ON THE IDENTIFICATION OF THE VARIOUS STRUCTURES IN THE YEAST CELL

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Summary

1. The nucleus of yeast could be fixed and stained selectively. The vacuoles are neo-formations. The vacuolar colloids often condense into granules which are transitory. This phenomenon could be accentuated by vital staining.

2. The centrosomes are identified purely on their position and role in the organization of the achromatic spindle. Available evidence suggests that the centrioles are not Feulgen positive.

3. The structure and behaviour of the nucleus of differentiated cells of higher organisms are entirely different from those of embryonic cells. The radically different reactions occurring in yeast under aero- and anaerobiosis render it essential to treat the cytological pictures observed in these two phases separately.

4. Actively dividing yeast cells in well aerated media show a homogeneous cytoplasm with no vacuoles or granules. The complete mitotic cycle could be mapped out by smearing at regular intervals samples from aerobic cultures.

5. Photomicrographs are presented showing the two chromosomes orientated on a spindle which has at its poles the centrioles with centrospheres. The centrosomes are not found in all the cells.

Introduction

Identification of the various structures in the yeast cell is a very easy matter if the approach is on orthodox lines. Lindegren and Rafalko (1950) after disagreeing with the majority of yeast cytologists (p. 181) offer a novel interpretation of the structure of the yeast nucleus. Not only that. They try to reinterpret the observations of the other investigators. This has posed a very simple question. Are all the other investigators wrong in their identification of the various structures in the yeast cell? Is it possible to confuse the nucleus with the centrosome and the vacuole with the nucleus?

Lindegren and Rafalko attribute all this confusion to the supposedly original wrong identification of Guilliermond (1920) which they claim has been perpetuated because of his great prestige. Anyone conversant with the

extensive work of Guilliermond on the "Cytoplasm of the Plant Cell" (1941) would disagree with the above conclusion. We had reason to dispute Guilliermond's identification of the nucleus in yeast (Ranganathan and Subramaniam, 1948). It was emphasized that 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) and Hirschbruch (1902) do not satisfy that definition. Guilliermond's claim was disputed because he repeatedly asserted (1940) that the nucleus of the yeast divided by amitosis. To identify any structure as the nucleus, a demonstration of mitosis is an essential prerequisite. Mitosis is defined as the "separation of the identical halves of the split chromosomes into two identical groups from which two daughter nuclei are reconstituted" (Darlington, 1937, p. 22). The body identified as the nucleus by Lindegren and Rafalko (1950) and Winge (1935) does not answer to the above definition.

Mlle. Cassaigne (1931) investigated the origin of the vacuoles in the yeast cell and described their mode of division in some cells. Her illustration (Fig. 1 *a*) shows a striking similarity to the division of the so-called yeast nucleus (Fig. 1 *b*) described by Lindegren (1945). A perusal of the

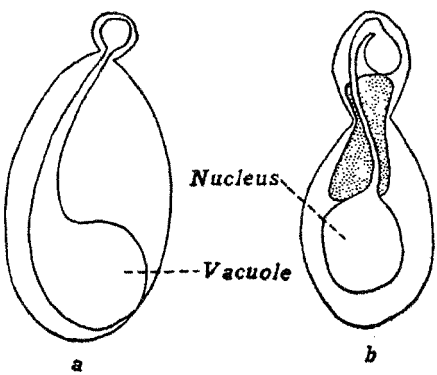

FIG. 1

figures shows that the same structure has been identified as the *vacuole* by Cassaigne and as the *nucleus* by Lindegren. Can such confusion be possible? For an elucidation of this question a clear idea of the general components of the cell and how they could be selectively stained is essential.

