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STUDIES ON MITOCHONDRIAL NUCLEIC ACIDS

IN MUCOR ROUXII

by

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Thesis Director's signature:

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PREFACE

I am grateful to Dr. Roger L. Storck for his interest, advice and encouragement during the period of this investigation. His advice on scientific and nonscientific problems made my graduate studies a rewarding experience.

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I dedicate this work to my parents, wife and children.

Vicente D. Villa

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May 1970

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INTRODUCTION

During the latter part of the nineteenth century, a number of cytologists observed granular bodies in the cytoplasm of a variety of eucaryotic cells. These granular bodies were assigned various names and functions. Undoubtly, some of these workers observed the organelles known today as mitochondria, and it is interesting that among the functions assigned to these organelles was that of carrying genetic information. In 1890 Altmann (1) suggested that these structures which he called "bioblasts" were bacteria-like and autonomous elementary living units capable of existing in the cytoplasm of the host cell, and he speculated on their genetic and metabolic roles in the host cell.

In 1913 Warburg (116) found cell respiration associated with granular insoluble components. In 1914 Lewis and Lewis (59) reported that the shape, size and location of mitochondria in chick embryo tissue cultures changed with time, and that these changes were a reflection of the cells' nutritional or endocrine state. These developments brought about an emphasis on the metabolic or respiratory function of the mitochondria, and the momentum of earlier speculations on the genetic role of mitochondria was lost to the extensive studies on the mechanisms by which mitochondria supply energy to the cell.

The evidence for the localization of cytoplasmic determinants in mitochondria was provided mainly by the work of Ephrussi and his collaborators (29) who discovered respiratory deficient mutants of Saccharomyces cerevisiae which they called "petites". They demonstrated that inheritable

changes in a series of respiratory enzymes (including cytochromes b and a) located in mitochondria were not transmitted according to classical Mendelian segregation. With the exception of the "segregational petite" mutant which segregates according to the classical Mendelian pattern, the petite determinants were demonstrated to be cytoplasmically inherited. This, thus, provided strong evidence for the semi-autonomous nature of mitochondria, and for the presence of extranuclear genetic determinants.

The "poky" mutants of Neurospora crassa obtained by Mitchell and Mitchell (68) have mitochondria deficient in cytochromes a, a₃ and b. In N. crassa, the male reproductive cells, unlike their female counterparts, do not contribute cytoplasmic factors to the formation of the diploid zygote. This fact enabled the Mitchells' to demonstrate that genetic transmission of the "poky" determinants followed a maternal or cytoplasmic inheritance pattern instead of the classical Mendelian pattern.

Garnjobst et al. (35) demonstrated that a wildtype or normal N. crassa strain changed to an abnormal one (abn-1) after the microinjection of cytoplasm from an abn-1 strain. Diacumakos et al. (26) found no such change occurred after microinjection of nuclei, but the wildtype changed to abn-1 after receiving purified abn-1 mitochondria.

The accumulation of evidence (29, 68) that genetic factors were not exclusively localized in the nucleus of the cell occurred almost simultaneously with the demonstration that deoxyribonucleic acid (DNA) is the carrier of genetic information (2, 45). However, the possibility for the existence of mitochondrial DNA (mit-DNA) was not considered seriously at this time. According to Borst et al. (9), the fact that cytoplasmic inheritance was considered as a sideline by most geneticists and that the

presence of DNA in mitochondrial preparations was taken as the result of nuclear contamination resulted in a lack of respect for the notion of a unique mit-DNA.

Only within the last decade have biochemical studies on the transmission and expression of genetic information by the mitochondrial system been taken seriously and the idea of a unique mit-DNA generally accepted. In 1959 Chevremont et al. (13, 14) demonstrated by autoradiography and cytochemistry the presence of DNA in mitochondria of tissue culture fibroblasts. In 1960 Chevremont et al. (15) found that the treatment of these cells with deoxyribonuclease (DNase) resulted in an increase of DNA content in the mitochondria as judged by Feulgen stain. Furthermore, they found that the treatment of the fixed preparations with DNase caused the removal of the mit-DNA. In 1963 Nass and Nass (73) demonstrated by electromicroscope that the fibrous inclusions frequently observed in the mitochondrial matrix were DNA fibrils.

Biochemical Isolation and Characterization of Mitochondrial DNA.

Although genetic and cytological evidence strongly suggested the presence of DNA in mitochondria, it remained nevertheless that direct isolation was essential to demonstrate unequivocally DNA in mitochondria. Schatz et al. (91) reported the association of DNA with S. cerevisiae mitochondria purified by density gradient centrifugation. Their claim was based on deoxyribose detection and digestion with pancreatic DNase. However, nuclear DNA (nu-DNA) contamination by adsorption could not be ruled out.

Luck and Reich (62) provided the first unequivocal proof for the existence of DNA in mitochondria. Their success resulted from the fact

that the DNA was extracted after DNase treatment of sucrose density gradient isolated mitochondria from N. crassa. This mit-DNA had a typical ultraviolet absorption spectrum, bound actinomycin D, and was sensitive to pancreatic DNase. Of greater importance was the fact that mit-DNA could be distinguished from nu-DNA by CsCl equilibrium density centrifugation since it had a buoyant density of 1.701 gm per cm³ versus 1.712 gm per cm³ for nu-DNA. Electromicrographs of the material showed long strands with the diameter of double stranded DNA and corresponding to a molecular weight of 13×10^6 .

The discovery that DNase treatment of mitochondria did not affect mit-DNA while it eliminated nu-DNA contamination resulted in the successful isolation of mit-DNA in a large number of organisms which included not only fungi, but other microbes, higher plants and animals.

The buoyant density of the mit-DNA in some higher animals such as rat, beef, mouse and guinea pig did not differ from that of its nuclear counterpart (21). However, mit-DNA in these species could be distinguished from the homologous nu-DNA by the fact that it renatured more readily after denaturation (21). The ability of mit-DNA to renature readily has been reported also in chicken (8, 78) and mouse (93). This unique characteristic was taken as an indication for a higher degree of homogeneity of mit-DNA preparations (21). This high degree of homogeneity of mit-DNA was also inferred from the narrowness of the bands observed in a CsCl gradient centrifugation profiles (21, 62) and also from the steepness of melting curves (80, 90, 111). Since the nu-DNA does not renature at all under the conditions in which mit-DNA does (21, 8), renaturation has been adopted as a useful criterion to distinguish the two

types of DNA's when their buoyant density is the same.

Another criterion used to distinguish between mit- and nu-DNA's is based on the differences in form and size of these molecules. Mit-DNA molecules have been found to be circular in the following: chicken (113, 114), L cells (72), mouse (93), S. cerevisiae (92, 95), monkey (106), sea urchin (25), human leukaemic leucocytes (19), normal human leucocytes and other mammalian tissues (18). Measurements of the size of circular mit-DNA filaments from electronmicrographs in a variety of organisms: chick, rat, beef, guinea pig, and mouse gave length values ranging only from 5 to 5.5 μ (94). On this basis, it was estimated that one circular mit-DNA molecule had a molecular weight of about 10^7 (93).

Biochemical Isolation and Characterization of RNA and Ribosomes in Mitochondria.

The demonstration of the presence in mitochondria of a DNA different from that found in the nucleus led to postulate the existence of ribonucleic acid (RNA) and protein synthesizing systems in mitochondria which would be independent from the nucleus and the cytoplasm. Active search for components of these systems was soon initiated in a number of laboratories. A DNA polymerase of mitochondrial origin has been reported for rat (67) and S. cerevisiae (119). Mitochondrial RNA polymerase activity has been detected in N. crassa (62), S. cerevisiae (100, 120), lamb (50), and a variety of mammals (74). The mitochondria of S. cerevisiae (118), N. crassa (3), and Tetrahymena pyriformis (104) have been shown to contain transfer RNA (t-RNA) and aminoacyl t-RNA synthetases. Epler (31) using reversed-phase chromatography showed in N. crassa distinct and separable species of mitochondrial and cytoplasmic t-RNA's for 15 amino

acids. In the same organism, Barnett et al. (4) found a full complement of mitochondrial aminoacyl t-RNA synthetases of which three acylated specifically mitochondrial t-RNA's. Two ribosomal RNA (r-RNA) molecules with bacteria-like sedimentation coefficient values (23S and 16S) have been detected in mitochondria from S. cerevisiae (84, 120) and N. crassa (54, 83). In addition, in N. crassa the mit- and cyt- types of r-RNA were found to differ in their nucleotide composition (54, 83). The results of several molecular hybridization studies in N. crassa (122), T. pyriformis (103) and S. cerevisiae (121) have led to the suggestion that mitochondrial r-RNA was synthesized with mit-DNA rather than with nu-DNA as a template.

The finding of high molecular weight RNA in mitochondria led to infer by analogy with the cytoplasm that this type of RNA was of ribosomal origin. The physico-chemical differences between mitochondrial RNA (mit-RNA) and cytoplasmic RNA (cyt-RNA) suggested that the mitochondrial ribosomes (mit-ribosomes) would be distinguishable from the cytoplasmic ribosomes (cyt-ribosomes) on the basis of their sedimentation velocity. Since the sedimentation coefficient of mit-RNA was less than that of the cyt-RNA and ribosomes from eucaryotes are known to sediment faster than those from procaryotes (77, 107) it was speculated that mit-ribosomes were of the bacterial type; thus, raising the question, which is far from being settled, on the possible parasitic origin of mitochondria.

Rifkin et al. (83) found no difference between cyt- and mit-ribosomes in N. crassa. Kuntzel and Noll (54) reported for the same organism values of 73S and 77S for mit- and cyt-ribosomes, respectively. Reports on mit-ribosomes from other sources gave values such as 55S for rat (76)

and 66S for corn (117). All these reports made clear that mit-ribosomes had a greater instability than their cytoplasmic counterpart as measured by the sensitivity to Mg^{2+} concentration variations. The fact remains that ribosomes were detected in the mitochondria of N. crassa, and that this plus the presence of the other macromolecular components listed above suggest that mitochondria are endowed with the apparatus for the transcription of genetic messages and for their translation into specific polypeptides.

Protein Synthesis in Mitochondria.

It has been found that isolated and washed rat liver mitochondria in a medium containing buffer, ATP, an amino acid mixture and succinate incorporated labelled amino acids, and that the first proteins to become labelled were insoluble and inner membrane-bound (6, 7, 48, 85, 86, 118). This suggested that mitochondria were able to synthesize their own structural proteins (48, 71). Grivell (39) found that isolated mitochondria from S. carlsbergensis incorporated labelled amino acids, and that this incorporation was resistant to ribonuclease (RNase), but sensitive to chloramphenicol (CAP). Henson et al. (44) studied the kinetics of incorporation in vivo of labelled phenylalanine into the subcellular and sub-mitochondrial fractions in S. cerevisiae during glucose derepression, and they reported a CAP sensitive incorporation into the mitochondrial fraction.

In the present work, it is shown that uniqueness of mit-DNA and mit-RNA is not limited to Neurospora and Saccharomyces but applies as well for several other fungi. Among these Mucor rouxii was selected for a detailed study of its mitochondrial nucleic acids.

M. rouxii is a dimorphic fungus because it grows yeast-like in anaerobiosis; whereas, in aerobiosis, growth is filamentous. Cells grown in anaerobiosis form germ tubes after 3 to 4 hours of exposure to air and progressively convert into filaments. In addition to the change in morphology, this conversion is also accompanied by a metabolic change resulting from the formation of active respiratory enzymes such as cytochrome c oxidase (41, 110).

In the present work, it is demonstrated that M. rouxii possess: (i) a mit-DNA which can be distinguished from its nuclear counterpart by its rate of renaturation after heat denaturation; (ii) a mit-ribosomal subunit not present in the cyt-ribosomes and monomers (monosomes) of great unstability; (iii) a mit-RNA population with a nucleotide composition significantly different from that of its cytoplasmic counterpart; (iv) a mit-RNA synthesizing system which respond to antibiotics during conversion in a different manner from the nuclear system; (v) a mitochondrial protein synthesizing system with a different sensitivity to antibiotics than the cytoplasmic system.

The present work also demonstrated an interdependence between the cytoplasmic and mitochondrial protein synthesizing systems in M. rouxii.

MATERIALS AND METHODS

A. The organisms used in this study are listed according to their taxonomy.

Zygomycetes	origin
<u>Cunninghamella echinulata</u>	(1)
<u>Mucor fragilis</u>	(1)
<u>M. rouxii</u> (NRRL 1894)	(3)
Ascomycetes	
<u>Ceratocystis ulmi</u>	(1)
<u>Chaetonium globosum</u>	(4)
<u>Gelasinospora autosteria</u>	(1)
<u>G. calospora</u>	(1)
<u>G. cerealis</u>	(1)
<u>G. tetrasperma</u>	(1)
<u>Neurospora crassa</u>	(2)
<u>N. sitophila</u>	(5)
<u>Sordaria macrospora</u>	(1)
Basidiomycetes	
<u>Daedalea confragosa</u>	(4)
<u>Schizophyllum commune</u>	(1)

Origin of the organisms: (1) Dr. C.J. Alexopoulos, Department of Botany, The University of Texas, Austin, Texas. (2) Dr. R.P. Wagner, Department of Zoology, The University of Texas, Austin, Texas. (3) Dr. C.W. Hesseltine, Northern Regional Research Laboratory (NRRL), Peoria, Illinois. (4)

American Type Culture Collection, Rockville, Maryland. (5) Fungal Genetic Stock Center, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire.

B. Composition of growth media

1. Synthetic Medium (BGA medium)

- a. Salt solution (fifty fold concentrated): 150.0 gm potassium phosphate (monobasic); 25.0 gm magnesium sulfate; 0.09 gm zinc sulfate ($7\text{H}_2\text{O}$); 0.05 gm ferrous sulfate ($7\text{H}_2\text{O}$); 0.015 gm manganous sulfate (H_2O); 0.02 gm cupric sulfate ($5\text{H}_2\text{O}$); 120.0 gm ammonium sulfate; distilled water up to 1,000 ml.
- b. Vitamin solution (stock solution): 100 mg thiamine-HCl; 100 mg nicotine acid; 100 ml distilled water. The pH was adjusted to 4.5 with KOH and the solution was sterilized by filtration through a $0.47\ \mu$ millipore membrane (Millipore Filter Co., Bedford, Mass.).
- c. Glucose solution: 20.0 gm D-glucose (Fisher Scientific Co., Fair Lawn, New Jersey) in 100 ml distilled water. The solution was sterilized by autoclaving for 15 minutes at 15 pounds pressure (121 C). In the respiratory adaptation experiments, varying concentrations of D-glucose were prepared and supplied in the same manner.
- d. Preparation of BGA liquid medium: 20 ml salt solution (50 fold concentrated); 2 gm vitamin-free casein hydrolysate (Difco); 900 ml distilled water. The pH was adjusted to 4.5 and the solution was sterilized by autoclaving (15 pounds pressure,

121 C for 15 minutes). After sterilization, 1 ml of the vitamin solution and 100 ml of 20% glucose solution were added.

The BGA "repressing" medium was prepared as indicated above, except that the final concentration of glucose was 5% instead of 2%.

The BGA "derepressing" medium was prepared in the same manner, except that the final glucose concentration was 0.5% instead of 2% and that the final vitamin-free casein hydrolysate concentration was 1% instead of 0.2%.

2. Complex Medium (YPG medium): 3 gm yeast extract (Fisher); 10 gm peptone (Fisher); 20 gm D-glucose; 1,000 ml distilled water. The pH was adjusted to 4.5 with 5N H₂SO₄, and the solution was sterilized by autoclaving. For solid YPG medium, 2.5% agar (Difco) was dissolved before sterilization.
3. Tomato Juice-Agar Medium: sporulation medium Libbey's tomato juice (500 ml) sterilized for 15 minutes; 5% agar in 500 ml distilled water sterilized for 30 minutes. The sterile, lukewarm, tomato juice and agar solutions were well mixed, and 200 ml were poured into sterile Roux bottles.

C. Growth conditions

1. Growth and harvest of spores. Tomato juice agar slants and YPG slants were used to maintain stock cultures. Roux bottles with tomato juice agar were inoculated with a diluted M. rouxii spore suspension acquired aseptically from a tomato juice agar slant.

The Roux bottles were incubated for about a week at 30 C, and the spores aseptically harvested by scraping the agar surface with a glass rod in sterile distilled water. The spore suspensions were filtered through six layers of sterile gauze to remove chunks of agar and mycelia. The spores were washed by centrifugation and resuspended in sterile distilled water. The final spore concentration was adjusted to approximately 1.0×10^9 spores per ml. The spores were counted under the microscope with a bacterial counting chamber (Bright-line Counting Chamber, American Optical Co., Instrument Division, Buffalo, New York). The suspension was distributed into several sterile screw cap tubes which were sealed and stored at 4 C. Spores stored in this manner were viable for several months. Normally the suspensions were kept for 3 months before a fresh batch of spores was harvested.

2. Aerobic cultures. All the organisms were grown aerobically in complex medium (YPG medium) at 25 C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N. J.) in baffled long-necked, two liter Erlenmeyer flasks. Cultures totaling six liters were inoculated with 1.0×10^5 spores per ml and incubated as indicated above for 16 to 18 hours. Cultures in which sporulation was poor or non-existent were inoculated with 30 ml of a 200 ml YPG starter culture which had been inoculated with the mycelium from a stock slant and incubated at 25 C for 12 hours on a rotary shaker (Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, N.J.). The cultures were harvested by filtration

through Whatman no. 4 filter paper. The mycelial pads were washed with one liter of cold 0.01M tris(hydroxymethyl)-amino methane (Tris), pH 7.9, containing 0.001M ethylenediamine-tetraacetate (EDTA) and 0.44M sucrose (TE buffer), the buffer used in the isolation of mitochondria for the purpose of extracting DNA.

3. Anaerobic cultures: M. rouxii

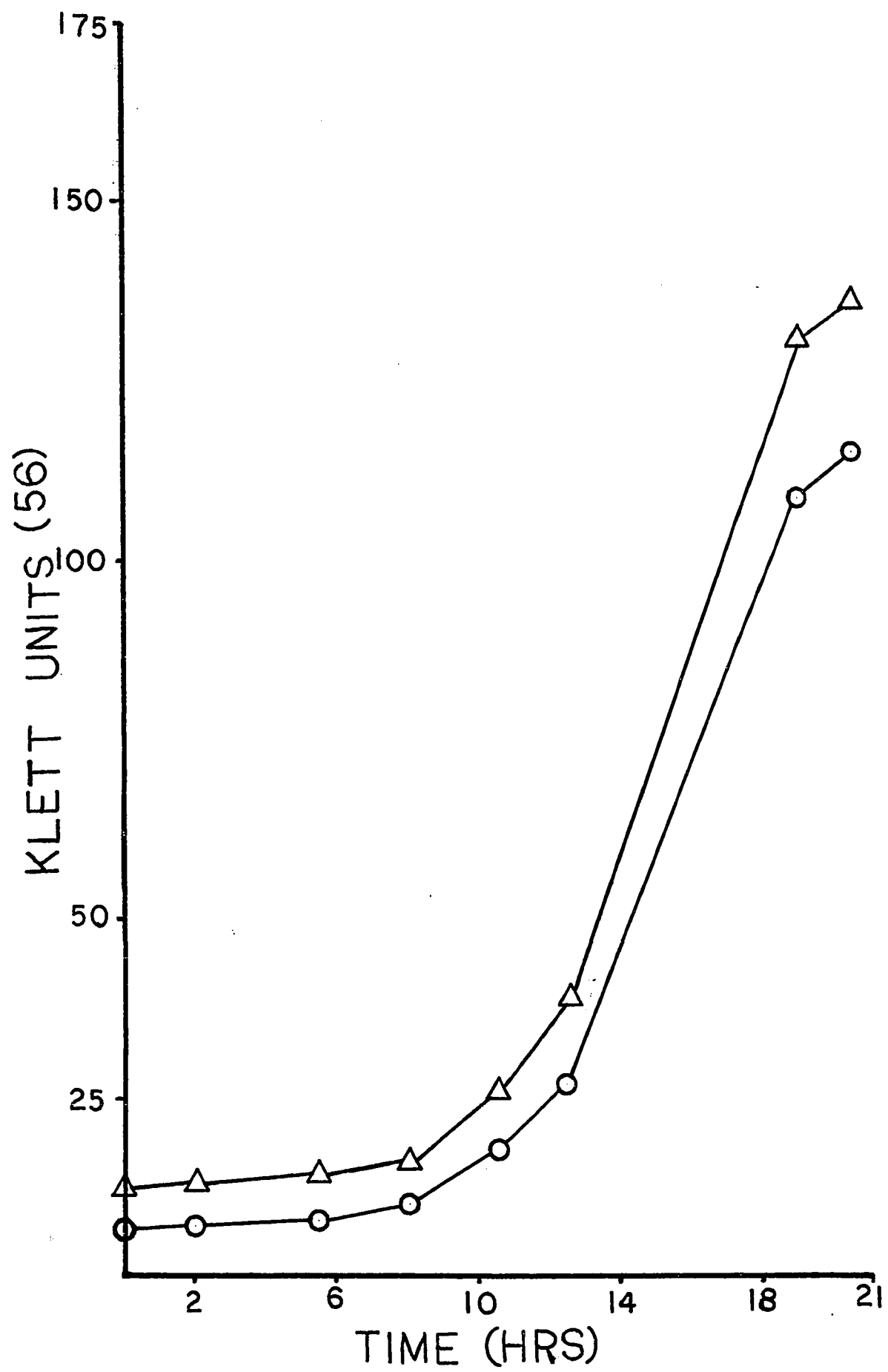
- a. In order to grow yeast-like cells, sporangiospores (6.0×10^5 spores per ml) were inoculated into BGA medium (5% glucose) under anaerobic conditions. The BGA medium was flushed with prepurified N_2 gas at 27 C with constant stirring by means of a magnetic rod. After inoculation, N_2 gas was flushed for an additional 15 minutes, and the cultures were then sealed except for an outlet which was submersed in water and served to relieve the CO_2 pressure in the cultures. The anaerobic cultures were incubated at 27 C and stirred with a magnetic rod for 16 hours.

M. rouxii sporangiospores began to swell in BGA medium (5% glucose) after 8 to 12 hours of incubation in anaerobic conditions. Budding occurred thereafter and lasted until the 19th hour at which time growth ceased. Fig. 1 suggests that the growth with inocula of 6.0×10^5 and 9.0×10^5 spores per ml was exponential. The time sequence of swelling and budding of the cells was the same with both inocula, and growth stopped at the same time. On the basis of these results, an inoculum of 6.0×10^5 spores per ml, and a growth duration

Figure 1

Growth of M. rouxii in anaerobiosis. BGA medium (100 ml per flask) was flushed with and sealed under prepurified N₂ gas. After inoculation by spore injection, the cultures were incubated at 27 C and stirred with a magnetic bar. Inoculum: 6.0×10^5 spores per ml

○——○ ; 9.0×10^5 spores per ml △——△ .



of 16 hours were routinely used.

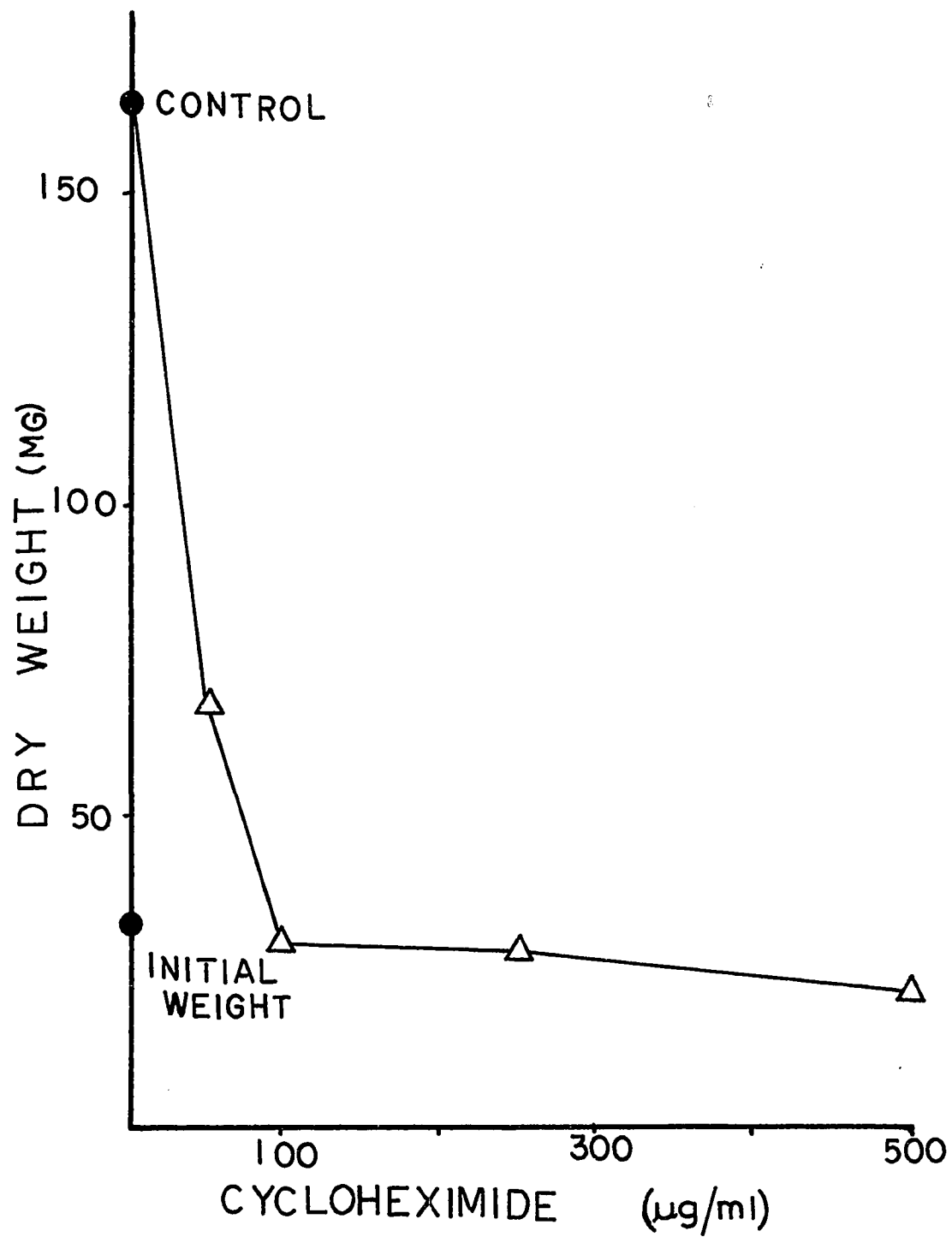
- b. Anaerobic growth for the analysis of total mit-RNA. Eight to ten liters of BGA medium (5% glucose) were incubated under the above conditions in a carboy equipped with a two hole rubber stopper having an inlet and an outlet to permit the flushing of the N_2 gas and relieve the CO_2 pressure upon growth. Nitrogen gas was flushed for 2 hours prior to the inoculation and for 15 minutes preceding the sealing of the cultures. At 16 hours, the cultures were either poisoned by injecting sodium azide (final concentration: $10^{-2}M$) in anaerobiosis, or else they were exposed to air for the desired period of time during conversion. The yeast-like cells were harvested by filtration through Whatman no. 1 filter paper and washed with one liter of cold 0.01M Tris, pH 7.5, containing 0.01M $MgCl_2$, 0.44M sucrose, 0.1% bovine serum albumin (Difco) and 0.01M sodium azide (TM buffer).
- c. Anaerobic cultures for the purpose of 3H -uridine incorporation during conversion were incubated under the conditions given above. These cultures consisted of 600 ml BGA medium (5% glucose) in a two liter Erlenmeyer flask, and N_2 gas was flushed for one hour prior to inoculation and for 15 minutes preceding the sealing of the cultures. At 16 hours, the cultures were harvested by filtration through millipore membrane, washed with 10 ml sterile distilled water, and transferred to BGA medium (0.5% glucose) containing the 3H -uridine.

4. Conversion of yeast-like cells to filaments. Yeast-like cells grown anaerobically for 16 hours were exposed to air and incubated at 25 C while stirred with a magnetic rod in order to get filamentous growth. The analyses of mit-RNA base composition during conversion were performed by exposing the anaerobically grown cells to air and flushing the N_2 - CO_2 mixture out of the carboy with a hair dryer. The cultures were then incubated for 40 minutes in the presence of air at 25 C with stirring in the same medium used for anaerobic growth.
5. Conversion of yeast-like cells to filaments in the presence of cycloheximide (Acti-dione, UpJohn Co., Kalamazoo, Mich.) and CAP (D(-)threo-Chloromycetin, Sigma Chem. Co., St. Louis, Missouri). Yeast-like cells grown by anaerobic incubation for 16 hours were aseptically harvested by filtration through sterile millipore membranes, washed with 10 ml sterile distilled water and transferred to BGA medium (0.5% glucose) containing varying concentrations of cycloheximide or CAP. The cultures were then incubated at 27 C in a water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Samples for dry weight determination were taken at different time intervals during conversion.

As shown in Fig. 2, cycloheximide inhibited increase in dry weight of converting cells, and inhibition was complete with 100 μ g per ml of cycloheximide. The experiments on the effect of cycloheximide on the different parameters studied during conversion were routinely done using 200 μ g per ml of cycloheximide in view of the fact that this concentration inhibited completely

Figure 2

Effect of cycloheximide concentration on M. rouxii converting cells.
Dry weight of the cells was determined after incubation in the presence of cycloheximide for 9 hours at 27 C in a shaking water bath.



increase in dry weight and germ tube formation.

Like cycloheximide, CAP inhibited increase in dry weight of converting cells. As shown in Fig. 3, this inhibition was concentration dependent. Inhibition was never complete (79%) even when concentrations as high as 4 mg per ml were used. Fig. 4 illustrates the inhibitory action of CAP on the dry weight increase during conversion, and also a small concentration dependency of this inhibition on CAP concentration. After 7 hours of contact with air and CAP, the inhibition was 79% and 89%, respectively with 3.5 mg per ml and 4.0 mg per ml. On the basis of these results, the experiments on the effect of CAP on the different parameters studied during conversion were routinely done using 4.0 mg per ml of CAP.

D. Preparation of mitochondria

1. Preparation of mitochondria for DNA extraction. The mycelial pads from six liters of aerobically grown cells were ground with acid-washed sea sand (E.H. Sargent Co., Dallas, Texas) and a small volume of TE buffer at 4 C. The mixture was initially a semi-dry paste, but as the grinding was performed the paste became wetter due to the breakage of the cells. Enough buffer was added to cover and resuspend the paste. A preliminary centrifugation at $1,500 \times g$ for 10 minutes in a Servall refrigerated centrifuge model RC-2 (Ivan Sorvall, Incorp., Norwalk, Conn.) served to remove the sand, whole cells and nuclei. The preliminary centrifugation was repeated to remove any remaining whole cells and nuclei. A crude mitochondrial pellet was collected from the homo-

Figure 3

Effect of CAP concentration on M. rouxii converting cells. Dry weight of the cells was determined after incubation in the presence of CAP for 7 hours at 27 C in a shaking water bath.

