selective (FYAD slants) medium and avoided as much as possible any further subculturing except the following four mutants 198-2B-C5, 198-2B-P8, 419-3C-E4, and 419-3C-P-F4 which I have extensively used and hence were subcultured several times.

Using a sterile toothpick I transferred cells of the resistant yeast clones from the FYAD slants to marked sectors on a series of plates with various Nystatin concentrations. Then using a sterile metal loop with small amount of sterile distilled water, I smeared the cells on the surface of the plate and incubated all the plates at 28°C for 48 to 72 hours.

I measured the amount of inhibition of the mutant strains by the same procedure used for the inhibition tests of the sensitive yeast strains (See section 4).

Results and discussion: Table 7 shows the mutation stability results and level of resistance of Nystatin resistant mutants retested two to nine months after isolation. Table 8 gives the inhibitory and lethal concentrations of Nystatin obtained for some of these mutants in complex liquid medium containing various levels of Nystatin.

Table 7. Growth of resistant mutants on solid media containing Nystatin.

Resistant[x] mutants of	No. mutants collected	No. clones growing on plates with the following Nystatin conc 500 units	1500 units	2000 units	2500 units	4000 units	No. clones[xx] that failed to grow on FYA 2 Glycerol
198-2B	40	39[+]/39	26/39	10/26	9/10	4/9	2/40
198-2B-P	18	18/18	15/18	14/15	13/14	5/13	18/18
419-3C	11	11/11	2/11	1/2	0/1	0	0/11
419-3C-P	6	6/6	6/6	6/6	6/6	2/6	6/6

Table 8. Inhibitory and lethal concentrations of Nystatin on mutant cells.

Mutant strain used	Min inhib con. Nystatin/PG	Units/ml Nystatin/H_2O	Lethal conc. Nystatin/PG	Units/ml Nystatin/H_2O
198-2B-C5	60-80	500	150	2000
198-2B-P8	30-40	120	100	1200
419-3C-E4	40-50	500	150	2000
419-3C-P-F4	50-55	120	100	1500

[+] Missing clone was discarded because of contamination.

[xx] Mutant cells were also grown on FYA 2G to check for spontaneously occurring Nystatin resistant "petite" clones among the "grande" mutants, and to double check the Nystatin resistant petite clones.

[x] I have simultaneously tested the four sensitive clones of these mutant cells and found that none of them grew on any of the Nystatin plates.

The Nystatin resistant mutants of the various yeast strains used exhibited stability and varying levels of resistance in both liquid and solid media containing Nystatin. These levels of resistance that we measured were not related to the Nystatin concentrations at which these isolates were originally isolated. Some resistant mutants isolated from a 1500 unit plate (e.g. the 198-2B-P mutants) showed resistance on the 2000 units or even the 4000 units plates, while others (e.g. the 419-3C mutants) failed to grow on this same concentration upon retesting. However, I have not observed in any of my resistant mutants any complete reversion to the sensitive state and as shown in Table 7 these mutants, that were found to be less sensitive upon retesting on Nystatin plates, retained their ability to grow on the 500 unit Nystatin plates.

As illustrated in Table 7, the great majority of the Nystatin resistant mutants are "stable" and do tolerate the 1500 unit level of resistance on solid medium. It also shows that the Nystatin resistant mutants of the two petite strains have higher levels of resistance than those of the grande strains on solid complex medium containing Nystatin.

The differences in response to Nystatin between the sensitive strains and their resistant mutants in complex liquid medium are shown in Tables 4 and 8 and Figures 1 and 2.

A close examination of these data leads to the following conclusions:

1. The resistant mutant 419-3C-E4 is more sensitive to Nystatin than 198-2B-C5 while the "petite" mutant 419-3CPF4 is less

sensitive to Nystatin than 198-2BP8.

2. The curves of the four resistant mutants, 198-2B-C5, 198-2BP8, 419-3C-E4, and 419-3C-P-F4 are steeper than those of their respective sensitive clones, which suggests a sharper margin between inhibitory and lethal concentrations of Nystatin in the case of the resistant cells.

3. The Nystatin resistant mutants respond to aqueous Nystatin inhibition and lethality in a remarkably different pattern than that to NPG (Table 8). This particular observation about the mutants response to NH_2O, where the lethal concentration was about 2000 units per ml (0.67 mgs Nystatin) as compared to 150 units per ml of NPG, points out the important and superior role played by Propylene glycol in this work. Thus aqueous Nystatin, which is widely used by many researchers, is less effective than NPG for resistance studies.

Another striking feature, that I have repeatedly noticed in my work with the mutant cells is the relatively poor growth of a small percentage of the mutants isolated on FYAD plates and in the complete W°hams medium. The majority of these poor growers on FYAD grow normally on FYAD plates containing low concentrations of Nystatin.

I will elaborate more on this feature in the coming sections and in section 8, which deals with the sterol content. I will show clearly that Nystatin resistance is accompanied by altered sterol metabolism.

Genetic Analysis of the Nystatin Resistant Mutants

Object: Resistance to Nystatin could be a nuclear or a cytoplasmic phenomenon. The only indubitable proof that such an altered phenotype is due to nuclear mutation is the demonstration that this trait segregates according to Mendel's First law. If resistance is a nuclear phenomenon, then either one or more genetic loci may be mutant and such mutations may either be dominant or recessive. Therefore, we made several crosses between mutant and non-mutant clones to determine if resistance to Nystatin is inherited in Mendelian fashion.

Methods: I crossed two of the mutant clones with the two original sensitive strains using the mass mating method of Pomper and Burkholder (1949), as shown in Table 9. All the diploids were prototrophic and sporulated well.

Table 9. Diploids resulting from mass mating of the haploid Sens. vs Res. clones.

	419-3C	419-3C-E_4
198-2B	Z843	Z844
198-2B-C5	Z845	Z846

The second step was testing the diploids for Nystatin resistance. I did this by preparing a FYAD master plate containing 68 colonies of each diploid. After 24 hours incubation at 28°C I replicated all master plates according to standard methods of replica plating. The media I used are shown in Table 10.

