STUDIES ON THE CYTOLOGY OF YEASTS.

V. Normal and Abnormal Mitoses in a Distillery Yeast.

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Contents.

Introduction.

The abnormal mitotic behaviour of a distillery yeast (Subramaniam and Ranganathan, 1946*a*, *b*) raised many problems of a fundamental nature. It was thought, therefore, that an extended and complete study of the mitotic cycle, if presented in the proper perspective, would be highly interesting. It has always been the custom to try to correlate and deduce abnormal behaviour of chromosomes in organisms, tissues or cells from normal patterns observed in innumerable plants and animals. The rapid advances in Cytology are only confirming the belief that there is some fundamental plan in the behaviour of chromosomes during mitosis and meiosis and that the different patterns in different cells when analysed resolve themselves into innumerable variations of a few basic procedures. After all, when widely different types of animals and plants like grasshoppers, salamanders, mice and lilies resemble each other in mitotic and meiotic phenomena, the above conclusion is inescapable.

The distillery yeast which forms the subject-matter of this paper shows heterotypical division of nuclei with the resultant formation of monosomic, trisomic and tetrasomic forms, which incidentally are also triploids, pentaploids and hexaploids, since the basic chromosome number in yeasts appears to be two (Subramaniam, 1946). In addition, as in the diploid brewery yeast, the tetraploid distillery yeast also shows somatic pairing during mitosis.

But before the relevant literature on the above problems could be cited, it appears necessary to review in detail the literature on the behaviour of the nucleus during cell division in yeasts.

Previous Work on the Cytology of Yeasts.

The Question of Identification of a Nucleus in Yeasts.—In a previous paper (Subramaniam, 1948*a*) it was emphasised that identification of nuclei in yeasts should be based on (1) a demonstration of mitosis and (2) a positive Feulgen reaction. Since the latter was introduced only in the year 1924, we can evaluate the earlier work on yeasts only on the basis of the first of these criteria.

The growth of our knowledge of the cytology of yeasts could be analysed under two distinct heads. They are: (1) identification of a particular organelle in the yeast cell as the nucleus and (2) the behaviour of the so-called 'nucleus' during budding and spore formation.

Earlier workers identified a homogeneous body in stained preparations of healthy yeast cells as the nucleus. It was suggested by Wager in 1898 that the homogeneous body is really the nucleolus. The 'vacuole' usually seen in close contact with the above and showing some granular contents was identified as the nucleus. The nucleus of yeast was thus conceived to be a compound body with the 'nucleolus' attached to one side of the nuclear vacuole and lying in the cytoplasm. The 'nucleolus' often showed granular contents and, according to Wager, its remarkable resemblance in such stages to granular nuclei may have misled the earlier workers to identify it as a nucleus.

Wager's interpretation, however, was not received with any enthusiasm by the other workers. Hoffmeister (1900) considered the 'nucleolus' of Wager to be the real nucleus and Guilliermond (1902, 1904) disputed Wager's identification on the following considerations: (1) The 'nucleolus' is not found in all cells in close association with the 'vacuole', (2) the 'nucleolus' itself, in well-stained preparations and in certain circumstances shows all the complements of a nucleus, viz. a membrane, a nucleoplasm, a network and occasionally a nucleolus, and (3) the granules inside the vacuole are not 'chromatin' but a reserve material of the nature of 'metachromatin'. The investigations of other workers like Barker (1902), Feinberg (1902), Hirschbruch (1902) and Marpmann (1902) concern the identification of the 'nucleolus' of Wager as the real nucleus and a discussion as to the minor details of its structure. Barker (1902) showed not only that the homogeneous body sometimes showed a granular structure, but that numerous vacuoles may occur in the same cell, one or all of which may show deeply staining granules. He saw such granules in the cytoplasm also near the 'nucleolus' of Wager.

Janssens (1902) suggested that the 'nucleolus' is always inside the nuclear vacuole and not outside it as suggested by Wager, and that its peculiar disposition observed by Wager is a fixation artefact. Hirschbruch saw a clear area around the homogeneous body but disagreed with Janssens and Leblanc (1898) that since the clear zone was not delimited from the cytoplasm by any membrane, it could not be considered a part of the nucleus. Feinberg (1902) could not observe the clear zone around the deeply staining body and hence was sceptical about its presence at all. He found certain granules inside the 'nucleus' which he thought corresponded to the nucleoli of *Amoeba* and *Sporozoa*. Kohl (1908) describes the nucleus as having a membrane, a fine chromatin net and a crystalloid in the nuclear sap.

As was shown in a previous paper (Subramaniam, 1948*b*), two different structures, viz. 'nuclear vacuole' and 'secretory vacuole', appear to have been confused by the earlier workers.

The work of Macallum (1899) could be made a convenient starting point for a critical analysis of the problem of identification of a nucleus in yeasts. He found in the cells vacuoles and granules. In early stages of fermentation, the granules

were blackened by the osmic acid in Flemming's fluid showing the presence of a lipoidal component. During the progress of fermentation this lipoidal component disappears from the granules which have a purely proteid constitution in later stages. Basing his observations on haematoxylin stained preparations, he suggests that since the cytoplasm during some stages, and granules during different stages, showed an affinity for the stain, the reactions may be attributed to the presence of chromatin. He was not prepared to identify the 'nucleolus' of Wager as either a nucleus or a nucleolus, since in his preparations he found cells entirely lacking such a structure or having more than one. Since the corpuscle ('nucleolus' of Wager) and granules in the vacuoles and cytoplasm showed the presence of masked organic phosphorus and iron, he was reasonably justified in considering them as chromatin in view of the limited knowledge then available regarding the chemistry of chromatin.

Macallum (1899), Guilliermond (1902, 1904 and 1910), Kohl (1908) and Wager and Peniston (1910) agree that the structure identified by them as the 'nucleus' of the yeast cell divides by amitosis. The descriptions, however, differ in minor details of the process. Even in this, there is remarkable agreement in the fact that the homogeneous body identified by Wager and Peniston as the 'nucleolus' and by Guilliermond (1910) as the 'nucleus' plays a prominent rôle. Macallum described the corpuscles elongating and constricting in the middle during budding and one or both products of division passing on to the bud. Guilliermond's description agrees with the above, while Wager and Peniston (1910) consider that the division of the 'nucleolus' is followed by that of the 'vacuole', and that one of the products of division of the 'nucleolus' along with a 'vacuole' containing chromatin granules passes into the bud.

