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OIL DROPS AND DE BARY "BUBBLES" IN ASCOSPORES

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Mycologists who have studied groups of Ascomycetes have usually included oil drops present in ascospores along with other spore characters of a species. If no distinct droplets appear in the spores this fact is ordinarily not mentioned. Boudier, in his Icones (1905–1910), beautifully illustrated ascospores with one or two oil drops in many species of Discomycetes. Rehm (1896) also regularly considered such droplets in ascospores as a diagnostic feature. Seaver, more recently (1928, 1951), pictured or mentioned oil drops in dozens of species of Discomycetes. This character is specific and not generic. For example, Seaver, covering the genus *Paxina*, reported oil drops present in 13 out of 14 species he placed in this genus.

De Bary (1884) pointed out that fatty substances may also appear as numerous small, yet distinct, bodies. They may also occur in an emulsified condition. Fatty matters in spores of *Neurospora* appear to be stored in both ways. A very high percentage of the ascospores of *Lachnea abundans*, however, show two very distinct oil droplets when the spores are mounted in water. These two types will be considered later.

Following his brief comments on the oil content of ascospores, de Bary (1866, 1884) continued with a paragraph reporting the results of some experiments dealing with the air and water content of the ripe ascospores. The 1884 edition covering this subject is essentially the same as the original edition. We may then believe that he still supported his original theory. Apparently contemporary mycologists and physiologists as well as later well-known students of the fungi, have either overlooked this paragraph or ignored it without comment. In any event, we are inserting below the paragraph as it appeared in the 1887 translation:

"The protoplasm of the spore in the young state is rich in water and when dry absorbs water rapidly from its environment. A spore lying in water appears under the microscope to be filled with it to turgescence. As it loses water it contracts, and if the wall is thin the membrane either sinks in irregularly or forms definite folds; round or ovoid spores take, therefore, the shape of a concave-convex lens, the edges of which are often bent over toward each other, and the spore has thus the form of a boat. Thick-walled spores do not change their form in drying, or change it but little. In many cases, an air bubble is formed inside the protoplasm as it parts with water, as in *Peziza abietina, P. melaena*, in *Sordaria*, etc. This is due to the fact that air, that is to say, some gas, is dissolved in the contents of the fresh

turgescent spore and is set free as soon as the quantity of water is brought down to a certain limit. The same result is produced if the spore in water is exposed to the influence of reagents like alcohol, glycerine or sulphuric acid which have the power of extracting fluids; the air bubble disappears when water replaces these agents."

De Bary seems not to have illustrated the bodies which he described so assuredly as air bubbles. Shear and Dodge (1927) published photographs of asci of three species of *Neurospora*. Some of the spores in each cluster of asci contained such bodies, but they made no comment on them. Faull (1930), however, in figure 1, F, gives us an excellent picture of a spore containing a body which she called an oil drop or oil globule. She had evidently not seen de Bary's discussion on such bodies, and she had overlooked the illustrations by Shear and Dodge.

Ninety years after de Bary had described this interesting phenomenon, Ingold (1956) gives us the results of his experiments working with *Sordaria fimicola*, *Pleurage curvicolla* and *Hypoxylon fuscum*. Ingold states that the separation of a gas phase in the formation of a gas bubble "is an invariable concomitant of drying." He suggests further that the "gas bubble" may originate within the spore just as it does during spore discharge in the annulus cell of a drying fern sporangium. This postulate is highly interesting and should be investigated further.

De Bary seems not to have discovered what, if anything, comes into view as the bubble, or whatever it is, gradually disappears following immersion of the spore in water. Ingold observed that if one examines a dry spore of a *Sordaria* a round paler zone in the center is always seen. Immersed in water the zone quickly appears as a "gas bubble" which then disappears within a few minutes. This behavior convinced Ingold that the gas phase is presumably water vapor. Neither de Bary nor Ingold reported the presence of typical oil drops or oil globules in spores of *Sordaria* and other species they studied. No one has mentioned, so far as has come to our attention, oil drops in normal spores of *Neurospora*. Oil or other fatty matters, however, must be present either as numbers of minute bodies, or in "emulsionized" form. See de Bary, the 1887 translation, also our own tests for oil in spores of *Neurospora* mentioned later. On the basis of our studies of *Neurospora* and other ascomycetes, we can accept, in principle, the view that formation of these "bubbles" involves at least two processes, alternate drying and wetting.

