A CONSIDERATION OF THE VACUOLE IN NEUROSPORA CRASSA

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Studies were carried out on the nature of vacuoles of *Neurospora crassa* Shear and Dodge. Morphological and cytochemical studies revealed that vacuoles are indeed sacs of watery fluid containing mitrochondria and numerous dissolved substances suspended in the viscous cytoplasm. Nutritional studies have revealed that under all conditions of growth, the vacuoles of type I are a constituent of the hyphae of the fungus while the vacuoles of type II are not.

Introduction

It was Zalokar[1] who first described the morphology of the hyphae of *Neurospora crassa* and discussed the vacuoles. Later on he [2,3] described by employing cytochemical techniques (before and after centrifugation) the distribution of some important biochemical constituents in the cells. His interest was largely concentrated on cell organelles other than the vacuoles, and, he made an incidental mention of some constituents of the vacuoles without any systematic attempt to understand their nature. The present study has been undertaken for a better understanding of the nature of vacuoles in *Neurospora crassa*.

Materials and Methods

An albino mutant of *Neurospora crassa* Shear and Doge (IMI—101718), isolated as a contaminant at the Cambridge Botany School in 1956 and identified by Professor A.M. Srb to be possessing certain peculiarities different from albino mutants of *N. crassa* already in his (Srb) possession has been used in this study.

The media used were the Czapek Dox agar[4] modified by the addition of 0.005% Difco yeast extract and Vogel's medium "N"[5] and were solidified with 1.5% Davis agar. Standard inoculations were made by a No. 3 cork boring from the edge of a 24-hour old colony. The inoculum were placed centrally or at the edge of a plate. Incubation was effected at 22°C.

Observations

I. Morphological and Cytochemical

Hyphal Contents and Vacuoles.—During the examination of growing hyphae under microscope, it has been noticed that the contents of the hyphal cells vary from the apical cells to the central cells (near the inoculum), and some six regions as detailed below proceeding from the periphery to the centre of the colony can be recognised (Table 1).

1. Those with the apical and sub-apical cells containing uniformly dense cytoplasm without a refractive body or bodies.

2. Cells with the contents still dense and uniform but having one rounded body or vacuole near the anterior septum. This type of vacuole shall hereinafter be referred to as the type-I vacuole.

Table 1.—Five Observations on the Main *Hyphae* of *Neurospora crassa*.

Observation/cell number	1	2	3	4	5
Homogeneous cytoplasm	1–5	1–5	1–5	1	1–2
One spherical type-I vacuole	6–17	6–32	6–15	2–22	3–30
One spherical type I vacuole and few small type-II vacuoles	18–38	33–40	16–40	23–47	31–50
One spherical type-I vacuole and numerous small type-II vacuoles	39–70	41–80	41–67	—	51–65
Large type-II vacuoles against wall of cell ..	71–95	81–96	68–94	48–160	66–102
Fewer type-II vacuoles against wall at anterior end of cell	96–125	97–144	95–107	161–170	103–106
Occasional type-II vacuoles	126–149	145–191	108–182	171–220	—
No vacuoles, clear streaming cytoplasm ..	150–366	192–314	183–260	221–237	107–147

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3. Cells with protoplasmic contents still uniform but numerous shiny inclusions and one type-I vacuole. These shiny bodies grow in size and number with the age of the cell. These will be referred to as the type-II vacuoles.

4. Region in which the contents of the cell appear to be full of large shiny bodies which are considered to be the enlarged type-II vacuoles. In between is a small amount of protoplasm.

5. In the protoplasm itself there are varying sizes of type-II vacuoles. They become fewer and smaller with the age of the cell. The protoplasm appears to be a fluid of very thin consistency.

6. Regions near the centre of the colony or inoculum, wherein the contents appear completely uniform.

The circular inclusion which has been referred to by Zalokar[1] as a vacuole, (here type-I vacuole), no doubt shrinks when hyphae are placed in hypertonic solution[6] presumably due to the loss of water. These type-I vacuoles are capable of movement along with the cell contents and can pass from one cell to another sometimes merging with the stationary spheres and at other times disintegrating during their passage through the septal pore but equally being formed by the coalescence of smaller bodies.

The smaller spherical bodies, the type-II vacuoles are prominent in the moving cell content from 16-50. Further back still there are rather large bodies (about 10μ) in the outermost part of the cell and pressed against the wall to give a semicircular appearance (Fig. 2a) and are called as here type-II vacuole. These large type-II vacuoles are occasionally seen to be moving along with the cell content but more often are found to be static apparently attached to the outer most part of the cell and with protoplasmic movement going on within the place between them.