The third step was the sporulation of the four yeast zygotes and the dissection of their asci to check for the segregation of Nystatin resistance and sensitivity among the isolated ascospore clones. We followed Johnston and Mortimer's (1959) method, using snail juice, the crop content of the garden snail Helix pomatia, to digest the ascus and then dissected the spores by means of five glass needle of a micromanipulator. We allowed ascospores to grow for 24 to 48 hours and then transferred the surviving ones to FYAD slants. Later the surviving clones were transferred to master plates and after incubation at 28°C for 24 hours, were replicated to appropriate plates (see Table 12) to test for the segregation pattern of the Nystatin resistant mutation.

Results and discussion:

Resistance of diploid clones: Table 10 shows the results of testing the four diploids for resistance to Nystatin.

Table 10. Resistance of diploid yeast clones to Nystatin.

Colonies from diploid strain #	No. of colonies surviving on (units Nystatin/plate)						
	FYAD	500 units Nys	1500 units Nys	2500 units Nys	Minimal	Min + Tween 80	Min + Erg
Z843	68	48/68	0/68	0/68	68/68	68/68	68/68
Z844	68	64/68	9/64	0/9	"	"	"
Z845	68	[x]49/68	18/49	1/18	"	"	"
Z846	68	68/68	67/68	5/67	"	"	"

The data in Table 10 show that:

1. All cells replicated are diploids (100% growth on minimal plates).
2. The 500 unit Nystatin plate is not a reliable level for measurement of resistance of diploid cells. As cells of Z843 grow perfectly well on this plate. This is explained by the vigorous growth rates of diploid cells which have no known nutritional requirements, and which contain high levels of Ergosterol as will be shown in the next section. The heavy growth of the diploids on the master plates probably results in "protection" of some of the replicated cells on the Nystatin plates; thus allowing them to grow

[x] On this plate 18 colonies were not replicated at all.

or to survive until the Nystatin decays. We observe this phenomenon with sensitive haploid cells at lower Nystatin concentrations .

3. Resistance to Nystatin seems to be a recessive characteristic as both Z844 and Z845, which are hybrids resulting from crosses between Nystatin sens and Nys res clones, turned out to be sensitive. The diploid Z846 resulting from a cross between the two resistant mutants 198-2B-C5 and 419-3C-E4 is resistant to Nystatin at 1500 units per plate, indicating that the mutations in both haploids involve the same locus if the mutations are nuclear.

4. That resistance to Nystatin in 198-CB-C5 and 419-3C-E4 may either be a) controlled by a single locus, and hence Z846 will be a homozygous recessive and is Nystatin resistant or b) that Nystatin resistance, which may be cytoplasmic or a physiological phenomenon, results from an additive effect of both resistant mutants.

Although these data give no evidence that Nystatin resistance is nuclear, they certainly rule out the possibility of the involvement of two different mutant loci in these crosses.

Spore germination: The results of the dissected ascospores that germinated after incubation for 72 hours at 28°C are shown in Table 11.

Table 11. Pattern of spore germination in dissected asci from diploids in Table 9.

Diploid	Total No. of asci dissected	Classification of asci as to No. of germinating spores per ascus				
		4/4	3/4	2/4	1/4	0/4
Z843	50	9	0	28	1	12
Z844	55	3	4	19	16	13
Z845	60	3	5	30	12	10
Z846	55	5	1	30	6	13
Totals	220	20	10	107	35	48
Percent of total		0.09	4.55	48.64	15.9	21.81

The total number of germinating ascospores was 359 out of an expected number of 880.

Table 11 shows that only 40.8% of all the isolated ascospores germinated. This high rate of nonviability observed in all four diploids was completely unexpected and rendered the analysis of our data very difficult. Only 9.09% of total asci dissected, of the four zygotes in question, gave rise to four (4/4) healthy ascospore clones per ascus.

Professor D.C. Hawthorne in a comment on the spore germination results wrote us saying "It looks like you are dealing with translocation heterozygotes. If one groups the 3/4 survivor class with the 4/4 and the 1/4 with the 2/4, then your data fit the expectations for a simple terminal translocation with at least one exchange point

showing no centromere linkage, 1(4/4): 4(2/4); 1(0/4).

This reciprocal translocation seems to be in the 198-2B strain and is independent of the Nystatin resistant characteristic, because it is also observed in the diploid Z843 which is a cross between the two sensitive parental clones 198-2B and 419-3C.

Thus for future work we will use another haploid yeast strain, that is free from this deleterious condition, in place of the 198-2B strain.

Segregation pattern of Nystatin resistance: I would like to note here that as a result of the inconveniences caused by the translocation heterozygote condition we treated the products of our dissected asci of each diploid as randomly isolated ascospores in our genetic analysis, i.e. we did not pay attention to types of asci because we obtained very few 4-spored surviving asci.

In Table 12 I will present the results obtained from the genetic analysis for segregation of Nystatin resistance in the diploid cells.

Table 12. Segregation of Nystatin resistance and sensitivity in the four diploids.

Ascospores from	No. of segregating ascospores as		
	Nys^s	Nys^r	Total
Z843	81	2^x	83
Z844	36	35	71
Z845	43	41	84
Z846	2^+	78	80

x Delayed growth, may be sensitive.

+ One of these from complete ascus, see discussion.

The main points that emerge from Table 12 are:

1. Nystatin resistance is a nuclear phenomenon in these mutants.
2. Segregation is normal and follows Mendel's first law. Both Z844 and Z845 show a 1:1 ratio of Nystatin resistance to sensitivity.
3. Nystatin resistance is controlled by one locus in each of the two mutants 198-2B-C5 and 419-3C-E4, as all but two of the ascospores produced by Z846 are Nystatin resistant.

The exceptional situation in the complete ascus 846-2A,B,C,D, where the resistance to sensitive ratio was 3:1 could be the result of a single cross over. We did not test for a possible double mutant because of the shortage of time. If this ascus did result from crossing over then the mutant genes controlling Nystatin resistance in 419-3C-E4 and 198-2B-C5 are a) functionally allelic i.e. members of the same cistron and b) heteroalleles i.e. genes having mutations at different sites.