As de Bary pointed out, any drying reagent such as alcohol or glycerine, may be used or the spores may first be suspended in water, then left to air-dry. We have used for many years the Shear Mounting Fluid, SMF, for making more or less permanent slide mounts. This consists of 300 cc. of 2% potassium acetate, 120 cc glycerine, 180 cc. alcohol.

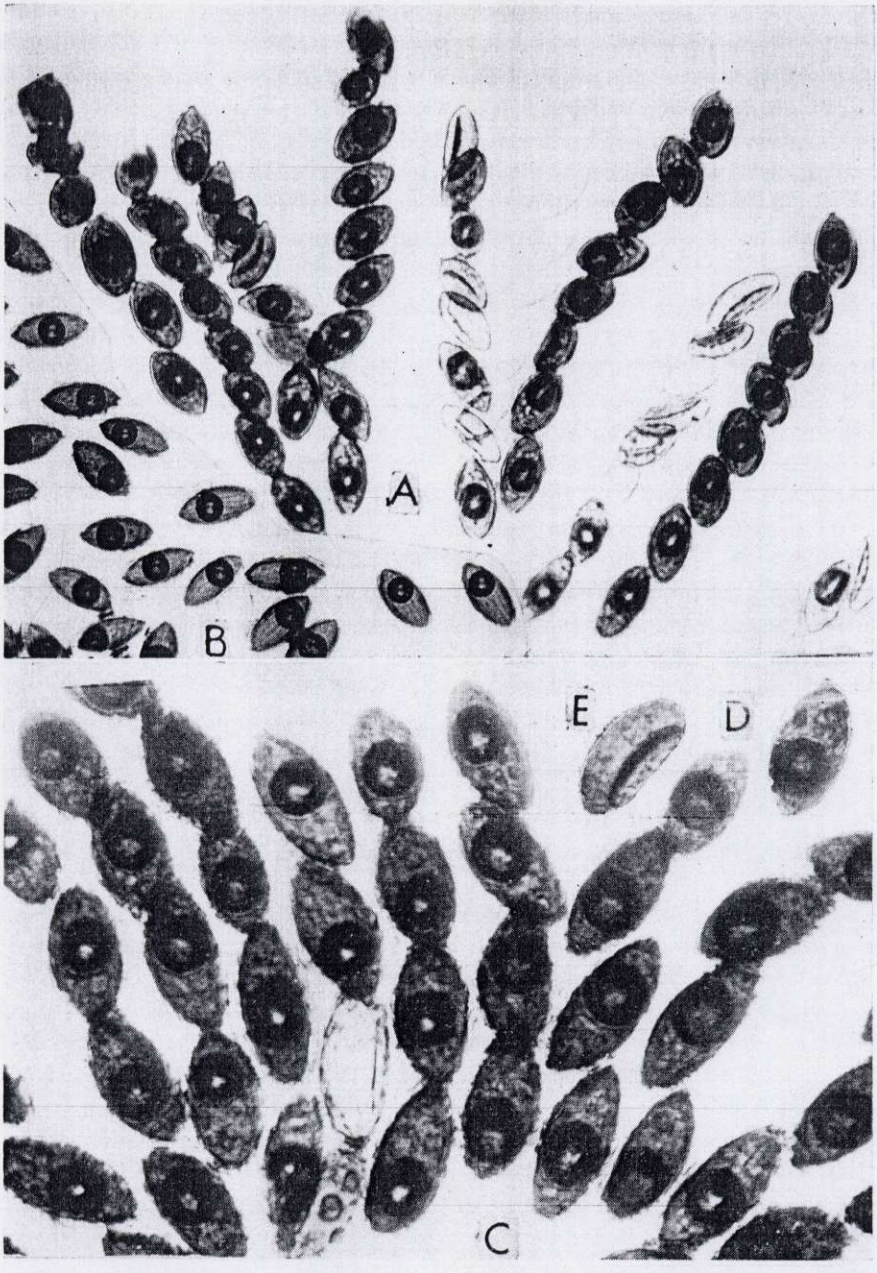

FIG. 1. *Neurospora tetrasperma.* All asci and spores had been immersed in the SMF. A. Part of a rosette of asci originally heterozygous, *AaEe*, for mating type *Aa* and *Ee* for 8-sporedness. Many spores contain one dBb each; other spores have collapsed due to the drying effects of the SMF reagent in which this rosette was mounted. Note that no collapsed spore contains a "bubble". B. From the same type of culture as A, except here the spores were from a spore-print and so were scattered about when mounted in the SMF. All these spores lie flat, having no doubt been pressed down by the cover-glass. The dBb in some spores is off-center. Such spores, if given space to move about, would have come to rest in an erect position. C. Part of a rosette of wild type asci. Except for a few spores that collapsed, each spore shows a single dBb. D. A spore with its dBb off-center. E. A collapsed spore. See text for further comment.

If one mounts mature spores of *Neurospora* in water he does not see any typical oil droplets, but there may be many granules present. Some of these may be the rib-forming bodies described by Dodge (1957); others may be fatty matters which some authors believe may represent stored food. If, now, one replaces the water in the mount with the SMF, it will be found that in a minute or so many ascospores will be forming conspicuous "bubbles", one in each spore. In such mounts the "bubbles" appear so quickly that it is not easy to see just where they came from. By diluting the SMF with water, a dilution (about 30%) can be made of such strength that the process can be followed very easily. Ingold (1956) reported that the "bubble" begins as a tiny one which may require half an hour to reach full size. With full strength SMF we have found that it requires only about a minute or so. These bubbles could consist of "some gas" as de Bary stated; or, as Ingold suggested, they may be a gas phase in the form of a bubble in which the gas is water vapor. Results of the following experiments would indicate that this question as to what these intracellular "bubbles" really are should be given a new look and further study. For the present at least, not knowing the true nature of these curious bodies, we shall refer to them as the dBb which the reader may translate either "de Bary bubbles" or "de Bary bodies", as he chooses.

