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NAME OF AUTHOR/*NOM DE L'AUTEUR*: Timothy William Flegel

TITLE OF THESIS/*TITRE DE LA THÈSE*: The Life-Cycle and Sexual Physiology of Sirobasidium magnum

UNIVERSITY/*UNIVERSITÉ*: Simon Fraser University

DEGREE FOR WHICH THESIS WAS PRESENTED/*GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE*: Ph. D.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE*: 1977

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE*: Dr. M. McClaren

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THE LIFE CYCLE AND SEXUAL PHYSIOLOGY OF *SIROBASIDIUM MAGNUM*

by

Timothy William Flegel

B.Sc. University of British Columbia 1965

M.Sc. University of British Columbia 1968

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the

Department of Biological Sciences

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Simon Fraser University April 1977

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ABSTRACT

In a search for a tool to study hormonally mediated yeast-hyphal dimorphism, jelly fungi were collected in Japan and the Philippines in 1968 and 1969. These were identified as samples of the Phragmobasidiomycete, _Sirobasidium magnum_. The conditions for growing this fungus on artificial media were determined and the stages in the life cycle were described. New techniques were developed for staining the nuclei of this organism and for observing the nuclei in living yeast cells. By microscopic examination, this dimorphic organism was found to have the following features. Dikaryotic mycelium formed following conjugation of uninucleate yeast cells. The dikaryotic mycelium, when incubated under coverslips for up to one month, formed a gelatinous fruiting body at the edge of the coverslip. In this fruiting body karyogamy and meiosis took place in obliquely septate catenulate basidia. Binucleate sterigmata were shed from the basidia and produced delayed uninucleate basidiospores which were subglobose and apiculate. These spores germinated by producing secondary basidiospores (germination by repetition) or by producing yeast cells by budding. The yeast cells grew predominantly by monopolar budding. Yeast colonies sparingly produced subglobose apiculate ballistospores which germinated by repetition or repeated the yeast phase directly by budding.

Growth of the yeast phase was studied in culture and various significant characteristics were shown. The cells were non-fermentative; they were encapsulated; they assimilated inositol. These features are characteristic of the imperfect yeasts in the genus *Cryptococcus* Kutzing emend Phaff et Spencer. However, the production of ballistospores by these yeasts is a feature that occurs in the imperfect yeast *Bullera* Derx. On the basis of morphological, cultural and assimilative characteristics the three yeast types were considered to be interrelated.

Mating between yeast isolates of *S. magnum* was demonstrated to be governed by multiple incompatibility factors which resulted in a modified tetrapolar heterothallism resembling that found in the genus *Tremella* Dill. ex Fr. by other workers.

Conditions for controlled study of conjugation were described. The process was shown to be mediated by diffusible substances called erogens. Cells were shown to be receptive to these erogens in G_1 of the cell cycle. The hormonal response was shown not to be mediated by 3':5'-cyclic adenosine monophosphate.

In conclusion, *S. magnum* was considered a tool with great potential for studying yeast-hyphal dimorphism specifically and for studying the processes of morphogenesis in general.

To my parents

ACKNOWLEDGEMENTS

I would like to thank my major supervisor Dr. M. McClaren for his invaluable assistance in the research for and the preparation of this thesis. I would also like to thank Dr. J.E. Rahe and Dr. R.J. Bandoni, the other members of my supervisory committee, for their continual interest in my work and their excellent reviews of the manuscript. In addition, I especially thank Dr. Bandoni for reviving the fruiting bodies that I sent from the Orient and for making yeast isolates from them.

I am indebted to the members of the Microbiology Department at Mahidol University in Bangkok who provided space and materials for the portion of this research done in Thailand.

I am most grateful to Arne McRadu for his exceptional graphs and illustrations, and to Ron Long for his assistance and advice in the preparation of the plates.

Finally, I would like to express my appreciation to the numerous members of the Faculty and Staff of the Department of Biological Sciences who contributed towards making my term at Simon Fraser University productive, academically stimulating, and personally rewarding.

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THE LIFE CYCLE AND SEXUAL PHYSIOLOGY OF SIROBASIDIUM MAGNUM

GENERAL INTRODUCTION

The Subdivision Basidiomycotina (sensu Ainsworth, 1966) is characterised by fungi with typically filamentous assimilative phases and perfect stage spores, basidiospores, which are borne externally on a dangeardien (Moreau, 1949), specifically termed a basidium. The largest Class of the Basidiomycotina is the Hymenomycetes (sensu Ainsworth, 1966) and within this Class the Subclass Phragmobasidiomycetidae (sensu McNabb, 1973) or Heterobasidiomycetidae (sensu Lowy, 1968) is of particular interest to this study. These fungi have divided basidia and a number of species in the Order Tremellales show dimorphic assimilative growth forms. The haploid assimilative stage of these dimorphic fungi grows in the laboratory as budding yeast cells. The yeast-like cells of this phase conjugate to form a dikaryotic mycelium which eventually produces a gelatinous basidiocarp. Some cultural studies have been done using these fungi (Brefeld, 1888, 1908; Möller, 1895; Kobayasi, 1937, 1939, 1962; Bandoni, 1957, 1963, 1965; Kobayasi and Tubaki, 1965; Flegel, 1968; Brough, 1970, 1974; Koske, 1972; Reid, 1975) but they have not been

extensive. Most of the research to date has been concentrated on species of <u>Tremella</u> Dill. ex Fr. and especially on <u>Tremella mesenterica</u> (S.F. Gray) Pers. and <u>Tremella</u> <u>fuciformis</u> Berk. of the Tremellaceae. The latter species is now cultivated commercially in Taiwan and can be purchased locally, canned or dried, as a Chinese specialty food.

The fungi of this group are particularly interesting because of the morphological transition that takes place during conjugation of compatible haploid yeast isolates. When such isolates are mixed under appropriate conditions, budding growth is eventually replaced by monokaryotic filaments or conjugation tubes (Bandoni, 1963; Kobayasi and Tubaki, 1962; Brough, 1974). In <u>Tremella</u> this transformation is mediated by diffusible substances which were termed erogens by Reid (1974) following the definitions by Machlis (1972). In <u>T. mesenterica</u>, Reid (1974) has stated that these substances are probably small peptides. Flegel (1968) has shown that these erogens could maintain monokaryotic filamentous growth if they were continuously supplied. Upon removal, budding cells (blastospores) were produced by the filaments and yeast growth resumed.

Such a system held great potential for morphogenetic studies on yeast to hyphal and hyphal to yeast transformations. The following sequence of investigations could be envisaged: the erogens could be extracted and identified; their intracellular origin and genetic determination could be ascertained; and their mode of action could be determined in the receptor cells. In addition to increasing the understanding of fungal dimorphism, this work would probably have some general application to other eukaryotic morphogenetic systems. All the necessary elements are present. There are naturally produced control substances which elicit a simply monitored morphological change. Investigations would be greatly facilitated by the yeast cells because they can be manipulated using standard bacteriological techniques.

The genus *Tremella* and particularly *T. mesenterica* was the most obvious candidate for this suggested work because of the great amount already known about it. However, it has not been possible, to date, to grow this fungus through its complete life cycle routinely in the laboratory. This would have to be possible before genetic analysis could be performed.

In searching for a more easily handled species from this group, I turned to the collection of a number of tropical species of jelly fungi. Among these was a species of the genus Sirobasidium Lagerh. et Pat. emend Bandoni from the monogeneric family Sirobasidiaceae. This genus was first characterised by Lagerheim and Patouillard (1892) when they described the two new species, S. albidum and S. sanguineum from Equador. The significant features of the genus as they saw it were a gelatinous basidiocarp, septate basidia borne in chains, and non-violently discharged basidiospores. During the years that followed, four other species were added to the genus: S. brefeldianum (Möller, 1895); S. magnum (Boedijn, 1934); S. indicum (Ramakrishnan and Subramanian, 1951); and S. japonicum (Kobayasi, 1962).

Re-examining the genus, Bandoni (1957) discovered the presence of forcibly ejected spores that had been missed by previous authors. He pointed out that the sessile "basidiospores" of Lagerheim and Patouillard were, in fact, deciduous sterigmata (epibasidia). They in turn produced delayed basidiospores discharged from secondary sterigmata and these spores germinated either by repetition or by budding.

The caducous sterigmata are an attractive bonus for genetic analysis. Being relatively large, they facilitate handling by micromanipulators and can be transferred to individual petri dishes for basidiospore retrieval.

The purpose of this study was to establish the identity of the species of Sirobasidium collected and then to determine the feasibility of using that organism as a tool for examining yeast-filament dimorphism. Chapter 1 describes the microscopic examinations, cultural procedures and mating tests done to determine the identity of the collections used. Chapter 2 is a description of the life cycle of this organism in culture. It includes a description of the cultural conditions devised to grow it and a description of the nuclear behavior in the cells during various stages of growth. Chapter 3 describes experiments dealing with the conjugation process.

CHAPTER ONE

SOURCE, DESCRIPTION AND IDENTIFICATION OF SPECIMENS

INTRODUCTION

When embarking on experimental work using a new species of organism it is essential that one be as certain as possible of its correct and valid taxonomic identity. The fungi used in this study were collected in Japan and the Philippines in late 1968 and early 1969 respectively and had not previously been reported from those countries. I have, therefore, been obliged to substantiate that the three collections studied were conspecific and to determine whether they constituted new species or corresponded to ones already described.

The characters used to delimit the genus Sirobasidium (Lagerheim and Patouillard, 1892; Bandoni, 1957) are distinctive enough to warrant separating it from the genus Tremella to which it is probably related. This relationship was recognized by Lagerheim and Patouillard (1892) when they placed it in the Tremellinees

(Tremellaceae) of Brefeld (1888). However, Möller (1895) considered the chain-linked basidia of sufficient importance to erect a new family, Sirobasidiaceen (Sirobasidiaceae), just for this genus. That separation has persisted until today and its general acceptance is apparent in recent works by Ainsworth (1971) and McNabb (1973).

In a recent review of the genus Sirobasidium, Kobayasi (1962) recognised six species. This excluded S. cerasi Bourd. et Galz. (1909) for reasons first outlined by Boedijn (1934), and it also excluded S. brunnea Lloyd (1923) because of its incomplete description. Kobayasi presented a table of characteristics for the six species but it contained information only from the original descriptions. A review of the relevant literature (Lagerheim and Patouillard, 1892; Möller, 1895; Coker, 1920, 1928; Boedijn, 1934; Martin, 1936; Olive, 1946, 1947; Ramakrishnan and Subramanian, 1951; Lowy, 1956, 1971; Bandoni, 1957) has led to the reconstruction of Kobayasi's table which is presented here in Table I. The terminology has been changed to agree with that presented in Chapter 2 of this thesis. It is apparent that the six species are not very distinctive one from the other.

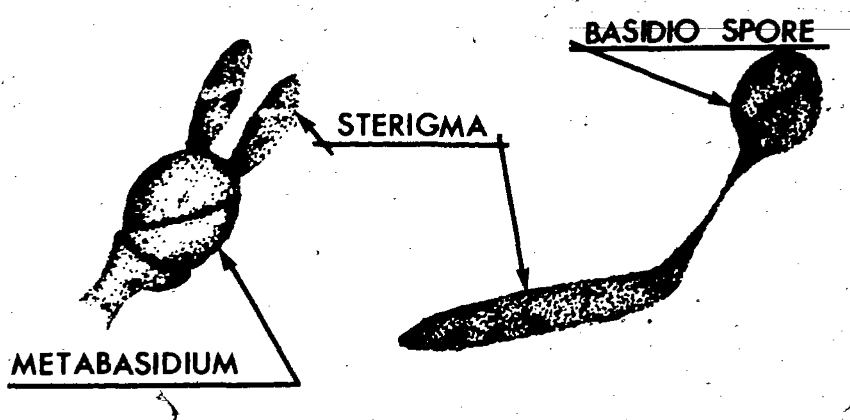


TABLE I

Summary of features of several species of Sirobasidium as derived from published reports.

	S. magnum	S. indicum	S. sanguineum	S. brefeldianum	S. albidum	S. japonicum
Basidia	7-10 X 17-20	8-13 X 12-32	11-13 X 13-20 12-15 X 24 10-12 X 18-20 14-17 X 15-18 10-12 X 18-20	11-18 X 17-49 11-13 X 22-40	14-20 X 15-22 12 X 15	4-7 X 15-37
Sterigmata	4-5 X 15-19	3-7 X 11-21	5-8 X 12-23 6-8 X 15-23 6-8 X 17-20 6-7 X 15-19 6-8 X 17-20	7-8 X 22-24 7-13 X 16-28 8 X 13-14 6-9 X 13-21	7-9 X 16-26 6-10 X 24-26	4-5 X 10-15
Spores	-	-	6-8 X 7-9 7-9 X 9-11 - X 9-11	- X 6-8	-	4-5 X 5-6
Cells per basidium	predominantly 2 up to 4	predominantly 2 up to 4	predominantly 4 predominantly 2 only 4	predominantly 2 up to 4 only 2	predominantly 4 only 4	2 to 4
Basidia per chain	2 - 8	2 - 5	2 - 4	2 - 6 2 - 7 3 - 10	2 - 8	2 - 4
Basidiocarp length	20-45 mm	3-5 mm	3-20 mm 12-37 mm 4-20 mm ?-20 mm	?-3 mm 0.4-1.3 mm 0.5-2 mm	2-4 mm	20-40 mm
Basidiocarp thickness	20-25 mm	1-1.5 mm	1-5 mm ?-4 mm	0.2-0.5 mm	-	-
Basidiocarp colour	tawny to ocraceous tawny	reddish brown	-buff-coloured to brownish -blood red -pale amber to reddish brick -ocraceous buff -light tan to reddish amber	-hyaline to whitish -white -watery-smokey to dusky white (flesh tint in some)	-hyaline to whitish	-milk white to partly orange-red

• Information for this table drawn from: Lagerheim and Patouillard, 1892; Möller, 1895; Coker, 1920, 1928; Boedijn, 1934; Martin, 1936; Olive, 1946, 1947; Lowy, 1956, 1970, 1971; Ramakrishnan and Subramanian, 1951; Bandoni, 1957. Unless otherwise indicated, measurements are given in um. Measurements that were reported in fractions of um were rounded to the nearest um.

were described before the nature of the basidiospores was discovered. That eliminates what could be a critical feature in distinguishing among species. Turning to the sterigmata (epibasidia), a great variation in size is evident even within a single fruiting body. As Martin (1936) pointed out for S. sanguineum, "Spores <sterigmata> borne on the lower basidia, hence deeply immersed in the gelatinous matrix, are long and narrow, 22-26 X 5-7 u while those borne at the surface are shorter and broader, 14-17 X 7-9 u." Between collections the range of sizes overlaps greatly. These facts make epibasidia a poor feature to use for species differentiation.

The same problems arise to a somewhat lesser degree with the sizes of the basidia proper. Nor is the number of cells per basidium very helpful, since quantitative information on the numbers of basidia with one, two, three, or four cells was not given. The fact that all types occur with regularity makes the the number of cells per basidium of questionable value for species differentiation. The displacement of septa within the basidium is of some value in S. japonicum where the majority seem to be transverse as they are in Auriculariaceous fungi. However, septation is of little value for distinguishing among the others.

The number of basidia in a chain overlaps for all the collections reviewed, except S. brefeldianum. In its case the feature seems worthy of note. However, even this may be shaky as Lagerheim and Patouillard (1892) state in their original description of S. sanguineum, "A la surface du Champignon, les filaments portent des chaînes de basides...mais ici, ces basides se séparent aisément les unes des autres lors de la formation des spores, de telle sorte qu'il ne nous a pas été possible d'observer le nombre d'articles composant chaque série; d'ordinare, on voit seulement de deux à quatre basides en place." The number of basidia per chain would also undoubtedly vary somewhat with the maturity of a fructification. The small differences reported between the five species excluding S. brefeldianum seem of little value for species delimitation.

This leaves shape and size of fructification, consistency, and colour to separate the remaining four collections one from the other. These features must be considered cautiously. Size can vary from substrate to substrate or under different environmental conditions. Without other supportive evidence of difference, it is a poor feature on which to erect a new species. Colour can vary greatly even within one species of jelly fungus. As Martin (1936) pointed out in describing some collections of what he considered to be S.

sanguineum, "No. 40 is obviously an older fructification than No. 41 and its pallid appearance may well be due to the fact that the color has been washed out, as not infrequently happens in the case of tremellaceous fungi with red or yellow tints." Brough (1974) in controlled laboratory cultures of *Tremella globospora* found striking colour differences in separate but genetically identical basidiocarps on a single agar plate. He urged caution in using colour as a feature for separating species of *Tremella*.

The white colour of *S. albidum* would separate it from the remaining three collections in the table. They in turn are all coloured brown with various degrees of reddish tone. The fruiting bodies of *S. magnum* are strikingly larger than those of *S. sanguineum* and *S. indicum*, but the latter two do not seem to differ essentially. It could be possible that these three are different collections of the same species. Without mating tests and basidiospore characteristics, a decision is difficult. It is clear that the taxonomy is in need of yet another examination.

Keys to the species of *Sirobasidium* have been presented by Ramakrishnan and Subramanian (1951) and by Lowy (1956, 1970, 1971) but they do not include the six species presently recognized. I have

revised these keys to include all the species of the genus *Sirobasidium* that have been reported.

REVISED KEY TO THE SPECIES OF *SIROBASIDIUM*

1. Metabasidia predominantly 3 or 4 celled 2
1. Metabasidia predominantly 2 celled 4
    2. Basidial septa predominantly transverse and oblique; basidia clavate to cylindric .... *S. japonicum*
    2. Basidial septa predominantly vertical; basidia globose to short fusiform 3
3. Basidiocarp hyaline to whitish when fresh .... *S. albidum*
3. Basidiocarp buff-coloured to reddish-brown when fresh .... *S. sanguineum*
    4. Fructification large, several cm long and 1-2 cm thick .... *S. magnum*
    4. Fructification small, several mm long and 1-2 mm thick 5
5. Basidia up to 10 or more per chain; basidiocarp hyaline to whitish when fresh .... *S. brefeldianum*
5. Basidia 5 or less per chain; basidiocarp reddish-brown when fresh .... *S. indicum*

Using this key, my collections fit the description of S. magnum. However, as I have already pointed out, the characteristics used to delimit the species of Sirobasidium are weak and it is possible that future mating tests will show that some of the above species are synonymous. For the present, I will conform to the published descriptions and accept S. magnum as a distinct species.

The purpose of this chapter was to compare my collections microscopically with each other and with syntype material of S. magnum from Java and to do mating tests between the yeast isolates derived from those collections. In this way I hoped to establish the identity of my collections with certainty.

MATERIALS AND METHODS

Yeast strains used were derived by spore fall from fruiting bodies TWF 11, which was collected at Shimoda, Japan, in November 1968, and TWF 56 and 58, which were collected at Bislig, Philippines, in February 1969. All were growing on decaying wood and were air dried after collection. Several weeks later, these basidiocarps were revived in distilled water and suspended over malt extract agar. Yeast cultures developing from single spores were numbered according to the collection from which they were obtained.

A syntype specimen of Sirobasidium magnum was kindly supplied by the Bogor Botanical Gardens, Java via Dr. R.J. Bandoni at the University of British Columbia. This specimen had been preserved in alcohol from which it had been removed and dried for mailing. I am uncertain as to whether basidiospores were still present in this material.

Microscopic features of the basidiocarps were measured on a Zeiss Standard Universal microscope using an oil immersion objective, phase optics and a calibrated eyepiece micrometer. The basidiocarps were revived in water for 1-2 hours and fragments were mounted in 1%

potassium hydroxide for observation. Twenty different examples were measured for each structure considered. These were then statistically treated for comparisons.

Yeast strains were maintained on a solid or liquid medium called yeast soytone broth or agar (YS) which contained: Difco yeast extract, 1.0 g; Difco soytone, 1.5 g; glucose, 10.0 g; magnesium sulphate septahydrate, 0.2 g; calcium chloride, 0.1 g; distilled water, 1 litre; Difco Bacto agar, 15.0 g for solid media. This medium was later simplified to yeast extract (YE) broth or agar (Difco yeast extract, 3.0 g; glucose, 10.0 g; distilled water, 1 litre; Difco Bacto agar, 15.0 g for solid media) with equally good results. A chemically defined medium was also used on occasion but only for the cultivation of the yeast cells. It was designated YNB and consisted of 6.7 g of Difco yeast nitrogen base (detailed contents given in the Difco Manual, Difco Laboratories, Detroit, Michigan) in 1000 ml distilled water with the addition of 10.0 g glucose. This was autoclaved before use. Mating tests were performed on conjugation medium (CJM) (Difco yeast extract, 3.0 g; glucose, 1.0 g; distilled water, 1 litre; Difco Bacto agar, 15.0 g for solid media). Before autoclaving, all media were adjusted to pH 6.8 with 0.1 N sodium hydroxide. Liquid cultures were incubated in 250 ml erlenmeyer flasks with 50 ml of medium. They

were held at 25 ^{o}C and shaken continuously in a PsycroTherm incubator (New Brunswick Scientific Corporation) at 50 rpm. Inoculum for liquid cultures consisted originally of a loop of cells from agar culture. Successive transfers consisted of 0.05 ml aliquots taken from stationary phase cultures (36-48 h).

Mating tests were performed by inoculating a speck of each of two yeast strains side by side near the circumference of a petri dish of CJM. After mixing the two strains thoroughly with an inoculating loop, three radiating streaks were drawn across the plate away from the area of mixing. Often, better conjugation occurred in the streaks than in the area of mixing. An alternate method which discouraged yeast cells from overgrowing the cross consisted of covering the cells, mixed on YE agar, with a coverslip which had previously been dipped in 95% ethanol and then flame sterilized. Using this technique, fruiting bodies developed when dikaryotic hyphae resulting from conjugation reached the edge of the coverslip.

In all cases crosses were incubated at 25-28 ^{o}C in a Percival controlled-environment chamber in the dark. Crossed isolates were examined for the presence of conjugation tubes after 12-24 hours, and for hyphae with clamp connections after 48 hours. If the

clamp-bearing mycelium was left to grow in the dark for approximately one month, fruiting bodies formed. Once basidiocarps had formed, the dishes were removed to a second incubator at the same temperature but with a 10-hour light and 14-hour dark regime. Light was supplied by one 25-inch cool-white fluorescent lamp of 20 watts power. This illumination gave better production of basidia and spores than occurred on basidiocarps incubated completely in the dark.

A limited number of assimilative tests was done employing isolate TWF 58-23 with Difco yeast nitrogen base for carbon compounds and Difco yeast carbon base for nitrogen compounds. Glucose, D-mannitol and D-xylose were obtained from Difco; D-cellobiose, from Sigma Chemical Corp.; and inositol from Eastman Organic Chemical Co. All carbon compounds were used to give 2 g carbon per litre and nitrogen compounds to give 0.2 g nitrogen per litre. For these tests the media used were filter sterilized and the pH was not adjusted.

More extensive assimilative tests were kindly performed by the Yeast Division of the Centraalbureau voor Schimmelcultures, Julianalaan 67a, Delft, Netherlands. The strains used were TWF 11-5, 11-7, 58-23 and 58-27 and they have been placed in the CBS culture

collection as CBS 6806, 6805, 6804 and 6803 respectively. In addition, strains 56-1, -2, -12 and -20 have been placed in the American Type Culture Collection, Washington, D.C. and their aquisition numbers are 34209, 34210, 34211 and 34212 respectively.

RESULTS

In gross aspect, the basidiocarps of the syntype of *S. magnum* and collections TWF 11, 56 and 58 were very similar. All three specimens were large, gelatinous, and *Tremella*-like, lobed and folded, and in the same colour range. Two are shown in Plate VI, Fig. 50. The syntype specimen was a somewhat lighter shade probably because it had been stored in alcohol. The fruiting bodies produced in laboratory culure were also similar. They were not as thick and the colour varied from light tan to reddish brown. One, arising from the cross of TWF 58-23 (A_1B_1) and TWF 11-2 (A_2B_3), was examined microscopically. It is shown in Plate VI, Fig. 49.

Microscopically, the basidia, sterigmata and basidiospores of the basidiocarps listed above gave the measurements shown in Figs. 1 and 2. Twenty examples of each structure were measured. Widths of the sterigmata and diameters of the basidiospores were measured at the widest point. A histogram of the results is shown in Fig. 1. The means and standard deviations are shown in Fig. 2. A multiple discriminant analysis gave the Mahalanobis D^2 values listed in Table II. A value in excess of 2 is considered a significant difference ($P = 0.05$).

Figure 1. Histograms of measures of microstructures of five different basidiocarps. Numbers of structures are given on the Y axes while measurements in micrometers are given on the X axes.

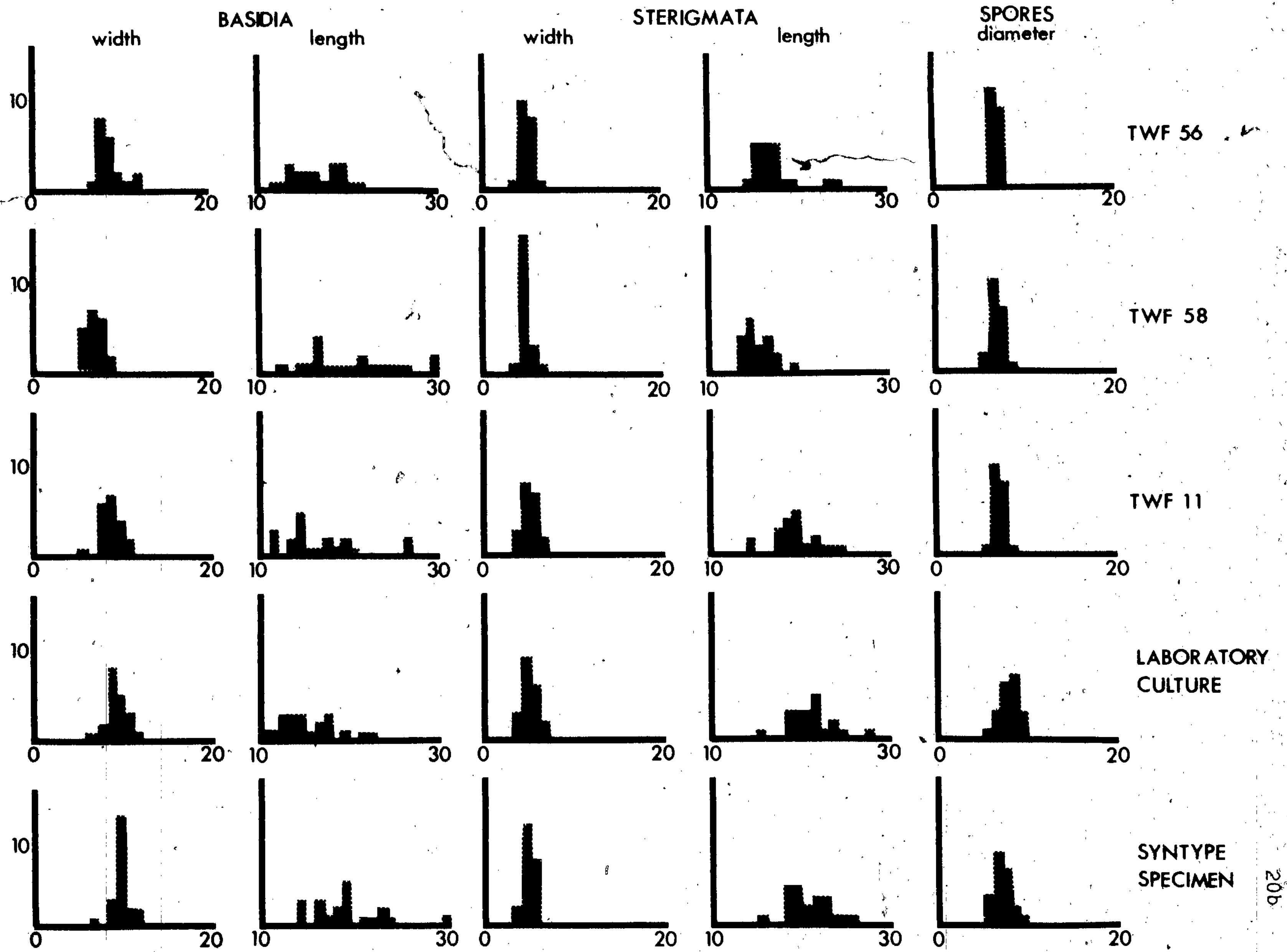
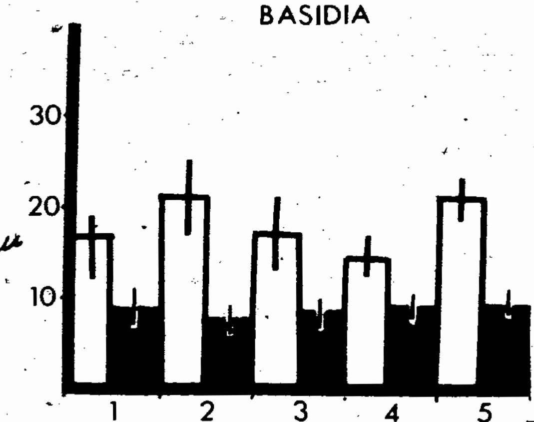
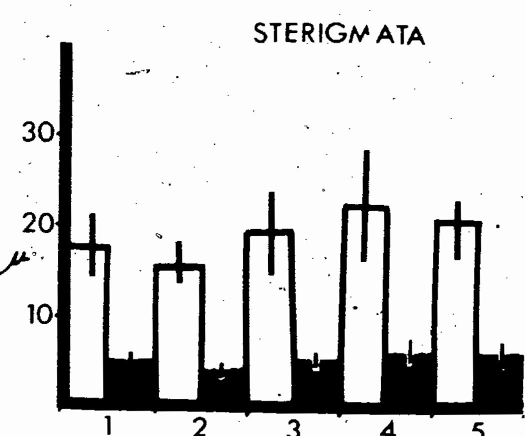
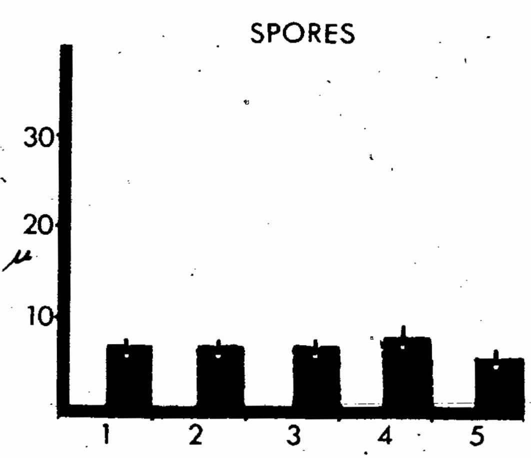

BASIDIA
width
length
STERIGMATA
width
length
SPORES
diameter
TWF 56
TWF 58
TWF 11
LABORATORY
CULTURE
SYNTYPE
SPECIMEN

Figure 2. Histograms of means and standard deviations for the measurements presented in Figure 1.