Historical Resumé

Cell Structures.—Apart from the nucleus, the bodies in the cytoplasm are classified into two categories. The Golgi apparatus and mitochondria are considered to be living inclusions while fat, yolk, plastids, volutin and vacuoles constitute the deutoplasm. If we fix *an yeast cell* in Carnoy's alcohol-chloroform-acetic acid mixture for one hour, the Golgi apparatus, the mitochondria, fat globules and plastids would not complicate the picture (*cf.* Table 737, p. 356, Gatenby and Cowdry, 1928). We are then left only with the nucleus, the vacuoles, the centrosome and instead of the yolk, the volutin or metachromatin. Of these, the vacuoles and the metachromatic granules in yeasts, are temporary structures (Caspersson and Brandt, 1941).

The Vacuoles.—Since the time of de Vries no one has questioned the existence of the vacuoles (Guilliermond, 1941). However, there has been no unanimity of opinion as to whether vacuoles originate from pre-existing ones or can be formed *de novo*. The majority veer to the view that it can arise *de novo*. Even those who believe that the vacuole is a permanent cell structure concede that in actively dividing meristematic cells, it has an entirely different appearance. It is only during the process of cell differentiation that the vacuole enlarges and becomes very conspicuous. In animals, on the other hand, there has been a controversy regarding the exact identification and role of the so-called "vacuome" (Parat, 1928; Gatenby, 1929, 1932; Ludford, 1930; Möllendorff, 1918).

That vacuoles may be formed as a reaction to an unfavourable environment was shown by me several years ago (Subramaniam, 1937). Thus vacuoles may be formed at specific stages during growth and differentiation and they also do appear as neo-formations when the environment is unfavourable. *The vacuolar contents differ from tissue to tissue and from plant to plant.* But whatever be the nature of the contents, the vacuoles have a definite wall (Chambers and Hofler, 1931). The vacuoles could be fixed and demonstrated with specific fixatives and stains. The necessity, however, for observations on living material has been emphasized by all investigators. In living cells, the colloidal contents of the vacuoles present different pictures at different stages. They may be homogeneous or there may develop in the vacuoles grains of different sizes. These grains are transitory. They may slowly disappear leaving the vacuolar contents once more homogeneous.

Vacuoles in Yeasts.—These changes could be accentuated by staining with vital dyes. It is a common observation that new granules originate in vacuoles when methylene blue is used as a vital stain. A host of investigators using a variety of vital dyes (Guilliermond, 1941) have observed the

same phenomenon. The results are much more clear-cut when "basic" dyes are employed. In *Saccharomycodes ludwigii*, Guilliermond (1941) describes the following changes. When a drop of neutral red solution is added: "it is observed that there are immediately produced in the vacuoles, a great number of granules strongly stained and showing Brownian movement. These are the result of precipitation of the vacuolar colloid through the action of neutral red. It sometimes happens that these precipitates, carried against the wall of the vacuole, pass through it and are deposited in the perivacuolar cytoplasm (Fig. 86) a phenomenon which is also caused by fixatives and which leads to errors of interpretation" (p. 136).

Another important observation of Guilliermond (1941) has to be taken into serious consideration. If the neutral red solution is more concentrated, "the granules quickly coalesce into a small number of large globules (sometimes into a single globule) which come to be closely appressed to the wall of the vacuole. Then they diminish little by little in volume and disappear, while the entire vacuole takes on a diffuse stain which later becomes more pronounced" (p. 137). Lindegren's original claim (1945) that the "*vacuole*" in the yeast cell is the "nucleus" is based on vital staining with methylene blue and toluidine blue. Though unaware of Guilliermond's investigations on identical lines, Lindegren has unwittingly confirmed Guilliermond's observations. Their interpretations of the same phenomena are different. While the movement of the smaller grains were described by Lindegren (1945) as reminiscent "of a flock of midges hanging in the summer air" (p. 774), the larger grains described by Guilliermond (1941) as coalescing during the progress of staining are considered by Lindegren (1945) to be the "nucleolus or the 'balled up' chromosomes (one cannot tell which)" moving sluggishly on the floor of the nuclear vacuole.

