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## WALL STRUCTURE OF ASCOSPORES OF NEUROSPORA TETRASPERMA

R. J. Lowry and A. S. Sussman<sup>1</sup>

THE DARK COLOR and tough exterior of *Neurospora* ascospores makes them difficult subjects for cytological investigation. With this in mind, these cells were treated with sodium hypochlorite in an attempt to bleach them and to soften their surface. Although this treatment proved ineffective for the intended purposes, the ribbed layer, for which the genus was named, became swollen as a result of the treatment and could be freed from the cell, thereby greatly simplifying its study.

Meanwhile, Dodge (1957) had called attention again to this layer of the ascospore surface whose formation and structure he had described previously (1928, 1934). Although these aspects of the ascospore surface have also been studied by others (Lindegren and Scott, 1937; Faull, 1930) the true nature of the ribs, their relation to the rest of the cell and their chemistry has remained undefined. The present work is concerned with the elucidation of some of these details and with an approach to the analysis of the entire ascospore surface.

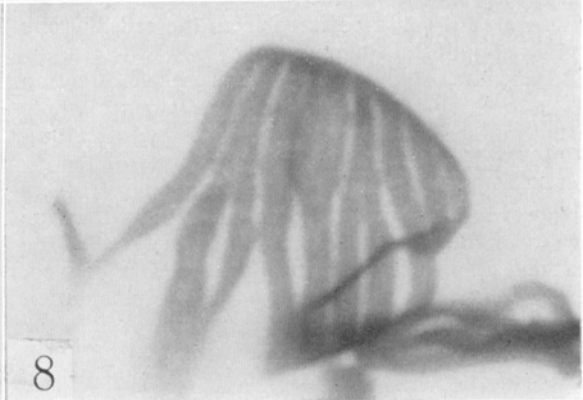
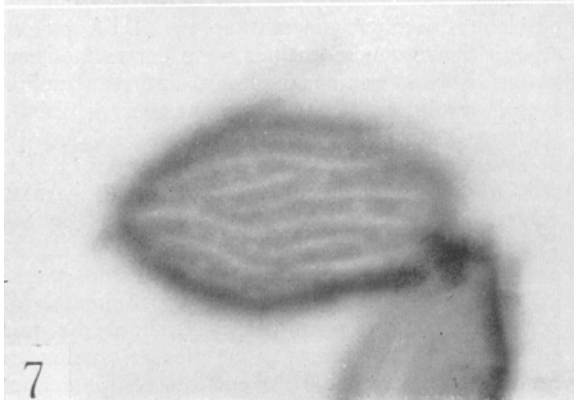
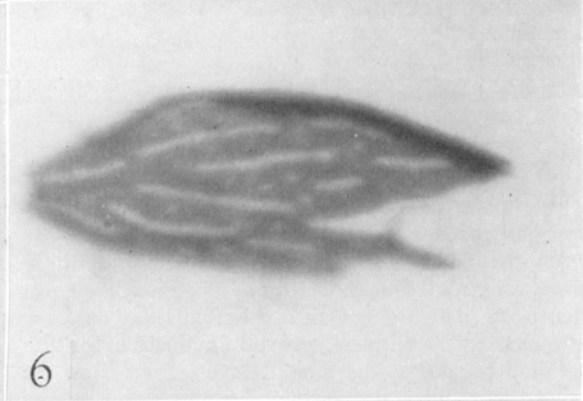
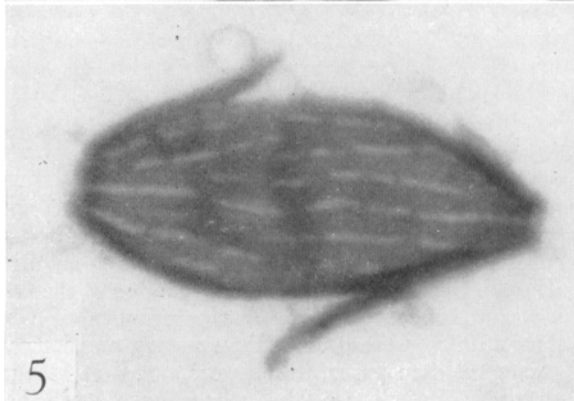
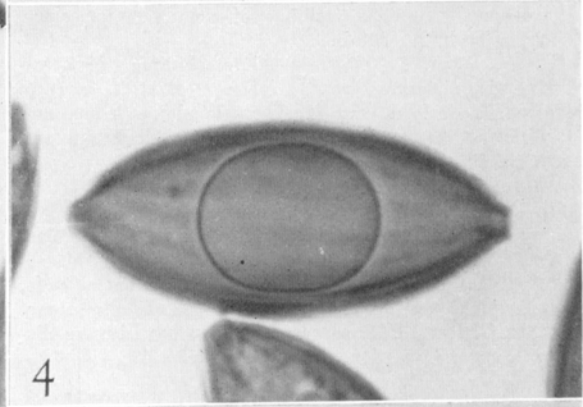
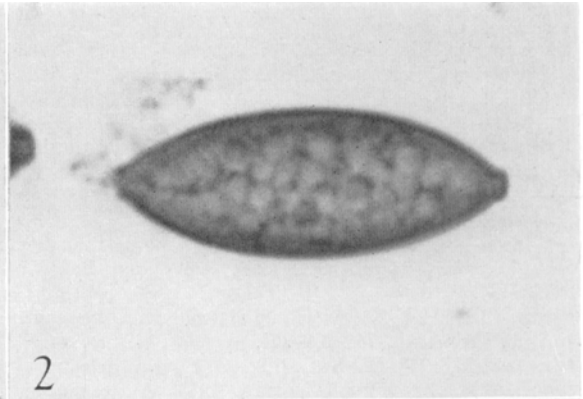
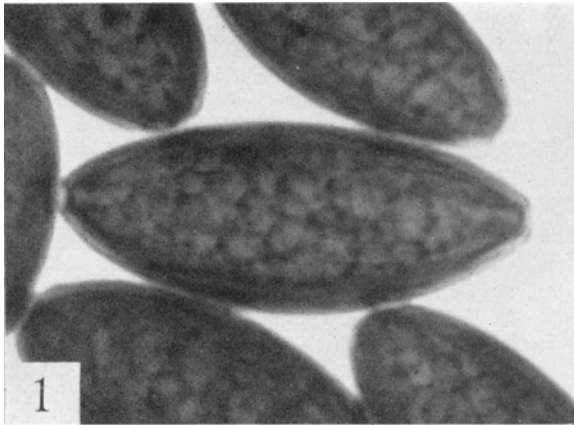
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**MATERIALS AND METHODS.**—Ascospores of *Neurospora tetrasperma* were obtained from a cross of strains 394.4 and 394.5 which were acquired through the kindness of Dr. B. O. Dodge of the New York Botanical Garden. The techniques of Goddard (1935) were used in the growing and harvesting of the ascospores, which were stored at 4°C. until used. Before use, the spores were washed in ethylene diaminetetraacetic acid (EDTA or Versene). Spore concentrations were determined, activation by heat was accomplished and germinability was ascertained in the same manner as used in previous studies (Sussman 1954).