1=TWF 56
2=TWF 58
3=TWF 11
4=Laboratory–cultured basidiocarp
5=Syntype _S. magnum_ basidiocarp

length

width

TABLE II

D^2 Mahalanobis statistic for distance between measured structures for various fruiting bodies of S. magnum.

	TWF 56	TWF 58	TWF 11	Lab culture	Syntype
TWF 56	-	-	-	-	-
TWF 58	9.796	-	-	-	-
TWF 11	1.020	7.937	-	-	-
Lab culture	3.072	19.321	1.496	-	-
Syntype of S. magnum	5.041	27.828	2.002	1.664	-

The "spores" shown for the syntype are questionable. The globose structures observed and recorded here lacked the conspicuous apiculus present in the spores of the other specimens.

After one week on YE agar, colonies of yeast strains derived by spore fall were mucous-slimy with a glistening surface. They were thick and white at first but turned creamy yellow after long incubation. The yeast cells were ellipsoid, ovoid or spheroid, and they were encapsulated when grown on the media used (Plate I; Figs. 3, 4). Budding was predominantly monopolar, successive buds arising from the same point. The size of the cells varied with the age of the culture and the medium used but generally fell within the range 3.5-6 X 5-7 u. When colonies from single isolates were inverted over fresh YE agar and incubated for up to one month, new colonies appeared in small numbers on the lower plate. These colonies arose from ballistospores which could germinate by budding or by repetition (Plate I; Fig. 7).

Results from the four strains tested for assimilation of carbon compounds as outlined by Van der Walt (1970a) are shown in Table III. Growth curves for compounds tested in this laboratory are shown in Figs. 53 and 54 of Chapter 3 and they agree with the results from the

TABLE III

Results of assimilative tests for various carbon compounds, using yeast isolates from basidiocarps TWF 11 and TWF 58.

Carbon Compound	58-27 CBS 6803	58-23 CBS 6804	11-7 CBS 6805	11-5 CBS 6806
glucose	+	+	+	+
galactose	-	-	-	-
L-sorbose	+	+	+	+
sucrose	-	-	-	-
maltose	-	-	-	-
cellobiose	+	+	+	+
trehalose	+	+	+	+
lactose	+	+	+	+
melibiose	-	-	-	-
raffinose	-	-	-	-
melezitose	-	-	-	-
inulin	-	-	-	-
soluble starch	-	-	-	-
D-xylose	+	+	+	+
L-arabinose	- or weak +	-	- or weak +	- or late +
D-arabinose	late +	late +	+	+
D-ribose	- or weak +	- or weak +	weak or late +	weak or late +
L-rhamnose	weak +	+	+	+
ethanol	+	+	+	+
glycerol	+	-	late +	weak or late +
erythritol	weak or late +	weak or late +	- or weak +	- or weak +
ribitol	+	+	+	+
galactitol	-	-	-	-
D-mannitol	+	+	+	+
D-sorbitol	+	+	+	+
-methyl-D-glucoside	-	-	+	-
salicin	late +	+	+	+
DL-lactic acid	-	-	weak or late +	weak or late +
succinic acid	-	-	-	-
citric acid	+	+	+	+
inositol	+	+	+	+
glucono- -lactone	+	+	+	+
2-keto-gluconate	-	-	+	+
5-keto-gluconate	-	-	+	+

Note: all experiments run for 21 days at 25 °C
+ = positive growth
- = no growth

Centraalbureau. In addition, all these strains were non- fermentative and growth at 37 °C was negative. Assimilation of potassium nitrate was negative while sodium nitrite was weakly positive and ammonium nitrate was positive. All these strains produced starchlike compounds when grown in acidic media. Work at the Centraalbureau showed aerobic mycelial growth on cornmeal agar and I have substantiated this result.

The outcome of crossing 24 isolates of TWF 58 and 56 in all combinations is shown in Table IV. Four different mating types are evident and they have been arbitrarily labeled A_1B_1, A_2B_2, A_1B_2, and A_2B_1. Half the crosses resulted in the formation of conjugation tubes, but only half of these in turn produced clamped mycelia. Blastospores were often produced at the ends of conjugation tubes if mating had not occurred (Plate I; Fig. 9). Conjugating pairs of cells are shown in Plate I, Fig. 10 and Fig. 11, while dikaryotic hyphae and clamp formation are shown in Plate I, Fig. 12 and Plate III, Fig. 25 respectively.

In addition to testing strains from the Philippines, 10 isolates of TWF 11 from Japan were crossed in all combinations with each other and with TWF 58-27 (A_1B_1) and TWF 58-23 (A_2B_2). The crosses within TWF 11 showed a similar pattern to those of the Philippine

TABLE IV

Results of crosses in all combinations of 24 isolates of *S. magnum* yeasts from collections TWF 56 & 58

		56–1	56–3	56–4	56–6	56–15	58–26	58–27	58–31	58–37	56–8	56–20	56–21	56–2	56–9	56–11	56–13	56–18	56–19	56–12	56–5	58–23	58–24	58–25	58–28
56–1	A_1B_1	–	–	–	–	–	–	–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
56–3			–	–	–	–	–	–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
56–4				–	–	–	–	–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
56–6					–	–	–	–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
56–15						–	–	–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
58–26							–	–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
58–27								–	–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
58–31									–	–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
58–37										–	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	+
56–8	A_1B_2										–	–	–	+	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)
56–20												–	–	+	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)
56–21													–	+	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)
56–2	A_2B_1													–	–	–	–	–	–	–	–	–	–	–	–
56–9															–	–	–	–	–	–	–	–	–	–	–
56–11																–	–	–	–	–	–	–	–	–	–
56–13																	–	–	–	–	–	–	–	–	–
56–18																		–	–	–	–	–	–	–	–
56–19																			–	–	–	–	–	–	–
56–12	A_2B_2																			–	–	–	–	–	–
56–5																					–	–	–	–	–
58–23																						–	–	–	–
58–24																							–	–	–
58–25																								–	–
58–28																									–

NOTE : – = no conjugation tubes

(+) = conjugation tubes but no clamped mycelium

\+ = conjugation leading to clamped mycelium

A_1B_1, A_1B_2, A_2B_1, A_2B_2 = arbitrarily assigned mating types

isolates (Table V). However, all crosses between the Japanese and Philippine isolates that resulted in conjugation tubes also led to clamped mycelia. This indicated the presence of at least a third and fourth incompatibility factor B_3 and B_4, and the isolates in Table V have been appropriately labeled.

Fruiting bodies resulting from crosses of 58-23 X 11-2 and 58-23 X 11-1 were resuspended over agar and yeast colonies arising from spore fall were mated. These crosses, in turn gave new fruiting bodies which also produced basidiospores.

TABLE V

Results of crosses in all combinations of 10 isolates of S. magnum yeasts from collection TWF II

	Mating type	11–5	11–10	11–1	11–4	11–11	11–2	11–6	11–7	11–8	11–9
11–5	A_2B_4	–	–	–	–	–	(+)	+	+	+	+
11–10			–	–	–	–	(+)	+	+	+	+
11–1	A_2B_3			–	–	–	+	(+)	(+)	(+)	(+)
11–4					–	–	+	(+)	(+)	(+)	(+)
11–11						–	+	(+)	(+)	(+)	(+)
11–2	A_1B_4						–	–	–	–	–
11–6	A_1B_3							–	–	–	–
11–7									–	–	–
11–8										–	–
11–9											–

NOTE: – = no conjugation tubes

(+) = conjugation tubes but no clamped mycelium

\+ = conjugation leading to clamped mycelium

A_1B_3, A_1B_4, A_2B_3, A_2B_4 = assigned mating types

DISCUSSION

The decision as to whether two organisms or populations of organisms are conspecific can be philosophically and technically complex. In the end no single piece of information is in itself sufficient. The trend with most of the larger fungi is to concentrate on morphological features and a limited number of chemical tests. When there are few morphological features, as with the yeasts, the trend is to concentrate on assimilative patterns. These trends are necessitated even for distinctions above the species level because of the lack of sexual stages in imperfect yeasts.

The value of these assimilative tests has been questioned for some yeasts (Storck, Alexopoulus and Phaff, 1969; Bandoni, Johri and Reid, 1975), and their meaning here should therefore be viewed cautiously. Where possible one should have, in addition to physiological and morphological features, information as to whether mating occurs between organisms; that is, mating which results in a fertile filial generation. Even here detailed cytology may be necessary to determine whether hybridization has taken place. However, as stated by Bandoni, Johri and Reid (1975) for

Sporobolomyces Kluyver et van Niel, "We assume that conjugation, formation of dikaryotic mycelia, and chlamydospore production can occur only if the mating strains are of the same species. Interspecific hybridisation is possible, but the large number of interacting genes required for production of the dikaryotic stage would make this possibility seem unlikely."

On the basis of size, colour and macroscopic morphology, the basidiocarps of TWF 11, 56 and 58 are little different each from the other or from the syntype of _S. magnum_ from Bogor. The histogram comparing the sizes of microscopic structures of the above three basidiocarps and of the one produced in the laboratory shows essential similarity. Those parameters with little variability are closely grouped together while those with great variation overlap considerably. Although the discriminant analysis showed significant differences between some of these parameters, the differences were as great between the four known to be conspecific by mating tests as they were between those and the syntype. However, the lack of apiculi on the globose structures assumed to be basidiospores in the Bogor material, could be critical. It is possible that they were indeed spores but that they were immature and were physically disloged during preparation of microscope mounts. This assumes that all the mature spores were washed off by the alcohol preservative.

On the strength of macroscopic and microscopic morphology, cultural characteristics, conjugation, dikaryon formation and basidiocarp formation, it is reasonable to conclude that collections TWF 11, 56, and 58 are conspecific. Their conspecificity with the syntype of S. magnum is strongly inferred on the basis of morphology. Absolute confirmation would require mating tests with living collections from Java.

The yeast phases of all the living collections were grossly similar in agar culture. On the basis of the assimilative tests performed and the production of globose ballistospores, these yeasts are similar to those in the imperfect genus Bullera Derx. (Phaff, 1970). In the absence of information as to the origin of these yeasts, the four strains in Table IV would probably be considered different species of the genus Bullera Derx. However, derivation of the isolates, conjugation, and the formation of dikaryotic mycelium and basidiocarps show this to be untrue.

The incompatibility pattern described here for S. magnum is a modified tetrapolar pattern that is identical to that described first for Tremella mesenterica (Bandoni, 1963) and later for T. globospora Reid (Brough, 1974). In addition, the physiology and morphology of

the cells are similar and the nature of the sexual reaction indicates the presence of a system of erogens similar to that of *T. mesenterica* (Bandoni, 1965). These features of close similarity reinforce the general consensus that these genera lie in closely allied families.

There are also certain similarities between the yeasts of the imperfect genus *Cryptococcus* Kutzing emend. Phaff et Spencer and those of *Tremella* and *Sirobasidium*. Kobayasi (1962), on the basis of cultural and physiological criteria, found that the yeast phase of *S. japonicum* Kobayasi coincided with *Cryptococcus diffluens* (Zach) Lodder et Kreger-van Rij. He did not seem to consider the fact that he had observed ballistospore production by the yeast phase. Also, a study of extracellular heteropolysaccharides from several species of *Tremella* and from *C. laurentii* (Kufferath) Skinner was found to reinforce the positive relationship between these yeasts that was suggested by morphology and physiology (Slodki, Wickerham and Bandoni, 1966).

Treating the yeast phase of *S. magnum* as a distinct entity, and using a taxonomic key (Lodder, 1970a), it can be identified as belonging to the imperfect genus *Bullera* rather than *Cryptococcus*. This is because the yeast phase of *S. magnum* produces subglobose apiculate

ballistospores. However, as Phaff (1970) states, should inositol-assimilating species of *Bullera* cease to produce ballistospores and be studied subsequent to the loss, they would be placed in the genus *Cryptococcus*. Such considerations had previously led Lodder and Kreger-van Rij (1955) to suggest that *Cryptococcus* might have arisen from ballistospore-forming yeasts by the loss of spore-forming ability.

The discovery of ballistospores from the yeast phase of *S. magnum* in addition to its affinities to *Tremella* is gratifying. The closeness of these two genera and their subsequent similarities to *Cryptococcus* and *Bullera* serve to increase the probability that the four genera are interrelated and that at least some species of *Cryptococcus* and *Bullera* have basidiomycetous origins.

CHAPTER TWO

THE LIFE CYCLE OF SIROBASIDIUM MAGNUM IN CULTURE

INTRODUCTION

Before describing work on the life cycle of *S. magnum*, it would be worthwhile to examine the terminology used in the description of phragmobasidiomycetous básidia. Because this group includes Basidiomycetes with highly variable basidia that may be transitional in form, it has been subject to considerable taxonomic revision over the years. These revisions have not been unanimously accepted and much controversy has surrounded them.

Any natural classification system is an attempt to arrange organisms in a way that reflects their evolutionary relationships. With vascular plants and vertebrates, this task has been considerably assisted by an extensive fossil record. Not so for the fungi. Classification schemes for this group have depended almost exclusively on interpretation of present day forms. The assumption is that at least some of these closely resemble the ancestral types from which

later forms arose. In existing floras, candidates for such "primitive" types are usually those with relatively simple and relatively unstable morphology. Certainly such fungi abound in the Phragmobasidiomycetidae, and the weight of present day opinion regards them as reminiscent of Homobasidiomycete ancestors. However, notable exceptions to this view have been proposed (Bessey, 1950; Rogers, 1934; Martin, 1945) and the whole question of Basidiomycete phylogeny is amply covered in reviews by Saville (1955, 1968).

Much attention, then, has been focused on the Phragmobasidiomycetidae for phylogenetic reasons. In an extensive review of such discussions, Talbot (1954) reiterates Rogers' opinion (1944) that, "..the basidium and associated characters are reliable indications of kinship, and that hymenial variations are comparatively recent and trivial." Micromorphology consequently engages the spotlight in these phylogenetic discussions and the terminology used to describe the basidium is crucial. It is not surprising that various terminologies have been suggested and that controversy has arisen around them. Talbot (1954) has summarised the various arguements and selected the terminology proposed by Donk (1931, 1954) as the most logical. He prepared a pictoral summary of the major terminologies and it has been reproduced in The Dictionary of the

Fungi (Ainsworth, 1971). Talbot's article sparked further controversy which spanned several years (Donk, 1956, 1958; Martin, 1957). The story is well chronicled by Lowy (1968) and Talbot (1973). Their terminology will be the one used here, and the pertinent words, as originally defined by Talbot (1954), are:

BASIDIUM - that organ of the Basidiomycetes which is partly the homologue of the ascus, and which following karyogamy and meiosis bears the basidiospores either directly or on extensions of the gonotocont wall, the sterigmata. The term is taken to include the probasidia, metabasidia and sterigmata as parts of the whole basidium.

PROBASIDIUM - that part or stage of the basidium in which karyogamy occurs, i.e. the primary basidial cell. Included also in this term are the teleutospores of rusts, the chlamydospores of smuts, and the more or less persistent or resistant cells in the same stage of development in the Auriculariaceae. The term is intended to denote the "first stage of the basidium" rather than "that which precedes the basidium".

METABASIDIUM - that part or stage of the basidium in which meiosis of the diploid nucleus occurs. In many basidia it obviously

replaces <follows in developmental sequence> the probasidium. The term is intended to denote the final stage of the basidium as an antithesis of the first stage, or probasidium.

STERIGMATA - those parts of the basidium which come between the metabasidium and the basidiospores, or the elongations of the metabasidium through which the nuclei migrate to the spores which are borne terminally. Each sterigma is composed of a basal, filamentous or inflated part called the protosterigma, and an apical point called the spiculum on which the spore is borne.

Two further terms in this original article were redefined by Talbot (1968) in response to an article by Lowy (1968). These redefinitions were necessary because of accumulating cytological evidence concerning primary septa (those arising as a result of nuclear division) and secondary or adventitious septa (those arising in the absence of nuclear division). These two terms were redefined as follows:

PHRAGMOBASIDIUM - a basidium whose metabasidium is divided by primary septa.

HOLOBASIDIUM - a basidium whose metabasidium is not divided by primary septa but may sometimes become adventitiously septate.

The fact that these terms of reference are used here should not be taken to imply that I accept totally the classification proposed by Talbot (1968). That the question is still open to discussion is emphasized by recent publications (Lowy, 1968, 1969; Talbot, 1968, 1970; Donk, 1972a, 1972b, 1973a, 1973b,1973c). The present sentiment is perhaps best expressed by Talbot (1970) in response to criticism from Lowy (1969). He writes, "Indeed I fully expect it <a classification of Basidiomycetes> to be found imperfect; my diffidence <in proposing it> however, was due to my temerity in suggesting any classification at all, in an area where much more knowledge is generally needed. Further arguement at this stage is pointless: what is required is work."

Cultural work on species of the genus *Sirobasidium* is extremely limited. Möller (1895) did a limited amount with *S. sanguineum*, reporting yeastlike growth on nutrient media. Later, Kobayasi (1962) examined the yeast phase of *S. japonicum* on nutrient media and reported that it sparingly produced ballistospores. He also noted seeing conjugation between one pair of yeast cells and he reported

growing immature basidiocarps on malt agar. Much more work has been done on other tremellaceous fungi and that work has some bearing on studies with Sirobasidium. I will, however, restrict my review to tremellaceous fungi with budding haploid assimilative stages. This will exclude fungi with yeast phases in the Septobasidiales, Auriculariales and Ustilaginales.

In a recent review, Kobayasi and Tubaki (1965) report budding growth in Sirobasidium, Tremella and Holtermannia Sacc. et Trav. Of these three genera, Tremella has been the most extensively studied in culture, beginning with the studies of Brefeld (1888, 1908) and Möller (1895) where basidiospores were germinated and budding yeast cells grown. These authors also diagrammed what we now know to be conjugation tubes arising from some of the yeast cells. Such descriptions were also forthcoming from Whelden (1934), Kobayasi (1937, 1939) and Bandoni (1961). However, Bandoni (1963) was the first to describe formation of dikaryotic clamped mycelia following conjugation and the modified tetrapolar incompatibility system governing its formation. Kobayasi and Tubaki (1965) later reported conjugation leading to dikaryotic mycelia in Tremella fuciformis and Holtermannia corniformis Kobayasi. In addition, they described the formation of fruiting bodies of T. fuciformis in culture, but they

did not describe any special technique for producing them. Fruiting bodies of this fungus are commercially produced on a rice husk mixture in Taiwan, but inspection of a sample of these showed them to be infertile (Brough, 1970). Basidiocarps of the parasitic fungus _Tremella uliginosa_ Karst. (Koske, 1972) have been produced in culture with its ascomycete host but basidiospore production could not be obtained and the life cycle could not be completed. The only reported species which did complete its life cycle in culture was _Tremella globospora_ (Brough, 1970, 1974). Although the fungus was parasitic, it easily grew axenically on synthetic media and produced fertile basidiocarps.

A review of cytological studies of the lower Hymenomycetes has been presented by Talbot (1954). Again, these have concentrated on dimorphic genera other than _Sirobasidium_. They have in addition emphasized nuclear events in basidiocarps. Few articles have appeared describing nuclear events during yeast growth and conjugation (Bandoni and Bisalputra, 1971).

Brough (1970) has diagramatically summarized the life cycle of _Tremella globospora_ and that diagram would generally apply to other dimorphic tremellaceous fungi.

The purpose of the work described in this chapter was to discover the conditions necessary to have S. magnum routinely complete its life cycle under controlled laboratory conditions. An additional purpose was to elucidate the nuclear events accompanying the various stages in the life cycle.

MATERIALS AND METHODS

Yeast cultures were maintained on YE agar and broth, or on YNB broth (Chpt. 1). Conjugation for cytological staining was carried out using equal numbers of two mating strains from 36-hour cultures in YE broth. These were mixed and diluted to 5-7 X 10^5 cells per ml in liquid CJM (Chpt. 1). One ml of this mixture was transferred to each of several 25 X 75 mm sterile glass microscope slides in sterile petri dishes. After incubation for 12 to 24 hours at 25-28 ^oC, the liquid was poured off the slides. Next, they were either air dried or not before fixing for 20-24 hours in 70% ethanol with 0.56 g potassium hydroxide per 100 ml (N/10). Slides were removed from the fixative and rinsed for 2-5 minutes with tap water before staining for 2 hours in propionic haematoxylin (Henderson and Lu, 1968). After staining, the slides were washed in tap water, covered, and observed.

The same fixing and staining routine was employed for other stages in the life cycle by using smears on slides, agar cultures on slides, or squash mounts of structures stained in agar blocks. Basidia developed around crosses of fully compatible isolates on YE agar or potatoe dextrose agar (PDA). These crosses and basidiocarp production were carried out as described in Chapter 1.

Ballistospores and basidiospores were collected by suspending yeast colonies or fruiting bodies respectively over agar coated slides in sterile petri dishes (Carmo-Sousa and Phaff, 1962). The slides were then air dried, fixed and stained as described above.

Nuclei of living cells were observed on Fortner slides (Robinow, 1975) with YE agar containing 20% maltose.

To test the effects of carbon dioxide concentration and anaerobic atmospheres on yeast and hyphal growth, Gaspac anaerobic jars were used in conjunction with Gaspac carbon dioxide generators and anaerobic environment packs (Becton, Dickinson and Co., Cockeyesville, Maryland, U.S.A.). Atmospheres of increasing carbon dioxide concentration were obtained by using increasing numbers of generators. These gaspac anaerobic jars were also used as candle jars (a candle was lit inside the container before it was closed).

RESULTS

Upon staining, yeast cells were found to be uninucleate except when producing daughter cells (Plate I; Figs. 5,8). Occasionally a budding cell showed a single nucleus which was present in the daughter bud (Plate I; Fig. 8). This suggests that these yeasts may have a mode of cell division similar to that described for several basidiomycete yeasts by McCully and Robinow (1972a, 1972b). Ballistospores arising from yeast colonies were at first uninucleate, but swelled greatly and became multinucleate (Plate I; Fig. 6). One of the spores was observed to contain five nuclei. The spores germinated by producing buds directly, by producing buds at the ends and sides of short germ tubes, or by producing sterigmata with secondary ballistospores (Plate I; Fig. 7). The source of the ballistospores produced in small numbers by yeast cells was not determined with certainty. Yeast cells with sterigmata were not observed, but in any yeast colony there was always a very small number of enlarged, elongated cells (Plate IV; Fig. 41). Judging from the size of the ballistospores, it is likely that the elongated cells are their source.

When compatible yeast isolates were mixed under appropriate conditions, conjugation tubes were produced and compatible cells fused (Plate I; Fig. 10; Plate V, Fig. 43). The inflated area at the point of tube fusion is an artifact of fixing and staining, but indicates the flexibility or softening of the cell walls that must occur upon fusion. If conjugation did not occur, cells were capable of producing successive blastospores from the tips of conjugation tubes (Plate I; Fig. 9; Plate V, Fig. 43). The cell, with its attached tube, remained uninucleate, as did the blastospores produced. The dikaryotic mycelium (Plate V, Fig. 44) produced after tube fusion arose either from one of the conjugating cells at a point on the cell wall opposite to its conjugation tube (Plate I; Fig. 10) or from the actual point of fusion of the conjugation tubes (Plate I; Fig. 11). The dikaryotic mycelium produced clamp connections (Plate I; Figs. 11, 12) either immediately or after several non-clamped septa had been formed (Plate IV; Fig. 39). These septa were formed to cut off evacuated cells and may have been formed without accompanying nuclear division (adventitious septa).

The dikaryotic mycelium produces regular clamp connections (Plate I; Fig. 12; Plate III; Fig. 25) but is quickly overgrown by rapidly multiplying yeast cells. These yeast cells inhibit the mycelial phase

and interfere with the formation of typical basidiocarps. Covering mated cells with a coverslip was found not to inhibit conjugation but did inhibit growth of the yeast phase. Inhibition of yeast growth in this way is illustrated in Plate VI, Fig. 46. With the yeast cells so inhibited, dikaryotic mycelium from mated cells grew to the edge of the coverslip and there produced a typical pigmented basidiocarp (Plate VI; Fig. 49). Anaerobic environments prevented growth of both the yeast and mycelial forms and also prevented conjugation. A candle jar (oxygen reduced to approximately 10% and carbon dioxide elevated to approximately 10%) still permitted yeast overgrowth of crosses. However, carbon dioxide concentrations of approximately 15% with oxygen unadjusted inhibited yeast cells but allowed the dikaryotic mycelium to grow (Plate VI; Fig. 48). A comparison of cells mated under coverslips (Plate VI, Fig. 47), and in atmospheres of approximately 15% carbon dioxide with oxygen normal (Plate VI; Fig. 48) shows that there is a difference between the two. The hyphae in the second are pigmented while those in the first are not.

Basidial development was observed in agar surrounding mated cells and in basidiocarps, but most of the illustrations presented here were derived from specimens removed from around matings on PDA. A squash mount of hymenium removed from a fruiting body arising from a cross of TWF 58-23 and TWF 11-2 is shown in Plate V, Fig. 45.

Metabasidia with attached sterigmata photographed in situ are shown in Plate II, Figs. 13 and 14. Probasidia are shown before karyogamy (Plate III; Fig. 26) and after karyogamy (Plate III; Figs. 27, 28). Catenulate probasidia are shown in Plate II, Fig. 16 and in Plte IV, Fig. 40. Septate metabasidia are shown in Plate II, Fig. 17 and Plate III, Fig. 29. The majority of the metabasidia were divided into two cells by an oblique septum but occasionally they were divided into three or four cells. Developing sterigmata (Plate III; Figs. 30 to 32) were generally binucleate and exhausted the metabasidium upon their formation (Plate II; Fig. 18). The sterigmata were released (Plate II; Figs. 15, 19) as free structures and were usually binucleate (Plate II; Fig. 24). They were occasionally seen with a single nucleus and once with three nuclei. If the sterigmata were submerged, they produced extended secondary sterigmata (Plate II; Fig. 19) that had the appearance of "germination tubes". In some cases secondary sterigmata were seen with attached basidiospores (Plate II; Fig. 20).

Released basidiospores (Plate II; Fig. 23) were subglobose, apiculate and could germinate by repetition (Plate II; Figs. 21, 22) or by budding. They were uninucleate (Plate III; Fig. 33) and produced short germ tubes which in turn budded off uninucleate yeast

cells (Plate III; Figs. 34 to 37). In one case, a dikaryotic clamped mycelial filament was seen arising directly from a basidiospore (Plate III; Fig. 38) and therefore the occasional basidiospore must be dikaryotic.

An examination of the dikaryotic cells in Plate III, Figs. 25 to 27 shows a noticeable difference in size between the two members of a nuclear pair. This correlates with a difference in sedimentation rates between A_1 and A_2 cells on sucrose gradients (Chpt. 3).

The results of this cultural work are summarized in the life cycle diagram presented in Fig. 51 while major nuclear events are shown in Fig. 52.

Figure 51. Diagram of the life cycle of *Sirobasidium magnum* as determined through work in laboratory culture:

A. gelatinous basidiocarp on decaying wood;

B. microscopic detail of the hymenium showing various stages in basidial development;

C. caducous sterigmata, two with secondary sterigmata and basidiospores;

D. basidiospores germinating by repetition;

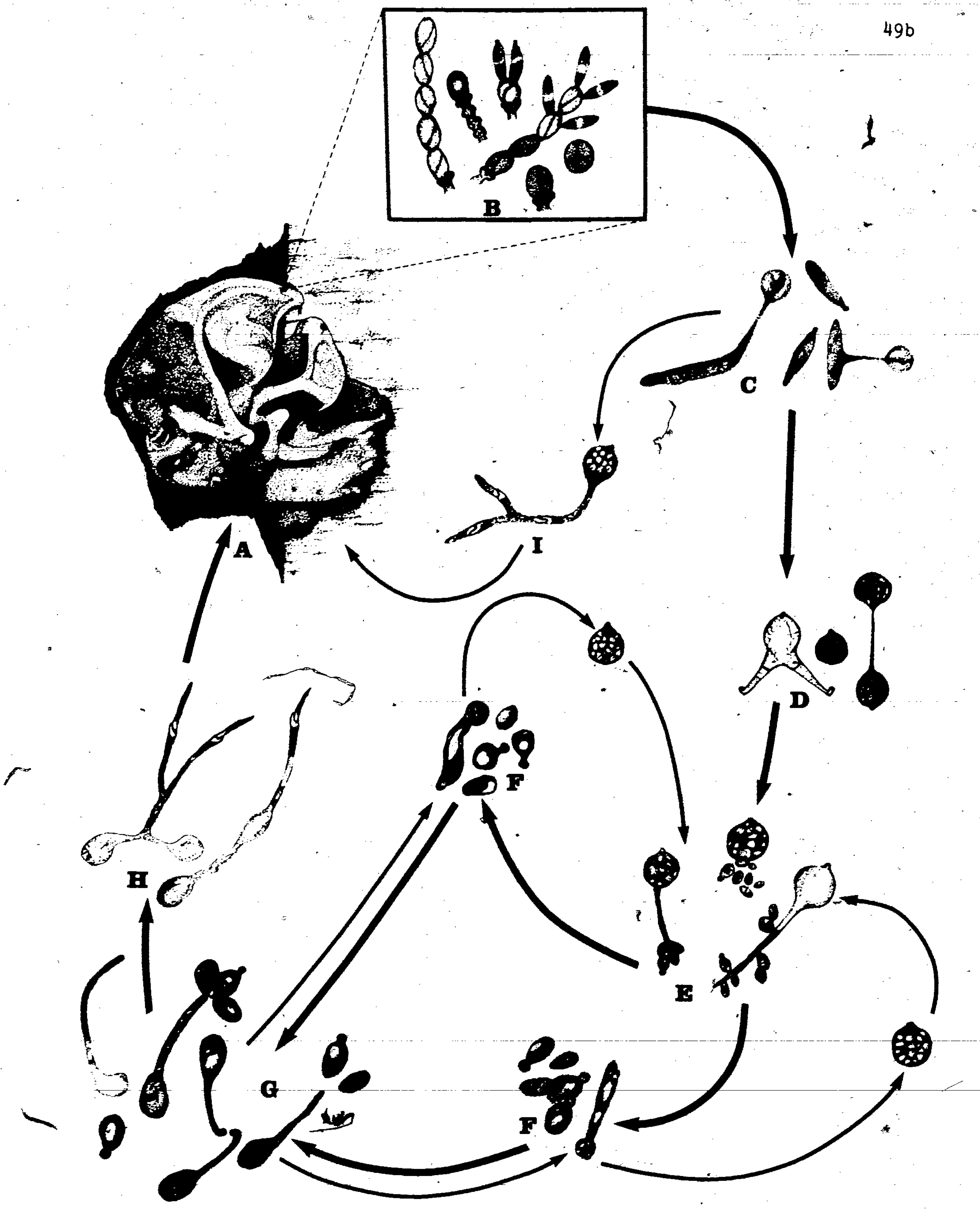
E. basidiospores germinating to produce yeast cells;

F. yeast strains which sparingly produce ballistospores from large inflated cells;

G. conjugation between compatible yeast cells some of which revert to budding growth if fusion with the opposite mating type has not occurred;

H. clamped mycelium arising from successful conjugants (This mycelium eventually produces a new basidiocarp.);

I. an occasional basidiospore which produces a clamped filament directly upon germination (This indicates a dikaryotic condition.)