Guilliermond describes another property. The vacuoles in the living cells are capable of excreting the accumulated dye. This excretion is said to be pronounced especially when the cells begin to bud. From photometric measurements, Guilliermond claims that the loss of staining by the vacuoles is not the result of a conversion of the dye into a colourless derivative but is an actual excretion. The fact that Lindegren (1945) and Lindegren and Rafalko (1950) have not taken into consideration the possible occurrence of vacuoles in yeast analogous to those observed in plant cells suggests that the vacuole has been confused with the nucleus.

The Centrosomes.—In the decade preceding the present century, the centrosomes were also conceived to have a permanence in the cell organization. The centrosomes are identified by their definite role during mitosis.

When present, they form the poles of the achromatic figure. Schrader (1946) defines the "Center" as the "morphologically distinguishable body toward which the spindle elements are orientated" (p. 7). In many animals and some plants, "the center is composed of the spherical centrosome in the middle of which lies a minute body, the centriole". The important point to be emphasized is that the centrosome is identified from its position and role in the organization of the achromatic spindle. The identification of centrioles and centrospheres in resting cells is based merely on an extension of the study of dividing cells. But the body identified by Lindegren and Rafalko (1950) plays no role either in the organization or the orientation of the spindle. In fact, they observed no spindle at all; neither does the centrosome produce an aster.

A perusal of the early work of Morgan and McClendon (Wilson, 1904) on the artificial production of cytasters would prove that their claim that the centrosomes can arise *de novo* is supported by the appearance of an aster with one or more grains in its centre. The need for a serious consideration of the possible *de novo* origin of centrosomes is based on the artificial induction of asters with centrioles and their role in the organization of the multipolar spindle in abnormally cleaving eggs. The suggestion of Lindegren and Rafalko that the centrosomes of yeast can show Feulgen positive bodies under specific conditions appears strange when we trace the general conception of the staining property of the centrosome. Darlington (1937), Schrader (1946), Pollister (1939) and Pollister and Pollister (1943) have suggested the probability that the centrioles may originate from the centromere. The centromere itself is not uniformly Feulgen-positive in all organisms (Lima-De-Faria, 1949). Spontaneous origin or artificial induction of cytasters entails the belief that in such cases the centriole should have a purely cytoplasmic origin. It need hardly be stated that cytoplasmic structures do not show a positive Feulgen reaction.

The nucleus.—The criteria for the identification of the nucleus have been elaborately described elsewhere (Subramaniam, 1948 *a*). These are (1) demonstration of an anaphase and (2) a positive Feulgen reaction. A very elementary distinction is necessary here. The structure and behaviour of the nucleus of differentiated cells are entirely different from those of embryonic cells. In *Drosophila*, the salivary chromosomes are entirely different in appearance from those seen during the division of embryonic cells.

Geitler's (1939) observations indicate that endopolyploid nuclei themselves could be classified into certain major groups according to their structure. These distinctions are not made by investigators on yeasts. The yeast

nucleus is claimed to be unique because attention has been confined to purely fermenting cells. The behaviour of the nucleus in cells proliferating under aerobic conditions has not been taken into consideration at all. This confusion could be traced to the vague terminology current in yeast literature.

Aerobic and anaerobic phases of yeasts.—Though Pasteur had clearly stated that during the aerobic phase, division of the yeast cells could be compared to those of higher organisms, this suggestion has been ignored by all the cytologists. Details have been given elsewhere (Subramaniam, 1948 *a*) as to how to obtain a purely aerobic culture for cytological investigations. There appears to be also a belief that the same cell can shift from the aerobic to the anaerobic type of metabolism without radical alterations in the nucleus or the cytoplasmic inclusions. How deep rooted is this conviction could be realized from the definition of "differential fermentation".

