7-17-1966

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MORPHOLOGICAL PATTERNS OF KARYOKINESIS IN *SACCHAROMYCES CARLSBERGENSIS*

by

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Submitted to the Faculty of Graduate Studies through the
Department of Biology in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario, Canada
1966

Approved:

ABSTRACT

Karyokinesis in S. carlsbergensis was studied using a newly developed staining procedure on cells from synchronously dividing cultures.

An imprint technique, involving either Helly's or osmium fixed cells was used. Treatment consisted of alcohol washings, imersion in distilled water, RNA extraction in a salt solution, hydrolysis to release aldehyde groups in the DNA and staining with azure A. The time required for the procedure was relatively short and yielded nuclei which are stained deeply blue and an almost colorless cytoplasm.

The staining revealed a pattern of division in which compact, inter-division nuclei became diffuse immediately before DNA replication. They then reassumed a compact shape but were slightly larger than previosly. A diamond shaped figure was then formed, which was thought ot represent the separation of the daughter nuclei. A wedge-shaped nucleus was formed in which a clear band could be seen apparently indicating the space between nuclei. The nucleus closest to the bud then proceeded into the growing cell while the other remained in the mother cell. The duration and timing of this sequence in relation to synchronized and unsynchronized growth patterns as well as its similarity to mitosis are discussed.

ACKNOWLEDGEMENTS

I wish to acknowledge with thanks, the direction and encouragement given by Dr. R.J. Doyle during the course of this work.

Special thanks are accorded to Dr. C.F. Robinow, University of Western Ontario for several helpful suggestions and to Dr. J.E.J. Habowsky and F.M. Lukas for their advice in preparing the photographs used herein.

Also thanks to Dr. H.C. M^{c}Curdy, Dr. R.J. Thibert and Dr. Habowsky for their suggestions and constructive criticisms in the preparation of this manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The study of cytokinesis and karyokinesis during asexual reproduction has long been of interest to the cytologist. Recent physiological and biochemical studies have complemented morphological investigations to give an increasingly clearer picture of the division process.

In the cells of higher plants and animals, a precise and consistent process labeled mitosis is operative during karyokinesis. As the period between division cycles ends, the nuclear membrane disappears. Then the deoxyribonucleic acid (DNA) elements of the nucleus become demonstrable as separate chromosomes, probably as a result of contraction. These are moved by spindle fibers to opposite poles of the dividing cell and the process terminates with the expansion of the chromosomes into a diffuse network and the formation of nuclear membranes in both mother and daughter cells.

This mode of division is often considered characteristic of all eukaryotic cells. Studies on the Eumycotina (true fungi), however, especially in the yeast genus Saccharomyces have cast doubt on the general validity of such a statement. These latter organisms are unicelluar Ascomycetes which reproduce asexually by budding during which the nucleus undergoes changes which are difficult to interpret as mitotic.

Although genetic data suggest the presence of chromosomes, they have yet to be demonstrated unequivocally by cytological methods. This has led to some debate regarding not only the pattern of division shown by these organisms but also as to the very identity of the nuclear structure.

This problem prompted the study to be described in this work in which new techniques were applied to supplement the more classical approaches to give a clearer result.

In reviewing the literature on the nuclear cytology of yeast, one is impressed by the lack of organization of the various stages of division into a definite chronological order. The recent development of a reproducible method of synchronizing yeast by Williamson and Scopes (1960) made possible the sampling, staining and observation of cells at well defined intervals of the budding cycle. Combined with physiological data already available regarding DNA, ribonucleic acid (RNA) and protein synthesis, as well as nitrogen uptake and oxygen uptake as determined by these authors, this cytological investigation provided an integrated picture.

The bulk of this material is based on the selective staining of DNA using a newly developed technique. In addition, staining by the methods of Feulgen and Giemsa as well as the study of the nucleus in live preparations were preformed as supplemental material. Recent findings revealing an intra-nuclear fibre apparatus prompted the study of this organelle also.

Thus, the morphology of the dividing yeast nucleus in a synchronized culture was studied using an original procedure for staining DNA.

CHAPTER II

LITERATURE REVIEW

A. General Structure of the Yeast Cell

Schwann (1837) is credited with one of the earliest descriptions of the yeast cell. The development of the compound microscope permitted many authors to study the cytology of this organism soon thereafter.

The progress since that time has been reviewed by Winge and Roberts (1958) and McClary (1964). Also there have been extensive studies combined with reviews by Nagel (1946) and Ganeson (1959).

The yeast cell has been shown to possess a thick outer wall composed of glucan-mannan polymers (Falcone and Nickerson, 1956; Korn and Northcote, 1960; Phaff, 1963). There are prominent birth (attachment to the mother) and bud (attachment to the bud) scars which can be distinguished on the basis of size (Barton, 1950; Northcote and Horne, 1952; Bartholemew and Mittwer, 1953; Agar and Douglas, 1955a). Within the cell is a cytoplasmic membrane which is believed to be composed of two poorly defined layers of protein separated by a well defined layer of lipid (Robinow and Murray, 1953; Hashimoto, Conti and Naylor, 1959). The cytoplasm is similar to that of other organisms and contains an endoplasmic reticulum which may be continuous with the cytoplasmic and nuclear

membranes (Linnanne and Vitols, 1962). Numerous granules are present including storage materials such as glycogen and metachromatic volutin (polyphosphate) bodies (Lindegren, 1949). Electron microscopy has revealed oval to circular mitochondria with cristae (Linnanne, Vitols and Nowland, 1963; Agar and Douglas, 1957; Mundkur, 1960). The most prominent structure is a membrane-encased central vacuole (McClary and Lindegren, 1959) which may contain particulate material and is closely associated with the nucleus (see Figure 1).

B. The Nucleus

1. Demonstration

The yeast nucleus is a strongly basophilic organelle and can be demonstrated with dyes such as haemotoxylin, methyl violet, methylene blue (Nagel, 1946) and even with Gram's iodine (Lindegren, 1949). Such methods are, however, handicapped by the basophilic nature of the cytoplasm which tends to obscure the nucleus and superior methods involve the use of nucleic acid specific dyes. Margolena (1932) used a classic Fuelgen technique but as recently demonstrated by Robinow and Marak (1966), the result is a faintly stained preparation. Giemsa was first used on microrganisms by Robinow (1942) and has since been widely applied to yeast (Ganeson, 1956; Lindegren, Williams and McClary, 1956; McClary 1958; McClary, Williams and Lindegren, 1957). The result of

Figure 1. Diagramatic representation of the typical yeast cell.

1. ----- cell wall
2. ----- nucleus
3. ----- cytoplasm
4. ----- vacuole
5. ----- storage granule
6. ----- ribosome
7. ----- mitochondria
8. ----- endoplasmic reticulum
9. ----- cell membrane

(x 2000)

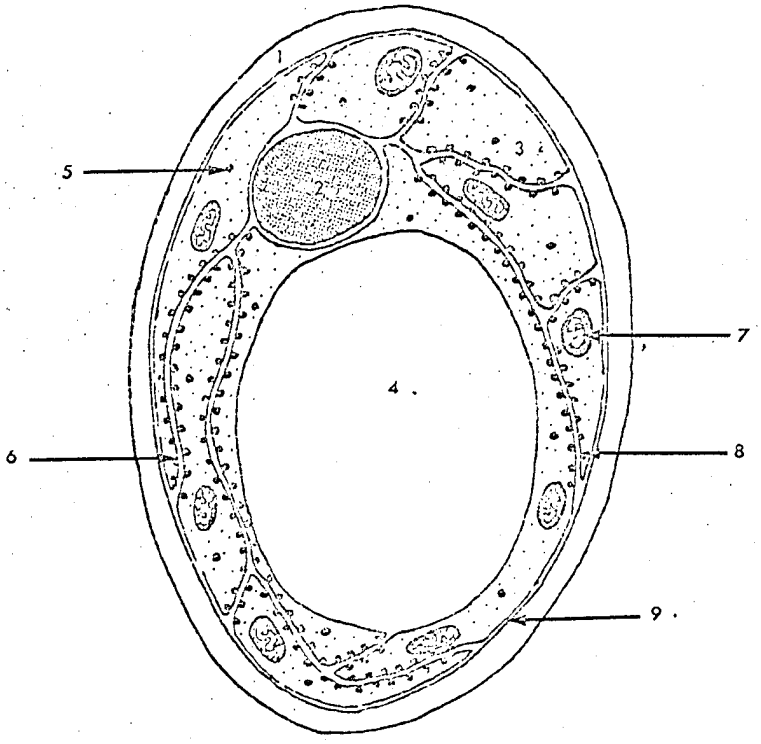


FIG 1

this stain is red deoxyribonucleic acid and purple-blue ribonucleic acid. Delamater (1951) recommended the use of Thionin or azure A with microorganisms but these have not been applied to any extent to yeast, although Huebschman (1952) has applied azure A to other fungi. Both of these above dyes theoretically undergoe a Schiff reaction but do not form a leucobase as found with Feulgen (Delamater, 1951).

Various fixatives have been employed in the study of the yeast nucleus. Navashin's, picric acid, iodine-formalin acetic acid, mercuric chloride (Nagel, 1946); Carnoy's (Lindegren et al., 1956); vapors of osmic acid (Delamater, 1951); Helly's (Robinow and Marak, 1966); alcohol-formalin-acetic acid (Yoneda, 1963); osmic acid-chloroform (Ganeson, 1959); Schaudin's (Widra and Delamater, 1955) and Zenker's (Ramirez and Miller, 1962) are included but the superiority of one method over the others is debatable.

The high RNA:DNA ratios (approximately 50:1 as estimated by McClary, 1964) found in the yeast cell necessitate rendering the RNA unstainable by hydrolysis in normal hydrochloric acid (Robinow, 1942), extraction with seventeen one-hundredths molar (.17M) sodium chloride (Ganeson, 1959), perchloric acid treatment (Lindegren et al., 1956) or use of RNAase (Ganeson,1959). All of these procedures when applied carefully result in deeply stained nuclei and a lightly stained cytoplasm but unfortunately all but the

RNAase result in considerable shrinkage of the protoplast.

Electron microscopy of the yeast nucleus has revolved primarily around the use of osmic acid as a combined fixative and stain (Agar and Douglas, 1957). Osmic acid-potassium permanganate (Tsukahara, 1963); potassium permanganate-uranyl acetate (Hashimoto, 1958a), and glutaraldehyde (Robinow and Marak, 1966) have also been used. A freeze drying technique has been developed by Mundkur (1959) in which after quick freezing, cells were fixed with alcohol vapors and cross linking protein fixatives. He also introduced gallocyanine- chromalum as a specific nucleic acid stain. Moor and Muhlethaler (1963) developed a freeze drying technique combined with etching rather than slicing of the specimen. Bartholemew and Mittwer (1954) used ultraviolet photolysis to render yeast cells transparent to the electron beam and Bradley (1955) has applied a carbon-replicating technique.

The general problems encountered in all such procedures were reviewed by Mundkur (1961a).

2. Interpretation

The use of various stains and fixatives has led to some diversity in the interpretation of the structure of the yeast nucleus. Consequently it is difficult to say which view represents the true case; however, in an effort to clarify the

issue, McClary (1964) has divided the therories which have arisen into three categories. In the first, the Fuelgen positive structure outside the vacuole is the nucleus and division is thought to take place by conventional mitosis (extra vacuolar-mitotic, Swellengrebel, 1905; Delamater, 1950; Ganeson, 1959; and Robinow, 1961). In the second category this same structure divides by a process other than mitosis (extra vacuolar-amitotic, Nagel, 1946; Mundkur, 1954; Conti and Naylor, 1954; Hashimoto, Conti and Naylor, 1958 and 1959). In the third and final category, the structure most often refered to as the vacuole is considered the nucleus; the Fuelgen-positive structure adjacent to this is a centriole and division is mitotic (nuclear vacuole concept, Lindegren 1949 and lindegren et al. 1956).

Listing the hypotheses of each investigator would lead to confusion but examination of the drawings and photomicrographs representing these therories permits abstraction of the general nuclear structure.

There is general agreement that the nuclear membrane is persistent during the division cycle (Agar and Douglas, 1957; Conti and Naylor, 1959; Hashimoto, 1958 and 1959; Moor and Muhlethaler, 1963; Mundkur, 1960; Robinow and Bakerspiegel, 1965). The nuclear membranes and the vacuolar membrane are thought by many to be distinct, but they may be interconnected (Agar and Douglas, 1957).

During the inter-division stage, the nucleus is quite homogeneous (Sinoto and Yuasa, 1941; Nagel, 1946; Agar and Douglas, 1957; Ganeson, 1958; Hasimoto, 1958a,1958b, and 1959; Ramirez and Miller, 1962; McClary, Bowers and Miller, 1962; Ferriera and Phaff, 1959). Delamater (1950) observed a nucleolus by light microscopy and Hirano (1962) saw a similar organelle using the electron microscope. Mundkur (1954) reported a "peripheral cluster" which may be equivalent to the nucleolus and Robinow and Marak (1966) have demonstrated the nucleolus in live cells as well as in stained and electron microscope specimens. Very recently Williamson (1966) has, using a slightly different technique, interpreted a very similar mass of material as chromosomes. Verification of the content of this structure can come only from cytochemical studies. The role of the nucleolus in division of the yeast nucleus is not understood. It does not seem to have a definte orientation on a chromosome but it is related to a fibre apparatus (Robinow and Marak, 1966).

From the data on killing by x-ray and by staining, Lindegren (1951) was able to show that the DNA of the cell doubled just prior to appearance of the bud. This fact has since been confirmed by the study of synchronized cultures (Williamson and Scopes, 1960).

Delamater (1951) postulated a sequence of events in which a dense homogeneous nucleus became less dense, even to the

point of being vesicular before it formed a "figure eight" shape during passage to the bud. Yoneda (1963) also reported such vesicular stages, as has Mundkur (1961b).

Levan (1946) using a squash technique on the dispersed stage, saw several chromosomes in metaphase. Several workers have reported nuclei which are composed of two separate and elongated bodies (Badian, 1937; Delamater, 1951; Ganeson, 1958). These have been interpreted as being chromosomes or clusters of chromosomes in telophase. Robinow and Marak (1966) have demonstrated that this may be in fact due to the extension of a non-staining fibre apparatus across the nucleus. Such a fibre is demonstrable by acid fuchsin staining and by electron microscopy and seems to arise from the centriolar plaque which is embedded in the nuclear membrane at an indentation. Hashimoto (1958b) has also seen such an indentation of the nucleus but was unable to demonstrate the centriolar plaque. On the level of the light microscope, Lindegren, Williams and McClary (1956) and Yoneda (1963) refer to spindle fibres which may be the same entity as that seen by Robinow and Marak, for in all cases demonstration is by an acid dye.