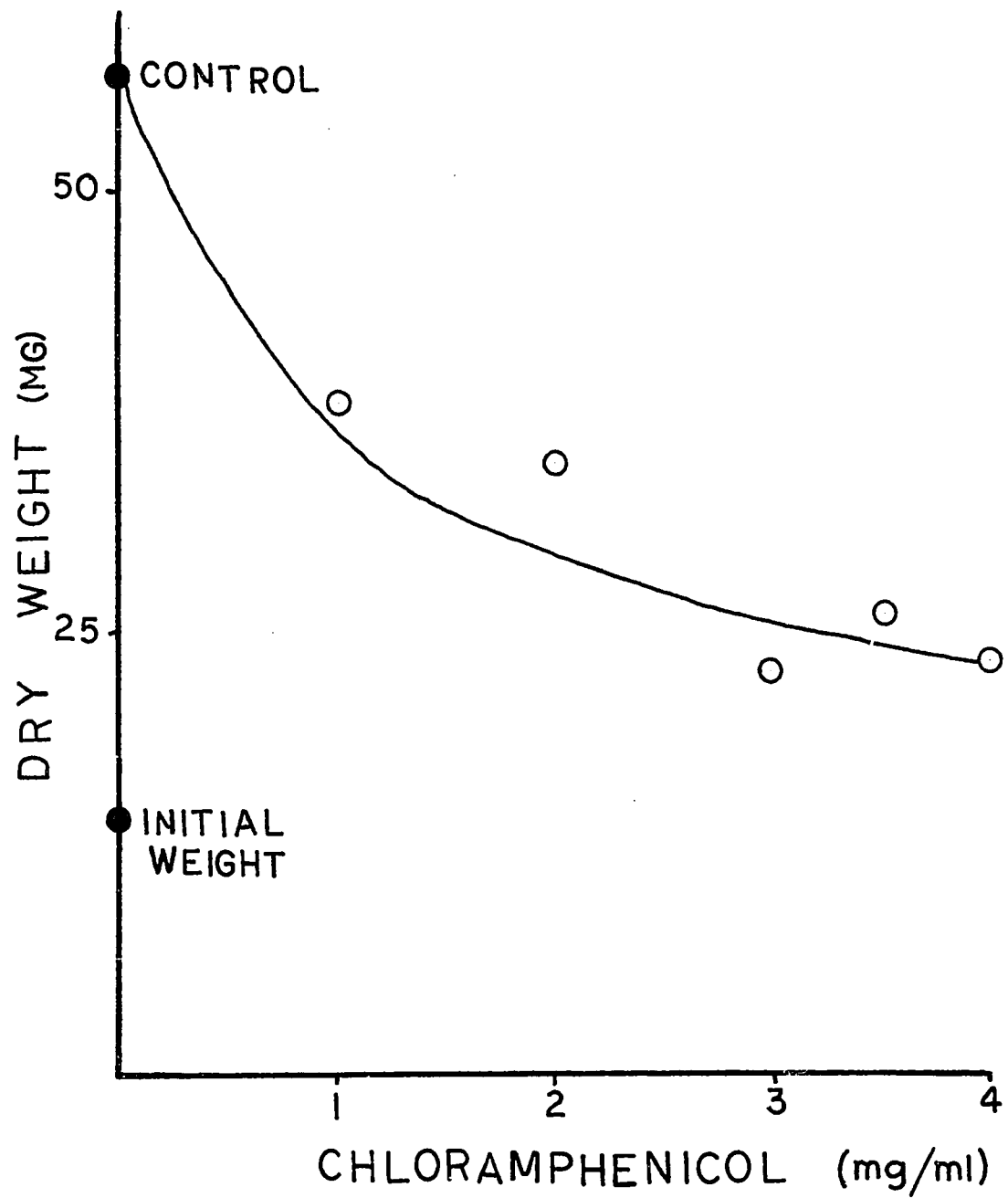
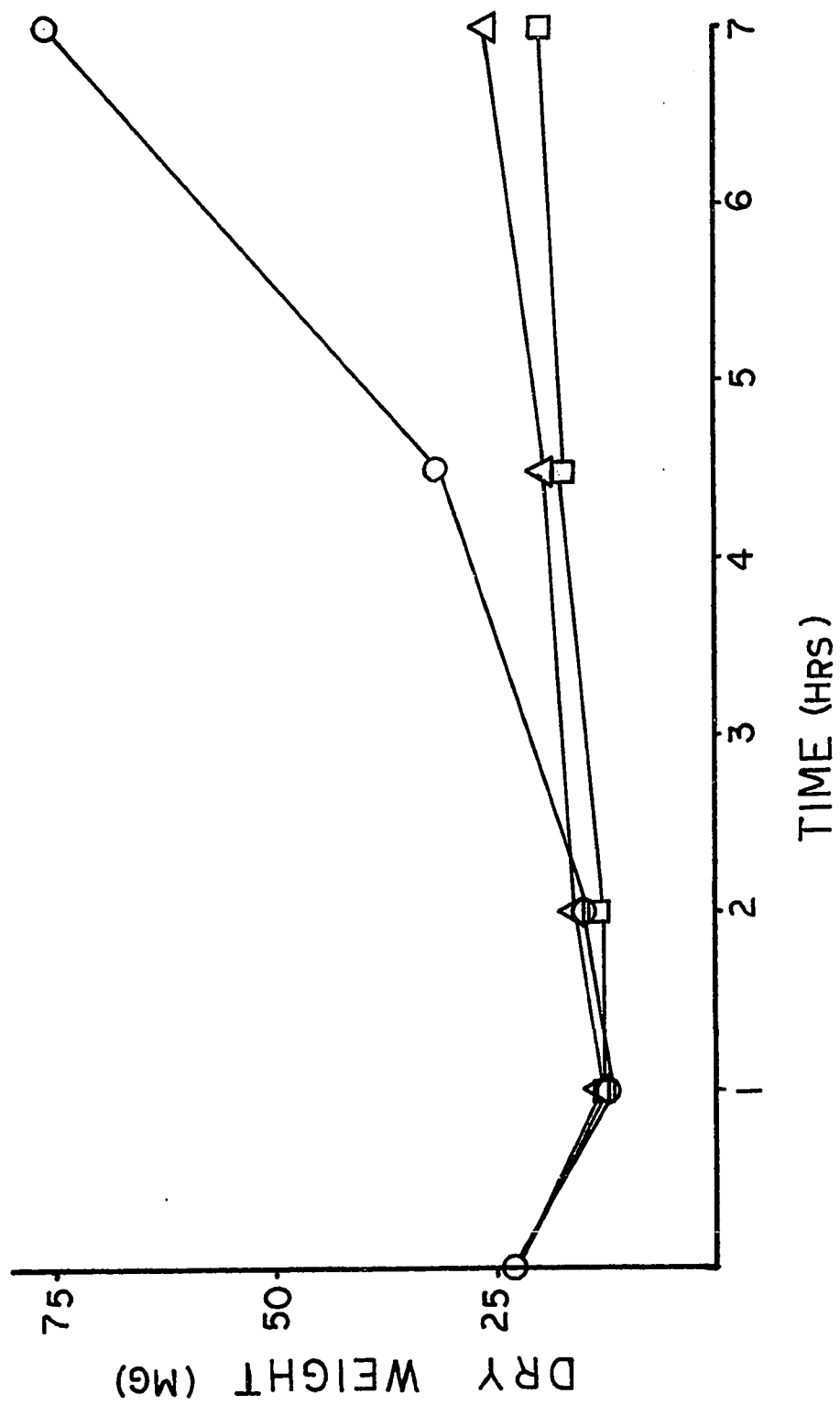


Figure 4

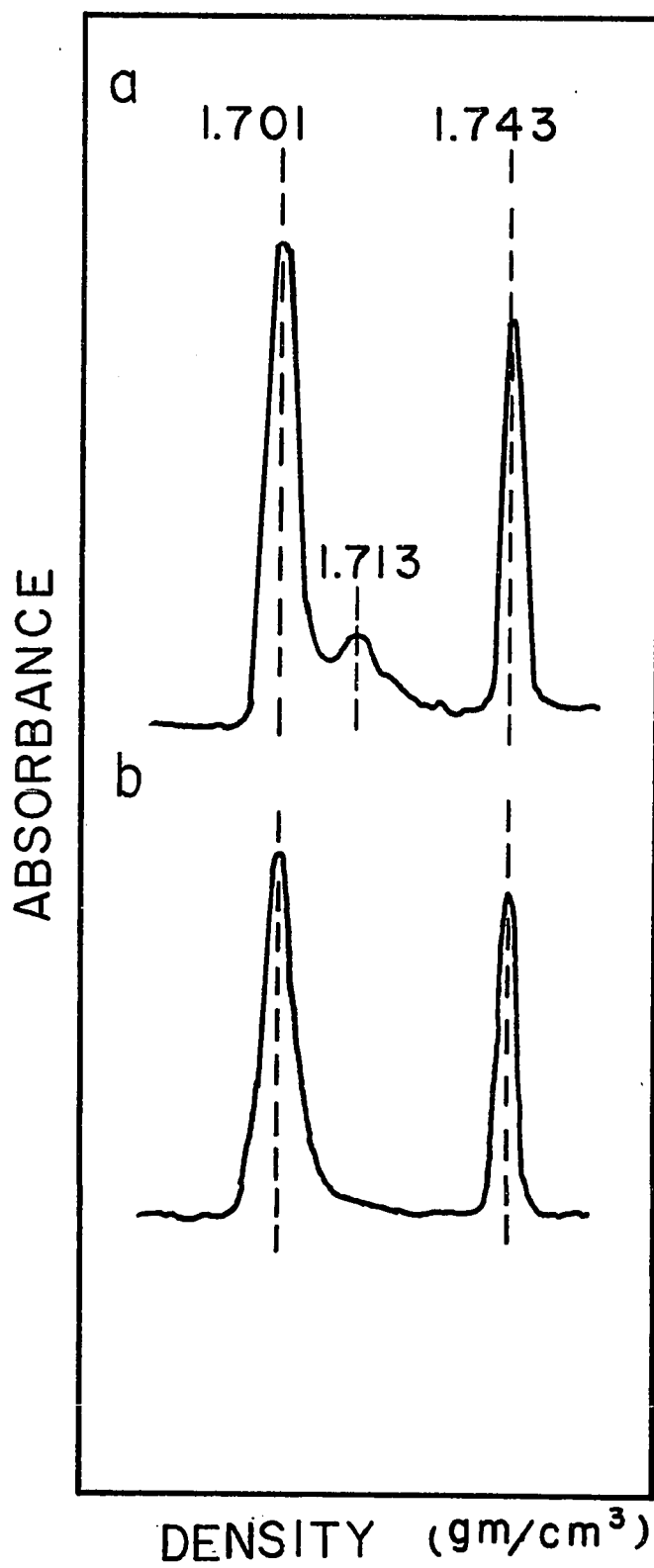
Effect of CAP on dry weight of M. rouxii converting cells during the first 7 hours during conversion. ○——○ control no CAP; △——△ 3.5 mg per ml CAP; □——□ 4.0 mg per ml CAP.



genate by centrifugation at 13,000 x g for 25 minutes in a no. 30 rotor of the Spinco model L preparative ultracentrifuge (Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, Calif.). The crude mitochondrial pellet was divided into two equal parts, one of which was resuspended in 3 ml of TE buffer, and the other was resuspended in 3 ml of 0.01M Tris, pH 7.9, containing 0.005M MgCl_2 and 0.44M sucrose (modified TE buffer). The mitochondrial fraction resuspended in modified TE buffer was treated with pancreatic DNase (twice crystallized, Worthington Biochem. Corp., Freehold, N.J.) 200 μg per ml for 45 minutes at 4 C; the control mitochondrial suspension in TE buffer was incubated under the same conditions in the absence of DNase. As shown in Fig. 5, pretreatment of mitochondria from Gelasinospora cerealis with DNase did not alter the integrity of mit-DNA but visibly eliminated nu-DNA. The 3 ml mitochondrial suspensions were layered on a 20 ml sucrose density gradient. The discontinuous gradients were prepared by the successive layering of 5 ml of TE buffer with the following sucrose concentrations: 2.2, 2.0, 1.9, and 0.97M. Fractions (1 ml) were collected by puncturing the bottom of the centrifuge tube after one hour of centrifugation at 25,000 rpm at 4 C in an SW25 rotor of a Spinco model L ultracentrifuge. The protein content of each fraction was determined by the method of Layne (56) with the use of the Folin-Ciocalteu reagent. Cytochrome c oxidase contents were determined according to the method of Haidle and Storck (41). A centrifugation profile showing the distribution of cytochrome c oxidase specific

Figure 5

Microdensitometer tracings of G. cerealis DNA photographed after 20-hour centrifugation at 44,000 rpm on an analytical ultracentrifuge. (a) DNA preparation obtained from mitochondria not treated with DNase prior to extraction shows contamination of mit-DNA (1.701 gm per cm³) by nu-DNA (1.713 gm per cm³). (b) DNA preparation after treatment of intact mitochondria with DNase. DNA from SP8 bacteriophage (1.743 gm per cm³) was used as reference.



activity (rate constant per mg protein) is shown in Fig. 6 for mitochondria extracted from N. crassa. Similar profiles were obtained with mitochondria preparations of: Cunninghamella echinulata, Gelasinospora autosteria, and M. rouxii. The fractions with the highest specific activity, for example, 13 to 17 in the case of Fig. 6, and which correspond to a distinct band visible in the gradients, were pooled and the mitochondria were pelleted by a 90-minute centrifugation at 30,000 rpm at 4 C in the no. 30 rotor of a Spinco preparative ultracentrifuge. For the organisms not listed above, mitochondria were purified as described except that an SW25.2 rotor was used, and thus twice as much material was layered on 56 ml of the sucrose gradient.

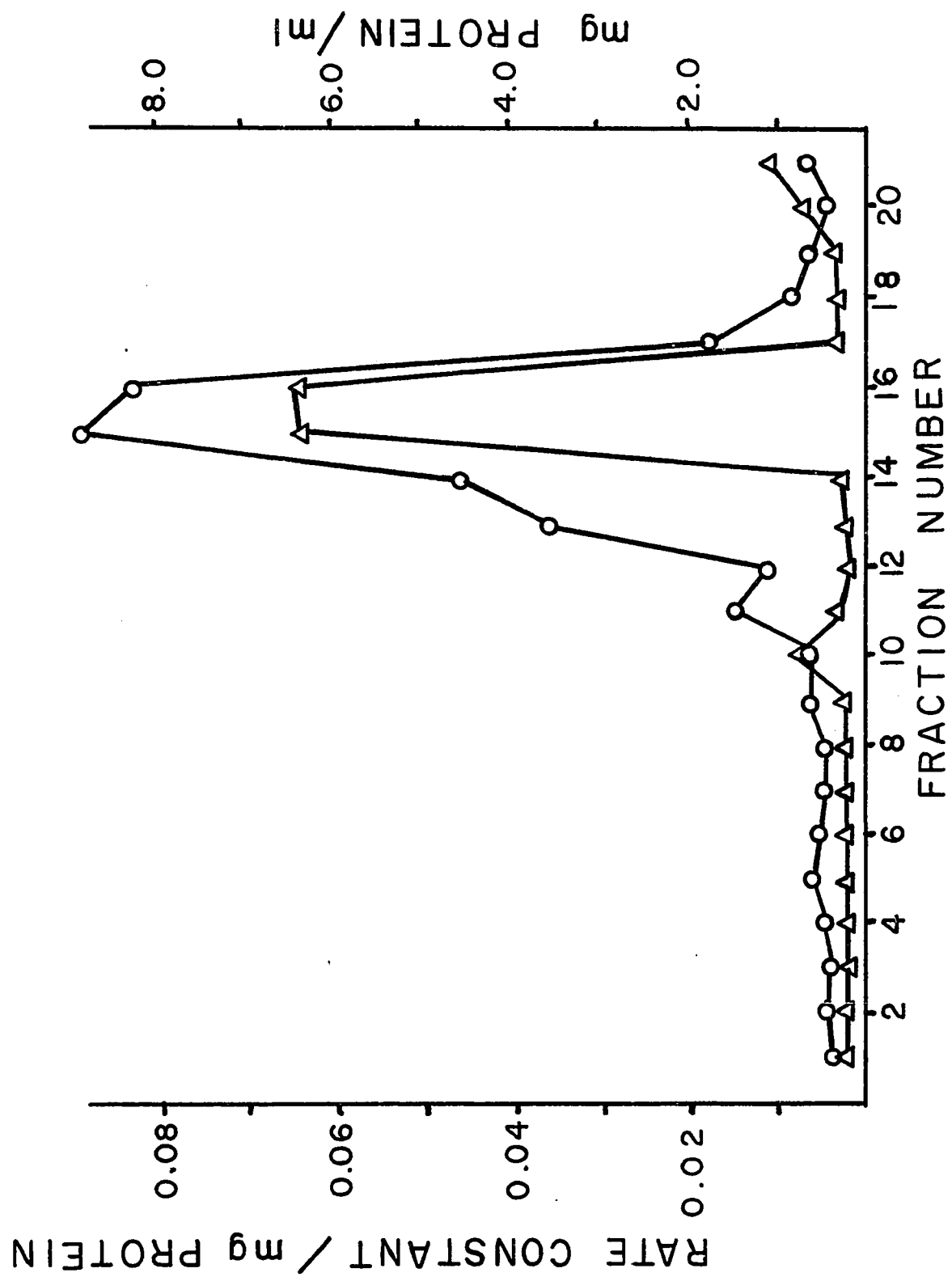
2. Preparation of mitochondria for the isolation of ribosomes.

Mitochondria were isolated and purified on discontinuous sucrose density gradients in the same manner as described for the DNA extraction, except that the following buffer solution (AMTP buffer) was used: 0.01M Tris, pH 7.5, containing 0.01M $MgCl_2$, 0.1M NH_4Cl , 0.44M sucrose, 0.1% bovine serum albumin and 20 μg per ml polyvinyl sulfate (K and K Laboratories, Plainview, N. Y.), and the mitochondria were not treated with nucleases.

3. Preparation of mitochondria for the determination of the RNA base composition. Mitochondria were isolated in TM buffer. Instead of using centrifugation to remove the sea sand after grinding, the resulting paste was washed with the buffer solution through a double layer of Kimwipe (Kimberly Clark Corp.) and filtered

Figure 6

Sucrose density gradient centrifugation profile of a N. crassa mitochondrial suspension showing the coincidence of the distribution of protein (Δ — Δ) and of cytochrome c oxidase (\bigcirc — \bigcirc). The discontinuous sucrose density gradient was centrifuged in a SW-25 rotor at 24,000 rpm for 60 minutes at 4 C in a Spinco model L ultracentrifuge.



with gentle suction. The crude mitochondrial pellet was re-suspended in the TM buffer solution plus 150 μg per ml RNase (Bovine pancreatic ribonuclease, type 1-A, Sigma Chem. Co.) and incubated at 4 C for 30 minutes. As shown in Fig. 7, the amount of RNA extractible from M. rouxii mitochondria remained constant when the concentration of RNase reached 200 μg per ml, and the immunity of mit-RNA to RNase treatment of mitochondria ceased when the temperature was raised from 25 C to 37 C. As shown in Fig. 8, treatment of M. rouxii mitochondria at 4 C with RNase had no strong effect on the amount of RNA that could be extracted from mitochondria. The use of RNase became useful for the elimination of RNA contaminating the mitochondria since RNase activity was not lowered significantly at 4 C. The mitochondria were then washed by centrifugation and purified on a discontinuous sucrose density gradient consisting of two shelves. Fig. 9 shows the centrifugation profiles for continuous and discontinuous sucrose density gradients used in preparing mitochondria for RNA extraction. The cytochrome c oxidase specific activity was expressed as rate constant per O.D.₂₆₀ absorbance measured on the RNA hydrolyzed by RNase in each fraction. As shown in Fig. 9, the cytochrome c oxidase specific activity peak in the discontinuous gradient was relatively higher and narrower than the peak in the continuous gradient. The profiles shown on Fig. 10 demonstrate that the distribution of RNA in each of the gradient type coincides with that of cytochrome c oxidase activity. The fact that there is a correspondence between RNA

Figure 7

Effect of pancreatic RNase concentration on RNA content of M. rouxii mitochondria after incubation for 30 minutes at various temperatures.

○——○ incubation at 4 C for 30 minutes; △——△ incubation at 25 C for 30 minutes; ●——● incubation at 37 C for 30 minutes.

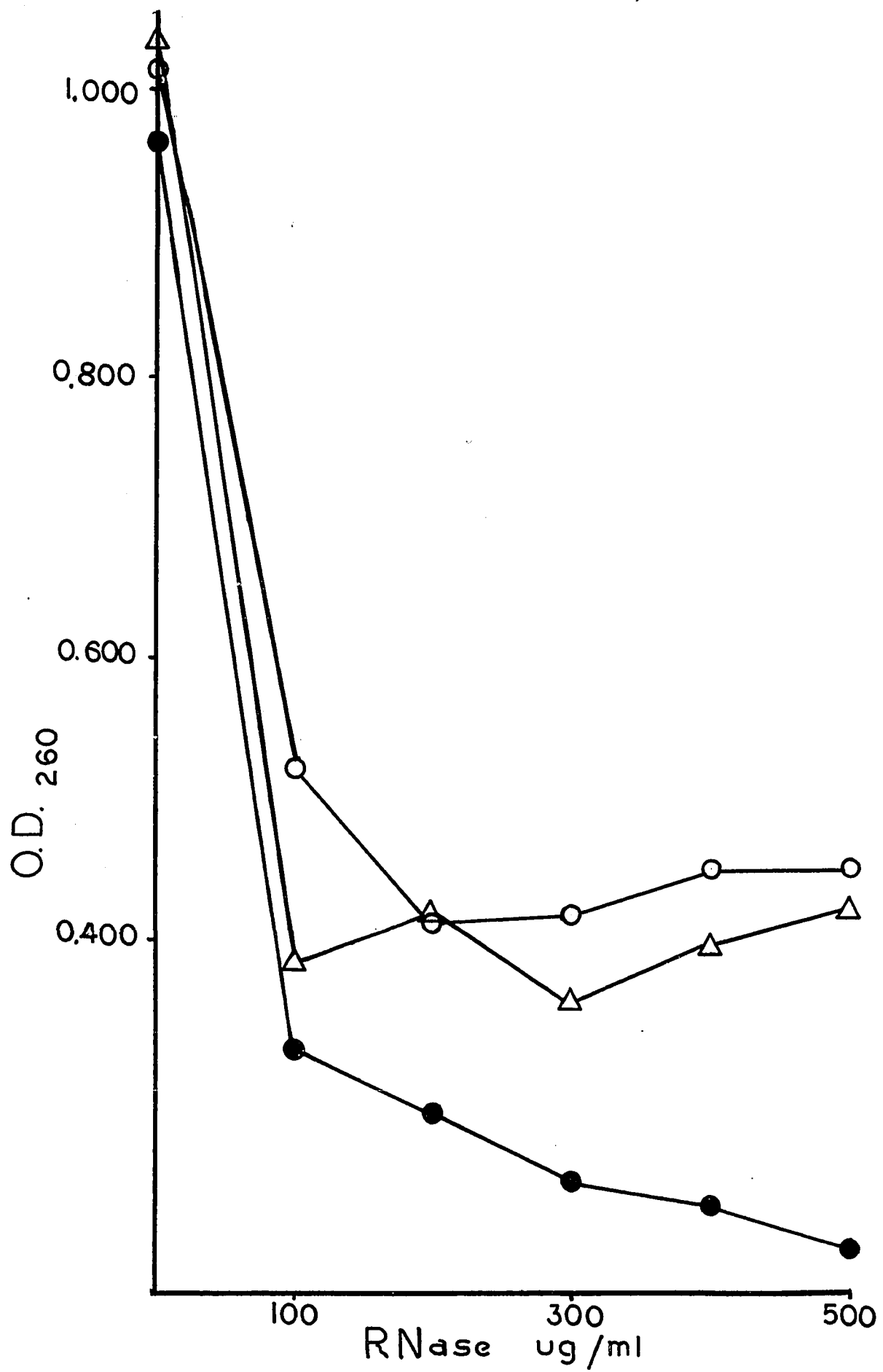


Figure 8

Sucrose density gradient centrifugation profiles of mitochondrial RNA (Δ — Δ) after pancreatic RNase treatment (150 μ g per ml at 4 C for 1 hour) of mitochondria from M. rouxii. \bigcirc — \bigcirc control mitochondrial suspension not treated with RNase.

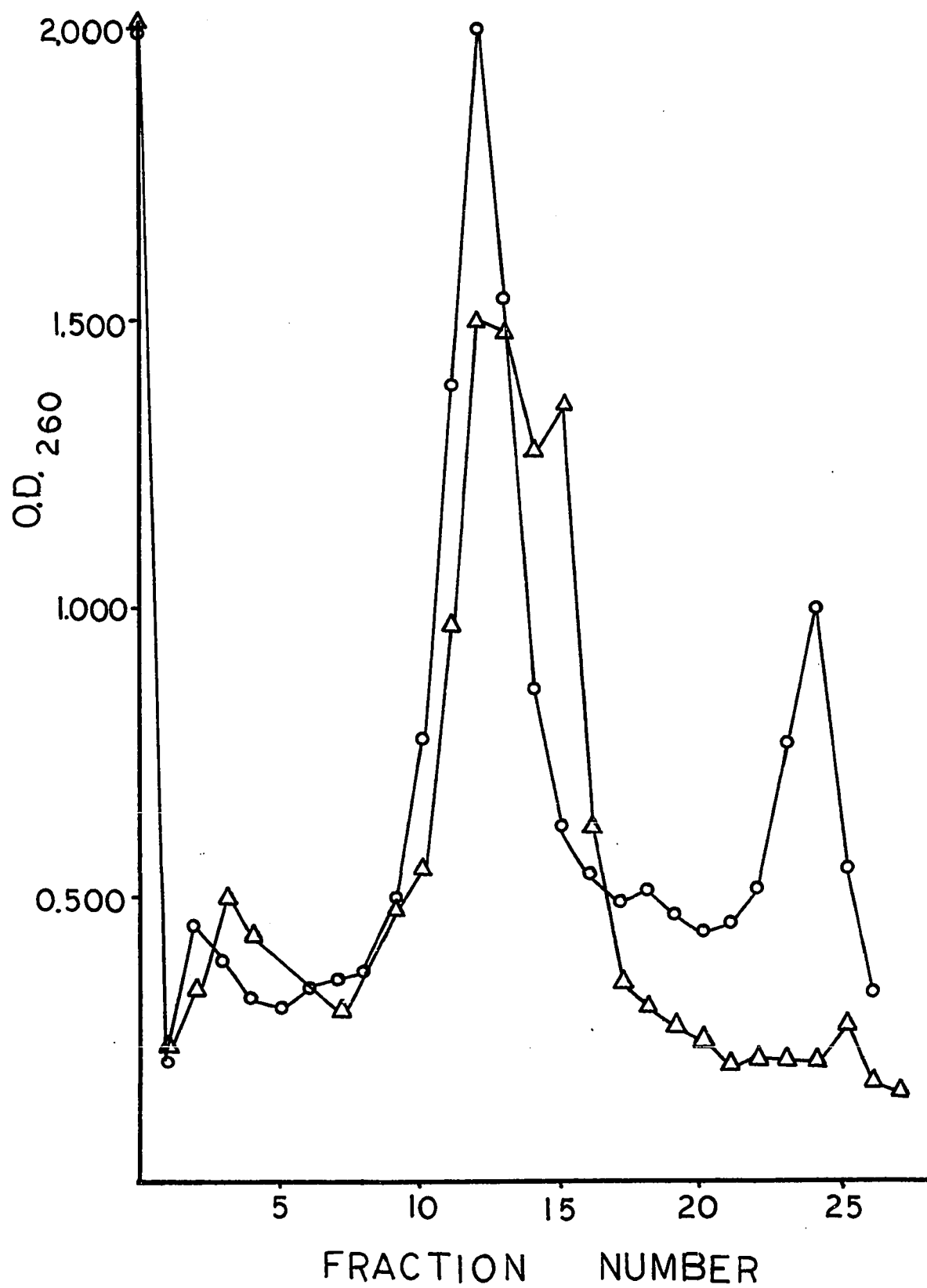


Figure 9

Sucrose density gradient centrifugation profiles of M. rouxii mitochondrial suspensions showing the distribution of mitochondria expressed as cytochrome *c* oxidase activity per O.D.₂₆₀ absorption of each fraction after RNase treatment. Δ — Δ discontinuous sucrose density gradient consisting of two shelves: 2.0M and 1.0M sucrose; \bigcirc — \bigcirc continuous, linear sucrose density gradient (1.0M and 2.0M sucrose). The gradients were centrifuged in SW-25.2 rotor at 6,000 rpm for 90 minutes at 4 C in a Spinco model L-2 ultracentrifuge.