"The theory of the differential principle of yeast nourishment was first applied, with indifferent results, in efforts to obtain higher yields of yeast and alcohol from strong spirit brews, but eventually the increasing demand for compressed yeast exceeded production from fermented distillery and vinegar brewery worts and the principle was combined with Pasteur's discovery of the effect of abundant aeration upon fermenting yeast and applied to the production of yeast from extremely dilute worts. The term "differential" fermentation *thus lost its fermentative significance* but survived to indicate the evolution of the process" (Walter, 1941, pp. 92–93).

It is curious that a parallel situation occurs in biochemical investigations also. Just as the students of cytology have confined their attention to fermenting cells, biochemists have also concentrated on "the carbohydrate metabolism and its relation to the reaction products, alcohol and carbon dioxide. Papers actually concerned with the reaction involved in the synthesis of yeast substance are on the other hand very few. In addition the majority of these latter investigations were unfortunately carried out under such varied conditions that it is hard to draw any general conclusions" (Menzinsky, 1950, p. 2). The last statement is only too true for cytological investigations.

The radically different reactions occurring in yeast under aero- and anaerobiosis render it essential to treat the cytological pictures observed in these two phases separately. When cells in a five-day-old fermenting culture were stimulated to divide by replacing the spent medium with fresh wort, the cytological pictures observed were entirely different from that seen during the aerobic phase. Following this procedure we were able to show that our strain, BY 1, which has two chromosomes during the aerobic phase

(Subramaniam, 1946; Duraiswami and Subramaniam, 1951) becomes highly endopolyploid (Subramaniam, 1948 *b*; Prahlada Rao and Subramaniam, 1952) under anaerobic fermentative conditions. Fermenting cultures show, however, a few normally dividing cells having two chromosomes, which are comparable to the " replacement " cells in glandular tissue. It is these cells which give rise to a new crop when conditions become suitable for aerobic proliferation, for, the majority of the endopolyploid cells become necrotic and disintegrate after varying periods of activity.

Observations

Actively dividing cells in well aerated media show a homogeneous cytoplasm with no granules or vacuoles (Photo 1). Cells from a five-day-old fermenting culture, on the other hand, show one or more conspicuous vacuoles (Photo 2). Refractile granules may or may not be present in such cells and when present are not necessarily confined to the vacuoles. They often occur outside the vacuole (Photo 2). If we take aerobic cultures as the standard for evaluation, it is logical to conclude that the vacuoles and granules in fermenting cells should be neo-formations. This conclusion is nothing novel since Guilliermond (1941) describes the following changes when the cells begin to bud.

He inoculated *Saccharomyces ellipsoideus* into a liquid medium containing 0·005% neutral red and observed that the cells accumulated the dye. " The accumulation is at its maximum at the end of half an hour, then, after about three hours (two hours for some yeasts), the cells lose their colour and it is only then that they begin to bud. The loss of colour is brought about by a process which is the converse of that by which the staining was accomplished. The homogeneously stained vacuolar sap loses its colour and there are seen to appear in the vacuoles intensely stained granules which little by little lose their colour and disappear " (p. 140). Caspersson and Brandt (1941) using an ultra-violet light and a quartz microscope observed granules in resting cells. These multiplied and disappeared when the cells began to grow. The vacuolated nature of the cells in fermenting cultures is a very characteristic feature.

If samples from an aerobic culture in which the cells show no vacuolation or granules are smeared at intervals of five minutes for a period extending over an hour and fixed in Carnoy's fluid, the complete mitotic cycle could be mapped out. The optimum time for fixation has been found to be one hour. The fixed material does not show any vacuoles and neither are there any granules in the cytoplasm. It is known that fixation for one hour in

Carnoy's fluid removes completely the Golgi apparatus and mitochondria and leaves only the nucleus and the centrosome, if the latter is present.

It is possible, therefore, to see the stages of division of the nucleus without any complicating structures in the cytoplasm. During the prophase, a single centrosome could be seen on one side of the nucleus. The nucleus itself appears as a stained mass. The centrosome is composed of a vesicular centriole, surrounded by a centrosphere. During the early metaphase, the two chromosomes could be seen on the equator of a thin spindle (Photo 3).

