STUDIES ON THE CYTOLOGY OF YEASTS

Part VII. Nuclear Phenomena in Cells from 24-Hour Agar Slants

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Introduction

Yeast could be cultured in liquid or solid media. It was shown in a series of contributions (Subramaniam and Ranganathan, 1946; Subramaniam, 1947; 1948 *a*; 1948 *b*; Ranganathan and Subramaniam, 1948) that the cytological picture changes with the physiological condition of the organism. Fermenting cells were shown to become endopolyploid. All the above investigations were confined to cultures grown in liquid media. Workers in yeast cytology appear to have made indiscriminate use of cultures of different ages grown on different media for investigations, under the assumption that uniform cytological behaviour would be exhibited by the organism irrespective of the cultural conditions. When one considers the fact that the cytological picture varied with the physiological and cultural condition of the organism, it will be apparent that much of the confusion in the literature is due to the above wrong assumption.

Nagel (1946) uses shaken and unshaken broth cultures of varying ages, material from young and old agar slants and even samples from giant colonies. It is too much to expect that the cytological behaviour of yeast should be identical whatever be the physiological condition of the cultures. The rational method of approach is to investigate the nuclear behaviour under each specific cultural condition. If the biochemical behaviour of fermenting yeast cells is entirely different from that during aerobic proliferation (Menzinsky, 1950), one fails to understand how reproducible results could be expected if either of these cultures is used indiscriminately for cytological investigations.

Winge (1935) claimed that Laustsen had developed a method for staining the nucleus of yeasts by the Feulgen technique. He stated in that paper that " the finer cytological details, concerning in particular the chromosomes

will be dealt with in subsequent reports" (p. 104). In 1948, Winge and Roberts make the following statement: "The experiments of the senior author in searching for the very difficultly observable chromosomes in yeasts have not led to a resumption of cytological investigations" (p. 311). Again in 1950 they assert that technical difficulties should have prevented the chromosomes in yeasts being counted satisfactorily (p. 79). In a recent paper, Winge (1951) while suggesting that our demonstration of two chromosomes in a brewery yeast is already anticipated by Badian in 1934 brings out the oft-repeated criticism of Badian's work that the chromosomes seen both in the haploid and diploid cells are identical in number. Ranganathan and Subramaniam (1948) discussed Badian's work. They state that the serious objection voiced against Badian's description and figures is capable of an easy explanation if one considers the chromosomes to be acrocentric. They invite attention to a similar state of affairs recorded in protozoa by Hall and others (Sharp, 1934). The omission by Winge (1951) of any reference to studies III, IV and V from this laboratory is rather significant. To him the whole field of yeast cytology appears dubious and hence he does not feel justified in coming to any conclusion either from his preparations or from those of others. It is interesting to note in this connection that Winge mentions only that the material for his investigations was from vegetatively growing cells in liquid culture. No mention is made of the age of the culture used for the study. One is rather surprised that the importance of the age of the material used should have been so completely ignored. Failure to assess the significance of the dependence of the cytological pictures on the age of the culture—not to mention the importance of the state of aero- or anaerobiosis prevalent during growth—has instead of clearing the confusion only led him to discard the published observations of other workers.

A Critique of Some Recent Publications

In a recent paper Lindegren and Rafalko (1950) reaffirm Lindegren's (1945) original identification of the vacuole in yeasts as the nucleus. They claim that the chromosomes in the vacuole could be demonstrated by the Feulgen method modified by Rafalko (1946). Nagel (1946) working in Lindegren's laboratory, arrives at a different conclusion. "The 'magnicorp' (vacuole or nuclear vacuole of most authors) is Feulgen negative" (p. 271). Lindegren and Rafalko comment that Guilliermond's original wrong identification of a particular structure as the yeast nucleus, is responsible for much of the confusion in yeast cytology. The same remark could be made regarding Lindegren's (1945) identification of the vacuole in the yeast cell as the nucleus (Subramaniam, 1952).

Nagel (1946) uses the terms ' parvicorp ' and ' magnicorp ' to denote the bodies identified as the nucleus by Guilliermond and the vacuole claimed to represent the nucleus by Lindegren respectively. The literature is already cluttered with superfluous terminology. Nagel coined the terms ' parvicorp ' and ' magnicorp ' which had no previous connotations, purely for descriptive purposes and regarded them as " independent of interpretations of nuclear organisation in yeast " (p. 254). These terms are also superfluous because the ' parvicorp ' alone is Feulgen positive and is said to show conventional configurations during the supposed prophase of meiosis. During spore formation the ' magnicorp ' is invisible and in Plate 17, Fig. 195, she illustrates a curious stage. " Several instances were observed in which the four spores were formed from the protoplasm at one end of a large cell. The other end was occupied by a body which resembled the magnicorp of other cells on the slide although no proof of its actual identity therewith was ascertained. A cell of this kind from a Giemsa-stained slide is illustrated in Plate 17, Fig. 195. Generally the magnicorp was not visible in the living cells during the process of sporulation " (p. 263). Logically the ' parvicorp ' which is said to take an active part during spore formation has to be identified as the nucleus. Since the ' magnicorp ' is either invisible or when rarely seen does not get included in the spores, the terms ' parvicorp ' and ' magnicorp ' do not serve any purpose.