B
C
I
A
D
F
E
H
G
F

Figure 52. Summary of nuclear events during the life cycle of Sirobasidium magnum:

A. uninucleate, haploid yeast cells that sparingly produce uninucleate ballistospores to repeat the yeast phase upon germination (For purposes of illustration, two fully compatible isolates are indicated.);

B. Conjugation between uninucleate yeast cells;

C. establishment of the dikaryotic stage in the life cycle;

D. growth of the dikaryotic clamped mycelium;

E. production of closely grouped dikaryotic cells as a prelude to the formation of catenulate probasidia which mature centrifugally;

F. karyogamy in the probasidium;

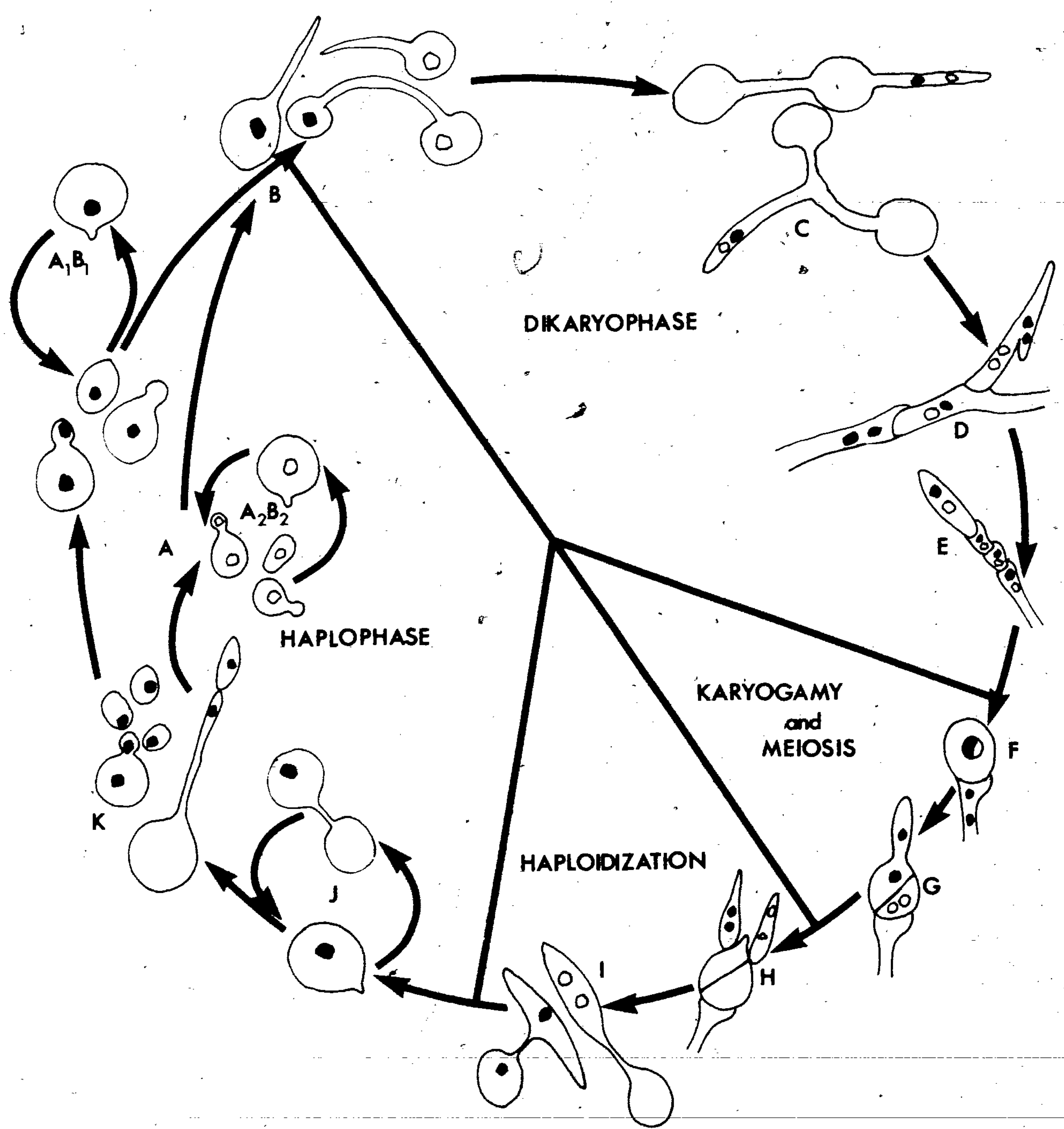
G. post-meiotic metabasidium with nuclei migrating into a developing sterigma;

H. mature metabasidium with caducous sterigmata ready to be shed;

I. sterigmata producing uninucleate basidiospores;

J. basidiospores germinating by repetition;

K. basidiospores germinating to reestablish the uninucleate yeast phase.


B
C
A_1B_1
DIKARYOPHASE
D
A
A_2B_2
E
HAPLOPHASE
KARYOGAMY
and
MEIOSIS
F
K
G
HAPLOIDIZATION
J
H
I

DISCUSSION

In their original description of the genus *Sirobasidium*, Lagerheim and Patouillard (1892) placed it in the family Tremellaceae as it had been delimited by Brefeld (1888). However, they noted two distinct differences from all other genera in the family, namely, "la desposition des basides en chapelets à développement basipète et l'absence de stérigmates". In addition, they likened a basipetally produced chain of basidia to a single basidium of auriculariaceous fungi on the assumption that the cells in a *Platygloea* Schroet. basidium get younger towards the base. This, they proposed, would show a relationship between the Auriculariaceae and the Tremellaceae. This arguement was eventually overthrown. Later cytological evidence summarized by Talbot (1954) and that presented here, show that the structures Lagerheim and Patouillard compared are not homologous. However, the relationship of *Sirobasidium* to the Auriculariaceae was raised again by Kobayasi (1962) when he described *S. japonicum* and pointed out the occurrence of cylindrical metabasidia with four cells created by three transverse septa. He further pointed out the range and variability of metabasidial septation in individual and different species of *Sirobasidium*. This, he claimed, varied from the

auriculariaceous type to the tremellaceous type. Using these arguments, Kobayasi placed an ancestral type resembling Sirobasidium in a key phylogenetic position. From this ancestral type he envisaged evolution of terminal sidelines with the Auriculariales leading to the Uredinales, and with the Tulasnellaceae leading to the Dacrymycetales. It is presumed that the arrow he drew above Sirobasidium indicates that a Sirobasidium-like ancestor led to the higher basidiomycetes through the Tremellales. Kobayasi's scheme seems to be an amalgamation and extension of schemes presented by Olive (1946) and Bandoni (1957).

Olive (1946) had suggested a link between Sirobasidium and auriculariaceous fungi on the basis of septal variations in basidia of S. sanguineum. He stated, "Therefore, from the standpoint of septation, the basidial forms range, in the same fungus, from the Tremella type to the Auricularia type. It is the occurrence of such Heterobasidiomycetes as the present species which tend to emphasize the phylogenetic relationships between tremellaceous and auriculariaceous forms."

Bandoni (1957) proposed yet another phylogenetic possibility, "..that both the tremellaceous and tulasnellaceous basidial forms may have evolved from an ancestral type posessing *Sirobasidium*-like basidia, the former through loss of the septa separating the epibasidia <sterigmata> from the hypobasidium <metabasidium>, and the latter through loss of the cruciate septation of the hypobasidium <metabasidium>. As has already been stated, caducous epibasidia <sterigmata> are sometimes found in species of *Tulasnella* Schroet. Therefore, the distinction between epibasidia <sterigmata> which are retained on the hypobasidium <metabasidium>, but separated from it by septa, and the type found in *Sirobasidium* would not appear to be great."

Bandoni's proposal is supported by the cytological evidence provided here. The septa formed between the metabasidium and sterigmata of *Tulasnella* are adventitious (Rogers, 1932; Khan and Talbot, 1976) as are those between the metabasidium and caducous sterigmata of *S. magnum* and probably other species of *Sirobasidium*. This fact certainly confirms that the structures are completely homologous. Although few would dispute the plausibility of Bandoni's arguement, it is doubtful that many would accept the more expansive one put forward by Kobayasi. Certainly his phylogenetic scheme is

unique and unlike all those presented by earlier authors (Gauman and Dodge, 1926; Rogers, 1934; Linder, 1940; Martin, 1945; Bessey, 1950) where Sirobasidium occupied a position on a side branch of the main phylogenetic trunk leading to the "higher" Basidiomycetes. However, it is true that such schemes preceded full understanding of the nature of the basidia and spores of the genus. In spite of this knowledge, though, Donk (1972b) considered Kobayasi's view as , "..overemphasizing a potential variability that is to be found in many tremellaceous species, although not so strongly developed as in the genus Sirobasidium as a whole." Donk's objection to Kobayasi's scheme is that it overemphasizes the great variability and instability of basidial characters; yet these are the very features one should look for in seeking out a "primitive" organism. Perhaps such phylogenetic speculation will never be resolved. In any case, it seems unquestioned that the Sirobasidiaceae, Tremellaceae and Tulasnellaceae at least are reasonably closely related, though the line of descent is far from settled. Donk (1972b) states that intermediate types such as Metaburdotia Olive (Olive, 1957) and Pseudotulasnella Lowy (Lowy, 1964) completely bridge the gap between the latter two families. This and other considerations have led him to suggest that all these families be included in the Auriculariales, along with the Auriculariaceae. It is doubtful that such a sweeping change would be well received.

Cytological studies of basidial development in tremellaceous fungi have been reviewed by Whelden (1934, 1935a, 1935b) and Talbot (1954). Of all the fungi discussed, those of the Tremellaceae show a pattern of development most similar to the one seen here for S. magnum. A dikaryotic hyphal cell, usually terminal, undergoes karyogamy followed by the first meiotic division. This division is accompanied by the formation of a basidial septum. In the Tremellaceae, this septum is usually vertical while in S. magnum it is usually oblique. From this point, the developmental patterns differ more. In the Tremellaceae, the second meiotic division is again accompanied by septal formation. In S. magnum it usually is not. The four haploid nuclei in Tremella, Exidia Fr., etc. then migrate with accompanying cytoplasm into four sterigmata which remain attached to the metabasidium. They do sometimes become adventitiously septate but this is rare. In S. magnum, the four products of meiosis migrate into two sterigmata in pairs, emptying the metabasidium of cytoplasm in the process. Septa then form, separating the sterigmata from the metabasidium and the sterigmata fall away. They act as disseminules and produce delayed basidiospores, while the basidiospores in the Tremellaceae are produced on the basidium. Phylogenetic schemes could be devised which derive either of these basidial types one from the other. However, the great plasticity

present in the genus Sirobasidium and even within individual species of the genus, argues in favour of it being the more primitive of the two.

Suppression of yeast growth by overlaying the cells with a coverslip could be most easily explained either by a decreased oxygen concentration, increased carbon dioxide concentration or both of these working in concert. Increased carbon dioxide seems to be the critical factor here and this is consistent with work reviewed by Tabak and Cooke (1968). They concluded that changes in carbon dioxide concentration generally had greater effects for smaller changes than did changes in oxygen concentration. It is noteworthy, however, that dikaryotic hyphae arising from crosses in 15% carbon dioxide differed from those under coverslips by becoming pigmented. This is most likely an oxygen-related effect since carotenoid pigment (Valadon, 1966, 1976; Simpson and Chichester, 1971) and quinone pigment production can be affected by concentrations of this gas.

Basidiocarp production at the edge of the coverslip is probably also an oxygen-related effect and may be related to phenyloxidase activity. Several studies have been done to show the effect of these enzymes on fruiting body formation in Schizophyllum commune Fr.

(Leonard and Dick, 1968; Leonard and Raper, 1969; Leonard, 1971; Leonard and Phillips, 1973; Phillips and Leonard, 1976).

Robinow (1975), in a discussion of light microscopy of yeasts described procedures for observing nuclei in living cells. These procedures involved various manipulations to obtain mounting media of the correct refractive index to make the nuclei visible. All those techniques failed with the yeasts of *S. magnum*, perhaps because of the heavy capsule that surrounds the cells. However, agar media with 18% to 28% maltose provided the required refractive index. It is possible that this procedure would work with other encapsulated yeasts such as those of *Tremella* and *Cryptococcus*.

Cultural studies using artificial media in the laboratory often raise the question whether the results are relevant to the organism "in nature". In this study, the problem does not arise with the basidiocarps. In the forest, they are erumpent from under the bark of fallen trees. This would seem somewhat comparable to basidiocarps arising at the edge of coverslips in agar culture. Basidia, sterigmata, and spores in both types of fruiting bodies were identical and undoubtedly serve the ends of genetic recombination and dissemination. One interesting variation does occur here however.

The formation of fruiting bodies occurred on relatively rich media and would not occur on less rich media. This is contrary to the situation in many fungi where fruiting is brought on by a decrease in nutrient supply (Hawker, 1950; Cochrane, 1958).

In nature the role of the haploid yeast phase which arises from the spores is not so clear. I am not aware of any field work which has shown the occurrence of tremellaceous yeasts. The explanation for the lack of reports is possibly that they have not been looked for or that they have been misidentified when found. In any case, the most straightforward proposal as to the function of the yeast phase is that they are more or less secondary disseminules which grow in plant exudates until they conjugate and penetrate the bark of decaying plants. Beneath the bark the dikaryotic mycelium would grow and spread until formation of a basidiocarp at a crack or fissure in the bark.

Another possible role for these yeasts was first suggested by Dr. R.J. Bandoni (personal communication). They may be associated with insects. The most striking case of heterobasidiomycetous yeasts associated with insects is that which occurs in the Septobasidiales. This group was first described by Patouillard (1892) and was later

studied in great detail by Couch (1938). As exemplified by Septobasidium curtsii, these organisms infect their insect hosts as yeast cells arising from basidiospores. It is possible that the yeast phase of Sirobasidium is associated with insects, because they could act as a vector for infection of decaying tree trunks. A review of yeasts found in the mycangia of beetles (Callaham and Shifrine, 1960), includes several species of the imperfect yeast genera Cryptococcus and Candida Berkhout. Both of these genera are now known to include basidiomycete species (Fell, 1970; Shadomy, 1970; Kwong-chung, 1975, 1976; Kwong-chung and Popkin, 1976) and it is not inconceiveable that some of those isolates described as Cryptococcus might turn out to be haploid yeasts of tremellaceous fungi. As pointed out in Chapter 1, yeasts strains of this group in particular could be easily misidentified as species of Cryptococcus.

A simple way to test this hypothesis would be to carry out mating trials between yeasts isolated from insects or plant exudates, with known mating-type strains of Tremella and Sirobasidium.

CHAPTER THREE

THE CONJUGATION PROCESS

INTRODUCTION

In this dissertation, I have used the term "yeast" to describe the haploid budding cells of S. magnum. This may give rise to some objections and since the budding cells are central to the discussion in this chapter, I feel obliged to justify applying this term to them.

Yeasts have been defined as fungi that exist predominantly in the single-celled state (Lodder, 1970b; Rose, 1975). However, this broad definition has led to some confusion. The difficulty is evident in the following quotation from Rose (1975): "....there are several genera of fungi which, although they contain species able to grow in the yeast state, are not considered true yeasts simply because they do not exist predominantly as single cells." This statement shows the importance of the term "predominantly" in the definition of yeasts given above. It also betrays an obvious distinction for its author between the adjective "yeast" and the noun "yeast". This distinction

makes it possible for a fungus to have yeast cells while it is not at the same time a yeast. The reason for this distinction is historically bound as is plainly recognized in the following apology: "For the authors it is, therefore most painful to have to state that it is not possible to give a satisfactory definition of the term 'yeasts' which fully embraces all the organisms which are more or less generally accepted as belonging to the yeast domain. We shall, therefore, have to confine ourselves to an attempt to retrace the historical development of the notion 'yeasts'" (Lodder and Kreger-van Rij, 1967). In the text following their apology, the authors show how this originally restrictive term expanded with time to include more and more species. I should make it clear that the term is applied at the specific level, i.e. "Saccharomyces cerevisiae is a yeast." It is equivalent to saying that Arbutus menziesii is a tree. In this latter statement, the single word "tree" conveys a considerable amount of information, i.e. the organism named is a vascular plant; it is either a Gymnosperm or an Angiosperm; it is large and it is woody. The same sort of convenience was once obtained from the term "yeast". At one time or another it carried any one or several of the following implications: the organism named was a fungus; it reproduced by budding; it was fermentative; it was a member of the order Saccharomycetales (Ascomycotina) or of the family Cryptococcaceae

(order Moniliales, Deuteromycotina). However, as time has passed, fewer and fewer of these implications follow. At present we are left with only "fungi predominantly in the single-cell state" and we are left to struggle with the word "predominantly".

I feel that most of the confusion here arises because of the previous taxonomic associations of the term "yeasts". All "yeasts" were once considered to be either full-fledged ascospore-forming Ascomycetes or imperfect yeasts that were "probably asporogenous Ascomycetes". This attitude spawned terms like, "sporidia" and "yeast-like cells" to cover budding cells in other non-Ascomycetous taxonomic groups and in dimorphic fungi.

Now, very recent information has shown that "sporidial" forms and "yeast-like" forms of dimorphic fungi exist in families and orders to which "true yeasts" have been transferred from the family Cryptococcaceae (reviews by Wickerham, 1969; Fell, 1970). Are we now to change from calling these transferred organisms "yeasts" to calling them "yeastlike"? Lodder (1970a) seems to suggest this in the following chapter heading: "Discussion of the yeast-like genera belonging to the Ustilaginales". However the author himself seems somewhat confused in that this title does not concur with the

following statement in his introduction: "Chapter IV deals with the genera belonging to the ascomycetous-yeasts, Chapter V with the basidiomycetous yeast genera Leucosporidium and Rhodosporidium,....". These organisms are referred to as "yeasts" and "yeast-like" in the same publication!

The whole problem hinges on the one word "predominantly" in the definition of yeasts. How does one determine predominance in a dimorphic fungus?

Problems such as this do not arise with the terms "filamentous" and "mycelial" because they do not construe taxonomic restrictions to nearly the same extent as the term "yeast". Yet the terms are somewhat antithetical. Surely, whether a fungus assumes one or the other of these growth forms is likely to be as much in response to an extant ecological role as to phylogenetic history. Consequently, I believe that the term "yeast" should be redefined as a general term as follows: A yeast is an assimilative fungal growth form which is unicellular and which reproduces by budding or by fission (eg. Schizosaccharomyces Lindner). This would be a more ecotypical designation. It would require compound terms such as ascomycetous yeasts, basdiomycetous yeasts and deuteromycetous yeasts, where

phylogenetic association was desired. It would dissociate the term "yeast" from a single taxonomic group and would make it a term describing a growth form. It would allow one to state for example, that the haploid phase of S. magnum is a yeast and that under appropriate conditions other species of fungi are yeasts.

This definition would have the advantage that the term "yeasts" could be applied with precision. It would remove taxonomic overtones and therefore encourage consideration of possible phylogenetic associations amongst various fungi that are dirmorphic or grow exclusively in yeast or filamentous form.

Nor would this definition cause undue difficulty for authors working in the area of "traditional yeast study". They could simply state which taxonomic groups were excluded from their particular treatise.

My contention is not without precedent. Donk (1972b), in a discussion entitled, "Budding in the Heterobasidiomycetes", makes the complete break with tradition that is required. He covers many hymenial Heterobasidiomycetes and refers to their budding phases as yeast forms rather than yeast-like forms. I completely concur with that designation and it will be the one followed in this dissertation.

Conjugation between yeast cells in S. magnum marks the transition from the budding assimilative phase to the filamentous assimilative phase in its life cycle. The process is a sexual one but does not differ morphologically from yeast-filament transitions which are non-sexual. It is possible that similar physiological events occur in these cells undergoing comparable changes in growth form. Consequently, I would like to review work concerning yeast-filament or yeast-hyphal dimorphism that is of the non-sexual type, and to follow it with a review of sexual conjugation between yeasts.

Yeast-hyphal dimorphism has been the object of considerable research in medical mycology because a number of human pathogens are dimorphic and grow pathogenically in one or the other of their two growth forms. It is possible that an understanding of the morphogenetic process could lead to prophylactic or therapeutic methods. Several of these pathogens have been studied (see Romano, 1965 for a review) but I will confine my coverage to Candida albicans (Robin) Berkhout, the organism most extensively studied from the standpoint of dimorphism. The relevance of Candida to this study is enhanced by work showing some members of the genus to be Basidiomycetes (Fell, Statzell, Hunter and Phaff, 1969).

Candida albicans is believed to be infective in the hyphal form although, like other pathogens, it is systemically dispersed in the yeast form. Nickerson (1953) found that the morphogenetic process in this pathogen was influenced by sulfur containing compounds and particularly by cysteine. Work on Candida with sulfhydryl compounds accumulated in the decade following Nickerson's work and it has been reviewed by several authors (Nickerson, 1963; Romano, 1965; Bartnicki-Garcia and McMurrough, 1971). This work culminated in a widely accepted hypothesis first proposed by Falcone and Nickerson (1959). They suggested that different morphology was based on the chemical structure of cell walls. A predominance of disulfide bonds between protein moieties of protein-mannan complexes was seen to produce filamentous growth. A reducing environment was supposed to reduce these disulfide bridges and lead to budding growth. The interconversion of sulfhydryl and disulfide bonds was proposed to be mediated by a protein disulfide reductase. This model has been questioned recently by Wain, Price, and Cawson (1975). Using standardized yeast phase cultures, these authors demonstrated a general inhibitory effect of cysteine on both yeast and hyphal forms of C. albicans. They could not substantiate the morphogenetic controlling effect of cysteine in their experimental system.

Other chemicals known to affect dimorphism in *C. albicans* are biotin (Yamaguchi, 1974) and glutamate (Nishioka and Silva-Hunter, 1974). The mode of action of these chemicals is as yet unclear, but they serve to show that the biochemical process which brings about morphogenetic change is likely to be a complex one. Whatever this process is in *C. albicans*, both chemical analysis and electron microscopical examination of cells walls show that the ultimate result is significant differences between the wall structures of the two growth forms. Marriott (1975) has recently suggested that these wall differences are accompanied by differences in the plasma membranes of the two forms. It remains to be established how these differences come about.

Another large body of work on yeast-hyphal dimorphism has been done with molds of the zygomycetous genus *Mucor* Mich. ex Fr. These fungi are so distantly related to basidiomycetous fungi that mention of them in the context of this thesis would seem superfluous except for the sheer volume of publication relating to dimorphism in them. Again, this work has been reviewed by Nickerson (1963), Romano (1965), and Bartnicki-Garcia and McMurrough (1971). Although many substances and environmental conditions have been shown to affect dimorphism in *Mucor*, no general dimorphic mechanism has been distilled which could

be compared to the model suggested for Candida albicans by Falcone and Nickerson (1959). Terenzi and Stork (1969) used phenethyl alcohol to induce yeast morphology in M. rouxii <Mucor rouxianus (Calmette) Wehmer> through inhibition of mitochondrial respiration. They concluded that yeast morphology in Mucor was always related to conditions which favoured fermentation. They wrote, "These examples demonstrate that inhibition of respiration and enhancement of fermentation always restricts morphological differentiation in filamentous fungi.....To conclude, we suggest that filamentous morphology in fungi might be regarded in many instances as a morphogenetic expression of the Pasteur effect. This concept is in agreement with the views of Warburg regarding oncogenesis, expressed in the following way: 'Respiration energy creates and maintains a high differentiation of body cells. Fermentation energy can only maintain a low differentiation. It follows that if respiration is replaced by fermentation in body cells, high differentiation must disappear'." This point of view assumes that hyphal morphology represents "high differentiation" when compared to yeast morphology. Yet Terenzi and Stork did not present any information to justify this point of view and I feel it would be difficult to do so. In any case, the dimorphic mechanism may not be so simply explained. As Bartnicki-Garcia and McMurrough (1971) point out, "Vegetative

morphogenesis of Mucor rouxii depends on a variety of environmental factors such as oxygen, carbon dioxide, hexoses, heavy metals, dicarboxylic acids, uncharacterized factors present in complex nutrients and others.....By suitably changing the concentration of any one of these factors, it is possible to induce either the development of yeast cells or hyphae. It would seem misleading, however, to assign to any one of these factors the causal rôle in dimorphic development, for the effect of each factor is conditioned by the concentration of the others."

Very recent work with M. racemosus Fres. reveals that the widely occurring control substance, 3':5'-cyclic adenosine monophosphate plays a rôle in its morphogenetic process (Carsen and Sypherd, 1974; Paznokas and Sypherd, 1975). Using a membrane permeable derivative of this chemical, the authors could induce either yeast or hyphal morphology under atmospheres of nitrogen. These results certainly dispense with the Terenzi-Stork hypothesis, but open a completely new line of investigation to be followed.

I think these examples are sufficient to show that the subject of yeast-filament dimorphism is a complex one where different mechanisms may operate in different fungi. However, the systems described above

suffer from a considerable disadvantage, in that highly artificial environments are often used to bring about morphogenetic change and it is difficult to relate these experimental conditions to what may occur with these fungi in a natural setting. However, _Mucor racemosus_ may prove to be an exception to this situation in that a recent report (Mooney and Sypherd, 1976) indicates that this fungus produces a volatile morphogenetic stimulator. In this report either yeast or hyphal morphology could be achieved simply by varying the gas flow rate in atmospheres of pure nitrogen.

Sexual conjugation between yeasts when a lengthy conjugation tube is produced can be viewed as a yeast-hyphal transition which does not involve the difficulties just outlined. Yeast or hyphal form can be elicited by the presence or absence of sexually compatible cells in otherwise identical culture media. No highly abnormal environmental manipulation is required.

Yeasts with relatively long conjugation tubes are to my knowledge always Basidiomycetes. They can be contrasted with ascomycetous yeasts where conjugation tubes are much shorter and where conjugation is often preceded by close contact through agglutination.

DeBary (1884) first recognized the sexual nature of conjugation between basidiomycetous yeasts in what he called the "sporidia" of smuts. Some of these grew on artificial media in what he called "yeast-like form". His interpretation that this phenomenon was sexual was later substantiated by cytological studies (summary by Fischer and Holton, 1957). Bauch (1925) proposed that conjugation in the smut *Ustilago* (Pers.) Roussel was mediated by specifically produced chemical substances which stimulated the formation of conjugation tubes. However, he was unable to prove experimentally the existence of these proposed substances.

Bandoni (1961) recognized sexual conjugation between yeasts in the Tremellales and later (Bandoni, 1963) described it in detail in *Tremella mesenterica*. Other tremellaceous fungi which were shown to exhibit conjugation between yeasts were other species of *Tremella* (Kobayasi and Tubaki, 1965; Flegel, 1968; Brough, 1970, 1974) and *Holtermania corniformis* (Kobayasi and Tubaki, 1965).

In 1967, Banno discovered that several strains of *Rhodotorula glutinis* (Fres.) Harrison were sexually active. Following conjugation of haploid yeast cells, sexual sporogenesis ensued which resembled that in the smuts. Banno proposed the new name *Rhodosporidium*

torruloides for this yeast and transferred it to the Ustilaginales. Since Banno's work, many yeasts formerly classified in the Cryptococcaceae have been shown to have basidiomycetous life cycles. In these life cycles, the sexual stage is always preceded by conjugation. Two of these other new genera are *Leucosporidium* (*Candida* species; Fell et al., 1969) and *Filobasidiella* (*Cryptococcus*; Kwon-chung, 1975, 1976; Kwon-Chung and Popkin, 1976). A similar condition seems to prevail for at least some strains of *Sporobolomyces* as shown by studies of Bandoni, Lobo and Brezden (1971) and Bandoni, Johri and Reid (1975). Van der Walt (1970b) has proposed the basidionym *Aessosporon salmonicolor* for *Sporobolomyces salmonicolor* (Fischer et Brebeck) Kluyver et van Niel but the details of his proposed life cycle are somewhat tentative. As they stand they are not compatible with the studies of Bandoni et al. Perhaps this can be attributed to differences between the yeast strains used.