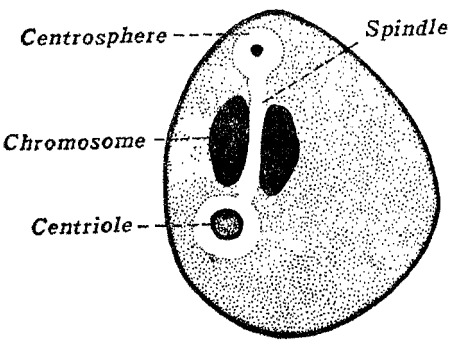

FIG. 2

The spindle appears unstained and shows no fibrillation. The two centrioles with their centrospheres are located at the poles of the spindle. One of the centrioles has a vesicular shape, while the other is granular. The chromosomes are identical in size. When the anaphase separation commences, the centrioles with their centrospheres disappear and the spindle alone persists between the daughter chromosomes. Often, the spindle remnant could be seen lying between the two nuclei as illustrated by Subramaniam (1946) in his Figs. 28 and 32. Lindegren and Rafalko (1950) have not reproduced these illustrations correctly. The stippled region representing the spindle remnant has been altered to give one an impression that the nucleus is dividing amitotically.

The chromosomes are disposed with their longitudinal axes directed towards the centrioles (Fig. 2). One can only surmise that the centromere is of a diffused nature. It is not surprising to find the chromosomes arranged parallel to the spindle. Schrader (1935) describes alterations in the disposi-

tion of the X chromosomes of *Protenor* in the two meiotic divisions. The daughter halves proceeded to the poles broadside on in the first spermatocyte division. In the second, the behaviour was different. During the anaphase, the longitudinal axis was parallel to that of the spindle instead of being at right angles. Centrosomes do not occur in all the cells. In fact, they could be observed only in a very small percentage. Cells at early metaphase showing two chromosomes, but having no centrosomes are illustrated in Photo 4. Having mistakenly identified the nucleus as the centrosome, Lindegren and Rafalko seem to have completely missed the real centrosomes in yeasts.

Discussion

Under aerobic conditions the cells of our two chromosome strain divide almost every hour. This is merely a confirmation of similar observations (*cf.* Walter, 1941, p. 93). Recent investigations in this laboratory have offered evidence that cultures growing on agar slants are composed of endopolyploid vacuolated cells (Prahlada Rao and Subramaniam, 1952) with a sprinkling of normally dividing cells. The unorthodox identifications of Lindegren and Rafalko (1950) are based on samples from 24-hour agar slant cultures incubated at 30° C. in a shaker for periods varying up to six hours. Proof that cells taken from the agar slants have not become endopolyploid is lacking. In fact one wonders whether these authors are aware even of the importance of endopolyploidy in yeasts.

If large numbers of endopolyploid cells are introduced into wort, shaking for six hours under purely aerobic conditions does not remove them from the culture. These endopolyploid cells show characteristic behaviour leading even to the suspicion that many attempt a somatic reduction in their chromosome complement. Without realising these facts, Lindegren and Rafalko claim that the so-called Feulgen positive bodies in the centrosome appear after growth for one and a half hours in the shaker. On the other hand, the bodies claimed to be "chromosomes" are seen only after the lapse of six hours. It is curious that for a period of six hours the cells have not divided even once. On this basis it is legitimate to question their claim that the cells are really diploid. It is quite likely that a variety of cell-types have been confused. The position of their so-called centrosome varies in different illustrations. "The centrosome, always closely associated with the nuclear vacuole, may actually be inside the nuclear membrane, a possibility suggested by the appearance of preparations stained with acid fuchsin" (p. 185). Thus the position of the so-called centrosome appears to be as variable a factor as the number of bodies identified by them as the chromosomes.