Lindegren (1951) demonstrated with the light microscope that the nucleus is not passed to the bud until the later is well developed. Conti and Naylor (1959) reached the same conclusion in a parallel situation with fission yeasts. With the light microscope, the nucleus seems to contain

several particulate objects which may be chromosomes while in the elongated form (Ramirez and Miller, 1962) but similar objects are visible in some non division stages (no bud visible) at which time no individual chromosomes should be seen (McClary, Williams and Lindegren, 1957). Such features are especially prominent in squashed preparations in which the wall has been digested with snail gut juice Robinow, personal communication).

Much of this data is from light microscope work. Results with the electron microscope have been disappointing because of a lack of techniques and little can be seen with this insrument which is not visible with the light microscope.

Several points are quite perplexing. For example, Delamater (1950) describes an early telophase in which the bud is receiving the nucleus from the mother cell and a late telophase where the nucleus is still in division within the mother cell. This is similar to the findings of Ramirez and Miller (1962) who noted a lack of synchronization between bud formation and nuclear division. Delamater (1960) and Ganeson (1954) have both demonstrated centrioles which are protrusions on the nuclear outline. These have little in common to the association of the centriolar plaques of Robinow and Marak (1966) with invaginations of the membrane.

From this Robinow (1965) has abstracted the view that, "There is no doubt that it (the yeast nucleus) contains

chromosomes in some form, but it is equally certain that their cycle of duplication and separation does not resemble the events of an ordinary mitosis".

In summary, the nuclear cycle of Saccharomycan seems to progress entirely within the nuclear membrane. The nucleus is quite homogeneous and the chromosomes when seen, seem to be randomly distributed and present as discrete units through out the entire cycle. There is a nucleolus and a fibre apparatus within the membrane, but little is known concerning thei function. Changes in the density of the nucleus have been postulated but definitive demonstration is lacking.

With this in mind, the study of the nucleus in synchronously dividing cultures was undertaken in the hope that a definite sequence of events for the divisional process could be found. The advantage of synchrony, is that it permits the study of a large number of cells which are in the same stage of division. Also, available biochemical data on the cells in synchrony enabled pinpointing of the stage at which DNA, RNA and protein increased and the correlation of this with the cytological data. A similar study was undertaken recently using the electron microscope (Williamson, 1966) but interpretation of his figures is incomplete.

C. Synchronization of the Yeast Culture

Yeast undergoe a natural synchrony when they are allowed to grow into a lag phase and then are placed in a rich fresh

medium. In the past, artifical procedures generally involved producing a stationary or log death culture by any of several methods including the use of starvation media. Williamson and Scopes (1960, 1962, and 1964) developed a synchronization procedure which included removal of all cells but those of a particular size group and successive feeding and starving of the culture. Utilizing this procedure they were able to study the patterns of synthesis found in the budding cycle. Some of their data are shown in Figure 2.

Figure 2. Graph illustrating the biochemical data of Williamson and Scopes (1961, 1962, 1964) in relation to synchrony as expressed in percent buds.

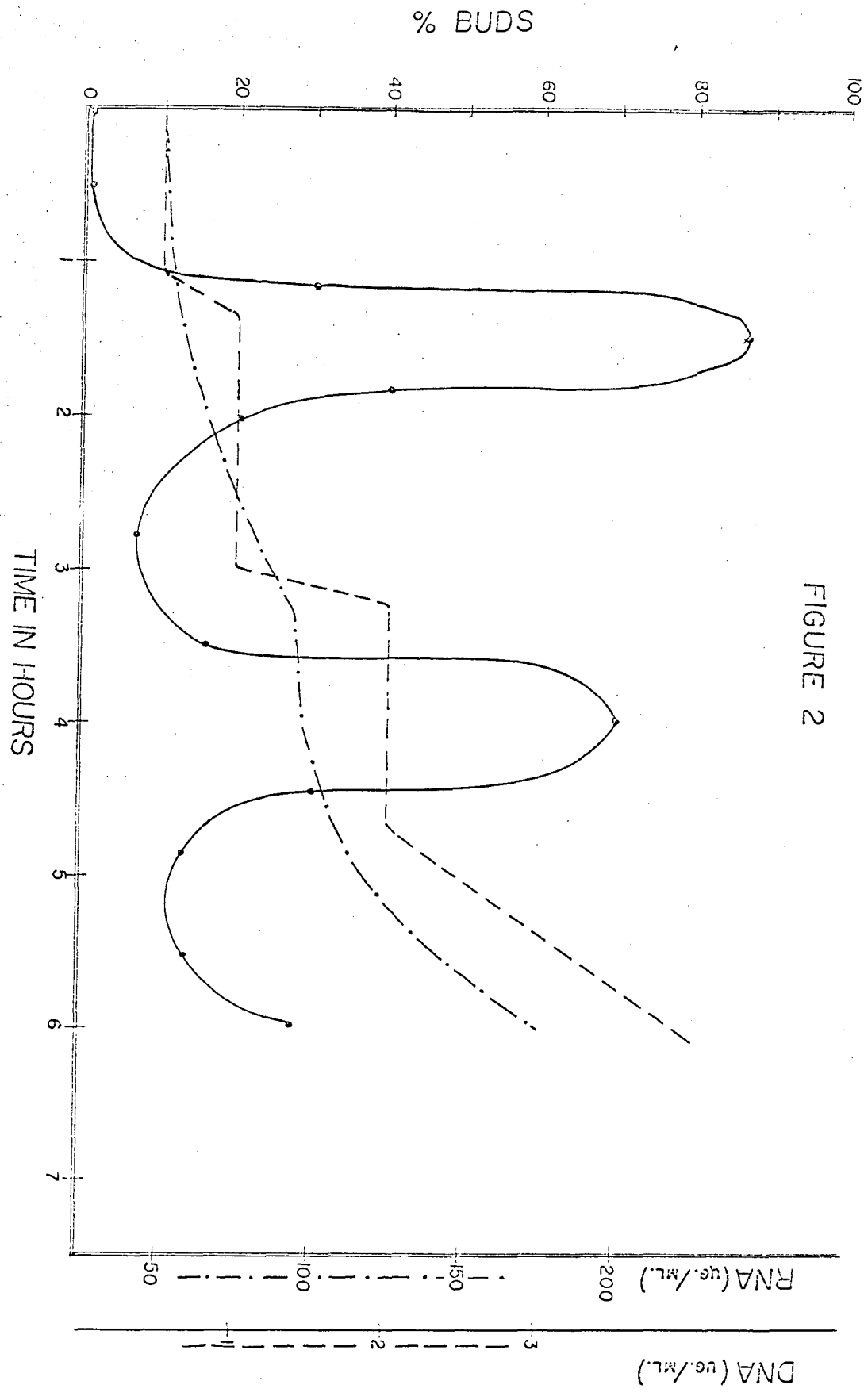


FIGURE 2

CHAPTER III

METHODS AND MATERIALS

A. Materials

The strain of yeast used was Saccharomyces carlsbergensis University of Windsor strain 195, which was obtained from L. J. Wickerham as his stock number 379. This yeast is quite similar to S. cerevisiae, differing only in raffinose fermentation.

The media used to cultivate the organism are given in Table I.

The fixatives employed were Carnoy's, Helly's and the vapors of osmic acid. The formulation of these is given in Table II.

The stains used were Giemsa, Azure A, Feulgen and Acid Fuchsin. The formulation of these are given in Table III.

B. Methods

1. Synchrony

The yeast cells were induced to divide synchronously by modification of a procedure described by Williamson and Scopes (1962). S. carlsbergensis was innoculated in 50 ml. of 2x MY in a 250 ml. screw capped flask and was incubated at 30° C with rapid shaking on a wrist action or reciprocating shaker. A budding index was determined by microscopic

TABLE I

Cultivation Media

No. 1

peptone	- 3.5 gm.
yeast extract	- 5.0 gm.
dextrose	- 40 gm.
KH_2PO_4	- 2.0 gm.
H_2O	- 1000 ml.

(C. R. Hebb, personal communication)

MY

malt extract	- 3.0 gm.
yeast extract	- 3.0 gm.
peptone	- 5.0 gm.
dextrose	- 10.0 gm.
H_2O	- 1000 ml.

(Malt extract medium of Wickerham (1951))

(2x MY contains twice the weight of each component still dissolved in 1000 ml. of water.)

W and S Starvation Medium

KCl	- 0.745 gm. (0.01 M)
$CaCl_2$	- 0.260 gm. (0.0024 M)
$MgCl_2 \cdot 6H_2O$	- 0.508 gm. (0.0025 M)
H_2O	- 1000 ml.

(Williamson and Scopes, 1962)

TABLE II

Fixatives

Carnoy's Fixative

absolute ethanol	-	6 parts
chloroform	-	3 parts
glacial acetic acid	-	1 part

This is made immediately prior to use.

Helly's Fixative

distilled H_2O	-	100 gm.
$HgCl_2$	-	5 gm.
$K_2Cr_2O_7$	-	5 gm.
neutral formaldehyde	-	4 ml. (just prior to use)

(N.B.: The usual Na_2SO_3 is absent.)

Osmic Acid Fixative

vapors of freshly prepared 1% solution in distilled water

TABLE III

Stains

Giemsa

Giemsa's stain, powder	-	3.8 gm.
glycerol, analytical grade	-	125 gm.
methanol	-	375 gm.

The glycerol is warmed to sixty degrees centigrade at which point the stain powder is added and mixed thoroughly. The methanol is added and mixed and the stain is allowed to stand overnight before filtering.

Gurr's Giemsa Buffer

$Na_2HPO_4 \cdot 2H_2O$	-	0.569 gm.
KH_2PO_4	-	0.594 gm.

Grind in mortar and dissolve in 200 ml. of H_2O, final pH 6.8. Final solution is 10 ml. of buffer plus 10 drops stain.

Azure A

azure A stain, powder	-	1.0 gm.
H_2O	-	100 gm.

Water is warmed to aid complete solution and then stain is filtered. Immediately before use add either 0.6 ml. of 1N HCl and 0.6 ml. of $K_2S_2O_5$, or 10 drops of thionyl chloride ($SOCl_2$).

Basic Fuchsin

basic fuchsin	-	1 gm.
H_2O	-	200 gm.
1N HCl	-	20 ml.
$NaHSO_3$	-	1 gm.

Water is heated to boiling point and dye is stirred into it. The solution is cooled to 50°C and the acid is added. It is further cooled to 25°C and the sodium bisulfite is added.

sulfurous acid for washing	-	H_2O	-	200 ml.
	-	1N HCl	-	10 ml.
	-	$Na_2S_2O_3$	-	1 gm.

TABLE III (Continued)

Acid Fuchsin

acid fuchsin, powder	-	0.1 gm.
H_2O	-	5000 ml.
glacial acetic acid	-	50 ml.

examination. After one day's incubation the budding index (fraction of the total cells in the budding process) was usually about .20-.25. This figure declined slowly and reached a low after six days of .02-.05 with a cell concentration of 2.5×10^8 per milliliter.

Ten milliliters of the cells at this stage were harvested by centrifugation at 1000 x g for five minutes. The supernatant was discarded and the cell pellet was washed once in distilled water. After this washing the cells were centrifuged at 300 x g in a 15% (w/v) mannitol solution to eliminate small cells from the culture. This washing was repeated until the supernatant showed little turbidity. The effect of this is to decrease the population frequency of cells 5-6 u in diameter by approximately 50%.

The cell pellet was then suspended in 10 ml. of MY medium and aerated as vigorously as possible in a 150 x 15 mm. test tube for thirty five minutes at 30^0 C. (An antifoam agent was tried in an effort to increase bubbling rate but surface active agents seemed to inhibit budding.) At the end of this period, the cells were centrifuged immediately to remove them from the rich medium and were then washed twice with Williamson and Scope's starvation medium. They were then aerated in this medium for five hours at 30^0 C in a similar tube to that previously used with the rich medium. The cells were then stored overnight at 4^0 c. The feeding

and starvation cycle was repeated on three more successive days.

On the fifth day, 4 ml. of this culture were suspended in 50 ml. of No. 1 medium in a 250 ml. screw-capped flask to give a cell concentration of about 1.8×10^6 per ml. This was incubated at 30^0C with rapid shaking and after a lag of approximately ninety minutes the cells began to bud. Counts were made as recommended by Williamson and Scopes (1960). Thirty minutes later the culture reached a budding index of .65-.80. A time course curve is given in the results (Figure III).

2. Study of Live Material

In an effort to complement the stained preparations, live material was also studied. Standard phase contrast of water mounts from young cultures proved sufficent to study the gross morphological changes accompanying the budding process.

In order to study the behavior of the nucleus in live preparations, it was necessary to use a procedure recently described by Robinow and Marak (1966). The procedure is designed to avoid the problems normally caused by the dense phase rings around the material. A slide was coated with a layer of medium containing 21% gelatin, 1% yeast extract and 2% glucose. This was inoculated with yeast cells and incubated at room temperature for four hours. This incubation resulted in elimination of granular material within the cell

which might have obscured the nucleus. After this incubation the slide was examined with phase contrast.

3. Treatment of Unsynchronized Material

Slides for preliminary studies generally consisted of unsynchronized material and were prepared as follows. A coverslip (No. 1-1/2) was coated with a thin layer of Mayer's albumin to which was added a thick drop of cell suspension. Without allowing this to dry (it was found that air drying caused considerable cell shrinkage), it was transferred to one of the several fixatives used. Carnoy's was used for a fixation period of ten minutes, Helly's for twelve minutes and vapors of osmic acid (1%) for two minutes. The coverslip was then transferred to 70% ethanol to remove excess fixative and then placed in .17M sodium chloride at 60°C for ten minutes. The coverslips were removed from the hydrolysis bath and dipped in water to terminate the extraction and were then placed in either azure A or Giemsa stain for from one and one half to two hours. Coverslips were then removed from the dye, washed in tap water and mounted over Diaphane or Permount. As a control on the specificity of the stain, some slides were treated with DNase (Sigma) at a concentration of .1 mg. per ml. in a 0.1M phosphate buffer pH 7.7, for one hour at 40°C.

Material to be stained with basic fuchsin was treated similarly except that it was washed first with sulfite water

after staining and then with tap water and was finally mounted over a drop of aceto-carmine.