As regards the cytological phenomena during sporulation also, there appears to be considerable divergence of opinion. Macallum (1899) described the cytoplasmic granules collecting near the 'corpuscle', which during the process showed also changes in its granular contents. The corpuscle then elongates and gets constricted in the middle. The above changes were compared by Macallum to the division of the nucleus in *Euglena* in view of the then belief that in *Euglena* as in other Protozoa, the division of the nucleus was by amitosis.

Wager and Peniston's (1910) descriptions follow that of Macallum. They found the 'nuclear vacuole' disappearing before the division of the 'nucleolus' and the surrounding chromatin granules into two equal portions. These divide again and sporogenous cytoplasm is said to accumulate around them as a prelude to transformation into spores. Guilliermond (1910), on the other hand, asserts that mitosis is the rule during spore formation.

In spite of the above apparent unanimity of opinion as regards the occurrence of amitosis during budding, this opinion was not shared by a minority even during the beginning years of the present century. Janssens and Leblanc (1898) and Janssens (1902), who perhaps were the first to recognise the nucleus of the yeast, claimed that its division is a simplified form of mitosis. Swellengrebel (1905) from his investigations on a compressed yeast confirmed Guilliermond's identification of the nucleus, but found the nuclear division to be mitotic. He observed four chromosomes arranged at the equator of a spindle, which later separated into two daughter groups passing to opposite poles. Swellengrebel, therefore suggested that the division of the yeast nucleus is reminiscent of that of the micronucleus in *Paramoecium*.

Fuhrmann (1906) arrives at a similar conclusion and his paper is well illustrated. He describes the resting nucleus as a small structure exhibiting a granular structure. Often, he could distinguish a large granule which he suggests may be the nucleolus. He could follow the various stages of mitosis and found not only the four chromosomes and spindle, but even structures which in all probability are the centrosomes.

Guilliermond (1910) claims to have observed some figures suggestive of mitosis in *Hansenula* (*Villia*) *saturnus*, but considers it to be an exception than the general

rule. On the above basis, he tries to explain away the observations of Swellengrebel and Fuhrmann as erroneous. Both Swellengrebel and Fuhrmann found the division of the nucleus preceding bud formation. Guilliermond, though admitting the independence of the two related phenomena, is not willing to admit the precedence of the nuclear phenomena to bud formation. On the above assumption he tries to suggest that the stages of mitosis seen by the two investigators are merely demonstrations of the variable structure of the nuclei which present at different stages different dispositions of the chromatin network.

Kater in 1927 investigated the cytological phenomena during budding and is emphatic that it is mitotic. He traces the various stages of the process and suggests that the chromosome number is probably eight.

Guilliermond (1940), it appears, saw some figures suggestive of mitosis in *Saccharomyces paradoxus*. From his investigations on *Saccharomyces ellipsoideus*, Renaud (1938) is emphatic that one cannot dream of counting the number of chromosomes. Still he asserts that it is not four as suggested by Fuhrmann (1906) but is in all probability eight. It is surprising to find a controversy as to the number of chromosomes characteristic of the different species. *It appears as if the workers* (Renaud, 1938; Levan, 1946) *have ignored altogether the question of polyploidy in yeasts.* Once existence of polyploidy is admitted, then, all the records of chromosome numbers in yeasts automatically arrange themselves as multiples of two (Subramaniam and Ranganathan, 1945*b*).

All the above investigations were carried out without any confirmation with the Feulgen technique. Under the circumstances, we have only one of the criteria to evaluate the validity of the identification of a particular structure in the yeast cell as the nucleus. *If we rigidly apply the definition that the 'nucleus is a cell body which arises or reproduces by mitosis'* (Darlington, 1937), *then the structures* claimed as 'nuclei' by Wager and Peniston (1910), Hoffmeister (1900), Feinberg (1902), Hirschbruch (1902), Marpmann (1902) and Kohl (1908), *do not satisfy that definition.*

The claims of Janssens and Leblanc (1898), Janssens (1902), Swellengrebel (1905), Fuhrmann (1906) and Kater (1927) appear to be valid, while Guilliermond's and Renaud's identifications may probably be considered as correct, even though, while admitting that division is by mitosis, they could not count the number of chromosomes.

The technique of handling yeasts for cytological investigations has already been described elsewhere (Subramaniam, 1948*a*).

Observations.

(*a*) *Carnoy-Iron-Haematoxylin Preparations.*

In two preliminary notes (Subramaniam and Ranganathan, 1946*a*, *b*) it was shown that the distillery yeast, which is the subject-matter of this paper, shows mitotic aberrations. Since the cells of the strain *SC 9* have only two chromosomes (Subramaniam, 1946), the distillery yeast which has four was taken to be a tetraploid. This regularly produces certain percentages of diploids, triploids, pentaploids and hexaploids. However, in order to interpret the structures seen during the pro- and metaphase stages, one has to keep in mind the fact that along with the cells having the typical tetraploid constitution, one may expect the other chromosomal mutants also to appear.

The changes in the nucleus become evident 40 minutes after changing the wort. In smears made between 40 and 50 minutes, the prophasic changes could be followed step by step. In Fig. 1 is shown a resting cell with a nucleus having a rhomboidal shape. No stained granule is seen in the cytoplasm or the nucleus and the nucleus itself appears as a clear area. A small granule appears inside the nucleus (Fig. 2) and it begins to increase in size (Figs. 2, 4 and 5). The nucleus also increases in size.

Pictures suggestive of the 'nuclear vacuole' of Wager and Peniston (1910) may occasionally be seen (Fig. 6). But their homologies are different. What is seen is an artefact resulting from the plastering of the chromatin mass to the nuclear membrane and distortion of the nucleus itself. Janssens (1902) suggested a similar explanation, but he appears to have been under the impression that the homologies of the structures seen by him were identical with that of Wager (1898). The chromatin mass shows signs of quadripartite division in Fig. 7, while in Fig. 8 it appears as two irregularly shaped unequal masses. Two masses of equal size may be seen plastered on the nuclear membrane (Fig. 9). The central chromatin mass shows signs of a bipartite division (Fig. 10) and the prophasic contraction of the two chromosomes of the diploid are seen in Figs. 11, 12 and 13. The two unequal and irregular masses shown in Fig. 8 seem to give rise to the two unequal rods shown in Fig. 14. By contraction of both and division of the longer one, the late prophase of the triploid (Fig. 15) appears to come into being, while the contraction and separation of the quartets in the quadripartite mass (Fig. 7) gives rise to the identical stage (Fig. 16) of the tetraploid. Fig. 17 shows the characteristic arrangements of the four chromosomes at metaphase, while in Fig. 18 each of the four chromosomes is double. A perusal of Figs. 17 and 18 would show that not only do the chromosomes show a 'somatic pairing', but that each chromosome presents also a duplex structure. The chromosomes have a vesicular shape and have a chromophilic rind and a chromophobic interior.