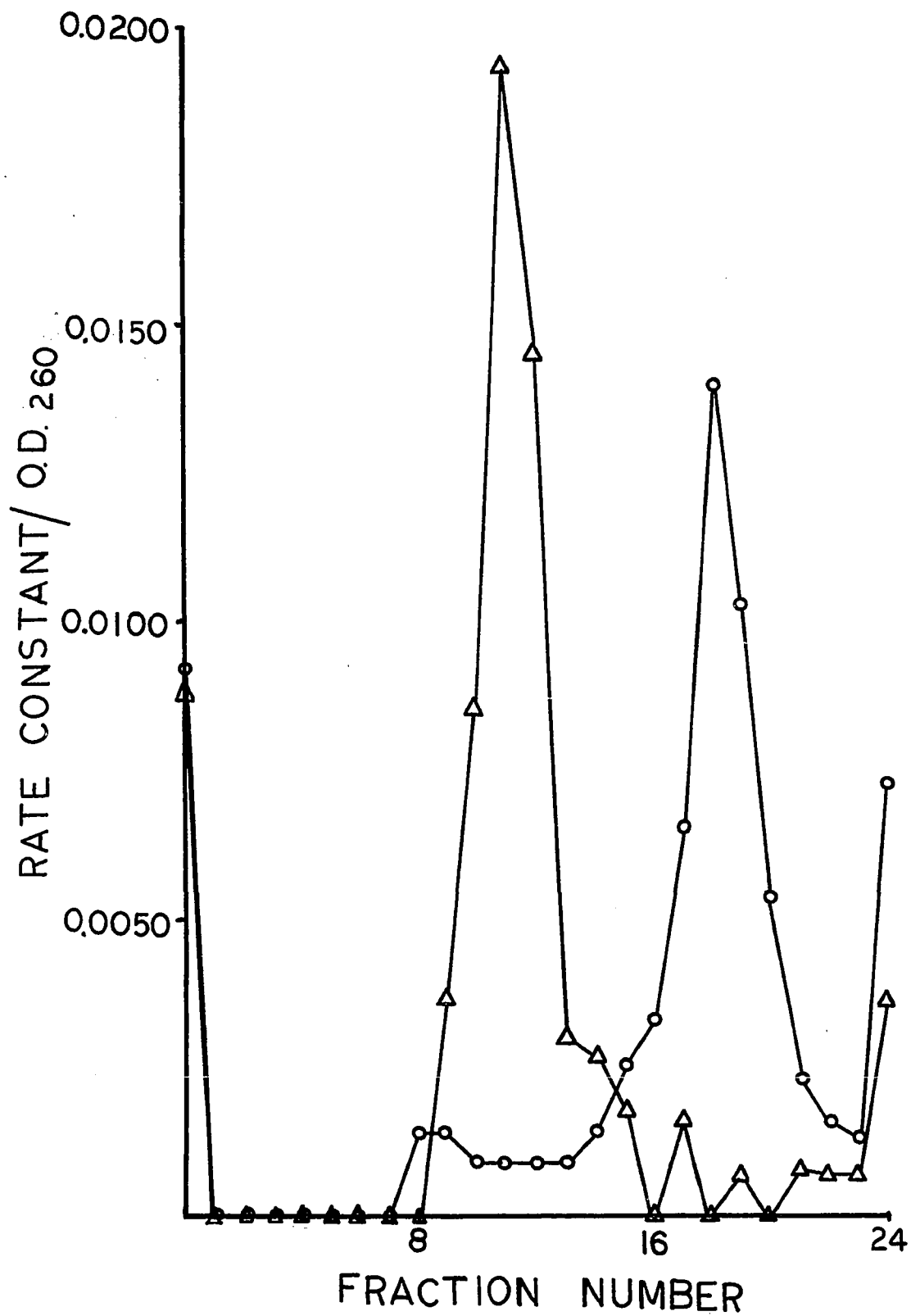
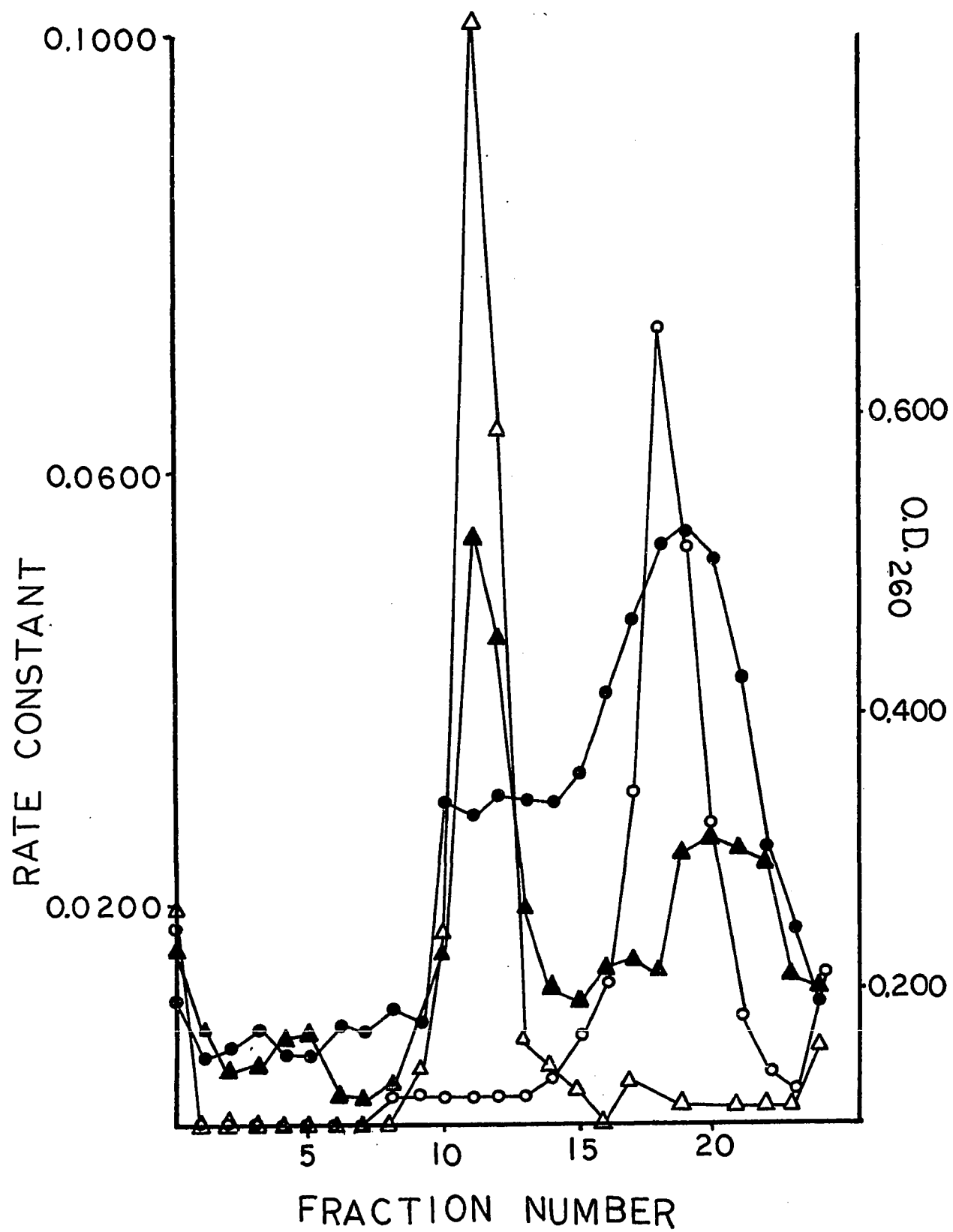


Figure 10

Sucrose density gradient centrifugation profiles of M. rouxii mitochondrial suspensions in discontinuous (Δ) and continuous (\circ) sucrose density gradients. Data from profiles in Fig. 9 have been plotted to show the coincidence between the RNA content (O.D._{260} closed circles and triangles) and cytochrome c oxidase activity per fraction (open circles and triangles) for the continuous and discontinuous gradients, respectively.



content and cytochrome c oxidase activity in each fraction suggests that mitochondria have their own RNA population. After centrifugation the distinct turbid bands visible in the discontinuous gradients were collected in one fraction which was centrifuged in the no. 30 rotor at 30,000 rpm for 90 minutes at 4 C in a Spinco preparative ultracentrifuge.

4. Preparation of mitochondria for the ^3H -uridine incorporation. Mitochondria were prepared by grinding the cells with acid-washed sea sand in TM buffer. The resulting paste was washed with buffer solution (total volume 100 ml) through a double layer of Kimwipe tissues by using gentle suction. The filtrate was centrifuged twice at 1,500 x g for 10 minutes, and the supernatant fraction was centrifuged at 13,000 x g for 25 minutes to yield the mitochondrial pellet. The mitochondrial pellet was resuspended in 1 ml of the buffer solution plus 0.2 units of bovine spleen phosphodiesterase II (Worthington Biochem. Corp., Freehold, N. J.) and were incubated at 4 C for 1 hour. The mitochondrial suspension was then diluted with the buffer solution and washed by centrifugation at 13,000 x g for 25 minutes.

As shown in Fig. 11, the constant amount of extractible mit-RNA after preincubation of *M. rouxii* mitochondria with spleen phosphodiesterase was higher than the amount of extractible mit-RNA after preincubation of mitochondria with pancreatic RNase. These results are in agreement with the sedimentation analysis evidence, reported by Leon and Mahler (58) which demonstrated that preincubation of yeast mitochondria with pancreatic

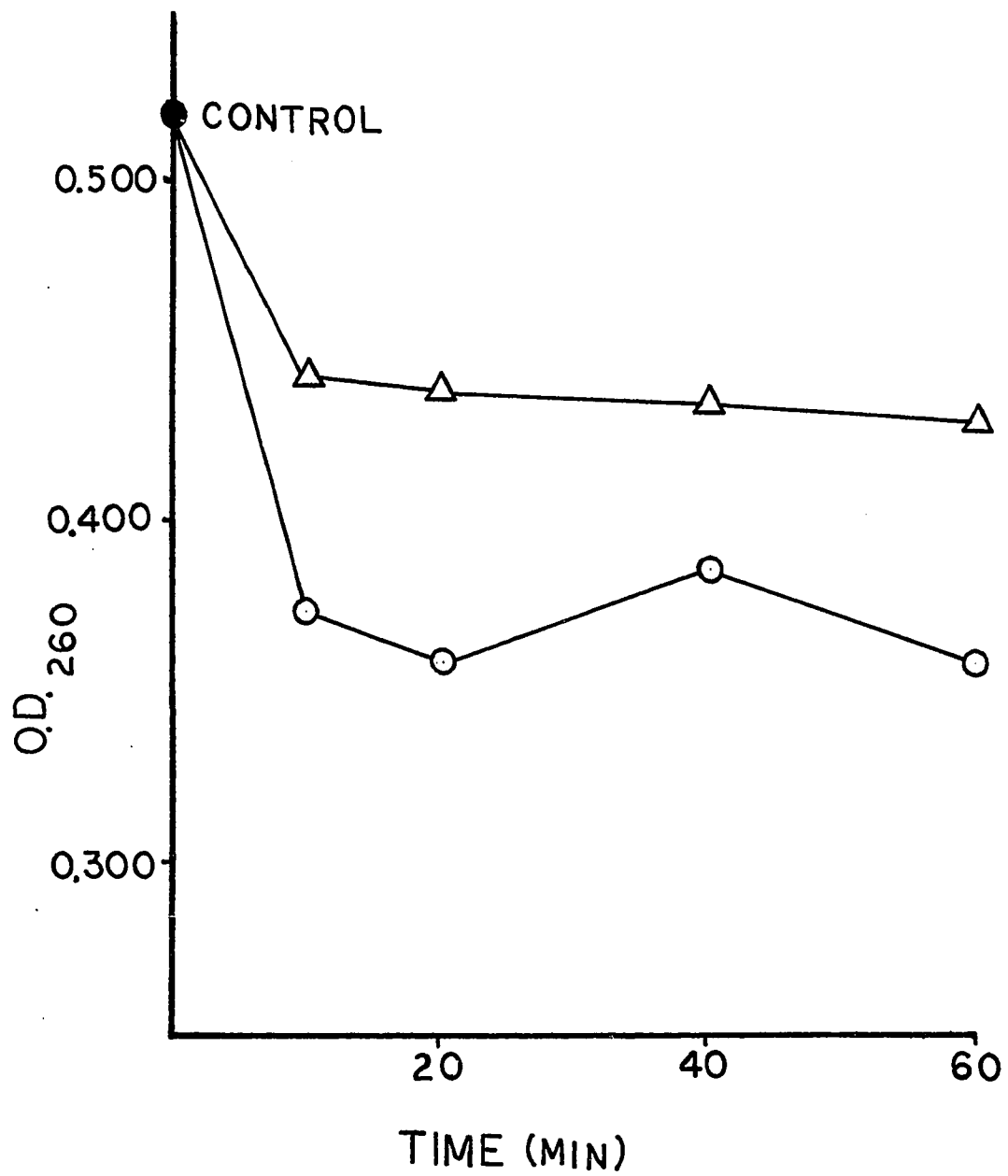
Figure 11

Effect of spleen phosphodiesterase and pancreatic RNase on M. rouxii mitochondrial suspension RNA content (O.D.₂₆₀) as a function of time.

(●) no nuclease; Δ — Δ spleen phosphodiesterase 0.2 unit per ml;

○—○ pancreatic RNase 100 μ g per ml.

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RNase, but not with spleen phosphodiesterase, resulted in mit-RNA degradation.

E. Characterization of DNA

1. Extraction and purification of mit-DNA. The purified mitochondrial pellet was suspended in 6 ml of 0.01M Tris, pH 7.9, containing 0.001M EDTA, 2.5% (w/v) sodium dodecyl sulfate and 0.2% (w/v) sodium deoxycholate. The suspension was frozen and thawed, and then was incubated at 37 C for 30 minutes. An equal volume of water-saturated phenol (Fisher Scientific Co., Pittsburgh, Pa.; certified for chromatography), pH 5.5, containing 0.001M EDTA was added. The mixture was shaken for 30 minutes and then centrifuged. The traces of phenol contaminating the aqueous phase were removed by shaking for 5 minutes with an equal volume of anhydrous ether. Two volumes of absolute ethyl alcohol were added to the aqueous phase after the removal of the ether by N₂ bubbling. The nucleic acid precipitation was completed after 1 hour at 0 C. The precipitate was collected by centrifugation and dissolved in 6 ml of a solution of saline and sodium citrate (SSC) (65). α -Amylase (B grade; Calbiochem., Los Angeles, Calif.) was added to a final concentration of 2 mg per ml. The mixture was incubated for 30 minutes at 37 C. RNase (five times crystallized; Sigma Chem. Co., St. Louis, Mo.) was added to a final concentration of 50 μ g per ml from a 2 mg per ml stock solution which had been previously heated at 80 C for 10 minutes to destroy possible contaminating DNase. After incubation at 37 C for

2 hours, pronase (B grade, Calbiochem.) was added to a final concentration of 50 µg per ml. The mixture was incubated for an additional 2 hours at 37 C. The DNA preparation was dialyzed for 24 hours at 4 C against 2 liters of SSC. DNA concentration was estimated by optical density measurement at 260 mµ (O.D.₂₆₀) with 1 O.D.₂₆₀ unit equivalent to 50 µg per ml.

2. Extraction and purification of total DNA. Mycelial pads were washed with a saline-EDTA solution (0.1M EDTA, 0.15M NaCl). The mycelial pads were frozen with liquid N₂ in a mortar. After the liquid N₂ had boiled off, grinding was carried out for 1 minute until caking of the resulting powder began to occur. More N₂ was added, and grinding was continued until microscopic observation showed that approximately 90% of the filaments had been ruptured. The dry frozen powder was transferred to a long-necked, round-bottom, distillation flask and covered with a solution of saline-EDTA. The procedure for DNA extraction was similar to that described by Cheng and Sueoka (12), but included in addition incubations with α-amylase and pronase as described for mit-DNA preparation. On completion of the incubations, an equal volume of a mixture of isoamyl alcohol, chloroform, and phenol (2:48:50) was added, and shaking was carried out for 30 minutes to remove the enzymes and the other residual proteins. The aqueous phase separated by centrifugation was deproteinized as above. The final clear aqueous solution was precipitated with 2 volumes of absolute ethyl alcohol. The precipitate was collected either by spooling onto a glass stirring rod or by allowing it to settle

after mixing of the aqueous and ethyl alcohol phases. Further purification of DNA was obtained by using the isopropyl alcohol precipitation technique described by Marmur (65).

3. Determination of mole per cent guanine plus cytosine. The method of Meselson, Stahl, and Vinograd (66) was used for the determination of the buoyant density of the DNA. The conversion into mole per cent guanine plus cytosine (%GC) was calculated according to Schildkraut, Marmur, and Doty (88). Sp8 bacteriophage DNA was used as reference. All DNA buoyant densities were related to that of Escherichia coli, which was taken to be 1.710 gm per cm³ (88).
4. Determination of renaturation rate. Nu-DNA and mit-DNA samples (2 ml SSC containing 20 µg per ml DNA) were denatured by incubation at 100 C for 10 minutes and then rapid cooling in ice. The DNA samples were denatured in the same manner for the renaturation studies, but instead of rapidly cooling, they were incubated at 60 C for 2 hours in 2x SSC and then allowed to cool slowly to room temperature.

F. Characterization of ribosomes and ribosomal RNA.

1. Preparation and purification of mit-ribosomes. The purified mitochondrial pellet was resuspended in cold 0.01M Tris, pH 7.5, containing 0.01M MgCl₂, 0.1M NH₄Cl, 20 µg per ml polyvinyl sulfate, and 2% triton x-100 (Ocyly Phenoxy Polyethoxyethanol, Sigma Chem. Co.) (AMTP buffer solution) to give a final concentration of 5 mg of protein per ml of AMTP buffer. The suspension was thoroughly mixed with a syringe at 4 C until the turbid suspension

cleared. The mit-ribosomes were purified by centrifugating 7 ml of lysate over 3 ml of 0.90M sucrose in AMTP buffer for 14 hours at 40,000 rpm in no. 40 rotor of the Spinco model L-2 ultracentrifuge. The ribosomal pellet was washed with a small volume of the AMTP buffer and then resuspended in 2 ml of the buffer solution.

2. Preparation and purification of cyt-ribosomes. The mycelial pads were ground with liquid N₂ as described for the extraction of total DNA, but the dry frozen powder was resuspended in AMTP buffer solution. The homogenate was centrifuged twice at 25,000 x g for 15 minutes to eliminate whole cells, nuclei, mitochondria and other heavy components. The supernatant was centrifuged at 40,000 rpm in no. 40 rotor of the Spinco model L-2 ultracentrifuge for 2 hours at 4 C. The ribosomal pellet was washed with a small volume of AMTP buffer and then resuspended in another small volume of the buffer solution.
3. Extraction of cytoplasmic and mitochondrial r-RNA's. Purified ribosomes were resuspended in 0.005M Tris, pH 7.5, containing 0.01M NaCl and 20 µg per ml polyvinyl sulfate (TNP buffer) and mixed with an equal volume of 4% SDS in TNP buffer. The mixture was incubated at 37 C for 10 seconds and then chilled in ice. The chilled material was overlaid on sucrose density gradients for the RNA sedimentation analyses.
4. Sucrose density gradient centrifugation. The sedimentation pattern of cyt- and mit-ribosomes was determined by centrifugation on a sucrose density gradient. Ribosomes suspended in AMTP

buffer were layered directly on a 5 to 20% sucrose-AMTP buffer linear gradient; 0.2 ml of the ribosomal suspension containing from 300 to 400 μ g RNA per ml was overlaid per 5 ml gradient. Fig. 12 shows the sedimentation pattern of M. rouxii cyt-ribosomes in linear (5 to 20% w/v) sucrose density gradient after centrifugation at 50,000 rpm in SW-50L rotor at 4 C for different lengths of time. Clearly, the optimum centrifugation time was 30 minutes. This duration was adopted for the centrifugation of both cyt- and mit-ribosomes. The bottom of the centrifuge tubes was pierced and the gradients were fractionated into 0.2 ml fractions. The absorbance at 260 m μ (O.D.₂₆₀) of each fraction was measured and a sedimentation profile was obtained by plotting O.D.₂₆₀ versus fraction number.

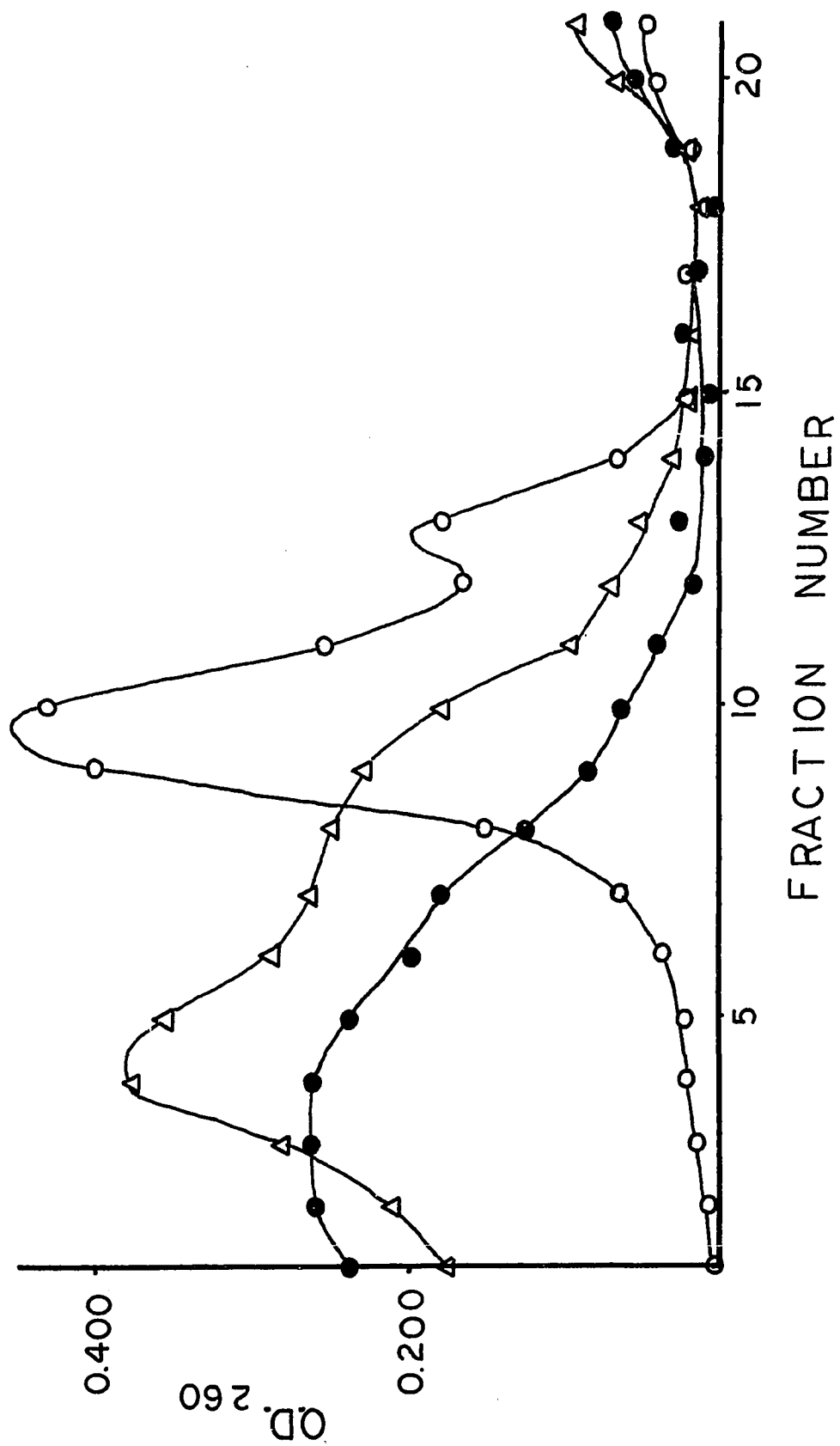
5. Ultracentrifugation of cyt- and mit-ribosomes. Three concentrations of washed ribosomes suspended in AMTP buffer were centrifuged at 20 C in a Spinco model E analytical ultracentrifuge equipped with Schlieren optics. The sedimentation coefficients expressed in Svedberg units (S) were obtained graphically by extrapolating to infinite dilution the linear relation of S versus concentration (87).

G. Nucleotide composition of cyt- and mit-RNA's

1. Fractionation of cell extracts. The procedure for the preparation of the mitochondria is given on page 23. The microsomal fraction obtained after pelleting the mitochondria (13,000 x g for 25 minutes) was used as the source of cyt-RNA. The microsomal fraction was repeatedly centrifuged at 13,000 x g for 25

Figure 12

Sucrose density gradient sedimentation profiles of M. rouxii cyt-ribosomes. The ribosomes (0.2 ml AMTP buffer containing an equivalent of 0.377 mg RNA) were layered on a 5 ml linear sucrose gradient (5 to 20% sucrose in AMTP buffer) and centrifuged at 50,000 rpm for various time intervals at 4 C in SW-50L rotor in a Spinco L-2 ultracentrifuge. ○——○ centrifugation for 30 minutes; △——△ centrifugation for 45 minutes; ●——● centrifugation for 60 minutes.



minutes to insure the removal of any remaining mitochondria.

2. RNA hydrolysis. The mitochondrial and cytoplasmic fractions were precipitated at 4 C with 0.5N perchloric acid (PCA), centrifuged and the supernatant fraction discarded. The pellets were washed with cold 0.5N PCA and centrifuged again. The pellets, free from cold PCA soluble material, were dissolved in 0.3N KOH and hydrolyzed at 37 C for 17 hours. The unhydrolyzed material was precipitated with 0.5N PCA and centrifuged. The supernatant fraction was brought to pH 10.0 with KOH and chromatographed.
3. Nucleotide separation by column chromatography (20). The nucleotides in the hydrolyzate (the equivalent of 3 to 5 mg RNA) were adsorbed on washed Dowex 1-X8, 200-400 mesh (J.T. Baker Chem. Co., Phillipsburg, N.J.) in the formate form. The column was 5 cm in length and 1 cm in diameter. The elution was performed as described by Henney and Storck (43). Impurities, cytidylic acid, adenylic acid, uridylic acid, and guanylic acid were eluted in that order by the stepwise addition of water, 0.005N, 0.025N, and 0.1N formic acid, 0.5N NH_4 formate-0.1N formic acid, respectively. The 0.D.₂₆₀ and 0.D.₂₈₀ of the effluent were recorded on a Gilford Multiple Sample Absorbance Recorder (Gilford Instruments, Oberlin, Ohio). The amount of nucleotide present in each peak was determined by integrating the area of the peak with a planimeter (101). The nucleotide content of each peak was also determined by recording the 0.D.₂₆₀ of the total effluent corresponding to the peak as described by Henney and Storck (43).

H. Incorporation of ^3H -uridine into cyt- and mit-RNA's during conversion.

Yeast-like cells (600 ml cultures) grown anaerobically, as described on page 15, were harvested after 16 hours and transferred to 600 ml of BGA derepressing medium containing 2.5×10^{-5} M uridine and 1 μ C per ml 3 H-uridine. The aerobic cultures in derepressing medium were incubated at 27 C and stirred with a magnetic rod. At different time intervals after exposure to air (30 minutes, 3 hours, and 6 hours), 200 ml aliquots of the culture were filtered through millipore membranes. The cells were washed with 10 ml sterile water and resuspended in a small volume of TM buffer in a cold mortar. The grinding of the cells and preparation of mitochondria were done as described on page 30. The 13,000 x g supernatant fraction, free of mitochondria, was used as the source of cyt-RNA. The prepared mitochondrial pellets and aliquots of the 13,000 x g supernatant fractions were precipitated at 4 C in 10% trichloroacetic acid (TCA). The specific activity of RNA in the TCA precipitable material was determined essentially by the procedure of Nomura, Hall, and Spiegelman (75). The TCA precipitates were sedimented by centrifugation at 5,000 rpm for 15 minutes in a refrigerated Servall RC-2 centrifuge and washed once with 5 ml of cold 10% TCA. The pellets were washed in 3 ml of 0.2M sodium acetate. Pancreatic RNase (0.2 ml of 100 μ C per ml) was added to the resuspended pellets and these were incubated at 37 C for 30 minutes with occasional stirring. The suspensions were then chilled in ice and 0.15 ml of 5M PCA (perchloric acid) was added. After incubating the suspensions at 4 C for 30 minutes, the precipitates were removed by centrifugation. The O.D.₂₆₀ and radioactivity of the resulting supernatant fractions

containing the RNase solubilized material were determined. A volume of 0.5 ml from each fraction was mixed with 15 ml of Bray's solution (10) in scintillation vials and the radioactivity measured in a Nuclear Chicago "Unilux II" liquid scintillation spectrophotometer. The specific activity of the RNA was expressed as counts per minute (CPM) per O.D.₂₆₀.

I. Respiration studies

1. O_2 uptake. The rate of O_2 uptake in converting cells was measured using the manometric techniques described by Umbreit et al. (112) in a Warburg respirometer equipped with 15 ml double sided-armed vessels. Culture samples (3 ml) were transferred directly to the main compartment of the vessel; the center well contained 0.2 ml of 10% KOH and a filter paper strip in order to absorb the CO_2 . After allowing for equilibration to 27 C, O_2 uptake was measured every 10 minutes for a duration of 40 minutes. The total content of the Warburg flask was chilled and cold TCA was added to a final concentration of 10%. The TCA precipitable material was used for the determination of the protein content in the 3 ml of culture. The O_2 uptake was calculated with the interval uptake method described by Umbreit et al. (112). The O_2 uptake was expressed as $\mu l O_2$ per hour per mg protein.
2. Induction of cytochrome c oxidase activity. The cytochrome c oxidase activity in converting cells was determined every 1 or 2 hours for the first 9 or 10 hours under various conditions: repression, derepression, presence of CAP (4 mg per ml), and presence of cycloheximide (200 μg per ml). The cytochrome c oxidase

assay was done essentially as described by Haidle and Storck (41). At designated time intervals, 50 ml samples were harvested by filtration through millipore membranes. The cells were washed with 10 ml of sterile water, and then ground with 6 gm of sea sand for 2 minutes in a cold mortar. The ground material was suspended in 3 ml of cold 0.1M phosphate buffer, pH 7.0, and the resulting homogenate was filtered through a double layer of Kimwipe tissue on a Buchner funnel with gentle suction. After removing the sand by filtration, the filtrate was spun at 2,000 rpm for 5 minutes at 4 C; the resulting supernatant fraction was used for the enzyme assay according to the procedure described by Smith (98). The protein content of the supernatant was also determined, and the enzyme activity was expressed as rate constant per mg protein.

3. Cytochrome spectra. The remaining supernatant fraction used to assay the cytochrome c oxidase activity was used in the analyses of cytochromes. The cytochromes in the supernatant fraction were solubilized by adding triton x-100 to a final concentration of 5%. The suspension was homogenized by repeated pipetting with a dispopipette and incubated at 4 C for 4 hours. The suspension was then centrifuged at 11,000 rpm for 30 minutes at 4 C in a Servall RC-2 centrifuge. The clear, yellowish supernatant fraction was scanned in 1-cm light path quartz cuvettes and the absorbance recorded with a Gilford automatic recording spectrophotometer. The difference spectrum was recorded by measuring the optical density of half of the sample reduced with trace

amounts of sodium dithionite, using the other half oxidized with potassium ferricyanide as a blank. When isolated mitochondria were used as the source of cytochromes, the procedure was basically as the one above, and as described by Terenzi and Storck (110).

J. Chemical analyses

1. Protein determination. Protein was determined by the method described by Layne (56) using the Folin-Ciocalteu reagent. Insoluble samples were solubilized with 0.33N NaOH at 100 C for 10 minutes prior to determination. A solution (1 mg per ml) of purified bovine serum albumin (J.T. Baker Chem. Co., Phillipsburg, N.J.) was used as a standard. Optical density at 750 m μ was read against a reagent blank.
2. RNA determination. RNA was determined by the orcinol method described by Schneider (92). A solution (1 mg per ml) of commercial RNA (K and K Laboratories, Inc., Plainview, N.Y.) was used as a standard. Optical density at 660 m μ was read against a reagent blank.
3. DNA determination. DNA was determined by the diphenylamine method described by Schneider (92). A solution of salmon sperm DNA (1 mg per ml) was used as a standard. Optical density at 600 m μ was read against a reagent blank.

K. Photomicrographs were taken on Kodak Plus-X Pan film (Eastman Kodak Co., Rochester, N.Y.) with a Nikon-AMF camera (Dolan Scientific Instruments, Houston, Texas) attached to a Bausch and Lomb microscope (Bausch and Lomb Optical Co., Rochester, N.Y.).

RESULTS

A. Base composition of nucleic acids in mitochondria of several fungi.

1. DNA. Fig. 13 shows a centrifugation profile of N. crassa mit-DNA and contaminating nu-DNA with respective buoyant density of 1.702 gm per cm³ and 1.713 gm per cm³. These values do not differ significantly from those reported by Luck and Reich (62). The bimodal distribution of the DNA preparations shown in Fig. 13 was found when mitochondria had not been preincubated with DNase, provided of course that there was a difference in the buoyant density of mit- and nu-DNA's. After DNase treatment, the DNA molecules were invariably distributed unimodally as shown in Fig. 14 for Ceratocystis ulmi. One should notice the sharpness of the mit-DNA band indicative of homogeneity.

The buoyant density values of nu- and mit-DNA's expressed as ρ_{GC} are listed, respectively, in columns 1 and 2 of Table I. Each value represents an average of at least two determinations of the buoyant density on the same DNA sample; in the case of M. rouxii and N. crassa the values represent an average of two determinations performed on different DNA extracts. To permit a comparison, values obtained from the literature for microbes and animals are listed on Table II.

Contaminating nu-DNA was never observed in preparations, not treated with DNase, from C. echinulata, M. rouxii, and M. fragilis, and the buoyant density was the same for total DNA and mit-DNA preparations. Since it had been reported (21, 90, 93)

Figure 13

Microdensitometer tracing of N. crassa DNA photographed after 20-hour centrifugation at 44,000 rpm on model E analytical ultracentrifuge. The DNA extracted from the mitochondrial suspension not treated with DNase prior to extraction shows contamination of mit-DNA (1.702 gm per cm³) by nu-DNA (1.713 gm per cm³). SP8 bacteriophage (1.743 gm per cm³) DNA used as reference.