Nagel's hesitation to identify definitely the parvicorp as the nucleus, seems to be due to Lindegren's claim (1945) that the vacuole is the yeast nucleus and that it is Feulgen negative. Since Lindegren now claims (Lindegren and Rafalko, 1950) that the yeast nucleus is Feulgen positive, the ' parvicorp ' of Nagel has to be identified as the nucleus of yeast. It is curious that while the Feulgen positive parvicorp does not show mitotic stages it is supposed to show ' meiosis '! If there is no mitosis, one fails to understand why there should be meiosis. The claim for meiosis is not based on cytological evidence. It is supposed to exist because regular Mendelian segregation has been demonstrated in yeasts by Winge and Lindegren.

These contradictory conclusions from the same laboratory are ignored by Lindegren and Rafalko. Still they (Lindegren and Rafalko, 1950) claim that Ranganathan and Subramaniam " mistook the centrosomes and described ' mitosis ' of the centrosomes probably because they consistently used Carnoy's fixation ". In making this statement they have completely ignored the criteria and definitions on which the chromosomes in yeasts were identified by Subramaniam (1948 *a*). That the chromosomes of yeasts give a positive Feulgen reaction was shown by Subramaniam and Ranganathan as far back as 1946. The interpretation by Lindegren and Rafalko of our results ignores

the elaborate discussions on methods of staining the chromosomes of yeasts by diverse techniques (Subramaniam, 1948 *a*) and as such does not conform to the published facts. Lindegren and Rafalko (1950) claim that the centrosome is Feulgen positive without offering any evidence for such a claim. Nagel (1946) remarks that the centriole is not usually Feulgen positive (p. 266). When that is the case Lindegren and Rafalko ought to have offered a logical explanation as to why the centrosome in yeasts alone are Feulgen positive.

Diploidy and Endopolyploidy

Our method of approach has been entirely different. During aerobic proliferation, the nucleus of yeasts divides mitotically. Our control strain has two chromosomes, and the various phases could easily be traced. Pl. IV, Photo 1 shows many cells at an early metaphase condition. The fixation is with Carnoy's fluid and the staining with iron-hæmatoxylin. It is immaterial whether the preparations are stained by the above technique or with Feulgen's leuco-basic fuchsin after fixation in osmic vapour (Subramaniam, 1948 *a*). On the other hand, if a five-day old fermenting culture is stimulated to divide by the addition of fresh wort (Subramaniam, 1948 *b*) and stained by the Feulgen method, the picture is entirely different (Pl. IV, Photo 2 and Pl. V, Photo 3, *cf.*, illustrations of Subramaniam, 1948 *b*). The cells show an ascending grade of polyploidy. At *a*, in Pl. IV, Photo 2, a diploid anaphase could be made out. As mentioned by Subramaniam (1948 *b*), fermenting cultures show a very small percentage of cells retaining their diploid condition. The anaphase seen at *a*, in Pl. IV, Photo 2, is crucial evidence that the mode of division of the *diploid cells* is the same irrespective of whether they occur in aerobic or anaerobic cultures (Subramaniam and Ranganathan, 1946). If attention had been confined to fermenting cells alone, the cytological pictures observed in Pl. IV, Photo 2 and Pl. V, Photo 3 should be rather confusing. If two investigators had confined their attention, one to the purely aerobic culture (Pl. IV, Photo 1) and the other to the anaerobic fermenting one (Pl. IV, Photo 2 and Pl. V, Photo 3), their results have necessarily to be contradictory. The cells shown at *b*, in Pl. V, Photo 3 cannot be compared with those shown at *a* in Pl. IV, Photo 2. The only logical interpretation for the ascending grade of complexity in the cells seen in Pl. IV, Photo 2 and Pl. V, Photo 3 is that the cells become endopolyploid during fermentation. Fermenting cells have been compared to secretory cells by Guilliermond (1920) and the occurrence of endopolyploidy in fermenting cultures is nothing surprising. Lindegren and Rafalko appear to have confused polyploidy with endopolyploidy. These are entirely different phenomena. Cells of our diploid and tetraploid strains become

endopolyploid during fermentation confirming the belief that these two phenomena are as distinct in yeasts (Mitra and Subramaniam, 1949), as in higher animals (Geitler, 1937). Highly endopolyploid nuclei in fermenting cells show amitosis-like phenomena (Pl. V, Photo 3), irregular segregation of chromosome complements and often micronuclei formation (Subramaniam, 1948 *b*).