Several reports of mating hormones in diverse fungi appeared in the years following Bauch's (1925) hypothesis that such substances regulated conjugation in *Ustilago*. These reports have been reviewed in recent articles by Machlis (1972), Gooday (1974) and Bu'Lock (1976). The first report which unequivocally demonstrated the existence of mating substances in Basidiomycete yeasts was made by

Bandoni (1965) for Tremella mesenterica. Since that original report, somewhat similar hormonal systems have been shown in other species of Tremella (Flegel, 1968; Brough, 1970) and in Rhodosporidium toruloides (Abe, Kusaka and Fukui, 1975). Reid (1974) has suggested that these substances are small peptides in Tremella mesenterica.

The only mating hormones from a yeast that have been purified and identified are from Saccharomyces cerevisiae Hansen. Two of these have been identified as small peptides (Duntze, MacKay and Manney, 1970; Duntze, Stotzler, Bucking-Throm and Kalbitzer, 1973; Shimoda, Yanagishima, Sakurai and Tamura, 1976). Another causing cell expansion, has been identified as n-octanoic acid (Sakurai, Tamura, Yanagishima, Shimoda, Hagiya and Takao, 1974). However, these substances do not induce filamentous conjugation processes.

The function of the mating hormones or erogens in Tremella mesenterica was investigated (Flegel, 1968) and a continuous supply appeared necessary for hyphal morphology to be maintained. Removal caused a reversal to budding growth. A similar phenomenon seems to occur in Tremella globospora (Brough, 1970). A comparison of the walls of yeast cells and conjugation tubes in T. mesenterica led Reid and Bartnicki-Garcia (1976) to conclude that, "The conjugation hormone

of *T. mesenterica* does not appear to act by inducing major changes in the polysaccharides of the cell walls. Perhaps its main action is to control, in some unknown way, the distribution of wall synthesizing enzymes over the cell surface."

The purpose of the work described in this chapter was to establish standardized conditions for conjugation in *S. magnum*; to determine the presence or absence of conjugation hormones; to discover whether response to the hormone was dependent on the cell cycle; and to determine whether the yeast to hyphal change was associated with changes in intracellular levels of 3':5'-cyclic adenosine monophosphate.

MATERIALS AND METHODS

The yeast strains of *S. magnum* used in studying the conjugation system were TWF 58-23, 58-27, 11-1, 11-2, 11-5 and 11-6. The media used for growth and maintainance were YS, YE or YNB. Conjugation trials were carried out in CJM. These media and cultural conditions are described in Chapter 1. Cells prepared for radioactive measurements were grown in Vitamin Free Yeast Broth (VFB) which contained Difco Vitamin Free Yeast Base, 6.7 g; glucose, 10.0 g; distilled water, 1 litre. This medium was adjusted to pH 6.8 using 1/10 N sodium hydroxide and autoclaved before use.

Cell concentrations were determined using a Bausch and Lomb Spectronic 20 colorimeter set at 560 nm. This was calibrated by haemacytometer counts (Appendix A) where a cell with an attached bud was counted as one cell.

Microscopic observations were made using a Zeiss Standard Universal Microscope with and without phase optics, or using a Reichert Zetopan research microscope set for transmitted light interference contrast. All photomicrographs were taken using a Reichert Photo- Automatic camera.

Conjugation was observed on Fortner slides (Robinow, 1975) or on open sterile microscope slides with a 17 mm circular depression. These slides were precleaned with chromic acid solution and rinsed for three hours in three changes of distilled water. They were dried and autoclaved in 10 X 100 mm glass petri dishes lined with a 100 mm filter paper. The filter paper was later wetted with 2 ml sterile distilled water. The depression slides were then inoculated with an appropriate volume of equally mixed cell isolates to give a final density of cells of approximately 300 cells/mm^2 in a total volume of 50 ul. Following inoculation, the slides were incubated in a Percival controlled-environment chamber at 25°C in the dark.

These depression slides were used to test the effect of various chemicals on the conjugation process. The chemicals were dissolved in CJM and filter sterilized using 13-mm Swinnex filters with Millipore filters of pore diameter 0.45 um (Millipore Corporation). Total volumes were made up to 50 ul using CJM also sterilized using Swinnex filters. Chemicals tested were, ethyl alcohol, caffeine, theophylline, adenosine 3':5'-cyclic monophosphate (cAMP), $N^6,O^{2'}$-dibutyryl adenosine 3':5'-cyclic monophosphate (dbcAMP), guanosine 3':5'-cyclic monophosphate (cGMP), and $N^2,O^{2'}$-dibutyryl guanosine 3':5'-cyclic monophosphate (dbcGMP) all obtained from Sigma

Chemical Corporation. The presence and permeability of erogens was determined using Spectrapor dialysis membranes 1 and 2 (Spectrum Medical Industries Incorporated) with molecular weight exclusion limits of 6,000 to 8,000 daltons and 12,000 to 14,000 daltons respectively. The techniques were modified from those used by Bandoni (1965). The membranes were steam sterilized and then used in either one one of the two following ways. (1) They were inoculated one on each side with 50 ul of CJM containing approximately 10^5 cells/ml of opposite mating types. Controls were membranes with the same mating type on both sides. (2) They were placed between two slides coated with CJM agar each inoculated with approximately 300 cells/mm^2 of opposite mating-type cells. Controls were identical except that identical mating types were on both slides. In both cases, the cells were incubated at 25°C in the dark. Observations were made using a microscope and cells could be identified by the focal plane in which they lay.

Attempts to isolate a cell free fraction of culture medium which was hormonally active included: using supernatant medium from centrifuged cultures of single mating types and from mixed conjugating cells; incubating live cells with heat killed cells (40°C for 2 hours) of the opposite mating type; using medium surrounding dialysis bags (Spectrapor 2) containing single mating types; agar blocks

removed from areas adjacent to single mating types. These trials were carried out with exponential phase and stationary phase cultures and the fractions were bioassayed at full strength and at various dilutions up to 1,000 X.

Discontinuous sucrose gradients were used for separation of cells in the exponential growth phase on various media. These gradients were prepared in 18 X 110 mm test tubes and they ran from 45% aqueous sucrose to 20% aqueous sucrose in 5% steps. Three ml of each sterile sucrose solution was used. Cells were harvested from 50 ml 24-hour cultures and centrifuged in sterile 50 ml centrifuge tubes for 10 minutes at 3000 rpm on a Model HT International centrifuge. The supernatant was discarded and the cells were resuspended in 2 ml of sterile distilled water. One ml was removed and layered onto the top of the sucrose gradient tube. The tubes were then centrifuged at 1500 rpm (approximately 350 g) on an International Centrifuge Model CS (swing rotor) for 10 minutes. One ml was removed containing the cells highest in the gradient. The cell concentration was then determined on a Bausch and Lomb Spectronic 20 colorimeter as outlined above.

Synthesis of DNA was measured using $H_3{}^{32}PO_4$ (80 Ci/mg P), <6-^{3}H> thymidine (2.0 Ci/mM), <6-^{3}H> thymine (25 Ci/mM) and <6-^{3}H> adenine (21 Ci/mM). These radiochemicals were obtained from Amersham/Searle Corporation. Gradient cells prepared from cultures on VFB were transferred to a 1000 ml erlenmeyer flask containing 150 ml VFB with 1 to 2 uCi/ml of the appropriate labeled compound added. Twenty-five ml of this inoculated solution was immediately removed to a separate flask in a water bath at 70°C. The master flask was incubated in a PsycroTherm reciprocating incubator shaker at 25°C with a 50 rpm shake. At 30 minute intervals, 4 one-ml samples were removed from the master flask and transferred to the freezer. One 1-ml sample was removed from the heated flask and also transferred to the freezer. At the end of the experimental period the samples were removed from the freezer. One ml of 2N KOH was added to three samples from each time interval and to all of the heat-treated samples. These were incubated at room temperature for 12 to 16 hours. Ten ml of 20% trichloroacetic was added immediatley to the rest of the samples. These samples were then filtered through 24 mm Whatman GF/C glass fibre filters pre-wetted with distilled water containing 0.2 g/l of the unlabeled tracer compound. The filters were then washed with three 10-ml aliquots of the pre-wetting solution before being transferred to scintillation vials. This procedure was repeated for the remaining vials after overnight incubation in the KOH solution.

The filters in the scintillation vials were treated with 1 ml each of NCS tissue solubilizer (Amersham/Searle Corporation) and incubated at 70°C for 1 hour in a water bath. Finally 1 drop of concentrated acetic acid was added along with standard toluene scintillation "cocktail" and radioactivity was measured on a Packard Liquid Scintillation Counter model 3003. A separate series of identical samples was run and half were treated with DNase to substantiate that the hydrolysis-stable acid-precipitatible material was DNA.

The effect of glucose on conjugation was measured on solid and in liquid media, and on Fortner slides (Robinow, 1975). Initial trials were done on solid and in liquid CJM and the number of cells with conjugation tubes out of the total number of cells was converted to a percentage (400 cells total/sample). Later trials were conducted using CJM on Fortner slides with 100 ml agar containing the following amounts of glucose: 0.1 g (5 X 10^{-3}M); 1.0 g (5 X 10^{-2}M); 5.0 g (0.28 M); 10.0 g (0.55 M) and 15.0 g (0.82 M). Controls consisted of similarly prepared slides with the same ascending molar concentrations of maltose and galactose (Sigma Chemical Corporation) in CJM agar.

Intracellular levels of cAMP were measured using a cAMP Radioimmunoassay Kit obtained from Schwarz/Mann. Cell extracts were prepared following the general method outlined by Paznokas and Sypherd

(1975). Stationary phase cells of opposite mating types (48-hour cultures in YE broth) were centrifuged and washed with sterile distilled water. A portion of these cells was added to a 250-ml erlenmeyer flask to give a final concentration of approximately 10^5 cells/ml in a total volume of 10 ml of liquid CJM. A total of six flasks was prepared: two each for single mating types and two for a mixture of the two types. After 10 hours stationary incubation at 25°C, the single isolates were combined to form two pairs. A 10 ml sample was removed from each of the now 4 flasks on ice and immediately filtered on 24 mm RA Millipore filter discs (Millipore Corporation; pore size 1.2 u). The filters were then immersed in 2.5 ml ice-chilled 1.0 N HCl in 15 X 150 mm test tubes. The cells were resuspended by agitation. The filters were left in the test tubes and the suspensions were frozen and thawed three times in dry-ice acetone. Cellular debris was removed by sedimentation at 13,000 rpm on a Sorval model RC2-B refrigerated centrifuge. A 1.0 ml sample of each supernatant was removed to another tube and evaporated to dryness at 60°C under an air stream. The residue was then dissolved in 1.0 ml of 0.05 M sodium acetate buffer at pH 6.2. Controls consisted of identically treated samples except that known amounts of cAMP were added to the cold HCl immediately before extraction by freezing and thawing.

Total protein was determined by collecting cells on 24 mm RA Millipore filters and resuspending them by agitation in a tube containing 3 ml of 1.0 N sodium hydroxide. The filters were removed and the suspensions were heated to 100 °C for 20 minutes. Debris was removed by centrifugation as above. Protein analysis was done on the supernatant using the method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin as the standard.

RESULTS

The assimilative tests outlined in Chapter 1 delimit some carbon compounds which are assimilated by yeast strains of S. magnum. However, these tests give simply + or - results and do not give any idea of how good these compounds are as energy sources. Fig. 53 shows growth curves for a number of carbon compounds in liquid YNB and for liquid YS. Optical density readings upon which these graphs were based are shown in Appendix B. It is clear that glucose provides for the fastest rate of increase. Also, it can be seen that these 50-ml cultures reach the stationary phase of growth after 36 hours. In media with NH_4NO_3 as the nitrogen source (eg. YNB), the pH of the culture medium fell during growth and the medium became quite acidic. In contrast, the pH of YS increased with time. Growth curves for nitrogen compounds in YCB are shown in Fig. 54. Optical density readings upon which these graphs were based are shown in Appendix C. Mean generation times (Morris, 1958) were calculated from the growth curves. They are shown on the graphs along with initial and final pH readings.

The effect of cell concentration on conjugation is shown in Plate VII, Fig. 55. This was a difficult phenomenon to measure precisely because the differences observed were largely qualitative. Certainly, at 15 cells/mm^2 and below, no conjugation occurred and at 30,000 cells/mm^2 and above it was severely limited. From 300 to 3000

Figure 53. Growth curves of isolate TWF 58-23 using various carbon compounds in YNB. These are compared to growth in YS broth. Mean generation times (gt) are given in brackets following the name of the carbon source. In addition, pH's of the growth medium are indicated where measurements were made. Controls consisted of YNB without any carbon compound added.

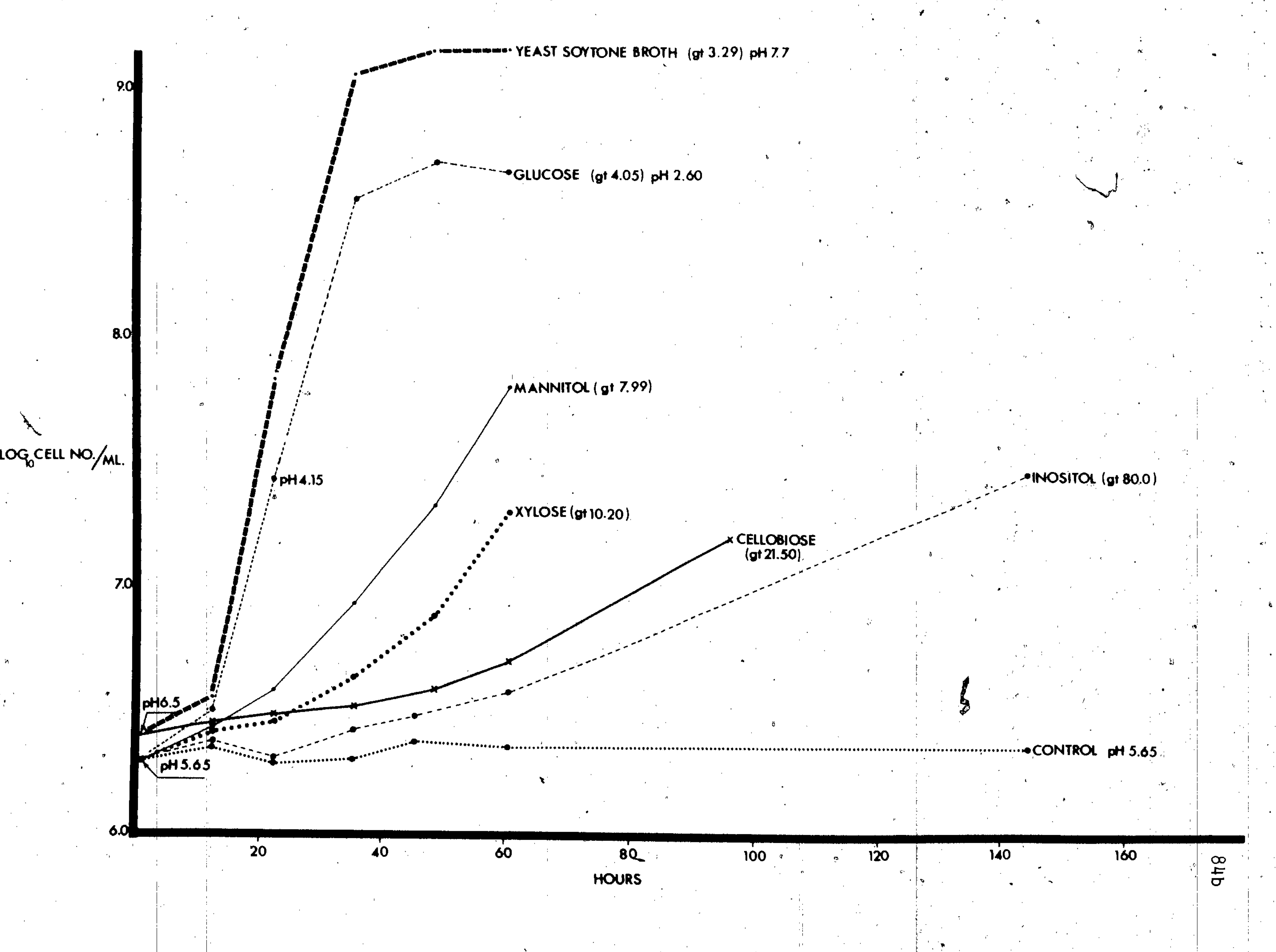
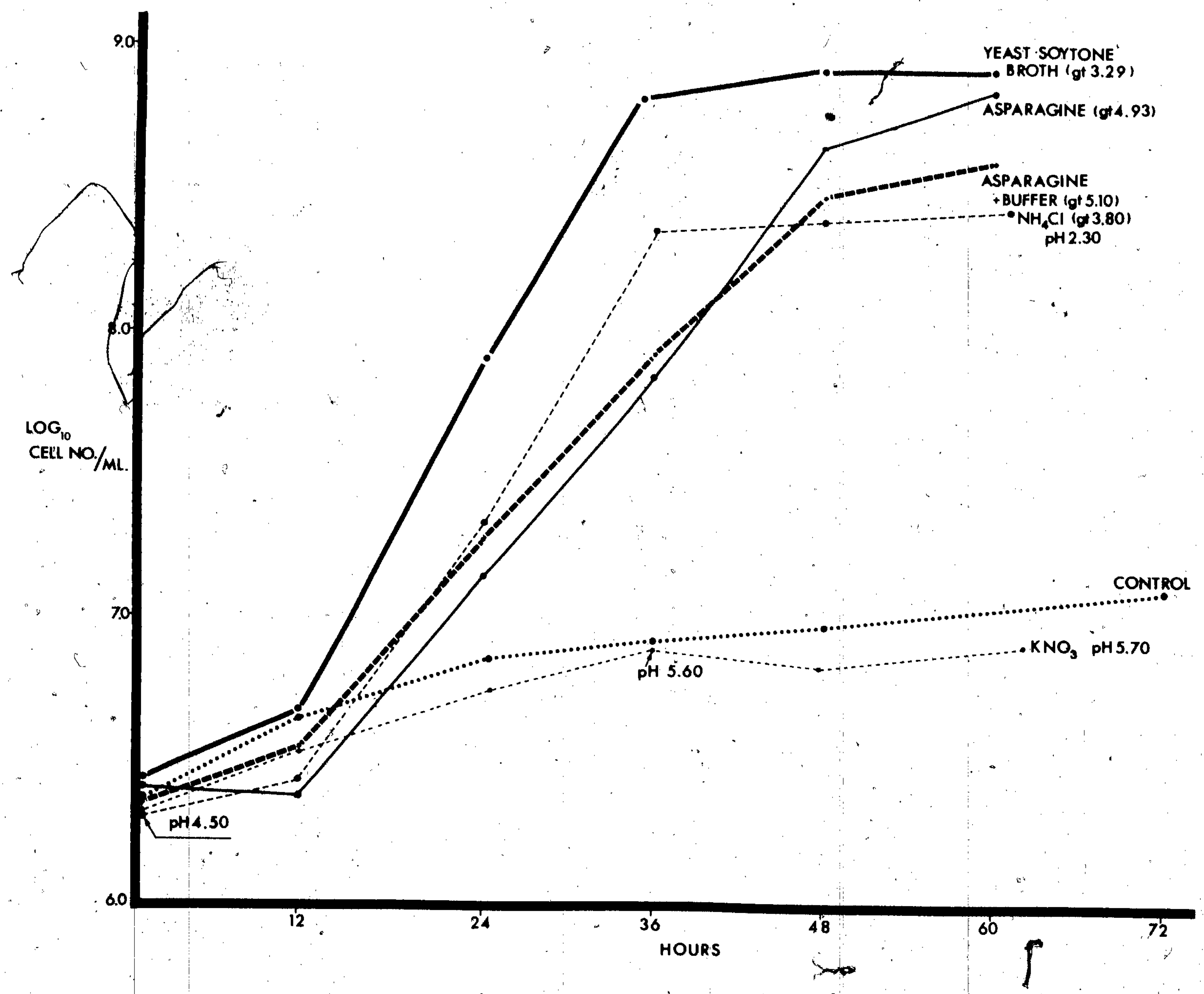

YEAST SOYTONE BROTH (gt 3.29) pH 7.7
GLUCOSE (gt 4.05) pH 2.60
MANNITOL (gt 7.99)
pH 4.15
XYLOSE (gt 10.20)
INOSITOL (gt 80.0)
CELLOBIOSE (gt 21.50)
CONTROL pH 5.65
pH 6.5
pH 5.65
LOG10 CELL NO./ML.
9.0
8.0
7.0
6.0
20
40
60
80
100
120
140
160
HOURS

Figure 54. Growth curves for TWF 58-23 in YCB containing various different nitrogen compounds. These are compared with growth in YS broth. Mean generation times (gt) are given in brackets following the name of the nitrogen source. In addition, pH's of growth media are indicated where measurements were made. Controls consisted of YCB without any nitrogen compound added.



YEAST SOYTONE BROTH (gt 3.29)
ASPARAGINE (gt 4.93)
ASPARAGINE +BUFFER (gt 5.10)
NH_4Cl (gt 3.80) pH 2.30
CONTROL
KNO_3 pH 5.70
pH 5.60
pH 4.50
9.0
8.0
7.0
6.0
LOG_{10} CELL NO./ML.
12
24
36
48
60
72
HOURS

cells/mm^2 the response was greatest and the working concentration of 300 cells/mm^2 was chosen because it ultimately gave the best development of dikaryotic mycelium following conjugation. The depth of liquid over the conjugating cells also affected the conjugation response. The greater the depth, the less the response even though the number of cells per mm^2 was held constant. However, as long as total depth was 5 mm or less, conjugation response was not noticeably decreased. Conjugation response on solid medium was always better than in liquid medium but on solid medium it was difficult to reproduce experiments quantitatively. This is why liquid trials were used in most conjugation tests.

Time-lapse photographic sequences of conjugation on CJM agar (Figs. 56 and 57 and Plate VIII) and budding on YE agar (Plates IX and X) are presented here. Several things are evident from these sequences. First, conjugation may take place between cells separated from each other by some distance. Second, the growth of the conjugation tubes is sharply directional just before end to end fusion. Some cells continue to bud throughout the whole incubation period even though they are close to other conjugating cells of both mating types. In the budding cells the average time from the emergence of one bud to the emergence of another was not constant for

Figure 56. Tracings from a time-lapse photographic series of conjugation between isolates TWF 58-23 and 58-27. The frames in each row were taken at the same time. The series is continued in Figure 57. Photographs were taken at the following times after the isolates were mixed together:

a. 1.0 h

b. 1.5 h

c. 1.8 h

d. 2.3 h

e. 2.8 h

f. 3.4 h

g. 4.0 h

h. 4.3 h

i. 4.8 h

a

b

c

d

e

f

g

h

i

Figure 57. Continuation of the time-lapse series of conjugation from Figure 56. The frames in each row were taken at the following times after the cells were mixed together:

j. 5.3 h

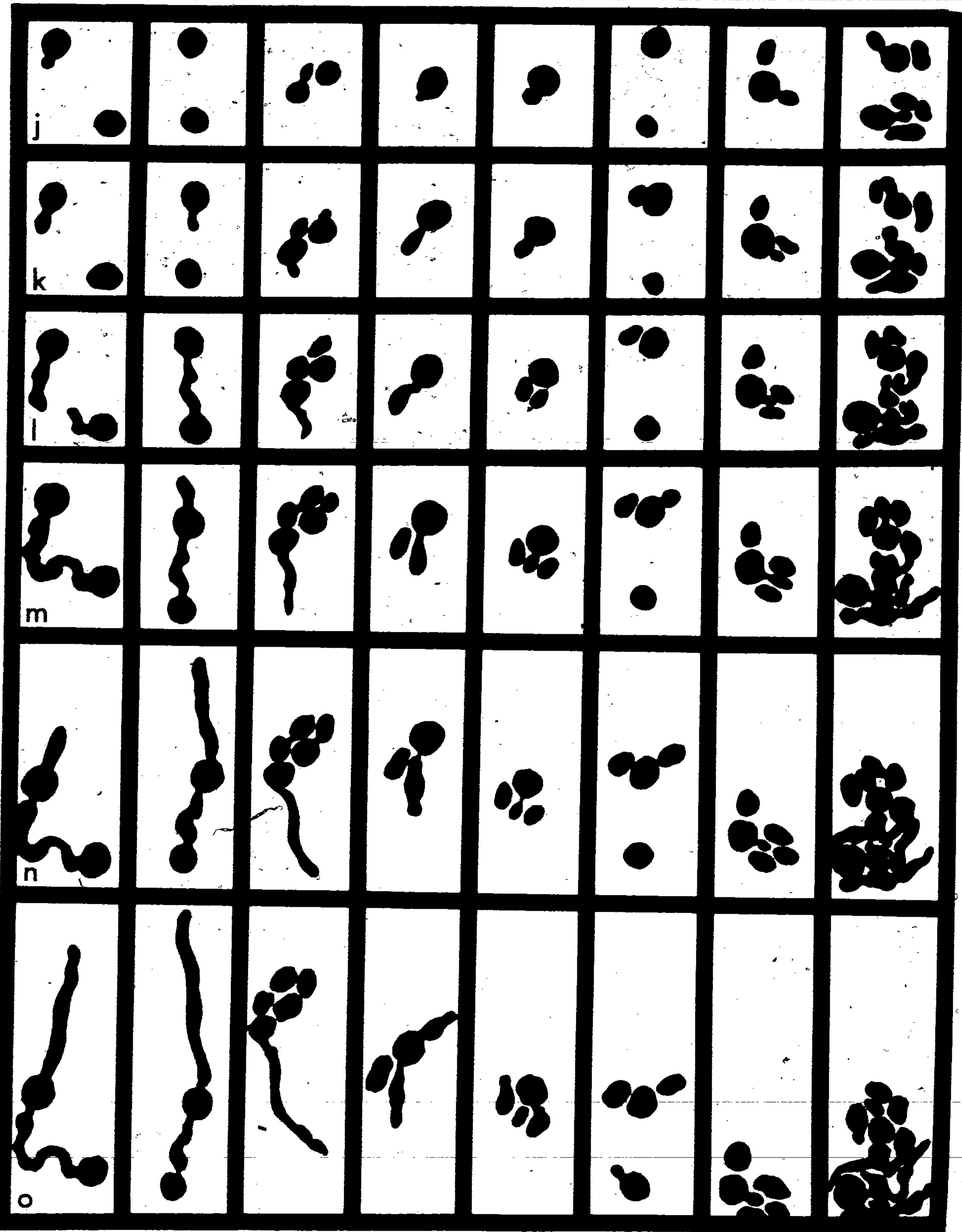
k. 6.3 h

l. 7.3 h

m. 8.3 h

n. 9.3 h

o. 10.3 h


j
k
l
m
n
o

all cells in the time lapse sequence (Table VI). Once budding had begun from any cell, the average time from one bud release to the next bud release was approximately 1.5 hours. However, for daughter cells, the time from release to first bud formation was an average of approximately 3.0 hours. Subsequent buds from these cells averaged approximtely 1.5 hours from release to release like the original mother cells. This evidence shows that mother and daughter cells in this particular yeast are not equivalent at the time of bud release. Some special aging seems to be required for the cells to obtain the ability to bud. From both the budding and conjugation sequences it is apparent that the smaller, newly released cells swell before buds or conjugation tubes are produced.

The fact that conjugation tubes arise from cells separated by a distance indicates that diffusible substances may induce their formation. The existence of such substances was substantiated by trials with dialysis membranes. Photographs of cells on each side of such membranes are shown in Plate XI, Fig. 61. On Spectrapor 1 (MW cutoff 6,000 to 8,000 daltons), the cells on each side of the membrane did not produce conjugation tubes but continued to bud. On Spectrapor 2 (MW cutoff 12,000 to 14,000 daltons) cells on both sides of the membrane produced conjugation tubes, although there was a

TABLE VI

Bud-release to bud-release times for S. magnum yeast cells during a time-lapse budding sequence.

	Mother-cell buds	Daughter-cell 1st buds	Daughter-cell 2nd and subsequent buds.
	3.5*	4.0	2.0
	1.5	3.5	1.5
	1.0	3.5	1.0
	1.5	3.5	1.5
	1.5	1.5**	1.5
	1.0	3.5	2.0
	1.5	3.5	1.5
	1.0	2.5	1.5
	1.5	2.5	
	1.0	3.0	
	1.5	3.5	
	2.0	2.5**	
	1.5	3.0	
	1.5		
	1.5		
	2.0		
	2.0		
	1.0		
	1.5		
	2.0		
	2.0		
	1.0		
	1.5		
Mean	1.57	3.08	1.56
S.D.	0.55	0.67	0.32
S.E.	0.11	0.19	0.11

Note: * This bud was produced at the poopsite pole to the first bud.

** These buds arose from 1st daughter cells whose release time could not be determined precisely since the 2nd daughter cell arose from the mother's opposite pole.

qualititative difference in the appearance of the tubes of the two mating types. Those of 58-27 (A_1B_1) were shorter and thicker than those from 58-23 (A_2B_2). All attempts to isolate a cell-free fraction of culture medium which was hormonally active failed.