It was stated above that ascospores of *Neurospora* mounted in water show no typical oil droplets. If the water is replaced with the SMF reagent, "de Bary bubbles" such as are shown in figure 1, A-E, quickly appear in the spores. If the drying reagent is then replaced with water, the dBb gradually decrease in size and finally disappear. This could be the end result so far as de Bary and Ingold carried on their experiments. However, if a particular spore is watched carefully as the dBb is gradually decreasing in size, there will be seen to appear, faintly at first, then more distinctly, one or two round bodies which we would all call oil drops. So, we would then see a spore containing the last vestige of the original dBb separate from and in addition to newly formed "oil drops" which are not characteristic of living normal spores of *Neurospora*. Finally, only the oil globules remain. This alternation of phases can be demonstrated in the reverse order. For example, start with spores, each with one or two oil globules still immersed in water. This water is replaced with the SMF reagent. In a short time the tiny dBb reappear, and as they get larger and larger it is the oil drops that begin to fade out and finally disappear altogether, at least as well defined oil drops. One can easily prove that ascospores of *Neurospora* do not normally and regularly contain typical oil drops, yet be rich in oil or other fatty matters. Take some perithecia from a culture eight or ten days old and make a squash mount of them in the S III solution. One will find a perithecium with a dozen or more asci with spores in various stages

of maturity. One ascus will have just delimited its spores while others may contain four dark-colored mature spores. Other asci will contain spores in intermediate stages of maturity. Up to the time of rib-formation all the contents of each spore will have been stained red, not just portions confined to oil drops. By adding SMF to these mounts they can be preserved, at least for some weeks.

Oil drops and dBb in a *Lachnea*. Other experiments were carried out using a little Discomycete, *Lachnea* (*Patella*) *abundans*. Dodge (1922) described how this species could be readily cultured. The dichotymously branched conidiophores produce the botryose conidia which serve as a good diagnostic character. A very striking feature is the long slender ascus with eight spores, each spore containing two very distinct oil droplets. Occasionally a spore may contain only one. The spores are hyaline but their walls are rather rigid.

