THE REACTION OF LIVING VEGETATIVE CELLS AND ZYGOTES OF *SACCHAROMYCES CARLSBERGENSIS* TO NEUTRAL RED

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ABSTRACT

1. The reaction of living vegetative cells from 24-hr. agar slants to different concentrations of neutral red has been investigated. Guilliermond's repeated emphasis that the vacuole is not the nucleus of yeast and that neutral red is specific for the study of the vacuole of living cells is confirmed and illustrated with photomicrographs.

2. A comparison of the reaction of living vegetative cells and zygotes without and with visible nuclei shows that neutral red produces neo-formations only inside the vacuole, thus emphasizing the unrelated nature of the vacuole and the nucleus.

INTRODUCTION

The demonstration that the living nucleus of yeast is an extra-vacuolar structure in vegetative cells (Royan and Subramaniam, 1956; Royan, 1956 *a*, *b*, *c*; Thyagarajan and Subramaniam, 1957 *a*) as well as zygotes (Thyagarajan and Subramaniam, 1957 *b*), has rendered possible an analysis whether the vacuole is also nuclear in character (Wager, 1898; Wager and Peniston, 1910; Lindegren, Williams and McClary, 1956).

The radical divergence of views between Wager (1898; Wager and Peniston, 1910) and Guilliermond (1920) centred round the question of the real nature of the yeast vacuole. Wager and Peniston (1910) conceived of nuclear continuity through spores as mediated by the extra-vacuolar structure which they termed the 'nucleolus'. Yet, Wager considered the vacuole also as having the status of a vegetative nucleus. Guilliermond (1920), on the other hand, identified the 'nucleolus' of Wager as the real nucleus of yeast and the vacuole as the homologue of the vacuoles of plants and animals.

During his extensive investigations on the cytoplasmic inclusions of plant cells, Guilliermond (1941) substantiated his contention that the reaction of the yeast vacuole to basic vital dyes is similar to that of vacuoles of plant cells in general.

Vital dyes have the property of penetrating living cells and colouring specifically certain cytoplasmic inclusions (Guilliermond, 1941). A very dilute solution of Janus Green B is said to stain the mitochondria of animal cells bluish green.

But this is transitory since the colour changes to pink and disappears from the mitochondria altogether as a consequence of its reduction to a leuco-base (Cowdry, 1924).

Neutral red, on the other hand, has been shown to be specific for the study of the vacuole since there is a progressive accumulation of the dye inside the vacuole. Because the nucleus is not stained by neutral red (Gatenby and Cowdry, 1928) it was thought that a study of the reaction of living cells of *Saccharomyces carlsbergensis* to neutral red would be interesting.

OBSERVATIONS

I. Cells from Agar Slants

The majority of the cells of *S. carlsbergensis* from 24-hr. wort agar slants show vacuoles as well as grains (Thyagarajan, 1956) and were therefore used for experiments with various dilutions of neutral red in distilled water. A small quantity of material from the agar slant was mixed well in a drop of neutral red on a slide and a loop of cells was transferred to another slide containing a fresh drop of neutral red to ensure a uniform scattering of the cells when sealed with paraffin under a coverslip. Preparations containing very few cells were chosen for study and a photographic record of the progressive changes in the vacuole was made.

A. Reaction of Cells to 0·1% *Neutral Red.*—When the cells are suspended in a 0·1% solution of neutral red, brightly stained granules exhibiting Brownian movement appear immediately inside the vacuoles. According to Guilliermond (1941) these granules are the vacuolar colloids precipitated and stained by neutral red. Photos 1–13 illustrate the various types of reaction of the vacuoles. A stained granule is seen in the vacuoles of cells shown in Photo 1. In cell *A* (Photo 1) the granule lies apposed to the vacuolar boundary, while in cell *B* it is partly inside and partly outside the vacuole. A pair of stained bodies are seen in the vacuoles of cells in Photos 2 and 3 and three such bodies may occur either in a row (Photo 4) or scattered inside the vacuole (Photo 5). These granules may vary not only in number (Photos 1–5) but also in size (Photos 6–8). There is a general tendency for these granules to fuse together into a large mass (Photo 9), or a single granule may enlarge in size with progress of time and fill up completely the area of the vacuole (Photo 10). The vacuolar boundary is well defined and suggestive of the presence of a membrane in Photos 2, 8 and 9. Photo 9 is interesting in that the beaded nature of the vacuolar boundary is reminiscent of a similar condition observed under dark ground illumination in unstained living cells (Photo 40 in Pl. XXI and p. 282, Thyagarajan, 1956).