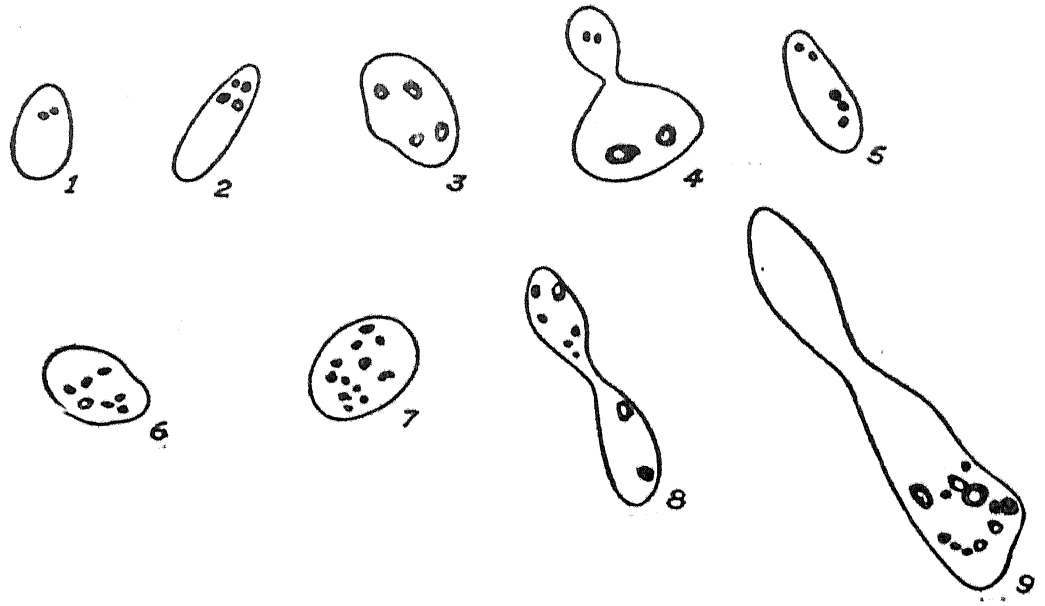
THE PROBLEM

Are cells growing on agar slants comparable to those proliferating aerobically in well-aerated liquid media? Two considerations seem to militate against such a possibility. The mere fact that the former is growing on a medium in the solid state in contrast to the latter which is submerged in a liquid, raises the question of differences in the easy availability of the nutrients to the cells. Apart from this, the difference in the diffusion of products of cellular metabolism may also be influential. For instance, if alcohol is being produced, in the liquid culture there is every chance for it to be diffused uniformly throughout the medium, whereas in the solid medium, *viz.*, the agar slant, due to the very slow diffusibility of the alcohol, local concentrations amounting to a toxic level could easily be set up. Under such circumstances, one could possibly expect cells growing on agar slants to resemble closely those in fermenting liquid cultures. If fermenting yeast cells resemble secretory cells and as a result show endopolyploidy, the cytological phenomena observed in cells growing on agar slants should be similar to that observed in fermenting cells. Similar should be the case in cells comprising the giant colonies also.

OBSERVATIONS

To check this possibility, samples from 24-hour agar slants were smeared, fixed in Osmic vapour, hydrolysed for six minutes and stained by Feulgen's leuco-basic fuchsin according to the method described by Subramaniam (1948 *a*). A variety of cytological pictures are observed in such Feulgen slides. At *a*, in Pl. VI, Photo 4 and Pl. VII, Photo 5, a normal diploid anaphase could be made out. In the cell at *b*, in Pl. VI, Photo 4, one pair has reconstituted into a nucleus, while a pair of chromosomes are lying free at the other end of the cell. In the other cells illustrated in the field there are varying number of bodies which because they show positive Feulgen reaction have to be identified as chromatin. If this is accepted, the different numbers are capable of being explained as the result of an ascending grade of endopolyploidy, the stained bodies of larger size originating by the fusion of a number of chromosomes. These are arranged in an orderly manner in the camera lucida illustrations. The normal diploid metaphase (Fig. 1) is

followed by the anaphase (Figs. 2 and 3). In cells showing su h anaphase configurations, all the daughter chromosomes may be identical in size (Fig. 3), or one pair may be larger (Fig. 2). The smaller pair is migrating to the bud in Fig. 4. The chromosomes in the mother cell have become vesicular and large, reminiscent of nuclei. In Figs. 5, 6 and 7 the cells show an increasing number of bodies. Naturally these should have originated by the division of the original two chromosomes without any bud formation. Even