Tests for linkage of Nys with Ad, try. and mating type (MT):

Table 13. Random ascospore analysis of haploid isolates for Z844 and Z845.

A. Z844 198-2B ad_2 Nys^s a x 419-3C-E4 try_1 Nys^r α

Sporeditype	Number of ascospore isolates locus pair		
	ad_2 Nys	try_1 Nys	a/α Nys
Parental	36	41	34
Non-parental	39	34	28
Total	75	75	62[x]

[x] We did not score the prototrophs isolated for "mating type".

B. Z845 198-2B-C5 ad2 Nys^r a x 419-3C try_1 Nys^s α

Sporeditype	Number of ascospore isolates locus pair		
	ad2 Nys	try_1 Nys	a/α Nys
Parental	44	53	42
Non-parental	44	35	25
Total	88	88	67[x]

Table 13 (A and B show no evidence for linkage between the Nystatin resistance and the Ad_2 locus in both Z844 and Z845 as there was no significant difference between the number of parental and non-parental spores segregating for the Ad_2 Nys pair locus. However, there is some indication that the Nystatin and the try_1 genes are linked loosely (Table 13B shows 18 parental ditype spores more than the recombinant ones). I am afraid that our data is not enough in order to make any conclusions about linkage of the Nystatin genes with any of the above mentioned known markers.

Ahmad and Woods (1967) very recently reported the isolation of stable Nystatin resistant mutants of S. cerevisiae from platings of a sensitive wild-type strain on low concentrations of the anti-biotic. These mutants were found to be resistant to 10, 15 or 60 units of drug per ml. They found 3 genes plus two modifiers for Nystatin resistance. The Nystatin resistance locus that we detected in our two mutants could be one of the three loci they reported.

[x] We did not score the prototrophs isolated for "mating type".

Ergosterol Determination in Yeast

Object: Ergosterol is the primary sterol in yeast and is by far the most readily isolated (Breivik and Owades, 1957). In these experiments we have determined the Ergosterol content of the following yeast clones:

1) Nys. sensitive	a) 198-2B	2) Nys. resistant	a) 198-2B-C5
	b) 198-2BP		b) 198-2B-101H
	c) 419-3C		c) 198-2B-P8
	d) 419-3CP		d) 419-3C-E4
	e) Z843		e) 419-3C-F_2
	f) Z844		f) 419-3C-F3
	g) Z845		g) 419-3CP-F_4
	h) Z846		

This determination enables us to check for any alterations in the sterol metabolism of the Nystatin resistant mutants.

Methods: We grew all yeast cultures in filter sterilized (Millipore filter HA, average pore size 0.45 u) FYe 2D.

We determined the Ergosterol content of our yeast samples using the method of Breivik and Owades (1957) . We digested the (dried and weighed) samples with alcoholic potassium hydroxide and extracted Ergosterol in a single extraction with spectrophotometric grade n-heptane. We determined the Ergosterol content (percent Ergosterol) of each sample from the 281.5 millimicrons absorbance measurements on the Beckman DU spectrophotometer.

Results and discussion: The remarkable results of this crucial experiment are found in Table 14 and in Figures 3, 4, 5, 6, 7, and 8. Examination of the results clearly indicates that all but one of the Nystatin resistant mutants tested have no detectable amounts of Ergosterol. We found differences in Ergosterol content between the sensitive parental clones and their "petite" mutants.

Figure 3 shows the vast difference between the amount of Erogsterol in the Nystatin sensitive strain 198-2B (curve A) and its two Nystatin resistant mutants 198-2B-C5 and 198-2B-101H (curves C and D). The extract of 198-2B has a typical Ergosterol absorbance maximum (curve B) at 281.5 millimicrons, while the resistant mutants fail to show any measurable absorption at this same wavelength. This suggests the absence of detectable amounts of Ergosterol in these two mutants (Table 14), however, the mutants show maximum absorbance at 205 millimicrons (peak off the scale) while 198-2B shows less absorbance (OD = 0.9) at this same wavelength. The 205 mu absorbing material in the mutant strains could probably be Ergosterol precursors or break-down products.

In Figure 4 (curve B) the resistant mutant 419-3C-E4 shows a minor Ergosterol absorbance peak (OD = 0.1) at 281.5 millimicrons, while in Figure 5-B and C the resistant mutants 419-3C-F3 and 419-3C-F2 which are more resistant to Nystatin than 419-3C-E4, show no Ergosterol peaks at all i.e. have no detectable amounts of Ergosterol (Table 14).

The content of Ergosterol in 419-3C-E4, which is 0.02% (Table 14), may account for its lower level of resistance (see Figure 2) when compared to 198-2B-C5 (see Figure 1). Table 14 shows

Table 14. Determination of Ergosterol content in the Nystatin sensitive and resistant strains.

Clone	Incubation period (hrs.)	Dry wt. cells (mg/ml culture)	Total Ergosterol ug/ml culture	Percent Ergosterol
Nys. Sensitive				
419-3C	24	5.72	75.4	1.32
		5.88	77.9	1.32
	36	8.30	105.4	1.27
	48	8.06	104.7	1.30
419-3CP	48	2.13	34.1	1.60
198-2B	24	3.74	34.2	0.91
		3.88	35.4	0.96
	36	7.16	67.8	0.95
	48	5.09	62.8	1.23
198-2BP	48	1.98	13.2	0.67
Z843	24	7.94	128.6	1.62
Z844	24	8.07	122.8	1.52
Z845	24	7.97	122.8	1.54
Nys. Resistant				
419-3C-E_4	24	5.72	0.83	0.01
		5.96	1.25	0.02
	36	7.20	3.37	0.05
	48	6.78	1.35	0.02
419-3C-F_2	24	2.67		
419-3C-F_3	24	5.29		
419-3CP-F_4	48	1.85		
198-2B-C5	24	2.24		
		2.31	None-detectable	
	36	5.86		
	48	4.86		
198-2B-101H	36	5.85		
198-2BP8	48	1.93		
Z846	24	8.09		