Acid fuchsin material was fixed in Helly's only and was stored in 70% ethanol until needed. The coverslips were then rinsed several times in 1% acetic acid and then stained for two and one half minutes. The slip was then washed several times in 1% acetic acid and mounted over the same.

4. Treatment of Synchronized Material

In the case of synchronized material, cells were taken as aliquots directly from No. 1 medium. Two methods were used depending on the fixative employed.

When osmic acid vapors were used, a drop of the culture was withdrawn by pipette and placed on a No. 1-1/2 coverslip which had previously been coated with a thin layer of Mayer's albumin. Without allowing this to dry, the coverslip was placed in a Coplin type dish in a finger bowl where it was exposed to the vapors of osmic acid for two minutes. It was then removed and stored at 4^{0}C until needed for staining.

For Helly's fixative, 5 ml. of the culture were withdrawn and pipetted into a screw capped test tube. The cells were rapidly centrifuged (1000x g) into a pellet and quickly re-suspended in 5 ml. of Helly's fixative. After twelve minutes the cells were recentrifuged and the supernatant discarded. The cells were washed once in 70% ethanol, twice in tap water

and then concentrated to a volume of .2 ml. This was spread on a plain agar plate (1.5% agar) and stored at 4^{0}C until staining.

When required, agar blocks about $5mm^{2}$ were cut and placed cells down on coverslips coated with albumin. With the side of the agar bearing the cells in contact with the albumin, the block was tapped down and then flipped off leaving an imprint of cells on the coverslip.

In either case, coverslips bearing the previously fixed cells were transferred to 70% ethanol where they were kept for five minutes. They were then transferred to distilled water for five minutes.

The next step was devoted to elimination of RNA. Cells were first extracted with .17M sodium chloride and were then hydolysed to specifically release aldehyde groups on DNA. Hydrolysis was carried out in 1N HCl at 60^{0}C for times varying from ten to twenty minutes depending on the division stage (see Table IV).

An alternative to this was the use of RNase (Sigma). This was made as a stock solution containing 2 mg. per ml. in pH 5.5 saline. For use this was diluted with 0.2M phosphate buffer pH 7.5 to a final concentration of 0.1 mg. per ml. Coverslips were placed in this solution and digestion was allowed to procede for one and one half hours at 30^{0}s.

TABLE IV

Treatment of Synchronized Material
(all times in minutes)

Synchrony time		HCl hydrolysis	Azure A	Giemsa
0		10	120	120
10		10	100	120
20		13	100	120
30		15	100	120
40		15	120	100
50		17	120	100
60		17	120	100
70		13	100	100
80		13	100	100
90	budding begins	17	100	100
100	DNA doubles	15	115	100
110		15	115	100
120	budding reaches peak	15	110	100
130		17	130	100
140	nuclear transfer	20	130	100
150		17	115	100
160	end of first cycle	15	115	100
170		15	100	110
180		17	100	110
190		15	115	110
200	budding reaches peak	15	115	110
210		15	130	120
220		17	130	120
230	nuclear transfer	20	115	120
240		17	115	120

Cells treated in this way took slightly longer to stain and even then gave faintly stained preparations. This procedure resulted in less shrinkage than extraction in sodium chloride.

Coverslips were then placed in Giemsa or azure A for the times given in Table IV. After staining the coverslips were washed several times in tap water, dried by blotting and mounted over Diaphane.

Photomicrographs were taken using a Leitz Ortholux research microscope equipped with apochromatic oil immersion objectives and either a Mikas 35 mm camera or a Bausch and Lomb Model L camera. The film used was Panatomic X.

CHAPTER IV

RESULTS

A. Synchrony

After preliminary studies to define the staining procedure, all slides described were prepared from five synchrony runs. Pooled data from the five is presented in Table V and Figure 3.

The degree of synchrony reached was slightly less than that obtained by Williamson and Scopes (1960) but still was satisfactory, varying from sixty-five to eighty percent. The number of buds (as determined by microscopic count) began to exceed the background level at ninty minutes. A budding peak was reached forty minutes after, that is at one hundred and thirty minutes after innoculation into No. 1 medium; almost immediately after which a second budding cycle began. The loss of synchrony in the second division was approximately twelve percent and the duration of the cycle was the same as the first. This gives a generation time of eighty minutes for the population which is comparable with the results of Williamson and Scopes (although our lag period was about twenty minutes longer). In that this is an average for the population, the generation time of an individual cell may be slightly different.

An attempt was made to follow synchrony by monitoring

TABLE V

Pooled Data of Five Synchrony Experiments

Time (min.)	Total Cells	1.0*	1.1	2.0	2.1	2.2	Other	Percent Buds
0	500	478	3	19	0	0	0	0.52
20	300	293	2	5	0	0	0	0.66
40	500	458	5	37	0	0	0	0.93
50	400	381	5	13	0	0	0	1.45
60	500	470	8	22	0	0	0	1.53
70	500	457	8	35	0	0	0	1.49
80	300	267	5	27	0	0	1	1.53
90	500	464	14	22	0	0	0	2.68
100	551	441	90	18	2	0	0	16.1
110	552	315	207	24	4	2	0	36.6
120	453	179	250	9	10	4	1	55.4
130	504	101	352	29	12	10	0	69.0
140	500	104	283	99	7	7	0	50.0
150	308	57	115	112	7	8	9	31.0
160	398	68	99	198	10	8	15	19.3
170	331	36	62	194	0	8	5	13.4
180	183	52	38	103	0	3	1	13.0
190	126	9	16	85	2	6	1	38.4
200	108	9	13	33	1	48	0	55.6
210	95	4	9	33	1	42	1	52.5
220	120	2	8	45	2	55	2	27.4
230	147	4	15	90	0	27	0	21.0

* 1.0 denotes a cell with bud
1.1 denotes a cell with a small bud
2.0 denotes a double cell
2.1 denotes a double cell with one bud
2.2 denotes a double cell with two buds

Figure 3. Graph showing the synchrony curve as expressed in percent buds, cell numbers (one cell being any unit one-half as large as a mother cell or larger) and turbidity.

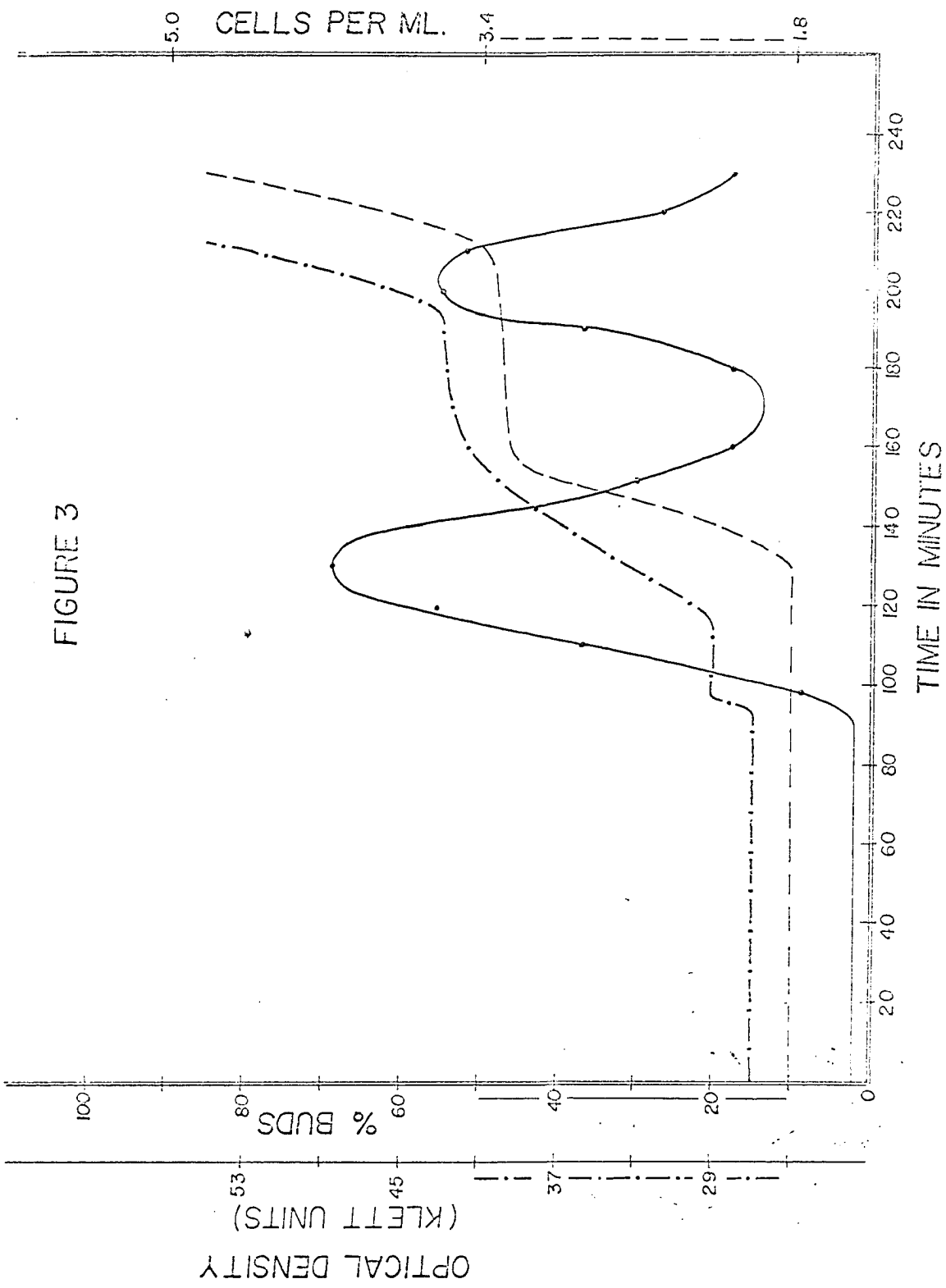


FIGURE 3

turbidity with a Klett colorimeter and the results are shown in Figure 3. As can be seen, variations in the curve are perceptible but slight. This may be due to the fact that the cell volumne increases at a relatively constant rate when compared to the budding index (Williamson and Scopes, 1964).

Studies of the division process revealed a similar pattern in the first and second divisions and thus only the first cycle will be discussed in relation to nuclear events.

B. Observations on Living Cells

Study of live material in the budding process with phase contrast gave no indication of the changes undergone by the nucleus but some general information was gained. A "resting cell" (Figure 4) shows a thick outer wall and large central vacuole clearly. Around the vacuole can be seen a thin dark rim of cytoplasm which is most abundant at the apex of the cell from which the bud will develop. There is a good deal of granulation within the cell. As the bud begins to develop, a cytoplasmic bridge between mother and daughter cells becomes apparent (Figure 4). Granulation at this point seems concentrated at the center of the cell. The bud grows until it is the same size as the mother cell but the two cells remain attached until they begin to bud. Each then separately then repeats an identical cycle.

Observations on live cells prepared by the gelatin technique described by Robinow and Marak (1966) show readily the

Figure 4. A series of photomicrographs showing the general morphology of the budding cycle. Phase contrast oil immersion objective (x90). Total magnification is x7000 .

4A

4B

4C

4D

advantages of this method. (See Figure 5.) In such preparations, the nucleus is clearly visible as is the cell wall and central vacuole. Before the bud appears (Figure 5A) the nucleus is circular and a clear central area is visible giving a vacuolar appearance. In the early budding stages, the nucleus appears to be rather diffuse (Figure 5B) having no definite outline. The nucleus then assumes a triangular or wedge shape when the bud is about one half the size of the mother cell. Close examination shows a faint linear band of low nuclear density at an angle perpendicular to the long axis of the mother cell. Two similar bands can be seen below this and at a right angle from it (Figure 5C). As the nucleus begins tranfer to the bud, a dark area can be seen in the nucleus in the region closest to the vacuole (Figure 5D). There also is a faint area originating in the isthmus between mother cell and bud and projecting into the nucleus. These may represent respectively, the nucleolus and fibre apparatus recently described by Robinow and Marak (1966). Transfer of the nucleus continues (Figure 5E) until equal amounts of nuclear material are present in both cells. The process terminates with complete separation of the two nuclei, one remaining in the mother cell and the other in the bud (Figure 5F).

Throughout the cycle, little can be seen of the internal structure of the nucleus with the exception of the aforementioned faint areas. Instead most of the changes which are

Figure 5. A sequence of photomicrographs showing the nucleus in live cells during the budding cycle. Phase contrast oil immersion objective (x90). Gelatin medium. Total magnification is x6000.

A. Vesicular stage. Arrow indicates central clear area in the nucleus.

B. Diffuse stage.

C. Divided stage. Arrow indicates faint clear linear area.

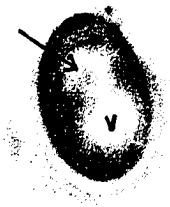
D. Transfer stage. Arrow indicates nucleolus.

E. Late Transfer stage.

F. Separated stage.

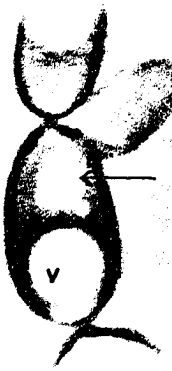
N = nucleus, V = vacuole. This notation will be used throughout.

Overleaf. Diagramatic representation of Figure 5.