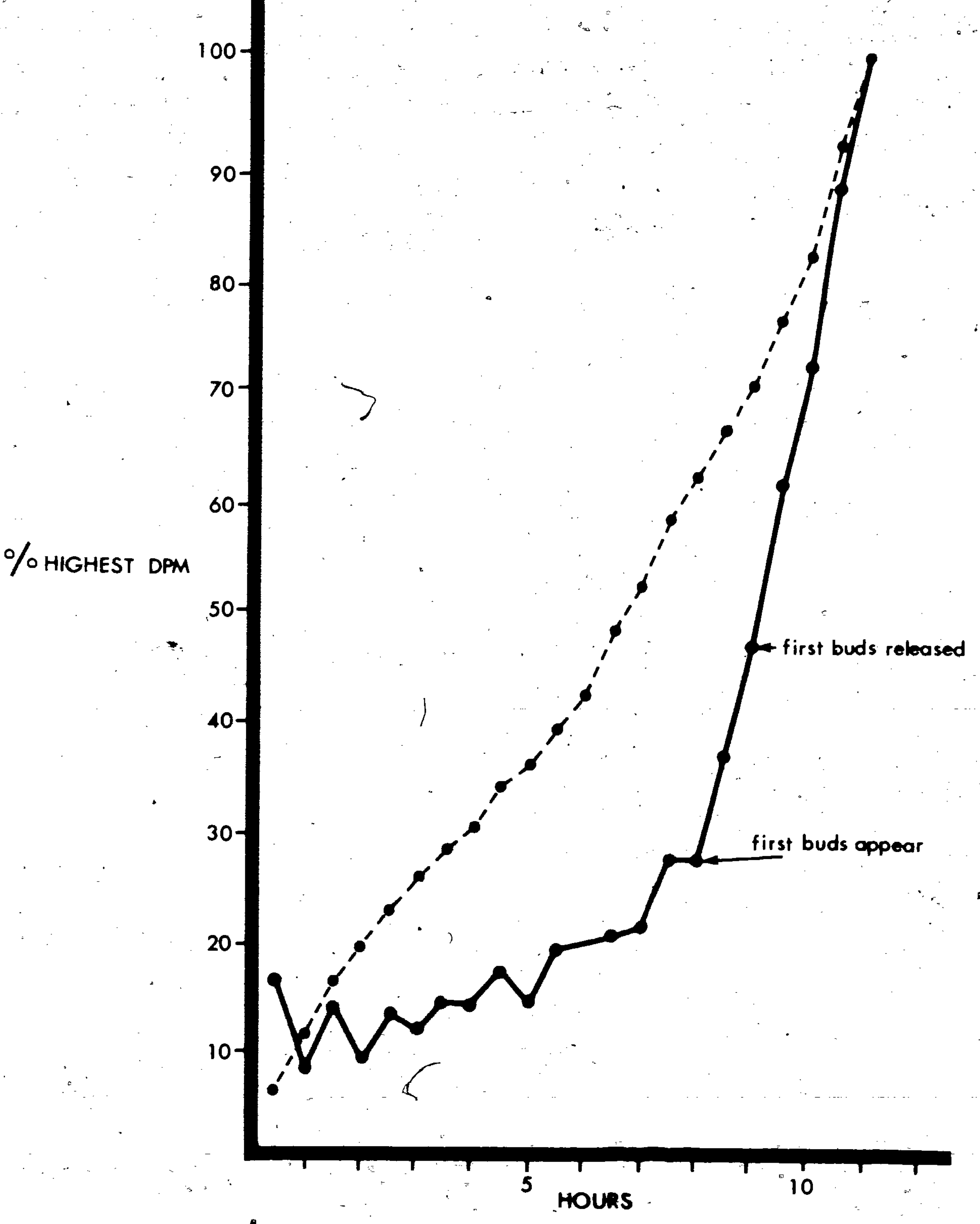
Initial results with conjugation tests showed that cells from stationary phase cultures gave a higher conjugation response than cells from exponential phase cultures. So also did cells from the tops of sucrose gradients (Plate XII, Fig. 62). Since cell growth in stationary phase is usually arrested in G_1 of the cell cycle, this evidence suggested that the cells were receptive to conjugation hormone only in G_1 of the cell cycle. To test whether cells from the top of a sucrose gradient were in G_1, they were transferred to fresh medium containing radioactive chemicals and uptake into DNA was measured. These gradient cells were 95-100% without buds and, judging from their sizes, consisted mostly of newly released cells. It is important to note that A_1 cells were more dense than A_2 cells and consequently moved further down the sucrose gradients than the A_2 cells did.

Tritiated thymine and thymidine were not incorporated into DNA by the yeasts of S. magnum. Uptake of $H_3{}^{32}PO_4$ is presented in Fig. 63 and uptake of <6-^{3}H> adenine is shown in Fig. 64. In

addition, these figures show the time when buds begin to appear and the total uptake of label before basic hydrolysis. It is clear that the cells enter DNA synthesis more or less synchronously. However, it is also clear that this synchrony is not maintained for even one division cycle. The apparent leveling at 8 hours in the ^{3}H adenine uptake is not supported by data on budding index and the leveling is also evident in the total uptake before basic hydrolysis. When the total uptake of 4×10^{6} dpm is compared to the total label used (2 uCi/ml) it is obvious that the cells have simply started to exhaust the supply of the labeled nutrient. Controls showing DNase treatment of hydrolysis-resistant acid precipitatable material are shown in Appendix D. They substantiate that most of the radioactivity in the alkaline stable precipitates could be attributed to DNA.

The quantitative effect of glucose concentration on conjugation is shown in Fig. 65 for both solid and liquid media. To determine whether this effect was simply osmotic, galactose and maltose media (sugars not assimilated) were compared to conjugation on glucose medium. The results are shown in Plates XIII to XV. Although all three sugars reduce conjugation with increasing concentration, only glucose succeeds in stopping it altogether at 0.82 M. This result was repeatable and suggested the possibility of catabolic repression of conjugation by glucose.

Figure 63. Uptake of $H_3{}^{32}PO_4$ by sucrose-gradient-selected cells of TWF 58-27. The broken line gives the total uptake of label into TCA-precipitatable material. The solid line gives the uptake of label into alkaline-stable, acid-precipitatable material. The highest total number of disintegrations per minute (DPM) was 320,425 while the highest DPM for the alkaline-stable material was 7,661.



100
90
80
70
60
50
40
30
20
10
% HIGHEST DPM
first buds released
first buds appear
5
10
HOURS

Figure 64. Uptake of <6-^{3}H> adenine by gradient-selected cells of TWF 58-27. The coarse broken line gives the total uptake of label into TCA-precipitatable material while the solid line gives the uptake of label into alkaline-stable, acid-precipitatable material. The highest total number of disintegrations per minute (DPM) was 4,321,451 while the highest mean DPM for the alkaline-stable material was 103,900. The light broken line shows the percentage of cells with attached buds (minimum number of cells counted per sample was 100). Standard deviations are indicated for means of the alkaline stable samples.

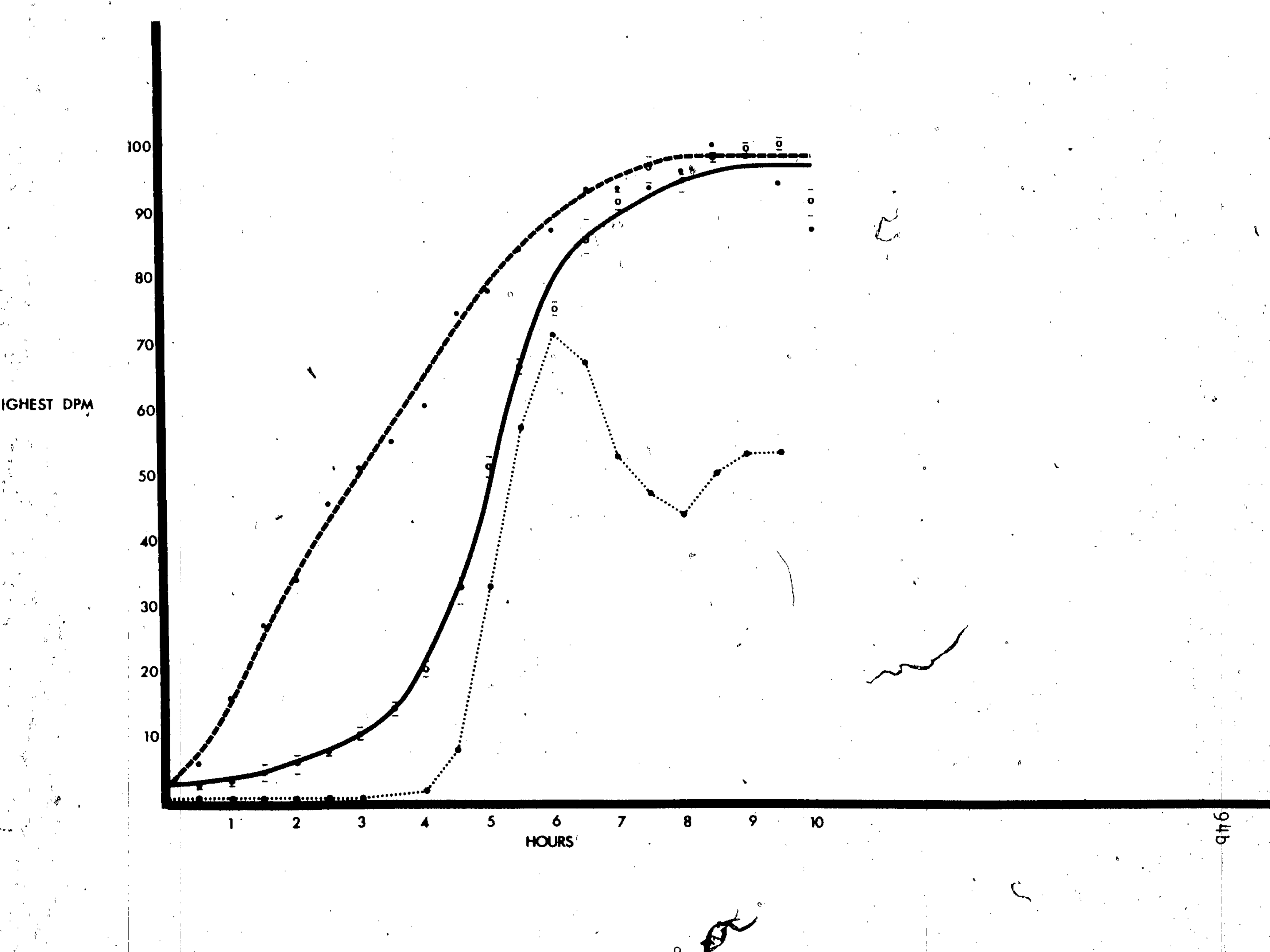
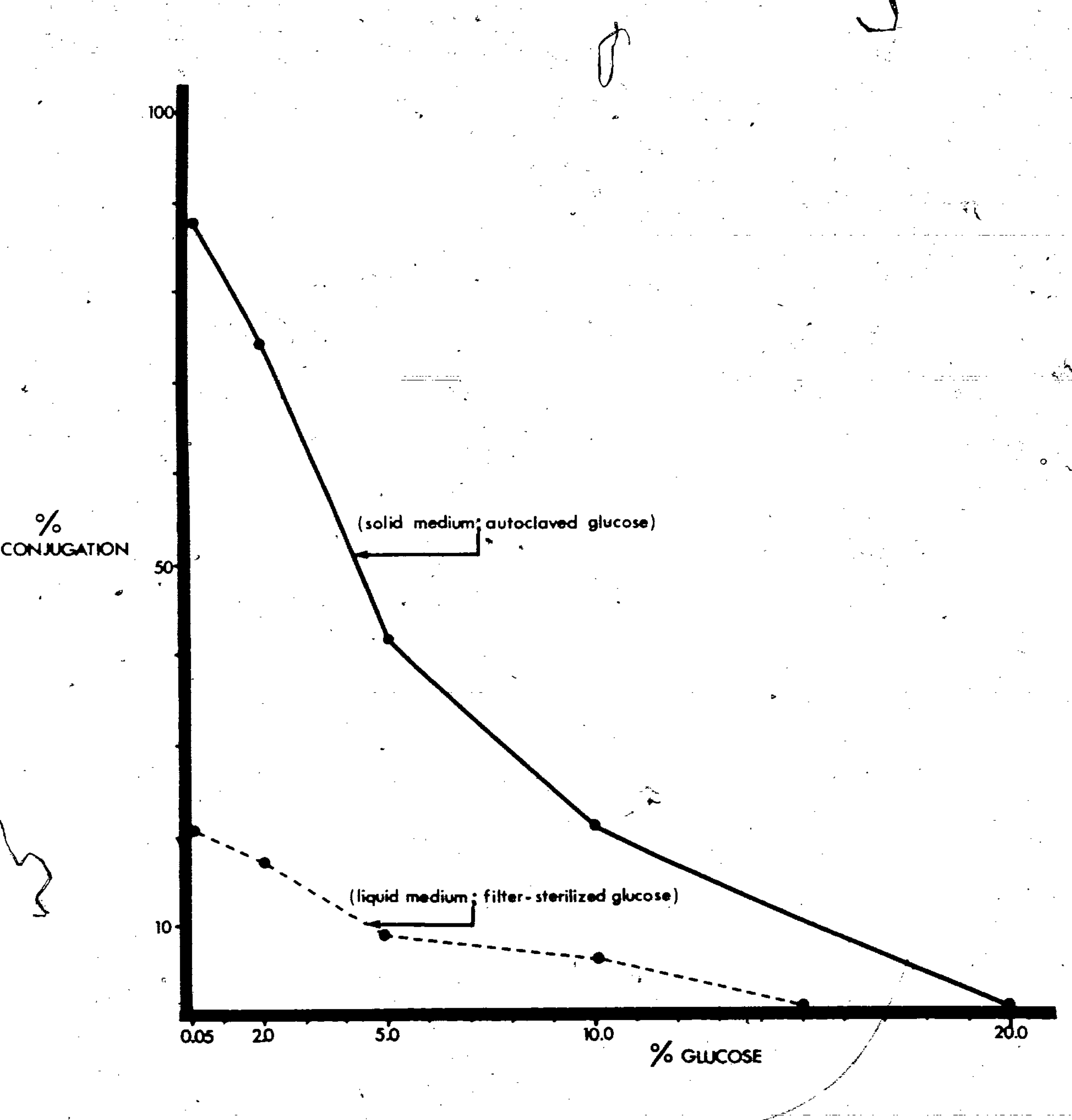

% HIGHEST DPM
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
HOURS

Figure 65. Quantitative effect of glucose on conjugation-tube formation. Trials were measured at 12 hours and the minimum total number of cells counted per sample was 400. Note the difference in response on solid and in liquid medium.


100
%
CONJUGATION
50
10
(solid medium; autoclaved glucose)
(liquid medium; filter-sterilized glucose)
0.05
2.0
5.0
10.0
20.0
% GLUCOSE

Growth and conjugation between yeast cells was morphologically unaffected by theophylline. However, solid media containing the related chemical compound, caffeine, produced large inflated yeast cells and some cells with hyphal extensions (Plate XVI). Some of these cells seemed to produce successive buds which did not become detached and irregular hyphal-like extensions resulted. Caffeine did not appear to inhibit or enhance conjugation between yeast cells on solid CJM but did appear to somewhat reduce the tendency of cells without conjugation tubes to bud and the tendency of cells with conjugation tubes to revert to budding growth. Ensuing development of dikaryotic mycelium was reduced when compared to development on CJM without caffeine.

The effect of alcohol on conjugation is shown in Plate XVII. These photographs were taken from one of the three replicates for each concentration tested. Qualitatively, the tests indicated that alcohol had a mild stimulatory effect on conjugation in liquid CJM.

The chemicals cAMP, dbcAMP, cGMP and dbcGMP were tested on single and mixed stationary isolates of 58-23 and 58-27 in the depression slide system. There was no morphological effect from concentrations ranging from 1 to 20 mM. Budding and conjugation proceeded normally as in the untreated controls.

Results from tests on intracellular levels of cAMP are shown in Table VII. The concentration (0.04 pM/mg protein, bovine serum albumin equivalent) was extremely low in both "conjugating" and non-conjugating cells, and it was equivalent in both.

TABLE VII

Levels of cAMP in conjugating and non-conjugating yeast cells of *Sirobasidium magnum*.

Cells	Total protein (mg/ml BSA equivalent)	Radio-immunoassay % label bound	Total cAMP pM/ml	pM cAMP per mg BSA equivalent
23 + 27	3.25	44	0.156	0.05
	4.00	45	0.164	0.04
23 x 27	4.50	46	0.148	0.03
	4.25	41	0.222	0.05

DISCUSSION

The fall in pH during growth in YNB and YCB-NH_4NO_3 broth was undoubtedly attributable to the selective uptake of ammonium ions from the medium (Hawker, 1950; Cochrane, 1958). This observation was consistent in all assimilative tests using YNB because the nitrogen source in that commercial preparation is NH_4NO_3. The rise in pH in YS broth and YE broth was probably due to the fact that nitrogen in these media was supplied predominantly by organic compounds. Some of these amimo acids could be deaminated and used as carbon sources. The result of the deamination would be accumulation of ammonia in the growth medium (Hawker, 1950; Cochrane, 1958). Attenuation of growth at approximately 36 hours in YNB, YE and YS broth may have been the result of accumulating staling products, diminishing nutrients, or a combination of both. No tests were done to determine the cause.

Time lapse sequences of budding can be used to determine volume changes and division timing in the life-cycle of yeast cells (Mitchison, 1971; Mitchison and Carter, 1975). Using this technique with *S. magnum* led to surprising results in that there was a difference between the bud production time of newly released cells and cells that had already produced their first daughter cell. This is

unlike many yeast cells where, as Morris (1958) states, "...the time taken for successive generations to be reproduced by two individual cells of the same age is of the same order." Certainly, in order to obtain cell synchrony over two or more generations this would be essential. The yeasts most heavily studied in cell-cycle analysis (Saccharomyces cerevisiae and Schizosaccharomyces pombe Lindner) definitely fall into this category. It is interesting to note that for these two yeasts, the mother and daughter cells are more or less equal in size at the time of separation. They are not in Sirobasidium magnum. It may be that the cell needs to reach a critical size before it can produce a bud and the daughter cells may thus have an extended G_1 period relative to their mother cells. It would not be surprising if this non-equivalence was a general feature of yeasts in which there is a noticeable difference in size between the mother and daughter cells at the time of bud separation. Such non-equivalence means that any exponential phase culture of cells consists really of two distinct cell types which produce buds at different rates. Consequently, mean generation times calculated from the growth curve cannot, in themselves, give a clear idea of the length of the cell cycle.

Conjugation between cells of S. magnum was reduced at both low and high concentrations of cells. A similar situation has been reported for conjugation in Tremella (Flegel, 1968; Brough, 1970). There could be several explanations for this phenomenon. Since the conjugation process is hormonally regulated, the effect could be a reflection of hormone concentration, i.e. in the same volume of test fluid, too few cells may not produce enough hormone in a requisite time to reach a required minimum threshold level while too many cells may produce too much hormone to elicit a response. Another possibility is that conjugation inhibitors are produced by the yeast cells. Reid (1973) in studying conjugation in T. mesenterica with semi-purified hormone preparations, noted a similar decrease in conjugation response with increasing concentrations of test cells. He suggested that this could be explained either by the presence of self-inhibitors or by competition between cells for available hormone. The latter possibility seems unlikely here since the ratio of cells (1:1) was held constant during the tests. Evidence for the inhibitor theory comes from the fact that low concentrations of mixed cells did bud over the test period even though they did not conjugate. These initially low concentrations of cells eventually reached the same density as the trials which had already conjugated well. Still, there was no resulting conjugation response. A preliminary test for the

existence of yeast-phase-produced inhibitors could be done by using additions of single-isolate cell-free culture medium to CJM containing mixed compatible isolates. In any case, the inhibition of dikaryotic filamentous growth by yeast cells as reported in Chapter 2 may be related to this inhibition of filamentous growth during mating.

Time-lapse sequences of conjugation in S. magnum show that in no case do conjugation tubes arise from cells with attached buds. Neither is there an example of a bud arising from a cell which is producing a conjugation tube. In Saccharomyces cerevisiae (Hartwell, 1973) this same feature is evident and it contrasts with that in Ustilago violacea (Pers.) Fuckel (Poon, Martin and Day, 1974) where cells with attached buds may take part in conjugation. It has been suggested that cells of S. cerevisiae must be in G_1 of the cell cycle in order to conjugate (Poon et al., 1974; Bucking-Throm, Duntze, Hartwell and Manney, 1973) while it has been shown that one mating type of Ustilago violacea is mating-competent throughout its cell cycle (Cummins and Day, 1973). The results presented here show that S. magnum most likely behaves in the same way as S. cerevisiae, i.e. both partners must be in G_1 of the cell cycle at the time of conjugation.

Also, as shown in the time-lapse sequences, some cells produce only successive buds even when they are in the vicinity of conjugating cells of opposite types. This could be a manifestation, on crude agar medium, of microenvironmental differences which prevent hormonal communication, or it could be that the cells are physiologically unreceptive to the conjugation hormones. None of the results here could distinguish between these alternatives. In addition to persistently budding cells, there were also some cells which neither budded nor conjugated. These cells were most likely dead cells, for as pointed out by Morris (1958), even exponentially growing cultures accumulate increasing numbers of these.

The reason why ethanol mildly stimulates conjugation is unclear. Although *S. magnum* yeasts do not grow anaerobically tests were not done to determine whether they produce ethanol during aerobic culture. Assimilative tests show that they can at least use it as the sole carbon source. They may, therefore, produce it in small amounts as other fungi do (Cochrane, 1958) when the oxygen supply is low but not absent. At the same time, this ethanol phenomenon may have some ecological significance. For example, Bandoni (1961) indicates that many species of *Tremella* are parasitic on or associated with other fungi. Since *S. magnum* grows on decaying wood as do many species of

Tremella, a similar sort of association may exist. If it does, and if the associated wood destroying fungus produces alcohol as some do (Cochrane, 1958), then it is possible that the ethanol could act as a stimulator of the process leading to the dikaryotic parasitic stage. A review of fungal interactions caused by volatile metabolites has been presented by Hutchinson (1971). Another review by Fries (1973) covers the effects of volatile organic compounds in general on the growth and development of fungi.

Preliminary evidence for the presence of conjugation hormones in S. magnum appears in the time-lapse sequences of conjugation. Tubes arise from cells separated from one another by distances of several microns. In addition, there is often an abrupt change in the directional growth of conjugation tubes when they are close, and this enables them to meet tip to tip. Such an abrupt directional change has been reported for Tremella (Bandoni, 1965; Brough, 1970) and it has been suggested (Bandoni, 1965) that it is a chemotropic response. If it is, it could either be a response to a concentration gradient of the tube induction hormone, or a response to a completely different substance (Raper, 1967). Day (1976) has hypothesized that directional changes in conjugation tubes in Ustilago violacea result from the tubes following fimbrae of the opposite mating type. These fimbrae or

surface filaments have been reported previously by Poon and Day (1975a) as occurring in many yeasts. These authors later presented models for the function of these filaments in sexual interactions in Ustilago violacea (Poon and Day, 1975a) and in two species of Saccharomyces (Day, Poon and Stewart, 1975). Conjugation competence and the presence of fimbrae are coincident and treatments which affect one of these also affect the other. However, coincidence is not cause and as of yet these structures have not been demonstrated conclusively to be the directors of conjugation tube production. Should Day's model prove correct for U. violacea, it is doubtful that the situation in Tremella mesenterica, at least, is the same. Reid (1974) has shown that the erogens are less than 1000 daltons in molecular weight and that would not be consistent with the length of the structures required to link cells separated by several microns.

In S. magnum, exclusion of the morphogenetic erogens by one membrane and not another indicate a molecular weight of between 8,000 and 14,000 daltons. Crandall (1977) has suggested that the inability to prepare an active isolate of this hormone shows that, "...each mating type may inactivate or inhibit the pheromone from the opposite type." A similar suggestion has been made for Saccharomyces cerevisiae

(Hicks and Herskowitz, 1976). These suggestions would be consistent with the results on conjugation versus cell concentration discussed above.

Failure of S. magnum to incorporate thymine or thymidine directly into DNA is a feature common to many yeasts (Hartwell, 1974) and may be the result of an absence of thymidine kinase in the cells (Grivell and Jackson, 1968). However, labelling experiments using $H_3{}^{32}PO_4$ and <6-^{3}H>-adenine showed that yeast cells from the top of a sucrose gradient were in G_1 of the cell cycle and that they entered the S phase of DNA synthesis more or less synchronously. The inability to maintain synchrony for even one cycle is probably due to the length of time taken for the cells to produce their first buds. Since the G_1 period is so extended (approximately 3 hours) there is a good deal of time for the cells to get out of phase with each other. Also, the fact that the total experimental time is 8 to 10 hours means that the fed label has had considerable time to become distributed through various pathways by H exchange. This is probably why DNase resistant acid-precipitatable material accumulated with incubation time. It may be more useful in future to select those cells from the gradient that have no attached buds but which have already produced at least one bud. In an exponential phase population, this would be a

relatively uniform-sized population somewhat larger than the cells used here and consequently lower in the sucrose gradient. They would have a much shorter cell-cycle length than their daughter cells, and, more particularly, a much shorter G_1 period. The total result should be closer synchrony throughout the cell cycle and a simultaneous entry into G_1 of the second cycle. Again, beyond one cycle, synchrony would not be possible because of the difference between mother and daughter cells at the time of separation.

It is worthy of note, at this point, that cells with the A_1 genotype (58-27 = A_1B_1; 11-6 = A_1B_3 and 11-2 = A_1B_4) moved further down the sucrose gradient than A_2 cells (58-23 = A_2B_2; 11-1 = A_2B_3 and 11-5 = A_2B_4). This correlates with the larger size of the former cells and with the larger nuclei of these cells pointed out in Chapter 2. This may indicate a difference in chromosomal number between the two cell types. It has the advantage that the specific nuclei can be identified in cytological staining of various stages in the life cycle.

The mode of operation of the conjugation hormones in _S. magnum_ can, at present, only be speculated upon. If the models presented for other yeasts all prove correct for their respective species, then the

mechanisms are indeed various. The chemical nature of the hormones, their numbers in any one species, their mode of production, and their effects on target cells may be entirely dissimilar. In addition, nothing has been done in this study or in the others discussed, concerning the reception of the conjugation hormones at the cell surface. This area of study has been the object of much attention in agglutinating yeasts but not in those which produce long conjugation tubes. The subject is covered in a recent review by Crandall (1977). Studies on internal cellular changes wrought by conjugation hormones are more common. In _S. magnum_ preliminary experiments to determine possible cyclic-nucleotide involvement gave mixed results. External application of these compounds and their fat-soluble analogues to single and mixed cell isolates were without visible consequence. This in itself did not exclude cyclic nucleotide involvement in morphogenesis for the results could simply have meant that none of the compounds tested could enter the cell.

Experiments with glucose and caffeine were at least positivley suggestive that cyclic nucleotides influenced morphogenesis in _S. magnum_. The inhibitory effect of glucose on conjugation was partially, though not wholly, a result of osmotic effects. In mammalian hormonal systems, adenyl-cyclase activity has been reported

to be subject to catabolite repression by glucose (Robinson, Butcher and Sutherland, 1971; Pastan and Perlman, 1971). In this way, glucose interferes with cAMP accumulation and aborts the normal response to hormonal stimulation. By analogy to S. magnum, one would expect a high concentration of cAMP to lead to hyphal growth, while a low concentration would lead to budding growth. This would be opposite to the situation reported for Mucor racemosa (Carsen and Sypherd, 1974; Paznokas and Sypherd, 1975).

Results from tests with caffeine were also suggestive of cyclic nucleotides being involved in morphogenesis in S. magnum. Again, in mammalian systems this compound has been reported to inhibit cAMP specific phosphodiesterase and to lead to accumulation of cAMP (Robinson et al., 1971; Pastan and Perlman, 1971). In S. magnum, treatment with this compound led to a relatively small but definite number of filamentous outgrowths from yeast cells and it also led to large inflated cells. If this were a cyclic-nucleotide effect similar to that occurring in mammals, then it would support the view that high internal concentrations of cAMP would result in hyphal growth while low concentrations would result in budding growth in S. magnum. On the other hand, the length of time taken to to gain this response was relatively long (up to 7 days) and consequently these growth forms may

be a manifestation of cellular changes unrelated to those caused by conjugation hormones. For example, Reid (1975) has reported the occurrence of inflated cells in Tremella mesenterica yeasts grown on media containing asparagine. Since caffeine is a nitrogen-containing compound, inflated cells in S. magnum may be a result of caffeine catabolism and not phosphodiesterase inhibition.

The limited tests carried out here to determine the levels of cAMP in conjugating and non-conjugating cells show that it is probably not a mediator in the yeast-hyphal transformation in S. magnum. This does not preclude the possibility that another cyclic nucleotide is involved.

SUMMARY

The purpose of this thesis was to demonstrate the usefulness of the dimorphic fungus _Sirobasidium magnum_ as a tool for studying yeast-hyphal transformation. Information from such specific study should have potential application to morphogenetic studies in eukaryotic organisms in general.

Because the organisms used in this study had not been found previously in the areas where they were collected, Chapter 1 described work done to establish the identity of the organisms. While carrying out this identification, aspects of the yeast cells and their mating system brought to light possible relationships between the genera _Sirobasidium_, _Tremella_, _Bullera_ and _Cryptococcus_.

Once the identity of the study material was established as _Sirobasidium magnum_, techniques were devised which brought the organism through its life cycle in the laboratory. This made genetic analysis feasible under controlled conditions. Techniques were also developed to observe the cellular nuclei at the various stages in the life cycle. Using these techniques, the times and places of dikaryotization, karyogamy, meiosis and monokaryotization were

established. This work was described in Chapter 2 and was necessary before a particular focus could be made on conjugation events leading to dikaryotization.

Chapter 3 addressed the question of whether conjugation in _S. magnum_ was a suitable process for the study of yeast-hyphal dimorphism. There it was shown that the yeast-hyphal transition (conjugation tube production) was mediated by diffusible substances to which yeast cells responded only during the G_1 phase of the cell cycle. Other tests showed, contrary to preliminary indications, that 3':5'-cyclic adenosine monophosphate was not involved as a secondary messenger in conjugation-tube formation.

In conclusion, this thesis shows that _S. magnum_ has considerable merit as a tool for studying yeast-hyphal transformation and that it deserves further attention in that regard. In a relatively simple system it makes possible the elucidation of the mechanisms of hormone production and of hormone action in target cells.

One limitation of _S. magnum_ for physiological studies arises from the demonstration that mother and daughter yeast cells are not equivalent at the time of separation. This means that synchronous

cell cultures cannot be maintained for more than one turn of the cell cycle.