(*b*) *Feulgen Preparations.*

Similar stages could be seen in Feulgen preparations. Since there was the possibility that the stained granules inside the nucleus may be the 'heterochromatin' persisting in the resting nuclei and since the possibility that its absence in the cell in Fig. 1 may be due to difficulties in staining, Feulgen preparations of growing cells between 20 and 25 minutes after changing the medium were carefully studied. In such preparations there occur only a small percentage of cells showing any positively stained structures. Fig. 19 shows a cell in which the nucleus could be distinguished as a slightly deeply stained area. The deeper staining is by light green, the nucleus contains no Feulgen stained granule, and is comparable to the stage illustrated in Fig. 1. The resting nuclei, therefore, apparently do not contain chromocentres and this seems to be confirmed by the absence of any stained structure in the nuclei immediately after completion of the telophasic changes (Figs. 40, 45 and 52). The stained granule appears, therefore, to be the fused heteropycnotic regions of chromosomes. In Fig. 20 is shown a granule similar to that seen in Fig. 2. The granule itself is surrounded by a clear space, and the area of the nucleus could be made out only by its slightly deeper staining with light green. As in iron-haematoxylin preparations (Figs. 2, 3, 4 and 8) in Feulgen slides also (Figs. 19, 20 and 21), an increase in the volume of the nucleus is followed later by a progressive reduction. The granule increases gradually in size (Figs. 20, 21 and 22) but there appears to be a change in the staining reactions of the nucleus. In Fig. 21 the nucleus is distinguished from the cytoplasm by its deeper shade of staining and the Feulgen positive grain appears surrounded by a halo. In Fig. 22, on the other hand, the nucleus itself appears as an unstained area containing the chromatin grain.

Metaphase stages occur in large numbers in preparations made between 50 and 55 minutes. But as pointed out elsewhere (Subramaniam, 1948*a*), other stages would also appear in such preparations in small percentages. Apart from the fact that the division of a small percentage of cells does not synchronise with that of the majority, there are also precocious ones as well as laggards. Thus, examination of any slide would show small percentages of earlier and later stages than the one seen in the majority of the cells. The descriptions hereafter are of stages seen in smears made between 50 and 55 minutes. In Fig. 23 the chromatin mass appears

vesicular having a Feulgen positive rind and a Feulgen negative interior. This appears to divide first into two (Fig. 24) and then into four (Fig. 25). There is a marked difference not only in the structure of the early developing chromosomes but also in their size in the two preparations. Fig. 24 may as well be considered the mid-prophase of the diploid. A similar stage of the triploid and hexaploid may be seen in Figs. 26 and 27. It is very difficult to conclude whether the mid-prophase stage of the hexaploid shown in Fig. 27 is preceded by that illustrated in Fig. 26. The probability is there, as the structure of the chromophile bodies would indicate. It is also very difficult to interpret correctly the appearances seen in cells where the four chromosomes appear in groups of three and one (Fig. 28) and where two of the chromosomes are connected together by a thin thread of stained material (Fig. 29). The dispositions of the chromosomes in Fig. 30 is likewise capable of an interpretation as an anaphase of the diploid.

The typical metaphases of the diploid (Fig. 31), triploid (Fig. 32), pentaploid (Fig. 33) and the hexaploid (Fig. 34) show the characteristic 'somatic pairing'. Owing probably to technical difficulties, all the chromosomes or some of them do not show the characteristic duplex structure (Figs. 33, 40, 46 and 49). As has been shown by Subramaniam (1947*a*) it appears that the stained rind of the chromosome of the distillery yeast corresponds to the euchromatin identified in higher animals and plants and the Feulgen negative interior of the chromosomal vesicle to the heterochromatin. The problem of heterochromatin in yeasts would be dealt with briefly in the discussion.

The sizes of the chromosomes appear to be almost identical in the meta- and anaphase stages in both iron-haematoxylin and Feulgen preparations (Figs. 17, 18, 35, 37 and 38). It is *ca.* 0·75μ. The tetraploid metaphase (Fig. 35) is of short duration and is succeeded by the early anaphase in which eight chromatids appear scattered in the cytoplasm (Fig. 36). The chromatids begin to separate into two equal groups (Fig. 37) and one of them may reconstitute a nucleus even before a bud begins to develop (Fig. 38). Or, the separation continues and is completed only when one group reaches the cytoplasm of the bud (Fig. 39). In cells which contain a reconstituted nucleus and four chromosomes, the latter seem to migrate to the bud (Fig. 40). In such cases, as would be evident from the figure (Fig. 40), the reconstituted nucleus lapses into the resting stage even before the telophasic changes commence in the bud. Thus, cells show differences in the behaviour of the daughter sets of chromosomes. The telophasic changes of the daughter nuclei may or may not synchronise. These differences in the behaviour of the daughter groups of chromatids have already been described by Subramaniam (1946). In fact, Subramaniam confirmed what has already been recorded some four decades back by Swellengrebel (1905) and Fuhrmann (1906). One of the main grounds on which Guilliermond (1910) dismissed lightly the observations of the above authors was on the basis that they describe nuclear divisions as preceding bud formation. One fails to understand the validity of his criticism since he himself admits that the phenomena of bud formation and nuclear division need not show any synchronisation.

(c) *Mitotic Aberrations.*

The description up till now dealt mainly with the normal mode of division. A considerable percentage of cells show mitotic aberrations. It would be desirable, therefore, to begin the description from the early anaphase stage shown in Fig. 36. Instead of the chromatids separating into two groups of four each, they may separate into groups of unequal numbers. This separation may be seen even when bud formation has not commenced as in Fig. 41, where two of the chromosomes lie separated from the rest. When a bud does begin to develop, the number in the bud and mother cell does not get equalised. Only the separated group passes into