Two means were used to study the nature of the ribs of the outer layer of the cell wall, hereafter referred to as the ribbed layer of the cell surface. These were the following:

1. Stationary incubation of the ascospores in 20 per cent commercial Clorox (approximately one per cent sodium hypochlorite)—It should be pointed out that Clorox seems to deteriorate on standing, for in our experience, different lots of this material gave different results. When newly opened bottles were used, even one tenth of the above amount



sufficed. It is necessary, therefore, to be empirical in approaching the use of Clorox in this way. As usually performed, our experiments consisted in mixing equal parts of Clorox at the right dilution, and a suspension of ascospores (2 mg. per ml.). The mixture was incubated for 30–60 min. at room temperature in order to affect the capsules so that their bond to the cell was loosened sufficiently for them to be removed. At this time the cells were washed by centrifugation in 3–5 changes of distilled water and concentrated in a small volume of water.

2. Shaking in a Mickle vibratory tissue homogenizer—Suspensions of ascospores and Clorox were prepared as above and placed in the vessels of the homogenizer. This material was agitated for 8–20 min., depending upon the strength of Clorox used, after which time the spores were washed as described before. When this method was used the ribbed layer was entirely dissolved and did not appear in the relatively intact form in which it was recovered as a result of treatment by stationary incubation.