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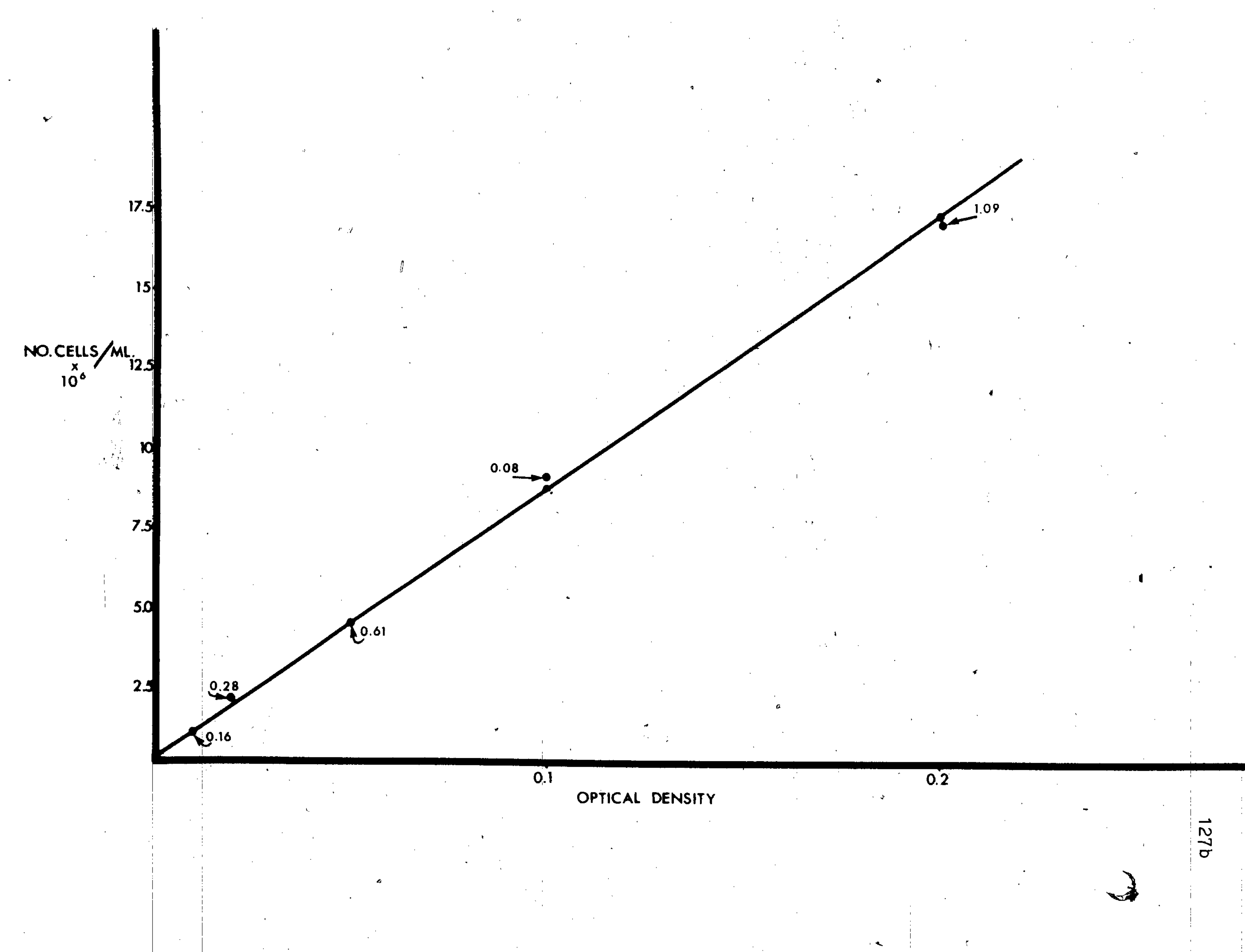
APENDIX A

Standard curve for cell number versus optical density. Eight haemacytometer counts were made for each dilution except for the greatest dilution where 16 counts were made. The standard deviations for the means of these counts are given in the figure. The equation used to draw a line through the means was $y/10^6 = 84x + 0.345$.


NO. CELLS/ML. x 10^6
17.5
15
12.5
10
7.5
5.0
2.5
1.09
0.08
0.61
0.28
0.16
0.1
0.2
OPTICAL DENSITY

APPENDIX B: Optical density readings for 58-23 grown on various carbon sources.

MEDIUM	0 Hr.	12 Hr.	24 Hr.	36 Hr.	48 Hr.	60 Hr.	96 Hr.	144 Hr.
	X1	X1	X10	X100	X100	X100	-	-
YNB-Glucose	0.020	0.035	0.033	0.021	0.030	0.029	-	-
	0.029	0.031	0.030	0.019	0.040	0.030	-	-
	0.022	0.035	0.032	0.022	0.030	0.028	-	-
	X1	X1	X1	X1	X1	X1	X1	-
YNB-Cellobiose	0.022	0.030	0.030	0.040	0.042	0.058	0.198	-
	0.018	0.025	0.039	0.030	0.044	0.063	0.208	-
	0.018	0.039	0.030	0.040	0.047	0.060	0.190	-
	X1	X1	X1	X1	X1	X10	-	-
YNB-Xylose	0.025	0.024	0.028	0.044	0.091	0.019	-	-
	0.028	0.022	0.029	0.048	0.088	0.020	-	-
	0.023	0.026	0.037	0.049	0.096	0.023	-	-
	X1	X1	X1	X1	X1 *	X1	-	X10
YNB-Inositol	0.017	0.020	0.020	0.030	0.031	0.042	-	0.035
	0.019	0.021	0.021	0.029	0.030	0.045	-	0.032
	0.018	0.019	0.020	0.025	0.035	0.038	-	0.025
	X1	X1	X1	X1	X10	X10	-	-
YNB-Mannitol	0.021	0.029	0.042	0.098	0.023	0.078	-	-
	0.020	0.030	0.041	0.098	0.023	0.070	-	-
	0.019	0.029	0.048	0.078	0.023	0.070	-	-
	X1	X1	X1	X1	X1 *	X1	-	X1
YNB-Control	0.018	0.020	0.017	0.020	0.022	0.021	-	0.025
	0.020	0.021	0.020	0.020	0.028	0.021	-	0.025
	0.020	0.022	0.019	0.021	0.025	0.023	-	0.021
	X1	X1	X10	X100	X100	X100	-	-
YS-Broth	0.026	0.042	0.095	0.080	0.100	0.100	-	-
	0.030	0.049	0.095	0.071	0.100	0.101	-	-
	0.022	0.042	0.100	0.074	0.101	0.100	-	-

*- measured at 45 rather than 48 hr.

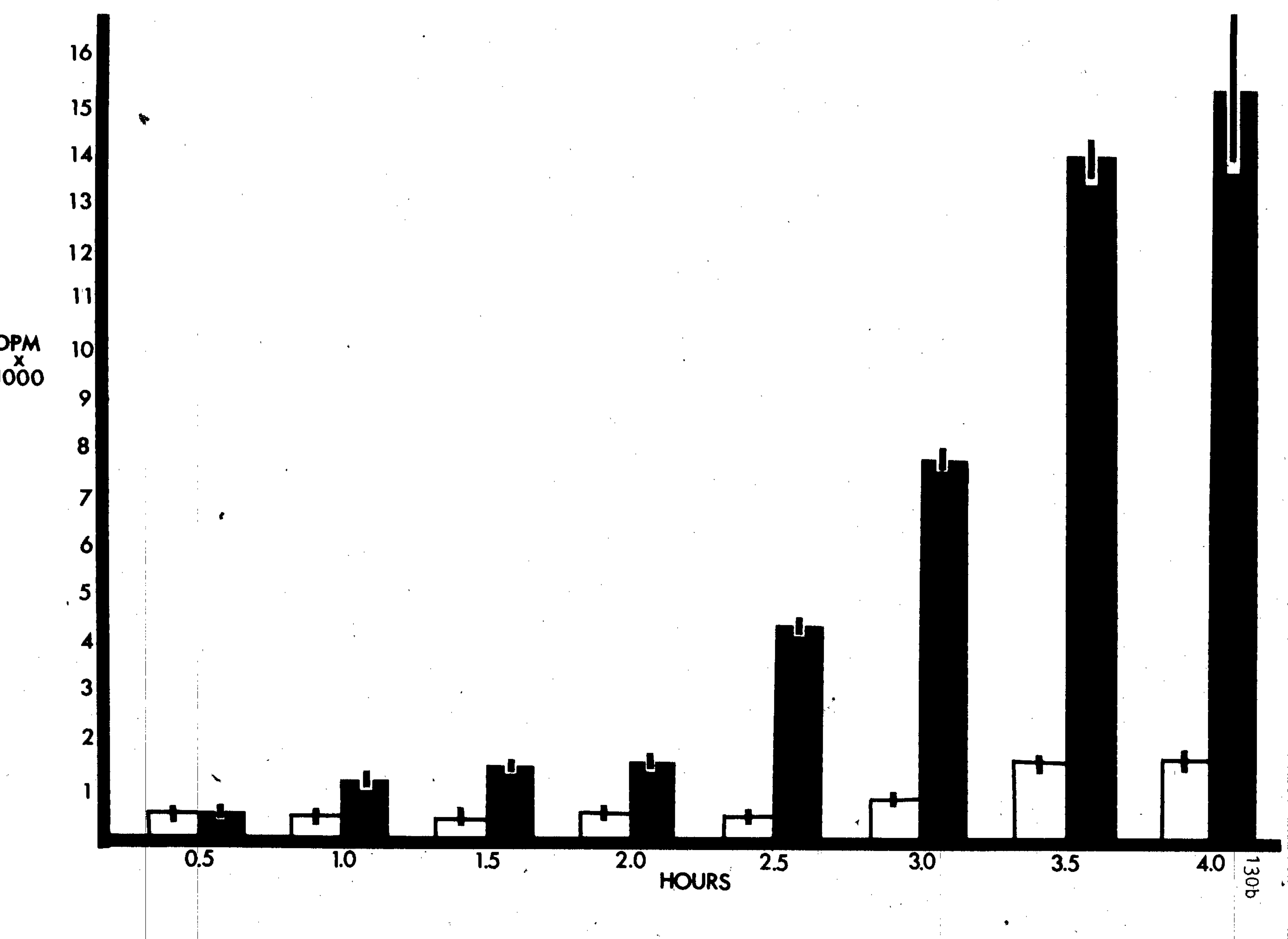
APPENDIX C: Optical density readings for 58-23 grown in YCB with various nitrogen sources.

MEDIUM	0 Hr.	12 Hr.	24 Hr.	36 Hr.	48 Hr.	60 Hr.	72 Hr.
	X1	X1	X1	X10	X100	-	-
Asparagine	0.028	0.021	0.120	0.105	0.040	-	-
	0.022	0.028	0.138	0.073	0.038	-	-
	-	0.021	0.171	0.068	0.040	-	-
	X1	X1	X10	X10	X100	X100	-
Asparagine (buffered)	0.022	0.031	0.019	0.092	0.026	0.045	-
	0.020	0.038	0.019	0.100	0.024	0.045	-
	-	0.040	0.019	0.105	0.029	0.041	-
	X1	X1	X10	X100	X100	X100	-
NH_4Cl	0.020	0.028	0.020	0.022	0.032	0.031*	-
	0.018	0.030	0.029	0.024	0.030	0.035	-
	0.020	0.030	0.020	0.022	0.031	0.031	-
	X1	X1	X1	X1	X1	X1	-
KNO_3	0.020	0.033	0.050	0.072	0.070	0.068*	-
	0.020	0.038	0.060	0.082	0.058	0.082	-
	0.020	0.030	0.048	0.062	0.065	0.085	-
	X1	X1	X1	X1	X1	-	X1
Control	0.023	0.040	0.075	0.080	0.090	-	0.100
	0.025	0.045	0.072	0.078	0.090	-	0.132
	0.023	0.048	0.071	0.078	0.091	-	0.140

* - measured at 62 rather than 60 hr.

APPENDIX D

Comparison of the activity in DNase treated and untreated filters with alkaline-stable acid-precipitatable material. Open bars show the activity of the DNase-treated samples while the solid bars show the activity of the untreated filters. Insets show standard deviations of the means for the triplicate samples.


DPM
x
1000
16
15
14
13
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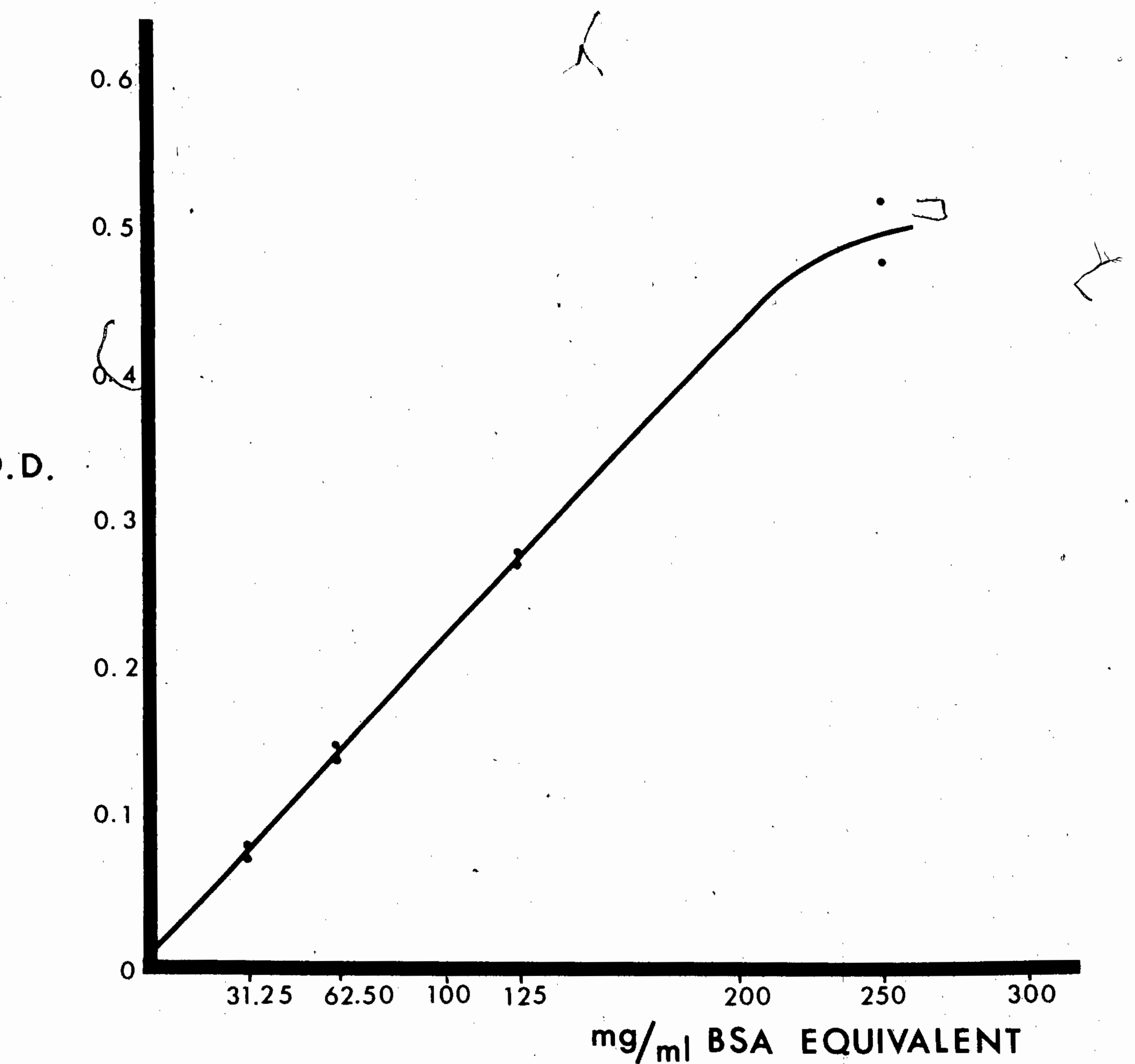
11
10
9
8
7
6
5
4
3
2
1
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
HOURS

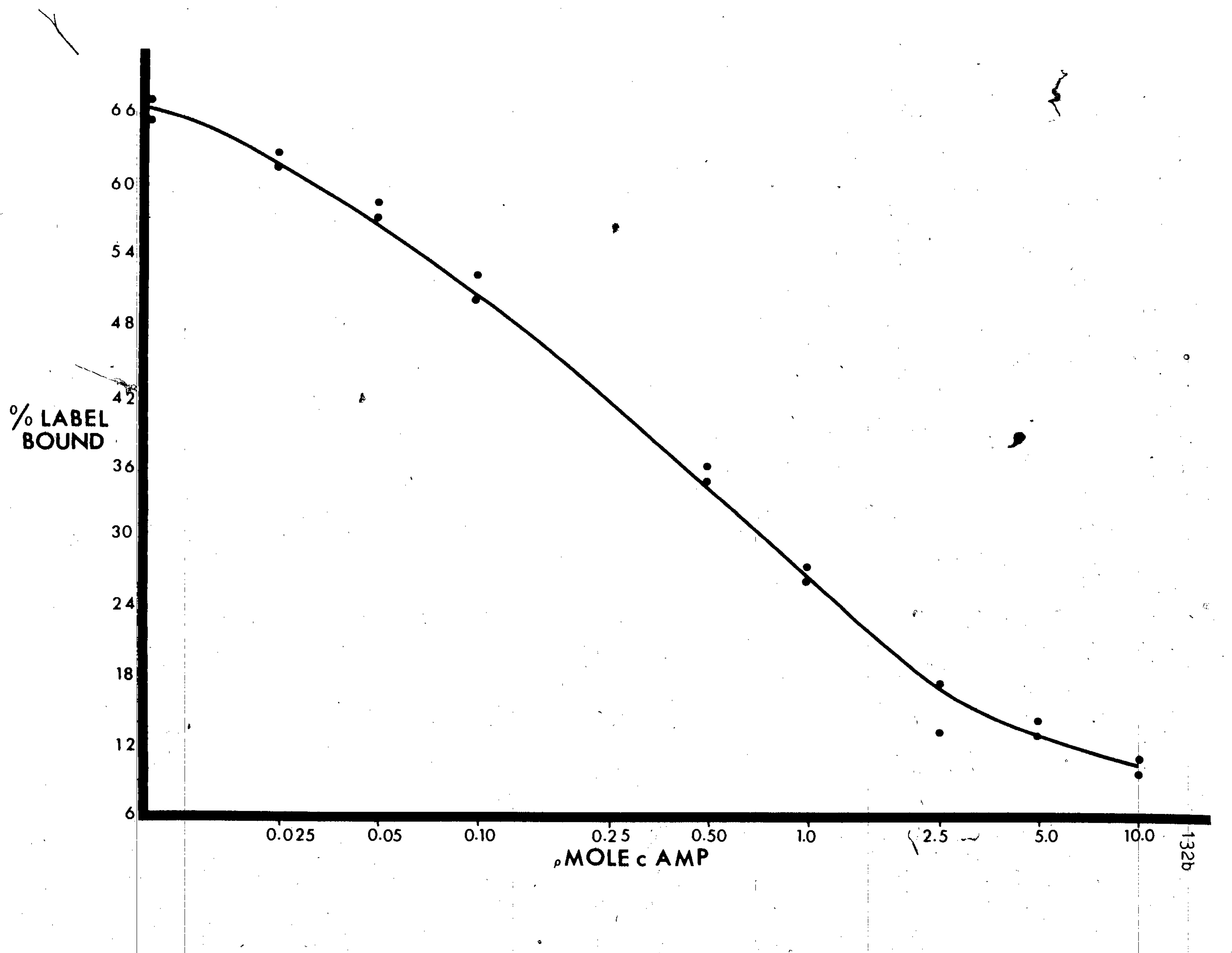
APPENDIX E

Calibration curve for optical density versus mg/ml bovine serum albumin (BSA).


O.D.
0.6
0.5
0.4
0.3
0.2
0.1
0
31.25
62.50
100
125
200
250
300
mg/ml BSA EQUIVALENT

APPENDIX F

Standard curve for radioimmunoassay of cAMP.


% LABEL BOUND
66
60
54
48
42
36
30
24
18
12
6
0.025
0.05
0.10
0.25
0.50
1.0
2.5
5.0
10.0
pMOLE c AMP

APPENDIX G

This appendix contains Plates I to XVII.

PLATE I

Figures 3-5. Stationary-phase cells of TWF 58-23.

Figure 3. Encapsulated cells from YS broth in an india-ink preparation (X1750).

Figure 4. Encapsulated cells from YCB with NH_4NO_3 as the nitrogen source. This is an india-ink preparation. (X1750)

Figure 5. Cells with haematoxylin-stained nuclei (X1750).

Figures 6 and 7. Inflated ballistospores from isolate 58-23.

Figure 6. Germinating spores containing one to three nuclei (X1750).

Figure 7. Spores germinating by repetition (X1750).

Figure 8. Exponential-phase yeast cells (TWF 58-23) with attached buds (X1750).

Figures 9-12. Cells from a cross of yeast isolates TWF 58-23 and 58-27.

Figure 9. A cell that has produced a conjugation tube and then reverted to budding .

Figure 10. A conjugating pair of cells showing the inflated area of conjugation-tube contact and the beginning of a dikaryotic hyphal strand arising from one of the conjugants (X1750).

Figure 11. A conjugating pair of cells stained with haematoxylin to show the two nuclei in the hyphal strand arising from the point of contact of two conjugation tubes (X1750).

Figure 12. The first branch of a dikaryotic hyphal strand arising from a conjugating pair of cells. The branch arose from a clamp connection and another clamp connection was then produced on the new branch (X1750).

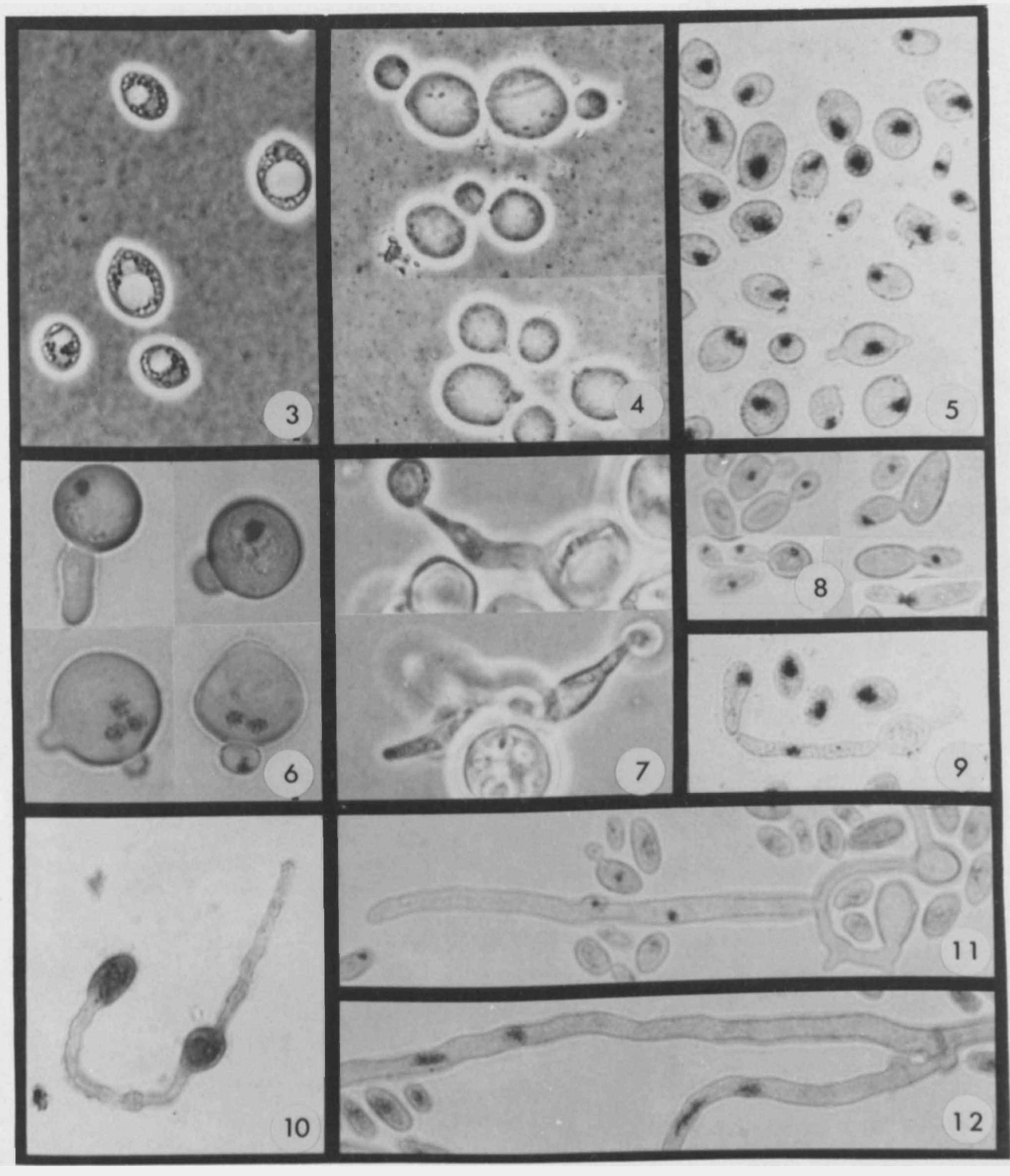

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PLATE II

Figures 13-19. Basidia and sterigmata from agar culture.

Figure 13. Metabasidia with attached sterigmata (X245).

Figure 14. Two sterigmata attached to a metabasidium empty of cytoplasm (X640).

Figure 15. Detached sterigmata (X1600).

Figure 16. Catenulate probasidia and metabasidia (X950).

Figure 17. Obliquely septate metabasidium (X950).

Figure 18. Pair of metabasidia, uppermost evacuated of cytoplasm but with the sterigmata still attached (X950).

Figure 19. Detached sterigmata producing secondary sterigmata (X950).

Figure 20. Detached sterigma with secondary sterigma and attached basidiospore (X1200).

Figures 21-23. Basidiospores

Figure 21. Basidiospores germinating by repetition (X1100).

Figure 22. Basidiospores germinating by repetition (X1100).

Figure 23. Ungerminated basidiospores (X1200).

Figure 24. Detached sterigmata with haematoxylin-stained nuclei (X1750).

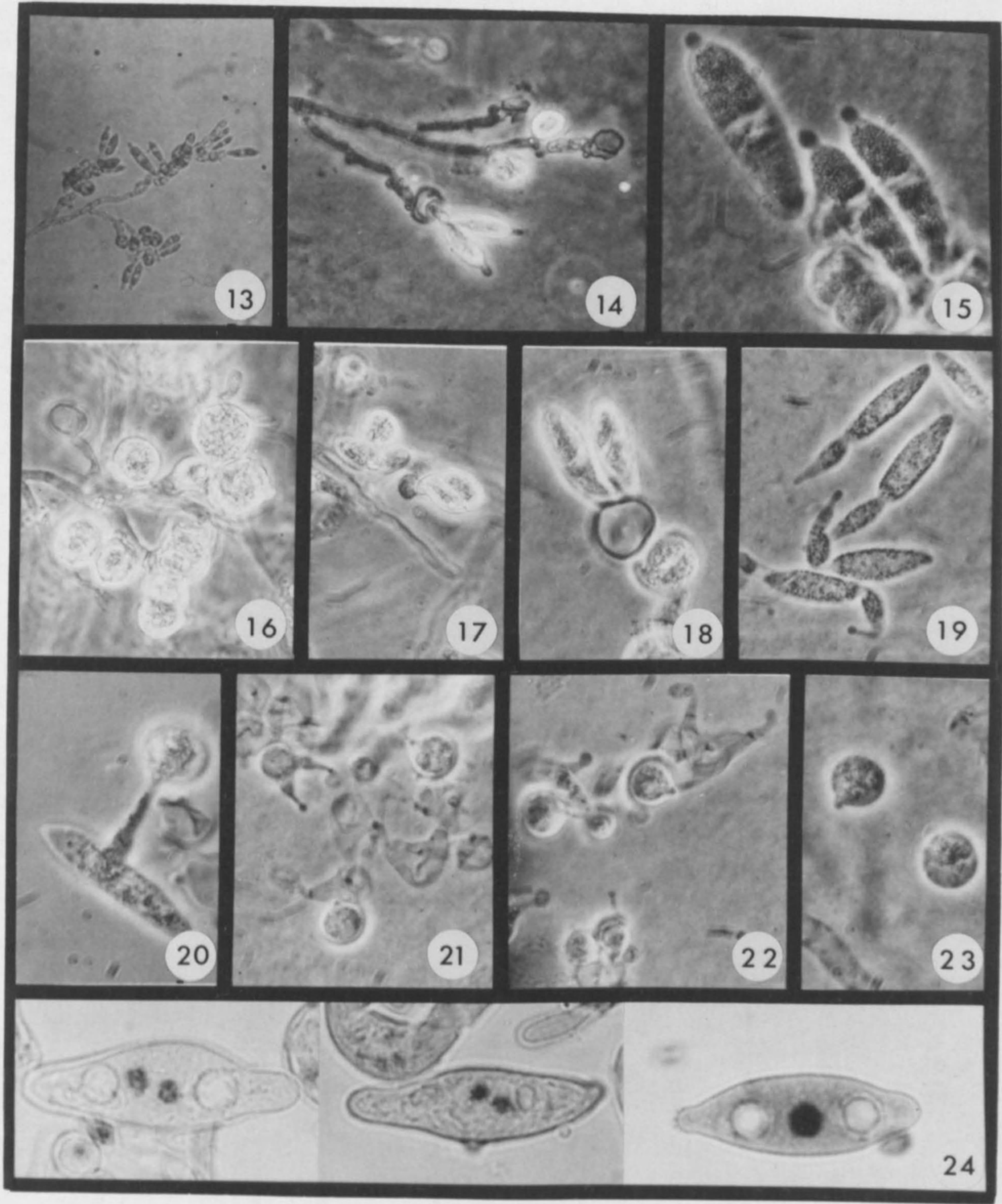
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Plate III

Figure 25. Formation of first clamp connection after conjugation of two compatible cells (X1750).

Figure 26-32. Stages in basidial development and maturation.

Figure 26. Probasidial cell before karyogamy (X1750).

Figure 27. Two probasidial cells, the upper one post karyogamy and the lower one still dikaryotic (X1750).

Figure 28. Two probasidia after karyogamy, both subtended by clamp connections (X1750).

Figure 29. Trinucleate metabasidium with oblique crosswall (X1750).

Figure 30. Metabasidium beginning sterigmatal formation (X1750).

Figure 31. Trinucleate metabasidium with unfilled sterigmata attached to the binucleate cell (X1750).