In cells containing two vacuoles, both of them show staining (Photos 11 and 12). The vacuoles of the mother and bud react in an identical fashion as those of other cells. Both show masses stained by neutral red in Photo 13.

B. Reaction of Cells to 0·01% *Solution of Neutral Red.*—The reaction of the cells to 0·1% solution of neutral red was rather quick and it was found difficult

to get a photographic record of the progressive changes in a vacuole. This was possible when the cells were suspended in a 0·01% solution of neutral red.

A series of exposures of the same group of cells over a period of six hours are illustrated in Photos 14–19. Ten minutes after mounting the cells in neutral red, cell *A* in Photo 14 shows two stained granules while the vacuoles of cells *B* and *C* are bereft of any such structures. The two granules in cell *A* fuse and at 15 min. a single body alone is seen in apposition with the vacuolar boundary (Photo 15). This grain assumes a crescentic shape lining the boundary of the vacuole after the lapse of one hour (cell *A*, Photo 16). At the end of two hours (Photo 17) while the stained crescent of cell *A* has slightly increased in area, neutral red granules have appeared in the vacuoles of cells *B* and *C* (Photo 17). The stained area in all the three cells are crescentic after four hours (Photo 18), the depth of staining being intense at the periphery and lighter in the centre. This progressive staining continues and six hours after mounting the cells in neutral red, the greater part of the vacuole in all the three cells (*A*, *B*, *C*, Photo 19) appears stained.

That this is not an isolated instance is illustrated by micrographs 20–24 of another group of cells, where attention was focussed on the dancing body of cell *A*. The dancing body has an affinity for neutral red and its gradual increase in size during a period of six hours is illustrated in Photos 21, 22, 23 and 24. Among the cells in the group, *B* and *C* of Photo 20 do not show any stained structures inside the vacuole. After an hour three crescents are visible in the vacuole of cell *C* (Photo 21). At the end of two hours while the stained area has increased in cell *C*, there are two bodies at the vacuolar periphery in cell *B* (Photo 22). The gradual increase in size of the stained areas are illustrated in Photos 23 and 24 taken after four and six hours respectively after the commencement of the experiment. Attention is invited to the fact that while the dancing body of cell *A* of Photo 20 shows a progressive increase in size during the course of six hours, greater areas of cells *B* and *C* are stained by neutral red even though the first appearance of such structures was later than in cell *A*. The vacuoles show a diffuse colouration at the end of twelve hours (Photo 25) and appear brightly tinted after 24 hours (Photo 26).

II. Cells from Wort

C. Reaction of Zygotes from Three-Day Old Cultures to 0·01% *Neutral Red.*—The nucleus is not visible in the zygotes observed three days after the inoculation of spores into wort. Since the pH of wort before inoculation was on the acid side (pH 4·6–4·8) the crop from the wort tube was centrifuged, washed and then suspended in a phosphate buffer at pH 7 for 15 min. before transferring the cells to 0·01% neutral red solution. The reaction of the zygotes to the dye was similar to those observed in vegetative cells from agar slants. Stained granules appear inside the vacuoles, get attached to the vacuolar boundary (Photos 27 and 28) and often appear as crescentic masses (Photos 29 and 30) at the vacuolar periphery (*cf.*, Photos 1–26).