VISUAL EXAMINATION

Examination of vacuoles of the type-I and type-II vacuoles in the 24-hour old hyphae of *Neurospora crassa* under the lower magnification shows them to be clear spaces in the more dense cytoplasm. Examination by highest power of the microscope and by the phase-contrast microscope fails to reveal any structure. The shrinkage of the vacuoles on immersion in hypertonic solutions suggests that the vacuoles contain water and other dissolved substances only. When the vacuoles

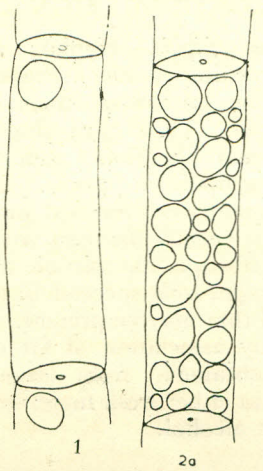

Fig. 1.—*Neurospora crassa*—Diagramatic representation of (1). apical cell with a vacuole of type-I near the septum, (2a). Middle region cell packed with vacuole type-II.

loose water in hypertonic solutions, observation suggest that they do not entirely disappear and that some part of the contents or the boundary-membrane remains as a lens-shaped body which grows in size and gives rise to the vacuole once more when the fungus is immersed in an isotonic solution.

FIXATION

Normal method of fixation using alcohol, acetic acid, chromic acid and formalin based fixative were employed. These lead to the collapse of the vacuoles but how far this is due to a general disruption of the cytoplasm or due to the destruction of the lining membrane of the vacuole is not clear. However, good fixation can be obtained by plunging the hyphae (growing on cellophane) into alcohol cooled to -70°C and later gradually warming to 0°C in cold alcohol saturated with picric acid. The fact that they can be fixed by some method at last shows that they are part of the cytoplasmic structure. This is further borne out by Zalokar's[7] electron micrographs of vacuoles.

CYTOCHEMICAL TESTS

The cytochemical tests for the vacuolar contents pose a number of problems. First of all, if the cytochemical tests are applied to the unfixed hyphae, the vacuoles cannot be recognised after treatment. If, on the other hand, fixation is resorted to, considerable washing to remove fixing material becomes necessary and contents of the vacuole might all be leached away. The following negative results have therefore to be treated with caution.

The methods employed published by Glick.[8]

1. *Fats and Lipids.*—Hyphae stained with Sudan IV according to the method of Key and Whitehead show numerous small fatty particle stained deep red. Their pore plugs also stained deep red confirm Shatkin's[9] description of the pore plug being lipoid in nature. There is some difficulty in applying this method for the examination of vacuoles because fixation with formalin as recommended disrupts the vacuole and treatment with alcohol might only succeed in removing any lipoid material that may be present. However, no evidence of any association of fat or lipid with vacuole was obtained from large number of attempts to stain either fresh material or that fixed in formalin or alcohol.

2. *Carbohydrates and Polysaccharides.*—Carbohydrates as detected by the Hotchkiss method were shown to be distributed over all the cytoplasm but were found to be absent in the vacuoles.

3. *Glycogen.*—Glycogen tested by the Bauer-Feulgen method and by the addition of iodine were found to be well distributed everywhere except in the vacuoles.

4. *Proteins.*—Arginine containing protein was tested for by the method of Serra. While the presence of such proteins was clearly indicated throughout the cytoplasm, no evidence, however, was seen of the material in the vacuoles.

5. *Deoxyribonucleic Acid.*—The hyphae were stained by the azur. A method of Huedschman[10] which is claimed to be specific for deoxyribonucleic acid. While the nuclei were stained prominently, no appreciable staining on any of the contents of the vacuole took place.

It appears therefore that all the staining procedures failed to show any identifiable material in the vacuoles. This agrees in general with Zalokar's finding. He[3] centrifuged the hyphae and observed a partition of materials, the fatty particles and the vacuolar material being the least affected by the centrifugal force. He tried many staining procedures on the material and could only give evidence for the presence in the vacuoles of ribonucleic acid, succinic dehydrogenases, cytochrome oxidase and alkaline and acid phosphatase, as distinct microscopic granules.