Figure 32. Binucleate sterigma attached to a metabasidial cell emptied of cytoplasm (X1750).

Figure 33. Uninucleate basidiospores (X700).

Figure 34. Germinating basidiospores; the lower one containing two nuclei; the inflated tip is a preparation artifact (X700).

Figures 35-37. Germinating basidiospores producing yeast cells at the tips and sides of germ tubes.

Figure 35. Cells stained with haematoxylin to show nuclei in the germ tube and in the yeast cells (X1750).

Figure 36. Yeast cells being produced at the sides and tip of a basidiospore germ tube (X1750).

Figure 37. Germ tube and yeast cells stained for nuclei (X1750).

Figure 38. Dikaryotic clamped mycelial tip which arose from a single basidiospore (X1750).

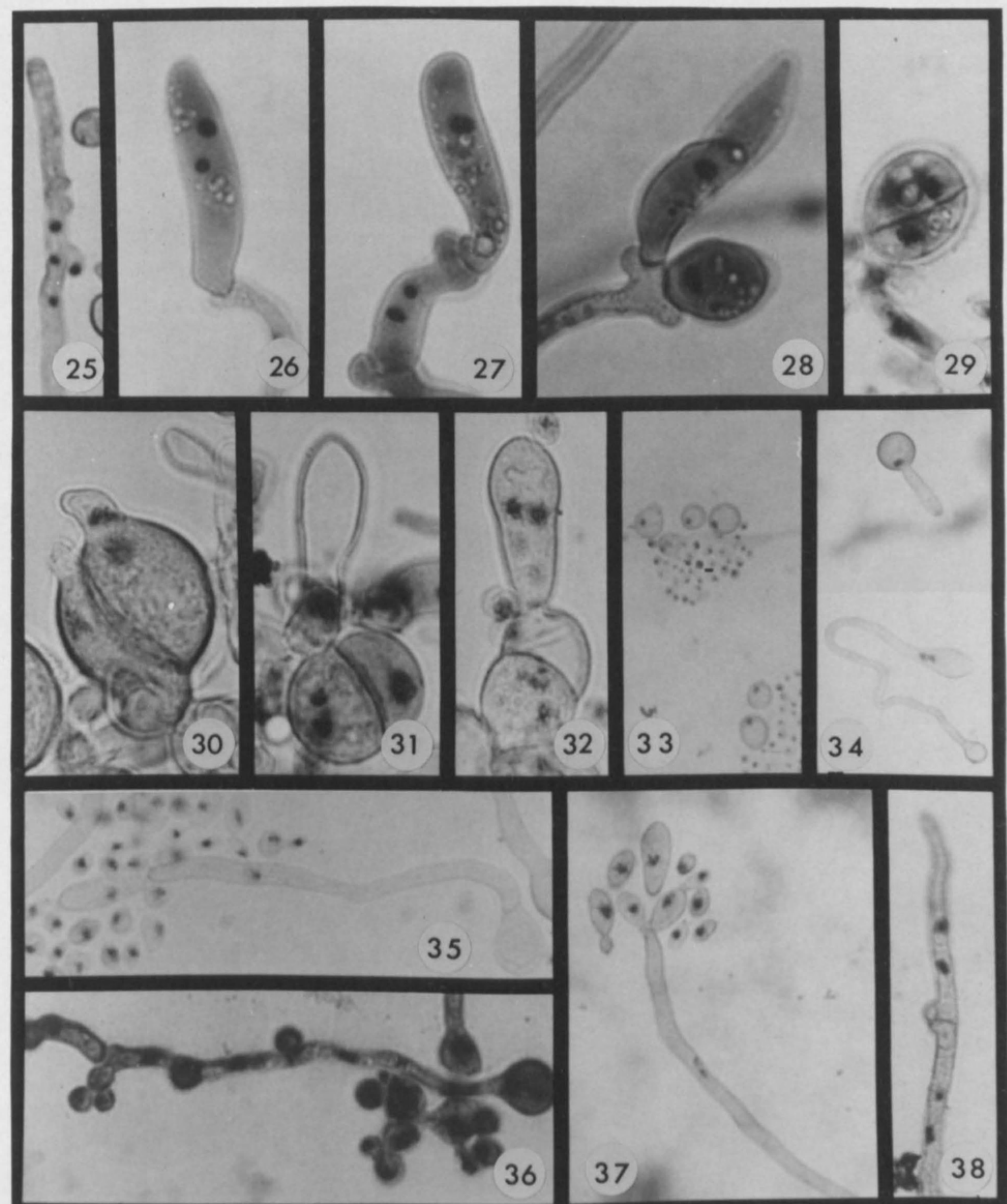
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PLATE IV

Figure 39. Conjugating cells of TWF 58-23 and 58-27 showing the cytoplasm withdrawing from the conjugating cells following formation of the dikaryon. The septum formed without an accompanying clamp connection is probably adventitious, i.e. it was probably formed in the absence of nuclear division.

Figure 40. Short clamped hyphal cells (probasidia) destined to become catenulate metabasidia (X2000).

Figure 41. Examples of the large elongated cells present in small numbers in yeast colonies. These are from isolate TWF 58-27. Such cells probably produce ballistospores (X950).

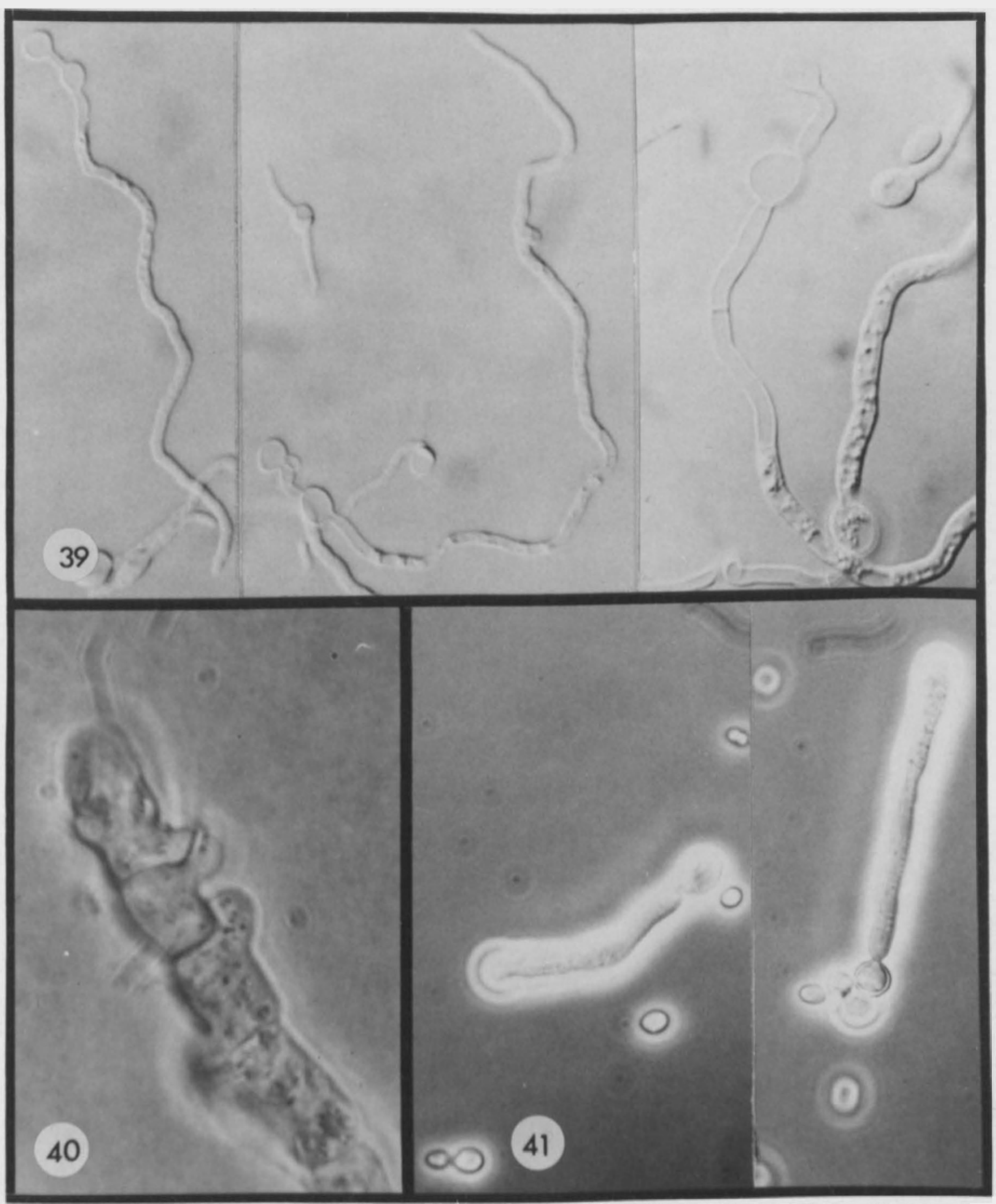
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PLATE V

Figure 42. A mixture of cells of isolates TWF 58-23 and 58-27 grown on YE agar with 20% maltose. The nuclei are visible as clear areas with a central dark nucleolus. These may be contrasted with the lighter-appearing vacuoles. (X1400).

Figure 43. A mixture of cells of isolates TWF 58-23 and 58-27 showing cells with conjugation tubes. Some of these cells have reverted to budding (X700).

Figure 44. Dikaryotic hyphae resulting from a cross of TWF 58-23 and 58-27. This was grown in YE broth. (X300)

Figure 45. Light and dark field photomicrographs of a squash mount from a laboratory-grown basidiocarp. It arose from a cross of TWF 58-23 and TWF 11-2. A well developed hymenial layer is evident with catenulate basidia and abundant released sterigmata. (X200)

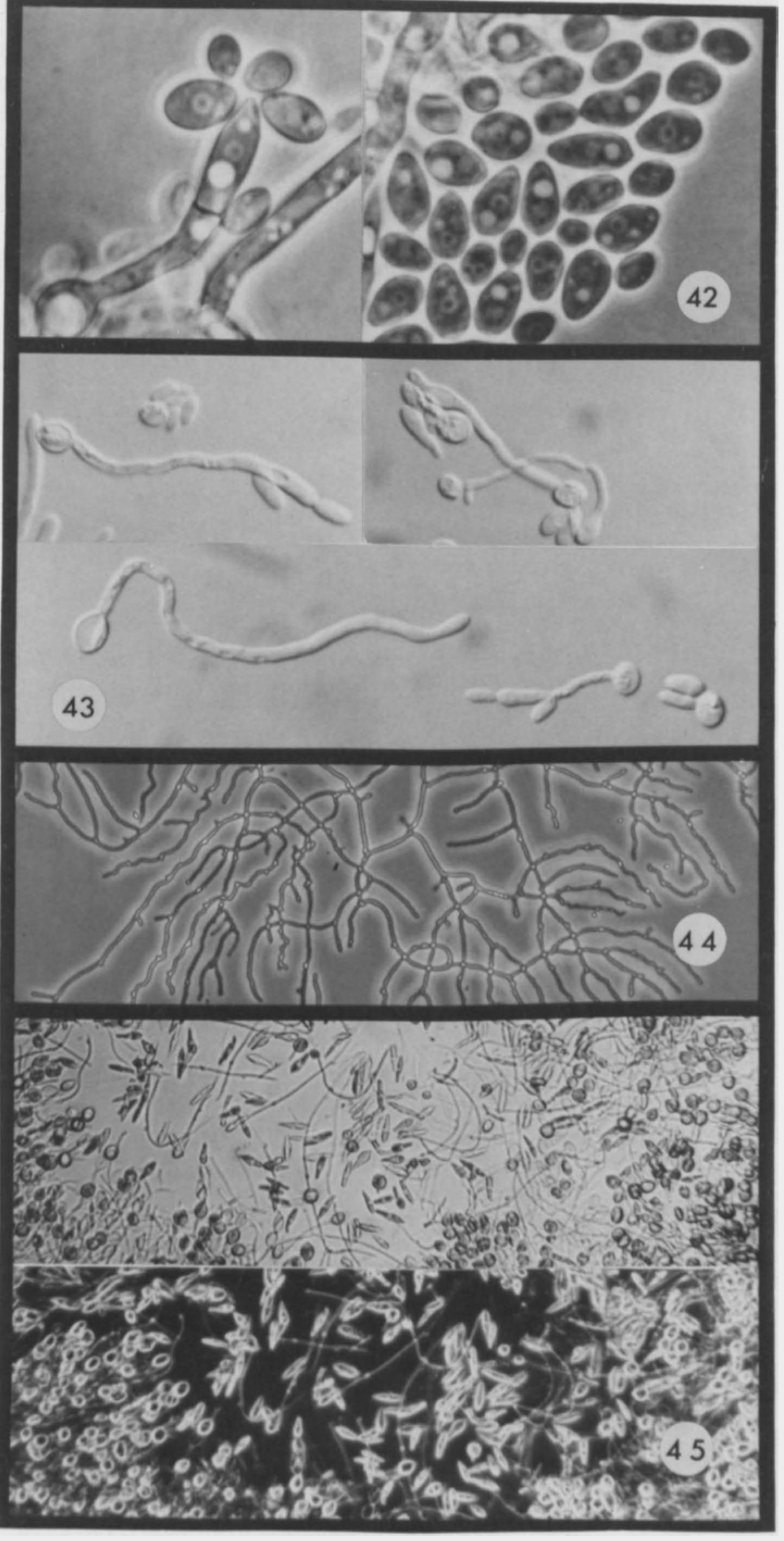

42
43
44
45

PLATE VI

Figure 46. Inhibition of yeast growth by an overlaying coverslip. A lawn of cells of TWF 58-23 was spread over the plate and a coverslip set over the centre of the lawn. (X2.70)

Figure 47. Dikaryotic hyphae growing under a coverslip. This mycelium arose from a cross of yeast strains TWF 58-23 and 58-27 that was overlaid with a coverslip. A basidiocarp has begun to form where the hyphae have reached the edge of the coverslip. (X1.36)

Figure 48. A cross of yeast strains TWF 58-23 and 58-27 grown in an atmosphere of approximately 15% carbon dioxide. Note the preponderance of hyphal growth and the pigmentation of the hyphae. (X1.36)

Figure 49. A basidiocarp arising at the edge of a coverslip from a cross of yeast isolates TWF 58-23 and 11-2. (X3.23)

Figure 50. Portions of basidiocarps from collections TWF 11 and TWF 58. TWF 11 is on the right and TWF 58 is on the left. These specimens were revived in distilled water before being photographed. The scale is numbered in centimetres. (X1.75)

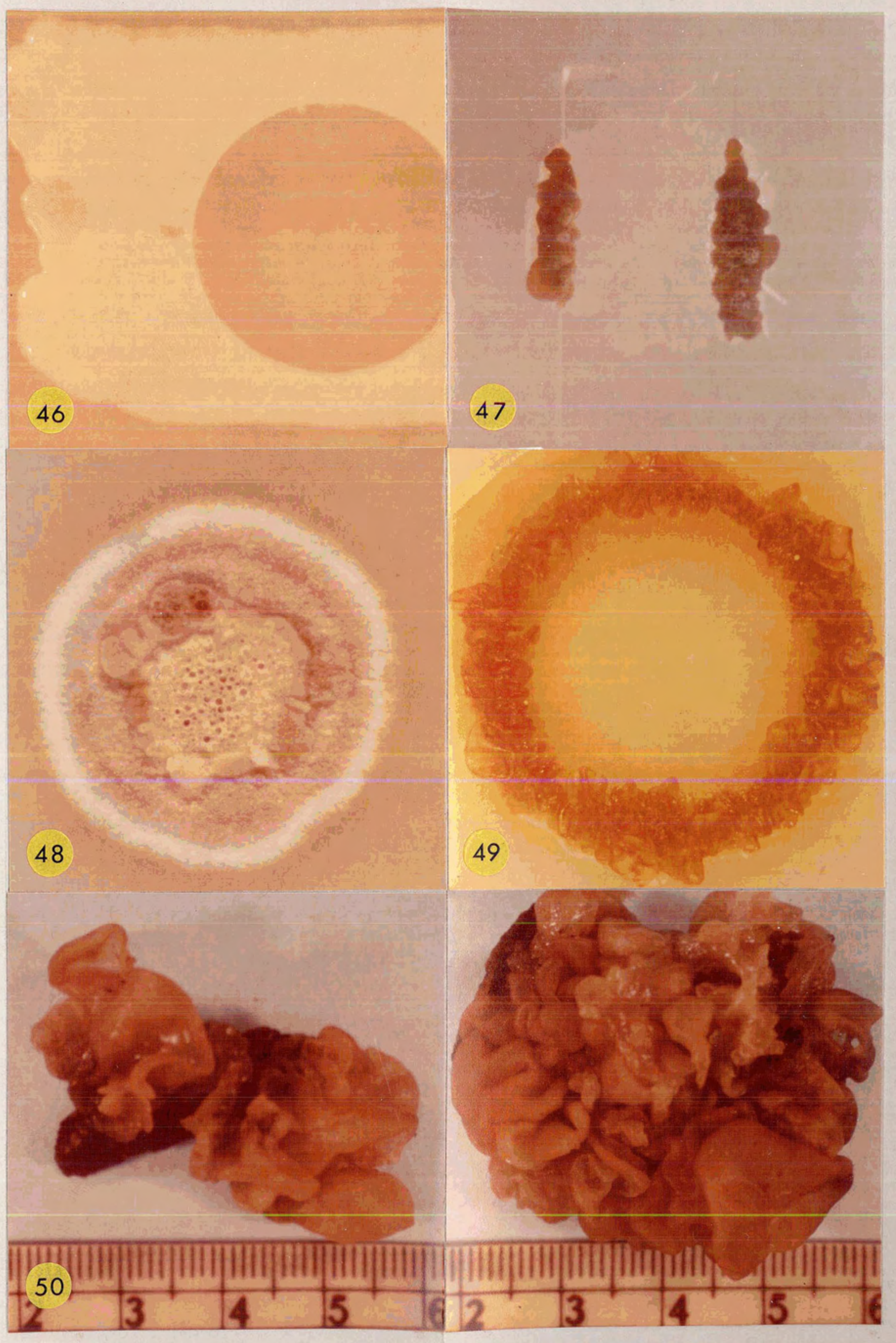

46
47
48
49
50

PLATE VII

Figure 55. Effect of cell concentration on conjugation between isolates TWF 58-23 and 58-27. Photographs were taken 30 hours after the two isolates were mixed, so dikaryotic mycelium has had time to develop. Initial densities of cells were:

a. 15 cells/mm^2

b. 30 cells/mm^2

c. 1.5×10^2 cells/mm^2

d. 3.0×10^2 cells/mm^2

e. 1.5×10^3 cells/mm^2

f. 3.0×10^3 cells/mm^2

g. 3.0×10^4 cells/mm^2

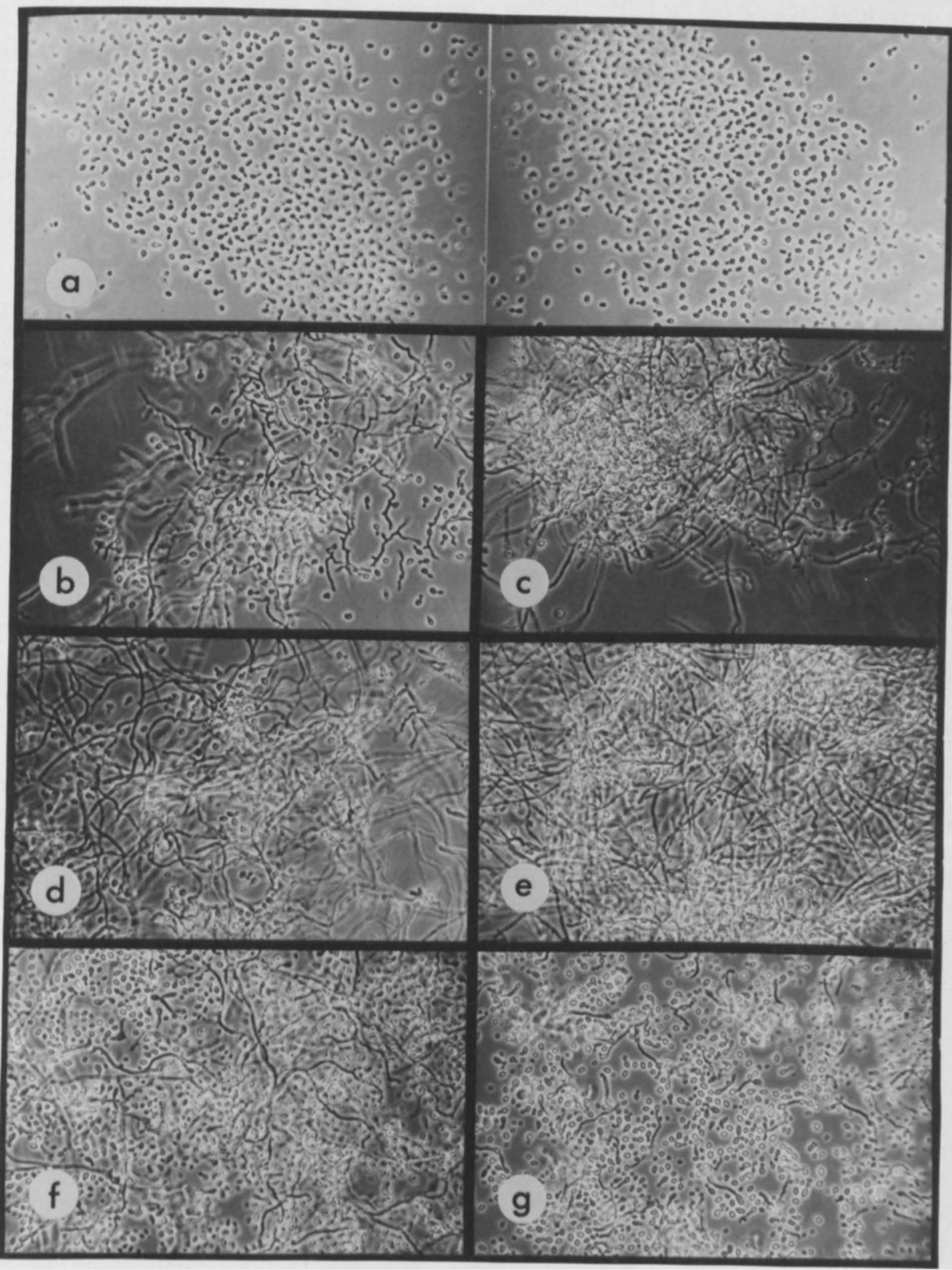

a
b
c
d
e
f
g

PLATE VIII

Figure 58. Time-lapse series of conjugation between isolates TWF 58-23 and 58-27. The frames in each row were taken at the same time. The photographs were taken at the following times after the cells were mixed together:

a. 0 h

b. 10.0 h

c. 10.5 h

d. 11.0 h

e. 11.5 h

f. 12.0 h

g. 12.5 h

h. 13.0 h

i. 13.5 h

j. 14.0 h

k. 14.5 h

l. 15.0 h

m. 15.5 h

n. 16.0 h

o. 16.5 h

p. 20.0 h

a
b
c
d
e
f
g
h
i
j
k
l
m
n
ø
p

PLATE IX

Figure 59. First 4.5 hours of a 9-hour time-lapse photographic sequence of budding with isolate TWF 58-27. The frames in each row were taken at the same time. The times for each row were as follows:

a. 0 h

b. 0.5 h

c. 1.0 h

d. 1.5 h

e. 2.0 h

f. 3.0 h

g. 3.5 h

h. 4.0 h

i. 4.5 h

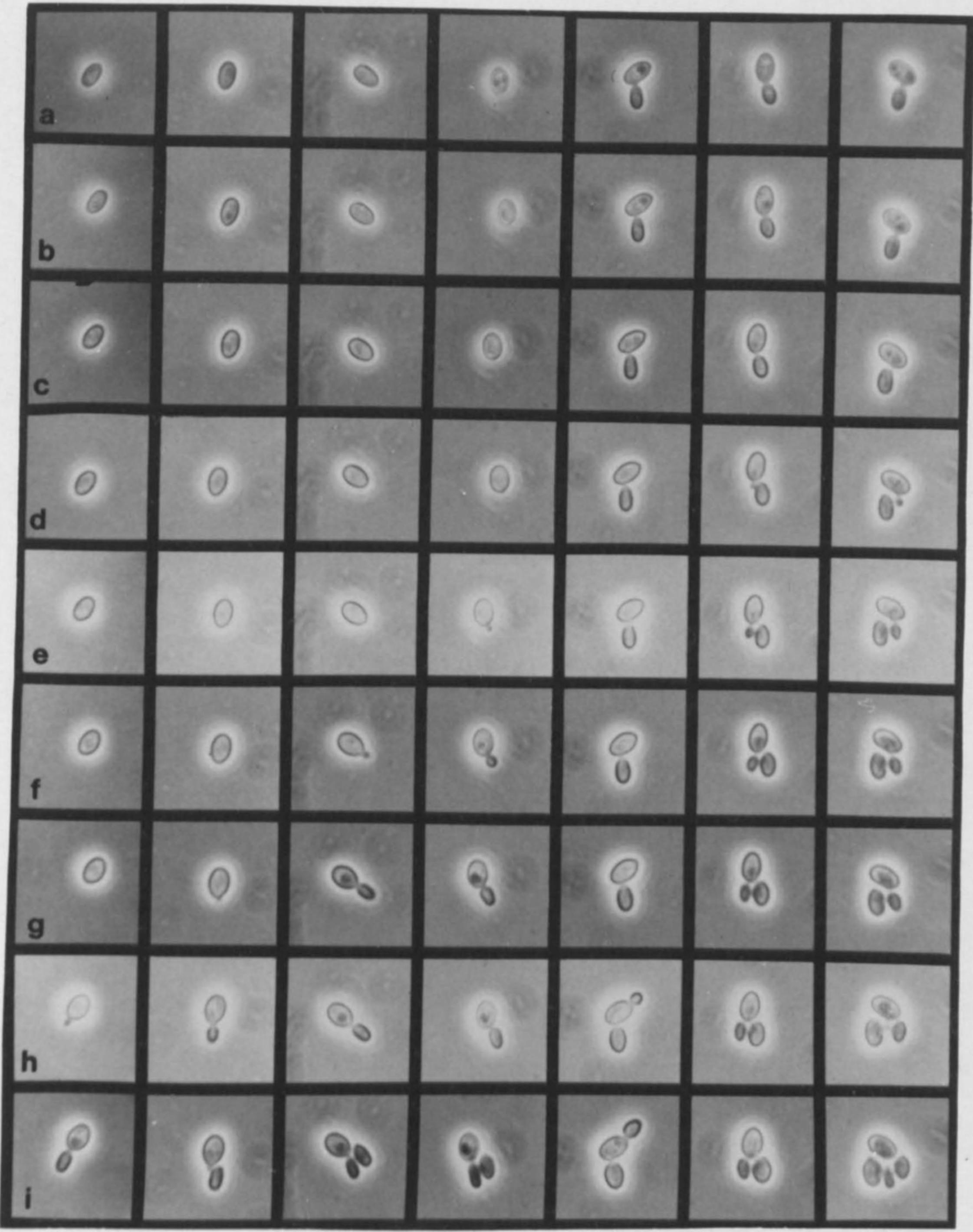

a
b
c
d
e
f
g
h
i

PLATE X

Figure 60. The second 4.5 hours of a 9-hour time-lapse photographic sequence of budding with isolate TWF 58-27. The frames in each row were taken at the same time. The times for each row were as follows:

j. 5.0 h

k. 5.5 h

l. 6.0 h

m. 6.5 h

n. 7.0 h

o. 7.5 h

p. 8.0 h

q. 8.5 h

r. 9.0 h

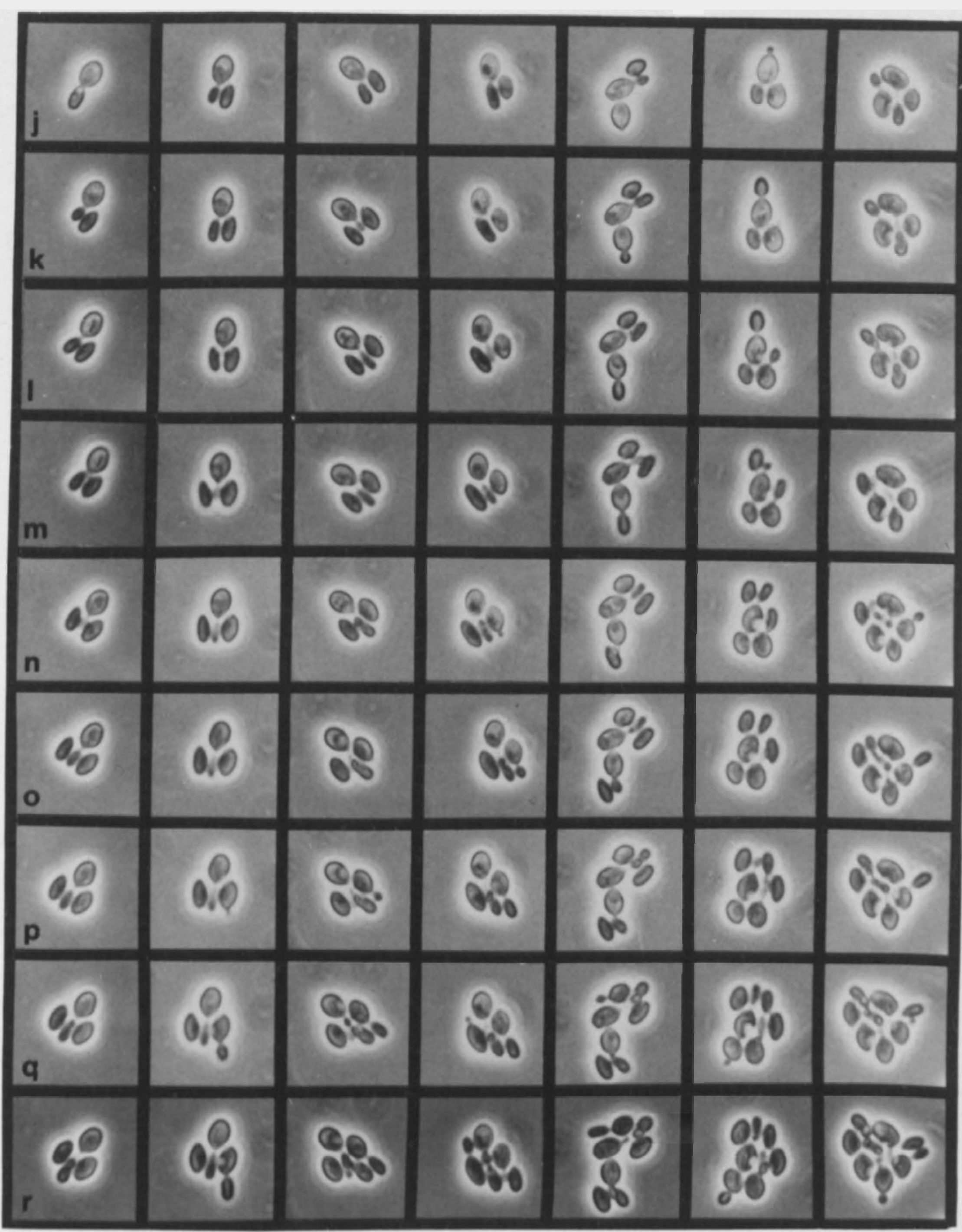

j
k
l
m
n
o
p
q
r

PLATE XI

Figure 61. Demonstration of diffusible conjugation-tube stimulators:

a. cells of isolate TWF 58-27 on the underside of the semipermeable membrane;

b. same field of view as in (a.) but this time focused on isolate 58-23 on the upper side of the semipermeable membrane.