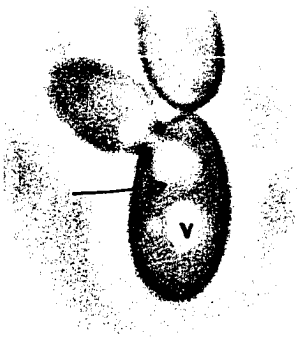


5A

5B

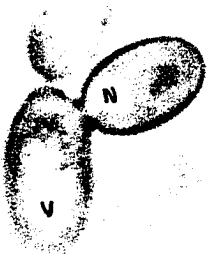


5C

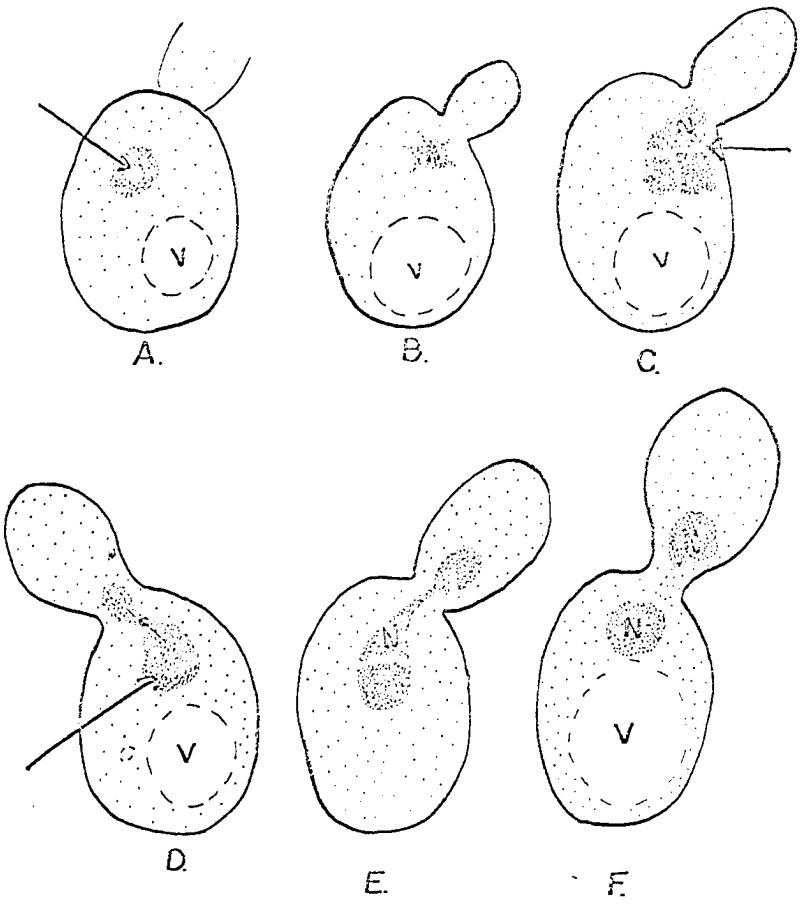


5D

5E



5F



5.

discernable are in the outline or shape of the nucleus. As will be seen, these changes also occur in stained preparations.

C. Study of Stained Unsynchronized Cells

Of the three fixatives (Helly's, Carnoy's and osmic acid) used in preliminary studies, osmium gave the least satisfactory results. Nuclei in cells treated with this fixative were generally quite compact (Figure 7A,B,C). This fixative has been critisized in the past regarding its application to nuclear material (Pearse, 1961; Siegel, 1964), and such an evaluation is not unfounded. Only in rare cases could any detail be seen with this fixative; most of the nuclear events to be described being obscured by its use. This was true in comparison to both azure A and Giemsa stained preparations.

Carnoy's fixative, though highly recommended in standard texts (Pearse, 1961) as a nuclear fixative was not as satisfactory as Helly's. Although it resulted in preparations of good nuclear detail, the rest of the cell - notably the vacuole - was not preserved as well as with Helly's. Use of the latter seemed to result in less cell shrinkage than found in Carnoy-treated preparations. Use of Helly's fixative, with hydrolysis in one normal hydrochloric acid followed by either Giemsa or azure A staining gave preparations of high quality (see Figure 8, 12 and 14).

Occasional cells showed dense cytoplasmic staining dee-

pite the selectivity of the stain. In an effort to alleviate this problem, most preparations were also extracted in .17M sodium chloride even though tis resulted in some shrinkage. This gave satisfactory results and led to an interesting discovery. In some extensively extracted preparations, there appeared a clear area around the nucleus (Figure 6A). This might be due to shrinkage of the nuclear material because of the ionic environment or removal of RNA at the immediate periphery of the nucleus or a combination of both.

RNAase (Sigma) was applied in an attempt to biochemically validate the action of azure A. Treatment with this enzyme resulted in preparations similar to those prepared using other methods of extraction of RNA. The only noticable difference was that RNAase treated preparations required slightly longer staining times (Figure 6B).

Pretreatment of the cells with DNase (Sigma) before staining with azure A, resulted in cells in which the cytoplasm and vacuole could be detected, but no nuclei could be seen (Figure 6C).

Application of the Feulgen technique to yeast cells resulted in very faintly stained preparations (Figure 7D). The nuclei appeared more delicate than in azure A preparations but was so faintly stained as to hinder meaningful study.

Generally speaking, a comparison of the Giemsa and azure

Figure 6. A. Helly's fixed, extensive sodium chloride extraction, acid hydrolysis, azure A stained. Note the clear area between the nucleus and the cytoplasm as indicated by the arrow (x2000).

B. Helly's fixed, RNAase extraction, azure A stained (x2500).

C. Helly's fixed, acid hydrolysis, DNAase extracted, azure A stained. Note the absence of nuclei. (x4000).

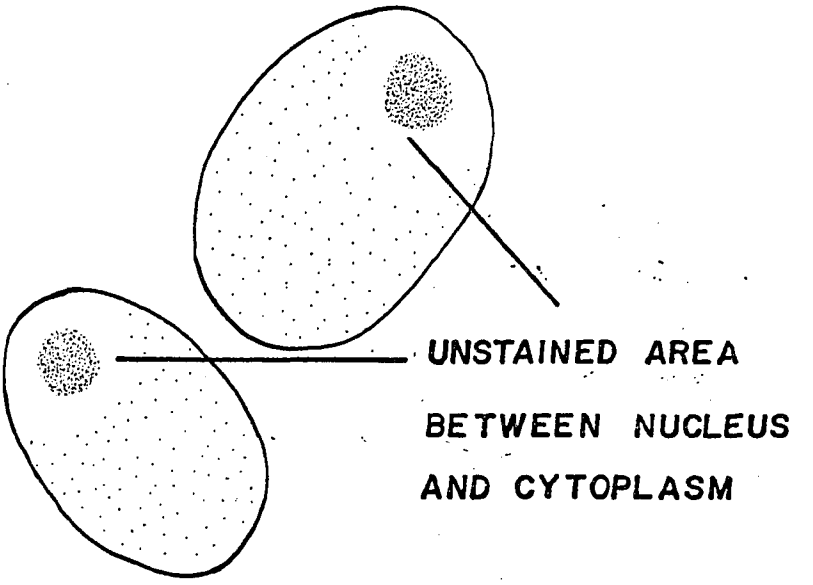
A-C. Bright field oil immersion objective (x100). All remaining photomicrographs were taken with this objective.

Overleaf. Diagramatic representation of Figure 6A.

6A

6B

6C



6A

Figure 7. A. Osmium fixed, acid hydrolysis, azure A stained. The nuclei are compact and homogeneous (x4000).

B. Osmium fixed, acid hydrolysis, Giemsa stained. Note the dense compact nuclei (x3000).

C. Osmium fixed, acid hydrolysis, Giemsa stained. (x6000).

D. Helly's fixed, acid hydrolysis, Feulgen stained. This illustates how weakly Feulgen positive the yeast nucleus is. (x9000).

7A

7B

7C

7D

A preparations shows that the latter are more delicate than those of the former, but still retain enough depth of staining to make detailed study possible. Giemsa preparations often appeared uniformly dense and did not show as much outline detail as did azure A stains. Also, the cytoplasm of Giemsa stained cells was colored deeper than that of azure A preparations. Thus it seemed that azure A was a more suitable stain for studying the yeast nucleus since it stained more intensely than Feulgen techniques and yet showed more detail than Giemsa. Reviewing this fact in the light of the work of Sachs and Hartman (1965) and Delamater (1951), it was decided to use azure A as the main stain with Giemsa serving only a secondary role.

D. Study of Stained Synchronized Cells

In studying the nucleus in synchronized cell populations, it was found that several morphological changes occured. From time 0 through time 30 (times refer to minutes after inoculation into No. 1 medium) the nuclei were compact and rounded (see Figure 8). The position of the vacuole could be seen and the cytoplasm was faintly stained, but no detail was visible in the nucleus. Projecting from the homogeneously darkly stained nucleus were thin elements of what appeared to be nuclear marerial. There were as many as three such protuberances (Figure 8D), but often there was only one. In some cases the projections terminated in granule-like bodies

Figure 8 Helly's fixed, sodium chloride extraction, acid hydrolysis, azure A stained. Synchronized material at time 0. Note the rounded small nuclei and the integrity of the vacuole.

A. (x1000)

B. Arrow indicates double projection of the nucleus (x2000)

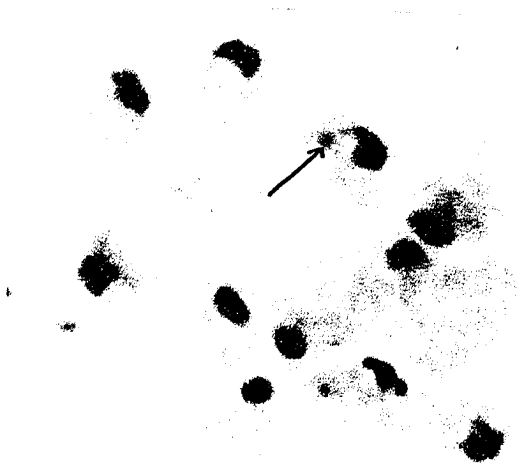
C. Arrow indicates long nuclear projection ending in a granule (x3000)

D. (x3500)

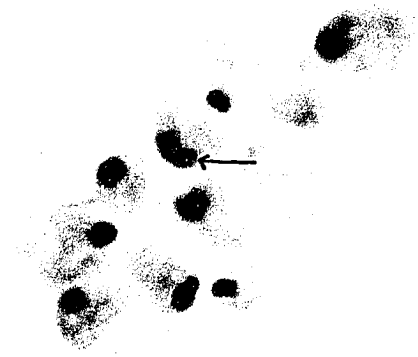
E. (x2000)

Overleaf: Diagramatic representation of Figures 8B, 8C and 8D.

8A

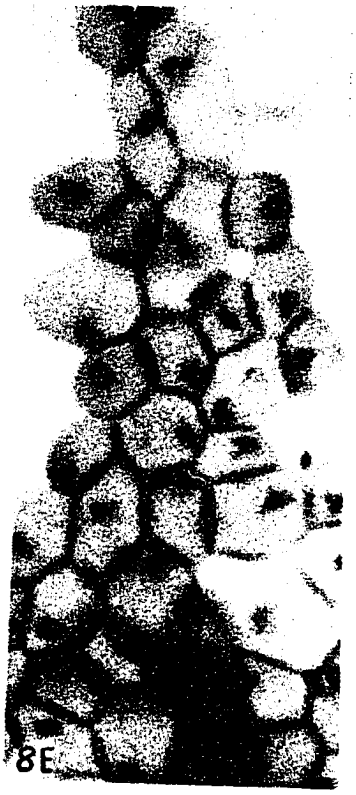


8C

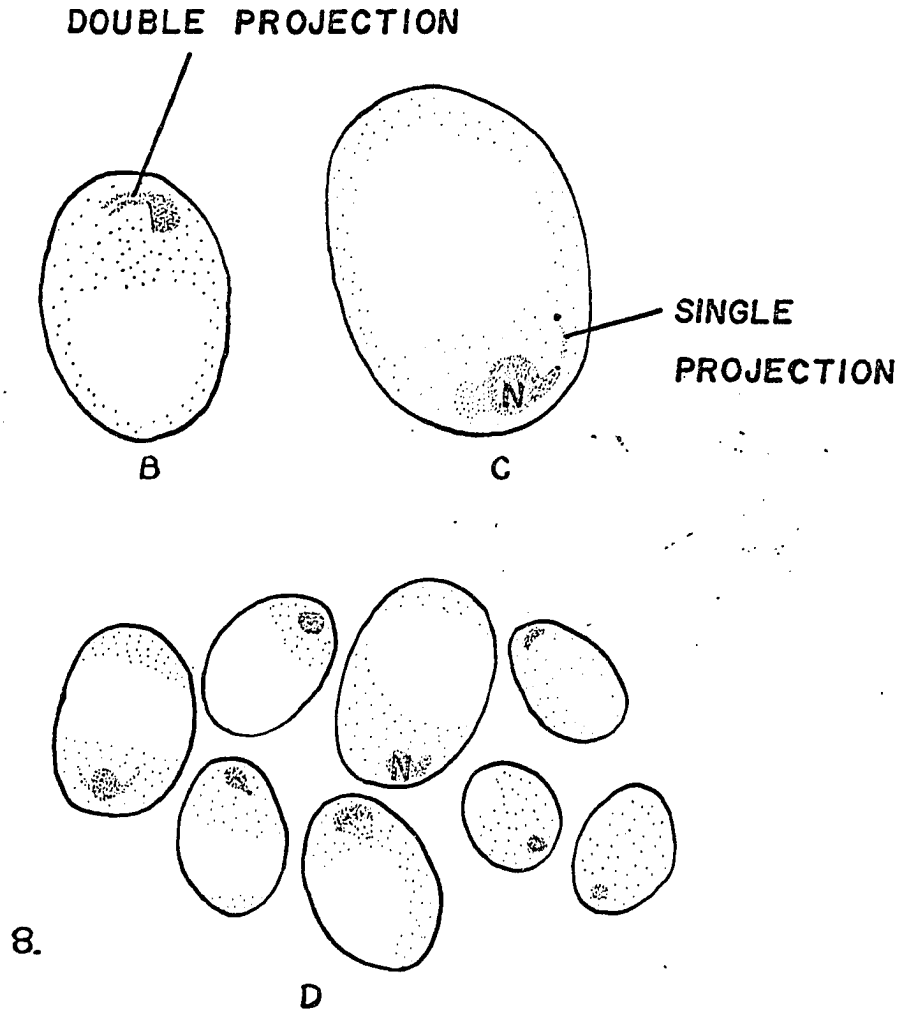


8B

8D



8E



8.

Figure 9. Drawing to illustrate the cap on the nucleus which often stained deeply with azure A at early synchrony times. It is not always orientated proximal to the vacuole.