the bud (Figs. 42 and 46). It does not appear necessary that the mother cell should always have the set composed of the larger number of daughter chromosomes. The complement that migrates to the bud is apparently determined by its proximity to the developing bud. Thus, while in Figs. 42 and 46 the mother and bud contain 6 and 2 and 5 and 3 chromosomes respectively, in Figs. 43, 47 and 49 the sizes of the complement of chromosomes are reversed. As in normal divisions, the telophasic changes in these heteroploid daughter nuclei may or may not show any synchronisation. There appears to be only a slight difference in the timing of the telophasic changes in the two daughter nuclei in Fig. 50. In Figs. 43, 44 and 49 the changes in the nucleus of the mother cell is far advanced, while that of the bud has yet to begin and the extreme condition is seen in Fig. 45 where the nucleus of the mother cell has lapsed into the resting stage while the chromosomes still remain discrete in the bud. The telophasic changes appear to be a reversal of those leading up to the metaphase. The chromosomes begin to lose their affinity for stains while remaining as discrete bodies (Fig. 43), or they may fuse in pairs (Fig. 43), the fused pairs may remain in close apposition (Fig. 48), or they may unite into a single mass (Fig. 50). The identical type of telophasic change may occur in the mother and bud (Fig. 50) or the mother cell may show one type and the bud another (Fig. 48). From Fig. 50 one could surmise that even after fusion of the Feulgen negative heterochromatic regions, the fused Feulgen positive euchromatic regions still furnish the outer rind. The irregularly shaped body formed as a result of the fusion of the chromosomes (Fig. 50) undergoes a progressive reduction in size (Figs. 51 and 52) until just prior to its disappearance from view, it may appear as a small granule surrounded by a clear area. The resting nucleus so formed is very refractory to stains and could be made out only in overstained cells. The progressive reduction in size of the fused chromosome mass and its final disappearance in stained preparations suggest that resting nuclei do not contain a chromocentre.

Discussion.

(*a*) *The Results of Feulgen Reaction in Yeasts.*

It appears that the only cytological method which differentiates the desoxyribose nucleic acid from the ribose type is Feulgen's nucleal reaction (Mirsky, 1943). While chromatin could be selectively stained by the Feulgen technique, this is not possible by ordinary staining procedures. Positive Feulgen reaction in yeasts has been observed by Voit, Jiroweck, Neumann, Peter and Imsenecki (1936 for previous references), while Margolena (1932), Rochlin (1933) and Robinow (1942) demonstrate Feulgen positive nuclei in yeasts. Delaporte and Roukhelman (1938) and Delaporte (1939) extracted ribose nucleic acid from the cytoplasm and desoxyribose nucleic acid from the nucleus of the yeast.

Badian (1937) claimed a demonstration of chromosomes in yeast by the Feulgen technique, but his views are vitiated by his attempt to compare the cytological behaviour of yeasts with that of bacteria. Yeasts are products of degenerative evolution from higher Fungi and hence such an approach does not appear justifiable. Owing probably to the above angle of approach his descriptions appear unconventional.

He also stained osmic fixed cells by a modified Giemsa method and found that during budding the two chromosomes divide longitudinally. The pairs of chromatids separate and serious objection has been voiced (Lindegren, 1945*a*, *b*) against his description and figures of their final separation, which appears to resemble a crude transverse fission. If one considers the chromosomes to be acrocentric, the objections automatically lose their validity. A separation of this type has been demonstrated in Protozoa. 'The chromosomes lying parallel to the axis of the division figure may break in two at the middle, but it has been shown by Hall and others

that in the prophase they are split and open out from one end, the supposed transverse division being merely the completion of the separation at the other end' (Sharp, 1934, p. 190). But Badian's observation that the spore chromosomes fuse end to end in the zygote, and that the haplophase and diplophase chromosome numbers are identical, requires confirmation before acceptance. The technical difficulties involved in investigations of such stages are enormous and until clear-cut confirmation is available, the above unconventional description can only be considered *sub judice*.

Thomas (1945) investigated the cytology of the giant strain of *Torulopsis utilis* produced by Thaysen and Morris (1943) by camphor treatment, and found that the nucleolus was four times larger in volume than that of the control strain. Both the control and the giant cells showed, however, only two chromosomes, which in the latter was 'greater in size'. One would have thought that while dealing with a controversial subject like the identification of chromosomes in yeasts, and especially when the observations are cited as evidence for some views on the causes of malignancy, Thomas would have supported his identification by demonstration of anaphase stages in the control as well as the mutant strains. Lacking such a demonstration his claim that the change induced by camphor on yeast 'although genetic, is probably not due to change in the chromosomes but rather to one in cytoplasmic determinants' does not appear justifiable. The statement that the chromosomes of the giant strain contain an increased nucleic acid charge is not explained by the suggested change in the cytoplasmic determinants in yeast induced by camphor. Biesele (1944*a*, *b*) states that the greater the size of the chromosomes in a tissue of the rat, the greater is the concentration of B-vitamins—with the exception of inositol—and that the chromosome size offers an indication of their synthetic activity in particular tissues of the rat. Are gene mutations the cause for an increase in chromosome size in the giant strain of *Torulopsis*? From a study of two rat neoplasms, Biesele (1944*b*) comes to the conclusion that the chromosome size in such cells are either the same as in rat organs with poor B-vitamin content, or double or four times as large. In such cells diploid metaphases far exceeded the number of polyploid ones. Since, however, a greater percentage of resting nuclei showed more plasmosomes than the regular diploid complement, it was considered that 'the chromosomes of double and quadruple size in the two neoplasms are probably composed of more discrete strands than is true in normal tissues.' The increase in volume of the nucleolus four-fold in the giant strain of *Torulopsis* suggests either that difficulties in handling did not enable the separation of the chromosomes or that the chromosomes are polytene.

Levan and Sandwall (1943) suggested without any cytological confirmation that since Bauch could isolate giant strains after camphor treatment of yeasts, the probability of a chromosome doubling by endomitosis in such cells was worth consideration. However, Levan in a recent contribution (1946) seems to have obtained no confirmation for the above suggestion but found mitotic irregularities when yeast cells were treated with camphor, butyl alcohol and benzene. Without presenting a careful description of the mitotic cycle in the control he suggests that these aberrations afford opportunities for estimating the chromosome size in yeasts. His illustrations when compared with ours suggest that his technique of handling is yet inadequate and appears capable of enormous improvement. The above may explain the tendency of the anaphase chromosomes to stick together. His generalisations on the basic chromosome number in yeasts appear, therefore, to be premature especially since he has studied only one strain.