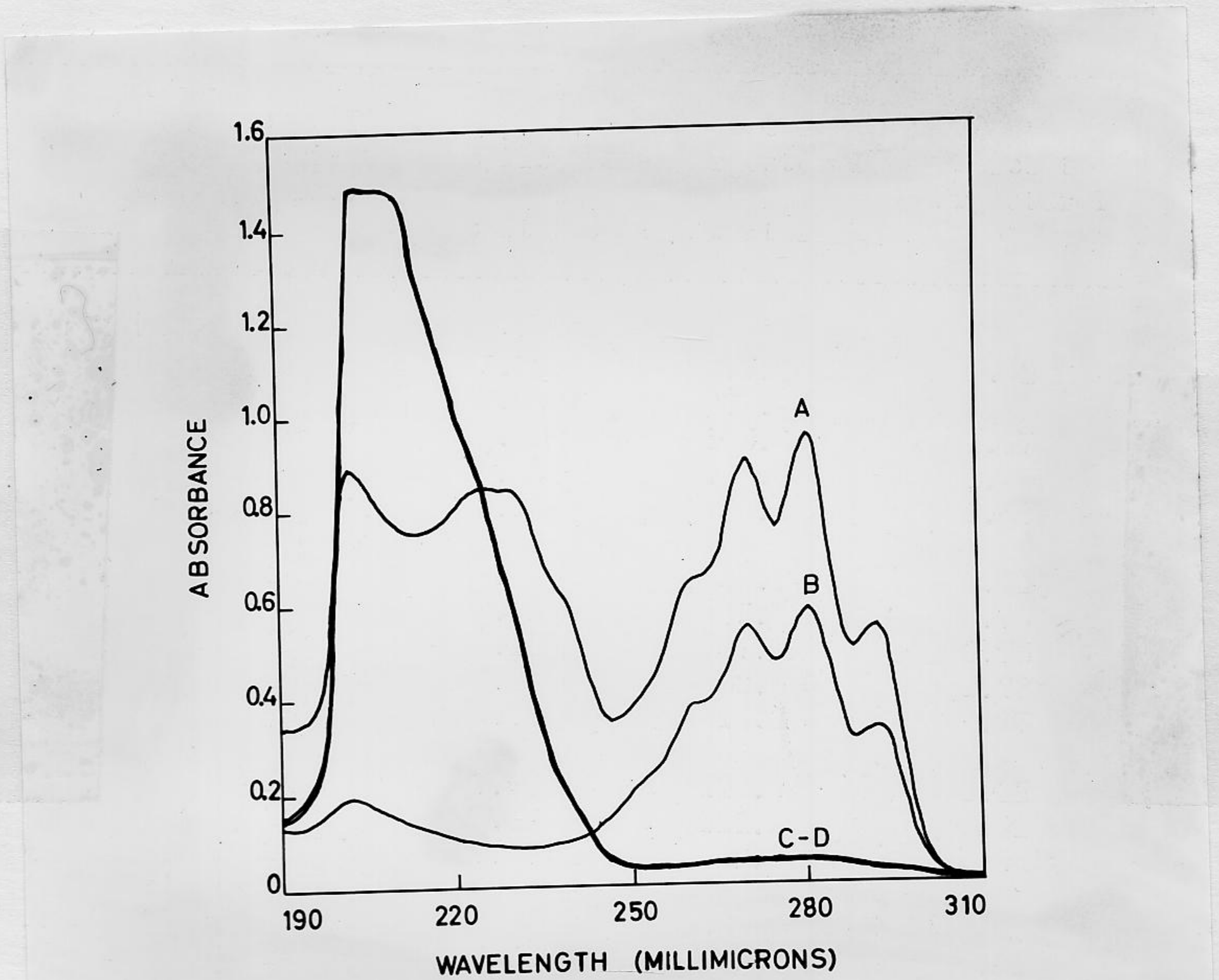

Figure 3. Ultraviolet spectra of yeast cell extract

(A) 198-2B 36 hrs. extract
(B) Ergosterol (20 ug/ml)
(C & D) 198-2B-C_5 and 198-2B-101H 36 hrs. extract