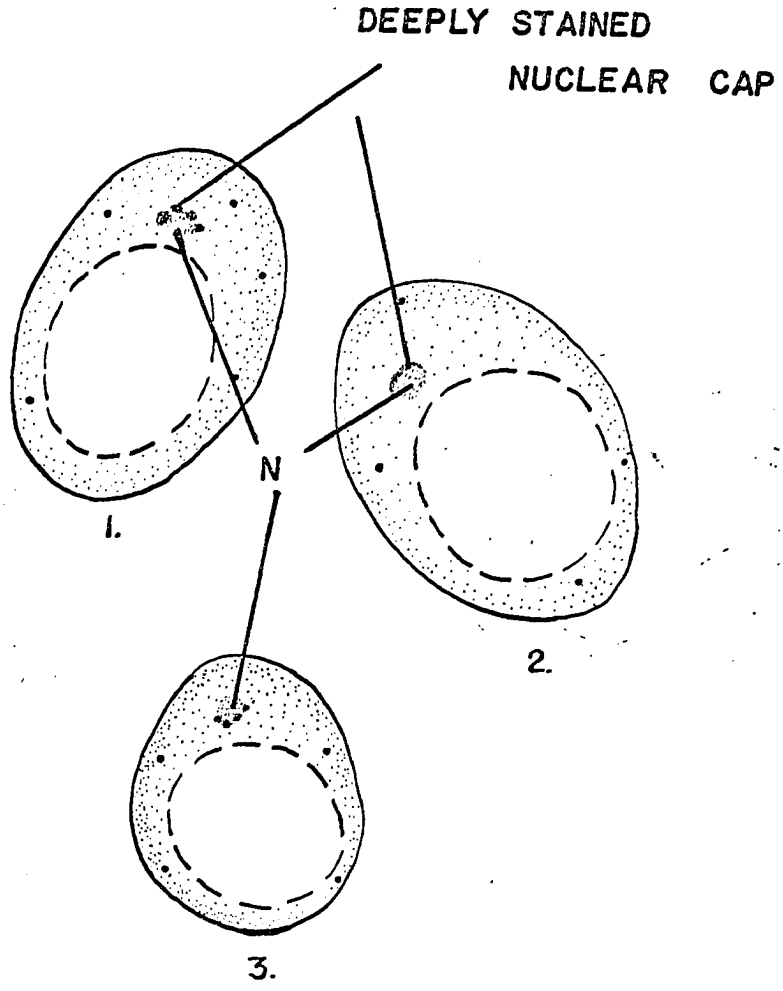


FIG. 9

(Figure 8C) which are similar to the centioles of Delamater (1950) and Ganeson (1959). Isolated nuclei showed tis projection to be doubled (Figure 8B). A cap of nuclear material was sometimes seen which stained more intensely than the rest of the nucleus. In the light of the specificity of azure A for DNA, this cap is not believed to be the nucleolus described by Robinow and Marak (1966) (Figure 9).

At time 30, the nucleus often assumed an elongated crescent shape and seemed to be located very near the central vacuole. It was not determined whether this represented an exaggerated state of the projections or a configuration different from this. The nucleus then assumed the shape of a holow sphere or vesicular body (Figures 10A and 5B). This figure was most prominent from forty to forty five minutes after the start of the synchrony experiment.

Beginning at time 60 and reaching a maximum frequency at time 80, the clear central areas disappeared and the nuclei decreased in their staining intensity. It appeared as if the nuclear material was spreading out or becoming more diffuse (Figures 10B, 10C and 11A). The clearly defined edges of the nuclei became indistinct and the diameter of the nuclei increased considerably. Some cells showed chromosome like bodies. At the same time the cytoplasm began to stain more densely and ther often appeared a single large, dark central area (Figure 11B).

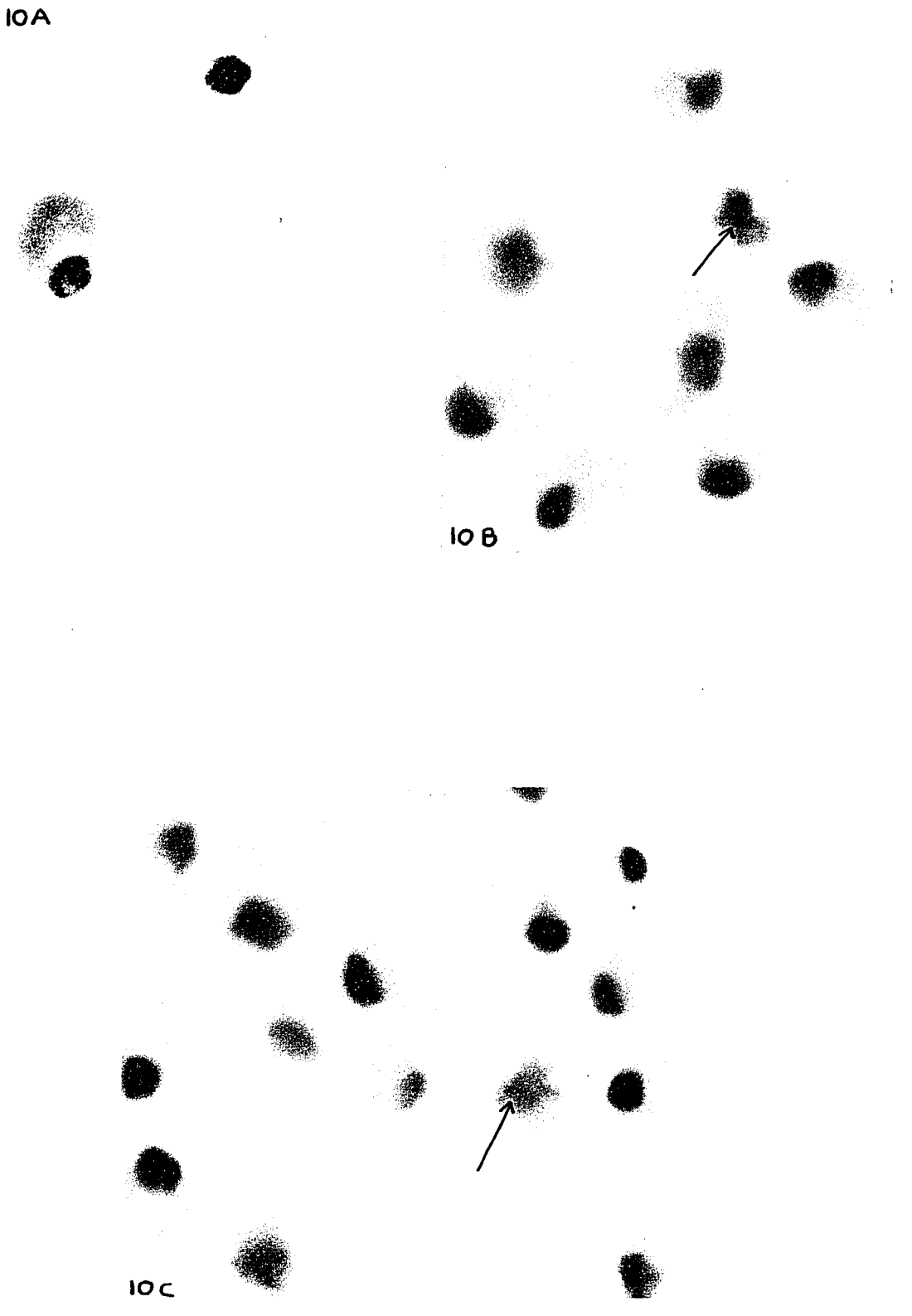
Figure 10. Helly's fixed, sodium chloride extracted, acid hydrolysis, azure A stained.

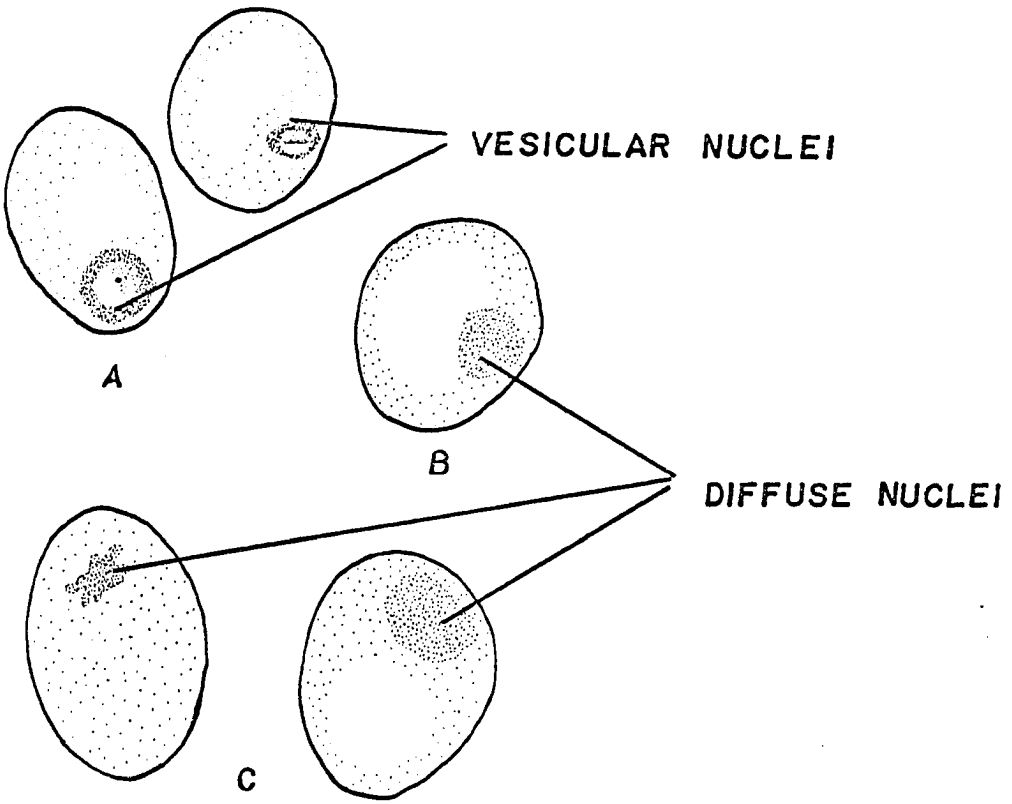
A. Vesicular stage. Synchrony time 45. (x3000) (x30000

B. Diffuse stage. Synchrony time 70. (x3000)

C. Diffuse stage. Synchrony time 80. (x5000) Note the faded edge and increased size of the nuclei.

Overleaf: Drawing to illustrate Figure 10A, B andC





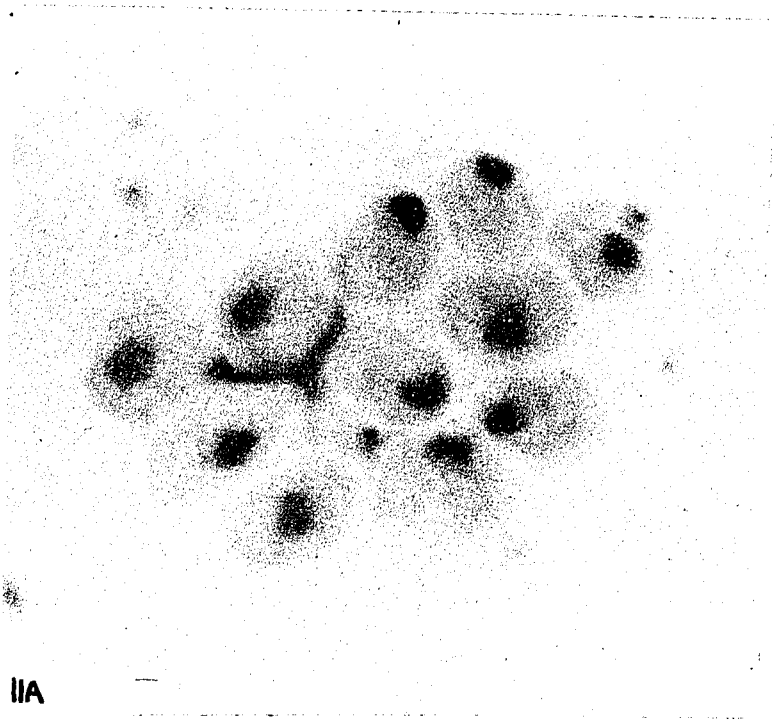
10.

Figure 11. Helly's fixed, sodium chloride extracted, acid hydrolysis, azure A stained.

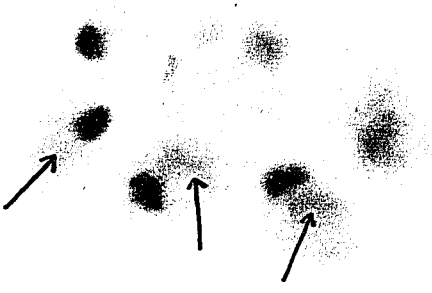
A. Diffuse stage. Synchrony time 85. (x4000)

B. Diffuse stage. Synchrony time 85. (x4000) Large central dark staining areas are visible and are indicated by the arrows.

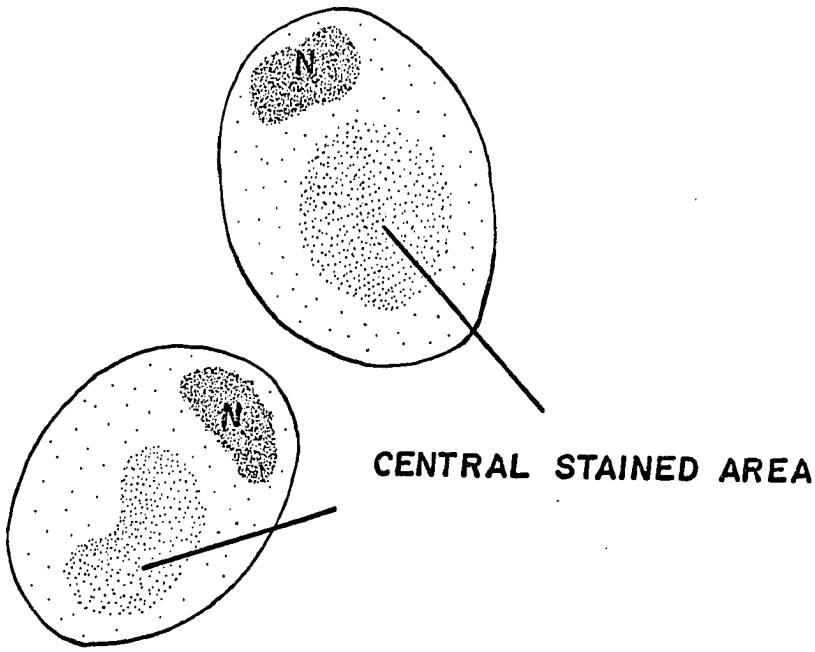
Overleaf: Diagramatic illustration of Figure 11B.



IIA



II B



II. B

At time 90 the cells began to bud. The nuclei at this stage reassumed a condensed configuration but did not become as dense as seen earlier in the experiment. The staining intensity reverted to its original state but the nuclei were slightly larger than at time 0. The nuclei then became oriented quite close to the bud. In a few cases, the nuclei could be seen at oppisite ends of the cell from the bud but in such cases it was not possible to determine if nuclear division and transfer did in fact occur.

Diamond-shaped nuclei could be seen 110-120 minutes after the start of the experiment. After, there appeared a dark band stretching across the nucleus while the remainder of the nuclear material stained faintly and was outlined. (see Figure 12A). Other figures could be seen during the same period in which a faintly staining area began to appear between two denser areas of deeply staining nuclear material. (Figure 12B).