While discarding a number of old slide-mounts of various fungi, it was noticed that one slide was labeled "October 1938, *Lachnea abundans*". This slide is still in perfect condition. The SMF had been used in preparing the mount. The interesting thing is that practically every ascospore contained a single dBb. Fortunately Dr. J. W. Groves was able to send us a fresh culture. It has proved to be most excellent material in many ways.

We continued to use the SMF as usual as a drying agent, supplementing this with a saturated solution of Sudan III in alcohol, this not only as a test for oil, but also as a drying agent at the same time. Small fragments of ascocarps were crushed in water under the cover-glass. Scores of asci showed that each ascus had eight spores and, as stated above, each spore had two oil globules, but no dBb. When the water was replaced by the SMF an intracellular dBb soon appeared in each spore. The most interesting thing that happened, but not at first realized, was that as the dBb was *forming*, the *two* oil drops were gradually *fading* away and, as distinct oil drops, they finally disappeared, leaving only the beautiful little dBb, *one* in each spore.

Reversing the treatment, this time replacing the SMF with water, the dBb very slowly decreased in size and finally disappeared. In the meantime, the two oil drops were gradually reappearing, but instead of each spore having, as usual, two oil drops, some spores now had only one (fig. 2). Sometimes the crushed fragments of the ascocarp were so thick that it required two or three days to replace all the SMF with water so that no longer could any dBb be found. Dodge, Singleton and Rolnick (1950) in their Fig. 2 C, show a cluster of asci of the 8-spored *N. tetrasperma* mutant in which two spores in each of two asci contained a very tiny dBb. It is impossible to say now whether those dBb were just appearing or just fading away. Several stages in this oil-drop "bubble" relationship in *Lachnea*

abundans are diagrammed in our Fig. 2 A-J. Those diagrams will be explained later.

Testing for oil in spores and asci. Because of the thick dark-colored spores of *Neurospora*, the oil tests were made mostly with *L. abundans*. Crushed fragments of an ascocarp had recently been immersed in the S III