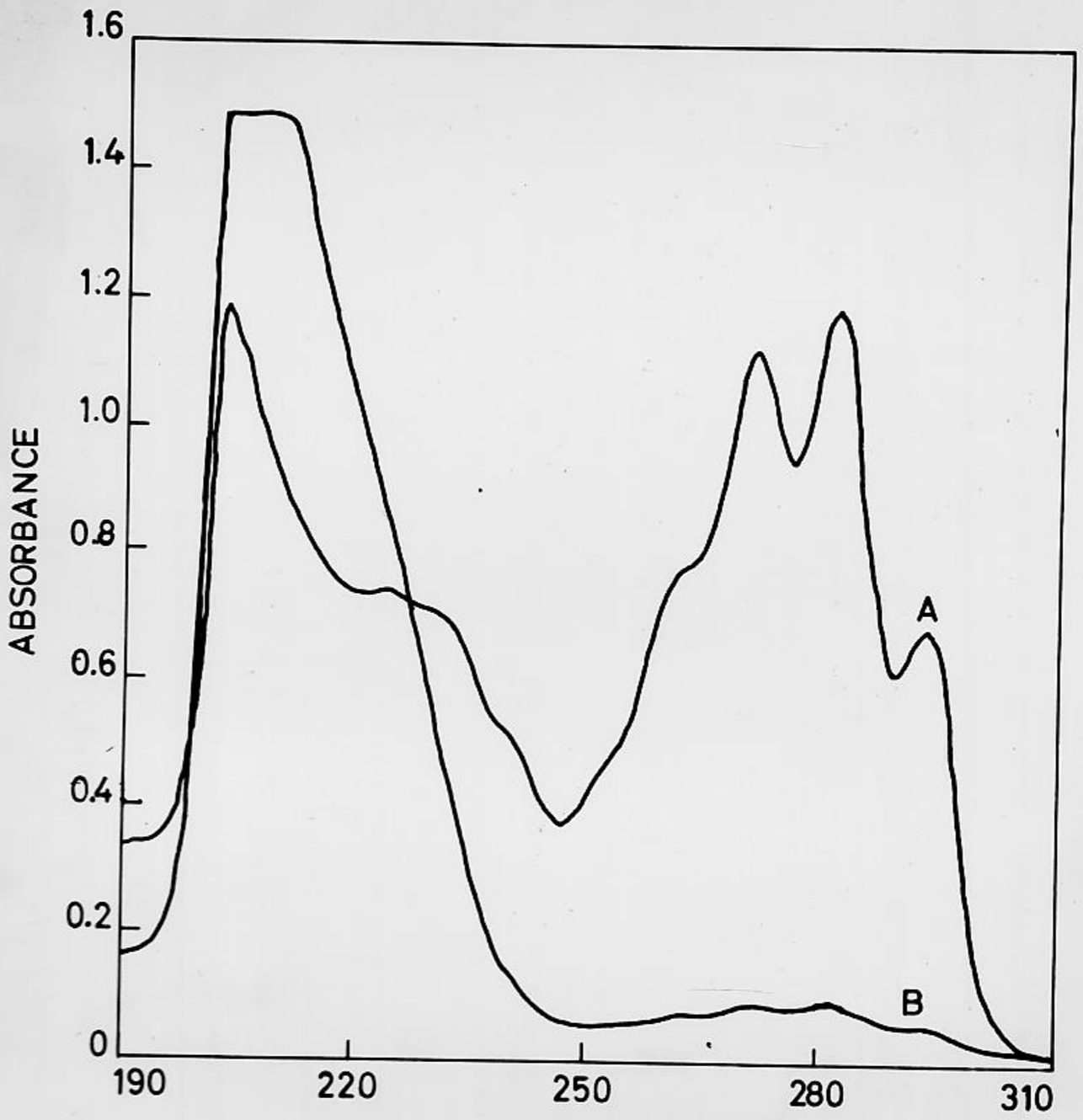

Figure 4. Ultraviolet spectra of yeast cell extract

(A) 419-3C 36 hrs. extract
(B) 419-3C-E_4 36 hrs. extract.

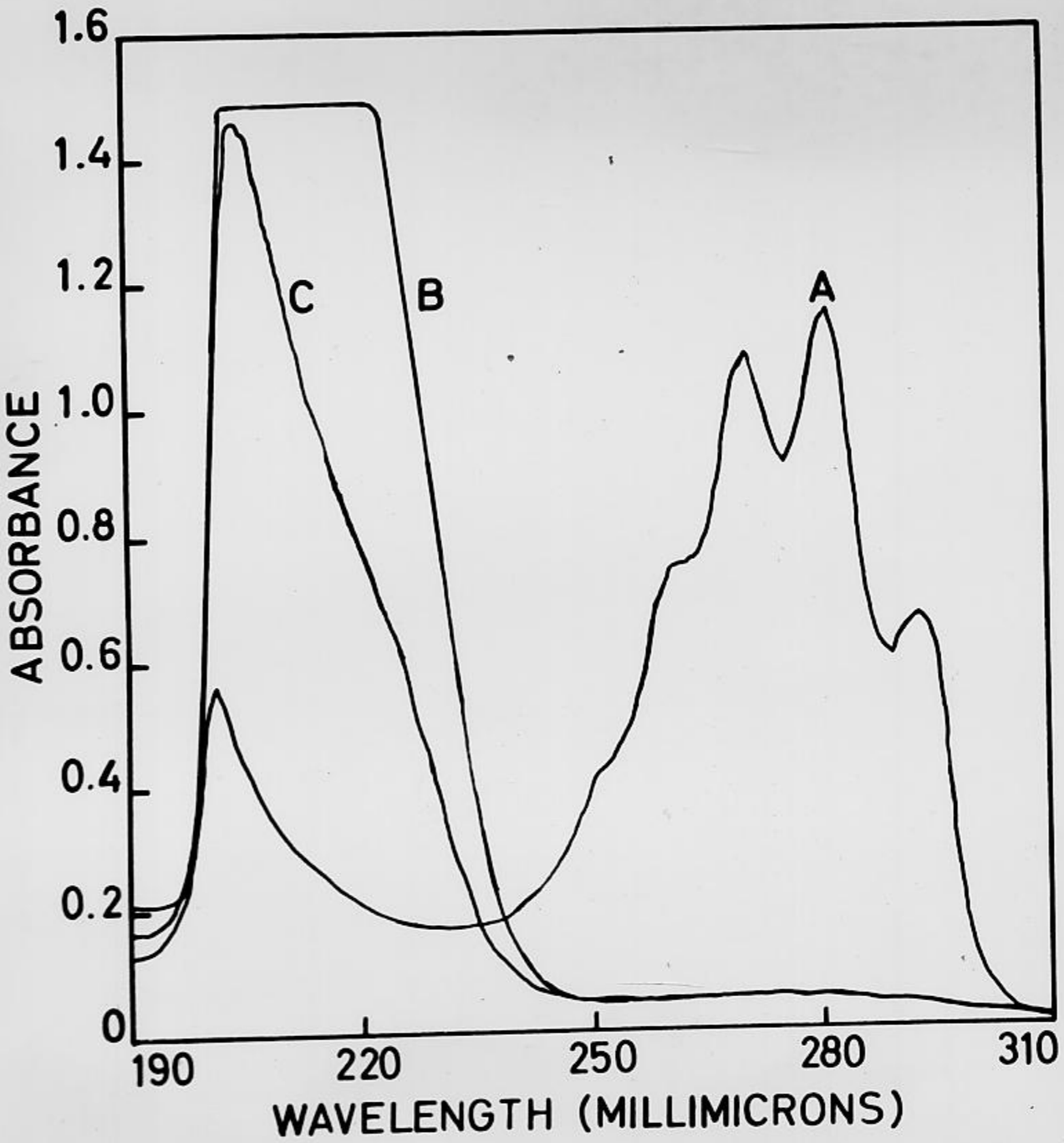

Figure 5. Ultraviolet spectra of yeast cell extract

(A) Ergosterol (20 ug/ml)
(B) 419-3C-F_3 48 hrs. extract
(C) 419-3C-F_2 48 hrs. extract.