FIGS. 1–9.—Fig. 1. Early metaphase. Fig. 2. Early anaphase. The chromosome pairs are unequal. (cf. *a*, in Photo. 4). Fig. 3. Late anaphase. (cf. *a*, in Photo. 5). The chromosomes are equal. Fig. 4. The bud contains a pair of chromosomes while the pair in the mother cell have become vesicular. Figs. 5, 6 and 7. The ascending grade in number of the chromosomes. Fig. 8. Segregation of the chromosomes into unequal complements. (cf. *c*, in Photo. 4). Fig. 9. A budding cell showing a large number of chromosomes some of which appear to be compound. (cf. *b*, in Photo. 5).

All drawings drawn at a magnification of × ca. 3,300.

endopolyploid cells exhibit the phenomena of budding (Figs. 8 and 9) but the segregation of chromosomes is into unequal complements as described by Subramaniam (1948 *b*) in fermenting cultures. In Fig. 8, there are six bodies of unequal size in the bud and only two in the mother cell. In Fig. 9 on the other hand, the chromosomes, simple and compound, are limited to the mother-cell (*b*, in Pl. VII, Photo 5). Pl. VII, Photo 6 is of living cells from a 24-hour agar slant. As could be made out very few of the cells show any vacuoles. These observations are of cells from 24-hour agar slants. As in fermenting cultures, with passage of time, the cells in agar slants should show increasing complexity. Thus the cytological observations if carried

out on material cultured under entirely different conditions would give rather confusing pictures. If, on the other hand, one attempts to correlate the changes starting with purely aerobic cultures, the varying pictures could be explained in a rational manner.

DISCUSSION

A comparison of Pl. IV, Photo 2, Pl. V, Photo 3, Pl. VI, Photo 4 and Pl. VII, Photo 5 with the illustrations of Levan (1947) shows remarkable similarity. Levan has apparently confined his attention to fermenting cells and it is not surprising that on the evidence available to him he concluded: " the low chromosome numbers earlier published for yeasts (Badian, 1937; Sinoto and Yuasa, 1941) may have been influenced by such fusions " (p. 464). If he had investigated the cytology of his strain under purely aerobic conditions, an entirely different picture would have been observed by him.

Lindegren and Rafalko (1950) have confined themselves to material from " a 24-hour agar slant and incubated at 30° C. for one to six hours on a shaker " (p. 170). As the results presented above show an ascending grade of endopolyploidy in cells growing on agar slants, it means that such endopolyploid cells were used by Lindegren and Rafalko as the starting material in their experiments in shaker flasks. The cytological behaviour of such cells on a shaker depends on (i) the quantity of the inoculum and (ii) the efficiency of aeration. If the inoculum is heavy, the cells would continue to be endopolyploid. If the aeration is not efficient, the results would be identical. These two factors are completely ignored by Lindegren and Rafalko. It is not surprising that the cells figured show different number of bodies identified by them as the chromosomes. They illustrate in their Fig. 9, four pairs in the mother cell and one in the bud. In Fig. 15 on the other hand, they illustrate ten bodies in the mother cell and eight in the bud. But in spite of this variability they claim that, " the chromosomes number four or five pairs " apparently not taking into consideration the enormous variability even in their illustrations. The results, as such, appear to be of questionable validity.

Theoretically, the yield of yeast under ideal conditions of aeration could be calculated. But even under ideal experimental conditions, the theoretical yield is generally not obtained (Menzinsky, 1950). Similarly, when yeast is grown aerobically for cytological investigations, it has been found impossible to avoid a few endopolyploid, fermenting cells. Under optimum conditions, the percentage of endopolyploid cells in smears is indeed low.

It is desirable to give a rational explanation as to why the behaviour of the yeast nucleus during aerobic proliferation alone should be taken as the standard for an evaluation of the changes under different cultural conditions. In higher animals and plants, only the embryonic cells show a regular constancy in the behaviour of the nucleus. It is this fixity and stability which has led to the formulation of the chromosome theory of inheritance. When, as in the salivary glands of *Drosophila* (White, 1945; Lorz, 1947), the nucleus shows an entirely different type of organisation, the interpretation of such alterations is based on a knowledge of the behaviour of the chromosomes in the embryonic cells.