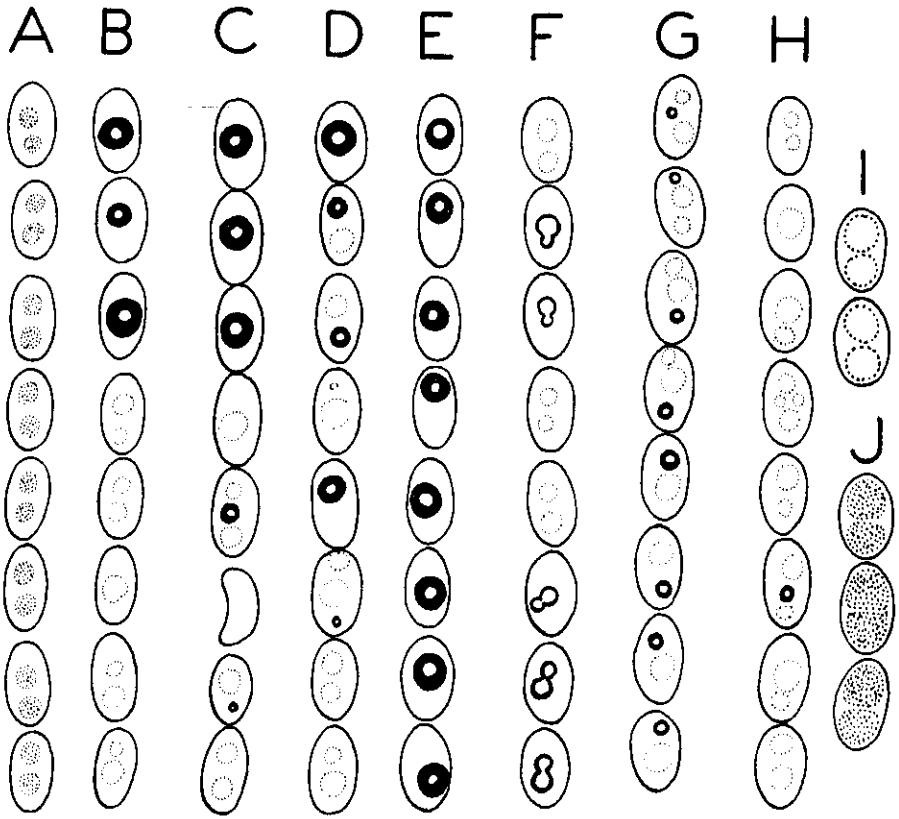

FIG. 2. *Lachnea abundans*. Diagrams of the eight spores in various asci. Asci A, I and J had been immersed in a Sudan III solution. All other asci had been at first immersed in SMF for some hours and then placed in water. A. Each spore had two drops stained reddish, reacting as oil drops. B. Three upper spores had each a single dBb, one of which was now shrinking. C. Again, each of the three upper spores contained one dBb while the 5th and 7th showed that as the dBb shrink, oil drops come into view. One spore had collapsed. D. The dBb in the 6th spore is about to disappear. E. All eight spores, one dBb each just before replacing the SMF with water. F. Interesting because the dBb in five spores seem to have blistered, a rare sight. G. After adding water, the dBb in each spore is on the way of disappearing as oil drops come into view. These dBb had all disappeared completely when examined half an hour later. H. Much like G but the dBb disappeared sooner. I. All eight spores contained two large unstained oil drops. J. Spores from the adjacent ascus I, but here the large oil drops had been stained dark-red.

solution and then left overnight in a damp chamber. It was then found that in 71 asci each spore contained a single dBb as shown in figure 2, E; in 20 other asci each spore had two oil drops which, in the meantime, had been stained some shade of red; in 15 asci each spore had two unstained oil drops; five asci had six spores, each with a single dBb but the other two spores had two colorless oil drops.

In another test, a fragment that had been mounted in water showed that each spore had two normal oil drops. The water was then replaced with the S III solution. In a very short time many spores began to develop the dBb. This also proved that the S III could serve very well as a drying agent for spores while the oil drops in certain other spores in the same mount were stained red.

A squash mount of a similar fragment was first immersed in S III for about an hour. Many spores soon began to develop the dBb. The squash mount was then flooded with water and left in a damp chamber for two days. When examined again, each of the eight spores in 38 asci now contained two unstained oil drops, while each spore in 22 other asci contained two large oil drops that had been stained some shade of orange or red. No spore now contained a dBb. Apparently, those spores that originally developed dBb could most likely have been the ones which, when immersed in water, were the same ones whose oil drops had not originally taken the S III red stain.

In another test, 26 asci showed a single dBb in each of the eight spores, while the oil drops in each of the spores of 20 other asci were found to be stained deep red. In still another mount, the two oil drops in each spore of 80 asci showed simply the large unstained oil drops while in the same squash mount the two oil drops in each spore of 41 asci showed some shade of the red stain. No doubt, if this same cluster of asci had been examined after having been subject to the drying effect of the S III for some hours and then immersed in water, the spores instead of showing *two* unstained oil globules each, would then have been seen to contain clear-cut dBb, *one* in each spore.

Figure 2, B-II, is made up of diagrams of several different asci, each containing eight spores. The mounts had first been immersed in SMF and later flooded with water. The purpose of the diagrams was two-fold. First, to show some of the stages in the shrinking and final disappearance of the dBb and, at the same time, indicate reappearance of oil drops; second, to show that one can find here and there during the tests spores with only a single oil drop. Each diagram was made from a sketch designed to show the presence of dBb and oil drops as they appeared at that particular time. No attempt was made to indicate other spore contents. Only very roughly do the diagrams indicate the true dimensions of dBb, oil drops, spores and asci. Three asci, A, I and J, in this figure had been immersed in S III. The oil

drops shown in the spores of ascus *A* were rather small, but they were stained rose-red, while the red oil drops in ascus *J* were large and were deeply stained red by the S III. The other spores in this ascus are not shown here. The large oil drops in all eight spores of ascus *I* were not stained even though asci *I* and *J* were adjacent in this mount.