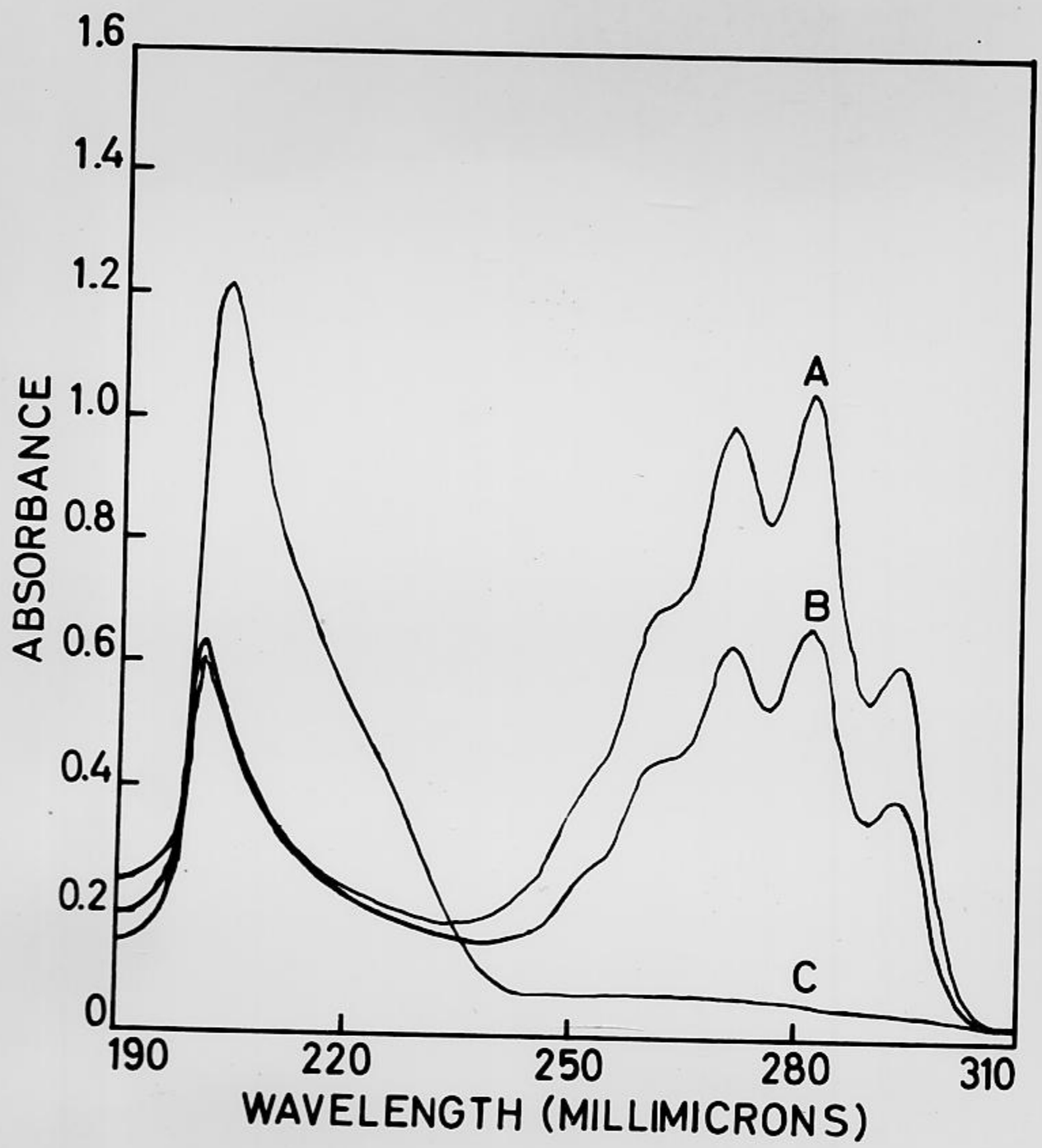

Figure 6. Ultraviolet spectra of yeast cell extract

(A) Ergosterol (40 ug/ml)
(B) 198-2BP 48 hrs. extract
(C) 198-2BP$_8$ 48 hrs. extract