D. Reaction of Cells with Visible Nuclei to 0·01% Solution of Neutral Red.—(i) *Vegetative cells.*—Though Guilliermond (1941) repeatedly emphasized that the vacuole is not the nucleus of yeast, the evidences adduced by him were not complete since his experiments with neutral red were carried out on cells in which the nucleus was invisible in the living condition. The observations presented in the preceding paras is an attempt at confirmation of Guilliermond's observations with photomicrographs (Photos 1–30). The nucleus is visible in a small percentage of the vegetative cells as well as zygotes from 7–10-day old wort cultures (Thyagarajan and Subramaniam, 1957 *a*, *b*). The crop from such a wort tube was centrifuged, washed and suspended in a phosphate buffer at pH 7 for 15 min. and then transferred to 0·01% neutral red solution. Photo 31 is that of an unstained living vegetative cell showing the extra-vacuolar position of the nucleus. The vacuole has a luminous boundary (Aswathanarayana, 1956 *a*, *b*; Royan, 1956 *b*; Thyagarajan, 1956) under dark ground illumination (Photo 32).

When the cells are suspended in neutral red there is occasionally a non-specific tinting of the nucleus as well as the cytoplasm in cells which appear to be senescent. Observations were therefore confined to those in which the nucleus as well as the cytoplasm were unstained. A stained granule has appeared inside the vacuole in Photo 33. In the case of bi-vacuolate cells, each vacuole shows a neutral red stained granule (Photo 34) at the beginning. With progress of time, more number of granules appear in the vacuole, exhibit Brownian movement (Photo 35), coalesce into large globules (Photos 36 and 37) and get attached to the vacuolar boundary (Photo 38). Stained crescents lining the vacuolar boundary are very common (Photo 39). Sometimes the stained structures may be seen migrating through the vacuolar boundary (Photos 40 and 41) and coming to rest in the cytoplasm (Photos 42 and 43).

These observations are reminiscent of the following description by Guilliermond (1941) of the reaction of *Saccharomycodes ludwigii* to 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). Micrograph 44 shows the final stage when the entire vacuole appears diffusely tinted. The vacuolar boundary, however, is brightly stained, but the accumulation of the dye at the periphery is irregular. The extra-vacuolar nucleus (Photos 33–44) is not stained but shows greater contrast when mounted in neutral red.

(ii) *Zygotes.*—The nucleus of the zygote (Thyagarajan and Subramaniam, 1957 *b*) lies usually between the two vacuoles (Photo 45). Like the vegetative cells, the two vacuoles of the zygotes have also luminous boundaries suggesting that they are well formed membranes (Photos 46 and 47). Phenomena comparable to those in vegetative cells occur on staining the zygotes with neutral red. A single granule has appeared in one of the vacuoles of the zygote in Photo 48.

The gradual increase in the number of these granules, their fusion and migration to the vacuolar boundary are illustrated in Photos 49, 51 and 52.

Occasionally the zygotes have only a single vacuole. As a consequence, the nucleus is shifted towards the end containing the cytoplasm. Such an example is illustrated in Micrograph 50. The large vacuole contains two bodies stained by neutral red. Only one of the vacuoles of the zygotes in Photos 53 and 54 exhibits a small neutral red stained crescent.

The two vacuoles often exhibit differences during the progress of staining. In the zygote in Micrograph 55, one of the vacuoles appears diffusely stained, while in the other there is an irregular accumulation of the dye at the periphery. During these changes inside the vacuole, the nucleus exhibits no visible alteration and neither could granules stained by neutral red be seen inside the nucleus.

Discussion

A vast amount of literature has developed on Neutral Red Cytology (Bowen, 1927; Parat, 1928; Ludford, 1930; Kœhring, 1930; Gatenby, 1931; Subramaniam, 1937; Guilliermond, 1941; Junquiera and Hirsch, 1956; Lacy and Challice, 1957) in consequence of attempts (*a*) to homologize the cytoplasmic inclusions of plant and animal cells and (*b*) to support or counter the claim of Parat (1928) that the Golgi apparatus described from fixed preparations of animal cells has no existence since it is the result of precipitation of silver or osmium in vacuoles stainable with neutral red in the living condition.