It has been shown that if the water in such a squash mount were displaced again by the S III the oil drops in such spores as shown in *I* would have disappeared, and a dBb would have in each case then come to view. We have, however, not yet proved that a dBb would have developed in an ascospore whose red oil drops had been subjected to another immersion in S III. No doubt if alcohol alone had been used as the drying agent, the S III in the oil drops would have been dissolved out so that dBb might then have developed in that spore. To repeat, it still remains to be found that the ascospore that contained oil drops that had absorbed the S III agent, may at the same time show a dBb. It is very easy to demonstrate that definite oil-drops and clear-cut dBb can exist *temporarily* in the same spore. Then why is it that in our *Lachnea*, when we dehydrate the spore, the two oil drops gradually disappear but only one, not two, dBb comes into view? One can't say that two dBb never appear in the same spore. We have actually seen two somewhat abnormal elongated dBb in a few large ascospores of a *N. tetrasperma* hybrid race. Normally, the size of the ascospore does not seem to make any difference. Spores of *N. tetrasperma* are much larger than spores of *N. sitophila*, yet only one dBb develops in each spore.

Oil or other fatty matters in aborted asci. Asci of *N. tetrasperma* that were originally homozygous *Aadd* for the recessive lethal *d* usually abort without spore formation (Dodge 1935). This type of abortion was at first described as deliquescent abortion because the ascus and its contents gradually disappear as the culture ages. Apparently, no one has investigated chemically this type of abortion in *Neurospora*. That such asci contain a great deal of oil or other fatty matters is easily proved by introducing S III under the cover-glass and so displacing the water of the squash mount. Almost at once the entire ascus contents become deeply stained some shade of red, often mixed with gold-yellow. Very often parts of the contents form globules as figured by Dodge. These take the stain very readily and so are apt to be mistaken for spores, "spores without nuclei!" Such deeply stained globules are readily distinguished from ascospores because, rather rarely, an ascus *Aadd* does delimit spores. In such instances, by comparison, no one could fail to distinguish the red-stained spores from the red globules.

The walls of indurated aborted asci of *Neurospora*, due to lethal *I* as well as phenocopies of them, develop dark brown walls with ribs similar in their origin and markings to ascospores themselves. As young asci develop, they become inflated. At this stage, they have thin walls and take the S III stain showing quantities of fatty matter irregularly distributed in globules.

Although many hundreds of the "mature" heavily-walled asci mounted in the SMF have been studied we have never seen a single dBb develop in such asci. By cracking these indurated asci one can prove the presence of some oily matter by using the S III stain.

The conidia of *Lachnea abundans*, when immersed in S III, stain dark red without showing either oil drops or dBb. The conidia do not shrink or collapse. They must be readily permeable to the alcohol carrying the S III. Some conidia of *Neurospora* collapse quickly, yet so far as is noted do not form distinct oil drops or dBb under the same conditions.

The results noted above suggested that squash mounts of normal or wild type perithecia of this species should be investigated by subjecting them to the S III test for fatty matters. When this was done it was discovered that the ascogenous cells, young asci, young spores, and even full-sized spores, take the stain if their walls have not turned olive-green or very dark colored so that the spores were opaque. Spores that were just begining to show the first stages in rib formation often took the red stain. Fully mature spores take no stain. Such spores, however, are the ones that may develop dBb if dehydrated with alcohol, glycerine, or certain other drying agents. This S III solution should prove to be an excellent stain for various stages in the origin and development of the perithecia. Young protoperithecia, as well as outgrowths and other hyphal branches, often take the red stain beautifully, showing the presence of fatty matters.

The following example illustrates the apparent inconsistency with which oil drops and dBb may respond to the S III reagent. Fragments of an ascocarp of *Lachnea abundans* had been mounted in water for some hours. The water was then replaced with the S III solution. Several hours later the S III was in turn replaced with water. When a certain ascus was first examined the top, or first, spore at that time contained two unstained oil drops; the second spore contained a single dBb, no distinct oil drops being present; spore number 3 contained a single large unstained oil drop; the adjacent spore below contained a single large oil drop but this one was stained deep-red; spore number 5 contained two large oil drops, both deeply stained red; spores nos. 6 and 7 each had a somewhat shrunken dBb, but no definite oil drops; spore No. 8 had two normal unstained oil drops. About half an hour later the picture of this ascus was practically the same except that the dBb in spores 2, 6 and 7 had shrunken to become mere vestiges of their original size and appearance. However, where there were at first no oil drops in these three spores, there now showed plainly two unstained oil drops along-side the remains of the dBb. In spores 2 and 6 the vestigeal dBb were now in between, but not touching, the oil drops. See Fig. 2, B–H for diagrams of comparable stages in the appearance and disappearance of oil drops and dBb where the reagent was SMF instead of S III.