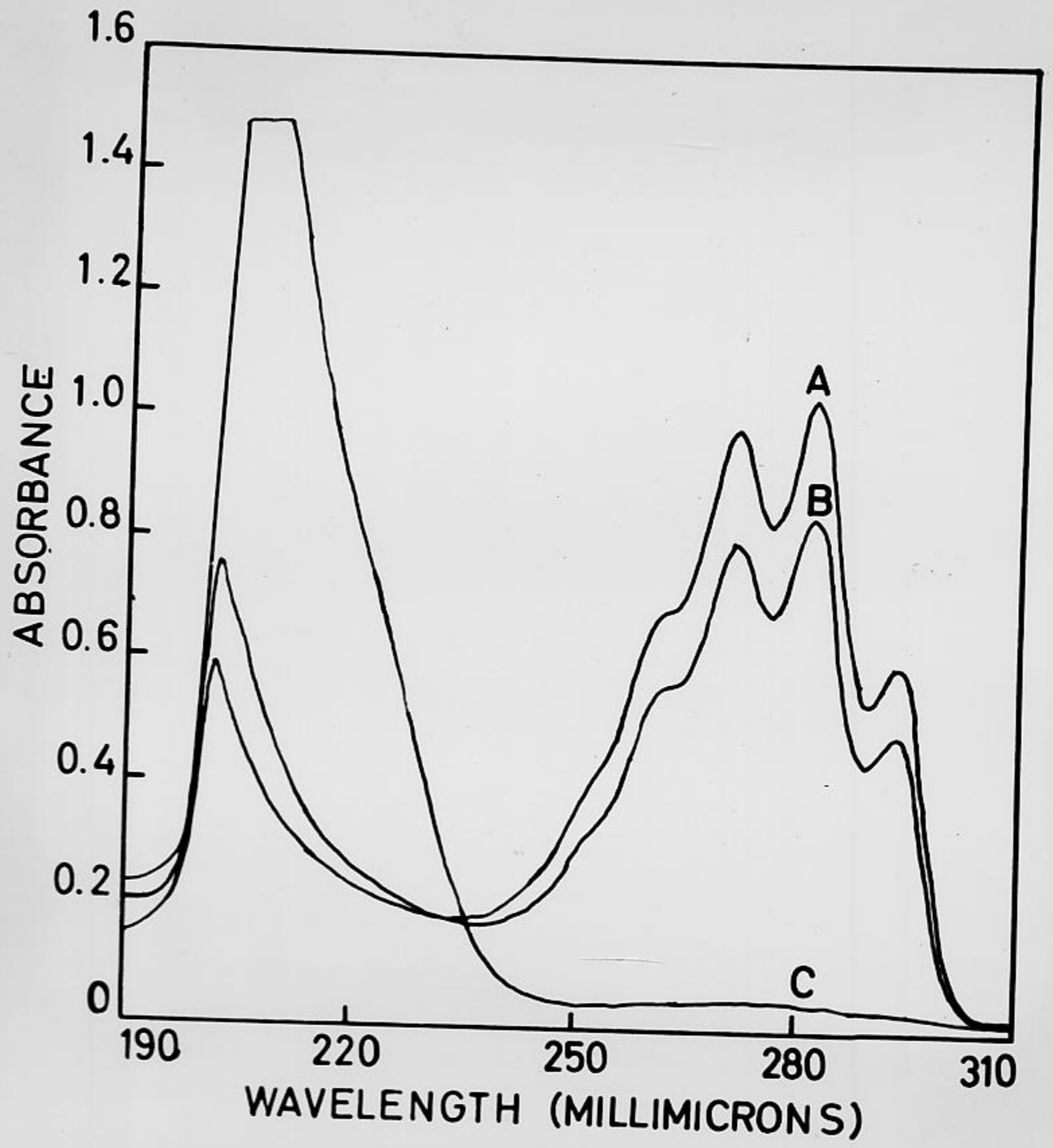

Figure 7. Ultraviolet spectra of yeast cell extract

(A) Ergosterol (40 ug/ml)
(B) 419-3CP 48 hrs. extract
(C) 419-3CP-F4 48 hrs. extract

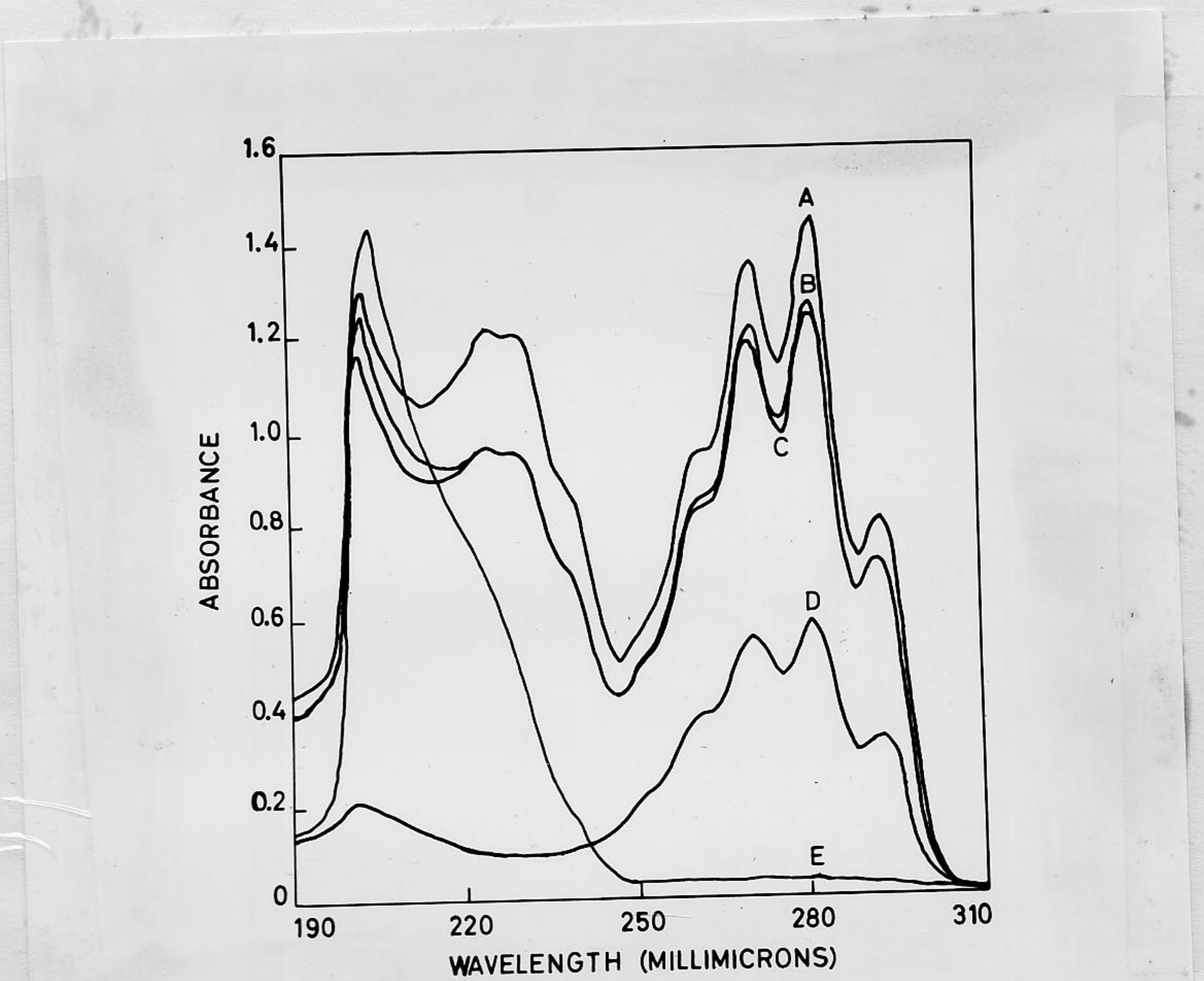

Figure 8. Ultraviolet spectra of yeast cell extract

(A)	Z843	24 hrs. extract
(B)	Z845	24 hrs. extract
(C)	Z844	24 hrs. extract
(D)	Ergosterol	(20 ug/ml)
(E)	Z846	24 hrs. extract.

that 419-3C, which is more sensitive to Nystatin than 198-2B, contains more Ergosterol than the latter, while 419-3CP, which is more resistant to Nystatin than 198-2BP contains more sterols than the latter. Thus it seems that resistance to Nystatin is independent of the Ergosterol content of the particular strains.