During the next interval of about twenty minutes (time 120 to 140), the nuclei assumed a wedge shape with the tip of the wedge quite close to the bud. In many nuclei, a faintly staining band covering an area which was perpendicular to the long axis of the mother cell could be seen (Figures 12C, 13A and 13B). This is very similar to the case described earlier for gelatin embeded material (Figure 5C).

At time 140 to time 160, transfer of the nucleus across the isthmus between mother cell and bud took place. A very

Figure 12. Helly's fixed, sodium chloride extracted, acid hydrolysis, azure A stained.

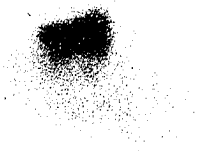
A. Diamond-like stage. Synchrony time 110 (x3000).

B. Diamond-like stage. Synchrony time 120 (x5000).

C. Divided stage. Synchrony time 125 (x6000) Arrow indicates linear unstained area across the nucleus.

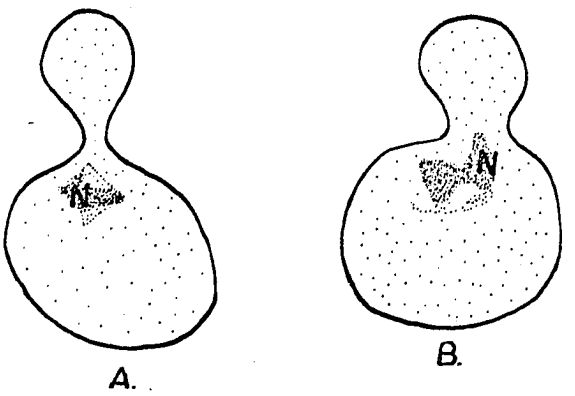
Overleaf: Diagramatic representation of Figure 12A, B and C.

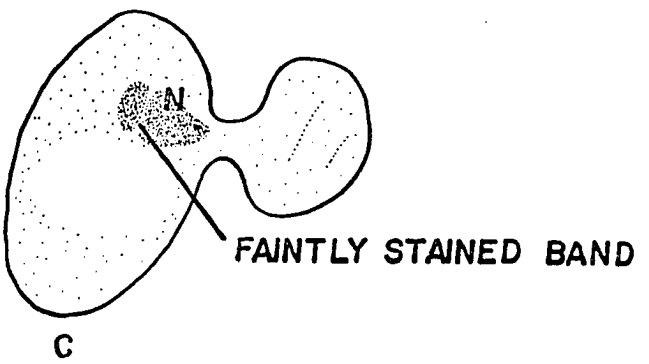
12A



12B

12C.





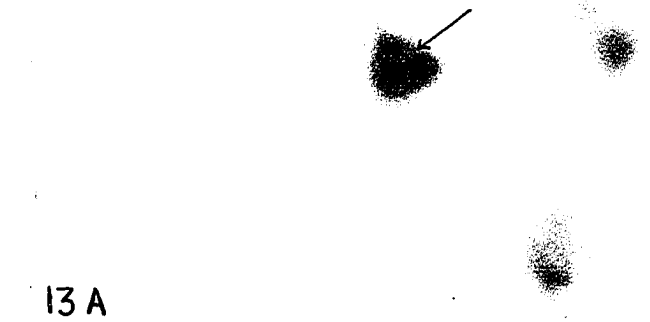
12.

Figure 13. Helly's fixed, sodium chloride extracted, acid hydrolysis, azure A stained.

A. Divided stage. Synchrony time 130 (x3500). Arrow indicates area unstained.

B Divided stage. Synchrony time 130 (x3500). Arrow indicates area unstained. Note that a thin extension of the nucleus has entered the bud.

Overleaf: Diagramatic representation of Figure 13A and B.



13 A

13 B

13.

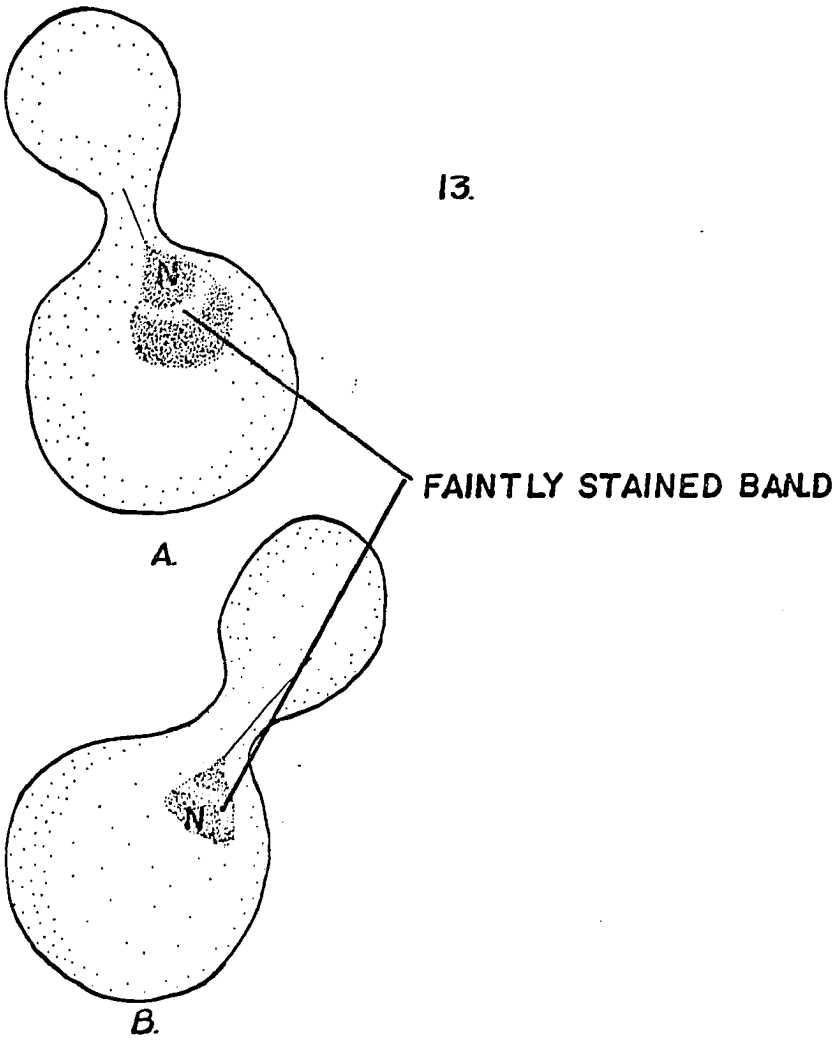


Figure 14. Helly's fixed, sodium chloride extracted, acid hydrolysis, azure A stained.

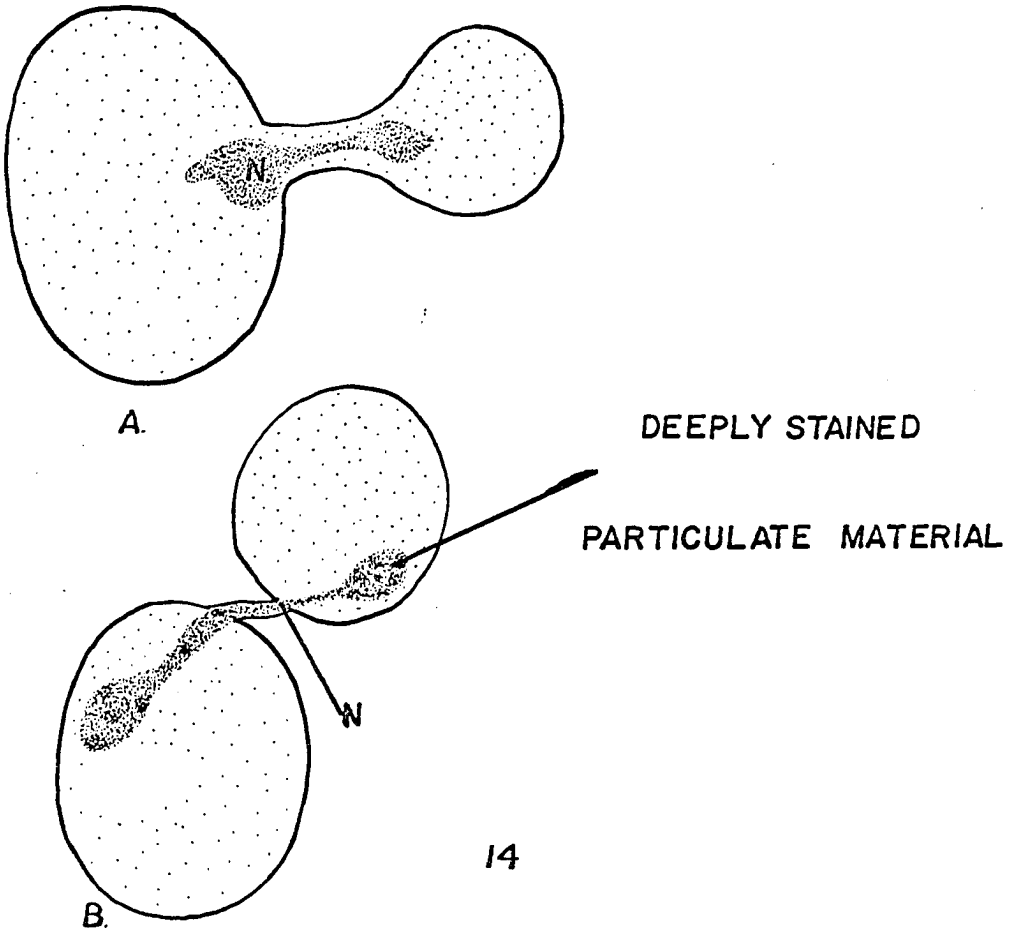
A. Transfer stage. Synchrony time 150 (x5000).

B. Transfer stage. Synchrony time 150 (x5000).

Overleaf. Diagramatic representation of figure 14A and B.

14A

14B



14

thin lightly staining thread first progressed into the bud (Figure 14A). Soon a large bulb of nuclear material appeared in both the mother cell and bud, the two being connected by a thin thread (Figure 14B). In some cases the thread appeared to consist of alternating light and dark areas suggesting the involvement of particulate material (see Ramirez and Miller, 1962). The thread was usually quite straight through out its length but in a few cases was straight for only short segments (Figure 16D).

At times 160 to 170, the nuclei could be seen as complete structures in both cells. These bodies were quite dense and stained evenly and at time 180, the cycle was repeated for the second division.

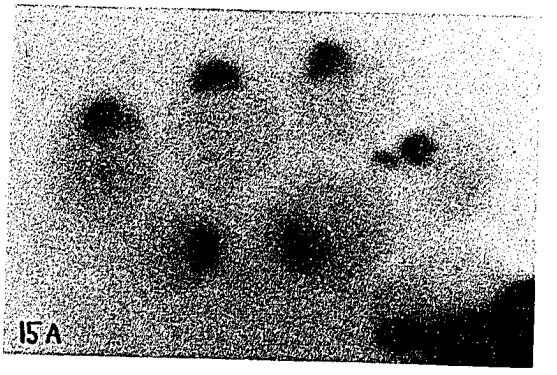
The study of synchronized material with Giemsa gave results in close agreement with those found using azure A. Again the nuclei were compact initially (Figure 15A) and then changed from a rounded to an elongated shape. Often a crescent was observed on the nucleus which was slightly different in color from the rest of the organelle. The vesicular nuclei seen with azure A were not seen in Giemsa preparations, possibly due to non-specific staining within the central area. The diffuse stage was clearly visible and again some cells showed chromosome like bodies (Figure 15B,15C). The nuclei became slightly more compact (Figure 16A) and then assumed a wedge shape (Figure 16B). The clear areas seen in azure A

Figure 15. Helley's fixed, sodium chloride extracted, acid hydrolysis, Giemsa stained.

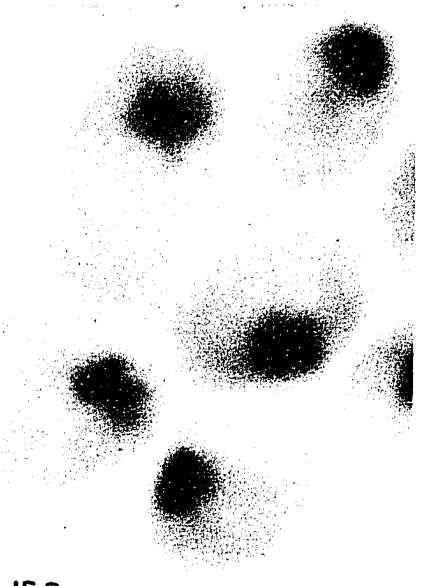
A. Compact stage. Synchrony time 20 (x3000).

B. Diffuse stage. Synchrony time 80 (x6000).

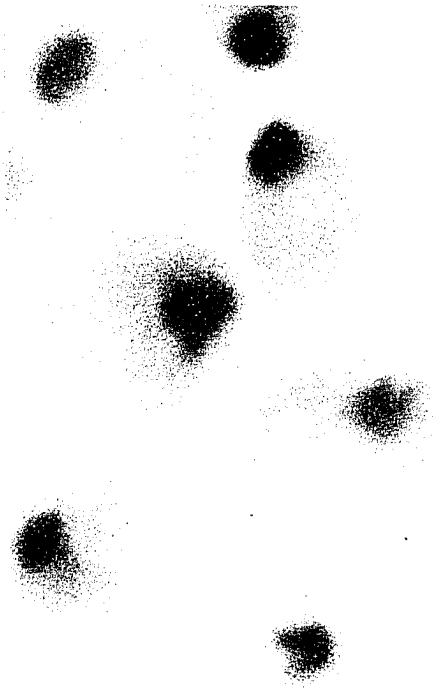
C. Diffuse stage. Synchrony time 90 (x6000).



15 A



15 B



15 C

Figure 16. Helly's fixed, sodium chloride extracted, acid hydrolysis, Giemsa stained.

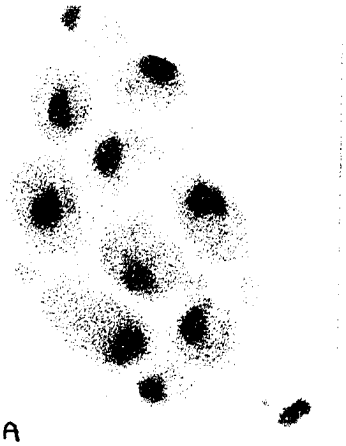
A. Compact stage following Diffuse stage. Synchrony time 100 (x3000).