RESULTS.—In the original generic description of *Neurospora* the ascospores were designated as being “black or greenish black when mature,” and “longitudinally ribbed” (Shear and Dodge, 1927). These ribs are visible in the untreated spore as diagonal lines which appear along its edge (fig. 1). Also visible is a hyaline layer which can be seen at each end of the spores, over the germ pore. The ribs which give the genus its name are shown in fig. 3, in which one may clearly observe the branching of the ribs and the clear areas between. The large central globule in the cell shown in this figure contrasts with the smaller and more numerous ones seen in the cells in fig. 1. Usually such large globules indicate that the cells have died. For purposes of comparison, the same cell illustrated in fig. 3 was photographed in a median optical section instead of at the surface, and the invisibility of the ribs suggests their location on the surface of the ascospore. The germ pores are very noticeable in this figure as flattened areas at the ends of the cell.

When spores were shaken in Clorox by the method described previously, in order to remove the ribbed layer, cells like the one shown in fig. 2 were obtained. It should be noted that the hyaline layer around the germ pores has been removed by this treatment, although the cell appears normal as judged by the small globules which are like those in the untreated cells in fig. 1. Their viability was verified by the fact that Clorox-treated cells, if

washed free of the oxidant and resuspended in water, germinated normally after activation.

Dodge (1957) alluded to the difficulties involved in describing the branching of the ribs on the ascospores. Therefore an attempt was made to remove the ribbed layer in order that its structure could be analyzed. This was accomplished by stationary incubation in hypochlorite for periods of time up to 45 min. After removal of the hypochlorite by washing in water, in order to stop the reaction, many ribbed layers could be found to be free in suspension along with cells which looked like those in fig. 2. Several of these ribbed layers are illustrated in fig. 5–8, in which it can be seen that they consist of flat strips of material with thin places in between. When the thin places are weakened by Clorox, tears develop in the interstitial areas and the tatters illustrated in fig. 8 are obtained. In addition, fig. 6 and 7 disclose that pores are present and are distributed at irregular intervals in the ribs of this layer.

Striking evidence of the presence of the ribbed layer and its surface localization is provided in fig. 9 and 10. Cells were treated in Clorox for 60 min. in standing solutions, after which they were resuspended in water. Although the swollen ribbed layer completely surrounds the cell in fig. 9, that around the cell in fig. 11 appears to have been retracted from the areas around the germ pores. Longer incubation in Clorox results in the complete removal of the ribbed layer from the cell.

After removal of the ribbed layer, the ascospores were still dark brown and rigid although somewhat lighter in color than before being treated with Clorox. In an effort to study the remainder of the spore surface, cells from which the ribbed layer had been removed by standing incubation in Clorox for 15 min. were mounted in water on a slide. A cover slip was then placed over the mount and used to squeeze the cells. As a result of such treatment the melanized layer of the spore surface was broken in some cases, disclosing the presence of still another well-defined surface layer beneath. This is illustrated in fig. 11–16 which demonstrate progressive stages in the exposure of the inner cell wall. The melanized wall appears to be somewhat brittle for it seems to fracture instead of tear. The flattened protuberances at the ends of the ascospore, through which the germinal buds penetrate upon germination, are part of the melanized wall for, as fig. 16 discloses, they disappear upon its removal. This figure also illustrates the inner cell wall which probably surrounds the protoplast.

Allusion has been made previously to the fact

Fig. 1–8.—Fig. 1. Untreated ascospores of *N. tetrasperma*.—Fig. 2. Ascospore from which the ribbed layer has been removed by treatment with 1% sodium hypochlorite.—Fig. 3. Ribs on surface of untreated spores. Photograph was taken by focussing on the upper surface of the spore.—Fig. 4. Same cell as in Fig. 3 except that it was photographed in median optical section.—Fig. 5–8. Ribbed layers removed from ascospores by action of sodium hypochlorite, and stained in 0.4% gentian violet.—All cells magnified approximately  $\times 2,000$ .