Discussion and conclusion. It was pointed out earlier in this paper that

ascospores of *Neurospora* do not show typical oil drops in more or less definite numbers when mounted in water or in SMF. Furthermore, when the young spores are mounted in S III the contents stain beautifully but the fatty mass is not delimited or confined to a definite number of distinct oil drops. The ascospores of *Lachnea abundans* old or young, mounted in water, usually show two clear-cut oil drops. Whether or not every oil drop will be stained red by the S III seems to depend on the age or maturity of the spore at the time. It was interesting to see in the same spore two oil drops and entirely separate and apart from them, a tiny dBb. In such instances, there is a critical period when, not knowing the set-up treatment, one could not tell whether the oil drops were just coming into view or were just beginning to fade out. In either case, the "gas bubble" would be doing just the opposite thing. Since the Sudan III is dissolved in alcohol, it first acts as a drying agent, often resulting in the formation of a single intracellular dBb in certain spores. Furthermore, some spores in the mount will be in that stage of maturity which favors the test for oil. In such spores one sees rose-colored or red oil drops. If the mount is a fresh one there will usually be two oil drops in each spore, occasionally only one. Some asci may be about to delimit the spores. The contents of such an ascus will then become rose-colored due to the S III. Squash mounts of pieces of ascocarps often contain a hundred or more asci. One is then apt to become bewildered seeing so many different types of reactions to the S III. Nevertheless, compared with species of *Neurospora*, this little *Lachnea* (*Patella*) gives us far superior material for the investigation of "bubbles" and oil drops in ascospores.

Those of us who have studied *Gelasinospora tetrasperma* have not reported seeing these curious bodies in ascospores. However, a re-examination of our slide mounts and photographs shows that this species reacts in much the same way as do species of *Neurospora* when their spores are immersed in the SMF reagent. A high percentage of the spores will contain a single dBb. In both cases, under certain conditions or treatments, many spores collapse. This effect was due no doubt, to the fact that such spores had thin walls, or that the drying agent was too strong.

Insert B in our figure 1 shows many spores, each with its single "bubble." The interesting feature here is that the dBb in several spores is off-center, that is, near one end. It was found that when care was taken to leave bits of mycelium or fragments of the perithecium on the slide so that space was left between the slide and the cover glass, the spores at first float around end over end then come to rest. Here and there one finds a spore standing on end as it were. Focusing the microscope, one will always find the dBb. By lightly tapping the cover glass the spore may tip over so that one can see that the dBb is near one end. Usually this spore will rather quickly

become erect again. The question is whether or not it is always the uppermost end of this erect spore that contains the "bubble". Not enough work has been done to warrant any conclusion on this point. De Bary would no doubt have explained this occasional erect position of the ascospores noted above as a state of equilibrium; the "air bubble" being lighter than the other contents of the spore would be in the top end. Ingold could very well say that in this two-phase system the water-vapor "bubble", being lighter, would account for the erect position of the spore.

Reconsider the situation where ascospores of *Lachnea abundans* are mounted in water. The two oil drops in a spore represent rather definite substances. Mounted in the SMF or in Sudan III, for example, the globules gradually lose their definite boundaries, become fainter and finally practically disappear. Meanwhile, beginning as a mere speck, a dBb comes to view and soon attains its full size. Replace the drying agent with water and now it is the dBb that gradually disappears and oil drops come into view. Much is yet to be learned regarding these concomitant activities. The most important questions to be answered seem to be: what is the true nature of these dBb and what, if any, relationship have they to the oil or other fatty matters? De Bary called them air bubbles. Ingold presumed them to be water vapor bubbles. We are leaving it to the microchemist to give us the true answers.

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