In Figure 5 C the mutant 419-3C-F_2, which is characterized by very poor growth on FYAD but which has no known additional growth requirements, shows a sharp peak at 200 to 205 millimicrons (O.D = 1.45) while 419-3C-F_2, which grows nearly normally on FYAD gives an off-scale peak around 215 mu. The different absorbances at different peaks suggest different kinds and amounts of extractable components, and it appears that the absence or presence of some of these components may result in poor growth.

It is very important to note here that these two mutants are highly resistant to Nystatin and that both grow normally on Nystatin plates?. Could the poor growth of 419-3C-F_2 be due to a requirement for Nystatin?. This same phenomenon, i.e. poor growth on FYAD followed by normal growth upon replication to Nystatin plates, was encountered also with some of the resistant clones derived from the isolated single spores.

Figure 6 shows the absorbance spectra for 198-2BP (curve B) and 198-2BP_8 (curve C) while Figure 7 shows spectra of 419-3CP (curve B) and 419-3CP-F_4 (curve C). Both of the Nystatin resistant "petite" mutants have no Ergosterol (Table 14) as illustrated also by their zero absorbance at 281.5 mu, however, both show a peak at 200 to 205 mu (198-2BP_8 has an absorbance of 1.2 while the 419-3CP-F_4

peak is off-scale). Surprisingly enough this same phenomenon is shared by 198-2BP (absorbance 0.64), 419-3CP (absorbance 0.76) and pure Ergosterol (40 ug/ml absorbance 0.6), but each has a different measurable amount of this same substance !. I think that this phenomenon can be explained in this particular situation by the presence of a compound in different amounts which is a breakdown product of Ergosterol as the same phenomenon is illustrated by the Ergosterol spectra, (Figures 6 A and 7 A). Note that the Ergosterol was treated in same way as the extracts.

Another interesting finding about the "petites" is shown in Table 14, where 198-2BP contains less sterols than its "grande" parent 198-2B while on the other hand the "petite" 419-3CP contains more sterols than its "grande" parental strain 419-3C. This finding supports Parks' and Starr's report that respiratory deficient yeast cells may contain or may synthesize more or less sterols than the wild type cells. I feel here that a large number of "petite" strains should be collected and investigated for their sterol content and Nystatin resistance before one makes any generalization in this respect. Whether this situation is dependent on or related to the nature of respiratory deficiency, which is not quite well understood, is very difficult to assert.

We did similar test for Ergosterol determination and spectral analysis with extracts from the four diploid clones.

Table 14 shows that Z844 and Z845, which are heterozygous for Nystatin resistance, have only slightly less Ergosterol than Z843, while they all have typical Ergosterol peaks (Figure 8 A,B,C) at

281.5 mu. However, Z846, which is a homozygous recessive for Nystatin resistance shows no peak at 281.5 mu (Figure 8 E), i.e. has no detectable amount of sterol by this method. Note that (Table 14) there is no difference in the dry weight (mg/ml culture) of these four diploids, i.e. they all have similar rates of growth including the Z846 which has the highest obtained dry weight (8.09 mg/ml culture) after 24-hours of growth. Yet this diploid is strikingly different in being devoid of Ergosterol.

Goint back to Figure 8, Z843, Z844 and Z845 show broad peaks at 230 mu suggesting the possible presence of dehydroergosterol and they also give sharp peaks at 202 to 204 mu. Z846 shows no dehydro-ergosterol peak either, but has a very sharp one at 205 mu (absorbance 1.45). However, unlike the haploid resistant strains it is not markedly higher.

These results with the four diploid clones confirm the previously described results with the haploid resistant mutants. Z846, 198-2B-C_5, 198-2B-101H, 419-3C-F_2 & F_3 and the petite Nystatin resistant mutants have no detectable amounts of Ergosterol while 419-3C-E_4 which is less resistant to Nystatin than 198-2B-C_5 has about 0.02% Ergosterol which amount to 1.5% of the Ergosterol content of its sensitive parental strain 419-3C.

These mutants do have an altered sterol metabolism as Nystatin resistance is accompanied by or is a result of the reduction of Ergosterol synthesis or its total stopping in such mutants.

IV. SUMMARY

The original purpose of this work was to isolate sterol requiring mutants of the yeast Saccharomyces cerevisiae. We, as many other investigators, failed to find any such mutants for reasons which we still do not understand. Therefore we attempted to find mutants resistant to the lethal and inhibitory effects of the polyene antifungal agent Nystatin which complexes with the sterols of yeast cells. We hoped that these Nystatin resistant mutants would show altered sterol metabolism.

We were able to isolate a number of Nystatin resistant mutants. We found spontaneously resistant mutants and induced mutants with Ethyl methane sulfonate and N-methyl-N-Nitro-N-nitrosoguanidine (EMS and NGu).

The stable mutants were resistant to 40 to 80 international units of Nystatin (dissolved in Propylene glycol) per ml in complex medium and were resistant to 120 to 500 units of Nystatin suspended in water.

None of these Nystatin resistant mutants showed requirement for or growth stimulation by added sterols or any other compounds although all the mutants failed to grow as well as their parental strains.

The mutants highly resistant to Nystatin contained no detectable sterols.

Resistant strains and sensitive clones were crossed and we found that Nystatin resistance is nuclear and controlled by one locus in mutants studied. All of the mutants studied had a recessive phenotype.

Some of the mutants isolated in the mutation hunts and some of the resistant ascospore clones grow poorly in complex medium without Nystatin but grow luxuriantly in the presence of Nystatin. This suggests a dependence on Nystatin for normal growth in these clones.

We know that Nystatin resistant mutants will be useful in the study of sterol metabolism in yeast and we believe that the existence of these apparently "sterol-less" mutants calls into question the necessity of sterols in aerobic growth .

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