B. Wedge shaped nucleus. Synchrony time 125. Arrow indicates vacuole which seems to be migrating to the bud. (x4000)

C. Transfer stage. Synchrony time 150 (x4000).

D. Transfer stage. Synchrony time 150 Note the short length of the straight segments. (x4000).

Overleaf. Diagramatic representation of Figure 16A and B.



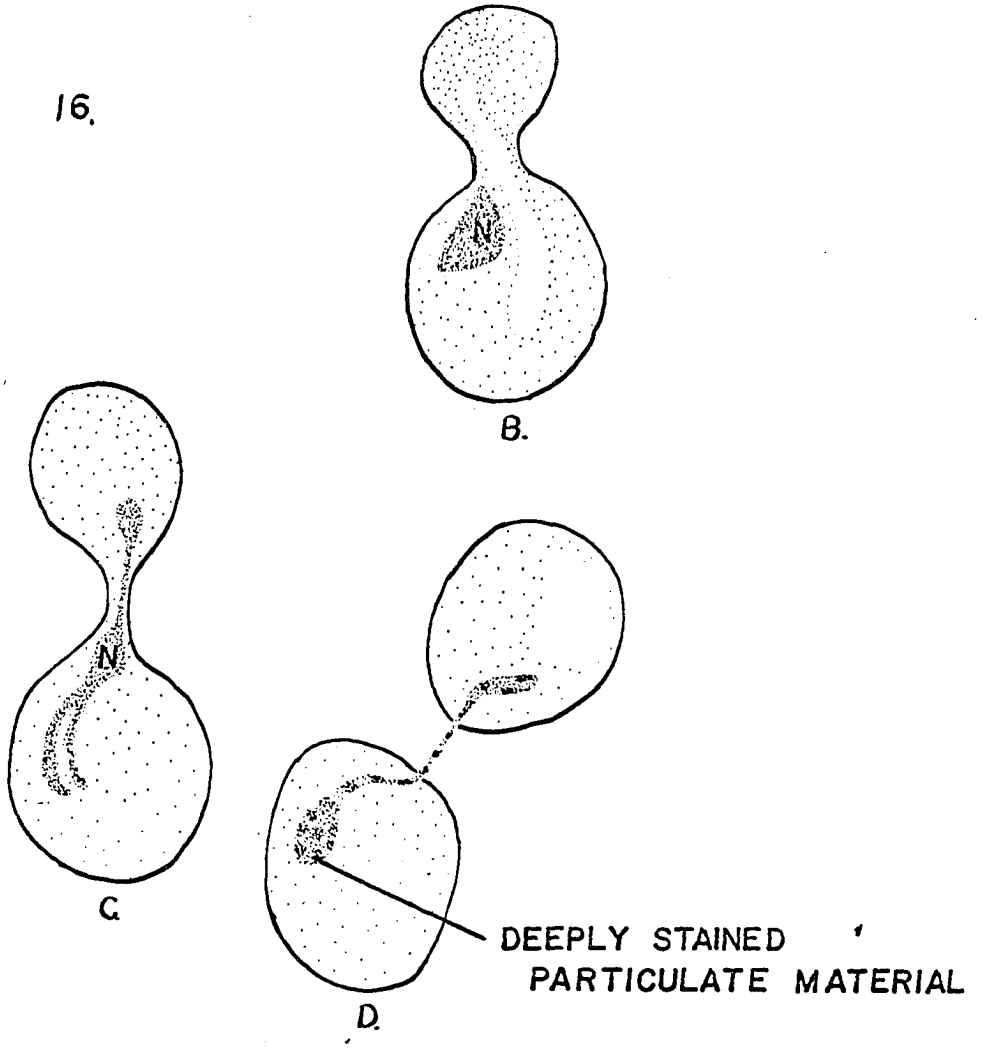
16A

16 B

16 C

16 D

16.



preparations are not evident in Giemsa stains probably for the same reason mentioned for the vesicular stage. Transfer of the nucleus proceeded according to the pattern described with azure A stained material (Figure 16C,16D).

It is interesting to note the position of the vacuole during division. Quite often in the bud several small vacuoles could be seen which later seemed to merge into a single vacuole. However, at times what appeared to be the vacuole could be seen moving intact across the isthmus (Figure 16B).

Using the method of storing fixed material on agar surfaces described previously, it was found that acid fuchsin preparations had crenated vacuoles and often stained poorly. Because of this, unsynchronized material was studied using the size of buds relative to the mother cell for determining the sequence of events. This stain is suggested for demonstration of the nucleolus and the fibre apparatus as described by Robinow and Marak (1966). The nucleolus occupies the periphery of the nucleus on the side proximal to the vacuole with the centriolar plaque and fibre rodlet being directly oppisite. This configuration remains until the nucleus assumes the wedge shape. At this time, the fibre apparatus rapidly stretches across the nucleus to the nucleolus. The fibre results in clear areas in azure A preparations which are parallel to and extend into the isthmus between mother cell and bud. The fibre projects into the bud before the

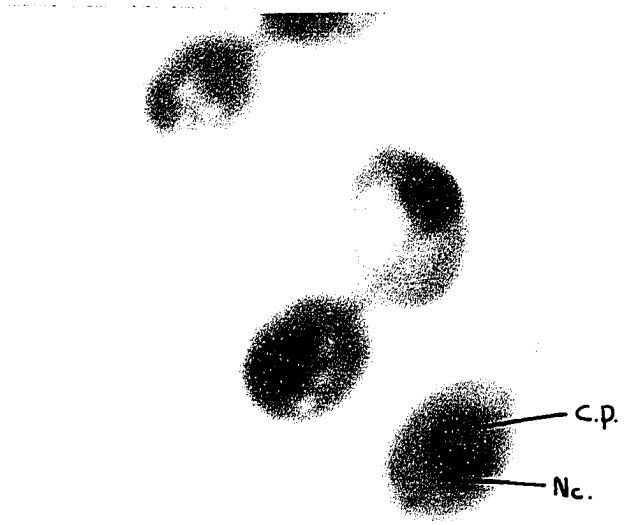
Figure 17. Helly's fixed, acid fuchsin stained. Unsynchronized. Note that the nucleus in some cases is stained. (x4000)

Ne = nucleolus

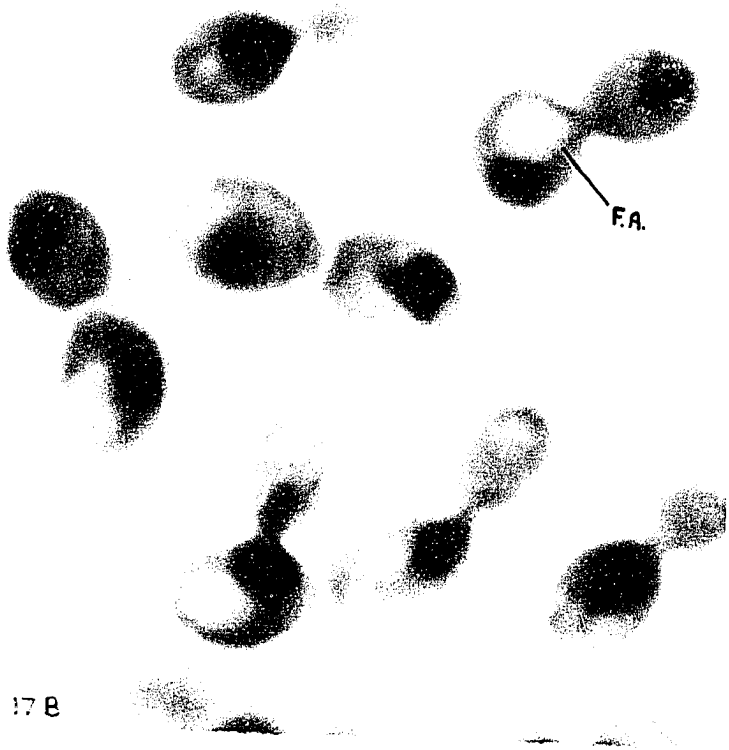
C.P. = centriolar plaque

F.A. = fibre apparatus

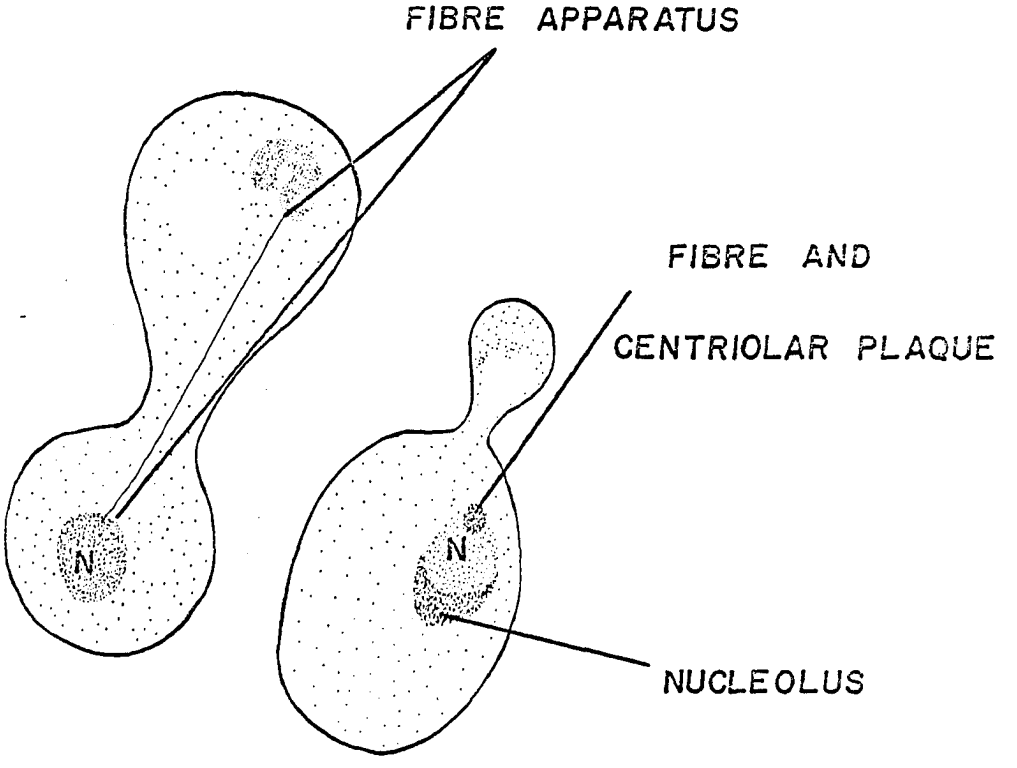
Overleaf. Diagramatic representation of Figure 17.



17 A



17 B



17. A

nuclear material as reported by Robinow and Marak (1966) and would be at aright angle to the clear area mentioned to exist at this time (Figure 17A,17B).

CHAPTER V

DISCUSSION AND CONCLUSIONS

As indicated in the literature review, there is a diversity of opinion on what constitutes the most advantageous combination of pretreatments and stains for the yeast nucleus.

Osmic acid is widley used as a fixative and stain in electron microscopy, but its use as a nuclear fixative in light microscopy has been criticized as it tends to render the nucleus structureless unless a pH of 6.1 is maintained (Pearse, 1962; Siegel, 1964; Kellenberger et al. 1958). In vapor fixed material it is impossible to maintain this condition and our experience has shown that this fixative leaves the nucleus quite compact and homogeneous appearing. Since osmium was unsatisfactory, the use of Carnoy's was attempted. Although Pearse (1962) strongly recommends Carnoy's as a nuclear fixative and some microbial cytologists (Lindegren et al., 1956) have found it satisfactory, Nagel (1946) and Yoneda (1963) have found it to be of little use with yeast in that it caused shrinkage of the cells. We also experienced this and thus found Carnoy's unsatisfactory also.

Helley's fixative minus the sulfite was found to give both nuclear detail and vacuolar preservation. Mundkur (1961) feels that the latter is an important criterion for judging artifact abscence. It was thus decided to use this in the

study of synchronized material even though it resulted in some cell shrinkage.

Lindegren et al. (1956) recommended use of perchloric acid rather than acid hydrolysis to remove RNA from the yeast cell, claiming that the latter destroyed the vacuole. This was shown to be false when Helley's fixative was used (Figures 8 and 10) and thus the vacuolar collapse experienced by Lindegren may have been due in part to his use of Carnoy's.

There would appear to be no simple explanation of the fact that optimum hydrolysis times during the synchrony cycle did not follow exactly the gradual increase in ribonucleic acid. The concommitant changes in cell size and amounts of bound RNA may account for this discrepancy. However, the general pattern was an increased hydolysis time for increased RNA concentration.

The fact that extensive sodium chloride extraction resulted in a non-staining area around the nucleus indicated that the nucleus may be contracted away from the cytoplasm by this treatment (Whitfield and Murray, 1953). An equally likely possibility exists that there may be a rim of RNA (the nucleolus ?) on the nucleus and its removal results in the clear area.

The comparison of Feulgen, Giemsa and azure A staining indicated that the latter was useful in studying the yeast

nucleus. In that it stained more intensely than basic fuchsin and more specifically than Giemsa, its use has obvious advantages. (It might be noted that the dye used was not entirely pure and therefore slight cytoplasmic staining may have been due to contaminent dyes of a similar structure such as methylene blue.) Certainly, the main problem in this and any similar study is to find a stain and staining conditions which develop enough depth of color in the object studied and yet doesn't obscure details by overstaining. The use of azure A has resulted in the observation of the various stages of karyokinesis described in the results section and the use of synchronized cultures has permitted the arrangement of these stages into a sequence. This led to the following hypothesis for nuclear division during asexual reproduction.

In Figure 18A is seen the interdivision nucleus. It is visible at synchrony times 0-40 but in a young nonsynchronized culture it lasts only five or ten minutes. The duration of its prescence was prolonged during the synchrony run due to the lag period. The nucleus at this stage was compact and rounded with the nucleolus occupying a position upon the nucleus proximal to the central vacuole. The centriolar plaque is directly opposite near the apex of the cell. This is seen in Figures 8A-D, 15A and 17A. The nucleus then elongates slightly as shown in Figure 18B. It is unclear whether this

reflects a change which always occurs or is due to the lag period. In any event, the duration of this stage is short.

Beginning at synchrony time 40 and lasting for between ten and twenty minutes, a Vesicular Stage was observed (Figures 18C, 5A and 10A). It is thought that this reflects the beginning of a dispersion of chromatinic material. At approximately synchrony time 80, this dispersion is complete and the Diffuse Stage becomes visible for a period of twenty minutes (Figures 18D, 10B-C, 11A-B and 15 B-C). These larger lighter staining forms are seen immediately prior to and during DNA synthesis as measured by Williamson and Scopes (1960) and postulated by Lindegren (1951). Therefore this change may indicate an unfolding of chromatinic material to facilitate the replication of DNA.