There is information available to correlate the observations recorded in this paper with the sequence of events observed on staining the cells of plants and animals with neutral red. The fact that, in *S. carlsbergensis*, neutral red stains a preformed structure like the dancing body and at the same time produces neoformations is reminiscent of Ludford's (1930) observations on animal cells and renders attractive Guilliermond's (1941) suggestion that, since the staining of the vacuole is the consequence of a precipitation of the vacuolar colloids, the dancing body itself should be the result of such a precipitation in normal living cells (*cf.*, Hartman and Liu, 1951).

It is only in yeast that the vacuole has been identified as the nucleus (Wager, 1898; Lindegren, 1949) and, as in the case of the animal Golgi apparatus, there has been sharp divergences of view between the investigators regarding the validity of such an identification (Guilliermond, 1920; Subramaniam, 1946; 1951; DeLamater, 1950; Lietz, 1951). It was shown earlier (Thyagarajan and Subramaniam, 1957 *a*, *b*) that the nucleus of the vegetative cells as well as zygotes of *S. carlsbergensis* is extra-vacuolar with a nuclear membrane enclosing formed structures inside. Under certain conditions the yeast vacuoles have also visible membranes (Aswathanarayana, 1956, *a*, *b*; Royan, 1956 *b*; Thyagarajan, 1956) under dark ground illumination. Evidence has been offered in this paper that both the vacuoles of the zygote have luminous boundaries (Photos 46 and 47).

Though the nucleus and the vacuole seem to be delimited from the cytoplasm by what appear to be formed membranes, their reactions to neutral red are different. While the nucleus remains unstained, neutral red produces neo-formations and, with progress of time, stains the vacuole completely.

The occasional migration of structures stained by neutral red from the vacuole to the cytoplasm requires consideration in view of the presence of a vacuolar membrane. Ludford (1931) noticed in cells stained with neutral red an alteration of the Golgi apparatus which was entirely different from that occurring during autolysis. Weiss (1955) reported a loss of definition of the ergastoplasm, the mitochondria, and possibly the Golgi complex in electron micrographs of neutral red stained cells and concluded that the dye is a cytoplasmic toxin. In the above context it is not difficult to visualise the disappearance of the vacuolar membrane in regions where the neutral red structures are in contact with it. At present it is difficult to confirm that possibility.

According to Marston (1923) azine and azonium bases form precipitates with proteolytic enzymes. There is even evidence that these precipitates retain their activity. The staining of mitochondria with Janus Green B was explained on that basis (Bourne, 1951). Kœhring (1930) suggested that though neutral red partially inactivates enzymes, yet, in sufficiently high dilutions, it stimulated further enzyme production as evidenced by the formation of a large number of vacuoles in Ciliates and by the acceleration in the rate of division of Amœbæ.

Subramaniam (1937) showed that neutral red vacuoles in the oocytes of *Meretrix casta* are neo-formations and reported the formation of similar unstained vacuoles under certain environmental conditions. Dustin (1947) mentions that exposure of red blood cells of frog to ammonium salts and amines results in the formation of vacuoles and considers that vital staining with basic dyes reveals the ribonucleoproteins occurring in very small quantities.

Thus whatever may be the nature of the substances stained by neutral red all are agreed that it is not the nucleus that is stained by the dye. Therefore, the reactions of living cells of *S. carlsbergensis* to neutral red reinforce the earlier work from this laboratory (Subramaniam, 1946; Royan and Subramaniam, 1956; Royan, 1956 *a*, *b*, *c*; Thyagarajan and Subramaniam, 1957 *a*, *b*) that the nucleus and vacuole of yeast are unrelated structures.

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DESCRIPTION OF PHOTOMICROGRAPHS

PLATES V AND VI

PHOTOS 1–13. Reaction of Living Cells from 24-hour Agar streaks to 0·1% Neutral Red.

The rapid appearance of granules stained by neutral red inside the vacuole, their increase in number and the final uniform staining of the vacuole are illustrated.

PHOTOS 14–26. Reaction of cells to 0·01% neutral red.

PHOTOS 14–19. The progressive changes in the vacuoles of cells *A*, *B* and *C* over a period of six hours culminating in the tinting of the greater part of the vacuole (Photo 19) could be observed.