Levan and Sandwall (1943) concede that camphor has to be classified along with colchicine and acenaphthene. Subramaniam (1947*a*) has recently demonstrated that a short treatment with acenaphthene of actively growing cultures of the two chromosome strain *BY 1* gives cells showing not only variable chromosome numbers but even inequality in the size of the chromosomes. But most of them

appear to be unbalanced since only a tetraploid and a mutant could be isolated after prolonged treatment. Two years ago we suggested (Subramaniam and Ranganathan, 1945*b*) that it would be possible to produce a tetraploid or octoploid by controlling the time of treatment and that the measurements of the chromosomes of the induced tetraploids need not agree with those of the diploids. It was also pointed out that 'in the light of the above discovery it appears probable that different observers have been investigating different races passing under the name of *S. cerevisiae*'. Levan (1946) has not adduced any evidence necessitating a change of our above suggestion. Even for an estimation of the sizes of the chromosomes, yeast cells undergoing treatment with chemicals are least suitable, since vigorous growth has been noticed in higher plants on return to normal environment. This has been the experience of Subramaniam (1947*a*) also in yeasts. Ostergren (1944) states: 'Colchicine and other C-mitotic substances are able to induce an extra contraction of the chromosomes. Often, they contract to nearly half the length of the normal metaphase chromosome' (p. 433). Under the circumstances, it is surprising to find Levan (1946) suggesting that mitotic disturbances observed in cells undergoing treatment with chemicals 'furnish an opportunity to estimate the size of single yeast chromosomes'. The sizes of the chromosomes, said to vary from 0·1μ to 0·5μ by Levan, can have, therefore, no relation to the actual size in normal cells. For a study of the chromosome size based on mitotic abnormalities, the distillery yeast offers ideal scope, since such abnormalities occur under normal conditions.

(*b*) *Causes for Confusion.*

It would be admitted that criticisms questioning the validity of certain identifications should necessarily be substantiated by evidence as to probable causes which may have led previous investigators in the wrong direction. For this, a brief résumé of the recent and very rapid advances in our knowledge of the rôle of nucleoproteins in heredity and cell metabolism appears necessary (Caspersson and Santesson, 1942; Darlington, 1942). Two types of nucleic acids occur in the cell and these are the desoxyribose type found only in the nucleus and particularly in the chromosomes, and the ribose type found in the nucleoli and the cytoplasm. The ribose type alone is said to be enzymatically active. During the prophase stages the protein fibres of the chromosomes get charged with the desoxyribose nucleic acid which is lost as soon as the daughter nuclei assume their resting condition. In the chromosomes themselves, the chromatin is composed of two distinct types, euchromatin carrying the major genes and heterochromatin carrying the 'polygenes' (Mather, 1944). These two types show differential staining behaviour. In many types of resting nuclei the nucleic acid is retained in the heterochromatic regions while the rest of the chromosomes lose their nucleic acid coats. In many plants and animals undergoing mitosis at low temperatures (Darlington and La Cour, 1940; Callan, 1942) the heterochromatic regions appear unstained. In actively growing and secreting cells the nuclei swell and a nucleolus composed of ribonucleoproteins makes its appearance. From his investigations, Caspersson (1941) suggests that while the higher proteins are synthesised by the euchromatin, the heterochromatin is responsible for the production of simple proteins like histones, which reaching the cytoplasm from the nuclei are responsible for the production of ribonucleic acid and proteins in the cytoplasm (Caspersson, 1947).

Thus heavy concentrations of ribonucleic acid are found in actively secreting and growing cells and in neoplasms. The nucleoli appear to be the main storehouses for this type of nucleic acid since they have been shown to contain considerable amounts in cells of the embryo sac which appear to be used up largely in the formation of the desoxyribose type of the nuclei of the developing embryos.

Judged from the above background, the positive reactions for organic phosphorus and masked iron exhibited by the corpuscle as well as the granules occurring

not only in the 'vacuole' but also in the cytoplasm of the yeast (Macallum, 1899; Wager and Peniston, 1910) have little significance since the two types of nucleic acid could only be distinguished by a Feulgen test. Wager and Peniston's description of the nuclear division during budding and their terminology appear strange to modern students of cytology. Since during spore formation, the 'nuclear vacuole' disappears and the daughter nuclei are the result of division of the 'nucleolus' and the 'chromatin grains' associated with it, these structures are not what they are claimed to be. The nucleoli, at least in higher plants, dissolve and disappear in the cytoplasm during the early prophase stages. The possibility of their 'nucleoli' being the real nucleus is there since it gives a Feulgen reaction (Lindegren, 1945*a*, *b*), while the vacuole does not form a portion of the nucleus at all. It appears rather strange, therefore, to see a revival of the ideas of Wager and Peniston (1910) and an attempt to re-interpret their 'nucleolus' as the 'centriole' and the vacuole as the 'nucleus' (Lindegren, 1945*a*, *b*). The significance of the *nuclear vacuole* of Wager and Peniston has already been discussed in a previous publication (Subramaniam, 1948*b*).

The selection of fermenting cells alone for cytological investigations has led to rejection, as artefacts, of pictures of mitotic division observed occasionally. Fuhrmann (1906) says that he used only young cultures, 20 to 24 hours old, which were grown on a nutrient medium containing 10% gelatin, 2% peptone, 1% grape sugar and 0·5% potassium chloride in his investigations. Without studying cells at identical stages of growth on media of identical composition used by Fuhrmann, Wager and Peniston (1910) remark: 'It is not uncommon for a new centre to arise at the side of the nucleolus opposite to the original patch, bringing about an appearance which strongly resembles the anaphase of a typical mitotic division (Figs. 55 and 60) and has evidently been mistaken for such by Swellengrebel (1905) and Fuhrmann (1906).' When it is realised that such pictures were seen only in cells taken either from the foam or after a limited period of growth in dilute wort, where in all probability the cells were growing aerobically, and when optimum conditions for aerobic growth, as in the manufacture of compressed yeast (Walter, 1941), are considered to be good aeration and dilute wort, the value of Wager and Peniston's criticisms could just be imagined.

(*c*) *Heterochromatin.*

The problem of heterochromatin in yeasts was recently discussed by Subramaniam (1947*a*) who adduced evidence of a change of euchromatin into heterochromatin and pointed out that the chromophobic core of the chromosomes correspond to the heterochromatin of higher plants and animals. Further, it was suggested that the peculiar position of the heterochromatin militated in no way against its identification.

In the distillery yeast also the chromosomes show a chromophilic cortex and a chromophobic interior. Further, some portions of the chromosomes are heteropycnotic in the early stages.

If we conceive that growth of the chromocentre shown in Fig. 2 is centrifugal, then this identical region in the chromosome shows a reversal of staining reaction, for they form the Feulgen negative core of the chromosomes. In telophase (Fig. 50) the mass formed by the fusion of the chromosomes shows a duplex structure and exhibits also a reversal of the staining reaction in that it becomes granular (Fig. 50 bud, Fig. 44) before losing its staining capacity altogether. In the mitosis of the diploid strain of the brewery yeast, Subramaniam (1946) describes and figures (his Fig. 4) a chromatin grain comparable to that illustrated in Fig. 2 of the present paper. Since the chromosomes during meta- and anaphase stages did not show any duplex structure and since such a duplex structure was evident in the chromosomes

when they were duplicated in the brewery strain *SC 9*, Subramaniam (1947*a*) appears to have concluded that some of the euchromatic genes have become heterochromatic.