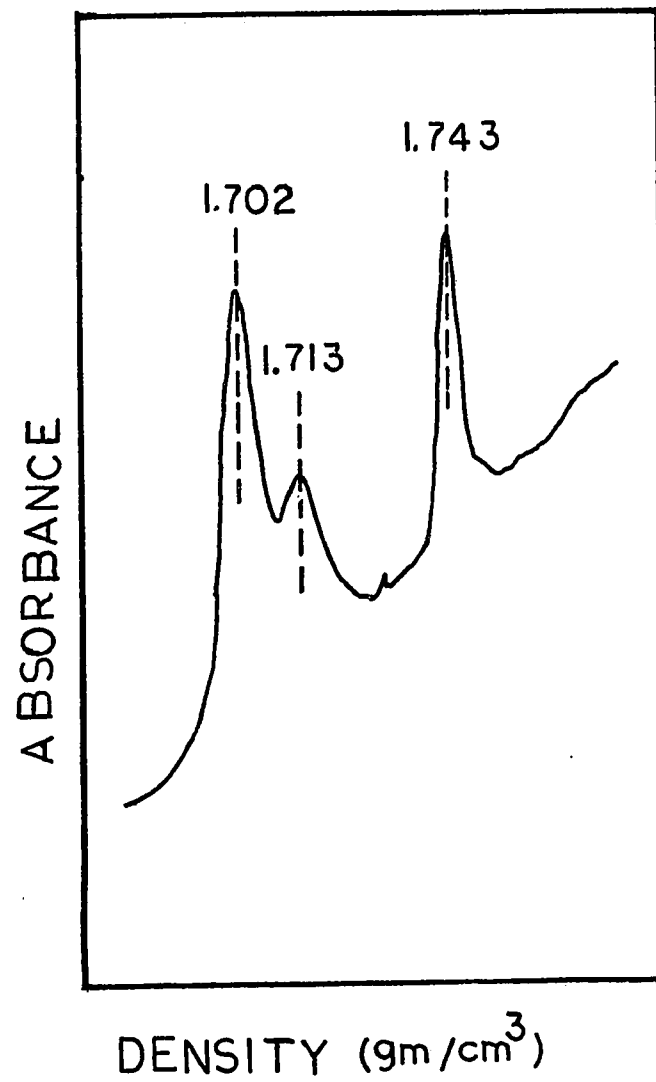


Figure 14

Microdensitometer tracing of C. ulmi DNA photographed after 20-hour centrifugation at 44,000 rpm on model E analytical ultracentrifuge.

The DNA was extracted from a mitochondrial suspension preincubated with DNase. SP8 bacteriophage ($1.743 \text{ gm per cm}^3$) DNA was used as reference.

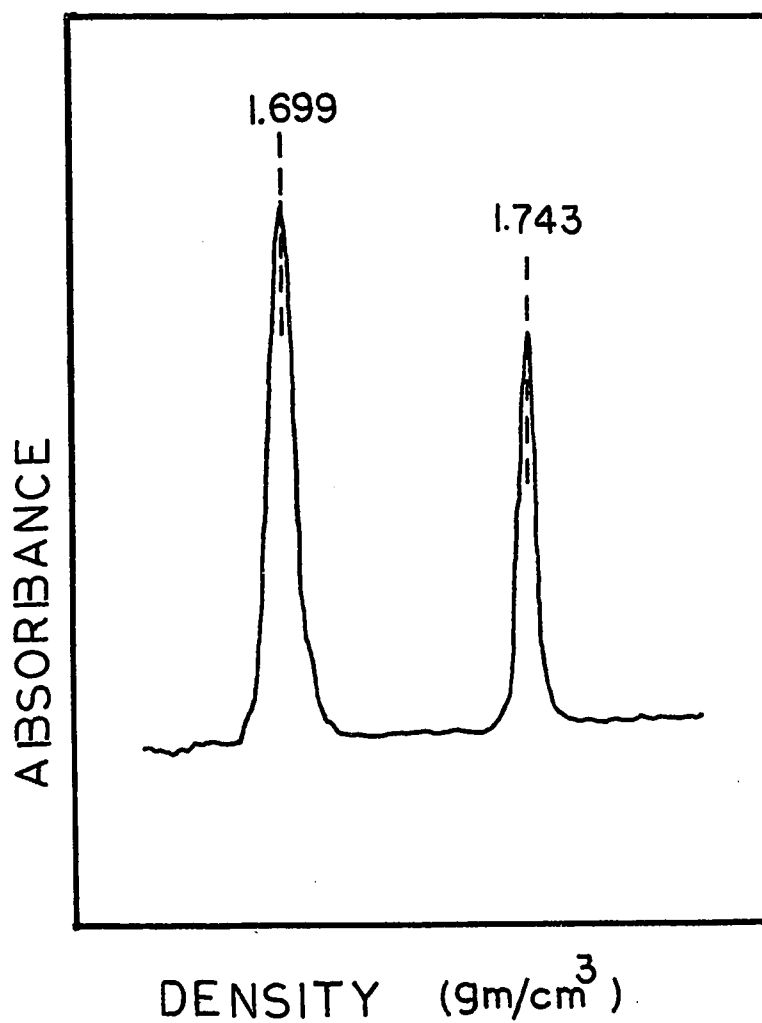


Table I. Guanine plus cytosine content in moles
percent of nuclear and mitochondrial DNA of fungi

Organism	(1) nu-DNA	(2) mit-DNA	(1-2)
ZYGOMYCETES			
<u>Cunninghamella echinulata</u>	34	34	0
<u>Mucor fragilis</u>	39	38	+ 1
<u>Mucor rouxii</u>	37	38	- 1
ASCOMYCETES			
<u>Ceratocystis ulmi</u>	56	40	+ 16
<u>Gelasinospora autosteria</u>	54	41	+ 13
<u>Gelasinospora calospora</u>	55	41	+ 14
<u>Gelasinospora cerealis</u>	55	42	+ 13
<u>Gelasinospora tetrasperma</u>	--	44	--
<u>Neurospora crassa</u>	54	42	+ 12
<u>Neurospora sitophila</u>	55	43	+ 12
<u>Sordaria macrospora</u>	54	42	+ 12
<u>Chaetonium globosum</u>	58	34	+ 24
BASIDIOMYCETES			
<u>Daedalea confragosa</u>	57	31	+ 26
<u>Schizophyllum commune</u>	61	28	+ 33

Table II. Guanine plus cytosine content in moles percent of nuclear and mitochondrial DNA from microorganisms and animals as reported in the literature*

Organism	(1) nu-DNA	(2) mit-DNA	(1-2)	Reference †
<u>Physarum polycephalum</u>	41	27	+14	32
" "	38	27	+11	40
<u>Saccharomyces cerevisiae</u>	38	22	+16	90
" "	41	26	+15	111
wild type	42	28	+14	69
nuclear mutant	42	28	+14	69
" "	42	28	+14	69
mitochondrial mutant	42	36	+ 6	69
" "	40	24	+16	69
<u>S. carlsbergensis</u>	40	24	+16	70
" "	40	25	+15	21
<u>Tetrahymena pyriformis</u> Syngens	26	26	0	102
" "	33	27	+6	102
" "	28	27	+1	102
" "	26	26	0	102
" "	31	25	+6	102
Strain	29	25	+4	102
<u>Paramecium aurelia</u>	30	43	-13	105

Continuation of Table II

<u>Euglena gracilis</u>	48	32	+16	28
<u>Rana pipiens</u>	43	43	0	24
<u>Xenopus</u>	43	45	- 2	24
Chicken liver	39	48	- 9	80
Mouse	40	40	0	93
Rat liver	44	42	+2	90
Guinea pig liver	41	41	0	21
Lamb heart	45	55	-10	51
Beef liver	44	56	-12	9

*When the author gave only buoyant density values, these were converted into %GC using the equation of Schildkraut, et al. (reference 88).

† The reference numbers correspond to those found in the Bibliography.

that mit-DNA renatured more readily than nu-DNA, the DNA preparations obtained from M. rouxii were analyzed for their ease of renaturation (Fig. 15 and 16). A solution of nu-DNA was heated at 100 C for 10 minutes and immersed in an ice bath. After it was chilled, it was mixed with a solution of native nu-DNA, and the mixture was analyzed by CsCl gradient equilibrium centrifugation. As shown in Fig. 15a, two peaks were present at equilibrium. The peak with a buoyant density of $1.714 \text{ gm per cm}^3$ corresponds to denatured nu-DNA and the peak with a buoyant density of $1.698 \text{ gm per cm}^3$ to native nu-DNA. The difference in buoyant density observed ($0.016 \text{ gm per cm}^3$) is close to the expected value of $0.015 \text{ gm per cm}^3$ (88). As shown in Fig. 15b, only one peak with a buoyant density of $1.714 \text{ gm per cm}^3$ was present with heat-denatured nu-DNA alone. A similar profile was observed (Fig. 15c) when the DNA solution in 2x SSC was incubated at 60 C for 2 hours (93) after denaturation at 100 C for 10 minutes. As illustrated in Fig. 16a, heat denaturation of mit-DNA from M. rouxii caused an increase in buoyant density from 1.697 to $1.713 \text{ gm per cm}^3$. However, if the mit-DNA solution in 2 x SSC was incubated at 60 C for 2 hours after heating at 100 C, it yielded a band with a buoyant density of $1.701 \text{ gm per cm}^3$ (Fig. 16b), which is only $0.004 \text{ gm per cm}^3$ higher than the buoyant density of native mit-DNA (Fig. 16c).

2. RNA. The results of the RNA nucleotide composition determinations are shown in Table III. In all instances, mit-RNA had a %GC lower than the corresponding cyt-RNA. The range in the difference

Figure 15

Microdensitometer tracings of M. rouxii nu-DNA photographed after 20-hour centrifugation at 44,000 rpm on an analytical ultracentrifuge. (a) A mixture of heat-denatured nu-DNA ($1.714 \text{ gm per cm}^3$) and native DNA ($1.698 \text{ gm per cm}^3$); (b) heat-denatured nu-DNA without mixing with native nu-DNA; (c) heat-denatured nu-DNA after 2 hour incubation at 60 C in 2 x SSC. The buoyant density, $1.714 \text{ gm per cm}^3$, is the same as in (b). DNA from SP8 bacteriophage ($1.743 \text{ gm per cm}^3$) used as reference.

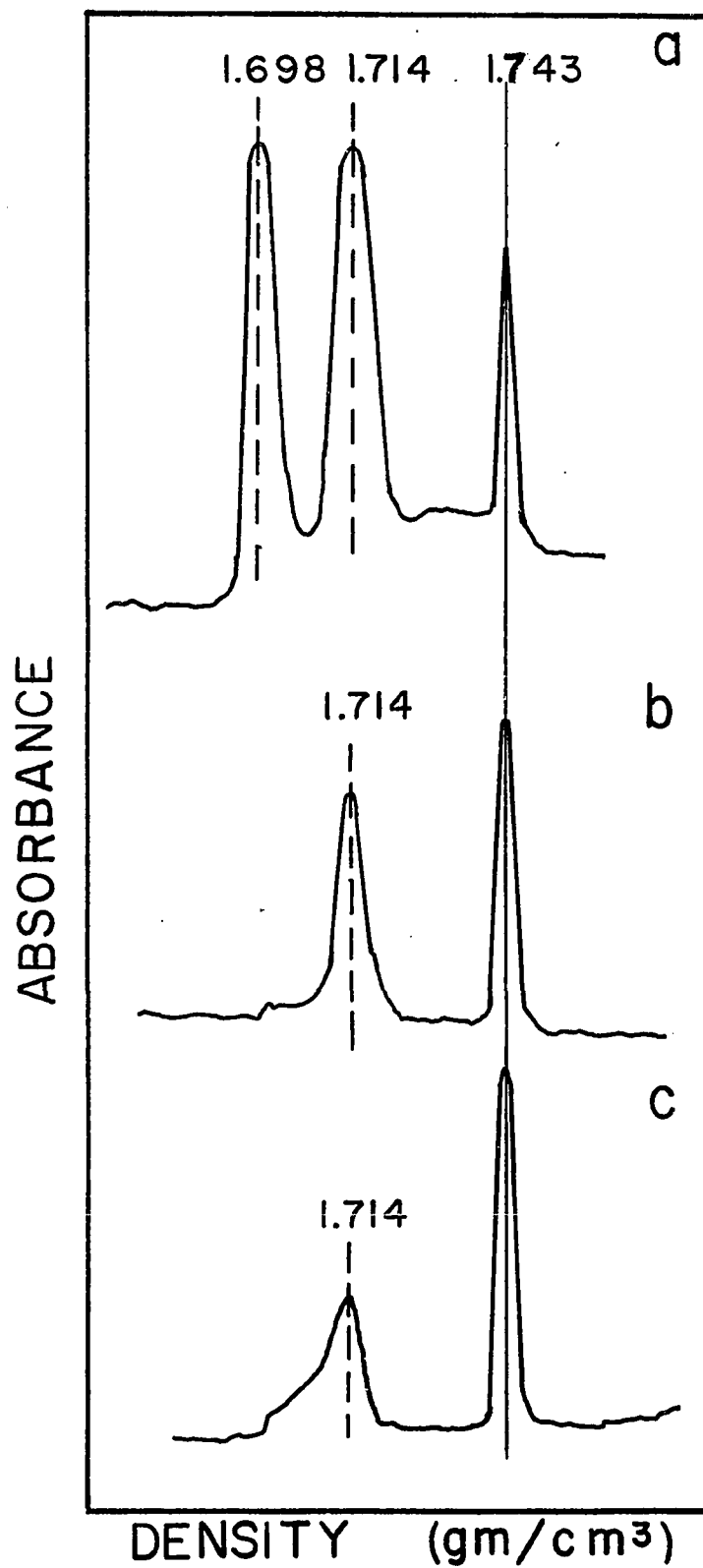


Figure 16

Microdensitometer tracings of M. rouxii mit-DNA photographed after 20-hour centrifugation at 44,000 rpm on an analytical ultracentrifuge. (a) heat-denatured mit-DNA ($1.713 \text{ gm per cm}^3$); (b) mit-DNA ($1.701 \text{ gm per cm}^3$) renatured by incubation at 60 C for 2 hours in 2 x SSC after heating at 100 C for 10 minutes; (c) native mit-DNA ($1.697 \text{ gm per cm}^3$). DNA from SP8 bacteriophage ($1.743 \text{ gm per cm}^3$) was used as a reference.

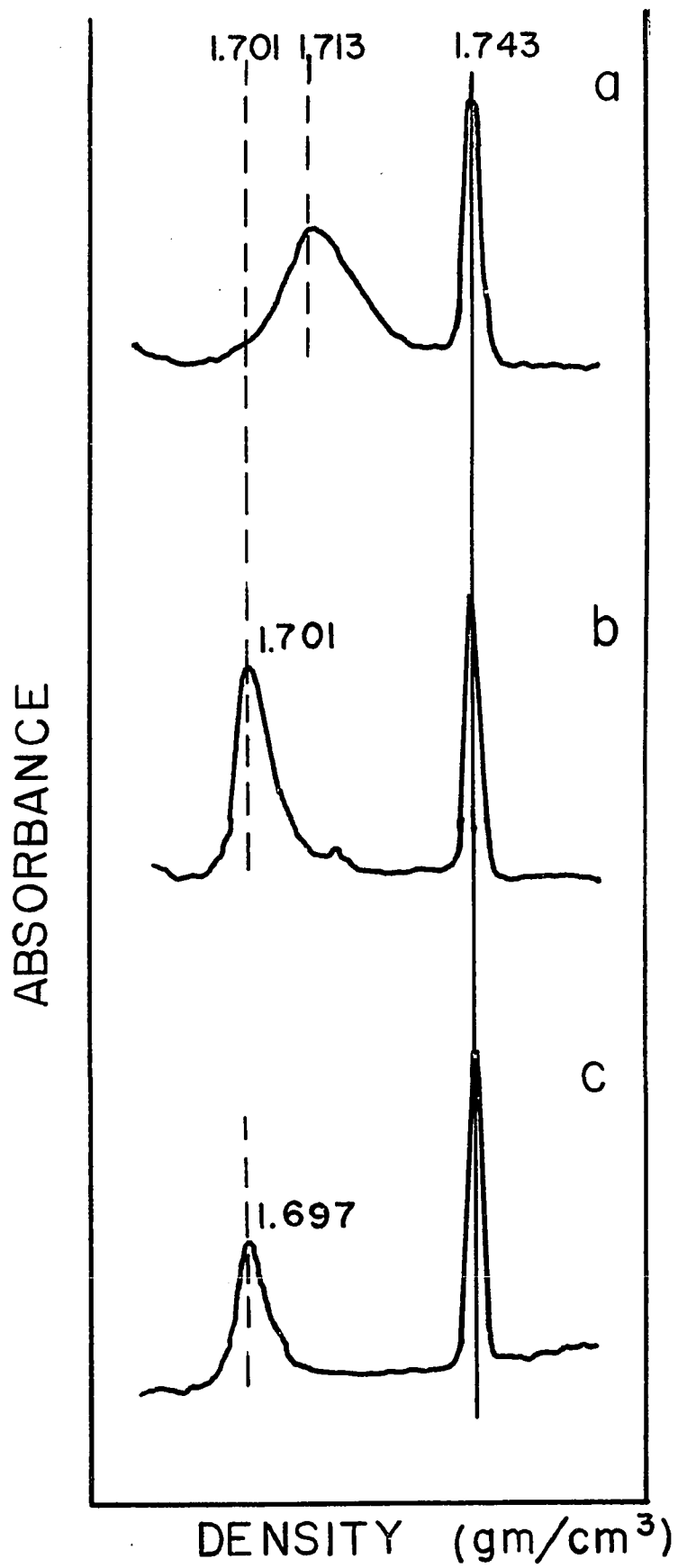


Table III. Nucleotide Composition of Mitochondrial and Cytoplasmic RNA in Fungi

Organisms	Moles per 100 moles of identified nucleotides						
	C	A	U	G	G+C	Pu/Py	6Am/6Ke A+U/G+C
ZYGOMYCETES							
<u>C. echinulata</u>							
mit-RNA	18.4	29.2	26.0	24.9	43.3	1.218	0.935 1.275
cyt-RNA	20.1	26.7	25.3	27.7	47.8	1.198	0.883 1.087
<u>M. rouxii</u>							
mit-RNA	22.0	32.9	28.2	16.9	38.9	0.990	1.220 1.567
cyt-RNA	22.2	26.9	26.3	24.5	46.7	1.060	0.967 1.139
ASCOMYCETES							
<u>C. ulmi</u>							
mit-RNA	22.2	26.2	23.0	28.6	50.8	1.212	0.938 0.968
cyt-RNA	22.6	22.1	23.0	32.4	55.0	1.195	0.807 0.820
<u>C. globosum</u>							
mit-RNA	22.6	26.2	23.5	27.6	50.2	1.167	0.955 0.990
cyt-RNA	23.9	23.7	21.1	29.2	53.1	1.176	0.946 0.844
BASIDIOMYCETES							
<u>S. commune</u>							
mit-RNA	20.3	26.5	23.8	28.6	48.6	1.249	0.893 1.035
cyt-RNA	22.8	26.0	21.7	29.1	51.9	1.238	0.961 0.919

(C, cytidylic acid; A, adenylic acid; U, uridylic acid; G, guanylic acid; Pu, purines; Py, pyrimidines; 6Am, adenylic and cytidylic acids; 6Ke, guanylic and uridylic acids) mit-RNA, mitochondrial RNA; cyt-RNA, cytoplasmic RNA

between the two types of RNA's extended from 7.8% (M. rouxii) to 2.9% (Chaetonium globosum). The values for the cyt-RNA of M. rouxii agree with those obtained by Storck (101).

No attempt can be made on the basis of this limited survey to correlate the nucleotide composition of mit-RNA and mit-DNA or to assess a possible taxonomic or phylogenetic significance to these results. However, one might perhaps note that mit-RNA of the Basidiomycetes and of the two Ascomycetes has a higher %GC than that of Zygomycetes.

- B. Sedimentation of ribosomes and RNA from M. rouxii. The centrifugation profile of freshly isolated M. rouxii mit-ribosomes in a sucrose density gradient is shown in Fig. 17. The fast sedimenting peak corresponds to 78.8S particles while the slow sedimenting major peak corresponds to 45.2S particles. These S values were determined by analytical ultracentrifugation as shown in Fig. 18. The sedimentation coefficient value (S) was obtained by analyzing several concentrations of ribosomes and extrapolating the regression line relating S value and concentration to the infinite dilution intercept. Fig. 18a shows that the Schlieren pattern mimics that in Fig. 17 both in broadness and relative amount of each of the two peaks. Fig. 18b shows the pattern of cyt-ribosomes with a 80.4S peak and a 58.4S peak. From these analyses, it appears that 80S ribosomes are present in mitochondria of M. rouxii. This is consistent with the findings of Rifkin et al. (83) for N. crassa.

The mit-ribosome preparations yielded a larger proportion of subunits than the cyt-ribosomes, and furthermore, the mit-ribosomal

Figure 17

Sucrose density gradient sedimentation profile of M. rouxii mit-ribosomes. The ribosomes (0.2 ml AMTP buffer containing 0.33 mg RNA) were layered on a 5 ml linear sucrose gradient (5% to 20% sucrose in AMTP buffer) and centrifuged at 50,000 rpm for 30 minutes at 4 C in a SW-50L rotor in a Spinco L-2 ultracentrifuge.

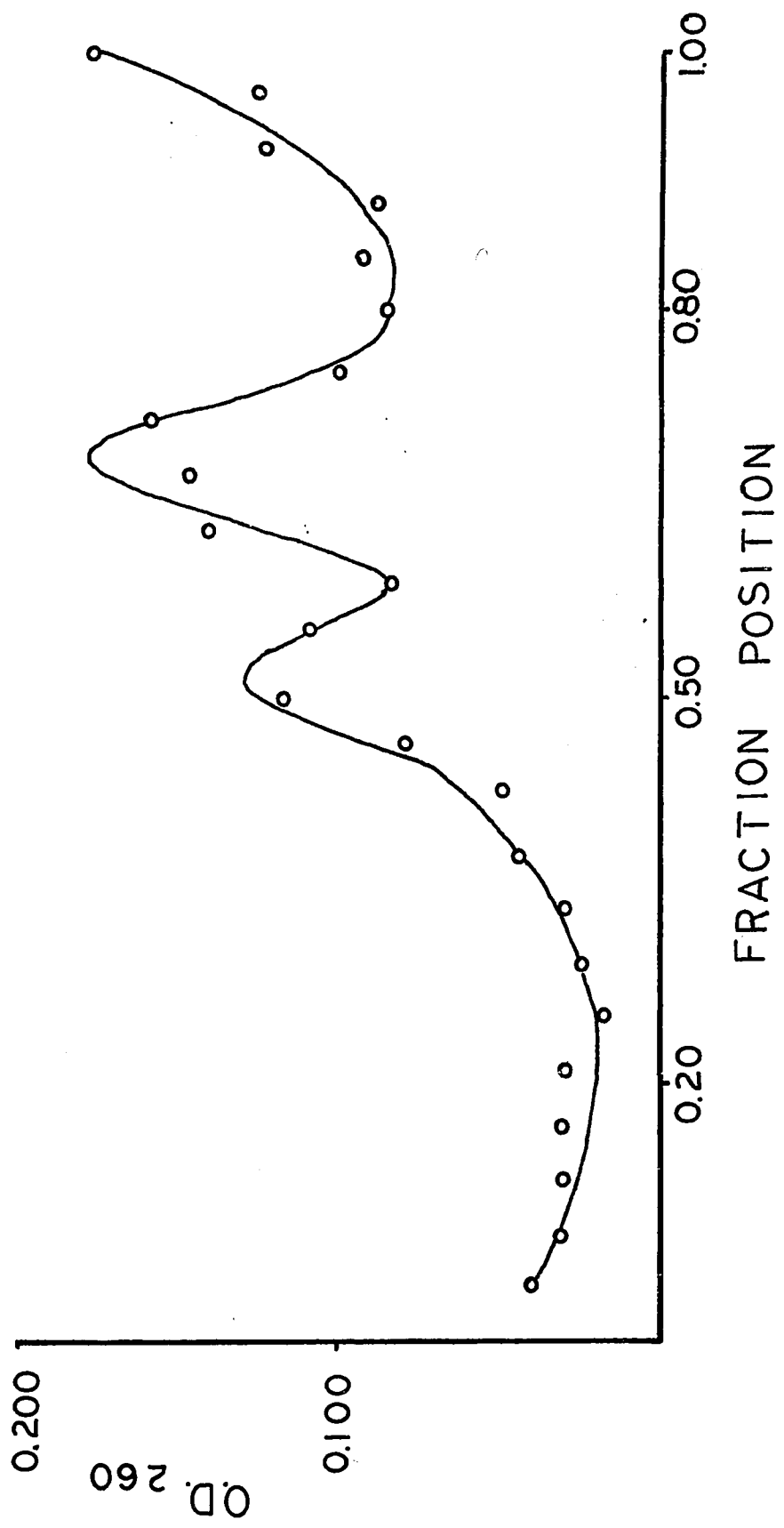
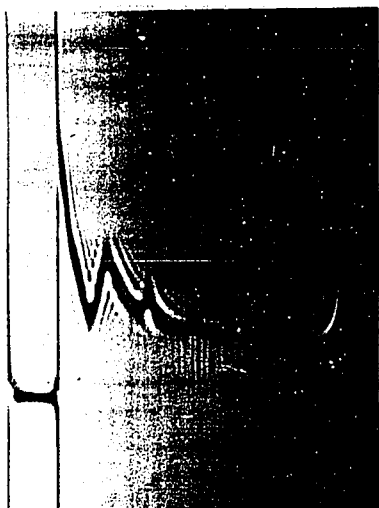


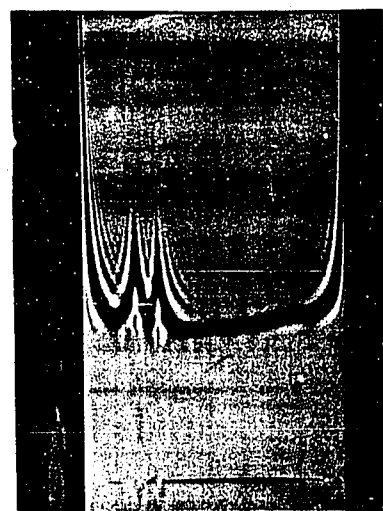
Figure 18

Schlieren profiles of M. rouxii mit- and cyt-ribosomal extracts. The extracts containing 3.5 mg per ml RNA in AMTP buffer were centrifuged at 40,000 rpm and 20 C for 10 minutes. (a) Mit-ribosomes; the small, fast moving peak corresponds to 78.8S ribosomes and the large, broad, slow moving one to 45.2S subunit. (b) Cyt-ribosomes; the fast moving peak corresponds to 80.4S ribosomes and the slow one to 58.4S subunit.

a



b



subunits were smaller (45.2S) than the cyt-ribosomal subunits (58.4S). This situation suggests, in agreement with observations made by others (52, 54, 83), that mit-ribosomes of M. rouxii are more unstable than their cytoplasmic counterparts. This is illustrated by a comparison of Fig. 17 and Fig. 19. Fig. 19 shows the sedimentation pattern for the same mit-ribosome preparation as in Fig. 17, but after storage for two days in the freezer. Fig. 19 shows that the mit-ribosomes present in the profile shown in Fig. 17 had disappeared. This is not the case for the control profile of cyt-ribosomes as shown in Fig. 19. However, as also shown in Fig. 19, if mit- and cyt-ribosome preparations were mixed and centrifuged, the cyt-ribosomes underwent dissociation. This observation suggested that a ribosome degrading factor (s) was present in mitochondrial preparations. In order to test this hypothesis, cyt-ribosomes were diluted with ribosome-free mitochondrial extracts. The sedimentation profiles are shown in Fig. 20, and it appears that a soluble factor present in mitochondrial extracts induces the dissociation of monomeric ribosomes.

Fig. 21 shows the sedimentation profiles of M. rouxii mit- and cyt-ribosomal RNA's alone and in a mixture. As can be seen, the mit-ribosomal RNA profile is devoid of molecules with the highest molecular weight. Of the two peaks, one is located on the light density shoulder of the small r-RNA and the other coincides with soluble RNA (s-RNA). These results clearly indicate a difference in the sedimentation pattern in a sucrose density gradient of cyt- and mit-ribosomal RNA.

Figure 19

Sucrose density gradient sedimentation profiles of M. rouxii cyt- and mit-ribosomes after two days of storage in the freezer. The ribosomes (0.2 ml AMTP buffer containing 0.344 mg RNA) were layered on a 5 ml linear sucrose gradient (5% to 20% sucrose in AMTP buffer) and centrifuged at 50,000 rpm for 30 minutes at 4 C in SW-50L rotor in a Spinco L-2 ultracentrifuge. ●——● cyt-ribosomes; △——△ mixture of cyt- and mit-ribosomes; ○——○ mit-ribosomes.

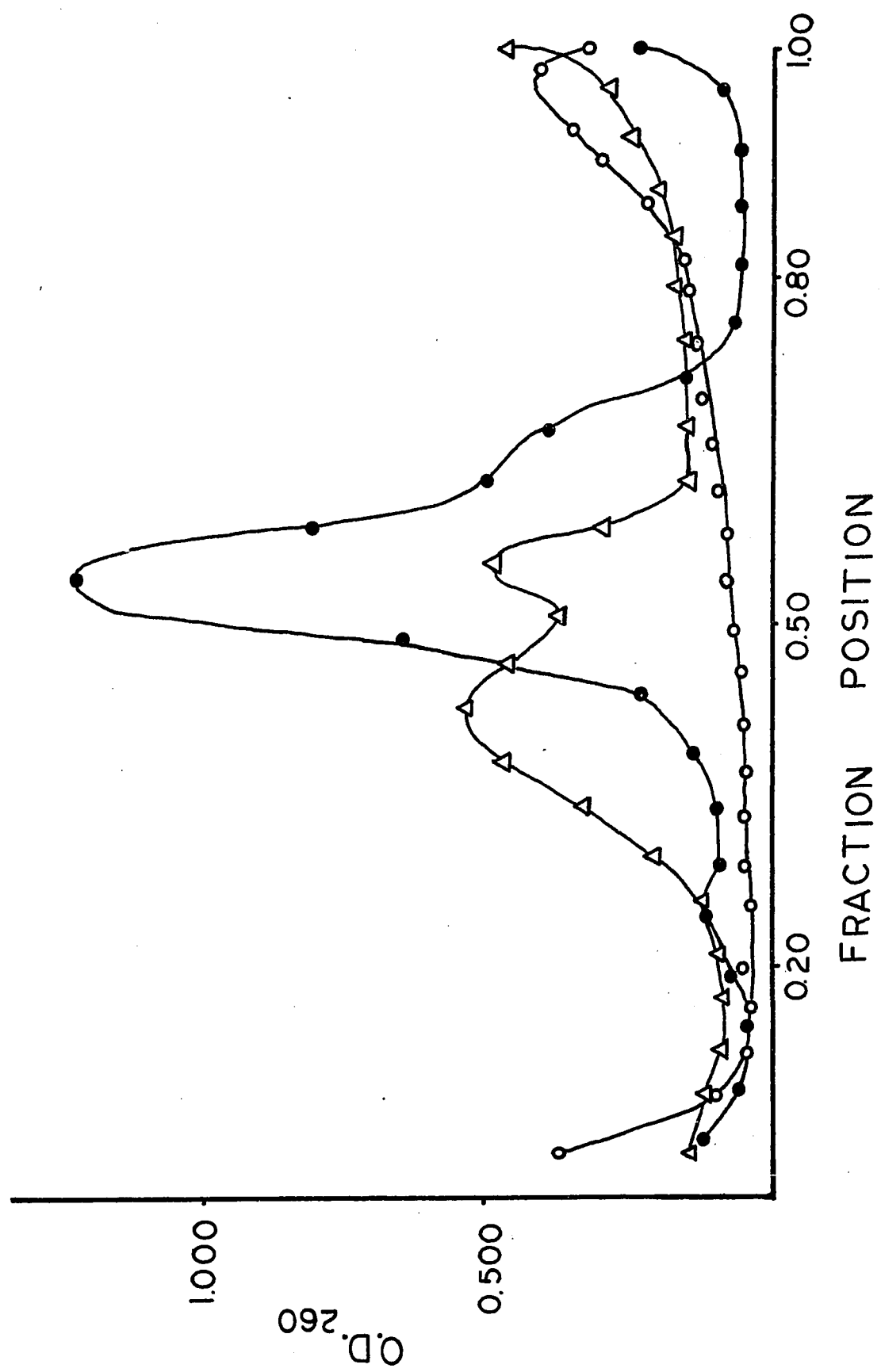


Figure 20

Sucrose density gradient sedimentation profiles of M. rouxii cyt-ribosomes. The ribosomes were layered and centrifuged as described in Fig. 19. ○—○ cyt-ribosomes (0.48 mg RNA) in AMTP buffer; △—△ cyt-ribosomes (0.48 mg RNA) containing supernatant fraction from mit-ribosome extract mixed before layering on the gradient; ●—● cyt-ribosomes (0.48 mg RNA) containing supernatant fraction from mit-ribosome extract, mixed and incubated at 25 C for 15 minutes before layering the material on the gradient.