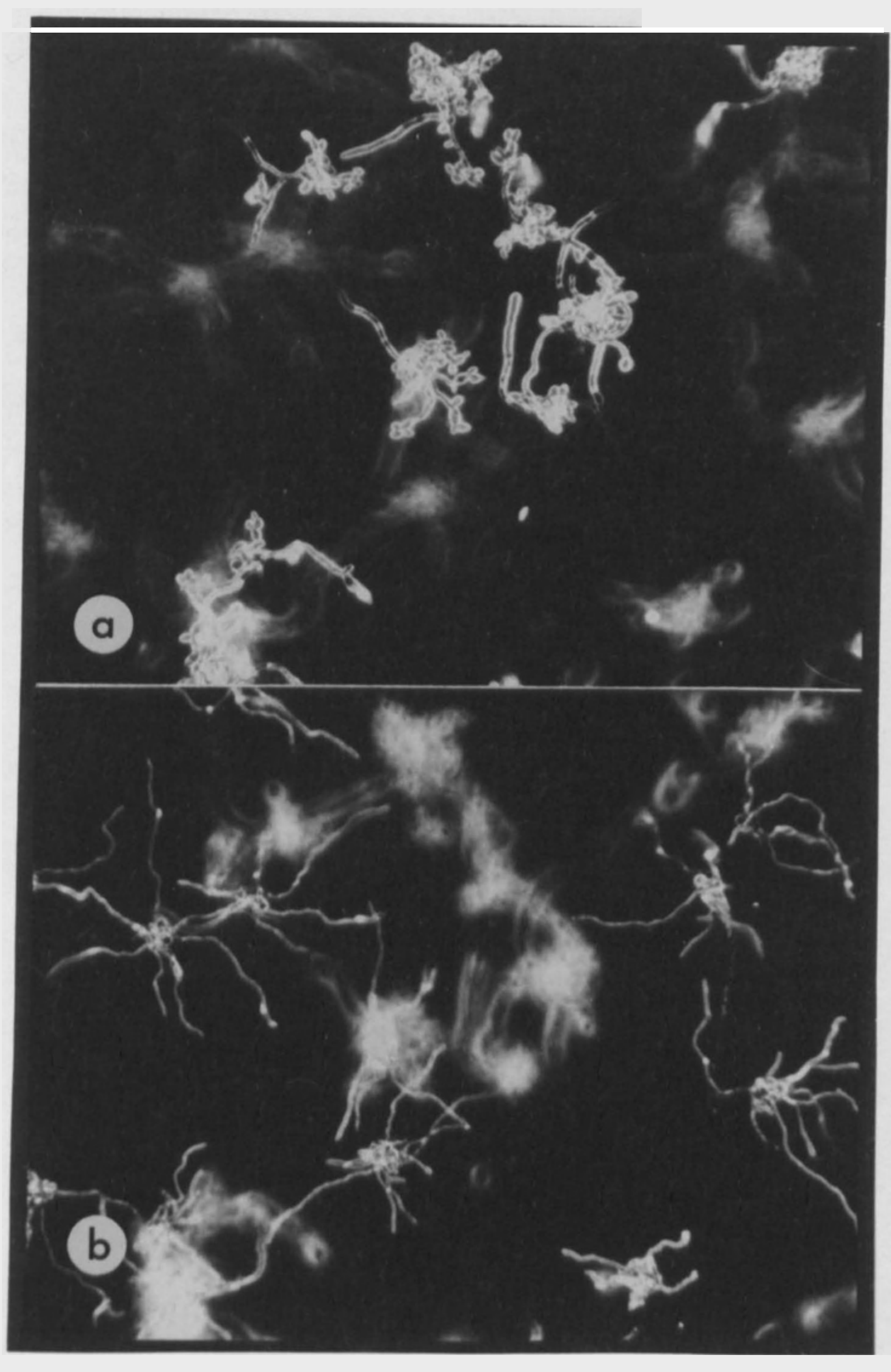

a
b

PLATE XII

Figure 62. Comparison of conjugation between gradient-selected cells and exponential phase cells from which the gradient cells were prepared. These photographs show approximately equal mixtures of isolates TWF 58-23 and 58-27.

a. Gradient-selected cells after 7 hours incubation.

b. Exponential phase cells (from which the cells in a. were selected) after 7 hours incubation.

c. Gradient selected cells at the start of incubation.

d. Exponential-phase cells at the start of incubation. They are from the culture from which the cells in c. were prepared.

e. Gradient-selected cells shown in c. after 8 hours incubation.

f. Exponential-phase cells shown in d. after 8 hours incubation.

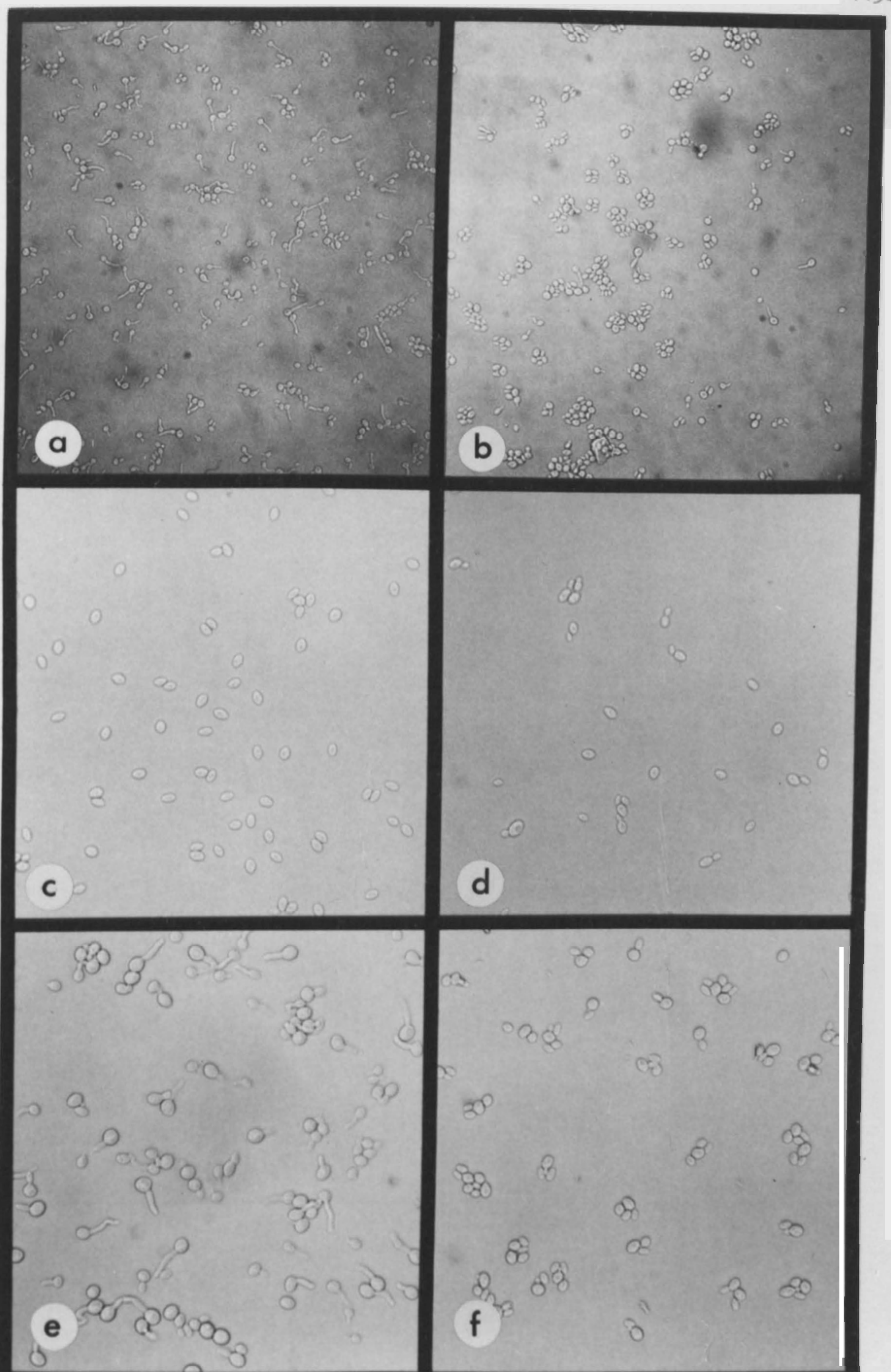

a
b
c
d
e
f

PLATE XIII

Figure 66. Effect of glucose concentration on conjugation. Photographs were taken after 36 hours so dikaryotic mycelium has developed. Concentrations of glucose were as follows:

a. 5.0×10^{-3} M/l

b. 5.0×10^{-2} M/l

c. 2.8×10^{-1} M/l

d. 5.5×10^{-1} M/l

e. 8.2×10^{-1} M/l

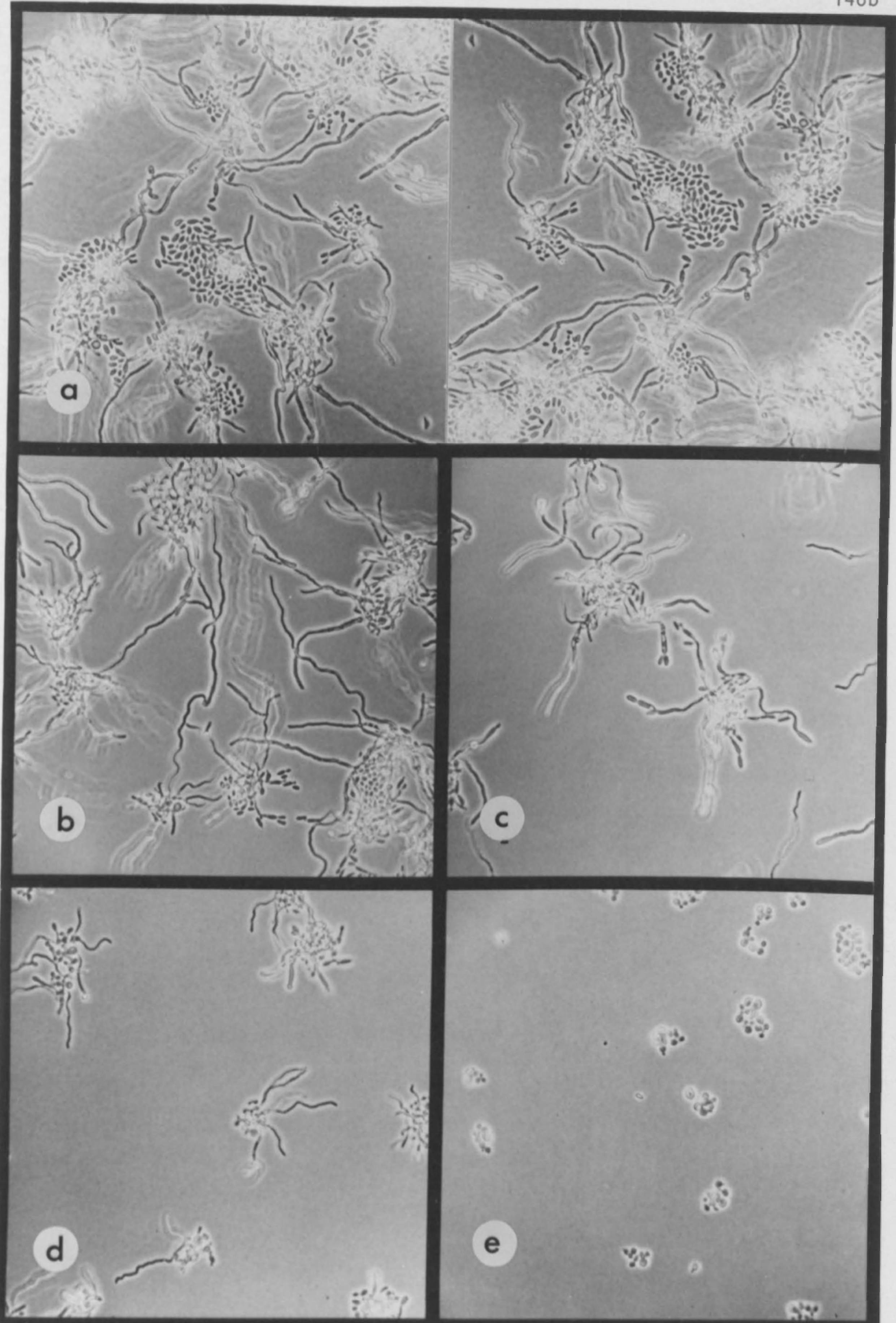

a
b
c
d
e

PLATE XIV

Figure 67. Effect of maltose concentration on conjugation. Photographs were taken after 36 hours so dikaryotic mycelium has developed. Concentrations of maltose were as follows:

a. 5.0×10^{-3} M/l

b. 5.0×10^{-2} M/l

c. 2.8×10^{-1} M/l

d. 5.5×10^{-1} M/l

e. 8.2×10^{-1} M/l

PLATE XV

Figure 68. Effect of galactose concentration on conjugation. Photographs were taken after 12 hours so there is no development of dikaryotic mycelium. Concentrations of galactose were as follows:

a. 5.0×10^{-3} M/l

b. 5.0×10^{-2} M/l

c. 2.8×10^{-1} M/l

d. 5.5×10^{-1} M/l

e. 8.2×10^{-1} M/l

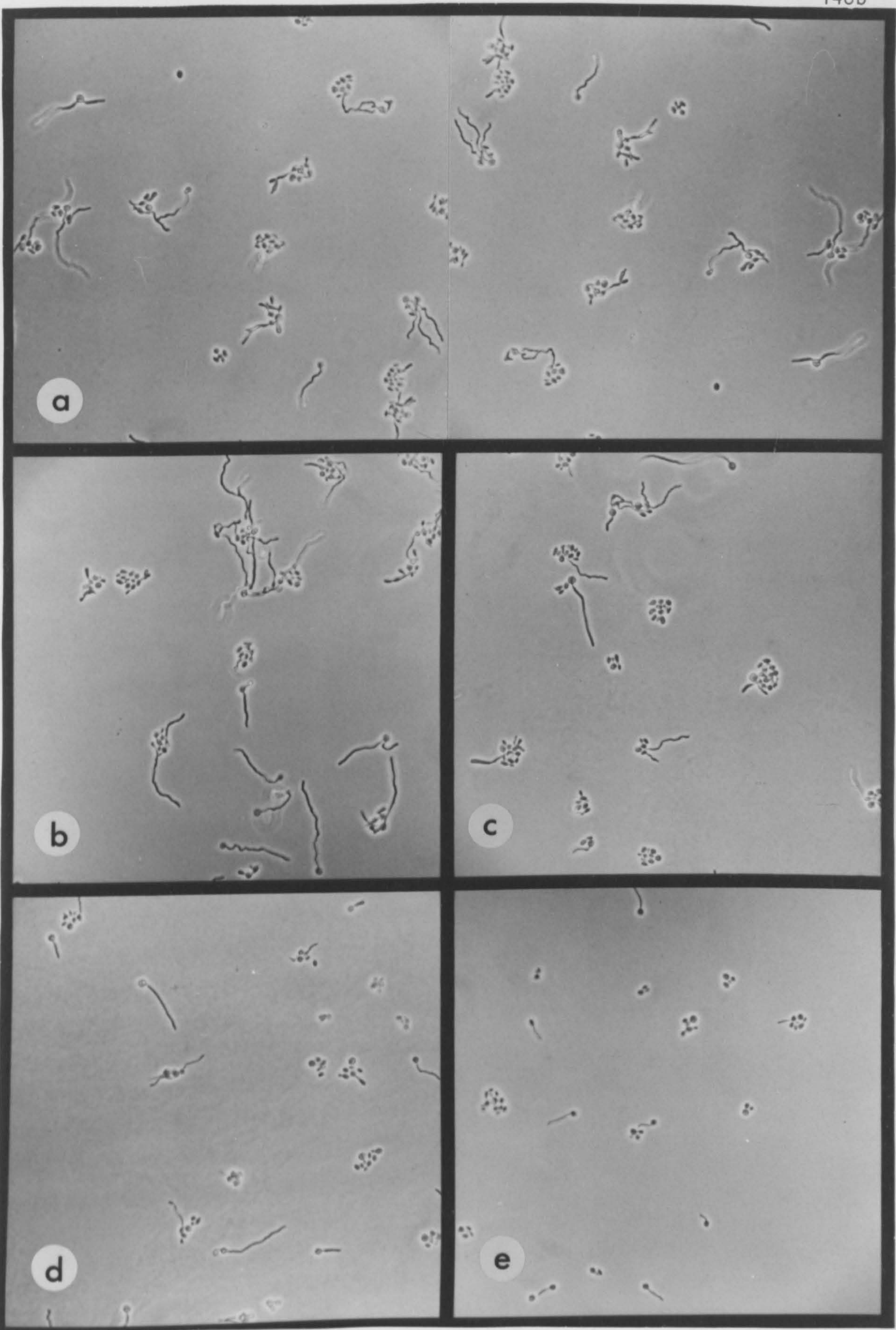

a
b
c
d
e

PLATE XVI

Figure 69. Effect of caffeine on cells of isolate TWF 58-23.

a. Normal cells on YE agar

b. Cells on YE agar with 2 mM/l caffeine

c. Cells on YE agar with 3 mM/l caffeine

d. Cells on YE agar with 4 mM/l caffeine

e. Cells on YE agar with 5 mM/l caffeine

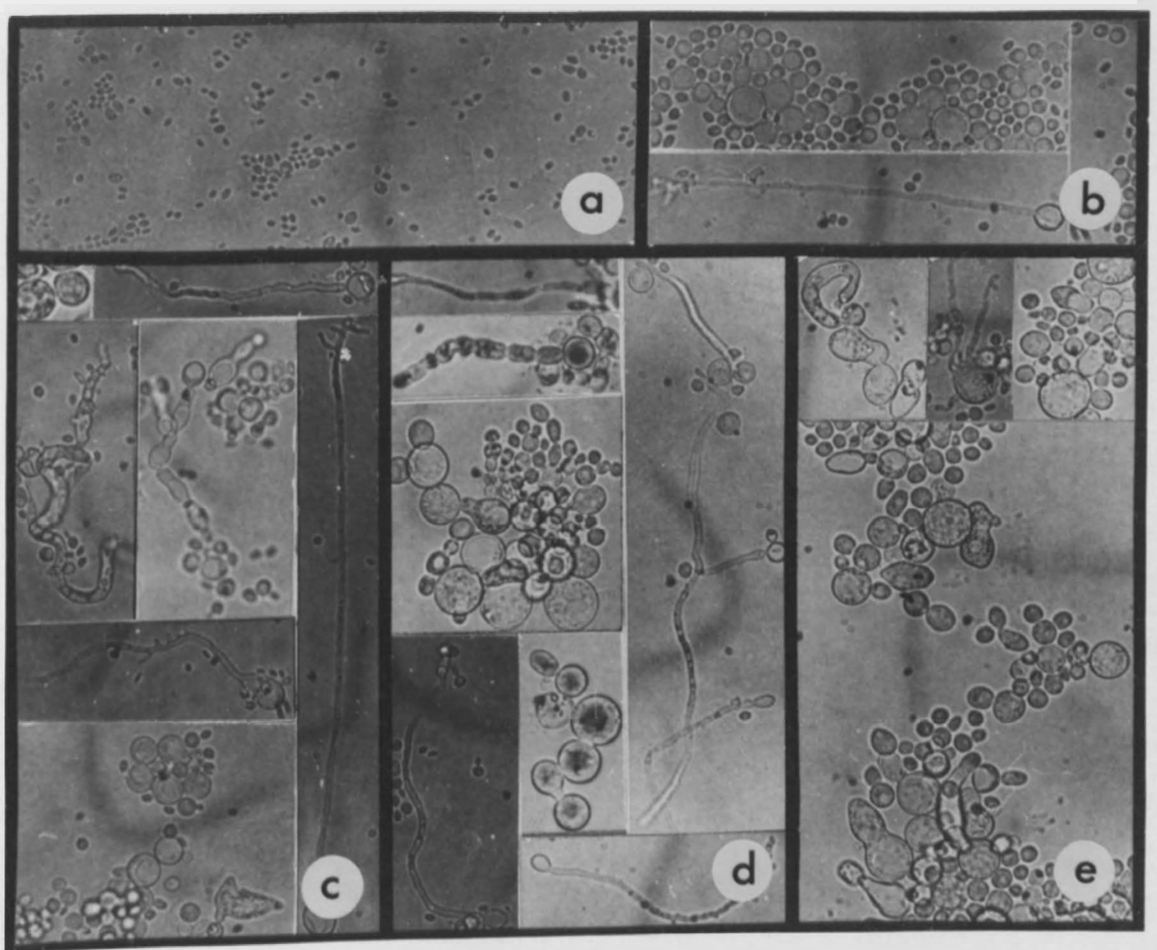

a
b
c
d
e

PLATE XVII

Figure 70. Effect of alcohol on conjugation. Amounts of 95% ethanol added to 100 ml autoclaved CJM were:

a. 0 ul

b. 10 ul

c. 20 ul

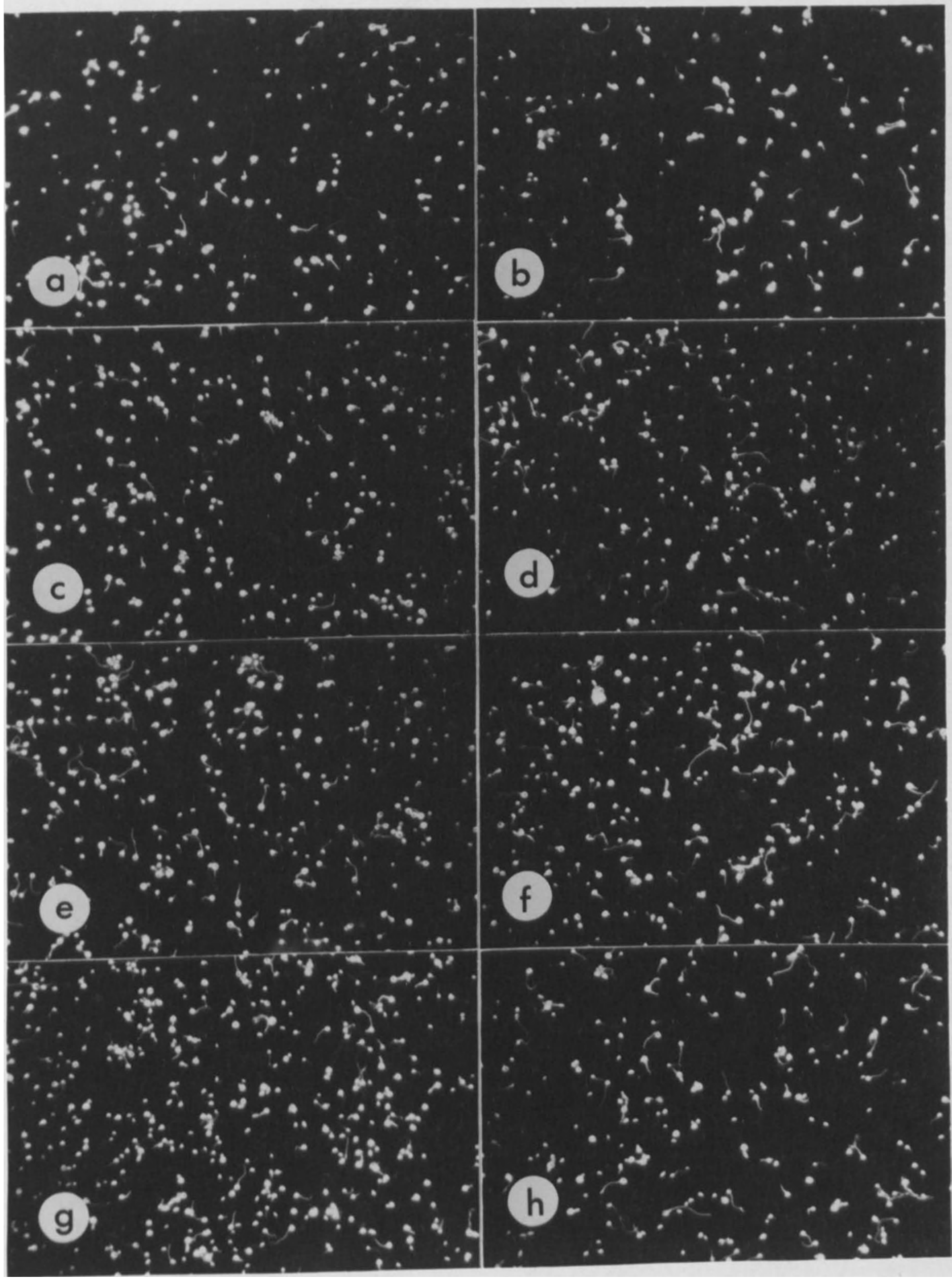
d. 75 ul

e. 325 ul

f. 650 ul

g. 2600 ul

h. 40 ul


a
b
c
d
e
f
g
h

CURRICULUM VITAE

NAME:	Timothy William Flegel
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BIRTHDATE AND PLACE:	February 18, 1943 at Trail, B.C., Canada
CITIZENSHIP:	Canadian
MARITAL STATUS:	Single
UNDERGRADUATE STUDIES:	1961-1965, University of British Columbia, Vancouver, B.C., Canada. Obtained B.SC. Degree in May 1965.
MASTERS DEGREE:	1965-1968, University of British Columbia, Vancouver, B.C., Canada. Obtained Degree in October 1968. Title of thesis was, Some aspects of conjugation in the genus Tremella Dill. ex Fr.
DOCTORATE:	1974 to present, Simon Fraser University, Burnaby, B.C., Canada. Title of thesis is, The life cycle and sexual physiology of Sirobasidium magnum.
SUBJECTS OF SPECIAL STUDY:	Mycology, Biochemistry and Genetics.
SCHOLARSHIPS:	Scholarship, Chris Spencer Foundation, 1961. Scholarship, University of British Columbia, 1961. Bursary, Government of British Columbia, 1961. Bursary, Imperial Order Daughters of the Empire, 1961-1966. Scholarship for Graduate Study, British Columbia Sugar Refining Company, 1966. President's Research Grant, Simon Fraser University 1977.
TEACHING AND RELATED EXPERIENCE:	Teaching Assistant, Biology, 1965-1967, University of B.C. Research Assistant to Dr. J.E. Bier, Forest Pathology, 1964, University of B.C. Research Assistant to Dr. G.H.N. Towers, Plant Biochemistry, 1965, University of B.C.

Demonstrator, Biology 101 and 102, Introductory Biology, Simon Fraser University, Burnaby, B.C., 1967.

Instructor, Biology 101, Introductory Biology, Simon Fraser University, Burnaby, B.C., 1968. In this appointment, I was responsible for teaching and administering the course with three Demonstrators and 100 students.

Lecturer, English Department, Chulalongkorn University, Bangkok Thailand, 1969-1970.

Lecturer, Scientific English Division, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, 1970-1974. In this appointment I was jointly responsible for writing materials and administering five part-time lecturers in the English course for junior and senior science students.

Guest Lecturer, Principles of Mycology, Mahidol University, Bangkok, Thailand, 1972.

Independent Research, Microbiology Department, Mahidol University, Bangkok, Thailand, 1970-1974.

Teaching Assistant, Department of Biological Sciences, Simon Fraser University, 1974-1976. One semester each for Introductory Ecology and The Biology of Vascular Plants. Two semesters each for Nonvascular Plants; Plants and Animals of B.C.; Ecology and the Population Explosion.

Lecturer, Ecology and the Population Explosion, Department of Continuing Education, Simon Fraser University, winter semester, 1974.

PUBLICATIONS AND PRESENTATIONS:

Berry, J.A. and T.W. Flegel, Properties of a sex hormone of Tremella mesenterica Fr., Proceedings of the Canadian Society of Plant Physiologists, Ottawa, Ontario, June, 1967.

Flegel, T.W. 1971. Conjugation in jelly fungi as a tool for morphogenetic studies. Seminar for Microbiology Department, Mahidol University, Bangkok, Thailand.

Flegel, T.W. 1974. English for Science - Where do we start?, Pasaa (Bulletin of the Central Institute of English Language, Bangkok), vol. 4, No. 1: 113-120.

Flegel, T.W. 1976. Conjugation and growth of Sirobasidium magnum in laboratory culture, Canadian Journal of Botany, 54: 411-418.

Elliott, C.R. and T.W. Flegel, New lessons in English for science and technology, English Language Services, Portland, Oregon, U.S.A., in press.

Flegel, T.W. Let's call a yeast a yeast. Canadian Journal of Microbiology, In press.