There appears to be still some confusion regarding the criteria on which a particular portion of a chromosome could be identified as 'heterochromatin'. Darlington's (Mather, 1944) suggestion that heterochromatic segments of chromosomes 'are liable to remain charged with thymonucleotides in the resting stage' does not appear to be of universal application since Manton (1935) classified nuclei into two types: 'prochromosomal' and 'solid', and states that in the latter the reticulum may or may not show chromocentres (p. 538). Heitz (1928), on the other hand, distinguished heterochromatin by its differential staining capacity. Heteropycnosis, while characteristic of sex chromosomes at certain stages in Orthoptera and Mammalia, cannot also be depended on since Darlington and La Cour (1940) and Callan (1942) located the differential segments in autosomes only in material undergoing mitosis or meiosis at low temperatures. Reversal of heteropycnosis is not also an unalterable characteristic, for White (1945) says that heteropycnosis itself is of two kinds, 'a kind that undergoes reversal in the course of spermatogenesis and a kind that does not' (p. 27). The above renders Darlington and La Cour's definition of heterochromatin, as those segments of chromosomes 'which have the property of failing to maintain the maximum nucleic acid cycle at mitosis', also unsatisfactory. It appears, therefore, that a number of factors have to be taken into consideration in identifying and locating heterochromatin in chromosomes. It is not surprising, therefore, that Mather (1944) comes to the conclusion: 'The failure to see heterochromatin may not be an indication of its absence.'

In yeasts volutin grains were considered by Caspersson and Brandt (1941) to correspond to the heterochromatin of higher organisms. Lindegren (1945*a*, *b*) after identifying the 'nucleolus' of Wager and Peniston (1910) as the 'centriole' has presented a further unconventional claim (Lindegren, 1947) that 'the desoxyribose nucleoprotein in the centriole is the equivalent of the heterochromatin in cells of higher organisms'. It was shown in a previous paper (Ranganathan and Subramaniam, 1947) that the 'centriole' described by Lindegren does not satisfy the criteria on which such structures are identified in animal cells. The criteria on which heterochromatin is identified in animals and plants discussed above makes any further comment on Lindegren's claim unnecessary.

There are no chances for confusion in the identification of heterochromatin in the chromosomes of the distillery yeast. These show (1) heteropycnosis at early prophase, (2) reversal of staining during late prophase, and (3) another reversal of staining during the telophase.

While Subramaniam (1947*a*) has been able to induce polyploidy in a brewery yeast, the distillery yeast is a naturally occurring polyploid. The similarity in structure of the chromosomes in these two polyploid strains is rather striking.

(*d*) *Mutations in Yeasts.*

Mutations appear to be as common in yeasts as in higher plants and animals. Owing to the rapidity of their life cycle yeasts offer unique opportunities for location and identification of mutations. As Winge (1944) remarks, experiments extending for a few weeks may not reveal these mutations, but if the experiments are continued over a long period of time, or if the same strain derived from a single spore or cell is studied after the lapse of a year or two, then the extensive nature of the mutational changes in yeasts become only too evident. While mutants may be easily overlooked in liquid cultures, the various types could easily be located in giant colonies, where they form 'sectors'. The changes in the cultures left standing for two years were so remarkable that Winge (1944) concludes that not only is it difficult to maintain the original culture unchanged over a period of time, but that it is even

difficult to conclude that sectors in such colonies, presenting an identical structure as the initial colony, have exactly the same hereditary complex.

As in higher plants one has to consider that mutations in yeasts may be of various types. These may be the result of mutations of genes, or due to loss or gain of either parts or entire chromosomes or due to polyploidy. In breweries and distilleries, the strains are always kept in an active condition and hence, while natural selection weeds out the less active ones, the vigorous types by their rapidity of division may completely replace in course of time even the parent strain. This may explain the constancy of the types employed in industry, where mutations apparently create no difficulties at all (Winge, 1944). Thus industrial types seem to have originated as a result of conscious as well as unconscious selection. The characteristics of industrial yeasts are that they have lost the power of spore formation. 'The property of being able to multiply by sporulation is of no practical importance to a brewer's yeast and brewer's bottom yeast will never in practice have access to such an amount of oxygen, and probably not even to such a high temperature as are required for spore formation. No wonder then that the capacity for sporulation is lost through mutation' (Winge, 1944, p. 92).

What is the nature of this mutation? Is it a gene or chromosomal mutation? It is known that sterility may be the result of hybridisation or polyploidy. According to Darlington (1937), a fertile diploid usually gives rise to a sterile polyploid and vice versa. Since we can eliminate the possibility of hybridisation, owing to the slender chances of such a probability under the conditions obtaining in breweries and distilleries, the question of polyploidy in yeast requires very serious consideration. It has been suggested that 'polyploidy favours the conditions of both diploid parthenogenesis (by suppression of meiosis) and apospory (by irregularity of meiosis)' (Darlington, 1937). A similar view is expressed by Sharp (1934) also.

It was discovered that there is a correlation between chromosome constitution and giant colony characteristics (Subramaniam and Ranganathan, 1948). The brewery yeast *SC 9* (Subramaniam, 1946) produces a giant colony showing folds on the surface, concentric striations at the periphery and a lobed margin. The auto-tetraploid produced by treatment with acenaphthene (Subramaniam, 1945) gives, on the other hand, a smooth giant colony having an almost uniform margin and showing only very faint striations. The strain employed in the cytological investigations presented in this paper, which is a distillery yeast, also produces a smooth colony showing only insignificant differences from the giant colony of the auto-tetraploid referred to above. The striking fact is that both are tetraploid. If on the basis of the above observation we conclude that polyploidy gives rise to smooth giant colonies, then it appears reasonable to conclude that *Saccharomyces unisporus* studied by Winge (1944), which forms a smooth giant colony, should also be polyploid. The giant colonies of single spores of the above strain were found by Winge to differ only slightly in appearance from colonies from single cells. This fact is in perfect consonance with the argument that the strain should be a polyploid one and its poor ability to form spores and 'direct diploidisation' only confirm the above impression. Thus even in published literature on yeasts evidences indicate that polyploidy is more common than is generally imagined (cf. Levan, 1946). Since yeast strains with a basic diploid set of two chromosomes are on record (Subramaniam, 1946), the distillery yeast strain used in the present investigations ought to be considered a naturally occurring tetraploid. The question is whether it is an auto- or an allo-tetraploid.