During aerobic proliferation, the yeasts show a comparable fixity and stability of the chromosomes. If the salivary chromosomes have to be interpreted in terms of those seen during mitosis, it stands to reason that a similar procedure should be followed in the case of yeasts also. Our control strain has two chromosomes. These could be easily demonstrated when cultured aerobically. The picture changes when cells from fermenting cultures or agar slants are examined. The ascending grade of complexity of the nuclear structure is evidenced by the Feulgen stained chromosomes seen in such cells. As in higher animals, therefore, this progressive complexity has to be interpreted as due to endopolyploidy. When samples from agar slants or fermenting cultures are grown in liquid cultures aerobically for a sufficiently long period, the population again shows typical mitosis. We have to presume therefore, that endopolyploidy is a specific modification to meet particular conditions of existence.

Summary

Much of the confusion in the interpretation of the cytological pictures observed in yeasts could be traced to the wrong assumption that cells from cultures under entirely different physiological conditions should exhibit uniform cytological behaviour.

The only rational method of approach will be to investigate the nuclear behaviour under each specific cultural condition. A critical evaluation of some recent publications is presented.

Photomicrographs showing the cytological pictures during the aerobic and anaerobic phases are presented to illustrate the confusion that would result if attention is confined to either of the above cultural methods, for evaluation.

The cytological pictures observed in cells taken from a 24-hour agar slant are entirely different from that observed in actively proliferating cells

in well aerated media. Photomicrographs and camera lucida drawings are presented as evidence that the varying number of bodies seen represent an ascending grade of endopolyploidy.

It is emphasized that the behaviour of the yeast nucleus under aerobic proliferation alone should be taken as the standard for the evaluation of the changes under different cultural conditions.

Endopolyploidy appears to be a specific modification to meet particular conditions of existence.