But this uncertainty has not deterred them from suggesting that Ranganathan and Subramaniam (1947) "mistook the centrosome for the nucleus and described 'mitosis' of the centrosome, probably because they consistently used Carnoy fixation" (p. 183). If the bodies identified by us as chromosomes are really portions of the centrosome, then what exactly are the bodies identified by us as the centrosomes? But this criticism is contradicted by another statement in the same paragraph. "Kater and Subramaniam observed mitochondria in the living cell, destroyed them by fixation, then stained, instead, the chromosomes, and on observation of the stained material assumed that the chromosomes which now appeared were the mitochondria they had seen in living cells" (p. 183). To say the least, the above criticisms are illogical. For a decade we were interested in the role of the Golgi apparatus and mitochondria (Subramaniam, 1934, 1935, 1937, 1939) and have even attempted to demonstrate the proteid matrix (Subramaniam, 1937, 1939) of these inclusions. Lindegren and Rafalko (1950) have confused issues without attempting a clarification of the problems involved.

The confusion in yeast cytology is more the result of a lack of organized attack on the problem on orthodox lines, than due to want of criteria. This confusion will persist so long as investigators try to treat yeast cells as unique and offer radical interpretations for simple structures. The photomicrographs presented in this paper should convince even a sceptic that the yeast cell is not a law unto itself.

From the commencement of investigations in this laboratory, we have been repeatedly emphasizing that there is little justification for treating the phenomena observed in yeasts as unique (Ranganathan and Subramaniam, 1947). We further observed: "The rapid advances in Cytology are only confirming the belief that there is some fundamental plan in the behaviour of the 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" (Ranganathan and Subramaniam, 1948, p. 389). It was surprising, therefore, to find that our attempts to explain the behaviour of the chromosomes in yeasts in the light of published literature on higher organisms, have been characterised as the result of "unrestrained fantasy"! (Winge, 1951). Distortion and misinterpretation of our results and statements have unfortunately been indulged in and these have been dealt with elsewhere (Subramaniam, 1950 *a*, *b*, *c*). We have been totally unsuccessful in our attempts to find any justification for the criticisms levelled against us.

While claiming to offer critical evidence for "direct diploidization", Winge and Laustsen (1937) identify certain bodies as nuclei which do not satisfy the criteria on which they are so identified in higher organisms. Based on such uncritical evidence they could, of course, speculate on cytoplasmic inheritance. The phenomenon described as "parthenogamy" by earlier workers (Sharp, 1934, p. 411) is made unique under the newly coined term "direct diploidization". Further, Winge could, without any cytological evidence, theorize on additional nuclear divisions, resorption and reassortment of nuclei to conveniently explain away the exceptions to Mendelian ratios in yeasts. But when we suggested the existence of mitotic and meiotic aberrations, these are characterized as "scientifically inexcusable"!

Winge repeats (1951 *a*, *b*) that he never denied the existence of polyploidy in yeasts. What we have been emphasizing is that he never took *into consideration* the possibility of polyploidy in evaluating his results. The following quotations from Skovsted (1948) and Winge and Roberts (1948) would justify our contention.

Bauch (1941) suggested the possibility of an induction of polyploidy in yeasts. Skovsted (1948) in repeating Bauch's experiments with camphor used a so-called "haploid" yeast and states: "Thus if camphor treatment produced polyploidy as suggested by Bauch, the new types would become diploid, *a process which is much easier to confirm on morphological character than the change from diploid to tetraploid*" (p. 250).

We presume that this is an admission that they had no valid criteria to differentiate polyploids from diploids. This is confirmed by a statement of Winge and Roberts (1950). "Be this as it may, we have here the unusual situation in which not less than four polymeric maltase genes exist in an organism, *about the polyploid nature of which we are totally ignorant*" (p. 48). They admit in the next page (p. 79): "As has been noted, polyploidy could satisfactorily explain the phenomenon." However, they do not interpret it on the polyploidy hypothesis but make the results unique by assuming the strain to be a diploid. The following statement would make this evident. "A similar case of four polymeric genes concerned with a qualitative character—here a specific fermentative ability—is unknown to us in the field of genetics" (p. 78). We admit that segregation in polyploids if assumed as occurring in diploids would really appear unique!