(*e*) *The Distillery Yeast an Auto-tetraploid.*

Since this yeast has been isolated from samples from distilleries, owing to the reasons adduced before, it appears likely that it is an auto-tetraploid. Evidence in favour of the above interpretation is afforded by the arrangement of chromosomes

in pairs during the various phases of mitosis. The 'somatic pairing' which has been described in various Diptera occurs in yeasts also. It is the homologous chromosomes that appear paired. Proof for such an assumption is afforded by Robertson's observations on a grasshopper, *Paratettix texanus*. 'In individuals produced by the union of two gametes in the usual way, the two parental chromosome sets tend to remain distinct in the developing tissues, whereas in individuals produced by parthenogenesis, the two sets (evidently formed here by chromosome division in the incomplete maturation of the egg) have a distinct paired arrangement' (Sharp, 1934, p. 126). Darlington (1937) mentions that during mitosis in polyploid plants 'somatic pairing' is conspicuous and that 'more than two chromosomes being attracted to one another, the groups lie radially instead of parallel' (p. 235). The arrangement of the chromosomes on the metaphase plate in the distillery yeast is suggestive of such an attraction between all the four chromosomes. It appears, therefore, that the distillery yeast is in all probability an auto-tetraploid.

(*f*) *Auto-tetraploidy, Somatic Pairing and Heteroploidy.*

Different types of association in different types of tissues in the metamorphosing *Culex* have recently been described by Grell (1946*a*, *b*). These associations range from mere close apposition without any visible contact of the homologous chromosomes of gonial and diploid divisions, each of which is composed of two chromatids, to the active somatic synapsis of the sister chromatids in the later divisions of the multiple complex cells. Grell (1946*b*) arrives at the conclusion that '(1) if chromosome reduplication occurs in the resting stage, active pairing stops and a process of unpairing begins; (2) if chromosome reduplication does not occur or is delayed, the active somatic pairing begun at anaphase continues into somatic synapsis in the one case and meiotic synapsis in the other' (p. 93).

In the brewery yeast (Subramaniam, 1946) and in the distillery yeast described in this paper, the chromosomes appear as a single chromatin mass which later separates into its component parts. Division into daughter chromosomes was seen only at metaphase in both these cases. It is true that such microscopic organisms as yeasts whose chromosomes have a size somewhere near the limit of visibility are least suited for any generalisation. But the fact remains that the suggestion of Grell that failure of reduplication of chromosomes during the resting stages leads to 'somatic synapsis' during the succeeding early prophase seems applicable to the case of yeasts also. Is it possible that this 'somatic synapsis' is followed by crossing-over in rare cases? Is it possible that the above is responsible for some of the occasional segregations seen in vegetative cells?

In *Culex* it appears that somatic pairing of 'sister chromosomes' gives place to pairing of chromatids and that attraction is always marked between the latest products. The attraction which becomes visible as the 'somatic pairing' should lapse during the metaphase, since the anaphase stages of somatic and gonial divisions in *Culex* show again an active association of sister chromosomes. A similar sequence of events seems also to take place in the normal division of the distillery yeast described in this paper. Normal separations, therefore, necessarily depend on a lapsing of the pairing force between the chromatids. If the timing of this loss does not synchronise in all chromatids or if there is a precocious resumption of the pairing force between sister chromosomes or groups of sister chromosomes, while separation into daughter groups is beginning, the result would be a mitotic aberration giving rise to daughter nuclei with different numbers of chromosomes.

It is only on this basis that the irregularities in mitosis in the distillery yeast could be interpreted. When even in extensively investigated auto-polyploids, the reason why four homologous chromosomes form either a quadrivalent, or a trivalent and a univalent, or two bivalents is not clear, any suggestion based on observations on yeast would be precarious.

Since genic mutations are as common in yeast as in other organisms, it appears likely that the presence of one or more such mutant genes in some of the chromosomes may explain the differences in the somatic pairing force between groups of chromatids resulting in irregularities of mitosis. It has been assumed (Sharp, 1934) that one of the main causes of heteroploidy is hybridisation. Such an assumption cannot be extended to the case of the distillery yeast under consideration. It appears that the main cause for mitotic aberrations in this yeast is the occurrence of auto-polyploidy and the resultant accentuation of the somatic pairing of homologous chromosomes.

Summary.

1. An extended and complete study of the normal and abnormal mitotic behaviour in a distillery yeast is presented.

2. Much of the confusion in earlier literature could be traced to the fact that observations were based solely on fermenting cells.

3. A critical analysis of the observations of earlier workers suggests that many of the claims of identification of a nucleus in yeast are not valid.

4. The various stages of division are described and illustrated from Carnoy-iron-haematoxylin, as well as from Feulgen preparations. Mitotic aberrations are common and give rise to nuclei with two and six and five and three chromosomes. The chromosomes during normal and abnormal mitoses show somatic pairing and exhibit a duplex structure.

5. Criteria for identification of heterochromatin in chromosomes are discussed and it is shown that there are no chances for confusion in the identification of heterochromatin in the chromosomes of the distillery yeast, since they show (i) heteropycnosis at early prophase, (ii) reversal of staining during late prophase, and (iii) another reversal of staining during the telophase.

6. The possibility of polyploidy in yeasts requires serious consideration, since many of the strains used in industry are sterile. Sterility may be the result of polyploidy or hybridisation. But since chances for hybridisation are very slender under the conditions obtaining in breweries and distilleries, auto-polyploidy appears highly probable. Evidence in favour of the above interpretation is afforded by the attraction between all the chromosomes in the distillery yeast.

7. There appears to be 'somatic synapsis' during early prophase stages. Normal separation of the chromatids necessarily depends on a lapsing of the pairing force during the metaphase. If the timing of this loss does not synchronise in all the chromatids, or if there is a precocious resumption of the pairing force between sister chromatids or groups of sister chromosomes, while separation into daughter groups is beginning, the result would be a mitotic aberration giving rise to daughter nuclei with different numbers of chromosomes.

8. Since genic mutations are as common in yeasts as in other organisms, it appears likely that the presence of one or more such mutant genes in some of the chromosomes may explain the differences in the pairing force between groups of chromatids resulting in irregularities of mitosis.

Acknowledgments.