PHOTOS 20–24. Exemplify the affinity of the dancing body of cell *A* in Photo 20 to neutral red and its gradual increase in size during a period of six hours. Photos 21–24 indicate that greater areas of cells *B* and *C* are stained by neutral red, even though the first appearance of such structures was later than in cell *A*.

PHOTO 25. The vacuoles appear diffusely stained at the end of twelve hours.

PHOTO 26. The vacuoles are brightly tinted at the end of 24 hours.

PLATE VII

PHOTOS 27–30. The reaction of living zygotes from 7-day wort cultures, in which the nucleus is not visible, to 0·01% neutral red.

PHOTO 27. Neutral red granules are seen only in one of the vacuoles of each zygote after 20 min. in the stain.

PHOTO 28. After 30 min. the vacuoles of the zygote as well as its bud show stained bodies.

PHOTOS 29 and 30. The prominent stained crescents at the vacuolar periphery at the end of 45 min. of exposure to the dye.

PHOTO 31. Living unstained vegetative cell showing the extra-vacuolar nucleus.

PHOTO 32. The boundary of the vacuole as seen under dark ground illumination.

PHOTOS 33–44. Reaction of living vegetative cells with visible nuclei, from 7-day wort cultures, to 0·01% neutral red.

PHOTOS 33–38. After 15 min. stained granules exhibiting Brownian movement are seen inside the vacuole.

PHOTO 38. A stained mass is seen at the vacuolar boundary.

PHOTO 39. The stained crescent at the vacuolar periphery (30 min.).

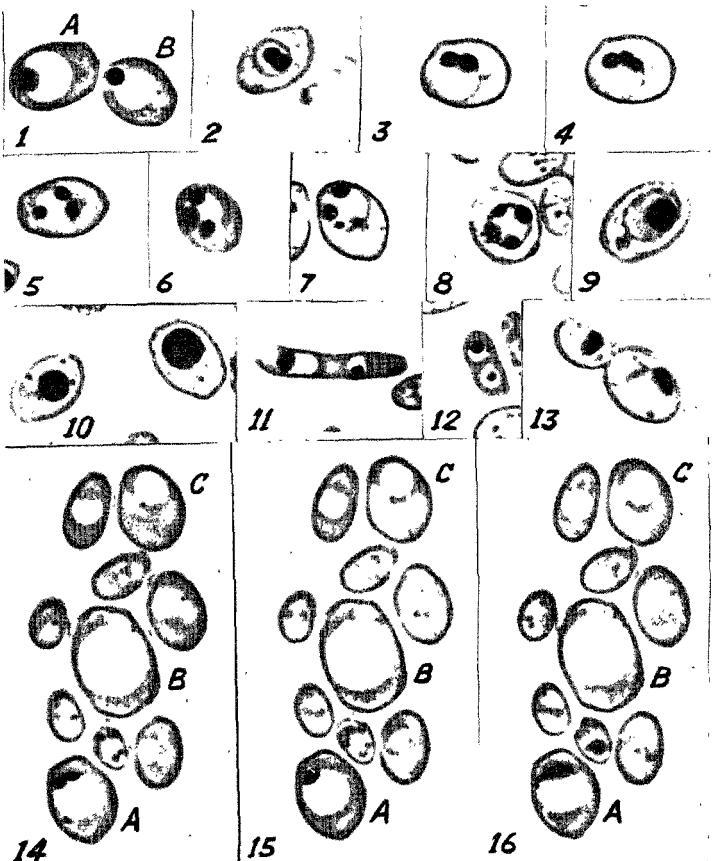
PHOTOS 40 and 41. Crescentic stained areas lying partly outside the vacuole (30 min.).

PHOTO 42. Neutral red stained granule lying in the cytoplasm (30 min.).