Near synchrony time 100, the nucleus again appears compact (Figure 18E and 16A). Close observation during this ten minute period indicates a slight increase in size of the nucleus and this is taken as a sign of completed chromatinic replication and the consequent increase in DNA.

A diamond-like figure becomes apparent between synchrony times 110 and 125 as shown in Figure 18F (see also Figure 12 A-B). The shape and position of the nucleus at this time when the nucleic acid has completed replication and the fact that a divided nucleus is seen immediately following, support the hypothesis that such a figure indicated separation of

the nuclei. Also, although between twenty-eight and thirty-two chromosomes would be expected (McClary et al., 1957; Ganeson, 1959) it was not possible to resolve anything resembling this number of discrete units. This was not unexpected in that the diameter of the nucleus is less than one micron in stained preparations. Most past authors have not postulated division of the nucleus to occur until much later in the cycle when the nucleus has completely moved to the bud. Here this later stage is recognized only as the terminus of the nuclear migration and not nuclear division. It would then be at approximately synchrony time 120 that actual mitosis occurs.

In Figure 18G, a wedge shaped nucleus is shown which is oriented quite close to the bud. Such a figure was apparent at synchrony time 125 and lasted less than twenty minutes (see Figures 5C, 12C and 13A-B). When one examines this figure closely, a clear linear area can be seen at an angle perpendicular to the long axis of the mother cell. This Divided Stage is thought to represent the two separated daughter nuclei which have just been formed witin a single intact nuclear membrane. The non-staining area represents the interspace. Such separation of the two nuclei witin the mother cell has been postulated by Ramirez and Miller (1962). Although such an interpretation may be premature, Williamson (1966) has shown an electron micrograph of a nucleus which

Figure 18. Diagramatic representation of the morphological pattern of karyokinesis in S. carlsbergensis.

A. Synchrony time 0. Compact Stage.

B. Synchrony time 20. Elongated Stage.

C. Synchrony time 40. Vesicular Stage.

D. Synchrony time 80. Diffuse Stage.

E. Synchrony time 90. Duplicated Stage.

F. Synchrony time 115. Diamond-like Stage.

G. Synchrony time 125. Divided Stage.

H. Synchrony time 135. Early transfer Stage.

I. Synchrony time 145. Transfer Stage.

J. Synchrony time 160. Separated Stage.

K. Synchrony time 180. Compact Stage.

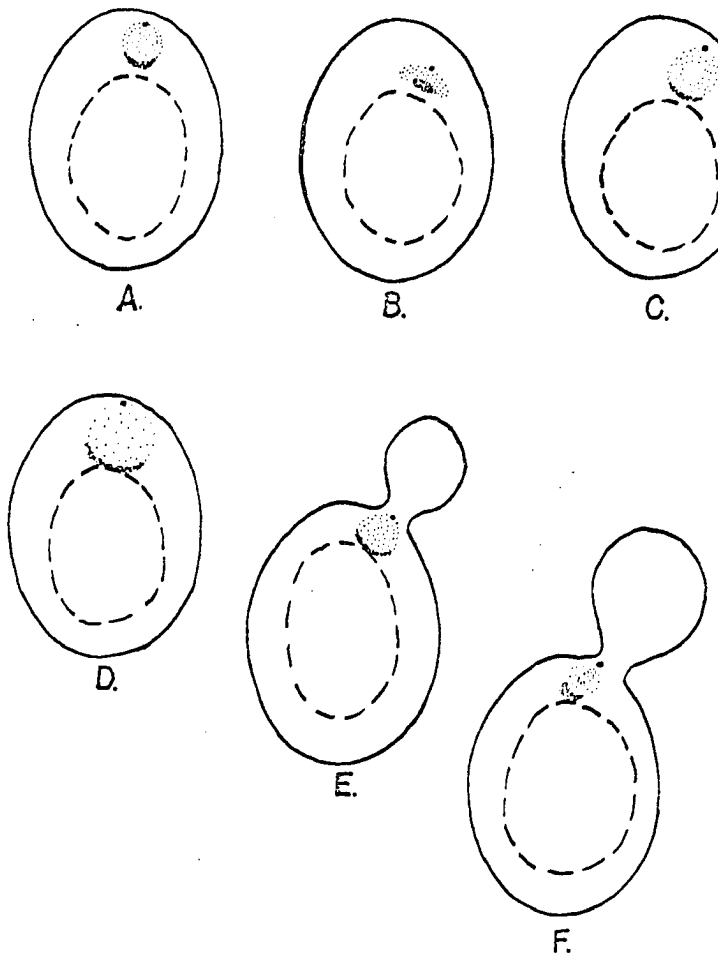


Figure 18

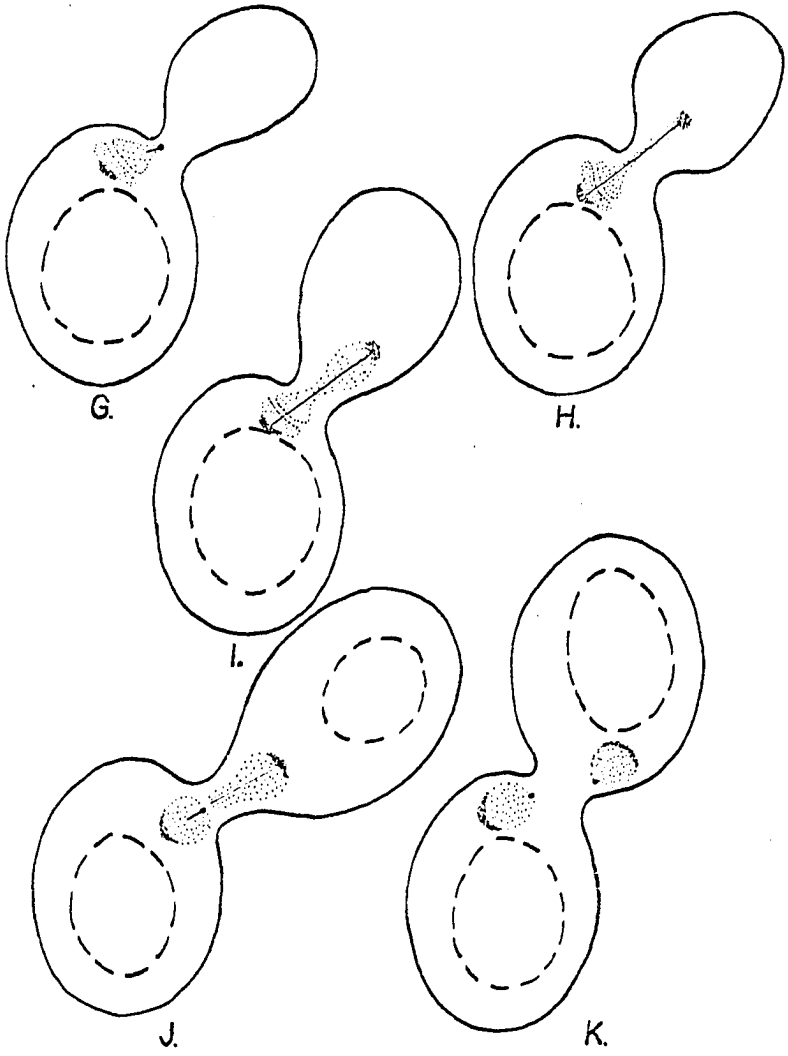


Figure 18 cont.

is generally similar and may be considered as cofirmatory.

However, it must be noted that Robinow and Marak (1966) have demonstrated a similar band and described it as being due to finger-like projections into the nucleus. Such indentations indicate the location of the centriolar plaque. Resolution of the question as to whether these stuctures are identical will likely depend on future electron microscope studies.

It is during this period that the fibre apparatus begins to elongate away from the centriolar plaque. This takes place quickly and by synchrony time 135 it is extended from a position within the bud to the nucleolus within the mother cell. As the fibre elongates, it seems to carry a small amount of chromatin with it into the bud, but the bulk of the nuclear material is left behind. It is important to see that while this fibre apparatus is extending between the mother cell and bud, the clear area between the separated nuclei is visible at an angle perpendicular to it (Figure 13B). Thus it would seem that the fibre cannot account for this latter faint linear area.

Figure 18I shows the movement of the nucleus progressing quite rapidly into the bud. This occurs at approximately synchrony time 145 and takes ten minutes to complete (Figure 14A-B and 16C-D). The fibre apparatus is still extended across the entire nucleus but the clear area is no longer

visible. This may be due to a movement of nucleolar material such that the area is covered.

The role of the fibre in the movement of the chromatin is unknown. The centriolar plaque and fibre apparatus may act as asters in determining the poles of the nucleus and thus determine contraction of the nuclear membrane in the region between nuclei. This would be similar in concept to the astral relaxation theory of mitosis postulated by Wolpert (1960). The chromatin would then be passively dragged into the bud by the nuclear membrane. It is possible, on the other handthat the chromatin attaches directly to the fibre apparatus and it than acts as a spindle for transfer of nuclear and nucleolar material. An intranuclear spindle has been found in other fungi, notably Allomyces (Robinow and Marak, unpublished) and Basidiobulus (Robinow, 1963). It is also possible that since the nuclear membrane remains intact, the chromatin may become attached and the membrane would act as a spindle. A further possibility is autonomous chromosome movement as found in protozoa (Grell, 1953; Leadale, 1963) especially in the light of the similarities between the yeast and protozoan nucleus.

Often the cleavage furrow in the membrane has been refered to as existing close to the bud side of the isthmus (Hashimoto, Conti and Naylor, 1958), but it is difficult to visualize how, at this stage when both cells are nearly equal

in size, one could determine which is the bud in a thin section. Thus, it is equally probable that the furrow exists near the mother nucleus as shown in Figure 18J.

The exact position of the centriolar plaque in the mother cell and the degree of fibre elongation at this Separated Stage is unclear. This is a difficult stage to study because of its short duration and the drawing (Figure 18J) is intended as a reminder of the existence of this structure and not its specific arrangement.

The division process terminates as the daughter nucleus moves entirely into the bud and both nuclei (one in the mother cell and the other in the bud) assume a configuration similar to that seen in Figure 18K and 18A.

The nucleolus can be seen as a dim gray crescent in live cells using phase contrast and gelatin embedding (Figure 5D) and can be demonstrated in fixed preparations with acid fuchsin. Although it has often been seen (Henneberg, 1915; Nagel, 1946; Lindegren et al., 1956; Robinow and Marak, 1966) its acceptance is only now becoming wide spread. It is interesting that such a structure, presumably composed of RNA-protein was not more easily recognized in Giemsa preparations. It has been our experience that Giemsa slides prepared from early times in the synchrony experiment show a crescent of slightly different color than the rest of the nucleus. This has also been reported by others (Nagel, 1946; Lindegren et

al., 1956; Yoneda, 1963). We have unexpectedly seen such a figure in azure A preparations (Figure 9). This raises the point of whether all these structures are the same structure. It would seem possible that they are not in that their orientation in respect to the vacuole is inconsistent. Perhaps the crescents seen in azure A preparations are early phases in the change of the nucleus to an elongated shape.

The Vesicular Stage during the division cycle is particularly interesting (Figures 5A and 10A). This was most often seen during the first division but other workers such as Yoneda (1963), Ferriera and Phaff (1959) and Delamater (1950) have reported similar figures in randomly dividing cultures. Such a figure may also be similar to the hollow spindle of Levan (1946).

The bluish color of the large central areas seen in the cytoplasm of azure A and Giemsa preparations at a time eighty minutes after the start of the experiment is indicative of large amounts of RNA, possibly in the form of ribosomes (Figure 11B). Biochemical data show at this time the RNA content of the cell is increasing. The fact that such areas resisted hydrolysis and sodium chloride extraction further supports that the RNA is in bound form and these dark areas may be identical to the "chromatin" seen by Lindegren et al. (1956) within the vacuole. A cytochemical study is needed to determine the composition of this material with certainty.

During actual transfer of the nucleus there is little detail to be seen (Figures 14A-B and 16C-D), but this might be expected if division had already taken place within the mother cell and only one nucleus was involved. The significance of the denser particulate bodies seen with Giemsa in some nuclei during the transfer process is unknown. If the previously mentioned dark rim in Giemsa preparations was in fact the nucleolus, this could be nucleolar material.

The demonstration of chromosomes in the yeast nucleus has chiefly involved the use of squashed preparations. Our experience with such preparations was limited, but we found it to be most successful when applied to the Diffuse Stage. It is difficult to accept the prescence of discrete chromosomes when the nucleus is diffuse and the DNA uncoiled to permit replication. Thus, it is thought that such figures do not really represent chromosomes but rather loops or extensions of nuclear material (Figures 10B-C and 15B-C), for even the counts reported by various authors vary greatly and are not entirely compatible with genetic data.

It is apparent that there are several points of difficulty in applying the term mitosis, in a classical sense, to asexual reproduction in yeast. First, the entire process occurs within the nuclear membrane while in mitosis the membrane is broken down at an early stage. The centrioles which determine the area of cleavage and the spindle fibres

connecting them with the chromosomes are lacking (although there is an intra-nuclear spindle of uncertain function). In fact there is even lack of conclusive evidence for the presence of chromosomes. Finally, there is a nucleolus but its position in the nucleus is quite different from that found in tissue cells.

Although many feel that the small size of the yeast nucleus is at fault for the lack of progress in revealing the divisional pattern, it is more likely that a lack of new techniques has been the primary problem. Although the literature is fairly extensive, it consists of a great deal of replication. Acid fuchsin staining, azure A staining and new methods of electron microscopy including glutaraldehyde fixation have recently given indications that resolution of the division process is imminent.

CHAPTER VI

SUMMARY

This study has shown that azure A has definite advantages over both Giemsa and Feulgen techniques for studying the yeast nucleus. It results in preparations which are stained deeply enough for easy observation and yet is specific in its action for deoxyribonucleic acid. Its application to synchronized cellular material has resulted in the delineation of a pattern for karyokinesis which receives considerable support from the literature. The result of combining this method with the biochemical data available on synchronized yeast cultures has lent even further support to the hypothesis.

The nucleus changes from compact to diffuse thereby facilitating the replication of DNA. Quite soon after duplication is completed, the two daughter chromatin bodies are separated while still remaining enclosed within a single membrane within the mother cell. One nucleus then migrates into the bud which is almost fully grown and the cycle is completed. During the process, the several successive stages of Compact, Elongated, Duplicated, Diamond-like, Divided Transfer and Compact can be distinguished.

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