Normally, *Nadsonia* is said to form only one spore. If it is the result of a meiotic division, the development of a single spore should be explained on a rational basis. We suggested that *Nadsonia* may be a diploid and that there may be no meiosis before spore formation. Winge's (1951) com-

ments on the above are rather revealing. He states: "On the other hand, Nadson and Konokotin (1911) demonstrated 40 years ago the formation of 4 nuclei in the ascus—*i.e.*, a reduction division" (p. 93). What are identified by Nadson and Konokotin as nuclei, do not answer to the definition of nuclei even though Winge may be willing to accept them as such. The mere presence of four bodies during sporulation does not necessitate an acceptance of their identification as nuclei. When Winge himself stated that the results of cytological investigations appeared to him to be dubious, it is peculiar that he should justify Nadson and Konokotin's rather vague identification of the bodies as nuclei. The criticism of our work by Winge turns out to be the expression of a personal opinion since it has no scientific justification. Personal predilection can never be a substitute for cold logic.

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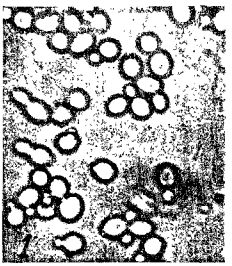

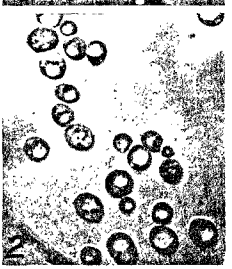

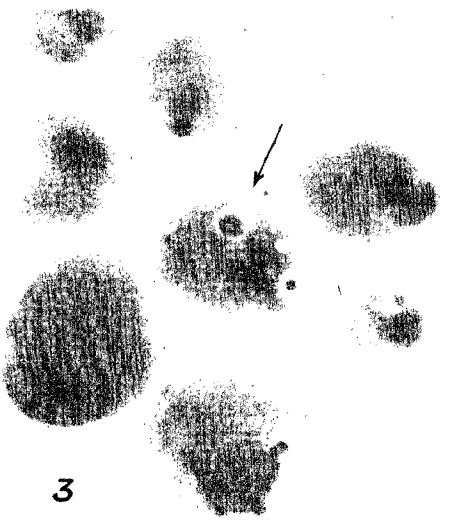
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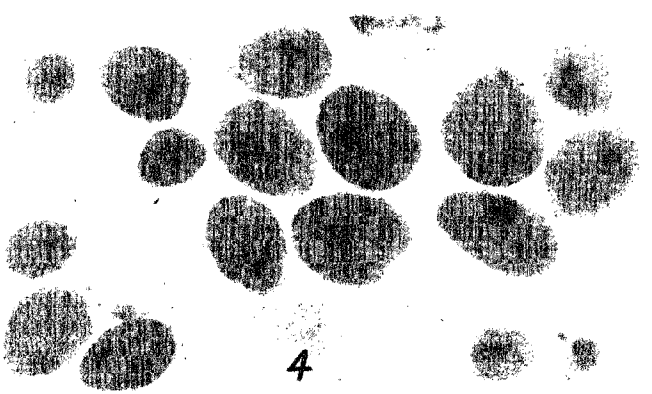
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——— and Roberts, C. (1950)	.. *Ibid.*, **25**, 35.

Description of Photographs

Photo 1. Cells of BY 1 from an aerobic culture showing no vacuoles, × 1,200.

Photo 2. Vacuolated cells from a five-day-old fermenting culture, × 1,200.

Photo 3. BY 1. Early metaphase showing the two chromosomes, the spindle and the centrosomes, × *ca.* 5,800.

Photo 4. Cells showing two chromosomes but no centrosomes, × *ca.* 5,100.