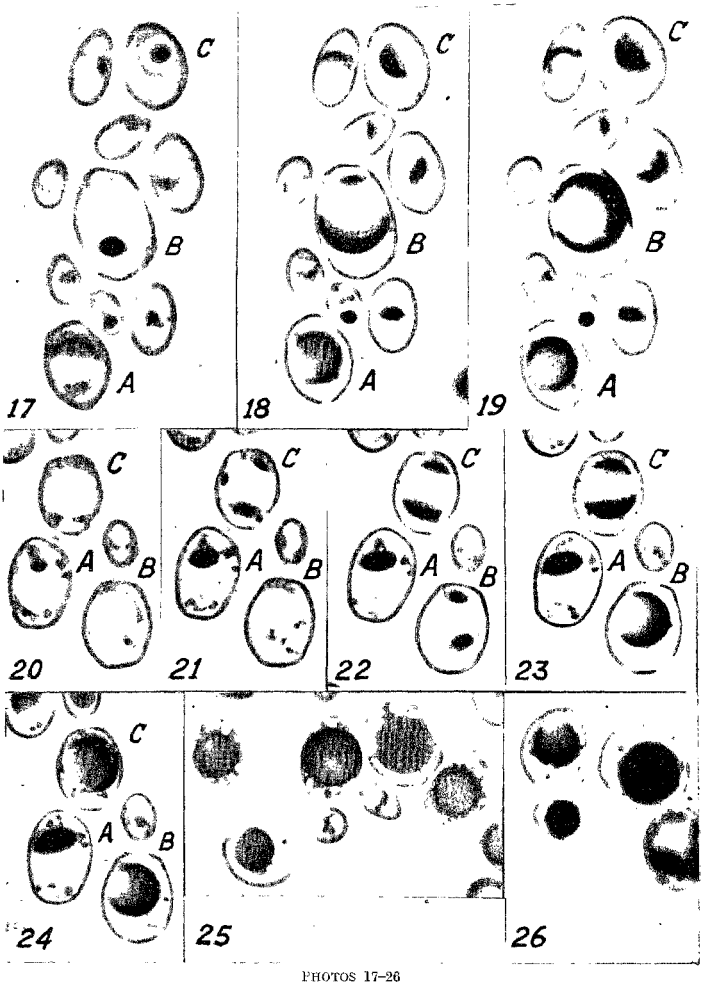
PLATE VIII

PHOTO 43. The big crescentic area seen in the cytoplasm at the end of one hour.

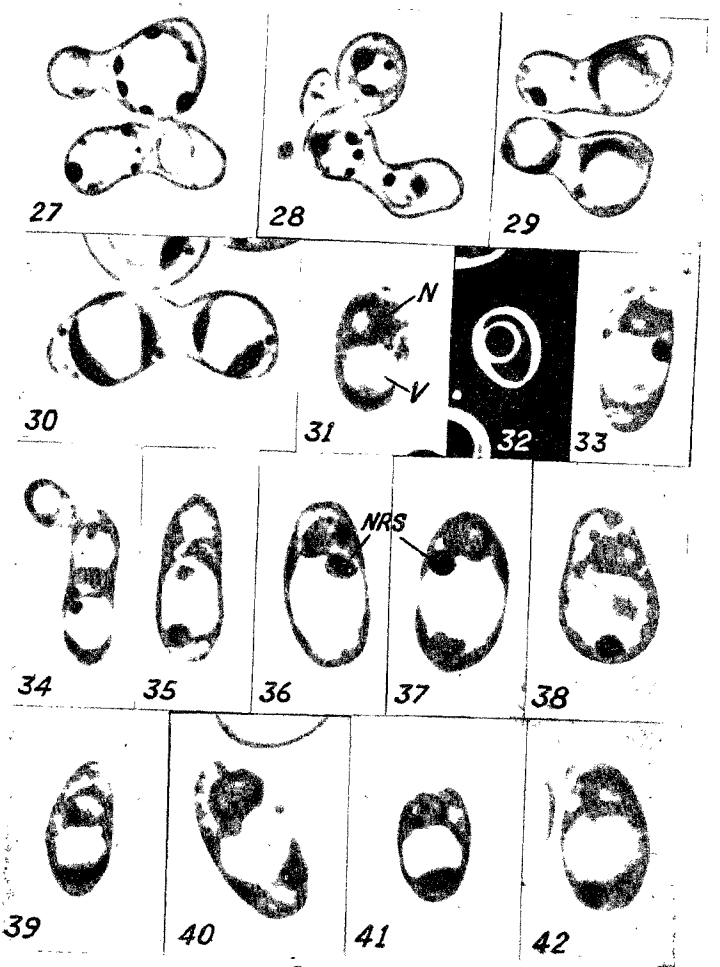
PHOTO 44. The entire vacuole is diffusely stained (Two hours). The irregular accumulation of the dye at the vacuolar boundary makes it appear brightly tinted.