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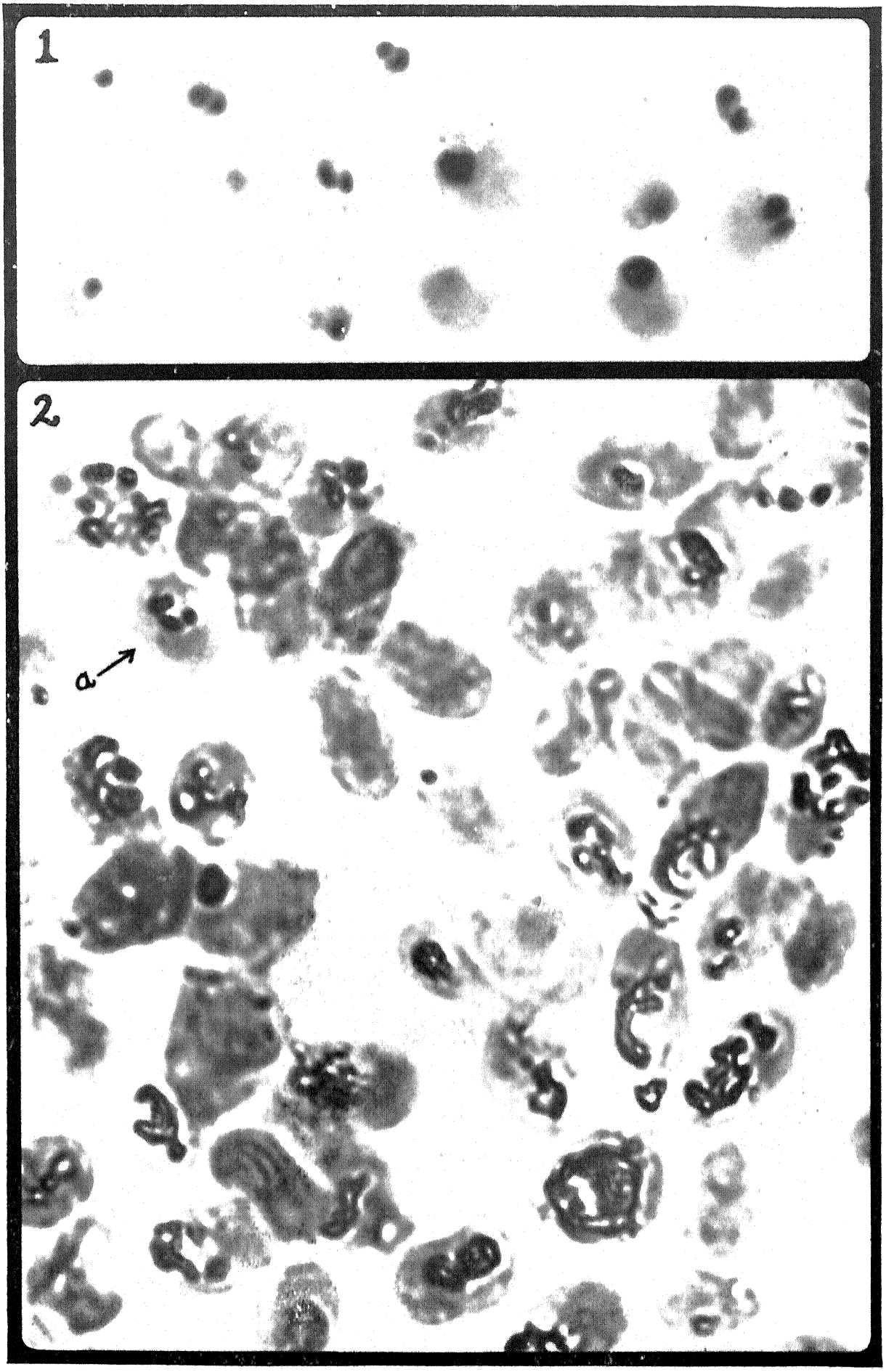
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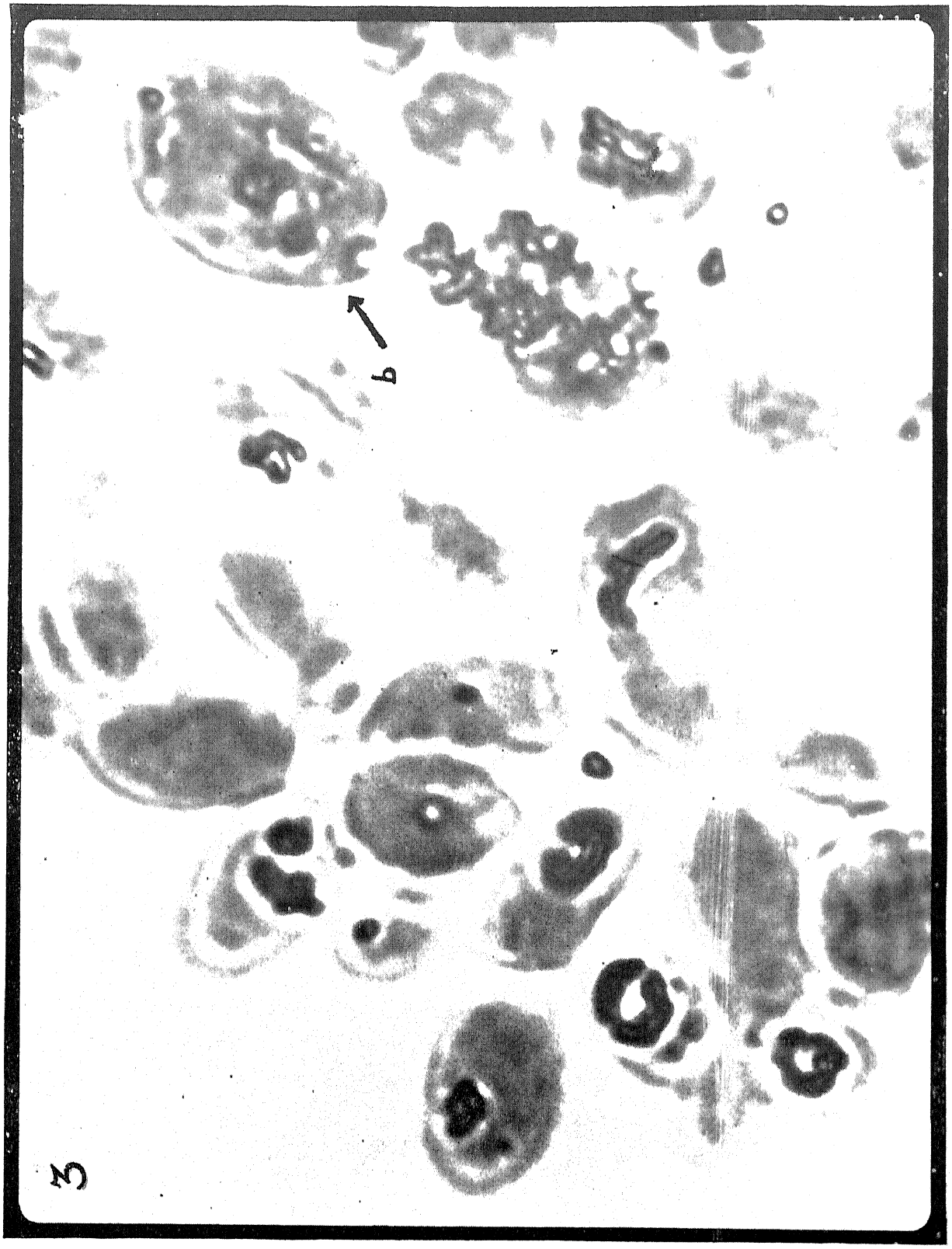
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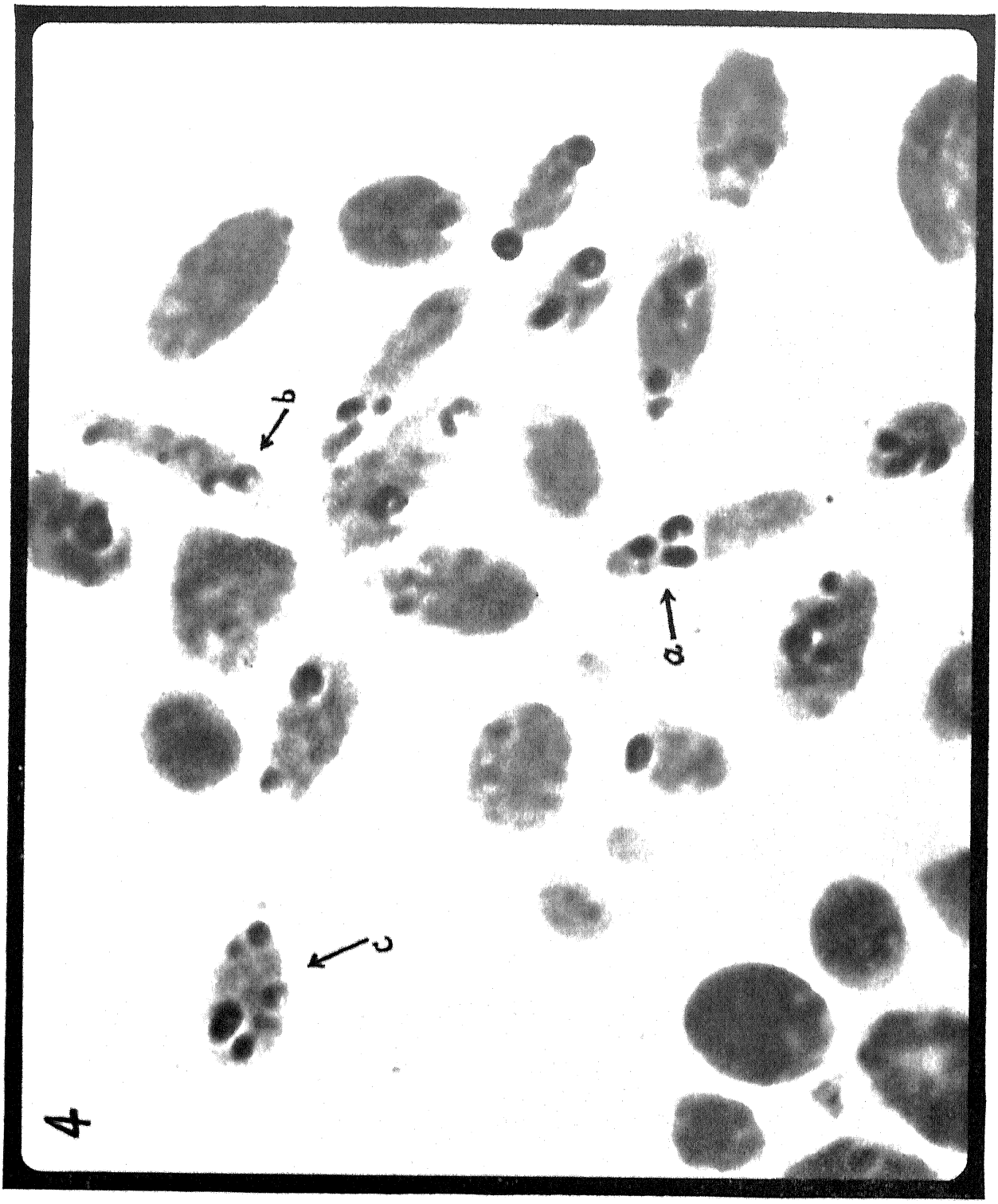
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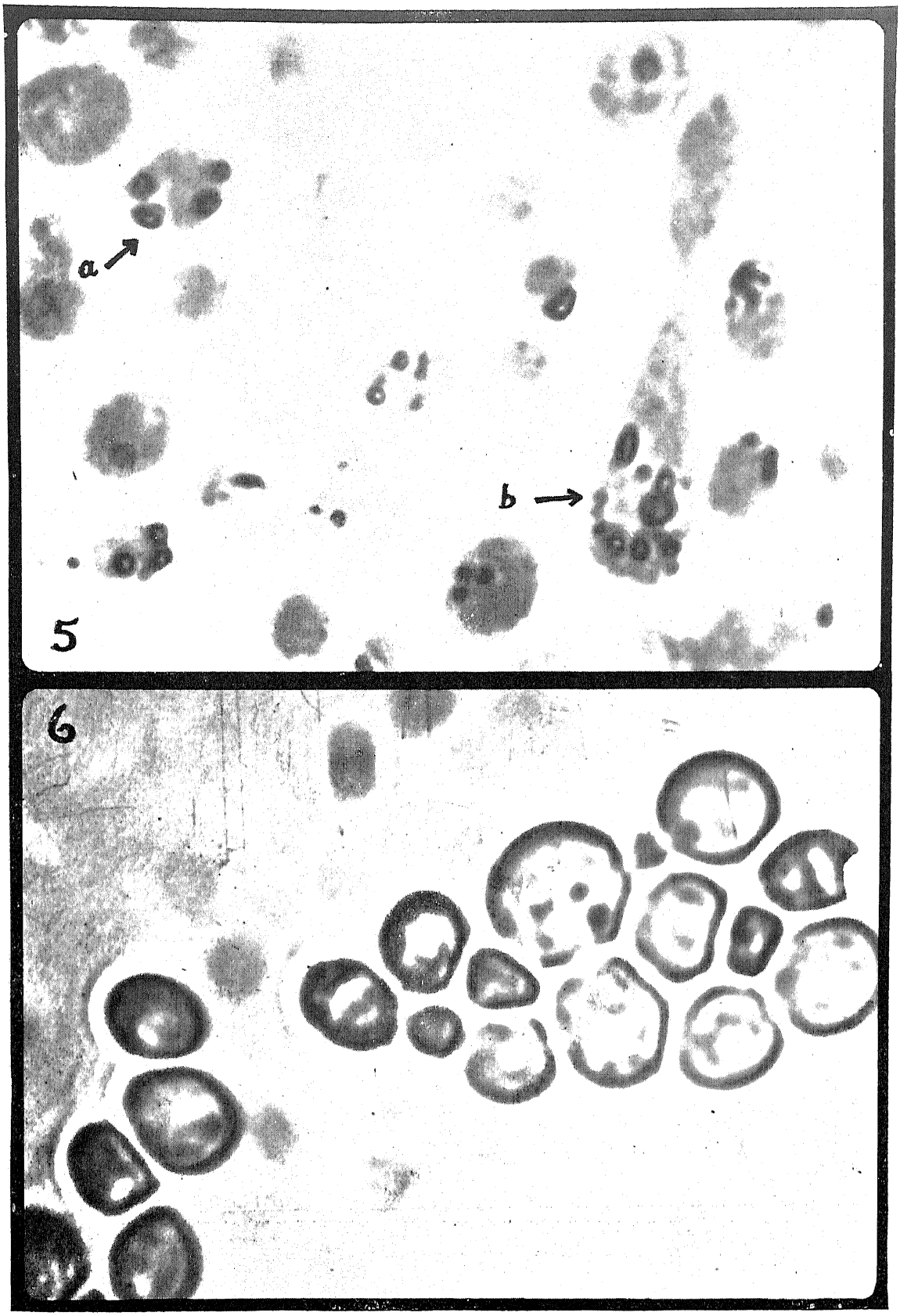
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Explanation of Plates

Photo 1. Cells at early metaphase showing the two chromosomes. Aerobic culture in wort. Carnoy-Iron Hæmatoxylin, × ca. 4,000.

Photo 2. A five-day old fermenting culture stimulated to divide by the addition of fresh wort. Cells show an ascending grade of polyploidy. Compare with illustrations presented in Plates 2 and 3 by Subramaniam (1948 *b*). At *a*, is a cell at early anaphase. Osmic fixation Feulgen's staining, × ca. 2,500.

Photo 3. A five-day old fermenting culture stimulated to divide by the addition of fresh wort. Compare with illustrations in Plate 3 by Subramaniam (1948 *b*). At *b*, is a highly endopolyploid cell. Osmic Feulgen, × ca. 5,000.

Photo 4. Cells from 24-hour agar slants. At *b*, is a cell in which there is a reconstituted nucleus at one end and a pair of chromosomes at the other end. Osmic-Feulgen, × ca. 4,500.

Photo 5. Cells from 24-hour agar slants. At *a*, is a cell at late anaphase. Osmic-Feulgen, × ca. 4,500.

Photo 6. Living cells from a 24-hour agar slant, × ca. 4,500.