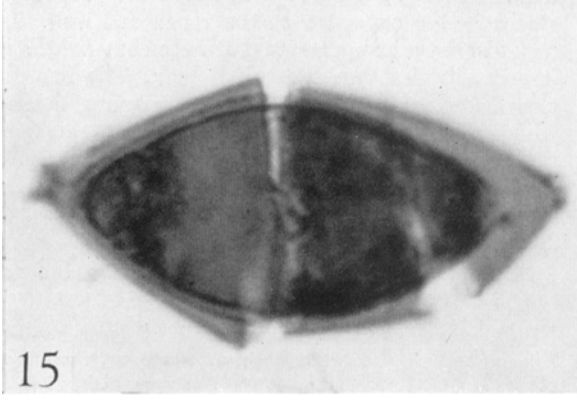
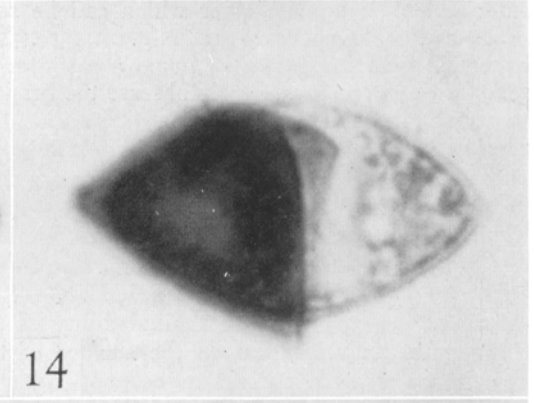
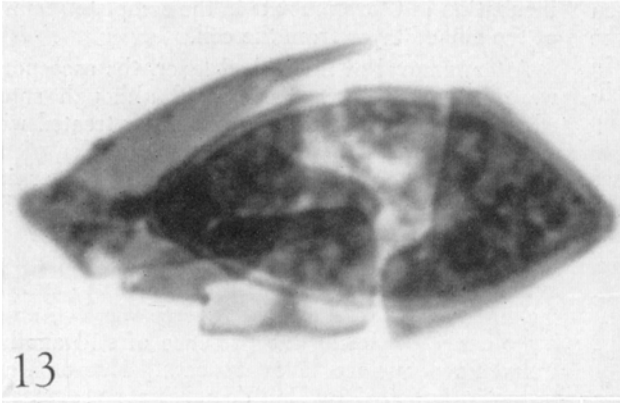
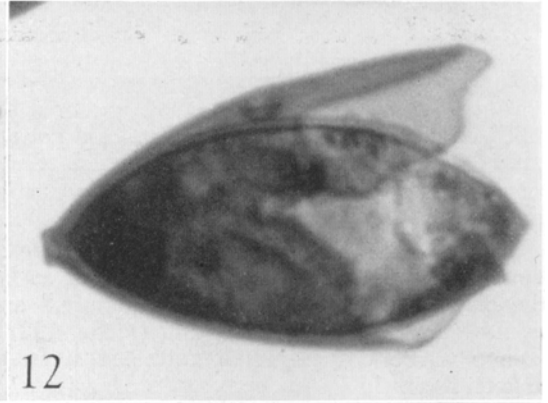
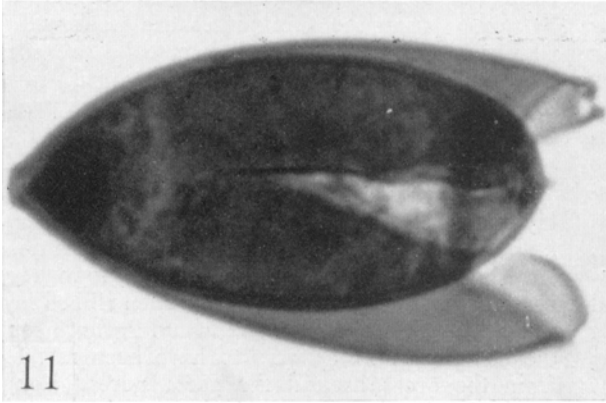
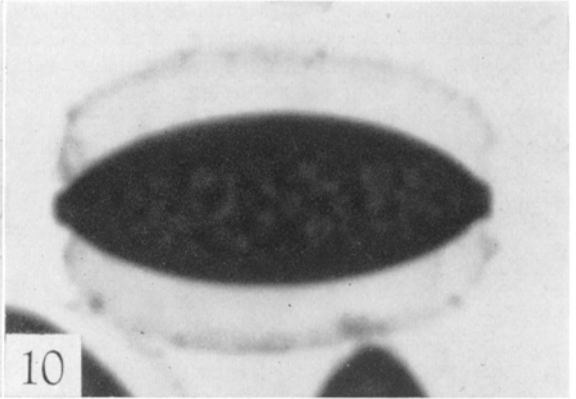
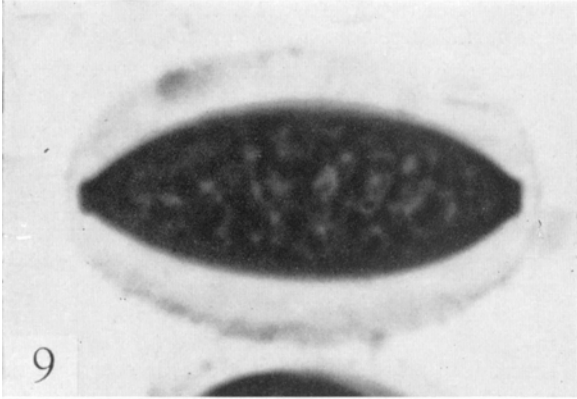