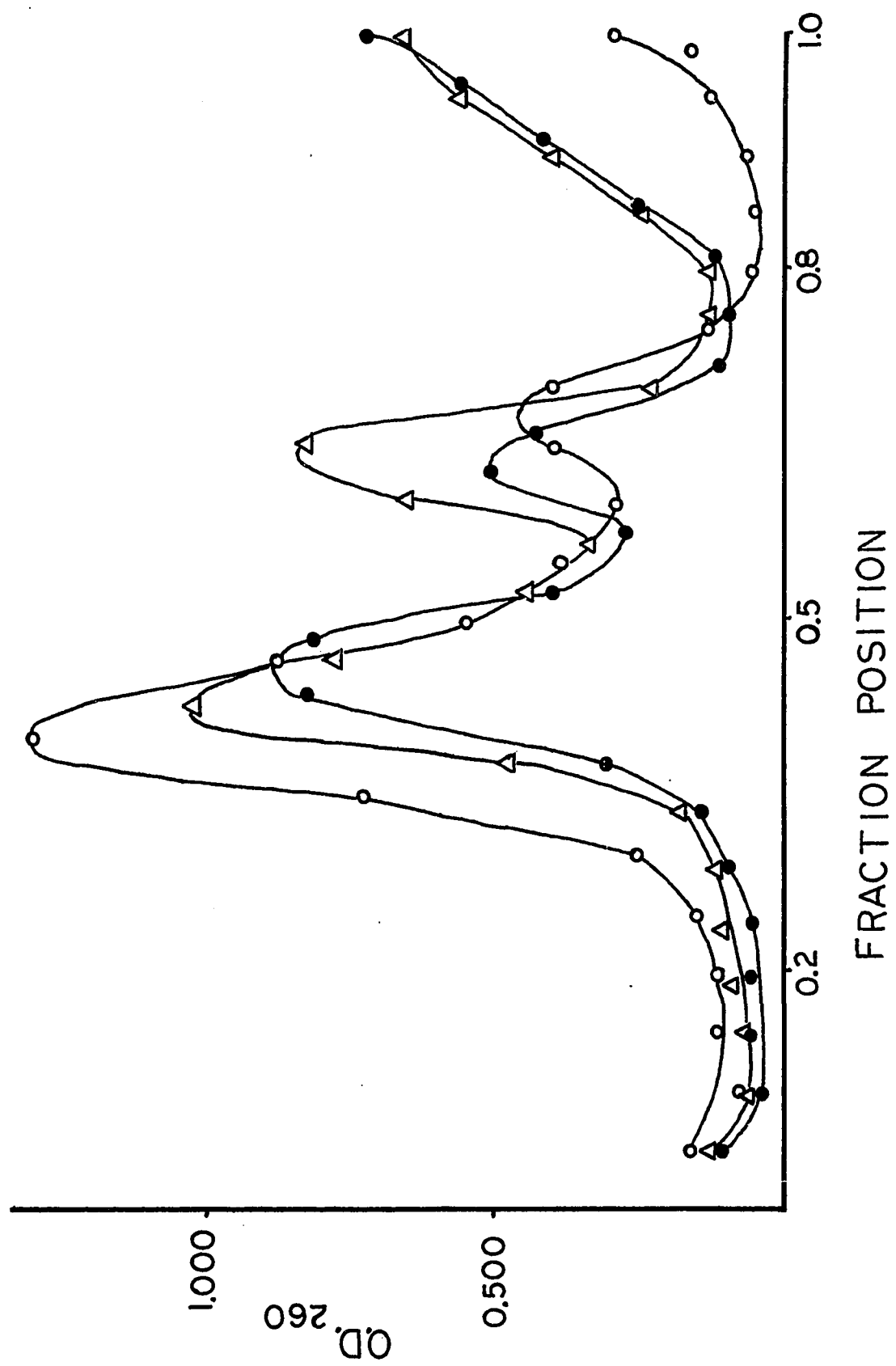
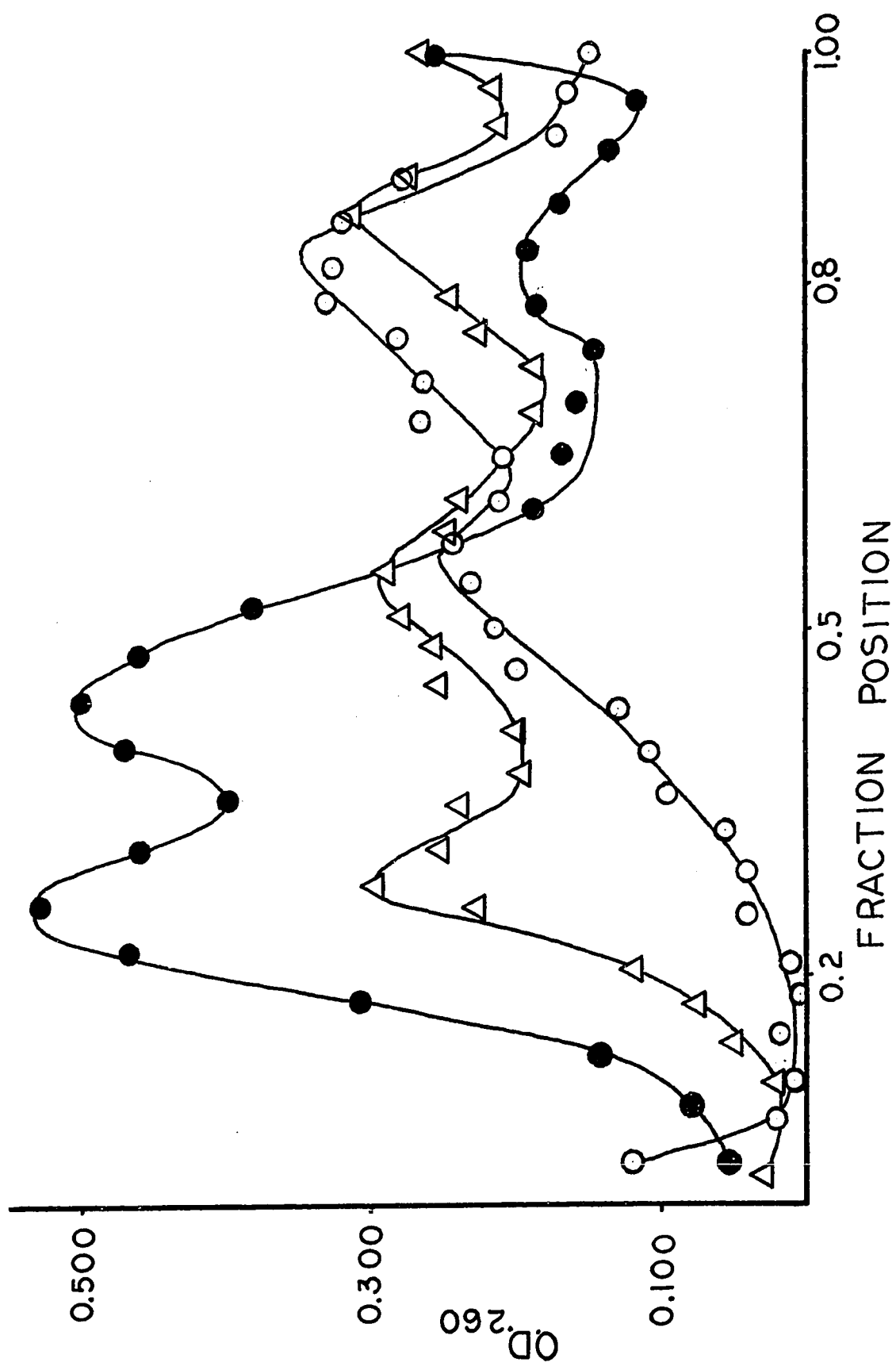


Figure 21

Sucrose density gradient centrifugation profiles of M. rouxii mitochondrial and cytoplasmic r-RNA's. The r-RNA (0.2 ml TNP buffer containing 0.3 mg RNA) was layered on 5 ml linear sucrose gradients (2.5% to 15% sucrose in TNP buffer) and centrifuged at 50,000 rpm for 3 hours at 4 C in SW-50L rotor in a Spinco L-2 ultracentrifuge.

○ — ○ mitochondrial r-RNA; △ — △ mixture of mitochondrial and cytoplasmic r-RNA's; ● — ● cytoplasmic r-RNA.



C. Conversion of yeast-like cells to filaments

1. Effect of glucose concentration on oxygen uptake. Fig. 22 shows that the rate of oxygen uptake (O_2 μ l per hour) was lower when yeast-like cells were exposed to air in medium containing 5% glucose than in one containing either 1% or 0.1%. Fig. 23 shows the increase in protein content for 3 ml samples as used in the Warburg flasks. In Fig. 24 the variation of O_2 uptake per mg protein per hour are shown as a function of time. One can see that increased glucose concentration has a marked inhibitory effect on the specific activity of O_2 uptake. At 8 hours, this uptake was repressed 35% and 60% with 1% and 5% glucose respectively, if the uptake with 0.1% glucose was taken as 100%. The O_2 uptake specific activity reached a peak around 2.5 to 4.5 hours after exposure to air, and then dropped to a constant level for each glucose concentration. The drop in the specific activity coincided with the initiation of germ tube formation shown in Fig. 31. This suggests, as shown in Fig. 22 and 23, that proteins other than respiratory enzymes were being synthesized. Fig. 24 also shows the specific activity of samples poisoned with cyanide after exposure to air for 1 hour and 7.5 hours, respectively. The % inhibition at 7.5 hours was 65% for cells in 5% glucose, 87% for cells in 1% glucose and 95% for cells in 0.1% glucose. In other experiments where the % inhibition by cyanide was determined at several times during a 10 hour incubation, the % inhibition increased with time in both low and high glucose cultures, but was always the highest for the cultures in low

Figure 22

Oxygen uptake (O_2 μ l per hour) of M. rouxii converting cells in BGA medium containing various glucose concentrations. ○——○ 5% glucose; △——△ 1% glucose; □——□ 0.1% glucose; the dotted line represents cyanide poisoned samples for each of the respective glucose concentrations.

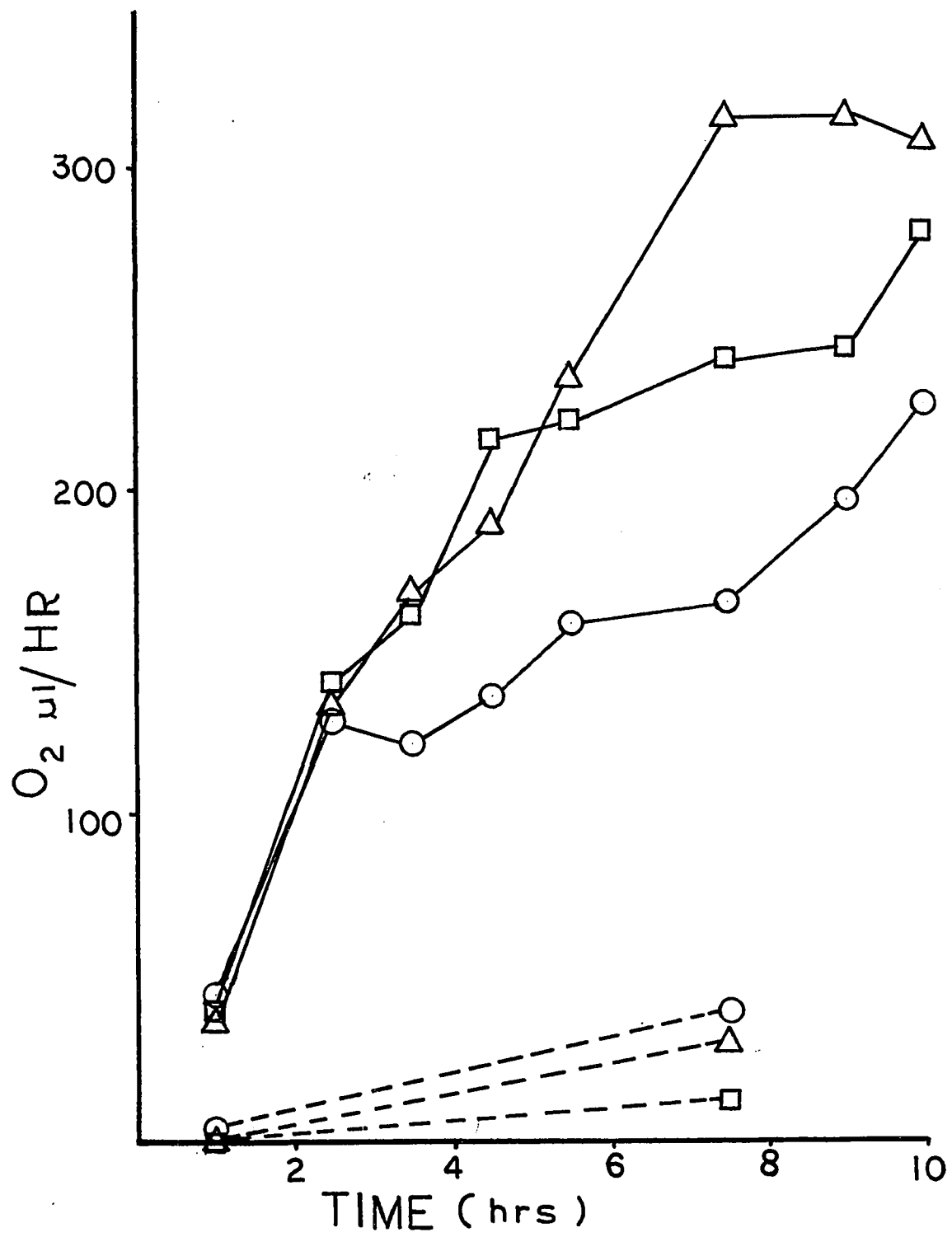


Figure 23

Increase in protein of M. rouxii converting cells (mg protein per 3 ml of cell suspension) in BGA medium containing various glucose concentrations. ○——○ 5% glucose; △——△ 1% glucose; □——□ 0.1% glucose; the dotted line represents cyanide poisoned samples.

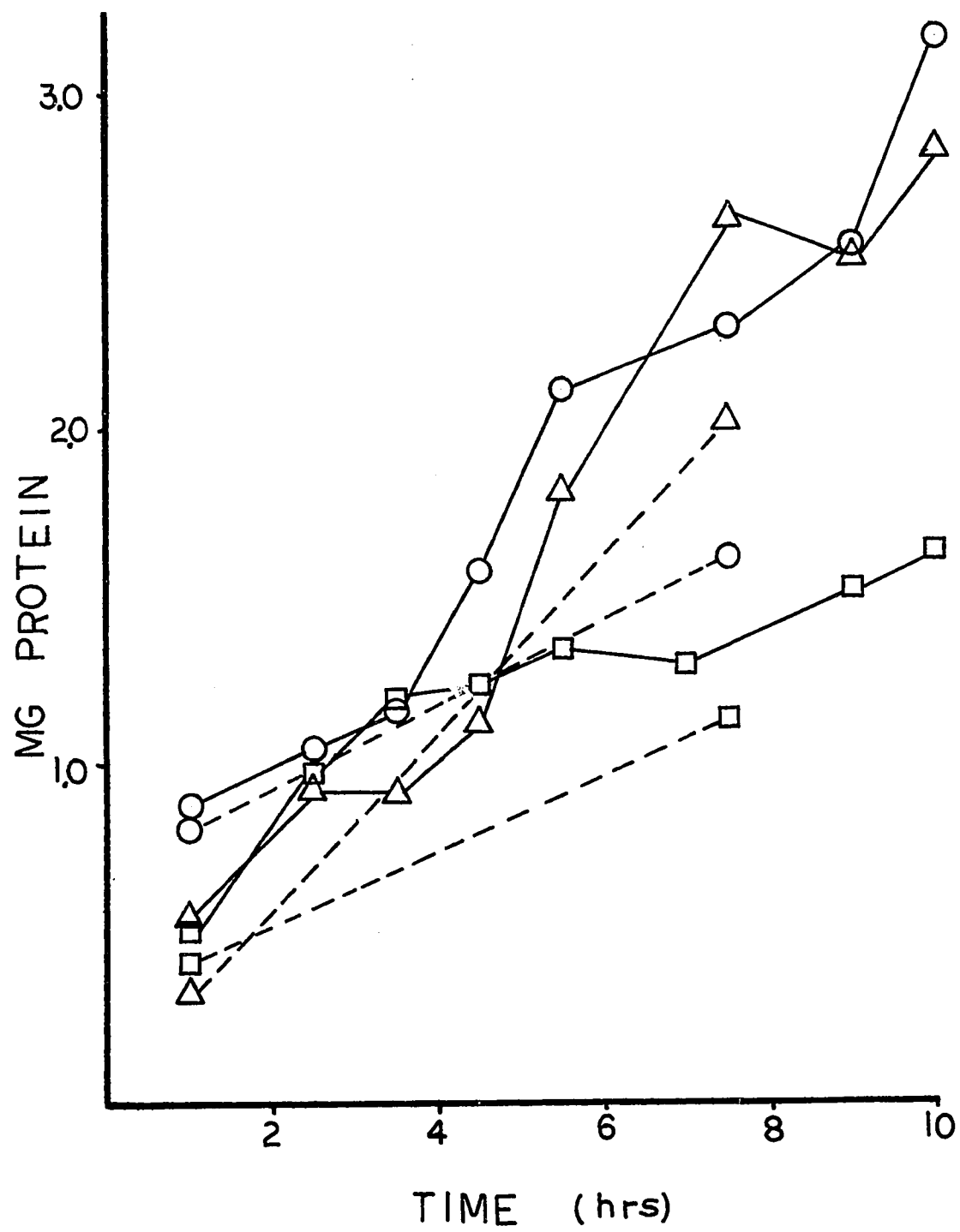
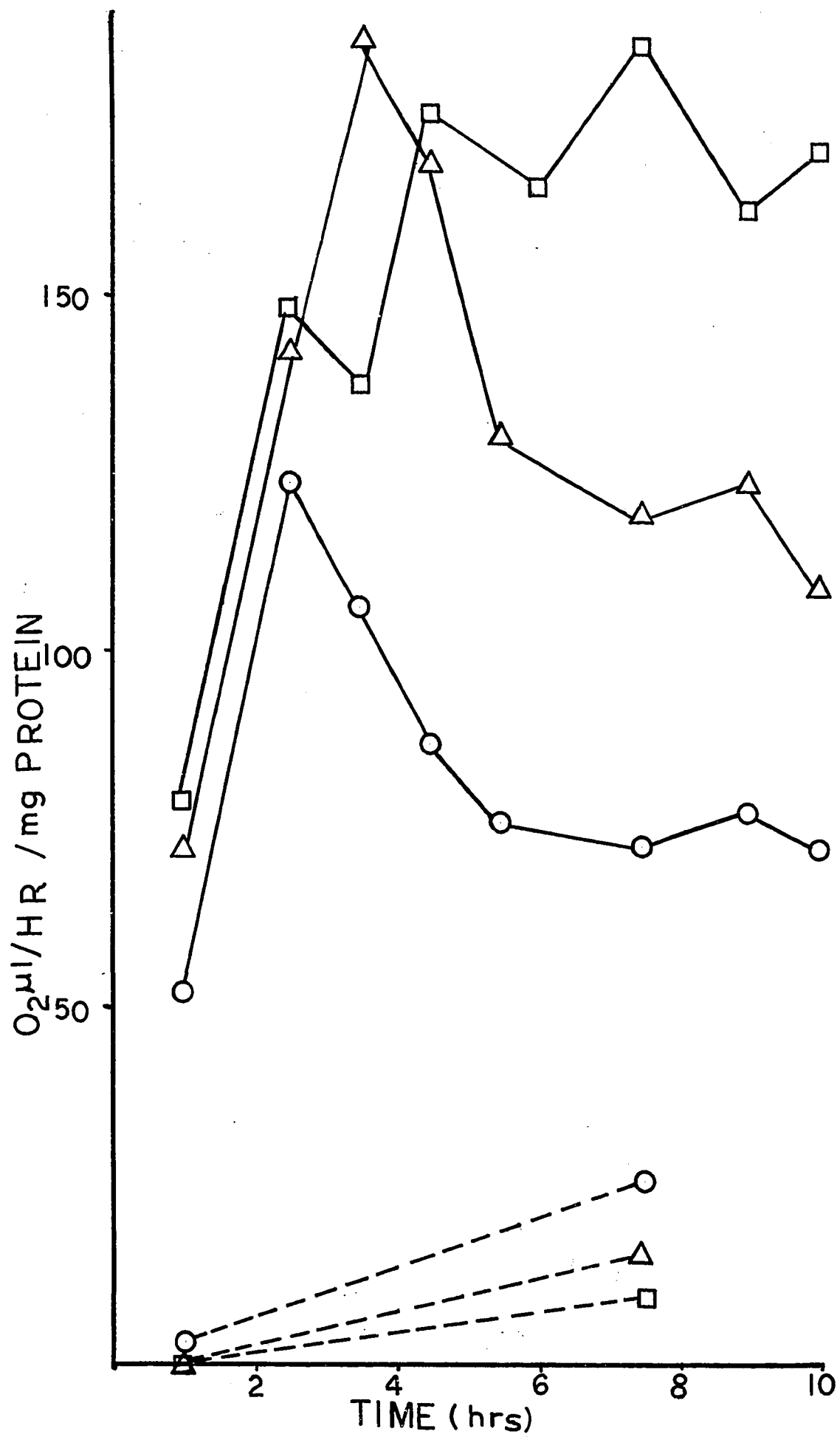


Figure 24

Oxygen uptake per mg protein of M. rouxii converting cells in BGA medium containing various glucose concentrations. ○——○ 5% glucose; △——△ 1% glucose; □——□ 0.1% glucose; the dotted line represents cyanide poisoned samples.



glucose concentrations.

2. Induction of cytochrome c oxidase activity.

Glucose repression. The finding that BGA medium with 5% glucose repressed oxygen uptake suggested that this repression took place at the level of the respiratory enzymes such as cytochrome c oxidase. Fig. 25 shows the specific activity (rate constant per mg protein) of cytochrome c oxidase in converting cells in BGA medium with 5% and 0.5% glucose. With 5% glucose the repression, expressed in % of the activity for cells in 0.5% glucose, was 66% at 4 hours, 47% at 5 hours, 37% at 6 hours, and 37% at 8.5 hours after exposure to air.

Cycloheximide inhibition. As shown in Fig. 26, the specific activity of cytochrome c oxidase in converting cells was completely inhibited by 200 µg per ml of cycloheximide. The % inhibition was 98.7% after 8 hours of contact with air and the drug, but decreased to 87% after 10.5 hours.

CAP inhibition. As shown in Fig. 27, the increase in cytochrome c oxidase activity in converting cells was inhibited by 4 mg per ml CAP. The % inhibition was 98% after 5 hours of contact with air and the drug, and decreased to 85% after 10 hours.

3. Cytochrome spectra.

Glucose repression. As shown in Fig. 28, cytochrome synthesis, like cytochrome c oxidase activity (Fig. 25), was repressed by 5% glucose after 8.5 hours of contact with air.

Cycloheximide inhibition. As shown in Fig. 29, cytochromes synthesis, like cytochrome c oxidase activity (Fig. 26), was

Figure 25

Repression by high glucose concentration of cytochrome c oxidase activity (rate constant per mg protein) induction in M. rouxii converting cells. Δ —— Δ BGA medium with 0.5% glucose; \circ —— \circ BGA medium with 5% glucose.

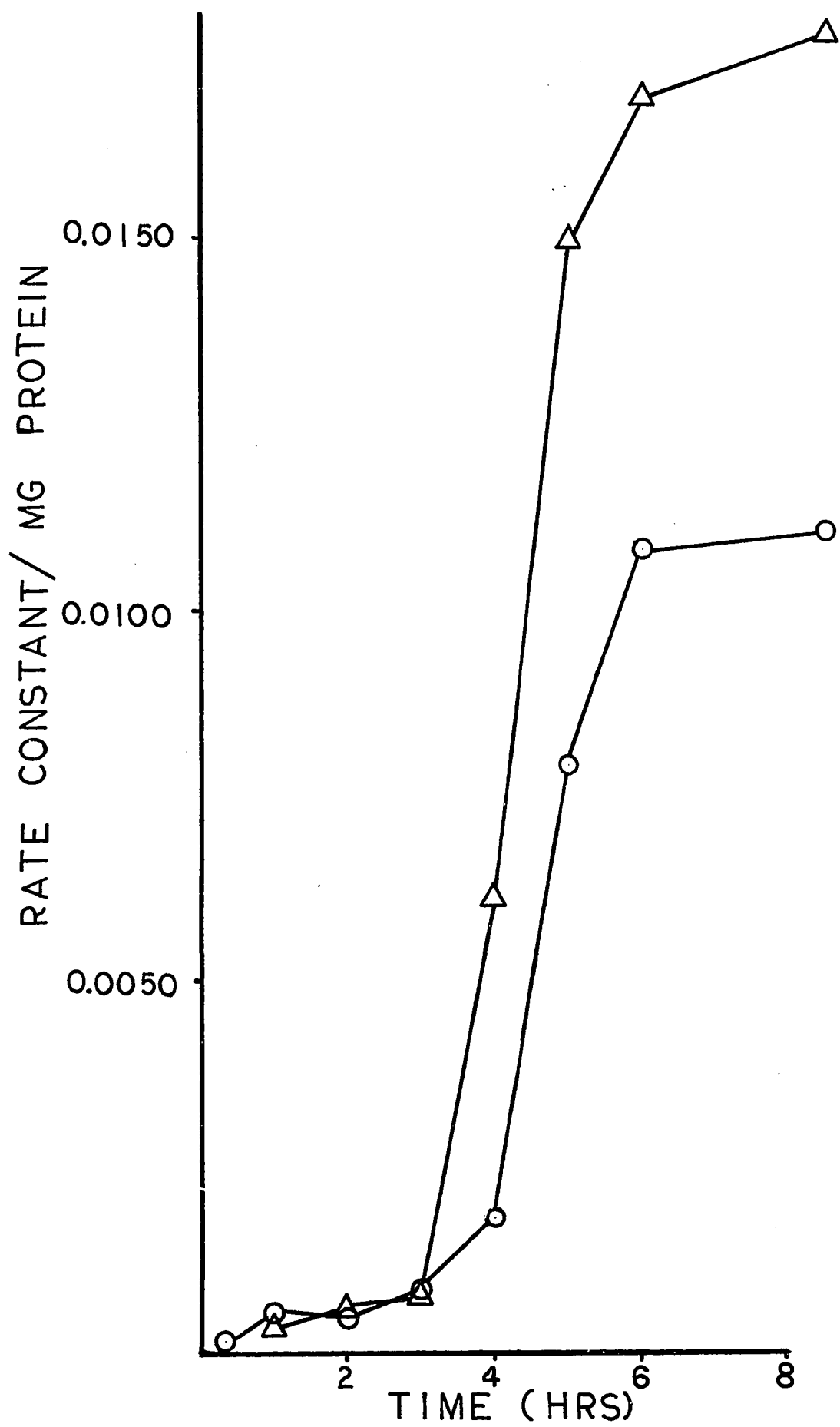


Figure 26

Inhibition by cycloheximide of cytochrome c oxidase activity (rate constant per mg protein) induction in M. rouxii converting cells.

○——○ no cycloheximide present; △——△ cells in contact with 200 µg per ml cycloheximide.

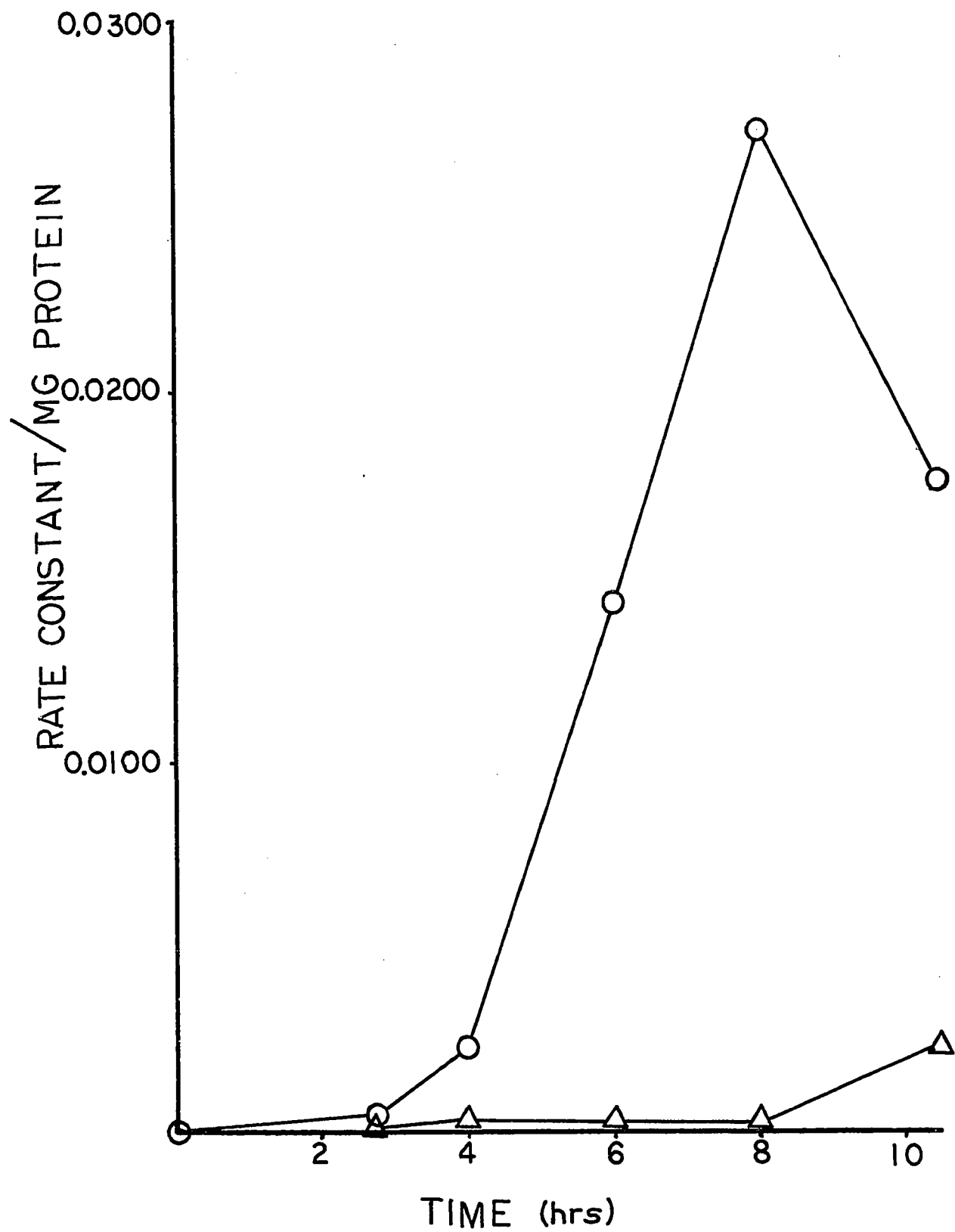


Figure 27

Inhibition by CAP of cytochrome c oxidase activity (rate constant per mg protein) induction in M. rouxii converting cells. ○ — ○ no CAP present; △ — △ cells in contact with 4 mg per ml CAP.