PHOTOS 1–16



PHOTOS 17–26



PHOTOS 27–42

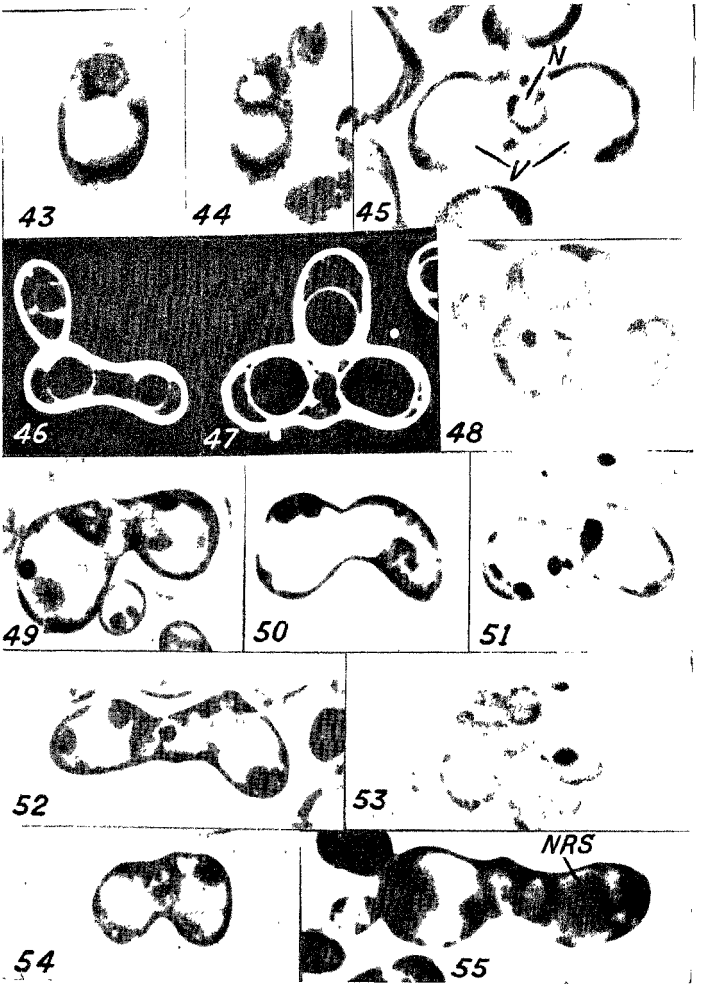

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PHOTO 45. Living unstained spore zygote. The nucleus is situated between the two vacuoles.

PHOTOS 46 and 47 The two vacuoles of the zygote have luminous borders under dark ground illumination.

PHOTOS 48–55. Reaction of living spore zygotes with visible nuclei to 0·01% neutral red.

PHOTO 48. A stained granule is seen in one of the vacuoles after 15 minutes.

PHOTOS 49–52. The changes observed in the vacuole at the end of 30 minutes.

PHOTOS 53 and 54. A neutral red stained structure is seen at the boundary of one of the vacuoles (45 min.).

PHOTO 55. One of the vacuoles is diffusely stained while the other shows only an irregularly stained region (two hours).

N, Nucleus; *V*, Vacuole; *NRS*, Structures stained by Neutral Red.

Magnification: Photos 1–26, × *ca* 2,500.

Photos 27–31, 33–45 and 48–55, × *ca* 4,500.

Photos 32, 46, and 47, × *ca* 2,700.