Such evidence as is available suggests that the vacuoles are indeed sacs of watery fluid containing a few metachromatic granules suspended in the more viscous cytoplasm, and as such they appear to be similar to the vacuoles of the higher plants.

NUTRITIONAL STUDIES

Whatever the nature of the vacuoles, its presence in the hyphae can be controlled to some extent by altering the medium. When a number of media were being investigated for their value for certain experimental procedures with *Neurospora crassa*, it was noticed that vacuoles of type-II were entirely absent from the main growing hyphae when the fungus was grown on Vogel's medium "N" with 1.6% sucrose and 0.005% yeast extract. Examination of main growing hyphae now showed that the apices and sub-apical cells appeared normal and completely filled with dense granular cytoplasm; the next cells in order showed the presence of a vacuole of type-I near the anterior septum of each cell; the remaining cells in the centre of the colony had no vacuoles and a less dense cytoplasm.

It became a matter of interest to discover what property of the medium might be important in controlling vacuole formation.

The first variation tried involved changing the carbon/nitrogen ratio by adding different amounts of sucrose and ammonium nitrate to the medium. This method showed a marked effect on the vacuoles of type-II. In Table 2 the observations

TABLE 2.—EFFECT OF THE CARBON/NITROGEN RATIO ON THE APPEARANCE OF THE *Hyphae* OF *Neurospora crassa*.

Medium		Distribution of vacuoles
Normal Vogel's medium		No vacuoles of type-II
Sucrose	1.6%	Vacuoles of type-I in cells 4-50 (approx.)
NH_4NO_3	0.2%	of main hyphae
Yeast extract	0.005%	
½ Carbon Vogel's medium		Vacuoles of type-II in smaller side branches only
Sucrose	0.8%	Vacuoles of type-I in cells 4-50 (approx.) of
NH_4NO_3	0.2%	main hyphae
Yeast extract	0.005%	
¼ Carbon Vogel's medium		Vacuoles of type-II common inside branches and occasionally present
Sucrose	0.4%	in main growing hyphae
NH_4NO_3	0.2%	Vacuoles of type-I in cells 4-50 (approx.)
Yeast extract	0.005%	of main hyphae
⅛ Carbon Vogel's medium		Vacuoles of type-II common inside branches and in
Sucrose	0.2%	main growing hyphae
NH_4NO_3	0.2%	Vacuoles of type-I in cells 4-50(approx.)
Yeast extract	0 005%	of main hyphae

have been presented from a study of a series of media derived from Vogel's medium by varying the carbon as sucrose and keeping the nitrogen as the ammonium nitrate constant; the pH was not adjusted. Each observation was obtained by scanning 5 plates for each treatment. It can be seen that with the reduction of carbon in the medium, the amount and the distribution of vacuoles of type-II increased until with the lowest proportion of carbon they were widespread. The fact that the ratio alone is not responsible is shown by an extension of this experiment where sucrose was maintained at $\frac{1}{4}$ of the normal level and ammonium nitrate was gradually reduced (e.g. $1, \frac{1}{2}, \frac{1}{4}$ and $\frac{1}{8}$). The vacuoles of type-II did not disappear as expected if only carbon/nitrogen balance was responsible.

The manipulation of the medium has number of other possible side effects on *e.g.*, pH, osmotic concentration, staling etc., and these have not been pursued. What is interesting is that the vacuole of type-II are not the constituents of the hyphae under all conditions of growth while the type-I vacuoles are.

Discussion

The vacuoles are of considerable interest. The fixed vacuoles (type-I vacuoles) which only occur at the anterior end of certain cells constitute a characteristic feature of *Neurospora crassa* and so far as the author is aware have not been reported for any other fungi. The moving vacuoles (type-II vacuoles) which are wide spread in the posterior cells are constantly moving in an anterior direction, apparently growing smaller and in some cases merging with a fixed vacuole. The problem of their nature so far has eluded the author but it is clear that they are composed of water and dissolved substances with only a few larger particles. The evidence from Zalokar's [3] cytochemical tests that they contain phosphatases of various sorts is suggestive. One other aspect of the vacuoles is interesting; Buller [11] and Zalokar[1] have suggested that the development and accumulation of vacuoles may be responsible, in whole or in part, for the forward movement of the cytoplasm. Not only is this not borne out by observation but also by the situation demonstrated where the fungus on particular media fail to develop vacuoles contradict these suggestions. This is not true only for *Neurospora crassa* but also for *Pyronema confluens*, a fungus with well-marked vacuoles which were commented upon by Buller himself.

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