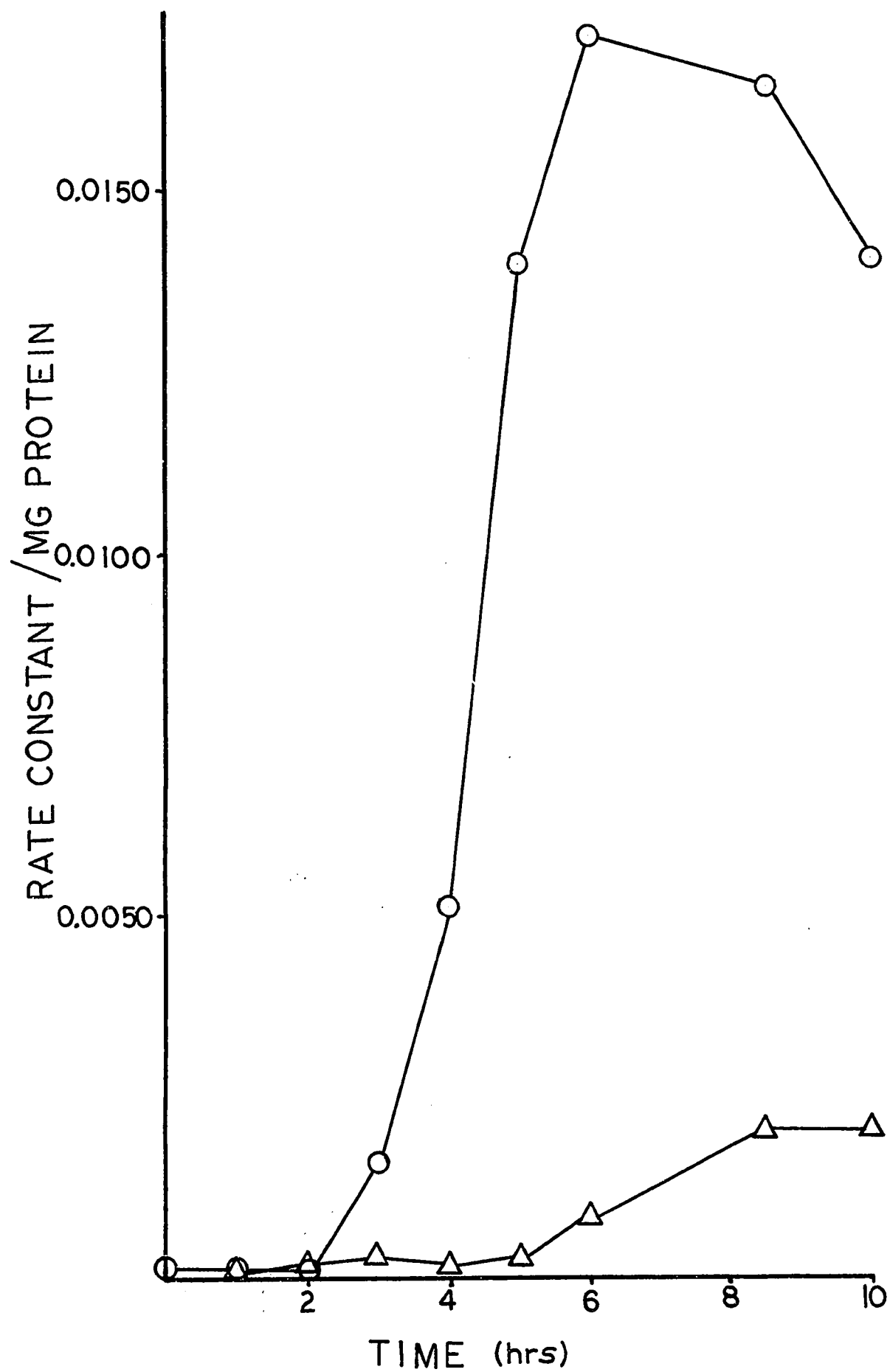


Figure 28

Repression by high glucose concentration of cytochromes synthesis in M. rouxii converting cells. (a) Reduced versus oxidized cytochrome difference spectra of cytochromes in converting cells in BGA medium with 0.5% glucose after 8.5 hours of contact with air; (b) Reduced versus oxidized cytochrome difference spectra of cytochromes in converting cells in BGA medium with 5% glucose after 8.5 hours of contact with air.

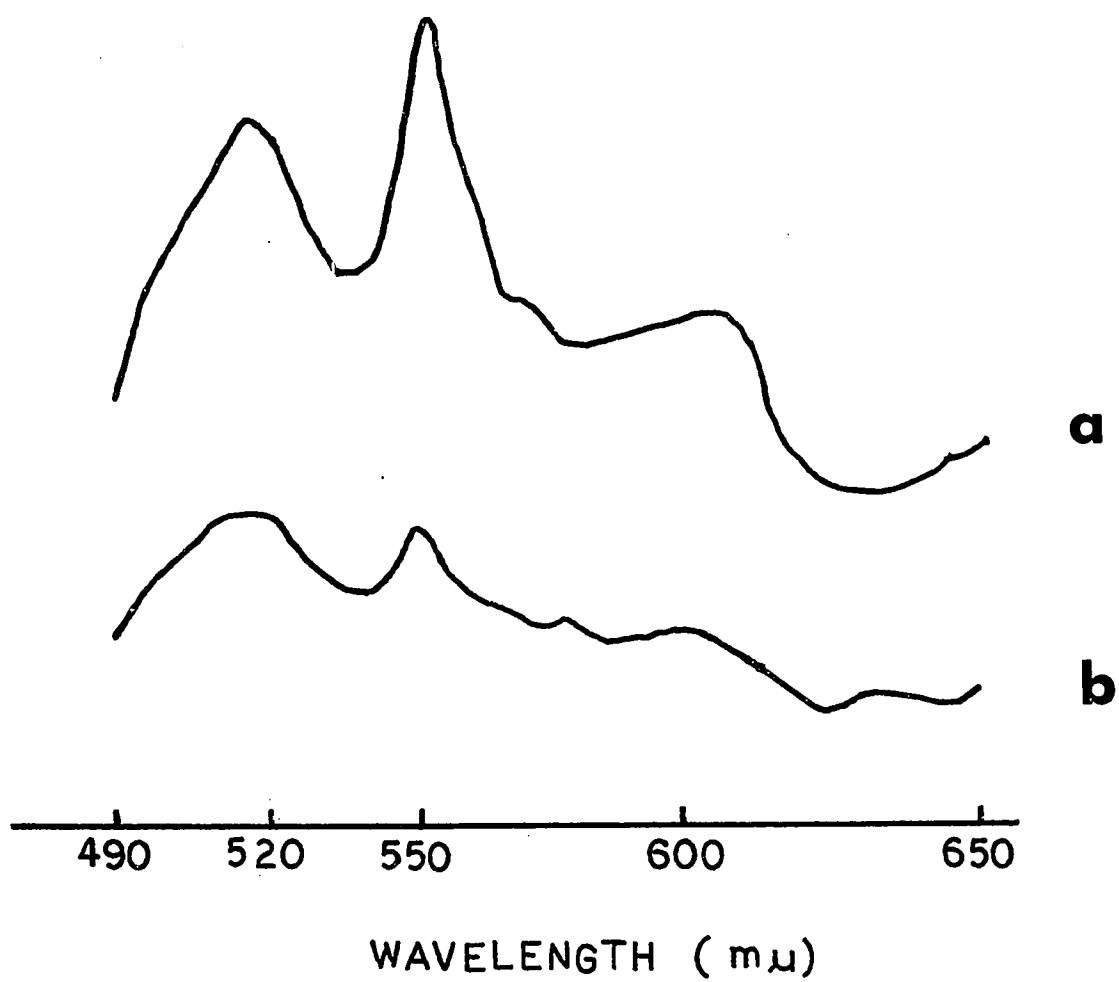
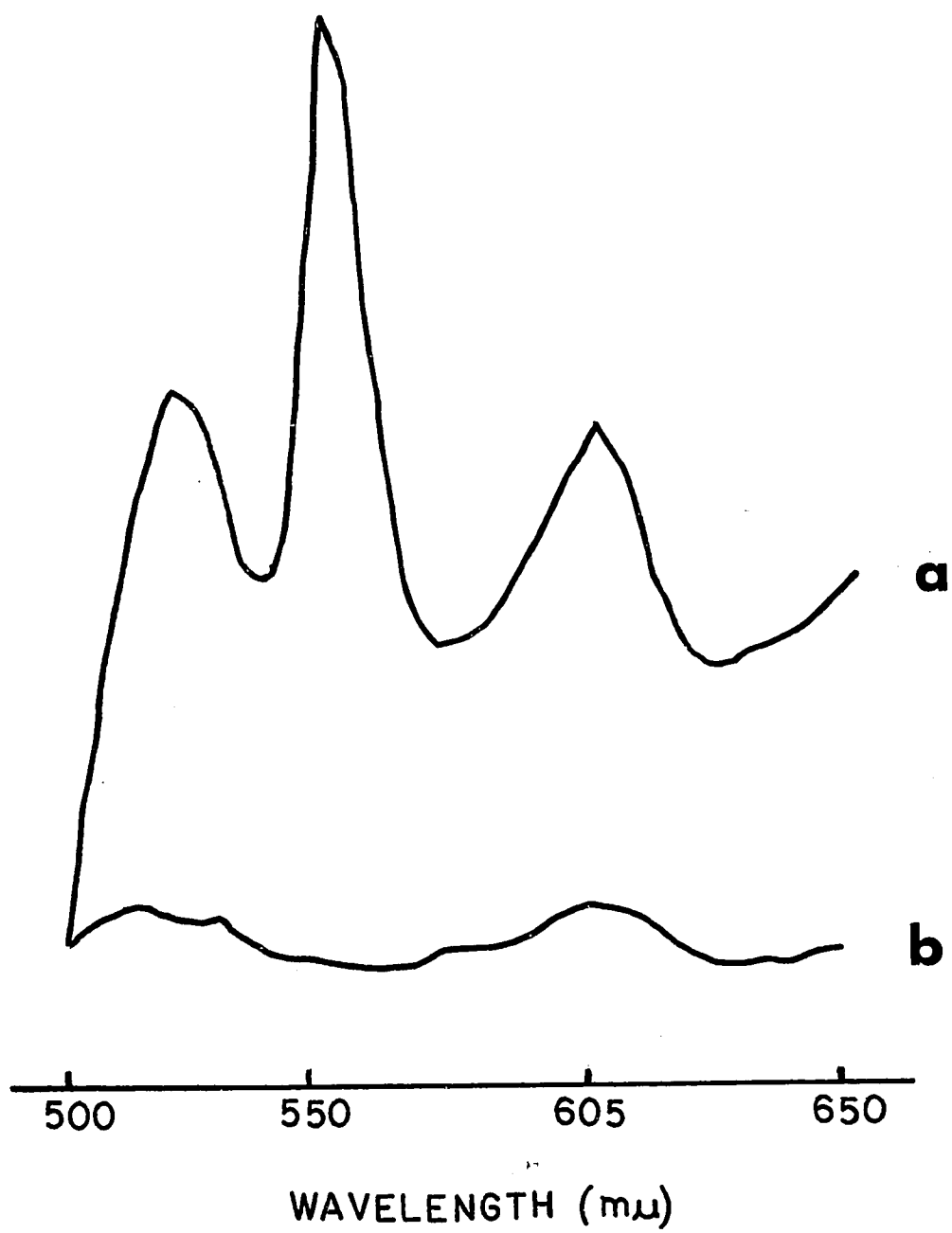


Figure 29

Inhibition by cycloheximide of cytochromes synthesis in M. rouxii converting cells. (a) Reduced versus oxidized cytochrome difference spectra of cytochromes from converting cells in absence of cycloheximide after 9 hours of contact with air; (b) Reduced versus oxidized cytochrome difference spectra of cytochromes from converting cells after 9 hours of contact with air and 200 μg per ml cycloheximide.



inhibited by treatment for 9 hours with 200 μ g per ml cycloheximide.

CAP inhibition. Unlike cycloheximide, CAP at a concentration of 4 mg per ml had a discriminatory effect on the synthesis of some cytochromes. Indeed, as shown in Fig. 30b, only the cytochrome a, a₃ complex (cytochrome c oxidase peak) was drastically reduced; whereas the other cytochromes did not appear to be affected.

4. Morphology.

Glucose effect. There was a gross morphological difference between converting cells incubated in BGA derepressing medium and BGA repressing medium. Fig. 31a shows the typical mixture of yeast-like cells and swollen spores after 16 hours of anaerobic growth. These cells were harvested by filtration, washed with sterile distilled water and resuspended in BGA medium with 1% glucose and in BGA medium with 5% glucose. Germ tube formation started after 2.5 hours, but the time sequence for germ tube elongation was, as shown in Fig. 31b to g, faster in the 1% glucose medium than in the 5% one. The high glucose concentration was responsible for the sparsed branching and the large diameter of the tubes. When cells were incubated in BGA medium containing either 0.5% or 0.1% glucose, the sequence of morphological changes during conversion was the same as with 1% glucose.

Cycloheximide inhibition. Fig. 32 reveals the effect of cycloheximide on the morphology and morphogenesis of converting cells. With 200 μ g per ml cycloheximide, germ tubes did not

Figure 30

Inhibition by CAP of cytochromes synthesis in M. rouxii converting cells. (a) Reduced versus oxidized cytochrome difference spectra of cytochromes from converting cells after 10 hours of contact with air in the absence of CAP; (b) Reduced versus oxidized cytochrome difference spectra of cytochromes from converting cells after 10 hours of contact with air and 4 mg per ml CAP.

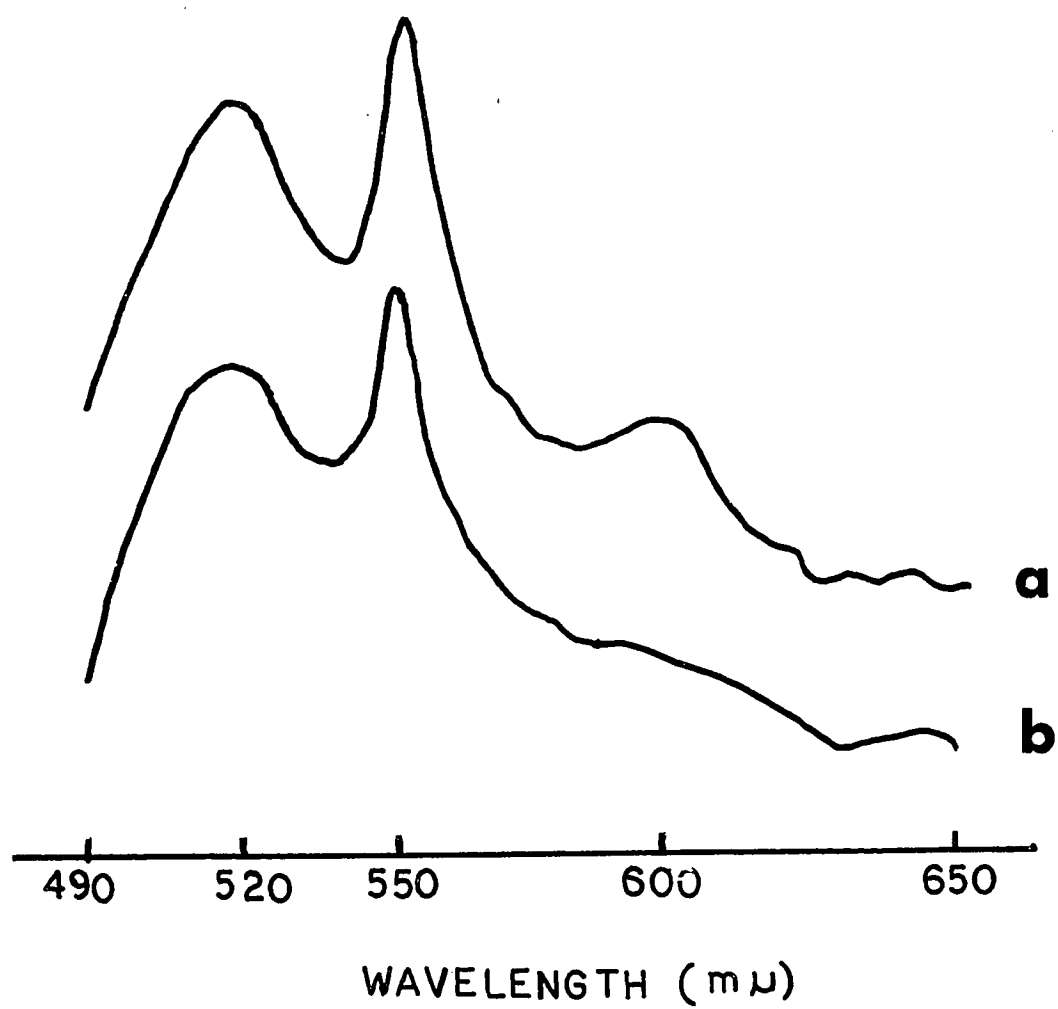
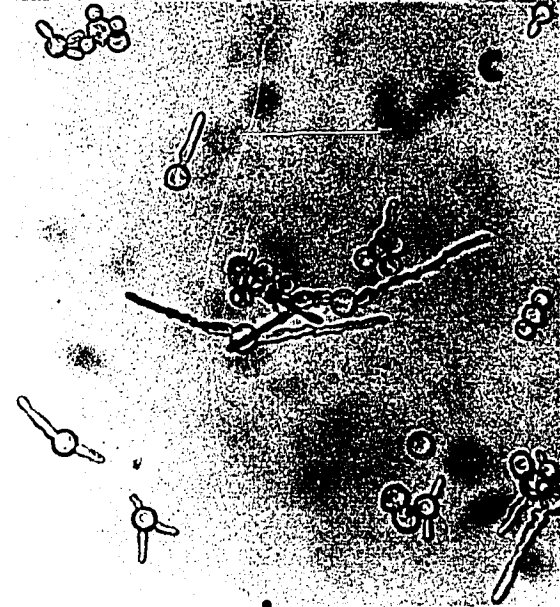
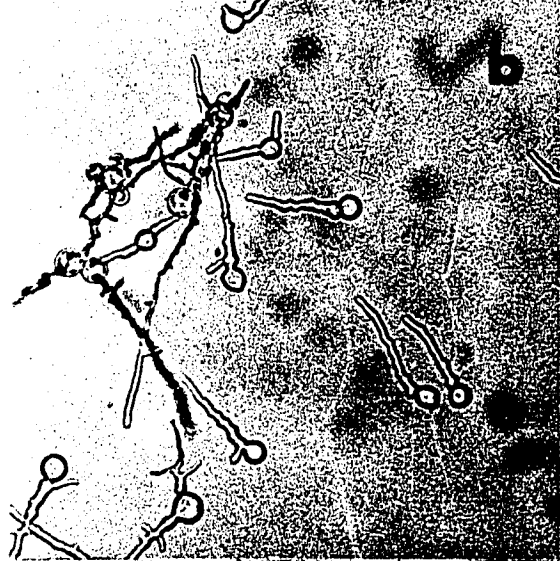
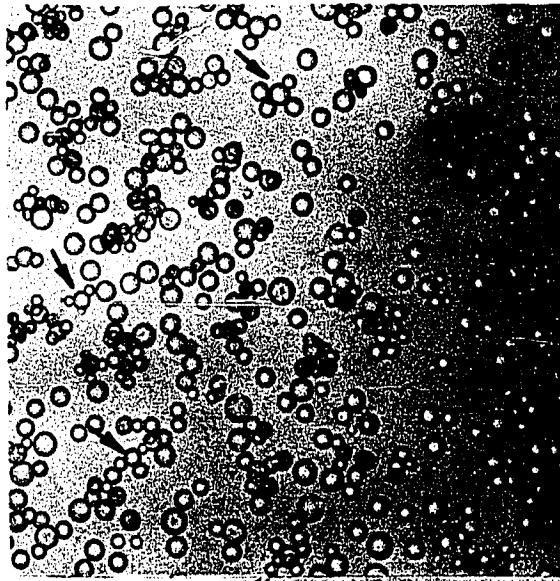


Figure 31

The effect of high glucose concentration on the changes in morphology of M. rouxii during conversion. (a) Cells grown in BGA medium with 5% glucose after 16 hours incubation in anaerobiosis, arrows pointing to typical yeast-like cells; (b) Cells in BGA medium with 1% glucose after 5.5 hours of contact with air; (c) Cells in BGA medium with 5% glucose after 5.5 hours of contact with air; (d) Cells in BGA medium with 1% glucose after 8.5 hours of contact with air; (e) Cells in BGA medium with 5% glucose after 8.5 hours of contact with air; (f) Cells in BGA medium with 1% glucose after 16 hours of contact with air; and (g) Cells in BGA medium with 5% glucose after 16 hours of contact with air.



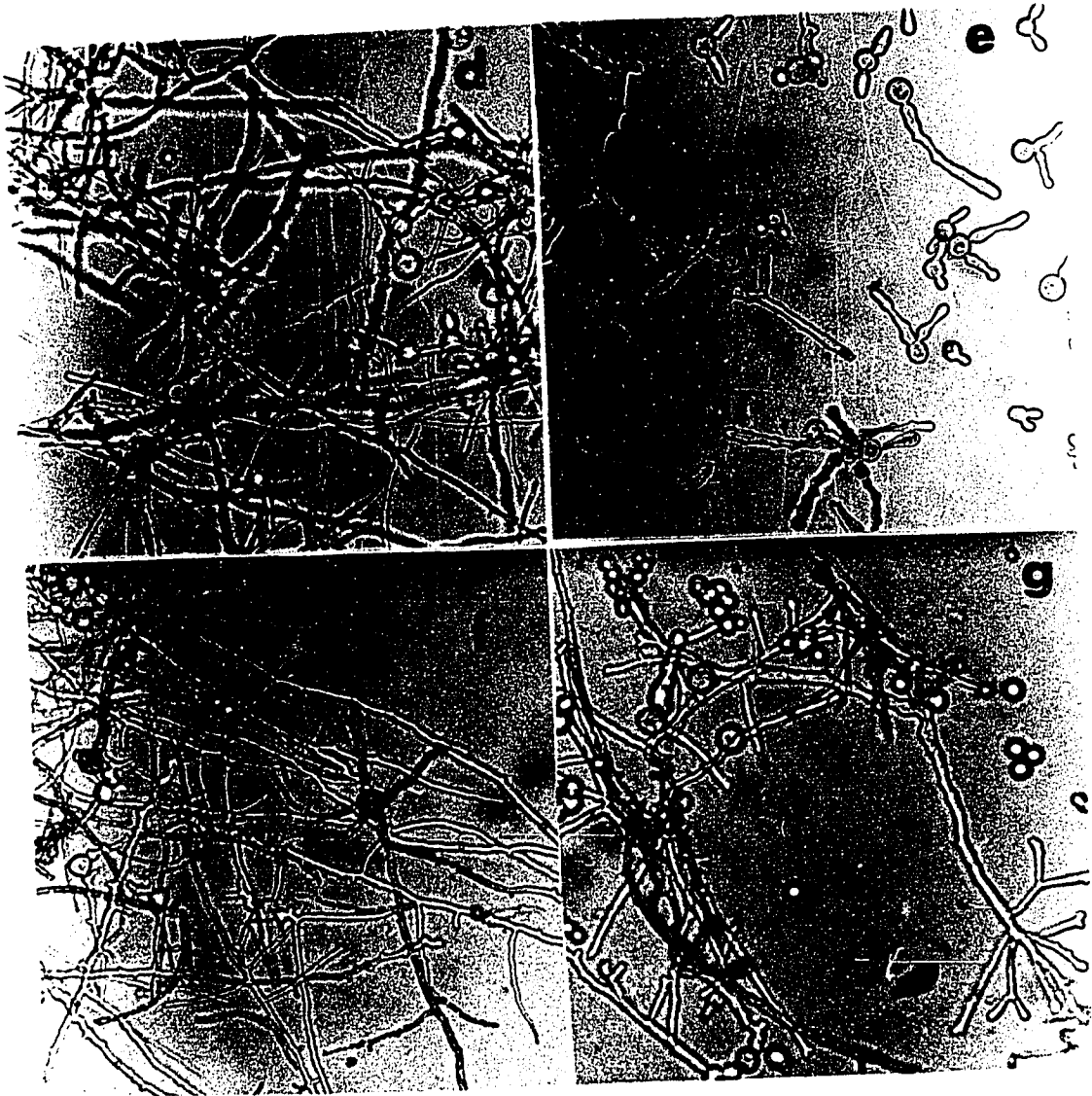
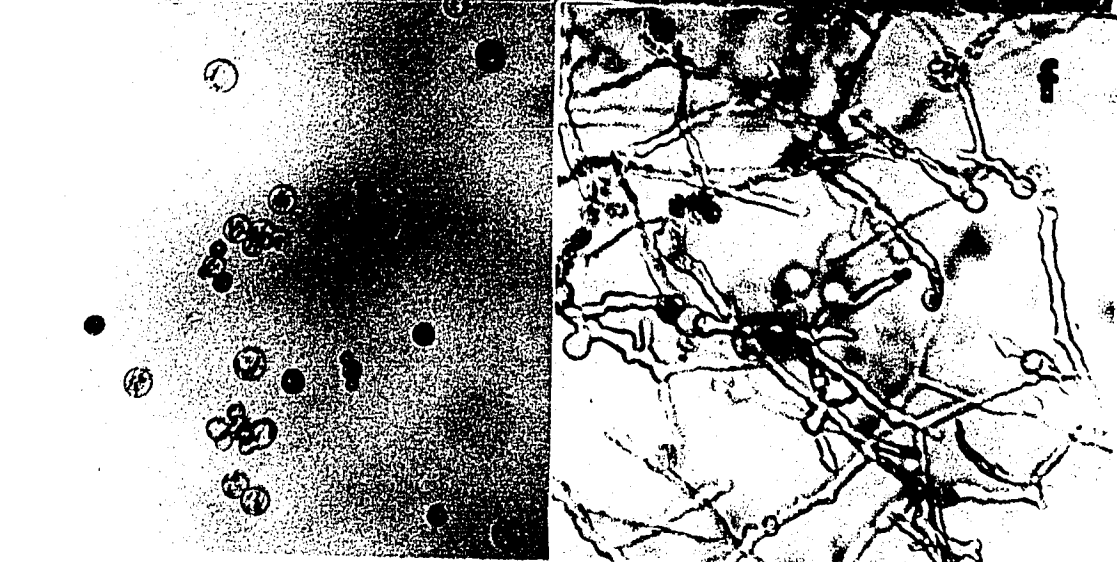
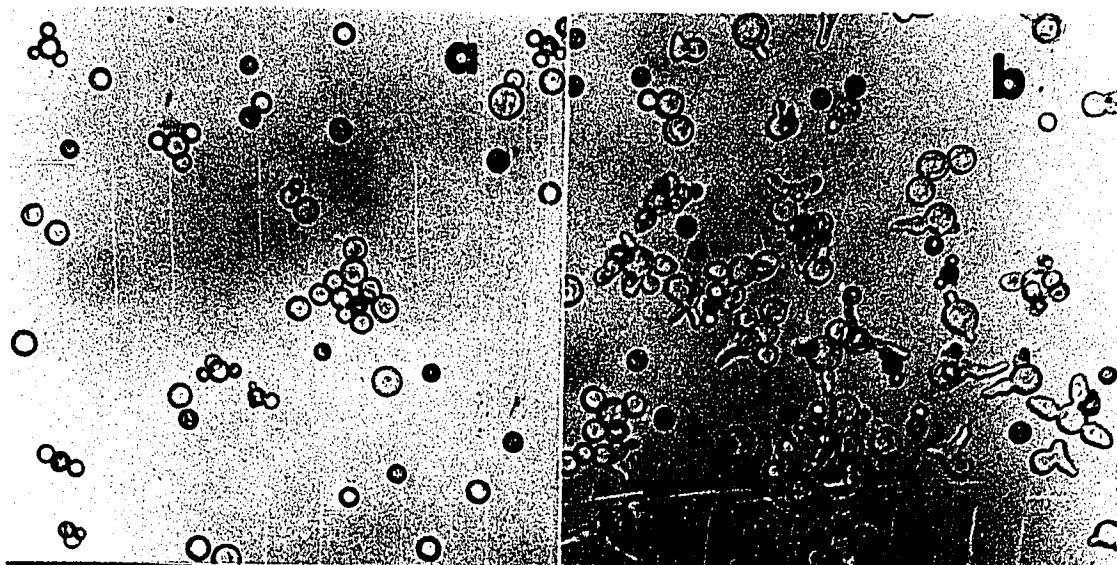


Figure 32

The inhibition by cycloheximide (200 μ g per ml) of changes in morphology of M. rouxii during conversion. (a) Cells after 4 hours of contact with air and cycloheximide; (b) Cells after 4 hours of contact with air in the absence of cycloheximide; (c) Cells after 8 hours of contact with air and cycloheximide; (d) Cells after 8 hours of contact with air in the absence of cycloheximide; (e) Cells after 10.5 hours of contact with air and cycloheximide; and (f) Cells after 10.5 hours of contact with air in the absence of cycloheximide.



emerge.

CAP inhibition. The effect of CAP on the morphology of converting cells after 3.5, 6.5 and 8.5 hours of contact with air and the drug is illustrated in Fig. 33b, d, and f, respectively. As can be seen by comparison with the control cells (Fig. 33a, c, and e), CAP did not prevent filamentation to the extent cycloheximide did.

5. Incorporation of ^3H -uridine.

Fig. 34 shows the rate of ^3H -uridine incorporation into mit- and cyt-RNA's and the corresponding absorption at 260 m μ (O.D.₂₆₀) after converting cells in BGA medium with 0.5% glucose were in contact with air for 30 minutes, 3 hours, and 6 hours. As shown in Fig. 35, the specific activity (CPM per O.D.₂₆₀) was almost the same for both mit- and cyt-RNA's.

Effect of cycloheximide on ^3H -uridine incorporation. Fig. 36 shows the rate of ^3H -uridine incorporation into mit- and cyt-RNA's and the corresponding O.D.₂₆₀ after converting cells were in contact with air and cycloheximide during the first 6 hours of conversion. As shown in Fig. 37, the specific activity for mit- and cyt-RNA's, unlike in the absence of cycloheximide (Fig. 35), differed considerably from each other when converting cells were in contact with air and the drug. As shown in Fig. 37, the specific activity of mit-RNA increased two-fold between 30 minutes and 3 hours and four-fold between 3 and 6 hours after contact with air and the drug; whereas the specific activity of cyt-RNA increased almost five-fold between 30 minutes and 3

Figure 33

The inhibition by CAP (4 mg per ml) of changes in morphology of M. rouxii during conversion. (a) Cells after 3.5 hours of contact with air in the absence of CAP; (b) Cells after 3.5 hours of contact with air and CAP; (c) Cells after 6.5 hours of contact with air in the absence of CAP; (d) Cells after 6.5 hours of contact with air and CAP; (e) Cells after 10 hours of contact with air in the absence of CAP; and (f) Cells after 10 hours of contact with air and CAP.