TABLE 1. Germination of ascospores of *N. tetrasperma* exposed to Clorox for varying periods of time. Equal parts of solutions containing 20% Clorox and 3 mg. per ml. of ascospores were mixed and incubated at room temperature. At the times noted below aliquots were removed and washed in 5 changes of distilled water after which they were activated and incubated.

Time in Clorox (min.)	Percentage germination
Stationary:	
10	86
20	79
30	81
45	56
60	32
90	23
Mickle homogenizer:	
10	91
20	79
30	0
40	0

that controlled treatment with Clorox did not result in the killing of the cells, as judged by their appearance. In order to check this more directly, the germination of ascospores after treatments of this kind was studied. Both stationary incubation and shaking in the Mickle vibratory tissue-homogenizer were used. At various times after the start of exposure to Clorox, 0.5 ml. aliquots were withdrawn and washed in five changes of distilled water. The volume was made up to 2 ml. and the spores were heat-activated, incubated at room temperature, and the percentage germination determined after 4 hr. The results provided in table 1 disclose that Clorox does not prevent germination after 30 min. of treatment by stationary incubation. However, beyond this time, a marked effect is apparent, although over 20 per cent germination is still obtainable after 90 min. On the other hand, treatment in the Mickle homogenizer results in a small decrease in germinability after 20 min. and in complete killing after 30 min. It should be noted that in the latter case all visible evidence of the presence of the ribbed layer had disappeared after 10 min. of shaking in Clorox.

Because the ribbed layer could be removed without impairing germination, it was possible to study the physiological effects of its removal. Previous work (Lowry et al., 1957; Sussman et al., 1957) had suggested that the surface of the ascospore acts to adsorb cations in much the same manner as an ion exchanger. Furthermore, the adsorbed cations were shown by indirect means to be arranged in depth on the surface rather than in a single plane.

TABLE 2. Effect of removal of ribbed layer upon the ability of ascospores of *N. tetrasperma* to adsorb cations. Cells with and without coats were exposed to  $1 \times 10^{-2}M$  solutions of  $UO_2(NO_3)_2$ ,  $AgNO_3$  and  $ThCl_4$  for 30 min. after which the residual salt was removed by centrifugation in 5 changes of distilled water. Activation, incubation and counting were performed as usual.

Treatment	Percentage germination		Relative germination	
	Controls	Minus ribbed layer	Controls	Minus ribbed layer
None	86	67	100	100
$UO_2^{++}$	16	11	18	16
$Ag^+$	0	0	0	0
$Th^{++++}$	57	64	66	95

Therefore, the relation of the ribbed layer to the adsorption of cations by Neurospora was studied.

One means of accomplishing this was by testing the effect of toxic cations upon the germination of ascospores. Adsorption of these was effected by mixing a suspension of 1 mg. per ml. of dormant ascospores with an equal volume of  $2 \times 10^{-2}M$  of various salts. This mixture was incubated at room temperature for 30 min. on a reciprocal shaking machine, after which the ascospores were washed free of the residual salt by centrifugation in five changes of distilled water. The spores were resuspended in water, activated and the percentage germination determined as before. It can be seen from the results in table 2 that the removal of the ribbed layer made no difference in the effect of  $UO_2^{++}$  and  $Ag^+$  upon spore germination. On the other hand, the toxicity of  $Th^{++++}$  was greatly diminished by this means.