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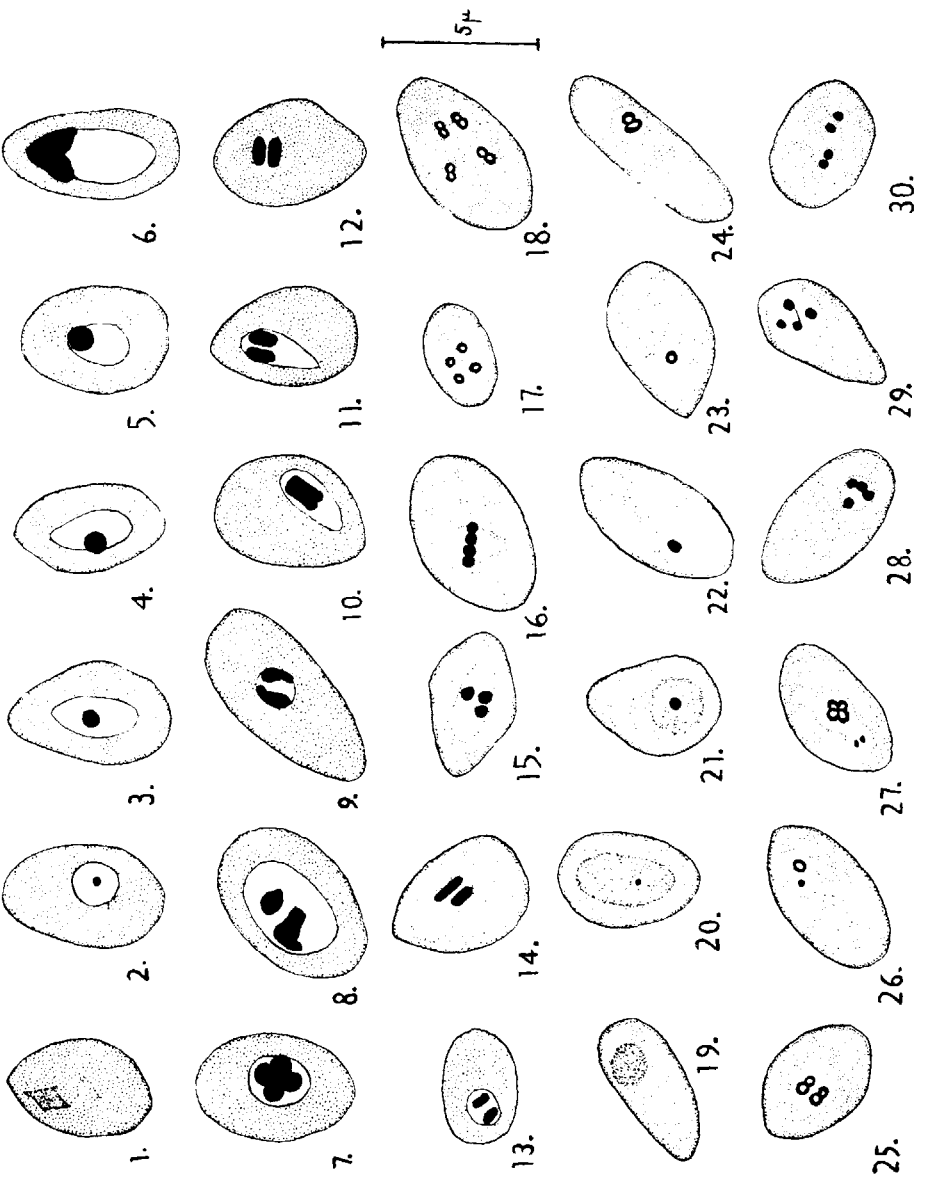
Description of Illustrations.

Figures 1 to 18 are from Carnoy-iron-haematoxylin and Figures 19 to 52 from Feulgen preparations.

Fig. 1. Resting cell showing the nucleus.
Figs. 2, 3, 4 and 5. Appearance of a stained granule inside the nucleus and its increase in size along with that of the nucleus.
Fig. 6. Picture suggestive of the nuclear vacuole and nucleolus of Wager and Peniston.
Fig. 7. Quadripartite division of the stained chromatin mass inside the nucleus.
Fig. 8. Two irregular chromatin masses inside the nucleus.
Fig. 9. Two chromatin masses plastered on to the nuclear membrane.
Fig. 10. Bipartite division of a chromatin mass.
Figs. 11, 12 and 13. Progressive decrease in size of the chromosomes of the diploid.
Fig. 14. Two rod-like masses of chromatin of unequal length.
Figs. 15 and 16. Early metaphase of the triploid and tetraploid respectively.
Fig. 17. Tetraploid metaphase.
Fig. 18. Each of the four chromosomes of the tetraploid appear double.
Fig. 19. Resting cell having a nucleus with no Feulgen positive bodies.
Figs. 20, 21 and 22. Increase in size of the Feulgen positive body inside the nucleus.
Fig. 23. Chromatin mass inside the nucleus showing a duplex structure.
Fig. 24. Bipartite division of the chromatin mass.
Fig. 25. Quadripartite division of chromatin.
Fig. 26. The chromatin appears as a grain and a vesicle inside the nucleus.
Fig. 27. A quadripartite chromatin mass and two chromatin grains inside the nucleus.
Fig. 28. Four chromosomes.
Fig. 29. Two of the chromosomes connected by a thin strand of stained material.
Fig. 30. Anaphase of diploid.
Fig. 31. Metaphase of diploid.

FIG. 32. Metaphase of the triploid.
FIG. 33. Metaphase of the pentaploid.
FIG. 34. Metaphase of the hexaploid.
FIG. 35. Metaphase of the tetraploid.
FIG. 36. Early anaphase of the tetraploid.
FIG. 37. Separation of the chromatids into two equal groups.
FIG. 38. Cell showing a reconstituted nucleus and four chromosomes.
FIG. 39. Mother and bud showing four chromosomes each.
FIG. 40. The nucleus in the mother cell has lapsed into the resting stage while the bud shows four chromosomes.
FIG. 41. Unequal separation of the eight chromatids forming two groups of two and six respectively.
FIG. 42. The bud shows two chromosomes while the mother cell has six.
FIG. 43. Early telophase in the mother cell while the chromosomes still remain discrete in the bud.
FIG. 44. The mother cell nucleus shows only a chromatin grain while in the bud the two chromosomes remain discrete.
FIG. 45. The bud shows two chromosomes while the nucleus in the mother cell has lapsed into the resting stage.
FIG. 46. The bud shows three chromosomes while the mother cell has five.
FIG. 47. The bud has five chromosomes while the mother cell has only three.
FIGS. 48, 49, 50, 51 and 52. Telophasic changes in the mother and bud.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