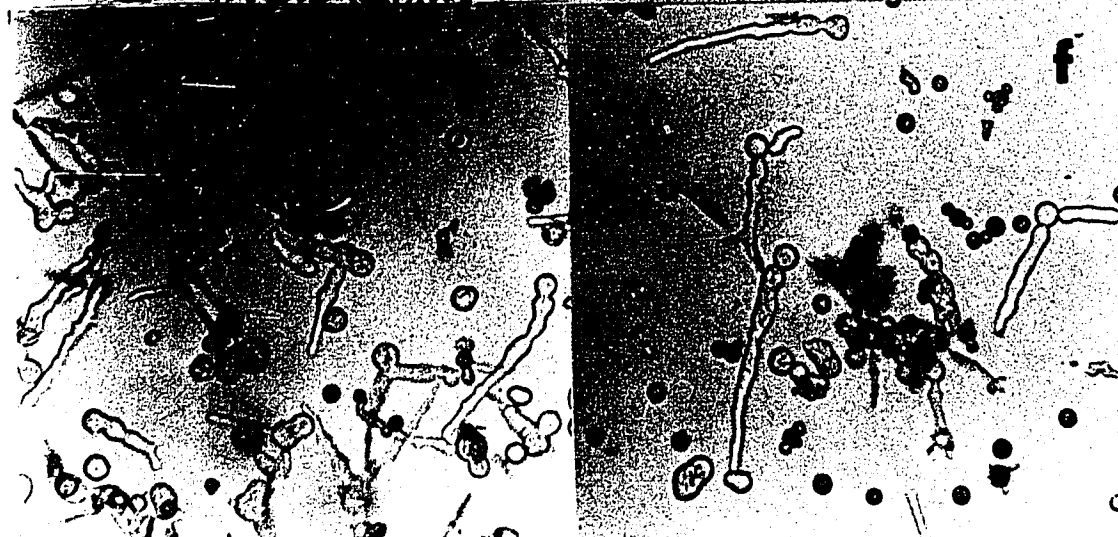
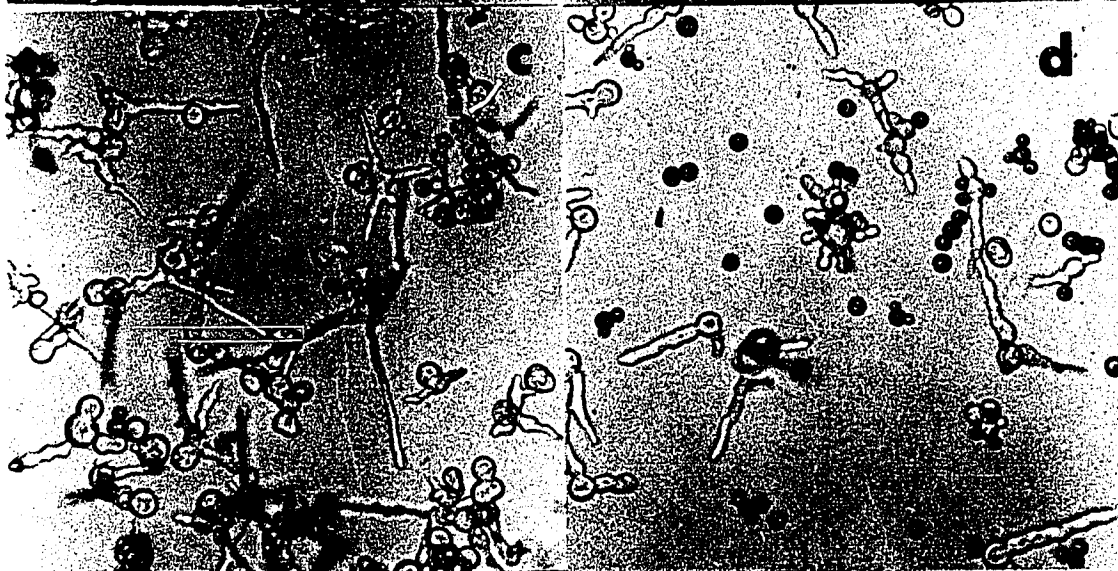
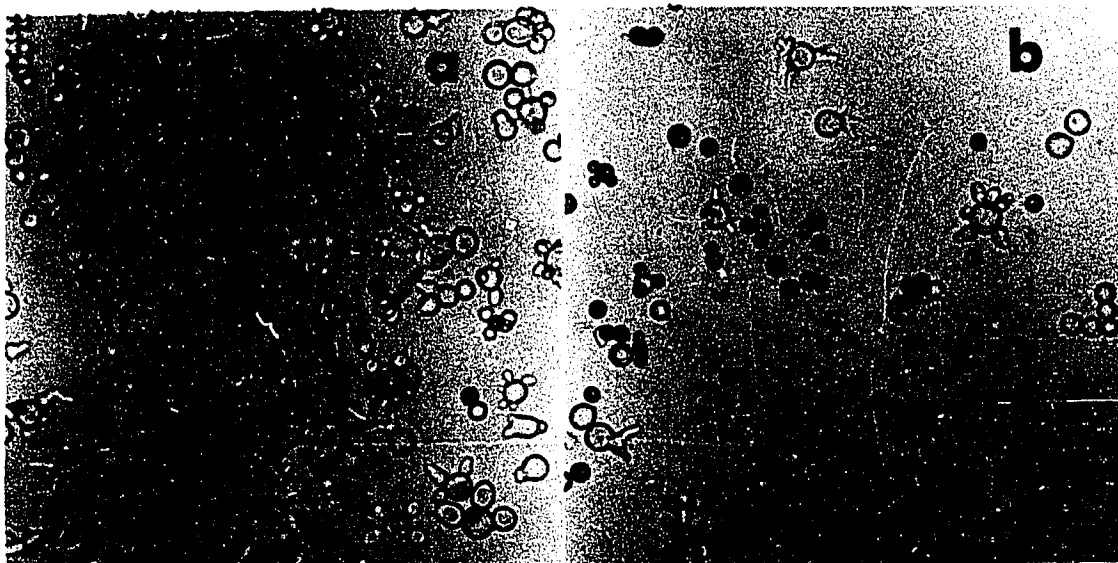


Figure 34

Rate of ^3H -uridine incorporation (CPM, dotted line) and increase in RNA content (O.D.260, solid line) of cytoplasm (\square) and mitochondria (Δ) of M. rouxii during the first 6 hours of conversion.

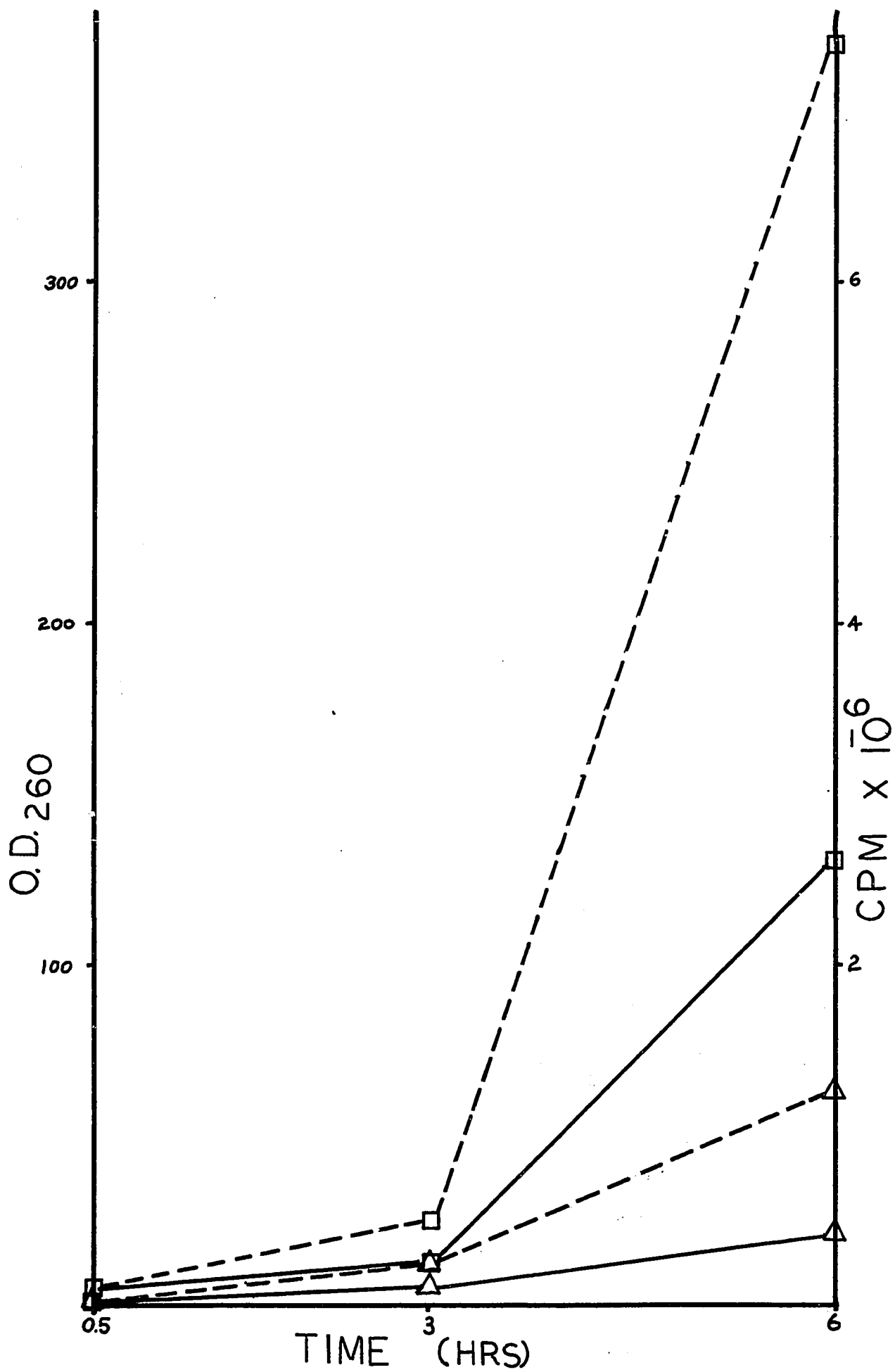


Figure 35

Specific activity (CPM per O.D.₂₆₀) of mit- and cyt-RNA's in M. rouxii during the first 6 hours of conversion. \triangle — \triangle mit-RNA; \square — \square cyt-RNA.

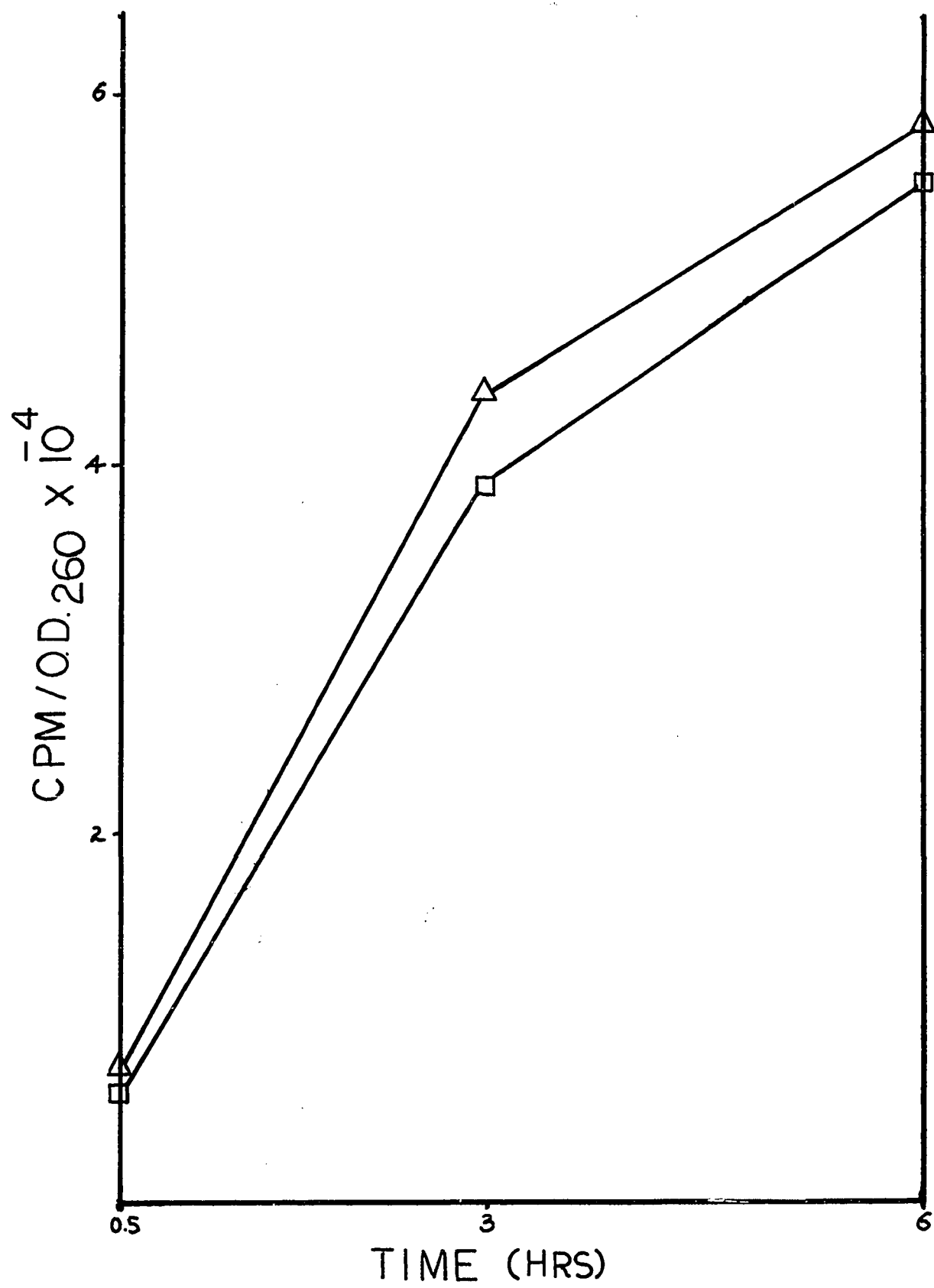


Figure 36

Effect of cycloheximide (200 μ g per ml) on the rate of ^3H -uridine incorporation (CPM, dotted line) and increase in RNA content (O.D.₂₆₀, solid line) of cytoplasm (\square) and mitochondria (Δ) of M. rouxii during the first 6 hours of conversion.

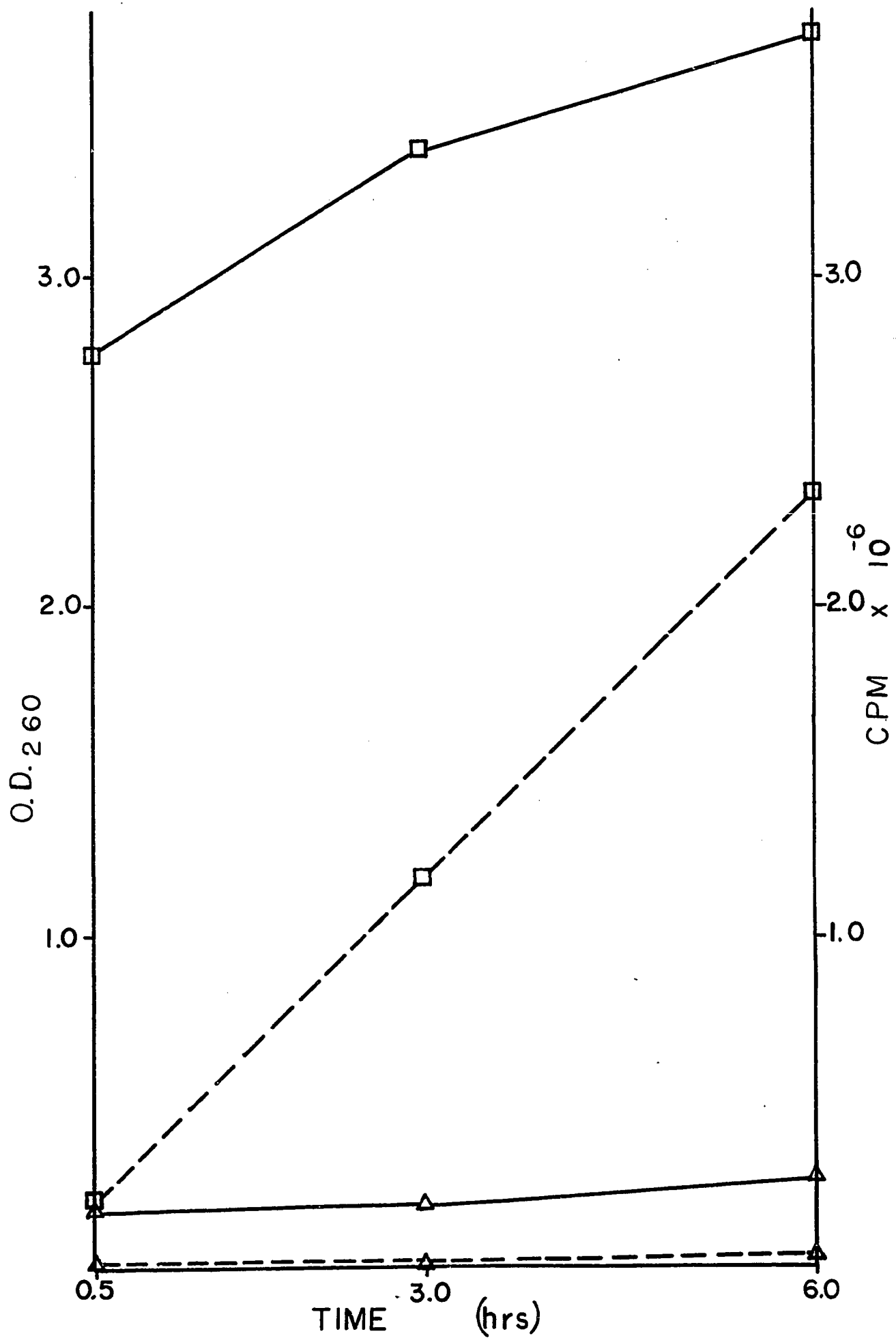
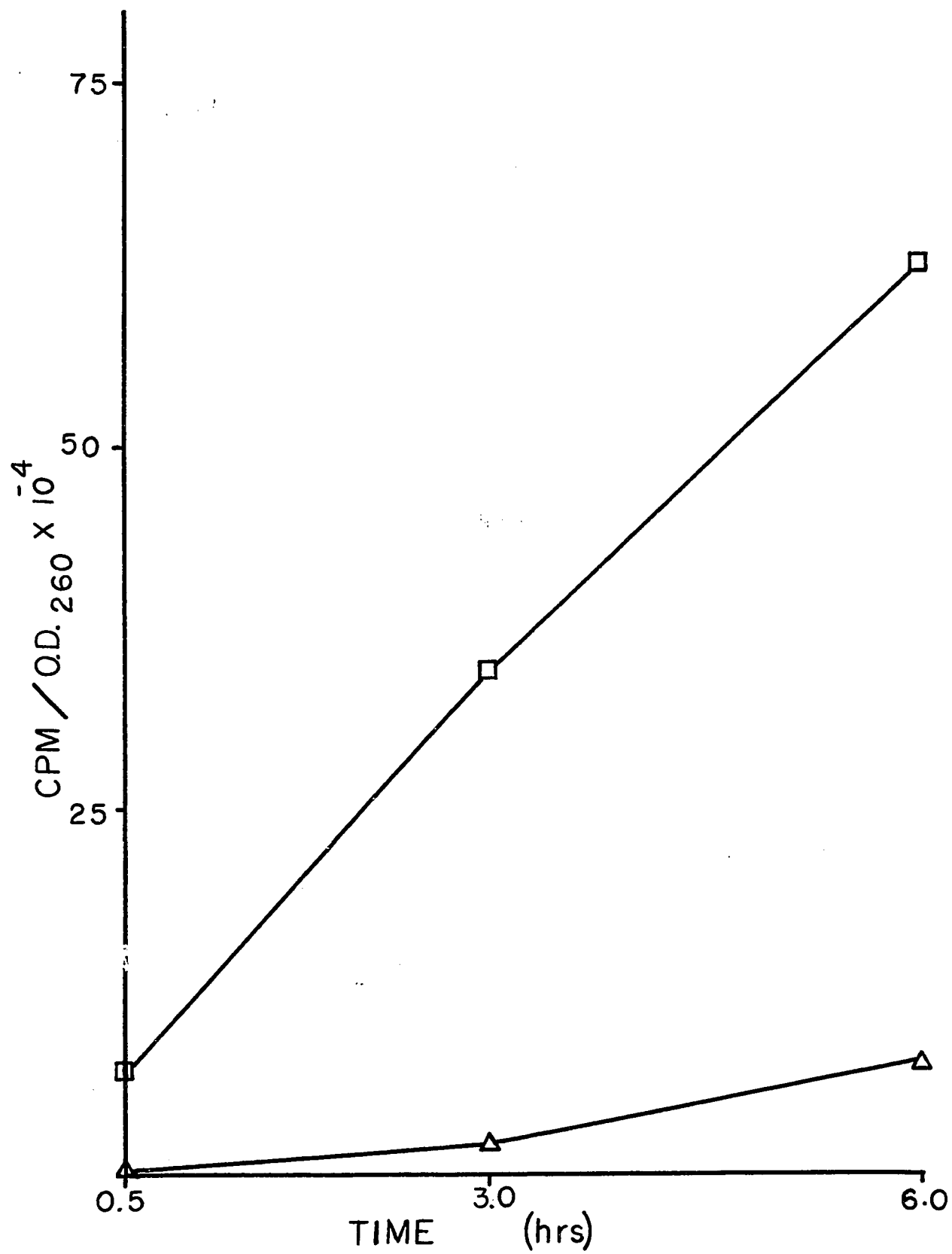


Figure 37

Effect of cycloheximide (200 μ g per ml) on the specific activity (CPM per O.D.₂₆₀) of mit- and cyt-RNA's in M. rouxii during the first 6 hours of conversion. \triangle — \triangle mit-RNA; \square — \square cyt-RNA.



hours and two-fold between 3 and 6 hours. A comparison of mit- and cyt-RNA's specific activity at 30 minutes, 3 hours and 6 hours in the absence (Fig. 35) and presence (Fig. 37) of cycloheximide shows that the specific activity of mit-RNA decreased in the presence of the drug while that of cyt-RNA increased.

Effect of CAP on the ^3H -uridine incorporation. Fig. 38 shows the rate of ^3H -uridine incorporation into mit- and cyt-RNA's and the corresponding O.D.₂₆₀ after converting cells were in contact with air and CAP during conversion. The specific activity (Fig. 39) for mit- and cyt-RNA's differed when ^3H -uridine incorporation was done in the presence of CAP. As shown in Fig. 39, the specific activity of both mit- and cyt-RNA's increased about 20-fold between 30 minutes and 3 hours after contact with air and CAP; whereas the mit-RNA specific activity decreased and that of cyt-RNA increased slightly between 3 and 6 hours. The CAP effect on the mit-RNA specific activity complemented that of cycloheximide, for with CAP the specific activity decreased between 3 and 6 hours after contact with air while with cycloheximide it was lowest between 30 minutes and 3 hours.

6. Nucleotide composition of mit- and cyt-RNA's.

Table IV lists the nucleotide composition of mit- and cyt-RNA's under aerobiosis, anaerobiosis, and conversion of yeast-like cells to filaments in the absence and presence of CAP and cycloheximide. The nucleotide composition of the mit-RNA was the same in aerobiosis (38.5% GC) and anaerobiosis and differed from that of cyt-RNA, 46.8% and 43.3% GC in

Figure 38

Effect of CAP (4 mg per ml) on the rate of ^3H -uridine incorporation (CPM, dotted line) and increase in RNA content (O.D.₂₆₀, solid line) of cytoplasm (\square) and mitochondria (Δ) of M. rouxii during the first 6 hours of conversion.

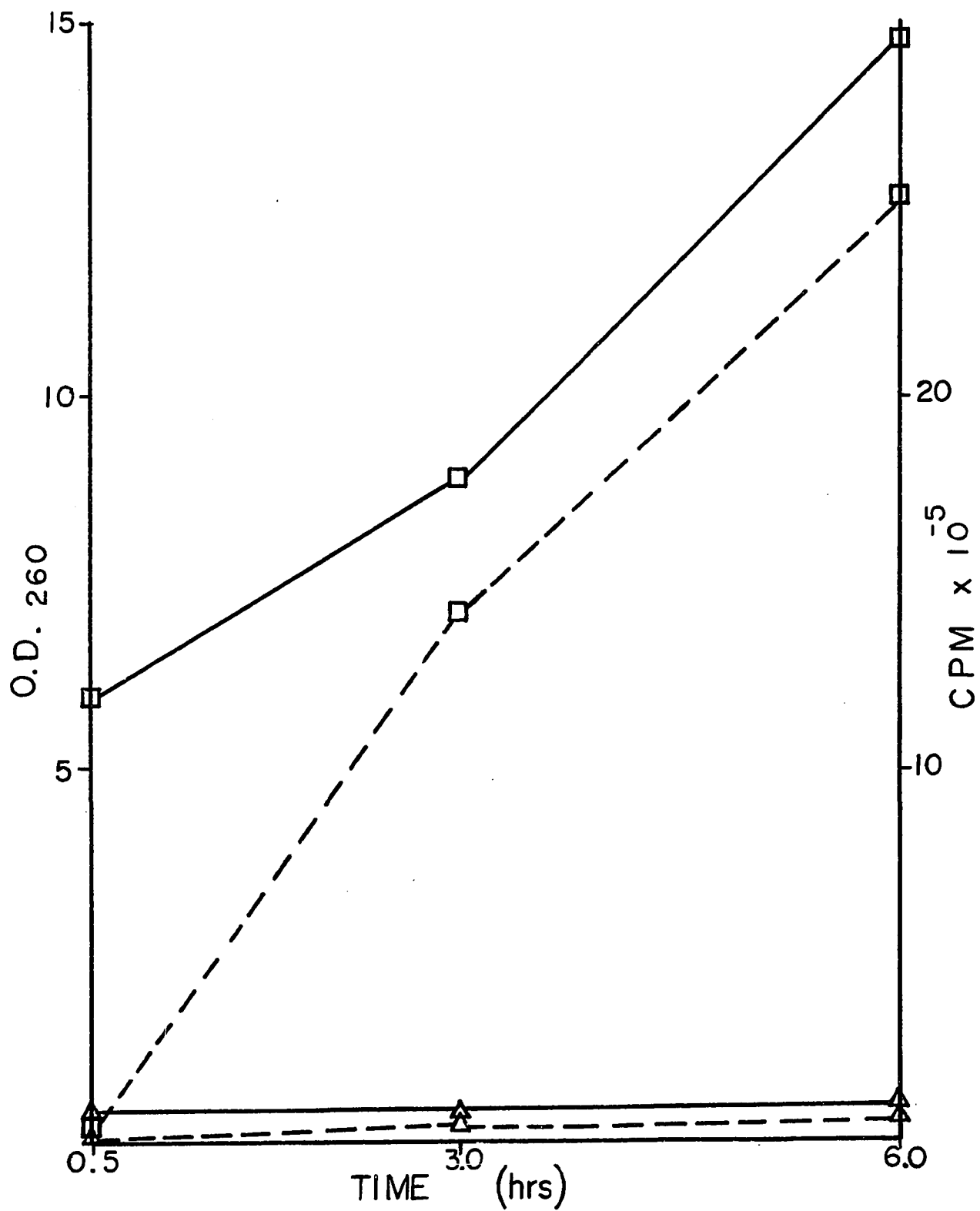


Figure 39

Effect of CAP (4 mg per ml) on the specific activity (CPM per O.D.₂₆₀) of mit- and cyt-RNA's in M. rouxii during the first 6 hours of conversion. \triangle — \triangle mit-RNA; \square — \square cyt-RNA.

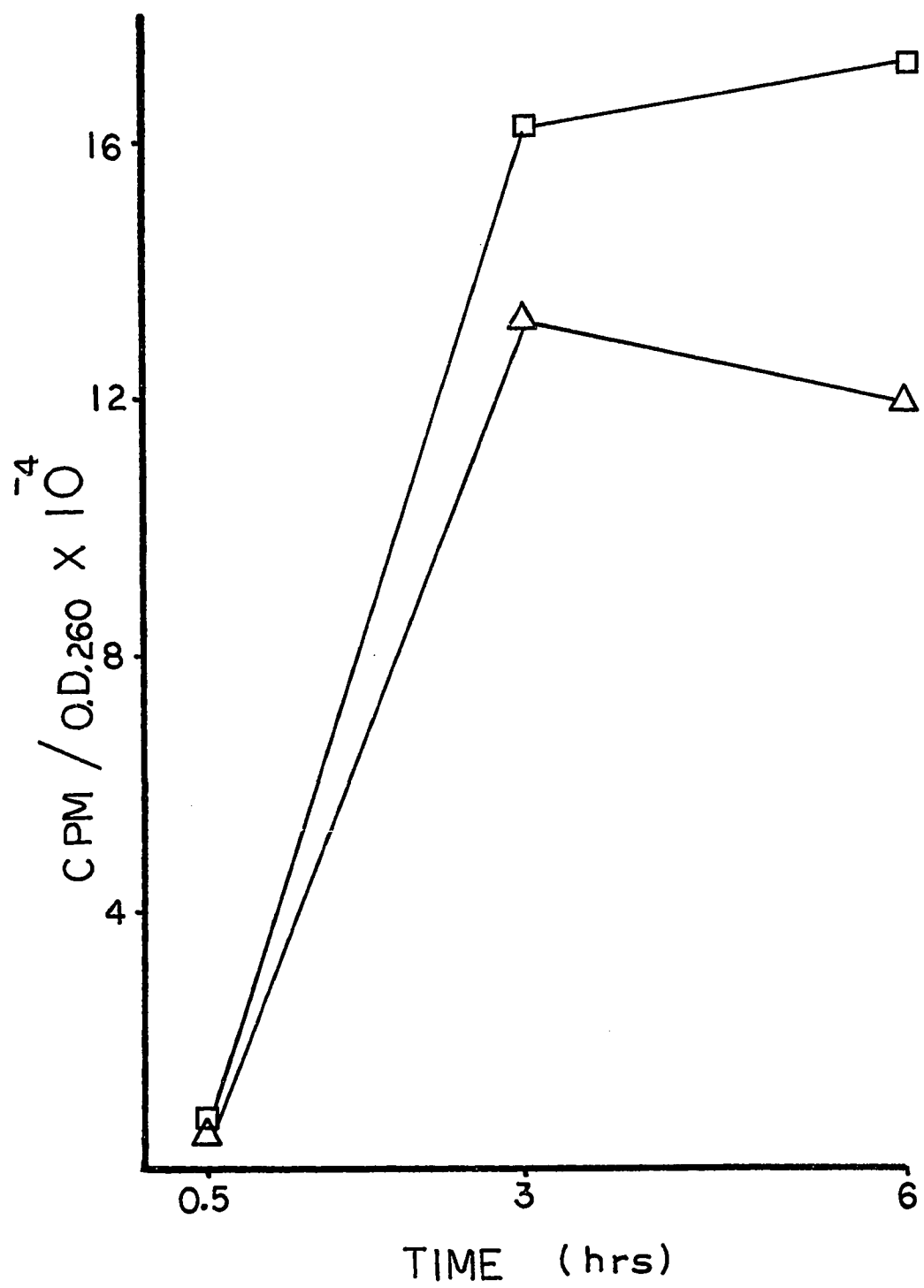


Table IV. Nucleotide Composition of Mitochondrial and Cytoplasmic RNA in M. rouxii

Source	Growth Conditions	Moles per 100 moles of identified nucleotides							
		C	A	U	G	G+C	Pu/Py	6Am/6Ke	A+U/G+C
mit-RNA cyt-RNA	aerobic	22.0	32.9	28.2	16.9	38.9	0.992	1.217	1.571
	"	22.2	26.9	26.3	24.6	46.8	1.062	0.965	1.137
mit-RNA cyt-RNA	anaerobic	22.3	33.5	28.0	16.2	38.5	0.988	1.262	1.597
	"	20.5	31.1	25.5	22.9	43.3	1.174	1.066	1.307
mit-RNA cyt-RNA	conversion	20.3	30.0	25.8	23.9	44.2	1.169	1.012	1.262
	"	21.6	26.8	25.5	26.1	47.6	1.123	0.938	1.099
mit-RNA cyt-RNA	conversion + cycloheximide	20.5	28.3	27.2	24.0	44.5	1.096	0.953	1.247
		20.8	28.1	27.5	23.6	44.3	1.070	0.957	1.255
mit-RNA cyt-RNA	conversion + CAP	19.8	32.0	24.1	24.1	43.8	1.278	1.075	1.281
		19.3	30.1	24.3	26.3	45.6	1.293	0.976	1.193

(C, cytidylic acid; A, adenylic acid; U, uridylic acid; G, guanylic acid; Pu, purines; Py, pyrimidines; 6Am, adenylic and cytidylic acids; 6Ke, guanylic and uridylic acids; mit-RNA, mitochondrial RNA; cyt-RNA, cytoplasmic RNA; CAP, chloramphenicol; conversion, exposure to air for 40 minutes)

aerobiosis and anaerobiosis, respectively. The %GC of the cyt-RNA was 3.5% higher under aerobic conditions than under anaerobic conditions. This change took place 40 minutes after transfer of yeast-like cells to air. After 40 minutes of contact with air, the %GC of mit-RNA (44.2%) differed significantly from that of mit-RNA after anaerobiosis for 16 hours (38.5%) and aerobiosis for 16 hours (38.9%).