A quantitative measure of the effect of removing the ribbed layer upon cation adsorption was obtained by determining the amount of methylene blue removed from solution by ascospores (Sussman and Lowry, 1955). This was done by mixing equal volumes of  $5 \times 10^{-5}M$  methylene blue and ascospore suspension (one mg. per ml.) followed by incubation at 20°C. for 10 min. on a shaking machine. Thereupon the spores were removed by centrifugation and the residual methylene blue determined as described before (Sussman and Lowry, 1955). As can be seen from the data in table 3, a large reduction in the amount of methylene blue adsorbed results from the removal of the ribbed layer. After 10 min. when the last visible evidence of the ribbed layer disappears, the reduction is greatest. With continued treatment, however, larger amounts of dye are adsorbed.

DISCUSSION.—The acidic nature of the surface of Neurospora ascospores is suggested by its affinity for methylene blue and other cations and by its

Fig. 9-16. Fig. 9, 10.—Ascospores treated with sodium hypochlorite in order to swell the ribbed layer. It has been swollen so that the germ pore is revealed.—Fig. 11-16. Progressive stages in the removal of the melanized wall of ascospores of *N. tetrasperma*. All cells magnified approximately  $\times 2,000$ .

TABLE 3. Uptake of methylene blue by intact ascospores of *N. tetrasperma* and by those without ribbed layers. Ribbed layers were removed by treatment in a Mickle homogenizer for the times indicated. After being washed in 5 changes of distilled water the spores were mixed with an equal volume of  $5 \times 10^{-5}M$  methylene blue and incubated at  $20^{\circ}C.$  for 10 min. on a shaker.

Time in hypochlorite (min.)	Residual methylene blue	Uptake of methylene blue	Percentage of control
control	$0.44 \times 10^{-5}M$	$2.06 \times 10^{-5}M$	---
10	$2.25 \times 10^{-5}M$	$0.25 \times 10^{-5}M$	12
20	$2.20 \times 10^{-5}M$	$0.30 \times 10^{-5}M$	18
40	$1.94 \times 10^{-5}M$	$0.56 \times 10^{-5}M$	44

over-all negative charge as determined electrophoretically (unpublished experiments of the authors). That the majority of the sites which are responsible for the adsorption of methylene blue are located in the ribbed layer is borne out by the data in table 3. This is in contrast to cations like  $Ag^+$  and  $UO_2^{++}$ , toxic amounts of which are adsorbed even in the absence of this layer. However,  $Th^{++++}$  seems to be rendered innocuous by the removal of the ribbed layer, leading to the conclusion that it is adsorbed primarily to this surface component. It should be emphasized that these conclusions are based upon previous work (Lowry et al., 1957) which has shown that cations are probably restricted to the exterior of the cell until shortly before protrusion of the germ tube. Therefore any toxic effect resulting from treatment of dormant cells must be due to adsorbed cations. Further evidence to support the suggestion that there are different adsorbing sites on the surface of the *Neurospora* ascospore may be found in the work of Lowry and Sussman (1956) with methylene blue and Polymyxin-B.

On the basis of the work reported herein, the surface of the ascospore appears to consist of at least three elements: a ribbed layer, a melanized rigid intermediate coat, and an inner wall which surrounds the protoplast. The description of these components of the surface was made possible by the action of Clorox in permitting the removal of the outermost layer. It was of interest, therefore, to compare these results with those of Le Gal (1947), who did extensive work on the surface ornamentation of operculate discomycetes. In common with our findings, Le Gal discovered that sodium hypochlorite (*Eau de Javel*) dissolved the elements on the spore surface which were responsible for their ornamentation. She refers to these as the callospectic compounds, but their chemical nature is still undefined. Other treatments which affected the surface included Schulze's solution (20 parts nitric acid to 3 of potassium chlorate) and, to a lesser extent, sulphuric acid. Here too the results parallel our own, for we have learned (unpublished results) that strong oxidants like potassium permanganate

act similarly to Clorox but that inorganic bases are ineffective under these conditions. Therefore the conclusion seems inescapable that Clorox reacts with the spore wall by virtue of its oxidizing capacity and not simply because of its basicity.