The nucleotide composition of mit-RNA was not affected after contact of the cells with air and cycloheximide for 40 minutes, for the transient change in %GC from 38.5% to 44.5% still occurred. On the other hand, cycloheximide prevented the change from 43.3% to 47.6% GC of cyt-RNA after contact with air and the drug for 40 minutes. The compositions of the mit- and cyt-RNA's did not differ significantly from each other when the cells were in contact with air and cycloheximide for 40 minutes.

Like cycloheximide, CAP had no effect on the transient change of mit-RNA nucleotide composition after contact of the cells with air and the drug for 40 minutes. Unlike cycloheximide, CAP did not prevent effectively the change from 43.3% to 47.6% GC of cyt-RNA after contact with air and the drug for 40 minutes.

DISCUSSION

Mitochondrial DNA. Mit-DNA from Saccharomyces (21, 69, 95, 111), Neurospora (62, 81) and Physarum (32, 40) had on the average a buoyant density of 0.015 gm per cm³ lower than that of the homologous nu-DNA.

In the present work 14 species of fungi distributed among Zygomycetes, Ascomycetes and Basidiomycetes were studied. In all cases, DNA could be isolated from purified mitochondria. Furthermore, the analyses of the %GC of both nu- and mit-DNA's demonstrated the following: (i) the compositional variation of mit-DNA, like that of nu-DNA, was minimal for organisms belonging to the same genus; (ii) the overall range of %GC for mit-DNA was less than for nu-DNA; (iii) the %GC of mit-DNA was either equal to that of its homologous nu-DNA or it was less, but it was never significantly higher; (iv) no simple correlation could be obtained from the comparison of nu- and mit-DNA's; (v) mit-DNA represented but a very small proportion of total DNA; and (vi) heat-denatured mit-DNA renatured more readily than nu-DNA.

A comparison of the values listed in Table I with those for Physarum polycephalum and Saccharomyces (Table II) indicated that among fungi the %GC range for mit-DNA (22 to 43%) was smaller than for nu-DNA (34 to 61%). However, if Tables I and II are combined, these ranges respectively became: 22 to 56 and 26 to 61. Therefore, it must be concluded that the compositional diversity of mit-DNA did not differ from that of nu-DNA.

The composition heterogeneity of mit-DNA, like that of nu-DNA, tended to be minimal for genera, with the exception of Tetrahymena and Saccharomyces. For S. cerevisiae the extreme values indicated in Table

II correspond to mitochondrial mutants having an altered respiratory activity (69). Such a modification in buoyant density could be due, according to Mounolou et al. (69), to a change in the extent of methylation or the presence of unknown components rather than a change in %GC. Henceforth, it appears that the taxonomic value of nucleotide of mit-DNA is as good as that of nu-DNA.

When the %GC of mit-DNA was the same as that of nu-DNA, as in the case of Mucorales (Table I) and some animal species (Table II), mit-DNA could, however, as shown here for M. rouxii, be distinguished from its nuclear counterpart by its ability to renature more readily after heat denaturation. This readiness to renature appears to be characteristic of all mit-DNA preparations (9). This property, together with the results of nearest-neighbor frequency analyses of mit-DNA from P. polycephalum (23), has led to the suggestion (23) that mit-DNA is similar to procaryotic DNA. This suggestion could be taken to mean that mitochondria have a parasitic origin.

Although the number of analyses of mit-DNA presented here is limited, it is nevertheless relevant to stress the fact that for Zygomycetes there was no difference between the %GC of mit-DNA and nu-DNA; whereas average differences of 15 and 29% were found for Ascomycetes and Basidiomycetes, respectively. It appears possible, therefore, that when more results become available the analysis of the base composition of mit-DNA, as well as that of nu-DNA, might have a phylogenetic significance.

Mitochondrial RNA. Evidence for the presence of RNA in mitochondria was first obtained by observing, as shown in Fig. 10, that cytochrome c oxidase activity and RNA (measured by ultraviolet absorption) occupied

the same position in the profile of sucrose density gradient centrifugation. That this RNA was truly mitochondrial was indicated by the fact that it still banded at the same position as mitochondria in sucrose gradient after RNase treatment (Fig. 8). Furthermore, the analysis of mitochondria from several fungal species revealed that mit-RNA had a nucleotide composition which differed significantly from that of the cytoplasmic counterpart (Table III).

Five species of fungi distributed among Zygomycetes, Ascomycetes and Basidiomycetes contained RNase resistant mit-RNA. The mit-RNA had a %GC range (39 to 51%) only slightly larger than that of the cyt-RNA (47 to 55%). For this limited number of fungi, the compositional diversity of mit-RNA did not differ much from that of cyt-RNA. No attempt can be made on the basis of this limited survey to correlate the nucleotide composition of mit-RNA and mit-DNA or to assess a possible taxonomic or phylogenetic significance to the results on Table III, but one might perhaps note that mit-RNA of the Basidiomycetes and of the two Ascomycetes had a higher %GC than that of the Zygomycetes.

Storck (101) analyzed 26 species of fungi distributed among Zygomycetes, Deuteromycetes, Ascomycetes and Basidiomycetes, and reported significantly overlapping in the %GC values of cyt-RNA between Zygomycetes and Ascomycetes, but no overlapping between Zygomycetes and Basidiomycetes. The results on Table III for the nucleotide composition of cyt-RNA are not significantly different from those listed for each respective class by Storck (101).

Mitochondrial ribosomes and ribosomal RNA. O'Brien and Kalf (76) found that rat liver mit-ribosomes with a sedimentation coefficient of 55S

were labelled with ^{14}C -leucine when mitochondria were pulsed labelled in vitro while 78S ribosomes associated with the mitochondria were not.

Rifkin et al. (83) reported 81S, 61S and 47S ribosomal particles in mitochondria of N. crassa, and suggested that the 55S particles detected by O'Brien and Kalf in rat liver might be a subunit. Eventhough the mit- and cyt-ribosomes had similar sedimentation rates, they differed in the chemical and physical properties of the RNA, and their sensitivity to Mg^{2+} ion concentration.

Kuntzel and Noll (54) reported that ribosomes from N. crassa mitochondria were 73S while the cyt-ribosomes were 77S. Kuntzel (52) demonstrated that mit-ribosomes in N. crassa had a procaryotic-like 50S subunit and the cyt-ribosomes had a eucaryotic-like 60S subunit, while the smaller subunit was 37S in both mit- and cyt-ribosomes. Kuntzel (53) also demonstrated that most of the ribosomal proteins from N. crassa mitochondria differed from the proteins of cyt-ribosomes when analyzed on a carboxy methyl cellulose column.

In the present work, M. rouxii mit-ribosomes were extracted using the procedure of Kuntzel and Noll (54). They were distributed among two boundaries: one with a sedimentation coefficient of 78.8S and the other with one of 45.2S (Fig. 18a). Thus, the only significant difference in this respect between mit- and cyt-ribosomes resolved in the fact that the smaller particles from mitochondria had an S value about 13S units lower than that of the cyt-ribosomal particles (Fig. 18a and b). Therefore, the most significant difference between mit- and cyt-ribosomal particles in M. rouxii appears to reside in the size of the subunits. From all the instances cited, it would appear that

mit-ribosomes possess or consist of 50S subunits which are absent in cyt-ribosomes. It is possible that the 78.8 component detected in mitochondria was a cytoplasmic contamination since the mitochondria were not treated with RNase. Therefore, it can only be stated that M. rouxii mitochondria possess a relatively stable 45.2S ribosomal particle which differed from the cytoplasmic 58.8S ribosomal particles and is close to the sedimentation coefficient reported for rat liver mit-ribosomes and the large subunit of N. crassa mit-ribosomes.

M. rouxii mitochondria appeared to possess, perhaps by contamination, a factor which degrades the ribosomal particles present in the extracts (Fig. 19). An endonuclease degrading both RNA and DNA of mitochondrial origin has been characterized in N. crassa (60). It is, therefore, possible that the factor associated with M. rouxii mitochondria may also be a mitochondrial nuclease or a nuclease due to lysosomal contamination.

Two bacterial type of r-RNA molecules (23S and 16S) have been detected in mitochondria from yeast (84, 121) and from N. crassa (27, 54, 83). It was shown in the present work that mitochondrial r-RNA from M. rouxii yielded after centrifugation in a sucrose density gradient a profile which differed significantly from that observed with cytoplasmic r-RNA. If one assumes that the peaks for cytoplasmic r-RNA correspond to 28S, 19S and 4S, then the peak for mitochondrial r-RNA would probably correspond to 16S RNA molecules. The centrifugation profiles for cytoplasmic and mitochondrial r-RNA's contained a slow sedimenting peak corresponding to RNA with molecular weights similar to that of t-RNA. More work needs to be done in order to characterize mit-ribosomes and

mit-RNA. It remains that the mit-ribosomes from M. rouxii appear to be highly susceptible to degradation by some factor (probably nucleases) associated with the mitochondrial fraction.

Glucose effect during conversion of yeast-like cells to filaments. Crabtree (22) reported the repression by glucose of the respiratory system in tumor cells. This phenomenon is known as the "glucose effect".

Lemoigne et al. (57) studied growth rates of S. cerevisiae under aerobic conditions and found that in high glucose concentrations there was an initial growth phase involving intensive fermentation followed by one involving respiration. The two distinct phases of growth did not occur with low glucose concentrations, and these workers (57) concluded that glucose concentration determines the breakdown pathway. Slonimski (96) found that at very low levels of glucose (less than 6.0×10^{-5} M) the rate of respiratory adaptation in S. cerevisiae increased with glucose concentration, but that at levels higher than 6.0×10^{-3} M, increased glucose concentration had a delaying effect on the onset of respiratory activity. In 1955 Ephrussi et al. (30) found that when S. cerevisiae cells fully adapted to aerobic conditions were incubated in the presence of 3% glucose, the fermentation rate increased markedly during exponential growth phase, while the rate of respiration decreased. At late exponential growth phase when glucose became limiting, fermentation dropped to a low with a simultaneous increase in the rate of respiration.

Glucose repression in S. cerevisiae decreased the number of mitochondria and membrane-delimited mitochondrial profiles (47, 123).

Jayarman et al. (47) demonstrated that high glucose concentrations repressed the activity of respiratory enzymes in mitochondria of yeast.

Reilly and Sherman (82) studied the cytochrome spectra in S. cerevisiae cells grown in high glucose concentrations and found that repressed cells had weak bands present, and concluded that repression did not block completely the formation of cytochromes.

Smith et al. (97) demonstrated that the amount of mit-DNA in S. cerevisiae decreased markedly as the concentration of glucose in the medium increased. They also demonstrated an increase in the relative concentrations of cytochromes concomitant with that of mit-DNA of cultures grown in various carbon sources. Rabinowitz et al. (79) reported that high glucose concentrations repressed the activity of the mit-DNA polymerase as well as that of respiratory enzymes in S. cerevisiae.

South and Mahler (100) reported that RNA polymerase activity in mitochondria of S. cerevisiae was repressed by high glucose concentrations, and the mit-RNA polymerase activity increased on release from glucose repression before the increase of other respiratory enzymes such as cytochrome c oxidase and L-malate dehydrogenase.

Haidle and Storck (41) studied the germination of M. rouxii spores during a period of anaerobic incubation followed by exposure to air, and demonstrated the synthesis of RNA, protein and the increase of cytochrome c oxidase activity during this period of respiratory adaptation.

In the present work, the "glucose effect" on the respiratory activity of converting M. rouxii cells was analyzed in order to find derepressed conditions permitting the study of the synthesis of mit-RNA during conversion. As shown in Fig. 24, 5% glucose inhibited 60% of the cyanide-sensitive respiratory activity found in 0.1% glucose converting cultures.

In Fig. 25 it was shown that the cytochrome c oxidase activity in converting cells incubated in 5% glucose was approximately 50% lower than in cells in 0.5% glucose. A similar finding was made in the case of S. cerevisiae (47).

Reilly and Sherman (82) demonstrated that glucose repressed the synthesis of cytochromes in S. cerevisiae. As shown in the present work, this is also the case for M. rouxii since the amount of the cytochromes as judged from Fig. 28 is about 50% less in repressed cells than in those grown in 0.5% glucose.

In addition to those biochemical effects, high glucose concentrations during conversion resulted in the formation of "abnormal" filaments (Fig. 31) which could be easily distinguished from those formed in low glucose concentrations. This morphological consequence of the glucose effect is inexistent in S. cerevisiae.

Terenzi and Storck (109, 110) found that phenethyl alcohol (PEA) induced yeast-like morphology in aerobiosis provided the glucose concentration was higher than 2%. They demonstrated that this aerobic yeast-like growth involved an increase in alcoholic fermentation and a concomitant decrease of the oxidative phosphorylation activity of mitochondria; thus, PEA was taken to act as an uncoupling agent and to stimulate a glucose effect. These results further suggested that in M. rouxii yeast-like morphology and fermentation were linked. Terenzi and Storck (110) calculated that 37 times more glucose was fermented in the presence than in the absence of PEA in cultures having 5% glucose. It was in cultures with 5% glucose and PEA that the yeast-like cells grew aerobically; therefore, it is not surprising that in the present work

where the glucose effect repressed 50% of the respiratory activity and supposedly enhanced fermentation to an equal extent, but not in the manner stimulated by PEA, the converting cells had an in-between morphology differing from the normal yeast-like cells and the slender filaments. It appears that the glucose effect alone in M. rouxii does not result in a level of fermentation needed to produce spherical, budding yeast-like cells aerobically, and therefore, it does not result in the complete inhibition but simply in a modification of the apical growth pattern.

In the present work, M. rouxii was grown anaerobically as yeast-like budding cells by using BGA medium with 5% glucose in a sealed flask under prepurified N₂ gas. The anaerobic culture yielded a mixture of swollen spores and yeast-like cells as shown in Fig. 31a. With lower glucose concentrations (1 to 2%), germ tube formation occurred in some of the cells. Bartnicki-Garcia (5) studied the effect of glucose concentration in anaerobic cultures of M. rouxii, and reported that the fungus could be grown into shapes covering its entire dimorphic spectrum simply by manipulating the glucose concentration in the medium. He reported that in liquid cultures continuously flushed with prepurified N₂ gas, the hyphae became progressively shorter and wider as the glucose concentration increased, until at 8% glucose all cells and progeny were spherical budding cells.

Effects of CAP and cycloheximide on yeast-like cells during conversion.

Mager (63) first reported the selective inhibition of mitochondrial amino acid incorporation in T. pyriformis by CAP. Low concentrations of CAP arrested the growth of the organism and inhibited strongly the incorporation of amino acids into isolated mitochondria; whereas incor-

poration in the cytoplasmic cell-free protein synthesizing system was insensitive to the drug.

Several workers (11, 99) demonstrated that cytoplasmic cell-free protein synthesis in S. cerevisiae was insensitive to CAP. Wintersberger (118) demonstrated that incorporation of amino acids into isolated S. cerevisiae mitochondria was inhibited by CAP. Subsequently, Linnane and coworkers (16, 17, 46, 55, 61) demonstrated that CAP did not inhibit the growth of S. cerevisiae in the presence of an excess of a fermentable substrate, but it completely inhibited the formation of cytochromes a, a₃ during active growth and inhibited markedly the formation of cytochromes b and c₁. Marchant and Smith (64) found that CAP inhibited the growth of the fungus Pythium ultimum, and that its mitochondria were devoid of cytochromes a, a₃ and b, but that the cytochrome spectrum indicated the presence of cytochrome c.

Clark-Walker and Linnane (16) demonstrated that cycloheximide, a drug characterized as inhibitor of cytoplasmic ribosomal protein synthesis, did not affect the yeast mitochondrial protein synthesizing system in vivo. A low concentration of cycloheximide (1 µg per ml) inhibited the growth completely, but the formation of cytochromes a, a₃ was not affected as determined by absorption spectrum.

Vary et al. (115) performed in vivo studies on the effect of CAP and cycloheximide on S. cerevisiae during respiratory adaptation, but unlike Clark-Walker and Linnane (46, 55) who determined the formation of cytochromes a, a₃, this group examined the effect of the drugs on the cytochrome c oxidase activity. Vary et al. (115) reported that CAP inhibited the activity of cytochrome c oxidase, preferentially to those

of malate dehydrogenase, fumarase, and succinate dehydrogenase were not inhibited. Cycloheximide inhibited the activity of all these enzymes including cytochrome c oxidase.

These in vitro and in vivo studies of S. cerevisiae on the effect of these inhibitors (cycloheximide and CAP) have suggested the presence of two protein synthesizing systems, one cytoplasmic and the other mitochondrial. The two systems are distinguishable from one another on the basis of the differential sensitivity to the two antibiotics. The results on the inhibitory effect of the antibiotics on the cytochrome c oxidase activity tend to suggest the existence of an interdependence of the mitochondrial and cytoplasmic protein synthesizing systems in the induction of cytochrome c oxidase activity.

Effect on M. rouxii. Haidle and Storck (42) demonstrated that cycloheximide inhibited protein synthesis, partial RNA synthesis, and the conversion of yeast-like cells to filaments. These results on the effect of cycloheximide on M. rouxii during conversion, and the evidence cited above on the S. cerevisiae cytoplasmic and mitochondrial protein synthesizing systems differential sensitivity to cycloheximide and CAP prompted the study of the effects of these two antibiotics on M. rouxii converting cells. The present work considers the effects of cycloheximide and CAP on (i) the formation and activity of cytochrome c oxidase; (ii) cyt- and mit-RNA's synthesis; (iii) nucleotide composition of the cyt- and mit-RNA's; and (iv) the morphological conversion during respiratory adaptation.

Effect on cytochrome c oxidase. Both cycloheximide and CAP inhibited markedly the cytochrome c oxidase activity during the first 10 hours of

conversion as was shown in Fig. 26 and 27, respectively. Notwithstanding these results on the enzyme activity inhibition, CAP indeed blocked the formation of cytochrome c oxidase (cytochromes a, a₃) to the extent that when determined spectrophotometrically the cytochromes a, a₃ peak (600-605 mμ) was nonexistent when compared to the control spectrum (Fig. 30); whereas cycloheximide blocked the formation of cytochromes c, c₁ and b (520, 550 and 562 mμ) peaks completely, and reduced cytochromes a, a₃ peak markedly (Fig. 29).

The observed effect of cycloheximide and CAP on the appearance of the cytochrome spectrum during respiratory adaptation suggests the existence of a cytoplasmic and mitochondrial protein synthesizing systems which as in S. cerevisiae (16, 46, 61) are not equally sensitive to cycloheximide and CAP. The inhibitory effect of both antibiotics on the cytochrome c oxidase activity increase suggests an interdependence in M. rouxii of the two protein synthesizing systems as is the case in S. cerevisiae (115).

This interdependence could be explained by suggesting that the dependence of cytochrome c oxidase activity on the mitochondrial protein synthesizing system (sensitivity to CAP) does not necessarily mean that the enzyme complex is synthesized there in toto. The different apoproteins and heme a (cytohematin) components could be synthesized separately inside and outside the organelle and assembled to form the active complex inside the mitochondria; therefore, inhibition of either the cytoplasmic or the mitochondrial protein synthesizing systems would result in the synthesis of only part of the complex. The fact that the formation of cytochrome a, a₃ was blocked by CAP (Fig. 30); whereas that of

cytochromes c, c_1 and b was not, indicates that cytochrome a, a_3 formation depends on the mitochondrial protein synthesizing system while that of the other cytochromes does not.

The effect of cycloheximide on the formation of the cytochromes indicates that c, c_1 and b are dependent on the cytoplasmic protein synthesizing system since their formation was completely blocked, while cytochromes a, a_3 formation was markedly but not completely blocked. The reduction of cytochrome a, a_3 formation also indicates the interdependence between the two protein synthesizing systems. The presence of the cytochrome a, a_3 peak (Fig. 29b), which virtually lacked enzymatic activity, but still had a cytochrome c oxidase-like spectrum in the presence of cycloheximide tends to indicate that to some extent the apoproteins and cytohemins necessary for the absorbance at 600-605 m μ were present in spite of the fact that the cytoplasmic protein synthesizing system was non-functional. Presumably, the mitochondrial system was at least partially functional. These results could be explained if the cytochrome c oxidase enzyme complex is synthesized by the mitochondrial system, but its assembly and activity depend on some prior or concomitant synthesis of proteins by the cytoplasmic system. The extremely low activity and low amount of cytochrome a, a_3 enzyme complex may be the result of the lack of interaction with proteins of cytoplasmic origin such as cytochrome c which is known to be synthesized by the cytoplasmic system and integrated into the inner membrane of mitochondria (33, 34, 38, 48, 49). When these cytoplasmic proteins are present, the mitochondrial protein synthesizing system may be stimulated to form additional cytochrome a, a_3 which integrated into the complete inner membrane may possess normal activity.

Effect on cytoplasmic and mitochondrial RNA's synthesis. Cycloheximide was shown (42) to inhibit protein synthesis and partially RNA synthesis in yeast-like cells that had been exposed to air for 45 minutes. The inhibition of ^3H -uridine incorporation was 30% of that in the control culture without the drug.

In the present work, the effect of both cycloheximide and CAP on the cyt- and mit-RNA's synthesis was determined during the first 6 hours of conversion. As was shown in Fig. 35, the specific activities of mit- and cyt-RNA's were almost the same in the absence of any drug. The two RNA synthesizing systems (nuclear and mitochondrial) were best differentiated by the ^3H -uridine incorporation in the presence of either cycloheximide or CAP.

The synthesis of mit-RNA during conversion in the presence of cycloheximide was drastically affected as compared to the synthesis of cyt-RNA. The incorporation of ^3H -uridine into mit-RNA was lower between 30 minutes and 3 hours than between 3 and 6 hours (Fig. 36). The increase in specific activity of the mit-RNA exhibited a lag between 30 minutes and 3 hours, and was much lower than the specific activity of the cyt-RNA throughout the 6 hour incubation period (Fig. 37). These results suggest that the synthesis of mit-RNA was dependent on the cytoplasmic protein synthesizing system which in this case was inhibited by cycloheximide. One possible explanation for this interdependence would be that the induction of a new RNA polymerase activity is required for the synthesis of mit-RNA during respiratory adaptation. Therefore, if the synthesis of mit-RNA polymerase takes place in the cytoplasm, this would result in the lowering of the specific activity of the mit-RNA

during conversion. The synthesis of the cyt-RNA during conversion may not require the induction of a new RNA polymerase activity, and for this reason the specific activity was not affected as that of the mit-RNA. Another possible explanation for the low specific activity of the mit-RNA may be the lack of a protein synthesized by the cytoplasmic system and which may participate in the transport of uridine into the mitochondria.

The specific activity of the cyt-RNA in the presence of cycloheximide (Fig. 37) was consistently higher than that in the absence of the drug (Fig. 35). If the accumulation of a high turnover RNA such as that reported in S. carlsbergensis (34) occurred in M. rouxii as a result of cycloheximide, the higher specific activity may be explainable. Haidle and Storck (42) reported that the RNA synthesized in the presence of cycloheximide had a composition that mimicked that of DNA. If the accumulation of this type of RNA occurs while the bulk of the r-RNA is not being synthesized but gradually turning over, this may explain the increase of the specific activity with time. The base composition of the cyt-RNA in the presence of cycloheximide indeed differed from the base composition of the RNA in the absence of the drug (Table IV).

Contrary to the effect of cycloheximide in which the synthesis of mit-RNA was lowest between 30 minutes and 3 hours, CAP produced a decrease in the rate of ^3H -uridine incorporation into mit-RNA between 3 and 6 hours (Fig. 39). The drop in specific activity reflected a lower incorporation of ^3H -uridine in proportion to the increase in the RNA. It is possible that between 3 and 6 hours in the presence of CAP some of the labelled RNA synthesized during the 30 minute to 3 hour period began to turnover and that the ^3H -uridine was lost and not available for

incorporation. CAP may have produced a change in the integrity of the mitochondrial membranes, particularly the inner membrane between 3 and 6 hour period so that the modified membrane resulted in the loss of ^3H -uridine from the mitochondrial pool. Such a dismantling of the inner membrane by CAP has been reported in S. cerevisiae mitochondria (61).

As was shown in Fig. 38, CAP did not seem to have a direct inhibitory effect on the mit-RNA synthesizing process since there was an increase in the amount of mit-RNA between the 3 and 6 hour period.

Effect on the nucleotide composition of the cyt- and mit-RNA's. Haidle and Storck (42) used ^{32}P to determine the nucleotide composition of cyt-RNA synthesized during the first 40 minutes of the conversion period, and reported that the cyt-RNA synthesized in the presence of cycloheximide differed in composition from that synthesized during the same period in the absence of the drug. As was shown in Table IV similar results were attained by simply determining the composition of the total RNA present in the cytoplasmic fraction. The 44.3% GC of the cyt-RNA in the presence of cycloheximide differed significantly from 47.6% GC in the absence of the drug, and on a nucleotide per nucleotide basis the cyt-RNA having 44.3% GC was closer to mimicking the composition of the M. rouxii nu-DNA. The nucleotide composition of the total cyt-RNA in the absence of cycloheximide (47.6% GC) resembled the values reported by Terenzi (108) for r-RNA (48.1% GC). Therefore, cycloheximide appeared to have inhibited the synthesis of r-RNA and t-RNA, while the synthesis of an RNA with a %GC closer to that of the nu-DNA (38% GC) did occur.

In spite of the fact that cycloheximide inhibited considerably the synthesis of mit-RNA during conversion (Fig. 36 and 37), the drug had

no effect on the change in the composition of mit-RNA from 38.5% to 44.5% GC. Cycloheximide limited the synthesis of mit-RNA, but it did not alter the types of mit-RNA synthesized since the composition of the total mit-RNA was the same in the absence or presence of the drug during the first 40 minutes of conversion. In this respect the effect of cycloheximide on the mit-RNA differed from that on the cyt-RNA where the drug enhanced the preferential synthesis of a messenger-like RNA (m-RNA).

CAP had no apparent effect on the nucleotide composition of the cyt- and mit-RNA's. Unlike cycloheximide, CAP had almost no effect on the change in the composition of cyt-RNA from 43.3% to 47.6% GC. The composition of cyt-RNA in the presence of the drug (45.6% GC) resembled more that in the absence of the drug (47.6% GC) than in anaerobiosis (43.3% GC).

Besides the effects of these two antibiotics, the present work (Table IV) demonstrates a transient change in the nucleotide composition of mit-RNA: 38.5% GC in anaerobiosis; 44.2% GC during conversion; and 38.9% GC in fully converted aerobic cultures. This transient change in the composition of mit-RNA during conversion may reflect the synthesis of a mit-RNA population which diminishes in anaerobic and fully converted aerobic cultures. It is possible that the bulk of the mit-RNA in anaerobic and fully converted aerobic cultures is of a ribosomal nature, and that the excessive synthesis of m-RNA and possibly t-RNA during conversion results in the change in composition of the total mit-RNA.

This may occur since this is a period of transformation of nonfunctional mitochondria, which seem to possess r-RNA, to a functional state; thus, the synthesis of m-RNA may occur in excess of the types of RNA carried

over from anaerobiosis. The composition of mit-RNA 40 minutes after exposure of the cells to air may simply result from a mixture of the three types of RNA (ribosomal, transfer and messenger) in which the amount of m-RNA is higher than the other types of RNA's.

The composition of cyt-RNA had a 43.3% GC in anaerobic cultures and 47.6% GC in cells exposed to air for 40 minutes. This change in the cyt-RNA, unlike that in mit-RNA, was not transient but of a permanent nature since total cyt-RNA from 16 hour aerobic cultures possessed the same composition (46.8% GC). The different composition of cyt-RNA in anaerobiosis may be the result of a difference in the ratio of the different types of RNA's present. It is possible that the composition of cyt-RNA in anaerobiosis may represent an RNA mixture considerably rich in m-RNA. This is in view of the fact that the composition of the cyt-RNA in anaerobiosis (43.3% GC) resembled that during conversion in the presence of cycloheximide (44.3% GC), and cycloheximide appeared to enhance the preferential synthesis of m-RNA. The shift in the composition of cyt-RNA from 43.3% to 47.6% GC during conversion suggested there is a considerable amount of cytoplasmic r-RNA synthesized during the early phase of conversion.

Effect on the morphology. Cycloheximide inhibited the conversion of yeast-like cells to filaments (Fig. 32) as Haidle and Storck (42) reported. The inhibition of conversion was expected since the bulk of the proteins are synthesized by the cytoplasmic protein synthesizing system which is inhibited by cycloheximide.

CAP inhibited the conversion of the majority of the cells (Fig. 33). The high concentration of CAP (4 mg per ml) needed to inhibit growth

suggested that cells and/or organelles were poorly permeable to this antibiotics.

In conclusion, it can be stated that the mitochondria in M. rouxii are similar to those in N. crassa, S. cerevisiae and other fungi in that they appear to possess the apparatus (DNA, RNA and ribosomes) needed to carry on protein synthesis. The studies on M. rouxii during respiratory adaptation have demonstrated that the mitochondrial system is not completely autonomous, for there seems to exist an interdependence between the mitochondrial and cytoplasmic protein synthesizing systems. This interdependence affects both protein and RNA synthesis, especially at the mitochondrial level. This complex relationship between the mitochondrial and cytoplasmic systems and the changes in nucleotide composition of the mit- and cyt-RNA's under different conditions (anaerobiosis, aerobiosis and conversion) serve to demonstrate that M. rouxii merits further investigation which is needed to understand better the events that occur at the cytoplasmic and mitochondrial levels during the conversion of yeast-like cells to filaments.

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