Although it is still too soon to make generalizations concerning the nature of the spore wall in various ascomycetes, it might be instructive to attempt to draw analogies between the surface structures of the discomycetes studied by Le Gal and those of *Neurospora*. That these structures may be of taxonomic and phylogenetic significance within the former group seems clear, and it may well be that wider application will be possible. For example, Rhoads (1918) has described a colorless "exospore" in *Daldinia concentrica* which dehisces after being treated with dilute alkali, while Yu (1954) illustrated a "perispore" in *Ascobolus stercorarius* which has been swollen by treatment of a similar kind. In another group, the work of Graham (1957) on the teliospore wall of *Tilletia controversa* seems directly pertinent, for he reports the presence of a reticulate surface structure which is swollen by treatment in sodium hypochlorite.

In view of the possibility that spore characters may become of increasing importance, we feel that the nomenclature of the surface layers must be clarified. The system which we follow is that described in detail by Le Gal (1947) on the basis of previous suggestions by Mattiolo (1921) and Malençon (1929). According to this nomenclature, the following structures may be delineated:

1. Endospore wall—that which is the most internal surface structure of the spore and which surrounds the protoplast.

2. Epispore wall—the layer which immediately surrounds the endospore. This includes, with the endospore, the essential elements of the spore. The epispore and endospore walls provide support and rigidity to the spore and maintain its shape.

3. Perisporic structures—"formations périsporiques"—this includes all the layers, or all other elements of the spore which surround the spore proper and which can be absent without interfering with the functioning of the spore. The perisporic structures include the *subperisporic* layer, which is often so narrow as to be inconspicuous, on the surface of which is a membranous pellicle, the *perispore*.

The perispore itself may be formed before the ornaments on the spore surface, in which case it is called the "*tunique externe de l'assise*." In this instance the ornamental material usually does not cross this layer. On the other hand, if the perispore arises simultaneously with the raised spore ornaments then it is called the "*coque interperisporique*."

In the case of the surface of ascospores of *Neurospora*, the interpretation of structure provided in fig. 17 is offered. The innermost wall is considered

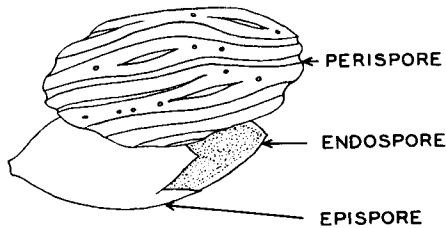


Fig. 17.—Diagrammatic representation of ascospore wall layers according to the terminology of Le Gal (1947).

to be the endospore, the melanized intermediate one is called the epispor, and the ribbed layer seems to be analogous with the "pellicule membranaire" of Le Gal, which would be the perispore.

Up to the present we have not been able to detect a sub-perispore layer, but its thinness often renders it imperceptible so that it would easily have been missed.

The chemical nature of the perispore of the ascomycetes is virtually unknown. Le Gal refers to it as consisting of callos-pectin because of its affinity for basic dyes like methylene blue and cotton blue and because of its failure to give a positive test for cellulose. These are at best very non-specific criteria, and all that is indicated is that an acidic polymer which does not seem to be cellulose may be involved. Graham's work (1957) and that of Prentice and Cuendet (1954) offer promise that in the basidiomycetes at least, the chemical nature of the spore walls will be analyzed. It is clear that

before the phylogenetic significance of the structures studied can be evaluated, chemical comparisons must be made. In addition, the complexity of the spore surface suggests that it will be necessary to analyze each of the separate layers before an understanding of their origin and interrelationships can be reached.

#### SUMMARY

The structure of the surface of ascospores of *Neurospora tetrasperma* has been shown to consist of at least three elements: 1. the perispore, an outermost layer which is ribbed and closely appressed to the epispor; 2. the epispor, an intermediate coat which is melanized and rigid and, 3. the endospore, an inner wall which surrounds the protoplast. The removal of the outermost layer was accomplished by the use of Clorox, thereby permitting the observation of its structure. Analogies are drawn with greatest amounts by this layer,  $\text{Ag}^+$  and  $\text{UO}_2^{++}$  are largely adsorbed by other elements of the spore surface work of Le Gal (1947) on discomycete spore walls, and certain parallels are suggested. The effect of the removal of the outermost layer of the spore surface was studied with respect to the ability of the cell to adsorb cations. Whereas methylene blue and  $\text{Th}^{++++}$  appear to be taken up in face. The presence of at least two different adsorbing sites is indicated.

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